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# Disturbed zinc homeostasis in diabetic patients by *in vitro* and *in vivo* analysis of insulinomimetic activity of zinc

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**Abstract**

Disturbances of zinc homeostasis have been observed in several diseases, including diabetes mellitus. To further characterize the association between zinc and diabetes, we recruited 75 patients with type 1 or type 2 diabetes and 75 nondiabetic sex-/age-matched control subjects in order to analyze differences concerning human zinc transporter 8 (hZnT-8) expression, single nucleotide polymorphisms (SNPs) in the genes of hZnT-8 as well as metallothionein 1A and serum/intracellular zinc. Furthermore, we investigated the relation between insulin and zinc homeostasis in type 2 diabetic subjects and consolidated our results by *in vitro* analysis of the effect of insulin on cellular zinc status and by analysis of the modulation of insulin signal transduction by intracellular zinc homeostasis. Concerning the expression of hZnT-8 and the SNPs analyzed, we did not observe any differences between diabetic and control subjects. Serum zinc was significantly lower in diabetic patients compared to controls, and intracellular zinc showed the same tendency. Interestingly, type 2 diabetes patients treated with insulin displayed lower serum zinc compared to those not injecting insulin. *In vitro* analyses showed that insulin leads to an increase in intracellular zinc and that insulin signaling was enhanced by elevated intracellular zinc concentrations. In conclusion, we show that type 1 and type 2 diabetic patients suffer from zinc deficiency, and our results indicate that zinc supplementation may qualify as a potential treatment adjunct in type 2 diabetes by promoting insulin signaling, especially in zinc-deficient subjects.


*Keywords:* Zinc; Diabetes mellitus; Zinc transporter; Single nucleotide polymorphism; Insulin signaling; Metallothionein

## 1. Introduction

Zinc is an essential trace element necessary for the function of more than 300 enzymes belonging to all enzyme classes. It plays an essential role in gene expression, cell proliferation and signal transduction [1–3]. Although the human body contains 2–4 g of zinc, only 12–16 μmol/L is present in plasma as a mobile zinc pool [4]. Considering the low plasma concentration of zinc and its importance for enzymatic function, it is not surprising that distribution and availability of zinc, especially free intracellular zinc, are tightly regulated by metal binding proteins such as metallothionein (MT) and by zinc transport proteins [5–8].

Two families of zinc transporters have been described so far. Import of zinc into the cytosol from either extracellular space or intracellular vesicles is mediated through the Zrt-/Irt-like protein (ZIP) or solute carrier (SLC) 39 family, which comprises 14 members (ZIP1–ZIP14). The zinc transporter (ZnT)/SLC30 family is responsible for zinc export from the cytosol into intracellular compartments like vesicles or the endoplasmatic reticulum on the one hand and extracellular space on the other hand. Ten ZnT members have been identified until today (ZnT-1–ZnT-10) [9].

Reduced serum zinc levels can be observed in numerous medical conditions such as diarrhea, rheumatoid arthritis, sickle cell anemia, cancer, impaired function of the immune system and diabetes mellitus [4,10–13]. The interaction between zinc homeostasis and diabetes has been thoroughly examined including the insulinomimetic effect of zinc on insulin signaling. Zinc has been shown to increase phosphorylation of the insulin receptor and to influence phosphoinositide-3-kinase, phosphoinositide-dependent kinase 1, Akt/protein kinase B and glycogen synthase kinase 3, which are part of the insulin signaling pathway [13]. However, effects of insulin on zinc homeostasis have only rarely been investigated [14].

Diabetes mellitus is characterized by chronic elevation of blood glucose concentrations, leading to a variety of potential metabolic, vascular and other complications. While type 1 diabetes is caused by immune-mediated destruction of insulin-producing beta cells, insulin resistance of target tissues and decreased beta cell function are

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considered the hallmarks of type 2 [15–18]. Due to its rising prevalence in recent years, diabetes mellitus has become a leading cause of morbidity and mortality in the Western population, resulting in significant reduction on life expectancy of affected diabetic individuals [19].

Studies analyzing zinc homeostasis in diabetes mellitus most consistently show that urinary zinc excretion is increased in diabetic patients, indicating loss of zinc [20–23]. The observations regarding plasma/serum zinc are contradictory since zinc in diabetic patients has been described to be decreased [24–26], normal [23,27] or increased [22,28], whereas the increased levels of serum/plasma zinc were detected in type 1 diabetic patients. Zinc in mononuclear cells, granulocytes, lymphocytes and leucocytes in general is reported to be lower in diabetic patients compared to controls [24,25,29]. Ecto 5′ nucleotidase activity, a sensitive marker of zinc deficiency [30], was shown to be reduced in women suffering from non-insulin-dependent diabetes compared to age-matched controls [31].

As possible functional index of zinc status, cytokine secretion by immune cells has been examined, which has been shown to be influenced by availability of zinc [32,33]. Comparably, a high-zinc diet given to diabetic rats attenuated the significantly lower lymphocyte proliferation in rats fed normal or low-zinc diets compared to controls [34]. This indicates that zinc status is reflected by the function of the immune system.

ZnT-8, a zinc exporter removing zinc from the cytoplasm, has been shown to colocalize with insulin in secretory granules of INS-1 cells, a model of β-cells, and in human islet cells [35,36]. In order to ensure proper storage and maturation of insulin in its secretory vesicles, zinc is needed for structural stability of the storage form of insulin, a solid hexamer requiring two zinc ions that tends to crystallize [37,38]. Considering localization and function of the ZnT-8 transporter as well as the finding that ZnT-8 is necessary for insulin crystallization [39,40], there might be a possible link to insulin secretion and diabetes mellitus worth being investigated.

This notion is supported by the finding that ZnT-8-overexpressing cells, compared to control cells, displayed enhanced insulin secretion as response to a high-glucose stimulus [36]. Accordingly, deletion of the mouse SLC30A8 gene encoding the ZnT-8 transporter and down-regulation of ZnT-8 expression in INS-1 cells led to impaired insulin secretion stimulated by glucose [41,42]. By now, ZnT-8 expression could also be detected in human peripheral blood mononuclear cells showing great variation in expression [7], in adipose cells [43], in cubical epithelium lining thyroid follicles and adrenal cortex cells [44] as well as in pancreatic alpha cells [40].

Within the last 2 years, several genome-wide association studies have identified a single nucleotide polymorphism (SNP) in the ZnT-8 gene SLC30A8, rs13266634 [C/T transition; arginine (Arg) (325) → tryptophan (Trp) (325)], which shows a clear association with diabetes mellitus type 2 [45–48]. Furthermore, the risk variant seems to be associated with reduced insulin secretion [49–51]. However, ZnT-8 does not only play a role in type 2 diabetes; it also represents a pancreatic autoantigen in type 1 diabetes leading to the production of anti-ZnT-8 antibodies [17].

Moreover, two polymorphisms in the gene of the zinc binding protein MT1A [MT1A 647, rs11640851, A/C transition, aspartate (Asp) → threonine (Thr); MT1A 1245, rs8052394, A/G transition, lysine (Lys) → arginine (Arg)] could recently be shown to be associated with type 2 diabetes, further supporting the role of zinc in diabetes mellitus [52,53].

In this study, the association between diabetes and the mRNA level of human ZnT-8 (hZnT-8) in human leukocytes is examined as well as the association between diabetes and the SNPs in SLC30A8 and the gene of MT1A, taking serum zinc as well as intracellular zinc in human lymphocytes into consideration. In order to further consolidate our results, the influence of insulin on intracellular zinc status is analyzed *in vitro* as well as the effect of insulin and zinc on insulin signaling using Akt and phospho-Akt (p-Akt) as indicators.

## 2. Methods and materials

### 2.1. Study design

A total of 156 subjects were recruited at RWTH Aachen University Hospital. Of those, six subjects were excluded: Clinical parameters were missing for two subjects, three subjects met an exclusion criterion which they reported after blood collection, and one subject had secondary diabetes due to growth hormone excess (acromegaly). The final study sample consisted of 150 subjects comprising 75 diabetic patients (22 type 1 diabetic patients and 53 type 2 diabetic patients) and 75 sex- and age-matched control subjects aged 22 to 82 years (overview Suppl. Tables 1–4). Inclusion criteria were age >18 years for diabetic as well as control subjects and type 1 or type 2 diabetes regarding diabetic subjects. Exclusion criteria comprised acute bacterial and viral infection, surgery within the last 3 months, cancer, liver disease/known clear elevation of liver enzymes and renal insufficiency. In female subjects, blood samples were not collected during menstruation because of changes concerning the number of lymphocytes observed during this period of the menstrual cycle [54]. Further exclusion criteria applying to control subjects were regular intake of acetylsalicylic acid/phenprocoumon and any type of diabetes mellitus.

In order to further characterize the participating individuals, we recorded body mass index (BMI) for all subjects as well as type of diabetes, duration of diabetes and insulin treatment for diabetic patients.

All subjects gave written informed consent to participate in this study. The ethical committee of the Medical Faculty at RWTH Aachen University approved the study protocol (EK089/08).

### 2.2. Blood collection

For analysis of hZnT-8 expression and hZnT-8-/MT1A SNPs, peripheral venous blood was collected in tubes containing ethylenediaminetetraacetic acid (EDTA) as anticoagulant (S-Monovette, Sarstedt, Nümbrecht, Germany). Sampling of blood for measurement of intracellular zinc was performed using lithium–heparin tubes without gel (S-Monovette, Sarstedt) in order to prevent chelation of zinc by EDTA.

Serum Monovettes (S-Monovette, Sarstedt) with or without gel allowed preparation of serum. After completion of the clotting process at room temperature (at least 30 min), centrifugation at 2000*g* for 10 min and serum decantation were carried out as soon as possible. Serum was stored at −20°C until analyzed. Fasting before blood collection was not required. Plasma zinc does not seem to be relevantly influenced by a nutrient-balanced meal [55].

### 2.3. Reagents and cell culture conditions

Zinc sulfate (Sigma-Aldrich, Steinheim, Germany) was stored as a stock solution of 100 mM, which was obtained by dissolving zinc sulfate in sterile water and subsequent sterile filtration. In order to reduce the concentration to 2 mM, as required for our experiments, the stock was further diluted in sterile water.

*N*,*N*,*N*′,*N*′-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN) (Sigma-Aldrich) was dissolved in sterile water to obtain a solution of 2 mM, which was ready for use after sterile filtration.

Sodium pyrithione (Sigma-Aldrich) was diluted in sterile water to obtain a stock solution of 50 mM. For experiments, this stock solution was further diluted to 5 mM in sterile water.

FluoZin-3 AM ester (Invitrogen, Karlsruhe, Germany; 50 μg) was dissolved in dimethyl sulfoxide, resulting in a stock solution of 1 mM, which was ready for use.

Human insulin (Sigma-Aldrich) was dissolved in RPMI 1640 medium (Cambrex, Verviers, Belgium) and stored as a stock solution of 1 mg/ml. This stock solution was then further diluted with RPMI 1640 medium to receive the desired concentrations. All other chemicals were from standard sources and of analytical quality.

HuT-78 cells, a T-cell lymphoma cell line, were obtained from DSMZ GmbH (Braunschweig, Germany) and cultured in a humidified 5% $CO_2$ atmosphere at 37°C in RPMI 1640 medium (Cambrex) supplemented with 10% heat-inactivated fetal calf serum (FCS; PAA Laboratories, Linz, Austria), 100 U/ml penicillin and 100 μg/ml streptomycin (all obtained from Cambrex).

### 2.4. Relative quantification of the hZnT-8 transporter

Total mRNA was extracted from whole blood using the QIAamp RNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Transcription of the isolated RNA into cDNA was performed using the Reverse Transcription System (Promega, Madison, WI, USA) according to the manufacturer's instructions.

A sequence detection system (ABI Prism 7000, Applied Biosystems, Foster City, CA, USA) was used for quantification of the hZnT-8 transporter mRNA levels. The oligonucleotide primers are located in adjacent exons; thus, amplification of genomic DNA can be excluded. These primers and the FAM/TAMRA-labeled TaqMan probes were described previously [7]. Additionally, mRNA of porphobilinogen deaminase

(PBGD) as a housekeeping gene was quantified. The sequences of the PBGD primers and the probe have previously been reported [56].

Standard curves were established for the hZnT-8 transporter as well as the housekeeping gene PBGD, and equations based on these standard curves were used to calculate the initial mRNA levels from the measured cycle threshold value.

In order to relatively quantify the results, the initial mRNA levels of the hZnT-8 transporter were normalized to, which means divided by, the initial mRNA levels of PBGD, hence used as endogenous reference. This allows the comparison of results obtained with probes from different individuals with possibly different amounts of total mRNA.

Each polymerase chain reaction (PCR) (50 μl) was performed using the following components: 24 μl deionized water ($dH_2O$), 5 μl PCR buffer (Qiagen), 5 μl of magnesium chloride (25 mM Qiagen), 1 μl dNTPs (10 mM; Applied Biosystems), 0.75 μl forward and reverse primer each (20 μM; Tib Molbiol, Berlin, Germany), 0.5 μl ROX (100 μM; Tib Molbiol), 0.5 μl probe (20 μM; Tib Molbiol), 0.5 μl HotStarTaq Polymerase (5 U/μl, Qiagen) and 12 μl of the respective cDNA or $dH_2O$ as negative control. As positive control, mRNA extracted from Jurkat cells (DSMZ GmbH), a T lymphocyte cell line, was reverse transcribed and amplified in every experiment. After HotStarTaq activation (15 min at 95°C), amplification was carried out running 45 cycles, each consisting of two steps, denaturing (15 s at 95°C) and annealing/extending (60 s at 60°C). All assays were run in triplicate.

### 2.5. Analysis of the hZnT-8 SNP (rs132666634)

Total DNA was extracted using the QIAamp DNA Blood Mini Kit (Qiagen) according to the manufacturer's instructions. The SNP PCR was carried out using the GeneAmp PCR System 2700 (Applied Biosystems). The ZnT-8 reverse primers chosen (ZnT-8_reverseC: 5′-gAACCACTTggCTgTCCCg-3′; ZnT-8_reverseT: 5′-gAACCACTTggCTgTCCCA-3′) contain a mismatch at their 3′- end. The sequence of the ZnT-8_forward primer is 5′-AgTTCCCATAgCgACAgggC-3′. Human growth hormone (HGH), a housekeeping gene, served as control within this multiplex PCR in order to detect PCR failures. The HGH primer sequences are as follows: HGH1, 5′-TTCCCAACCATTCCCTTATCCAgg-3′; HGH2, 5′-TCCACTCACggATTTCTgTTgTgTTTC-3′.

For each PCR, two Master Mixes were pipetted, one containing the ZnT-8_reverseC-primer and one containing the ZnT-8_reverseT-primer. The following reagents were employed for each assay: 36.75 μl/27.75 μl $dH_2O$, 5 μl PCR buffer (Qiagen), 2 μl dNTPs (10 mM; Applied Biosystems), 1 μl ZnT-8_forward-primer, 1 μl ZnT-8_reverseC- and 1 μl ZnT-8_reverseT-primer, respectively (20 μM; Tib Molbiol), 0.5 μl HGH_forward-primer, 0.5 μl HGH_reverse-primer, 0.25 μl HotStarTaq Polymerase (5 U/μl) and 6 μl/12 μl of the respective DNA (100 ng/200 ng) or $dH_2O$ as a negative control. After activation of the HotStarTaq Polymerase for 15 min at 95°C, each of the 30 cycles consisted of three steps, denaturing (30 s at 95°C), annealing (30 s at 64°C) and elongation (30 s at 72°C). For determination of genotypes, PCR products were analyzed by agarose gel electrophoresis and subsequent ethidium bromide staining.

### 2.6. Analysis of the MT1A SNP (rs11640851, rs8052394)

Two SNPs were screened and found in dbSNP database (http://www.ncbi.nlm.nih.gov/SNP/) (PubMed Reference rs11640851, rs8052394) corresponding, respectively, to an A/C transition at +647 nt position and to an A/G transition at +1245 nt position in the coding region of human MT1A gene. The first SNP is characterized by an Asp27Thr amino acid substitution, while the second one is characterized by a Lys51Arg amino acid change. We performed a PCR restriction fragment length analysis using the following primers: +647 A/C MT1A: sense 50-ACTTGGCTCAGCCCCAGATT-30, antisense 50-CACTCAGCTGGCAGCATTTG-30; +1245 A/G MT1A: sense 0-ATCTCCCATCTCCGACAGTG-30, antisense 50-AAAGAAAACCAGAGCCAGCA-30.

Genomic DNA from peripheral blood leukocytes was amplified with the primers described above. Conditions of amplification were previously reported [52].

### 2.7. Determination of zinc

Serum zinc was determined from hemolysis-free serum by atomic absorption spectroscopy (Varian SpectrAA 220 FS, reference values: 11–23 μmol/L).

For analysis of intracellular zinc concentration in lymphocytes, we applied the procedure previously published by Haase et al. with some modifications [57]. Briefly, after loading 500 μl of whole human blood with 10 μM of the cell-permeable FluoZin-3 AM ester, the assay was incubated at 37°C in a 5% $CO_2$-enriched atmosphere for 30 min. Subsequently, 100 μl of blood was distributed into each of three FACS tubes and was then stimulated with (a) 200 μM TPEN to obtain a minimal zinc concentration and (b) 1 mM $ZnSO_4$ and 50 μM sodium pyrithione to obtain a maximal zinc concentration, and (c) the third FACS tube remained without stimulant.

After another incubation step at 37°C in a 5% $CO_2$-enriched atmosphere for 30 min, 2 ml of 1:10 diluted BD FACS Lysing Solution (Biosciences, San Jose, CA, USA) was added to each FACS tube which were then incubated at room temperature for 10 min in the dark. Then, the cells were pelleted and washed with 2 ml RPMI 1640 medium (Cambrex) supplemented with 10% heat-inactivated FCS (PAA Laboratories), 100 U/ml penicillin, 100 μg/ml streptomycin and 1% (2 mM) L-glutamine (all obtained from Cambrex). For measurement of fluorescence, the pelleted cells were dissolved in 300 μl of the supplemented RPMI 1640 medium described above.

Fluorescence was detected after incubation for 30 min at 4°C using a flow cytometric analyzer (FACScan, Becton Dickinson, Heidelberg, Germany). Since the cells of every subject were gated using identical gates, precise gating of granulocytes was impossible. Therefore, we decided to include only lymphocytes in our analysis, which could be gated satisfactorily. Intracellular zinc concentration was calculated as previously described [57].

### 2.8. Influence of insulin on free intracellular zinc measured by fluorescence multiwell plate reader

For this experiment, HuT-78 cells were used. One million cells per milliliter were incubated with 1 μM FluoZin-3 AM ester in a buffer containing 5 mM glucose (Merck, Darmstadt, Germany), 1 mM magnesium chloride (Merck), 1 mM sodium dihydrogen phosphate (Sigma-Aldrich), 1.3 mM calcium chloride (Merck), 15 mM HEPES (Merck), 120 mM sodium chloride (Merck), 5.4 mM potassium chloride (Merck) and 20% FCS (pH 7.35) (PAA Laboratories) at 37°C in a shaking water bath for 30 min. After pelleting, cell density was adjusted to $2\times10^6$ cells/ml in the buffer described above. Then, 100 μl of cells per well was distributed into a 96-well plate, and three wells were stimulated with zinc (100 μM) plus sodium pyrithione (50 μM) or TPEN (50 μM), respectively, in order to obtain a maximal and a minimal zinc concentration. After 15 min of incubation at 37°C in a 5% $CO_2$-enriched atmosphere, fluorescence was detected with a TECAN 340 fluorescence multiwell plate reader (Tecan, Crailsheim, Germany) every 2 min for 10 min at 37°C. The remaining wells were then stimulated with insulin (10 μg/ml; 5 μg/ml) and boiled insulin (10 μg/ml; 5 μg/ml), respectively. Unstimulated wells served as negative control. All assays were run in triplicate. Fluorescence was detected every 2 min over a period of 1 h at 37°C. An excitation wavelength of 485 nm and an emission wavelength of 535 nm were used. Intracellular zinc concentration was calculated as previously described [57]. We used the fluorescence detected after 16 min in order to compare the intracellular zinc concentrations induced by the different stimulants since, after this time, a plateau was reached.

### 2.9. Influence of TPEN preincubation on intracellular zinc measured by fluorescence multiwell plate reader

Hut-78 cells ($1\times10^5$/ml) were incubated in medium supplemented with 1 μM TPEN for 72 h; cells cultured in regular medium served as control. Then, cells were pelleted, resuspended in regular medium and incubated at 37°C in a 5% $CO_2$-enriched atmosphere for a further 1 h. One million cells per milliliter were incubated with 25 μM Zinquin ethyl ester (Alexis Biochemicals, San Diego, CA, USA) in a buffer containing 5 mM glucose (Merck), 1 mM magnesium chloride (Merck), 1 mM sodium dihydrogen phosphate (Sigma-Aldrich), 1.3 mM calcium chloride (Merck), 15 mM HEPES (Merck), 120 mM sodium chloride (Merck), 5.4 mM potassium chloride (Merck) and 20% FCS (pH 7.35) at 37°C in a shaking water bath for 30 min. Then, 100 μl of cells per well was distributed into a 96-well plate and stimulated with zinc (100 μM) plus sodium pyrithione (50 μM) or TPEN (50 μM) in order to obtain a maximal and a minimal zinc concentration; the remaining wells were left untreated. All assays were run in triplicate. After 15 min of incubation at 37°C, fluorescence was detected with a TECAN 340 fluorescence multiwell plate reader every 2 min at 37°C. Excitation and emission wavelengths were 340 nm and 480 nm, respectively. We used the fluorescence detected after 20 min in order to compare the intracellular zinc concentrations induced by the different stimulants. The fluorescence obtained by TPEN stimulation was subtracted from the fluorescence measured in unstimulated wells.

### 2.10. Immunoblotting

As described above, Hut-78 cells ($1\times10^5$/ml) were incubated in medium supplemented with 1 μM TPEN for 72 h; cells cultured in regular medium served as control. After pelleting, cells were resuspended in regular medium without FCS, adjusted to a concentration of $1\times10^6$ cells/ml and incubated at 37°C in a 5% $CO_2$-enriched atmosphere for 3 h. For stimulation, cells were adjusted to a concentration of $2\times10^6$/ml without changing medium and stimulated with boiled insulin (10 μg/ml), insulin (5 μg/ml, 10 μg/ml), zinc (30 μM) and zinc (30 μM) plus insulin (5 μg/ml, 10 μg/ml) for 15 min. Regarding stimulation with zinc plus insulin, cells were preincubated with zinc at 37°C for 15 min before insulin was added. Then, HuT-78 cells were lysed using buffer consisting of 62.5 mM Tris-HCL (pH 6.8), 10% glycerol, 2% (w/v) sodium dodecyl sulphate, 0.01% (w/v) bromophenol blue, 0.1% (v/v) 2-mercaptoethanol and 1 mM sodium orthovanadate, sonicated for 10 s and boiled at 95°C for 5 min. An equivalent of $3\times10^5$ cells per lane was electrophoretically separated on 10% polyacrylamide gels and blotted onto nitrocellulose membrane (BioRad, München, Germany). Staining with Ponceau S (Sigma-Aldrich) ensured equal protein loading of gels. Subsequently, membranes were blocked with 5% fat-free dry milk in TBS-T [20 mM Tris-HCL (pH 7.6), 136 mM NaCl, 0.1% (v/v) Tween 20] for more than 1 h and incubated with primary antibodies directed against p-Akt and total Akt, respectively (1:1000) (all from Cell Signaling Technology, Frankfurt, Germany), at 4°C, gently shaking for at least 3 h. Membranes were then washed three times with TBS-T for 5 min and incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG secondary antibody (1:2000) and HRP-coupled anti-biotin antibody (1:1000) for detection of biotin-labeled MW standard for more than

1 h followed by detection with LumiGlo Reagent (Cell Signaling Technology) on a LAS-3000 (Fujifilm Lifescience, Düsseldorf, Germany). Density of bands was semiquantified using Adobe Photoshop Elements (Version 1.0).

### 2.11. Statistical methods

Since not all parameters measured are available for every subject, we analyzed four sex- and age-matched populations, one for each of the following parameters: SNPs (hZnT-8 and MT1A), hZnT-8-expression, serum zinc and intracellular zinc in lymphocytes (overview Suppl. Tables 1–4).

Statistical analysis was performed using SPSS 17.0. Significance of differences was calculated by paired (for matched samples) and unpaired Student's $t$ test for intracellular zinc and serum zinc as well as by nonparametric tests such as Wilcoxon signed ranks test (for matched samples) and Mann–Whitney test for hZnT-8-expression due to the lack of a normal distribution. For comparison of serum zinc of differently treated type 2 diabetic subjects and controls, which implicates a comparison of more than two independent groups, one-way analysis of variance (ANOVA) was performed using Dunnett T3 test for post hoc analysis since homogeneity of variances was not given. Genotype distribution of SNPs was analyzed by $\chi^2$ test. Analysis of variance in order to adjust for covariates was performed using the general linear model including Bonferroni adjustment applying repeated measures for matched samples. For *in vitro* analysis of intracellular zinc or the fluorescence of Zinquin in HuT-78 cells, paired Student's $t$ test was applied. Quantified results of immunoblotting were also analyzed by one-way ANOVA (Dunnett T3); comparison of the effect of each stimulant on cells preincubated in medium supplemented with TPEN and those preincubated in regular medium was performed by paired Student's $t$ test. $P$ values <.05 were considered statistically significant.

## 3. Results

### 3.1. Analysis of differences between diabetic patients and controls regarding hZnT-8 expression

In order to examine the association between hZnT-8 expression and diabetes mellitus, we compared expression profiles of diabetic patients and healthy controls. Expression was highly variable interindividually (range of relative fold expression 0.000–88.959).

There is no apparent difference in the level of hZnT-8 expression between diabetic patients and matched controls ($n$=148, $P$=.511), type 2 diabetic patients and matched controls ($n$=104, $P$=.443) or type 1 diabetic patients and matched controls ($n$=44, $P$=.935). Differences of hZnT-8 expression between male diabetic patients and male controls as well as between female diabetic patients and female controls remain insignificant (males: $n$=74, $P$=.765; females: $n$=74, $P$=.460). Similar results are obtained when the two types of diabetes are analyzed separately for differences between male/female diabetic individuals and their matched controls (type 2 diabetes — males: $n$=52, $P$=.778, females: $n$=52, $P$=.397; type 1 diabetes — males: $n$=22, $P$=.929, females: $n$=22, $P$=.929).

Different levels of hZnT-8 expression cannot be observed when comparing male diabetic patients with female diabetic patients ($n$=74, $P$=.113), male type 2 diabetic with female type 2 diabetic patients ($n$=52, $P$=.288), male type 1 diabetic patients with female type 1 diabetic patients ($n$=22, $P$=.158) or male control subjects with female control subjects ($n$=74, $P$=.245).

### 3.2. Analysis of the association between diabetes and the following SNPs: rs13266634 (hZnT-8), rs11640851 (MT1A), rs8052394 (MT1A)

Since SNPs in the genes of MT1A and hZnT-8, genes that are involved in zinc homeostasis, have been shown to be associated with type 2 diabetes [45–48,52], we analyzed those SNPs in our study samples ($n$=150; type 1 diabetic patients and controls, $n$=44; type 2 diabetic patients and controls, $n$=106). However, none of these SNPs show a significant difference concerning distribution of genotypes between diabetic type 1 and/or type 2 patients and control subjects (data not shown).

### 3.3. Serum zinc

We determined serum zinc as indicator of zinc deficiency in order to analyze differences concerning diabetic patients and controls. Serum zinc is significantly lower in diabetic patients compared to sex- and age-matched controls. When type 2 and type 1 diabetic patients are considered separately, in both groups, serum zinc concentration is significantly decreased compared to controls (Table 1).

This finding does not seem to be dependent on sex since, in male as well as in female diabetic patients, serum zinc was significantly lower compared to their corresponding controls. A similar result is obtained for male and female type 2 diabetic patients compared to matched controls, but regarding patients suffering from type 1 diabetes, the differences are not significant (Table 1). Deficiency of serum zinc (<11 μmol/L) was only observed in diabetic patients (10 type 2 diabetic patients: five males and five females; one female type 1 diabetic patient) and not in control subjects (Table 1). Comparison of male with female subjects does not display any significant difference (Table 1).

Table 1
Serum zinc

| | Type 1 and type 2 diabetes | | | Type 1 diabetes | | | Type 2 diabetes | | |
|---|---|---|---|---|---|---|---|---|---|
| | Patients | Controls | $P$ value | Patients | Controls | $P$ value | Patients | Controls | $P$ value |
| Males and females (mean±S.D.) | 11.81±2.18 ($n$=42) | 13.88±1.53 ($n$=42) | <.001[a] | 11.75±1.49 ($n$=8) | 13.50±1.60 ($n$=8) | .026[a] | 11.82±2.33 ($n$=34) | 13.97±1.53 ($n$=34) | <.001[a] |
| Males (mean±S.D.) | 11.76±2.00 ($n$=21) | 14.00±1.27 ($n$=21) | <.001[a] | 11.50±0.58 ($n$=4) | 14.00±1.83 ($n$=4) | .080 | 11.82±2.22 ($n$=17) | 14.00±1.17 ($n$=17) | .003[a] |
| Females (mean±S.D.) | 11.86±2.39 ($n$=21) | 13.76±1.79 ($n$=21) | .005[a] | 12.00±2.16 ($n$=4) | 13.00±1.41 ($n$=4) | .252 | 11.82±2.51 ($n$=17) | 13.94±1.85 ($n$=17) | .009[a] |
| $P$ value males vs. females | .889 | .621 | – | .670 | N/A | – | 1.00 | N/A | – |
| Adjusted for the difference in BMI between diabetics and controls (BMI diabetics−BMI controls) | | | | | | | | | |
| Males and females (mean±S.E.)[b] | 11.81±0.34 | 13.88±0.24 | <.001[a] | 11.75±0.35 | 13.50±0.57 | .031[a] | 11.82±0.41 | 13.97±0.26 | <.001[a] |
| Males (mean±S.E.)[b] | 11.76±0.42 | 14.00±0.27 | <.001[a] | 11.50±0.33 | 14.00±0.95 | .115 | 11.82±0.51 | 14.00±0.28 | .002[a] |
| Females (mean±S.E.)[b] | 11.86±0.53 | 13.76±0.39 | .006[a] | 12.00±0.49 | 13.00±0.74 | .125 | 11.82±0.63 | 13.94±0.46 | .012[a] |
| Adjusted for age and BMI (and duration of diabetes in patients) | | | | | | | | | |
| Males (mean±S.E.)[b] | 11.78±0.53 | 14.01±0.35 | N/A | 11.57±0.63 | N/A | N/A | 11.72±0.68 | N/A | N/A |
| Females (mean±S.E.)[b] | 11.84±0.53 | 13.75±0.35 | N/A | 11.93±0.63 | N/A | N/A | 11.92±0.68 | N/A | N/A |
| $P$ value males vs. females | .931 | .613 | N/A | .719 | N/A | N/A | .852 | N/A | N/A |

Values of serum zinc in μmol/L; $n$: number of subjects; N/A: not applicable.
[a] Statistically significant.
[b] Only standard error available for adjusted values.

### 3.4. Intracellular zinc in lymphocytes

In addition to serum zinc, which might not be suitable to detect subclinical zinc deficiency [58], we determined intracellular zinc in lymphocytes as a parameter of the body's zinc status. Intracellular zinc shows a tendency towards lower values in diabetic patients of both types compared to matched controls, but these differences do not reach statistical significance (Table 2).

Divided by sex, the tendency of lower intracellular zinc in diabetic patients is only present in males. In females, intracellular zinc in diabetic patients is even slightly higher compared to that in controls. None of these differences are significant (Table 2).

There are no significant differences concerning intracellular zinc in males versus females. Intracellular zinc is slightly higher in female type 2 diabetic patients compared to males, whereas in type 1 diabetic patients, male subjects show a tendency of higher intracellular zinc. After adjustment for age, BMI and duration of diabetes, the difference between intracellular zinc of type 2 diabetic males and females becomes significant, with females showing higher intracellular zinc (Table 2).

### 3.5. Influence of insulin therapy on zinc parameters in vivo

To investigate the potential influence of insulin on the zinc status of diabetic patients, we compared levels of serum zinc and intracellular zinc between type 2 diabetic patients with and without insulin treatment. Interestingly, those individuals treated with insulin show significantly lower serum zinc compared to type 2 diabetic patients not receiving insulin therapy ($P$=.027). Controls matched to type 2 diabetic patients display higher serum zinc compared to either group of type 2 diabetic subjects, although only the difference between controls and insulin-treated type 2 diabetic patients is significant ($P$<.001; controls vs. type 2 diabetes without insulin $P$=.112) (Fig. 1). Similar significant differences are not observed regarding intracellular zinc in lymphocytes, although the same tendency is displayed (one-way ANOVA $P$=.743). Consequently, post hoc testing was not performed.

### 3.6. The effect of insulin therapy on zinc parameters in vitro

To further consolidate our findings concerning the influence of insulin on zinc homeostasis, we examined the effect of insulin on cellular zinc status *in vitro*. Intracellular zinc in Hut-78 cells significantly increased after stimulation with 5 μg/ml insulin ($P$=.002) as well as with 10 μg/ml insulin ($P$=.003). In order to account for the fact that insulin contains zinc, we also stimulated the cells with boiled insulin, thereby inactivating insulin and leaving zinc as an active component. Boiled insulin (5 μg/ml, 10 μg/ml) did not lead to a significant increase in intracellular zinc in any of the concentrations used ($P$=.114; $P$=.084) (Fig. 2).

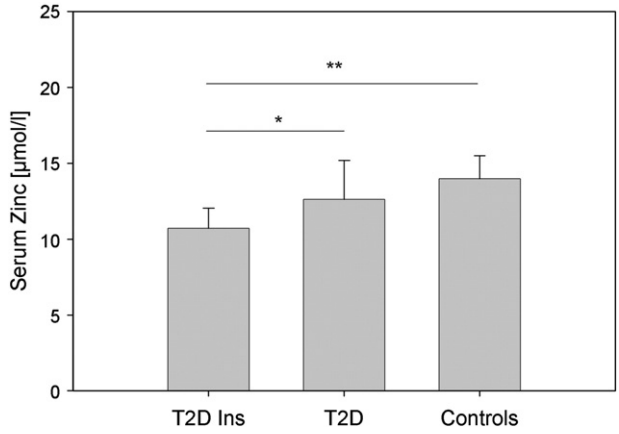


Fig. 1. Serum zinc in type 2 diabetic patients according to insulin therapy and in controls. Serum zinc was determined from hemolysis-free serum by atomic absorption spectroscopy. Bars represent mean±standard deviation (S.D.). Insulin-treated type 2 diabetic patients (T2D Ins): $n$=14, type 2 diabetic patients not treated with insulin (T2D): $n$=20, controls matched to type 2 diabetic patients (Controls): $n$=34. *$P$<.05; **$P$<.01.

### 3.7. Impact of zinc depletion on intracellular zinc signals

As diabetic patients are mostly found to be zinc deficient [24–26], we investigated the impact of zinc depletion on cellular zinc homeostasis *in vitro*. Cells preincubated with the zinc chelator TPEN showed significantly higher intracellular zinc after resuspension in regular medium compared to control cells ($P$=.038). This indicates an increased zinc uptake or increased release of bound zinc by cells deprived of zinc (Fig. 3).

Table 2
Intracellular zinc in lymphocytes

| | Type 1 and type 2 diabetes | | | Type 1 diabetes | | | Type 2 diabetes | | |
|---|---|---|---|---|---|---|---|---|---|
| | Patients | Controls | $P$ value | Patients | Controls | $P$ value | Patients | Controls | $P$ value |
| Males and females (mean±S.D.) | 3.09±1.43 ($n$=39) | 3.57±1.65 ($n$=39) | .206 | 3.10±1.18 ($n$=13) | 3.45±1.17 ($n$=13) | .456 | 3.08±1.56 ($n$=26) | 3.63±1.86 ($n$=26) | .301 |
| Males (mean±S.D.) | 2.76±1.09 ($n$=18) | 3.97±2.26 ($n$=18) | .064 | 3.29±1.21 ($n$=6) | 4.39±0.96 ($n$=6) | .183 | 2.50±0.97 ($n$=12) | 3.76±2.71 ($n$=12) | .173 |
| Females (mean±S.D.) | 3.37±1.63 ($n$=21) | 3.22±0.75 ($n$=21) | .712 | 2.95±1.22 ($n$=7) | 2.63±0.55 ($n$=7) | .524 | 3.59±1.81 ($n$=14) | 3.51±0.66 ($n$=14) | .900 |
| $P$ value males vs. females | .184 | .192 | – | .626 | N/A | – | .075 | N/A | – |
| Adjusted for the difference in BMI between diabetics and controls (BMI diabetics−BMI controls) | | | | | | | | | |
| Males and females (mean±S.E.)[b] | 3.09±0.23 | 3.57±0.27 | .212 | 3.10±0.25 | 3.45±0.33 | .449 | 3.08±0.31 | 3.63±0.37 | .310 |
| Males (mean±S.E.)[b] | 2.76±0.26 | 3.97±0.54 | .066 | 3.29±0.52 | 4.39±0.40 | .240 | 2.50±0.29 | 3.76±0.80 | .179 |
| Females (mean±S.E.)[b] | 3.37±0.36 | 3.22±0.12 | .696 | 2.95±0.24 | 2.63±0.22 | .405 | 3.59±0.46 | 3.51±0.15 | .887 |
| Adjusted for age and BMI (and duration of diabetes in patients) | | | | | | | | | |
| Males (mean±S.E.)[b] | 2.70±0.36 | 3.95±0.39 | N/A | 3.39±0.53 | N/A | N/A | 2.05±0.44 | N/A | N/A |
| Females (mean±S.E.)[b] | 3.43±0.33 | 3.23±0.36 | N/A | 2.86±0.49 | N/A | N/A | 3.97±0.40 | N/A | N/A |
| $P$ value males vs. females | .156 | .182 | N/A | .511 | N/A | N/A | .007[a] | N/A | N/A |

Values of intracellular zinc in nmol/L; $n$: number of subjects; N/A: not applicable.
[a] Statistically significant.
[b] Only standard error available for adjusted values.

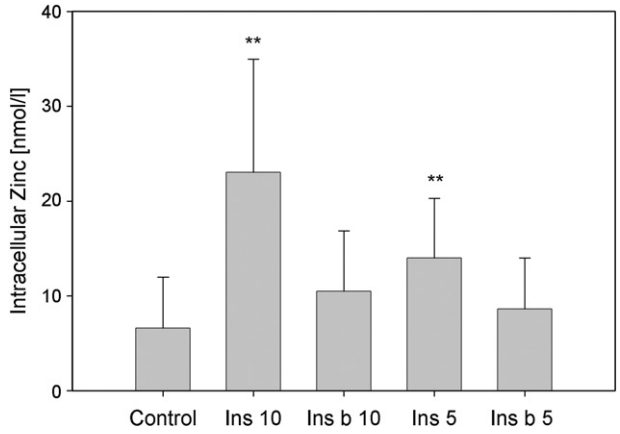


Fig. 2. Intracellular zinc in HuT-78 cells after stimulation. FluoZin 3-AM ester was used to selectively detect free intracellular zinc by a fluorescence multiwell plate reader. After determining a minimal and a maximal zinc concentration by adding zinc plus pyrithione and TPEN, respectively, HuT-78 cells were stimulated with 5 μg/ml and 10 μg/ml insulin (Ins) as well as 5 μg/ml and 10 μg/ml boiled (inactivated) insulin (Ins b). Bars represent mean±S.D., $n$=8. **$P$<.01 compared to control.

### 3.8. Effect of zinc and insulin on insulin signal transduction in cells cultured in zinc-depleted and zinc-sufficient medium

We further investigated the effect of insulin and zinc on zinc-deficient and zinc-sufficient cells *in vitro*, thereby aiming to imitate the influence of insulin and zinc on zinc-deficient cells in type 2 diabetic patients. Hut-78 cells were preincubated in regular medium or in medium containing TPEN and stimulated as indicated in Fig. 4. Then they were lysed, and p-Akt as indicator of insulin signal transduction was semiquantified after immunoblotting. Cells preincubated in regular medium showed a significantly higher amount of p-Akt/Akt when stimulated with 5 μg/ml insulin ($P$=.034), 10 μg/ml insulin ($P$=.009), zinc plus 5 μg/ml insulin ($P$=.031) and zinc plus 10 μg/ml insulin ($P$=.011) compared to control cells just stimulated with medium. The same differences were observed when the stimulants enumerated above were compared to boiled insulin ($P$=.049, $P$=.011, $P$=.038, $P$=.012). Stimulation with zinc resulted in significantly lower phosphorylation of Akt compared to 5 μg/ml insulin ($P$=.040), 10 μg/ml insulin ($P$=.011) and zinc plus both insulin concentrations ($P$=.034, $P$=.012).

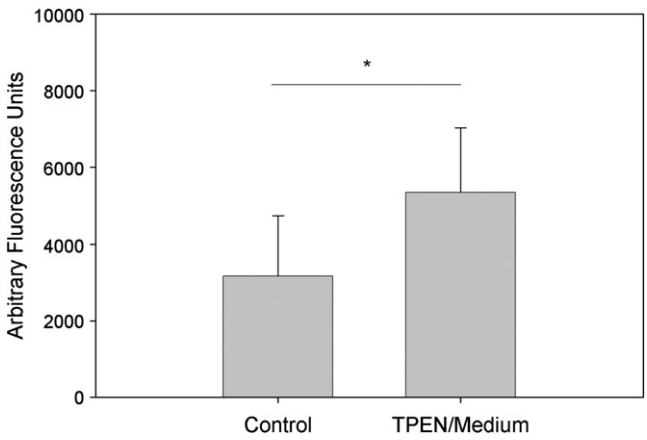


Fig. 3. Effect of preincubation with TPEN on intracellular zinc. The association between intracellular zinc of HuT-78 cells cultured in regular medium (Control) and those preincubated in medium containing the zinc chelator TPEN (TPEN/Medium) was estimated using Zinquin to selectively detect zinc. The resulting fluorescence was measured by a fluorescence multiwell plate reader. Bars represent mean±S.D., $n$=5. *$P$<.05.

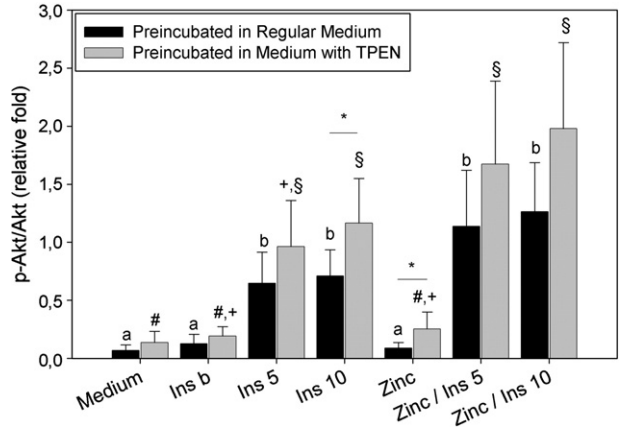


Fig. 4. Influence of zinc and insulin on insulin signaling (p-Akt/Akt) in Hut-78 cells preincubated in medium +/− TPEN. Cells were stimulated with boiled (inactivated) insulin (Ins b; 10 μg/ml), insulin (Ins; 5 μg/ml, 10 μg/ml), zinc (30 μM) and zinc (30 μM) plus insulin (5 μg/ml, 10 μg/ml) for 15 min. After immunoblotting, the resulting bands indicating the protein amount of p-Akt and Akt were semiquantified. Values are displayed as p-Akt/Akt. Bars represent mean±S.D., $n$=6. (a, b) Means of p-Akt/Akt of cells preincubated in regular medium differ if they lack a common symbol. (#, +, §, ~) Means of p-Akt/Akt of cells preincubated in medium containing TPEN differ if they lack a common symbol. Significant differences between cells preincubated in medium without TPEN and those preincubated in TPEN-containing medium stimulated with the same stimulant are indicated by *; *$P$<.05.

Hut-78 cells that were preincubated with TPEN showed a similar pattern. Stimulation with 5 μg/ml insulin ($P$=.036), 10 μg/ml insulin ($P$=.011) and zinc plus both of these insulin concentrations ($P$=.034, $P$=.018) led to greater Akt phosphorylation compared to control cells treated with medium. Similarly, p-Akt/Akt was significantly higher after stimulation with 10 μg/ml insulin ($P$=.016), zinc plus 5 μg/ml insulin ($P$=.041) and zinc plus 10 μg/ml insulin ($P$=.021) compared to boiled insulin. Zinc as stimulant resulted in significantly lower Akt phosphorylation compared to 10 μg/ml insulin ($P$=.017), zinc plus 5 μg/ml insulin ($P$=.046) and zinc plus 10 μg/ml insulin ($P$=.023).

In both assays — preincubation in medium supplemented with TPEN or in regular medium — a tendency towards promotion of insulin signaling by addition of zinc was observed, although differences are not significant (regular medium: 5 μg/ml insulin vs. 5 μg/ml insulin+zinc $P$=.545, 10 μg/ml insulin vs. 10 μg/ml insulin+zinc $P$=.264; medium with TPEN: 5 μg/ml insulin vs. 5 μg/ml insulin+zinc $P$=.566, 10 μg/ml insulin vs. 10 μg/ml insulin+zinc $P$=.434).

Preincubation in medium with TPEN instead of regular medium resulted in overall higher Akt phosphorylation, although this was only significant for cells stimulated with zinc ($P$=.010) and 10 μg/ml insulin ($P$=.029) (Figs. 4 and 5).

## 4. Discussion

Disturbances of zinc homeostasis have been observed in many medical conditions, such as infectious diseases, cancer and diabetes mellitus [11–13].

The zinc transporter hZnT-8 seems to be potentially involved in the development and/or progression of diabetes mellitus as can be assumed from its localization in insulin secreting pancreatic vesicles [35], from the SNP within its gene predisposing to type 2 diabetes mellitus [46–48] and from its role as an autoantigen in type 1 diabetes mellitus [17]. We suggested that the level of hZnT-8 expression might play a role in diabetes mellitus insofar as low hZnT-8 expression might lead to decreased accumulation of zinc and subsequently to reduced insulin storage and secretion, finally resulting in diabetes.

Since biopsies of human pancreas pose an ethical problem, we determined the expression of hZnT-8 in leukocytes [35]. Our results

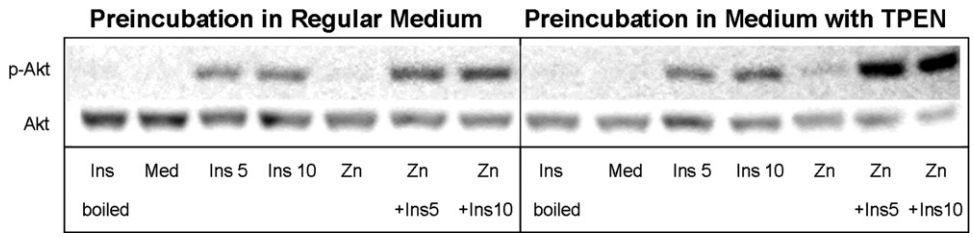


Fig. 5. Influence of zinc and insulin on insulin signaling (p-Akt/Akt) in Hut-78 cells preincubated in medium +/− TPEN. Cells were stimulated with boiled (inactivated) insulin (10 μg/ml), insulin (5 μg/ml, 10 μg/ml), zinc (30 μM) and zinc (30 μM) plus insulin (5 μg/ml, 10 μg/ml) for 15 min. Then they were lysed; proteins were electrophoretically separated and blotted onto nitrocellulose membrane. One representative example out of six immunoblots is shown.

do not show any significant differences between type 1 and/or type 2 diabetic patients compared to matched controls. This is partly due to high interindividual variability. In diabetic mice, the expression of ZnT-8 was shown to be down-regulated during the early stages of diabetes, supporting our initial thesis, but being opposed to our results [59]. Possible explanations for these contradictory results might be differences in species including differences in nutrition and genetic background, as well as the fact that we did not measure ZnT-8 expression in pancreatic cells, but in leukocytes.

Recently, it could be shown that down-regulation of ZnT-8 in INS-1 cells leads to reduced zinc uptake and reduced insulin secretion induced by a hyperglycemic stimulus [41]. However, mice lacking the ZnT-8 transporter did not consistently show an impairment in glucose clearance despite disturbed zinc accumulation in pancreatic islets [40,42] and lower plasma insulin levels [42]. In mice with β-cell specific knockout of ZnT-8, first-phase glucose-stimulated insulin secretion was found to be reduced *in vitro*. Although those mice were shown to be glucose intolerant, the levels of fasting glucose as well as plasma insulin during oral glucose challenge were similar between knockout mice and control mice [60]. Despite partly contradictory results of these studies, which cannot be extensively discussed here [40,42,60], it can be assumed from the findings discussed above that ZnT-8 influences glucose metabolism and thus development or progression of diabetes. The effect of ZnT-8 seems to be rather modest, though, with possible compensation for the missing transporter.

Consistently, in ZnT-8 knockout mice, glucose tolerance and beta cell function could be shown to be normal under standard laboratory conditions, but a high-fat diet as metabolic stressor can lead to failure of beta cells and deterioration of glucose homeostasis [39]. Similarly, Nicolson et al. observed that a high-fat diet given to ZnT-8 knockout mice leads to greater gain in body weight compared to controls and to disturbances of glucose homeostasis. Additionally, sex and age seem to modulate the impact of the lack of ZnT-8 [40]. This underlines the interaction between genetic predisposition and environment.

The rather modest effect of hZnT-8 is further emphasized by genome-wide association studies that were in need of great study populations in order to show a significant association between the SNP rs13266634 and type 2 diabetes [45–48]. We also determined the SNP in SLC30A8 (rs13266634) as well as two SNPs in the MT1A gene (MT1A 647, rs11640851; MT1A 1245, rs8052394) which have been found to be associated with type 2 diabetes [52,53]. We did not observe an association between any of these SNPs and diabetes, possibly due to our relatively small sample size.

Taken together, modification of expression or function of the hZnT-8 transporter seems to be one of multiple risk factors modestly contributing to diabetes mellitus.

Previous studies examining type 1 and type 2 diabetic patients have reported reduced serum/plasma zinc compared to healthy controls [24–26], but in type 1 diabetic subjects, normal or elevated serum zinc concentrations could also be observed [22,23,28]. In our study, a significant reduction of serum zinc in type 2 diabetic patients and type 1 diabetic subjects, analyzed separately or combined, can be observed in comparison to healthy controls. This stands in contrast to the studies showing elevated or normal serum zinc in type 1 diabetic patients which might be due to differences concerning study populations: the studies cited, for instance, included generally younger patients, did not match for sex and age and compared unequal numbers of patients and controls [22,23,28].

In addition to serum zinc, which is not suitable for detection of slight zinc deficiencies [58], we analyzed intracellular zinc in lymphocytes.

In line with previous studies reporting decreased intracellular zinc in leukocytes of diabetic patients [24,29], we see a consistent but insignificant reduction of intracellular zinc concentration in lymphocytes of type 1 and type 2 diabetic individuals in comparison to matched controls.

These results are partly opposed to the results of other groups who could show that changes of intracellular zinc in response to zinc depletion are observed earlier than changes in plasma zinc [30,61]. Thus, intracellular zinc seems to be a more sensitive indicator of zinc deficiency than plasma/serum zinc, and one would expect a significant instead of insignificant difference concerning intracellular zinc considering the significant difference in serum zinc in our study.

These inconsistencies might be due to the methods of zinc measurement used. Prasad et al. determined intracellular zinc by atomic absorption spectrophotometry after isolation of leukocyte subsets [61]. We did not isolate lymphocytes but lysed erythrocytes, which might influence the amount of intracellular zinc measured by FACS analysis. However, Williams et al. observed significantly reduced plasma zinc in diabetic patients combined with insignificantly reduced zinc in mononuclear cells/granulocytes determined by atomic absorption spectrometry. These results are consistent with our findings despite differences in methods [25].

A further explanation might be up-regulation of zinc transporters as a response to zinc depletion, leading to increased zinc uptake in order to maintain intracellular zinc homeostasis [7]. Thus, the relation between intracellular and serum/plasma zinc might change over time.

The most surprising result, which to our knowledge has not been described so far, is the association between insulin therapy and serum zinc concentration. Since the majority of commercially available insulin suspensions contain zinc, one would rather expect an increase in serum zinc due to insulin administration [62]. However, among type 2 diabetic patients, who do not necessarily require insulin therapy, a significant decrease in serum zinc can be observed in patients receiving insulin compared to those not injecting insulin.

There are at least two possible explanations. First, the low serum zinc in type 2 diabetic patients treated with insulin could be due to severity of disease since insulin is usually administered when lifestyle modifications combined with oral antidiabetic therapy are not sufficient to maintain glycemic control. Another explanation for low serum zinc in type 2 diabetic subjects receiving insulin therapy could be a direct effect of insulin on serum zinc especially since, in type 2

diabetes, high concentrations of insulin are required because of the characteristic insulin resistance [18].

Using HuT-78 cells, we could show such a direct effect of insulin on intracellular zinc homeostasis *in vitro*: Insulin led to a significant increase in free intracellular zinc. Whether insulin functions as a carrier or zinc transporters are involved in this process requires further research. Although the origin of this zinc still has to be determined, it might enter cells from extracellular space, thereby reducing zinc in extracellular fluid. This could possibly explain our finding that insulin-treated type 2 diabetic patients show reduced serum zinc compared to type 2 diabetic patients not receiving insulin. The observation that free intracellular zinc tends to be lower in lymphocytes from type 2 diabetic patients receiving insulin compared to type 2 diabetic subjects not treated with insulin is not necessarily contradictory since regulation of free intracellular zinc might be cell type specific as indicated by Haase et al. [14]. Zinc might also be bound to proteins, thus not being detected by our fluorescence probe.

The finding that zinc seems to enhance insulin-induced phosphorylation of Akt, a component of the insulin signaling pathway, indicates that zinc could help to ameliorate the disturbed response to insulin in type 2 diabetic patients. Akt phosphorylation induced by zinc has been shown to involve activation of insulin-like growth factor-1 receptor tyrosine kinase as well as inhibition of serine/-threonine protein phosphatases [63].

Since we could show that a short-time zinc deficiency leads to increased uptake of supplemented zinc into cells, thus promoting insulin signaling, the entity of these findings might allow the following conclusion: In patients with type 2 diabetes, the lower serum zinc level of insulin-treated patients could be caused by insulin therapy shifting zinc into cells. Moreover, this low serum zinc, since it indicates a state of zinc deficiency, could trigger increased uptake of supplemented zinc into the probably also relatively zinc-deficient cells, thus ameliorating insulin signaling and compensating for insulin resistance in type 2 diabetes. Consequently, zinc supplementation might improve glucose homeostasis in those patients by providing more zinc to insulin-responsive cells.

To further address the benefit of zinc supplementation in diabetes, a therapeutical trial of zinc supplementation would be helpful. The dosage of zinc leading to improvement of disease will have to be determined by monitoring parameters of glycemic control, zinc status and eventually further functional parameters of zinc homeostasis such as cytokine secretion [64,65].

In conclusion, our results indicate that the expression profile of hZnT-8 is at most weakly associated with diabetes mellitus in humans. We confirm that type 1 and type 2 diabetic patients suffer from zinc deficiency, supporting the idea of possible beneficial effects of zinc supplementation which could especially be effective in supporting type 2 diabetes therapy by promoting insulin signaling.

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jnutbio.2011.09.008.

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