Title
Investigation of Prolactin-related Vasoinhibin in Sera from Patients with Diabetic Retinopathy

Short running title
Prolactin-related Vasoinhibin in patients with diabetic retinopathy

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Abstract

Objective

In vitro experiments and in vivo studies on rodents demonstrate that N-terminal 14, 15, 16, 17 and 18 kDa fragments (prolactin-related vasoinhibin) of human Prolactin (PRL) are natural inhibitors of neovascularization in the retina and elsewhere. These N-terminal PRL-fragments belong to a family of peptides named vasoinhibins, which act as endogenous regulators of angiogenesis and vascular function. These observations led to the hypothesis that prolactin-related vasoinhibin could play a role in the pathophysiology of diabetic retinopathy in humans. The purpose of this study was to investigate whether patients with diabetes mellitus and diabetic retinopathy have aberrant concentrations of prolactin-related vasoinhibin in the circulating blood.

Research Design

We performed a case-control study and developed a new technique to semi-quantitatively determine prolactin-related vasoinhibin in serum samples from 48 male subjects. The case group consisted of 21 patients with diabetes mellitus and proliferative or non-proliferative diabetic retinopathy. The control group consisted of 27 healthy subjects with no history of diabetes mellitus.

Methods

For the detection of prolactin-related vasoinhibin we developed a new analytical method, consisting of immunologic and laser-induced fluorescence techniques.

Results

The case-group had significantly lower prolactin-related vasoinhibin serum concentrations than the control group (p=0.041). There was no significant difference between patients with proliferative and those with non-proliferative diabetic retinopathy.

Conclusion

We conclude that given the antiangiogenic and antivasopermeability actions of prolactin-related vasoinhibin, the decreased serum levels of prolactin-related vasoinhibin in patients with diabetes mellitus could contribute to the development and progression of diabetic retinopathy.
Investigation of Prolactin-related Vasoinhibin in Sera from Patients with Diabetic Retinopathy

Prolactin-related vasoinhibin in patients with diabetic retinopathy

From the Department of Gastroenterology and Endocrinology, Georg-August-University, Goettingen, Germany

Introduction

Diabetic retinopathy is a common microvascular complication in patients with diabetes mellitus. Whereas early, non-proliferative stages of diabetic retinopathy are characterized by microaneurysms, hemorrhages, intraretinal microvascular abnormalities and other pathological processes, the major characteristic of more advanced, proliferative stages is neovascularization in the retina. Despite the identification of associated factors, such as chronic hyperglycemia (1), the underlying pathophysiological mechanisms leading to the development and progression of diabetic retinopathy are not fully understood (2).

Research in this field has revealed that besides its production and systemic release by the pituitary gland, Prolactin (PRL) is also locally produced in multiple human tissues such as endothelial cells (3), where it acts as a cytokine (4). Studies revealed that full-length PRL is proteolytically cleaved to various N- and C-terminal fragments (5, 6). Further investigations showed that the N-terminal, in contrast to the C-terminal fragments (7), referred to as prolactin-related vasoinhibin, have antiangiogenic properties and belong to a family of antiangiogenic peptides that are also derived from growth hormone and placental lactogen (8,9). Based upon their functional and structural similarities, these peptides from different sources are classified as vasoinhibins and are characterized as endogenous regulators of angiogenesis and vascular function (10).

In vitro experiments and in vivo studies in rodents revealed that prolactin-related vasoinhibins inhibit neovascularization by several mechanisms (11), such as apoptosis-mediated vascular...
regression, thus being a potent inhibitor of angiogenesis in the retina (12) and elsewhere. In addition, prolactin-related vasoinhibin can inhibit VEGF-induced (Vascular Endothelial Growth Factor) endothelial cell proliferation (8) and VEGF-induced vasopermeability in the retinal vessels of diabetic rats (13), both believed to be important factors in the pathogenesis of diabetic retinopathy. These observations led to the hypothesis that prolactin-related vasoinhibin could be involved in the development and progression of diabetic retinopathy in humans (14, 10, 11).

We performed a case-control study to investigate whether the serum levels of prolactin-related vasoinhibin correlate with the presence of diabetic retinopathy. Additionally, we report on the development of a highly sensitive and semi-quantitative method to detect prolactin-related vasoinhibin in human serum.

**Subjects and Methods**

**General technical aspects.** For the detection of prolactin-related vasoinhibin and the systematic screening of the samples, we used a combination of immunologic and laser-induced fluorescence techniques. During this procedure, protein samples obtained by immunoprecipitation are loaded on a commercial, micro fluidic based lab-on-a-chip device. Electrokinetic forces move the samples through a network of microchannels containing a gel/dye mixture which function as a protein separation matrix with a resolution comparable to a 4-20% gradient gel. The dye intercalates directly with the protein SDS-micelles and generates fluorescence, which is detected by a red laser. The amount of the protein is determined by the detection of the intensity of the fluorescence and the corresponding molecular weights are determined by an automated comparison to an internal standard.
**Immunoprecipitation.** 150µl serum and 400µl PBS (Phosphate Buffered Saline) containing protease inhibitors (Cat. No.: 04693124001, Roche, Mannheim, Germany) was incubated with 50µl Protein A-Agarose (Cat. No.: 11134515001, Roche) for 1h at 4°C under rotation. The sample was centrifuged (centrifuge 5415R with rotor F45-24-11, Eppendorf, Hamburg, Germany) for 30s at 6000 rpm (rounds per minute) and 15µl of the mouse monoclonal antibody 5602 against hPRL (Cat. No.: CAN-hPRL-4100-12, Diagnostics Biochem Canada, London, Canada) was added to the supernatant. After incubation for 6 hours at 4°C under rotation, the sample was incubated with 50µl Protein G-Agarose (Cat. No.: 11719416001, Roche) for 16 hours under the same conditions. The sample was centrifuged for 5min at 6000 rpm and the supernatant was discharged. The pellet was washed with PBS and then resuspended in 25µl PBS. 6µl of the suspension was analyzed with the Bioanalyzer 2100 (Agilent Technologies, Waldbronn, Germany).

**Bioanalyzer 2100.** Adhering to the manufacturer’s instructions for the Protein 80 Kit (Cat. No.: 5067-1515, Agilent Technologies), but with slight modifications, 6µl of the sample was combined with 2µl denaturation solution from the kit containing 3.5 v/v% b-mercaptoethanol. The vial was placed on a heating block for 5 minutes at 95°C. 6µl of the sample was loaded on the chip (Cat. No. 5067-1515, Agilent Technologies) and underwent analysis by the device.

**Positive Controls.** For positive controls, we used recombinant human prolactin (rhPRL) from E.Coli (Cat.No.: 40-267, NatuTec, Frankfurt, Germany). 5ng rhPRL was loaded on the chip and was analyzed by the device (Figure 2A). Proteolysis of full-length PRL to the N-terminal PRL-fragments was conducted according the method of Piwnica et al (6). 10ng Cathepsin D from human liver (Cat. No.: C869-25UG, Sigma, Saint Louis, U.S.A.) was incubated with 500ng of rhPRL in 100µl citrate-buffer at pH 3 and 37°C for 1h. The reaction was terminated by adding 100µl of SDS-PAGE sample buffer. 4µl diluted in 8µl sample buffer and
denaturation solution underwent analysis with the Bioanalyzer 2100 (Figure 2B). The 
immunoprecipitation of a serum sample was also conducted with another mouse monoclonal 
antitype on hPRL (SC-80303, Santa Cruz Biotechnologies, 
Santa Cruz, California, USA).

**Negative Control.** For the negative control, we conducted the immunoprecipitation with a 
mouse monoclonal antibody (Ab 5601) against hPRL with an epitope at the C-terminal end 
of PRL (Cat. No.: CAN-hPRL-4100-11, Diagnostics Biochem Canada) This antibody is 
specific to a c-terminal epitope (amino acids 150-199 of full length hPRL) which is not 
present on the N-terminal 16K PRL fragment. Consequently, the antibody binds to full-length 
but not to N-terminal PRL fragments.

**Non-reducing conditions.** Immunoprecipitation and subsequent analysis of the sample with 
the Bioanalyzer 2100 under non-reducing conditions was conducted without the addition of b-
mercaptoethanol or any other reducing agent (Figure 3C).

**ECLIA** (Electro-Chemiluminescence Immunoassay). In addition to the determination of PRL 
in our assay, the concentration of full-length PRL was measured with a commercial ECLIA 
(Roche Diagnostics, Mannheim, Germany, Device: Cobas Modular Analytics E170, Kit: 
prolactin II, Cat. No.: 03203093 190). The sensitivity of the assay was 0.047ng/ml.

**Western Blot analysis.** 100µl of serum was incubated with 450µl PBS containing protease 
inhibitors (Roche) and 50µl Protein A-Agarose (Roche) for 1h at 4°C under rotation. The 
sample was centrifuged for 20s at 6000 rpm and 10µl of a rabbit polyclonal antibody against 
human prolactin (A0569, DakoCytomation, Carpinteria, California, U.S.A.) was added. After 
3h at 4°C under rotation, 50µl Protein A-Agarose was added and the sample was incubated 
for 12h. Following centrifugation (20s at 6000rpm), the supernatant was discharged and the 
pellet was washed thrice in PBS. The pellet was then resuspended in 30µl SDS-PAGE loading
buffer containing b-mercaptoethanol, boiled for 5min and then left on ice for 5min to cool down. After centrifugation (20s at 6000rpm), the supernatant was electrophoresed on a 17% SDS-PAGE and an electroblot was performed on a PVDF membrane. The amersham low-range rainbow molecular weight marker (3500-40.000 Da, Product Code: RPN755E) has been used to determine the molecular weights. The transfer was conducted semi-dry at 0.8mA/cm². The membrane was blocked for 12h with TBS containing 8% dry milk. Next, the membrane was incubated with a 1:500 dilution of another rabbit polyclonal antibody against hPRL (Cat. No.: Ab1971, Abcam, Cambridge, Massachusetts, U.S.A.) for 1 hour at 37°C and afterwards with a 1:1000 dilution of a peroxidase-conjugated swine anti-rabbit antibody (Cat. No.: P0399, DakoCytomation). ECL Western Blotting Reagents (GE Healthcare, Freiburg, Germany) were used to visualize the immunocomplexes (Figure 7).

**Subjects.** The case group consisted of 21 patients with diabetes mellitus with either proliferative (n=14) or non-proliferative (n=7) diabetic retinopathy, assigned to a respective subgroup. For the control group, 27 healthy control subjects with no history of diabetes mellitus were enrolled. Patients of the case group were recruited at the Eye Clinic of the University of Goettingen. Ophthalmologic diagnosis was acquired through indirect ophthalmoscopy according to the criteria of the Early Treatment Diabetic Retinopathy Study (15). All control subjects were ambulant patients, recruited in a practice for internal medicine in Bochum, Germany. Institutional ethics committee approval and written informed consent from all participants was obtained.

Exclusion criteria for all patients were medications and conditions known to increase or decrease prolactin levels: a medical history of prolactinoma, hypothyroidism, chronic renal failure, liver cirrhosis, lesions of the chest wall during the last 3 month before recruitment and treatment with cimetidine, cyproheptadine, MAO-inhibitors, meprobamate, methyldopa, metoclopramide, antipsychotic drugs, opiate, estrogen, prostaglandin, reserpine, sulpiride,
tricyclic antidepressants or verapamil. Blood samples were drawn from the cubital vein between 06:00 and 12:00 a.m. and underwent screening for full-length prolactin and 16K PRL.

**Statistical analysis.** Descriptive statistics were used to report the demographic and clinical characteristics of the study population. Age and years since diagnosis of the patients are presented as means in years ± standard deviation (SD). Prolactin concentrations are presented as means and their standard error (SE). The signals of 16K PRL were read by signal strength, defined as peak height in fluorescence units (FU) shown in the electropherograms of the Bioanalyzer 2100 device. The Wilcoxon-Mann-Whitney test was used to determine significant differences. Differences in means with a p-value <0.05 were considered statistically significant. Statistical analysis performed made using Prism 4 GraphPad Software (GraphPad Software, La Jolla, California, USA).

**Results**

**Immunocomplex Constituent Analysis.** To identify the signals of the immunoprecipitate measurement and to evaluate the characteristics of each component in the Bioanalyzer 2100, we conducted an immunocomplex constituent analysis. Protein G-Agarose, the affinity chromatography matrix used for the immunoprecipitation, was found to cause no peaks within the detection area. Analysis of the mouse monoclonal antibody (anti-hPRL antibody 5602) under reducing conditions showed migration of the immunoglobulin light chains corresponding to a molecular weight of approximately 26-28 kDa and the heavy chain at approximately 65 kDa (Figure 1), thereby not interfering with PRL signals. Recombinant human PRL was determined to migrate at approximately 22kDa (Figure 2A). Cathepsin D-cleaved PRL-fragments were determined to migrate at 11, 15, 16.5 and 17 kDa (Figure 2B).
Signal Identification of the immunocomplex measurement. Epitope mapping of the monoclonal antibody 5602 by Piwnica et al. (5) demonstrates that this antibody binds to the N-terminal residues 1-9 of human prolactin. It does not detect Δ1-9-hPRL (lacking the nine n-terminal residues). The immunoprecipitation is specific to n-terminal and full-length PRL but not C-terminal fragments. Thus, the 16-17 kDa component of the immunocomplex, consistent with positive and negative controls, was identified to represent N-terminal prolactin-related vasoinhibin and not a C-terminal PRL-fragment (Figure 2C). In accordance with recombinant human PRL as a positive control, we identified the signal at 22 kDa to represent full-length PRL (Figure 2C). A Western Blot analysis of the immunoprecipitates, using a rabbit polyclonal antibody against human prolactin (A0569, DakoCytomation, Carpinteria, California, U.S.A.), also confirmed the identity of the 22kDa-peaks but did not show any immunoreactive bands at 16-17kDa (data not shown). Immunoprecipitation with another mouse monoclonal antibody (SC-80303), binding to a different epitope on hPRL, and successive measurement of the immunoprecipitate according to the method described above, confirmed prolactin-related vasoinhibin and full-length PRL signals (Figure 3A). This antibody binds to an epitope corresponding to amino acids 29-151. For the negative control, the immunoprecipitation was conducted with a mouse monoclonal antibody against hPRL with an epitope at the C-terminal end of PRL (Ab 5601). Analysis of a sample, previously shown to contain prolactin-related vasoinhibin by immunoprecipitation with two monoclonal antibodies with N-terminal epitopes (CAN-hPRL-4100-12, DBC; SC-80303, SCB) and subsequent analysis with the Bioanalyzer 2100 revealed a 23K PRL but no prolactin-related vasoinhibin signal(Figure 3B).

Analysis of prolactin-related vasoinhibin under non-reducing conditions. Measurement of the immunoprecipitate under non-reducing conditions revealed, compared to analysis under reducing conditions, a similar pattern (Figure 3C).
Generation of PRL fragments. Incubation of recombinant human prolactin with Cathepsin D resulted in a proteolytic cleavage of full-length PRL into an 11, 15, 16,5 and 17 kDa PRL fragment(Figure 2C).

Demographic and clinical characteristics of the study population. The patients in the case group had a mean age of 64.3±11.8 years. The mean duration since the diagnosis of diabetes mellitus (Type I or II) was 19.6±10.0 years. 2 Subjects (10%) in the case group had Type I diabetes mellitus and 19 (90%) Type II diabetes mellitus. 17 Individuals (80%) were treated with insulin and 4 (20%) received oral hypoglycemic medication. 14 patients (66%) had proliferative diabetic retinopathy (Figure 4) and 7 (33%) non-proliferative diabetic retinopathy. The mean age of the patients in the control group was 54.0±18.3 years. The demographic and clinical characteristics of the study population are summarized in Table 1.

Serum prolactin-related vasoinhibin and 23K PRL concentrations. Statistical analysis revealed that the case group had significantly lower prolactin-related vasoinhibin concentrations than the control group (p=0.041). The case group had a mean prolactin-related vasoinhibin concentration of 2.39±0.59 FU whereas the control group had a concentration of 3.64±0.51 FU (Figure 5, Table 2). Subgroup analysis showed no significant difference between either of the subgroups. Patients with proliferative diabetic retinopathy had a mean prolactin-related vasoinhibin concentration of 2.51±0.85 FU and patients with non-proliferative diabetic retinopathy a mean prolactin-related vasoinhibin concentration of 2.15±0.56 FU (Table 2). Figure 6 demonstrates representative electropherograms of a patient of the control group (Figure 6A) a patient with retinopathia diabetic a simplex (Figure 6B) and a patient with retinopathia diabetic a proliferans (Figure 6C). Comparison of 23K PRL serum concentrations, measured by ECLIA, revealed no significant difference between either of the groups. The patients of the case group had a full-length PRL concentration of 238.6±19.38 mU/l; analysis of the control group revealed a concentration of 222.1±19.02 mU/l (p = >
Subgroup analysis of the case group showed that patients with proliferative diabetic retinopathy had a concentration of 233.5±26.20 mU/l and those with non-proliferative diabetic retinopathy had a concentration of 248.9±27.49 mU/l (p = > 0.05; Table 2). Western blot analysis of the showed reproducible 23K PRL bands in all serum samples, but only weak, non-reproducible bands at 16kDa which were not sufficient for densitometric analysis (Figure 7).

Discussion

It is a widely accepted model in the understanding of angiogenesis that neovascularization, in health and disease, is controlled by pro- and antiangiogenic factors (16, 17). There has been an ongoing effort during the last decades to identify these factors and investigate their relevance in pathophysiological conditions (18). Prolactin-related vasoinhibin has been identified as natural inhibitor of angiogenesis in the retina (12), by inhibiting VEGF-mediated retinal vasopermeability (13) and antagonizing proangiogenic effects of VEGF (8). Angiogenesis and increased vasopermeability of retinal vessels are pathologic processes occurring in diabetic retinopathy. In this context, a role of prolactin-related vasoinhibin in the pathogenesis of diabetic retinopathy in humans is being discussed (14, 10). The present study supports that theory and provides evidence that patients with diabetic retinopathy have, compared to healthy subjects, lower levels of prolactin-related vasoinhibin in circulating blood. In view of the fact that prolactin-related vasoinhibin has antiangiogenic properties and a preventive effect on retinal vasopermeability, the lower concentration of prolactin-related vasoinhibin could contribute to the development and progression of diabetic retinopathy. Our results corroborate the assumption that pathological neovascularization in the eyes of patients with diabetes mellitus could be based on a systemic shift in the equilibrium of pro- and anti-angiogenic factors in favor of enhanced angiogenic potential.
Several studies show that patients with diabetes mellitus have higher levels of vascular endothelial growth factor in serum and vitreous fluid (19). Since prolactin-related vasoinhibin can inhibit VEGF-stimulated endothelial cell proliferation (8, 20), the lower levels of prolactin-related vasoinhibin could result in decreased VEGF-inhibition, thereby contributing to a pro-angiogenic environment.

Investigating patients with Preeclampsia, Leaños-Miranda and colleagues reported that patients with acute renal failure exhibited higher urinary PRL concentrations and higher urinary frequencies of antiangiogenic PRL-fragments than patients without diminished renal function (21). Since impaired renal function is common in patients with diabetes mellitus, increased renal elimination could explain lower systemic concentrations of prolactin-related vasoinhibin. According to Ben-Jonathan et al. glycosylation may alter proteolytic cleavage of PRL (22). This may result in the decreased proteolytic production of prolactin-related vasoinhibin, and thus enhanced glycosylation due to hyperglycemia could explain decreased levels of prolactin-related vasoinhibin.

Besides a small study population, there are other limitations to our investigation. Clearly, as the blood samples were collected from the cubital veins, we cannot state any information of prolactin-related vasoinhibin distribution in the eye e.g. ocular fluid. Nonetheless, systemic prolactin can enter the eye (23) and potentially act in concert with, or in addition to, local factors. Although prolactin is expressed by retinal tissues and prolactin-related vasoinhibin has been detected in the rat retina (24), the origin of ocular prolactin could also be systemic. Furthermore, most of the information about prolactin-related vasoinhibin comes from either in vitro studies or in vivo studies on rodents. However, the antiangiogenic effects of prolactin-related vasoinhibin has been demonstrated for both rat and human prolactin. Moreover, the variability in post-translational modifications between PRL from these species are not substantial (22).
Since, according to the manufacturer (Agilent Technologies, Waldbronn, Germany) of the device agilent 2100 Bioanalyzer, the sizing resolution is 10% and the sizing accuracy has a 10% CV (coefficient of variation), we cannot discriminate between 16, 16.5 or 17 kDa PRL. To our knowledge, there is currently no method or assay available which is capable of isolating, semi-quantitatively measuring and discriminating between 16, 16.5 and 17 kDa-PRL.

Measurement of the immunoprecipitate under non-reducing conditions revealed, compared to analysis under reducing conditions, a similar pattern. (Figure 3C). Several measurements with and without the reducing agent (b-mercaptoethanol) demonstrate that it does not affect the immunocomplexes. When using the agilent 2100 Bioanalyzer, the addition of the reducing agent does not change or improve the migration properties. We think that similar results under reducing and non-reducing condition show that the addition of a reducing agent does not introduce laboratory-made artifacts.

The western blot analysis showed reproducible 23K PRL bands in all samples, but only weak, non-reproducible bands at 16kDa which were not sufficient for densitometric analysis (Figure 7). The two immunoreactive bands correlate close to the 24 kDa band and the 17 kDa band of the marker, consistent with full-length PRL and prolactin-related vasoinhibin. In addition to Ab1971, we used the monoclonal antibody mAb 5602 and the polyclonal antibody A0569 in multiple western blots, attempting to detect prolactin-related vasoinhibin. Both antibodies repeatedly and reproducibly detected full-length PRL but failed to show immunoreactive bands at 16 kDa. Ab1971 was the only antibody showing immunoreactive bands at 16kDa, but even this signal was not reproducible and therefore not suitable for a quantitative analysis (optical densitometry). A reproducible qualitative and quantitative analysis of prolactin-related vasoinhibin from human serum by immunoprecipitation and subsequent western blot has not been described to our knowledge. We suspect the major
reason for this is the extremely low amount of antigen. We recognized this by unsuccessfully attempting multiple western blots with dozens of variations in order to detect prolactin-related vasoinhibin, and subsequently started to combine immunoprecipitation with the laser-induced fluorescence technique instead of western blot.

Prolactin-related vasoinhibin has been linked to the pregnancy-associated diseases postpartum cardiomyopathy (25) and preeclampsia (21). In our investigation, the detection of prolactin-related vasoinhibin in serum samples with immunoprecipitation procedures followed by western blot analysis was either unsuccessful or not reproducible. In the study by Hilfiker-Kleiner et al. (25) the detection of prolactin-related vasoinhibin in serum samples by western blot analysis was only successful in samples from lactating women who have, due to its function in milk production and lactation, high systemic PRL levels. In our study we detected prolactin-related vasoinhibin in a series of serum samples from male subjects with full-length PRL levels within the normal range. Therefore we believe that the use of immunoprecipitation in combination with laser induced fluorescence is a new technique which could facilitate further research in this important field.

This study aimed to show whether prolactin-related vasoinhibin serum levels correlate with the presence of diabetic retinopathy. In this context, we chose a control group of healthy subjects. A control group of patients with diabetes mellitus but no microvascular complications was considered inferior since a monocausal role of prolactin-related vasoinhibin in the development and progression of diabetic retinopathy is unlikely. However, since we found decreased levels of prolactin-related vasoinhibin in patients with diabetic retinopathy, the investigation of prolactin-related vasoinhibin levels in diabetic patients without microvascular complications is an important question which requires further research.

In summary, we show that patients with diabetes mellitus and diabetic retinopathy have, compared to healthy subjects, decreased serum levels of prolactin-related vasoinhibin in
systemic blood. In view of the antiangiogenic and antivasopermeability actions of prolactin-related vasoinhibin, we conclude that the decreased vasoinhibin serum levels could contribute to the development and progression of diabetic retinopathy. Future investigations, disclosing prolactin-related vasoinhibin levels in patients with diabetes mellitus and no microvascular complications, will help to further evaluate the role of prolactin-related vasoinhibin in diabetic retinopathy.

Declaration of interest

The Authors state that they have no conflict of interest to declare.

Funding

This research did not receive any specific grant from any funding agency in the public, commercial or not-for-profit sector.

Acknowledgements

This study was conducted in cooperation with the Eye Clinic of the University of Göttingen. We gratefully acknowledge our colleague Dr. M. Tondrow for his expert opinion and support with patient recruitment. We thank Ph.D. J. Dudas from our department for excellent technical assistance and D. Suan M.D. for the critical review of the manuscript.

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**Figure and Table Legends**

**Figure 1.** Electropherogram of the immunocomplex constituent analysis. The mouse monoclonal antibody 5602 and protein-G agarose were analyzed under reducing conditions. The light chains migrate at a molecular weight of approximately 26-28kDa and the heavy chain at approximately 65kDa. Protein-G agarose causes no peaks.

**Figure 2A.** Positive Control. 5ng recombinant human PRL (rhPRL). **2B.** 500ng rhPRL was incubated with 10ng cathepsin D from human liver in 100µl citrate-buffer at pH 3 and 37°C for 1 h. Analysis of 4µl diluted in 8µl sample buffer shows proteolytic cleavage of full-length PRL into PRL fragments. **2C.** Analysis of a serum-sample, immunoprecipitated with the anti-hPRL mouse monoclonal antibody 5602 shows Prolactin-related vasoinhibin - and 23kDa-PRL signals.
**Figure 3A.** Electropherogram of a serum-sample, immunoprecipitated with a different mouse monoclonal antibody specific to hPRL, SC80303, confirms prolactin-related vasoinhibin - and 23kDa-PRL signals. **3B.** Negative control. Analysis of a serum-sample, immunoprecipitated with a mouse monoclonal antibody, specific to an epitope at the C-terminal end of PRL, Ab 5601, shows a 23kDa-PRL- but no prolactin-related vasoinhibin signal. **3C.** Analysis of a serum-sample immunoprecipitated with the antibody 5602 under non-reducing conditions, demonstrates, compared to analysis under reducing conditions, a similar pattern.

**Figure 4.** Fluorescein angiography of the retina of a patient from the case group diagnosed with proliferative retinopathy. Neovascularization and leaking vessels are seen around the papilla nervi optici.

**Figure 5.** Serum prolactin-related vasoinhibin concentrations. The bars represent prolactin-related vasoinhibin (PRL-V) signals in fluorescence units (FU), mean and standard error, disclosed by immunoprecipitation of serum samples and subsequent analysis with the Bioanalyzer 2100. The case group had significantly lower prolactin-related vasoinhibin concentrations than the control group (p=0.041). The case group had a mean prolactin-related vasoinhibin concentration of 2.39±0.59 FU whereas the control group had a concentration of 3.64±0.51 FU.

**Figure 6.** Representative electropherograms of a patient of the control group (6A) a patient with retinopathy diabetica simplex (6B) and a patient with retinopathy diabetica proliferans (6C).

**Figure 7.** Western Blot Analysis. Immunoprecipitation of serum samples followed by western blot analysis under reducing conditions revealed clear 23kDa-PRL (23K PRL) but only weak and non-reproducible prolactin-related vasoinhibin (PRL-V) bands. Samples 1-3: serum from patients with proliferative diabetic retinopathy, samples 4-6: samples from healthy controls. MWM = molecular weight marker.

**Table 1.** Demographic and clinical characteristics of the study population. Data are presented as number of individuals or mean years ± standard deviation. No. = number of individuals, yr = years, DM = diabetes mellitus, RDP = Retinopathia Diabetica Proliferans and RDS = Retinopathia Diabetica Simplex.

**Table 2.** Serum prolactin concentrations. The values are presented as means ± standard error. Serum PRL concentrations were determined with a commercial ECLIA (Electro-Chemiluminescence Immunoassay). Serum prolactin-related vasoinhibin (PRL-V) concentrations were determined by immunoprecipitation of serum samples and subsequent measurement with the Bioanalyzer 2100. Statistical analysis with the Wilcoxon-Mann-Whitney test revealed that the case group had significantly lower prolactin-related vasoinhibin concentrations than the control group (p=0.041). RDP denotes Retinopathia Diabetica Proliferans and RDS Retinopathia Diabetica Simplex.
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<td>Subgroup - RDP</td>
<td>2.51±0.85</td>
<td>2.15±0.56</td>
</tr>
<tr>
<td>Serum PRL (mU/l)</td>
<td>238.6±19.38</td>
<td>222.1±19.02</td>
</tr>
<tr>
<td></td>
<td>233.5±26.20</td>
<td>248.9±27.49</td>
</tr>
</tbody>
</table>
Ab 5602 / Protein-G Agarose

light chains

heavy chain

FU

kDa [kDa]
**Recombinant human prolactin**

![Graph showing full-length PRL](2A)

**Cleaved recombinant human prolactin**

![Graph showing cleaved PRL](2B)

**Immunoprecipitate Ab 5602**

![Graph showing immunoprecipitate](2C)
Immunoprecipitate SC-80303

prolactin-related vasoinhibin → full-length PRL

Immunoprecipitate Ab 5601

full-length PRL

Immunoprecipitate non-reducing conditions

prolactin-related vasoinhibin → full-length PRL
pseudoprolactin-related vasoinhibin

full-length PRL

FU

6A

6B

6C