

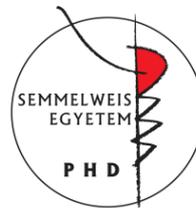
**Determination of DPP4 enzyme activity and GLP-1 hormone level in neonates  
born from pregnancies complicated with gestational diabetes mellitus and the role  
of certain maternal gene variants**

Doctoral thesis

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## List of abbreviations

AGA	Appropriate for gestational age
AT	Austrian pregnant population
BMI	Body Mass Index
CI	Confidence interval
DPP4	Dipeptidyl peptidáz-4 enzyme
EFSD	European Foundation for the Study of Diabetes
ELISA	Enzyme-linked immunosorbent assay
FPG	Fasting plasma glucose
GDM	Gestational Diabetes Mellitus
GLP-1	Glucagon like peptide-1
HUN	Hungarian pregnant population
HWE	Hardy-Weinberg equilibrium
IADPSG	International Association of Diabetes and Pregnancy Study Groups
IDF	International Diabetes Federation
IFCC	International Federation of Clinical Chemistry
KASP™	Kompetitive Allele Specific PCR
LGA	Large for gestational age
MAF	Minor allele frequency
MTNR1B	MTNR1B gene
OGTT	Oral glucose tolerance test
OR	Odds ratio
PG	Plasma glucose
sDPP4	Serum DPP4
SGA	Small for gestational age
SNP	Single-nucleotide polymorphism
UC	Umbilical cord
WHO	World Health Organization

## 1. Introduction

Globally, according to IDF data, one in seven births is affected by GDM. According to the most recent Hungarian study the prevalence varies between 8.1-14.8% depending on the diagnostic criteria applied. [1]

GDM is hyperglycaemia that is first detected during pregnancy. GDM is the most common form of diabetes occurring during pregnancy (~90%) which has to be differentiated from diabetes forms diagnosed before pregnancy (pregestational diabetes mellitus) and from diabetes in pregnancy (overt diabetes) categories. [2] The diabetogenic effect of pregnancy is attributable to several factors, including physiological hormonal changes during pregnancy and as a consequence that for the 2<sup>nd</sup> and 3<sup>rd</sup> trimester of pregnancy an insulin resistant condition develops. The increasing insulin demand associated to the decreasing insulin sensitivity is rising almost until the end of pregnancy (34-36<sup>th</sup> gestational week). Development of GDM might be expected in those pregnant women in whom adaptation through beta-cell plasticity can not overcome the insulin resistance occurring due to the pregnancy itself and due the commonly associated/ pre-existing obesity and the insulin secretion might not cover for the increased insulin demand.

The possibility of physiological adaptation is determined by several factors, such as the incretin hormone systems, DPP4 through degradation and inactivation of incretins, adipokine hormones in addition to the genetic factors.

Substantive clinical studies were not available before our study regarding the role of DPP4-incretin system in the development of GDM associated neonatal complications. On the other hand the role of *MTNR1B* gene rs10830963 variant *G* allele carrying (nearly half of the European population is carrier) in deterioration of early phase insulin response was previously described, but was observed as a genetic risk factor in GDM development predominantly in Asian studies.

## 2. Objectives

### ***I. Determination of DPP4-incretin system in neonates born from GDM and control (normal carbohydrate metabolisms during 75 g OGTT)***

Related to this we proposed the following aims:

I/1. DPP4 Umbilical cord (UC) serum:

- determination of serum DPP4 enzyme activity in UC blood serum of neonates born from GDM pregnancies and the DPP4 enzyme activity in UC blood serum of neonates born from non GDM pregnancies
- comparison of enzyme activity values in the two groups if sDPP4 has measurable enzyme activity in UC blood serum

I/2. GLP-1 UC plasma:

- related to this the determination of the active GLP-1<sup>7-36</sup> concentration in UC blood plasma of neonates born from GDM pregnancies and the active GLP-1<sup>7-36</sup> concentration in UC blood plasma of neonates born from non GDM pregnancies
- comparison of plasma concentrations in the two groups provided that active GLP-1<sup>7-36</sup> has measurable concentration in UC blood plasma.

### ***II. Determination of the role of maternal MTNR1B rs10830963 gene variant in GDM***

I had the opportunity to join a larger international study (EFSD New Horizons), which proposed the examination of the role of 77 gene variants, previously described in association with type 2 diabetes, GDM and/or other relevant metabolic traits, in the development of GDM in an Austrian-Hungarian pregnant population. Through my participation in this study I have the chance to present in Hungarian language the results related to the role of *MTNR1B* rs10830963 gene variant as a complementary part of my doctoral thesis.

### 3. Methods

#### 1. *Serum DPP4 enzyme activity and active GLP-1<sup>7-36</sup> plasma concentration in UC blood of neonates born from GDM and non-diabetic pregnancies*

##### 3.1.1. Patients included

The participants were enrolled in Budapest from Semmelweis University 2<sup>nd</sup> Department of Internal Medicine and 1<sup>st</sup> Department of Obstetric and Gynaecology and latter from the Obstetrics and Gynaecology Department of Szent Imre Teaching Hospital (ETT TUKEB number: 485/PI/11., 15100-2/2011-EKU). Due to the international cooperation a proportion of the participants were enrolled into the study from the University of Vienna (AKH).

We have enrolled 568 (210 GDM, 358 control) pregnant women during the OGTT performed between the 24-28<sup>th</sup> gestational week after reading the written information and signing the approval declaration to this study part. Unfortunately sample collection was possible only in less neonates because of different reasons: withdrawal of approval, missed UC puncture, obstetrical or other technical situations. Determination of serum DPP4 activity was eventually possible in 270 (159 control, 111 GDM) UC serum samples. We have collected plasma samples from 112 (40 GDM, 72 control) neonates for the determination of biologically active GLP-1<sup>7-36</sup> levels. [3]

##### 3.1.2. Diagnosis of GDM and control group

Pregnant women were enrolled and categorised in GDM or control group based on the results of the 75 g OGTT performed between the 24<sup>th</sup> and 28<sup>th</sup> gestational week, where control means that the OGTT result was not abnormal nor GDM or overt diabetes was diagnosed later during the pregnancy. 75 g OGTT screening was routinely performed in Hungary and Austria also. GDM was diagnosed according to the modified 1999 WHO criteria (FPG  $\geq 6.1$  mmol/L, 120' PG  $\geq 7.8$  mmol/L), in Austria according to the 2011 IADPSG criteria (FPG  $\geq 5.1$  mmol/L, 60' PG  $\geq 10.0$  mmol/L, 120' PG  $\geq 8.5$  mmol/L). [3]

### **3.1.3. Methods**

Maternal blood samples were obtained from cubital vein.

After clamping the UC blood sample was collected right after the delivery from the umbilical vein. Blood samples used for the determination of DPP4 enzyme activity were collected in native tubes, not containing anticoagulant and after centrifugation we used the serum. Blood sampling for the determination of active GLP-1<sup>7-36</sup> plasma concentration was performed in a special tube containing EDTA, sitagliptin and a protease-inhibitor cocktail. We separated the plasma at most 30 minutes with centrifugation (20 minutes, 2000 rpm) which was stored temporarily at -20 °C. The samples were marked with barcode and were stored anonymised according to the ethical authorisation.

### **3.1.4. Determination of cord serum DPP4 enzyme activity**

Serum DPP4 activity was determined in a continuous monitoring assay in a microplate reader (Thermo, Varioskan Flash) at 405 nm on 37 °C for 30 min. All DPP4 assays were run in duplicates: 9.4 µL serum and 115.6 µL assay buffer (10 mM Tris-HCl, pH 7.6) containing 2 mmol/L Gly-Pro-paranitroanilide tosylate substrate (Bachem, Bubendorf, Switzerland) was pipetted into 96 microplate wells. DPP4 enzyme activity was expressed in nmol/mL/min (U/L) of Gly-Pro-PNA hydrolysed.

### **3.1.5. Determination of cord plasma GLP-1<sup>7-36</sup> concentrations**

Cord plasma active GLP-1 concentration was determined using a fluorescence ELISA method and the EGLP-35K kit (Millipore, Billerica, MA, USA) under conditions as recommended by the manufacturer with fluorescence reads at the excitation/emission wavelength of 355 nm/460 nm on a Varioskan Flash reader (Thermo Fisher Scientific, Waltham, MA, USA). The detection of the biologically active form of GLP-1 requires an intact (uncleaved by DPP4) N-terminal end of the GLP-1 protein, and this could be detected using an N-terminal specific antibody against the GLP-1 hormone. Therefore, the method we used was appropriate for the detection of the plasma active GLP-1 concentration [including GLP-1(7-36 amide) and GLP-1(7-37)], but we could not detect those inactive GLP-1 forms that were cleaved by the DPP4 (e.g. GLP-1 9-36-amide). All GLP-1 samples were run in duplicates, using 100 µL sampled cord plasma with

standards of GLP-1 with the concentration range between 2 and 100 pM. Active GLP-1 concentration in the cord plasma is expressed in pmol/L.

### **3.1.6. Statistics**

Both the Shapiro–Wilks and the Kolmogorov–Smirnov tests were used to assess normality. Mann–Whitney U-test (MWU) was used to compare means and detect differences in case of nonparametric distributions, and 2-tailed T-test with independent variables was used when the distribution was normal. Kruskal–Wallis with the Newman–Keuls post hoc test was used for multiple comparisons, and Pearson’s test as well as the Kendall’s Tau test was used for analysing potential correlations. The Statistica (release 10, StatSoft) software was used for these calculations.

## ***II. Determination of MTNR1B rs10830963 gene variant***

### **3.2.1. Patients included**

In the framework of the EFSD New Horizons international study 960 (820 after exculsion from study and dropouts) pregnant women were enrolled from three centers in two countries. Patients were reclassified according to both GDM diagnostic criteria of the two countries (Hungary, Austria). After this the case numbers varied as follows: according to the modified 99’ WHO criteria 303 GDM, 517 control, and according to the IADPSG criteria 287 GDM, 533 control women were enrolled in the case-control study. [4]

### **3.2.2. DNS isolation method**

Genomic DNA was isolated using a DNA isolation magnetic bead based mag™ kit (LGC) robotized approach (Hamilton Robotics, Magna Starlet) using totally 200 µL EDTA-anticoagulated whole blood samples. The protocol is based on a frequently used technology during which the DNA binds to magnetic particles. The advantage is that it is a fast, easily automatisable method and lower amount of blood sample is needed compared to the manual methods and greater amount of DNA can be isolated.

### **3.2.3. *MTNR1B* genotyping**

PCR based KASP genotyping probe (LGC Genomics, UK) was used for the bi-allelic discrimination of the 77 SNPs. The overall call rate for all SNPs assessed exceeded the 97% and no discordant genotypes were identified in the control samples of which genomic DNA were isolated in two different runs and subsequently genotyped separately in duplicates for all SNPs. Kompetitive Allele Specific PCR genotyping system (KASP™) is a homogene end-point genotyping technique using fluorescence. The KASP genotyping method is compound of two parts:

- SNP specific KASP assay mix: two different, allelespecific, forward and one reverse primer
- the allelespecific forward primers are marked by different fluorophores (eg. FAM and HEX)
- universal KASP Master mix: FRET cassette and Taq polimerase containing optimised buffer solution.

Components of the assay: DNS sample, KASP assay mix and KASP master mix.

The first step of the PCR is coupling of allele-specific primers and target SNP containing DNA sequences and amplification of the targeted region by reverse primer. After this a complementary copy of the allele is made. In the following steps of PCR the allele-specific sequences are elongated. The marked region of the FRET cassette is complementary with the new allele sequence, binds to this and the appropriate signal can be detected.

### **3.2.4. Statistics**

We analyzed the *MTNR1B* genotype results and binary outcomes (GDM/ non-diabetic) using the logistic regression method and the qualitative traits using linear regression method. The analysis was performed under both the dominant and the additive genetic models. We calculated odds ratio (OR), genetic effect size and p-value for every SNP and used the Benjamini-Hochberg p-correction method to minimize false discovery. Deviations from the Hardy-Weinberg equilibrium in the genotype distributions were assessed for all SNPs using Chi Squared test.

## 4. Results

### I. UC DPP4 serum activity and active GLP-1<sup>7-36</sup> plasma concentration levels

#### 4.1.1. Clinical characteristics of the participating mothers and neonates

##### Maternal clinical results

The most important maternal clinical results (0', 60', 120' PG values measured at 75 g OGTT, pre-pregnancy BMI, average maternal age, weight gain during pregnancy, HbA<sub>1c</sub>) are shown in table 1.

Table 1.: Maternal clinical results

	Sampling at 75g OGTT / Study Group	n	Mean	95% CI
75 g OGTT plasma glucose values GDM group (mmol/L)	0'	199	5	4,91-5,09
	60'	114	9,49*	9,11-9,89
	120'	199	7,78*	7,55-8,02
75 g OGTT plasma glucose values control group (mmol/L)	0'	330	4,40*	4,35-4,45
	60'	136	6,77*	6,51-7,02
	120'	330	5,48*	5,36-5,60
Pre-pregnancy BMI (kg/m <sup>2</sup> )	GDM	195	27,77*	26,15-29,41
	Control	326	23,49	23-23,98
Maternal age (years)	GDM	210	32,85**	32,09-33,60
	Control	358	31,44	30,89-32,00
Weight gain during pregnancy (kg)	GDM	181	10,15***	8,78-11,51
	Control	326	11,94	11,22-12,66
HbA <sub>1c</sub> [% (IFCC units - mmol/mol)]	Hungary	43	5,14 (32,7)	5,03-5,25 (31,5-33,9)
	Austria	70	5,35 (35)	5,25-5,45 (33,9-36,1)

\* p<10<sup>-4</sup> (T-test, MWU), \*\* p=0,0031 (2-tailed T-test), \*\*\* p=0,012 (2-tailed T-test)

## Neonate clinical results

### *Proportion of SGA, AGA, LGA neonates in the control and GDM groups*

The proportion of SGA, AGA, LGA neonates in the Hungarian and Austrian GDM and control groups is shown in table 2.

Table 2.: Proportion of LGA, AGA, SGA neonates in the control and GDM group in the two countries

Hungary (99' mWHO)	LGA (%)	AGA (%)	SGA (%)	Austria (IADPSG)	LGA (%)	AGA (%)	SGA (%)
GDM (n=90)	22,22	72,22	5,56	GDM (n=120)	24,17	70,0	4,17
Control (n=194)	23,71	70,62*	5,67	Control (n=164)	20,12	77,4*	2,44

\*Later - after extension of study - (genetic study) the significant difference found in birth weight percentile distributions between the two countries was ceased.

### *C-peptid*

There was no significant difference in UC serum C-peptide values in the GDM (mean=1.46 ng/mL, 95% CI: 1.04-1.88 ng/mL, n=87) compared to the control (1.29 ng/mL, 95% CI: 1.16–1.43, n=159) group (MWU test).

#### **4.1.2. Umbilical cord serum DPP4 enzyme activity**

The mean DPP4 activity was significantly lower in UC serum of neonates born to women with GDM (mean=28.07 U/L, 95% CI: 26 32–29 82 U/L, n=111) compared to the activity in samples of neonates born to non-diabetic women (mean=31 61 U/L, 95% CI: 29 93– 33 29 U/L, n=159, p=0.0015, MWU-test) (Fig. 1). The non-diabetic UC blood sDPP4 activity values are near to the normal adult control (40.3 U/L, 95% CI: 37.8-42.8) serum activity ranges. We performed a power calculation to assess this difference, and this indicated an 81% statistical power.

The mean LnDPP4 value remained significantly (MWU-test, p=0.0015) lower in the GDM (mean=3.29) than in the control (mean=3.41) group. [3]

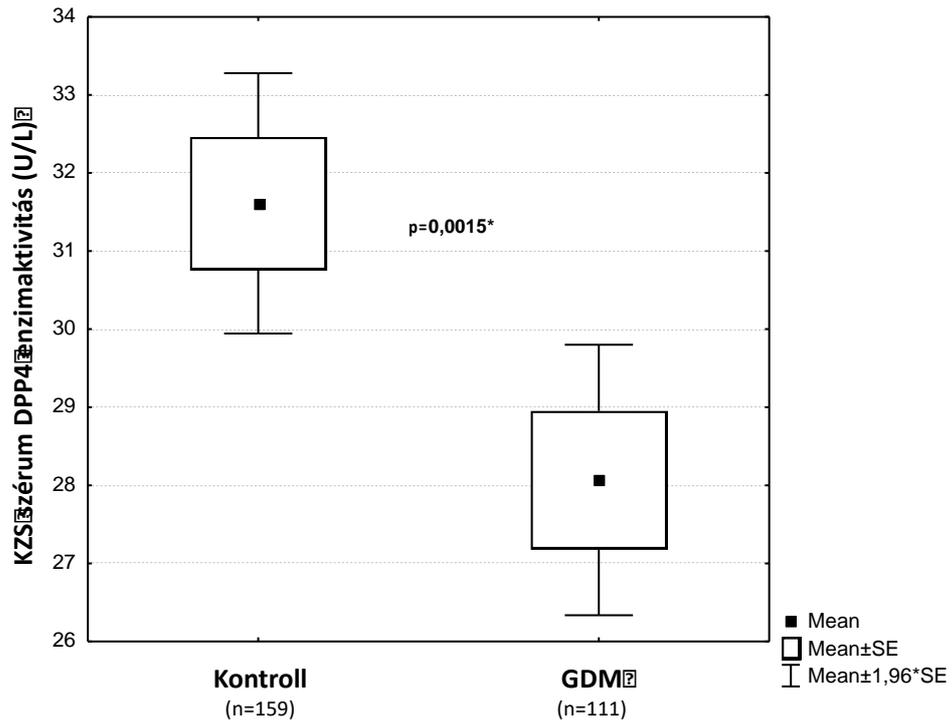


Figure 1.: DPP4 activity in UC blood serum of neonates born from control and GDM pregnancies

We performed a subgroup analysis according to treatment type (insulin therapy or diet alone) and found significant differences among groups with the lowest LnDPP4 values in the GDM on insulin group (mean=3.245, 95% CI: 3.17– 3.32, n=39) higher values in the GDM managed with dietary measures only group (mean=3.32, 95% CI: 3.25–3.40, n=72) and highest values in the nondiabetic control group (mean=3.41, 95% CI: 3.36– 3.45, n=159) (Fig. 2).

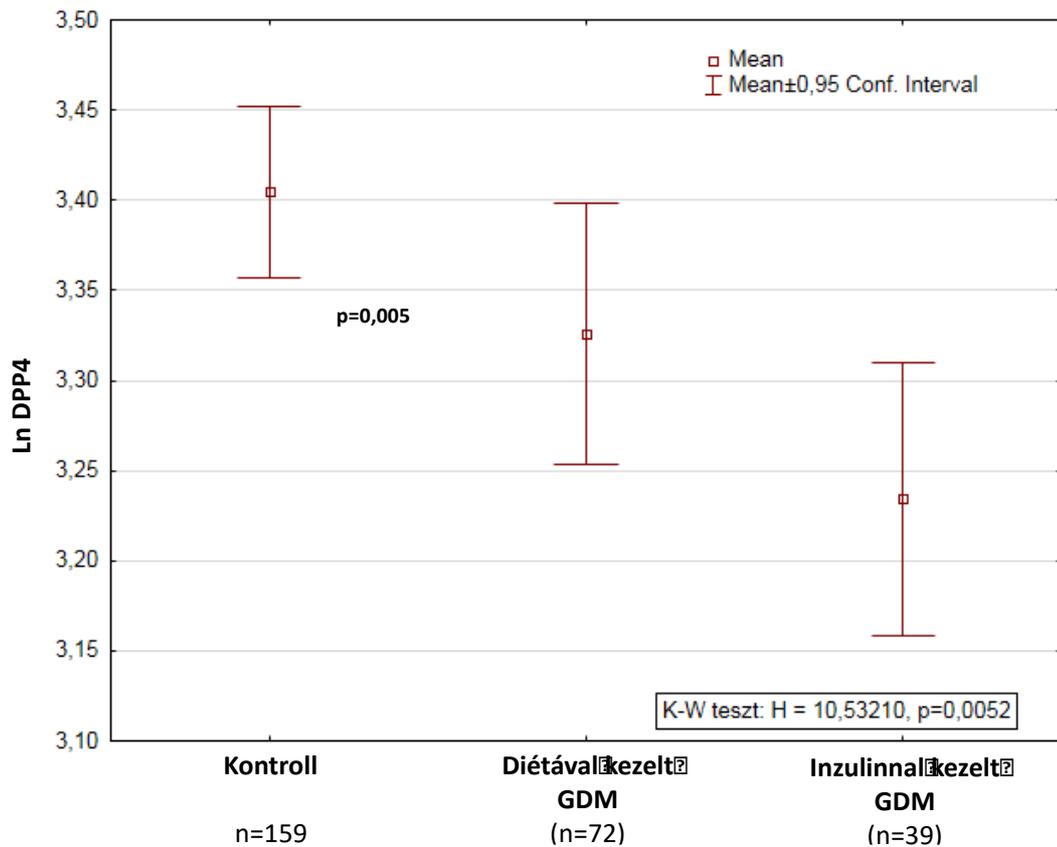


Figure 2.: DPP4 activity in UC blood serum of neonates born from control and GDM pregnancies according to treatment type

To assess the potential impact of the differing diagnostic criteria, we have recalculated the difference found between the GDM and the control group in the cord serum DPP4 activity after regrouping the data both using the modified 1999 WHO criteria for the Austrian population and vice versa using the IADPSG criteria FG and 120' PG level limits at OGTT for the Hungarian population. The described difference in cord serum DPP4 activity remained significant both when the modified 1999 WHO criteria [GDM: n=77, mean LnDPP4 = 3.29, (95% CI: 3.22–3.36); control: n=171, mean LnDPP4=3.39 (95% CI: 3.35–3.44, p=0.0048] and when the IADPSG criteria [GDM: n=71, mean LnDPP4=3.27 [95% CI: 3.20–3.34]; control: n=177, mean LnDPP4=3.4 (95% CI: 3.35–3.44), Kruskal-Wallis, Newman-Keuls post hoc, p = 0.0019] were applied. The difference in diagnostic criteria applied has not had a significant impact on the finding that in the GDM group the cord serum DPP4 activity is lower. [3]

### 4.1.3. Umbilical cord plasma GLP-1<sup>7-36</sup> concentration

Cord plasma active GLP-1<sup>7-36</sup> concentrations were close to the lower detection limit (2 pmol/L) and were not significantly altered in samples of neonates born to mothers with GDM (n=40, mean=3.61 pM, 95% CI: 2.96–4.28 pM) compared to the control samples (n=72, mean=3.43 pM, 95% CI: 3.04–3.82 pM, – MWU-test, p=0.6). The active umbilical cord blood plasma GLP-1 levels were typically low. [3]

## II. Results of the *MTNR1B* rs10830963 maternal genotyping

### 4.2.1. Clinical characteristics of pregnant included in the genetic study

#### *Fasting and 120' plasma glucose values*

We found differences in the Hungarian (HUN) population plasma glucose values at 75g OGTT measured between the 24-28<sup>th</sup> gestational week in the control (n=408) and GDM (n=195) groups: 0' PG 4.52 mmol/L vs. 4.96 mmol/L (difference between GDM and control group 95% CI: 0.34-0.54, p<10<sup>-4</sup>), 120' PG 5.45 mmol/L vs. 8.72 mmol/L (difference between GDM and control group 95% CI: 3.06-3.47, p<10<sup>-4</sup>), respectively.

The OGTT values measured between the 24-28<sup>th</sup> gestational week showed differences also in the Austrian (AT) control (n=183) and GDM (n=147) groups: 0' PG 4.38 mmol/L vs. 5.14 mmol/L (difference between GDM and control group 95% CI: 0.63-0.87, p<10<sup>-4</sup>), 60' PG 6.80 mmol/L vs. 9.68 mmol/L (difference between GDM and control group 95% CI: 2.47-3.28, p<10<sup>-4</sup>), 120' PG 5.42 mmol/L vs. 7.38 mmol/L (difference between GDM and control group 95% CI: 1.61-2.30, p<10<sup>-4</sup>), respectively.

We also found differences when we compared the 75 g OGTT and other clinical data between the two countries:

- in the controls in the 0' PG values (HUN>AT, 4.52 mmol/L vs. 4.38 mmol/L, p<0.05), weight gain during pregnancy in the controls (HUN>AT 13.80 kg vs. 9.47 kg, p<0.05)
- in the GDM groups in the 0' PG values (AT>HUN 5.14 mmol/L vs. 4.96 mmol/L, p<0.05) and 120' PG (HUN>AT 8.72mmol/L vs.7.38mmol/L, p<0.05) values and age at delivery (HUN>AT 33.7 years vs. 32.04 years, p< 0.05).

### ***BMI***

Beside the significant differences in OGTT plasma glucose values, the pre-pregnancy BMI (HUN: n=195/408, GDM 26.78 kg/m<sup>2</sup> vs. control 23.32 kg/m<sup>2</sup>, difference between GDM and control group 95% CI: 2.55-4.36, AT: n=147/183, GDM 28.31 kg/m<sup>2</sup> vs. control 23.40 kg/m<sup>2</sup>, difference between GDM and control group 95% CI: 2.72-7.09, p<10<sup>-4</sup>) according to our expectations and previous literature data showed significant differences between the GDM and control groups in both countries.

### ***Maternal age***

There were significant differences between GDM and control groups in both countries in the maternal age also (HUN: GDM 33.7 years vs. control 31.25 years, difference between GDM and control group 95% CI: 1.54-3.36, AT: GDM 32.04 years vs. control 30.51 years, difference between GDM and control group 95% CI: 0.08-2.97, p<0.05).

### ***HbA<sub>1c</sub>***

In general the conclusions based on HbA<sub>1c</sub> values are limited: on one hand because of the altered red blood cell turnover in pregnancy, on the other hand because in 53.1% in the Hungarian GDM population and 63.2% in the Austrian GDM population underwent HbA<sub>1c</sub> measurements. The mean HbA<sub>1c</sub> value in the Hungarian GDM population was 5.20 % [33 mmol/mol, 95% CI: 5.10-5.30 (32-34)], and 5.3% in the Austrian GDM population [34 mmol/mol, 95% CI: 5.21-5.38 (33-35)]. [3]

#### **4.2.2. *MTNR1B* rs10830963/G risk variant allele association with GDM development (as binary trait) in the international case-control study**

The genetic association study was performed from several aspects. On one hand we had the opportunity to perform associational study using the dominant and the additive genetic models for all the 77 SNPs regarding the association with GDM development. On the other hand – because of the different diagnostic criterias used in the two countries – we performed the association study after reclassification of patients according to both GDM diagnostic criteria.

Furthermore, the genetic association results are reported in the original article for all the 77 gene variants assessed after adjustments to maternal age and to maternal age and

BMI also. We found no significant deviation in the genotype distributions from the HWE in any case of the 77 gene variants assessed.

In the present scale I have the opportunity to present in Hungarian language the results regarding the *MTNR1B* rs10830963 gene variant *G* risk allele which demonstrated the most robust association with GDM development.

The minor allele frequency (MAF) of the *MTNR1B* rs10830963 gene variant *G* risk allele was higher in the GDM group compared with the control group using both diagnostic criteria (IADPSG: 36% vs. 28%, m99'WHO: 36% vs. 28%; GDM vs. control, respectively).

In order to assess the effect of *MTNR1B* rs10830963 genotype on GDM development the odds ratio (OR) values (reflecting the genetic effect sizes) were calculated after reclassification according to both diagnostic criteria, results are shown in Table 1.

<b>OGTT diagnostic criteria</b>	<b>OR</b>	<b>p</b>	<b>Genetic model</b>	<b>Adjustment</b>
<b>m 99' WHO</b>	1.67	$3 \times 10^{-3*}$	D	maternal age
<b>IADPSG</b>	1.80	$3 \times 10^{-3*}$	D	maternal age
<b>m 99' WHO</b>	1.41	$5 \times 10^{-3}$	A	maternal age
<b>IADPSG</b>	1.47	$2 \times 10^{-3}$	A	maternal age
<b>m 99' WHO</b>	1.64	$6 \times 10^{-3*#}$	D	maternal age and pre-pregnancy BMI
<b>IADPSG</b>	1.85	$7 \times 10^{-4*+}$	D	maternal age and pre-pregnancy BMI
<b>m 99' WHO</b>	1.39	0,012	A	maternal age and pre-pregnancy BMI
<b>IADPSG</b>	1.48	$3 \times 10^{-3}$	A	maternal age and pre-pregnancy BMI

\* The association remained significant after Benjamin-Hochberg p-correction also ( $p < 0,05$ ). + Statistical power = 90%, # Statistical power = 73%. D=dominant genetic model, A=additive genetic model

From the results the following can be observed:

Regarding *MTNR1B* rs10830963 genotype and GDM development the genetic effect size is greater using the dominant model compared to the additive model after adjustment to maternal age and to the maternal age and pre-pregnancy BMI.

Although the task of my thesis is not consisting all the result of the genetic study, in general we can observe that the *MTNR1B* genotype has the most robust effect (and the largest genetic effect size) out of the 77 gene variants, furthermore this effect remained significant after the Benjamini-Hochberg p-correction procedure rate and the statistical power was also appropriate. [4]

#### **4.2.3. Association between the *MTNR1B* rs10830963/G risk allele and the PG values at standard 75g OGTT**

The associations specified below and the related genetic effects are applicable for the whole pregnant population because 75 g OGTT is performed in every pregnant women between the 24-28<sup>th</sup> gestational week in Hungary and Austria also.

Carrying the *MTNR1B* rs10830963 *G* risk variant was the most significantly associated with FPG values from the 77 SNPs (mean effect size in *G* allele carriers compared to the *CC* genotype: 0.21 mmol/L (95% CI: 0.11-0.3 mmol/L) increase,  $p < 5 \times 10^{-4}$ ).

The *MTNR1B* rs10830963 *G* risk allele was the most significantly associated SNP with the 2 hour PG levels (mean effect size 0.61 mmol/L (95% CI: 0.3-0.89 mmol/L) increase,  $p = 5 \times 10^{-4}$ ) also. [4]

## 5. Conclusions

### I. Determination of umbilical cord serum DPP4 enzyme activity and active GLP-1<sup>7-36</sup> plasma concentration

- The serum DPP4 enzyme has measurable activity in the UC blood of neonates born from GDM and non-diabetic pregnancies.
- UC blood sDPP4 activity is significantly decreased in neonates born from GDM pregnancies (11% decrease) compared to non GDM neonates.
- The above-mentioned differences in the UC blood sDPP4 activities are not fully elucidated. The decrease sDPP4 UC enzyme activity may potentially be the result of an adaptive fetoplacental response leading to a decrease in the soluble form DPP4 as an adipokine hormone and/or part of a complex regulatory mechanism driven by the intrauterine metabolic environment.
- The described decrease in UC sDPP4 activity –theoretically – may contribute to some neonatal clinical complications seen in GDM (eg. hypoglycaemia, polycythaemia), but our first results regarding this can not be interpreted as direct evidence.
- The active GLP-1<sup>7-36</sup> umbilical cord plasma levels are low, close to the lower detection limit.
- With the present (limited) sample sizes significant difference was not detected in UC active GLP-1<sup>7-36</sup> plasma levels in neonates born from GDM and non GDM pregnancies.
- The GLP-1<sup>7-36</sup> and the incretin regulatory system (baseline secretion is possible) – based on the low hormone levels – is functionally unlikely to be fully active before the initiation of oral feeding, but further data from the postnatal period would be needed.

## II. Results of *MTNR1B* rs10830963 maternal genotyping

- In the EFSD New Horizons study out of the assessed 77 single-nucleotide polymorphisms the *MTNR1B* rs10830963/ *G* allele carrying has the most robust genetic effect on development of GDM in the Hungarian and Austrian pregnant women.
- The odds ratio of *MTNR1B* rs10830963 *G* allele carrying on GDM development – independent of the diagnostic criteria – exceeded (OR 1.6-1.9) the level of typical degree of risk increase seen in common polygenic diseases (OR below 1.5).
- The *MTNR1B* rs10830963 *G* allele carrying significantly increases both the fasting and 2 hours PG levels at 75 g OGTT between 24-28<sup>th</sup> gestational week. This effect clinically may also be considered significant, due to that increase in the 2 hours PG value is exceeding the 0.5 mmol/L difference. The *MTNR1B* rs10830963 *G* risk allele frequency is high (MAF 36% in GDM, and 28% in the non-diabetic population) in Hungary, concordant with the minor allele frequencies measured in the 1000 genomes program (MAF, weighted Hungarian population mean: 29%). Accordingly nearly half (49%) of the Hungarian women carry the *MTNR1B* rs10830963 *G* risk allele.
- Based on the observed genetic effect size and the MAF of the *MTNR1B* rs10830963 gene variant, the risk *G* allele may have substantial role in the development of GDM in our country also. Potentially – based also on our results – the *MTNR1B* rs10830963 gene variant might have a role in GDM precision medicine in the future.

## **6. Bibliography of the candidate's publications**

### **Original research publications related to the PhD thesis**

*Al-Aissa Z, Rosta K, Hadarits O, et al.:* Cord serum dipeptidyl-peptidase 4 activity in gestational diabetes. *Eur J Clin Invest*, 2015, 45(2), 196-203. IF: 2.714

Rosta K, *Al-Aissa Z, Hadarits O, et al.:* Association Study with 77 SNPs Confirms the Robust Role for the rs10830963/G of MTNR1B Variant and Identifies Two Novel Associations in Gestational Diabetes Mellitus Development. *PLoS One*, 2017, 12(1), e0169781. IF: 2.806

Firneisz G, Rosta K, *Al-Aissa Z, et al.:* The MTNR1B rs10830963 Variant in Interaction with Pre-Pregnancy BMI is a Pharmacogenetic Marker for the Initiation of Antenatal Insulin Therapy in Gestational Diabetes Mellitus. *Int J Mol Sci*. 2018 Nov 23; 19(12). IF: 3.687

### **Review articles related to the PhD thesis, but no original research data presented**

*Al-Aissa Z, Hadarits O, Rosta K, et al.:* A brief history of gestational diabetes mellitus, risk factors and current criteria of diagnosis. *Orv Hetil*, 2017, 158(8), 283-290. IF: 0.349

### **Original research publications not related to the PhD thesis**

Zóka A, Barna G, Somogyi A, Múzes G, Oláh Á, *Al-Aissa Z, Hadarits O, Kiss K, Firneisz G:* Extension of the CD4(+) Foxp3(+) CD25(-/low) regulatory T-cell subpopulation in type 1 diabetes mellitus. *Autoimmunity*, 2015, 48(5), 289-297. IF: 2.917

Zóka A, Barna G, Hadarits O, *Al-Aissa Z, Wichmann B, Múzes G, Somogyi A, Firneisz G:* Altered crosstalk in the dipeptidyl peptidase-4-incretin-immune system in type 1 diabetes: A hypothesis generating pilot study. *Hum Immunol*, 2015, 76(9), 667-672. IF: 2.127

Hadarits O, Zoka A, Barna G, et al.: Increased Proportion of Hematopoietic Stem and Progenitor Cell Population in Cord Blood of Neonates Born to Mothers with Gestational Diabetes Mellitus. *Stem Cells Dev*, 2016, 25(1), 13-17. IF: 3.562

**Review articles not related to the PhD thesis, but no original research data presented**

Zóka A, Müzes G, Somogyi A, Varga T, Szémán B, *Al-Aissa Z*, Hadarits O, Firneisz G: Altered immune regulation in type 1 diabetes. *Clin Dev Immunol*, 2013, 2013, 254874. IF: 2.934

**7. Bibliography**

1. Kun A, Tornoczky, J., Sudar, Z., Kerényi, Z., Tabak, A.G.: Pregnancy outcomes of women with untreated GDM (according to the WHO 2013 diagnostic criteria). European Association for the Study of Diabetes 51th Annual Meeting; 14-18 September, 2015; Stockholm 2015. *Diabetologia*2015.
2. Al-Aissa Z, Hadarits O, Rosta K, et al.: [A brief of gestational diabetes mellitus, risk factors and current criteria of diagnosis]. *Orv Hetil*, 2017, 158(8), 283-290.
3. Al-Aissa Z, Rosta K, Hadarits O, et al.: Cord serum dipeptidyl-peptidase 4 activity in gestational diabetes. *Eur J Clin Invest*, 2015, 45(2), 196-203.
4. Rosta K, Al-Aissa Z, Hadarits O, et al.: Association Study with 77 SNPs Confirms the Robust Role for the rs10830963/G of MTNR1B Variant and Identifies Two Novel Associations in Gestational Diabetes Mellitus Development. *PLoS One*, 2017, 12(1), e0169781.