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Plasma matrix metalloproteinases and tissue inhibitors of metalloproteinases explored in relation to the severity and progression of diabetic retinopathy in patients with type 1 diabetes: baseline and prospective analyses

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ABSTRACT

Aims To explore whether circulating matrix metalloproteinase-2 (MMP-2), MMP-9, MMP-9/neutrophil gelatinase-associated lipocalin, MMP-9/tissue inhibitor of metalloproteinase-1 (TIMP-1), MMP-14, TIMP-2 and TIMP-3 were associated with the severity and progression of diabetic retinopathy (DR) in patients with type 1 diabetes (T1D).

Methods Baseline and prospective analyses were conducted over a period of 10.5 person-years. In 2009, recruitment and biochemical analyses (MMPs, TIMPs, glycated haemoglobin (HbA1c), serum creatinine, macroalbuminuria) were performed. Fundus photography, performed at baseline and at follow-up in accordance with the regional screening programme, was compared after being categorised according to the International Clinical Diabetic Retinopathy Disease Severity Scale. 'DR progression at least one leve' was calculated. High MMP-2 was defined as ≥178 ng/mL (≥75th percentile) and high TIMP-2 as ≥205 ng/mL (≥75th percentile). DR was dichotomised as 'at least moderate DR' or 'no/mild DR'.

Results The study included 267 participants, 57% of whom were men. At baseline, the prevalence of high MMP-2 (p=0.001) and high TIMP-2 (p=0.008) increased with the severity of DR. 'At least moderate DR' (adjusted OR (AOR) 2.4, p=0.008) and macroalbuminuria (AOR 3.6, p=0.025) were independently associated with high MMP-2. 'At least moderate DR' (AOR 2.3, p=0.009) and macroalbuminuria (3.4, p=0.031) were independently associated with high TIMP-2. DR progression occurred in 101 (46%) patients (p<0.001). HbA1c≥53 mmol/mol was associated with the progression of DR (crude OR 3.8, p=0.001). No other MMPs or TIMPs were linked to the severity or the progression of DR.

Conclusions High levels of MMP-2 and TIMP-2 indicated more severe DR or diabetic nephropathy. Only HbA1c was associated with the progression of DR in 267 patients with T1D.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Several matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) have been demonstrated in the retina, the vitreous or in the epiretinal membranes in patients with severe diabetic retinopathy (DR) and may contribute to the development of DR.

WHAT THIS STUDY ADDS

⇒ More severe DR and diabetic nephropathy were independently associated with high levels of plasma MMP-2 and high levels of plasma TIMP-2.

⇒ Plasma levels of MMP-9, MMP-9/neutrophil gelatinase-associated lipocalin, MMP-9/TIMP-1, MMP-14 and TIMP-3 did not differ between the five levels of DR.

⇒ None of the explored MMPs or TIMPs can be used as a prognostic biomarker for the progression of DR.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ In further research, it would be of interest to explore whether plasma MMP-2 and TIMP-2 reflect the intravitreous levels and whether plasma MMP-2 and TIMP-2 could be used for therapeutic drug monitoring.

INTRODUCTION

Diabetic retinopathy (DR) is a major cause of blindness among people over 50 years.[1] Vision impairment and vision loss are linked to the terminal stages of DR, proliferative (P) DR or macular oedema.[2] Hyperglycaemia contributes to the development of DR, and glycated haemoglobin (HbA1c) is a well-established systemic prognostic biomarker of DR progression.[2–5] We have recently shown in a prospective study that high levels of soluble (s)CD163 and HbA1c were independently

associated with the progression of DR in this cohort of patients with type 1 diabetes (T1D).[5]

The progression of DR to PDR includes preretinal neovascularization and the synthesis of extracellular matrix (ECM).[6] Degradation of ECM is exerted by matrix metalloproteinases (MMPs), whose activity is regulated by tissue inhibitors of metalloproteinases (TIMPs).[6 7] TIMPs are multifunctional proteins with both cell growth promoting and inhibitory functions.[6 8] In patients with PDR, MMP-2, MMP-9 and TIMP-3 have been demonstrated in the retina where they normally are not expressed.[6] Increased levels of MMP-2, MMP-9 and MMP-14 have been found in the vitreous,[8 9] and TIMP-2 have been found in PDR membranes.[6] Previous research has shown that MMP-2,[10] MMP-9, TIMP-2[6] and MMP-14[9 11] are involved in neovascularisation of the retina. Increased levels of plasma MMP-2 have been demonstrated in patients with T1D with more severe levels of DR or diabetic nephropathy.[12 13] MMP-9 is considered to have strong impact on DR progression,[1] and increased levels of circulating MMP-9 levels have been found in T1D patients with DR.[14] Neutrophil gelatinase-associated lipocalin (NGAL) can bind to MMP-9, and the complex MMP-9/NGAL inhibits degradation of MMP-9, which leads to sustained proteolytic activity.[15] TIMP-1 is a specific inhibitor of MMP-9 which can form a complex with MMP-9 (MMP-9/TIMP-1).[16] TIMP-2 serves as an inhibitor of both MMP-2 and MMP-9.[8] TIMP-3 has shown anti-inflammatory and antiangiogenic effects on diabetic retina both in vitro and in vivo.[17] A schematic overview of the functions of the included MMPs and TIMPs is presented in figure 1.

We have previously explored several MMPs and TIMPs in relation to cardiovascular disease, and we found that MMP-14 was associated with cardiovascular disease in this cohort.[18]

We hypothesised that MMPs and TIMPs are involved in the development of DR and that they may constitute valuable biomarkers for the severity and progression of DR in patients with T1D in addition to HbA1c.

The aims of this study were to explore whether plasma MMP-2, MMP-9, MMP-9/NGAL, MMP-9/TIMP-1, MMP-14, TIMP-2 and TIMP-3 were associated with the more advanced forms of DR at baseline, and with the progression of DR in T1D patients with no apparent DR or non-proliferative diabetic retinopathy (NPDR) at baseline.

MATERIALS AND METHODS

Prospective design

At baseline, patients with T1D were recruited consecutively between March and December 2009 at a specialist

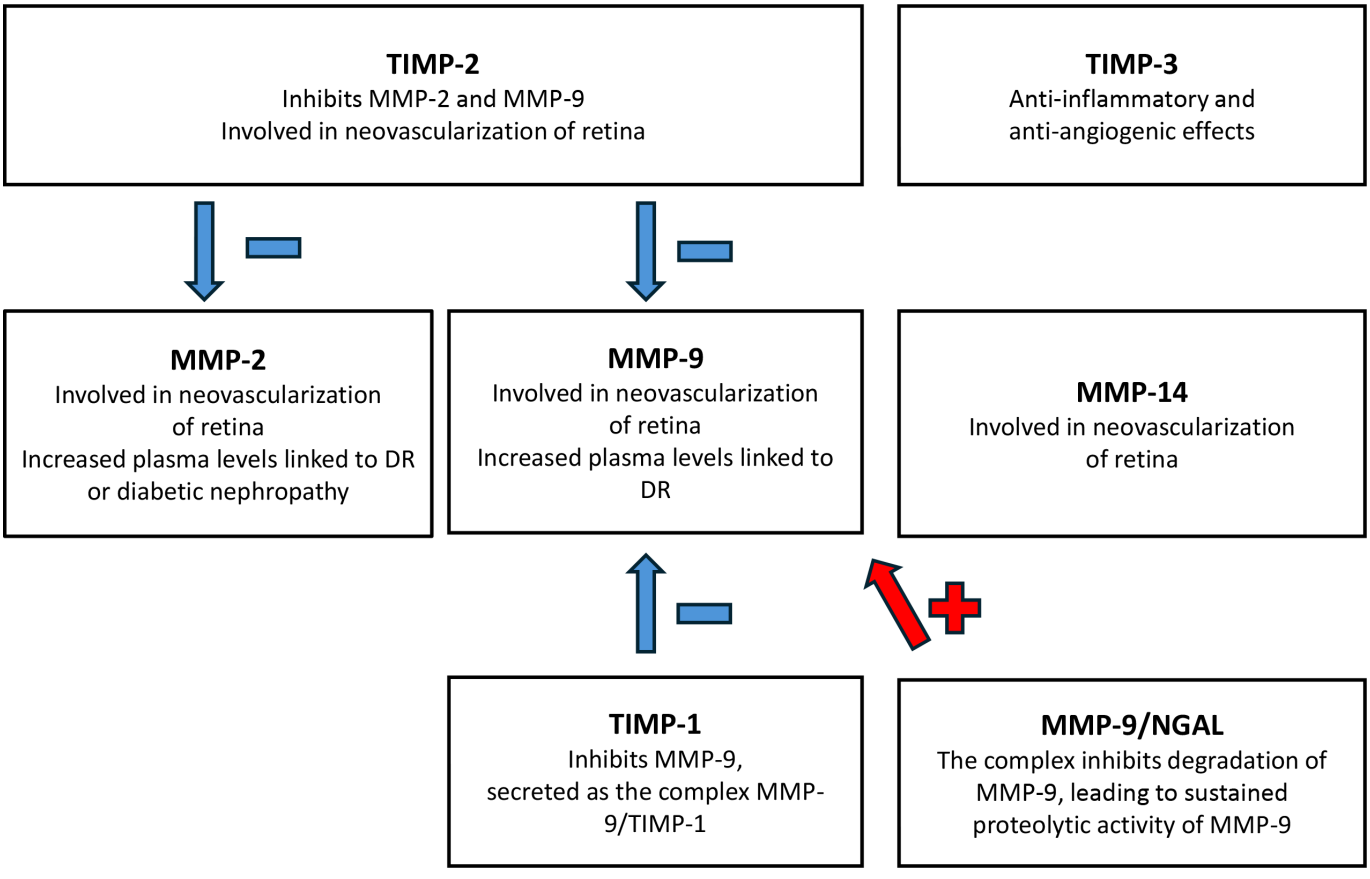

Figure 1 Basic summary of what is known from previous research about the included MMPs and TIMPs. DR, diabetic retinopathy; MMPs, matrix metalloproteinases; NGAL, neutrophil gelatinase-associated lipocalin; TIMPs, tissue inhibitor of metalloproteinases.

diabetes outpatient clinic in Växjö, Region Kronoberg, Sweden.[5 19] The study was terminated in November 2021. The plasma collection was performed at the time of recruitment in 2009, and the fundus photography was performed according to the regional screening programme for fundus photography as in our previous prospective study of sCD163 and DR.[5] All fundus photography was performed at the ophthalmology clinic at two sites, Ljungby and Växjö, Region Kronoberg, Sweden.[5 20] Inclusion criteria were: (1) adult patients with T1D who participated in our cross-sectional study of sCD163 and DR at baseline (n=290)[20]; (2) biochemical analyses were performed in 2009 of the selected MMPs and TIMPs; and (3) at least one follow-up fundus photography was performed at the clinic. Inclusion criteria 1 and 3 were the same as in our previous prospective study of DR, but not inclusion criterion 2 as no MMPs or TIMPs were included in the previous study.[5] 17 patients either declined to participate in the follow-up, had died before any follow-up fundus photography was performed or could not be contacted and were therefore excluded. Six patients did not fulfill criteria 2 or 3. Altogether, 267 (92%) patients with T1D were included. Exclusion criteria at baseline were end-stage renal disease, cancer, hepatic failure, pregnancy, psychotic disorders, severe substance abuse or cognitive deficiency.[5] For the purpose of this study, an ophthalmologist re-examined all fundus photography at baseline and at follow-up, categorising the results according to the International Clinical Diabetic Retinopathy Disease Severity Scale.[5 21] All data, except the follow-up fundus photography, were collected in 2009. The ophthalmologist was blinded to the results of the biochemical analyses. We controlled for sex, age, diabetes duration, follow-up period, kidney function, HbA1c, blood pressure (BP) and antihypertensive drugs (AHD).[5] No imputation of data was performed. Missing variables are presented in tables 1 and 2. Patients or the public were not involved in the design, conduct, reporting or dissemination plans of our research.

Screening for DR and quality assurance processes

In Sweden, all patients with T1D (≥10 years) are referred to ophthalmology clinics for regular screening. In Region Kronoberg, T1D patients with no DR are screened every second year. Patients with mild or moderate NPDR, independent of diabetes duration, are screened every year or every 6 months if the changes are close to the macula. Patients with severe NPDR will have a medical assessment within 3–6 months. Patients with PDR will have medical assessment within 2 weeks to 12 months depending on the severity and activity of the retinal disease.[5] Screening fundus photographs were identified from baseline (performed up to a year after plasma collection in 2009, or otherwise the last fundus photography performed prior to plasma collection) until follow-up (at the latest in November 2021). Patients who had not followed the screening programme were invited to have a follow-up fundus photograph.[5] Patients with lower-grade DR were usually photographed using scanning laser ophthalmoscopy (SLO) through an undilated pupil. The device used was an Optos California model: Panoramic Ophthalmoscope P200Dtx which produces an image of the retina in 200°. All patients with DR levels 3 or 4 were photographed using a Topcon fundus camera through a dilated pupil. The SLO has a greater field of view and in cases when the wider field of view in the SLO photo would affect the level of DR, the assessed area was restricted to match the previously investigated area using the Topcon fundus camera. The SLO produces an image with slightly lower contrast and resolution than conventional fundus photography.[5] All fundus photographs were graded into five levels, no apparent DR, mild, moderate or severe NPDR or PDR.[5 21] For this prospective study, an ophthalmologist re-examined all fundus photographs. As all images were previously graded by ophthalmologists at the clinic, the two results were compared. The results did not match in 15 cases. These were re-examined by an additional ophthalmologist and a consensus decision was taken between the two examiners. The re-examining ophthalmologist was masked to the MMPs, TIMPs and HbA1c levels, as well as all other data, when examining the photographs.

The five levels of DR were included in the analyses, but DR was also dichotomised as 'at least moderate DR' (moderate or severe NPDR or PDR) or 'no/mild DR'.

The progression of DR at least one level

The progression of DR at least one level from baseline to follow-up was calculated for patients with no apparent DR, mild, moderate or severe NPDR.[5]

Biochemical analyses

Plasma levels of the MMPs and TIMPs were analysed by using commercial human DuoSet ELISAs,[5 18 20] and supplementary ancillary kit (R&D Systems, Minneapolis, Minnesota, USA). Before the analyses, the plasma samples were diluted in phosphate-buffered saline supplemented with 1% bovine serum albumin and were run in duplicates. The dilution factors were for MMP-2: 1:30; MMP-9: 1:400; MMP-9/NGAL: 1:10; MMP-9/TIMP-1: 1:10 (1:25/1:50); MMP-14: 1:3 (1:25/1:50); TIMP-2: 1:400; and TIMP-3: 1:16. The ELISA analyses were performed according to the manufacturer's instructions. Absorbance was measured at 450–580 nm in a FLOUstar optima plate reader (BMG Labtech Gmbh, Ortenberg, Germany). Concentrations of unknown samples were calculated using a 4-parameter logistic regression curve. The intra-assay coefficients of variation (CVs) were for MMP-2: 3.7%; MMP-9: 2.2%; MMP-9/NGAL complex: 3.3%; MMP-9/TIMP-1 complex: 3.8%; MMP-14: 2.8%; TIMP-2: 2.0%; and TIMP-3: 1.6%. Exact values of MMP-14 were measured for 173 patients, but for 95 patients the MMP-14 values were under the detection limit of 0.154 ng/mL. The undetected values were approximated by 'zero' as in our previous study of

Table 1 Baseline characteristics, biochemical variables and medication compared between the five levels of diabetic retinopathy for 267 patients with type 1 diabetes

		All patients*	Diabetic retinopathy at baseline (according to the International Clinical Diabetic Retinopathy Disease Severity Scale)					
			No apparent DR†	Non-proliferative DR†			Proliferative DR†	
				Mild	Moderate	Severe		P value‡
n		267	73	54	63	30	47	–
DR (any level)		194 (73)	–	–	–	–	–	–
Sex	Women	114 (43)	33 (45)	31 (57)	23 (36)	10 (33)	17 (36)	0.075
	Men	153 (57)	40 (55)	23 (43)	40 (64)	20 (67)	30 (64)	
Age (years)		42 (18–59)	37 (26–50)	35 (28–47)	45 (36–53)	44 (32–49)	46 (42–55)	<0.001§
Diabetes duration (years)		20 (1–55)	9 (6–13)	17 (10–24)	22 (16–31)	26 (21–32)	35 (26–42)	<0.001§
HbA1c≥53 mmol/mol		218 (82)	54 (74)	38 (70)	57 (90)	26 (87)	43 (92)	0.002
MMP-2 (ng/mL)		154 (66–434)	146 (119–166)	143 (122–175)	162 (137–180)	152 (129–197)	164 (135–203)	0.012§
High MMP-2 (≥178 ng/mL)		66 (25)	10 (14)	10 (18)	19 (30)	10 (33)	17 (36)	0.001
TIMP-2 (ng/mL)¶		179 (0.0–468)	165 (138–195)	170 (147–188)	185 (167–212)	182 (151–209)	191 (171–220)	<0.001§
High TIMP-2 (≥205 ng/mL)¶		67 (25)	15 (20)	6 (11)	20 (32)	8 (27)	18 (38)	0.008
Combined high MMP-2 and high TIMP-2 (ng/mL)¶		40 (15)	5 (7)	3 (6)	11 (18)	7 (23)	14 (30)	< 0.001
MMP-9 (ng/mL)**		536 (222–1936)	552 (419–701)	536 (394–790)	535 (435–716)	566 (417–730)	497 (401–769)	0.94§
MMP-9/NGAL (ng/mL)††		21 (3–96)	24 (15–36)	24 (14–33)	20 (15–32)	22 (11–31)	18 (10–31)	0.34§
MMP-9/TIMP-1 (ng/mL)‡‡		15 (1–103)	15 (8–27)	14 (8–31)	16 (11–29)	17 (8–28)	14 (8–25)	0.79§
MMP-14§§		1.5 (0.0–447.0)	1.8 (0.0–10.1)	1.6 (0–3.3)	1.7 (0.0–4.3)	1.2 (0.0–6.7)	1.2 (0.0–5.9)	0.72
TIMP-3 (ng/mL)¶¶		8.2 (1–486)	9.8 (6.1–13.8)	7.5 (5.2–14.6)	7.0 (5.7–10.4)	7.7 (5.2–10.4)	8.8 (6.4–10.5)	0.17§
Serum creatinine (µmol/L)***		70 (28–182)	70 (62–78)	68 (58–78)	70 (61–76)	70 (62–80)	72 (64–88)	0.16§
Macroalbuminuria†††		15 (6)	0	0	4 (7)	5 (14)	7 (16)	<0.001
Systolic BP>130 mm Hg		44 (16)	5 (7)	5 (9)	14 (22)	6 (20)	14 (30)	<0.001
Diastolic BP>80 mm Hg		13 (5)	3 (4)	1 (2)	4 (6)	3 (10)	2 (4)	0.46
Continuous subcutaneous insulin infusion‡‡‡		23 (9)***	3 (4)	2 (4)	9 (14)	1 (3)	8 (17)	0.018
Antihypertensive drugs		89 (33)	7 (10)	7 (13)	27 (43)	18 (60)	30 (64)	<0.001

*Data are presented as n (%) or median (min–max).
† Data are presented as n (%) or median (q_1, q_3).
‡Linear-by-linear association was used unless indicated.
§ Kruskal-Wallis test.
¶Missing value (n): 2.
**Missing value (n): 5.
††Missing value (n): 4.
‡‡Missing value (n): 4.
§§Missing value (n): 9.
¶¶Missing value (n): 6.
***Missing value (n): 13.
†††Missing value (n): 15.
‡‡‡Otherwise multiple daily insulin injections.
BP, blood pressure; DR, diabetic retinopathy; HbA1c, glycated haemoglobin; MMP, matrix metalloproteinase; NGAL, neutrophil gelatinase-associated lipocalin; PDR, proliferative diabetic retinopathy; TIMP, tissue inhibitor of metalloproteinase.

Table 2 Comparisons of baseline variables between high and low levels of MMP-2 and TIMP-2

		MMP-2			TIMP-2		
		Low (<178 ng/mL)	High (≥178 ng/mL)	P value [a]	Low (<205 ng/mL)	High (≥205 ng/mL)	P value [b]
n		201	66		198	67	
Sex	Women	81 (40)	33 (50)	0.17	80 (40)	33 (49)	0.20
	Men	120 (60)	33 (50)		118 (60)	34 (51)	
Age (years)		40 (30–50)	46 (40–50)	0.001 [c]	40 (30–50)	45 (37–55)	0.001 [c]
Diabetes duration (years)		18 (9–27)	28 (18–35)	<0.001 [c]	18 (9–28)	26 (18–35)	<0.001 [c]
DR	Proliferative	30 (15)	17 (26)	0.001 [d]	29 (15)	18 (27)	0.008 [d]
	Severe non-proliferative	20 (10)	10 (15)		22 (11)	8 (12)	
	Moderate non-proliferative	44 (22)	19 (29)		42 (21)	20 (30)	
	Mild non-proliferative	44 (22)	10 (15)		47 (24)	6 (9)	
	No apparent	63 (31)	10 (15)		58 (29)	15 (22)	
DR, at least moderate		94 (47)	46 (70)	0.001	93 (47)	46 (69)	0.002
HbA1c (≥53 mmol/mol)		165 (82)	53 (80)	0.74	158 (80)	58 (87)	0.22
Serum creatinine (µmol/L) [e]		70 (61–78)	70 (64–79)	0.36[c]	70 (61–77)	74 (65–82)	0.016 [c]
Macroalbuminuria [f]		6 (3)	9 (14)	0.003 [g]	6 (3)	9 (14)	0.004 [g]
Systolic BP>130 mm Hg		13 (15)	13 (20)	0.42	30 (15)	14 (21)	0.28
Diastolic BP>80 mm Hg		10 (5)	3 (5)	0.89	10 (5)	3 (4)	0.85
TIMP (ng/mL) [h]		170 (148–191)	211 (187–232)	<0.001 [c]	–	–	–
High TIMP-2 (ng/mL) [h]		27 (14)	40 (61)	<0.001	–	–	–
MMP-2 (ng/mL)		–	–	–	143 (120–165)	187 (163–216)	<0.001 [c]
High MMP-2 (ng/mL)		–	–	–	26 (13)	40 (60)	<0.001
CSII		18 (9)	5 (8)	1.00 [f]	17 (9)	6 (9)	1.00 [f]
Antihypertensive drugs		61 (30)	28 (42)	0.071	58 (29)	31 (46)	0.011

[a, b] Data are presented as n (%) or median (q_1, q_3). [a, b] Chi-Square Test was used unless otherwise indicated. [c] Mann-Whitney U Test. [d] Linear-by-linear Association. Missing values: [e] 13/[f] 15. [g] Fisher's Exact Test. Missing values: [h] 2.
BP, blood pressure; CSII, continuous subcutaneous insulin infusion; DR, diabetic retinopathy; HbA1c, glycated haemoglobin; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase.

cardiovascular disease.[18] The analyses were performed at the Diabetes Laboratory, BMC, Lund University, Lund.

High MMP-2 was defined as ≥178 ng/mL (≥75th percentile) and high TIMP-2 as ≥205 ng/mL (≥75th percentile). A combined variable with high levels of both high MMP-2 (≥178 ng/mL) and high TIMP-2 (≥205 ng/mL) was constructed.

The biochemical analyses of HbA1c and serum creatinine were previously described.[5] HbA1c was dichotomised at ≥53 mmol/mol (≥7.0%).[3]

Macroalbuminuria was defined as ≥300 mg albumin/g creatinine.[22]

Blood pressure

BP was measured according to standard procedures by a nurse.

Medication

The patients used either multiple daily insulin injections or continuous subcutaneous insulin infusion. The indications for AHD were previously described.[5] AHD was dichotomised into users and non-users.

Follow-up periods

The follow-up periods were calculated, and subanalyses were performed for diseased patients.[5]

Statistical analysis

Histogram revealed that age, MMPs, TIMPs and serum creatinine were not normally distributed. Analyses were performed with Mann-Whitney U test or Kruskal-Wallis test. Data was presented either as median (min–max) or

median (quartile (q)$_1$, q$_3$). Linear-by-linear association, χ^2 test or Fisher's Exact test was used for categorical data.

Crude ORs (CORs) and multiple logistic regression analysis (Backward: Wald) were performed with high MMP-2 (three models), high TIMP-2 (three models) and the combined variable 'high MMP-2 and high TIMP-2' (one model) as dependent variables. Nagelkerke R^2 was used to evaluate each model. Linear regression (Backward) was used with diabetes duration as dependent variable. CIs of 95% were used. P<0.05 was considered statistically significant. SPSS V.23 was used.

RESULTS

In this study of DR, 267 patients with T1D participated (57% men). At the time of recruitment, the patients were 18–59 years old and diabetes duration ranged from 1 to 55 years. The total prevalence of DR at baseline was 194 (73%) and at follow-up, it increased to 220 (82%). Progression of DR at least one level occurred in 101 out of 220 patients (46%) (p<0.001).

95% of the baseline fundus photographs were performed during a period from 1 year prior to plasma collection until 1 year after plasma collection, and 98% were performed from 2 years prior to plasma collection until a year after plasma collection. The median follow-up period was 10.5 person-years for all 267 patients. The follow-up period for 258 non-diseased patients was (median (q$_1$, q$_3$); min–max) (11 (11, 11); 1–12) years. For nine patients who died before the study was terminated, the follow-up period was 1–10 years. Six of these patients had PDR at baseline, one patient acquired DR progression and two patients did not.

In table 1, baseline characteristics, the biochemical analyses and medication are compared between the five levels of DR. The 47 patients with PDR were the oldest, had the longest diabetes duration, had the highest prevalence of the combined variable 'high MMP-2 (≥178 ng/mL) and high TIMP-2 (≥205 ng/mL)', and macroalbuminuria (all p<0.001); high MMP-2 (≥ 178 ng/mL) (p=0.001); HbA1c≥53 mmol/mol (p=0.002); and high TIMP-2 (≥ 205 ng/mL) (p=0.008). The prevalence of the combined variable 'high MMP-2 and high TIMP-2' was 4.3 times higher in patients with PDR than in patients with no apparent DR, whereas the prevalence of high MMP-2 was 2.6 times higher, and the prevalence of high TIMP-2 was 1.9 times higher in patients with PDR than in patients with no apparent DR.

In table 2, comparisons are performed between patients with high and low MMP-2 levels and between patients with high and low TIMP-2 levels. Patients with high MMP-2 levels compared with patients with low MMP-2 levels, had longer diabetes duration and higher prevalence of high TIMP-2 levels (both p<0.001); were older and had higher prevalence of moderate and severe NPDR, and PDR (p=0.001); and macroalbuminuria (p=0.003). Patients with high TIMP-2 levels compared with patients with low TIMP-2 levels, had longer diabetes duration and higher prevalence of high MMP-2 levels (both p<0.001); were older (p=0.001); higher prevalence of macroalbuminuria (p=0.004); had higher prevalence of moderate and severe NPDR, and PDR (p=0.008); and higher serum creatinine levels (p=0.016).

In table 3, associations with high MMP-2 are presented for three models. In model 1, PDR (AOR 3.1, p=0.022), severe NPDR (AOR 3.1, p=0.041), moderate NPDR (AOR 2.5, p=0.042) and macroalbuminuria (AOR 5.2, p=0.003) were associated with high MMP-2. In model 2, at least moderate DR (AOR 2.4, p=0.008) and macroalbuminuria (AOR 3.6, p=0.025) were associated with high MMP-2. In model 3, diabetes duration (per year) (AOR 1.06, p<0.001) and macroalbuminuria (AOR 3.8, p=0.020) were associated with high MMP-2.

In table 4, associations with high TIMP-2 are presented for three models.

In model 1, macroalbuminuria (AOR 3.4, p=0.032) was associated with high TIMP-2, but not any of the five levels of DR. In model 2, at least moderate DR (AOR 2.3, p=0.009) and macroalbuminuria (AOR 3.4, p=0.031) were associated with high TIMP-2. In model 3, diabetes duration (per year) (AOR 1.02, p<0.001) and macroalbuminuria (AOR 3.7, p=0.022) were associated with high TIMP-2.

The associations between the combined variable 'high MMP-2 and TIMP-2' and the following variables were for PDR (AOR 5.2 (95% CI 1.5 to 17.8), p=0.009), severe NPDR (AOR 4.2 (95% CI 1.1 to 16.3), p=0.040), moderate NPDR (AOR 3.2 (95% CI 0.9 to 10.7), p=0.062), mild NPDR (AOR 1.1 (95% CI 0.2 to 5.0), p=0.94), no DR (1) and macroalbuminuria (AOR 4.3 (95% CI 1.4 to 13.3), p=0.011). Nagelkerke R^2 0.16.

Associations with diabetes duration are presented for two models. In model 1 (n=252, R^2 0.47), age (unstandardised coefficient B (UCB) 0.004, p<0.001), at least moderate DR (UCB 11.0, p<0.001) and high MMP-2 (UCB 3.7, p=0.006) were independently associated with diabetes duration, but not macroalbuminuria (p=0.62) or AHD (p=0.16). In model 2 (n=250, R^2 0.47), age (UCB 0.36, p<0.001), at least moderate DR (UCB 11.2, p<0.001) and high TIMP-2 (UCB 3.1, p=0.023) were independently associated with diabetes duration, but not macroalbuminuria (p=0.72) or AHD (p=0.14).

HbA1c≥53 mmol/mol was the only variable associated with 'progression of DR at least one level' (COR 3.8 (95% CI 1.8 to 8.1), p=0.001).

DISCUSSION

The main findings of this study were that patients with moderate NPDR, severe NPDR or PDR had higher prevalence of both high MMP-2 (≥178 ng/mL) and high TIMP-2 levels (≥205 ng/mL) compared with patients with no or mild DR at baseline. The highest prevalence of high MMP-2 and high TIMP-2 was observed in patients with PDR. Further exploration showed that 'at least moderate DR' and macroalbuminuria at baseline were independently associated with high MMP-2 and high TIMP-2 levels. However, after adjustments for diabetes

Table 3 Associations with high MMP-2 presented for three models

		High MMP-2 (≥178 ng/mL)							
				Model 1		Model 2		Model 3	
		COR (95% CI)	P value	AOR (95% CI)	P value [a]	AOR (95% CI)	P value [b]	AOR (95% CI)	P value [c]
Sex (women)		1.5 (0.8 to 2.6)	0.17	–	–	–	–	–	–
Age (per year)		1.04 (1.02 to 1.07)	0.001	–	–	–	–	1.02 (0.98 to 1.05)	0.33
Diabetes duration (per year)		1.06 (1.03 to 1.08)	<0.001	–	–	–	–	1.06 (1.03 to 1.08)	<0.001
DR	Proliferative	3.6 (1.5 to 8.7)	0.005	3.1 (1.2 to 8.1)	0.022	–	–	–	–
	Severe non-proliferative	3.2 (1.1 to 8.7)	0.026	3.1 (1.0 to 8.9)	0.041	–	–	–	–
	Moderate non-proliferative	2.7 (1.2 to 6.4)	0.022	2.5 (1.0 to 6.2)	0.042	–	–	–	–
	Mild non-proliferative	1.4 (0.6 to 3.7)	0.46	1.5 (0.5 to 4.0)	0.46	–	–	–	–
	No apparent	1		1		–	–	–	–
DR at least moderate		2.6 (1.4 to 4.7)	0.001	–	–	2.4 (1.2 to 4.5)	0.008	1.3 (0.6 to 2.9)	0.50
Macroalbuminuria		5.2 (1.8 to 15.3)	0.003	5.2 (1.8 to 15.3)	0.003	3.6 (1.2 to 10.8)	0.025	3.8 (1.2 to 11.9)	0.020
Antihypertensive drugs		1.8 (1.00 to 3.2)	0.051	–	–	–	–	0.7 (0.4 to 1.5)	0.41

[a, b, c] Multiple logistic regression (Backward Wald): n = [a, b, c] 252; Nagelkerke R Square [a] 0.05/[b] 0.09/[c] 0.15.
AOR, adjusted OR; COR, crude OR; DR, diabetic retinopathy; MMP-2, matrix metalloproteinase-2.

Table 4 Associations with high TIMP-2 presented for three models

		High TIMP-2 (≥205 ng/mL)							
				Model 1		Model 2		Model 3	
		COR (95% CI)	P value	AOR (95% CI)	P value [a]	AOR (95% CI)	P value [b]	AOR (95% CI)	P value [c]
Sex	(women)	1.4 (0.8 to 2.5)	0.21	–	–	–	–	–	–
Age (per year)		1.04 (1.02 to 1.07)	0.001	–	–	–	–	1.02 (0.98 to 1.05)	0.32
Diabetes duration (per year)		1.05 (1.03 to 1.08)	<0.001	–	–	–	–	1.05 (1.02 to 1.08)	<0.001
DR	Proliferative	2.4 (1.1 to 5.4)	0.036	2.2 (0.9 to 5.4)	0.08	–	–		
	Severe non-proliferative	1.4 (0.5 to 3.8)	0.50	1.4 (0.5 to 4.0)	0.53	–	–		
	Moderate non-proliferative	1.8 (0.8 to 4.0)	0.12	2.0 (0.9 to 4.5)	0.10	–	–		
	Mild non-proliferative	0.5 (0.2 to 1.4)	0.18	0.6 (0.2 to 1.7)	0.34	–	–		
	No apparent	1		1	–	–	–		
DR, at least moderate		2.5 (1.4 to 4.4)	0.002	–	–	2.3 (1.2 to 4.3)	0.009	1.3 (0.6 to 2.7)	0.52
Macroalbuminuria		4.9 (1.7 to 14.4)	0.004	3.4 (1.1 to 10.5)	0.032	3.4 (1.1 to 10.2)	0.031	3.7 (1.2 to 11.3)	0.022
Antihypertensive drugs		2.1 (1.2 to 3.7)	0.012	–	–	–	–	1.0 (0.5 to 2.0)	0.98

.[a, b, c] Multiple logistic regression (Backward Wald): n = [a, b, c] 250; Nagelkerke R Square [a] 0.10/[b] 0.09/[c] 0.13.
AOR, adjusted OR; COR, crude OR; DR, diabetic retinopathy; TIMP-2, tissue inhibitor of metalloproteinase-2.

duration, the associations did not remain significant due to the associations between diabetes duration and high MMP-2 and high TIMP-2. Diabetes duration was independently associated with 'at least moderate DR', high MMP-2 and high TIMP-2. HbA1c≥53 mmol/mol was the only variable associated with the progression of DR. No other MMPs or TIMPs included in the study were linked to the severity or progression of DR.

The interpretation of these findings is that high MMP-2 and high TIMP-2 at baseline indicated ongoing microvascular complications, either as more severe levels of DR or diabetic nephropathy with macroalbuminuria, which implies that neither MMP-2 nor TIMP-2 can differentiate between DR and diabetic nephropathy. As neither MMP-2, TIMP-2 nor the combined variable 'high MMP-2 and high TIMP-2' were independently associated with the progression of DR, this study does not support that just single baseline measurements could be used as prognostic biomarkers for the progression of DR. Maybe repeated measurements could provide more information. However, we cannot dismiss that MMP-2 and TIMP-2 may contribute to the development of DR. In previous research, it has been demonstrated that MMP-2 can induce injury to the tissues in the eye,[8 10] and vascular endothelial growth factor promotes increased MMP-2 expression and retinal neovascularisation.[10] MMP-2 has been confirmed in the retina, the vitreous and in epiretinal membranes of patients with PDR.[6 8 10] Previous research has shown that high plasma levels of MMP-2 were associated with both more severe DR and with diabetic nephropathy particularly in patients with longer diabetes duration,[12 13] which is in line with our findings. Despite being an inhibitor of MMP-2 and MMP-9,[8] it has been suggested that TIMP-2 is involved in the pathological processes leading to retinal neovascularisation,[6] which also is in line with our findings. The clear associations between diabetes duration and high levels of MMP-2 and TIMP-2 may be explanatory factors to why longer diabetes duration is linked to the development and progression of DR.[23] The association between HbA1c and the progression of DR is in line with previous research.[2–5] Our findings differ from previous research where plasma MMP-9 levels were increased in T1D patients with DR,[14] and where increased plasma levels of MMP-14 showed short-time predictive value for DR progression.[11]

The clinical grading of DR is sometimes difficult. It would have been valuable to be able to distinguish between severe NPDR and PDR by using biomarkers, but not even the combined variable 'high MMP-2 and high TIMP-2' could clearly distinguish between severe NPDR and PDR.

Strengths of this study were that several quality assurance measures were performed. All fundus photographs were re-examined. The ophthalmologist was blinded to the results of the biochemical analyses beforehand. The severity of DR was graded according to the International Clinical Diabetic Retinopathy Disease Severity Scale, a validated scale recommended for clinical classifying of DR.[21] Another strength was that precise ELISA techniques were used which showed low intra-assay CVs for the MMPs and TIMPs. We controlled for potential confounders previously linked to DR or diabetic nephropathy.[2–4 23 24] We did not include body mass index, serum lipids, lifestyle factors or cardiovascular disease, as we have previously shown in this cohort that these variables were not associated with the severity or progression of DR.[5] We estimate that the follow-up period of 10.5 person-years was adequate, as there was clearly significant progression of DR. Subanalyses of the diseased patients were performed.

One weakness of this study was that two imaging modalities were used. The resolution and contrast are slightly lower in SLO imaging compared with conventional fundus photography. This might have underestimated the number of patients with progression from level 0 to level 1. The SLO was only used in level 0 and level 1 and did therefore not affect the level of DR in the higher levels of DR.

In further research, it would be of interest to explore whether the intravitreous and plasma levels of MMP-2 and TIMP-2 are associated, and whether plasma MMP-2 and TIMP-2 could be used for therapeutic drug monitoring. It would be of great value if new techniques could be developed for in vivo detection of MMPs and TIMPs locally in the retina.

In conclusion, high plasma levels of MMP-2 and TIMP-2 levels indicated ongoing microvascular complications at baseline but did not differentiate between DR and diabetic nephropathy. No other MMPs or TIMPs included in the study were linked to the severity or the progression of DR. HbA1c was the only variable associated with the progression of DR.

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