

## Influence of digoxin-like immunoreactive factor on late complications in patients with diabetes mellitus

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**Abstract.** The aim of this study was to compare the intensity of typical late complications in diabetic patients ( $n = 65$ , 28 type I, 37 type II) who were not on glycoside drugs with low vs. high serum levels of digoxin-like immunoreactive factor (DLIF: group I,  $n = 42$ ,  $DLIF \leq$  the detection limit of  $0.2 \text{ ng ml}^{-1}$ ; and group II,  $n = 23$ , mean  $\pm$  SEM:  $1.17 \pm 0.31$  [ $0.25\text{--}4.96$ ]  $\text{ng ml}^{-1}$ ). For detection of nephropathy, urinary albumin excretion (24 h) and creatinine clearance tests were used. For coronary heart disease a questionnaire and standard ECG; for peripheral occlusive vascular disease a questionnaire; for eye disease a funduscopy; for neuropathy a neurological score system; and for autonomic neuropathy a standardized test battery was employed. Patients with high DLIF levels showed better test results in vibratory perception ( $95.7 \pm 1.5$  vs.  $82.8 \pm 3.8\%$ , normal finding = 100%,  $2p = 0.016$ ), had better percentile localizations concerning maximal pupillary area in darkness ( $28.4 \pm 6.6$  vs.  $8.1 \pm 1.8\%$ ,  $2p = 0.0004$ ), contraction velocity at 1 s ( $21.5 \pm 5.8$  vs.  $8.0 \pm 2.2\%$ ,  $2p = 0.012$ ), and dilation velocity at 6 s ( $23.0 \pm 6.8$  vs.  $10.5 \pm 2.5\%$ ,  $2p = 0.041$ ), had less retinopathy (with retinopathy:  $26.1\%$  vs.  $64.3\%$ ,  $2p = 0.0028$ ), and better percentile localizations in the respiratory sinus arrhythmia test ( $68.4 \pm 7.3$  vs.  $44.1 \pm 4.9\%$ ,  $2p = 0.0064$ ). There was no difference concerning nephropathy, blood pressure, coronary heart disease and peripheral vascular disease. Separate analysis according to the type of diabetes confirmed the results in each group. This would indicate that DLIF is a possible protective endogenous substance for eye disease, and peripheral (large sensory fibres) and autonomic neuropathy in diabetic subjects independent of the type of diabetes.

**Keywords.** Diabetic cardiovascular disease, diabetic nephropathy, diabetic neuropathy, diabetic retinopathy, digoxin-like immunoreactive factor.

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### Introduction

The sodium-potassium pump is responsible for the gradient of monovalent ions between intracellular and extracellular fluids. Three sodium ions are translocated outwards against two potassium ions inwards per one ATP molecule hydrolysed [1]. This ubiquitous transport system plays a major role in regulating body water content and several cell functions including control of cell volume, excitability and nutrition. Dysfunctions of the pump stem either from intrinsic membrane alterations, action of humoral factors, or both dysfunctions. Endogenous pump inhibitors, the so-called digoxin-like immunoreactive factors (DLIF), have been described in several tissues, such as brain [2–4], kidney [5], adrenal gland [6], and in biological fluids [7–9]. Furthermore, DLIF was found in several physiological and pathophysiological conditions including newborn infants [10], pregnant women [11], hypertensive pregnant women [12], renal failure [13,14], liver disease [15–17], essential hypertension [18–23], diabetes [24–26], obstructive sleep apnoea [27], acromegaly [28] and aneurysmal subarachnoid haemorrhage [29].

With the exception of a recently found endogenous ouabain-like compound [30], the chemical nature of digoxin-like immunoreactive factors is not yet known. Therefore, it is not possible to perform specific RIA assays for DLIF by use of antisera against DLIF, with DLIF as standard. However, it was found that there exists a good correlation between endogenous digoxin-like immunoreactivity measured by digoxin RIA and  $^3\text{H}$ -ouabain displacement on erythrocyte membranes in humans [31]. In accordance with Clerico *et al.* [32], it is thought that digoxin RIA methods can be useful for DLIF determination because they are generally more sensitive, precise and simple than other techniques used to detect endogenous digitalis-like factors. However, immunological methods should be used only as preliminary tests to detect the possible presence of DLIF in human biological fluids.

In this respect it was the aim of the study to compare the intensity of typical late complications in diabetic patients, with and without measurable

serum digoxin, using a standard immunological method. Known interference from drugs or severe disease states have been widely ruled out.

## Patients and methods

### Patients

Sixty-five diabetic outpatients (28 type I, 37 type II) with a mean age of  $47.1 \pm 2.2$  years (14–76 y) were studied after they had given informed consent. The clinical parameters of patients under study are shown in Table 1. None of these 65 patients received cardiac glycosides, spironolactone, prednisone or cortisone which is known to interfere with detection of digoxin immunoreactivity. Because of possible false positive measurements, all the patients were ensured to have near normal levels of serum triglycerides, serum cholesterol, haemoglobin A1c, serum sodium, serum potassium, serum bilirubin, serum protein, serum albumin, haemoglobin, leucocytes, platelets and haematocrit. The groups did not differ statistically significantly in the above-mentioned laboratory parameters. High sodium intake is known to increase the plasma concentration of Na/K-ATPase inhibitor [21]. Therefore, sodium diet might influence the results. Hence, 24 h urinary sodium and potassium excretion have to be similar in the two study groups (Na: group I vs. group II:  $137.4 \pm 11.8$  vs.  $140.7 \pm 14.0$  mmol d<sup>-1</sup>; K:  $53.7 \pm 5.9$  vs.  $61.9 \pm 5.4$  mmol d<sup>-1</sup>). None of the patients had severe pre-terminal renal failure (exclusion of patients with serum creatinine greater than 3.0 mg dl<sup>-1</sup>, serum urea greater than 100 mg dl<sup>-1</sup> and/or nephrotic proteinuria) or hepatic disease or pregnancy, and all had normal serum electrolyte levels. Furthermore, the presence of ventricular dysfunction was widely ruled out and from clinical aspects the patients had mild to moderate congestive heart failure distributed equally in both groups (exclusion of patients with heart failure of more than NYHA functional class II). The patients were

then divided into two groups according to their level of digoxin immunoreactivity. The first group consisted of patients with a DLIF level  $\leq$  the detection limit of 0.2 ng ml<sup>-1</sup> ( $n = 42$ ) and the second group of patients with DLIF above 0.2 ng ml<sup>-1</sup> ( $n = 23$ ). They were not different in clinical parameters (Table 1).

### Patients' specimens

On the day the patients returned their 24 h urine collection (08:00 hours), which they undertook a day before, a urine specimen was taken for the determination of urine creatinine, urine albumin, urine sodium and urine potassium. Furthermore, blood was drawn at the same time for the determination of serum cholesterol, serum triglycerides, haemoglobin A1c, serum creatinine for endogenous creatinine clearance, serum sodium, serum potassium, serum bilirubin, total serum protein, serum albumin, haemoglobin, leucocytes, platelets and haematocrit using standard techniques.

### Measurement of digoxin-like immunoreactive factor

Several studies showed that the TDX system (Abbott GmbH Diagnostika, Wiesbaden, Germany) can measure DLIF, but compared to other immunological techniques the interference was generally less than that seen in commonly used RIAs for digoxin [33, 34]. This may be due to the recommended removal of serum proteins for the use of this technique [35]. The cross-reactivity of the TDX system for cholesterol, cortisol, dehydroepiandrosterone sulfate, estriol, prednisolone, progesterone, 11- $\alpha$ -hydroxyprogesterone, 17- $\alpha$ -hydroxyprogesterone and testosterone was below 1%. The coefficient of variation for this kit was clearly below 8% and the average analytical recovery of digoxin was about 100%. Hence, in order to minimize interferences the TDX system was used in this study.

Before digoxin immunoreactivity measurement by

**Table 1.** Clinical parameters (mean  $\pm$  SEM) of patients under study. Percentages are given in parentheses and ranges in brackets. DLIF, digoxin-like immunoreactive factor

	DLIF $\leq$ the detection limit of 0.20 ng ml <sup>-1</sup>	DLIF above detection limit of 0.20 ng ml <sup>-1</sup>
DLIF [ng ml <sup>-1</sup> ]	$\leq 0.20$ ng ml <sup>-1</sup>	$1.17 \pm 0.31$ [0.25–4.9]
<i>n</i>	42 (64.6%)	23 (35.4%)
Age [y]	$48.8 \pm 2.6$	$43.9 \pm 4.1$
Female	30 (71.4%)	14 (60.9%)
Male	12 (28.6%)	9 (39.1%)
Body mass index [kg m <sup>-2</sup> ]	$25.6 \pm 0.8$	$24.4 \pm 1.1$
Duration [y]	$14.4 \pm 1.5$	$15.8 \pm 2.6$
Type [I/II]	18/24 (42.9%/57.1%)	10/13 (43.5%/56.5%)
Smoker	9 (21.4%)	7 (30.4%)
Daily insulin need [IU] (all types of diabetes)	$28.7 \pm 3.6$	$25.5 \pm 3.9$
Patients under sulphonylureas	11 (26.2%)	6 (26.1%)
Patients under metformin	2 (4.8%)	1 (4.3%)

fluorescence polarization immunoassay (FPIA), serum protein was precipitated using 5-sulfosalicylic acid in equivolume methanol/water in accordance with the manufacturer's recommendations. This mixture was centrifuged at  $9500 \times g$  at  $20^\circ\text{C}$  and digoxin was measured in the resulting supernate. All the digoxin determinations were carried out with the Abbott TDX analyser with original Abbott FPIA reagents. FPIA digoxin immunoreactivity in patients' samples was extrapolated from fluorescence polarization data stored in the TDX, consisting of analytical standards from Abbott: 0.0, 0.5, 1.0, 2.0, 3.0 and  $5.0 \text{ ng ml}^{-1}$ . Standard TDX software compensated for twofold dilution of the sample with 5-sulfosalicylic acid in reporting digoxin results. The detection limit for digoxin recommended by the manufacturer for 5-sulfosalicylic acid precipitation and FPIA method was  $0.2 \text{ ng ml}^{-1}$ . This detection limit is confirmed by others [36].

#### *Evaluation of diabetic late complications*

For the detection of nephropathy, 24 h urinary albumin excretion and endogenous creatinine clearance are used; for coronary heart disease a questionnaire and standard 12-lead electrocardiograms at rest; for peripheral occlusive vascular disease a questionnaire; and for eye disease the evaluation of the fundus by an ophthalmologist were used. Autonomic neuropathy was assessed by a battery of tests including standardized cardiovascular (variation coefficient of R-R intervals, respiratory sinus arrhythmia test = RSA, Valsalva test lying-to-standing test; [37]) and pupillometric investigations (latency time of pupillary light reflex, maximal pupillary area in darkness, contraction velocity at 1 s, dilation velocity at 6 s [38]). Peripheral neuropathy was assessed by measuring test groups as vibratory perception with a tuning fork (left and right: great toe, medial malleolus, medial epicondyle of the femur), temperature perception by means of a cold ( $22^\circ\text{C}$ ) and warm ( $30^\circ\text{C}$ ) metal disc (left and right: back of the foot, tibia, back of the hand, fingers) and reflexes (biceps brachii, triceps brachii, quadriceps femoris, triceps surae). On each of the mentioned three test groups a patient can obtain 100 points which indicates a completely normal function (=100% function). According to an international consensus conference, patients with 24 h urinary albumin excretion of more than  $30 \text{ mg d}^{-1}$  were said to have albuminuria [39].

#### *Statistical analysis*

Multiple group means were compared by ANOVA (SPSS/PC+ Advanced Statistics V4.0, SPSS Inc., Chicago, IN, USA). For autonomic function tests, the exact percentile localization derived from data of earlier studies were used [37,38]. Values are expressed as mean  $\pm$  SEM unless otherwise stated and  $2p=0.05$  is the significant level.

## **Results**

### *Frequency and level of measurable DLIF*

From a total of 28 type I diabetic patients 10 (35.7%) and from 37 type II diabetic patients 13 (35.1%) had a measurable DLIF level (all patients together: 35.4%). The average level in the 10 type I diabetic patients was  $1.1 \pm 0.4 \text{ ng ml}^{-1}$  (0.25–4.6) and  $1.2 \pm 0.4 \text{ ng ml}^{-1}$  (0.25–4.96) in the 13 type II diabetic patients, respectively. The average level in all patients with a measurable DLIF was  $1.2 \pm 0.3 \text{ ng ml}^{-1}$ .

### *Diabetic late complications*

Table 2 summarizes the data of patients with an undetectable DLIF level and those with a measurable DLIF level. There was no difference in endogenous creatinine clearance, 24 h urinary albumin excretion, frequency of peripheral occlusive vascular disease (pOVD) and coronary heart disease (CHD) between the two groups. Furthermore, the two groups were not different in systolic and diastolic blood pressure but a trend towards higher values in the group with low DLIF level seemed to exist. Retinopathy was clearly more frequent in patients with a low DLIF level ( $2p=0.003$ ). Beyond that, pupillometric parameters as maximal pupillary area ( $2p=0.0004$ ), contraction velocity at 1 s ( $2p=0.012$ ), dilation velocity at 6 s ( $2p=0.041$ ) and the result of the cardiorespiratory RSA test ( $2p=0.006$ ) were much better in patients with a measurable DLIF than in those with an undetectable level. The clinical neurological tests revealed a better test result in sensory large fibre function (vibration perception) in patients with high DLIF levels ( $2p=0.016$ ). In all the neurological tests (autonomic and peripheral) where no significant difference could be found, a trend towards better test results in diabetic patients with a high DLIF level seemed to exist. This is also true for the 24 h urinary albumin excretion, which differed nearly statistically significantly in type II diabetic patients (patients with a 24 h urinary albumin excretion of more than  $30 \text{ mg d}^{-1}$ : group I vs. II: 73.9% vs. 41.7%,  $2p=0.067$ ).

Figure 1 shows the results of fundoscopy and vibration perception testing of type I and type II diabetic patients with a high and low DLIF level. Type I diabetic patients with high DLIF levels had significantly less retinopathy than patients with low DLIF levels (30.0% vs. 70.6%,  $2p=0.042$ ). This could also be confirmed in type II diabetic patients (23.1% vs. 62.5%,  $2p=0.022$ ). Considering vibratory perception, type II diabetic patients with high DLIF level had better sensory large fibre function than patients with low DLIF (94.5 vs. 78.6%,  $2p=0.036$ ). This was not statistically significant in the smaller group of type I diabetic patients but there was a similar trend.

With regard to autonomic neuropathy, the maximal pupillary area was higher in type I and type II

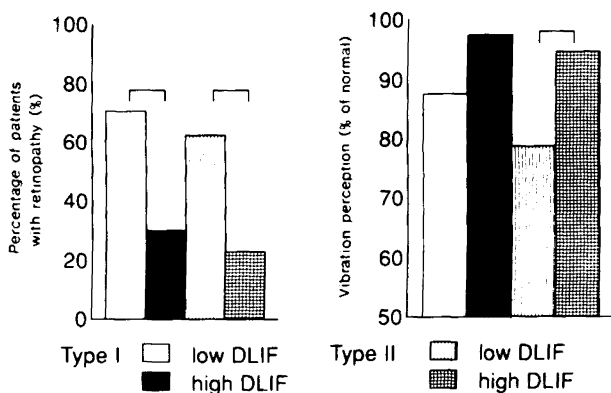
**Table 2.** Parameters (mean  $\pm$  SEM) of diabetic late complications of patients under study. Percentages are shown in parentheses. DLIF, digoxin-like immunoreactive factor; pOVD, peripheral occlusive vascular disease; CHD, coronary heart disease; RSA, respiratory sinus arrhythmia; Pe, percentile localization in [%]

	DLIF $\leq$ the detection limit of 0.20 ng ml <sup>-1</sup>	DLIF above detection limit of 0.20 ng ml <sup>-1</sup>	2P
<b>Kidney disease</b>			
Patients with a creatinine clearance of less than 70 ml min <sup>-1</sup>	21.9%	13.0%	0.389
Patients with a 24 h urinary albumin excretion of more than 30 mg d <sup>-1</sup>	65.0%	45.5%	0.140
<b>Cardiovascular disease</b>			
Systolic blood pressure [mmHg]	138.0 $\pm$ 3.5	132.6 $\pm$ 4.0	0.341
Diastolic blood pressure [mmHg]	80.3 $\pm$ 1.9	77.2 $\pm$ 2.3	0.316
Patients with a systolic blood pressure of more than 140 mmHg	38.1%	30.4%	0.554
Patients with a diastolic blood pressure of more than 90 mmHg	11.9%	4.4%	0.322
With pOVD	7 (16.7%)	5 (21.7%)	0.621
With CHD	10 (23.8%)	6 (26.1%)	0.842
<b>Eye disease</b>			
With retinopathy (all stages)	27 (64.3%)	6 (26.1%)	0.003
<b>Autonomic neuropathy</b>			
<i>Pupillary</i>			
Pe. in latency time	24.0 $\pm$ 4.3	21.1 $\pm$ 4.3	0.665
Pe. in maximal pupillary area	8.1 $\pm$ 1.8	28.4 $\pm$ 6.6	0.0004
Pe. in contraction velocity at 1 s	8.0 $\pm$ 2.2	21.5 $\pm$ 5.8	0.012
Pe. in dilation velocity at 6 s	10.5 $\pm$ 2.5	23.0 $\pm$ 6.8	0.041
<i>Cardiorespiratory</i>			
Pe. in RSA test	44.1 $\pm$ 4.9	68.4 $\pm$ 7.3	0.006
Pe. in var. coeff. of R-R intervals	33.8 $\pm$ 5.1	44.8 $\pm$ 6.5	0.196
Pe. in Valsalva test	27.6 $\pm$ 4.1	30.5 $\pm$ 5.9	0.676
Pe. in Ewing test	20.9 $\pm$ 4.4	28.4 $\pm$ 6.2	0.324
<b>Neuropathy</b>			
Vibratory perception [%]	82.8 $\pm$ 3.8	95.7 $\pm$ 1.5	0.016
Reflex status [%]	78.3 $\pm$ 3.2	80.4 $\pm$ 5.1	0.713
Temperature perception [%]	83.8 $\pm$ 2.7	89.4 $\pm$ 4.1	0.240

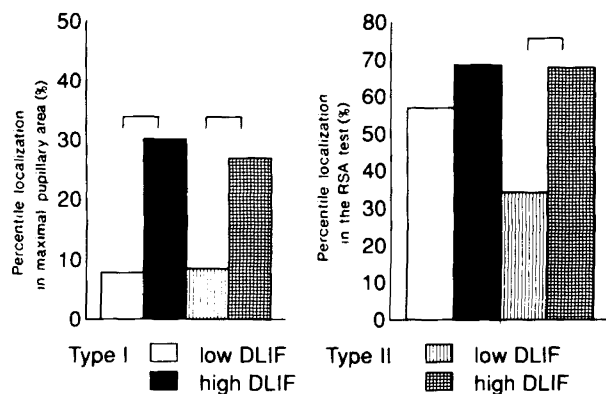
diabetic patients with high compared to low DLIF levels (type I: 30.1 vs. 7.8% percentile,  $2p = 0.031$  and type II: 27.0 vs. 8.5% percentile,  $2p = 0.007$ , Fig. 2). This could be confirmed in type II diabetic patients considering the test results in RSA test (high DLIF: 68.1 vs. low DLIF: 35.7% percentile,  $2p = 0.007$ ).

## Discussion

65 type I and type II diabetic patients were screened for digoxin-like immunoreactive factor. About 35% of all patients showed a measurable DLIF level with an average of 1.2 ng ml<sup>-1</sup>. It was shown that patients with high DLIF levels in serum had less severe late complications compared to those with a low DLIF



**Figure 1.** Results in patients with high and low levels of digoxin-like immunoreactive factor (DLIF) in type I and type II diabetic patients. (Left) Percentage of type I and type II diabetic patients with retinopathy. (Right) Result in the vibratory perception test in type I and type II diabetic patients. According to the definition, a finding of 100% is normal and a finding below 80% is neuropathic.



**Figure 2.** Results in patients with high and low levels of digoxin-like immunoreactive factor (DLIF) in type I and type II diabetic patients. (Left) Average percentile localization of maximal pupillary area in darkness. (Right) Average percentile localization in the autonomic function test of respiratory sinus arrhythmia.

serum level, especially regarding retinopathy and autonomic and peripheral neuropathy. This could also be confirmed when dividing the total group in type I and type II diabetic subgroups. Retinopathy, peripheral and autonomic neuropathy seem to be the only complications linked with the DLIF levels. However, the correlation between nephropathy and DLIF seems to exist at least in form of a trend. Moreover, if coronary heart disease and peripheral occlusive vascular disease were assessed with the same accurate techniques, which were not available, compared to the assessment of neuropathy, retinopathy and nephropathy a difference between the two groups might be demonstrable concerning the two late complications. To our knowledge, this is the first study that compares late complication in diabetic patients with respect to different DLIF serum levels. There have been several reports showing that DLIF was high in patients with essential hypertension [18–23] and one group had shown that type II diabetic patients with a family history of hypertension had higher DLIF levels [25]. It has been shown that patients with acromegaly and chronic volume expansion have decreased levels of DLIF and plasma volume after pituitary adenectomy [28]. Furthermore, pregnant women and their newborn infants have high DLIF levels which decrease rapidly after birth [10,11]. This may be also due to volume expansion in the perinatal period. From animal experiments it is known that volume expansion and salt-loading increases DLIF levels [4,7,8]. This could also be confirmed in humans [40]. From this point of view, DLIF levels seem to be increased secondarily in salt-loaded and volume-expanded subjects and probably not vice versa. Nevertheless, it should be mentioned that there are some studies which argue against the volume expansion hypothesis. It is said that DLIF lead to exaggerated vascular smooth muscle contraction and to an elevation of peripheral vascular resistance due to an increase in cytosolic free calcium concentration [41]. Furthermore, reducing the sodium pump activity may exaggerate neural stimulation and norepinephrine overflow [42] which, together with increased vascular resistance, might explain, at least in part, hypertension. But it is still a matter of debate whether DLIF is primarily high in some subjects then perhaps leading to hypertension, or secondarily as a consequence of volume expansion to counteract hypertension.

In diabetes mellitus there are some important factors for volume expansion which are increased insulin level, increased growth hormone level, increased sympathetic activity and high water intake because of high blood glucose levels [2,8,20,30,43–52]. Moreover, it is possible that inadequately high cortisol production rates as found in obese subjects [53] may contribute to volume expansion in type II diabetic patients, too. In later disease states, severe diabetic nephropathy also leads to volume expansion, but this appeared to be of no importance in this study.

These mechanisms have to be counteracted by a feedback control system that protects the subject from hypertension. The antagonistic factors leading to volume contraction are inhibition of the secretion of vasopressin, renin and aldosterone, stimulation of the secretion of atrial natriuretic peptide and digoxin-like immunoreactive factor(s), and local renal prostaglandin (E1, E2, I2) mechanisms [2,20,30,44,52].

In conclusion, and under consideration of the recent report showing that DLIF may be in part endogenous ouabain [30], lack of DLIF secretion in diabetic patients may be a factor leading to alterations in regulation of body water content and several cell functions including control of cell volume, excitability and nutrition due to intracellular and extracellular volume expansion. This in fact may lead to a diffusion barrier for vital nutrients in susceptible bradytrophic tissues such as retina and nerve. Hence, diabetic patients with low DLIF of unknown origin may be more susceptible for late complications due to volume expansion and lowered tissue perfusion. The somewhat speculative question arises as to whether administration of low doses of digoxin or ouabain may be protective in diabetic patients. Before administration of such drugs in a controlled prospective study, a larger cross-sectional survey would have to confirm our findings.

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