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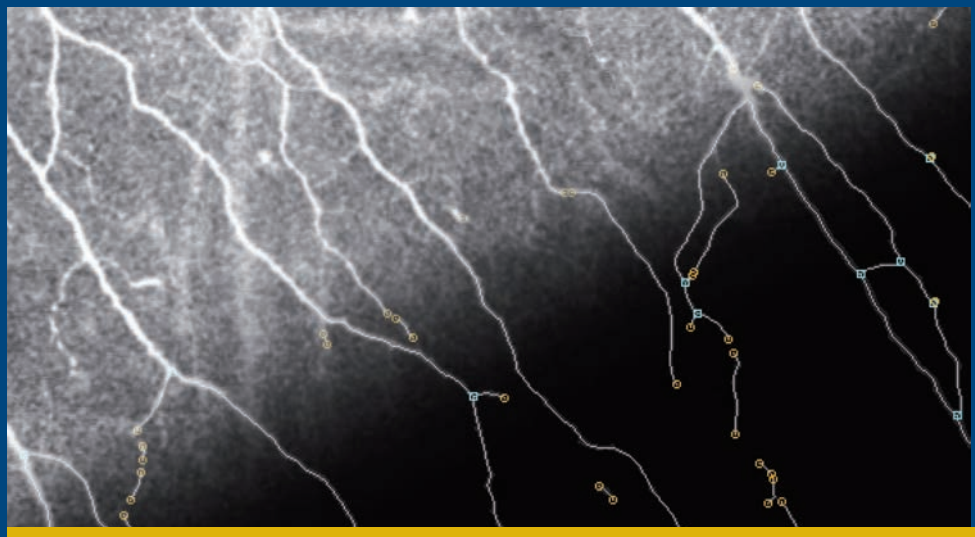
Vision and Diabetes

Rudolf F. Guthoff and Peter Wiedemann (Eds.)



Deutsche Akademie der Naturforscher Leopoldina –
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NEUE FOLGE NUMMER 401 BAND 119

Vision and Diabetes

Leopoldina Symposium in Cooperation with the University of Rostock and the University of Leipzig under the Auspices of the German Ophthalmological Society (DOG)

Rostock
March 15–16, 2013

Editors:

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With 71 Figures and 12 Tables

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Cover:
Image of the corneal sub-basal nerve plexus reconstructed from *in vivo* three-dimensional confocal microscopy of the cornea; the computer graphic illustrates the automatic segmentation and morphological analysis of the corneal nerve fibres (see KÖHLER et al. in this volume p. 127).

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Inhalt

Session 3: Diabetes Care – Today and Tomorrow

Opening Remarks

Rudolf F. Guthoff ML (Rostock)

According to estimates published in 2010, there are 285 million people with diabetes worldwide (of whom 90 % have type 2 diabetes). The number of people suffering from diabetes is set to continue to rise rapidly, with Asia and Africa being particularly affected. The main objective of the 2-day symposium "Vision and Diabetes" was to analyse the scientific interfaces between endocrinology and ophthalmology and to develop ideas for further collaboration.

To date, the close links between the fields of diabetology and ophthalmology have focused primarily on the diagnosis and treatment of diabetic microangiopathy of the retina – although there still exists no consensus concerning the relevant metabolic parameters for the prophylaxis of diabetic retinopathy.

In recent years major advances have been made in treatment appropriate for this disease stage – especially with regard to damage to the central retina and the threat to visual acuity – thanks to administration of drugs into the vitreous chamber and new microsurgery techniques. As a result there has been a marked decline in the incidence of blindness in the industrialized nations where such pharmacological and technological modalities are available.

International health research studies have shown that there is a major divide between optimal clinical care and current healthcare in Sub-Saharan Africa. Religious beliefs and attitudes to health play a crucial role, particularly when established treatment methods are transplanted from one medical culture for implementation in another. When introducing new treatment strategies it is important to acquire fundamental knowledge about the target population, the local culture and prevailing attitudes towards health issues.

The epidemiological importance of diabetes mellitus was outlined in introductory lectures delivered by Professor Doblhammer-Reiter of the Max-Planck-Institute for Demographics Rostock and Professor Paul Sieving (ML) of the US National Institutes of Health. Further sessions explored (*1*) advances in diabetic research, (*2*) the importance of novel ophthalmological imaging techniques – the eye as a biomarker for neuropathy, and (*3*) diabetic care – today and tomorrow.

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The Demography of Diabetes mellitus

Gabriele Doblhammer,[1,2,4,5] Thomas Fritze,[3,5] and
Anja Noerenberg[6] (Rostock)

With 3 Figures and 1 Table

Abstract

Against the background of ageing societies, diabetes is a major risk factor regarding mortality, disability and poor quality of life with high costs for the health care system. We find specific regional patterns of diabetes mellitus in Germany with the highest prevalence rates in the north-east and the lowest in the south west. Among others, age and life-style aspects are important known risk factors, however, living-circumstances in terms of economic conditions at the time of birth are also relevant for the risk of suffering from diabetes later in life.

Zusammenfassung

Vor dem Hintergrund der demografischen Alterung ist Diabetes eine große Belastung für Gesundheit, Wohlbefinden und Lebensqualität jedes Einzelnen, aber auch der Versorgungssysteme insgesamt. Zum einen beschreiben wir die spezifische regionale Verteilung der Diabetesprävalenz in Deutschland mit den höchsten Werten im Nordosten und den niedrigsten Werten im Südwesten. Zum anderen diskutieren wir verschiedene bekannte Risikofaktoren wie Alter und Lebensstil. Wir zeigen aber auch den Einfluss früher Lebensumstände auf das Risiko des Auftretens von Diabetes im späteren Leben am Beispiel ökonomischer Bedingungen zum Zeitpunkt der Geburt.

1. Introduction

While the reduction of cardio-vascular mortality is one of the main drivers of the increasing life expectancy, the prevalence of diabetes mellitus, one of the most important underlying causes of cardio-vascular disease, has been steadily increasing, and it is now one of the most frequent metabolic diseases in Europe as well as worldwide (Heidemann et al. 2011). Against the background of increasing life expectancy this article provides a general description of trends and patterns of diabetes mellitus in the industrialized world. We then explore age-specific and regional patterns in Germany using newly available data from the largest

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German public health insurance company. Finally, we turn to the question whether early-life living-circumstances influence the occurrence of diabetes mellitus later in life. We explore cyclical variations in the business cycle in eleven European countries in the first part of the 20th century and link this information with data on the occurrence of diabetes in a Europe-wide survey on health of elderly people.

2. Trends in Life Expectancy

The 20th century has been the century of unprecedented and continuous mortality decline resulting in ever increasing life expectancy in the industrialized world. In 1900 average life expectancy at birth was about 45 years, in 2009 Japanese women had worldwide the highest life expectancy with 86.5 years. In the last 160 years life expectancy has been increasing in average by three months a year among the record holding countries (Christensen et al. 2009, Oeppen and Vaupel 2002), which are the countries with the worldwide highest life expectancy at a given point in time. The increase was similar among the industrialized countries that did not have record life expectancy themselves (Rau et al. 2008). This remarkable upward trend was interrupted only briefly by the two World Wars and by the Spanish Flu in 1918, which, however, did not alter the pace of the steady increase. Up to now, there has been no deceleration in the increase in record life expectancy, and the rise has been steady and almost linear (Oeppen and Vaupel 2002).

These findings are based on the measure of period life expectancy, which is life expectancy estimated for a particular year combining the mortality regime of a new-born up to the oldest person. The measure of period life expectancy assumes that the mortality regime remains unchanged over the coming years. Since it is plausible to expect that mortality will also decline in the future, period life expectancy is most probably an underestimation of the true life expectancy. An alternative measure is cohort life expectancy where the mortality regime of a birth cohort is followed over time. The complete mortality experience is, however, only available for cohorts born 1920 and earlier, where most of the members have already died. Newer findings indicate that, when cohort rather than period life expectancy is considered, we see an even greater increase in record life expectancy, of more than five months per year (Shkolnikov et al. 2011). Regardless of whether cohort or period life expectancy is used, a further increase in life expectancy is expected for both the best-practice countries and for all other (developed) countries.

In the second part of the 20th century a new pattern of mortality decline has been evolving. The gains in life expectancy primarily stem from ages 60 and above with a large fraction contributed by the old and oldest-old (Christensen et al. 2009). Today an 80-year-old German woman has the same remaining life expectancy as her 75-year-old counterpart would have had 50 years ago. Her probability of dying at age 80 has more than halved.

In the first half of the 20th century the increase in life expectancy was due to the reduction of infant and child mortality as well as the successful fight against infectious disease. In the period 1850–1900 about 62 % of the increase in record life expectancy was due to the reduction of mortality at ages 0 to 14; in the years 1990 to 2007 they only contributed 6 %. In comparison, the oldest-old aged 80 and above contributed 0.87 % to the increasing life expectancy between 1850 and 1900, but 42 % between 1990 and 2007. Since 1990 about 79 % of the increase comes from ages 65 and above (Christensen et al. 2009). All major groups of

causes of death, with the exception of lung cancer among women, contributed to this mortality decline (Janssen et al. 2004, 2005).

Better living conditions, higher education and income, life-style factors such as smoking, diet and physical activity, and advances in medical technology are important determinants of the mortality decline (Meslé and Vallin 2002, Robine et al. 2005, Vallin 2005, Vallin and Meslé 2001). The first phase of the mortality reduction, frequently named the first epidemiological transition (Omran 1971), was caused by the reduction of infectious diseases. Many scholars argue that better living conditions in terms of nutrition, sanitation and public health measures were the driving factors. The second phase is related to the reduction of cardio-vascular mortality starting in the middle of the 1960 decade and medical technology as well as changing life style factors have significantly contributed to the decline. A future third phase in the mortality development might be related to the smoking patterns among women, where countries with high female smoking prevalence rates such as The Netherlands, Denmark and the United States fared worse in terms of mortality than e.g. France or Japan (Janssen et al. 2004, Nusselder and Mackenbach 2000). Also the trends in neurodegenerative diseases such as dementia might be important for future gains in life expectancy.

3. The Definition and Occurrence of Diabetes mellitus

Diabetes mellitus comprises different metabolic disorders but does not include a consistent clinical picture. It is moreover a syndrome with different etiology, epidemiology, and pathogenesis (Rosak 2003). Chronic hyperglycaemia and a high risk of malfunction of important organs are common symptoms of the syndrome. The reasons for the lack of function are microvascular (nephropathy, retinopathy, neuropathy) und macrovascular (atherosclerosis, myocardial infarction, stroke) complications (Ginter and Simko 2010). A major cause of all symptoms is a reduced insulin secretion or a deficient insulin action or both (Waldhäusl et al. 2004). According to the American Diabetes Association four different types of diabetes mellitus exist: type 1 diabetes, type 2 diabetes, gestational diabetes, and a group of other specific types of diabetes (e.g. diseases of the exocrine pancreases, endocrinopathies or chemically induced diabetes) (Sierra 2009).

Diabetes is a cost-intensive disease. In the year 2012 diabetes caused more than 471 billion US-Dollars in healthcare expenditures to cover the direct and indirect costs of the treatment. That is more than 11% of the total costs for healthcare for the age group 20 to 79 *(International Diabetes Federation 2011*). The costs in Europe amount to about 130.6 billion US-Dollars and in North America and Carribean it is approximately 223.5 billion US-Dollars in 2011 (Tab. 1).

In 2012, 371 million people worldwide suffer from diabetes (*International Diabetes Federation* 2011). More than 90% of the diabetes cases stem from type 2 diabetes, indicating the crucial role of this particular diagnosis for the patients and the health care system. There is a general consensus that the number of cases with type 2 diabetes will increase in almost all countries (Oputa and Chinenye 2012). In 2030, the projected number of people with diabetes will be about 552 million (age 20–79) starting from 366 million in 2011. The global age-standardized prevalence will increase from 8.5% in 2011 to 8.9% in 2030. Europe has a comparatively low age-standardized prevalence, which will increase from 6.7% (52.8 million people) in 2011 to 6.9% (64.2 millions) in 2030. The age-standardized prevalence in North

Tab. 1 Statistics of population, diabetes prevalence, healthcare expenditures, and diabetes mortality in Europe and North America and Carribean (Source: *International Diabetes Federation* 2012)

	Europe		North America and Carribean	
	2011	2030	2011	2030
Population				
Total population (millions)	899	931	481	558
Adult population (20–79 years, millions)	653	673	322	386
Diabetes (20–79 years)				
Regional prevalence (%)	8.1	9.5	11.7	13.2
Age-standardized prevalence (%)	6.7	6.9	10.7	11.2
Number of people with diabetes (millions)	52.8	64.2	37.7	51.2
Healthcare expenditures due to diabetes				
(20–79 years; USD)				
Total healthcare expenditure (billions)	130.6	157.0	223.5	283.9
Diabetes mortality (20–79 years)				
Number of deaths, men (thousands)	282.4		150.6	
Number of deaths, women (thousands)	317.6		130.2	
Number of deaths, total (thousands)	600.0		280.9	

America and Carribean is comparatively high, and it will increase from 10.7 % in 2011 (37.7 millions) to 11.2 % in 2030 (51.2 millions) (*International Diabetes Federation 2011*) (Tab. 1).

A problem when estimating the prevalence of diabetes is the high number of undiagnosed cases. Estimates suggest that worldwide about 50 % of the people suffering from diabetes do not receive a diagnosis by a physician. Therefore, an estimated 183 million might be unaware of their illness. While the proportion of undiagnosed cases is particularly high in Sub-Saharan Africa (81.2 %) and the Western Pacific area (57.9 %), also in Europe (38.6 %) and North America and Caribbean (29.2 %) a sizeable proportion remains undiagnosed. Reasons for the lack of diagnosis may be the unawareness or misdiagnosis of symptoms, or that affected people do not show any symptoms (*International Diabetes Federation 2011*).

Estimating the number of deaths due to diabetes is challenging. Most often there are no data on diabetes-related mortality or health statistics underestimate the number of deaths. *The International Diabetes Federation* (2011) estimates a number of 4.8 million deaths related to diabetes. Cardiovascular disease as one of the major causes of death for people with diabetes can account for about 50 % of the above mentioned number of deaths. There seems to be no gender difference.

4. Age-Specific Profile and Regional Patterns of Diabetes mellitus in Germany

Age is a significant risk factor of diabetes and the incidence and prevalence of diabetes increases with age (Heidemann et al. 2011). Increasing life expectancy resulting in population aging with ever more people at higher ages is thus a major factor behind the increase in the number of people with diabetes, particularly in the industrialized world.

We use newly available data of the "Allgemeine Ortskrankenkasse" (AOK) to explore age-specific prevalence rates of diagnosed diabetes for ages 60 and above in Germany. The AOK is the largest German public health insurance covering about one-third of the total population. Among the oldest-old, the AOK covers more than 50 % of the population. The claims data include complete records of the stationary and ambulatory treatment from each insured person with at least one day of insurance coverage (Schulz and Doblhammer 2012). An age-stratified sample was drawn from all insured persons of birth cohorts 1957 and earlier in the first quarter of 2007. The sample comprises 1,565,450 persons aged 60 and above.

Diabetes is defined based on the ICD numbers E10 to E14 according to the ICD-10 classification of diagnoses and diseases. We do not differentiate between different etiologies but combine all ICD numbers into one group which we call "diabetes".

Information about the population at risk is also based on the stratified sample and contains the number of AOK-insured person-years aggregated by sex, and age x. We calculate the prevalence-rates of diabetes as follows:

$$\text{Prevalence}_x = \frac{\text{insured person-years with diabetes diagnosis}_x}{\text{total insured person-years}_x} \qquad [1]$$

In the claims data, age-specific prevalence rates at age 60 start at a level of 18,208 cases per 100,000 person-years for men and 14,360 for women.[7] They increase until the age of 75–79 among men (30,450 cases) and 80–84 among women (24,825 cases). The increase is followed by a plateau, and from age 85–89 onwards they decrease for both sexes (Fig. 1). The plateau and the following decline is not unusual for an age-specific profile of disease, similar features exist in the age-specific profile of mortality (Vaupel et al. 1979), but also e.g. dementia (Daviglus et al. 2010, Doblhammer et al. 2012). Three different explanations might account for the plateau and the following decline. *First*, diabetes mellitus might be related to a certain age due to age-specific changes in biological and environmental risk factors. Once the high-risk age is surpassed, the prevalence of diabetes decreases. *Second*, selective mortality related to diabetes changes the composition of the population and at higher ages predominantly individuals free from diabetes survive. *Third*, medical doctors refrain from diagnosing diabetes with increasing age.

The claims data also permit the regional analysis of the diabetes pattern in Germany. We explore age-standardized prevalence rates using the German population aged 60+ for both sexes combined as the standard population (*HMD* 2013). Figure 2 shows the prevalence rates of diabetes in Germany on a 2-digit postal code level for the population at age 60 and above. The results reveal striking regional differences with the highest standardized prevalence rates in the north-east of Germany. We find the lowest rates in the south-western regions and especially in the north-western areas.

Similar results are reported by Schipf et al. (2012) who use a sample of 11,688 study participants and 1,008 persons with diabetes. The subjects aged 45–74 years stem from five regional population-based studies: SHIP in the northeast, CARLA in the east, HNR and DHS in the west, and KORA S4 in the south of Germany. Additionally, data from the nationwide German National Health Interview and Examination Survey 1998 (GNHIES 98) were includ-

7 In the remainder of the text we omit the qualification per 100,000 person years.

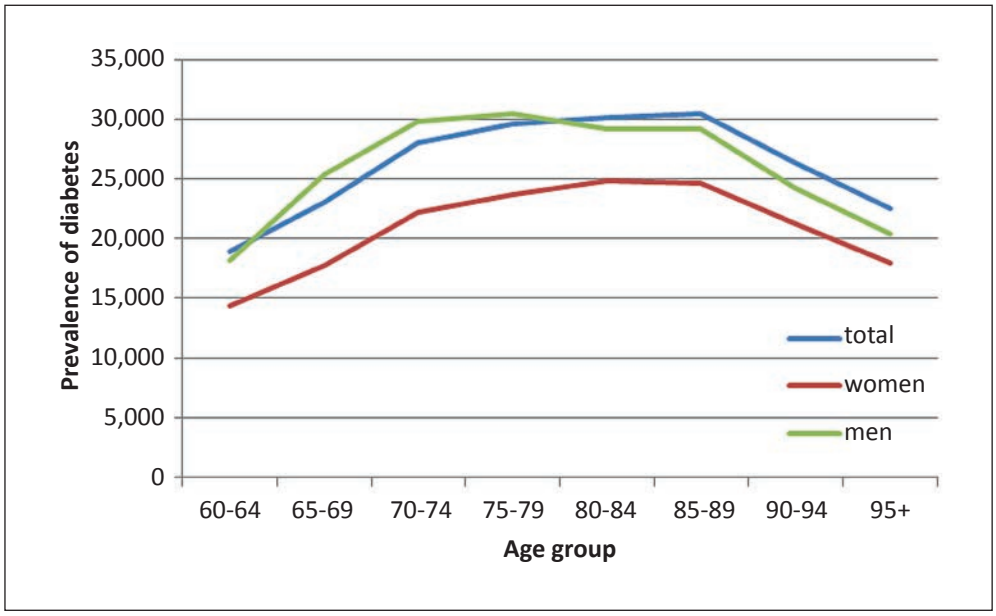

Fig. 1 Age-specific prevalence rates of diabetes, Germany, 2007 (Data source: Claims data AOK 2007; Prevalence per 100,000 person-years)

ed. All of the studies used similar methods regarding the study design, selection of the study population, and the definition of type 2 diabetes resulting in similar response rates. Type 2 diabetes was defined based on self-reported diabetes or self-reported diabetes treatment with drugs and age at diagnosis. The estimated regional prevalence rates have been directly standardized to the German adult population (31 December 2007). The results show a south-west-to-northeast gradient with the highest prevalence in the east (12.0 %) and the lowest in the south (5.8 %). The SHIP study in the northeast shows a value of 10.9 %, the western studies have a prevalence of 9.3 % (DHS) and 7.2 % (HNR). The results are consistent with results from our nationwide study.

The regional patterns in the prevalence of diabetes are in agreement with regional differences in cardio-metabolic risk factors such as obesity and lifestyle-habits. The prevalence of e.g. hypertension or smoking is higher in the northeast than in the south and west of Germany. Further, the regional differences might be explained by differences in health care (see Schipf et al. 2012 for more details and the according literature).

5. Risk Factors of Type 2 Diabetes

Next to age, several risk factors, especially for type 2 diabetes, are well established in the literature. This includes late-life aspects as well as conditions early in life.

Obesity and little physical activity are important risk factors (Ginter and Simko 2010) with obesity being part of the metabolic syndrome. This syndrome is a generic term for the co-occurrence of different diseases (Rosak 2003) and includes central obesity, impaired

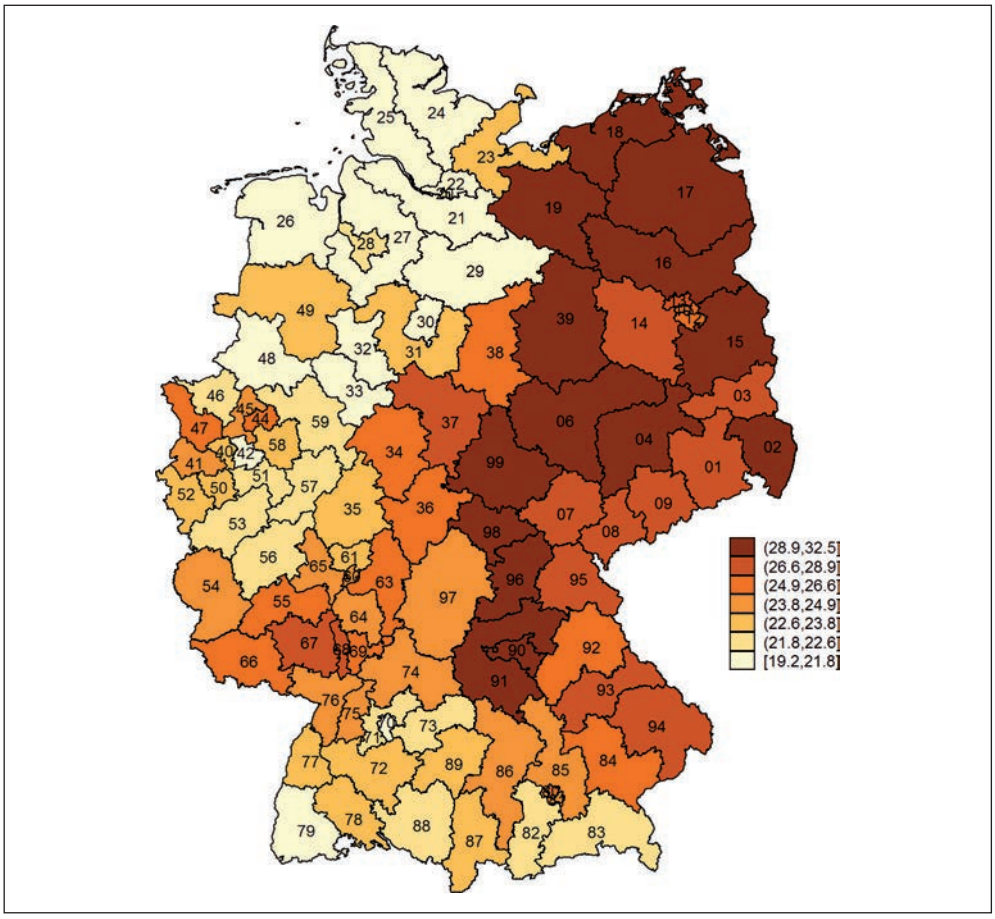

Fig. 2 Regional prevalence of diabetes in Germany (2007), ages 60+ (Data source: Claims data AOK 2007; Age-standardized; 2-digit postal codes; Cases per 100 person-years)

glucose tolerance, essential arterial hypertension and dyslipoproteinaemia (Haak and Palitzsch 2012). All of these affections go hand in hand with a high morbidity and mortality by coronary, cerebral, and peripheral vascular diseases (Rosak 2003).

In recent years the prevalence of obesity has grown rapidly in the developed countries. In 80 to 90 % of all cases people with type 2 diabetes are obese at the time of diagnosis (Haak and Palitzsch 2012). This increase reflects the lifestyle changes in the "western world" including a lack of exercise and the increase of hyper-caloric diet with high fat and low fibre content (Waldhäusl et al. 2004). The excessive intake of food leads to an elevated secretion of insulin, which than has a peripheral insulin resistance as consequence (Arastéh and Baenkler 2013). The risk of developing type 2 diabetes is three times higher with a weight gain of 8 to 10 kilograms and five times higher with a gain of 11 to 20 kilograms (Hien and Böhm 2010).

Further risk factors of type 2 diabetes include alcohol consumption, smoking (Rosak 2003), low education and low income (Heidemann et al. 2011). Furthermore, several ethnic

groups have a higher prevalence for diabetes. This holds true for East Asians, the Afro-Americans, the indigenous population and the Hispanics. The described effect is based on so-called "thrifty genes". These genes guarantee the optimal use of food and the fat deposition for difficult periods. On the contrary, these genes lead to a rapid increase of weight and the developing of adiposity in times with enough food (Waldhäusl et al. 2004). Haak and Palitzsch (2012) found that gestational diabetes in the past may also lead to a higher risk of type 2 diabetes. 50 % of the women who suffer from diabetes during pregnancy will have type 2 diabetes at a higher age (Oputa and Chinenye 2012).

A genetic predisposition might necessary for environmental risk factors to lead to an occurrence of diabetes. It concerns to a polygenetic, multifactorial inheritance whose genes are only partly identified. Twin studies show, that monozygotic twins have a concordance up to 90 % in developing diabetes (Haak and Palitzsch 2012).

An extensive literature exists about the effects of early-life factors on later-life morbidity and mortality. The underlying idea is that developing systems permanently modify their settings in response to social and biological cues during critical periods early in life (Kuzawa and Quinn 2009). One pathway may then exist through childhood exposure to diseases. The risk for type 2 diabetes and also for cardiovascular diseases and the metabolic syndrome may be increased by exposure to infectious diseases during early life (McDade et al. 2010). This may lead to a chronic activation of inflammatory pathways affecting morbidity and mortality in adulthood (Crimmins and Finch 2006, Finch and Crimmins 2004).

Another pathway may go through reduced fetal growth in combination with adult obesity (Crowther 2012) which may lead to an insufficient insulin secretion with the consequence of hyposomia and diabetic metabolism in the course of life. One explanation of the reduced fetal growth is the intrauterine determined abnormal development, respectively, dysfunction of the beta cells of the pancreas (Haak and Palitzsch 2012). Furthermore, type 2 diabetes is associated with a low birth weight (Hales et al. 1991, Lithell et al. 1996). The cardiovascular, obesity and diabetes effects of reduced nutrition *in utero* have been shown to be stronger if the affected individuals are exposed to a more favourable environment later in childhood (Eriksson et al. 2003, Schulz 2010).

6. Impact of Economic Conditions at the Time of Birth

Using the data of the Survey of Health, Aging and Retirement in Europe (SHARE)[8] allows us to analyse the risk of diabetes dependent on the economic conditions at the time of birth for the population at age 60 or older in Europe. We include 21,634 respondents with birth years between 1900 and 1945 from ten countries (Austria, Belgium, Denmark, France, Germany, Italy, The Netherlands, Spain, Sweden, and Switzerland) and link their information to the macro-economic deviations in the year of birth.[9]

We use the business cycle as an indicator for the economic conditions early in life following studies that focus on the effects of conditions at birth on later-life mortality (van den Berg et al. 2006, 2009, 2011). The business cycle describes the cyclical component of the natural logarithm of real GDP per capita for each country in our analysis. The GDP data are

8 See further information on SHARE at www.share-project.org.

9 A general description of the data can be found in Doblhammer et al. 2011.

provided by Maddison (2008). We construct a new indicator by computing the quartiles of the cycle. The lowest quartile indicates a "recession" period while the highest quartile includes "boom" periods. We comprise the second and third quartile into one category namely "average" periods. We link the year of birth with the according cyclical component of the same year. Depending on the precise date of birth in relation to the start of a boom or recession period, our early-life economic indicator covers most or all of the first year of life of an individual, or the period *in utero* including up to three months before conception. Birth in a recession period is then related to adverse economic conditions in many households with a low quality and quantity of nutrition, poor housing conditions, an enhanced stress level in the household, or a more difficult access to health care (van den Berg et al. 2006, 2009, 2011). We estimate a fixed-effects logistic regression model controlling for age, sex, and country of birth and find that people born in a recession period have a significantly increased risk of diabetes later in life (OR = 1.09, p = 0.09) compared to those born in a boom period (Fig. 3).

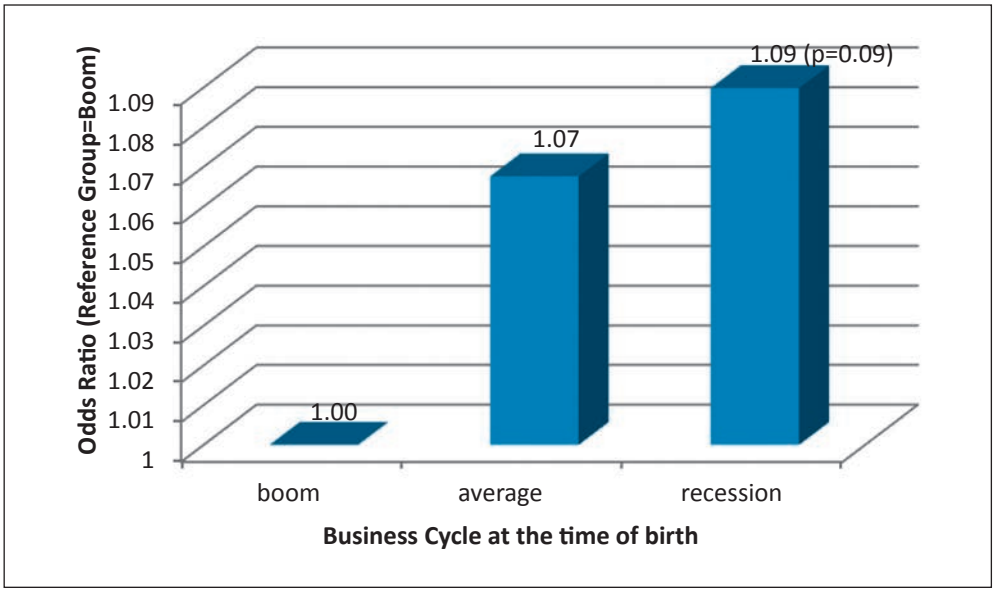

Fig. 3 Risk of diabetes by economic conditions in the year of birth, ages 60+ (Data source: Survey of Health, Aging and Retirement in Europe, waves I, II & IV; controlled for age, sex and country)

The impact of economic conditions on pre-natal and early natal nutrition is likely to be of major importance. While a flourishing economy allows a better living standard for the majority of the society, recessions, especially before 1945, lead to an income loss for many households and, therefore, to poor nutrition. The thrifty phenotype hypothesis of Hales and Barker (1992) proposes a relationship between poor fetal and infant growth and a higher risk of the metabolic syndrome and type 2 diabetes later in life, resulting from insufficient nutrition early in life. The latter led to enduring changes in the glucose-insulin metabolism which include reduced capacity for insulin secretion and insulin resistance. These two, combined with obesity and little physical activity, are the most important risk factors of type 2 diabetes.

7. Conclusion

The continuing increase in life expectancy will also lead to an increasing number of people suffering from diabetes. Large regional differences in the prevalence of diabetes point to the importance of preventive measures related to the life-style at adult ages. The relationship of the risk of diabetes at old age with economic circumstances at the time of birth shows, however, that public health measures should not be confined to adult ages alone but should start early in life. Diabetes does not only lead to lower life expectancy but also negatively affects health and well-being at old age. Reducing the burden of diabetes will therefore not only contribute to further gains in life expectancy but also to a higher quality of life at old age.

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Diabetic Retinopathy: Past, Present and Future

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With 4 Figures

Abstract

Diabetes and diabetic retinopathy cause major morbidity in the human population. Diabetes was described as long ago as 1552 B. C. in the *Papyrus Ebers*, and it affects many organ systems of the body, including the eye where it leads to vascular leakage and haemorrhage and results in blurred vision and ultimately total vision loss from proliferative retinopathy. Diabetic retinopathy was reported for perhaps the first time by Appolinaire Bouchardat in 1846. Three major stages of understanding this disease have occurred since then. The first phase began with the first description in 1846 and continued into the middle of the 20th century when various clinical aspects of the conditions were described. The second stage began in the 1970s with a series of interventional treatments that began with pan-retinal photocoagulation, developed through the National Eye Institute (NEI) Diabetic Retinopathy Study. This remains the mainstay of medical treatment for the ocular component of advanced diabetes. We have now entered a third phase, which portends the future based on biologic intervention at the molecular level. This work has been pioneered through the Diabetic Retinopathy Clinical Research Network (DRCR.net), established in 2002, which recently demonstrated that intraocular anti-vascular endothelial growth factor (VEGF) treatment was effective for diabetic macular edema. Success of this molecular approach will gather speed in the years ahead as we begin to understand the pathology at a cellular and system biology level.

Zusammenfassung

Diabetes mellitus wurde bereits im *Papyrus Ebers* im Jahr 1552 v. Chr. dokumentiert. Die Erkrankung betrifft das gesamte Nerven- und Gefäßsystem des Körpers und damit auch das Auge. Diabetische Retinopathie ist eine häufige Folgeerkrankung von Diabetes mellitus und stellt die häufigste Ursache der Erblindung in den westlichen Industriestaaten dar. Die pathologischen Veränderungen am Auge führen zu Wasseraustritt, Blutungen und Gefäßverschlüssen, die eine Sehverschlechterung und bei der proliferativen Form Sehverlust verursachen. Zum ersten Mal wurde die diabetische Retinopathie im Jahre 1846 von Appolinaire Bouchardat beschrieben. Seitdem unterscheidet man drei verschiedene Zeitabschnitte, die dem Verstehen der Erkrankung dienten. Der erste Zeitabschnitt begann bereits mit der ersten Beschreibung 1846 und hat sich mit den klinischen Aspekten der Retinopathie befasst. Diese Phase dauerte bis zur Mitte des 20. Jahrhunderts an. Erst ab 1970 hat ein neuer Zeitabschnitt begonnen, in dem die interventionellen Strategien, inklusive panretinaler Photokoagulation (Diabetische Retinopathie-Studie, *National Eye Institute*), entwickelt wurden. Die dritte Phase weist auf biologische Interventionen auf Molekularebene hin. Das im Jahr 2002 etablierte Netzwerk *Diabetic Retinopathy Clinical Research Network* hat den Weg für die molekularbiologischen Ansätze gebahnt, indem es die positiven Effekte von intraokulärem VEGF (*anti-vascular endothelial growth factor*) auf das Makulaödem nachgewiesen hat.

1. Introduction: Setting the Stage: Diabetes and Diabetic Retinopathy

In discussing diabetic retinopathy, one must first understand something of the history of diabetes mellitus. The term has a Greek origin meaning “syphon”, or copious urination, and “honey”, characterizing the loss of sugars in the urine. The historical record of diabetes traces back to 1552 B. C. when Hesa-Ra, an Egyptian physician, described a disease involving

urination and emaciation, as recorded in the *Papyrus Ebers*. Over the subsequent 2000 years, this condition was further described, and by 500 A. D. it had been differentiated into type 1 disease, which becomes apparent in young individuals, and type 2 disease, which affects adults and is related to weight. Worldwide, increasing economic success, first of industrialized countries and now extending even to developing countries, has led to a weight-related explosion of individuals affected. Current estimates indicate that approximately 300 million individuals are affected worldwide (Danaei et al. 2011). This presents a tremendous burden and cost to society, estimated at $245 billion in the U. S. alone in 2012 (*American Diabetes Association* 2013). As the world population increases, one can fully expect that millions more will be affected in the coming years.

Diabetes mellitus affects the health of many organ systems, including the eyes. Effect on the retinal vasculature causes microvascular angiopathy and leads to vascular leakage of serum and blood and, ultimately, proliferation of microvasculature in the late stage of diabetic retinopathy. It is estimated that 10 % of individuals have severe vision impairment, and 2 % are legally blind after 15 years of diabetes. According to a 2006 survey by Lions Club International, patients with diabetes are concerned by the potential for ocular disease, which they score as their top concern for the sequel of diabetes to cause impaired vision and to go blind.

2. Diabetic Retinopathy: Developing an Understanding

The first seminal event in developing a medical and clinical understanding of diabetic retinopathy came with the invention of the ophthalmoscope by Hermann von Helmholtz in 1851. This instrument allowed physicians to directly view the ocular fundus to observe the integrity of the blood vessels. By the turn of the 20th century, vascular problems were clearly known to be associated with longer-duration diabetes and ultimately these changes were associated with capillary micro-aneurisms seen in post-mortem retinal tissue from affected individuals.

Gerhard Meyer-Schwickerath, a German ophthalmologist, made a seminal observation in 1950 when he observed spots of retinal damage in individuals who had viewed a solar eclipse without appropriate visual screening of the intense light. He then looked for ways to apply light deliberately to destroy lesions in the retina by photocoagulating retinal tissue in a controlled fashion. Ultimately, he used high-intensity focused light from a xenon-arc lamp to photo-cauterize retinal tissue and blood vessels. In the 1960s, the development of concentrated light from the ruby laser was applied to the retina, and William P. Beetham and Lloyd M. Aiello recognized that photocoagulation seemed to be effective in treating neovascular diabetic retinopathy. This led to the pioneering large-scale ophthalmic clinical trial, termed the Diabetic Retinopathy Study (DRS), initiated in the 1970s. This randomized controlled trial enrolled approximately 1700 patients with advanced proliferative retinopathy at 15 participating centres. Eyes were randomized to receive photocoagulation, and the fellow control eyes were followed but not treated. A large reduction of risk for progression to severe vision loss was found for photocoagulated eyes, with a greater than 50 % risk reduction by pan-retinal photocoagulation (Figs. 1 and 2, *The Diabetic Retinopathy Study Research Group* 1978). This success spawned additional randomized controlled trials for diabetic retinopathy, the Diabetic Retinopathy Vitrectomy Study in 1976 and the Early Treatment Diabetic Retinopathy Study of 1979. The outcomes of these three studies have made a major impact on ameliorating risk of blindness from diabetic retinopathy and are still the mainstay of current treatment.

Question

- Does photocoagulation prevent severe vision loss from proliferative retinopathy?

Trial Description

- The randomized, controlled trial included more than 1700 patients at 15 centres.
- Eyes were randomized to immediate argon laser or xenon arc photocoagulation.
- The control fellow eye was not treated.
- Visual acuity was measured at four-month intervals for more than five years.

Results

- Argon laser and xenon photocoagulation conferred greater than 50 % risk reduction of severe visual loss.
- Photocoagulation benefit outweighs the risk for high-risk proliferative retinopathy.

Fig. 1 Diabetic Retinopathy Study

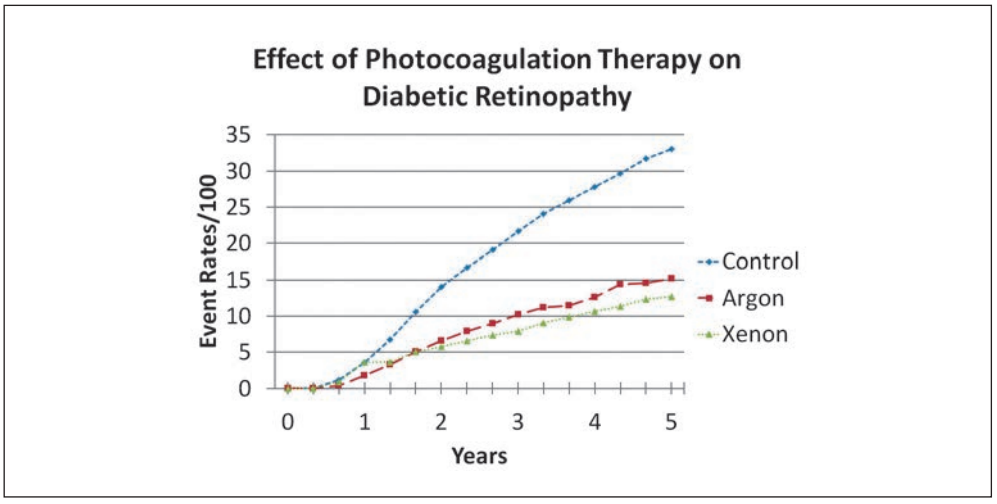

Fig. 2 The NEI Diabetic Retinopathy Study Research Group showed that photocoagulation therapy significantly reduced the cumulative event rate (oddurence of vision loss). A greater than 50 % risk reduction for progression to severe vision loss was found for eyes treated with pan-retinal argon laser or xenon arc phocoagulation (*The Diabetic Retinopathy Study Research Group* 1978).

Clinical trials for diabetic retinopathy then moved into a new phase in which attention shifted to controlling the underlying diabetes to learn whether this lessened the risk of vision impairment from diabetic retinopathy. The first of these trials was launched in 1983: the Diabetes Control and Complications Trial (DCCT), the Epidemiology of Diabetes Intervention and Complications Trial, and the United Kingdom Prospective Diabetes Study. These demonstrated that tight control of glucose reduced diabetic complications, particularly when stratified by maintaining a hemoglobin A1c value of 7 % or less. This was as-

sociated with substantial reduction in progression of retinopathy. Variations on this theme were conducted.

These series of trials included the Diabetes Prevention Program Outcome Study of 1996, which found that lifestyle modification was more powerful at reducing the conversion rate from a pre-diabetes to type 2 diabetes when compared with metformin, a standard oral medication taken to normalize systemic glucose levels and reduce hyperglycaemia. This trial was stopped before it ran the full course because of the power observed in risk reduction by exercise and weight loss. It is interesting to consider that this trial in the second millennium A. D. confirmed the apostolate of Indian physicians Sushruta and Charaka, identified between 400–500 A. D., that type 2 adult diabetes had an overt connection with overweight individuals. The strong environmental and lifestyle components of diabetes and diabetic retinopathy become confounding factors when considering a purely pharmaceutical intervention for this condition and when attempting to elucidate underlying causative factors.

3. Diabetic Retinopathy: The Present

The current era of diabetic retinopathy treatment entered a new phase with the formation of the Diabetic Retinopathy Clinical Research Network (DRCR.net). This multi-centre clinical research network was initiated in 2002 by the National Eye Institute with support from the National Institute of Diabetes, Digestive and Kidney Diseases at NIH. By 2012, it had 868 investigators at 249 sites. DRCR.net seeks to identify clinical opportunities to design and implement trial protocols for successful intervention in diabetic retinopathy. A key component of this effort is a set of standardized key study procedures that allow accurate data pooling across a large number of participating physicians. Without this standardization, the intrinsic noise in uncoordinated data would prevent successful study.

The modern era of treatments for diabetic retinopathy involves intervention at the molecular and cellular level, and major success has been achieved by attention to the microvascular angiopathy, which causes the diabetic macular edema that reduces visual acuity due to vascular leakage as serum collects in the macular retinal tissues. Recently, new compounds have been developed and received FDA certification for clinical use evaluation to treat angiopathy in solid tumour cancers (bevacizumab). Subsequently, this approach was also used for vasculopathy from age related macular degeneration (ranibizumab). Through the DRCR.net, this quickly led to consideration of anti-VEGF treatment for diabetic macular edema, and a study was begun, which ran from 2007–2010. The study design competed ranibizumab treatment, with or without immediate laser, *versus* laser retinal photocoagulation, with or without concomitant intraocular steroid (Triamcinolone). The anti-VEGF treatment provided clear benefit compared to laser treatment, with improvement in visual acuity compared to laser treatment and also the benefit that fewer eyes suffered visual acuity impairment compared to laser treatment. This work sets the stage for recognition that hypotheses-driven therapeutic interventions based on knowledge of underlying biology is a powerful approach for treatment in diabetic retinopathy and presumably as a generalizable strategy for medical disease.

4. Diabetic Retinopathy: The Future

As demonstrated by the DRCR.net, anti-VEGF treatment for diabetic macular edema provides clear benefit to patients. In addition, there is an equally important lesson to understand that treatments that focus on the underlying pathophysiology of disease hold great promise for the future. However, one must first reach a critical level of understanding the cellular pathways involved in disease ideology and progression. And this will be difficult because of the complexities of the underlying pathophysiology, in this case of diabetes and the inciting of factors that trigger diabetic retinopathy. Not all individuals with long-standing diabetes progress to developing diabetic retinopathy. A recent meta-analysis of 35 population-based studies of diabetics worldwide indicates that about one third of diabetic individuals have some degree of diabetic retinopathy, and fewer than 10 % have either diabetic macular edema or proliferative diabetic retinopathy (Fig. 3, Yau et al. 2012). This means that a substantial number of individuals with underlying diabetes do not progress to overt diabetic retinopathy. Consequently, one of the important facets of research for the future must come to understand the triggering events that lead to developing retinopathy. One pathway is through the use of molecular genetics to identify factors that contribute risks for conversion from diabetes to diabetic retinopathy.

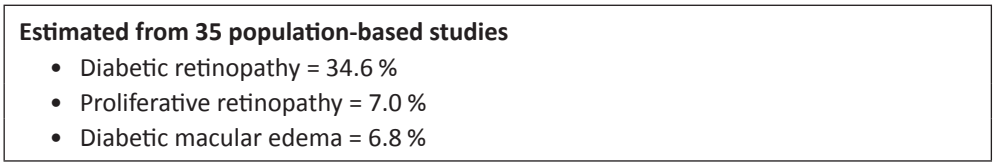

Fig. 3 Global prevalence of diabetic retinopathy among people with diabetes. Data from Yau et al. 2012

5. Diabetic Retinopathy: Genetics of Common Complex Diseases

The possibility of underlying genetic susceptibility is intimated from observing high risk populations, such as the Pima Indians in the U. S., who have high rates of diabetic retinopathy. Pima Indians are an isolated population of about 11,000 individuals residing on the Gila River Indian Reservation in Arizona, U. S. They have high rates of diabetes: about 50 % of Pima Indian youth have type 2 diabetes, which most commonly affects adults. The cumulative incident of proliferative retinopathy after 20 years is 14 % (Looker et al. 2003). Studies of 103 affected sib-pairs showed genetic linkage to chromosomes 3 and 9 in a study published in 1998 (Fig. 4, Imperatore et al. 1998). However, subsequent work has been unable to refine these loci. A potentially complicating factor is the recognition that diabetes prevalence of the youth of this tribe has increased two-fold since 1967, indicating that any underlying susceptibility will be compounded by environmental and lifestyle factors in studying individuals in this tribe.

A second example of a potential underlying genetic susceptibility for diabetic retinopathy comes from the Mexican-Americans in Starr County, Texas (Fu et al. 2010). This was a Genome Wide Association Study (GWAS) screen of 286 Mexican-Americans with type 2 diabetes. Two genetic markers showed strong association with advanced diabetic retinopathy, one mapping to chromosome 5 at an intron of calcium/calmodulin-dependent protein kinase 4 gene and a second at chromosomal location 15q13 within the formin 1 gene.

High Rate of Diabetic Retinopathy

- 11,000 members of the Gila River Indian Reservation in Arizona were studied for 30 years.
- 50 % of Pima Indians, including children, has type 2 diabetes.
- Pima Indians have a 14 % cumulative incidence of proliferative retinopathy after 20 years with diabetes.
- The prevalence of diabetes among Pima youth has increased twofold since 1967.

A 1998 study of 103 affected sib-pairs showed genetic linkage to chromosomes 3 and 9.

Fig. 4 Pima Indians in the U. S.

Despite these promising early indications of genetic association, identifying genes strongly associated with diabetic retinopathy has proven difficult. This may stem from several factors. Perhaps each gene makes only a small contribution that segments the risks and thereby requires a large number of cases for genetic analysis. Thus far, population sizes for retinopathy gene searches have been in the hundreds, *versus* the thousands of cases that have been employed for gene searches in diabetes and other systemic conditions. Other factors complicating the gene search would include diverse clinical features at different stages of disease progression. Or perhaps the environmental component is actually so large that it swamps the genetic determinants. And any role for epigenetics has not yet been studied.

Despite the lack of conclusive genetic etiologic factors, the preliminary analysis of GWAS studies indicates that a number of pathways and gene families may be involved in conversion to diabetic retinopathy and in its progression, including the glycemic pathway UDP-glucuronosyltranferases, *Wnt* signalling pathway, and crystallins, among others. This is the current state of future-directed genomic analysis of disease: searching for cellular ties into disease biology and thereby providing the next powerful development in epidemiology.

There are certainly other approaches to understanding the cellular basis of disease as it will develop in the future. Animal models exist for diabetes. Even though they do not perfectly recapitulate all facets of human disease, they nevertheless warrant careful biologic dissection and attention. They may provide clues as to which cells are affected early during disease progression.

The triggers for vascular changes clearly warrant attention. Throughout the decades and centuries and millennia of observing diabetes mellitus, vascular changes clearly are involved front and centre, as they also are in diabetic retinopathy. Hence, attention to human biomarkers for vasculopathy need further study. Inflammatory signals, both systemic and possibly those with a local retinal origin are warranted, given the treatment benefit of intravitreal steroid application in modifying transient diabetic retinopathy. And, attention to the remarkable biological tools that have been developed just in the decade since 2000 warrant consideration, such as silencing and micro RNAs.

6. Diabetic Retinopathy: Continuing Medical Needs and Opportunities

Meanwhile, as physicians, we still have the task of treating our patients with diabetic retinopathy to preserve vision. How can physicians best help patients? Clear management guidelines

exist for treating diabetic retinopathy (*American Academy of Ophthalmology Retina Panel* 2008). The outcomes developed from the DCCT provide clear indication that patients benefit from managing hyperglycaemia, hypertension, and serum lipids. Further, all patients with diabetes should seek regular ophthalmic clinical care to monitor early signs of development and progression of retinopathy. Much has been learned in managing life-threatening diabetes and vision-threatening diabetic retinopathy over the past half century. As physicians, we need to provide such care routinely to diabetic individuals.

And, what of the future? How can physicians help research? Beyond the care of individual patients, we still need to study disease manifestations and interventions. Ophthalmic physicians might well consider participating in large consortia to study diabetic retinopathy as a disease. For instance, the DRCR.net in the U. S. and comparable clinical networks outside the U. S. provide critical organizational resources and structure to make progress in treating this disease. Ultimately, these large-scale consortia will need to move into biologically-based hypothesis-driven therapeutic trials, and one way to identify cellular mechanisms is through participation in large-scale genetics initiatives.

7. Summary

Viewing diabetes and diabetic retinopathy across several millennia provides a perspective of our current unique position in biologic history. Medicine has clearly begun to study disease pathophysiology at the cellular level and to intervene with knowledge of how the biological system is failing at a systems level. Many of these techniques and biologic insights come only with years of scientific training and years of carefully developed research methodologies. At the other end of the spectrum, human disease develops in human patients and requires careful investigation by clinical research physicians. The future will require increased collaborative interactions between basic scientists and clinical investigators to build a strong platform that supports clinical research. Collaboration across the spectrum from disease biology to disease medicine can only come when scientists and clinical practitioners have opportunity to communicate. We must continue to foster such interactions to ameliorate vision loss and to restore and preserve health in diabetic retinopathy and diabetes.

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Session 1:
Advances in Diabetes Research

Nova Acta Leopoldina NF *119*, Nr. 401, 31–36 (2014)

Neurodegeneration – Lessons for the Diabetic Retina

Hans-Peter Hammes and Stephanie Busch (Mannheim)

With 3 Figures

Abstract

Diabetic retinopathy is characterized by changes of the neurovascular unit, involving damage and loss of vascular cells, activation of the (micro-)glia and progressive neurodegeneration. Intercellular communication is a fundamental requisite for vascular patency which is increasingly impaired, both in the diabetic and in the neurodegenerative retina. Non-hyperglycemic models contribute to the understanding of the molecular mechanisms involved in vasoregression, and may help to identify novel targets for prevention.

Zusammenfassung

Die diabetische Retinopathie wird durch Veränderungen der neurovaskulären Einheit, bestehend aus dem Verlust vaskulärer Zellen, der Aktivierung der (Mikro-)glia und der zunehmenden Neurodegeneration charakterisiert. Die interzelluläre Kommunikation ist wichtig für eine ungestörte Gefäßfunktion, die bei diabetischer Retinopathie und auch bei Retinadegeneration zunehmend gestört wird. Nicht-hyperglykämische Modelle tragen zum Verständnis der molekularen Mechanismen enorm bei, die bei Vasoregression beteiligt sind, und können dabei helfen, neue Zielstrukturen für eine Prävention zu finden.

1. Introduction

From the diabetologist's point of view, diabetic retinopathy is part of the triopathy which characterizes the simultaneous development of microvascular damage to the eye, the kidney, and the nerve (Friedman und L'Esperance 1980). Early and rapid development of retinopathy is a strong indicator of concomittant kidney disease in diabetes, and of increased cardiovascular morbidity and mortality (Klein 2006). A staging system for diabetic retinopathy has been successfully used for decades (*Early Treatment Diabetic Retinopathy Study Research Group* 1991), which is exclusively based on changes in the (micro)-vasculature (Fig. 1).

The focus on retinal vessels and their diagnostic use is at least in part the result of the fact that the neuroretina is translucent, and that changes induced by hyperglycaemia are not identifiable with direct ophthalmic inspection. The consistency between hyperglycaemia-induced changes in the eye and the kidney, characterized by increased vascular permeability and progressive vasoregression stimulated research of a shared underlying pathogenesis (Brownlee 2001). To this end, some overlapping mechanisms in the biochemistry and cell biology between the diabetic eye and kidney have been identified, but evidence that organ pathologies are under strong control of tissue specific mechanism is also strong (Forbes and

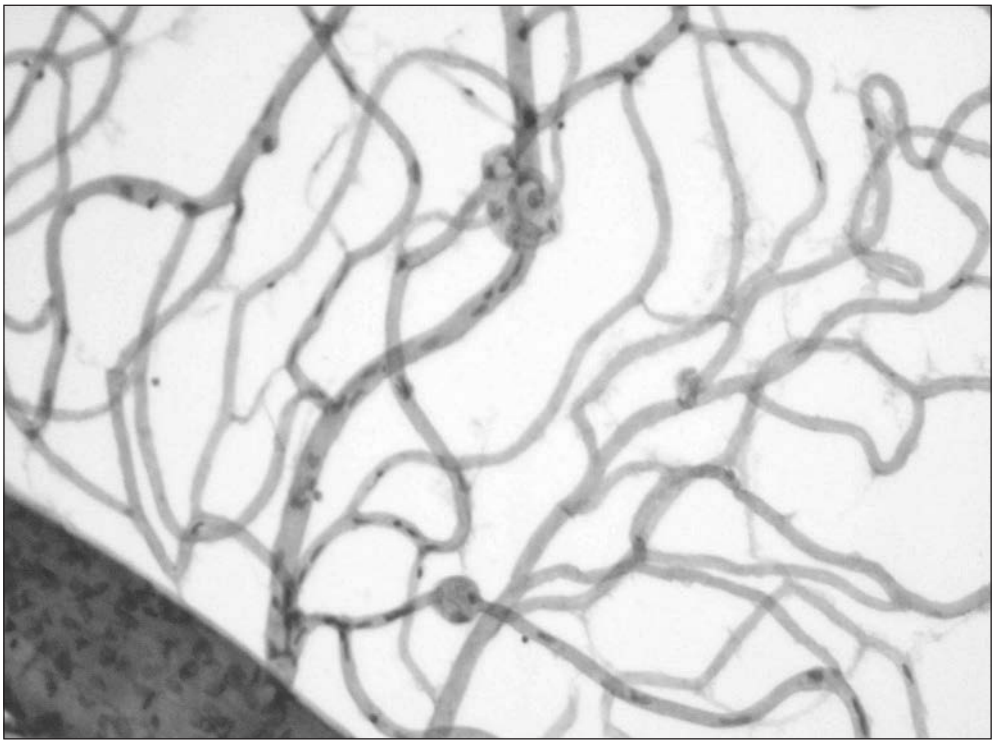

Fig. 1 Photomicrograph of a retina of type 2 diabetic patient with large areas of vasoregression and 2 microaneurysms. Original magnification 250×.

Cooper 2013). The development of novel technologies has shed light on the changes that the neuroretina undergoes immediatly after diabetes onset, putting emphasis on the role of the neurovascular unit as the determinant of retinal diabetic pathology, and, at the same time, the distinctive feature between eye and kidney in diabetes (Jackson et al. 2012). The neurovascular unit (NVU) of the retina, consisting of vascular, neuronal, glial and microglial cells has gained particular interest because of its role in specific functions such as the blood-retinal barrier, and in disease development and propagation (Fig. 2).

2. The NVU in Health and Diabetic Retinopathy

During development of brain and retina, sprouting angiogenesis and vasculogenesis form the vascular network without interfering with the integrity of the surrounding neuroglia structures. The close communication between the cells involved ensures proper vessel integrity, neuronal function, and immunological surveillance. Retinal glia serves an important role in providing simultaneous supportive function for both neurons and vascular cells. Excellent recent reviews describe the molecular machineries that form the basis of the blood retinal barrier, whose breakdown is one important feature of diseases in particular in diabetic retinopathy.

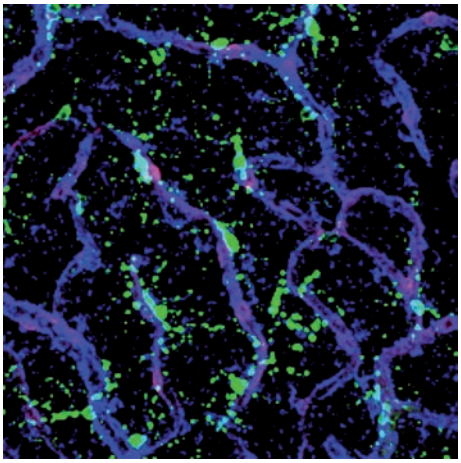

Fig. 2 Confocal photomicrograph of a rat retina depicting the deep capillary layer (*blue*) with pericytes (*pink*) and microglia (*green*) representing part of the neurovascular unit. Original magnification 200×.

Many of the mechanisms identified in experimental diabetic retinopathy affect the neurovascular unit. The earliest morphological sign is the loss of pericytes (Pfister et al. 2013). The underlying mechanisms comprise apoptotic cell death and migration, both of which are likely to be triggered by chronig hyperglycaemia and/or reactive intermediates (Pfister et al. 2008, Zheng et al. 2004). Vasoregression is the most important sign of hyperglycaemia-mediated microvascular damage and develops when also endothelial cells are damaged and lost in the retinal capillaries (Hammes et al. 2011). Reactive alterations in the retinal glia occur early in the diabetic retina (Hammes et al. 1995). It can be both, a consequence of, and a contributor to vascular pathology in the diabetic retina suggesting a strong interaction of cells in the neurovascular unit. Factors involved in hypoxia-induced angiogenesis such as VEGF-A and angiopoietin-2 are produced by Müller cells in response to various stimuli characteristic for the diabetic retina such as glucose, advanced glycation end products or reactive oxygen species (Hammes et al. 1998, 2004). Microglial cells become activated in the human diabetic retina, and in experimental animals, and may be involved in the damage signal to the microvessels (Krady et al. 2005, Zeng et al. 2008). The secretion of inflammatory cytokines, either induced by hyperglycaemia, or by the interaction of leucocytes adhering to the activated endothelium in capillaries and larger vessels, or from the bone marrow may cause endothelial damage (Li et al. 2012, Kern 2007). Neuroglial apoptosis is observed in diabetic retinae of rodents and patients indicating that diabetic retinopathy shares features of neurodegenerative retinal diseases (Hammes et al. 1995, Antonetti et al. 2012). Since treatment with neurotrophins whose cognate receptors are not expressed in vascular cells protects from diabetic vasoregression, it is likely that the neuroglia protects diabetic microvessels from damage through mechanisms which interfere with the biochemistry. The concept claims that hyperglycaemia induces mitochondrial overproduction of reactive oxygen species inhibiting the glycolytic flux in cells exposed to high glucose. Subsequent spillover of reactive intermediates at the level of the hexosamin pathway and the AGE pathway join to induce an upregulation of angiopoietin-2 in Müller cells (Brownlee 2001).

3. Neurodegeneration can Cause Secondary Vasoregression

According to novel insight from patients, neuronal damage in the diabetic retina can develop in the absence of vascular damage (Jackson et al. 2012). It is thus the question to what extent diabetic retinopathy is a specialized form of diabetic neuropathy. The question can experimentally become addressed in two ways: experimental diabetes induction in a model whose retina is anangiotic, such as the guinea pig, is assessed for retinal dysfunction that resembles human diabetic retinopathy. Alternatively, experimental neurodegeneration in an euangiotic model such as the rat is assessed for retinal vasoregression. Such a model has been generated through introduction of a mutated polycystin 2 gene (Gallagher et al. 2006). The transgene is expressed in the cilia-carrying parts of the photoreceptors leading to progressive neurodegeneration (Fig. 3). At the age of 7 months, the rats display large areas of vasoregression. In time course studies, the photoreceptor dysfunction was identified starting between 4 and 8 weeks of life, followed by strong activation of Müller glia (Feng et al. 2009). Among the strongest regulated Müller cell proteins is glial fibrillary acidic protein (GFAP). Novel data indicate that aquaporins, essential for the maintenance of proper water and mineral balance, are redistributed in the polycystic kidney disease (PKD) rat and that this redistribution resembles to some extent changes which accompany diabetic glial activation (Pannicke et al. 2006, Vogler et al., PlosOne, in press).

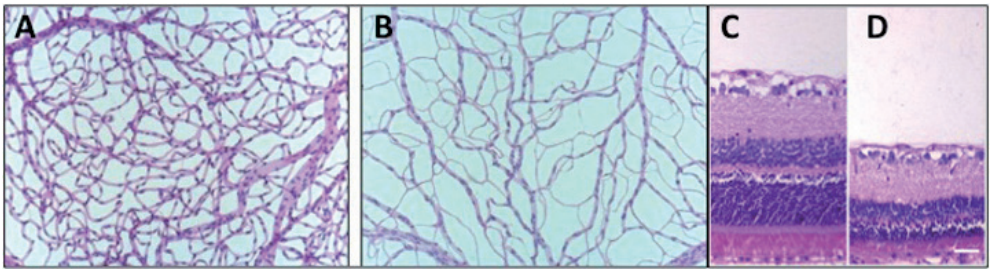

Fig. 3 Vasoregression and neurodegeneration in the PKD rat. Retinal digest preparation of a normal (panel A) and PKD (panel B) rat of identical age (7 months). Note the large areas of vasoregression in the PKD rat. Severe neurodegeneration (panel D) in the PKD rat, compared with normal retinae (panel C) at same age (7 months). From Gallagher et al. 2006.

In parallel, first signs of vasoregression are observed with a loss of pericytes and endothelial cells. Microarray analysis showed a strong up-regulation of components of the innate immunity system and the complement system. Cellular localization studies revealed that CD 74 positive microglial cells colocalized with acellular capillary segments suggesting that microglia may be candidate mediators of the neurovascular damage in this model, if the M1 phenotype was prevailing (Feng et al. 2011). Pharmacological reduction of reactive microglia reduces the formation of acellular capillaries along with a modulation of inflammatory cytokines. Thus, microglia can play an important role in the neurovascular unit to link neurodegeneration with secondary vasoregression.

4. Experimental Protection from Neurodegeneration Protects from Vasoregression

We have recently demonstrated that low-dose erythropoietin inhibits mechanisms related to the pathogenesis of diabetic retinopathy, including oxidative stress, formation of advanced glycation endproducts and inflammation (Wang et al. 2011). Receptors of erythropoietin are expressed on cells of the neurovascular unit and long-term administration of suberythropoietic concentrations of erythropoietin prevented both, the formation of acellular capillaries and neurodegeneration. Using this approach in the PKD rat, we identified a distinct protective effect on vasoregression. In the analysis of the neurodegeneration, low-dose erythropoietin protected from photoreceptor death and from degeneration of the outer retinal layers, in which the transgene is expressed, and where the first retinal lesions occur.

There are other diseases in which the neurovascular unit is severely affected. For example, Alzheimer's disease is characterized by neuronal injury, activation of microglia and glia, deposition of danger-associated molecular patterns (DAMPS) and secondary vasoregression (Zlokovic 2008). In the PKD rat, we found a deposition/accumulation of beta-Amyloid 1–42 and of HMGB-1 in astrocytes and in the vicinity of blood vessels of the superficial vascular network before vasoregression starts. As the phenotypic parallels, i.e. the sequence of neuronal damage, followed by glial and microglial activation, pericyte dropout and, ultimately, vasoregression between Alzheimer's disease and the PKD retina are striking, the model may well serve as a novel experimental tool to understand the shared pathogenesis of neurodegeneration and the role of the neurovascular unit that can be experimentally targeted (Busch et al. 2012).

In summary, as diabetic retinopathy is increasingly appreciated as a disease of the neurovascular unit, understanding the complexity of its pathogenesis can be improved by learning from models of retinal degeneration. One model is the PKD rat which shows that neurons determine vascular survival, and that activated microglia is one propagator of vasoregression.

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Diabetic Neuropathy – Diagnostic and Therapeutic Considerations

Dan Ziegler (Düsseldorf)

With 3 Tables

Abstract

Approximately one in three people with diabetes is affected by diabetic distal symmetric sensorimotor polyneuropathy (DSPN), which represents a major health problem as it may present with excruciating neuropathic pain and is responsible for substantial morbidity, increased mortality and impaired quality of life. Neuropathic pain causes considerable interference with sleep, daily activities, and enjoyment of life. Treatment is based on four cornerstones: (*a*) intensive diabetes therapy and multifactorial risk intervention; (*b*) treatment based on pathogenetic mechanisms; (*c*) symptomatic treatment; and (*d*) avoidance of risk factors and complications. Recent experimental studies suggest a multifactorial pathogenesis of diabetic neuropathy. From the clinical point of view, it is important to note that, based on these pathogenetic mechanisms, therapeutic approaches could be derived, some of which are currently being evaluated in clinical trials. Management of chronic painful DSPN remains a challenge for the physician and should consider the following practical rules: the appropriate and effective drug has to be tried and identified in each patient by carefully titrating the dosage based on efficacy and side effects; lack of efficacy should be judged only after 2–4 weeks of treatment using an adequate dosage. Analgesic combination therapy may be useful, and potential drug interactions have to be considered given the frequent polypharmacy in people with diabetes. Not only increased alcohol consumption but also the traditional cardiovascular risk factors such as visceral obesity, hypertension, hyperlipidaemia and smoking have a role in the development and progression of diabetic neuropathy and hence need to be prevented or treated.

Zusammenfassung

Etwa jeder dritte Diabetiker ist von der distal-symmetrischen sensomotorischen Polyneuropathie (DSPN) betroffen, die unter Ausbildung von einerseits teils quälenden neuropathischen Schmerzen und andererseits schmerzlosen Fußulzera mit erheblicher Einschränkung der Lebensqualität einhergeht. Neuropathische Schmerzen beeinträchtigen den Schlaf, die Alltagsaktivitäten und die Lebensqualität. Die Therapie der diabetischen Neuropathie umfasst vier Ansätze: (*a*) Kausale Therapie mit dem Ziel einer Nahe-Normoglykämie, (*b*) pathogenetisch begründbare Therapie, (*c*) symptomatische Therapie neuropathischer Schmerzen und (*d*) Vermeidung von Risikofaktoren und Komplikationen. Neuere experimentelle Studien legen eine multifaktorielle Pathogenese der diabetischen Neuropathie nahe. Vom klinischen Standpunkt ist wichtig, dass aus diesen pathogenetischen Mechanismen therapeutische Ansätze abgeleitet werden konnten, die derzeit in klinischen Studien evaluiert werden. Die Behandlung der chronisch schmerzhaften DSPN bleibt eine ärztliche Herausforderung und sollte die folgenden praktischen Regeln berücksichtigen: Jeder Patient benötigt eine individuelle Dosierung nach sorgfältiger Titration unter Berücksichtigung von Wirkung, Nebenwirkungen und Komorbiditäten; die Wirkungslosigkeit des Medikamentes sollte erst nach mindestens 2–4 Wochen Therapie bei ausreichender Dosierung beurteilt werden; analgetische Kombinationstherapie ist sinnvoll; vor dem Hintergrund der häufigen Polypharmazie bei Diabetikern sind potentielle Arzneimittelinteraktionen zu berücksichtigen. Nicht nur ein übermäßiger Alkoholkonsum, sondern auch die traditionellen kardiovaskulären Risikofaktoren wie viszerale Adipositas, Hypertonie, Hyperlipidämie und Rauchen spielen eine Rolle bei der Entwicklung und Progression der diabetischen Neuropathie und sind daher zu verhüten oder zu behandeln.

1. Clinical Impact and Epidemiology

Diabetic neuropathy has been defined as a demonstrable disorder, either clinically evident or subclinical, that occurs in the setting of diabetes mellitus without other causes for peripheral neuropathy. It includes manifestations in the somatic and/or autonomic parts of the peripheral nervous system (*San Antonio Conference* 1988) which are being classified along clinical criteria. However, due to the variety of the clinical syndromes with possible overlaps there is no universally accepted classification. The most widely used classification of diabetic neuropathy proposed by Thomas (Thomas 1973) has subsequently been modified (Sima et al. 1997). This proposal differentiates between rapidly reversible, persistent symmetric polyneuropathies and focal or multifocal neuropathies.

Diabetic distal sensorimotor polyneuropathy (DSPN) represents the most relevant clinical manifestation affecting approximately 30 % of the hospital based population and 20 % of community based samples of diabetic patients (Shaw et al. 2003). DSPN is a symmetrical, length-dependent sensorimotor polyneuropathy attributable to metabolic and microvessel alterations as a result of chronic hyperglycaemia exposure and cardiovascular risk covariates (Tesfaye et al. 2010). There is emerging evidence to suggest that pre-diabetes is associated with an increased risk of DSPN. In the general population (region of Augsburg, Southern Germany), the prevalence of DSPN was 28.0 % in diabetic subjects, 13.0 % in those with impaired glucose tolerance (IGT), 11.3 % in those with impaired fasting glucose (IFG) and 7.4 % in those with normal glucose tolerance (NGT) (Ziegler et al. 2008). The incidence of DSPN is approximately 2 % per year.

The most important aetiological factors that have been associated with DSPN are poor glycaemic control, visceral obesity, diabetes duration, height, hypertension, age, smoking, hypoinsulinaemia, and dyslipidaemia (Shaw et al. 2003). DSPN is related to both lower-extremity impairments such as diminished position sense and functional limitations such as walking ability (Resnick et al. 2000). Older patients with DSPN perform worse on tests of walking speed, static and dynamic balance, and coordination than those without DSPN (Resnick et al. 2002, Strotmeyer et al. 2008), while the association between reduced peripheral nerve function and poorer lower extremity strength is not explained by diabetes (Strotmeyer et al. 2009). In women above 85 years, diabetes still contributes to large-fibre peripheral nerve dysfunction which is markedly accelerated by age, but no synergistic effect of age and diabetes was observed (Resnick et al. 2001).

There is accumulating evidence suggesting that not only surrogate markers of microangiopathy such as albuminuria but also those used for DSPN such as nerve conduction velocity (NCV) and vibration perception threshold (VPT) predict mortality in diabetic patients (Forsblom et al. 1998, Coppini et al. 2000). Elevated VPT also predicts the development of neuropathic foot ulceration, one of the most common causes for hospital admission and lower limb amputations among diabetic patients (Abbott et al. 1998). Two recent studies underline the major impact of DSPN on morbidity and mortality. In the DIAD study (Young et al. 2009), both sensory deficits and neuropathic pain were independent predictors of cardiac death or nonfatal myocardial infarction. History of neuropathy was the most important predictor for increased mortality in type 2 diabetic subjects allocated to a very intensive diabetes therapy aimed at HbA1c <6.0 % in the ACCORD trial (Calles-Escandón et al. 2010).

Pain is a subjective symptom of major clinical importance as it is often this complaint that motivates patients to seek health care. Pain associated with diabetic neuropathy exerts a sub-

stantial impact on the quality of life, particularly by causing considerable interference in sleep and enjoyment of life (Galer et al. 2000). However, in one UK survey only 65 % of diabetic patients received treatment for their neuropathic pain, although 96 % had reported the pain to their physician (Daousi et al. 2006). While 77 % of the patients reported persistent pain over 5 years, 23 % were pain free over at least 1 year (Daousi et al. 2006). Thus, neuropathic pain persists in the majority of diabetic patients over periods of several years.

Chronic painful DSPN is present in up to 26 % of diabetic patients (Daousi et al. 2006, Davies et al. 2006, Ziegler et al. 2009b, c). In the general population (region of Augsburg, Southern Germany), the prevalence of painful PN was 13.3 % in the diabetic subjects, 8.7 % in those with IGT, 4.2 % in those with IFG, and 1.2 % in those with NGT (Ziegler et al. 2009b). Among survivors of myocardial infarction (MI) from the Augsburg MI Registry, the prevalence of neuropathic pain was 21.0 % in the diabetic subjects, 14.8 % in those with IGT, 5.7 % in those with IFG, and 3.7 % in those with NGT (Ziegler et al. 2009c). Thus, subjects with macrovascular disease appear to be prone to neuropathic pain. The most important risk factors of DSPN and neuropathic pain in these surveys were age, obesity, and low physical activity, while the predominant co-morbidity was peripheral arterial disease, highlighting the paramount role of cardiovascular risk factors and diseases in prevalent DSPN.

Persistent or episodic pain that typically may worsen at night and improve during walking is localized predominantly in the feet. The pain is often described as a deep-seated aching but there may be superimposed lancinating stabs or it may have a burning thermal quality. In a clinical survey including 105 patients with painful DSPN, the following locations of pain were most frequent: 96 % feet, 69 % balls of feet, 67 % toes, 54 % dorsum of foot, 39 % hands, 37 % plantum of foot, 37 % calves, and 32 % heels. The pain most often described by the patients as 'burning/hot', 'electric', 'sharp', 'achy', and 'tingling' was worse at night time and when tired or stressed (Galer et al. 2000). The average pain intensity was moderate, approximately 5.75/10 on a 0–10 scale, with the 'least' and 'most' pain 3.6 and 6.9/10, respectively. Allodynia (pain due to a stimulus which does not normally cause pain, e.g. stroking) may occur. The symptoms may be accompanied by sensory loss, but patients with severe pain may have few clinical signs. Pain may persist over several years (Boulton et al. 1983) causing considerable disability and impaired quality of life in some patients (Galer et al. 2000), whereas it remits partially or completely in others (Young et al. 1988, Benbow et al. 1993), despite further deterioration in small fibre function (Benbow et al. 1993). Pain remission tends to be associated with sudden metabolic change, short duration of pain or diabetes, preceding weight loss, and less severe sensory loss (Young et al. 1988, Benbow et al. 1993).

2. Pathogenetic Mechanisms

Recent experimental studies suggest a multifactorial pathogenesis of diabetic neuropathy (Tomlinson and Gardiner 2008). Most data have been generated in the diabetic rat model on the basis of which two approaches have been chosen to contribute to the clarification of the pathogenesis of diabetic neuropathy. *Firstly*, it has been attempted to characterize the pathophysiological, pathobiochemical, and structural abnormalities that result in experimental diabetic neuropathy. *Secondly*, specific therapeutic interventions have been employed to prevent the development of these alterations, to halt their progression, or to induce their regression despite concomitant hyperglycaemia. At present, the following six pathogenetic mechanisms

are being discussed which, however, in contrast to previous years are no longer regarded as separate hypotheses but in the first place as a complex interplay with multiple interactions between metabolic and vascular factors:

– Increased flux through the polyol pathway that leads to accumulation of sorbitol and fructose, *myo*-inositol depletion, and reduction in Na^+-K^+-ATPase activity.
– Endoneurial microvascular deficits with subsequent ischaemia and hypoxia, generation of reactive oxygen species (oxidative stress), activation of the redox-sensitive transcription factor nuclear factor κB (NF-κB), and increased activity of protein kinase C (PKC) and poly(ADP-ribose) polymerase (PARP).
– Disturbances in n-6 essential fatty acid and prostaglandin metabolism which result in alterations of nerve membrane structure and microvascular and haemorrheologic abnormalities.
– Deficits in neurotrophism leading to reduced expression and depletion of neurotrophic factors such as nerve growth factor (NGF), neurotrophin-3 (NT-3), and insulin-like growth factor (IGF) and alterations in axonal transport.
– Accumulation of non-enzymatic advanced glycation end-products (AGEs) on nerve and/or vessel proteins.
– Immunological processes with increased systemic inflammation and autoantibodies to vagal nerve, sympathetic ganglia, and adrenal medulla as well as inflammatory changes (Tomlinson and Gardiner 2008, Cameron and Cotter 2008).

From the clinical point of view it is important to note that, based on these pathogenetic mechanisms, therapeutic approaches could be derived, some of which have been evaluated in randomized clinical trials (see treatment section).

3. Diagnostic Aspects

Due to the increasing recognition of diabetic neuropathy as a major contributor to morbidity and the recent burst of clinical trials in this field on one hand, but the lack of agreement on the definition and diagnostic assessment of neuropathy on the other hand, several consensus conferences were convened to overcome the current problems, the most recent of which has re-defined the minimal criteria for the diagnosis of typical DSPN (Tesfaye et al. 2010)

– *Possible DSPN*: The presence of symptoms or signs of DSPN may include the following: symptoms-decreased sensation, positive neuropathic sensory symptoms (e.g., "asleep numbness," prickling or stabbing, burning or aching pain) predominantly in the toes, feet, or legs; or signs-symmetric decrease of distal sensation or unequivocally decreased or absent ankle reflexes.
– *Probable DSPN*: The presence of a combination of symptoms and signs of neuropathy include any ≥2 of the following: neuropathic symptoms, decreased distal sensation, or unequivocally decreased or absent ankle reflexes.
– *Confirmed DSPN*: The presence of an abnormality of nerve conduction and a symptom or symptoms or a sign or signs of neuropathy confirm DSPN. If nerve conduction is normal, a validated measure of small fibre neuropathy (SFN) (with class 1 evidence) may be used. To assess for the severity of DSPN, several approaches can be recommended: the graded

approach outlined above, various continuous measures of sum scores of neurologic signs, symptoms or nerve test scores, scores of function of acts of daily living or of predetermined tasks or of disability.

– *Subclinical DSPN*: The presence of no signs or symptoms of neuropathy are confirmed with abnormal nerve conduction or a validated measure of SFN (with class 1 evidence). Definitions 1, 2, or 3 be used for clinical practice and definitions 3 or 4 be used for research studies.
– *Small fibre neuropathy (SFN)*: SFN should be graded as follows: (*a*) possible: the presence of length-dependent symptoms and/or clinical signs of small fibre damage; (*b*) probable: the presence of length-dependent symptoms, clinical signs of small fibre damage, and normal sural nerve conduction; and (*c*) definite: the presence of length-dependent symptoms, clinical signs of small fibre damage, normal sural nerve conduction, and altered intraepidermal nerve fibre density (IENFD) at the ankle and/or abnormal thermal thresholds at the foot (Tesfaye et al. 2010).

The basic neurological assessment comprises the general medical and neurological history, inspection of the feet, and neurological examination of sensation using simple semi-quantitative bed-side instruments such as the 10 g Semmes-Weinstein monofilament, e.g. the Neuropen (Paisley et al. 2002) (touch), NeuroQuick (Ziegler et al. 2005) or Tiptherm (Viswanathan et al. 2002) (temperature), calibrated Rydel-Seiffer tuning fork (vibration), pin-prick (pain), and tendon reflexes (knee and ankle). In addition, assessment of joint-position and motor power may be indicated. The normal range for the tuning fork on the dorsal distal joint of the great toe is ≥5/8 scale units in persons aged 21–40 years, ≥4.5/8 in those aged 41–60 years, ≥4/8 in individuals aged 61–71 years, and ≥3.5/8 scale units in those aged 72–82 years (Martina et al. 1998). An indicator test for the detection of sudomotor dysfunction is the Neuropad which assesses plantar sweat production by means of a colour change from blue to pink. The patch contains the complex salt anhydrous cobalt-II-chloride. In the presence of water, this salt absorbs water molecules, normally changing its colour from blue to pink. If the patch remains completely or partially blue within 10 min, the result is considered abnormal (Papanas et al. 2013).

Clinical assessment should be standardized using validated scores for both the severity of symptoms and the degree of neuropathic deficits such as the Michigan Neuropathy Screening Instrument (MNSI) (Feldman et al. 1994), Neuropathy Symptom Score (NSS) for neuropathic symptoms, and Neuropathy Disability (NDS) for neuropathic deficits (impairments) (Young et al. 1993) which appear to be sufficiently reproducible. The neurological history and examination should be performed once a year. Minimum criteria for the clinical diagnosis of neuropathy according to the NSS and NDS are: (*a*) moderate signs with or without symptoms, (*b*) mild signs with moderate symptoms. However, this means that the exclusive presence of neuropathic symptoms without deficits is not sufficient to diagnose DSPN. Therefore, early stages of DSPN or a painful small fibre neuropathy without or with minimal deficits can only be verified using more sophisticated tests such as thermal thresholds or skin biopsy. Skin biopsy has become a widely used tool to investigate small calibre sensory nerves including somatic unmyelinated intraepidermal nerve fibres (IENF), dermal myelinated nerve fibres, and autonomic nerve fibres in peripheral neuropathies and other conditions (Lauria et al. 2010a, b).

A definition of peripheral neuropathic pain in diabetes, adapted from a definition proposed by the International Association for the Study of Pain (Treede et al. 2008), is "pain

arising as a direct consequence of abnormalities in the peripheral somatosensory system in people with diabetes" (Tesfaye et al. 2010). The intensity (severity) of neuropathic pain and its course should be assessed using an 11-point numerical rating scale (Likert scale) or a visual analogue scale. Various screening tools (with or without limited bed-side testing) such as the PainDetect, LANSS, NPQ, DN-4, ID-Pain have been developed to identify neuropathic pain. These questionnaires use verbal descriptors and pain qualities as a basis for distinguishing neuropathic pain from other types of chronic pain such as nociceptive pain (Bennett et al. 2007).

The following findings should alert the physician to consider causes for DSPN other than diabetes and referral for a detailed neurological work-up: (*a*) pronounced asymmetry of the neurological deficits, (*b*) predominant motor deficits, mononeuropathy, cranial nerve involvement, (*c*) rapid development or progression of the neuropathic impairments, (*d*) progression of the neuropathy despite optimal glycemic control, (*e*) symptoms from the upper limbs, (*f*) family history of non-diabetic neuropathy, and (*g*) diagnosis of DSPN cannot be ascertained by clinical examination.

The most important differential diagnoses from the general medicine perspective include neuropathies caused by alcohol abuse, uraemia, hypothyroidism, vitamin B12 deficiency, peripheral arterial disease, cancer, inflammatory and infectious diseases, and neurotoxic drugs.

4. Microscopy for Morphologic Assessment of Small Fibre Neuropathy

Skin biopsy has become a widely used tool to investigate small calibre sensory nerves including somatic unmyelinated IENF, dermal myelinated nerve fibres, and autonomic nerve fibres in peripheral neuropathies and other conditions. Different techniques for tissue processing and nerve fibre evaluation have been used. For diagnostic purposes in peripheral neuropathies, a recent guideline has recommended a 3-mm punch skin biopsy at the distal leg and quantifying the linear density of IENF in at least three 50-mm thick sections per biopsy, fixed in 2 % PLP or Zamboni's solution, by bright-field immunohistochemistry or immunofluorescence with anti-protein gene product (PGP) 9.5 antibodies. Quantification of IENF density appeared more sensitive than sensory nerve conduction study and sural nerve biopsy in diagnosing SFN. A normative database has recently been published (Lauria et al. 2010a, b).

Corneal confocal microscopy (CCM) is a noninvasive method for the study of human cornea *in vivo*. It has increasingly been used to assess the morphology of the subbasal corneal nerve plexus. CCM has good reproducibility and has been shown to contribute to the early detection of diabetic polyneuropathy (Ziegler et al. 2014). It may also be useful to document favourable changes in nerve fibre structure early after therapeutic intervention. Corneal nerve pathology is more pronounced in patients with diabetic polyneuropathy and is associated with its clinical severity. The sensitivity and specificity of CCM for the diagnosis of polyneuropathy is moderate to high. CCM now merits further use in large longitudinal studies to provide more information on the natural history of diabetic neuropathy and effects of treatment. Moreover, there is a need for a larger normative database. Finally, technical progress is expected to enable visualization of larger corneal areas and improve nerve fibre quantification, increasing diagnostic accuracy (Papanas and Ziegler 2013, Zhivov et al. 2010).

5. Treatment

5.1 Role of Intensive Diabetes Therapy in Treatment and Prevention of Diabetic Neuropathy

Several long-term prospective studies that assessed the effects of intensive diabetes therapy on the prevention and progression of chronic diabetic complications have been published. The large randomized trials such as the Diabetes Control and Complications Trial (DCCT) and UK Prospective Diabetes Study (UKPDS) were not designed to evaluate the effects of intensive diabetes therapy on DSPN, but rather to study the influence of such treatment on the development and progression of the chronic diabetic complications (*The Diabetes Control and Complications Trial Research Group* 1993, *UK Prospective Diabetes Study [UKPDS] Group* 1998). Thus, only a minority of the patients enrolled in these studies had symptomatic DSPN at entry.

Studies in type 1 diabetic patients show that intensive diabetes therapy retards but not completely prevents the development of DSPN. In the DCCT/EDIC cohort, the benefits of former intensive insulin treatment persisted for 13–14 years after DCCT closeout and provide evidence of a durable effect of prior intensive treatment on polyneuropathy and cardiac autonomic neuropathy ("hyperglycaemic memory") (Albers et al. 2010, Pop-Busui et al. 2009), similar to the findings described for diabetic retinopathy and nephropathy.

In contrast, in type 2 diabetic patients, who represent the vast majority of people with diabetes, the results were largely negative. The UKPDS showed a lower rate of impaired VPT (VPT > 25 V) after 15 years for intensive therapy (IT) *versus* conventional therapy (CT) (31 *versus* 52 %). However, the only additional time point at which VPT reached a significant difference between IT and CT was the 9-year follow-up, whereas the rates after 3, 6, and 12 years did not differ between the groups. Likewise, the rates of absent knee and ankle reflexes as well as the heart rate responses to deep breathing did not differ between the groups (*UK Prospective Diabetes Study [UKPDS] Group* 1998). In the ADVANCE study including 11,140 patients with type 2 diabetes randomly assigned to either standard glucose control or intensive glucose control, the relative risk reduction (95 % CI) for new or worsening neuropathy for intensive *versus* standard glucose control after a median of 5 years of follow-up was −4 (−10 to 2), without a significant difference between the groups (*The ADVANCE Collaborative Group* 2008). Likewise, in the VADT study including 1,791 military veterans (mean age 60.4 years) who had a suboptimal response to therapy for type 2 diabetes, after a median follow-up of 5.6 years no differences between the two groups on intensive or standard glucose control were observed for DSPN or microvascular complications (Duckworth et al. 2009). In the ACCORD trial (Ismail-Beigi et al. 2010), intensive therapy aimed at HbA1c <6.0 % was stopped before study end because of higher mortality in that group, and patients were transitioned to standard therapy after 3.7 years on average. At transition, loss of sensation to light touch was significantly improved on intensive *versus* standard diabetes therapy. At study end after 5 years, MNSI score >2, loss of sensation to vibration and light touch were significantly improved on intensive *versus* standard diabetes therapy. However, due to the premature study termination and the aggressive HbA1c goal, the neuropathy outcome in the ACCORD trial is difficult to interpret.

In the Steno 2 Study (Gaede et al. 2008), intensified multifactorial risk intervention including intensive diabetes treatment, angiotensin converting enzyme (ACE)-inhibitors, antioxidants, statins, aspirin, and smoking cessation in patients with microalbuminuria showed no effect on DSPN after 7.8 (range: 6.9–8.8) years and again at 13.3 years, after the patients

were subsequently followed observationally for a mean of 5.5 years, whereas the progression of cardiac autonomic neuropathy (CAN) could be retarded.

However, it has to be acknowledged that, in contrast to the DCCT, the aforementioned trials conducted in type 2 diabetic patients have used only a few clinical endpoints of DSPN or VPT rather than an array of quantitative measures including NCV. Thus, there is no firm evidence that intensive diabetes therapy or a target-driven intensified intervention aimed at multiple risk factors favourably influence the development or progression of DSPN as opposed to CAN in type 2 diabetic patients. However, there is general agreement that not only hyperglycaemia and excessive alcohol consumption but also the traditional cardiovascular risk factors such as visceral obesity, hypertension, hyperlipidaemia and smoking have a role in the development and progression of diabetic neuropathy and hence need to be prevented or treated on an individual basis.

5.2 Treatment Based on Pathogenetic Concepts

Recent experimental studies suggest a multifactorial pathogenesis of diabetic neuropathy. From the clinical point of view it is important to note that, based on the various pathogenetic mechanisms, therapeutic approaches could be derived, some of which have been evaluated in randomized clinical trials including the aldose reductase inhibitors (alrestatin, sorbinil, ponalrestat, tolrestat, epalrestat, zopolrestat, zenarestat, fidarestat, ranirestat), the antioxidant α-lipoic acid (thioctic acid), essential fatty acid (γ-linolenic acid), ACE inhibitors (trandolapril), prostacyclin (PGI_2) analogs (iloprost, beraprost), prostaglandin derivatives (PGE_1.αCD), nerve growth factor (NGF), PKCβ inhibitor (ruboxistaurin), C-peptide, vascular endothelial growth factor (VEGF) (Boulton et al. 2005), benfotiamine (vitamin B1 derivative), and actovegin (Tab. 1). These drugs have been designed to favourably influence the underlying neuropathic process rather than for symptomatic pain treatment. Since, in the foreseeable future, normoglycaemia will not be achievable in the majority of diabetic patients, the advantage of the aforementioned treatment approaches is that they may exert their effects despite prevailing hyperglycaemia. For clinical use only α-lipoic acid, benfotiamine, and actovegin are licensed and used for treatment of symptomatic DSPN in several countries, while epalrestat is marketed in Japan and India.

5.2.1 Benfotiamine

The lipid-soluble thiamine derivative benfotiamine inhibits three of the major biochemical pathways implicated in the pathogenesis of hyperglycaemia-induced vascular damage (hexosamine pathway, AGE formation pathway, and diacylglycerol (DAG)-protein PKC pathway) by activating transketolase in retinas of diabetic animals and also prevents experimental diabetic retinopathy (Hammes et al. 2003). Plasma thiamine concentration is decreased by 76 % in type 1 and 75 % in type 2 diabetic patients, associated with 24-fold (type 1) and 16-fold (type 2) increased thiamine clearance (Thornalley et al. 2007). In the BENDIP study neuropathic symptoms tended to improve after 6 weeks of treatment with benfotiamine 300 mg bid (Stracke et al. 2008). Further longer-term studies are required to confirm this finding.

5.2.2 α-Lipoic Acid

According to a meta-analysis comprising 1258 patients, infusions of α-lipoic acid (600 mg i.v./day) ameliorated neuropathic symptoms and deficits after 3 weeks (Ziegler et al.

Tab. 1 Treatment of diabetic neuropathy based on the putative pathogenetic mechanisms

Abnormality	**Compound**	**Mechanistic aim of treatment**	**Status of RCTs**
Polyol pathway ↑	Aldose reductase inhibitors	Nerve sorbitol ↓	
	Sorbinil		Withdrawn (AE)
	Tolrestat		Withdrawn (AE)
	Ponalrestat		Ineffective
	Zopolrestat		Withdrawn (marginal effects)
	Zenarestat		Withdrawn (AE)
	Lidorestat		Withdrawn (AE)
	Fidarestat		Effective in phase II trials, withdrawn
	Ranirestat		Phase III trials ongoing
	Epalrestat		Marketed in Japan and India
***myo*-Inositol ↑**	*Myo*-Inositol	Nerve *myo*-inositol ↑	Equivocal
GLA synthesis ↓	γ-Linolenic acid (GLA)	EFA metabolism ↑	Withdrawn (effective: deficits)
Oxidative stress ↑	α-Lipoic acid	Free radicals ↓	Effective in RCTs
	Vitamin E	Free radicals ↓	Effective in 1 small RCT
Nerve hypoxia ↑	Vasodilators	NBF ↑	
	ACE inhibitors		Effective in phase II trial
	Prostaglandin analogs		Effective in phase II trial
	$PhVEGF_{165}$ gene transfer	Angiogenesis ↑, NBF ↑	Effective in phase II trial
Protein kinase C ↑	PKC β inhibitor (ruboxistaurin)	NBF ↑	Ineffective
Poly(ADP-ribose) ↑	Actovegin	Poly(ADP-ribose) ↓, apoptosis ↓	Effective in 1 large RCT
C-peptide ↓	C-peptide	NBF ↑	Effective in phase II trials
Neurotrophism ↓	Nerve growth factor (NGF)	Nerve regeneration, growth ↑	Ineffective
	BDNF	Nerve regeneration, growth ↑	Ineffective
LCFA metabolism ↓	Acetyl-L-carnitine	LCFA accumulation ↓	Ineffective
NEG ↑	Aminoguanidine	AGE accumulation ↓	Withdrawn

NEG: non-enzymatic glycation; AGE: advanced glycation end products; EFA: essential fatty acids; LCFA: long-chain fatty acids; AE: adverse events; NBF: nerve blood flow; RCTs: randomized clinical trials; BDNF: brain-derived neurotrophic factor.

2004). Moreover, the SYDNEY 2 Trial suggests that treatment for 5 weeks using 600 mg of α-lipoic acid orally q. d. reduces the chief symptoms of DSPN including pain, paresthesias, and numbness to a clinically meaningful degree (Ziegler et al. 2006). In a multicentre, randomized, double-masked, parallel-group clinical trial (NATHAN 1) including 460 diabetic patients with stage 1 or stage 2a DSPN were randomly assigned to oral treatment with

α-lipoic acid 600 mg q. d. (n = 233) or placebo (n = 227) for 4 years. After 4 years, Neuropathy Impairment Score (NIS), but not NCV, was improved, and the drug was well tolerated throughout the trial (Ziegler et al. 2007a). A response analysis of clinically meaningful improvement and progression in the NIS and NIS of the lower limbs (NIS-LL) by at least 2 points showed that the rates of clinical responders were significantly higher and those of progressors were lower with ALA *versus* placebo for NIS (p = 0.013) and NIS-LL (p = 0.025), respectively. Clinical and post-marketing surveillance studies have revealed a highly favourable safety profile (Ziegler 2004).

5.2.3 Actovegin

Actovegin is a deproteinized haemoderivative produced from calf blood by ultrafiltration that contains low-molecular weight compounds of up to 5000 Da. A recent *in vitro* study using freshly prepared primary rat neurons showed that actovegin increases the number of neuronal cells, the total number of synaptic connections of neurons, inhibits apoptosis as measured by caspase-3 activity and reduces oxidative stress in neurons, as measured by the concentration of reactive oxygen species (Elmlinger et al., unpublished observations). In STZ-diabetic rats, reduced SNCV and IENFD were significantly ameliorated by actovegin treatment. Moreover, actovegin markedly decreased apoptotic cell death in sciatic nerves from STZ-diabetic rats as assessed by poly(ADP-ribose) content (Dieckmann, unpublished observations). Thus, actovegin improves experimental diabetic neuropathy possibly via anti-apoptotic mechanisms including poly(ADP-ribose), providing a rationale for treatment of human disease.

The efficacy and safety of actovegin in patients with diabetic polyneuropathy was recently evaluated in a multicentre, randomized, double-blind trial including 567 patients with type 2 diabetes who received 20 intravenous infusions of actovegin (2000 mg/day) or placebo once daily followed by three tablets of actovegin (1800 mg/day) or placebo three times daily for 140 days. Total symptom score (TSS) of the lower limbs and vibration perception threshold (VPT) measuring five sites per foot were used as co-primary outcome measures comparing the averaged values of each group over the trial period. In the primary analysis, TSS was significantly lower during actovegin treatment compared with placebo (p = 0.0003), while the improvement in VPT compared to baseline was significantly better with actovegin than with placebo (P = 0.017), but did not reach statistical significance when computed as averaged values (P = 0.084). Among the secondary endpoints, NIS-LL sensory function was significantly improved with actovegin *versus* placebo as was quality of life assessed by the SF-36 mental health domain. There were no differences in the incidence of adverse events between the groups (Ziegler et al. 2009a) confirming a favourable safety profile of this drug.

5.3 Pharmacological Treatment of Painful Neuropathy

Painful symptoms in DSPN may constitute a considerable management problem. The efficacy of a single therapeutic agent is not the rule, and simple analgesics are usually inadequate to control the pain. There is agreement that patients should be offered the available therapies in a stepwise fashion (Finnerup et al. 2005, 2010, Dworkin et al. 2007, 2010). Effective pain treatment considers a favourable balance between pain relief and side effects without implying a maximum effect. The following general considerations in the pharmacotherapy of neuropathic pain require attention:

– The appropriate and effective drug has to be tried and identified in each patient by carefully titrating the dose based on efficacy and side effects.
– Lack of efficacy should be judged only after 2–4 weeks of treatment using an adequate dose.
– Because the evidence from clinical trials suggests only a maximum response of ≈ 50 % for any monotherapy, analgesic combinations may be useful.
– Potential drug interactions have to be considered given the frequent use of polypharmacy in diabetic patients.

A stepwise rational treatment algorithm is summarized in Table 2. The advantages and disadvantages of the various drugs and drug classes used for treatment of painful diabetic neuropathy under consideration of the various comorbidities and complications associated with diabetes are summarized in Table 3. Prior to any decision regarding the appropriate treatment, the diagnosis of the underlying neuropathic manifestation allowing to estimate its natural history should be established. In contrast to the agents that have been derived from the pathoge-

Tab. 2 Treatment options for painful diabetic neuropathy

Approach	Compound/measure	Dose per day	Remarks	NNT
Optimal diabetes control	Lifestyle modification OAD, insulin	Individual adaptation	Aim: $HbA_{1c} \leq 6.5\,\%$	–
Pathogenetically oriented treatment	α-Lipoic acid (thioctic acid)§	600 mg i.v. infusion 1200–1800 mg orally	Duration: 3 wk Favourable safety profile	6.3* 2.8–4.2*
Symptomatic treatment	*Tricyclic antidepressants* (*TCA*)			
	Amitriptyline	(10–)25–150 mg	NNMH: 15	2.1
	Desipramine	(10–)25–150 mg	NNMH: 24	2.2/3.2
	Imipramine	(10–)25–150 mg	CRR	1.3/2.4/3.0
	Clomipramine	(10–)25–150 mg	NNMH: 8.7	2.1
	Nortriptyline	(10–)25–150 mg	plus Fluphenazine	1.2**
	SNRI			
	Duloxetine+	60–120 mg	NNT 120 mg, 60 mg	5.3, 4.9
	Anticonvulsants			
	Pregabalin+	300–600 mg	NNT 600 mg, 300 mg	5.9, 4.2
	Gabapentin	900–3600 mg	Unpublished trials	6.4
	Weak opioids			
	Tramadol	50–400 mg	NNMH: 7.8	3.1/4.3
	Local treatment			
	Capsaicin (0,025 %) cream	q.i.d. topically	Max. duration: 6–8 wk	5.7
Pain resistant to standard pharmacotherapy	*Strong opioids*			
	Oxycodone		Add-on treatment	
	Electrical spinal cord stimulation (ESCS)		Invasive, specialist required	2.6

§Available only in some countries; + licensed in US and EU; ns: not significant; *≥ 50 % symptom relief after 3 and 5 weeks; **combined with fluphenazine; OAD: oral antidiabetic drugs; CRR: concentration-response relationship; NNMH: number needed for major harm; SNRI: selective serotonin norepinephrine reuptake inhibitors; NNT: number needed to treat.

netic mechanisms of diabetic neuropathy, those used for symptomatic therapy were designed to modulate the pain, without favourably influencing the underlying neuropathy.

Tab. 3 Differential treatment of painful neuropathy considering frequent co-morbidities and side-effects.

	Duloxetine	Pregabalin	Tricyclics	Opioids	α-Lipoic acid
Depression	+*	n*	+	n	n
Obesity	n	–	–	n	n
Generalized anxiety disorder (GAD)	+	+	na	na	na
Sleep disturbances	+	+	+	+	na
Coronary heart disease	n	n	–	n	n
Autonomic neuropathy	na	na	–	–	+
Fasting glucose	(–)	n	–	n	n*
Hepatic failure	–	n	§	§	n
Renal failure	–	adapt dose	§	§	n
Drug interactions	–	n	–	n	n

Effect: + = favourable; – = unfavourable; n = neutral; na = not available
* Slight decrease possible; § dependent on individual agent

The relative benefit of an active treatment over a control in clinical trials is usually expressed as the relative risk, the relative risk reduction, or the odds ratio. However, to estimate the extent of a therapeutic effect (i.e. pain relief) that can be translated into clinical practice, it is useful to apply a simple measure that serves the physician to select the appropriate treatment for the individual patient. Such a practical measure is the "number needed to treat" (NNT), i.e. the number of patients that need to be treated with a particular therapy to observe a clinically relevant effect or adverse event in one patient. This measure is expressed as the reciprocal of the absolute risk reduction, i.e. the difference between the proportion of events in the control group (Pc) and the proportion of events in the intervention group (Pi): NNT = $1/(Pc–Pi)$. The 95 % confidence interval (CI) of NNT can be obtained from the reciprocal value of the 95 % CI for the absolute risk reduction. The NNT and NNH (number needed to harm) for the individual agents used in the treatment of painful diabetic neuropathy are given in Table 2. Usually, drugs with NNTs exceeding 6 for ≥50 % pain relief are regarded as showing limited efficacy. However, some authors have cautioned that summary NNT estimates may have limited clinical relevance, due to problems of heterogeneity (Edelsberg and Oster 2009).

5.3.1 Tricyclic Antidepressants

Psychotropic agents, among which tricyclic antidepressants (TCAs) have been evaluated most extensively, constitute an important component in the treatment of chronic pain syndromes since more than 30 years. Putative mechanisms of pain relief by antidepressants include the

inhibition of norepinephrine and/or serotonin reuptake at synapses of central descending pain control systems and the antagonism of N-methyl-D-aspartate receptor that mediate hyperalgesia and allodynia. Imipramine, amitriptyline, and clomipramine induce a balanced reuptake inhibition of both norepinephrine and serotonin, while desipramine is a relatively selective norepinephrine inhibitor. The NNT (CI) for a ≥50 % pain relief by TCAs in painful neuropathies is 2.1 (1.9–2.6). The number needed to harm (NNH) in patients with neuropathic pain for one drop out of the study due to adverse events is 16 (11–26) (Finnerup et al. 2010).

The most frequent adverse events of TCAs include tiredness and dry mouth. The starting dose should be 25 mg (10 mg in frail patients) and taken as a single night time dose one hour before sleep. It should be increased by 25 mg at weekly intervals until pain relief is achieved or adverse events occur. The maximum dose is usually 150 mg per day.

TCAs should be used with caution in patients with orthostatic hypotension and are contraindicated in patients with unstable angina, recent (< 6 months) myocardial infarction, heart failure, history of ventricular arrhythmias, significant conduction system disease, and long QT syndrome. Their use is limited by relative high rates of adverse events and several contraindications. Thus, there is a continuing need for agents that exert efficacy equal to or better than that achieved with TCAs but have a more favourable side effect profile.

5.3.1.1 Selective Serotonin Reuptake Inhibitors (SSRI)

Because of the relative high rates of adverse effects and several contraindications of TCA, it has been reasoned whether patients who do not tolerate them due to adverse events could alternatively be treated with selective serotonin reuptake inhibitors (SSRI). SSRI specifically inhibit pre-synaptic reuptake of serotonin, but not norepinephrine, and unlike the tricyclics they lack the postsynaptic receptor blocking effects and quinidine-like membrane stabilization. However, only weak effects on neuropathic pain were observed after treatment with fluoxetine, paroxetine, citalopram, and escitalopram. The NNT (CI) for a ≥50 % pain relief by SSRI in painful neuropathies is 6.8 (3.9–27) (Finnerup et al. 2010). Because of these limited efficacy data, SSRI have not been licensed for the treatment of neuropathic pain.

5.3.1.2 Serotonin Noradrenaline Reuptake Inhibitors (SNRI)

Because SSRI have been found to be less effective than TCAs, recent interest has focused on antidepressants with dual selective inhibition of serotonin and noradrenaline such as duloxetine and venlafaxine. The efficacy and safety of duloxetine was evaluated in 3 controlled studies using a dose of 60 and 120 mg/Tag over 12 weeks (Kajdasz et al. 2007). In all three studies, the average 24 hour pain intensity was significantly reduced with both doses as compared to placebo treatment, the difference between active and placebo being achieving statistical significance after 1 week. The response rates defined as ≥50 % pain reduction were 48.2 % (120 mg/day), 47.2 % (60 mg/day) und 27.9 % (Placebo), giving a NNT of 4.9 (95 % CI: 3.6–7.6) for 120 mg/day and 5.3 (3.8–8.3) for 60 mg/day. Pain severity, but not variables related to diabetes or neuropathy, predicts the effects of duloxetine in diabetic peripheral neuropathic pain. Patients with higher pain intensity tend to respond better than those with lower pain levels (Ziegler et al. 2007). The most frequent side effects of duloxetine (60/120 mg/day) include nausea (16.7/27.4 %), somnolence (20.2/28.3 %), dizziness (9.6/23 %), constipation 14.9/10.6 %), dry mouth (7.1/15 %), and reduced appetite (2.6/12.4 %). These adverse

events are usually mild to moderate and transient. To minimize them the starting dose should be 30 mg/day for 5–7 days. In contrast to TCAs and some anticonvulsants duloxetine does not cause weight gain (Gaynor et al. 2011), but a small increase in fasting blood glucose may occur (Hardy et al. 2007).

In a 6-week trial comprising 244 patients the analgesic response rates were 56 %, 39 %, and 34 % in patients given 150–225 mg venlafaxine, 75 mg venlafaxine, and placebo, respectively. Because patients with depression were excluded, the effect of venlafaxine (150–225 mg) was attributed to an analgesic, rather than antidepressant, effect. The most common adverse events were tiredness and nausea (Rowbotham et al. 2004). Duloxetine, but not venlafaxine, has been licensed for the treatment of painful diabetic neuropathy.

5.3.2 Anticonvulsants

5.3.2.1 Calcium Channel Modulators (α2-δ Ligands)

Gabapentin is an anticonvulsant structurally related to γ-aminobutyric acid (GABA), a neurotransmitter that plays a role in pain transmission and modulation. The exact mechanisms of action of this drug in neuropathic pain are not fully elucidated. Among others, they involve an interaction with the system L-amino acid transporter and high affinity binding to the α2-δ subunit of voltage-activated calcium channels. In an 8-week multicentre dose-escalation trial including 165 diabetic patients with painful neuropathy 60 % of the patients on gabapentin (3600 mg/day achieved in 67 %) had at least moderate pain relief compared to 33 % on placebo. Dizziness and somnolence were the most frequent adverse events in about 23 % of the patients each (Backonja et al. 1998). The NNT (CI) for a ≥50 % pain relief by gabapentin in painful neuropathies is 6.4 (4.3–12) (Finnerup et al. 2010). Due to this relatively high NNT and publication bias towards unpublished negative trials (Landefeld and Steinman 2009), the overall level of evidence in favour of gabapentin in painful DSPN is modest.

Pregabalin is a more specific α2-δ ligand with a 6-fold higher binding affinity than gabapentin. The efficacy and safety of pregabalin was reported in a pooled analysis of 6 studies over 5–11 weeks in 1346 diabetic patients with painful neuropathy. The response rates defined as ≥50 % pain reduction were 46 % (600 mg/day), 39 % (300 mg/day), 27 % (150 mg/day) and 22 % (Placebo), giving a NNT of 4.2, 5.9, and 20.0 %. The most frequent side effects for 150–600 mg/day are dizziness (22.0 %), somnolence (12.1 %), peripheral oedema (10.0 %), headache (7.2 %), and weight gain (5.4 %) (Freeman et al. 2008). The evidence supporting a favourable effect in painful diabetic neuropathy is more solid and dose titration is considerably easier with pregabalin than with gabapentin.

5.3.2.2 Sodium Channel Blockers

Although carbamazepine has been widely used for treating neuropathic pain, it cannot be recommended in painful diabetic neuropathy due to limited data from two very small studies (Rull et al. 1969, Wilton 1974). Its successor drug, oxcarbazepine (Beydoun et al. 2006, Grosskopf et al. 2006), as well as other sodium channel blockers such as valproate (Kochar et al. 2004), mexiletine (Jarvis et al. 1998), topiramate (Thienel et al. 2004), and lamotrigine (Vinik et al. 2007), showed only marginal efficacy and have not been licensed for the treatment of painful diabetic neuropathy. The NeuPSIG guidelines recommended that these

third-line medications (e.g., carbamazepine, lamotrigine, oxcarbazepine, topiramate, and valproic acid) should generally be reserved for patients with neuropathic pain who cannot tolerate or who do not respond adequately to first- and second-line medications (Dworkin et al. 2010). Accordingly, the EFNS guidelines have assigned carbamazepine a Level C rating (established as possibly useful/predictive or not useful/predictive) (Attal et al. 2010).

5.3.2.3 Lacosamide

Lacosamide is a novel anticonvulsant which selectively enhances the slow inactivation of voltage-dependent sodium channels but, in contrast to the aforementioned sodium channel blockers, does not influence the fast sodium channel inactivation. Its second putative mechanism is an interaction with a neuronal cytosolic protein, the collapsin response mediator protein 2 (CRMP-2) which plays an important role in nerve sprouting and excitotoxicity. Lacosamide has been evaluated in several studies in painful diabetic neuropathy (Ziegler et al. 2010). However, the drug was not approved by the FDA and EMEA for painful diabetic neuropathy in 2008, but further clinical trials may follow in the future.

5.3.3 Local Treatments

Capsaicin (trans-8-methyl-*N*-vanillyl-6-nonenamide) is an alkaloid and the most pungent ingredient in the red pepper. It depletes tissues of substance P and reduces neurogenic plasma extravasation, the flare response and chemically induced pain. Substance P is present in afferent neurones innervating skin, mainly in polymodal nociceptors, and is considered the primary neurotransmitter of painful stimuli from the periphery to the central nervous system. Several studies have demonstrated significant pain reduction and improvement in quality of life in diabetic patients with painful neuropathy after 8 weeks of treatment with capsaicin cream 0.075 % (Mason et al. 2004). It has been criticized that a double-blind design is not feasible for topical capsaicin due to the transient local hyperalgesia (usually mild burning sensation >50 % of the cases), it may produce as a typical adverse event. Treatment should be restricted to a maximum of 8 weeks, as during this period no adverse effects on sensory function (due to the mechanism of action) were noted in diabetic patients. The 8 % capsaicin patch (Qutenza) which is effective in postherpetic neuralgia (Backonja et al. 2008), is contraindicated in painful diabetic neuropathy due to desensitization of nociceptive sensory nerve endings which may theoretically increase the risk of diabetic foot ulcers.

The topical lidocaine patch 5 % (Lidoderm), a targeted peripheral analgesic was associated with relief of pain and tactile allodynia with a minimal risk of systemic adverse effects or drug-drug interactions in patients with post-herpetic neuralgia (PHN) (Davies and Galer 2004). Controlled clinical trials in patients with DSPN are underway.

Recent experimental evidence suggests that botulinum toxin type A may not only inhibit the release of acetylcholine at the neuromuscular junctions, but also modulate afferent sensory fibre firing, thereby relieving neuropathic pain. In a pilot study 12 one-time intradermal injections of botulinum toxin type A significantly reduced diabetic neuropathic pain over 4–12 weeks and transiently improved sleep quality (Yuan et al. 2009). Large-scaled studies are required to confirm this finding.

5.3.4 Opioids

Tramadol acts directly via opioid receptors and indirectly via monoaminergic receptor systems. Because the development of tolerance and dependence during long-term tramadol treatment is relatively uncommon, and its abuse liability appears to be low, it is an alternative to strong opioids in neuropathic pain (Harati et al. 1998). One conceivable mechanism for the favourable effect of tramadol could be a hyperpolarization of postsynaptic neurons via postsynaptic opioid receptors. Alternatively, the reduction in central hyperexcitability by tramadol could be due to a monoaminergic or a combined opioid and monoaminergic effect.

Most severe pain requires administration of strong opioids such as oxycodone. Although there is little data available on combination treatment, combinations of different substance classes have to be used in patients with pain resistant to monotherapy. Several add-on trials have demonstrated significant pain relief and improvement in quality of life following treatment with controlled-release oxycodone, a pure μ-agonist in patients with painful DSPN whose pain was not adequately controlled on standard treatment with antidepressants and anticonvulsants (Watson et al. 2003, Gilron et al. 2005). As expected, adverse events were frequent and typical of opioid-related side effects. A cross-over study examined the maximum tolerable dose of a combination treatment of gabapentin and morphine as compared to monotherapy of each drug. The maximum tolerable dose was significantly lower and efficacy was better during combination therapy than with monotherapy, suggesting an additive interaction between the two drugs (Gilron et al. 2005). The results of these studies suggest that opioids should be included among the therapeutic options for painful DSPN, provided that careful selection of patients unresponsive to standard treatments, regular monitoring, appropriate dose titration, and management of possible opioid-specific problems (analgesic misuse or addiction, tolerance, opioid-induced hyperalgesia) are ensured. Recent recommendations have emphasized the need for clinical skills in risk assessment and management as a prerequisite to safe and effective opioid prescribing (Dworkin et al. 2010). Treatment of painful DSPN with opioid agonists should generally be reserved for patients who have failed to respond to or cannot tolerate the first-line medications.

Tapentadol is a novel centrally active analgesic with a dual mode of action: μ-opioid receptor agonist and norepinephrine-reuptake inhibitor. A recent phase III, randomized-withdrawal, placebo-controlled trial evaluated the safety and efficacy of tapentadol extended release (ER) in painful diabetic DSPN. Patients with at least a ≥1-point reduction in pain intensity at the end of a 3-week open-label titration phase were randomized to receive placebo or the optimal fixed dose over 12 weeks. Compared with placebo, tapentadol ER 100–250 mg bid was associated with a statistically significant difference in the maintenance of the initial improvement and was well-tolerated (Schwartz et al. 2011). Further studies to confirm this finding are underway.

The response rates to analgesic monotherapy in painful diabetic DSPN are only around 50 %. Therefore, combination pharmacotherapy is required in patients who have only partial response or in whom the drug cannot be further titrated due to intolerable side effects. A recent trial showed that the combination of nortriptyline and gabapentin at the maximum tolerated dose was more effective than either monotherapy despite a lower maximum tolerable dose as compared with monotherapy (Gilron et al. 2009). Appropriate analgesic combinations include antidepressants with anticonvulsants or each of these with opioids. Some patients may even require a triple combination of these drug classes.

In summary, although several novel analgesic drugs have recently been introduced into clinical practice, the pharmacologic treatment of chronic painful diabetic neuropathy remains a challenge for the physician. Individual tolerability remains a major aspect in any treatment

decision. Little information is available from controlled trials on long-term analgesic efficacy, head-to-head comparisons of individual analgesics, and only a few studies have used drug combinations. Combination drug use or the addition of a new drug to a therapeutic regimen may lead to increased efficacy.

5.4 Non-pharmacological Treatment of Painful Neuropathy

Because there is no entirely satisfactory pharmacotherapy of painful diabetic neuropathy, non-pharmacological treatment options should always be considered. As for the pharmacological treatment, considerable efforts must also be made to develop effective non-pharmacological approaches. A recent systematic review assessed the evidence from rigorous clinical trials and meta-analyses of complementary and alternative therapies for treating neuropathic and neuralgic pain. Data on the following complementary and alternative medicine treatments were identified: acupuncture, electrostimulation, herbal medicine, magnets, dietary supplements, imagery, and spiritual healing. The conclusion was that the evidence is not fully convincing for most complementary and alternative medicine modalities in relieving neuropathic or neuralgic pain. The evidence can be classified as encouraging and warrants further study for cannabis extract, magnets, carnitine, and electrostimulation (Pittler and Ernst 2008).

5.4.1 Psychological Support

A psychological component to pain should not be underestimated. Hence, an explanation to the patient that even severe pain may remit, particularly in poorly controlled patients with acute painful neuropathy or in those painful symptoms precipitated by intensive insulin treatment. Thus, the emphatic approach addressing the concerns and anxieties of patients with neuropathic pain is essential for their successful management (Tesfaye 1998).

5.4.2 Physical Measures

The temperature of the painful neuropathic foot may be increased due to arterio-venous shunting. Cold water immersion may reduce shunt flow and relieve pain. Allodynia may be relieved by wearing silk pyjamas or the use of a bed cradle. Patients who describe painful symptoms on walking likened to walking on pebbles may benefit from the use of comfortable footwear (Tesfaye 1998).

Older type 2 diabetic patients often exhibit greater impairments in posture and gait and are typically at increased risk of falling. Following a 6-week balance training programme, significant improvements in leg strength, faster reaction times, decreased sway, and, consequently, reduced falls risk were observed in patients with type 2 diabetes aged 50–75 years (Morrison et al. 2010).

5.4.3 Acupuncture

In a 10-week uncontrolled study in diabetic patients on standard pain therapy, 77 % showed significant pain relief after up to 6 courses of traditional Chinese acupuncture without any side effects. During a follow-up period of 18–52 weeks, 67 % were able to stop or significantly reduce their medications and only 24 % required further acupuncture treatment (Abuaisha et al. 1998). Controlled studies using placebo needles should be performed to confirm these findings.

5.4.4 Electrical Stimulation

Transcutaneous electrical nerve stimulation (TENS) influences neuronal afferent transmission and conduction velocity, increases the nociceptive flexion reflex threshold, and changes the somatosensory evoked potentials. In a 4-week study of TENS applied to the lower limbs, each for 30 min daily, pain relief was noted in 83 % of the patients compared to 38 % of a sham-treated group. In patients who only marginally responded to amitriptyline, pain reduction was significantly greater following TENS given for 12 weeks as compared with sham treatment. Thus, TENS may be used as an adjunctive modality combined with pharmacotherapy to augment pain relief (Kumar et al. 1998).

One randomized controlled study showed a better effect of high-tone external muscle stimulation (HTEMS) than TENS on neuropathic symptoms after 1 week (Reichstein et al. 2005). A 3-month cross-over trial in diabetic patients with symptomatic DSPN is currently underway. Frequency-modulated electromagnetic nerve stimulation (FREMS) resulted in a transient reduction in neuropathic pain, without an effect on NCV in symptomatic diabetic neuropathy (Bosi et al. 2013).

In diabetic painful neuropathy that was unresponsive to drug treatment, electrical spinal cord stimulation (ESCS) with electrodes implanted between T9 and T11 resulted in a pain relief >50 % in 8 out of 10 patients. In addition, exercise tolerance was significantly improved. Complications of ESCS included superficial wound infection in 2 patients, lead migration requiring reinsertion in 2 patients, and "late failure" after 4 months in a patient who had initial pain relief (Tesfaye et al. 1996). This invasive treatment option should be reserved for patients who do not respond to drug treatment.

5.4.5 Surgical Decompression

Surgical decompression at the site of anatomic narrowing has been promoted as an alternative treatment for patients with symptomatic DSPN. It has been suggested that peripheral nerve decompression is reserved for the patient with neuropathy who has a demonstrable compression of a peripheral nerve in a known site of anatomic narrowing (Dellon 2008). However, none of the studies identified by a recent Cochrane review were randomized and none reported the criteria used for diagnosis of DSPN. Except for the presence of Tinel's sign, there was no other direct clinical or nerve conduction data given to identify the presence of compressive neuropathies (Chaudhry et al. 2008). Given the current evidence available, this treatment alternative should be considered unproven (Level U). Prospective randomized controlled trials with standard definitions and outcome measures are necessary to determine the value of this therapeutic intervention (Chaudhry et al. 2008, *Therapeutics and Technology Assessment Subcommittee of the American Academy of Neurology et al.* 2006).

6. Conflict of Interest

The author has received honoraria for speaking and consulting activities from Lilly, Pfizer, Daiichi-Sankyo, Merck Serono, Meda, Takeda, Eisai, Wörwag, Berlin-Chemie, Glenmark, Trigocare.

7. Disclosure

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Early Detection of Glial Dysfunction in Diabetic Retinopathy

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With 6 Figures

Abstract

Diabetic retinopathy is characterized by vascular abnormalities, neuronal dysfunction, and glial reactivity. Reactive alterations in retinal glia occur early in diabetic retinopathy and are a consequence of, and a contributor to, vascular abnormalities. Gliosis results in a dysregulation of various neuron-supportive functions of Müller glial cells including the maintenance of the retinal potassium and water homeostasis, the glial uptake and metabolism of glutamate, and the production of antioxidants. A better understanding of the gliotic mechanisms in the diabetic retina will help to develop efficient therapeutic strategies which increase the beneficial, and decrease the destructive, effects of reactive gliosis.

Zusammenfassung

Reaktive Veränderungen der retinalen Glia sind ein frühes Kennzeichen der diabetischen Retinopathie. Gliazellen reagieren auf die vaskulären Veränderungen und tragen zu den vaskulären und neuronalen Veränderungen bei. Die Gliose der Müllerzellen führt zu einer Dysregulation der retinalen Kalium-, Wasser- und Glutamathomöostase und zu einem Verlust des Schutzes gegen oxidativen Stress. Diese Veränderungen bewirken eine neuronale Dysfunktion und die Entwicklung eines Ödems. Ein besseres Verständnis der Gliosemechanismen ermöglicht die Entwicklung neuer therapeutischer Strategien zur Neuroprotektion in der diabetischen Netzhaut.

1. Introduction

Diabetic retinopathy is the leading cause of visual disability and acquired blindness among working-age adults in the industrialized world. Although diabetic retinopathy is primarily a microangiopathy, reactive changes in retinal neurons and glia occur early in the course of the disease and precede the onset of clinically evident vascular injury. Loss of colour and contrast sensitivity, abnormalities in the electroretinogram, and degeneration of retinal neurons and photoreceptors were observed in patients before vascular changes became obvious (Fletcher et al. 2007). Early neuronal and glial alterations, including decreases of electroretinogram components, increased apoptosis rates of retinal neurons, and reactivity of retinal glia, are also observed in experimental diabetic retinopathy (Fletcher et al. 2007). Apparently, diabetic retinopathy is a multifactorial disease that involves vasculature, neurons, and glia. Glial activation and dysfunction is a consequence of and a contributor to vascular abnormalities and neuronal dysfunction.

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The human retina contains three types of glial cells: microglial cells and two types of neuron-supporting macroglial cells, astrocytes and Müller cells (Bringmann et al. 2006). Microglial cells are blood-derived resident immune cells and play important roles in the host defense against invading microorganisms and the initiation of inflammatory processes. Astrocytes are localized to the nerve fibre and ganglion cell layers (Fig. 1) where their processes wrap around blood vessels. Müller cells are specialized radial glial cells which span the entire thickness of the neural retina (Fig. 1).

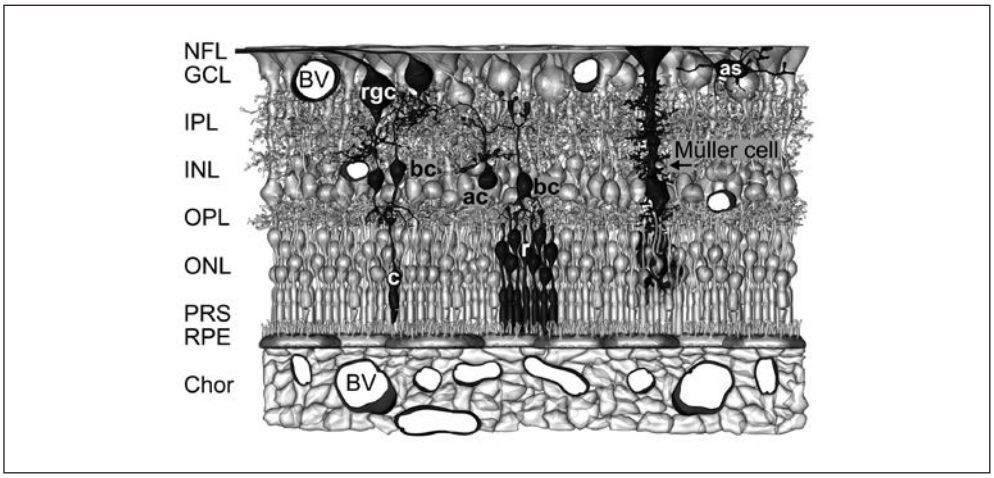

Fig. 1 Müller cells span the entire thickness of the neuroretina. Schematic drawing of the cellular constituents and basic neuronal circuits of a human retina. Astrocytes (as) are localized in the nerve fibre (NFL) and ganglion cell layers (GCL). The perikarya of Müller cells are localized in the inner nuclear layer (INL). From the perikaryon, two stem processes of Müller cells extend towards the inner (*top*) and outer (*bottom*) surfaces of the neuroretina. The funnel-shaped endfeet of Müller cells form (in association with a basement membrane) the inner surface of the retina. In the outer (OPL) and inner plexiform layers (IPL), side branches which form perisynaptic membrane sheaths originate at the stem processes. In the outer nuclear layer (ONL), the stem process of Müller cells forms membraneous sheaths which envelop the perikarya of rods (r) and cones (c). Microvilli of Müller cells extent into the subretinal space which surround the photoreceptor segments (PRS). The neural retina contains three interconnected plexuses of blood vessels (BV). The superficial vascular plexus is located in the NFL/GCL. The inner plexus and the deep plexus are located at the inner and outer borders of the INL, near the plexiform layers. Müller cells and astrocytes ensheath the superficial vessels while the deeper vessels are surrounded by Müller cells. ac, amacrine cell; bc, bipolar cell; Chor, choroid; rgc, retinal ganglion cell; RPE, retinal pigment epithelium.

They constitute an anatomical and functional link between retinal neurons and the vitreous and blood vessels. Most nutrients, waste products, ions, and other molecules are transported through Müller cells between the inner retinal blood vessels and the neurons. Müller cells support the the survival, functioning, and metabolism of photoreceptors and neurons, are responsible for the structural stabilization of the retina, and are active players in normal retinal function and retinal degeneration (Bringmann et al. 2006, 2009a). They are involved in retinal glucose metabolism, provide trophic substances to neurons, remove metabolic waste, mediate the retinal potassium, water, and acid-base homeostasis, and regulate the retinal blood flow and the barrier properties of vascular endothelia (Bringmann et al. 2006). Müller cells act as living optical fibres which guide light towards the photoreceptors (Franze et al. 2007,

Agte et al. 2011). Their processes function as soft, compliant embedding for neurons which supports synaptic plasticity (Lu et al. 2006). Müller cells support the neuronal activity by the supply of neurons with precursors of neurotransmitters, and are more directly involved in the regulation of synaptic activity by the rapid uptake of neurotransmitters, which terminates the postsynaptic glutamate action in inner retinal neurons (Matsui et al. 1999), and by the release of gliotransmitters (Newman 2004, Bringmann et al. 2009b).

2. The Janus Face of Gliosis

Müller cells are targets of, and players in, diabetic changes in the retina. Reactive Müller cells are neuroprotective but may also quit supporting the neurons, and rather contribute to neuronal degeneration (Bringmann et al. 2009a). Early after injury, gliosis is thought to be neuroprotective, as a cellular attempt to limit the extent of injury, e.g., by the release of neurotrophic factors. However, at later stages, the formation of glial scars and the expression of inhibitory molecules on the surface of reactive glial cells inhibit tissue repair as well as regular neuroregeneration. Moreover, functional changes of reactive glial cells, which in some aspects reflect a de-differentiation of the cells, may more directly contribute to further tissue damage, e.g., via disturbance of ion and water homeostasis, and dysregulation of neurotransmitter removal. It is noteworthy that the same gliotic reaction may exert biphasic effects, depending on time and/or amplitude. For instance, the expression of acute-phase proteins (e.g., of proteins with antioxidant activity) in Müller cells of diabetic rats may represent an adaptive response to maintain homeostasis and to protect neurons (Gerhardinger et al. 2005). On the other hand, persistent overexpression of acute-phase proteins may cause tissue damage including endothelial dysfunction and angiogenesis. Another example is the production of nitric oxide (NO). Retinal glial cells upregulate the expression of the inducible form of NO synthase under diabetic conditions (Abu-El-Asrar et al. 2001). Though NO increases the local retinal perfusion by dilating blood vessels, prevents platelet aggregation, and protects neurons from glutamate toxicity via closure of *N*-methyl-*D*-aspartate receptor channels (which are protective effects), higher concentrations of NO are toxic to retinal neurons, via the production of cytotoxic prostaglandins and the formation of nitrotyrosine that inactivates cellular proteins (Goureau et al. 1999, Du et al. 2004). In addition, the diabetic loss of functional hyperaemia, i.e., the dilation of arterioles in response to neuronal activity, was attributed to high NO levels resulting from the upregulation of the inducible NO synthase in glial cells (Mishra and Newman 2010). Soluble factors released by Müller cells such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), interleukin (IL)-1β, IL-6, and tumour necrosis factor (TNF) have neuroprotective effects (Faktorovich et al. 1990, Diem et al. 2001, Foxton et al. 2013) but are also involved in disease progression by inducing vascular leakage, neovascularization, and downregulation of the enzyme, glutamine synthetase (d'Amore 1994, Kruchkova et al. 2001, Shen and Xu 2009).

3. Müller Cell Proliferation and Degeneration

Müller cell gliosis is a response to changes occuring in the diabetic retinal milieu, caused by ischaemia-hypoxia, vascular leakage, oxidative stress, and inflammation. Accumulation of

advanced glycation and lipoxidation end products (AGEs/ALEs) is a further causative factor of Müller cell gliosis (Curtis et al. 2011). In the diabetic retina, Müller cells proliferate and undergo apoptosis (Hammes et al. 1995, Rungger-Brändle et al. 2000). Müller cell apoptosis is induced, at least in part, by hyperglycaemia and extravasated, highly oxidized glycated lipoproteins (Kusner et al. 2004, Wu et al. 2012).

4. Upregulation of Glial Intermediate Filaments

A hallmark of reactive Müller cell gliosis is the upregulation of the intermediate filaments vimentin, nestin, and glial fibrillary acidic protein (GFAP) (Fig. 2*A*) (Bringmann et al. 2009a). While astrocytes decrease their expression of GFAP, the GFAP expression in Müller cells is elevated early in experimental diabetes, well before overt vascular changes become demonstrable (Rungger-Brändle et al. 2000). The upregulation of intermediate filaments alters the biomechanical properties of Müller cells. Normally, Müller cells are about twice as soft as retinal interneurons (Lu et al. 2006). The inner and outer stem processes of Müller cells are even softer than the endfoot (Fig. 3*A*) and the soma. Because Müller cells are softer than neurons, they may act as soft, compliant embedding for neurons. Growing neurites prefer soft substrates (Fig. 3*B*) (Flanagan et al. 2002); neuronal growth cones are actively guided by soft substrates and avoid rigid substrates (Franze et al. 2009). Thus, Müller cells may act as deformable substrates for neurite outgrowth and branching implicated in both retinal development and adult synaptic plasticity (Lu et al. 2011). In addition, Müller cells may protect neurons from mechanical stress as shock absorbers which might be caused, for example, by the movements of the shrinked vitreous body in elderly.

Intermediate filaments contribute to the biomechanical properties of Müller cells. In mice lacking GFAP and vimentin, mechanical challenge revealed an enhanced fragility of Müller cells (Lundkvist et al. 2004). Basically, Müller cells of GFAP- and vimentin-knockout mice have similar biomechanical properties as cells from wildtype mice (Fig. 3*A*) (Lu et al. 2011). This reflects the relative scarcity of intermediate filaments in normal Müller cells. Reactive gliosis alters the biomechanics of Müller cells. The stiffness of the endfeet and inner stem processes of gliotic Müller cells is strongly increased as compared to that of control cells; the increased stiffness correlates with the increased density of intermediate filaments in the cells (Fig. 3*A*) (Lu et al. 2011). In contrast, Müller cells of mice that lack intermediate filaments do not undergo alterations of their biomechanics under pathological conditions. This suggests that the upregulation of intermediate filaments is one main factor which defines the viscoelastic properties of reactive Müller cells. Rigid glial scars will impair neurite growth and may contribute to the poor regenerative capabilities of the retina. The enhanced stiffness of reactive Müller cell endfeet and inner processes may be also, at least in part, responsible for the fact that, in diabetic retinopathy, new blood vessels grow towards the vitreous rather than within the retinal tissue. In GFAP- and vimentin-knockout mice, intraretinal rather than intravitreal vessel growth was observed, suggesting that reactive Müller cells provide a physical barrier to the growth of new blood vessels (Lundkvist et al. 2004). Therapeutic suppression of the upregulation of intermediate filaments in reactive Müller cells may facilitate the regeneration of injured retinal tissue.

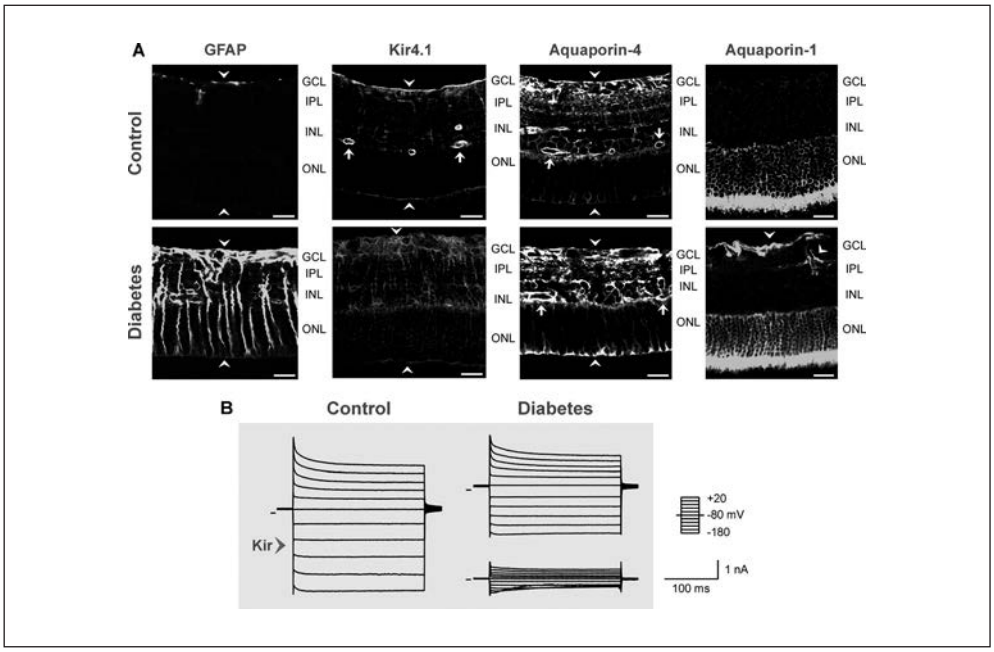

Fig. 2 Retinal gliosis in experimental diabetes is associated with alterations in the retinal distribution of the Kir4.1 potassium channel and aquaporins, and with a decrease in the potassium conductance of Müller cells. (*A*)*:* Immunostaining of retinal slices derived from control (*above*) and 6-months diabetic rats (*below*). Retinal gliosis is indicated by the upregulation of the intermediate filament glial fibrillary acidic protein (GFAP). In the control tissue, GFAP is restricted to astrocytes in the nerve fibre/ganglion cell layers (GCL). In the diabetic retina, GFAP is also expressed by Müller cell fibres that traverse the entire thickness of the neural retina. In the control retina, the Kir4.1 protein displays a prominent localization around the blood vessels (*arrows*) and at both limiting membranes of the retina (*arrowheads*). In the diabetic retina, the Kir4.1 protein is redistributed from these prominent expression sites, and is localized diffusely to Müller cells. In contrast, the retinal distribution of the glial water channel protein aquaporin-4 remains largely unaltered in the course of diabetes. On the other hand, the water channel protein aquaporin-1 is expressed by glial cells in the nerve fibre/ganglion cell layers and around the vessels in the inner plexiform layer (IPL; *arrowheads*) which is not observed in control retinal tissues. In the control retina, aquaporin-1 is expressed mainly by photoreceptor cells in the outer nuclear layer (ONL). INL, inner nuclear layer. Bars, 20 μm. (*B*)*:* Examples of whole-cell potassium currents recorded in Müller cells from control and 6-months diabetic rats. The Kir currents are depicted *downwardly*. Cells from diabetic animals display a reduction in the potassium conductance when compared to cells from control animals, with a substantial variation of the current amplitude in different cells.

5. Malfunction of the Glial Glutamate Uptake and Metabolism

Glutamate toxicity is a major cause of neuronal loss in diabetic retinopathy (Ola et al. 2013). The neurotoxic effect of glutamate is normally blocked by Müller cells, in particular via the uptake of glutamate from the extracellular space (Kawasaki et al. 2000). In Müller cells, glutamate is converted to glutamine which is released from the cells and taken up by retinal neurons as precursor for their synthesis of glutamate and γ-aminobutyric acid (Bringmann et al. 2009b). In diabetic retinopathy, the retinal glutamate metabolism is impaired, as manifested by an increased level of retinal glutamate and a reduced capability to convert glutamate into glutamine (Lieth et al. 1998). Increased levels of retinal glutamate have been proposed to be

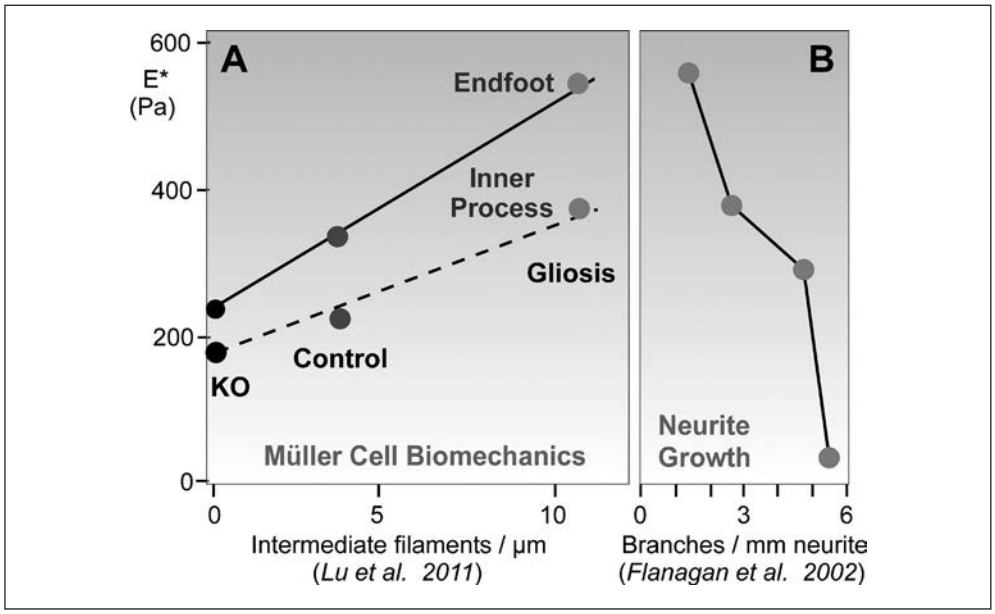

Fig. 3 Stiffness of murine Müller cells (*A*) and dependence of neurite branching on the stiffness of the culture substrate (*B*). The stifness (elastic modulus, E*) of Müller cells was measured at the inner stem process (localized in the inner plexiform layer *in situ*) and the endfoot of isolated cells derived from control retinas, from retinas of knockout (KO) mice which lacked intermediate filaments GFAP and vimentin, and in gliotic Müller cells. Müller cell gliosis was induced by high-intraocular pressure-induced transient retinal ischaemia for 1 h; the cells were isolated 6–8 days after ischaemia. Gliosis results in increased density of intermediate filaments in Müller cells. There is a linear regression between the elastic modulus of the endfoot and inner process and the density of intermediate filaments in the cells.

mainly caused by a malfunction of glial glutamate uptake. The major glutamate transporter of Müller cells is the electrogenic glutamate-aspartate transporter (GLAST) (Sarthy et al. 2005). A decrease in the GLAST-induced membrane currents in Müller cells (Fig. 4) was observed early in experimental diabetes (Li and Puro 2002). The reduced efficiency of the glial glutamate transport was attributed to reactive oxygen and nitrogen species which inhibit the GLAST-mediated glutamate transport (Trotti et al. 1996). However, further factors may contribute to the inhibition of the glial glutamate uptake. Because the electrogenic glutamate uptake is voltage-dependent (Fig. 4), a depolarization of Müller cells decreases the efficiency of the uptake. Depolarization of Müller cells can be induced by various mechanisms. The activity of the Na, K-ATPase, which is essential to maintain membrane hyperpolarization, decreases very rapidly under diabetic conditions (Macgregor and Matschinsky 1986). Inflammatory lipid mediators such as arachidonic acid and prostaglandins, which are produced under hyperglycemic conditions, are potent inhibitors of the Na, K-ATPase. In addition, arachidonic acid directly inhibits the GLAST transporters (Barbour et al. 1989). The very negative membrane potential of Müller cells depends also on the ample expression of inwardly rectifying potassium (Kir) channels, in particular Kir4.1 (Kofuji et al. 2000). Human Müller cells from patients with proliferative diabetic retinopathy display a depolarization as consequence of a functional inactivation or downregulation of Kir channels (Fig. 5*A*) (Bringmann

et al. 2002). The age-dependent decrease of Kir currents in human Müller cells (Fig. 5*B*) (Bringmann et al. 2003) will lower the threshold for membrane depolarization in cells of elderly patients.

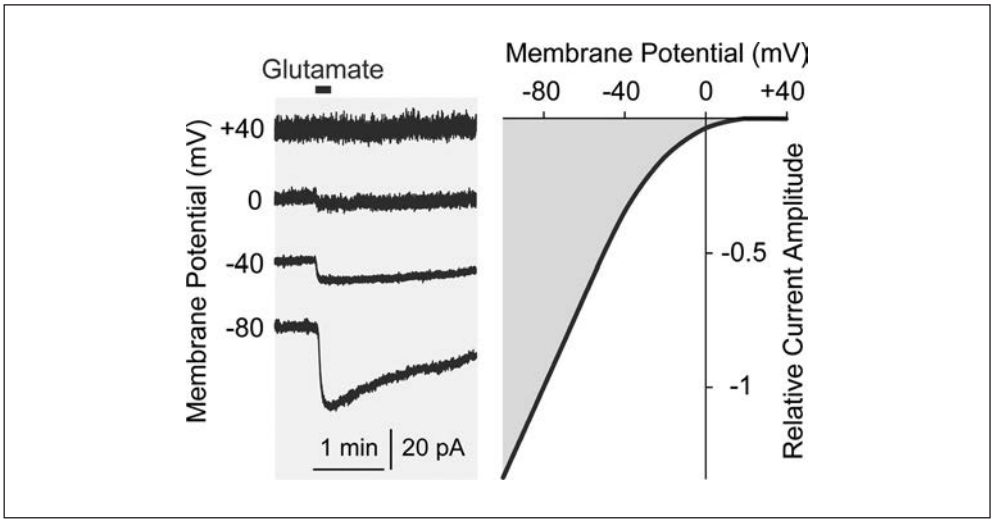

Fig. 4 GLAST-mediated electrogenic glutamate transport in Müller cells. Whole-cell records of membrane currents were made in acutely isolated cells. *Left:* Administration of glutamate (1 mM) to a Müller cell of the rabbit retina evokes inward currents at negative membrane potentials. *Right:* The current-voltage relation of the glutamate transporter currents in guinea-pig Müller cells shows that the efficiency of the glutamate transport increases with increasing (i.e., more negative) membrane potential.

A decrease in the glutamine synthesis was observed in the diabetic retina (Lieth et al. 1998); it might be caused by tyrosine nitration of the glutamine synthetase induced by reactive nitrogen species (Görg et al. 2007). When the retinopathy develops to a proliferative state, the glutamine synthetase of Müller cells will be down-regulated by the action of soluble factors such as bFGF and IL-1ß which inhibit the glucocorticoid-induced expression of the enzyme (Kruchkova et al. 2001, Shen and Xu 2009). Though bFGF is one of the major neurotrophic factors which support neuronal survival in the retina (Faktorovich et al. 1990), the bFGF-induced downregulation of glutamine synthetase might rather aggravate the process of neuronal degeneration.

6. Malfunction of the Glial Glutathione Synthesis

Reactive oxygen species – generated at high glucose levels and resulting from ischaemia-hypoxia and inflammation – are considered as a causal link between elevated glucose and the pathways of development of diabetic complications (Kowluru and Abbas 2003). The level of oxidative-nitrosative stress is positively correlated with the level of neuronal cell death in the diabetic retina (Ali et al. 2008). Müller cells produce various antioxidant molecules, in particular reduced glutathione, a tripeptide synthesized from glutamate, cysteine,

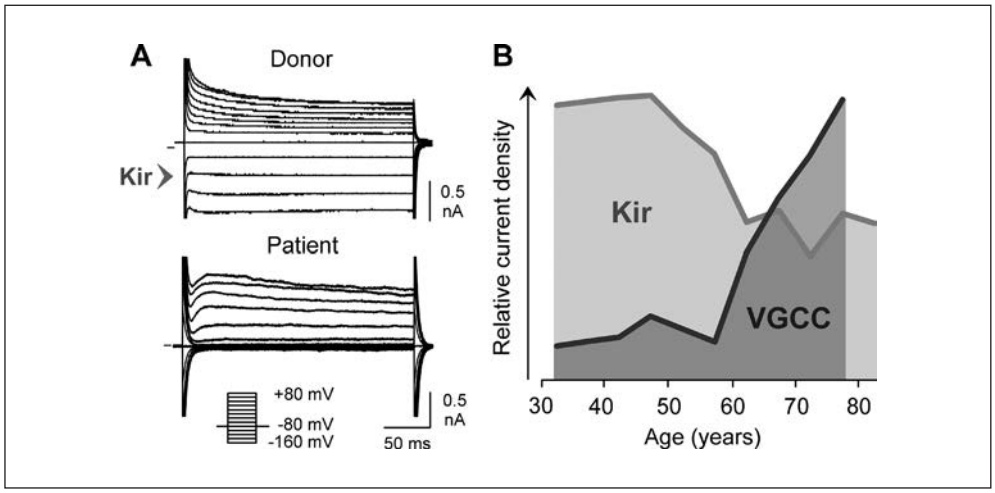

Fig. 5 Diabetic and age-dependent alterations of the membrane conductance of human Müller cells. (*A*)*:* Records of the transmembrane potassium currents in representative Müller cells from a post-mortem donor without apparent eye diseases and a patient with proliferative diabetic retinopathy. The Kir currents (evoked by membrane hyperpolarization from a holding potential of –80 mV) are depicted *downwardly*. Note the full absence of Kir currents in the current traces of the patient's cells. (*B*): Age-dependent alterations in the densities of Kir currents and currents through L-type voltage-gated calcium channels (VGCC) in human Müller cells. Whereas the Kir currents display an age-dependent decrease, the currents through VGCC increase in the course af aging.

and glycine (Huster et al. 2000). The increased oxidative stress and polyol pathway flux of glucose result in an enhanced consumption of reduced glutathione in the diabetic retina. In addition, the decreased glutamate uptake reduces the glutathione synthesis in Müller cells (Kern et al. 1994). Müller cells from aged animals contain reduced levels of glutathione; this is associated with mitochondrial damage, membrane depolarization, and reduced cell viability (Paasche et al. 2000). The age-dependent decrease in retinal glutathione may accelerate the pathogenesis of diabetic retinopathy in elderly patients. Externally applied radical scavengers like *Ginkgo biloba* extract enhance the intrinsic glutathione content of aged Müller cells and protect the mitochondria from the damaging action of free radicals (Paasche et al. 2000).

7. Dysregulation of the Retinal Potassium Homeostasis

A major function of Müller cells is the spatial buffering of the extracellular potassium concentration (Bringmann et al. 2006). Light-induced neuronal activity causes increases in the extracellular potassium level in the plexiform (synaptic) layers. If uncorrected, this will cause neuronal depolarization and hyperexcitation, resulting in glutamate toxicity. Müller cells take up excess potassium from the plexiform layers and release a similar amount of potassium into fluid-filled spaces outside the neural retina, i.e., the blood, vitreous, and subretinal space (Fig. 6*B*). Passive currents through Kir channels, in particular Kir4.1 (Kofuji et al. 2000), play a major role in the spatial buffering of the extracellular potassium

concentration. The Kir4.1 channel protein is expressed in a polarized fashion in the plasma membrane of Müller cells. Kir4.1 is mainly localized in membrane domains across which the cells dispose excess potassium, i.e., in perivascular membranes and at the limiting membranes of the retina (Fig. 2*A*) (Nagelhus et al. 1999).

In retinas of diabetic rats, there is a dislocation of the Kir4.1 channel protein, as indicated by the absence of the prominent expression of Kir4.1 at the limiting membranes of the retina and around the blood vessels; rather, Kir4.1 expression is more evenly distributed along the Müller cell fibres throughout the thickness of the retinal tissue (Fig. 2*A*) (Pannicke et al. 2006). The dislocation of the Kir4.1 channel protein is accompanied by a decrease in the Kir channel-mediated currents across Müller cell membranes (Fig. 2*B*), suggesting a functional inactivation of the channels. The inactivation and redistribution of Kir4.1 will cause a dysregulation of the retinal potassium homeostasis because the downregulation of perivascular Kir4.1 deteriorates the release of excess potassium into the blood, and the upregulation of Kir4.1 in Müller cell processes in the plexiform layers will allow unregulated potassium efflux into perisynaptic spaces. A similar dislocation of the Kir4.1 protein was observed in retinas of mice which carry a genetic inactivation of the dystrophin gene product Dp71, a protein involved in the plasma membrane clustering of Kir4.1. In these mice, the dislocation of Kir4.1 is associated with an enhanced vulnerability of retinal ganglion cells under ischaemic conditions (Dalloz et al. 2003). The redistribution of Kir4.1 from perivascular glial processes is, at least in part, caused by AGEs/ALEs (Curtis et al. 2011). The age-dependent decrease in the Kir currents (Fig. 5*B*) (Bringmann et al. 2003) increases the likelihood of Müller cell dysfunction when additional pathogenic factors such as oxidative stress and inflammation are present. This contributes to the increased vulnerability of the retinal tissue to pathogenic factors in elderly patients.

8. Glial Contribution to Retinal Edema

Retinal edema is the main cause of severe visual deterioration in non-proliferative diabetic retinopathy. Edema is characterized by the accumulation of water in the retinal parenchyma resulting in a thickening of the tissue. Generally, water accumulation within the retinal tissue results from an imbalance between the fluid influx from the blood into the retina and the fluid clearance from the retinal tissue into the blood (Bringmann et al. 2006). The water flux is driven by hydrostatic and osmotic gradients between the blood and the retina (Stefánsson 2009). Diabetic retinal edema is thought to be primarily caused by a breakdown of the blood-retinal barrier, resulting in increased vascular permeability (vasogenic edema). However, it has been shown that an impairment of fluid absorption from the retinal tissue is an essential step in edema formation. In the preclinical stage of diabetic retinopathy, there are two types of increased retinal thickness and intraretinal cyst formation that may be or not associated with vascular leakage (Lobo et al. 2000).

8.1 Contribution to Vasogenic Edema

Retinal capillaries are endowed with vascular endothelial cells and contractile pericytes which are covered by a basement membrane, and are ensheathed by glial cell processes arising from astrocytes and Müller cells. The inner blood-retina barrier is formed by tight

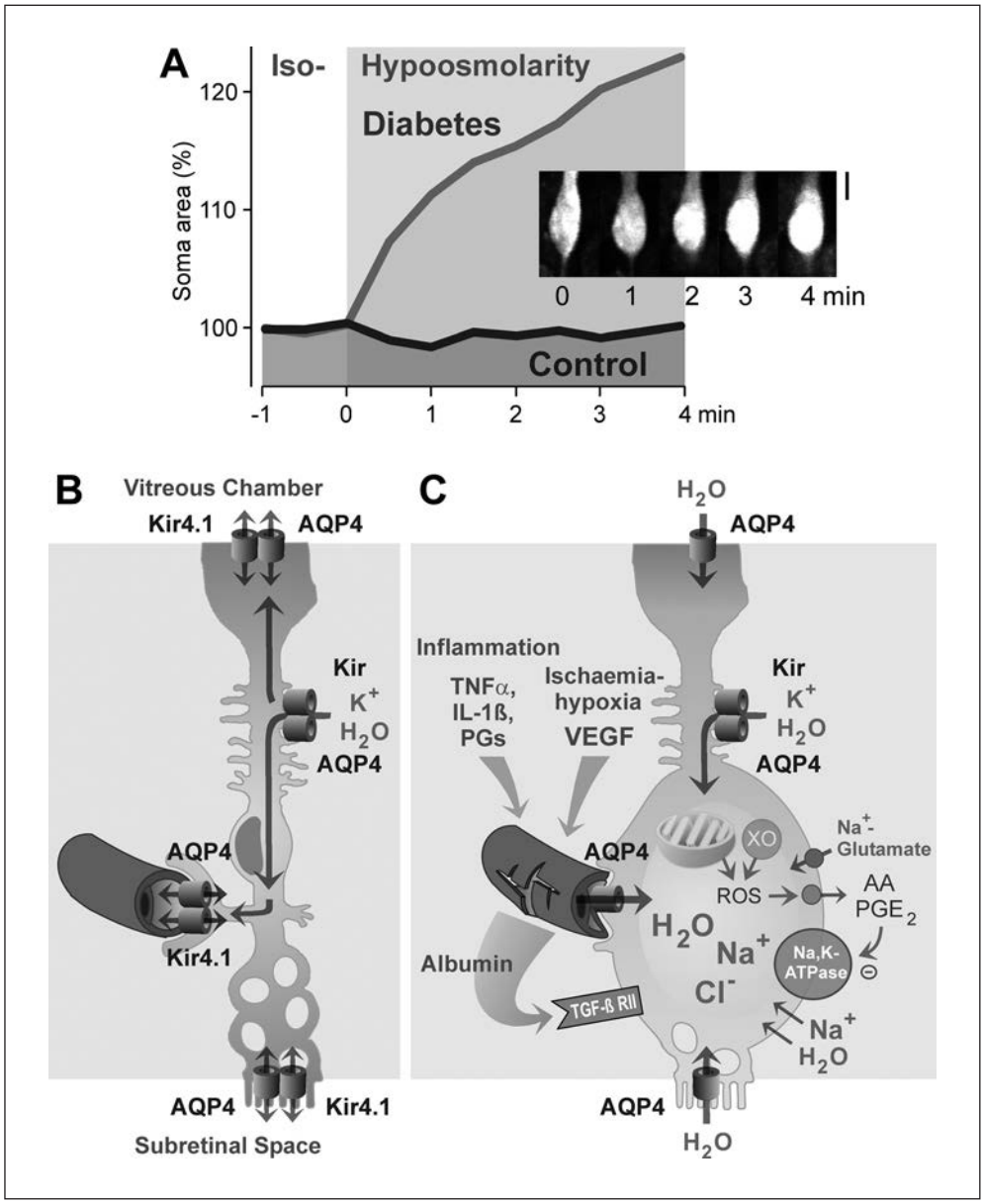

Fig. 6 Diabetes alters the osmotic swelling properties of Müller cells. (*A*)*:* Under hypoosmotic conditions, Müller cells in retinal slices from diabetic rats display a rapid time-dependent swelling of their somata, a response not observed in cells from control animals. The cross-sectional area of Müller cell somata was measured in freshly isolated retinal slices which were superfused with a hypoosmotic solution (60 % of normal osmolarity). *Inset*, example of a Müller cell soma from a diabetic animal before (*left*) and during (*right*) hypoosmotic exposure. Bar, 5 µm. (*B, C*)*:* Potential mechanisms of osmotic Müller cell swelling in the retina of diabetic rats.(*B*)*:* Under normal conditions, Müller cells mediate the fluid clearance from the retinal tissue into the blood mainly by a transcellular co-transport of water (facilitated by aquaporin-4 water channels, AQP4) and distinct osmolytes, predominantly of potassium ions (facilitated by Kir channels, in particular Kir4.1). (*C*): In diabetes, vascular leakage occurs due to the action of

junctions between the non-fenestrated endothelial cells. In hyperglycemic rats, the disruption of the inner blood-retina barrier is caused by opening of the tight junctions between vascular endothelial cells and by an increased vesicular transport of serum proteins across the endothelia. Müller cells enhance the endothelial cell barrier function under normoxic conditions but impair the barrier function under hypoxic conditions (Tretiach et al. 2005). Müller cell-derived factors which enhance the barrier function of endothelial cells are, for example, glial cell line-derived neurotrophic factor, neurturin, and pigment epithelium-derived factor (PEDF). The role of Müller cells in the establishment of the blood-retinal barrier is underlined by a recent study showing that conditional ablation of Müller cells in transgenic mice results in vascular telangiectasis, breakdown of the blood-retina barrier, and intraretinal neovascularization (Shen et al. 2012).

A breakdown of the blood-retina barrier may occur in the presence of mechanical stress to Müller cells after posterior vitreous detachment (Schubert 1989). The vitreous body adheres to the retinal tissue at the peripheral retina, the major superficial retinal vessels, the optic disc, and the macula. Pathological processes at the vitreo-retinal junction result in increased adhesiveness of the vitreal collagen fibres to the retinal internal limiting membrane. In cases of vitreous shrinkage and partial posterior vitreous detachment, tractional forces result in a chronic irritation of Müller cells and a local release of factors that, in turn, induce Müller cell gliosis and vascular leakage.

The major vessel-permeabilizing factor induced by hypoxia is VEGF (Schlingemann and van Hinsbergh 1997). In addition, other cytokines such as bFGF and inflammatory factors like TNFα, IL-1β, and prostaglandins enhance the permeability of retinal vessels (Derevjanik et al. 2002). Müller cells, astrocytes, and retinal neurons increasingly express VEGF in non-proliferative diabetic retinopathy, at times when there is no anatomical evidence of retinal malperfusion (Amin et al. 1997). Conditional disruption of the VEGF expression in Müller cells reduces the vascular leakage in the diabetic retina (Wang et al. 2010).

8.2 Alterations in Water Channel Expression

The water transport through Müller cells, which is involved in the resolution of osmotic gradients in the retinal tissue and across the glio-vascular interface, is facilitated by water-selective channels, the aquaporins (AQPs). In the neuroretina, AQP1 is mainly expressed by photoreceptor cells while AQP4 is expressed by Müller cells and astrocytes (Fig.

inflammatory factors and VEGF. Vascular leakage results in serum influx into the retinal parenchyma. In the presence of osmotic gradients, extravasated serum albumin induces a swelling of Müller cells via activation of the transforming growth factor-β receptor type II (TGF-β RII). Albumin is internalized by receptor-mediated endocytosis; endocytosis is associated with the generation of oxygen radicals and activation of phospholipase A_2. Osmotic stress induces the production of reactive oxygen species (ROS) in the mitochondria and by the xanthine oxidase (XO). ROS stimulate the production of arachidonic acid (AA) and prostaglandins (PGs) which inhibit the Na, K-ATPase. Inhibition of the ATPase results in sodium influx which is associated with a water influx into the cells. In the normal, healthy retina, the sodium influx can be compensated by a potassium efflux through Kir4.1 and (after autocrine activation of adenosine A_1 receptors) two-pore domain potassium channels. Müller cells of diabetic animals down-regulate the expression of functional Kir4.1 channels, which impairs the compensatory efflux of potassium ions under hypoosmotic conditions. An influx of sodium via electrogenic glutamate uptake carriers may further enhance the osmotic pressure of the cell interior. The downregulation of Kir4.1 impairs the glial release of potassium into the blood and the resolution of osmotic gradients across the glio-vascular interface.

2*A*) (Nagelhus et al. 1999, Iandiev et al. 2005). AQP4 knockout mice display reduced electroretinogram b-waves, suggesting that the Müller cell-mediated water transport is involved in mediating the neuronal signal transduction (Li et al. 2002). Diabetes alters the retinal distribution of AQP1 and AQP4. In diabetic retinas, an additional strong expression of AQP1 is observed in glial cells located in the innermost retinal layers and around the innermost blood vessels (Fig. 2*A*) (Iandiev et al. 2007). The superficial retinal vessels are surrounded by AQP4 in control retinas, and by AQP1 in diabetic retinas. The alteration in the type of perivascular AQPs was found to coincide with the occurrence of neuronal apoptosis in the ganglion cell and inner nuclear layers of the diabetic retina (Fukuda et al. 2010). The functional significance of the switch in perivascular AQPs remains to be determined. Possibly, the expression of AQP1 may represent an attempt of glial cells to facilitate the water clearance around leaky vessels.

8.3 Neuronal Edema

Water accumulation in retinal neurons and glial cells may contribute to the development of diabetic retinal edema. In the diabetic retina, the swelling of ganglion cell bodies and their processes precedes the loss of these cells and the gliosis in the inner retinal layers (Duke-Elder and Dobree 1967). Diabetic retinopathy is characterized by increased levels of retinal glutamate. Glutamate induces a thickening of the inner retinal tissue, caused by a swelling of retinal neurons and synapses (Uckermann et al. 2004). Over-stimulation of ionotropic glutamate receptors drives sodium, chloride, and water into the postsynaptic structures. Over-stimulation of ionotropic glutamate receptors during an ischaemic episode results in long-term depolarization of retinal neurons that causes an opening of voltage-gated calcium channels and neuronal calcium overload. The long-lasting calcium overload activates the apoptosis machinery of the cells, resulting in neuronal cell death. Müller cells may contribute to the neuronal cell swelling and degeneration, in part by the malfunction of the glial glutamate uptake (see above).

Müller cells may contribute to the glutamate-induced swelling and degeneration of retinal neurons also by providing the water required for the rapid ion flux into activated neurons (Bringmann et al. 2005). The plexiform (synaptic) layers of the retina are high-resistance barriers for paracellular fluid movement (Antcliff et al. 2001). Because synapses are closely ensheathed by Müller cell membranes, the water that flows into the neurons during activation of ionotropic glutamate receptors and voltage-gated ion channels will be delivered predominantly from the Müller cell interior through AQP4 water channels (Bringmann et al. 2005). For osmotical reasons, a simultaneous water influx from the blood into the Müller cells will occur. An inhibition of the rapid water transport through Müller cells should delay the ion flux into neurons, resulting in lower levels of neuronal cell swelling and apoptosis. Indeed, disruption of the AQP4 gene in mice was shown to protect against impaired retinal function and cell death after retinal ischaemia (Da and Verkman 2004).

8.4 Glial Cell Edema

Under diabetic conditions, the rapid water transport through Müller cells is disturbed, as indicated by an alteration in the osmotic swelling characteristics of the cells. Müller cells in retinal slices from adult healthy animals keep their volume constant for at least 10 min

when the osmolarity of the extracellular medium abruptly decreases (Fig. 6*A*) (Wurm et al. 2008a). However, Müller cells in retinal slices from diabetic animals swell immediately upon hypoosmotic challenge (Fig. 6*A*) (Pannicke et al. 2006, Wurm et al. 2008a). This change in the osmotic swelling characteristics of Müller cells suggests that the transglial water transport driven by osmotic gradients is dysregulated in the retina of diabetic animals. Whether an intracellular edema or even a swelling of Müller cells contribute to diabetic macular edema remains controversial. It has been shown that experimental diabetic retinopathy is associated with a water accumulation within Müller cells and in the interstitial spaces of the retina, resulting in extended extracellular spaces and edematous Müller cell endfeet and perivascular processes (Kumar et al. 2013). Water influx into Müller cells occurs when the intracellular osmotic pressure is increased as compared to the osmotic pressure of the extracellular fluid, blood, and/or vitreous. Activity-dependent osmotic gradients are present in the retinal tissue under normal and pathological conditions. Under normal conditions, Müller cells are surrounded by a hypoosmotic extracellular fluid during periods of intense neuronal activity (Dmitriev et al. 1999). In addition, the uptake of neuron-derived osmolytes such as potassium and sodium-glutamate may increase the osmotic pressure of the Müller cell interior relative to the extracellular fluid. Thus, neuronal activity in the retina generates an osmotic gradient that favours water flux from extracellular to intracellular spaces. These osmotic conditions are exacerbated under pathological conditions which are characterized by glutamate-induced hyperexcitation and an impairment of Müller cells to redistribute osmolytes, e.g., potassium, into the blood and vitreous (see above). A decrease in blood osmolarity, e.g., in cases of hyponatraemia and hypoalbuminaemia, produces an osmotic gradient across the glio-vascular interface resulting in a swelling of perivascular glial processes.

Further mechanisms may contribute to intracellular water accumulation in Müller cells. Ion efflux through plasma membrane channels, resulting in equalization of the transmembrane osmotic gradient, is one mechanism that prevents cellular swelling under hypoosmotic conditions. The major ion channel that mediates passive ion efflux from Müller cells is the Kir4.1 potassium channel (Pannicke et al. 2006). The functional inactivation of Kir4.1 channels in Müller cells of the diabetic retina (Fig. 2*B*) impairs the channel-mediated efflux of potassium in response to a decreased extracellular osmolarity. The importance of Kir4.1-mediated potassium efflux for the prevention of Müller cell swelling is indicated by the correlation between the amplitude of osmotic Müller cell swelling and the decrease in the potassium conductance of the cells found in experimental retinal ischaemia (Wurm et al. 2011). Potassium efflux through Kir4.1 will also compensate the increased sodium influx into the cells caused by the uptake of sodium-glutamate and the decreased Na, K-ATPase activity.

The action of arachidonic acid and of its metabolites, in particular prostaglandin E_2, was causally implicated in the development of retinal edema (Guex-Crosier 1999). Oxidative stress and the generation of inflammatory lipids are causative factors of Müller cell swelling in the retina of diabetic rats (Pannicke et al. 2006, Wurm et al. 2008a, Krügel et al. 2011). Apparently, osmotic challenge causes oxidative stress in Müller cells which stimulates the production of arachidonic acid and prostaglandins. These mediators inhibit the Na, K-ATPase, resulting in intracellular sodium overload, water influx, and cellular swelling (Fig. 6*C*). One major source of reactive oxygen species involved in the induction of Müller cell swelling are dysfunctional mitochondria (Krügel et al. 2011). In addition, superoxide produced by the enzyme xanthine oxidase is a factor that contributes to the osmotic Müller cell swelling in retinas of diabetic rats.

Vasogenic edema is associated with extravasation of serum proteins such as albumin. Serum albumin induces Müller cell swelling in the presence of osmotic gradients (Löffler et al. 2010). Albumin activates the transforming growth factor (TGF)-β receptor type II (Fig. 6*C*); activation of the receptor results in oxidative stress, the production of arachidonic acid and prostaglandins, and intracellular sodium overload (Löffler et al. 2010). Extravasated albumin may represent one factor that links vascular leakage, Müller cell edema, and dysregulation of retinal fluid clearance in the diabetic retina.

A further factor that contributes to the alteration in the osmotic swelling properties of Müller cells in the diabetic retina is the inactivation of a purinergic signalling cascade that normally prevents Müller cell swelling. This signalling cascade is initiated by a consecutive release of ATP and adenosine from the cells which results in autocrine activation of $P2Y_1$ and adenosine A_1 receptors, respectively (Uckermann et al. 2006, Wurm et al. 2008a). The final step in this signalling cascade, activation of A_1 receptors, results in the opening of potassium and chloride channels in the Müller cell membrane; the transmembrane ion flux equalizes the osmotic gradient across the plasma membrane and, thus, prevents the swelling of the cells under hypoosmotic conditions. The purinergic signalling cascade can be activated by two mechanisms: either by osmotic stress which induces a release of ATP from Müller cells, or by activation of metabotropic glutamate receptors by neuron- or Müller cell-derived glutamate (Uckermann et al. 2006, Wurm et al. 2008a). Müller cells release glutamate upon activation of various different receptor types. VEGF, neuropeptide Y, and various other receptor ligands activate the volume-regulatory cascade upstream of glutamate release (Uckermann et al. 2006, Wurm et al. 2008b). Membrane stretch induced by osmotic pertubations may trigger a release of ATP from Müller cells which activates $P2Y_1$ receptors in an autocrine fashion. The osmotic swelling of Müller cells in diabetic retinas (Fig. 6*A*) suggests that the endogenous purinergic swelling inhibition in response to osmotic/mechanical stress is abrogated. However, the ligand-induced inhibition of osmotic swelling is still functional, i.e., administration of exogenous ATP, adenosine, glutamate or VEGF prevents the swelling (Wurm et al. 2008a, b). This suggests that the purinergic receptors involved in cell volume regulation are functional, while no ATP is released in response to osmotic/mechanical stress. The functional relevance of the abrogation of the osmotic release of ATP from Müller cells in the diabetic retina remains to be determined. Such an abrogation might prevent excess release of glial ATP which would otherwise disturb regular neuronal information processing and may even induce neuronal death (Zhang et al. 2005) under conditions of increased osmotic imbalances as occurring in the diabetic retina. The abrogation of the osmotic release of ATP from Müller cells might be also glioprotective, as it prevents the $P2Y_1$-mediated cytotoxic calcium overload and an excessive ATP-induced release of growth factors from the cells (see below).

Although intracellular edema of Müller cells caused by water accumulation within the cells was described to occur in animal models of diabetic retinopathy (Kumar et al. 2013), a swelling of Müller cells was rarely observed. This may suggest that endogenous mechanisms are present that inhibit the swelling of Müller cells despite the presence of osmotic gradients that favour water influx into the cells. Though the osmotic/mechanical release of ATP is abrogated in Müller cells of diabetic retinas, the ligand-induced inhibition of osmotic swelling is functional. Many of the receptor ligands which were shown to inhibit osmotic Müller cell swelling, including VEGF and neuropeptide Y, are upregulated in the ischaemic retina (Yoon et al. 2002). Thus, the ligand-induced inhibition may represent one reason for the fact that Müller cells usually do not swell despite the presence of intracellular edema.

9. Glial Contribution to Retinal Neovascularization

After formation of large hypoxic areas in the retina, the expression of angiogenic factors that initiate vascular growth determine further progression of the disease towards a proliferative retinopathy. Fibrovascular tissues grow from the retina into the vitreous, representing an aberrant attempt to reoxygenate the retinal tissue. VEGF is the most relevant angiogenic factor (Schlingemann and van Hinsbergh 1997). The balance between angiogenic factors, particularly VEGF, and antiangiogenic factors is thought to be essential for angiogenic homeostasis in the retina. The major anti-angiogenic factor is PEDF. The expression of PEDF is reduced in Müller cells of the diabetic retina (Shen et al. 2011) which results in upregulation of VEGF (Zhang et al. 2006). However, the role of Müller cells in inducing retinal neovascularization is incompletely understood. In the healthy retina, Müller cells provide a permanent anti-proliferative condition for vascular endothelial cells, by the release of soluble anti-angiogenic factors such as PEDF, thrombospondin-1, and TGF-β (Eichler et al. 2001). In spite of the observation that hypoxia enhances the expression of VEGF and downregulates the expression of PEDF and TGF-β in Müller cells, conditioned media of Müller cells grown under hypoxic conditions failed to stimulate the proliferation of vascular endothelial cells (Eichler et al. 2001). The angiostatic role of Müller cells may represent one reason for the fact that, in diabetes, newly formed retinal vessels grow toward the vitreous but not into the ischaemic retinal tissue.

10. Formation of Epiretinal Membranes

The main factor that causes a decrease in visual acuity in proliferative diabetic retinopathy is the formation of fibrovascular epiretinal membranes. New vessels grow from superficial veins and venules into the posterior vitreous cortex. Tractional forces resulting from contraction of the extracellular matrix or of individual cells can emanate from the epiretinal tissue; they distort the anatomical position of the retina and lead to recurrent traction retinal detachment. Müller cells are the principal glial cells in fibrovascular membranes (Nork et al. 1987). Müller cells de-differentiate, migrate within and out of the retina, and display cellular hypertrophy. Müller cells in diabetic epiretinal membranes may transdifferentiate into myofibroblasts; these cells generate tractional forces in response to growth factors in the vitreous, thus causing traction retinal detachment (Guidry 2005).

It has been suggested that the breakdown of the blood-retina barrier and vitreous haemorrhage are pathogenic factors of epiretinal membrane formation (Bringmann and Wiedemann 2009). The presence of serum and blood cell-derived growth factors and cytokines, of inflammatory blood-borne cells, and of cell debris in the vitreous, triggers Müller cell process extension and proliferation. The precise mechanisms of how Müller cells respond to vitreous haemorrhage are still unclear. One mechanism may include the phagocytosis of blood-borne substances and of blood cell debris. In addition, AGEs localized to the vitreous cavity and internal limiting membrane (Barile et al. 2005) may activate Müller cells to proliferate towards the vitreous. A further factor that may cause proliferation of Müller cells are focal epiretinal tractional forces at vitreoretinal junctures. Müller cells sense tractional forces by calcium-dependent mechanisms. Stretching the retinal tissue or mechanical stimulation of Müller cells induce calcium responses, upregulation of the transcription factor c-Fos and

of bFGF, and release of ATP from Müller cells (Newman 2001, Lindqvist et al. 2010). ATP induces activation of matrix metalloproteinases and the relesase of further mitogenic factors from Müller cells such as platelet-derived growth factor (Milenkovic et al. 2003). Thus, mechanical stress triggers molecular responses in Müller cells (e.g. release of bFGF) that prevent neuronal degeneration and that induce vascular permeabiliy and proliferation of Müller cells. The calcium-dependent Müller cell responses, which are involved in the release of growth factors and Müller cell proliferation, are facilitated in elderly people because the currents through voltage-gated calcium channels display an age-dependent increase (Fig. 5*B*) (Bringmann et al. 2003).

11. Concluding Remarks

The gliotic dysfunction of Müller glial cells may contribute to the progression of diabetic retinopathy, as it exacerbates vascular abnormalities, retinal edema, and neuronal dysfunction, and contributes to the development of fibrovascular epiretinal membranes. At present, there is no established neuroprotective treatment that avoids visual disturbance in patients with diabetic retinopathy. Common treatments of diabetic retinopathy include laser photocoagulation to improve tissue oxygenation, intravitreal corticosteroids to reduce inflammation, intravitreal anti-VEGF agents, and vitreoretinal surgery. These treatments are applicable only at advanced stages of the disease, and are associated with adverse effects. The loss of retinal neurons early in diabetic retinopathy is the main cause why patients with diabetic retinopathy lose vision even if the retinal edema is treated with photocoagulation or vitrectomy (Gardner et al. 2002). Therefore, new pharmacological treatments for the early stages of the disease are needed. A proper understanding of the network of gliotic responses in the diabetic retina and of their beneficial *versus* damaging effects appears to be essential for the development of new therapeutic strategies to treat diabetic retinopathy. It may be conceivable, for example, that a supression of the inhibitory action of gliotic Müller cells on tissue regeneration may direct the growth of newly formed vessels into the ischaemic retinal tissue rather than into the vitreous. Allowing intraretinal neovascularization would result in restoration of the physiological network of capillaries in avascular retinal areas. Though the last two decades watched a huge increase in our knowledge regarding glial reactivity in diabetic retinopathy, there remain many open questions. The molecular and cellular events involved in the pathogenesis of diabetic retinopathy are still poorly understood. Much of the current knowledge about glial cell dysfunction was obtained in animal models and on cultured cells and thus awaits confirmation on human cells/tissue *in situ*. A better understanding of the molecular mechanisms of gliosis in the diabetic retina would help to develop therapeutic agents that support the protective, and diminish the damaging, effects of gliosis.

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Obesity and Vascular Damage

Matthias Blüher (Leipzig)

With 3 Figures

Abstract

Obesity represents a major health burden affecting more than 20 % of Western populations with steadily increasing incidence. Thus, projections from the World Health Organization predict for the first time a decline in the mean life expectancy as a consequence of obesity-associated comorbidities such as atherosclerosis, type 2 diabetes and specific types of cancer. However, the mechanisms linking increased adipose mass in obesity to these disorders are not fully understood. There may be direct and indirect mechanisms underlying the increased risk of obese individuals to develop vascular damage. Direct mechanisms may include the release of adipokines affecting the vasculature. Obesity may also indirectly via increased blood pressure, changes in glucose and lipid metabolisms as well as insulin resistance contribute to dysfunction of the vasculature.

Zusammenfassung

Adipositas stellt ein großes Gesundheitsproblem dar, das über 20 % der westlichen Bevölkerung mit stetig steigender Inzidenz betrifft. Adipositas führt laut einer WHO-Vorhersage zu einem Rückgang der Lebenserwartung aufgrund zunehmender Adipositas-assoziierter Begleiterkrankungen wie Arteriosklerose, Typ-2-Diabetes und maligne Neubildungen. Allerdings sind die Mechanismen, über die eine vermehrte Fettmasse zu diesen Erkrankungen führen kann, nicht vollständig verstanden. Wahrscheinlich spielen sowohl direkte als auch indirekte Mechanismen eine Rolle beim erhöhten Risiko für Gefäßerkrankungen bei Personen mit Adipositas. Dabei könnten Adipokine eine direkte schädigende Wirkung auf das Gefäßsystem haben. Zusätzlich könnte Adipositas auch über eine Erhöhung des Blutdrucks, Veränderungen im Lipid- und Glukosestoffwechsel sowie durch Insulinresistenz zu Schädigungen im Blutgefäßsystem beitragen.

1. Introduction

Obesity represents one of the five major health risks in modern societies with a frequency of more than 20 % of the population in developed countries (*WHO* 2007). Obesity increases the risk for type 2 diabetes, fatty liver disease, hypertension, coronary heart disease, stroke, dementia, obstructive sleep apnea and several types of cancer (Blüher 2010, Van Gaal et al. 2006, Le Roith et al. 2008). For instance, ~30 % of obese men and women develop type 2 diabetes, the incidence of hypertension and arthrosis among obese patients is approximately 50 % (Mokdad et al. 2003). Increased total body fat mass is associated with impaired insulin sensitivity, increased blood pressure, altered concentrations of serum lipids and markers of inflammation (Blüher 2010). Due to these relationships, weight loss usually results in marked improvement in the metabolic abnormalities associated with obesity and improves insulin

sensitivity (Uusituba et al. 2003), blood pressure (Stevens et al. 2001), and concentrations of serum lipids (Dattilo et al. 1992) and circulating markers of inflammation (Ziccardi et al. 2002). Interestingly, significant reduction in subcutaneous fat mass by liposuction does not improve circulating metabolic and inflammatory parameters (Klein et al. 2004), whereas reduction of visceral fat mass by omentectomy in addition to gastric banding has significant beneficial and long-term effects on measures of glucose metabolism and insulin sensitivity in obese individuals (Thorne et al. 2002). Therefore, the pathogenic link between increased adipose tissue mass and higher risk for obesity related disorders is not necessarily directly related to fat mass. Adipose tissue dysfunction and ectopic fat accumulation seem to be important factors determining the individual risk to develop metabolic and cardiovascular co-morbidities of obesity (Blüher 2009). There may be direct and indirect mechanisms underlying the increased risk of obese individuals to develop vascular damage (Fig. 1). Concerning direct mechanisms, it has been recognized during the past decade that adipose tissue is an endocrine organ secreting a number of bioactive molecules, so called adipokines, but also other molecules (Fig. 2).

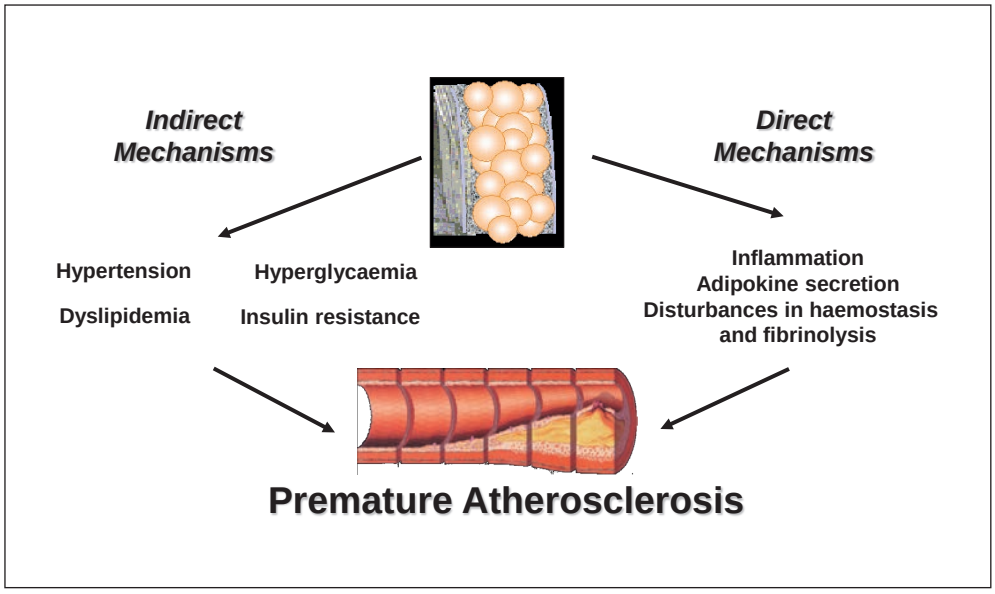

Fig. 1 Mechanisms linking obesity vascular damage

Adipokines participate in various metabolic processes including the regulation of appetite control, insulin sensitivity and insulin secretion, energy expenditure and inflammation. With the development of adipose tissue inflammation and dysfunction adipokine secretion is significantly altered (Blüher 2009). These changes in adipokine secretion are very likely to link impaired adipose tissue function to vascular damage (Blüher 2009, Van Gaal et al. 2006). After the discovery of leptin as an adipose tissue-derived hormone (Zhang et al. 1994) several other adipokines have been discovered and found to contribute to cardiovascular risk (Van Gaal et al. 2006).

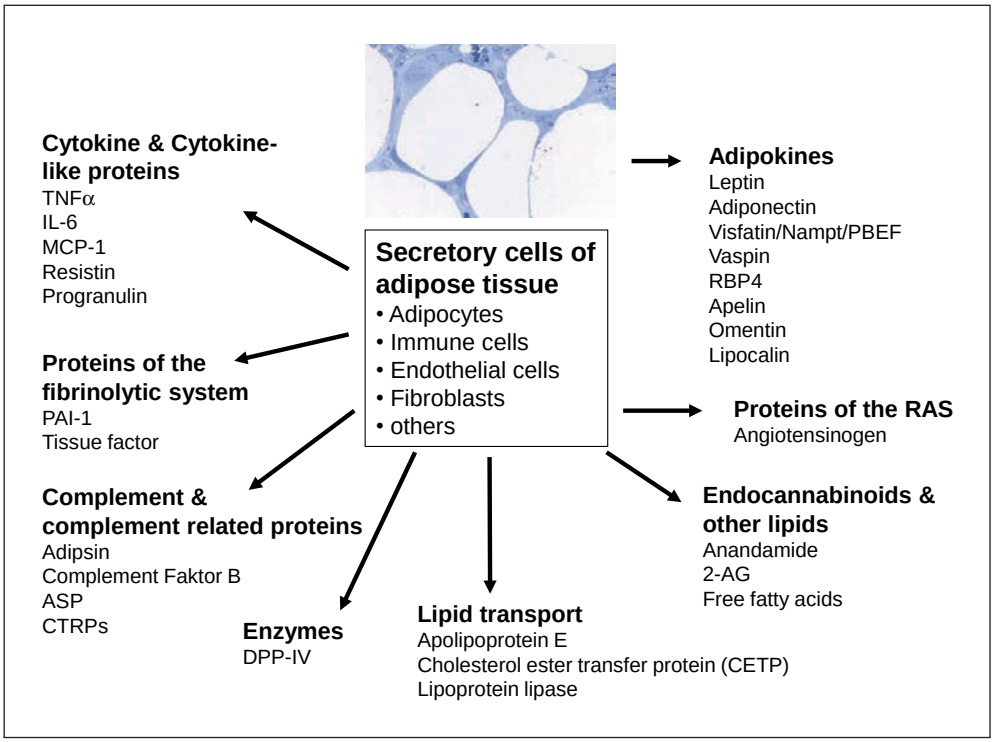

Fig. 2 Adipose tissue as an endocrine organ. Examples for endocrine adipose tissue derived molecules, which contribute to the inflammatory process in obesity. Abbreviations: 2-AG, 2-Arachidonoylglycerol, ASP, acylating simulation protein; MCP-1, monocyte chemotactic protein-1, PAI-1, plasminogen activator inhibitor-1, RAS, renin angiotensin system, RBP-4, Retinol binding protein-4

2. Indirect Mechanisms Linking Obesity to Vascular Damage

In addition to the proposed direct mechanisms, obesity may indirectly contribute to vascular damage by increasing the risk for hypertension, insulin resistance, visceral and ectopic fat accumulation, dyslipidaemia, a pro-coagulatory and pro-inflammatory state, hypofibrinolysis, reduced fitness level, glucose and lipid toxicity, decreased expression of protective factors and several others (Fig. 3).

To which extent these factors might contribute to an independent, additional risk among obese individuals is unclear (reviewed in Van Gaal et al. 2006). Dyslipidaemia in obesity is characterized by increased levels of very low-density lipoprotein (VLDL) cholesterol, triacylglycerols and total cholesterol, an increase in small dense LDL particles, and lower high-density lipoprotein (HDL) cholesterol levels (reviewed in Van Gaal et al. 2006). However, it is not clear whether hypercholesterolaemia further increases the risk of cardiovascular disease in obese individuals. But the insulin-resistant state of abdominal obesity adds substantially to the risk of atherosclerosis of patients with familial hypercholesterolaemia (Gaudet et al. 1998). Obesity also limits the beneficial effects of lipid-lowering strategies (Nicholls et al. 2006). However, aggressive reduction of lipids with the use of statins can

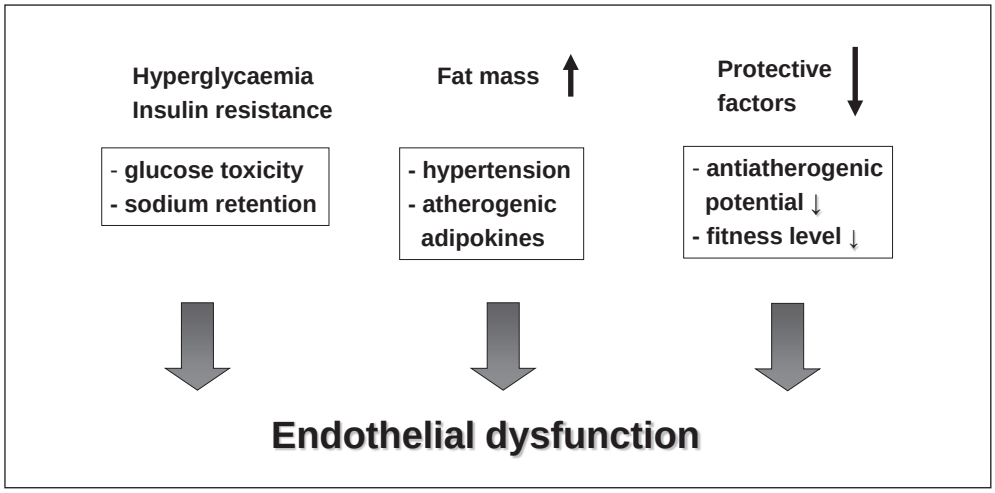

Fig. 3 Indirect mechanisms of obesity related vascular damage

have a beneficial effect on coronary plaque development in obese individuals (Nicholls et al. 2006). Obesity induces several cytokines and inflammatory markers that might contribute to vascular damage in obese people. It is also associated with increased levels of endothelial cell secretion products including adhesion molecules. Since indirect mechanisms linking obesity to vascular damage have been extensive discussed elsewhere, this review focuses on the concept that secreted products from adipose tissue may directly affect the vasculature.

3. Altered Adipokine Secretion May Represent a Direct Link between Obesity and Vascular Damage

During the past decade it became clear that in addition to cells of the immune system and the liver, adipose tissue also expresses many pro- and anti-inflammatory factors and could therefore contribute to increased levels of inflammatory markers in the circulation of obese individuals (reviewed in Blüher 2010). Adipose tissue was shown to produce and secrete pro-inflammatory cytokines and adipokines including TNFα, transforming growth factor β (TGFβ) and interferon-γ, C-reactive protein (CRP), interleukins (IL) -1, -6, -8, -10, plasminogen activator inhibitor-1 (PAI-1), retinol binding protein-4 (RBP4), vaspin, fibrinogen, haptoglobin, angiopoietin-related proteins, metallothionein, complement factor 3, serum amyloid A (SAA) protein, anandamide and 2-AG as well as chemoattractant cytokines, such as monocyte chemotactic protein-1 (MCP-1), progranulin and macrophage inflammatory protein-1α (reviewed in Blüher 2010). Production of molecules, which are considered as anti-inflammatory mediators including adiponectin and IL-10 are reduced in obese states (reviewed in Blüher 2010). In the majority of obese patients, adipose tissue dysfunction develops as a primary defect in obesity and may mechanistically link obesity to increased risk of cardiovascular disease. Genetic and environmental interactions cause dysfunction of adipose tissue by initiating a sequence of adipocyte hypertrophy, hypoxia, several stresses and inflammatory

processes (Blüher 2009). As a consequence, impaired adipose tissue function contributes to a proinflammatory, atherogenic, and diabetogenic state and may be mechanistically linked to the development of obesity associated vascular damage. As a symptom of adipose tissue dysfunction, adipokine secretion is shifted towards a proinflammatory and atherogenic pattern. Although it is difficult to determine the quantitative contribution of adipose tissue to the low grade inflammatory state in obesity, increased production of pro-inflammatory adipokines significantly contribute to maintain the inflammatory process in obesity and may cause insulin resistance in the liver, muscle and other organs (Blüher 2010). The etiological importance of adipokines in the pathogenesis of metabolic and cardiovascular diseases was demonstrated for several adipokines (reviewed in Van Gaal et al. 2006). The role of the adipokines leptin, adiponectin and several others as mediators linking increased fat mass and/or impaired adipose tissue function to metabolic and cardiovascular diseases has been extensively characterized during the past years (reviewed in Van Gaal et al. 2006). There are several other adipose tissue derived molecules including tumour necrosis factor -α (TNFα), interleukin-6 (IL-6), IL-8, IL-10, omentin, monocyte chempattractant protein-1 (MCP-1), osteonectin, plasminogen activator inhibitor-1 (PAI-1) and more which are extensively discussed elsewhere (Kralisch et al. 2007). The most recent adipokines to emerge as contributors to obesity-related diseases are chemerin, apelin, retinol-binding protein-4 (RBP4) and progranulin (Blüher 2010).

4. Does Leptin Link Obesity to Vascular Damage?

Leptin is secreted from adipocytes, controls food intake and energy expenditure and has atherogenic and growth properties (Ahima and Flier 2000). Leptin decreases orexigenic and increases anorexigenic peptide synthesis in the hypothalamus thereby decreasing appetite (Ahima and Flier 2000). Leptin was cloned in 1994 as the protein product of the ob gene mutation, which leads to extreme obesity in the *ob/ob* mouse model (Zhang et al. 1994). The importance of altered leptin signalling for the development of obesity and diabetes is further supported by the discovery that a mutation in the leptin receptor gene causes obesity and diabetes in *db*/*db* mice (Chen et al. 1996). Human obesity is associated with increased circulating leptin levels, which have been proposed to play a role in insulin resistance and metabolic syndrome (Ahima and Flier 2000). In common obesity, leptin levels are already high and further augmentation by exogenously administered leptin does not significantly influence appetite and body weight most likely due to central leptin resistance (Ahima and Flier 2000). In addition to effects of leptin on insulin sensitivity (Blüher 2010), there may be a direct link between high circulating leptin concentrations and increased cardiovascular risk. Leptin may enhance platelet aggregation and arterial thrombosis (Konstantindis et al. 2001), promote angiogenesis, impair arterial distensibility and induce proliferation and migration of vascular smooth muscle cells (Singhal et al. 2002). In addition to these mechanisms, leptin seems to enhance the calcification of vascular cells (Parhami et al. 2001).

5. Does Adiponectin Link Obesity to Vascular Damage?

Adiponectin is highly expressed in adipocytes and has important antidiabetic, anti-atherogenic and anti-inflammatory properties (Turer and Scherer 2012). In contrast to many other

adipokines, adiponectin serum concentration is decreased in obesity, type 2 diabetes and other insulin resistant states (Turer and Scherer 2012). Furthermore, adiponectin expression and secretion increase upon improved insulin sensitivity and weight loss (Turer and Scherer 2012). Insulin-sensitizing TZDs probably mediate part of their effect via adiponectin since they increase plasma concentrations of this adipokine in both, subjects with normal insulin sensitivity and type 2 diabetics *in vivo* (Turer and Scherer 2012). In contrast, various hormones associated with insulin resistance and obesity including catecholamines, insulin, glucocorticoids, TNFα and IL-6 down-regulate adiponectin expression and secretion in fat cells *in vitro* (Turer and Scherer 2012). Besides its peripheral effects, adiponectin acts in the brain to increase energy expenditure and cause weight loss. The role of adiponectin as an endogenous insulin sensitizer is supported by knockout experiments in mice. Two independent studies demonstrate impaired insulin sensitivity in adiponectin knockout mice as compared to wild type controls (Kubota et al. 2002, Maeda et al. 2002). In transgenic mice, which overexpress adiponectin in adipose tissue, adiponectin was shown to have anti-obesity effects due to enhanced energy expenditure and impairment of adipocyte differentiation (Bauche et al. 2007). Adiponectin was shown to play a direct role in improving insulin sensitivity on the whole body level (reviewed in Turer and Scherer 2012). Adiponectin may have anti-atherogenic effects, and low adiponectin serum concentrations are associated with increased risk for cardiovascular disease (reviewed in Turer and Scherer 2012). Endothelium-dependent vasoreactivity is impaired in people with low adiponectin levels, which could contribute to the development of hypertension in visceral obese individuals (Ohashi et al. 2006). In addition, it has been suggested that adiponectin protects plaque rupture by the inhibition of matrix metalloproteinase function (Kobayashi et al. 2006), because adiponectin increases the expression of tissue inhibitor of metalloproteinase in macrophages and selectively suppresses endothelial cell apoptosis (Kobayashi et al. 2006). Adiponectin also inhibits the expression of adhesion molecules through the inhibition of nuclear factor-B activation and suppresses foam-cell formation (reviewed in Turer and Scherer 2012).

6. Does RBP4 Link Obesity to Vascular Damage?

Under normal conditions, retinol-binding protein-4 (RBP4) is predominantly secreted from the liver, but also expressed in adipocytes (Yang et al. 2005). However, increased RBP4 serum concentrations in patients with obesity and type 2 diabetes have been shown to be the result of increased RBP4 expression in visceral adipose tissue (Klöting et al. 2007). RBP4 was identified as a highly expressed circulating adipokine in mice lacking the glucose transporter 4 in adipose tissue (Yang et al. 2005). The effects of RBP4 may be mediated through retinol-dependent via retinoic acid receptors and retinoic acid-X receptors to regulate gene transcription or retinol-independent mechanisms. It has been demonstrated that RBP4 can induce the retinoid-regulated gene encoding the gluconeogenic enzyme PEPCK, increase basal glucose production, and reduce insulin action to suppress glucose production in hepatocytes *in vitro* (Yang et al. 2005). RBP4 has gained a lot of attention after the first notion that it is elevated in the serum of insulin resistant humans and mice (Yang et al. 2005) and that increased RBP4 serum concentrations are associated with many components of the metabolic syndrome including visceral fat accumulation (Klöting et al. 2007). Therefore, increased RBP4 serum concentrations might causally link (visceral) obesity to insulin resistance and its associated

metabolic and cardiovascular diseases. RBP4 was in addition to its metabolic associations shown to be associated with endothelial dysfunction in patients with newly diagnosed type 2 diabetes (Park et al. 2009). In another recent study, circulating RBP4 concentrations were inversely associated with intima-media and plaque echogenicity in carotid arteries, suggesting that RBP4 could be involved in the development of atherosclerosis (Ingelsson et al. 2009).

7. Does Chemerin Link Obesity to Vascular Damage?

Chemerin (RARRES2 or TIG2) is highly expressed in liver and white adipose tissue and secreted as 18-kDa inactive pro-protein which undergoes extra-cellular protease cleavage generating the 16-kDa active chemerin molecule (reviewed in Roman et al. 2012). It exerts potent anti-inflammatory effects on activated macrophages expressing the chemokine-like receptor-1 (CMKLR1) or the ChemR23 receptor (Blüher 2010). Chemerin plays an important role in adipocyte differentiation and modulates the expression of adipocyte genes involved in glucose and lipid homeostasis (Blüher 2010). Chemerin expression was shown to be decreased in adipose tissue of *db*/*db* mice compared with controls (Takahashi et al. 2008). In contrast, chemerin expression was significantly higher in adipose tissue of impaired glucose tolerant and diabetic *Psammomys obesus* compared with normal glucose tolerant sand rats (reviewed in Blüher 2010). ChemR23 knockout mice are unresponsive to chemerin and displayed an increased neutrophil infiltrate following LPS challenge (Luangsay et al. 2009). In a mouse model of acute lung inflammation induced by LPS, chemerin displayed potent anti-inflammatory properties, reducing neutrophil infiltration and inflammatory cytokine release in a ChemR23-dependent manner (Luangsay et al. 2009).

In humans, chemerin levels did not differ significantly between subjects with type 2 diabetes and normal controls (Bozaoglu et al. 2007). However, in normal glucose tolerant subjects, chemerin levels were significantly associated with BMI, triglycerides, and blood pressure (Bozaoglu et al. 2007). Moreover, adipose tissue explants from obese patients have significantly higher chemerin secretion than those obtained from lean controls (Sell et al. 2009). Sell et al. (2009) found in addition that chemerin release is correlated with BMI, waist-to-hip ratio, adipocyte volume as well as insulin resistance at the level of lipogenesis and insulin-induced antilipolysis in adipocytes (Sell et al. 2009). Importantly, adipocyte-derived secretion of chemerin may be involved in the negative crosstalk between adipose tissue and skeletal muscle contributing to the negative relationship between obesity and insulin sensitivity (Sell et al. 2009). Although circulating chemerin levels are significantly associated with markers of inflammation, visceral obesity and insulin resistance, circulating chemerin does not seem to predict coronary atherosclerosis (reviewed in Roman et al. 2012). More studies are necessary to elucidate whether chemerin contributes to the altered communication between adipose tissue and other organs in obesity.

8. Does Apelin Link Obesity to Vascular Damage?

Apelin, the endogenous ligand of the APJ receptor, has been identified in a variety of tissues, including stomach, heart, skeletal muscle, and white adipose tissue (reviewed in Castan-Laurell 2005). Apelin is a 36 amino-acid peptide identified in 1998 as the endog-

enous ligand of the orphan G protein-coupled receptor, APJ (reviewed in Castan-Laurell 2005). Apelin and APJ mRNA are widely expressed in several rat and human tissues and have functional effects in both the central nervous system and periphery (Castan-Laurell 2005). The cardiovascular system appears to be an important source of apelin since both apelin and its receptor are present in heart, large or small conduit vessels and endothelial cells (Castan-Laurell 2005). Apelin gene-deficient mice developed progressive impairment of cardiac contractility associated with systolic dysfunction, suggesting that apelin is crucial to maintain cardiac contractility in pressure overload and aging (Kuba et al. 2007). The observed changes in cardiac contractility were associated with concerted up regulation of genes involved in extracellular matrix remodelling and muscle contraction (Kuba et al. 2007). Apelin serum concentration was shown to be higher in patients with obesity and insulin resistance (reviewed in Castan-Laurell 2005). Recently, higher apelin serum concentrations were found to be associated with liver cirrhosis both in rats and humans. Moreover, treatment of rats with cirrhosis with an apelin receptor antagonist showed diminished hepatic fibrosis and loss of ascites suggesting the hepatic apelin system as a novel therapeutic target in liver disease (Principe et al. 2008). In addition, apelin has been shown to be involved in the regulation of cardiovascular function, fluid homeostasis, vessel formation and cell proliferation (reviewed in Castan-Laurell 2005). Recently reported glucose lowering effects of apelin seem to involve endothelial NO synthase, AMP-activated protein kinase, and Akt further suggesting that apelin might link obesity to insulin resistance, endothelial dysfunction and cardiovascular disease.

9. Conclusions

Adipose tissue is not only an energy storage organ, but is appreciated increasingly as an endocrine organ and part of an innate immune system. Bioactive molecules, so called adipokines, are secreted from adipose tissue and considerably contribute to the regulation of metabolism and inflammatory responses. Altered adipokine expression and circulating levels have been frequently reported in patients with cardiovascular and metabolic diseases including obesity and type diabetes. Altered adipokine profiles may well be a symptom of adipose tissue dysfunction. Genetic and environmental interactions may cause dysfunction of adipose tissue by initiating a sequence of adipocyte hypertrophy, hypoxia, several stresses and inflammatory processes. As a consequence, adipose tissue produces a proinflammatory, atherogenic, and diabetogenic adipokine profile, which may be mechanistically linked to the development of obesity associated disorders. Identification of novel adipokines which may link obesity to vascular damage represents a hot topic in obesity research. Our understanding of the complex effects of distinct adipokines and the interactions between these bioactive mediators is still incomplete.

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Mutations in the mtDNA as a Cause of Blindness and Diabetes

Simone Baltrusch and Markus Tiedge (Rostock)

With 6 Figures

Abstract

Most of the mitochondrial proteins are encoded by the nuclear genome. However, the genetic information for 13 polypeptides, which are integral parts of the respiratory chain complexes and the ATP synthase, is located on the maternally-inherited mitochondrial DNA (mtDNA). Pathogenic mtDNA mutations in the NADH-ubiquinone-oxidoreductase (complex I) cause Leber Hereditary Optic Neuropathy (LHON), a disease characterized by acute or sub-acute midlife blindness. The analysis of the disease mechanism is difficult, because mtDNA mutations cannot be experimentally generated. Cybrid models with mixed pathological mitochondria showed higher generation of reactive oxygen species (ROS) in retinal ganglion cells as a central trigger of apoptotic cell loss. However, this cell model did not allow analysing the impact of mtDNA mutations on complex organ function in mammals. In this review we show that conplastic mouse strains, which differ only in a single mtDNA mutation on a common nuclear genomic background open new perspectives for the investigation of the biochemical consequences of such variations. Interestingly, conplastic mice carrying a mutation in the ATP synthase (complex V) showed a fragmented mitochondrial phenotype with lower ATP levels and higher mitochondrial ROS generation and metabolic dysfunction.

Zusammenfassung

Die Information für fast alle mitochondrialen Proteine befindet sich im nukleären Genom. Allerdings werden 13 Polypeptide, die zentrale Untereinheiten von Atmungskettenkomplexen und der ATP-Synthase bilden, durch die maternal vererbte mitochondriale DNA (mtDNA) kodiert. Pathogene mtDNA-Mutationen in der NADH-Ubiquinon-Oxidoreduktase (Komlex I) führen zur Leberschen Optikusatrophie (*Leber Hereditary Optic Neuropathy*, LHON), einer Erkrankung, die akut oder schleichend zur Erblindung im mittleren Lebensalter führt. Da die Herstellung von mtDNA-Mutationen experimentell nicht möglich ist, ist die Aufklärung des zugrundeliegenden Mechanismus der Erkrankung schwierig. Unter Zuhilfenahme des Cybrid-Modells konnte eine erhöhte Bildung von reaktiven Sauerstoffspezies (ROS) als Auslöser der Apoptose und des Untergangs der Ganglienzellen der Retina ermittelt werden. Allerdings kann mittels dieses Zellmodells nicht der Einfluss von mtDNA-Mutationen auf Organebene in Säugetieren untersucht werden. Kürzlich wurden konplastische Mausstämme etabliert, die sich nur in einer einzigen mtDNA-Mutation unterscheiden und damit die Möglichkeit eröffnen, den Einfluss einer solchen Veränderung auf den Gesamtorganismus zu untersuchen. Interessanterweise konnte so gezeigt werden, dass Mäuse, die eine Mutation in der ATP-Synthase (Komplex V) tragen, nicht nur fragmentierte Mitochondrien und einen niedrigeren ATP-Gehalt aufweisen, sondern auch mehr mitochondriales ROS generieren und eine metabolische Fehlfunktion zeigen.

1. Introduction

Mitochondria play a crucial role in cellular bioenergetics. In contrast to traditional textbook knowledge these organelles are not autonomous, but act within a highly dynamic network. Fusion between mitochondria, subsequent fission as well as mitophagy are important pathways

to maintain mitochondrial function (Liesa et al. 2009, Westermann 2002). Depending on the cell type and the metabolic requirements of an organ, mitochondria appear different in shape, number and network structure. Mitochondria consist of two membranes, an outer and inner one, separating the intermembrane space (Fig. 1). Due to its cristae structure the inner mitochondrial membrane has a high surface area and hosts the enzyme complexes of the electron transport chain and oxidative phosphorylation as well as several substrate transport systems. Enzymes of the TCA cycle and fatty acid oxidation are located in the mitochondrial matrix.

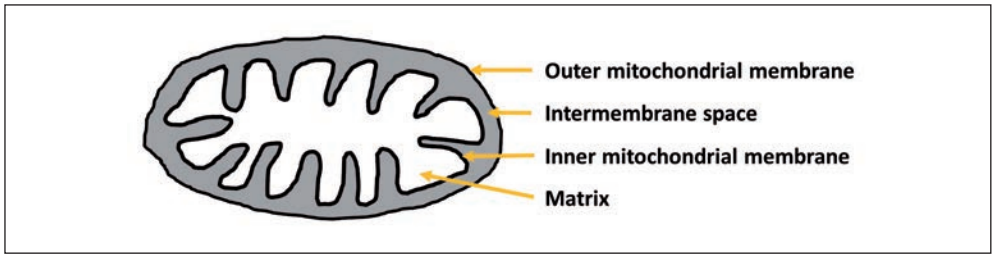

Fig. 1 Structure of mitochondria

2. Key Subunits of the Respiratory Chain Complexes and the ATP Synthase are Encoded by the Mitochondrial DNA (mtDNA)

The mitochondrial matrix contains thousands of copies of the maternally-inherited circular, double-stranded mitochondrial DNA (mtDNA) (Anderson et al. 1981). The mtDNA codes for 13 subunits of the respiratory chain complexes and the ATP synthase as well as the tRNAs and rRNAs necessary for mitochondrial protein synthesis (Fig. 2). All other subunits and assembly factors of the respiratory chain complexes, the ATP synthase and other mitochondrial proteins, are encoded by the nuclear genome. Thus, mitochondria are not self-replicating organelles. It is a key-topic of mitochondria research to elucidate how transcription and replication of mtDNA and nuclear DNA (nDNA) are coordinated, and functional complexes in the mitochondrial membrane are correctly assembled and adapted to metabolic requirements and cellular stressors (Angerer et al. 2011, Chatre and Ricchetti 2013, Koopman et al. 2010). Complex I is a NADH-ubiquinone-oxidoreductase, and seven subunits of this electron transporter are encoded by the mtDNA, namely ND1, ND2, ND3, ND4, ND4L, ND5, and ND6. Complex III is an ubiquinol-cytochrom c oxidoreductase responsible for the so-called Q-cycle of electron transfer with cytochrome b subunit encoded by the mtDNA. Complex IV is the cytochrome c oxidase eventually transferring the electrons to O_2. Three subunits of complex IV, COI, COII, and COIII, are encoded by the mtDNA. By the electron flow through complex I, III and IV, protons are pumped in the intermembrane space thereby generating a proton and charged gradient. This chemiosmotic force is used by the ATP synthase (complex V) to form ATP from ADP and Pi. ATP6 and ATP8 are polypeptides of complex V, which are encoded by the mtDNA. In contrast to control and repair mechanisms of the nDNA, mtDNA is poorly protected against mutations by reactive oxygen species (ROS) or chemical stressors. Thus, mtDNA mutations can easily accumulate and affect mitochondrial function. In addition to the known pathogenic mtDNA mutations, variations in the mtDNA are currently

discussed to be associated with complex polygenic diseases (diabetes, neurodegeneration), which appear bioenergetic in origin (Larsson and Rustin 2001, Taylor and Turnbull 2005, Wallace 2013).

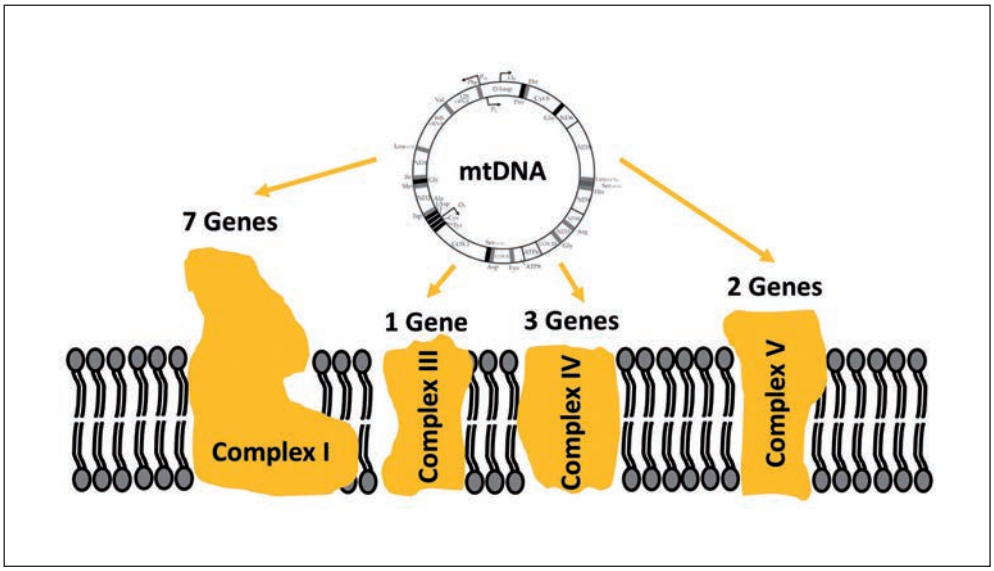

Fig. 2 The respiratory chain complexes and the ATP synthase are in part encoded by the mitochondrial DNA.

3. Pathogenic mtDNA Missense Mutations

In 1871 the German ophthalmologist Theodor Leber firstly described a genetically-inherited syndrome of visual loss resulting from cell loss in the optical nerve. In honor of this seminal achievement the syndrome was named Leber Hereditary Optic Neuropathy (LHON). However, it took more than 100 years to identify the molecular cause of this disease as mutations in ND4, but also ND1 and ND6 of complex I are responsible for the severe central vision loss (Huoponen et al. 1991, Johns et al. 1992, Wallace et al. 1988). Thus, LHON is a mitochondrial disease resulting from stable mtDNA mutations. In addition to rare variants in single families, three mutations are most frequently the cause of LHON. ND4 11 778 G to A nucleotide exchange resulted in an arginine to histidine exchange at position 340 in the polypeptide of ND4 (Wallace et al. 1988). ND1 3460 G to A nucleotide exchange resulted in an alanine to threonine exchange at position 52 in the polypeptide of ND1 (Huoponen et al. 1991). ND6 14 484 T to C nucleotide exchange resulted in a methionine to valine exchange at position 64 in the polypeptide of ND6 (Johns et al. 1992). Although the mode of inheritance of the disease is clearly maternal, male carriers of the mutation have a higher risk to develop clinical symptoms of LHON (Sadun et al. 2011). Notably, the severity of LHON is related to the percentage of mutated mtDNA per cell (Fig. 3). Patients with a higher copy number of mutated mtDNA and in particular ND6 14484C carriers also develop generalized central nervous system symptoms with movement disorder, impaired speech and mental retardation

(Chalmers and Schapira 1999). Why only retinal ganglion cells collapse eventually leading to blindness although the mutated mtDNA is also present in other tissues is hitherto unknown due to lack of corresponding animal models (Kirches 2011). Studies demonstrating reduced activity of the NADH-ubiquinone-oxidoreductase (Complex I) were performed in blood platelets and lymphocytes, but not in retinal ganglion cells (Carelli et al. 1997).

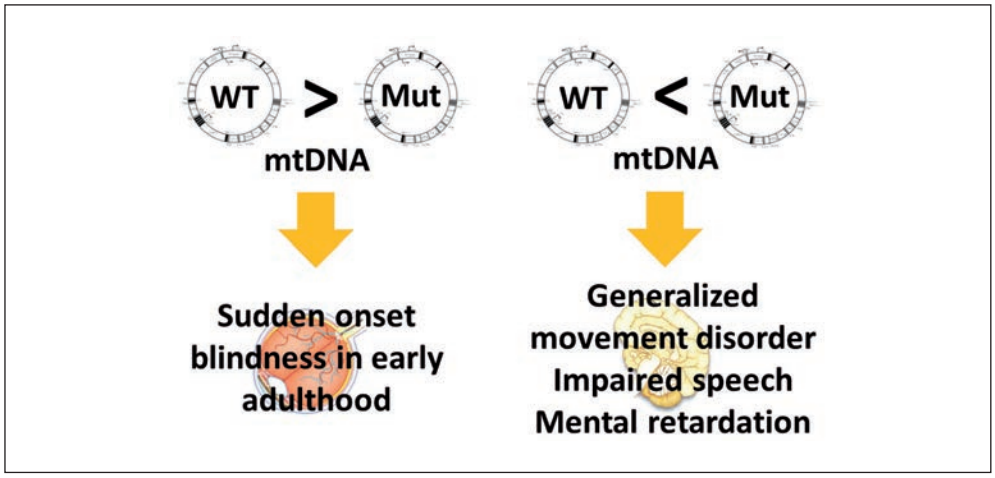

Fig. 3 Severity of Leber hereditary optic neuropathy (LHON) depends on the percentage of mutated mtDNA.

4. Cybrid Model Studies Revealed Higher ROS Production in Cells Carrying a Pathogenic mtDNA Mutation

To characterize the effect of a LHON mtDNA mutation on cellular function and viability in more detail the cybrid model (King and Attardi 1989) (Fig. 4) has been applied in different studies. The DNA-intercalating agent ethidium bromide treatment is applied to tumour cell lines for several passages resulting in depletion of mtDNA due to insufficient repair mechanisms in mitochondria. In these so-called Rho-0 cells ATP is exclusively generated by glycolysis, because the oxidative phosphorylation capacity is significantly reduced. Thereafter mtDNA of interest can be artificially introduced in Rho-0 cells via cytoplasts, cellular components which contain mitochondria but are free of nuclear compartments. By this way transmitochondrial cytoplasmic hybrids or shortly cybrids carrying LHON mtDNA mutations have been developed (Danielson et al. 2002, Ghelli et al. 2003, Wong et al. 2002). Due to leakage in the electron transport chain, especially by transfer of one electron to O_2 from the stable semiquinone, oxygene radicals are inevitably formed in mitochondria. Dysfunction of complex I in mitochondria with LHON mtDNA mutations increases ROS production concomitantly with a decline in the ATP production. This could be the trigger together with impaired axonal distribution of mitochondria, increased Ca^{2+} sensitivity and increased permeability transition probability for destruction of retinal ganglion cells (Kirches 2011). However, using the cybrid model the pathomechanism of an mtDNA mutation can only be analysed on the single cell level, but not on the level of tissues and organs. In particular it is

not possible to generate mice that are "transgenic" for a defined mitochondrial DNA mutation. The solution of this problem is the generation of conplastic mice following the strategy to combine stable mtDNA phenotypes with a defined nuclear background.

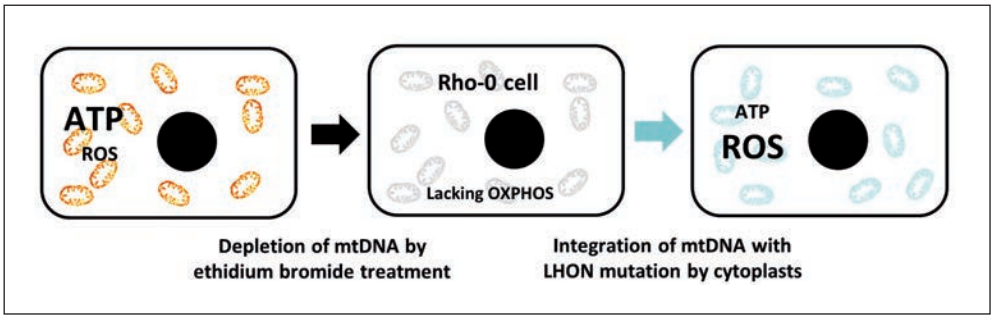

Fig. 4 Generation of cybrid cells

5. Conplastic Mice as a New Model to Study the Effect of mtDNA Mutations

It could be shown by sequence analysis that mtDNA from most common inbred mouse strains that have been generated during the last 100 years are descended from a single female (Goios et al. 2007, Yu et al. 2009a). A phylogenetic tree of mtDNA of inbred mouse strains revealed that a couple of mtDNA mutations occurred spontaneously during breeding, which resulted in characteristic mtDNA patterns for the different inbred mouse strains (Goios et al. 2007, Yu et al. 2009a). However, a specific phenotype cannot be directly related to an mtDNA mutation, because the mouse strains differ also in the nuclear genome. Conplastic mice are generated by choosing a defined background strain and breeding a male of this background strain with a female of an inbred mouse strains carrying the mtDNA mutation of interest. Offspring of the first generation carry mitochondria with this mtDNA mutation due to the maternal inheritance. In this first generation the nuclear genome is a mixture of both strains. After ten and more generations of backcrossing with males of the background strain, the conplastic mouse strain differs from the background strain only in the mtDNA (Fig. 5) (Yu et al. 2009a). Thus, this technique paves the way to analyse the effect of a stable homoplasmic mtDNA mutation on the level of living mammals.

6. An mtDNA Mutation in the ATP8 Gene Causes Higher ROS Level in Insulin-Secreting Pancreatic Beta Cells

Our group investigated the conplastic mouse strain C57BL/BTac-mt$^{FVB/NJ}$ in more detail due to its obvious ROS-generating phenotype (Weiss et al. 2012, Yu et al. 2009b). This conplastic mouse strain harbors the nt7778T polymorphism inside the ATP8 gene (complex V, ATP synthase) of the mtDNA from the FVB/NJ strain resulting in an amino acid exchange from aspartic acid to tyrosine (Yu et al. 2009a). This mouse strain showed a higher susceptibility to autoimmune diseases and fragmented mitochondria were visible in isolated cells from dif-

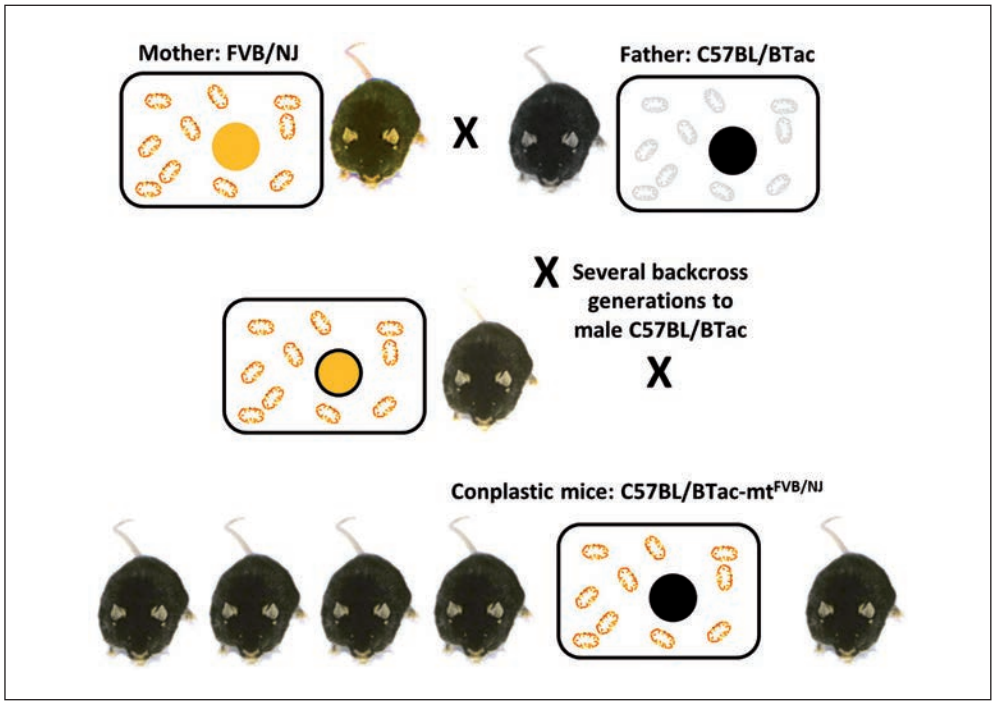

Fig. 5 Generation of a conplastic mouse strain

ferent tissues (Weiss et al. 2012, Yu et al. 2009b). As glucose-induced insulin secretion crucially depends upon metabolic stimulus-secretion coupling and thus mitochondrial functions, we then studied ROS generation in beta cells. The mitochondrial generation of ROS was measured in living insulin-secreting beta cells isolated from pancreatic islets after MitoSox® staining using fluorescence microscopy (Fig. 6).

In comparison to the control, beta cells from C57BL/BTac-mt$^{FVB/NJ}$ mice showed increased ROS generation and reduced ATP levels and glucose induced insulin secretion (Weiss et al. 2012). This raised the question of the metabolic impact from this secretory dysfunction. Whereas the glucose tolerance was still normal under control conditions in C57BL/BTac-mt$^{FVB/NJ}$ mice, feeding a high-fat diet for three months the animals develop impaired glucose tolerance (Weiss et al. 2012). Thus, the mtDNA mutation in the ATP synthase resulted in a high susceptibility to metabolic stress, which contributes to the manifestation of diabetes. On the other hand ROS generation in C57BL/BTac-mt$^{FVB/NJ}$ mice induced a significant proliferation of beta cells in response to the stressor, a phenomenon summarized as mitohormesis. The C57BL/BTac-mt$^{FVB/NJ}$ mouse is an interesting model to study a defined mitochondrial phenotype on a common nuclear genomic background. Ongoing experiments are focusing on mitochondrial dynamics (fission/fusion) and the effects on mitochondrial gene expression as a crosstalk with the nuclear genome. Conclusively, conplastic mouse strains will help to understand the impact mtDNA mutation as a trigger of adaptive processes during lifetime on complex metabolic and neurodegenerative diseases.

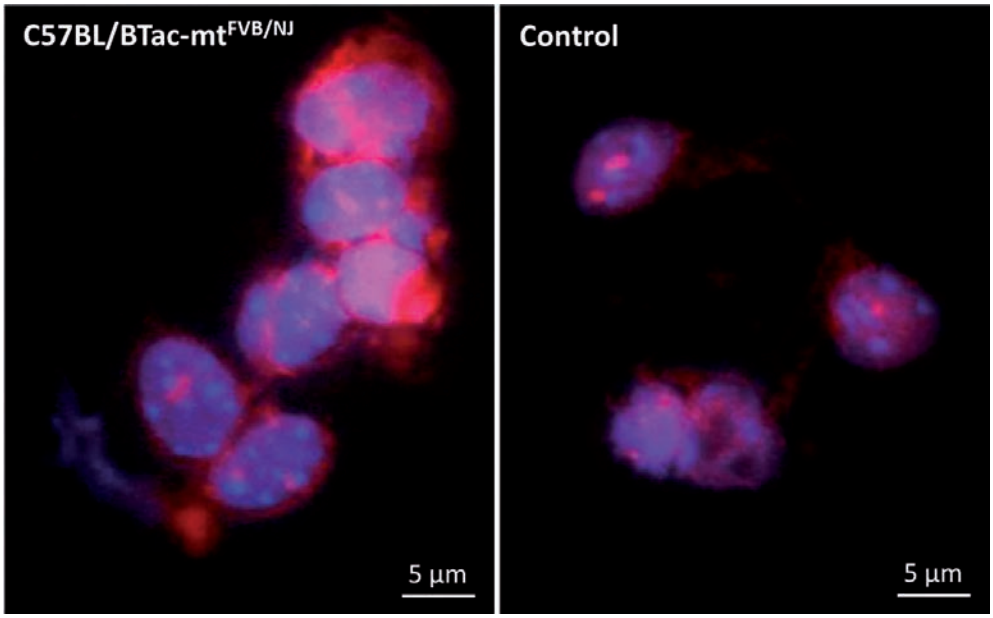

Fig. 6 Pancreatic beta cells of C57BL/BTac-mt$^{FVB/NJ}$ (*left*) and control (*right*) mice stained with MitoSox® (red colour indicates ROS) and DAPI (blue, nucleus) and cultured at 5 mmol/l glucose.

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Diabetic Complications: A Disease of Reactive Metabolites

Peter Paul Nawroth,[1] Stefan Herzig,[1, 2] and Thomas Fleming[1]

With 4 Figures

Abstract

Recent data indicate that glucose is only partially responsible for the development of late diabetic complications; however, reactive metabolites such as reactive oxygen species and reactive carbonyl species have been shown to play an important role in late diabetic complications via post-translational modification of proteins. Animal experiments and clinical studies have started to provide evidence that reactive metabolites, independent of glucose, can mediate late complications in diabetes.

Zusammenfassung

Nach neuesten Daten ist Glukose nur zum Teil verantwortlich für diabetische Spätschäden, jedoch sind reaktive Metabolite wie reaktive Sauerstoffspezies und Alphadicarbonyle durch posttranslationale Modifikation von Proteinen an der Entstehung diabetischer Spätschäden beteiligt. Tierexperimente und klinische Studien haben gezeigt, dass reaktive Metabolite unabhängig von Glukose als Mediatoren zur Entwicklung diabetischer Spätschäden an Organen beitragen.

1. Introduction

Diabetes has been defined clinically by a correlation between plasma glucose or glycated haemoglobin (HbA1c) and the development of late complications, such as microvascular (retinopathy, nephropathy and neuropathy) and macrovascular diseases (heart disease and stroke). Within this context, diabetes may therefore be viewed not as a disease, but as an epidemiological risk assessment for glucose. This has lead to lowering of blood glucose as the prevailing clinical strategy for prevention and development of these complications in both type 1 and 2 diabetics.

However, this simple cause-and-effect relationship is not necessarily as clear cut as we once thought. A revaluation of the Diabetes Control and Complications Trail (DCCT) has shown that although an effect of glucose lowering on complications is present, the effect is much less than expected. It was shown that HbA1c in combination with duration of diabetes

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could only predict *ca.* 11 % of diabetic patients at risk of microvascular complications (*The Diabetes Control and Complications Trial Research Group* 1995, Lachin et al. 2008). In an additional study from the DCCT, it was shown that whilst intensive insulin therapy can reduce the burden of renal failure in type 1 patients, it was only achieved in less than 1 % of patients over a 22 year period (*The Diabetes Control and Complications Trial Research Group* 2011). Several studies in type 2 diabetic patients have shown that not all patients benefit from better glucose control and/or reduction of HbA1c to within normal levels (Currie et al. 2010, UK *Prospective Diabetes Study Groups* 1998, Selvin et al. 2010, Matsushita et al. 2010). Whilst in the ADDITIONS study, it has been shown that there is a sub-group of diabetic patients with late complications, identifiable by high HbA1c despite normal oral glucose tolerance (Skriver et al. 2010). It is clear from these studies that the relationship between glucose and development of complications in diabetes is not linear, suggesting the involvement of factors and/or pathways other than hyperglycaemia in the development of complications.

For both type 1 and 2 diabetic patients, it has been shown that they are more susceptible to the formation of metabolic intermediates generated in glycolysis, in particularly trioses phosphate intermediates (glyceraldehyde-3-phosphate and dihydroxyacetone phosphate) (Fleming et al. 2012). The net concentration of trioses phosphates was found to correlate positively with the formation of methylglyoxal (MG); reactive metabolite and potent glycating agent formed the non-enzymatic degradation of the trioses phosphates (Fig. 1*A* and *B*). Interestingly, this correlation was only observed with respect to diabetes and was not present to such an extent in the healthy control subjects, and when normalized to intracellular glucose concentration, showed that diabetic patients could be characterized by dramatic increased intracellular reactive metabolites (Fig. 1*C* and *D*). This would suggest that within the context of diabetes, it is not the level of glucose, but how the system handles and processes the glucose which is important to the development of diabetic complications.

2. Formation of Reactive Metabolites

Reactive metabolites can be defined as a heterogeneous group of endogenous produced metabolites and compromise of two distinct but interrelated species; reactive oxygen species (ROS) and reactive carbonyl species (RCS). The formation of these reactive metabolites is inextricably and insidiously linked to the rate of metabolic flux and/or increased dependence on glycolysis for an energy supply within the cell (Fleming et al. 2010). In situations of increased energy demands (such as aging, tumour development and cancer) and in metabolic disorders with excessive energy supply (such as diabetes) the metabolic flux is enhanced. Under normal physiological conditions, the metabolic flux is controlled by a network of rate-limiting enzymes ensuring energy supply without an accumulation of reactive metabolites (Fleming et al. 2010). In situations of increased energy availability or energy demand, however, the consequences of increasing concentrations of highly reactive metabolites, which cannot be readily detoxified and/or metabolized by key enzymes of the metabolic network, is the inactivation of pathways, a dysregulation of the cellular energy homeostasis and substrate inhibition of key regulator enzymes, such as glyceraldehyde-3-phosphate dehydrogenase (Du et al. 2003, Lee et al. 2005, Beisswenger et al. 2003).

The production of ROS, which compromise superoxide anions (O_2^-), hydroxyl radical (HO·) and hydrogen peroxide (H_2O_2), is largely the result of living in an aerobic environment

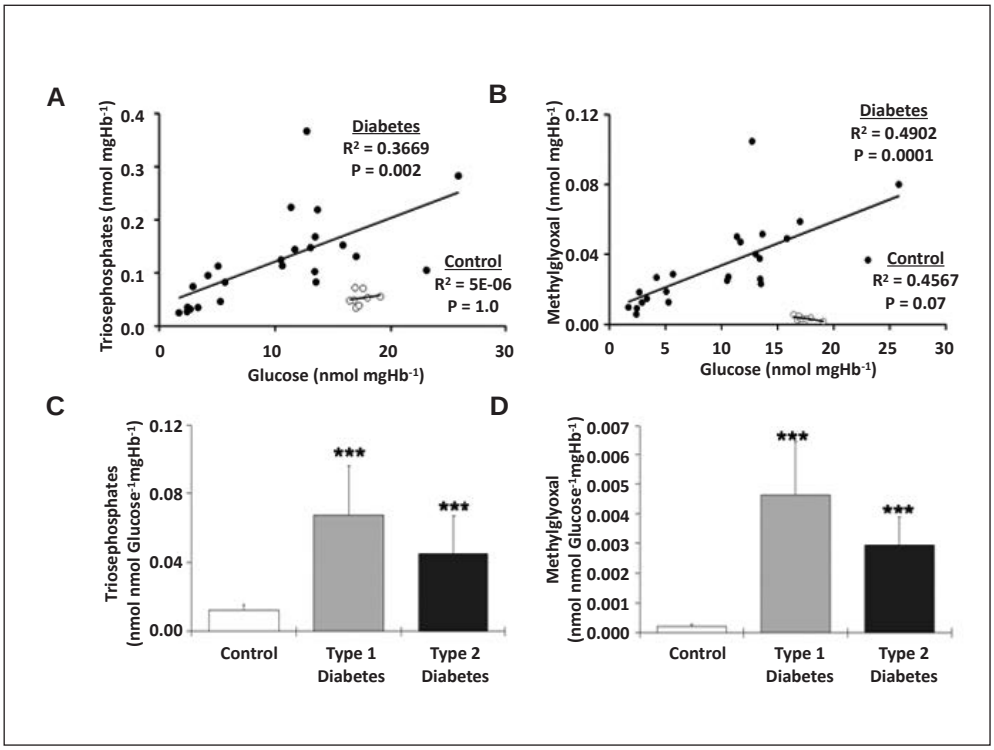

Fig. 1 Correlation of total trioses phosphate intermediates and intracellular glucose in haemolysate from healthy control (n = 10; *white dots*) and diabetic patients (n = 24, *black dots*). Correlation of MG and intracellular glucose in haemolysate from healthy control (n = *10*; *white dots*) and diabetic patients (n = 24, *black dots*). The net trioses phosphate pool normalized to intracellular glucose concentrations in healthy controls (n = 10), type 1 (n = 12) and type 2 (n = *12*) diabetic patients. Methylglyoxal normalized to intracellular glucose concentrations in healthy controls (n = *10*), type 1 (n = *12*) and type 2 (n = *12*) diabetic patients. Adapted from Fleming et al. 2012.

(Davis 1995), and the production is a natural by-product of the mitochondrial electron transport chain (Fig. 2*A*). It is estimated from *in vitro* experiments using isolated mitochondria that 0.12–2 % of respiration goes to O_2^- production, however, these values cannot be extrapolated to the *in vivo* situation, where mitochondrial O_2^- production would be considerable lower (Finkel and Holbrook 2000, Murphy 2009). Once formed, ROS can interact and damage a number of biological macromolecules, despite a short half-life and a limited sphere of influence (*ca.* five molecular diameters before it oxidizes a target) (Pryor 1986).

RCS are a heterogeneous group of small molecular weight carbonyls activated by α,β-unsaturation as in 4-hydroxynonenal and acrolein, α-oxo-substitution as in glyoxal and MG, and β-oxo-substitution as in malondialdehyde (Fig. 2*B*). These compounds are formed endogenously by lipid peroxidation, carbohydrate metabolism and autoxidation of reducing substrates (O'Brien et al. 2005). As with ROS, RCS exhibit a facile reactivity with various biomolecules, including proteins, DNA and phospholipids, generating stable products at the end of a series of reactions that are thought to contribute to the pathogenesis of vascular diseases such as atherosclerosis and diabetes. In addition, it has been recently found that some

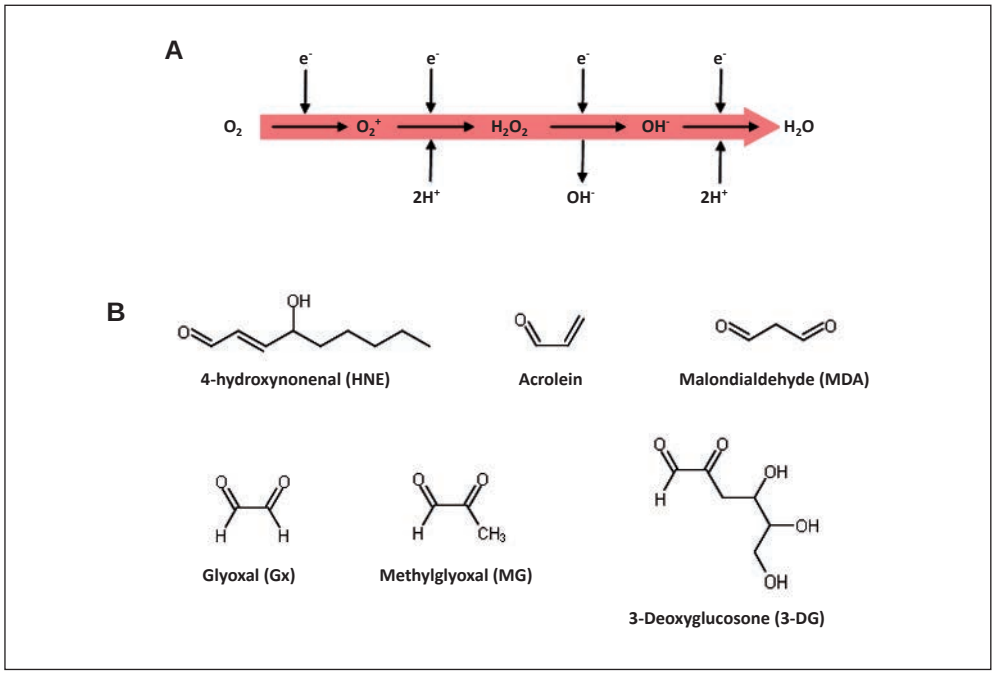

Fig. 2 Formation of reactive oxygen species from the univalent reduction of oxygen. The structures of reactive carbonyl species (RCS) generated from lipid peroxidation and carbohydrate metabolism and autoxidation.

of the RCS are responsible for the effects of lipid peroxidation and carbohydrate metabolism on signalling/transcription regulation suggesting that RCS may play a role as regulatory molecules of vascular dysfunction (O'Brien et al. 2005). These effects are similar to the detrimental effects caused with ROS accumulation and have in the past been either mistaken or overlooked. Compared to ROS, aldehydes are stable and can diffuse within or even escape from the cell and attack targets far from the site of their formation. Furthermore, several of the most reactive RCS are derived from glucose metabolism, such as MG, which belongs to the highly reactive class of RCS, known as the dicarbonyls (Thornalley 2005, 2008). As ROS require three stages of metabolism before production, RCS can be viewed as providing a more direct insult to the macromolecular integrity of the cell.

3. Detoxification

To minimize the production of reactive metabolites and subsequent damage induced, the body has evolved a multifaceted, intricate, response known as the antioxidant defence system. This is composed of enzymatic (superoxide dismutase, catalase, glutathione peroxidase and peroxyredoxins) and low molecular mass ROS scavengers (such as glutathione and vitamins) (Finkel and Holbrook 2000). One such defence mechanism with respect to MG involves limiting the availability of trioses phosphates which could spontaneously form MG. This is achieved by maintaining low concentrations of trioses phosphates during steady state gly-

colysis by feedback inhibition of phosphofructokinase 1 and capping the active site in trioses phosphate isomerase, preventing the fragmentation of the phosphoendiolate intermediate to MG and phosphate. Although effective in decreasing the formation of MG, some trioses phosphates does escape and as such cells have developed mechanisms for detoxification. MG can be metabolized by MG reductase to lactaldehyde, by aldose reductase to hydroxyacetone and by 2-oxoaldehyde dehydrogenase to pyruvate. However, the system that handles most of the cellular MG is the glyoxalase system (Thornalley 2003) (Fig. 3*A*).

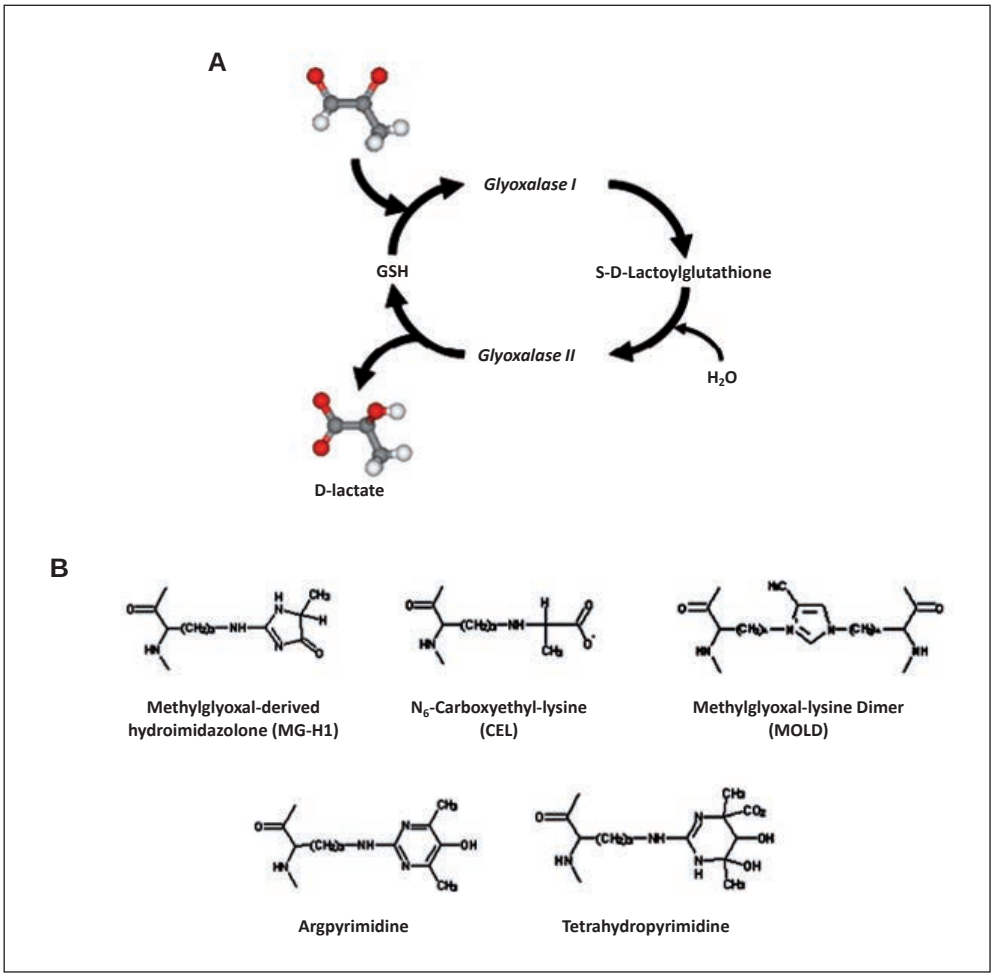

Fig. 3 The glyoxalase system. The major MG-derived AGEs

Whilst these defences are effective in reducing the daily production of reactive metabolites, when they become diminished, impaired or overwhelmed, then an imbalance will occur resulting in an accumulation of reactive metabolites, giving rise to a state of cellular stress (Davis 1995, Finkel and Holbrook 2000).

4. Role of Methylglyoxal in Mediating Late Diabetic Complications

MG like most reactive metabolites can react with proteins, specifically at arginine, lysine and cysteine residues, in process referred to collectively as glycation to form advanced glycation endproducts (AGEs) (Fig. 3*B*). The physiological importance of protein glycation and AGEs remains under investigation. Particularly damaging effects are produced by covalent crosslinking of proteins which confers resistance to proteolysis (Thornalley 2008). Protein modification is also damaging when amino acids residues are located in sites of protein-protein interactions, enzyme-substrates interactions and protein-DNA interaction. Studies looking at the modification of model proteins such as human serum albumin (Ahmed et al. 2005) and vascular basement membrane type IV collagen by MG (Dobler et al. 2006) have shown that formation of AGEs causes structural distortions, loss of side chain charge and functional impairment.

Recently, we have shown that elevated MG in diabetes leads to the modification of arginine and/or lysine residues within voltaged-gated sodium channels (VGSCs), in particularly the tetrodotoxin-resistant (TTXr) $Na_v1.8$ and the tetrodotoxin-sensitive (TTXs) $Na_v1.7$. Modification of $Na_v1.8$ was associated with increased electrical excitability and facilitated firing of nociceptive neurons, whereas modification of $Na_V1.7$ promoted slow inactivation (Bierhaus et al. 2012). In mice, treatment with MG reduced nerve conduction velocity, facilitated neurosecretion of calcitonin gene-related peptide, increased cyclooxygenase-2 expression and evoked thermal and mechanical hyperalgesia, as well as increasing blood flow in brain regions involved in pain processing (Fig. 4).

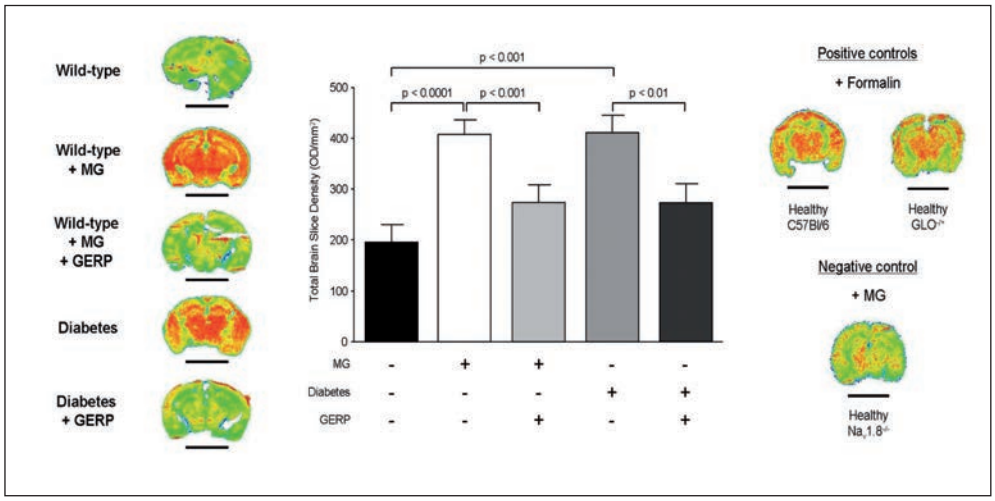

Fig. 4 Methylglyoxal induced thermal hyperalgesia results in increased brain activity. Sample colourized coronal sections showing differences in brain activation after heat stimulation; increased cerebral blood flow is indicated in red. Each image is taken from a single brain section in the individual mice. Data represent the mean SD. (N = 4 mice per group). Adapted from Bierhaus et al. 2012.

Similar changes were also observed streptozotocin-induced and genetic mouse models of diabetes but not in $Na_v1.8$ knockout mice. Several strategies including the use of a novel peptide

scavenger for MG were shown to be effective in reducing MG- and diabetes-induced hyperalgesia (Bierhaus et al. 2012). These finding provide not only a novel, reactive metabolite mechanism for development of diabetic neuropathy but also provide an explanation for the coexistence of positive and negative clinical symptoms in diabetic neuropathy. Similar findings have also been reported for TRPA1 (Eberhardt et al. 2012) and TRPV1 (Onkawara et al. 2012), as well as in diabetic retinopathy (Hammes 2003).

5. Conclusion

Hyperglycaemia is a fundamental clinical characteristic of diabetes, but it is not the direct mediator of late diabetic complications, given the less than anticipated benefit which comes from lower blood glucose. A new definition of diabetes is required which takes into account the balance between excess reactive metabolite formation and reduced detoxification. Such an approach will provide new therapeutic approaches to the yet unsolved problem of late diabetic complications.

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Session 2:
Ophthalmic Imaging:
The Eye as a Biomarker for Neuropathy

Nova Acta Leopoldina NF *119*, Nr. 401, 109–113 (2014)

Novel Imaging Technique

Jens Dawczynski (Leipzig)

With 5 Figures

Abstract

Within the last years different new retinal imaging technologies have been developed and published. For routine use in daily practice an additional benefit for instance image resolution or early changes detection is necessary. Optical coherence tomography (OCT) becomes more and more a routine diagnostic tool and will be used for retina imaging. Special devices like dynamic vessel analysis (DVA) and dual wavelength oximetry may be used for early detection of retinal changes and do have a potential as screening technologies in the future.

Zusammenfassung

In den vergangenen Jahren wurde eine Vielzahl neuer Imaging-Verfahren im Bereich der Netzhaut entwickelt. Für die breite Anwendung in der Praxis ergibt sich immer die Frage des zusätzlichen Nutzens bzw. der möglichen Anwendung als Screening-Technologie. Die optische Kohärenztomographie (OCT) erlangt auf Grund ihrer einfachen Anwendung und exzellenter Auflösung zunehmende Bedeutung in der Routinediagnostik. Spezielle Verfahren wie die dynamische Gefäßanalyse (DVA) und die Zweiwellenlängenoximetrie haben ihren Stellenwert in der Frühdiagnostik von Netzhautveränderungen.

New imaging technologies of the eye and especially of the posterior part of the eye become more and more popular within the last years. There are several new developments and some of them are already at the market and commercially available. From a clinical point of view the question is always what are the benefits of this new techniques. Of special interest is always the matter, if there are any new, additional information with the new techniques and if it is possible to use them as early screening techniques.

If it would be possible to design a "perfect" imaging technique for the eye it should:

- be easy to use,
- give clear distinctions of findings,
- be applicable for elderly and handicapped people, too,
- ease to use,
- affordable.

For decades ophthalmoscopy and fluorescence angiography have been the gold standard for investigation of changes in the posterior region of the eye. And also today, these techniques are common used and necessary. But because of possible anaphylactic reaction, the uses of invasive methods like fluorescence angiography should be questioned in each case.

For routine retina diagnostic spectral domain high resolution optical coherence tomography (SD-OCT) becomes more and more popular. It allows a non-invasive and often non-mydriatric assessment of the retina and optic disc (Fig. 1). Especially vitreoretinal ad-

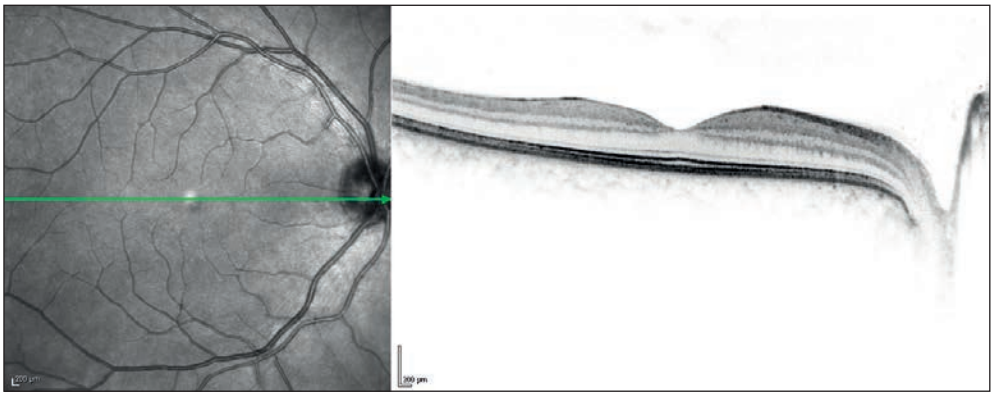

Fig. 1 Optical coherence tomography of healthy subject (central retina area with macular region and optic disc)

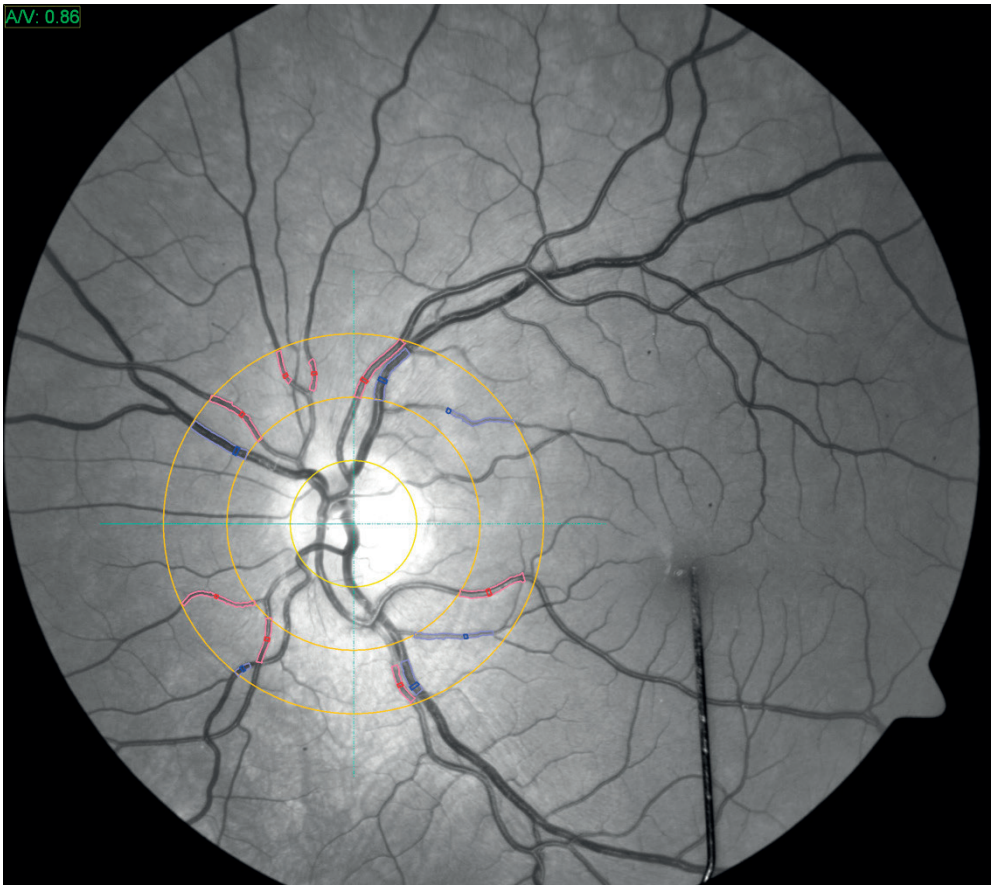

Fig. 2 Static and dynamic vessel analysis of venous (*blue*) and arterial (*red*) retinal vessels

hesions and intraretinal changes like edema may be detected very precisely. SD-OCT is also for follow-up investigations a very useful tool. Within the next years new OCT developments will enter the market, which may allow estimating also deeper areas like choroidea and will also offer higher resolutions.

New imaging techniques could offer new opportunities for early detection of vascular changes, e.g. in early diabetic retinopathy (Fig. 2 and 3). Dawczynski et al. (2007) demonstrated that endothelial dysfunction of retinal vessels is detectable by flicker provocation test. Healthy retinal vessels show a transient dilation after flicker light stimulation. This reaction, triggered by the release of nitric-oxidase, gives indirect evidence for endothelial function of the vessel. In diabetic retinopathy with disturbed endothelial function a decrease in vessel dilation could be observed.

Furthermore, Mandecka et al. (2007) demonstrated not also an increasing decrease in vascular reaction with increasing severity of diabetic changes (Fig. 4). They also found a decrease in arterial diameter change in diabetic patients without visible signs of diabetic retinopathy. Therefore, dynamic vessel analysis might also be considered as screening method for early diabetic changes of the retina.

Hammer et al. (2011) developed a non-invasive technique for determination of haemoglobin oxygenation of retinal vessels. By the so-called dual-wavelength oximetry transmission spectra of reduced (Hb) and oxygenated (HbO_2) haemoglobin as well as filter and spectral sensitivity of the red and the green camera channel are used to appreciate arterial and venous haemoglobin oxygenation. By combination with dynamic vessel analysis (Fig. 5) changes after flicker stimulation could be detected. In patients with diabetic retinopathy Hammer et al. (2012) demonstrated an increase of retinal venous oxygen saturation.

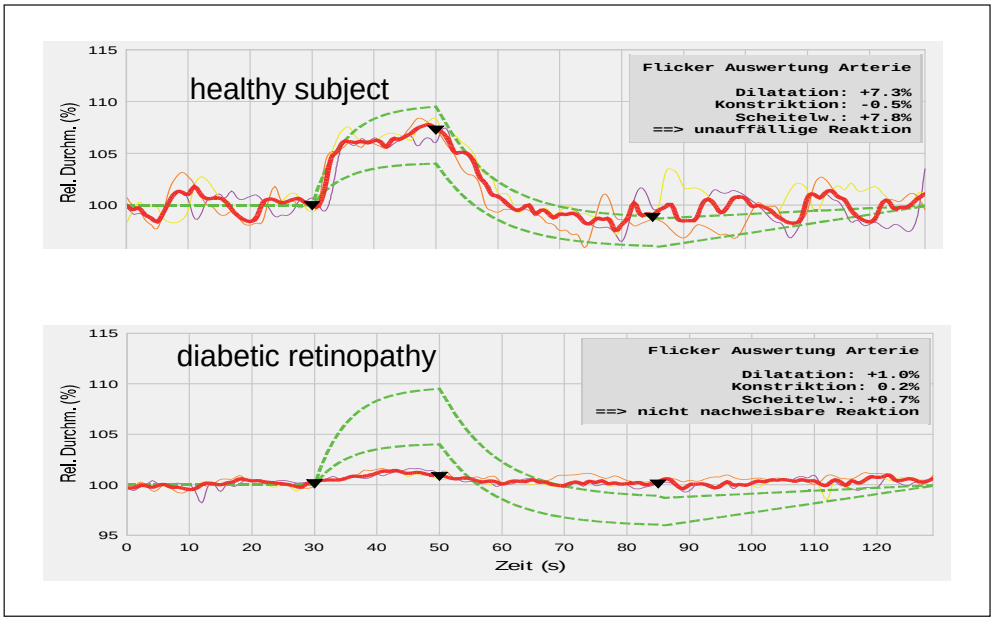

Fig. 3 Dynamic vessel analysis in healthy subject with normal vessel dilation to flicker light and decreased vessel dilation in patient with diabetic retinopathy

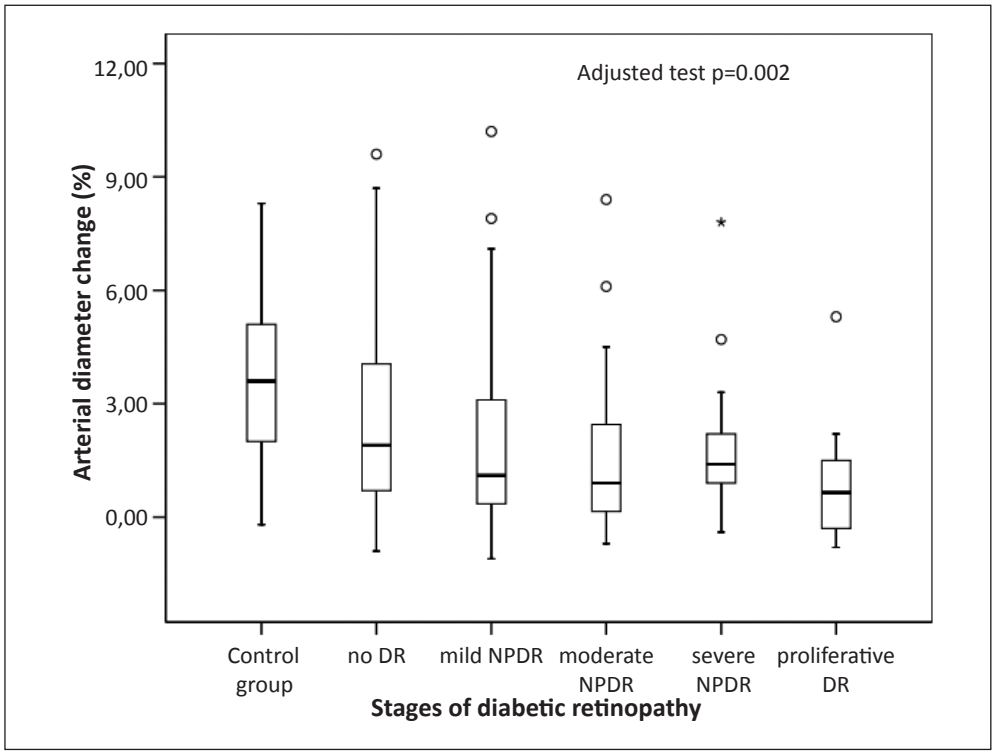

Fig. 4 Dynamic vessel analysis: arterial diameter changes at stages of diabetc retinopathy (DR), Mandecka et al. 2007

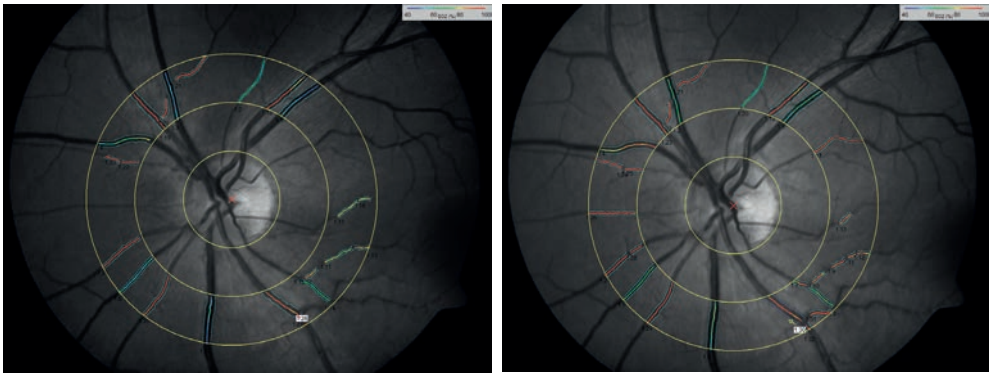

Fig. 5 Dual wavelength oximetry: colour-coded oxygen saturations before (*left*) and during flicker stimulation in a healthy subject

Very recent results by Jian et al. (2013) demonstrate a possible further direction of retinal imaging in the future. By combination of adaptive-optics system with optical coherence tomography a new quality of resolution for *in vivo* imaging could be reached.

In Conclusion there are many new imaging technologies especially for detection of early changes in diabetic retina. Combination of high resolution imaging techniques (SD_OCT) and dynamic tests (dynamic vessel analysis/dual wavelength oximetry) could be used as early screening method. In near future new devices with even higher resolution will be available.

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Nachhaltigkeit in der Wissenschaft

Leopoldina-Workshop
am 12. November 2012 in Berlin

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Im Mittelpunkt der weltweiten Überlegungen zur Bewältigung zentraler Herausforderungen des 21. Jahrhunderts steht das Konzept der Nachhaltigkeit. Damit dieses Prinzip sich in konkreten Handlungsvorschlägen widerspiegeln kann, bedarf es der Präzisierung. Der Band untersucht daher die Nachhaltigkeit in der Wissenschaft, der wichtigsten Informationsquelle der Gesellschaft. Dabei wird Nachhaltigkeit sowohl der Strukturen als auch der Aktivitäten in Forschung und Lehre betrachtet. Behandelt werden die „Erforschung von Nachhaltigkeit“, die Strategien zum besseren Verständnis liefern soll, der Komplex „nachhaltig forschen“, der Voraussetzungen, Verläufe und Folgen von Forschung gemäß den Kriterien der Nachhaltigkeit analysiert, und die „Nachhaltigkeit von Forschung“, die Wesensprinzipien der Wissenschaft – etwa die Falsifizierbarkeit ihrer Resultate – im Lichte der Idee der Nachhaltigkeit untersucht. Schwerpunkte der Analyse bilden in allen Bereichen einerseits das Spannungsverhältnis zwischen Freiheit und Nachhaltigkeit der Wissenschaft sowie andererseits die Auswirkungen der Debatte auf die Strukturen des Wissenschaftssystems.

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Advances in *In vivo* Imaging of Corneal Nerves

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Karsten Winter,[3] and Bernd Köhler[4]

With 10 Figures

Abstract

The demands of contemporary corneal imaging have evolved from its descriptive findings using the slit lamp to *in vivo* assessment of changes at the cellular level. Currently, the latter can be provided by *in vivo* confocal microscopy. This article gives an overview of corneal nerve imaging using the up to date techniques, describes the milestones in confocal imaging of corneal nerves, and illustrates the possibilities and difficulties to translate this technology from the laboratory into clinical practice.

Zusammenfassung

Die Anforderungen der kornealen *In-vivo*-Bildgebung der Gegenwart haben sich gravierend gewandelt, ausgehend von einer deskriptiven Beschreibung durch die Spaltlampe hin zu einer *In-vivo*-Beurteilung der Hornhaut auf zellulärer Ebene. Die konfokale *In-vivo*-Mikroskopie erweist sich hier als exzellente Untersuchungsmethode. Dieser Artikel gibt einen Überblick über die Entwicklung der konfokalen *In-vivo*-Laserscanningmikroskopie und beschreibt die Meilensteine bei der Darstellung kornealer Nerven. Weiterhin werden die Möglichkeiten und Schwierigkeiten diskutiert, diese Technologie vom Labor in die klinische Praxis zu überführen.

1. Introduction

In ophthalmology, primary diagnosis largely depends on the availability of reproducible and reliable data concerning changes in normal structures during health and disease. In this process, the importance of 'biomicroscopy' to understand the morphologic phenotype of the pathologic condition has been extensively dominated by slit-lamp examinations. Later in this series of developments, despite the established methods of imaging the living structure and after the wide acceptance of published images of tissue microstructure, *in vivo* confocal microscopy largely remained a tool for research laboratories with an interest in the clinical applications of prototype instruments.

In vivo confocal laser scanning microscopy (CLSM) has been used as a powerful tool for the non-penetrative, real-time visualization and analysis of living corneal tissues at the cellular level of both laboratory animals (Cavanagh et al. 1993) and in humans (Cavanagh et al.

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2000, Eckard et al. 2006, Elder 1961, Grupcheva et al. 2002). By using this versatile tool, several working groups have described 2-dimensional mapping (Patel and McGhee 2005, Yokogawa et al. 2008, He et al. 2010) of the living human ocular surface and cornea *in vivo*. In all these studies this complex procedure was executed off-line with the use of external software support for the very time-consuming image post-processing.

2. Milestones of *In vivo* Corneal Confocal Microscopy

A sequence of continuous and interlinked technical improvements over the years has led to the development of present day *in vivo* confocal microscopy. At the beginning, a real-time tandem scanning confocal microscopy (CM) was introduced by Petran and Hadravsky, in which the basic part of the system was contributed by Nipkow, to provide real-time point illumination and point detection. The main properties of the system include video rate, true colour and direct observation in combination with a marginal image quality based on the low intensity of the reflected light (Böhnke and Masters 1997). Advancement was marked by the use of one sided Nipkow disc CM based on the work of Xiao et al. (1988) and Xiao et al. (1990). A Nipkow disc is a mechanical scanner consisting of a rotating disk with small holes upon its periphery through which narrow beams of light pass. This simple design suffered from the disadvantage of low intensity of illumination during image formation. A clinical version of this original microscope was first manufactured by the Tandem Scanning Corporation (Reston, VA, USA) and later by the Advanced Scanning Corporation (New Orleans, LA, USA).

The time required for scanning can be strikingly reduced by using a slit aperture where all points in the axis of the slit will be scanned at the same time. Here, the adjustable slit height permits the user to vary the thickness of the optical section, and also allows to control the amount of light that reaches the cornea. Because of the use of the slit instead of a pinhole, this microscope is truly confocal only in the axis perpendicular to the slit and the contrast of the system is very low. Slit scanning *in vivo* CMs are available commercially from different manufacturers like Tomey Corporation (USA), Nidek Technologies (Japan) and Helmut Hund (Wetzlar, Germany).

Rapid advances in laser technology have enabled the development of a completely other generation of confocal microscopes. The CM, invented by Minsky, was designed mainly for the clinical examination of the human eye and enabled clinicians to observe unstained, living ocular tissue at the cellular level (Young and Roberts 1951, Davidovits and Egger 1971, 1973). Subsequently, based on Minsky's design, Webb developed a laser scanning ophthalmoscope for the observation of human retina in real-time (Webb et al. 1980, 1987, Webb 1990), where a coherent laser is used as a high intensity light source, and the laser beam is scanned over the microscope focal plane by a set of galvanometer scanning mirrors to enable fast scanning in x and y directions.

Webb's concept was implemented later in the development of the Heidelberg Retina Tomograph (HRT) (Heidelberg Engineering, Heidelberg, Germany), one of the well-established *in vivo* confocal imaging systems in clinical ophthalmology. The HRT device uses a coherent 670 µm diode laser source instead of white-light, to acquire and evaluate topographic measurements of the optic nerve head to detect glaucomatous damage. The HRT was further modified by Stave et al. (2002) (University of Rostock, Germany) who developed a detachable objective system mounted onto the HRT, named 'Rostock Cornea Module' (RCM),

transforming the HRT into a high-resolution CLSM to enable the visualization of the ocular anterior segment (Fig. 1). At present, application of CLSM in ophthalmology is not restricted to corneal research, but also includes the evaluation of non-transparent tissues of the ocular surface including conjunctiva, sclera, limbal region, lacrimal gland, and tear film as single anatomical units and parts of eye-associated lymphatic tissue (Zhivov et al. 2006).

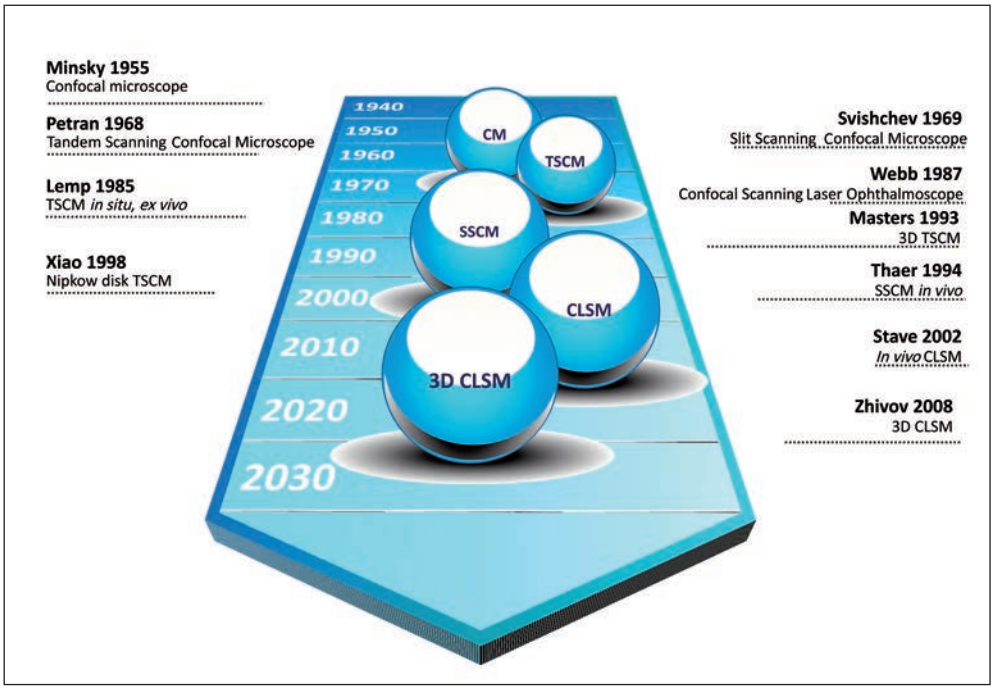


Fig. 1 Milestones in corneal confocal imaging

In summary, confocal microscopy is established as a valuable tool for acquiring high resolution images and allowing 3-D reconstructions of topologically complex biological specimens. Investigation of the cornea with *in vivo* confocal microscopy has been very useful in expanding our understanding of nerve and keratocyte anatomy in healthy and diseased human corneae. The representation of nerve fibres leashes running parallel to Bowman's layer can be visualized and depicted effortlessly whereas stromal fibres can be seen only as short reflected lines due to their oblique orientation. In conclusion, *in vivo* CLSM enables imaging of the corneal nerves comparable to histological sectioning (Fig. 2).

3. Disadvantages of CLSM Imaging

Even though CLSM has proved to be a great tool for analysing living corneal tissues in laboratory animals and humans, its principal limitation is the small field of view for imaging (HRTII-RCM 400x400 µm, i.e. 0.160 mm^2; Nidek Confoscan 4460 × 345 µm, i.e.

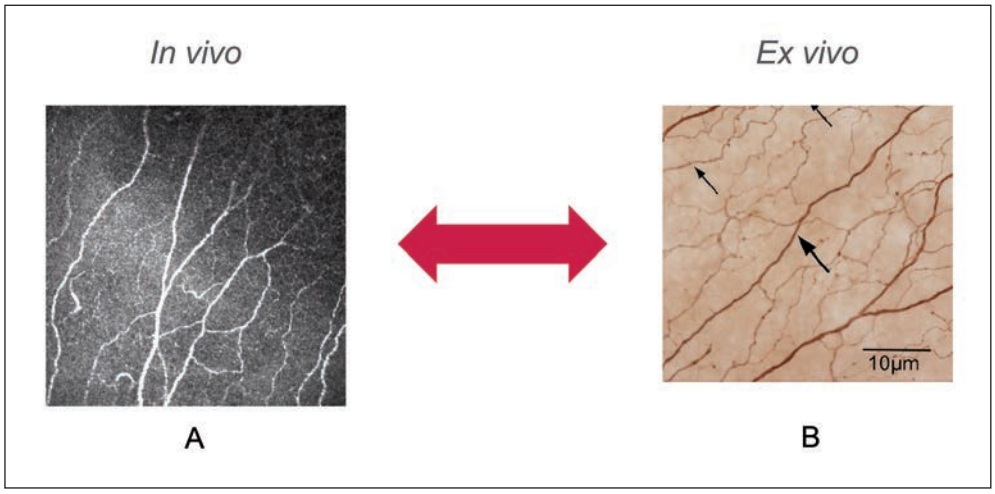


Fig. 2 Comparison of the images of (*A*) *in vivo* CLSM (Reichard et al. 2010) and (*B*) histology (Marfurt et al. 2010) focused on corneal nerveimaging.

0.159 mm²). The HRTII-RCM field of view in comparison to the ocular surface is given in Figure 3. Secondly, during CLSM imaging, so far the reproducible examination and quantification of the same area is very unlikely and almost impossible. Also, techniques have to be established to create two-dimensional maps of the corneal sub-basal nerve plexus.

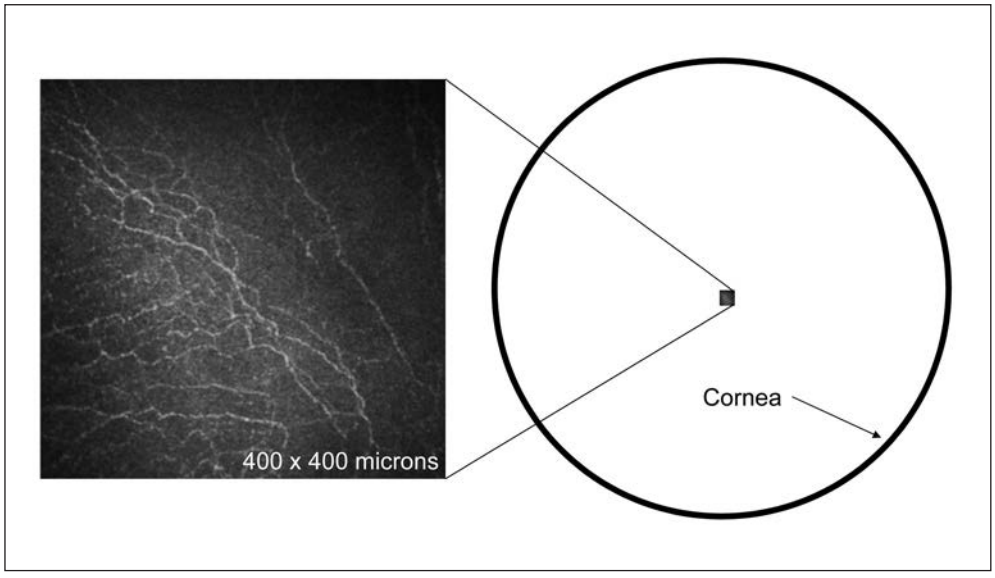


Fig. 3 The CLSM field of view (400 × 400 microns) in comparison to the surface area of cornea (from Zhivov et al. 2010).

4. History of Corneal Nerve Plexus Reconstruction

4.1 Manual Mapping

Several research groups have published reports of two-dimensional reconstruction mapping of stained corneal whole mounts (Al-AqabaL et al. 2010) as well as of the living human cornea (Stachs et al. 2010, Patel et al. 2005, 2006, Yokogawa et al. 2008). In addition, our own group has published results of *in vivo* three-dimensional reconstruction of the ocular surface and cornea (Stachs et al. 2007, Zhivov et al. 2006, 2009).

One of the simplest ways to make a 2D reconstruction of the nerve plexus is a manual mapping with the aid of computer-based tools. Figure 4 depicts a two-dimensional mapping (slice alignment, intensity adjustment, resampling), which was performed manually by using the software AMIRA (TGS Inc, San Diego, Calif.) and STITCHER (RealVIZ S.A., San Francisco, Calif.). Even though a number of papers has been published using this technique, the main limitation of manual mapping is the time required for the image reconstruction and processing which is a major limitation of its application in clinical practice and trials.

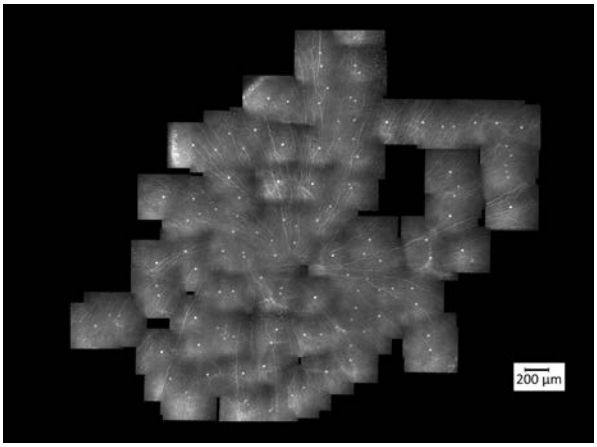


Fig. 4 Manual assembly of 150 images depicting the architecture of the sub-basal nerve plexus in a 30-year-old healthy male volunteer (Zhivov et al. 2009).

4.2 Real-Time Mapping of the Corneal Sub-basal Nerve Plexus

Recently we have published a method to generate two-dimensional reconstruction maps of the SNP in the living cornea in real-time by *in vivo* laser scanning confocal microscopy (Zhivov et al. 2010). CLSM source data (frame rate 30 Hz) was used to create large-scale maps of the scanned area by means of the ART (automatic real time) composite mode (Guthoff et al. 2010). The algorithm attempts to align single live image onto the so far mapped composite image by means of landmark feature based image processing using an affine transformation. The six transformation parameters (2×2 transformation matrix and translation in x and y) between the actual live image and the composite image are calculated using automatically extracted landmark features in both images. Because of the four additional degrees of freedom

(compared to using only translations between images), this spatial transformation is better capable of aligning corresponding corneal structures in the transformed live image and the composite image. Initially a single live image is used as the first instance of the composite image. The successive transformed live images then add to the composite image (Fig. 5). Live images are discarded if transformation parameters cannot be reliably determined.

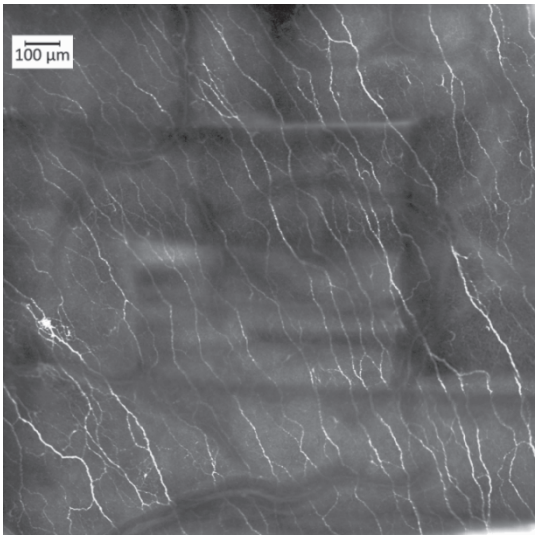


Fig. 5 A real time reconstruction using the ART composite mode of the HRT.

4.3 Offline Mapping

Even though the described ART composite mode is an immense improvement over the manual generation of SNP mosaic images used before, both in terms of time required and of the possibility to handle motion artefacts, the affine transformation used can only reduce, not entirely correct the motion-induced image distortions. To be precise, it can fully compensate linear, but not accelerated movements. Because erratic involuntary and unavoidable (non-linear) eye movements always occur during the image acquisition process, Allgeier et al. (2010) developed an elastic image registration method that has been specifically adapted to the image acquisition process of the CLSM. In theory the proposed image registration method is capable of correcting any motion-induced image distortions. The resulting SNP mosaic images (Fig. 6) verify that motion artefacts are in fact significantly reduced compared to an ART composite map. Because the used computation is time-consuming and includes a global optimization step to minimize alignment errors over the entire image sequence, the mapping process can only be operated offline.

4.4 Volumetric Image Analysis

The most secure and effective way for large scale SBP mosaicking is the use of volumetric information, SBP reconstruction followed by image fusion. This approach was implemented

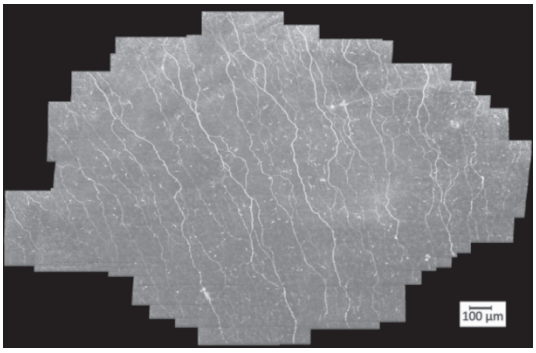


Fig. 6 Mosaic image generated offline using the registration method developed by ALLGEIER et al. 2010.

by KÖHLER et al. (2010) and ALLGEIER et al. (2012) according to the pipeline depicted in Figure 10 *left*. The proposed technique features the reconstruction of SNP images from CLSM volume scans and the generation of mosaic images to provide an expanded view of the SNP. An example for a successful reconstruction can be found in Figure 7.

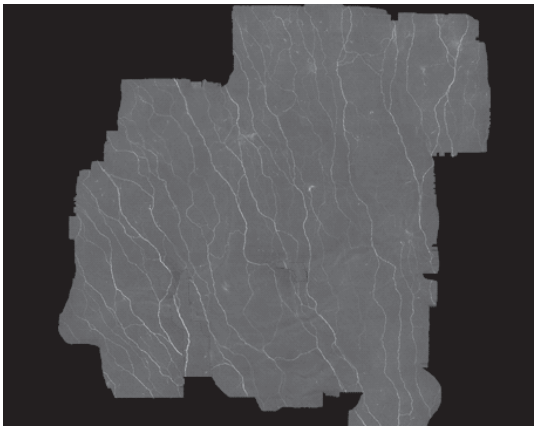
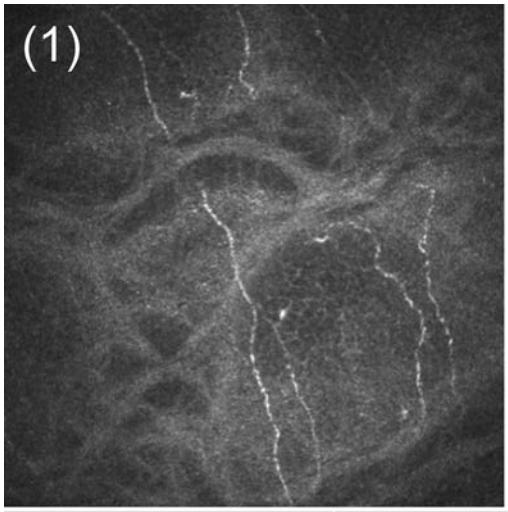
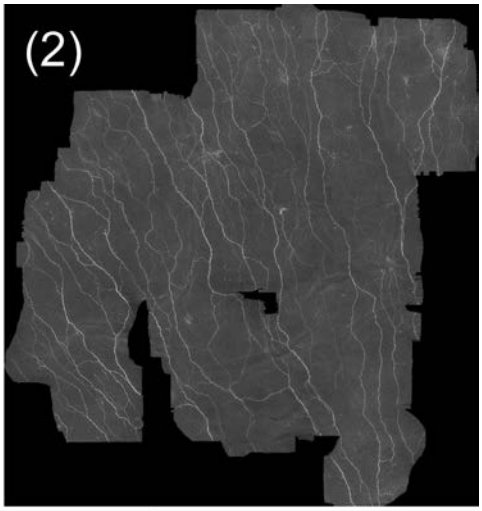


Fig. 7 Nerve plexus reconstruction and subsequent image fusion from volume data sets according to the technique developed by ALLGEIER and KÖHLER (ALLGEIER et al. 2012, KÖHLER et al. 2010).

5. Efforts and Outcome of the Different Image Sources for Quantification

In conclusion, there are diverse image acquisition sources available for nerve fibre quantification which can be classified into two main categories: "uncorrected single image analysis" and "advanced image analysis" using SNP reconstruction in combination with a large scale image fusion. Despite the availability of different sources, the effort and outcome of the final quantification is very diverse. So, towards getting reliable and repeatable quantifications, future studies should concentrate on showing what is stringently necessary to calculate robust





	(1) Single image analysis	(2) Advanced image analysis
Technique	image analysis using single uncorrected source images	advanced image analysis using corrected and merged large scale images
Examination time	comparable	
Nerve reconstruction	no	yes
Image fusion	not necessary	yes
Time effort	fast	time consuming

Fig. 8 Image quantification: Sources, efforts and outcome of different image sources for quantification

data for statistical analysis. Moreover, the final accepted image reconstruction technique has to be implemented into the laser scanning image device for its use in larger clinical trials.

6. State of the Art – SNP Quantification

Corneal nerve fibres quantification with minimal manual intervention is a major task in using CLSM during clinical practice. At present, different groups are working on the advancement of this subject using different approaches. Kallinikos et al. (2004) were the first to describe a semi-automated technique for quantifying SBP tortuosity. Even though the degree of agreement with subjective grading had not been assessed, this method is very good to support the intra- and inter observer repeatability for single images or image sets. But at the clinical level, due to its poor intra- and inter observer reliability of recurrent patient examinations this method was not successful.

To facilitate computerized tracing of nerves and nerve tortuosity, Scarpa et al. (2011) have established an algorithm for the automated identification of corneal nerve structures. The reliability of this system was also confirmed by its comparison to subjective tortuosity grading. Sindt et al. (2013) have developed a rapid image evaluation system for corneal *in vivo* confocal microscopy. By taking less than 10 s per scan, the software could be able to calculate rapidly and accurately the number, mean length of nerves and immune cells as well as the number and mean density of immune and wing cells providing quantitative results describing nerve, dendritic and wing cells.

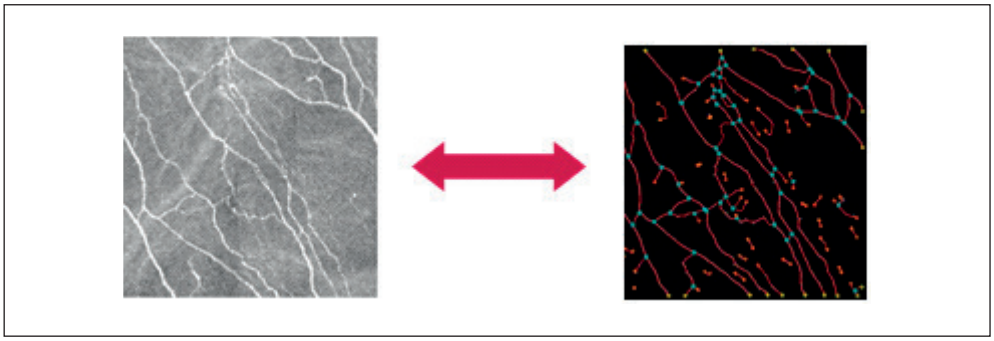


Fig. 9 Example of an image analysis. All components of the network are skeletonized and undirected graphs were generated.

Our group is developing a quantification pipeline according to the concepts developed by Winter et al. (2010). The main steps of this approach are visualized in Figure 10. Ongoing work is focused on minimizing manual adjustments and to speed up the overall quantification process to implement this into the CLSM device.

7. Clinical Applications

Corneal nerve function is involved in diverse important ocular surface processes such as mechanical, thermal and chemical signal monitoring, preservation of ocular surface integrity by modulation of epithelial cell proliferation and migration, release of trophic factors (neuropeptides, substance P, growth factors) and lacrimation reflex. The integrity and function of corneal nerves are affected in the course of different pathological situations including diabetes, infection, limbal stem cell deficiency, lysosomal storage diseases as well as during corneal surgery, keratoconus and keratitis. Since cornea is the only structure which allows the direct, non-invasive observation of nerves using confocal microscopy, a number of prospective clinical observations can be addressed and evaluated depending on corneal confocal nerve imaging. Currently, corneal nerve imaging in different kinds of neurodegenerative diseases is one of the potential clinical applications of *in vivo* CLSM.

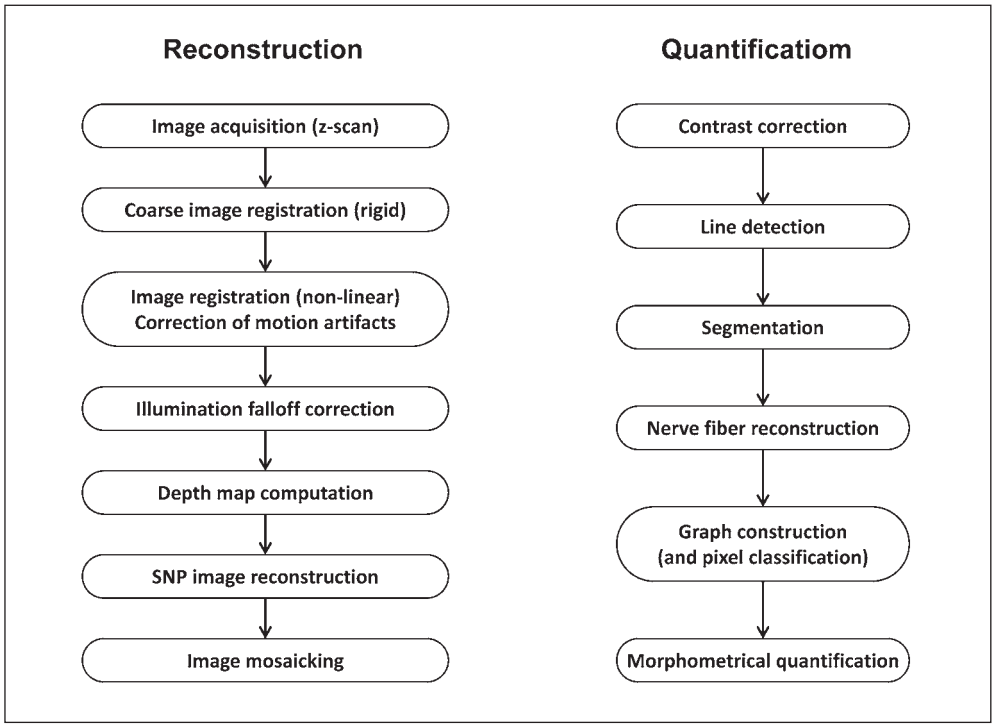


Fig. 10 Main steps in CLSM image reconstruction and quantification pipeline developed by Allgeier and Köhler (Allgeier et al. 2012 and Köhler et al. 20102) and Winter (Winter et al. 2010).

8. Conclusion

In vivo CLSM imaging of corneal nerves is possible for different clinical applications and can be used as a biomarker for a wide range of corneal and systemic diseases. Several characteristic morphologic quantities can be examined, calculated and determined using different data acquisition pipelines. Further effort is necessary to standardize these technologies and it is of the utmost importance to optimize the imaging and quantification procedure for its universal applicability to strengthen multidisciplinary clinical trials. Nevertheless, *in vivo* CLSM can already be successfully applied to assess microstructural changes of the corneal innervation in normal and various corneal disorders.

Acknowledgement

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Software-based Imaging and Quantitative Analysis of the Corneal Sub-basal Nerve Plexus

Bernd Köhler,[1] Stephan Allgeier,[2] Oliver Stachs,[3] Karsten Winter,[4] and Georg Bretthauer[1,2]

With 10 Figures

Abstract

The corneal sub-basal nerve plexus (SNP) possesses a high potential for the early diagnosis of diabetic peripheral neuropathy. Early morphological changes of the sub-basal nerves can be assessed *in vivo* using confocal laser scanning microscopy (CLSM). However, current study results cannot be compared easily because of the lack of a standardized image acquisition and analysis protocol. We propose and discuss an approach to such a standardized procedure. It focuses on specially developed image processing techniques in order to achieve a high degree of automation and generate robust, reproducible results. The proposed technique features the reconstruction of SNP images from CLSM volume scans, the generation of mosaic images to provide an expanded view of the SNP, and automatic segmentation and quantitative morphological analysis of the sub-basal nerve fibres.

Zusammenfassung

Der sub-basale Nervenplexus der Kornea (SNP) besitzt ein großes Potenzial zur frühzeitigen Diagnose einer diabetischen peripheren Neuropathie. Frühe morphologische Veränderungen der sub-basalen Nerven können mithilfe der konfokalen Laser-Scanning-Mikroskopie (CLSM) *in vivo* beurteilt werden. Aufgrund fehlender standardisierter Aufnahme- und Analyseprotokolle lassen sich die Ergebnisse aktueller Studien jedoch nur schwer miteinander vergleichen. Wir beschreiben und diskutieren einen Ansatz einer solchen standardisierten Vorgehensweise. Der Fokus liegt dabei auf speziell entwickelten Bildverarbeitungsalgorithmen, um einen hohen Automatisierungsgrad und robuste, reproduzierbare Ergebnisse zu erzielen. Die beschriebene Methode beinhaltet die Rekonstruktion von SNP-Bildern aus CLSM-Fokusserien, die Erzeugung von Mosaikbildern zur Darstellung eines erweiterten SNP-Bereichs sowie die automatische Segmentierung und quantitative morphologische Analyse der sub-basalen Nerven.

1. Introduction

For some years now, confocal laser scanning microscopy (CLSM) has been applied for high-resolution *in vivo* imaging of tissue structures of the cornea. The cornea is densely innervated and the only point in the human body, at which nerve fibres can be analysed *in vivo* and non-invasively by optical imaging methods, such as CLSM. The corneal sub-basal nerve plexus (SNP) – located directly on the anterior side of the basal membrane of the epithelium and, hence, mainly parallel to the surface of the cornea – is particularly suited for such

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investigations. The SNP is a dense network of thin, unmyelinated nerve fibres (Guthoff et al. 2005). Many CLSM studies have examined changes of the sub-basal nerve fibres in case of various ocular or systemic diseases involving the peripheral nerve system (Guthoff et al. 2009, Niederer and McGhee 2010, Patel and McGhee 2009, Malik et al. 2005). Presently, several studies are concentrating on diabetic peripheral neuropathy (DPN), one of the most frequent long-term consequences of diabetes mellitus. They suggest that the thin, unmyelinated nerve fibres exhibit pathological changes in a very early stage of DPN already (Malik et al. 2005). The assessment of alterations of the thin corneal nerve fibres by CLSM appears to be an ideal means for the early diagnosis of DPN and therapy control.

Most examinations of sub-basal nerve structures in case of DPN indicate characteristic alterations that correlate significantly with the degree of neuropathy. With increasing degree of neuropathy, the density of nerve fibres appears to decrease, while their tortuosity increases (Mehra et al. 2007, Kallinikos et al. 2004, Quattrini et al. 2007, Rosenberg et al. 2000). While the results largely agree in tendency, agreement of the absolute values is smaller. Morphometric nerve parameters vary in a wide range of values within a group of patients with a comparable stage of neuropathy. Even more importantly, value ranges of differently staged patients show considerable overlaps, leading Holmes et al. (2010) to conclude that the definition of absolute value ranges for the healthy state or various stages of neuropathy might not be possible.

Apart from certainly existing individual differences of nerve fibre morphology, we assume that part of the high variance and overlap of measured morphological values is also due to an insufficient size of the evaluated SNP area, as in most previous studies morphological parameters are based on the analysis of a single (often manually selected) CLSM image per patient. Considering a typical field of view of about 0.4×0.4 mm^2 (area 0.16 mm^2) in high-resolution *in vivo* CLSM, a very small area of the cornea (about 110 mm^2) only is represented in a single image. As the local density of the SNP nerve structures may vary considerably over the corneal surface area (He et al. 2010, Patel and McGhee 2005), evaluation of a single image is not sufficient for a reliable characterization of the SNP nerve fibre density; this was also demonstrated quantitatively by Vagenas et al. (2012).

In addition, ridge-like tissue deformations in Bowman's membrane and the adjacent tissue layers can cause further difficulties in the reliable assessment of sub-basal nerve structures by CLSM. These deformations may have variable extents and are characteristic of a phenomenon referred to as anterior corneal mosaic (ACM) by Bron (1968) and Bron and Tripathi (1969). Kobayashi et al. (2006) first described CLSM images of the ACM ridges and the collagen fibre bundles (called K-structures by the authors) found beneath them. Due to these deformations, the tissue layer of the SNP often is visible in partial areas of the CLSM images only (Fig. 1), which further reduces the assessable area of the SNP.

To obtain objective, reproducible, statistically reliable, and therefore better comparable nerve fibre morphology data that are largely independent of the examiner for diagnosis, standardized imaging conditions and automatic evaluation of a maximum SNP area are indispensable.

To address the above difficulties and constraints, we developed image processing software to reconstruct two-dimensional images of the SNP from CLSM volume scans, generate mosaic images with an extended field of view, and automatically segment the corneal sub-basal nerve fibres in these mosaic images for the reliable assessment of sub-basal nerve morphology.

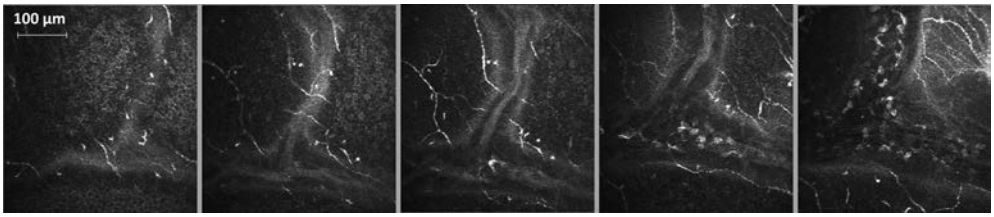

Fig. 1 CLSM images in various focus depths in the area of the SNP with ACM deformation

2. Software-based Imaging of the Sub-basal Nerve Plexus

Software-based imaging of the sub-basal nerve plexus can be divided into several process steps: image data acquisition, CLSM image registration, SNP reconstruction, and fusion of SNP images.

2.1 Image Data Acquisition

The image data are recorded using a confocal laser scanning microscope HRTII with RCM (Heidelberg Retina Tomograph II with Rostock Cornea Module; Heidelberg Engineering GmbH, Heidelberg; hereinafter referred to as HRT). To reduce eye movements during the examination the subjects are instructed to keep their gaze at a fixed point.

The HRT is operated in a special oscillating volume scan (OVS) mode, in which focus image stacks with an axial inter-image distance of about 0.5 µm are recorded of the basal epithelium through the SNP and Bowman's membrane to the anterior stroma in alternating directions. The stack size is 96 images, representing a partial volume of 48 µm in height. If this turns out to be insufficient to capture the entire height of present ACM ridges, the scan depth is increased to 120 images, corresponding to a height of the imaged partial volume of 60 µm.

2.2 CLSM Image Registration

The recorded image data are processed in a subsequent offline process. In a first step, every focus image stack is registered completely, i.e. the individual images are transformed into a common three-dimensional coordinate system. This step is required, as the eyes move permanently during recording even in case of excellent patient compliance. As a result, successive images are shifted by a certain vector.

Moreover, eye movements also cause characteristic distortion artefacts in the images because of the way the images are acquired sequentially pixel by pixel. Motion of the eye in horizontal direction (nasal-temporal) leads to horizontal shear, motion in vertical direction (inferior-superior) causes vertical compression or strain of image information (Fig. 2). Image registration therefore has to correct both the lateral offset of the individual images of a focus image stack by appropriate translations and the movement-caused distortions.

In principle, the absolute extent of motion-induced distortions is reduced with shorter recording intervals. Individual image lines (recorded in about 65 µs each) can be assumed to be free of distortions in good approximation. The task of image registration can then be described as determining offset vectors for all image lines, which compensate motion-caused distortions and correctly align the individual images. By the comparison of two directly suc-

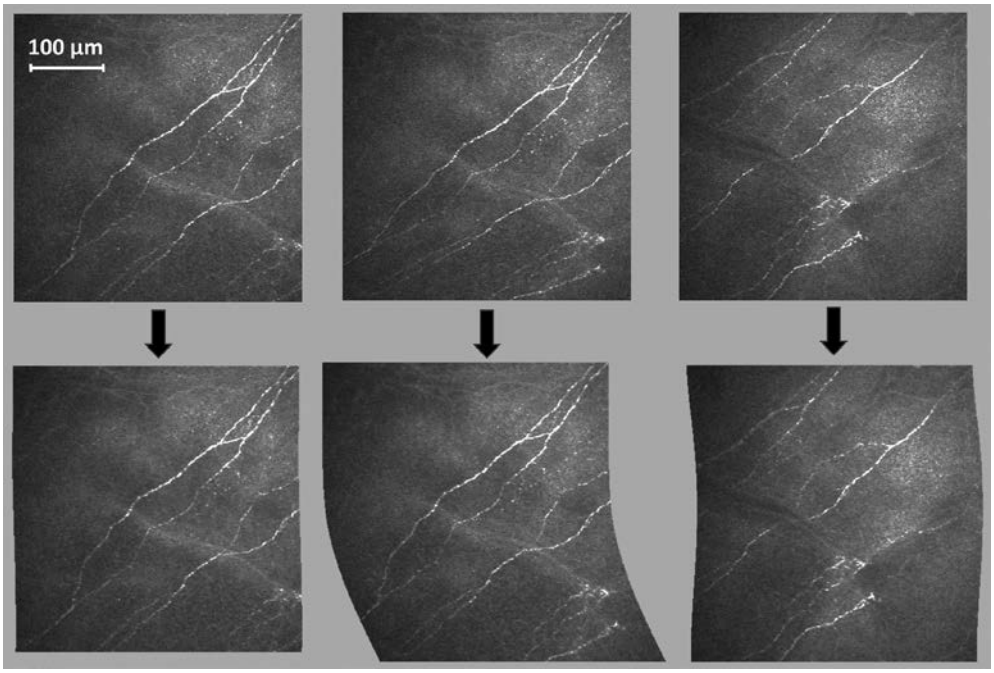

Fig. 2 Directly adjacent individual images of a focus image stack in the area of the SNP with strong motion-induced distortion in the second image; *top row*: Prior to correction of motion artefacts; *bottom row*: After correction of motion artefacts.

ceeding individual images, partial areas can be related to each other. Ideally, this might be done for every single image line, which would directly yield the offset values of the image lines of one of the images in relation to the other image. Unfortunately, the image information of a single image line is not sufficient to make a robust correlation. Every image is therefore decomposed first into strip-shaped partial images of a certain height. With the help of the phase correlation function (Takita et al. 2003), the corresponding image area in the previous image is identified for every partial image (Fig. 3).

The registration result of every partial image directly relates to a position change of the eye between the known recording times of the two corresponding image areas. These relations can be translated into a system of linear equations from which the complete motion of the eye during the recording of the focus image stack can be estimated using a mathematical minimization approach (Allgeier et al. 2010, 2011a). Taking into account the model for the development of motion artefacts explained above, the correction vectors of all image lines can be derived directly from the calculated motion trajectory of the eye and the motion artefacts can be corrected (Fig. 2).

2.3 SNP Reconstruction

The anterior surface of Bowman's membrane – near which the SNP is located – has a much higher reflectivity than adjacent tissue layers. This boundary layer is visible as a light area

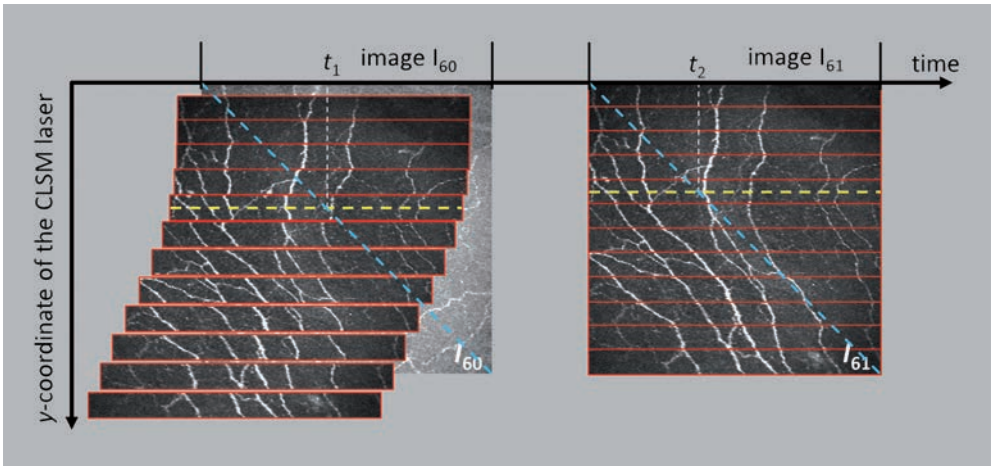

Fig. 3 Schematic representation of non-linear registration; the strip-shaped partial images of the second image are matched in the best possible way with the previous image using a correlation method.

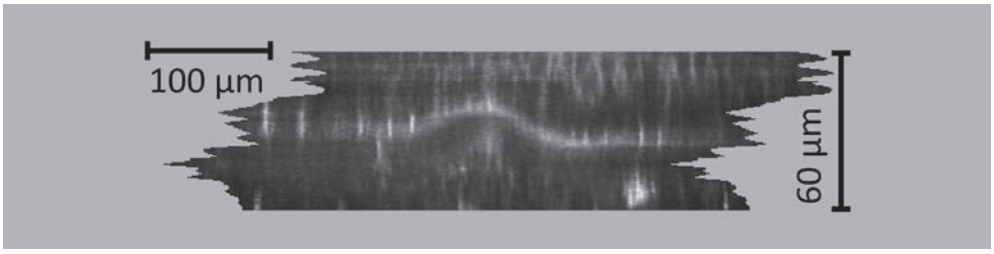

Fig. 4 Section in the x-z-plane of a reconstructed volume with ACM deformations; the boundary surface between the basal epithelium and Bowman's membrane is visible as a highly reflective layer.

in section images along the z-axis of the volume reconstructed from the registered image stack (Fig. 4).

By tracking this highly reflective layer, starting from an automatically identified seed point, a depth map of the SNP can be calculated (Allgeier et al. 2011b, Köhler et al. 2010). Using this depth map, a two-dimensional image of the epithelial basal membrane can be reconstructed from the registered image stack. It shows the SNP over the complete area, also in case of ACM deformations (Fig. 5).

2.4 Fusion of SNP Images

The image registration and SNP image reconstruction process described in sections 2.2 and 2.3 is carried out for all acquired image stacks of a subject. The intermediate result at this point is a set of two-dimensional reconstructed SNP images with the sub-basal nerve structures visible over the entire field of view. The visible area of the SNP in each of these images is usually approximately equal to the size of the field of view of the microscope, but it can be

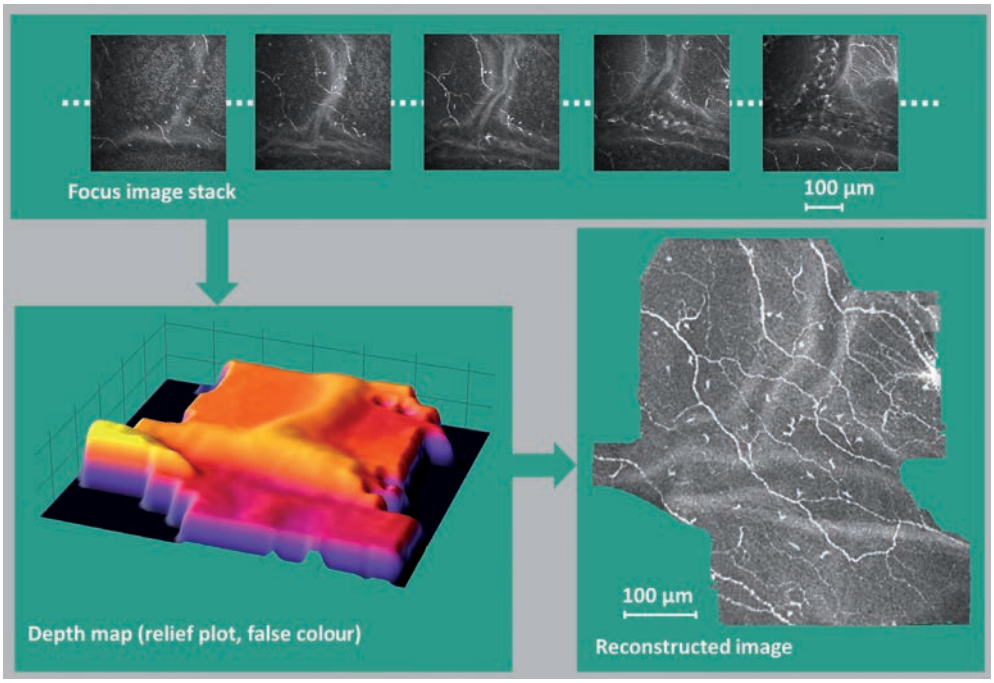

Fig. 5 Overview of the SNP reconstruction steps; top: Five individual images of a focus stack; bottom left: Depth map calculated from the focus image stack (rendered as relief plot and with colour coding of depth values); bottom right: Reconstructed projection image of the SNP.

increased or decreased, depending on the local arrangement of the ACM and the eye movements during the scan.

As the microscope is not moved during the examination of a subject and the subjects themselves are instructed to keep their gaze at a fixed point, positional changes of the imaged part of the SNP can be attributed almost exclusively to unavoidable unconscious eye movements. The reconstructed SNP images usually show a certain amount of partially overlapping areas and can therefore be combined in a larger mosaic image in principle. This first requires them to be aligned to each other and after this image registration, they are fused into the resulting mosaic image.

Although motion artefacts have already been corrected during CLSM image registration (see section *2.2*), small distortions may still remain in the reconstructed SNP images. Thus, simple rigid approaches will not be sufficient for the image registration task. To compensate the remaining image distortions, we are currently using the same image registration algorithm for the reconstructed SNP images as for the CLSM image stacks.

The fusion of the registered images is finally done by calculating a weighted average of all overlapping image areas. Weights are introduced, because the signal-to-noise ratio is higher in central areas of the images and decreases towards the image borders. The weight of a pixel is then defined by a linear function of its distance to the closest image border, reaching a maximum weight of 1 at a certain distance threshold. All pixels with a border distance exceeding the threshold are equally weighted with 1. Figure 6 shows a mosaic image generated by 61 reconstructed SNP images.

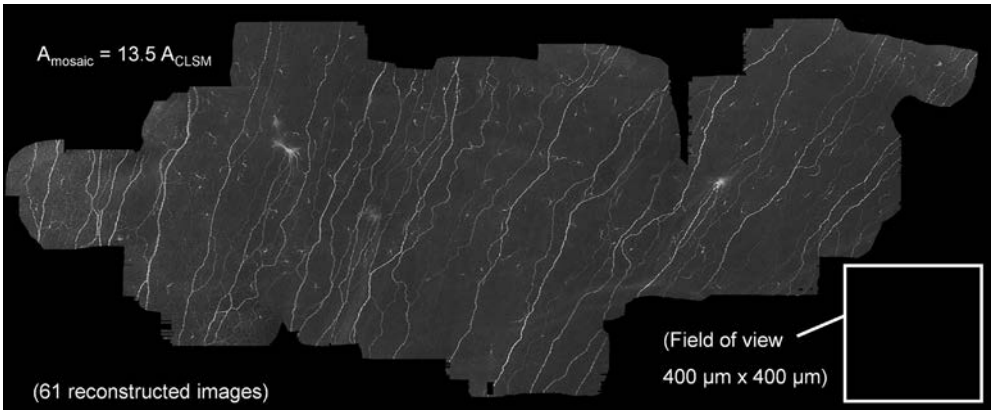

Fig. 6 Result of image fusion from 61 focus image stacks; the white frame shows the size of an individual HRT image (400 × 400 µm²) for comparison.

3. Software-based Analysis of the Sub-basal Nerve Plexus

3.1 Image Preprocessing

A series of image preprocessing steps have to be carried out before analysis of the fused reconstructed SNP images can be performed. In general, this comprises three consecutive steps: Processing of irregular image borders as well as holes in the image area, standardization of the gray-scale information within the image area along with removal of image artefacts, and image segmentation, including skeletonization and restoration of disconnected nerve fibre progressions in order to obtain the best possible segmentation quota of nerve fibres.

Due to the inherently unavoidable effects of eye movement in *in vivo* CLSM images of the cornea, the reconstructed SNP images and, hence, also the mosaic images generally have irregularly shaped image borders. Furthermore, the mosaic images may possess holes which are enclosed portions of the image area where no image information from the SNP layer is present. These border phenomena must be addressed, because they can interfere with subsequent image processing steps. This especially applies when employing algorithms based on image filters with larger window widths. In a first step all image features are extrapolated outwardly from image borders into the surrounding black area or inwardly into image holes. The width of this "safety image margin" is defined by 5 pixels using a distance transform-based operation that closes sharp or narrow border incisions as well as small holes effectively. Image features are then extrapolated into this defined area. This approach ensures morphological and topological correctness of the medial axis for segmented nerve fibres in a later thinning process beyond the actual image area. Larger border notches or image holes cannot be reconstructed accurately due to lacking data in the specific image areas.

Processing of borders and holes is followed by processing of actual image information. SNP reconstruction largely eliminates contrast changes based on ACM, but its features may still be visible faintly. These can be seen as gentle and large-scaled brightness variations. Unshading and local adaptive elimination of these variations clear the image and enhance relevant thin image features (nerve fibres) which are depicted as sudden and narrow bright-

ness changes in the image area. Basically, this process can be described as a high-pass filter operation. The resulting image is then convolved with a series of line-detecting Gabor kernels (Jain and Farrokhnia 1991) which additionally enhance nerve fibres and especially fibre branches. This method is suited better than methods based on non-linear anisotropic diffusion systems (Winter et al. 2010), where in some cases branches were eliminated by cutting off branching fibres due to the constraint of only one valid diffusion direction. The result of this line detection approach considerably depends on the kernel parameters of the Gabor filter, which were adjusted to occurring fibre properties and to image properties resulting from prior preprocessing steps.

The filtered image is segmented using a minimum-error thresholding method by Kittler and Illingworth (1986). In some areas of the resulting image, a variety of wrongly segmented image components may appear. On the one hand, they are induced by artefacts in the reconstructed mosaic image of the SNP (sudden changes in brightness, blurring, or doubling of image features) and on the other hand, segmentation errors are caused by low contrasting fibres or contrast-changing progressions of fibres. The segmented image has to be corrected by removing artefacts and reconnecting actual nerve fibre progressions. Segmentation artefacts are removed based on the size and shape of the objects, their underlying gray-scale values, and their distance to other fibre structures. Interrupted nerve fibre segments are reconnected in a series of sub-steps. In this way, the segmented nerve fibre network is skeletonized and fibre endpoints are identified. The pixel coordinates of the corresponding fibre sections (medial axes) are interpolated with B-splines (Dierckx 1993) which are continued towards the estimated endpoint direction and widened with a search cone. The seeking process advances iteratively over a defined maximum reconnection distance for all endpoints in parallel and can connect two endings as well as one ending to a fibre in order to create a branch. The thickness of such reconstructed fibres is estimated on the basis of the original fibre sections involved. After this series of preprocessing steps (Fig. 7), the resulting image is ready for image analysis.

3.2 Image Analysis

Comprehensive quantitative analysis of segmentation-corrected images is based on characteristic morphological and topological quantities (Zhivov et al. 2011, 2013). In order to obtain these parameters, the segmented nerve fibre network is projected onto the original image area and skeletonized. Subsequently, an undirected graph of all resulting medial lines is generated and special types of skeleton pixels are identified (Fig. 8). Branches are skeleton pixels with typically three (or less frequently, four) neighbouring skeleton pixels. They indicate the centres of nerve fibre branches. Endpoints are skeleton pixels with only one neighbouring skeleton pixel and they indicate the end of a nerve fibre. Connection points are special endpoints that are located in direct vicinity of the image border, suggesting continuation of the fibre beyond the imaged area.

Suitable parameters would be the coverage of the image area with nerve fibres [%] in segmented images and the normalized nerve fibre length [mm/mm²] in skeletonized images. The skeleton can be analysed for the number of branchings and connection points, thus revealing information about the topological embedding into the non-imaged surrounding nerve fibre network. Furthermore, the spatial arrangement of different point types can be explored, revealing homogeneous or clustered distributions. Nerve fibres itself can be analysed regarding their thickness, tortuosity, and general orientation.

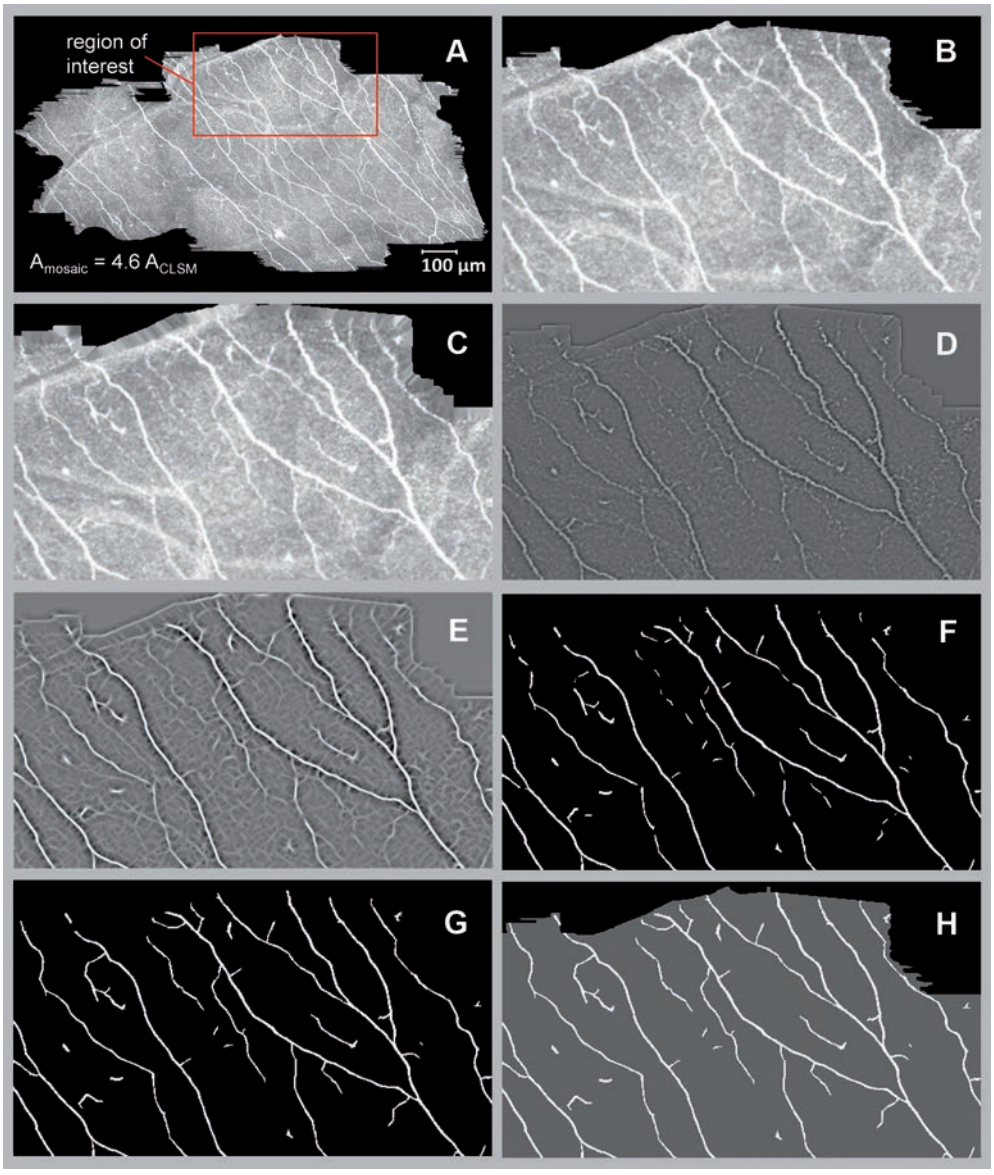

Fig. 7 Illustration of image preprocessing steps: (*A*) Original mosaic image, (*B*) ROI of the mosaic image, (*C*) distance transform and feature extrapolation, (*D*) unshading and local adaptive elimination of brightness variations, (*E*) line detection with Gabor kernels, (*F*) segmentation and artefact removal, (*G*) reconnection of nerve fibre progressions, (*H*) nerve fibres with image area.

Parameters should always be used carefully, since their reliability is influenced by the size of the underlying imaged area as well as by the irregular shape of image borders. In accordance with Vagenas et al. (2012), our studies show that parameters become more robust with increasing image area.

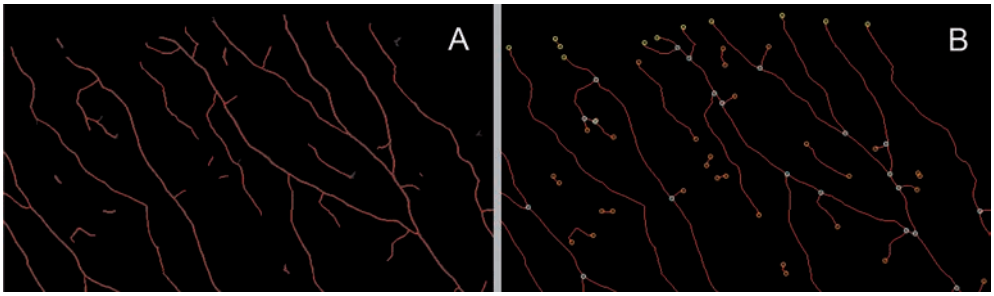

Fig. 8 Result images of the image analysis applied to the image of Fig. 7, (*A*) all components of the network are skeletonized, (*B*) generated undirected graph.

4. Applications

The image processing technique described in sections 2 and 3 has been used extensively with volume scans taken from 163 subjects. A total of 6444 focus stacks have been recorded, an average of 39.5 per subject. Not all of them could be further processed due to several reasons. Some could not be fully registered due to extreme motion artefacts, some did not pass the SNP reconstruction, and some had to be excluded, because they did not contain images from the SNP (e.g. because of loss of contact between the eye and the microscope). Of the 6444 original image stacks, a total of 4872 (approx. 75.6 %) reconstructed SNP images have been generated, with an average of 29.9 per subject.

Using the fusion process described in chapter 2.4, an average of 10.1 mosaic images have been generated for every subject, and the mosaic image with the most expanded area is used by default for the analysis. For 32.4 % of the subjects the automatically chosen mosaic image contained too many artefacts for the analysis process. In these cases, another suitable mosaic image has been selected manually. Figure 9 shows the distribution of the sizes of the mosaic images used for the analysis. About 80 % of all mosaic images show an area of at least twice the size of the field of view of the HRT, for about 40 % of all mosaic images the depicted area is increased by a factor of at least 4, and about 10 % are expanded by a factor of more than 6.

Segmentation and reconnection of nerve fibres using the described image processing steps is reliable even for image areas with poor contrast. All images that passed SNP fusion could be processed and evaluated. Parallelization of algorithms involved in image analysis improves calculation time dramatically.

5. Discussion

5.1 Image Reconstruction

To our knowledge, the reconstruction of two-dimensional SNP images presented here is the first approach to addressing the problem of ACM deformations. Surprisingly, this phenomenon is hardly mentioned in the literature relating to *in vivo* CLSM examinations of the cornea (except for Kobayashi et al. 2006 and Yokogawa et al. 2008, both of which are specifically dedicated to this topic), although it was encountered in many of the subjects during our exam-

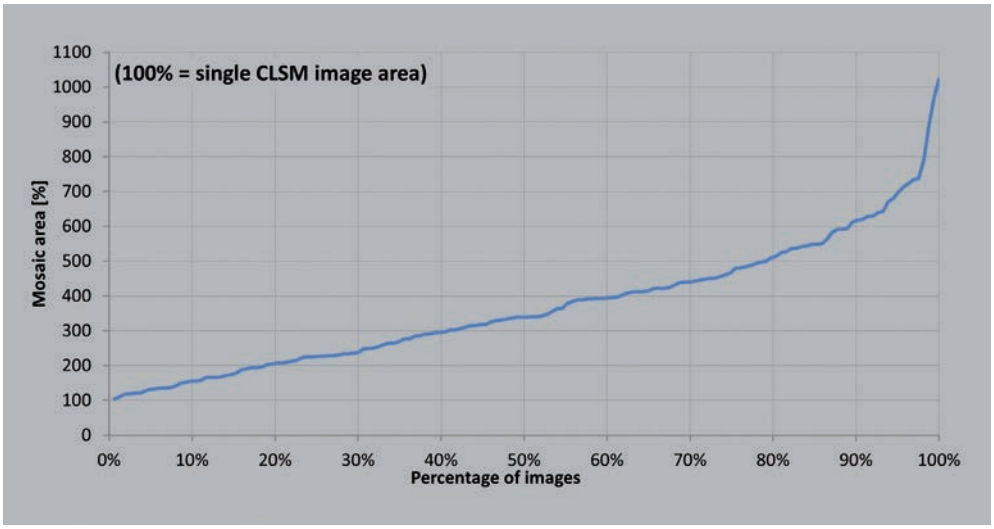

Fig. 9 Distribution of the sizes of the mosaic images generated in a study with 163 subjects.

inations. The measured mean axial extension of the ridges (25.3 µm) is far above the depth of focus of the HRT of 7–8 µm (Zhivov et al. 2009). Our experience so far shows that in exceptional cases only the ACM deformations are weak enough to ensure a reliable representation of the SNP over the complete image area.

In most studies published the analysed images are selected by experts from a larger number of images according to certain obviously subjective criteria. One of these criteria may be the size of the image area actually showing the basal membrane (Holmes et al. 2010). Aside from the subjectivity introduced by the manual selection of single images from a larger set of images, this approach does not guarantee images free of ACM deformations. Yokogawa et al. (2008) have pointed out that the ACM forms a meshed network, and that the average mesh area is smaller than the field of view of a single CLSM image. In our opinion, a reliable examination of the SNP, in particular for the measurement of nerve fibre morphology, is possible only, if the ACM phenomenon is considered. Recording and evaluation of focus image stacks within the framework of the method presented here allow for a complete reproduction of the SNP in the imaging area, also in case of ACM deformations. The algorithm developed produces good results, but still requires expert knowledge and manual interventions (e.g. deletion of incorrectly extracted tissue structures at the borders of the reconstructed images; inspection of the automatically identified seed point for calculating the depth map). Work of our team is presently concentrating on automating these steps or on preventing them in the first place by improving the SNP extraction process.

In fact, pursuing a three-dimensional imaging approach might be advantageous even in situations with minimal to no ACM ridges, especially against the background of an increasing level of automation in the overall imaging and image analysis process. Presented with a CLSM image of the SNP from a region with only small ridges, a human observer will likely have no problems discerning the larger and medium-sized nerve fibres and might be able to intuitively identify some smaller, maybe slightly defocused ones as well. Automatic nerve

fibre segmentation algorithms, on the other hand, provide much better segmentation results of the nerve fibres, if they are ideally focused rather than defocused. The same argument would be true for plane areas that have been imaged slightly oblique. Finally, even in the case of perfect imaging conditions (no ACM ridges, non-oblique imaging), the presented approach (but using a smaller scan depth) would eliminate the always subjective selection of a single focus depth in which to acquire the images or the subjective decision of which images are suited best for processing. We are currently planning statistical investigations of how much the resulting morphometric parameters differ when using single CLSM images *versus* using reconstructed SNP images and how much they are influenced by the exact choice of the focus depth.

5.2 Mosaicking

The first publication of SNP mosaic images composed of a number of CLSM images was made by Patel and McGhee (2005). For the first time, the authors described the large-area arrangement of sub-basal nerve fibres in healthy test persons, which run from all peripheral directions towards a vortex-like structure located inferior of the corneal apex. The mosaic images were composed in an extremely complex software-supported, but manually performed process. Since then, these or similar techniques have been used by various groups to study the dynamics of the SNP (Patel and McGhee 2008), the large-area structure of the SNP in case of keratoconus (Patel and McGhee 2006), post-operative regeneration of the SNP (Stachs et al. 2010), and the arrangement of the ACM (Yokogawa et al. 2008). Efron (2011) describe a semi-automatic process reducing the time needed for the generation of the mosaic image by up to 80 %, and Zhivov et al. (2010) mention the ART-composite recording mode of the HRT as a method for the automatic generation of mosaic images of the SNP. As none of the publications mentioned contain details on image registration, it may be assumed that either rigid or, at best, affine standard image registration processes were applied, which are not able to correct non-linear motion-induced distortions in the images.

The approach to generating large-area images of the SNP presented here differs from the publications mentioned above in several respects. *Firstly*, the method includes non-linear image registration to correct motion artefacts in the focus image stacks. *Secondly*, the mosaic image is not composed directly from the individual images, but from previously generated SNP reconstruction images. As a result, the SNP is shown in an undisturbed way, also in case of ACM deformations. The extension of the image area compared to the image field of the microscope, however, exclusively results from the erratic and involuntary eye movements of the examined person and is not accomplished specifically, but randomly.

The statistical analysis of Vagenas et al. (2012) shows that the field of view of a single image is insufficient for reliable morphometrical assessment of the SNP; Figure 10 also illustrates the inhomogeneous distribution of the corneal nerve fibres. A critical look at our results so far (see section 4) – and taking into consideration the results of Vagenas et al. (2012) – reveals that relying solely on erratic eye movements for lateral expansion of the imaged area does not generally yield a large enough mosaic image of the SNP for reliable morphometrical assessment. Any resolution of this challenge must fulfil two (opposing) constraints: On the one hand, the desired large-area reconstruction of the SNP requires a larger (and specific) relative movement of eye and microscope. On the other hand, a minimum overlap of the images to be composed is required.

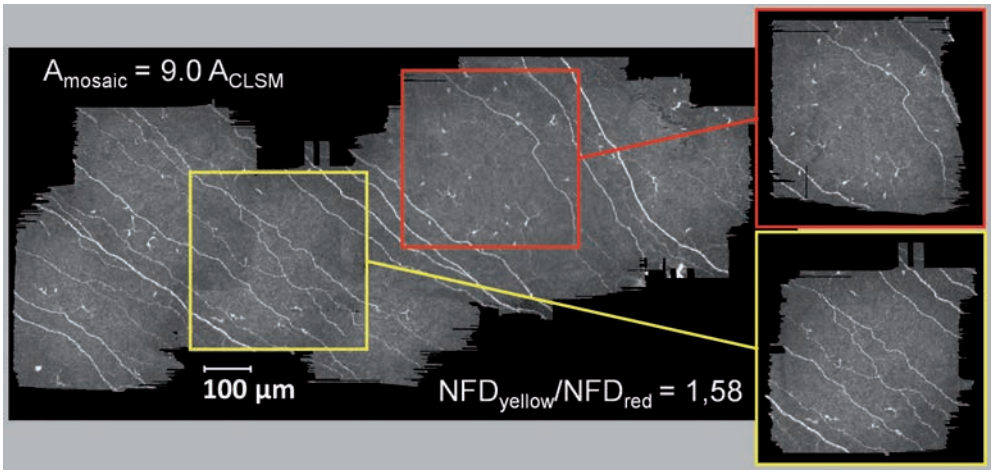

Fig. 10 Resulting image produced by the fusion of 14 focus image stacks; the coloured frames show the positions of two selected reconstruction images; *right*: Separate representation of the corresponding reconstruction images.

A potential solution of this problem may be an automatically controlled imaging technique, with the view direction of the examined person being guided by a moved fixation target in front of the non-examined eye. By a real-time-capable registration of the images, the change of the view direction is pursued constantly and controlled via the position of the fixation mark. This approach has been evaluated in a feasibility study and is currently being developed.

5.3 Analysis

In the last years, a large variety of image processing algorithms for segmentation of nerve fibres and similar structures (mostly retinal blood vessels) were presented. To name only a few, an approach based on extraction of image ridges was presented by Staal et al. (2004). Line operators and support vector classification were used by Ricci and Perfetti (2006). Winter et al. (2010) used coupled anisotropic nonlinear diffusion, and Ferreira et al. (2010) presented a method based on contrast-limited adaptive histogram equalization and phase symmetry. Dabbah et al. (2009) presented a method based on Gabor filters, a dual-model automatic detection of nerve fibres (Dabbah et al. 2010) and a multi-scale adaptive dual-model detection algorithm (Dabbah et al. 2011).

We implemented and used the described segmentation procedure because of its potential when applied to images of different quality. The background brightness of SNP fused images can vary and, based on the original imaging conditions, range from rather dark to very bright. In addition, contrast of nerve fibres is also inhomogeneous between different images to, and nerve fibre brightness inhomogeneities within the same image occur frequently. Compared to other strategies, the Gabor filtering approach may sometimes result in interrupted fibre progressions, but this flaw is more than compensated by our subsequent implementation for fibre progression reconnection. Combination of these two steps creates a powerful tool for nerve fibre detection. Furthermore, our implementation works fast because of the possibility to parallelize employed algorithms for the use on multi-core machines.

Our analysis of the sub-basal nerve plexus employs generally accepted parameters like nerve fibre density, number of branches, thickness of nerve fibres, nerve fibre tortuosity, and fibre orientation. Additionally, we introduced connection points as a topological parameter to determine their capability to describe the topological embedding of the detected nerve fibre network within the non-observed surroundings by its degree of linkage. It still remains to be examined to what an extent this characteristic quantity can be reasonably applied to differently sized and irregularly shaped images or how a potential dependence on image size and shape might be removed. We are currently investigating further parameters for their feasibility to characterize the topological properties of the SNP in depth.

6. Conclusion

The method presented for the generation of images of the SNP represents an important step towards an automatic and, hence, objective and reproducible assessment of the morphology of the SNP. Still, manual intervention is required, but work is aimed at complete automation. The new method includes non-linear registration of the image data to correct unavoidable motion artefacts. The registered image stack is a basis for the extraction of the basal membrane of the epithelium and the reconstruction of a two-dimensional projection image of the SNP. This solution of the ACM deformation problem and the generation of larger-area mosaic images are supposed to allow for an assessment of the state of sub-basal nerve structures that is statistically more reliable than analysis based on individual images.

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Corneal Confocal Microscopy: A Surrogate Endpoint for Diabetic Neuropathy

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With 4 Figures and 2 Tables

Abstract

The accurate quantification of peripheral nerve damage and repair in diabetic patients is critical to identify those with early subclinical damage to enable more aggressive multifactorial intervention to limit progression. Furthermore, accurate assessment of repair allows the development of surrogate endpoints to establish the efficacy of new therapies. The neurological examination is commonly employed, but has poor repeatability and the assessment of vibration perception or insensitivity to the 10 g monofilament only identifies advanced neuropathy i.e. the at risk foot. Techniques which assess early neuropathy include neurophysiology, but they only assess large fibres, whilst quantitative sensory testing (QST) assesses small fibres, it can be highly subjective. More objective techniques which quantify structural damage such as skin biopsy for the evaluation of intra-epidermal nerve fibre density (IENFD) are invasive and not widely available. The emerging ophthalmic technique of corneal confocal microscopy allows rapid, non-invasive and reproducible quantification of corneal nerve morphology enabling early diagnosis, stratification of severity and the ability to detect early nerve fibre repair to assess therapeutic benefit in diabetic patients with peripheral neuropathy. The present review provides a detailed critique of the rationale, practical approach and diagnostic ability of CCM to diagnose diabetic and other peripheral neuropathies.

Zusammenfassung

Eine präzise Quantifizierung der peripheren Nervenschädigung und Regeneration eröffnet eine Möglichkeit, diabetischen Patienten mit subklinischer Beeinträchtigung zu identifizieren, und ermöglicht somit eine frühzeitige multifaktorielle Intervention, um die Progression des Diabetes und der Neuropathie zu stoppen. Darüber hinaus kann die akkurate Quantifizierung der Nervenfasern als nützliches klinisches Instrument dienen, um die Effekte der Therapie auszuwerten. Die häufig angewendete neurologische Untersuchung ist nur schlecht reproduzierbar. Die Vibrationsmessung mittels Stimmgabel und das Testen der Empfindlichkeit mittels Monofilament erkennen Veränderungen nur in fortgeschrittenen Stadien der Erkrankung. Im frühen Verlauf wird die Neuropathie neurophysiologisch bewertet. Die Neurophysiologie evaluiert jedoch die „Large fibre"-Neuropathie, im Gegensatz zur quantitativen sensorischen Testung (QST), und kann somit sehr subjektiv sein. Eine objektive Methode stellt die Hautbiopsie zur Messung der intraepidermalen Nervenfaserdichte dar. Leider ist der Gebrauch dieser Technik aufgrund ihrer Invasivität eingeschränkt. Die *in-vivo*-konfokale Laserscanningmikroskopie erlaubt die Darstellung der Morphologie der Hornhautnerven und deren schnelle und reproduzierbare Quantifizierung. Somit eröffnet diese Technik die Möglichkeit einer Frühdiagnose der peripheren Neuropathie und einer Beurteilung der Therapiewirkung. Die vorliegende Übersichtsstudie konzentriert sich auf den Einsatz der *In-vivo*-Hornhautmikroskopie zur Erkennung und Messung der peripheren Neuropathie bei der diabetischen und anderen Neuropathien.

1. Introduction

Diabetic neuropathy is a global problem affecting ~50 % of the 366 million people worldwide with diabetes. It is the most common and costly complication of diabetes leading to painful

neuropathy (~21 %) (Abbott et al. 2011) and a 23.3-fold increased relative risk of foot ulceration and amputation (Holman et al. 2012). Diabetic peripheral neuropathy is an essential prerequisite for foot ulceration with the annual incidence rising from < 1 % in those without neuropathy to > 7 % in those with established neuropathic deficits (Abbott et al. 1998). Furthermore, it has recently been shown to be an independent predictor for all-cause hazards ratio (HR = 4.4) and diabetes-related (HR = 11.82) mortality (Hsu et al. 2012).

Management is difficult as even tight glycaemic control, a cornerstone for the management of diabetes, has been shown at best to limit progression of neuropathy in patients with type 1 (Albers et al. 2010) but not type 2 diabetes (Ismail-Beigi et al. 2010, Gaede, et al. 2008, Dumont et al. 2009, Zoungas et al. 2009). There is no Food and Drug Administration (FDA) approved disease modifying therapy to prevent or reverse human diabetic neuropathy. Moreover, the development of disease modifying drugs for diabetic peripheral neuropathy (DPN) has stalled completely. There are many potential reasons/excuses for the multiple failed trials, not least of which is premature testing in patients with limited experimental data, but also testing interventions in those with advanced neuropathy. However, it is increasingly apparent that there are significant issues with the endpoints deployed in clinical trials of human diabetic neuropathy. Indeed a recent two-step hierarchical cluster analysis has revealed that neurophysiological tests do not aggregate by typical "small", "large", or "autonomic" nerve fibre subtypes (Gibbons et al. 2010). The latest recommendations advocate a combination of symptoms and signs, QST and electrophysiology for the "diagnosis" of diabetic neuropathy (Tesfaye et al. 2010). Despite spectacular failures in several recent major clinical trials, by default, rather than design, these same measures have been adopted as surrogate endpoints to establish the benefits of therapeutic intervention (Tesfaye et al. 2007, Ziegler et al. 2011). Hence, it is no surprise that we currently have no major development of new disease modifying therapies for human diabetic neuropathy.

No doubt, symptoms and neurological deficits have direct relevance to patients, but they are excessively variable with poor reproducibility (Dyck et al. 2010). Similarly, QST is subjective, highly variable and has limited reproducibility (Freeman et al. 2003). Neurophysiology is objective and reproducible, but does not assess small fibres, which are the earliest to be damaged and show repair (Quattrini et al. 2007). Small fibres can be assessed objectively by quantifying IENFD in skin biopsies, however, this is an invasive procedure which requires expert laboratory assessment and has considerable variability even amongst controls (Lauria et al. 2010, Engelstad et al. 2012). Therefore, effective treatments may have failed not due to a lack of efficacy, but due to an inability of the currently advocated endpoints to detect improvement in clinical trials of diabetic neuropathy (Dyck et al. 2007). A summary of the advantages and limitations of the present techniques to quantify nerve fibre damage in diabetic neuropathy is presented in Table 1.

Hence, there is an urgent need for a non-invasive, sensitive surrogate marker in clinical trials of diabetic neuropathy. There is accumulating and strong evidence that the ophthalmic technique of *in vivo* corneal confocal microscopy (IVCCM) might be such an ideal surrogate endpoint for DPN.

2. Morphology of Human Corneal Innervation

The cornea is the most densely innervated tissue in the body (Müller et al. 2003). Corneal nerves are derived from the ophthalmic division of the trigeminal nerve, enter the cornea

Tab. 1 Advantages and disadvantages of tests assessing diabetic neuropathy

Method	Advantage	Disadvantage
Clinical/Neurological Examination	Simple, easy to perform, does not require special equipment	Not sensitive, not reproducible
Nerve Conduction Studies (NCS)	Sensitive, objective, currently the gold standard for diagnosis	Assesses only large fibres, requires special equipment and expertise
QST	Evaluates large and small nerve fibres, quantitative, relatively easy to perform	Subjective, moderate reproducibility, requires special equipment
Sympathetic Skin Response (SSR)	Simple, fast, objective	Semi-quantitative, normal or abnormal, low sensitivity
Quantitative Sudomotor Axon Reflex Test	Sensitive, objective, reproducible	Requires special equipment, time-consuming
Autonomic Testing	Objective, quantitative	Moderate sensitivity, requires special equipment
Sural Nerve / Skin biopsy	Quantitative, sensitive, currently the gold standard to quantify small fibres	Invasive, costly, risk of infection at the site of biopsy, requires specialist histological techniques
Non-Contact Corneal Aesthesiometry (NCCA)	Non-invasive, quantitative	Subjective, moderate sensitivity
IVCCM	Non-invasive, rapid reproducible, sensitive and quantitative	Requires special equipment and expertise

in the middle third of the stroma and run forward anteriorly in a radial fashion towards the centre where they lose their myelin sheath. The human cornea contains myelinated Aδ fibres, which are large-diameter (6 μm), straight nerves that respond primarily to mechanical stimuli, and unmyelinated C fibres which are small-diameter (2–4 μm) nerves that respond to thermal and chemical stimuli (Beckers et al. 1994) (Fig. 1). Detailed knowledge of corneal nerve architecture and morphology has been provided by studies employing light (Müller et al. 1997, Jones and Marfurt 1998, Guthoff et al. 2005) and electron (Beckers et al. 1994, Marfurt et al. 2010) microscopy and more recently IVCCM (Møller-Pedersen et al. 1998, Oliveira-Soto and Efron 2001, Patel and McGhee 2005, 2006, Mannion et al. 2007, Yokogawa et al. 2008).

Corneal innervation plays an important role in regulating epithelial cell growth, proliferation and differentiation in normal physiological states or in response to corneal disease, trauma or surgery through the release of several growth factors, cytokines and neurochemicals (Gallar et al. 2004). *In vitro* co-culture studies suggest that neurones release substance P that stimulates epithelial cell growth, proliferation, differentiation and type VII collagen production (Müller et al. 2003). Thus patients with impaired corneal innervation may be at increased risk of ulceration due to impaired trophic support provided by the corneal nerves and a reduced tear film with diminished corneal healing (Allen and Malinovsky 2003, Yamada et al. 2003). This has parallels with foot ulceration; however, the incidence of corneal ulceration is much lower, only because the possibility of trauma and undue or unperceived pressure on the cornea is limited. Of course in the foot the daily pressure associated with

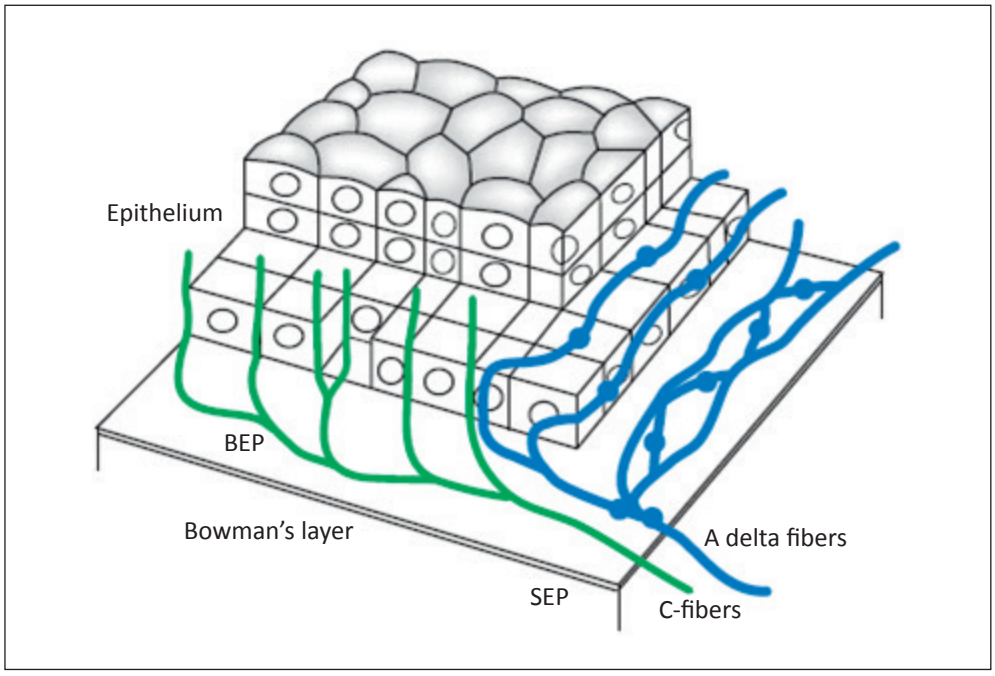

Fig. 1 Three-dimensional representation of the innervation of the human cornea (Tavakoli et al. 2012) (BEP: basal epithelial plexus, SEP: sub epithelial plexus).

ambulation and inappropriate foot wear can result in ulceration due to small fibre dysfunction and blunted pressure induced vasodilation (Fromy et al. 2002, 2012).

3. *In vivo* Corneal Confocal Microscopy

In vivo Corneal Confocal Microscopy (IVCCM) is a relatively new technique which has evolved rapidly from an ophthalmic research tool to a diagnostic tool with a variety of clinical applications in ocular and more recently neurological disease. The non-invasive nature and rapid image acquisition time of the technique has made it an ideal method to quantify a range of microstructures of the cornea, including the epithelial cell layer, Bowman's membrane, sub-basal nerve plexus, stroma and endothelium (Patel and McGhee 2013).

3.1 Image Acquisition

The type of IVCCM used can significantly affect the quality of images. As a result, studies using a laser scanning confocal microscope (LSCM; e.g. Heidelberg Retina Tomograph III Rostock Cornea Module [RCM], Heidelberg GmbH, Heidelberg, Germany), have reported higher sub-basal nerve densities compared to studies using a Tandem scanning (TSCM) or a slit scanning confocal microscopy (SSCM; e.g. Nidek Confoscan 4, Nidek Technologies, Padova, Italy and Tomey Confoscan P4, Tomey, Erlangen, Germany) due to differences in

the light source, contrast and resolution (Tavakoli et al. 2012). Furthermore, studies have employed a range of scanning, image sampling and quantification methodologies. There is no consensus regarding the minimum number of images required for representative quantitative analysis. The majority of published studies have used up to five images per eye for analysis and reassuringly a recent study has suggested that 5–8 images are optimal, depending on the parameter being assessed (Vagenas et al. 2012).

3.2 Image Quantification

The quality of the selected images is extremely important, and once image selection is complete, all images should be de-identified and randomized by an independent investigator prior to analysis to avoid observer bias. The majority of studies have defined sub-basal nerve density as the total number of nerves in each image, which allows quantification of the nerve density in an area (no/mm^2) (Malik et al. 2003, Chang et al. 2006, Messmer et al. 2010, Tavakoli et al. 2010b, Ahmed et al. 2012, Edwards et al. 2012, Tavakoli et al. 2013). Others have presented the data as the number of nerves per image (Midena et al. 2006) or the total length of the nerves within a frame (Grupcheva et al. 2002, Erie et al. 2005), but have, nevertheless, referred to the measure as a nerve density, which to the non-expert reader can be extremely confusing.

Adaptation of a global protocol to quantify corneal nerve morphology is of paramount importance as it will enable the direct comparison of results from different studies and allow multicentre interventional studies. To date most studies have employed semi-automated image analysis to assess sub-basal nerve alterations, a labour-intensive task, which is also subjective and time consuming. Recently, studies from several different centres have assessed the impact of inter- and intra-observer variability on the quantification of corneal nerve morphology using IVCCM, and have reported excellent reproducibility amongst patients with diabetes and controls (Smith et al. 2013, Efron et al. 2010, Hertz et al. 2011, Petropoulos et al. 2013). Very good reproducibility has been shown using a clinically relevant "study-level" protocol of subject re-examination (intra-observer intra-class correlation coefficient 0.72; inter-observer intra-class correlation coefficient 0.73; Hertz et al. 2011). Inherent inter-observer differences and experience were identified as the main causes of variation, especially for the parameter of nerve branch density suggesting the need for a fully-automated image analysis system to eliminate inconsistencies and expedite image analysis time. Such software has been recently developed (Scarpa et al. 2008, Dabbah et al. 2011, Sindt et al. 2013).

Stromal nerves have been studied less extensively with IVCCM. Few studies have quantified the density and the diameter of the stromal nerves (Oliveira-Soto and Efron 2001, Hosal et al. 2004, Simo et al. 2005). However, a wide range of results have been reported that may be due to inconsistency in capturing stromal nerves due to their orientation and sparse distribution (Hosal et al. 2004). Stromal rather than sub-basal nerves appear more robust in surviving *post-mortem* change (Simo et al. 2005), therefore, *in vitro* studies should focus on stromal nerves, whilst *in vivo* studies using IVCCM should focus on sub-basal nerves.

4. Corneal Nerve Changes in Diabetic Neuropathy

Over the past decade there has been increasing research interest in modelling the relationship between corneal nerve fibre loss and neuropathy (Efron 2012, Papanas and Ziegler 2013).

An association between neurotrophic corneal ulcers and diabetes was reported as early as 1977 (Hyndiuk et al. 1977). Subsequently, a reduction in corneal nerve density was demonstrated in experimental diabetes *ex vivo* (Yamada et al. 2003). The cornea, due to the unique property of transparency, allows direct, non-invasive, *in vivo* imaging of the small unmyelinated nerve fibre bundles. The first study using NCCA in diabetes was by Rosenberg et al. in 2000 showing sub-basal nerve alterations and a reduction in corneal sensitivity in patients with diabetic neuropathy (Rosenberg et al. 2000). However, since then a burgeoning literature has shown that IVCCM can quantify peripheral neuropathies, in particular diabetic neuropathy (Rosenberg et al. 2000, Malik et al. 2003, Chang et al. 2006, Midena et al. 2006, Messmer et al. 2010, Tavakoli et al. 2010, Smith et al. 2013) (Tab. 2).

We have demonstrated that IVCCM quantifies early small nerve fibre damage (Malik et al. 2003, Hossain et al. 2005, Tavakoli et al. 2010b) with good sensitivity and specificity (Tavakoli et al. 2010b) (Fig. 2). Others have confirmed that IVCCM detects mild neuropathy (Edwards et al. 2012), and corneal nerve fibre length in particular has a high sensitivity (91 %) and specificity (93 %) for identifying diabetic sensorimotor polyneuropathy (Ahmed et al. 2012).

Furthermore, a reduction in corneal nerve fibre length has been related to elevated HbA_{1c} even in normal subjects, suggesting that IVCCM may detect early sub-clinical pre-diabetic nerve injury (Wu et al. 2012). In a study of patients with idiopathic small fibre neuropathy (ISFN) we have demonstrated significant corneal nerve damage which was related to higher triglycerides (Tavakoli et al. 2010a). In a recent study we have also shown that IVCCM can be performed in children with diabetes (Sellers et al. 2013). Importantly, we have shown that corneal nerve damage assessed using IVCCM relates to the severity of intra epidermal nerve fibre loss (gold standard for small fibre damage) in foot skin biopsies (Quattrini et al. 2007). More recently corneal nerve fibre length has been shown to correlate significantly with three independent measures of small fibre function: cold detection thresholds, laser Doppler imager flare and heart rate variability (Sivaskandarajah et al. 2013). The further significant potential of IVCCM as a viable surrogate endpoint has been evidenced by demonstrating that IVCCM detects nerve fibre regeneration within 6 months of simultaneous pancreas kidney transplantation (SPK) whilst neurological deficits, QST, NCS and IENFD remain unchanged in diabetic patients (Mehra et al. 2007, Tavakoli et al. 2013).

Of further clinical relevance we have also demonstrated an improvement in corneal nerve fibre density after improvement in glycaemia, blood pressure and lipids in diabetic patients (Tavakoli et al. 2011).

A potential limitation of IVCCM is the speed of analysis; however, automated image analysis has been developed for the rapid quantification of corneal nerve images. Indeed we have developed an automated image analysis system, which shows extremely high correlation with manually assessed corneal nerve fibre density and length (Dabbah et al. 2010, 2011, Petropoulos et al. 2014, Dehghani 2014).

Arguments against IVCCM have to date revolved around the relatively short nerves being studied and the fact that the cornea is avascular which is in contrast to the long somatic nerves and the compelling evidence for a vascular basis of diabetic neuropathy (Malik et al. 1989, 1992). However, corneal nerve pathology has been found to correlate with IENFD loss in biopsies from the dorsum of the foot (Quattrini et al. 2007) and, more recently, a significant correlation has been shown between corneal nerve fibre length and a range of small fibre measures of diabetic neuropathy (Sivaskandarajah et al. 2013). Furthermore, recent studies

Tab. 2 Summary of the results of quantitative corneal nerves assessment using IVCCM in diabetic neuropathy

Studies	n (yrs)	Age of	Type Method / IVCCM	Acquisition Fibre Density images assessed per subject	Corneal Nerve Branch Density (CNFD)	Corneal Nerve Fibre Length (CNBD)	Corneal Nerve (CNFL)	Study Limitations
Sellers et al. 2013	12	14.8 ± 2.1	LSCM	Section / 5 images	24.1 ± 3.1 no/mm^2	43.7 ± 13.7 no/mm^2	18.2 ± 2.4 mm/mm^2	Small sample size
Zhivov et al. 2013	18	68.8 ± 8.8	LSCM	Section / not specified	0.006 ± 0.002 mm/mm^2	25.3 ± 28.6 no/frame	6222 ± 2419 µm	Small sample size
Ahmed et al. 2012	33	50.0 ± 14.3	LSCM	Volume / 2 images	28.0 ± 9.0 no/mm^2	17.0 ± 12.0 no/mm^2	11.1 ± 3.6 mm/mm^2	Image selection criteria
Edwards et al. 2012	88	58.0 ± 9.0	LSCM	Section / 8 images	–	graphical	graphical	CNFD not presented
Nitoda et al. 2012	139	63.0 ± 2.0	LSCM	Sequence / 3 –5 images	23.3 ± 0.8 no/mm^2	31.8 ± 2.6 no/mm^2	12.5 ± 2.6 mm/mm^2	–
Tavakoli et al. 2011	25	52.0 ± 2.0	SSCM	Section / 3 –5 images	18.8 ± 2.1 no/mm^2	6.9 ± 1.5 no/mm^2	8.3 ± 0.9 mm/mm^2	Small sample size
Hertz et al. 2011	26	43.0 ± 16.9	LSCM	Volume / 2 images	32.5 ± 9.7 no/mm^2	26.0 ± 17.1 no/mm^2	13.6 ± 3.5 no/mm^2	Image selection criteria
Ishibashi et al. 2012	38	46.7 ± 1.6	LSCM	Not specified / 4 –5 images	25.3 ± 1.0 no/mm^2	–	9.8 ± 0.3 mm/mm^2	–
Tavakoli et al. 2010b	101	58.3 ± 2.2	SSCM	Section / 3 –5 images	24.1 ± 2.6 no/mm^2	10.3 ± 1.7 no/mm^2	4.9 ± 0.5 mm /mm^2	–
Messmer et al. 2010	67	54.0	LSCM	Volume and Sequence / 5 images	16.5 no/mm^2	17.5 no/mm^2	10.2 mm/mm^2	Sample demographics
De Cillà et al. 2009	50	62.6 ± 6.0	LSCM	Not specified / 1 image	2.4 ± 1.0 no/frame	–	–	Image selection and analysis criteria
Midena et al. 2006	42	–	SSLM	– no/frame	2.2± 0.3 (degree)	0.8±0.1	–	–
Chang et al. 2006	42	63.8 ± 7.2	SSCM	Not specified no/mm^2	16.1 ± 5.7 no/mm^2	24.9 ± 7.7	–	Image selection and analysis criteria
Quattrini et al. 2007	54	58,0 ± 10.9	SSCM	Section / 3 –5 images	23.7 ± 3.2 no/mm^2	7.31 ± 1.98 no/mm^2	3.94 ± 0.63 mm/mm^2	–
Mocan et al. 2006	35	58.4 ± 10	SSCM	Not specified / 1 image	28.3 ± 10.4	39.7 ± 13.2 no/mm^2	–	Image analysis criteria
Malik et al. 2003	18	57 ± 12.8	SSCM	Section / 3 –5 images	27.8 ± 6.5 no/mm^2	27.2 ± 13.2 no/mm^2	7.5 ± 1.1 mm/mm^2	Sample size
Rosenberg et al. 2000	23	46 ± 8.3	TSCM	Section / 2 images	3.1 ± 1.2 no/frame	–	–	Type of IVCCM

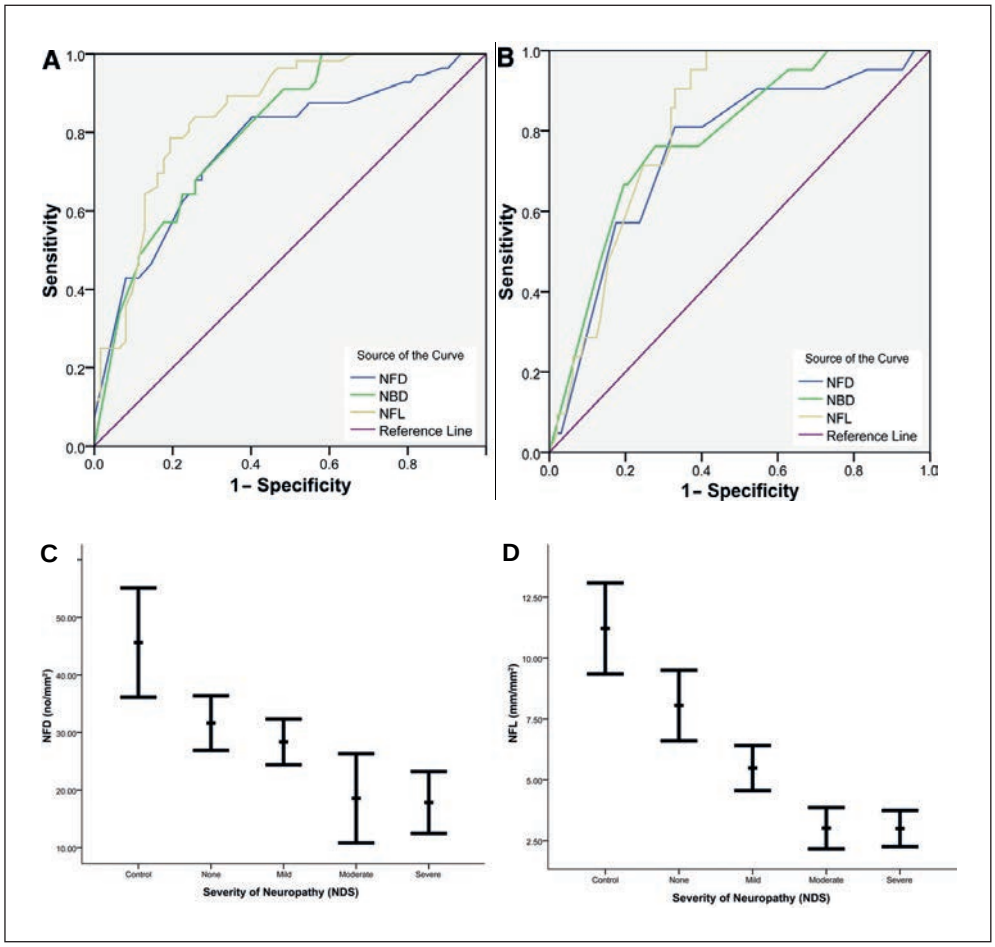

Fig. 2 Receiver operating characteristic curves for the diagnostic validity of NFD, NBD, and NFL for (*A*) NDS > 3 and (*B*) NDS > 6. Corneal nerve morphology in control subjects and diabetic patients with increasing neuropathic severity: (*A*) NFD ($P < 0.0001$) and (*B*) NFL ($P < 0.0001$) (Tavakoli et al. 2010b).

in animal models of diabetic neuropathy using IVCCM have shown a significant reduction in blood flow in the posterior ciliary artery and corneal nerve fibre loss with an improvement in both blood flow and corneal innervation after intervention with a vasopeptidase inhibitor (Davidson et al. 2012a, b).

5. Summary

In conclusion IVCCM appears to be an ideal non-invasive clinical technique, which can assess alterations in corneal cellular pathology and in particular has been used to quantify small nerve fibre pathology in relation to diabetic neuropathy. With the development of automated

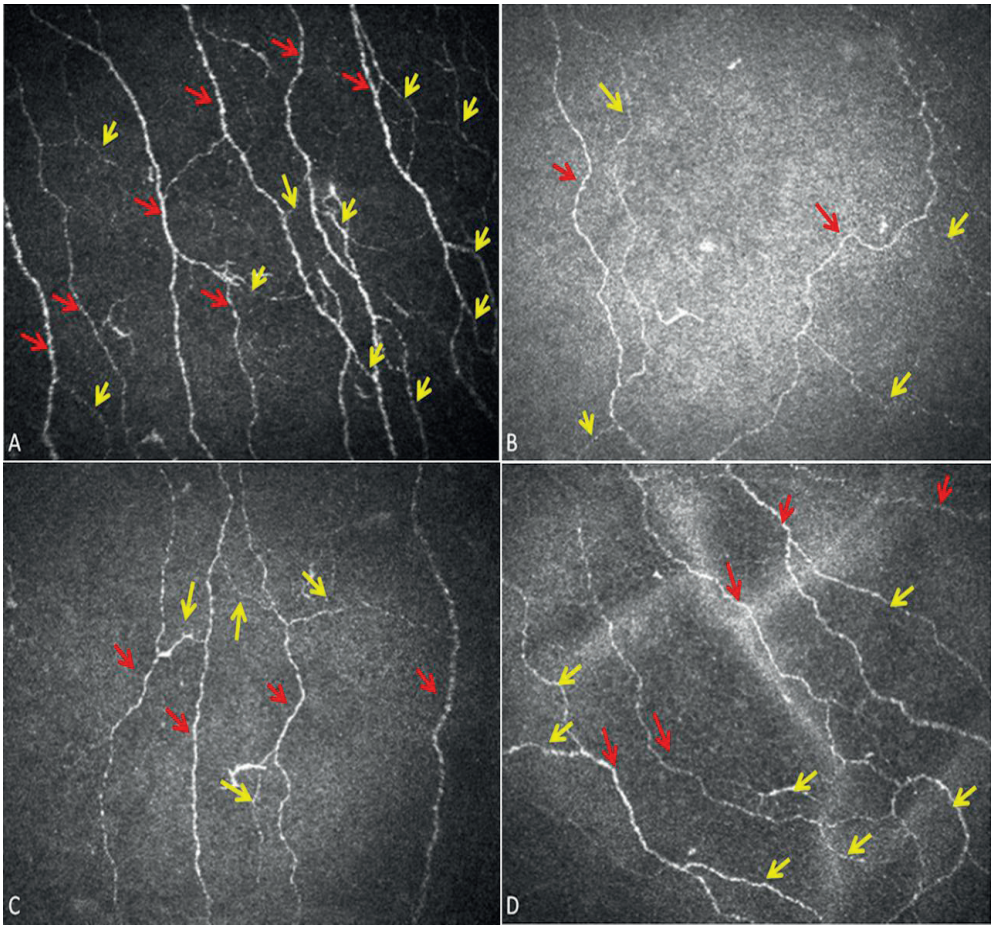

Fig. 3 Subbasal nerve images from the cornea of: a control subject (*A*) and a patient with type 1 diabetes at baseline (*B*) and at 6 (*C*) and 12 (*D*) months after SPK. The red arrows indicate main nerve fibres, and yellow arrows indicate branches (Tavakoli et al. 2013).

image analysis we predict a rapid increase in the clinical utility of IVCCM in the assessment of diabetic neuropathy and a range of peripheral neuropathies. In this review we have considered the considerable potential of this powerful technique to undertake detailed morphological analysis of corneal nerves to act as a surrogate measure of peripheral neuropathy. It appears that the widest application of IVCCM may well be in the field of metabolic or neurological disease, particularly as it may provide a non-invasive means to identify patients with minimal neuropathy, quantify the severity of neuropathy and follow progression or assess therapeutic response, in not only diabetic neuropathy, but also a range of other neuropathies. There is clearly a need to establish a normative data set taking into account age and to standardize the method of capturing, sampling and analysing the images in order to use IVCCM in longitudinal prospective or interventional multi centre studies.

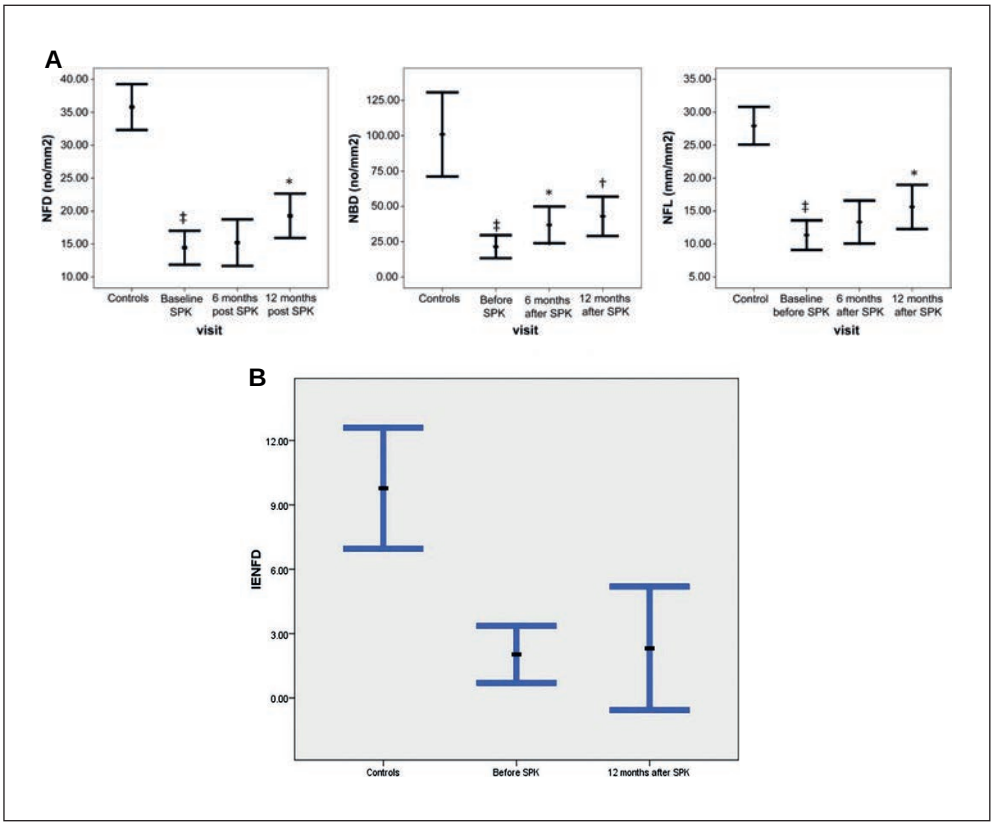

Fig. 4 (*A*) CNFD (*left*), CNBD (*middle*), and CNFL (*right*) in diabetic patients at baseline and at 6 and 12 months after SPK where significant regeneration is recorded**.** In (*B*): IENFD in control subjects and in diabetic patients at baseline and 12 months after SPK showed no significant improvement (Tavakoli et al. 2013).

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Pathogenesis of Corneal Nerve Damage

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With 6 Figures

Abstract

Cornea is the most densely innervated tissue in the human body. Cornea is mainly innervated by the sensory nerve fibres of the ophthalmic branch of the trigeminal nerve, and also by the less numerous sympathetic as well as parasympathetic nerve fibres. In addition to their well-known sensory function, corneal nerves help maintain the integrity of ocular surface by releasing epitheliotrophic substances and contribute to the lacrimation reflex. Corneal nerves are of great interest to clinicians and scientists due to their significant roles in regulating ocular surface integrity. Changes in corneal nerve morphology have been studied in animal models using light and electron microscopy. More recently, *in vivo* confocal microscopy (IVCM) has been used as a non-invasive technique to study variances in affected corneal nerves during different pathologies. IVCM provides high-resolution images of corneal structures in the living human eye and enables the examination of the same cornea repeatedly over time. Using these techniques it has been repeatedly shown that corneal nerves are affected in keratoconus, dry eye, diabetes mellitus, various infectious, age-related processes and in refractive surgery. The purpose of the present study is to describe corneal nerves alteration in different pathological conditions and to discuss mechanisms possibly contributing to these degenerative changes.

Zusammenfassung

Die Hornhaut des Auges ist eines der am dichtesten innervierten Gewebe des menschlichen Körpers. Sie wird überwiegend von sensorischen Nervenfasern durchzogen, die dem Nervus ophthalmicus, einem Ast des Nervus trigeminus, entspringen. Zu einem kleinen Teil wird die Hornhaut von sympathischen und parasympathischen Nervenfasern innerviert. Die Nervenfasern der Hornhaut sind für die starke Berührungs-, Temperatur- und Schmerzempfindlichkeit verantwortlich. Auch für den Hornhautreflex ist eine normale Innervation der Hornhaut Voraussetzung. Darüber hinaus exprimieren die Hornhautnerven Wachstumsfaktoren, die für die Proliferation und Migration der Epithelzellen verantwortlich sind. Die pathologischen Modifikationen in der Struktur der Hornhautnerven führen zu einer Verminderung und häufig sogar zu einer Aufhebung der Hornhautsensibilität sowie Funktionsstörungen. Die Veränderungen der Hornhautnerven wurden intensiv mittels Licht- und Elektronenmikroskopie im Tiermodell untersucht. In den letzten Jahren hat die nicht-invasive konfokale Mikroskopie neue Möglichkeiten eröffnet, die Hornhaut *in vivo* zu untersuchen. Sowohl invasive als auch nicht invasive Techniken haben pathologische Veränderungen der Hornhautnerven im Alter, bei Keratokonus, Diabetes mellitus, Infektionen und nach der refraktiven Chirurgie demonstriert. Die vorliegende Arbeit diskutiert mögliche Mechanismen, die zu einer Beeinträchtigung der Morphologie und der Funktion der Hornhautnerven bei verschiedenen Pathologien führen.

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1. Nerve Supply of the Cornea

The cornea is one of the most heavily innervated tissues in the body. Most corneal nerve fibres are sensory in origin and are derived from the ophthalmic branch of the trigeminal nerve; however, in some cases the inferior cornea receives some of its innervation from the maxillary branch of the trigeminal nerve.

The nasociliary nerve, originating from the ophthalmic division of the trigeminal nerve, travels through the superior orbital fissure, enters the orbit, runs inferotemporally to the superior rectus muscle and the optic nerve, and then, after branching into long ciliary nerves, penetrates the sclera a few millimetres away from the optic nerve. As it travels through the suprachoroidal space, the nerve branches multiple times, creating a loose network of axons, which penetrate the limbal area in a circumferential manner (Kim and Dohlman 2001). The nerve bundles lose their perineurium and myelin sheaths within approximately 1 mm of the limbus and continue into the cornea surrounded only by Schwann cell sheaths (Muller et al. 2003). The absence of a myelin sheath on central corneal axons is necessary to maintain corneal transparency. After entering the cornea the nerve trunks travel in a semiradial direction through the middle third of the stroma, and form a dense subepithelial plexus (SEP) (Müller et al. 1997). After penetrating Bowman's membrane, these nerves form a dense subbasal nerve plexus (SNP), the branches of which terminate in all layers of the corneal epithelium (Marfurt et al. 2010).

The nerve endings, known as axon terminals in the corneal epithelium, are structurally and functionally similar to those of myelinated A-delta and unmyelinated C-fibre types (Guthoff et al. 2005). The axon terminals from one axon represent the free nerve endings of the epithelium and are arranged in a leash-like fashion with others to span several hundred micrometres along the surface of the basal cell layer in a parallel fashion. Branching from the basal plexus, single beaded fibres travel perpendicularly toward the wing cells, thus innervating all layers of the epithelium (Auran et al. 1995, Guthoff et al. 2005).

All mammalian corneas also receive sympathetic innervation from the superior cervical ganglion, exhibiting significant inter-species differences in innervation density and distribution (Marfurt and Ellis 1993). A modest parasympathetic innervation from the ciliary

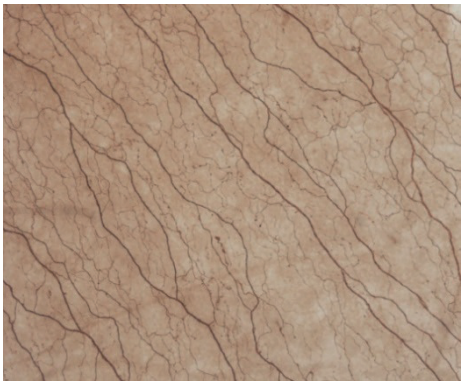

Fig. 1 Human corneal SNP visualized with beta III-tubulin staining (Marfurt, unpublished).

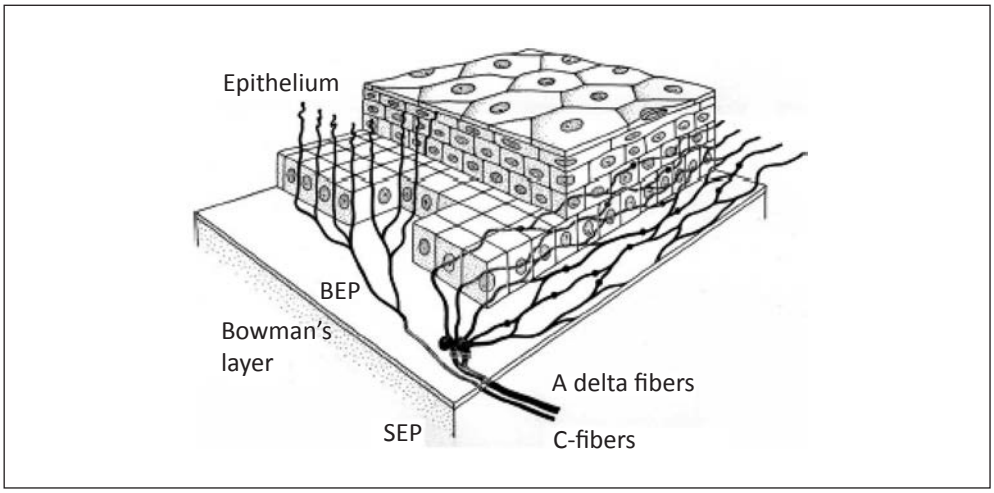

Fig. 2 Schematic drawing of corneal epithelial innervation (Guthoff et al. 2005). Reprinted with permission from Wolters Kluwer Health.

ganglion has been demonstrated in corneas of some mammals (Marfurt et al. 1998). As for the human cornea it remains unclear whether it receives parasympathetic innervation.

Until now, 17 different neuropeptides and neurotransmitters have been described in the corneal nerves, including acetylcholine, catecholamines, calcitonin gene-related peptide, cholecystokinin, substance P, galanin, and pituitary adenylate cyclase-activating peptide (reviewed in Muller et al. 2003).

The corneal nerves have a variety of sensory and efferent functions. Mechanical, thermal and chemical stimulation of the corneal nerves produce predominantly a sensation of pain in humans (Lele and Weddel 1959). The dense population of corneal nerves has a critical role in protecting the cornea and the rest of the eye from the external environment. Corneal nerves transmit impulses to brainstem centres that mediate reflex lacrimation and blinking and thus help maintain proper hydration of the ocular surface. Furthermore, corneal nerve fibres exert important trophic and mitogenic influences on the corneal epithelium and contribute to the integrity and maintenance of a healthy ocular surface. Corneal nerve-derived trophic factors contribute to trophic and regulatory processes in the epithelium, and stimulate epithelial cells to proliferation, differentiation and migration.

Damage to corneal nerves leads to diminished corneal sensitivity and possible transient or long-term alterations in the functional integrity of the ocular surface. When the cornea is injured either accidentally or as a consequence of its surgical manipulation, the corneal nerves are severed to a variable degree. Taken into consideration their relevance for ocular surface health, the corneal nerves have gained an increasing interest for clinicians and researchers.

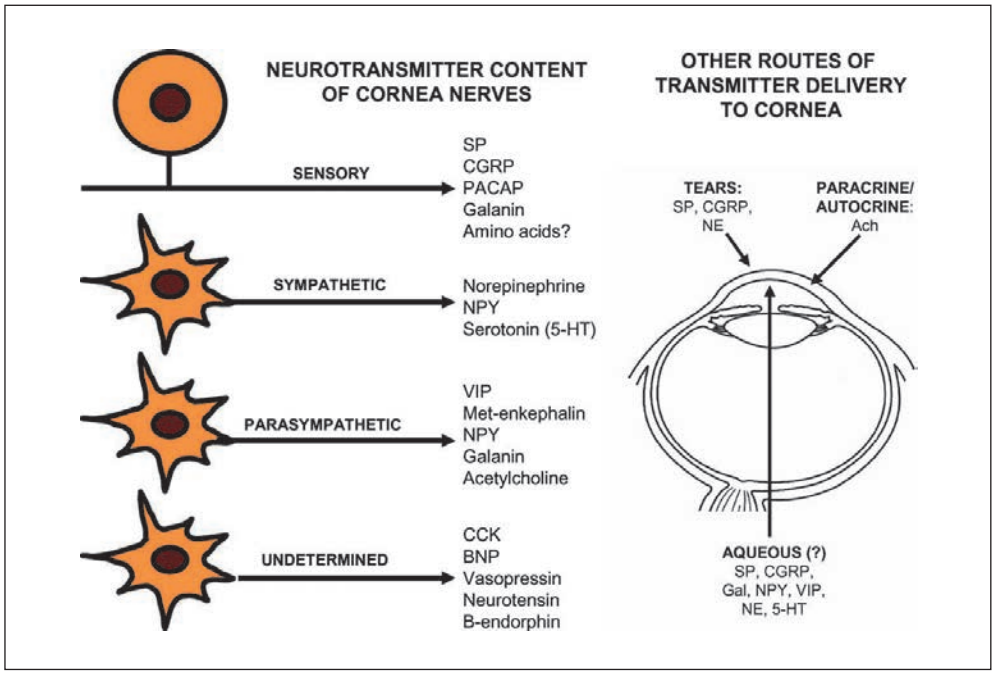

Fig. 3 Neurochemistry of the corneal innervation (Muller et al. 2003). Reprinted with permission from Elsevier.

2. Age-related Changes

Age-related changes in corneal nerve density are well known and must be considered when interpreting long-term SNP nerve data after refractive surgery and in pathologic corneal conditions.

Studies performed in normal healthy subjects demonstrated that corneal sensation decreases with age, with the greatest reduction during the fourth and fifth decades (Millodot 1977). The cause of the decline in corneal sensitivity in the aging eye remains to be investigated. It is not fully understood whether this reduction occurs due to thickening of the fibrous structure of the cornea, to a decrease of water content, or to an atrophy of nerve fibres.

There are only a few studies addressing age-dependent alterations of corneal nerves, and even these sparse data are controversial. The disparity between findings results from the different microscopy techniques used and from how the nerve density was calculated from the obtained images. On the one hand Grupcheva and co-workers found a small age-associated decrease in SNP density ranging from a mean density of 632 mm/mm^2 in a young group (mean age 22 years) to 582.6 mm/mm^2 in an older group (mean age 74 years) (Grupcheva et al. 2002). On the other hand, using *in vivo* tandem scanning microscopy, Erie and colleagues found no age-associated change in the density and orientation of the SNP in the central human cornea (Erie et al. 2005a). Using confocal laser-scanning microscopy (Heidelberg retina Tomograph/Rostock Cornea Modul, HRT/RCM) another group was able to show a subtle, but still statistically significant decline in SNP density with age (Niederer et al. 2007).

The calculated reduction in the latter study was 0.9 % per year. Even after exclusion of three oldest cases, a significant correlation was still observed between age and SNP density. This reduction did not correlate with epithelial basal cell density, as could be expected, taken into consideration the metabolic interplay between the epithelium and SNP. However, in this study a positive correlation could be detected between SNP and keratocytes density, most pronounced in the anterior stroma, where keratocyte loss averaged 0.9 % per year. Keratocyte density has been reported to reduce with age also in other *in vivo* studies, and this reduction in keratocyte density with age has subsequently been confirmed by *ex vivo* studies (Berlau et al. 2002, Hahnel et al. 2000). It has been proposed, that the keratocyte decline in the anterior stroma could play a role in the diminishment of the SNP, because these cells are situated in close proximity to the nerves and are known to be metabolically very active and release trophic substances (for example, NGF), needed for nerve growth and sprouting.

An *ex vivo* study confirmed a SNP decline with age, using an immunohistochemical technique (He et al. 2010). Here, there were no differences in SNP density between genders, but there was a progressive nerve density reduction concomitant with aging, mainly in eye samples of donors' 70-years of age and older. Furthermore, a very interesting observation was made in that study, revealing irregular lesions of nerve fibres in the superficial stroma beneath Bowman's layer, and this was seen in donor corneas of 70 years of age and older (Fig. 4). These lesions can be ascribed to the accepted increased lipid deposition in the cornea over age. This accumulation clinically manifests as so called Arcus senilis an opaque ring in the corneal periphery. The authors proposed that the SNP density decrease was associated with observed abnormalities, occurring in the peripheral cornea.

In conclusion, there is a confirmed age-related loss of intact nerve endings from the ocular surface, and this loss can, at least partially, be responsible for corneal sensitivity decrease and retarded epithelial wound healing in elderly patients.

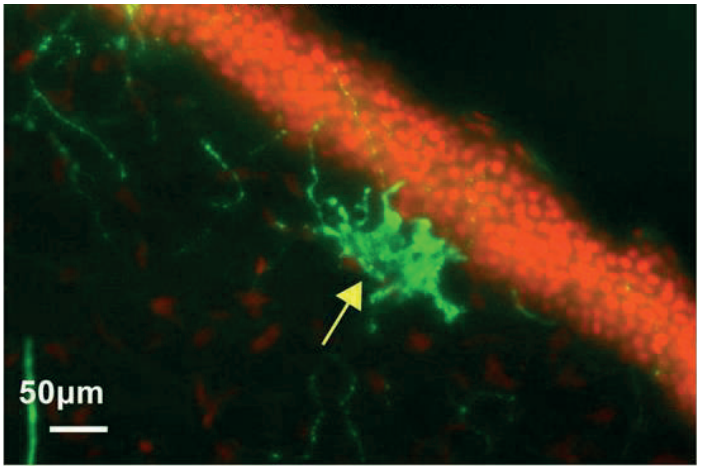

Fig. 4 Transected view showing a nerve lesion (green, beta III tubulin staining) localized in the anterior stroma beneath the epithelial basement membrane (He et al. 2010). Reprinted with permission from Elsevier.

3. Dry Eye Disease

Dry eye disease (DE) is one of the most common ocular conditions worldwide. DE refers to a spectrum of ocular surface diseases with multiple etiologies. Regardless of the initiating causes, a vicious cycle of inflammation can develop on the ocular surface in DE that leads to ocular surface disease. Ocular irritation in DE may cause considerable discomfort and negatively affect the patient's quality of life. Epidemiologic studies indicate that up to 33 % of the population aged 50 years or more experience DE symptoms (Bourcier et al. 2005). There are many causes for dry eye syndromes. One cause is functional alteration of the nerves innervating the lacrimal gland and the ocular surface (Dartt 2004). In a mouse model of DE an almost complete absence of parasympathetic nerves in the lacrimal glands was demonstrated using immunohistochemistry, as opposed to wild type mice with clearly identifiable parasympathetic nerves in the lacrimal glands (Song et al. 2003). The cornea is known to be affected through increased production or decreased elimination of inflammatory cytokines and proteolytic enzymes.

In DE, corneal pathological alterations have been observed in corneal epithelial, stroma and nerves. The distinct corneal features in DE include irregular and disorganized epithelium, altered corneal epithelial barrier function, activation of anterior keratocytes, abnormal nerve morphology and presence of inflammatory cells (Tuominen 2003). Also, the overall corneal epithelial thickness is significantly decreased in DE (Liu and Pflugfelder 1999). Furthermore, decreased density of conjunctival goblet cells and decreased production of mucus by the ocular surface epithelium are characteristic features of DE.

Corneal sensation was shown to be significantly reduced in DE patients when compared to healthy age-matched individuals (Benítez del Castillo et al. 2004). This reduction correlated with certain clinical parameters such as tear production and the state of the ocular surface, and was documented by the lower number and density of nerves at the sub-basal level, as shown by using IVCM. Along with the decrease of nerve fibres, higher numbers of beadings, presence of nerve sprouts, and increased tortuosity were observed by several authors. These parameters are indices of increased metabolic activity, and suggest a response directed to repair the pathological alterations. These findings were confirmed by another group also using IVCM (Zhang et al. 2005). Importantly, this study showed a direct correlation between nerve fibre density and integrity of the ocular surface (shown by Bengal rose and fluorescein staining). On the other hand, the integrity of the ocular surface correlated inversely to beading of nerves. Our group studied different groups of patients with dry eye (Erdélyi et al. 2007). Here, the density of superficial and intermediate epithelial cells was smaller compared with the normal participants, which is possibly caused by the enlargement of those cells due to metabolic dysfunction. In accordance with other findings also here a decreased nerve fibre density in SNP and increased tortuosity could be demonstrated.

The demonstration of the existence of nervous alterations in patients with DE has led to the use of neuroprotective and neurotrophic eye drops for the treatment of this frequently occurring disease. Thus, topically applied nerve growth factor (NGF) accelerated the recovery of corneal sensitivity and ameliorated the symptoms of DE in a rabbit model of LASIK (Joo et al. 2004).

In a human cornea, substance P-derived peptide and insulin-like growth factor I have been shown to restore corneal nerve morphology and function (Benítez del Castillo et al. 2005). Taken together, the corneal nerves are involved in the pathogenesis of DE, and their recovery can partially reduce DE symptoms.

4. Keratoconus

Keratoconus is a debilitating corneal ectasia that principally affects young people in the second or third decade of their lives (Sherwin and Brookes 2004). Biomechanically, the keratoconic cornea is weaker and more elastic compared to the normal cornea. Measurements of rigidity, stress and strain to failure, and energy absorption for keratoconic cornea revealed that all these parameters were significantly lower when compared to controls (Andreassen et al. 1980). Clinically, keratoconus manifests as progressive corneal instability characterized by abnormal thinning and steepening of the cornea (Rabinowitz 1998). This abnormal curvature of the cornea changes its refractive power, often resulting in myopia and irregular astigmatism and leading to mild to marked impairment in the quality of vision. Visual loss occurs primarily from irregular astigmatism and myopia and secondarily from corneal scarring (Jhanji et al. 2011). The definitive cause underlying the development of keratoconus remains unclear. However, it appears to be a heterogeneous condition that may be produced by a variety of unrelated abnormalities of a metabolic and biochemical nature. Keratoconus has been reported to be associated with other syndromes (Kenney et al. 2000). Keratoconus affects virtually any structure of the cornea. Epithelial cell apoptosis, keratocytes damage, increased levels of destructive enzymes, degradation of collagenous matrix, and digestion of Bowman's layer have been shown in keratoconic human corneas (Rabinowitz 1998, Sherwin and Brookes 2004). The corneal nerves undergo significant changes already in early stages of keratoconus. Whether these nerve alterations are primary or secondary pathological manifestations remains unclear. It has been suggested that the nerves may be mediating a feedback mechanism of epithelial initiation and keratocyte activation, trapping the cornea in a destructive cycle of matrix remodelling and wound healing. Brooks and associates have demonstrated the involvement of corneal nerves in progression of keratoconus using immunohistochemistry (Brookes et al. 2003). Here, the authors have shown that the destructive processes in keratoconus involves fragmentation of plexus and increased levels of proteolytic enzymes cathepsin B and D, produced by nerves and associated Schwann cells. The nerves in the normal cornea also express these enzymes, but not nearly as extensively as during active keratoconus.

Morphologically, the corneal nerves showed pronounced changes, both in the SNP and stroma, as shown by the acetylcholine esterase technique (Al-Aqaba et al. 2011). Keratoconic corneas exhibited a significant alteration in corneal nerves at the corneal apex, whereas the peripheral nerves showed normal morphologic features in most cases similar to the architecture of nerves in the control subjects. Nerves in the SNP showed loss of their radial orientation and demonstrated a very tortuous course. Alterations in stromal nerve morphology included thickening, increased tortuosity and nerve spouting. The thickness of central stromal nerves in keratoconic corneas was 19 μm, which was almost twice as thick as that in the central stromal nerves in the controls.

Also, IVCM revealed lower SNP nerve density and thicker nerve fibres in the stroma (Mocan et al. 2008). A two-dimensional reconstruction map of the living keratoconic cornea revealed abnormal SNP architecture and significantly reduced density compared to normal corneas (Patel and McGhee 2006). At the apex of the cone, a tortuous network of nerve fibre bundles was noted, while at the topographic base of the cone, nerve fibre bundles appeared to follow the contour of the base, with many of the bundles running concentrically in this region. The SNP reduction was shown to correlate with corneal sensation and keratoconus stage (Patel et al. 2009).

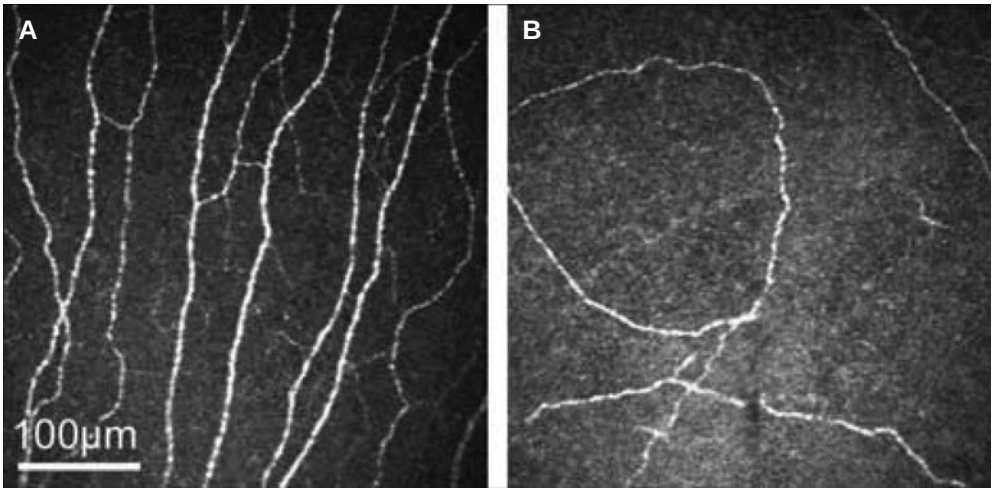

Fig. 5 IVCM demonstrating SNP in normal (*A*) and keratoconic (*B*) corneas (Patel et al. 2009). Permission obtained from Nature Publishing Group.

In conclusion, there is histological and *in vivo* evidence of the involvement of corneal nerves in the pathologic process of keratoconus, suggesting that they may play a role in the pathophysiologic features and progression of the disease.

5. Diabetes mellitus

The cornea is significantly affected in diabetes mellitus, and the pathological changes to the corneal cellular structures often cause visual impairment. Diabetic corneas exhibit reduced numbers of haemidesmosomes, basement membrane abnormalities, altered growth factor levels, epithelial cellular enlargement, edema, and delayed wound healing resulting in persistent epithelial defects (Cavallerano 1992). The most recognized diabetic complications in the cornea include neurotrophic corneal ulcers, filamentous keratitis, decreased corneal sensation, and a characteristic epithelial dystrophy, which is referred to as diabetic keratopathy (Kenney et al. 2005). At first, the involvement of corneal nerves in diabetes pathogenesis was shown in experimental studies with diabetes induction. Thus, corneal innervation was studied by light and electron microscopy in rats with streptozocin-induced diabetes, using the nonspecific cholinesterase reaction, gold chloride impregnation, and plastic-embedded sections (Ishida et al. 1984). In that study axonal degeneration could be demonstrated along with increased beading and irregularities in the basal lamina of Schwann cells. More recently, another *ex vivo* study was published in which investigation of qualitative and quantitative parameters of SNP and stromal nerves was performed *ex vivo* on human corneal sections (corneas obtained from the eye bank), using immunohistochemical staining with beta III tubulin antibody, which is a well-recognized pan-neuronal marker (He and Bazan 2012). The authors of this study provided an entire view of the nerve architecture in human diabetic corneas, showing a significant decrease in SNP density, which was noted already at early stages

of the disease and correlated with diabetes duration. The nerves coursing from the periphery to the central cornea displayed increasing tortuosity and irregular nerve beading. The latter is a recognized parameter for some cases of compensatory nerve regeneration.

In recent years, IVCM, as a rapid, non-invasive clinical examination technique, has opened new opportunities for investigation of corneal nerves in human diabetic patients. The first publication on this issue is a clinical study involving patients with type 1 diabetes mellitus (Rosenberg et al. 2000). The authors observed reduction of SNP in all patients, and this occurred even in patients with mild to moderate neuropathy, whereas corneal mechanical sensitivity was reduced only in patients with severe neuropathy, suggesting that decreases in nerve fibre bundle counts precede impairment of corneal sensitivity. Furthermore, the decrease of SNP correlated positively with epithelial thinning. Also in type 2 diabetes mellitus the corneal SNP displayed significant changes in terms of nerve fibre density, nerve branch density, and increased tortuosity coefficient (Chang et al. 2006). The authors of this work revealed also a significant reduction of corneal basal epithelial cell density, which correlated with SNP impairment. The alterations in corneal innervations and basal epithelial cell density could be demonstrated for different neuropathic stages.

These findings were later confirmed in numerous *in vivo* studies utilizing laser-, tandem- or slit-scanning confocal microscopes (reviewed by Efron 2011). Various parameters of SNP fibre morphology have been investigated, including nerve fibre count, length, branching, beading, width, tortuosity, orientation, and reflectivity. The absolute counts of nerve parameters differed among studies. Discrepancies in absolute values reported by different research groups can be attributed to the use of different instruments and different approaches to stereological analysis of confocal images. Nevertheless, most reported findings on SNP parameters are in good agreement with each other, showing overall a significant decrease in SNP length and density. The reduction of SNP density along with progressive decrease in corneal sensation was shown to correlate with increasing severity of diabetic neuropathy (Tavakoli et al. 2011). Notably, simultaneous pancreas–kidney transplantation restored SNP parameters; significant improvements in corneal nerve fibre density, branch density, and length could be

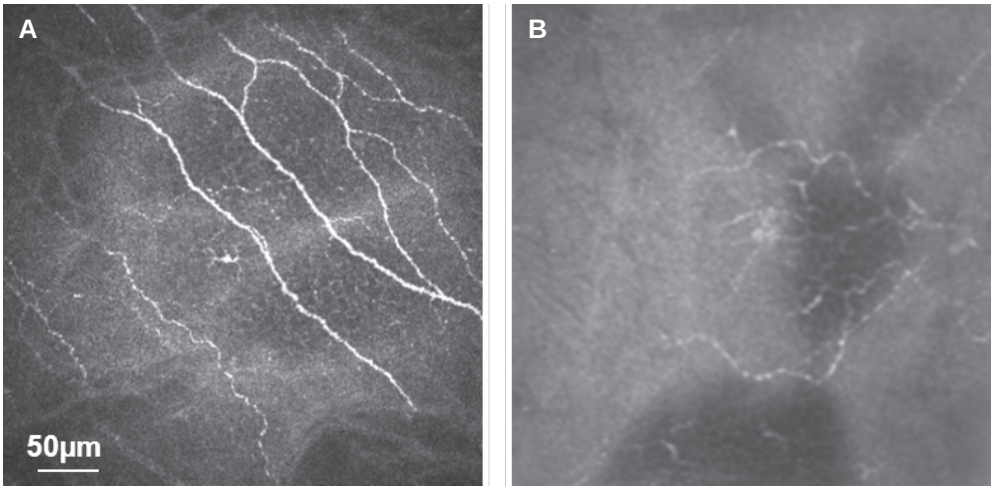

Fig. 6 IVCM demonstrating the SNP in normal (*A*) and diabetic (*B*) corneas (Zhivov, unpublished)

shown 12 months after surgery. It is noteworthy to mention that normalization of glycaemia after transplantation showed no significant improvement in neuropathy assessed by the neurologic deficits (Tavakoli et al. 2013), suggesting that not only the early nerve degeneration, but also the regeneration, that was missed by neurophysiological assessment techniques, can be detected and accurately measured by IVCM.

More recently, our group examined corneal innervation using an automated analysis of pre-segmented SNP structures (Zhivov et al. 2013). We analysed 10 SNP parameters and correlated the findings with neuropathic status and diabetic retinopathy. The nerve fibre density, total fibre length and nerve branches were found to be significantly decreased in diabetic patients, and this reduction correlated with neuropathic status. The patients exhibited also reduced corneal sensation. Interestingly, the patients without diabetic retinopathy demonstrated significant SNP changes, suggesting that corneal nerve alterations precede retinal microvascular changes.

In conclusion, the SNP is negatively impacted in both types of diabetes mellitus. SNP changes become obvious even at early stages of diabetes and prior to manifestation of neurological symptoms. This fact has made the SNP a surrogate marker for diabetic neuropathy. SNP pathology is associated with a reduction of corneal basal epithelial cell density, which seems completely logical taken into consideration the tight interplay between corneal epithelial cells and nerves.

6. Corneal Surgery

The effect of cutting and laser ablation of the cornea in various procedures has drawn much attention to the corneal innervation. Several treatment approaches, including Laser Epithelial Keratomileusis (LASEK), laser *in situ* keratomileusis (LASIK) and photorefractive keratectomy (PRK) disrupt the integrity of the corneal sensory innervation reducing corneal sensation and trophic function. This can lead to epithelial disintegrity, disturbed wound healing and epitheliopathy (Wilson 2001).

Corneal nerves are disrupted during LASEK surgery and the procedure results in a significant reduction in corneal sensation. The depressed corneal sensation after LASEK was shown to improve during the next few months after surgery (Herrmann et al. 2005). The reduction of sensitivity after LASEK revealed a direct correlation with the number of nerves in the SNP, as shown by IVCM, and also the regeneration of corneal nerves correlated strongly with the recovery of corneal sensation (Lee et al. 2006). On the other hand, another group demonstrated that SNP density did not return to preoperative levels 6 months after surgery, while corneal sensitivity returned to normal levels 3 months after surgery (Darwish et al. 2007).

Degeneration of nerve structures in the SNP starts just a few hours after LASIK. Although long SNP fibres are still visible in the central cornea 3 days post-LASIK, they are significantly reduced in number, or even entirely absent by 1 week (Linna et al. 2000, Stachs et al. 2010). The first very thin nerve fibres could be visualized 1 month after the procedure, following by gradual recovery over time, and an SNP incorporating parallel and branched fibres began to be visualized 1 year postoperatively (Stachs et al. 2010). In this study we demonstrated, that despite continuous increases in density over time, the SNP appears to remain incomplete for up to 2 years after LASIK.

SNP nerve density after LASIK has been reported to be reduced by 51 %, 35 %, and 34 % at 1, 2, and 3 years, respectively (Erie et al. 2005b).

Photorefractive keratectomy (PRK) also affects the corneal nerves in a drastic way. Corneal sensitivity is known to be significantly reduced at week 1, with a further significant reduction at week 2 (Murphy et al. 1999). A gradual recovery in sensitivity then followed to reach preoperative levels by 1 year. After PRK, mean SNP density was reduced by 59 % at 1 year when compared with preoperative values. By 2 years, SNP density was not significantly different from density before PRK and remained unchanged over the following 5 years (Erie et al. 2005b).

Penetrating keratoplasty involves transection of all corneal nerves in both the host and donor cornea. The subsequent recovery is a prerequisite for normal corneal function. In a longitudinal study, corneas were examined using IVCM over 12 months after keratoplasty (Darwish et al. 2007). Interestingly, corneal sensation was apparently recovered by 1 year, while at that time point almost no nerves in the SNP could be observed. The authors suggested that this discrepancy might be a methodologic phenomenon related to the inability of IVCM to image fine regenerating nerves that mediate corneal sensibility. Our group has demonstrated that the first finest branches of the SNP become obvious 12 months keratoplasty.

Previously, it was shown histochemically, that neither the SNP nor stromal nerves had completely regenerated by 3 years post-keratoplasty (Tervo et al. 1985). The study with the longest follow-up period reported re-innervation of the central cornea with sub-basal nerves and stromal nerves occurring at 2 years and 7 months postoperatively, respectively (Richter et al. 1996). Taken together, the findings on corneal re-innervation after keratoplasty are relative inconsistent. The extent and time of re-innervation in corneal grafts has been shown to be unpredictable and varies in individual patients, depending on the patients' age and preoperative diagnosis.

Taken into consideration the key role of corneal nerves for ocular surface integrity, attempts have been made to accelerate corneal nerve regeneration after injury. The corneal regenerative response is known to correspond with the coordinated expression of neurotrophins and regeneration-associated genes (Huebner and Strittmatter 2009). Neurotrophins have been repeatedly reported in the literature to have beneficial effects on corneal nerves, predominantly in animal models. Topically-applied NGF was beneficial in the early recovery of corneal sensitivity after LASIK (Joo et al. 2004) and PRK (Esquenazi et al. 2005) in rabbits.

More recently, we created a new murine model to study corneal nerve regeneration predominantly at the SNP level. With a guided trephine system a circular incision through the corneal epithelium and anterior stroma was generated to selectively cut the SNP nerves while leaving the deeper lying stromal nerves intact. In contrast to other injury models, involving epithelial removal, our model did not cause excessive inflammation and any epithelial defects. On this model we further examined the possible positive influence of ciliary neurotrophic factor-CNTF, which was applied topically. Our model was sensitive enough to differentially quantify the morphological parameters in 2 groups, treated after mechanical nerve cutting either with vehicle or with CNTF. In this study we were able to ascertain more facilitated regeneration in the CNTF-group, suggesting that this cytokine might have also therapeutic usefulness in clinical practice.

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Diabetic Maculopathy – Can Optical Coherence Tomography Guide the Management?

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With 2 Figures and 1 Table

Abstract

The use of optical coherence tomography (OCT) has allowed major progress in the evaluation of diabetic maculopathy. The OCT examination is fast, non-invasive and gives a biomicroscopic section through the retina. We describe 5 parameters for OCT evaluation which determine the visual prognosis of the patient and can be used to develop a new classification of diabetic maculopathy for future individualized treatment of this disabling disease.

Zusammenfassung

Die Optische Kohärenztomographie (OCT) führt zu einem deutlichen Fortschritt in der Diagnostik der diabetischen Makulapathie. Die OCT-Untersuchung ist schnell und nicht-invasiv und ergibt einen biomikroskopischen Schnitt durch die Netzhaut. Wir beschreiben fünf Auswertungsparameter, die die Sehschärfe beeinflussen und für eine neue Klassifikation benutzt werden können. Damit könnte in Zukunft eine individuelle Therapieentscheidung mit objektiven Daten getroffen werden.

1. Introduction

Many diabetic patients lose their vision through complications of proliferative retinopathy, but the major cause of visual impairment is diabetic macular edema (DME).

The clinical examination of a diabetic patient's eyes is done by examining the best corrected visual acuity (BCVA) and then by stereoscopic ophthalmoscopy, documenting changes by photography. Additional methods of specific value are fluorescein angiography (FAG) and optical coherence tomography (OCT).

FAG gives information which cannot be obtained by ophthalmoscopy as various stages of circulation and the competence of the blood retinal barriers can be examined. It identifies abnormalities of perfusion and is the only tool to find an ischaemic maculopathy with areas of non-perfusion. It is not possible to predict the foveal avascular zone outline and size based solely on the volume and thickness measurement or retinal structure evaluation on OCT. FAG gives permanent documentation in the form of a time resolution film or set of photographs. The invasive FAG procedure is regarded as safe but rarely severe reactions have been recorded.

OCT is a high speed diagnostic imaging method that generates high resolution two-dimensional cross sectional images through detection of reflections of infrared light has become an essential tool in ophthalmology. OCT fundus images provide an accurate spatial

colocalization of retinal features observed on the *en face* and cross sectional images. The datasets can be displayed as a volume image and therefore allow for the three-dimensional examination of the retina. The OCT examination gives detailed information about the retinal structure over large areas and holds the promise for an unprecedented ability to describe and monitor changes in the local geometry of the retina. The OCT image closely approximates the histologic appearance of the macula and for this reason has been referred to as an *in vivo* optical biopsy. Commercial instruments have an axial resolution of approximately 5 µm. For clinical purpose the examination time is limited by the patient's ability to avoid eye movements. OCT has great potential as it is non-invasive and delivers digital data which can be analysed and stored.

Tab. 1 Comparison of different method for evaluation of DME

	Vision	Ophthalmoscopy	FAG	OCT
Cost	++	++		
Invasiveness	++			++
Morphology				++
Perfusion			++	
Telemedicine		++		++

All methods have advantages and disadvantages: They differ in invasiveness and in the capacity to detect a specific pathological feature: e.g. retinal ischaemia can only be recognized and proven by FAG.

2. Results

DME, defined as retinal thickening in the posterior pole results from vascular hyperpermeability and other alterations in the retinal microenvironment and represents a common cause of vision loss among diabetics. It can occur in perfused and ischaemic areas and may or may not be characterized by intraretinal cyst formation. The natural history is variable. For the exact localization and quantitative assessment of DME by OCT we have developed 5 rules:

– *Central subfield mean thickness*: Central subfield mean thickness is the preferred OCT measurement for the central macula because of its higher reproducibility and correlation with other measurements of the central macula (Browning et al. 2008a, b, Chan et al. 2006). The normal retina has a central thickness of 250 µm. Modern treatment with anti-VEGF injections reduces the thickness and is partially guided by the thickness.
– *Vitreoretinal interface*: The importance of the vitreomacular interface has only recently been fully acknowledged since the natural history and pathology of a posterior vitreous detachment (PVD) were understood. PVD is not an acute process but a protracted event (Johnson 2010). A higher prevalence of perifoveolar PVD with foveolar attachment is seen in diabetic patients with macular edema. A perifoveolar PVD may have a role in the development of this complication (Gaucher et al. 2005). Diabetic eyes without macular edema have a significantly higher rate of PVD than those with macular edema (Nasral-

LAH et al. 1988). Vitreous attachment is a risk factor for DME. This is due to traction and probably an altered concentration of biologic mediators at the border between vitreous and retina (KISHI et al. 2003). A high prevalence of vitreomacular interface abnormalities in eyes with persistent DME was demonstrated after focal laser treatment and underscores the superiority of OCT in detecting these abnormalities (GHAZI et al. 2007).

Fig. 1 Diabetic macular edema with epiretinal membrane

On the other side a PVD can result in resolution of DME in 55 %. Findings suggest that vitreomacular separation may promote the spontaneous resolution of DME and consequently improve visual acuity (HIKICHI et al. 1997).

– *Intraretinal changes*: OCT findings showed that the cystoid spaces in the outer plexiform layer (OPL) were accompanied by photoreceptor damage beneath the cystoid spaces in DME (MURAKAMI et al. 2012). Hard exudates in the macular region even at a distance from the foveal centre affect macular function with DME (HOLM et al. 2010) and associate with locally reduced microperimetric sensitivity. Thus, the lesions associated with reduced sensitivity (macular hard exudates and cystoid oedema) for a white-on-white stimulus were such lesions that cause light to be blocked or scattered before it reaches the photoreceptors, suggesting that optical effects are a major cause of sensitivity loss (SOLIMAN et al. 2010).
– *Photoreceptor integrity*: There have been many attempts to predict function (BCVA and microperimetric sensitivity) and morphology. The best correlation between morphology and function in different macular diseases seems to be with photoreceptor integrity as evaluated by OCT.

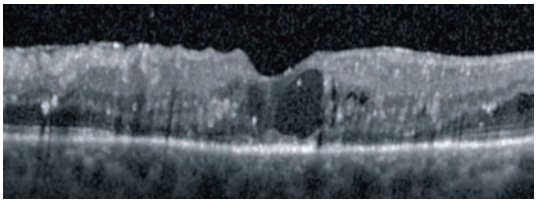

Fig. 2 Loss IS/OS line under cystoid spaces in DME

(*a*) OCT showed that the cystoid spaces in the OPL were accompanied by photoreceptor damage beneath the cystoid spaces in DME (Murakami et al. 2012).
(*b*) The presence of hyperreflective foci in the outer retina is closely associated with a disrupted external limiting membrane (ELM) and inner segment/outer segment (IS/OS) junction on the OCT image and decreased BCVA in DME (Uji et al. 2012).
(*c*) IS/OS and ELM are useful hallmarks for use in evaluation of foveal photoreceptor layer integrity, and are closely associated with final BCVA in DME (Shin et al. 2012). Pretreatment BCVA and photoreceptor status can predict potential restoration of photoreceptor integrity and subsequent visual recovery in DME (Shin et al. 2012). Postoperative visual acuity of eyes with complete IS/OS after resolution of DME was significantly better than that without complete IS/OS, though macular edema was completely resolved in both groups (Sakamoto et al. 2009).
(*d*) In particular, photoreceptor outer segment (POS) thickness seems to be an important predictor of function and visual acuity in patients with DME (Alasil et al. 2010). POS length can be quantitatively assessed using OCT. The strong correlation of POS length with visual acuity suggests that the POS measures may be directly related to visual function (Forooghian et al. 2010).

– *Subretinal changes*: Subretinal changes such as a fibrosis make a visual recovery impossible.

3. Discussion

OCT is a useful tool to detect and measure DME without the need for pupillary dilatation (Medina et al. 2012).

Little evidence exists that characteristics of DME described by the terms „focal“ or „diffuse“ help to explain variation in visual acuity or response to treatment (Browning 2008a). The established classifications of retinal edema were not developed with the modern OCT technology and need to be adapted to include the new findings.

A new classification of diabetic maculopathy is now possible. The new classification should consider:

– Localization of the edema (DME with or without centre involvement);
– vascular condition (vasogenic, ischaemic);
– central subfield mean thickness;
– vitreoretinal interface abnormalities (vitreomacular adhesion or traction);
– intraretinal changes (macular hard exudates and cystoid edema, quantity and pattern);
– photoreceptor integrity;
– subretinal changes.

Diabetic maculopathy is a different disease process at each stage, and each patient is different. In the future the OCT may allow a more personalized treatment, which may impact treatment selection and patient subgroup stratification.

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Session 3:
Diabetes Care – Today and Tomorrow

Nova Acta Leopoldina NF *119*, Nr. 401, 179–186 (2014)

Early Detection of Neural Dysfunction in Diabetic Retinopathy

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With 2 Figures and 1 Table

Abstract

Multiple studies have shown that diabetes impacts the structure and function of the neurosensory retina, yet there is little understanding of how to use them as diagnostic tools in clinical research to define disease state and predict progression, or to implement them into clinical practice. The evolution of diabetes into a world-wide epidemic requires more complete understanding of the neurosensory component of diabetic retinopathy and a diagnostic approach that suits the patient population of the twenty-first century.

Zusammenfassung

Zahlreiche Studien belegen den Einfluss des Diabetes mellitus auf die Struktur und Funktion der neurosensorischen Retina. Es fehlt ein durchgehendes Verständnis, wie dieses Wissen in der klinischen Forschung, zur Festlegung des Krankheitsstatus, dessen Progression oder in der praktischen klinischen Arbeit zu nutzen ist. Aufgrund der weltweiten Diabetesepidemie ist es erforderlich, ein umfassendes Wissen der neurosensorischen Komponenten der diabetischen Retinopathie zu erlangen, um angemessene diagnostische Vorgehensweisen, die der Diagnostik des 21. Jahrhunderts entsprechen, zu entwickeln und diese für die Patienten verfügbar zu machen.

1. The Evolution of Diabetic Retinopathy Research

The understanding and classification of diabetic retinopathy has been conventionally understood in terms of the vascular features since its definition over 160 years ago. That is, ophthalmoscopy reveals signs of leakage, occlusion, and proliferation of abnormal new vessels leading to microaneurysms, haemorrhages, macular edema, and neovascularization, respectively. During the last 60 years advanced stages of diabetic retinopathy have been treated with surgical approaches such as lasers and vitrectomy in persons whose vision is threatened.

The focus on vascular features provided a useful means to classify patients, and to develop clinical trial endpoints and outcomes for regulatory approval of treatments. Late stage diabetic retinopathy causes severe impairment of vision as assessed by the Early Treatment Diabetic Retinopathy Study (ETDRS) visual acuity chart that was developed in the 1980s (Ferris et al. 1982). The EDTRS chart was derived from the 1968 Airlie House Classification (Goldberg and Fine 1969) when pituitary ablation was the only available therapy for proliferative diabetic retinopathy. Today, the standard of care for PDR is pan-retinal photocoagulation which prevents severe vision loss as assessed by visual acuity, but laser therapy decreases the overall quality of vision by damaging the visual field and night vision. To achieve better patient out-

comes in the 21st century greater emphasis must be placed on diagnosing and treating the earlier stages of diabetic retinopathy. Improved medical therapies are required to meet the demand created by an increasing population of patients with type 2 diabetes. Visual acuity as a clinical trial endpoint is impractical for evaluating treatments for early diabetic retinopathy therapies so new approaches are required to deliver the promise of improved patient outcomes.

In the six decades since the development of these early classifications and destructive treatments for diabetic retinopathy, the efficacy of diabetes care has improved markedly, advancing from a point in the late 1970s in which urine glucose testing was the sole means for patients to test their blood sugar concentrations, and most patients received one or two daily injections of long-acting insulin (often of bovine or porcine origin), to the routine use of home glucose monitors, insulin pumps, improved blood pressure and lipid control. This more aggressive approach developed from the Diabetes Control and Complications Trial (*The Diabetes Control and Complications Trial Group* 1993) and United Kingdom Prospective Diabetes Study (*UK Prospective Diabetes Study Group* 1998) that proved intensive metabolic control reduces all complications compared to the standard control of the period. This revolution has led to marked reduction in the risk of complications (Nathan et al. 2009, Klein et al. 2010). Screening programmes that facilitate earlier diagnosis of retinopathy when vision is largely intact has been successful in some countries (Britain, Denmark, Iceland) through screening of populations of persons with diabetes.

The understanding of the pathophysiology of the vascular components of diabetic retinopathy has also expanded with the definition of the role of vascular endothelial growth factor (VEGF) and the use of VEGF antagonists to improve macular edema and possibly proliferative retinopathy (Antonetti et al. 2012). Over the same decades, there has been slow but substantial expansion of investigations into the impact of diabetes on the neurosensory retina. Now these collective advances allow greater understanding of the pre-clinical and early pre-clinical stages of diabetic retinopathy when visual acuity is largely intact. At this point patients have minimal symptoms and may have only a few microaneurysms and haemorrhages, and have not yet developed macular edema or neovascularization.

Therefore, it is useful to consider the global impact of diabetes on the retina, how these impacts of diabetes can be measured, and how these measurements can quantify the effects of treatments. These efforts may lead to further improvements in the visual outcome of persons with diabetes. Useful perspective on neuroretinal dysfunction in diabetes may be gained by the examples of retinitis pigmentosa and glaucoma. In these conditions, understanding the role of photoreceptor and ganglion cell degeneration, respectively, has led physicians to evaluate patients on the basis of tests that reflect the nature of the disease pathogenesis-visual fields and electroretinography – rather than on central visual acuity which is preserved until late in the disease course. In addition, genotype-phenotype correlations have led to better understanding of distinct types of retinitis pigmentosa and glaucoma that give rise to disease staging, understanding of prognosis, and individualized treatments. These examples suggest the possibility that the impact of diabetes on the neurosensory retina assessed by tests other than visual acuity may lead to better understanding of the pathophysiology and clinical impact of diabetes on the retina.

Clinic and laboratory based studies of the impact of diabetes on visual function and the neurosensory retina now exceed 200 publications. Well-recognized changes in visual function include reduced contrast sensitivity, blue-yellow colour perception, dark adaptation, photostress responses, photopic visual fields and electroretinographic responses (Roy et al. 1986,

Della Salla et al. 1985, Frost-Larsen et al. 1981a, Agardh et al. 2006, Simonsen 1974, Holfert et al. 2011). Studies of retinal cellular alterations include retinal endothelial cell tight junction proteins and leukostasis, decreased intermediate filament protein expression in astrocytes, progressive apoptosis of retinal neurons including ganglion cells, amacrine and bipolar cells, and loss of photoreceptors; loss of synaptic protein expression; microglial cell activation; Müller cell dysfunction that includes increased expression of intermittent filament proteins, impaired glutamate-glutamine metabolism and decreased Kir 4.1 potassium channel expression; and disruption of pigment epithelial cell tight junction integrity (Antonetti et al. 2012, Stitt et al. 2013). Thinning of the retina has been detected by optical coherence tomography (van Dijk et al. 2010, Peng et al. 2009). These changes have been appreciated mostly within the last 15 years. However, the first report of neuronal defects in the retina were described in 1927 (Donato 1927). Bloodworth (1962) and Wolter (1961) described retinal ganglion loss in the early 1960s, and Simonsen (1969, 1974) pioneered the use of electroretinography to reveal Müller cell defects. Bresnick emphasized the role of the involvement of the neurosensory retina 25 years later (Bresnick 1986, Bresnick and Palta 1987), and Frost-Larsen et al. (1981b) showed that a photostress, the recovery of visual acuity after light adaptation could predict incident DR. Adams and colleagues (Bearse et al. 2004, 2006, Ng et al. 2008) have detailed reduction in multifocal ERG implicit times that reveal loss of function of the peripheral retina in early diabetes and the ability to predict development of retinal vascular lesions. Agardh et al. (Bengtsson et al. 2005) showed that sensitivity using short-wave automated and white-on-white perimetry correlate with ETDRS retinopathy grade. Several studies have shown that diabetes reduces frequency doubling perimetry sensitivity, indicative of the function of the inner retina, including magnocellular ganglion cells, in persons with diabetes without retinopathy and with mild nonproliferative retinopathy (Realini et al. 2004, Parikh et al. 2006, Parravano et al. 2008, Jackson et al. 2012, Wang et al. 2012). Taken together, these functional assays indicate that diabetes impairs the function and interactions of inner retinal neurons and glia, as well as the photoreceptor-pigment epithelial complex (Jackson and Barber 2010). However, most of these studies examined a single endpoint in modest-sized cohorts in a cross-sectional manner, and the metabolic characterization of the subjects has been limited. The uses of these tests and their relative merits in diabetes are shown in Table 1.

Jackson et al. (2012) studied six visual function endpoints in normal controls, persons with diabetes without retinopathy and persons with mostly mild to moderate non-proliferative diabetic retinopathy. These endpoints included ETDRS visual acuity, contrast sensitivity, dark adaptation, frequency doubling perimetry, standard white-on-white photopic visual fields, and dark-adapted (scotopic) visual fields. The patients were similar in age and had visual acuity of 20/30 or better. Results show that 83 % of patients with diabetes or diabetic retinopathy exhibited significantly reduction in foveal sensitivity with the matrix frequency doubling perimeter, and 47 % showed reductions in scotopic visual field sensitivity, 33 % in photopic visual fields, and 25 % each in contrast sensitivity and dark-adaptation, but only 17 % had subnormal visual acuity (Fig. 1). To the best of our knowledge, this is the first multi-modality study of visual function in diabetes and the results show that patients with non-proliferative diabetic retinopathy had a 50 % reduction in FDT sensitivity and scotopic sensitivity indicating both inner and outer retinal defects. The FDT impairment was three times greater than visual acuity loss. Together, these findings indicate that both the inner and outer retina are affected by diabetes.

Tab. 1 Visual Function Tests Relative to Diabetic Retinopathy

Test name	Retinal region(s) evaluated	Advantages	Disadvantages	Time required	References
Visual acuity	spatial resolution by foveal cone photoreceptors	rapid, repeatable	can be normal until late stage disease; does not reflect extrafoveal involvement	5–15 min	Ferris et al. 1982
White on white perimetry (photopic visual sensitivity)	macular and peripheral cone photoreceptors	quantitative, widely available	variable responses; tiring for patients	5–20 min/eye	Agardh et al. 2006
Full-field ERG	whole retina cone and rod photo-receptors and their interactions	sensitive to early changes in retinal function; predicts progression from NPDR to PDR	requires dark adaptation, expensive equip-ment, highly trained staff, corneal electrodes, high degree of standardization	≈ 40 min	Roy et al. 1986, Agardh et al. 2006, Simonsen 1974, Holfort et al. 2011, Bresnick and Palta 1987
Multifocal ERG	whole retina cone photoreceptors	predicts development of NPDR	requires expensive equipment, highly trained staff, corneal electrodes, high degree of standardization	30 min	Bearse et al. 2004, Bearse et al. 2006, Ng et al. 2008
Contrast sensitivity	inner retinal cells of fovea	rapid, readily available	not well standardized for clinical trials	10–30 min	Della Salla et al. 1985
Photostress	foveal cone photoreceptors	predicts development of NPDR	instrument not currently available	20 min	Frost-Larsen et al. 1981a
Frequency doubling perimetry	magnocellular ganglion cells of inner retina responsible for the central 24° of visual field	can be used in clinic settings no pupil dilation required; minimal technician training required	not standardized for clinical research	5 min/ eye	Realini et al. 2004, Parikh et al. 2006, Parravano et al. 2008, Jackson et al. 2012
Dark adapto-metry	cone and rod function	new instruments permit rapid testing	pupil dilation	5–20 min	Frost-Larsen et al. 1981b

The question of reversibility of functional changes has yet received little attention. However, Holfort and colleagues (2011) followed subjects with type 1 diabetes following the initiation of continuous subcutaneous insulin therapy and found significant improvement in rod photoreceptor dark adaptation and dark-adapted b-wave amplitudes were seen after 52 weeks, time to a standardized rod intercept, dark-adapted rod b-wave full-field amplitude, standard combined rod-cone b-wave amplitude, but no detectable change in cone adaptation, electroretinographic cone function or retinopathy. This is, to our knowledge, the first longitudinal study that demonstrates the ability of the retina to improve in response to enhanced metabolic control. The results further suggest the possibility that retinal function tests may be used to

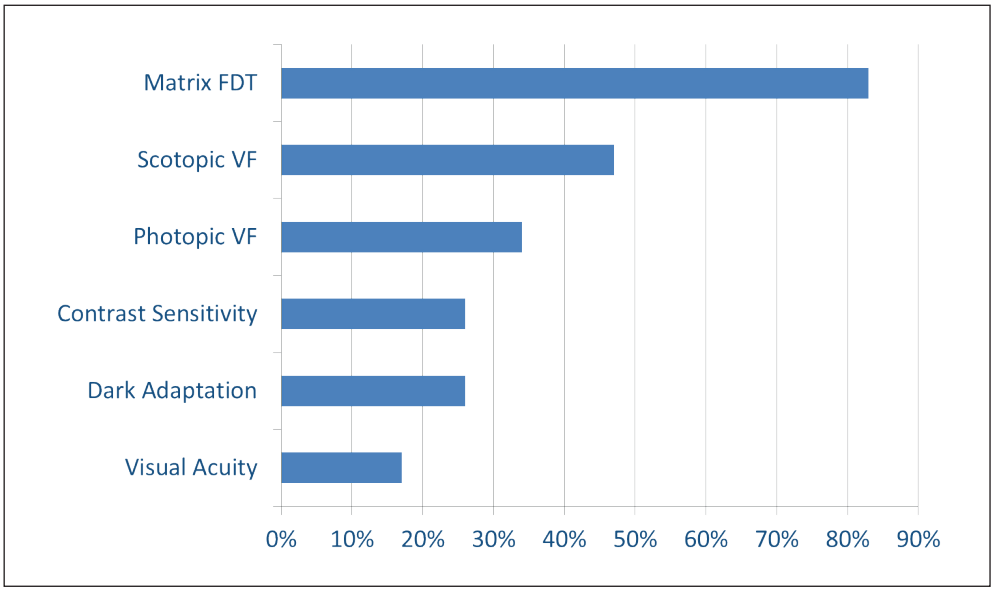

Fig. 1 Percentage of subjects falling outside of the normal reference range. From Jackson et al. 2012

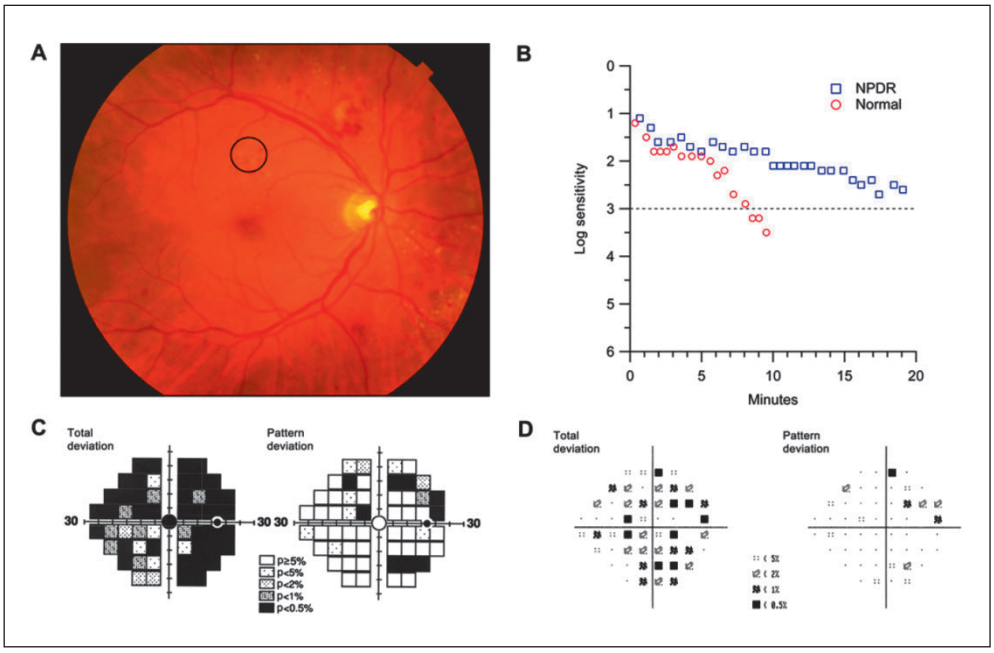

Fig. 2 A patient with 21 years of type 2 diabetes mellitus, 20/25 visual acuity and moderate non-proliferative diabetic retinopathy (NPDR) exhibits gross visual function abnormalities (*A*). Dark adaptation (*B*) was grossly impaired compared with the typical normal adult. The frequency doubling technology field (*C*) exhibited quite deep deficits in sensitivity that were exhibited to a lesser but significant extent on the light-adapted visual field (*D*). From Jackson et al. 2012.

monitor retinal status independently of fundus imaging and to reveal subtle features of retinal pathophysiology that have not been discernible in the past.

2. A Path Forward

Numerous studies over the past several decades provide incontrovertible evidence that visual function tests reveal information about the retina in persons with diabetes, just as they have for retinitis pigmentosa and glaucoma. Next steps must include: *First*, longitudinal studies that assess multiple aspects of visual function in persons with well-characterized diabetes; and *second*, use of visual function tests as endpoints in therapeutic clinical trials. These studies will require the collaboration of multiple centres and sufficient funding to ensure conclusive results so that the flood tide of diabetic retinopathy in the 21st century can be stemmed.

3. Conclusion

Collectively, numerous studies have shown that diabetes affects the entire retina including the entire neurovascular unit, and the impact appears to be slightly greater in the inner retina than the outer retina. Additional longitudinal studies are now needed to determine how these visual function defects correlated with additional structural changes and if they parallel or predict development of typical microvascular lesions. Such studies will be essential tools for the development of therapeutic trials to continue to reduce the burden of diabetic retinopathy.

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Treatment of Diabetes mellitus Type 2 – A Practical Approach

Hans-Christof Schober and Daniel Paschke (Rostock)

With 2 Figures and 3 Tables

Abstract

Sedentary lifestyle, obesity and an ageing society combine an increase in the prevalence of type 2 diabetes, a condition in which the morbidity and mortality from cardiovascular disease overcomes all other reasons for dying. The most effective way to lower cardiovascular risk in type 2 diabetes is to combine treatment of blood pressure, lipids and glucose. Accompanying problems in elderly people like adherence, depression, multimorbidity, and polypharmacy should be implemented in treatment decisions. It is time to move from glucose lowering to an individualized approach implementing different treatment targets and to keep people moving.

Zusammenfassung

Bewegungsarmut, Übergewicht und Adipositas sowie eine deutlich älter werdende Gesellschaft führen zu einem Anstieg der Prävalenz des Diabetes mellitus Typ 2. Makroangiopathische Veränderungen wie Herzinfarkt, Schlaganfall und arterielle Verschlusskrankheit bestimmen in höherem Maße Morbidität und Mortalität als mikroangiopathische Veränderungen. Das auf diese Veränderungen bezogene Therapieziel der Blutzuckersenkungen ist nicht so effektiv wie die Kombination aus Fett-, Blutdruck- und Blutzuckersenkung. Hinzu kommen insbesondere bei betagten Patienten Probleme wie Medikationstreue, Depression, Multimorbidität und Polypharmazie. Alle diese Faktoren erfordern eine individualisierte, einzelfallbezogene Therapie, in deren Zentrum nicht die Blutzuckersenkung, sondern die individuellen Patienteninteressen stehen sollten. Ein zentraler Stellenwert kommt dabei der physischen Aktivität zu. Diese wirkt präventiv und therapeutisch.

1. Introduction

Diabetes mellitus is a common disease in western countries and it is a relevant cardiovascular risk factor. The aims of treatment are therefore the reduction of cardiovascular morbidity and mortality. A huge number of studies are available concerning glycaemic management in type 2 diabetes. Concerning cardiovascular morbidity and mortality different therapeutic options have been developed as shown by the Steno-2 study (Gaede et al. 2003). The most effective treatment is the combination of lowering blood pressure, lipids and glucose. Intensified glucose lowering is more difficult to achieve and able to induce a negative impact in quality of life (Huang et al. 2007). Our society is an aging society, with an increasing number of frail people, taking polypharmacy due to multimorbidity. Therefore, the background risks of each patient are different.

2. Questions and Results

In daily clinical settings question arises: Are these facts taken into account in the recent studies concerning treatment of diabetes type 2? Previous opinions like to control glycaemic parameters have to be discussed. In order to deal with these problems, a critical approach is essential. There seems to be a need to reverse previous opinions. Two cases may illustrate these problems: Inhospital patients allocated due to diabetic problems are usually older (Tab. 1).

Tab. 1 Cases of inhospital patients allocated due to diabetic problems

	Case (*1*), Mr. E. T., male	Case (*2*), Ms. A. F., female
Age (a)	82	76
Diabetes duration (a)	40	25
HbA1c (%)	11,0	13.0
Glucose (mmol/l)	5.1 – 31	9.0 – 21
BMI (kg/m^2)	28	34
Blood pressure (mmHg)	160/90	165/95
Total cholesterol (mmol/L)	3.6	7.2
Triglycerides (mmol/l)	2.7	4.1
Abdominal circumference (cm)	114	118
Number of drugs	8	12

Both cases illustrate – these patients do not take their medication correct. The diabetes is permanently decompensated, the problems "surrounding" the diabetes are as important if not more relevant than glycaemic control. Therefore, the questions are:

– What about reasonable (meaningful) treatment in this age group?
– What about adherence and compliance in older patients?
– Is less more?

How many drugs to elderly patients usually take? – In average 8, median 11, from 2 to 25 (O'Sullivan et al. 2013). Three recently published studies concerning glycaemic control and associated co-morbidities (Patel et al. 2008, Duckworth et al. 2009, *Action to Control Cardiovascular Risk in Diabetes [ACCORD] Study Group* 2008) do not meet the patients characteristics described above:

Age (a):
VADT: 60.3 ± 9.0
ADVANCE: 66.0 ± 6.0
ACCORD: 62.3 ± 6.8

HBA1c (%):
VADT: 9.40 ± 2.0
ADVANCE: 7.51 ± 1.57
ACCORD: 8.30 ± 1.0

There are a number of guidelines developed in view of the complexity in the treatment of diabetes type 2:

- Concerning Diabetes: HbA1c (%) < 6,5?
- Concerning LDL (mmol/L): < 1.6
- Concerning blood pressure (mmHg): 130/85
- Concerning CHD: Aspirin
- Concerning Dyslipidaemia: Statins

To fulfil these treatment goals a widening array of pharmacological agents is available. By using all these possibilities the hope is to avoid the diabetic damages like micro- and macroangiopathy.

Concerning the older patients, the effectiveness of these approaches has to be determined, a risk benefit ratio would be helpful. A recently published paper (Yudkin et al. 2010) provides same numbers need to treat in order to achieve guideline goals.

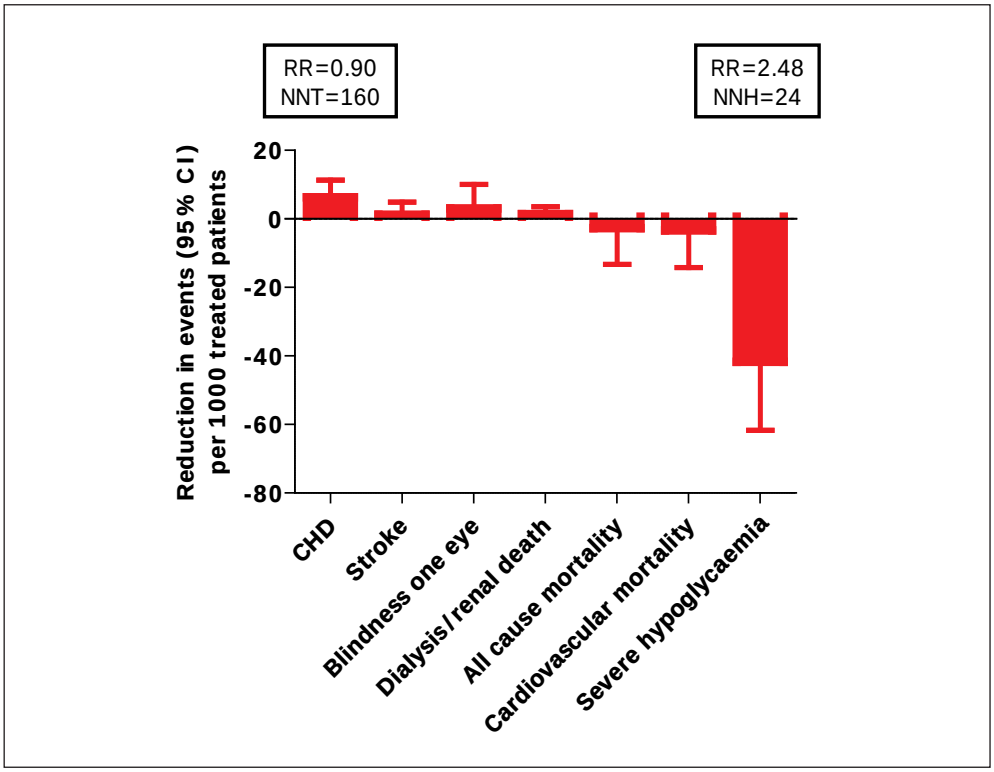

Fig. 1 Number needed to treat *versus* number needed to harm lowering glycaemia

In order to prevent one event by lowering cholesterol 1 mmol/l the number need to treat is 44 over 5 years. Lowering blood pressure by 10/5 mmHg this number is 33.6 and for lowering the HbA1c by 0.9 % 118 for 5 years in each case. These numbers depend on the background risk of the population (Oliver 2009).

The achievement of rigorous targets has to be questioned, according to the individual situation of a patient. Therefore which treatment goals have to be defined?

Tab. 2 Possible treatment goals

Glycaemia?	Symptoms?
Blood pressure?	Physical activity?
Lipid – levels?	Social activity?

Different scenarios have to be discussed (Nathan 2012, Margolis and O'Connor 2012): What is relevant for the patient, especially in consideration of his/her background risk.

Tab. 3 Treatment goals and relevance for the patient and the physician

What is relevant for the patient?	What is relevant for the physician?
Quality of life	Fulfil the guidelines
Life expectancy	Treatment along published data

There are some more problems in treating elderly people like adherence to anti-diabetic and other drugs. The 10 % of the elderly diabetics, suffering depression, is independently associated with poor adherence (Kilbourne at al. 2005). There are data that adherence is of more importance than the use of statins (Balkrishnan et al. 2003*).* Every 10 % increase in the medical possession ratio (staying on the described drug) can decrease the annual medical cost from 28.9 to 8.6 % in a single patient.

In a so-called shared decision approach together with the patient a personalized medicine has to be established. The objectives are: To take into account the specific requirements and the specific characteristics of each individual patient.

Alternative models of thinking focus on moderate and vigorous physical activity as the dominant health-related aspect of human movement (Katzmarzyk 2010). Humans are designed for movement. The Aerobic Center Longitudinal Study (ACLS) analysed data of 10,000 men and 3000 women followed for 8 years for all causes of mortality in relation to initial levels of cardiorespiratory fitness. Men and women in the lowest quintile were 3.44 (95 % CI 2.05 – 5.77) and 4.65 (2.22 – 9.75) times more likely to die compared with men and women in the upper quintile, respectively (Blair et al. 1989). A decrease in HbA1c similar to all modern oral drugs (– 0.62 %) can be achieved in exercise groups (Thomas et al. 2006).

The above mentioned knowledge has led to a more patient-centred approach "providing care that is respectful of and responsive to individual patient preferences, needs, and values and ensuring that patients values guide all clinical decisions" (*Commitee on Quality of Health Care in America* 2001). The American Diabetes Association (ADA) and the European Association for the Study of Diabetes (EASD) has written a statement incorporating the best available evidence taking into account all the new insights. This results in a new approach to management of hyperglycaemia (Inzucchi et al. 2012, Ismail-Beigi et al. 2011).

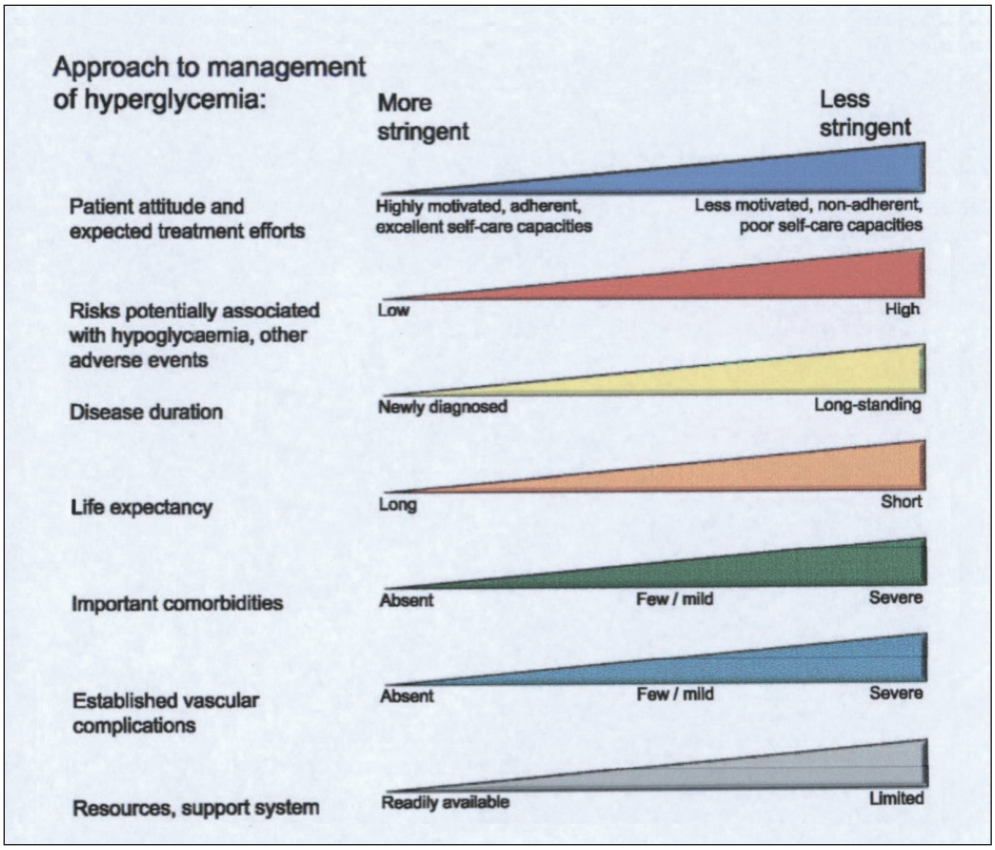

Fig. 2 Approach to management of hypoglycaemia (Inzucchi et al. 2012)

3. Conclusion

From a practical point of view the recommendation could be the following:

- Not more than 4 drugs for a better adherence and for lesser side effects (Chowdhury et al. 2013, Chapman et al. 2008).
- The physician has to ask what the patients want: Take something against hypertension (140/90 mmHg), take statins (?), take 1 antidiabetic drug and take something for well-being (against pain or depression).
- If possible recommend physical exercise as the best prevention and treatment for obesity and diabetes type 2.

This approach has to be adapted on a heterogeneous diabetic population different in age, severity of the disease, and individual background risk with special emphasis on patient individual characteristics.

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Rolle der Wissenschaft im Globalen Wandel

Vorträge anlässlich der Jahresversammlung
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Gesellschaftliche Probleme verlangen heute sehr häufig eine Widerspiegelung im Bereich der Wissenschaften. Als Nationale Akademie der Wissenschaften ist die Leopoldina in zunehmendem Maße gefordert, auch Beratung bei Fragen zu liefern, die über Länder und Kontinentgrenzen hinausgreifen: Klimawandel, der Einsatz erneuerbarer Energien, Fragen der Gesundheitsversorgung, die Einrichtung einer effektiveren Landwirtschaft zur Bekämpfung von Hunger in Krisengebieten und die sich wandelnde Altersstruktur von Bevölkerungen in vielen Staaten sind nur einige Beispiele für entsprechende Gebiete mit dringendem Forschungsbedarf. Sie bilden Herausforderungen für die Gesellschaften, die nur in internationaler, oft globaler Zusammenarbeit zu bewältigen sein werden. Daher wählte die Leopoldina 2012 das Thema „Rolle der Wissenschaft im Globalen Wandel“ für ihre Jahresversammlung. Der Band umfasst Beiträge zu den Themenkomplexen „Die Erde im Globalen Wandel“, „Herausforderungen des Globalen Wandels“ und „Lösungswege von Problemen des Globalen Wandels“ sowie zu den gesellschaftlichen und politischen Implikationen der mit dem globalen Wandel verbundenen Prozesse.

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Treatment of Diabetic Retinopathy

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Abstract

Diabetic Retinopathy (DR) is the main cause of impaired vision in people between 25 and 74 years old in developed countries. Metabolic control is still considered the first way to prevent the incidence and the progression of DR. Nevertheless, specific treatments are needed as soon as DR sight-threatening complications appear. Recent advances in understanding DR pathophysiology have led to an important improvement in the management of DR. Specific treatment should combine different approaches, including laser, medical therapy, and surgery, addressed to all the different pathogenetic mechanisms of DR. New drugs are under investigations and further results are expected from future trials, in an attempt to provide answers to the many clinical questions, especially regarding doses, frequency and correct regimen of each treatment.

Zusammenfassung

Die diabetische Retinopathie ist in den Industriestaaten die häufigste Ursache einer schweren Visusminderung bei 25- bis 74-Jährigen. Um die Inzidenz und Progression dieser Erkrankung zu reduzieren, wird vor allem eine bessere Einstellung des Stoffwechsels empfohlen. Dennoch wird bei visusbedrohenden Komplikationen der diabetischen Retinopathie eine spezifische Therapie notwendig. Neueste Erkenntnisse über die Pathophysiologie der diabetischen Retinopathie führten zu bedeutenden Fortschritten in der Therapie. Abhängig von den verschiedenen Pathomechanismen stehen unterschiedliche Therapiekonzepte, inklusive Laser, intravitreale Injektionen, chirurgische Maßnahmen oder auch Kombinationen aus diesen, zur Verfügung. Neue Medikamente werden untersucht und erkenntnisbringende Ergebnisse aus zukünftigen Studien werden erwartet, um viele klinische Fragen, wie die der erforderlichen Dosierung, Intervallzeiten zwischen den Anwendungen und das korrekte Therapieregime, zu beantworten.

1. Introduction

Diabetic retinopathy (DR) is one of the microvascular complications of diabetes as the consequence of chronic hyperglycaemia on retinal neuronal and endothelial cells. DR is the principal cause of impaired vision in people between 25 and 74 years old, since the worldwide prevalence of diabetes mellitus has been increasing during last decades. This disease affects one third of people with diabetes mellitus, and still represents the most common cause of blindness in diabetic patients. Visual loss from diabetic retinopathy may be related to diabetic macular edema (DME), vitreous haemorrhage, retinal detachment, or neovascular glaucoma. A comprehensive therapeutic approach should match with any of these specific manifestations of DR, in addiction to strict systemic glycaemic control.

2. Classification

The Early Treatment Diabetic Retinopathy Study Group (ETDRS) classified DR based on the identification of clinically significant alterations, defined by standard stereo fundus photographs. Non-proliferative retinopathy (NPDR) is characterized by microaneurysms, dot, blot or flame haemorrhages, venous beading, and intraretinal microvascular abnormalities. This stage is graded as absent/mild/moderate/severe/very severe according to the extent and localization of these abnormalities. If left untreated, very severe NPDR may evolve to proliferative retinopathy (PDR). PDR is characterized by the development of abnormal neovascularization (NV) arising from the anterior and/or posterior segment, in response to angiogenic growth factors (VEGF and other molecules) up-regulated by non-perfused retina. Endothelial walls of NV are more fragile than normal vessel and can easily break down, causing pre-retinal or vitreous haemorrhages, and subsequent fibrous proliferation and tractional retinal detachment, that feature the high-risk PDR stage (HR-PDR). DME is the major cause of visual impairment in people with DR, and it is the result of blood-retinal barrier disruption and extravascular inflammation. It may arise both in the setting of non-proliferative and proliferative diabetic retinopathy, and can be simply classified into vasogenic, characterized by focal vascular leakage, non-vasogenic, tractional, and mixed. The ETDRS introduced the definition of clinically significant macular edema (CSME), including three different forms of DME involving the macular area.

3. Treatment

Several studies, including the Diabetes Control and Complications Trial (DCCT) for type 1 and the United Kingdom Prospective Diabetes Study (UKPDS) for type 2 diabetic patients, show that primary prevention, including strict blood glucose, blood pressure, and serum lipids control, reduces the incidence and severity of DR and the need of laser therapy. The recent advances in understanding DR pathophysiology have led to an important improvement in DR therapeutic approach that includes laser photocoagulation, intraocular injections of steroids or anti-VEGF molecules, and surgery.

3.1 Laser Photocoagulation

Laser photocoagulation is currently considered the standard of care for both PDR and DME, even if new approaches based on intravitreal injection have significantly revolutionized the DR management. The mechanism of action of laser is mainly based on destruction of ischaemic retina, leading to improved inner retinal oxygenation, reduced production of pro-angiogenetic factors and greater release of neovascularization inhibitors from RPE cells.

Different types of laser can be used: Argon green (514 nm) or dye yellow (577 nm) lasers are the conventionally used wavelengths, but krypton red (647 nm) and diode (810 nm) lasers have also been effectively employed. Panretinal photocoagulation (PRP) is the standard of care for high-risk PDR, but also should be considered for patients with severe NPDR and early PDR, particularly in case of patients with type 2 diabetes mellitus, significant visual loss due to PDR in the fellow eye and/or if follow-up cannot be assured. In 1976, the Diabetic Retinopathy Study (DRS) set the standards for argon PRP and demonstrated that PRP re-

duced the risk of severe visual loss (SVL) in eyes with PDR by 50 % or more during a 5-year period of follow-up, compared with no treatment. Among patients with HR-PDR, the benefits of treatment definitely outweighed the risks (5 year rate of SVL in such eyes was reduced from 50 % without treatment to 20 % with treatment).

ETDRS was a multicentre, randomized clinical trial designed to evaluate the best timing for PRP in the management of NPDR or early PDR. The study demonstrated that early treatment was associated with a smaller incidence of severe visual loss, compared to deferred laser treatment, achieving NV regression in 60 % of patients after 3 months. Longer-term reports showed that long-lasting effectiveness of PRP treatment is dose related, and supplemental PRP is often required following primary treatment in order to achieve complete disease regression. Laser photocoagulation plays a fundamental role also in the treatment of DME: focal treatment is directly addressed to individual leakage points (microaneurysms, intraretinalmicrovascular abnormalities or short capillary segments) identified on pre-treatment fluorescein angiography; grid treatment, instead, is applied to areas of diffuse leakage. The ETDRS reported the standard guidelines for macular argon photocoagulation, stating that prompt laser treatment reduced the risk of moderate visual loss (losing >15 letters of VA) at 3 years by 50 %, compared with deferred treatment. Complications associated with conventional lasers are progressive restriction of visual field, colour vision, night vision, and contrast sensitivity impairment, as burn-induced scars tend to expand over time. Other side effects may include pain, choroidal or vitreous haemorrhage, DME worsening and epiretinal membrane formation.

New laser technologies have been proposed, differing in pulse power, time of exposure, spot size, or pattern distribution from conventional treatment, in order to selectively target retinal pigment epithelium and spare surrounding tissue. Light laser treatment, based on spot power ranging from 50 to 100 mW instead of 100 to 250 mW, has been used for grid treatment, showing no significant difference in BCVA, OCT, contrast and central sensitivity, compared with ETDRS grid laser standards. Micropulse diode laser (MPD) technique uses a train of repetitive short laser pulses, with an energy lower than the threshold needed to produce visible retinal whitening. This approach is able to reduce the collateral thermal diffusion to adjacent cells and the consequent chorioretinal damage. It has been shown to be as effective as conventional argon laser, without laser scar development. Pattern Scan Laser (PASCAL) system is a frequency-doubled Nd:YAG 532 nm-wavelengthed laser, that allows a simultaneous application of a grid of multiple laser pulses, each 1 magnitude shorter in duration than standard pulse.

The Manchester Pascal Study, a randomized clinical trial, reported that PASCAL 20-millisecond single-session PRP may be significantly shorter, less painful but as effective as conventional multiple-session PRP, and no additional adverse events (CRT increase, visual acuity or visual field impairment) have been observed in the short-term. Chappelow et al. (2012) evaluated the efficacy of PASCAL PRP in treating newly diagnosed high-risk proliferative diabetic retinopathy (PDR), compared to that performed with traditional argon laser, after 6 months follow up. When using traditional laser settings, giving patients treated with each type of laser a similar number of spots (1438 vs 1386; $P = .59$), PASCAL PRP seemed to be less effective in producing the regression of retinal neovascularization within 6 months of initial treatment (73 % of persistence or recurrence of NV vs 34 %; $P < .0008$). Number, spot size, or duration of laser burns of PASCAL PRP should be increased to improve its efficacy.

3.2 VEGF-Inhibitors

Starting from the evidence that laser alone improves vision acuity only in a small group of eyes, and many patients reported visual loss despite treatment, the first line treatment for DME especially characterized by centre involvement and vision loss, can be the therapy based on the anti-VEGF injections. Several randomized, multicentre clinical studies demonstrated that VEGF inhibitors result in a significant gain of visual acuity in eyes with DME, associated with reduced risk of progression toward more advanced forms of diabetic retinopathy. However, intravitreal anti-VEGF molecules, compared to laser treatment, have shorter duration of action and higher recurrence rate of the disease. This may lead to more frequent treatment, with consequent increased risk of adverse events related to intraocular injections, including endophthalmitis, retinal tears and holes, and vitreous haemorrhage.

Overall, anti-VEGF therapy shows a favourable tolerability profile in diabetic patients; patients with DME and co-existing proliferative diabetic retinopathy because have an increased risk of tractional retinal detachment secondary to fibrosis. Damaged ischaemic retinal tissue releases VEGF, and its expression is up-regulated by high glucose level, protein kinase C activation, and glycation end-products formation. VEGF promotes angiogenesis and affects vaso-permeability, increasing the intraretinal leakage. VEGF class includes 5 members: placental growth factor, VEGF-A, VEGF-B, VEGF-C and VEGF-D. VEGF-A, especially the $VEGF_{165}$ isoform, plays the most important role in the pathogenesis of diabetic retinopathy. Several randomized, multicentre clinical studies demonstrated that VEGF inhibitors have significant therapeutic effects in eyes with DME, resulting in a significant gain of visual acuity. Moreover, eyes assigned to anti-VEGF injection are less likely to show a progression toward the more advanced forms of diabetic retinopathy. VEGF-inhibitors that have been studied for diabetic retinopathy are pegaptanib, ranibizumab, bevacizumab and aflibercept. At present, these medications are administered into the eye by intravitreal injection. Pegaptanib is a pegylated anti-VEGF aptamer, a single strand of nucleic acid that binds with high specificity to $VEGF_{165}$. Macugen Diabetic Retinopathy Study (MDRS), a phase II, randomized, multicentre clinical trial, studied the effects on CSME of three different doses of pegaptanib (0.3 mg, 1.0 mg, 3.0 mg), compared to placebo. After 36 weeks follow up, patients treated with pegaptanib significantly gained visual acuity (+4.7 letters *versus* −0.4 letters, $P = 0.04$) and experienced a central retinal thickness (CRT) reduction, comparing to those treated with sham injections. No significant difference was found in the 0.3 mg, 1.0 mg and 3.0 mg groups.

Ranibizumab is a monoclonal antibody fragment that binds and inhibits multiple variants of VEGF-A molecule; it derives from bevacizumab, but it is smaller than the parent molecule. Ranibizumab therapy has been supported by the data from READ-2, RESOLVE, RESTORE, DRCRnet.

The READ-2 was a phase II study, designed to compare ranibizumab injections with focal/grid laser monotherapy and combination therapy in patients with CSME. The change in best corrected visual acuity (BCVA) from baseline to month 6 achieved with ranibizumab injections monotherapy was higher (+7.24 letters) than laser monotherapy (−0.43 letters) and combination therapy (+3.8 letters). At month 24, the 101 patients who remained in the study showed mean BCVA improvements of 7.7 letters (ranibizumab-only group), 5.1 letters (laser group), and 6.8 letters (combination therapy group). However, combination therapy arm resulted in lesser need of ranibizumab injections without a major disadvantage in visual outcome at 2 and 3 years.

The RESOLVE study was a 12-month study with 151 eyes randomly assigned to intravitreal ranibizumab (0.3 or 0.5 mg; n = 51 each) or sham (n = 49). The treatment schedule comprised 3 consecutive monthly injections, after which repeated treatment or rescue laser photocoagulation could be performed in refractory cases. At 1 year follow up, mean BCVA improved from baseline by 10.3±9.1 letters with ranibizumab and declined by 1.4 letters with sham. Only 4.9 % of the patients receiving ranibizumab required rescue laser compared with 34.7 % of those receiving sham treatment. The RESTORE, a phase III study, demonstrated the superiority in 2-year follow-up of ranibizumab 0.5 mg, both as monotherapy and combined with prompt or deferred ETDRS laser treatment, compared with laser treatment alone. At month 12, CRT was significantly reduced from baseline with ranibizumab alone (–118.7 μm) or combined with laser (–128.3 μm) *versus* laser only (–61.3 μm). There was a significantly greater BCVA improvement in the ranibizumab monotherapy (6.1 letters) and the combined group (5.9 letters) compared with the laser arm (0.8 letter).

The Diabetic Retinopathy Clinical Research Network (DRCR.net) study evaluated the effects of intravitreal ranibizumab with prompt or deferred (≥24 weeks) focal/grid laser treatment and intravitreal triamcinolone with prompt laser, compared to effects of laser treatment alone. Intravitreal ranibizumab with prompt or deferred laser and triamcinolone with prompt laser, provided superior visual acuity outcomes (respectively +9 letters, +9 letters and +4 letters) compared to prompt laser monotherapy (+3 letters) for at least 2 years. There was a greater risk of 10- and 15-letter vision loss in the laser-only cohort (13 %, 8 %, respectively) *versus* the ranibizumab cohorts (4 %, 2 %, respectively).

Bevacizumab is an anti-cancer molecule, used off-label in non-malignant eye diseases, including diabetic macular edema. The DRCR.net study randomly assigned 109 patients with DME in one of five groups: focal photocoagulation at baseline (N = 19, Group A), intravitreal injection of 1.25 mg bevacizumab at baseline and 6 weeks (N = 22, Group B), intravitreal injection of 2.5 mg bevacizumab at baseline and 6 weeks (N = 24, Group C), intravitreal injection of 1.25 mg bevacizumab at baseline and sham injection at 6 weeks (N = 22, Group D), or intravitreal injection of 1.25mg bevacizumab at baseline and 6 weeks with photocoagulation at 3 weeks (N = 22, Group E). CRT and BCVA were measured at baseline and after 3, 6, 9, 12, 18, and 24 weeks. A CST reduction was present at 3 weeks in 43 % bevacizumab-treated eyes (Groups B–D) and in 28 % eyes treated with laser alone (Group A), and at 6 weeks in 37 % and 50 % eyes, respectively. BCVA followed the same trend. No difference was found between 2.5 mg and 1.25 mg bevacizumab groups and between bevacizumab monotherapy and laser combined therapy.

Recently, the Pan-american Collaborative Retina Study Group (PACORES), a retrospective, multicentre study, evaluated the anatomical and functional outcomes at 24 months in patients with diffuse DME treated with bevacizumab, alone or associated to grid laser photocoagulation, *versus* laser alone. Decrease in CRT and improvement in BCVA turned out to be higher in bevacizumab treated groups with respect to laser monotherapy.

Moreover, BOLT study, a 24-month trial comparing bevacizumab monotherapy with laser, demonstrated the superiority of bevacizumab injections compared to grid laser treatment. 80 patients with centre-involved DME were randomized to receive either 1.25 mg intravitreal bevacizumab (IBV), or macular laser therapy (MLT). At month 12, the bevacizumab group gained a median of 8 letters compared with a median of –0.5 letters in the laser group. At month 24, IVB group gained a mean of 9 letters and MLT group gained a mean of 2.5 letters.

Aflibercept (Trap-eye) is a recombinant fusion protein that contains the extracellular domains of human VEGFR1 and VEGFR2, fused to the constant region (Fc) of human IgG1, functioning as a soluble decoy receptor. Clinical effects of aflibercept were investigated in DA VINCI study. The results showed that aflibercept produced a statistically significant improvement in visual acuity and a clinically relevant reduction in central retinal thickness, compared to macular laser photocoagulation.

Overall, anti-VEGF therapy shows a favourable safety and tolerability profile in diabetic patients. Caution should be exercised in patients who have DME with co-existing proliferative diabetic retinopathy because of the increased risk of tractional retinal detachment secondary to fibrosis, and in patients with a history of stroke, even if no increased rate of cardiovascular or cerebrovascular events has been found. Ranibizumab should not be used during pregnancy, unless the expected benefits from treatment outweigh the potential risks to the fetus.

3.3 Intravitreal Corticosteroids

Another approach for the management of DME is represented by intravitreal injections of corticosteroids. Several studies have confirmed their efficacy in reducing DME improving visual function. More specifically, the patients with persistent DME (refractory to other treatments) and pseudophakic eyes should undergo intravitreal corticosteroids injections (triamcinolone acetonide, dexamethasone, fluocinoloneacetonide). These molecules reduce the inflammatory reaction of the retina, and seem to inhibit neoangiogenic pathways. However, intravitreal corticosteroids are associated with cataract progression and intraocular pressure elevation (IOP), and consequent antiglaucoma medication or surgery. The DRCR.net investigated the effects of triamcinolone acetonide compared to macular laser photocoagulation: after an initial effect on BCVA and OCT, laser result overcame those of triamcinolone acetonide at the end of 3-year follow up. In the laser group, the change in BCVA letter score from baseline to 3 years was 5, while in each IVTA group was 0. Dexamethasone is injected with 0.7 mg slow-release drug delivery devices (OZURDEX), offering a more long-lasting effect and reducing the need of intravitreal injections required. The safety and efficacy on BCVA of OZURDEX for the treatment of persistent DME (lasting more than 90 days) has been demonstrated comparing with 0.350 mg dexamethasone and placebo, at 180 days follow up, with a small reduction after 3 months.

The FAME studies, two double-masked, phase III efficacy trials, demonstrated anatomic and funtional improvement in patients with laser resistant DME, after sustained-delivery devices of fluocinoloneacetonide (ILUVIEN®) injections at 3-year follow up. Two doses of fluocinoloneacetonide (FAc) have been studied (0.45 μg per day and 0.23 μg per day). At month 36, the percentage of patients in who gained ≥15 in letter score in the FAc group was 28.7 % (lower dose) and 27.8 % (higher dose), compared with 18.9 % in the sham group.

3.4 Pars Plana Vitrectomy

Surgical approach, including pars plana vitrectomy (PPV), is generally used to manage tractional DME and severe complications of DR such as non-clearing vitreous haemorrhage, severe fibrovascular proliferation, and retinal detachment. It is considered an option also for patients with DME or PDR not responding to laser photocoagulation. Cataract, retinal or choroidal detachment, vitreous haemorrhage, endophthalmitis, increased IOP, iris rubeosis, and neovascular glaucoma are the most common postoperative complications.

4. Conclusions

Over the past decade great strides have been made in the management of DR. It is also clear that DR is a chronic disease with variable clinical manifestations, and, therefore, a single treatment cannot be enough for the entire course of the disease. Metabolic control is the first way to prevent the incidence and to slow-down progression of DR. Specific treatment should combine different approaches, such as laser, surgery and medical therapy, in order to act on all the different pathogeneticmechanisms of DR. New drugs are under investigations and further results are expected from the future trials, trying to give an answer to the clinical open questions, regarding the doses, the frequency and the correct regimen of each treatment.

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Assessment of Diabetic Neuropathy in Diabetes mellitus Type 2 Patients in Democratic Republic of the Congo

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With 1 Figure and 1 Table

Abstract

The aim of the paper was to study the changes of sub-basal nerve plexus (*SNP*) in type 2 diabetic Congolese patients and to correlate these changes with the severity of diabetic neuropathy, diabetic retinopathy and grades of diabetic foot syndrome. 28 patients aged 59 ± 8.1 yrs. with diabetes-related foot ulcerations (duration of diabetes mellitus 13.4 ± 7.4 yrs., HbA1c 9.9 ± 3.6 %) were evaluated. The patients were ranked in Wagner 0–1 (mild manifestations, 46.4 %), Wagner 2–3 (moderate, 35.7 %) and Wagner 4–5 (severe, 17.9 %). Corneal SNP was studied using confocal laser scanning microscopy. Corneal sensitivity using Cochet-Bonnet aesthesiometer, neuropathy symptom score (*NSS*), neuropathy disability score (*NDS*), ankle-brachial index (*ABI*) and ophthalmological status were also evaluated. Significant differences were observed between Wagner 0–1 and Wagner 4–5 in confocal parameters of SNP (decrease of number of branches, connectivity points, nerve fibre density) and ABI increase as well as between Wagner 2–3 and Wagner 4–5 in confocal parameters. The increase of NDS and corneal sensation reduction were significant between Wagner 0–1 and Wagner 2–3. The patients with diabetic retinopathy have significantly higher diabetes duration and NDS score; there was no difference in SNP morphology or corneal sensation. Taken together, the grade of diabetic foot syndrome correlates with corneal nerve changes and decreased corneal sensation

Zusammenfassung

Ziel der Studie war es, Änderungen des subbasalen Nervenplexus (*SNP*) bei Typ-2-Diabetes-Patienten aus der Demokratischen Republik Kongo zu untersuchen und diese Änderungen mit der Schwere der diabetischen Neuropathie, der Retinopathie und dem Grad des diabetischen Fußsyndroms zu korrelieren. 28 Patienten im Alter von 59 ± 8.1 Jahren mit diabetischen Fußveränderungen (Dauer des Diabetes mellitus 13.4 ± 7.4 Jahre, HbA1c 9.9 ± 3.6 %) wurden untersucht. Entsprechend der Fußveränderungen wurden die Patienten in Wagner 0–1 (milde Manifestationen des diabetischen Fußsyndroms, 46.4 %), Wagner 2–3 (moderat, 35.7 %) und Wagner 4–5 (schwer, 17.9 %) eingestuft. Der subbasale Nervenplexus der Kornea wurde mittels der *in-vivo*-konfokalen Laserscanning-Mikroskopie (*CLSM*) untersucht. Weiterhin wurde der Neuropathie-Symptom-Score (*NSS*), der Neuropathie-Defizit-Score (*NDS*), der ABI (Knöchel-Arm-Index) und der ophthalmologische Status ermittelt und die Hornhautsensibilität mittels Cochet-Bonnet-Ästhesiometrie bestimmt. Signifikante Unterschiede wurden zwischen den Gruppen Wagner 0–1 und 4–5 in den SNP-Parametern (Abnahme der Nervenfaserzweige, Anzahl der Verbindungspunkte, Nervenfaserdichte) sowie dem ABI beobachtet. Ebenso zeigten sich signifikante Unterschiede in der SNP-Morphologie zwischen Wagner 2–3 und Wagner 4–5. Die Abnahme des NDS und der Hornhautsensibilität waren signifikant zwischen Wagner 0–1 und Wagner 2–3. Die Patienten mit diabetischer Retinopathie hatten eine deutlich höhere Diabetesdauer sowie einen deutlich höheren NDS ohne Unterschiede in der SNP-Morphologie oder Hornhautsensibilität. Zusammenfassend korreliert der Grad des diabetischen Fußsyndroms mit Veränderungen des subbasalen Nervenplexus und einer Abnahme der Hornhautsensibilität.

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1. Introduction

About 4.3 % of African adult population are suffering from diabetes (*International Diabetes Federation* 2012). The absence of financial support results in low health care making the effective diagnostic and therapy impossible. The Democratic Republic of the Congo (*DRC*) is the low-income country with severe underweight and malnutrition problems (de Onis et al. 2000, Hansson and Zanchetti 1994), about 50 % of diabetes mellitus type 2 patients in Congo are uncontrolled with consequential severe diabetic neuropathy (*DN*) (Longo-Mbenza et al. 2011).

About a half of diabetes-related in-patient treatment is due to the diabetic foot ulcerations as a consequence of DN and peripheral vascular disease (*PVD*) (Kumar et al. 2009). The up-to-date standard diagnostic of small fibre neuropathy is the measurement of intradermal nerve fibre density in skin biopsy specimens. Recent studies have shown the possibility of non-invasive and reliable assessment of the small fibre neuropathy evaluating the unmyelinated fibres of sub-basal nerve plexus (*SNP*) using *in vivo* confocal microscopy. The micromorphology of SNP correlates with the grade of DN as well with the changes of the intradermal nerve fibre density. The complex relation between impairment of SNP, decrease of corneal sensation as well as development of diabetic retinopathy (*DR*) and DN was also evaluated (Maser et al. 1991).

Since 2002 there is a well-established partnership programme between the Departments of Ophthalmology in Kinshasa (*DRC*) and Rostock (Germany). The primary goal of this collaboration was the diagnosis and therapy of diabetes mellitus patients in cooperation with the local diabetes ambulance. Interestingly, the huge discrepancy between the advanced foot ulceration and mild to moderate DR was characteristic for most of patients (Knappe et al. 2013). Our intension was to assess the peripheral DN, DR and morphology of SNP in diabetes mellitus type 2 patients using *in vivo* confocal microscopy, established screening methods and diagnostic possibilities for the first time in Sub-Saharan Africa.

2. Materials and Methods

2.1 Patients

This study was approved by the local ethics review board of Rostock University as well as authorities of St. Joseph Hospital in Kinshasa and performed in respect to the *Declaration of Helsinki*. The study was carried out in joint team of internists and ophthalmologists in St. Joseph Hospital in Kinshasa (*DRC*) in 2012. The patients with diabetes mellitus type 2 were recruited from the diabetic out-patient department.

2.2. Internal Medical Status

Demographic data, data about onset of diabetes or onset of symptoms, respectively, type of diabetes, current diabetes management and treatment was collected. Glycaemic control was evaluated using HbA1c and blood sugar (capillary blood glucometer DCA 2000+, Siemens, Germany).

The Neuropathy symptom score (*NSS*) and Neuropathy disability score (*NDS*) were performed by S. P. and H.-C. S. and classified as described elsewhere (Young et al. 1993b, Quattrini et al. 2007, Haslbeck et al. 2004; Fig 1).

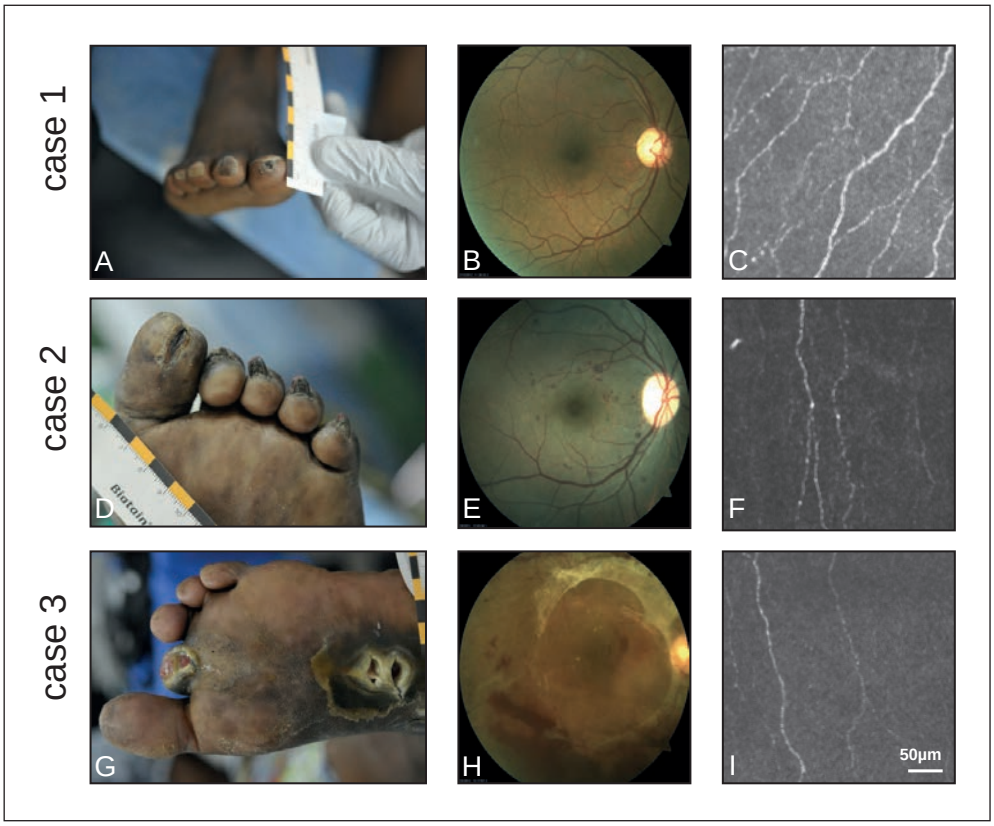

Fig. 1 Case 1: 62 yrs. old male patient, Diabetes duration 9 yrs, Hba1c 6.5 %, NDS 4 pts; Wagner 1 (*A*), no signs of DR (*B*), normal morphology of SNP (NFD = 0.011µm/mm²) (*C*), Case 2: 62 yrs. old female patient, Diabetes duration 7 yrs, Hba1c 9.4 %, NDS 5 pts; Wagner 2 (*D*), advanced non-proliferative DR (*E*), moderate decrease of confocal parameters of SNP (NFD = 0.010 µm/mm²) (*F*), Case 3: 44 yrs old female patient, Diabetes duration 9 yrs, Hba1c 11 %, NDS 9 pts; Wagner 5 (*G*), proliferative DR with retina detachment (*H*), moderate decrease of confocal parameters of SNP (NFD = 0.011 µm/mm²) (*I*)

The ankle-brachial index (*ABI*) was evaluated in order to screen the peripheral vascular disease (*PVD*). It was obtained by S. P. and H.-C. S by measuring the systolic blood pressures in the ankles (dorsalis pedis and posterior tibial arteries) and arms (brachial artery) using a handheld Doppler (Huntleigh Doplex D900, Cardiff, United Kingdom) and then calculating a ratio (Young et al. 1993a). The ABI <0.9 was interpreted to PVD.

The Wagner classification of foot ulceration based on wound depth and the extent of tissue necrosis (Wagner 1987) was used. The ulcers were measured with a tape and photographed with a digital camera.

2.3 Ophthalmological Status

Ophthalmological status was evaluated by S. P. The staging of diabetic retinopathy was performed in mydriasis as described elsewhere (Wilkinson et al. 2003).

2.3.1 Corneal Sensation

Corneal sensation was measured by a Cochet-Bonnet esthesiometer in central and peripheral cornea (Luneau Ophthalmologie, Paris, France; diameter of monofilament 12/100). The filament was applied with a complete length of 60 mm, by the negative response the length was reduced consequently on 5 mm till the positive answer was achieved.

2.3.2 *In vivo* Confocal Laser-scanning Microscopy (CLSM)

The patients' corneas were investigated *in vivo* by the HRT II in combination with the Rostock Cornea Module-RCM (Heidelberg Engineering, Heidelberg, Germany). The HRTII/RCM system uses as a laser source a diode laser with a wavelength of 670 nm and is equipped with a water contact objective (63×/0.95W, 670 nm; Zeiss, Jena, Germany). The distance from the cornea to the microscope is kept stable by a single-use contact element in sterile packaging (Tomo-Cap). Coupling between the patient's cornea and the cap is facilitated with a thin lubricant layer of Vidisic gel (Bausch & Lomb/Dr. Mann Pharma, Berlin, Germany; refractive index 1.35). The eye to be examined was anesthetized by instilling Proparakain 0.5 % eye drops (Ursapharm, Saarbrücken, Germany).

Image acquisition of the central cornea was performed in z-scan of automatic volume scan mode (30 images, volume depth 60 µm, constant interslice distance 2 µm). The acquired images have a definition of 384 × 384 pixels over an area of 400 × 400 µm. Confocal microscopy was performed in the region of interest, i.e. at the level of basal cells, SBP, Bowman's membrane and anterior stroma at depths from 30 to 90 µm. At least three scans were performed. The total duration of microscopy was about 15 minutes.

2.4 Quantification of Micromorphological Parameters

The evaluation of SBP on the basis of best artefact free single image was performed automatically on pre-segmented images using in house developed software tool (Boulton and Vileikyte 2000) and was based on morphological (length, diameter, density) and topological (continuity and connectivity) parameters. In order to illustrate the most representative data the number of analysed parameters were reduced to these with significant differences between the different groups ($p<0.05$). That means the average single fibre length [µm], nerve fibre density [mm/mm²], connectivity points [n], and numbers of branches [n] were evaluated in the current study.

3. Results

Twenty-eight diabetes mellitus type 2 patients (59 ± 8.1 years; 12 male/16 female; diabetes duration 13.4 ± 7.4 yrs) with diabetes-related foot ulcerations were evaluated. The ABI was 1.1 ± 0.3 eliminating the PVD as an ulcus origin. The peripheral DN was characterized with an NSS of 5.9 ± 2.8 pts. and NDS of 6.5 ± 3.0 pts. The demographic and clinical data of patients are presented in the Table 1.

According to the stage of diabetic retinopathy the patients were stratified in two groups: "no retinopathy" (NDR) – 11 patients (39.3 %) and with DR – 17 patients (60.7 %). These

Tab. 1 Clinical and demographic data of participants (data presented as mean ± SD) divided in different groups; ABI = Ankle Brachial Index, NDR = No diabetic retinopathy, DR = Diabetic retinopathy

Group	ALL (n=28)	NDR (n=11)	DR (n=17)	Wagner 0–1 (n=13)	Wagner 3–4 (n=10)	Wagner 4–5 (n=5)
Age [yrs.]	59±8.1	60.1±7.3	58.2±8.7	57.2±6.2	63.4±6.7	54.6±12
Duration of DM [yrs.]	13.4±7.4	10.5±7.6	15.3±7	13.9±9	14.1±7	10.8±2.8
HbA1c [%]	10.7±2.5	9.8±2.1	11.2±2.6	11.2±2.4	10.4±2.7	9.8±2.1
NSS [pts.]	5.9±2.8	6.6±1.6	5.5±3.3	6.2±2.9	5.4±3.3	6.4±0.9
NDS [pts.]	6.5±3.0	5.3±2.8	7.3±2.9	6.2±2.9	5.4±3.3	6.4±0.9
Corneal sensation [mm]	44±20	49±22	42±19	55±10	36±24	4.6±2.1
ABI (ulcer)	1.1±0.3	1,±0.4	1.1±0.2	1.0±0.3	1.1±0.3	1.3±0.1
Nerve fibre density [mm/mm²]	0.015±0.008	0.016±0.007	0.014±0.008	0.018±0.01	0.014±0.004	0.008±0.003
Nerve branches [n]	180.7±152.3	185.9±103.4	177.3±180.1	237.4±197.4	163.1±74.6	68.3±11.8
Connectivity points [n]	19.8±12.9	18.6±9.2	20.6±15.1	24.7±14.3	19.6±10.4	7.4±2.6
Average single fibre length [µm]	22.4±5.5	25.2±4.5	20.5±5.3	23.6±5.1	20.7±5	22.7±7.4

two subgroups differed in duration of diabetes ($p = 0.027$) and NDS ($p = 0.011$), but not in Hba1c and age. Corneal sensation was not significantly different in both groups (Tab. 1).

The same cohort was divided in 3 subgroups according to foot ulceration (Fig. 1): mild manifestations (Wagner 0–1) – 46.4 %, moderate (Wagner 2–3) – 35.7 % and severe (Wagner 4–5) – 17.9 %. These subgroups did not differ in duration of diabetes, HbA1c and age. The NDS was significantly increased ($p = 0.001$) and the corneal sensation decreased ($p = 0.032$) comparing Wagner 2–3 and Wagner 0–1. The ABI was significantly higher ($p = 0.03$) comparing Wagner 4–5 to Wagner 0–1.

The comparison between patients with grade 0 (pre-ulcerative site, or healed ulcer) and grade 1–5 (any ulcer) presented solely the increased NDS. The comparison of Wagner 1–5 patients with DR with the group Wagner 0 without DR showed increased NDS and decreased corneal sensation.

In vivo confocal microscopy showed the alterations in SNP morphology between the different groups (Fig. 1 and Tab. 1). The significant difference between the patients with and without DR was only the average single fibre length ($p = 0.02$). The comparison of Wagner 1–5 group with DR and Wagner 0 with NDR presented the decrease of nerve fibre density ($p = 0.04$). The reduction of confocal parameters (nerve fibre density, number of nerve branches, number of connectivity points) were noted both in Wagner 4–5 compared to Wagner 2–3 ($p = 0.01$; $p = 0.003$; $p = 0.005$ accordingly) as well as in Wagner 4–5 compared to Wagner 0–1 ($p = 0.03$; $p = 0.01$; $p = 0.001$ accordingly).

4. Discussion

In this study we evaluated clinically the function of large fibres (vibration perception with tuning fork, 10-g monofilament) as well as small fibre function (hot/cold rods) with resulting NSS of 5.9 ± 2.8 pts. and NDS of 6.5 ± 3.0 pts. indicating moderate to severe peripheral neuropathy. The theoretically possible electrophysiological investigations in order to determine the surrogate endpoints for foot ulceration as well as the evaluation of small fibre neuropathy with skin punch biopsy were not yet performed in Africa. We include the confocal microscopy of sub-basal nerve plexus (*SNP*) as a clinically established parameter which correlates well with the PDN and could substitute the time and resource consuming electrophysiology or skin biopsy.

Peripheral neuropathy is the leading cause of the foot ulceration in diabetic patients. The diabetes mellitus type 2 patients have a 15 % cumulative lifetime incidence of foot ulceration (Boulton and Vileikyte 2000, Boulton et al. 2004). The prevalence of peripheral neuropathy in Africa varies from 10 to 36 % (Mbanya and Mbanya 2003), it seems to be present even at the early diabetes due to the very low level of glycaemic control.

One third of all foot ulcerations are caused by peripheral vascular disease (PVD) cases. (Carrington et al. 2002). The prevalence of PVD is about 10 % among diabetes mellitus type 2 patients comparing to 4 % in general population of Europe (Potier et al. 2011). PVD in diabetic patients has significantly higher amputation and mortality rate comparing to the non-diabetic patients (Jude et al. 2001). According to the literature, the prevalence of low limb arterial disease in Africa contributing to the development of diabetic foot syndrome varies from 4 to 28 % (Mbanya and Mbanya 2003).

Although the strength of ABI measurements is affected by the presence of diabetes and resulting to the decrease of ABI sensitivity to 53 % and specificity to 95 % (Potier et al. 2011) due to the secondary medial artery calcification (*MAC*) (Young et al. 1993b), the high levels of ABI are considered to be typical for diabetic patients and the ones with MAC have a risk of 1.7 to develop retinopathy (Everhart et al. 1988). Our patients show the ABI of 1.1 ± 0.3 (in 57.1 % the ABI was over 1.10 and in 17.8 % less then 0.9) indicating the mediasclerosis rather then PVD as an genesis of foot ulceration; 53.6 % of patients had a moderate to severe diabetic foot ulceration.

The foot ulcer classification allows the subdivision of patients in three groups with resulting significant differences: the significant decrease of corneal sensation and NDS from Wagner 0–1 to Wagner 2–3 following by significant increase of ABI (indicating mediasclerosis) as well as significant decrease of confocal parameters (nerve fibre density, nerve branches and connectivity points) from Wagner 0–1 to Wagner 4–5. The significant decrease of the confocal parameters was also shown from Wagner 3–4 to Wagner 4–5. These data point out that the changes of SNP morphology correlate with the grade of foot ulcer. On the other hand, Tavee described the changes of SNP (decrease of nerve density, number of branches, single nerve fibre length, tortuosity) and its correlation with established electrophysiological parameters as well as with the result of the skin biopsy (Tavee and Zhou 2009).

Our results show a substantial inconsistency between partly extensive foot ulcerations and only mild to moderate diabetic retinopathy in most of the patients: 17 out of 28 patients (60.7 %) have the signs of DR by duration of diabetes by 15 ± 3 yrs. and HbA1c of 11.2 ± 2.6 %. The prevalence of DR increases with the duration of disease (55 % by patients with over five years of diabetes duration; Unwinn et al. 1999) as well as with the poor glucose

control (77 % of patients by HbA1c >8 %; Chadli et al. 2000). The NDR group of our study showed significantly lower diabetes duration without changes in HbA1c level. Barr et al. stated a 2.5 odds ratio for the development of DR in the presence of PDN (Barr et al. 2006). In accordance with it, our data show the increased NDS score in DR group emphasizing the higher level of the PDN. Comparing the subgroups of Wagner 1–5 and DR with Wagner 0 and NDR we stated the increase of NDS as well as decrease of corneal sensation. There were no changes in terms of morphology between the groups with and without DR with a single exception of an average single fibre length. The significant nerve fibre density decrease was stated in the group of Wagner 1–5 and DR comparing to the patients with Wagner 0 and NDR.

The studies of peripheral neuropathy, foot ulceration as well as evaluation of ophthalmological status of diabetic patients using the up-to-date diagnostic and technical methods are possible in Sub-Saharan Africa. The results of our study underline the primary development of foot ulceration due to the diabetes mellitus and emphasize the polyetiological development (peripheral neuropathy, peripheral vascular disease, low hygiene standards, etc.) of the disease. The decrease of NDS is characteristic for the patients with superficial foot ulceration with or without diabetic retinopathy. On the contrary, the decreased NDS as well as corneal sensation and nerve fibre density of the SNP were found out in the group of severe foot ulceration and DR. So far, the grade of diabetic foot syndrome correlates with corneal nerve changes and corneal sensation. The further studies have to be the platform for both scientific knowledge and practical skills exchange between the local and European specialists making the health care system in Africa better.

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Diabetes Research in Ghana

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Abstract

Type 2 diabetes is on the increase in Ghana fuelled by rising urbanization and obesity. There is also substantial pre-diabetes. There is little information on diabetes types such type 1 diabetes, ketosis prone diabetes and MODY. Gestational diabetes and diabetes in pregnancy appear to be associated with poor maternal and foetal outcome. Diabetes is associated with serious morbidity and premature mortality. There are major knowledge gaps that may be addressed by multidisciplinary and international research teams drawn from within and outside Ghana. For example there is a need to better characterize the various forms of diabetes, their complications and to study their links with genes and the environment. Also surveys and registries may provide data for policy and planning. Such international research partnerships may help build diabetes research capacity for the country.

Zusammenfassung

Für den Diabetes mellitus Typ 2 findet sich eine zunehmende Inzidenz und Prävalenz in Ghana, verursacht durch Verstädterung und damit einhergehende Gewichtszunahmen und Fettsucht. Eine Zunahme des Prädiabetes wird beobachtet. Aus Ghana liegen nur wenige Angaben zum Diabetes mellitus Typ 1, zu Ketoazidosen und zu anderen Diabetesformen wie MODY vor. Gestationsdiabetes und Schwangerschaft mit Diabetes führen zu einer erhöhten Morbidität und Mortalität bei Mutter und Kind. Die vielen fehlenden Informationen zu den oben genannten Themen könnten durch interdisziplinäre internationale Forschergruppen in und außerhalb von Ghana gewonnen werden. Es ist erforderlich, die verschiedenen Diabetesformen und deren Komplikationen besser zu charakterisieren und Beziehungen zu genetischen Grundlagen und den Umwelteinflüssen herzustellen.

Register und Populationsanalysen können die Datengrundlage für politische Entscheidungen und Planungen liefern. Eine derart gestaltete internationale Partnerschaft kann die Forschungskapazität in Bezug auf den Diabetes in Ghana deutlich voranbringen.

1. Introduction

Until recently, diabetes was thought to be rare in sub-Saharan Africa (SSA) and that it was a disease of Caucasians which only affected affluent native Africans. It is now clear that diabetes is a worldwide condition that afflicts disproportionately those in the developing world including those in SSA (King and Rewers 1993). In this long abstract we summarize original works on diabetes in Ghanaians together with existing gaps in knowledge.

2. Search Strategy

A systematic review of all scientific articles published on diabetes in Ghana between the time of independence in 1957 and to the end of 2012 and available on PubMed and Google Scholar was carried out. Articles providing data on Ghana in respect of diabetes prevalence, aetiopathogenesis, outcomes, complications, morbidity, and mortality; diabetes diagnosis, healthcare costs and care were searched for. Searches included the use of the following keywords: Diabetes, Ghana, Research, Epidemiology, Pathogenesis. Published local works of interest in references of the primary searches were also reviewed. Sources of grey articles from local non-indexed sources, conference abstracts and articles available at websites on the internet were also included in the review.

3. Results

3.1 Type 1 Diabetes

Type 1 diabetes is poorly characterized with a paucity of information regarding the aetiology, immunology and epidemiology of this disease in SSA. In a clinical study in the early 1990s at the Diabetes Clinic in Accra, Ghana, type 1 diabetes formed 7 % of the cases seen (Amoah 1994). Agyei-Frempong et. al. (2008) found a latent autoimmune diabetes in adults (Lada) prevalence of 0.75/1000 in Kumasi. There is no information on type 1 diabetes in Ghanaian children. Type 1 diabetes requires to be characterized phenotypically, biochemically, immunologically and genetically.

3.2 Type 2 Diabetes

Diabetes is on the increase in Ghana. Within four decades the prevalence of type 2 diabetes (T2DM) increased considerably from less than 0.5 % to 6.4 % (Dodu 1958, Amoah et al. 2002a). This is partly due to an increase in urbanization from 20 % at independence to over 50 % presently and rising obesity (Amoah 2003). Life expectancy has also increased from about 40 years at independence to nearly 60 years presently. Further, there is a large body of pre-diabetes waiting to convert to full blown diabetes (Amoah et al. 2002a). The condition is projected to nearly double in the next couple of decades. Thus translation of diabetes prevention strategies is needed in the country to halt or slow down the epidemic. The relatively large rate of undiagnosed diabetes of 70 % (Amoah et al. 2002a) may reflect a poorly developed health infrastructure in Ghana. Facilities and personnel for diabetes care have been reported to be severely limited in Ghana (Amoah et al. 1998). Similar and higher rates of undiagnosed diabetes have been noted in other parts of SSA (Balde et al. 2007).

Vuvor et al. (2011) noted a positive association between diabetes and known risk factors including physical inactivity. The strongest association, however, has been reported with waist-to-hip ratio with the waist-to-hip ratio being the best discriminator for diabetes in Ghanaians (Frank et al. 2012). Type 2 diabetes in Ghanaians is characterized by impaired first and second phase insulin secretion (Amoah et al. 2001). In Ghanaians and Nigerians linkage to four regions of chromosome 12, 19, and 20 (Rotimi et al. 2004) have been found with two loci, 12q24 and 20q13.3, which had previously been reported to harbour diabetes

susceptibility genes in several other ethnic groups, showing the highest LD scores. Also, linkage with variants of the transcription factor 7-like 2 gene (TCF7L2), seen in Europeans, have also been noted in this cohort of west Africans (Tong et al. 2009). These results have considerable relevance in the study of type 2 diabetes in other African ancestry populations, most particularly, African Americans, considering the history of forced migration of West Africans during the slave trade.

3.3 Gestational Diabetes and Diabetes in Pregnancy

In a small prospective study (n = 37*)* by Amoah et al. (2003a), generally, Ghanaian pregnant women reported late for antenatal care with only 11 % first accessing care in the first trimester. Not surprisingly, the women had relatively poor obstetric outcomes. Maternal complications in pregnancy were 16.2 % hydramnios and 10.8 % pre-eclampsia; 24.3 % had pre-pregnancy hypertension (Amoah et al. 2003b). Fetal distress occurred in one pregnancy, 24 % of babies had macrosomia, two were still born, one died in the neonatal period, one had congenital malformation and 10.8 % had hypoglycaemia (Amoah et al. 2003b).

3.4 Other Specific Types of Diabetes

To the best of our knowledge this topic has hardly been studied. In a clinical study this group was found to account for 3 % of individuals with diabetes receiving care at the Korle Bu Teaching Hospital. The entity known as ketosis prone diabetes is yet to be identified and researched.

3.5 Pre-Diabetes

Fasting pre-diabetes (impaired fasting glucose, IFG) in the Greater Accra region was 6.1 % and impaired glucose tolerance (IGT) was 10.7 with 3.8 % having combined IFG and IGT (Amoah et al. 2002a). The total combined burden of pre-diabetes in the Ghanaian study was relatively high (20.6 %). This relatively high pre-diabetes rate in association with moderate diabetes prevalence (6.4 %) may indicate a relatively advanced stage of the diabetes epidemic in Ghana and calls for intervention to avert the epidemic.

3.6 Diabetes Complications, Morbidity and Mortality

In an eight year retrospective morbidity/mortality study 1678 persons with diabetes with a mean age of 50.1 ± 0.4 years were admitted; the male to female ratio of 1:1 (Amoah et al. 2003). Diabetes accounted for 6.8 % of adult medical admissions and was responsible for 7.3 % of adult medical deaths. Diabetes was associated with a relatively high case fatality rate of 22 %. This compares (24 %) with an earlier study covering a shorter period (Adubofour et al. 1993).

Diabetes ketoacidosis (DKA) accounted for a third (34.5 %) of diabetes medical admissions and over a third (38.7 %) of deaths in subjects with diabetes. The case fatality for DKA in the Amoah et al. (2003) study was 26.1 % which is about half that of an older study of Adubofour et al. (1993). There were no data on hyperglycaemic hyperosmolar state or hypoglycaemia. Major co-morbid conditions in admitted diabetes patients included infections

(malaria n=67, respiratory infection n=58, cellulitis n=54, septicaemia n=25, HIV n=8, tuberculosis n=7); circulatory disorders (stroke n=87, heart failure n=35, coronary artery disease n=8, and chronic renal failure n=20) (Amoah et al. 2003).

In a cohort of diabetes type 2 patients from Ghana and Nigeria the prevalence of diabetic retinopathy was 17.9 % and the odds for nephropathy increased 3-fold from the highest fasting plasma glucose compared to the lowest fasting plasma glucose (Rotimi et al. 2003). Adinortey et al. (2011) found among 288 patients with type 2 diabetes, 28 % with neuropathy, 17 % with nephropathy and 9 % with diabetic retinopathy in Kumasi (Adinortey et al. 2011). A relatively high rate of 70 % sexual dysfunction has also been reported in Ghanaian men with diabetes. Amidu et al. (2010) found 14.2 % with severe difficulty, avoidance (10.9 %), premature ejaculation (8.8 %), non-sensuality (7.3 %), infrequency (4.0 %), dissatisfaction (3.6 %) and non-communication (3.3 %).

Very little data exists on diabetes macrovascular disease. In patients with coronary artery disease evaluated at the national referral centre for cardiac disease in Accra, the second most prevalent risk factor after hypertension was diabetes (Amoah and Kallen 2000). Yeboah et al. (2012) found using the ankle brachial index method in 300 patients with type 2 diabetes, aged 40 – 89 years, rates of moderate and severe peripheral arterial disease (PAD) of 2.0 and 21.7 %, respectively. This compares with PAD rates of 19 % in US diabetes adults over 40 years (Gregg et al. 2004). Medial sclerosis (ABI >1.3) was observed in 11.3 % of patients. Our study may thus have under estimated the prevalence of PAD using ABI. Doppler wave form analysis may have improved the diagnostic yield. After adjustment for age, gender, diabetes duration, smoking and alcohol status, percent body fat, visceral fat and systolic blood pressure correlated negatively with ABI. Associated risk factors for peripheral arterial disease were total cholesterol (positively) and HbA_{1c} (negatively) after adjustment for age, gender, diabetes duration, smoking and alcohol intake. There were no significant association of PAD with LDL- and HDL-cholesterol (Yeboah et al. 2011).

Out of 966 surgical patients seen at the Military Hospital in Accra, 80 (8.3 %) had diabetes mellitus with soft tissue infection or foot disease. The peak age of presentation with soft tissue infection or foot disease was 50 – 59 years. Diabetic foot disease (53.0 %) was the commonest followed by cellulitis of the leg and other soft tissue infections. Overall amputation rate was 33.3 % while mortality was 8.8 % (Asumanu et al. 2010). Presently there are no systematic foot care programmes. Podiatrists are non-existent in the health system. Professional and degree programmes may therefore be established in the Schools of Allied Health Sciences to develop much needed capacity to reduce limb loss. Meanwhile, programmes to train health providers to care for the feet of patients with diabetes may prove beneficial.

3.7 Diabetes Education, Care and Management

To the best of our knowledge Ghana was the first country to implement a comprehensive national multidisciplinary diabetes care programme in SSA (Amoah et al. 2000). Between 1995 – 1998 Diabetes Teams (doctor, nurse educator dietician/medical nutrition counsellor and pharmacist) were trained for all teaching hospitals, all 10 provincial hospitals and 63 % of sub-regional/district government hospitals. Among diabetes subjects, the main sources of diabetes information were diabetes nurse/dietherapist, 95.7 %; radio 94.2 %; posters 92 %, and television 84.9 % (Amoah et al. 2003). Advantage may be taken of these electronic media with high patronage and wide coverage to implement a diabetes education and awareness

programme to enhance diabetes knowledge and awareness in the light of the high morbidity and mortality burdens from diabetes.

3.8 Diabetes Control

Most diabetes patients cannot afford home blood glucose monitoring as the cost of the glucose meters and test strips are expensive for patients who have to pay for these out of pocket. Further, HbA_{1c} is neither widely available nor affordable. At the Tamale Teaching Hospital in the northern part of Ghana, among 240 subjects with diabetes, (60.8 %) had good preprandial glucose levels (<7.2 mmol/L) whilst 39.2 % had high (>7.2 mmol/L) (Titty et al. 2010). Glycaemic control as assessed by A1c was good (HbA1C <7.5 %) in 40 % of the patients and poor in the remaining 60.0 % (Titty et al. 2010). Lack of adherence may be contributory with 42.4 % of 140 diabetes patients missing clinic appointment, 27.9 % skipping taking diabetes medication, and with 25 and 75 % following their dietary regime when at home and away from home, respectively (Amoah et al. 2003).

3.9 Psychosocial Perspectives

Daily drug and dietary routines impose a considerable psychosocial burden on patients with diabetes in Ghana. Additionally, the relatively high cost of diabetes medication and recommended foods tends to undermine the commitment of patients to biomedical care. It has been estimated that rural diabetes patients on the minimum daily wage could be using up to 60 % of their monthly income for a month's supply of insulin (de Graft-Aikins 2003). Further, de Graft-Aikins (2005) showed that most persons with diabetes are aware that diabetes was not curable. However, their hope for cure, tended to set them on a passive cure seeking course with intermittent healer shopping during acute phases of diabetes.

4. The Way Forward

Type 2 diabetes is on the increase in Ghana fuelled by rising urbanization and obesity in association with a large burden of pre-diabetes. There is little information on diabetes types such type 1 diabetes, ketosis prone diabetes and MODY. Gestational diabetes and diabetes in pregnancy appear to be associated with poor maternal and foetal outcome. Diabetes is associated with serious morbidity and premature mortality. There, however, exist major knowledge gaps that require to be addressed. Ghana presently lacks resident indigenous research expertise to bridge these gaps. Bridging the knowledge gaps will therefore require multidisciplinary and international research teams drawn from within and outside Ghana. Opportunity exist in such research partnerships to build much needed middle and senior level diabetes research capacity in Ghana for now and the future.

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Sepsis – A Translational Approach

Leopoldina-Symposium
am 25. November 2011 in Münster

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Die Sepsis erfordert eine besonders kostenintensive Behandlung im Krankenhaus. Relativ häufig entwickelt sich sehr rasch aus der Sepsis eine schwere Sepsis und schließlich ein septischer Schock, der besondere Anforderungen an die Ärzte stellt. Gerade auch die Behandlung schwerer Fälle hat in den letzten Jahren deutliche Fortschritte durch ein besseres Verständnis der Mechanismen der Erkrankungsausprägung gemacht. Neue therapeutische Ansätze wurden entwickelt und eingesetzt. Die Beiträge behandeln an der Nahtstelle von Grundlagenforschung und klinischer Anwendung diese neuen Entwicklungen von innovativen Therapiestandards und ihren Nutzen für das Überleben der Patienten. Die Beiträge sind in englischer Sprache verfasst.

Wissenschaftliche Verlagsgesellschaft Stuttgart

Closing Remarks

Peter Wiedemann ML (Leipzig)

Diabetes is a global epidemic with significant morbidity. The prevalence of diabetes type 2 in our adult population is 5–8 %. Diagnosis in good time and the avoidance of complications is the medical goal in the initially symptomless disease. Diabetic retinopathy is the specific microvascular complication of diabetes and affects 1 in 3 persons with diabetes. Diabetic retinopathy remains a leading cause of vision loss in working adult populations. Patients with severe diabetic retinopathy have a poorer quality of life and reduced levels of physical, emotional and social well-being, and they use more health care resources.

Studies have shown that optimal control of blood glucose, blood pressure, and blood lipids can reduce the development of a diabetic retinopathy and slow its progression. Since visual loss may not be present in the earlier stages of diabetic retinopathy, regular screening is essential to enable early intervention.

In the Leopoldina Symposium "Vision and Diabetes" it was our wish to show the interaction between ophthalmology and general especially internal medicine in the treatment of diabetic patients. During the development of this programme we saw that ophthalmology has to learn a lot from general medicine to best help the patients and prevent a further progress of this disease. But it also showed that ophthalmology may help in the early diagnosis of this disease by advanced imaging technology which shows early deficits and morphological changes in the cornea and retina as surrogate of diabetic damage and biomarker for diabetic neuropathy.

In the sessions current treatment procedures were reported for diabetic retinopathy and systemic disease, and special emphasis was given to the African experience.

And therefore we hope that having an eye on "Vision and Diabetes" our symposium may help to improve the situation worldwide for patients suffering from diabetes and diabetic retinopathy.

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Forschung und Verantwortung im Konflikt?

Ethische, rechtliche und ökonomische Aspekte der Totalsequenzierung des menschlichen Genoms

Symposium
vom 15. bis 16. März 2012 in Heidelberg

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Die Möglichkeit, das komplette Genom von Individuen zu sequenzieren, ist seit einigen Jahren vorhanden und wird stetig weiterentwickelt. Damit werden nicht nur neue Diagnose- und Therapieformen zukünftig verfügbar, sondern es gelangen auch gesellschaftliche Probleme und neue ethische Fragen in den Fokus. Die ethischen und rechtlichen Aspekte der Totalsequenzierung des menschlichen Genoms bedürfen vor diesem Hintergrund einer wohldurchdachten interdisziplinären Auseinandersetzung. Die Beiträge des Bandes beleuchten den Konflikt zwischen Forschungsdynamik, ärztlichem Handeln und Patientenversorgung aus verschiedenen Perspektiven und wollen Orientierungspunkte für die aktuelle Debatte sein. Das Spektrum der Ansätze reicht von gesundheitsökonomischen und gesellschaftswissenschaftlichen Überlegungen über Regulierungsfragen bis zu grundlegenden ethischen Reflexionen.

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