The aspects of hyperglycaemia during the perinatal period and childhood

Hyperglycaemia during the perinatal period

Foetal type lymphocytes in type 1 diabetes mellitus during childhood

Ph.D. Thesis

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I. Introduction

The importance of hyperglycaemia is increasing nowadays because of its high prevalence and elevated incidence of complications on short and long term basis. In the past decade, besides internalists, paediatricians and neonatologists meet more and more frequently with transient or permanent disturbances of glucose homeostasis.

During childhood hyperglycemia is mostly associated with Type 1 diabetes mellitus (T1DM), an organ specific autoimmune disease with the permanent disturbance of glucose homeostasis. Due to the inflammatory conditions in the first, asymptomatic phase of the disease the B-cells are permanently damaged. Several lines of evidences suggest the role of genetic, environmental factors and T lymphocytes in the pathogenesis of T1DM. Most of our knowledge is generated from studies investigating the antigen-specific elements of the immune-response, while limited data are available on the role of non-conventional lymphocytes - such as γδ T, CD5+ B and NK T cells- in the development of the autoimmune illness. These subsets of the lymphocytes form a bridge between inherited and acquired immunity, and play a crucial role role in the maintenance of the immune-tolerance and regulation of the immune-response. Since T1DM is characterized with complex disturbance of the immune-regulation both γδ T and CD5+ B lymphocyte subtypes may play a role in the pathogenesis of T1DM.

Less attention is paid to hyperglycemia as a temporary condition during childhood or neonatal period; however, its incidence is growing among severely ill children and newborn infants. Moreover, the duration of hyperglycemia is closely related to survival rate and hospital stay. In critically sick adult patients the strict control of glucose homeostasis resulted in better outcome emphasizing the importance of euglycemia.

As a result of the improving survival rate of extremely low gestational age infants the number of extremely low birth weight infants among NICU patients increased dramatically representing a new challenge for neonatologists. Until the seventies the majority of the neonatal handbooks referred only transient neonatal diabetes, as a neonatal disease with hyperglycemia. However, Dweck et al. observed an increasing incidence of hyperglycemia among preterm infants already in 1974. It is interesting to note that the prevalence depends on the definition and measurement used and the late complications have not been revealed. Hyperglycemia itself and the applied insulin therapy as well might influence hormonal and immunological changes during the neonatal period, which may affect the glucose homeostasis

and body composition in the adulthood, thus the impact of neonatal hyperglycaemia projects beyond the neonatal period.

II. Aims

II/1. Investigation of glucose-homeostasis and neonatal hyperglycaemia in preterm neonates during the perinatal period

II/1.1 Currently limited data are available concerning incidence rate, main risk factors and consequences of hyperglycaemia in preterm neonates, particularly in our country. We aimed to

- investigate the incidence rate of neonatal hyperglycaemia in the subsets of our preterm neonates,
- reveal the main risk factors of hyperglycaemia, and
- analyze the potential complications associated with elevated blood glucose level.

II/1.2. Despite of intensive research into aetiologies of retinopathy of prematurity (ROP), there have been no new interventions that have been resulted in decline in the incidence of ROP since the identification of oxygen as an offending pathogen. In 2003 *Garg et al* demonstrated that hyperglycaemia is associated with the development of severe ROP in extremely low birth weight infants (ELBWI). Confirmation of this novel observation and establishment of pathogenetic role of hyperglycaemia could improve prevention of retinopathy in preterm neonates. The purposes of our examination were to

- evaluate the association between hyperglycaemia and development of ROP at any stage in very low birth weight infants (VLBWI),
- analyze further risk factors of retinopathy of prematurity,
- investigate, whether the risk factors of ROP vary in different birth weight categories of the preterm babies.

II/1.3. In diabetes mellitus several glycaemic markers are used to monitor glucose homeostasis. During the perinatal period limited relevant data are available regarding glycaemic markers, including fructosamine. Serum fructosamine level correlates directly with average glucose concentration during the preceding 3 weeks. Our aims were to

- reveal, whether serum fructosamine during the perinatal period provide a new information about glucose homeostasis of the preterm and term neonates as well as of the foetus,
- compare the serum fructosamine levels of the normoglycaemic and hyperglycaemic preterm infants,
- answer the question, whether serum fructosamine is a suitable marker to predict the development of hyperglycaemia-related morbidities in preterm neonates.

II./1.4. Leptin is known to fulfil the role of an adipostat, a sensor and regulator of body adiposity in adults. Its level is lower in preterm neonates as compared to term infants, besides, its concentration increasing with advancing age. Theoretically, blood glucose may regulate

leptin-secretion, thus hyperglycaemia during the early postnatal period may have long term effect on the body composition and on the development of obesity, as well as type 2 diabetes mellitus. We made an attempt to confirm the first part of this hypothesis experimentally. Our purposes were to

- investigate the leptin-secretion of isolated rat adipocytes in response to the various concentration of glucose ,
- study influence of energy-producing substrates on the leptin-secretion of isolated fat cells,
- reveal, whether the insulin has additional effect on the energy producing substrate-mediated leptin secretion *in vitro,*
- investigate *in vitro* the stimulus-secretion coupling mechanism of the glucose+insulin induced leptin-secretion, and to determine if intracellular calcium do play any role in the process.

II/.2. Investigation of circulating γδ T and CD5+ B lymphocytes in T1DM during childhood

Several data support that foetal type lymphocytes -γδ T and CD5+ B cells- play crucial role in the maintenance of immune-tolerance, however, their involvement in the pathogenesis of T1DM has not been established. We

II/2.1.

- investigated the peripheral proportion of the CD5+ B and γδ T lymphocytes in newly diagnosed T1DM children and in diabetic patients' ICA positive non-diabetic 1st degree relatives ("prediabetics") in comparison to healthy control subjects.

II/2.2.

- studied quantitative alteration of particular γδ T lymphocyte subsets that were determined on the basis of T cell receptor (TCR) chain usage and cytokine production of the cells. We also investigated, whether could be detected any association between the relative proportion of circulating γδ T lymphocyte subsets and the clinical parameters of T1DM patients.

III. Material and methods

Ad II/1.1 Retrospective study was conducted with participation of 99 ELBW preterm neonates and 115 preterm neonates with birth between 1000-1500 g (gestational age (GA): 26.6±2.3 vs. 29.8±2.0 weeks, birth weight (BW): 803±158 vs. 1260±171 g [mean±S.D.]), who were born and treated between January 1, 2000 and December 31, 2001 at the Department of Obstetrics and Gynaecology, University of Pécs, Medical School. Incidence

rate, potential risk factors and consequences of the hyperglycaemia were studied. We recorded the Clinical Risk Factor for Babies (CRIB) score – attributed as a marker of illness severity-, the presence of intraventricular haemorrhage (IVH) and sepsis, the rate of glucose infusion, medication (steroid, xantin) and insulin treatment. Hyperglycaemia was defined if blood glucose level was repeatedly 8.5 mM or higher. Glucose values consisted of both bedside whole blood glucose and laboratory serum glucose determination. Unless condition of the patient required otherwise, glucose measurements were scheduled routinely at least every 8-12 h during the first week, or when receiving parenteral nutrition, or being under medication which may influence glucose homeostasis, later on at least twice a week. Parenteral aminoacid was introduced on the 2-3rd day of life, enteral feeding was started on the 2-4th day. Insulin treatment was applied (0.01-0.1U/kg/dosi or 0.01-0.1U/kg/h) if blood glucose level was repeatedly higher than 10 mM in spite of reduction of the rate of glucose infusion. T student test and χ^2 test were applied during the statistical analysis. Additionally, we also determined incidence rate of the hyperglycaemia analyzing data of 89 VLBWI (58 moderately- and 22 extremely-preterm infants) prospectively, who were born between January 1, 2006 and December 31, 2006 at the same department. During the later study period there were some alterations in the general care of the neonates, the definition of the hyperglycaemia was basically unchanged (see *ad II/1.3. chapter).*

Ad II/1.2. A retrospective case-control study was conducted of all VLBWI born at the Department of Obstetrics and Gynaecology, University of Pécs, Medical School between January 1, 2000 and December 31, 2002. We analyzed the data of 201 neonates who survived the neonatal period (GA: 29.1±2.6, BW: 1143±241 g [mean±S.D.]). The relationship between ROP at any stage and occurrence of hyperglycaemia, as well as GA, BW, Apgar score at 1 and 5 minutes, CRIB score was investigated. Ophthalmoscopic screening was performed on a weekly basis from the 4th postnatal week, and the findings were classified according to the International Classification of ROP. χ^2 test and logistic regression analysis were performed to investigate association between the variables. Mann Whitney U test was applied to compare continuous variables. ROP as a response variable was dichotomized into two groups (no ROP and ROP at any stage) and hyperglycaemia was included as a categorical variable taking two values (above or below 8.5 mM).. We divided the group of the VLBWI into two subgroups (ELBWI and infants with birth weight 1000-1499 g.: n: 63 vs. 138, GA: 26.7±2.0 vs. 30.1±2.1 weeks, BW: 844±118 vs. 1280±136 g)

Ad II/1.3.) Out of 89 preterm neonates who were treated in our NICU between 1st of January and 31st of December, 2006, and eligible on the basis of inclusion criteria (BW < 1500 g,

GA:≤32 weeks) 60 preterm infants (GA: 28.4±2.3 weeks [mean±SD]) were enrolled in the study. Exclusion criteria were congenital malformation, birth weight < 500 g, maternal diabetes, absent of informed consent. Two ELBWI, who died within the first week were excluded; data of 58 preterm neonates (22 extremely- and 36 moderately-premature infants; GA: 25.9±1.0 vs. 29.9±1.3 weeks, BW: 893±157 vs. 1339±313 g) were analyzed. Twenty-six healthy term neonates (GA: 39.1±1.3 weeks, BW: 3380±392 g) were also enrolled, as controls. A 0.5 ml blood sample was taken from each baby on day 3(±3) (week 1 sample), and additional samples were taken on day 24(±3) (week 4 sample) and day 45(±3) (week 7 sample) from preterm neonates. Fructosamine measurement was performed by a colorimetric method, all measured values were corrected for serum albumin according to the suggested correction formula. Hyperglycaemia was defined if blood glucose level was repeatedly 8.5 mM or higher, cut-off value for the hypoglycaemia was 2.6 mM. Glucose values consisted of both bedside whole blood glucose and laboratory serum glucose determinations. Unless condition of the infant required otherwise, glucose measurements were scheduled routinely at least every 8 h during the first postnatal week, or when receiving parenteral nutrition, or being under medication, which may influence glucose homeostasis, later on at least twice a week. Parenteral aminoacid was introduced on the 1-2nd day, enteral feeding was started on 1-5th day. Insulin treatment was applied (0.01-0.1U/kg/dosi or 0.01-0.1U/kg/h) if blood glucose level was repeatedly higher than 10 mM, in spite of reduction of the rate of glucose infusion. Demographic characteristics of the neonates, as well as the number of the days with hyper- and hypoglycaemia, CRIB score, presence and severity of IVH and ROP, length of hospital stay and total number of days on positive pressure ventilation and on supplemental oxygen were recorded. Serum protein-albumin- and fructosamine-levels of the "week 1 samples" were compared. We also investigated postnatal alteration of the fructosamine level in preterm neonates, as well as its association with the hyperglycaemia and related complications. Student t-test and Wilcoxon exact tests, χ^2- test, linear correlation and logistic regression were applied. Statistical significance level was set at level of $p<0.05$. The study protocol was approved by Regional- and Hungarian Research Ethical Committee of Health Sciences.

Ad II/1.4.) Rats were anesthetized with Metofane before decapitation and adipocytes were isolated from the basis of the epididymal fat pad. Animals of similar sizes were used because cell size varies directly with fat stores of the animal. Aliquots of the adipocytes (3-5*10^6 cell/ml) were incubated first in base DMEM (Sigma) medium (contains no glucose, pyruvate and L-glutamine), and then with or without of insulin (100 ng/ml) in the absence or presence of various concentrations of glucose (5, 15, 25 mM), or 25 mM concentrations of galactose,

fructose, alanine, pyruvate, or 2-deoxyglucose. (The 2-DG is an energy depleting substrate). After 4 h incubation medium were withdrawn to measure leptin level by Rat Leptin RIA Kit (Linco Research). To determine the effect of intracellular calcium on the glucose+insulin induced leptin-secretion, the adipocytes were incubated for 1 h in calcium-free MEM medium in the presence of 1 mM pyruvate, next, aliquots of the cells were supplemented with equal volumes of MEM alone, MEM and glucose+insulin, MEM and calcium or MEM and glucose+insulin+calcium. Final concentrations were 25 mM glucose, 100 ng/ml insulin and 1 mM calcium. We also investigated the effect of intracellular calcium chelator (BAPTA-AM) on the glucose+insulin induced leptin-secretion. The adipocytes were pre-incubated with BAPTA for 1 h, then washed and incubated with DMEM medium, with or without glucose+ insulin (25 mM, 100 ng/ml). Influence of potassium and calcium channel modulators on the leptin response was investigated. Fat cells were incubated in DMEM medium, and then supplemented with or without glucose+insulin (25 mM, 100 mg/ml) in the various concentrations of the ion channel modulators glibencamid, nimodipine, verapamil, cadmium chloride ($CdCl_2$), nickel chloride ($NiCl_2$) or diazoxid. Because of the variation in the absolute amount of leptin released in control cells, alterations in the leptin secretion were expressed as a degree of increase or decrease in leptin concentration in medium bathing cells treated with or without leptin secretagogues or inhibitors (experimental vs. control group). To determine if the change was statistically significant one-sample t test and unpaired t test were applied. Statistical significance level was set at level of $p<0.05$.

Ad II/2.1.) Twenty two recently diagnosed T1DM patients (age: 12.8±0, 9 years [mean±SD] were recruited from the Department of Paediatrics, Medical School, University of Pécs, Hungary before the introduction of the insulin treatment. Thirteen healthy 1st degree relatives of T1DM patients (16.6±2.24 years), who had high Islet Cell Antibody (ICA) titer ("prediabetics") and 43 healthy control subjects (12.9±1.2 years) were also recruited. Having informed consent of the parents 7 ml blood sample was taken and peripheral blood mononuclear cells (PBMC) were separated on Ficoll-Paque gradient. The fixed cells were labelled by indirect immuno-peroxidase staining using anti-CD22, anti-CD5 and anti-pan-TCR γδ monoclonal antibodies. The immunostaining method was amplified by silver intensification. The percentage of the CD5+ B and γδ T lymphocytes was expressed as a proportion of the total lymphocyte count. The relationship between the variables was assessed by correlation and linear regression. Results were considered statistically significant at p value of equal or less than 0.05. The study protocol was approved by Regional Research Ethical Committee.

Ad II/2.2.) Twenty two recently diagnosed T1DM patients (age: 11.9±3.0 years [mean±SD]) and 24 age–matched non diabetic control subjects (age: 12.2.±3.3 years) were recruited from the Department of Paediatrics, Medical School, University of Pécs. All patients were sampled within 12.6±10.6 weeks from establishing the diagnosis. Having informed consent of the parents 6 ml blood sample was taken from each participant. Clinical parameters of the patients, such as duration of the diabetes, age at the onset, HbA1c level and mean daily insulin requirement at the time of sampling were recorded. PBMCs were freshly separated and non-stimulated cells were directly labelled with fluorochrom conjugated monoclonal anti-TCR-Vγ9 (PE), anti-TCR-Vδ2 (FITC), anti-TCR-Vδ1 (FITC), and anti-CD3 (PE) antibodies. To investigate the cytokine production of the lymphocytes separated aliquots of the cells were stimulated according to the distributor's guidelines, then labelled with anti–IFNγ (APC) and anti-IL10 (APC) monoclonal antibodies, and finally labelled with anti-TCR-Vγ9 (PE), anti-TCR-Vδ2 (FITC), anti-TCR-Vδ1 (FITC) and anti-CD3 (PE) antibodies. Phenotypic analysis of the cells was carried out by Becton–Dickinson flow-cytometer. Data were analyzed using CellQuest software of Becton Dickinson. Relative proportion of the TCR-Vγ9+, TCR-Vδ2+, TCR-Vδ1+, TCR-Vγ9+/Vδ2+ and CD3+ cells was determined, besides fraction of the IFNγ+ and IFNγ-, as well as that of the IL10+ and Il10- subsets were established. Comparison of the study groups was performed by Mann-Whitney U and Student t tests; correlation of the data was assessed by Spearman's non-parametric correlation. Statistical significance level was set at $p<0.05$. The study protocol was approved by Regional Research Ethical Committee.

IV. Results

Ad II/1.1.) In association with remarkable increase in the relative proportion of the ELBWI as well as with their improving survival rate the incidence of hyperglycaemia tripled between January 1, 1998 and December 31, 2001 at our NICU among ELBW preterm neonates. During the period between January 1, 2000 and December 31, 2001 29.3% of the ELBWI and 17.5% of the VLBWI was detected temporally with hyperglycaemia. The incidence rate was 4.1 times higher in ELBWI neonates as compared to infants with BW with 1000-1499 g ***(Fig 1)***.

During our prospective study conducted between January 1, 2006 and December 31, 2006 we detected 50% incidence rate of hyperglycaemia in VLBWI, which is higher as it was detected during the previous study period. The difference could have been related to a higher rate of the glucose infusion during the later period, however, the difference was not statistically significant (6.6±1.3 mg/kg/min, maximum 8.6±1.6 mg/kg/min vs. 7.0±1.2 mg/kg/ min,

maximum 9.1±1.5 mg/kg/min) [means±S.D.]). More importantly, mean number of the daily blood glucose determinations were higher during the later study period (5 (3-9) vs. 2 (1-4) during the 1st week in ELBWI). Since 2003 blood glucose levels have been determined during the blood gas analysises resulting in increased number of the blood glucose values available. The hyperglycaemia was detected mainly during the first two weeks in ELBWI, its prevalence decreased afterward, however, in one third of the neonates still remained a problem temporally after 4 weeks of age.

The hyperglycaemic episodes of the first two weeks were almost exclusively coincidental with glucose infusion in ELBWI; after this period the impact of parenteral glucose administration decreased. Although, the rate of the glucose infusion did not differ significantly in hyperglycaemic and normoglycaemic neonates ***(table 1)***. Sixty percent of the hyperglycaemic episodes between the 2nd and 4th weeks, 72% of those after the 4th week occurred under steroid treatment. The steroid therapy, which was initiated because of bronchopulmonary dysplasia (BPD), showed significant correlation with the development of neonatal hyperglycaemia (OR: 2.6, 95% CI: 1.05-8,1; $p<0.05$). We did not find association between xantin administration and hyperglycaemia (OR: 1.2; 95% CI: 0.48-9.71; ns).

Ninety two percent of the hyperglycaemic infants required insulin therapy at least one occasion to correct elevated blood glucose level. Majority (91% and 83 %) of the hyperglycaemia during the 1st and 2nd week of life required insulin, however, after this period high blood glucose level normalized without insulin in 50% of the hyperglycaemic patients. Severe hypoglycaemia was not detected as a side effect of the insulin treatment, but repeated administration of the drug was associated with hypokalaemia.

We did not detect difference between hyperglycaemic and normoglycaemic ELBWI concerning CRIB score (7.0±2.9 vs. 5.9±2.1, ns; mean±S.D.), maximal percentile postnatal weight loss (14.4±2.3 vs. 14.9±1.9 %; ns), and duration to regain birth weight (17.6±5.8 vs. 16.1±4.0 days; ns). The hyperglycaemia was closely related to sepsis (OR: 6.18; 95% CI: 1.2-34.6, $p<0,01$), as well as to IVH grade III-IV (OR: 11.4; 95% CI: 2.8-52.4; $p<0,01$, but did not affect mortality rate (OR: 1,4; 95%-os CI:0,54-3,66, ns).

Ad II/1.2.) The incidence of ROP and hyperglycaemia was 35.3 and 19.4%, respectively between January 1, 2000 and December 31, 2002 in the VLBWI who survived the perinatal period. The incidence of the hyperglycaemia and the CRIB score were significantly higher, the GA, BW and Apgar scores at 1 and 5 minutes were lower in ROP patient, than those without the disease ***(table 2).*** Investigating the independent effect of these factors on ROP

development, only GA and hyperglycaemia were proven to be significant risk factors after logistic regression analysis ***(table3).***

The group of VLBWI was divided according to the BW (infants with BW below 1000 g and between 1000-1499 g). Analyzing the data of the subgroups separately we found, that GA and BW were lower, the CRIB score and the incidence of hyperglycaemia were significantly higher among infants with ROP in both weight-groups ***(table 4)***. The CRIB score of hyper- and normoglycaemic ROP patients did not differ significantly in either subgroup. Using a logistic regression model, in the ELBWI GA and CRIB score, in 1000-1499 g cohort GA and hyperglycaemia were found to be significant contributors of the ROP development (***table 5)***

Ad II/1.3. We investigated the serum fructosamine-, albumin and protein levels of 22 extremely preterm, 36 moderately preterm and 26 term neonates. Serum protein and albumin concentrations were significantly lower in extremely compared to moderately preterm or term infants. (albumin: 25.9±1.3 vs. 29.6±1.3 or 32.5±1.0 g/l; $p<0.05$, $p<0.001$; protein: 41.3±1.7 vs. 50.5±2.3 or 54.9±3.2 g/l; $p<0.05$, $p<0.001$), thus all measured fructosamine were corrected for albumin according to the suggested formula.

Fructosamine levels during the 1st week of the life were significantly higher in both groups of the premature neonates compared to term ones; there was no difference between the two subgroups of the preterm infants ***(Fig 2)***. Fructosamine concentration declined over the first 7 weeks of life in preterm neonates ***(Fig 3)***.

Analyzing fructosamine levels of the 4th week samples, we did not find any difference between preterm infants with or without hyperglycaemia during the first 3 weeks of life (217.0±9.8 vs. 209.4±8.4 μM; NS, mean±SE). Besides, its level was similar on the 7th week in the preterm infants who had hyperglycaemia during the preceding 3-week period (week 4-7) and in the normoglycaemic neonates (188.4±10.6 vs. 198.1±10.7 μM; NS). Fructosamine concentrations of the 4th and 7th week samples did not show correlation either with the number of hyperglycaemic days of the 3-week period, that preceded the samplings, or with CRIB score, presence of severe ROP or ROP at any stage, severe IVH or IVH of any grade, number of days on mechanical ventilation and on supplemental oxygen. Fructosamine level in the 1st week samples was associated with the number of hypoglycaemic days during the first 3 weeks of life (r: 0.498, $p<0.02$), additionally its level on the 4th week correlated with length of hospital stay (r: 0.285, $p<0.05$).

The incidence of hyperglycaemia during the first 3 weeks of life was 77 and 33% in extremely- and moderately premature infants; the mean number of days with hyperglycaemia was 2.3 (range 1-9) and 0.5 (range 1-4), respectively. By the end of the sampling period, the

incidence decreased up to 32 and 8.3%. The occurrence of hyperglycaemia showed association with development of ROP at any stage, or severe ROP ((χ^2 =4.41, d.f.=1, p<0.05; χ^2 =8.03, d.f.=1, p<0.01), as well as with the presence of IVH (χ^2 =4.46, d.f.=1, p<0.05). The number of hyperglycaemic days inversely related to GA and BW, directly to CRIB score, length of mechanical ventilation and supplemental oxygen; however in a logistic regression model only GA remained significant (r=-0.495; p<0.01).

Ad II/1.4. Isolated rat adipocytes were incubated with various concentrations of glucose to determine, if energy-producing substrates stimulates leptin secretion *in vitro.* Glucose increased leptin-release in the medium significantly in a dose-response manner; 25 mM glucose raised leptin-secretion by ~3.5fold ***(Fig 4)***. Other substrates -fructose, alanine and pyruvate- raised leptin-release 1.80±0.15 (p<0.05), 1.74±0.13 (p<0.05) and 1.70±0.18 (p<0.05)- fold. Substrates that cannot be metabolized by cells (L-glucose, galactose) did not influence the leptin-release. Incubation of the cells with 2-DG, which caused energy depletion of the cells, resulted in significant decrease in the secretion ***(Fig 5)***. Leptin-release of the cells treated with alanine and pyruvate could have been stimulated further with insulin (100 ng/ml) ***(Fig 5).*** In this particular experiment insulin did not increase significantly leptin-release of the glucose treated cells, however, when we combined results of several experiments with adipocytes incubated with 25 mM glucose with or without insulin (100 ng/ml), insulin increased leptin secretion by an additional 1.26±0.08-fold (p<0.01).

In the absence of calcium, glucose+insulin (25 mM, 100 ng/ml) resulted in about 75% increases in the leptin-release, while in the presence of calcium, glucose+insulin stimulated leptin secretion by approximately fivefold. Calcium alone raised leptin-release by twofold ***(Fig 6)***. Chelating intracellular calcium by preincubation with BAPTA-AM did not prevent leptin-release, but inhibited substrate mediated (25 mM glucose+100 ng/ml insulin) leptin secretion by ~50% (p<0. 001). The non-specific calcium channel blockers $NiCl_2$ and $CdCl_2$ prevented glucose+insulin mediated leptin-release; 1 mM $NiCl_2$ and 10 mM $CdCl_2$ concentration reduced leptin-release by 50% (p<0.01). Nimodipine and verpamil had no significant effect on the secretion. Stimulation of ATP-dependent potassium channels by diazoxid inhibited glucose+insulin mediated leptin secretion in a dose-dependent manner.

Ad II/2.1.) The relative proportion of peripheral blood CD5+ B lymphocytes decreased rapidly by advancing age in newly diagnosed T1DM children, in ICA+ 1st degree relatives of T1DM patients (r: -0.30; p<0.01), as well as in healthy control subjects (r:-0.65; p<0.001). Similar negative age-trend was found concerning γδ T lymphocytes in the group of diabetic and "prediabetic" subjects (r: -0.35; p<0.01), as well as in healthy controls (r: -0.72; p<0.001).

However, the age-dependent decline in the ratio of particular lymphocyte subsets was more prominent in the control group.

Recently diagnosed T1DM patients and "prediabetic" children had significantly higher percentage either of the CD5+ B lymphocytes or of the γδ cells as compared to control subjects. ($p<0.0001$, $p<0.03$). Besides, we did not find any difference between diabetic and "prediabetic" subjects ***(Fig 7-8)***.

Ad II/2.2.) Investigating TCR chain-usage of circulating T lymphocytes significant augmentation of the TCR Vγ9+/Vδ2+ (double positive) lymphocyte subset was detected in diabetic children as compared to controls (2.66±0.52 vs. 1.27±0.22%; $p<0.05$ [mean±SE].). We were unable to detect any difference between diabetic and control subjects in the percentage of the single positive TCR/Vδ1+, TCR/Vδ2+, and TCR/Vγ9+ cells ***(Fig 9)***. The relative proportion of TCR Vγ9+/Vδ2+ cells showed a strong linear correlation with the percentage of single positive TCR Vγ9+ (r:0.971; $p<0.001$ and r:0.857; $p<0.001$) and of the TCR Vδ2+ cells (r: 0.975; $p<0.001$ and r:0.501; $p<0.05$) both in diabetic and control subjects. The majority of the Vγ9+ chains combined with Vδ2+ chains both in patients and controls (77% and 61%). According to the percentage of the Vγ9+/Vδ2+ cells the patients could be divided into two separate groups. A small subgroup of the patients had higher percentage of the circulating Vγ9+/Vδ2+ cells as upper 95% confidence limit established for non-diabetic controls (T1DM-"high-group"), while majority of the patients had similar percentages of the Vγ9+/Vδ2+ cells as controls.

In T1DM subjects we did not find any alteration in the percentage of the CD3+T cells (70.44±1.76 vs. 71.77±1.53; NS), as well as in their IFNγ (17.87±2.25 vs. 16.50±1.65; NS) or IL10 production (2.37±0.42 vs. 1.57±0.30; NS.). The IL10+ fraction of the TCR/Vγ9+ cells expanded significantly in diabetic children as compared to controls (6.48±2.38 vs. 4.09±2.18 %; $p<0.05$), whereas percentage of the cells expressing IFNγ tended to be lower (31.15±3.27 vs. 38.35±5.05 %, NS) ***(Fig 10)***. Among total lymphocytes the relative proportion of the IL10+/Vγ9+ and IFNγ-/Vγ9+ cells displayed elevation (0.16±0.03 vs. 0.09 ±0.03% ; $p<0.01$; 2.38±0.38 vs. 1.24±0.15%; $p<0.05$).

Diabetic patients with "high" percentage of the Vγ9+/Vδ2+ cells had an increased proportion of the IFNγ producing T lymphocytes, but no alteration in the Vγ9+ cells' cytokine-profile, while those patients who had almost "normal" percentage of the Vγ9+/Vδ2+ subset, displayed expansion of the IL10+ cells among Vγ9+ lymphocytes, but no change in the cytokine

production of the CD3+ T cells was observed ***(Table 6)***. Comparing the subgroups of the patients with "normal" and "high" percentage of the Vγ9+/Vδ2+ cells we did not find association with clinical parameters, such as duration of diabetes, age at onset as well as Hba1c level at the time of sampling. Mean daily insulin requirement tended to be lower in those patients who had "high" percentage of the Vγ9+/Vδ2+ cells (0.87±0.08 vs. 1.02±0.04 U/kg/day; p: 0.065)

VI. Summary and practical application of the results

Investigation of glucose-homeostasis and neonatal hyperglycaemia in preterm neonates during the perinatal period

- Postnatally we observed a *transient hyperglycaemia in about 50% of the VLBWI. The incidence* -may be as high as 70% in immature preterm infants-, is *inversely related to BW and GA, and decreasing with advancing postnatal age*. *To get a true picture, investigation of blood glucose level is necessary at least 3-5 times/day within the first week of life* or during the period of medication which may influence glucose homeostasis.

- *During the first two weeks of life hyperglycaemia occurred almost exclusively when glucose infusion was applied.* However, the rate of parenteral glucose infusion was not higher in the hyperglycaemic newborns than in normoglycaemic infants. *There was a relationship between steroid therapy and hyperglycaemia* suggesting the need of close glucose control in steroid treated preterm infants. *We also observed a correlation between sepsis and hyperglycaemia in the population studied.*

- Among ELBWI there was no relationship between hyperglycaemia and the mortality rate or the CRIB score characterizing the severity of illness. However*, we detected a higher rate of intracranial bleeding among preterm infants suffering from hyperglycaemia.*

- We concluded that *in preterm infants with a birth weight of less than 1500 g the GA and the hyperglycaemia are independent risk factor for the development of ROP at any stage.* Stratifying the VLBWI according to BW we observed that in the 1000-1499 g cohort the hyperglycaemia and the low GA both may influence ROP development. In ELBWI lower GA and higher CRIB score were independent risk factors for ROP. *Based on these results we conclude that hyperglycaemia is a new potential risk factor for ROP development, and more attention should be paid to eye examination of hyperglycaemic preterm infants*.

- We were the first to report the usefulness of fructosamine detection in the analysis of glucose homeostasis in preterm infants. *Immediately after delivery we detected higher fructosamine levels in preterm compared to term infants that refers to higher glucose level of*

fetuses with lower gestational age in utero. Postnatal decrease of the fructosamine levels was observed in preterm infants during the first 7 weeks of life, which may reflect lower blood sugar levels.

- We were unable to detect any difference in fructosamine levels of VLBWI with or without hyperglycaemia. Fructosamine levels did not correlate with the duration of hyperglycaemia and the hyperglycaemia-related complications. *Determination of the fructosamine levels provided new information about perinatal glucose homeostasis; however, it failed as a marker of predicting late complications of neonatal hyperglycaemia.*

- In vitro we investigated the effect of different glucose concentrations on the leptin- secretion of rat fat cells *and found a dose-related increase*. It was also proved that *glucose induced leptin secretion may be further increased by insulin*. According to our results ATP dependent K channels and Ca channels are involved into this process, and the *Ca influx into the cells is the stimulus-secretion coupling mechanism of the glucose-insulin induced leptin-secretion.*

Circulating γδ T and CD5+ B lymphocytes in T1DM during childhood

- We detected *increase*d *percentage of circulating γδ T and CD5+ B lymphocytes in recently diagnosed T1DM patients, as well as in ICA+ 1st degree relatives of T1DM patients* as compared to controls. The quantitative alteration of subgroups of γδ T cells could also be detected for several weeks after the diagnosis. We conclude that quantitative alterations of *the CD5+ B and γδ T lymphocytes may be related to autoimmune nature of the disease.*

- Investigating TCR chain usage and cytokine production of the γδ T lymphocyte, *we observed significant augmentation of the Vγ9+/Vδ2+ subset, as well as of the IL10 producing fraction of the Vγ9+ cells in T1DM children* as compared to control subjects.

- According to the percentage of the Vγ9+/Vδ2+ cells, T1DM children could be divided into two separate groups which were accompanied with differences in the cytokine production of the Vγ9+ cells and the CD3+ T cells. Diabetic patients with high percentage of the Vγ9+/Vδ2+ cells had an increased proportion of the IFNγ producing T lymphocytes, but no alteration of the Vγ9+ cells' cytokine profile, while those patients who had almost "normal" percentage of the Vγ9+/Vδ2+ subset, displayed expansion of the IL10+ cells among Vγ9+ lymphocytes, and no change in the cytokine production of the CD3+ T cells.

- We did not find significant association between the relative proportion of particular subsets of γδ cells and the clinical parameters of the patients.