Diabetologia (2004) 47:878–884
DOI 10.1007/s00125-004-1388-5

Diabetologia

Genotypic and phenotypic differences between Arabian and Scandinavian women with gestational diabetes mellitus

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Abstract

Aims/hypothesis. Gestational diabetes mellitus is a heterogeneous disorder characterised by impaired insulin secretion and action. Our aim was to study whether autoimmunity, variations in genes affecting insulin secretion and action, or both, contribute to the development of gestational diabetes and whether the pathogenesis of the disease differs between women with a Scandinavian or Arabian background.
Methods. We studied a total of 500 unrelated women with gestational diabetes (400 Scandinavian and 100 Arabian) and 550 unrelated pregnant non-diabetic control women (428 Scandinavian and 122 Arabian) matched for ethnicity.
Results. Arabian women with gestational diabetes were 50% more insulin resistant for the same BMI compared with Scandinavian women with the disease (homeostasis model assessment [HOMA-IR]; 3.2±0.3 vs 2.2±0.2, p=0.02). Both Scandinavian (4.2% vs 0.9%, p=0.008) and Arabian (4.6% vs 0.0%, p=0.03) women with gestational diabetes had a higher frequency of GAD antibodies (GAD65Ab) than the matched controls. The frequency of *HLA-DQB1* risk genotypes was slightly higher in Scandinavian women with gestational diabetes than in the Scandinavian controls (46.3% vs 38.8%, p=0.03) but no significant difference was found between the Arabian women with gestational diabetes and the Arabian controls (47% vs 51.6%, p=0.47). There were no significant differences in the frequency of the insulin gene variable number of tandem repeat (*INS VNTR*) alleles and genotypes or the peroxisome proliferator-activated receptor-gamma 2 (*PPARγ2-Pro12Ala*) polymorphism between the women with gestational diabetes and the control women either in Arabian or in Scandinavian women.
Conclusions/interpretation. Gestational diabetes mellitus was associated with the presence of GAD65Ab in both study groups. Scandinavian women with gestational diabetes may share some genetic features with Type 1 diabetes. In addition, Arabian women with gestational diabetes are more insulin resistant than Scandinavian women with gestational diabetes and with the same BMI.

Keywords Arabian · Autoimmunity · GAD65Ab · Gestational diabetes mellitus · *HLA-DQB1* · Insulin resistance · *INS VNTR* · mtDNA · *PPARγ2* · Scandinavian

Received: 11 December 2003 / Accepted: 1 March 2004
Published online: 17 April 2004

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Abbreviations: GAD65Ab, GAD antibodies · GDM, Gestational diabetes mellitus · INS VNTR, Insulin gene variable number of tandem repeat · mtDNA, Mitochondrial DNA · PPARγ2, Peroxisome proliferator-activated receptor-gamma2

Introduction

Gestational diabetes mellitus (GDM) is defined as carbohydrate intolerance with onset or first recognition during pregnancy [1]. It is characterised by impaired insulin secretion and action [2, 3]. Gestational diabetes complicates about 1 to 3% of all pregnancies in the western world [4], whereas higher rates are reported among small ethnic groups [5]. There is no international consensus regarding the definition of diagnostic criteria for GDM. In Sweden the diagnosis of GDM is based on a 75-g OGTT and defined as a 2-h capillary glucose concentration of at least 9 mmol/l. According to these criteria approximately 1.2% of pregnant wom-

en in Sweden develop GDM [6]. In Arabian women a GDM prevalence from 5 to 38% has been reported [7, 8]. Although most women with GDM revert to normal after delivery, impaired glucose tolerance and/or diabetes develop in about 50% within 10 years postpartum [9, 10]. Women with GDM often have a history of maternal diabetes, which suggests a genetic component for the disease [11]. Moreover, the offspring of women with abnormal glucose tolerance during pregnancy are at a higher risk of developing insulin resistance, obesity or diabetes at an early age [12]. To date, several genetic studies have been carried out to identify susceptibility genes predisposing for the development of GDM. Associations have been reported between GDM and variants in the glucokinase [13], mitochondrial DNA [14, 15], β_3-adrenergic receptor [16], sulphonylurea receptor 1 (*SUR1*) [17], insulin receptor and insulin-like growth factor 2 (*IGF2*) genes [18]. Some of these associations have not been replicated [19, 20]. This inconsistency may be due, in part, to ethnic heterogeneity between different populations.

HLA class *II* alleles on the short arm of chromosome 6 and autoantibodies including islet cell antibodies (ICA), GAD65 autoantibodies and insulin autoantibodies (IAA) are strongly associated with immune-mediated Type 1 diabetes, which is characterised by beta-cell destruction and absolute insulin deficiency [21]. Increased frequencies of HLA-risk antigens and high prevalence of ICA, insulinoma-associated antigen 2 (IA-2) and GAD antibodies have also been reported in women with GDM [22, 23].

Studies have shown that variation in the variable number of tandem repeat (*VNTR*) mini-satellite located in the promoter region of the insulin gene (*INS*) is associated with several diseases or phenotypes including Type 1 diabetes, central obesity, insulin resistance, polycystic ovary syndrome, birth weight and Type 2 diabetes [24, 25, 26]. Depending on the number of repeats, *INS VNTR* can be divided into class *I* (26–63 repeats), *II* (64–140 repeats) and *III* (141–209 repeats) [27]. The number of repeats is considered to influence expression of the insulin gene in both the thymus and the pancreas [28, 29]. Whereas the class *I* allele has been associated with increased risk of Type 1 diabetes, the class *III* genotype has been suggested to increase risk of Type 2 diabetes. Cross-sectional studies have shown that the protective *Ala* allele of the *PPARγ2-Pro12Ala* polymorphism is associated with reduced risk of Type 2 diabetes [30]. The maternally inherited mutation *A3243G* in the mitochondrial $tRNA^{leu\ (UUR)}$ gene is associated with maternally inherited diabetes and deafness (MIDD), which is characterised by pancreatic beta cell dysfunction [31].

We investigated whether autoimmunity, variations in genes affecting insulin secretion and action, or both, contribute to the development of GDM and whether GDM pathogenesis differs between women with a Scandinavian or Arabian background.

Subjects and methods

Study population

All pregnant women in the southern part of Sweden are routinely offered a 75-g OGTT at 27 to 28 weeks of pregnancy. The tests are carried out in the local antenatal care clinics, using HemoCue devices (HemoCue, Ängelholm, Sweden) for capillary whole blood analysis. Women at high risk (previous GDM or a family history of diabetes) are also offered an OGTT at 12 to 13 weeks of pregnancy. GDM is defined as a 2-h capillary glucose concentration (double-test) of at least 9 mmol/l. We recruited 500 unrelated GDM women (400 Scandinavian and 100 Arabian) and 550 unrelated non-diabetic pregnant controls (428 Scandinavian and 122 Arabian) consecutively from the screening procedure in southern Sweden. The Arabian women were immigrants from most of the Arab countries (Iraq, Lebanon, Morocco, Palestine, Syria, etc.). The reason for the different sample size between the two populations was the limited number of Arabs living in Sweden. The clinical and metabolic characteristics were available only for GDM women living in the city of Malmö who were invited to take part in a 5-year follow-up study with repeated OGTTs at 1, 2 and 5 years postpartum. The population in the southern part of Sweden is very homogenous and we therefore considered this subset to be representative of the larger group of 500 women with GDM. Before participating in the study, the purpose, nature and potential risks were explained, and informed written voluntary consent was obtained from each subject. The study protocol was approved by the ethics committee of Lund University.

Genetic analyses

A3243G mutation in the mitochondrial tRNAleu gene. Total DNA was isolated from peripheral blood lymphocytes or blood samples were collected as dried blood spots on Whatman filters (VWR International, Stockholm, Sweden), and punch-outs in 96-well plates were soaked directly in PCR amplification buffer. PCR was carried out using primers specific to mtDNA [31]. A 427-bp fragment was digested overnight with ApaI (New England Biolabs, Beverly, Mass., USA) at 37 °C. Samples were electrophoresed on 5% polyacrylamide gel under non-denaturing conditions and stained with ethidium bromide to visualise the fragments using GELSCAN2000 (Applied Biosystems, Foster City, Calif., USA).

HLA-DQB1 genotyping. The second exon of the *DQB1* gene was amplified using biotinylated PCR primers as described previously with modification of the forward primer (5′-CA TGT GCT ACT TCA CCA ACG G) [32]. After amplification, DNA was captured onto streptavidin-coated microtitre wells and denatured using mild alkaline solution. Hybridisation was done with a panel of lanthanide-labelled probes specific for *HLA-DQB1* alleles and with a probe controlling DNA amplification. We used five probes to distinguish *DQB1* alleles. Of them, four (0602/3, 0201, 0301 and 0302) have been described previously [32] in addition to (0603/4; 5′-TTG TTA CCA GAC ACA). After washing and adding the enhancement solution, several fluorescent signals were detected simultaneously by time-resolved flurometry using Victor 2 (Wallac Oy, Turku, Finland).

Hph1 polymorphism genotyping of the INS VNTR. The *T/A* polymorphism located 23 bp 5′ of the start codon is in link-

Table 1. Clinical characteristics of Arabian and Scandinavian women with GDM

Variable	Scandinavian (*n*)	Arabian (*n*)	*p* value
Age (years)	32.4±0.4 (400)	31.9±0.6 (100)	0.8
BMI (kg/m^2)	28.9±0.5 (111)	30.9±0.6 (51)	0.004
HbA_1c (%)	4.1±0.1 (111)	4.3±0.1 (49)	0.2
Fasting plasma glucose (mmol/l)	4.9±0.1 (68)	5.7±0.2 (20)	0.002[a]
P-glucose 30-min (mmol/l)	8.5±0.1 (59)	9.2±0.4 (16)	0.05[a]
P-glucose 2-h (mmol/l)	9.2±0.2 (64)	10.3±0.6 (20)	0.07
Fasting serum insulin (mU/l)	10.0±0.7 (64)	12.9±1.3 (20)	0.2[a]
S-insulin 30-min (mU/l)	44.7±3 (55)	40.7±4 (16)	0.7
S-insulin 2h-min (mU/l)	71.5±4.7 (57)	82.3±10.8 (16)	0.3
FS-C-peptide (nmol/l)	0.47±0.02 (63)	0.53±0.04 (22)	0.2
HOMA-IR	2.2±0.2 (63)	3.2±0.3 (20)	0.02[a]
I/G30 (mU/mmol)	9.8±1.0 (53)	8.3±0.8 (16)	0.9
(I/G30)/HOMA-IR	5.7±0.6 (53)	3.3±0.6 (16)	0.01[a]

Data are means ± SEM. As all clinical data were not available from all study subjects, the number of individuals is given in parentheses. [a]After adjustment for BMI (ANCOVA)

age disequilibrium with *VNTR* alleles. The *T* allele is in linkage disequilibrium with the short (Class *I*) and the *A* allele with the long (Class *III*) *VNTR* alleles [24]. We used a restriction fragment length polymorphism method involving digestion of the PCR-amplified DNA with HphI (New England Biolabs, Beverly, Mass., USA) enzyme [33]. The *VNTR* classes were inferred directly from the *Hph1* genotypes. The *T/T* genotype was referred to as *I/I*, the *T/A* as *I/III* and the *A/A* as *III/III*.

PPARγ2-Pro12Ala genotyping. The exon B of the *PPARγ2* gene was genotyped by PCR-RFLP using primers 5′-GAT AGA GAC AAA ATA TCA GTG (forward primer) and 5′-GTA TCA GTG AAG GAA TCG CTT TCC G (reverse primer). PCR was carried out with 25 ng genomic DNA or dried blood spots in a total volume of 20 µl containing 1×$(NH_4)_2$ SO_4-buffer [16 mmol/l $(NH_4)_2SO_4$, 67 mmol/l TRIS pH 8.8, 0.01 TWEEN 20], 10 µmol/l each dNTP, 2.4 mmol/l $MgCl_2$, 0.5 U *Taq* polymerase (Amersham Pharmacia Biotech, Uppsala, Sweden), 1.5% Formamide and 10 pmol of each primer. The cycling conditions were 94 °C for 5 min, 35 cycles of 94 °C for 30 s, 56 °C for 30 s and 72 °C for 60 s followed by final extension at 72 °C for 10 min. PCR was followed by digestion with BstUI (New England Biolabs, Beverly, Mass., USA) at 60 °C for 2 h, and digests were separated on 4.5% aragose gel (SeaKem, Rockland, Me., USA) and stained with ethidium bromide to visualise the fragments. The *Pro12* allele gives a 113-bp fragment, whereas the *Ala12* allele gives fragments of 87 bp and 25 bp.

GAD65 autoantibodies (GAD65Ab). GAD65Ab were measured by a radio-immunoprecipitation assay using ^{35}S-labelled recombinant human GAD65 produced by coupled in vitro transcription-translation as described [34]. Punch-outs from dried blood spots were incubated in assay buffer overnight to elute antibodies [34]. The results are expressed as relative units (RU): RU=(sample cpm−mean cpm of three negative controls)/(cpm of a positive internal reference−mean cpm of three negative controls)×100. The cut-off limit for positivity was 5 RU. According to standardised international units, 5 RU is equal to 32 U/ml. At the Combined Autoantibody Workshop [35], the specificity and sensitivity of the GAD65Ab assay were 99 and 75% respectively. GAD65Ab were analysed in all control subjects and in 376 GDM women (289 Scandinavian and 87 Arabian).

Metabolic measurements

Blood glucose was measured using HemoCue devices or by a glucose oxidation method. Serum insulin concentrations were measured using an enzyme immunoassay from Dako (Cambridgeshire, UK). BMI was calculated as weight/height^2 (kg/m^2). Homeostasis model assessment (HOMA-IR; fasting serum insulin × fasting plasma glucose/22.5) was used to estimate the degree of insulin resistance [36]. Beta cell function was estimated as the insulinogenic index during the first 30 min of the OGTT (I/G30: serum insulin 30 min−fasting serum insulin/plasma glucose 30 min−fasting plasma glucose) [37]. Since insulin resistance is known to modulate insulin secretion, we adjusted insulin secretion measured as I/G30 for insulin resistance by dividing I/G30 by the HOMA-IR [38].

Statistical analyses

Clinical data are presented as means ± SEM. Significance of differences between group means was tested by the Mann-Whitney U test or analysis of variance or covariance (ANCOVA) with BMI and *PPARγ2* genotype as covariates. Logarithmic transformation was used for data with right-skewed distribution. Allele and genotype frequencies were compared between groups by chi square or Fisher's exact test. The statistical analyses were carried out using the Number Cruncher Statistical Systems (NCSS, Kaysville, Utah, USA) and BMDP Statistical Software, Version 1.12 (BMDP, Los Angeles, Calif., USA). Two-sided *p* values of less than 0.05 were considered statistically significant.

Results

The Arabian GDM women had a higher HOMA-IR index (3.2±0.3 vs 2.2±0.2, p=0.02) and a lower disposition index, i.e. their beta cell compensation for the degree of insulin resistance [(I/G30)/HOMA-IR] was impaired (3.3±0.6 vs 5.7±0.6, p=0.01), compared with Scandinavian GDM women after adjustment for BMI (Table 1).

Table 2. *HLA-DQB1* genotype frequencies in Scandinavian and Arabian women with and without GDM

HLA-DQB1 Genotype	Scandinavian women		Arabian women	
	GDM *n* (%)	Controls *n* (%)	GDM *n* (%)	Controls *n* (%)
02/X	98 (24.5)	85 (19.9)	29 (29)	38 (31.2)
0302/X	63 (15.8)	59 (13.8)	12 (12)	18 (14.8)
02/0302	24 (6)	22 (5.1)	6 (6)	7 (5.7)
02/X or *0302/X* or *02/0302*	185 (46.3)[a]	166 (38.8)	47 (47)	63 (51.6)
0602(3)/X	85 (21.3)	102 (23.8)	13 (13)	11 (9)

X means either a homozygous allele or any allele other than 02, 0302 or 0602(3)
[a] p=0.03 (corrected p value for multiple comparisons p>0.1), Scandinavian GDM women vs Scandinavian control women

Table 3. The *PPARγ2-Pro12Ala* genotype and allele frequencies in Scandinavian and Arabian women with and without GDM

	Scandinavian women		Arabian women	
	GDM *n* (%)	Controls *n* (%)	GDM *n* (%)	Controls *n* (%)
Genotype				
Pro/Pro	286 (71.5)	317 (74.1)	91 (91)	106 (86.9)
Pro/Ala	111 (27.7)	105 (24.5)	9 (9)	15 (12.3)
Ala/Ala	3 (0.8)	6 (1.4)	0 (0.0)	1 (0.8)
Allele				
Pro	683 (85.4)	739 (86.3)	191 (95.5)	227 (93)
Ala	117 (14.6)	117 (13.7)	9 (4.5)	17 (7)

Table 4. *INS VNTR* genotype and allele frequencies in Scandinavian and Arabian women with and without GDM

	Scandinavian women		Arabian women	
	GDM *n* (%)	Controls *n* (%)	GDM *n* (%)	Controls *n* (%)
HphI Genotype				
I/I	202 (50.5)	214 (50)	61 (61)	80 (65.6)
I/III	169 (42.3)	185 (43.2)	34 (34)	38 (31.1)
III/III	29 (7.2)	29 (6.8)	5 (5)	4 (3.3)
HphI Allele				
I	573 (71.6)	613 (71.6)	156 (78)	198 (81.1)
III	227 (28.4)	243 (28.4)	44 (22)	46 (18.9)

GAD65 autoantibodies. The presence of GAD65Ab was associated with GDM in both study populations. Among Scandinavian women with GDM, 12/289 (4.2%) were positive for GAD65Ab compared with 4/428 (0.9%, p=0.008) in the controls. Similar frequency was observed in Arabians where 4/87 (4.6%) of GDM women were positive for GAD65Ab compared with 0/122 (0.0%, p=0.03) in the controls.

HLA-DQB1 genotypes. The frequency of *HLA-DQB1 *0201/0302* or **0201/X* or **0302/X* (X excludes *0602/3*) risk genotypes was slightly higher in Scandinavian women with GDM than in the Scandinavian controls (46.3% vs 38.8%, p=0.03; corrected p value for multiple comparisons p>0.1) but no significant difference was seen between Arabian women with GDM and the Arabian controls (47% vs 51.6%, p=0.47) (Table 2). In Scandinavian GDM patients, the presence of GAD65Ab was associated with *HLA-DQB1* risk genotypes (p=0.04).

PPARγ2. The *Pro12Ala* allele and genotype frequencies of the *PPARγ2* gene are shown in Table 3. There was no significant difference in the frequency of the *Pro12Ala* variant between Arabian or Scandinavian women with GDM and the controls matched for race. We also tested whether, as previously shown, there was a difference in HOMA-IR between carriers of the different *PPARγ2* genotypes. In this study, HOMA-IR also differed significantly between carriers of the *Ala/Ala or Pro/Ala* and *Pro/Pro* (1.9±0.1 vs 2.5±0.2, p=0.11; one-tailed p value <0.05) genotypes. However, HOMA-IR still differed significantly between Arabian and Scandinavian GDM women after adjusting for the *PPARγ2-Pro12Ala* genotype (p=0.02).

INS VNTR. There were no significant differences in the frequency of the *INS VNTR* alleles or genotypes between GDM and control subjects in either Arabian or Scandinavian women (Table 4).

The *A3243G* mutation in the mitochondrial *tRNA^{leu}* gene was rare in the study populations. It was found in only one Arabian (1.0%) and one Scandinavian (0.3%) woman with GDM but not in the controls. The Arabian GDM woman had a maternal history of diabetes. She was 38 years old at the time of diagnosis, had a fasting C-peptide concentration of 0.28 nmol/l and was GAD65Ab negative. She had no hearing loss. The Scandinavian woman had no family history of diabetes. She was 34 years old at diagnosis and also GAD65Ab negative. She had no hearing loss.

Discussion

We demonstrate that the relative distribution of genotypes conferring risk for Type 1 diabetes and variants known to impair insulin secretion and action differ between Scandinavian and immigrant Arabian women living in Sweden. Our finding that Scandinavian women with GDM have a higher frequency of GAD65Ab than Scandinavian control women supports a Finnish study that concluded that GDM in some Scandinavian women may represent an autoimmune form of diabetes [39]. A similar difference was observed between Arabian GDM and control women, suggesting that autoimmunity may contribute to the development of GDM in Arabian women as well. To our knowledge, this is the largest report on GAD65Ab in GDM and control women and the first report studying the potential role of GAD65Ab in Arabian GDM women. Whether Type 1 diabetes-associated markers such as GAD65Ab, ICA and insulin autoantibodies are associated with GDM is, however, still controversial. A lower frequency (2.2%) of GAD65Ab was reported in GDM women from other Scandinavian countries [40]. The frequency of GAD65Ab has been shown to vary between different populations. In Maine (USA), about 6% of the women with GDM were positive for GAD65Ab [41], whereas the frequency in GDM women from Germany was as high as 9.5% [23]. In Italy, the frequency of GAD65Ab varied from 0 to 3.6% in GDM women [42, 43]. Although the confidence interval for these frequencies may overlap, it suggests a significant contribution of Type 1 diabetes in the GDM population in some but not all populations. These discrepancies between studies might be due to differences in selection criteria, in ethnic background of the subjects and in GAD65Ab assay methodology. In our study, GDM women were recruited irrespectively of the type of treatment or family history of diabetes.

In a previous smaller study, we found that the *HLA-DQB1 *02/X* (*X* excludes *0302* or *0602/3*) was significantly increased in Swedish GDM women who had a family history of diabetes compared with subjects with NGT, but no significant difference was observed in the frequency of GAD65Ab [44]. In the present study, Scandinavian GDM women had a slightly higher frequency of *HLA-DQB1* risk genotypes than the Scandinavian controls. However, these differences were not statistically significant after adjustment for multiple comparisons (corrected p value for multiple comparisons $p>0.1$). This may, however, represent an over correction, as the *HLA* genotypes tested are in strong linkage disequilibrium [45] and thereby do not represent independent observations. A report showed a two-fold increase in the frequency of HLA-DR3 and -DR4 antigens in GDM compared with the controls matched for race, and the increase was statistically significant in black women from the United States [22]. Another study found no significant difference in the frequency distribution of *HLA-DQB1*, *-DQA1* and *-DRB1* alleles between Caucasian GDM and control women from Germany [46].

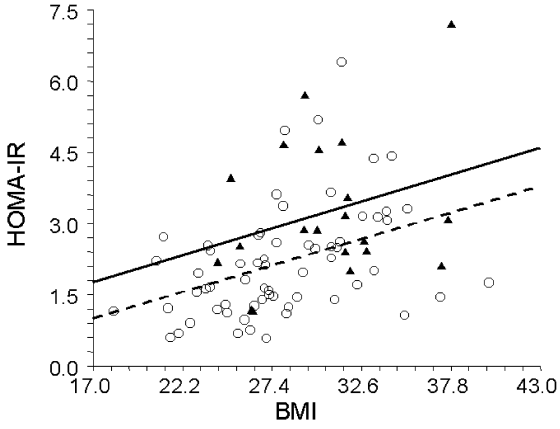

Fig. 1. Relation between HOMA-IR and BMI in Arabian (*solid triangles and solid line*) and Scandinavian (*empty circles and dashed line*) women with GDM

A higher frequency of GDM in populations with a high frequency of Type 2 diabetes has been reported [47]. As Type 2 diabetes is more common in the Arabian population [48] compared with Scandinavians [49], we hypothesised that Arabian GDM women would be more insulin resistant than Scandinavian GDM women. This was the case; the Arabian GDM women were 50% more insulin resistant than Scandinavian GDM women, as judged from the HOMA-IR index (3.2±0.3 vs 2.2±0.2, p=0.02). Importantly, this difference was not due to differences in BMI (Fig. 1). We did not observe a significant difference in the frequency of the *Pro12Ala* variant between the GDM women and the controls in either Arabian or Scandinavian women. This may simply represent a power issue, as the sample size required to demonstrate associations with a susceptibility allele with a relative risk in the range of 1.2 clearly exceeds the numbers included in this study and most studies on GDM. Given the previous data on a genotype–phenotype correlation between the *Pro12Ala* polymorphism of the *PPARγ2* gene [30, 50, 51] and the current finding of a difference in HOMA-IR between carriers of the *PPARγ2* genotypes, this polymorphism may partly explain the difference seen in HOMA-IR between the Arabian and Scandinavian women with GDM. However, since adjusting the ethnic difference in HOMA-IR for genotype did not abolish the difference between the two groups, other factors must also contribute to the difference.

A possible association between *INS VNTR* and GDM has only been investigated in GDM women from Greece. The *INS VNTR III/III* genotype was shown to be more frequent in GDM women than in the controls (8.7% vs 2.7%, p=0.02) [52]. In our study, there were no differences in allele or genotype

frequencies of the *INS VNTR* between the GDM women and the controls in either, Arabian or Scandinavian women. This discrepancy between the results may be due to ethnic differences and the use of different diagnostic criteria.

The role of mitochondrial mutations in the pathogenesis of GDM has also been studied in different populations. The A3243G mutation was reported in one of twelve Japanese women with GDM [15]. A T to C substitution at nucleotide 3398 in the mitochondrial *ND1* gene was associated with GDM in women from Singapore [14]. The frequency of the A3243G mutation in mitochondrial $tRNA^{leu}$ gene was rare in our study in women with GDM, thus excluding it as an important susceptibility factor for GDM, which is consistent with previous observations in other populations [14, 19].

In conclusion, we demonstrate in a large study that GDM is associated with the presence of GAD65Ab in both study populations. Scandinavian women with GDM may share some genetic features with Type 1 diabetes. In addition, Arabian women with GDM were approximately 50% more insulin resistant than Scandinavian women with GDM and with the same BMI.

Acknowledgements. This work was supported by grants from the JDF Wallenberg Foundation (99JD-12812), the Swedish Medical Research Council (VR 72X-14064), EC grant (GIFT), the Novo Nordisk Foundation and grants to the Diabetes Prediction in Skåne (DiPiS) study. We thank the patients for their participation, and the DiPiS research group for helping with recruitment of some of the GDM and control women and for helping with HLA typing and GAD65 antibody analyses.

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