Genetic and immunological risk factors of gestational diabetes mellitus

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ACADEMIC DISSERTATION

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“And of knowledge, you (mankind) have been given only a little”

The Holy Quran, Surat Al Isra’, verse (85)

To my beloved parents *Nasser* and *Shafwa*
To my brothers and sisters
To my uncle Shaker Al Bornow
To Dr. Osama Al Rayyes

CONTENTS

ABBREVIATIONS

ADA	American Diabetes Association
ADRB 3	beta3-adrenergic receptor
APM1	adiopnectin gene
BMI	body mass index
CAPN10	calpain 10 gene
CVD	cardiovascular disease
DBS	dried blood spots
EASD	European Association for the Study of Diabetes
ENPP1	ectonucleotide pyrophosphatase/phosphodiesterase 1 gene
FOXC2	forkhead transcription factor gene
GAD65Ab	glutamic acid decarboxylase-65 antibodies
GCK	glucokinase gene
GCT	glucose challenge test
GDM	gestational diabetes mellitus
HFE	hemochromatosis gene
HLA	human leukocyte antigen
HNF1A	hepatocyte nuclear factor-1α gene
HOMA-IR	homeostasis model assessment
IA-2Ab	tyrosine phosphatase antibodies
IAA	insulin antibodies
ICA	islet cell antibodies
IDF	International Diabetes Federation
IGF2	insulin-like growth factor 2 gene
INS	Insulin gene
INSR	insulin receptor gene
IRS1	insulin receptor substrate 1 gene
KCNJ11	potassium inwardly-rectifying channel, subfamily J, member 11 gene
Kir6.2	pancreatic beta-cell ATP-sensitive K+ (K_{ATP}) channel subunit
LADA	Latent autoimmune diabetes in adults
LEP	leptin gene
MBL2	mannose-binding lectin 2 gene
MHC	major histocompatibility complex
MODY	Maturity onset diabetes of the young
MtDNA	mitochondrial DNA
NCEP ATPIII	National Cholesterol Education Program Adult Treatment Panel III
NDDG	National Diabetes Data Group
NGT	normal glucose tolerance
OGTT	oral glucose tolerance test
OR	odds ratio
PBEF1	pre-B-cell colony enhancing factor 1 gene

PPARG	peroxisome proliferator-activated receptor gamma2 gene
PPARGC1	PPAR-gamma coactivator 1, alpha gene
SEM	standard error of mean
SLC2A4	solute carrier family 2 (facilitated glucose transporter), member 4 gene
SNP	single nucleotide polymorphism
SUR1	Sulfonylurea receptor 1 gene
UCP2	uncoupling protein 2 gene
WHO	World Health Organisation
VNTR	variable number of tandem repeat

LIST OF ORIGINAL PAPERS

This thesis is based on the following papers, referred to in the text by their Roman numerals I-IV.

I. **Shaat N**, Ekelund M, Lernmark A, Ivarsson S, Nilsson A, Perfekt R, Berntorp K, Groop L (2004) Genotypic and phenotypic differences between Arabian and Scandinavian women with gestational diabetes mellitus. *Diabetologia*: 45:878-884*

II. **Shaat N**, Ekelund M, Lernmark A, Ivarsson S, Almgren P, Berntorp K, Groop L (2005) Association of the E23K polymorphism in the *KCNJ11* gene with gestational diabetes mellitus. *Diabetologia* 48:2544-2551*

III. **Shaat N**, Karlsson E, Lernmark A, Ivarsson S, Lynch K, Parikh H, Almegren P, Berntorp K, Groop L (2006) Common variants in MODY genes increase the risk for gestational diabetes mellitus. *Diabetologia, in press*

IV. **Shaat N**, Lernmark A, Karlsson E, Ivarsson S, Parikh H, Almegren P, Berntorp K, Groop L Association testing of common variants predisposing to the metabolic syndrome or related traits with gestational diabetes mellitus. *Submitted to JCEM*

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Abstract

Gestational diabetes mellitus (GDM) is a heterogeneous disorder that is defined as carbohydrate intolerance with onset or first recognition during pregnancy. Impaired beta-cell function and insulin resistance are the hallmarks of GDM. The overall aim of this thesis was to study the genetic and immunological risk factors that increase susceptibility to GDM. First, we investigated whether autoimmunity and genetic variants affecting insulin secretion or action, or both, contribute to the development of GDM. We found that GDM was associated with the presence of glutamic acid decarboxylase-65 antibodies (GAD65Ab) in Arabian and Scandinavian women. In addition, Scandinavian women with GDM were found to share some genetic features such as HLA DQB1 risk genotypes (odds ratio [OR] 1.36, [95% CI 1.03−1.79], p=0.03) with type 1 diabetes. Furthermore, Arabian women with GDM were more insulin resistant than Scandinavian women with GDM and with the same BMI. We also investigated whether GDM has a similar genetic predisposition as type 2 diabetes by studying common genetic polymorphisms that have previously been associated with type 2 diabetes. Among 5 studied polymorphisms, we found that the E23K polymorphism of the potassium inwardly-rectifying channel, subfamily J, member 11 (KCNJ11) gene was associated with a modestly increased risk (1.17, [1.02 −1.35], p=0.027) of GDM. This is compatible with its effect on insulin secretion and the crucial role of impaired beta-cell function in the pathogenesis of GDM. Additionally, we studied whether common variants in MODY [Glucokinase (GCK), hepatocyte nuclear factor 1-alpha (HNF1A), and HNF4A] genes also increase the risk of GDM. We found that the A-allele of the –30G>A polymorphism in the beta-cell-specific promoter of the GCK increases the risk of GDM with a modest OR of 1.28 ([1.06 −1.53], p=0.008). Moreover, the HNF1A I27L polymorphism was also associated with an increased risk of GDM (1.16 [1.001−1.34], p=0.048). All these variants are supposed to influence beta-cell function. Finally, we tested whether common genetic variants that have been associated with the metabolic syndrome or its components would also confer risk for GDM. We found that the T-allele of the +276G>T polymorphism of the adiponectin (APM1) gene, which has previously been associated with insulin resistance, increases the risk of GDM with an OR of 1.17 ([1.01−1.36], p=0.039).
We conclude that common variants in several type 1 and type 2 diabetes candidate genes in addition to immunological factors increase susceptibility to heterogeneous GDM.

Key words: Association, autoimmunity, GDM, genetics, gestational diabetes mellitus, metabolic syndrome, MODY, risk factors, type 1 diabetes, type 2 diabetes.

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REVIEW OF THE LITERATURE

INTRODUCTION

History

In 1824, Heinrich Bennewitz defended his doctoral thesis in which he described a woman with diabetes during pregnancy, although he thought that diabetes was a symptom of her pregnancy [1]. Glycosuria, which disappeared postpartum, was the only biochemical evidence of diabetes [1, 2]. In 1882, Matthews Duncan reported an increased risk of foetal death in pregnancies complicated by diabetes, and the mother herself died from diabetes within a year in most cases [3]. In the middle of the 20th century, several studies reported an increased likelihood of perinatal mortality and delivery of large infants to mothers who subsequently developed diabetes in middle age [4-7]. These early reports formed the concept of gestational diabetes as a "pre-diabetic state".

Definition

The term "Gestational diabetes" was probably first used by O´Sullivan in 1961 [8] replacing the term meta-gestational diabetes proposed by Hoet in 1954 [9]. Also, Jorgen Pedersen used the term gestational diabetes in 1967 [10]. However, a definition of gestational diabetes mellitus (GDM) was first provided at the 1st International Workshop-Conference on GDM in 1980, in which it was defined as "carbohydrate intolerance with onset or first recognition during pregnancy" [11]. The diagnosis of GDM applies regardless of whether insulin is used or the condition persists after pregnancy [12]. However, GDM does not apply to pregnant women with previously diagnosed diabetes, but it does not exclude the possibility that unrecognized glucose intolerance may have antedated the pregnancy [12]. GDM represents approximately 90% of all pregnancies complicated by diabetes [13].

Inheritance

In 1985, Martin et al. demonstrated that women with a maternal family history of diabetes have an increased risk of developing GDM and suggested that this might be due to exposure to an abnormal environment during intrauterine development [14]. However, subsequent studies have consistently shown that women with a family history of diabetes have an increased risk of GDM irrespective of whether of maternal or paternal origin [15-19]. These results suggest a genetic component of the disease.

EPIDEMIOLOGY

Prevalence of GDM

The GDM epidemic is underway with a progressively increasing prevalence during the last decades [20-24]. The prevalence of GDM varies markedly between different ethnic populations. Whereas high rates have been reported in Asian (~5-10%), Hispanic/Mexican-American (~5-7%) and Arab (~5-7%) populations, the prevalence among Caucasians is approximately 2-4%. These differences might also be attributed partially to the usage of different diagnostic criteria. Table 1 summarizes the prevalence of GDM in women from different populations.

Recurrence of GDM

GDM usually reverts to normal glucose tolerance (NGT) after delivery, but it may reappear in subsequent pregnancies. The recurrence rate of GDM varies between 17-70% in different populations [18, 25-35]. This may reflect true heterogeneity but may also be attributed to the use of different diagnostic criteria. Several factors in the index pregnancy predispose to the recurrence of GDM in subsequent pregnancies such as advanced maternal age (> 30 years old), obesity (BMI ≥30 kg/m^2), early deterioration of glucose tolerance (gestation age <24 weeks), need for insulin treatment and delivery of macrosomic infant. Weight gain between pregnancies, multiparity, short interval between pregnancies, and being a member of an ethnic group with high prevalence of diabetes are also associated with recurrence of GDM [26, 27, 30-36].

Of note, Moses et al. found that women with recurrent GDM during a subsequent pregnancy had higher fat intake when compared with women in whom GDM did not recur [37]. This may suggest that dietary modification of fat intake before and during pregnancy may reduce the recurrence rate of GDM [37].

Screening for and diagnosis of GDM

Screening for GDM is recommended in all pregnancies unless the pregnant woman is at low risk. Women at high risk of developing GDM should undergo glucose testing during the first trimester. If they are not diagnosed with GDM at their initial screening, they should be retested between 24 and 28 weeks of gestation [35, 38]. Risk factors for GDM include old age, obesity, multiparity, family history of GDM or diabetes, previous poor obstetric outcome, chronic hypertension, multiple pregnancy as well as high-risk ethnicity such as women of Hispanic, African, Native American, Asian, Pacific Islands or Indigenous Australian ancestry origin, particularly when they reside in Western countries or in an urban setting.

Table 1. Prevalence of gestational diabetes mellitus among different populations.

Country of investigation	Population/ Ethnic group	Diagnostic criteria for GDM	Prevalence (%)	Nr. of participants	Period of investigation	Ref.
America						
Canada	Aboriginal Cree	NDDG	12.8	579	1995-1996	[39]
USA	Non-Hispanic white	NDDG	1.9 -3.4	21,444	(1994/1996-2000/2002)	[24]
USA	Hispanic	NDDG	2.8 -5.1	5920	(1994/1996-2000/2002)	[24]
USA	African American	NDDG	2.5-4.6	2293	(1994/1996-2000/2002)	[24]
USA	Mexican-American (85%)	Carpenter-Coustan & NDDG	6.8	6857	1995–1999	[40]
Brazil	Brazilian	WHO (1985 & 1998)	7.6	5004	1991-1995	[41]
Europe and Australia						
Italy	Italian (Sicilian)	Carpenter-Coustan	4.6	2554	1990- 2000	[42]
Italy	Italian	Carpenter-Coustan	8.7	3806	1995-2001	[43]
UK	Caucasian	EASD	1.2	315	1991-1992	[44]
Denmark	Predominantly Danish	WHO 1985 or local	3.2	6158	1995-1997	[45]
Sweden	Swedish (85%)	EASD	1.2	12,382	1995-1998	[46]
New Zealand	European	Local	3.3	1623	1994-1995	[47]
New Zealand	Maori	Local	7.9	1297	1994-1995	[47]
New Zealand	Pacific Islanders	Local	8.1	1513	1994-1995	[47]
Australia	European	Local or WHO 1985	5.2	2749	1979-1988	[20]
Australia	Australian & New Zealand	Local or WHO 1985	4.3	23,257	1997-1988	[20]
Asia						
Sri Lanka	Sri Lankan	WHO	5.5	721	1998 [b]	[48]
UAE	Indian subcontinent	ADA (100-g OGTT)	35.3[a]	419	1998-2000	[49]
China	Chinese	WHO 1998	2.3	9471	1998-1999	[50]
Australia	Chinese	Local or WHO 1985	13.9	653	1979-1988	[20]
Australia	Vietnamese	Local or WHO 1985	7.3	1300	1979-1988	[20]
Australia	Indian subcontinent	Local or WHO 1985	15	440	1979-1988	[20]
Taiwan	Taiwanese (Taipei)	WHO 1985	0.6	872	1993[b]	[51]
Thailand	Thai	NDDG	10.2	1200	2001	[52]
Japan	Japanese	Local	2.9	749	1999-2001	[53]
Korea	Korean	NDDG	2.2	3581	1991-1993	[54]
Turkey	Turkish	NDDG	1.2	807	2003 [b]	[55]
Pakistan	Pakistani (Karachi)	Carpenter-Coustan	3.5	2230	1992 [b]	[56]
UK	Asian	EASD	5.8	49	1991-1992	[44]
USA	Asian	NDDG	6.3 -8.6	1465	(1994/1996-2000/2002)	[24]
India	Indian (Kashmiri)	Carpenter-Coustan or WHO 1998	3.8	2000	1999-2002	[57]
Iran	Iranian	Carpenter-Coustan	4.8	1310	1999-2001	[58]
Africa						
Ethiopia	Rural Ethiopian	WHO 1985	3.7	890	1999 [b]	[59]
UK	African/ Afro-caribbean	EASD	2.7	300	1991-1992	[44]
Australia	African	Local or WHO 1985	9.4	309	1979-1988	[20]
Arabs						
UAE	Arab	ADA (100 g OGTT)	30.9[a]	1098	1998-2000	[49]
Australia	Arab	Local or WHO 1985	7.2	836	1979-1988	[20]
Bahrain	Predominantly Arab	–	5.4	5199	1989 [b]	[60]

[a] Participants were women at risk for GDM or with a positive glucose challenge test (GCT). [b] year of publication.

The American Diabetes Association's (ADA) Fourth International Workshop-Conference on GDM held in 1997 recommended a one or two-step screening procedure for GDM [38]. The one-step procedure implies a diagnostic oral glucose tolerance test (OGTT) administered to all women, while in a two-step procedure, a 50 g oral glucose challenge test (GCT) is followed by a diagnostic 75 g or 100 g OGTT if 1-h plasma glucose concentration ≥ 7.8 mmol/l (≥140 mg/dl) [38].

The first criteria for the diagnosis of diabetes during pregnancy were proposed by O'Sullivan and Mahan in 1964 [61], and subsequently modified by Carpenter and Coustan [62]. In the United States, the most commonly used diagnostic criteria are those recommended by the ADA or the National Diabetes Data Group (NDDG) [38, 63]. The ADA supports the use of the Carpenter-Coustan diagnostic criteria for 100 g OGTT [38] or an alternative use of 75 g OGTT modified from Sacks et al. [64]. The NDDG criteria are also based on 100 g OGTT, but with cut-off values higher than those recommended by the ADA [63]. The most widely used criteria for the diagnosis of GDM in the other parts of the world are the World Health Organisation (WHO) criteria for diabetes in non-pregnant adults, which are based upon a 75 g OGTT [65, 66]. According to the Diabetic Pregnancy Study Group of the European Association for the Study of Diabetes (EASD), GDM is defined as a 2-hour capillary glucose concentration (double-test) ≥ 9 mmol/l (≥ 162 mg/dl) [67]. The different criteria used worldwide for the screening and diagnosis of GDM are summarized in Table 2.

Table 2. Criteria for screening and diagnosis of gestational diabetes mellitus.

	WHO (1985)		**WHO (1998)**		**EASD**	**NDDG**	**ADA**		
	IGT	**Diabetes**	**IGT**	**Diabetes**					
Glucose load for OGTT	75	75	75	75	75	100	100	75	50*
Fasting Glucose	< 7.8 (140)	≥ 7.8 (140)	< 7.0 (126)	≥ 7.0 (126)	-	≥ 5.8 (105)	≥ 5.3 (95)	≥ 5.3 (95)	-
1-h	-	-	-	-	-	≥ 10.6 (190)	≥ 10 (180)	≥ 10 (180)	≥ 7.8 (140)
2-h	7.8-11.0 (140-198)	≥ 11.1 (200)	7.8-11.0 (140-198)	≥ 11.1 (200)	≥ 9.0 (162)	≥ 9.2 (165)	≥ 8.6 (155)	≥ 8.6 (155)	-
3-h	-		-	-	-	≥ 8.1 (145)	≥ 7.8 (140)	-	-

Values are presented as mmol/l (mg/dl). *50 g GCT is used for screening purposes only (see text for details). All tests are performed after overnight fasting except the 50 g GCT test. Criteria are based on venous plasma concentrations except for the criteria by the EASD, which are based on capillary blood. Two or more values should be met or exceeded for the diagnosis of GDM according to NDDG [63] or ADA [38]. According to WHO (1985), one or both values should be met or exceeded for the diagnosis of GDM [65]. According to WHO (1998), pregnant women are diagnosed with GDM if criteria for the diagnosis of diabetes or IGT are met (one or both values should be met or exceeded for the diagnosis of "Diabetes" and both for the diagnosis of "IGT") [66].

PATHOPHYSIOLOGY OF GDM

Beta-cell function and GDM

During a normal pregnancy, several physiological alterations occur, providing a metabolic environment that initially favours maternal fat deposition and later optimizes foetal growth [68]. As gestation progresses, insulin secretion increases, reaching a maximum in the third trimester in both normal and GDM pregnancy [69-72]. However, the relative increase in insulin secretion is significantly less in women with GDM than in healthy pregnant women [71, 73, 74]. Studies have demonstrated that impaired beta-cell function in women with GDM is mainly attributed to decreased early-phase insulin secretion [75-77]. Moreover, when insulin secretion was adjusted for the degree of insulin resistance, women with GDM had severe reduction in beta-cell function compared to normal pregnant women [77]. Whereas some studies have reported that women with GDM had higher second-phase insulin response to glucose as compared to pregnant controls [72, 76] others have reported similar response [75].

Several research groups have demonstrated that insulin secretion was substantially decreased in normal healthy women with a history of GDM as compared to matched controls after pregnancy [78-81]. In addition, impaired beta-cell function in women with GDM during pregnancy predicts the development of diabetes in both early postpartum ($\leq$ 6 months) [82, 83] and in the long-term after delivery [84, 85]. Furthermore, it has been shown that women with GDM have increased proinsulin concentrations as well as an increased proinsulin-to-insulin ratio [76, 86], which persists postpartum [86]. This is consistent with the observation that hyperproinsulinaemia is associated with beta-cell dysfunction in patients with T2D [87] and predicts development of diabetes in non-diabetic subjects [88].

Insulin resistance and GDM

Insulin sensitivity decreases progressively by about 70% with advancing normal gestation [70-72, 75, 89-92]. In normal pregnancy, beta cells compensate for the increased insulin resistance to control blood glucose [75, 90]. However, in a pregnancy complicated by GDM, the physiological insulin resistance occurs on a background of chronic insulin resistance, leading to a deterioration of glucose tolerance [71, 89]. In 1985, Ryan et al. were among the first to demonstrate an increased insulin resistance in women with GDM [89]. They reported a decrease in glucose infusion rate during euglycaemic clamp in women with GDM by 40-60% compared to pregnant non-diabetic controls and by 60-70% compared to non-pregnant controls [89]. Furthermore, increased endogenous glucose production has been demonstrated in women with GDM compared to healthy pregnant controls [71, 72, 77]. This could be due to excess release of free fatty acids (FFA) from adipose tissue, as a correlation has been shown

between endogenous glucose production and circulating FFA, re-emphasizing the stimulatory role of FFA on gluconeogenesis [77]. Other studies have consistently shown that women with GDM exhibit decreased insulin sensitivity compared to pregnant control women [91, 93].

Though insulin resistance returns to normal levels after normal pregnancy, it does not abate completely in women with GDM after pregnancy [78, 94-96]. This likely contributes to the increased risk of developing T2D [97] and/or the metabolic syndrome (MetS) [98-100] later in life.

COMPLICATIONS OF GDM

GDM is associated with an increased risk for pregnancy-related complications in the mother, such as hypertensive disorders (gestational hypertension and pre-eclampsia) as well as an increased need for caesarean delivery [35, 101].

Infants of women with GDM are often born large for gestational age (macrosomia), which may result in birth traumas. They are also prone to other complications such as hyperinsulinaemia, polycythaemia, hypocalcaemia, and hyperbilirubinaemia [102]. The prevalence of congenital malformation in infants of women with GDM is still controversial [35]. Some studies have found an increased frequency of congenital malformations, whereas others reported a malformation rate similar to that in the general population [35]. Furthermore, the offspring of women with diabetes during pregnancy are at an increased risk of developing obesity, impaired glucose tolerance (IGT), and T2D later in life [103, 104].

GDM AND METABOLIC DISORDERS

Type 2 diabetes

Type 2 diabetes is a heterogeneous disorder associated with premature death and development of late complications such as cardiovascular disease, end-stage renal disease, blindness and limb amputations [105, 106]. It is characterized by impaired insulin secretion and action, both of which precede, by several years, and predict the development of the disease [107, 108]. The prevalence of T2D is progressively increasing and is estimated to affect approximately 220 million people by the year 2010 worldwide [105].

Type 2 diabetes results from interaction between common genetic variants and environmental factors. There is compelling evidence that T2D is inherited [109, 110]. The finding of different concordance rates between monozygotic and dizygotic twins supports this concept [111, 112]. Also, the relative risk (λs) for a sibling to a patient with T2D is about 3.5 [113]. In addition, the association of common polymorphisms (e.g. *PPARG* Pro12Ala, *KCNJ11* E23K, *CAPN10*

SNP43, -SNP44 or their combinations) in candidate genes with a modest increased risk of the disease is consistent with the polygenic nature of the disease [110, 114]. The fact that factors such as sedentary lifestyle, obesity and dietary intake also increase the risk of T2D [105, 115, 116] demonstrates the important role of non-genetic factors.

GDM and type 2 diabetes

Epidemiological studies suggest an association between several high-risk prediabetic states, GDM, and T2D (figure 1) [35]. The prevalence of GDM is increasing in direct proportion to the prevalence of T2D in a given population or ethnic group [20-24, 35, 105]. In addition, it has been shown that 2.6-70% of women with GDM developed T2D over 28 years postpartum [97]. The progression of GDM to T2D increases steeply within the first 5 years after delivery and then appears to plateau after 10 years [97]. The differences in the prevalence of T2D in women with GDM might be attributed to differences in ethnic background, various lengths of follow-up among studies as well as differences in diagnostic criteria and selection of the initial population with GDM [20, 97, 117]. GDM and T2D also share some traditional risk factors such as age, obesity and high fat diet [35, 105].

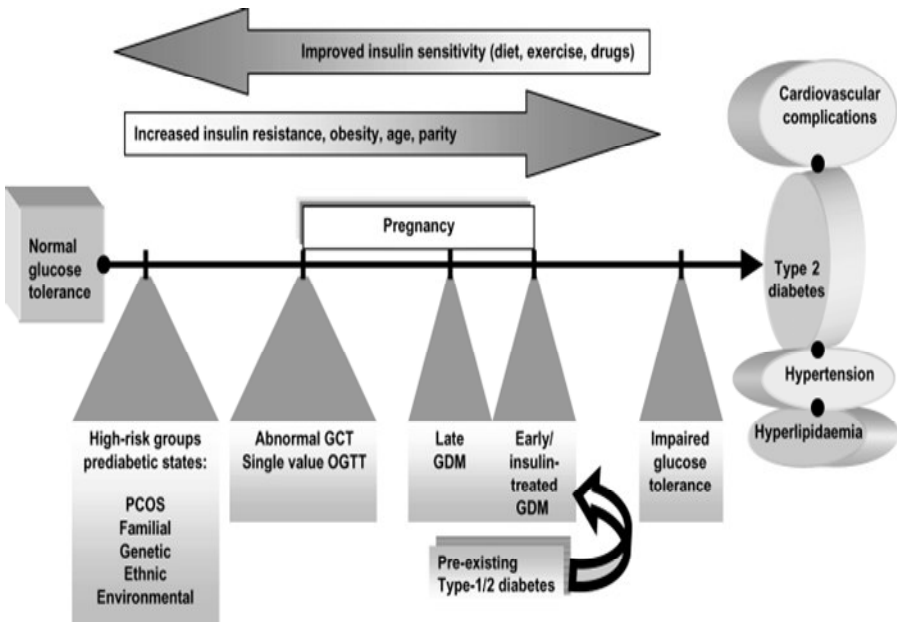

Figure 1. The progression from NGT to T2D may be accelerated by factors that increase insulin resistance and attenuated by life-style modifications and insulin-sensitizing drugs (such as metformin). Pregnancy is a period of increased insulin resistance and the clinical manifestations may vary from NGT to GDM. Early onset of GDM, in the first half of pregnancy, and the need for insulin treatment may offer a greater risk of future development of T2D. Pre-existing T1D or T2D should also be considered. *With permission from Diabetic Medicine; Ben-Haroush et al, 21 (2), 103-113.*

Certain phenotypic features can identify GDM women who are at high risk of developing diabetes after pregnancy. This includes family history of diabetes, old age, multiparity, recurrence of GDM, insulin requirements during pregnancy, pre-term delivery, pre-pregnancy and postpartum obesity as well as belonging to an ethnic group with high prevalence of T2D [82, 97, 117-121].

Maturity Onset Diabetes of the Young (MODY)

Maturity onset diabetes of the young (MODY) was first described by Fajans & Conn in 1960 [122]. MODY is a monogenic autosomal dominant form of diabetes with onset before the age of 25 years [123, 124]. It is characterized by pancreatic beta-cell dysfunction and accounts for about 2-5% of all cases of diabetes [123, 124]. So far, six MODY genes have been identified. Except MODY2, all forms of MODY are caused by mutations in transcription factors [123, 124].

MODY2 was the first described MODY gene. Heterozygous mutations in the gene encoding the glycolytic enzyme Glucokinase (*GCK*) were found to cause the disease [125, 126]. It is characterized by a glucose sensing defect, which leads to increased glucose threshold for stimulation of insulin secretion, and in turn to mild chronic hyperglycaemia [127]. It accounts for 20-30% of all MODY subtypes [128, 129]. Subsequently researchers have found that mutations in hepatocyte nuclear factor 4-alpha (*HNF4A*) and *HNF1A* cause MODY1 and 3, respectively [130, 131], both of which are characterized by severe beta-cell dysfunction [132, 133]. MODY1 accounts for about 5%, while MODY 3 accounts for approximately 65% of all MODY subtypes [128, 129]. Mutations in the insulin promoter factor 1 (*IPF1*/ MODY4), transcription factor 2 (*TCF2*/ MODY5), and neurogenic differentiation factor 1 (*NEUROD1*/ MODY6) have also been shown to cause rare forms of MODY [134-136]. Of note, a homozygous mutation in *IPF1* has been shown to cause pancreatic agenesis [137], whereas MODY 5 is characterized by both diabetes mellitus and non-diabetic renal disease, particularly renal cystic disease [138].

GDM and MODY

Both GDM and MODY are characterized by defective beta-cell function [123, 124, 139]. As early as in 1993, in parallel with the characterization of the MODY 2, mutations in *GCK* have been identified in women with GDM [140, 141]. This was confirmed and supported by other studies in which a wide variation in the prevalence of *GCK* mutations (1.5-80%), depending on the selection criteria, has been reported [142-144]. In addition, a common polymorphism (−30G>A) in the beta-cell-specific promoter of *GCK* has been associated with impaired beta-cell function [145] and increased fasting glucose levels during pregnancy [146].

Already Lehto et al. suggested that *HNF1A* could play a role in the predisposition for GDM by demonstrating that 38% of diabetic women with

linkage to the *MODY 3* (*HNF1A*) gene had a history of GDM [133]. In addition, a mutation in *HNF1A* has been described in a Swedish woman with GDM, who developed diabetes one year postpartum [144]. Mutations in *IPF1* have also been reported in Swedish and Italian women with GDM [144, 147]. *In vitro*, the mutation found in Italian women (P33T) resulted in reduction in DNA-binding and transcriptional activation of the mutant protein [147].

Taken together, rare mutations and common variants in MODY genes seem to predispose to GDM at least in a subset of pregnant women.

Metabolic syndrome

The metabolic syndrome (MetS) is a major health problem worldwide affecting about 30% of the adult population [148-150]. It was earlier described as a syndrome of insulin resistance and compensated hyperinsulinaemia designated as ”Syndrome X” by Reaven in 1988 [151]. The metabolic syndrome is currently defined as a cluster of metabolic abnormalities associated with increased risk for cardiovascular disease (CVD) and subsequent T2D [152, 153] with insulin resistance as the main underlying pathophysiological feature [154]. The most commonly used definitions for MetS are those from the WHO [66], National Cholesterol Education Program (Adult Treatment Panel III) (NCEP ATPIII) [155], and International Diabetes Federation (IDF) [156]. All these definitions agree that hyperglycaemia, obesity, dyslipidaemia, and hypertension are core components of the syndrome but do not give equal weight to the different components.

There is increasing evidence that MetS has a genetic component. Several studies have shown that components of MetS are heritable [157-159]. In addition, common genetic variants (e.g. *APM1* +276G>T, *PPARG* Pro12Ala, *PPARGC1A* Gly482Ser, *FOXC2* −512C>T, and *ADRB3* Trp64Arg) have been associated with increased risk for MetS or its components [160-164]. Environmental factors such as obesity and sedentary life style also increase the risk of the syndrome [165].

GDM and the metabolic syndrome

A possible link between GDM and MetS has been suggested as insulin resistance is a common pathophysiological feature of both disorders [139, 154]. Clark et al. suggested that GDM might be considered as a component of MetS [166]. The authors showed that traits of MetS (e.g. high pre-pregnancy BMI, insulin, triglycerides, and low HDL-C) were predictive of GDM.

Women with GDM are at increased risk of developing MetS later in life [98, 99, 167], with obesity being the best predictor [98, 99, 167, 168]. In addition, women with prior GDM show more abnormalities in the components of MetS (i.e. higher BMI, waist:hip ratio, blood pressure, glucose, insulin, triglycerides

as well as lower levels of HDL-C) as compared to healthy control women [98, 167, 168]. Interestingly, the offspring of women with GDM are at high risk of developing MetS in childhood [169].

AUTOIMMUNITY

Type 1 diabetes

Type 1 diabetes (T1D) is a complex disease that results from autoimmune destruction of the pancreatic beta-cells, which in turn leads to absolute insulin deficiency and insulin requirement for survival [66]. The majority of patients with T1D have one or more markers of immune destruction [i.e. antibodies against islet cells (ICA), insulin (IAA), glutamic acid decarboxylase-65 (GAD65Ab) or protein tyrosine phosphatase (IA-2A)] [170]. Type 1 diabetes is the most common form of diabetes among children and young adults of Caucasian origin [171]. It accounts for 10-15% of diabetes in Caucasians, with the highest incidence reported in Finland and Sardinia followed by Sweden [171, 172].

It has been shown that the sibling relative risk (λs) for T1D is approximately 15 [173]. In addition, twin studies have reported a concordance rate of 40-50% in monozygotic twins but only 11% in dizygotic twins [174, 175]. These observations support the view that T1D has a genetic component. Genetic susceptibility to T1D is determined by several chromosomal loci. The HLA (*IDDM1*) region is a cluster of genes located within the major histocompatibility complex (MHC) on 6p21. This region has shown the strongest association with T1D, particularly the HLA-DQ haplotypes [DQ2 (*DQA1**0501–*DQB1**0201) and DQ8 (*DQA1**0301–*DQB1**0302)] or in combination with HLA-DR alleles [170, 176]. In addition, the insulin (*INS*) gene on chromosome 11 and at least 16 other chromosomal regions have also been implicated in the genetic susceptibility of the disease [170, 176, 177].

There are also environmental factors seem to contribute to the disease risk. They include gestational infections, short period of breast feeding and thereby early introduction of supplementary milk products, stress events and many others [178].

GDM, HLA and type 1 diabetes

Pregnancy is a unique immunologic condition where normally the placenta acts as an immunological barrier between two different HLA genotypes. Against this background, autoimmunity could play a role in the pathogenesis of GDM.

The first study on a possible association between GDM and the HLA region was performed more than two decades ago, and demonstrated that HLA -DR3 and -DR4 antigens were associated with ICA in women with GDM [179].

However, no significant difference in the frequency of these antigens was observed between GDM and control subjects [179]. Later, Freinkel et al. reported a two-fold increase in the frequency of HLA -DR3 and -DR4 antigens in GDM women compared to racially matched controls, but the differences were significant only in a subgroup of black subjects [180]. In a German study, no significant differences in the frequency of HLA -DR or -DQ alleles were observed between GDM and control subjects [181]. However, the DR3 allele was significantly increased in GDM women with islet autoantibodies (ICA, GADAb and/or IA-2A), particularly in those with GADAb [181]. In addition, in GDM women with GADAb, the frequencies of DR4 and *DQB1**0302 alleles were significantly higher than in controls [181]. It has also been shown that women with GDM who were positive for at least one antibody (ICA, GAD65Ab or IA-2A) had significantly higher frequency of *HLA* DR3-DQ2/X or DR4-DQ8/X compared to healthy control subjects from Sweden [182]. Moreover, women with GDM who were negative for those antibodies also had an increased frequency of *HLA* DR7-DQ2/X, DR9-DQ9/X and DR14-DQ5/X compared to controls [182]. Of note, decreased frequency of *HLA* DR2 alleles has been reported in Chinese women with GDM compared to pregnant controls [183]. On the other hand, some studies failed to find significant differences in the distribution of HLA alleles or antigens between GDM and control subjects [184, 185].

Ferber et al. demonstrated that GDM women with *HLA* DR3 or DR4 alleles have an increased risk of developing T1D postpartum [181].
Taken together, these studies suggest that *HLA* contributes to GDM, but the exact mechanism remains to be determined.

GDM, islet autoantibodies and type 1 diabetes

Based upon the presence of autoantibodies, GDM can be divided into an autoimmune and a non-autoimmune form.
A wide range in the prevalence of **ICA** has been reported in women with GDM (1.5%-38%), with the highest prevalence in women from the USA [179] and the lowest in women from Germany (Table 3) [186]. Of note, ICA-positive GDM women had lower frequency of high titres (> 80 JDF units) but higher frequency of low titres (< 20 JDF units) than ICA-positive subjects with T1D at diagnosis [187]. Five per cent of Finnish women with GDM had **GAD65Ab** [188]. As for ICA, the frequencies have varied widely from 0 to 9.5% (Table 3). The prevalence of GAD65Ab seems to be similar in European and in Asian and African women. Relatively low titres and low prevalence (0-3%) of **IAA** have been reported in GDM (Table 3). The same pattern was seen for **IA-2A** with a prevalence ranging from 0 to 6.2% (Table 3).

In 1980, Steel et al. reported that 3 out of 5 ICA-positive women with GDM developed T1D during the first year after pregnancy [189]. This has been confirmed in subsequent studies [190-192]. Interestingly, women who were

ICA-positive at diagnosis of GDM but had NGT after pregnancy showed decreased insulin response to glucose compared to controls postpartum [193].
In the Finnish study, the presence of GAD65Ab was a strong predictor of T1D with a sensitivity of 82% [188]. In addition, in Danish [194, 195] and German [192] women with GDM, GA65Ab positivity during pregnancy, at delivery or postpartum conferred an increased risk of developing T1D. Furthermore, GAD65Ab and ICA in non-diabetic women during pregnancy also predict T1D [196]. In German women with GDM, IA-2A predicted the development of T1D with a low sensitivity of 34% [192].

It is obvious that a subset of women have an autoimmune form of GDM. The course of the autoimmune destruction of the residual beta cells seems to continue after delivery, which may eventually progress to Latent Autoimmune Diabetes in Adults (LADA) or T1D.

GENETICS

Overview

Genetic variations

Genetic variations are differences in the sequence of DNA from one person to another. Most of the variations are single base changes called single nucleotide polymorphisms (SNPs) found at 1250 bp (base pair) intervals in the genome [214]. Other changes include deletions or insertions of one or more bases. Microsatellites are polymorphic short tandem repeats of two to four nucleotides, which are dispersed throughout the genome every few thousand base pairs [214].

Search for genes predisposing to polygenic diseases

Identifying genes underlying susceptibility to complex diseases represents a major challenge of current research. There are several approaches to search for such genes and a combination of several approaches is necessary.

- Linkage studies

Linkage analysis seeks to identify disease-gene localization when there is no *priori* knowledge about the underlying genetic defect of the disease. Traditionally, this is performed by genotyping of highly polymorphic microsatellite markers (400-500) covering the entire genome (so called *genome-wide scan*) in families with clusters of the disease [215, 216]. When there is evidence of regions of excess allele sharing in affected family members, the next step would be *fine mapping* by genotyping additional markers to narrow

Table 3. Prevalence of islet autoantibodies in women with gestational diabetes mellitus.

Country of investigation	Subjects (n)	ICA (%)	GADAb (%)	IA2-Ab (%)	IAA (%)	Ref.
USA	88	35				[197]
	52	38.5				[179]
	160	7.5				[180]
	187	1.6[a]				[198]
	181[b]	2.8				[199]
	100		6			[200]
Europe and Australia						
Australia	734		1.8[a]			[201]
UK	50	10				[189]
	173[c]		4.6[a]			[202]
Germany	437	8.5	9.5	6.2		[192]
	68	1.5				[186]
Italy	68	2.9			1.5	[203]
	70	2.8	1.4	0		[204]
	123	6.5	4.1			[205]
	83		3.6			[206]
	145	10	0	0	3	[207]
	39	5				[208]
Spain	534	13				[193]
	203				1	[209]
Scandinavia						
Finland	112		5			[188]
	98	3	4	1		[210]
	385	12.5	5.9	4.7	1	[211]
Sweden	66		3			[144]
	199		6[d]			[182]
Denmark	139	2.9			0	[191]
	139		2.2			[194]
	453		4.9[a]			[195]
Asians, Arabs and Afro-Caribbean						
Saudi Arabia	90		2.2	0		[212]
	55	1.8[a]				[184]
UK (South-Asian women)	86		3.5[a]			[202]
UK (Afro-Caribbean women)	62		3.2[a]			[202]
Southern India	86		41[e]			[213]

[a] GADAb were measured in women with GDM postpartum. [b] Black women from USA. [C] Caucasian women from UK. [d] Women who were positive for at least one antibody (ICA, GAD65Ab or IA-2A). [e] Women who were positive for GAD65Ab or IA-2Ab.

the region(s) further [216]. These regions often encompass a large number of genes and choosing candidate genes for association studies has been proven to be a difficult task.

- Expression studies

The majority of genes are transcribed (expressed) to mRNA. Differences in gene expression are responsible for both morphological and phenotypic differences. Gene expression changes rapidly in response to cellular events or external stimuli. There are several methods to measure mRNA abundance including Northern blotting, polymerase chain reaction after reverse transcription of RNA (RT-PCR), clone hybridization, differential display, and others. New technologies using high density oligonucleotide arrays or cDNA arrays make it possible to evaluate the expression of thousands of genes simultaneously, which will give insight to disease-associated pathways, thereby identifying candidates for association studies [217].

- Association studies

Association studies seek to identify susceptibility genes for the disease. *Candidate* genes are selected based on assumptions that the known or presumed function of the gene might contribute to the pathogenesis of the disease [216]. Variants (mostly SNPs) in these genes are tested for association with the disease by analyzing the allele distributions in population-based (*case-control*) or family-based (i.e. *transmission disequilibrium test* [*TDT*]) samples [218]. The problem with interpretation of an association is that a SNP can either be the cause of the disease (causative SNP) or a marker of the disease. This occurs when the disease susceptibility allele and the marker allele are so close to each other that they are inherited together, a situation called *linkage disequilibrium* (*LD or allelic association*) [219].

- **Animal models**

Animal models are widely used to identify novel genes that may contribute to the development of diseases in humans. Such models also provide a valuable tool for studying the function of discovered genes since both the genetic and environmental factors of the experimental animals can be closely monitored. The use of knockout and transgenic mice has become a cornerstone in the field [220].

Genetics of GDM

Identification of the underlying genetic causes of GDM will eventually give a better view of the mechanisms that contribute to the pathophysiology of the disease. In addition, it may improve options to possibly prevent GDM and complications for the mother and her child. So far, few genetic association

studies, expression profiling and functional studies have been carried out to dissect the genetics of GDM. However, linkage studies have not been performed in GDM owing to the difficulty to collect family-based samples. Figure 2 shows a schematic representation of strategies to search for genes predisposing to GDM.

The following genes have shown a potential role in susceptibility to GDM:

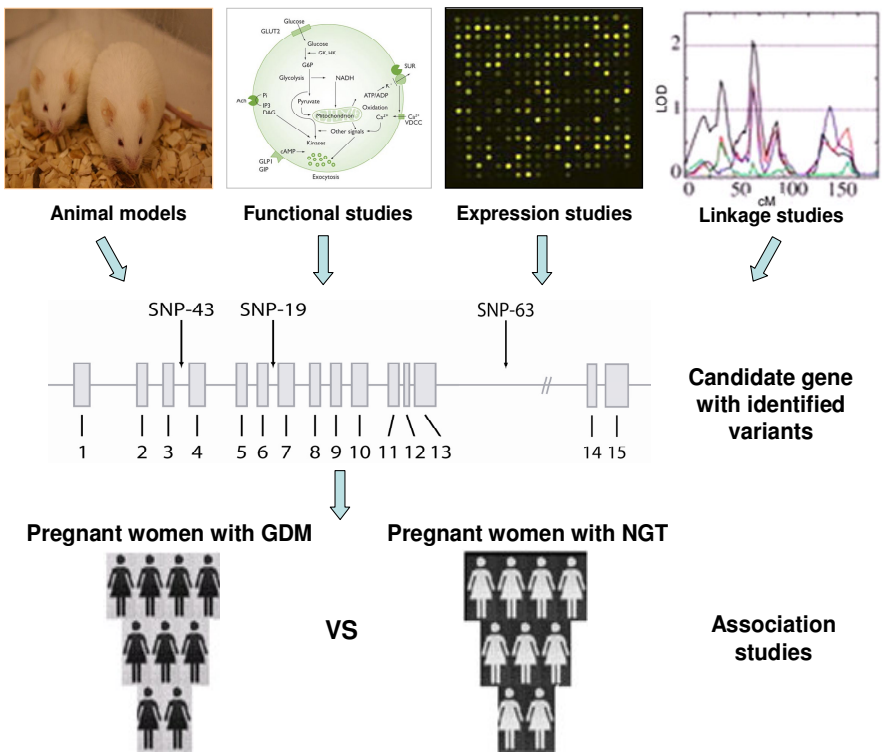

Figure 2. A schematic representation of how to find genes predisposing to GDM. Candidate genes are selected from linkage studies (e.g. genome-wide scans), functional studies (e.g. insulin secretion or insulin-signalling pathway), expression profiling (e.g. cDNA microarray) and animal models (e.g. *Lepr*$^{db/+}$ *mice*). Association studies are carried out with variants (mostly SNPs) across the candidate gene. The allele frequencies of these SNPs are compared between women with GDM and pregnant healthy controls to assess whether these variants are associated with increased or decreased risk for GDM.

Calpain-10 (CAPN10)

The gene encoding CAPN10, a cysteine protease, is located on 2q37 and is expressed in many tissues including pancreas, muscle and adipose tissues [221, 222]. *CAPN10* is the first T2D gene identified by positional cloning [221, 223]. In the original study, three intronic variants (SNP43, SNP19 and SNP63) were found to be associated with increased risk of T2D in Mexican-American, Finish and German populations [221]. In addition, a haplotype combination (121/112) defined by these SNPs was associated with an increased risk of the disease [221]. As usual in genetic association studies, some but not all subsequent studies could replicate this finding [110, 224, 225].

These three variants have also been studied in Austrian Caucasian women with GDM [226]. SNP63 but neither SNP43 nor SNP19 was associated with GDM [226]. A haplotype combination (121/221) was also associated with an increased risk of GDM [226]. This suggests that different risk alleles may be operative in T2D and GDM.

Sulfonylurea receptor 1 (SUR1 or ABCC8)

The ATP-sensitive potassium channels are composed of two components: the sulfonylurea receptor (SUR1) and the inwardly rectifying potassium channel (Kir6.2) (Figure 3) [227, 228]. Mutations in *SUR1* are associated with hyperinsulinaemic disorders [229, 230]. Furthermore, common variants in *SUR1* have been associated with T2D in different populations [231-235].

Rissanen et al. studied the role of several variants in *SUR1* on the risk of GDM in Finnish subjects [236]. The (cagGCC→tagGCC) in exon 16 splice acceptor site and the R1273R (AGA→AGG) variants were more common in women with GDM than in NGT subjects [236]. However, both variants were in linkage disequilibrium and risk alleles differed between populations. This may suggest that the reported associations are caused by a variant in linkage disequilibrium with these polymorphisms [236]. These results are in line with the findings in T2D [231, 233, 237]. Also, R1273R has been associated with hyperinsulinaemia in NGT subjects [238].

Hemochromatosis (HFE)

The hemochromatosis (*HFE*) gene is located on chromosome 6. Mutations in *HFE* cause the hereditary form of hemochromatosis, which is an autosomal recessive disorder of excess iron storage in different organs [239, 240]. Diabetes is a common consequence of hemochromatosis [239, 240].

Cauza et al. studied whether two mutations (C282Y and H63D) known to cause hemochromatosis also increase the risk of GDM. The 282Y allele was more common in 98 European women with GDM than in 102 matched pregnant

controls, whereas no significant difference was observed for the H63D mutation [241]. The allele frequency of both mutations did not differ significantly between 96 women with GDM as compared to 62 matched controls from Mediterranean countries [241]. Interestingly, serum ferritin levels were higher in women with GDM than in controls irrespective of the HFE-genotype [241]. However, no significant impact of these mutations on the risk of T2D was observed in recent meta-analyses [242].

Mannose-binding lectin 2 (MBL2)

Mannose-binding lectin (MBL) is an acute phase protein that is synthesized mainly in the liver and is considered a key molecule in innate immunity [243, 244]. It is encoded by *MBL2* on chromosome 10 [244]. Concentration of MBL is genetically determined and its deficiency predisposes to recurrent infections and autoimmune diseases such as systemic lupus erythematosus [244]. Several common polymorphisms including R52C and G54D in *MBL2* have been associated with low levels of MBL [244].

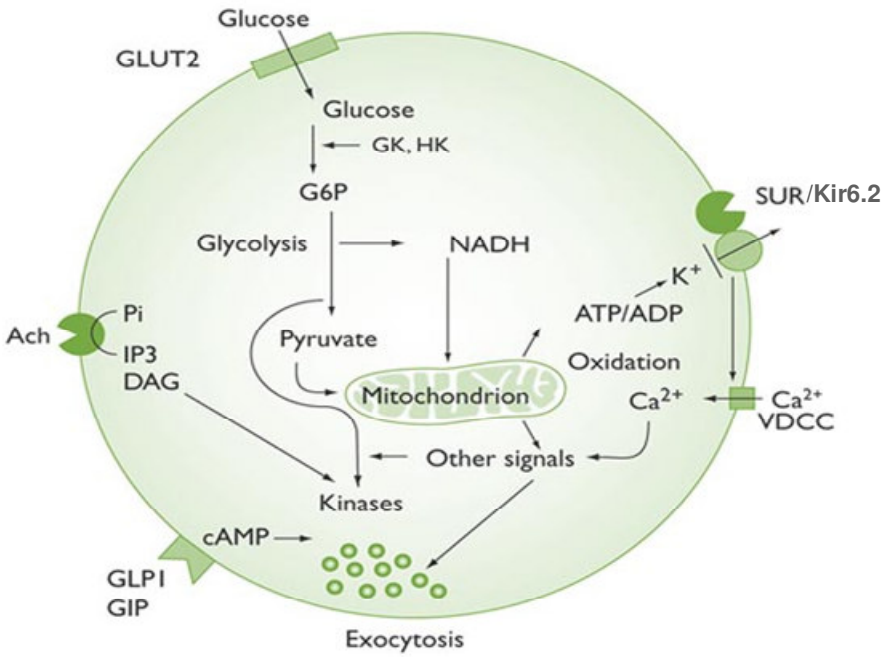

Figure 3. The role of K^+ ATP-sensitive channels in insulin secretion. K^+ATP-sensitive channels are composed of two subunits (SUR1/Kir6.2). Glucose enters the beta-cells through glucose transporters that allow rapid equilibration between extra- and intracellular glucose concentrations. Glucose oxidation in beta-cells leads to a rise in ratio of adenosine triphosphate (ATP) to adenosine diphosphate (ADP). This inhibits the SUR1/Kir6.2 channels activity, which leads to depolarization and opening of voltage-dependent calcium (Ca^{2+}) channels with Ca^{2+} entry, which in turn triggers insulin exocytosis. *With permission from Current Medicine Group.*

Megia et al. studied the R52C and G54D polymorphisms as well as plasma MBL levels in 105 women with GDM and in 173 healthy pregnant women from Spain [245]. The G54D polymorphism was associated with an increased risk of GDM with an OR of 2.03, whereas no evidence of association was seen for the R52C polymorphism. In addition, among women with GDM, carriers of the G54D polymorphism had higher glucose levels, were treated with insulin more frequently and had heavier infants compared to wild-type carriers. The mechanism by which this polymorphism may predispose to GDM is not known. However, the importance of MBL role in inflammation [246] might shed light on this mechanism since low-grade systemic inflammation has been shown to be a risk factor for GDM [247].

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β3-adrenergic receptor (ADRB3)

The β3-adrenergic receptor (ADRB3) is a pivotal receptor mediating catecholamine-stimulated thermogenesis and lipolysis [248]. In humans, *ADRB3* is expressed in various tissues including adipose tissue, skeletal muscle and pancreatic beta cells [249-251]. The *ADRB3* maps to the short arm of chromosome 8. A common polymorphism (Trp64Arg) has been originally associated with abdominal obesity, insulin resistance and early onset of T2D [164, 252]. In a recent analysis of published results on this polymorphism, we observed a consistent association with features of MetS [110]. Moreover, the Arg64 variant seems to affect insulin secretion in *vivo* and *vitro* [251, 253, 254]. It was also associated with a decrease in energy expenditure [255] and a marked decrease in ADRB3 function (i.e. agonist sensitivity) [256].

The putative role of Trp64Arg polymorphism in the pathogenesis of GDM has also been investigated. In Austrian Caucasian women, it has been associated with mild GDM defined by 60-min post-load glucose during OGTT [257]. However, it could not be replicated in Greek [258] or Taiwanese [259] women. Of note, it was associated with increased weight gain and fasting insulin during pregnancy [257, 259].

Glycoprotein PC-1 (*ENPP1*)

The class II transmembrane glycoprotein PC-1 is encoded by *ENPP1* (ectonucleotide pyrophosphatase/phosphodiesterase 1) located on chromosome 6. *ENPP1* is expressed in several tissues including skeletal muscles and adipose tissue [260, 261]. It has been considered a potential candidate gene for insulin resistance because it inhibits insulin receptor tyrosine kinase (IRTK) activity [262]. In addition, a common variant (K121Q) in *ENPP1* (PC-1) has been associated with insulin resistance and T2D [263-267].

Shao et al. showed that the PC-1 protein content in skeletal muscle was 63% greater in women with GDM compared to pregnant controls [268]. In addition, PC-1 content negatively correlated with insulin receptor phosphorylation and

IRTK activity [268]. These findings suggest that *PC-1* may have an important role in the pathogenesis of GDM by inducing insulin resistance during pregnancy.

Mitochondrial DNA (mtDNA)

The human mitochondria have a unique DNA (mtDNA), which is a small double-stranded circular molecule (16,569 bp), encoding 2 ribosomal RNAs, 22 tRNAs and 13 subunits of the respiratory chain enzyme complex [269]. An A to G substitution at nucleotide position 3243 (A3243G) in the mitochondrial $tRNA^{leu}$ causes maternally inherited diabetes and deafness (MIDD), which is characterized by impaired insulin secretion [270-272]. The mutation also causes a variety of phenotypes including MELAS (mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes) syndrome [273].

The A3243G mutation has also been studied in women with GDM. No evidence of association was found neither in Singaporean women nor in predominantly Caucasians women from the USA [274, 275]. However, it was found in 1 of 12 Japanese women with GDM [276]. On the other hand, the T3398C mutation in the mitochondrial *ND1* was more common in Singaporean women with GDM compared to pregnant controls (2.9 vs. 0%) [275]. Seventy five per cent (3/4) of women carrying the T3398C mutation had a maternal history of diabetes [275], but no information was given about deafness or neurological status of these women. In fact, this mutation has also been described in patients suffering from MELAS, cardiomyopathy and diabetes [277], but no data about the functionality of this mutation. The C3254A mutation in $tRNA^{leu}$, as well as the G3316A, T3394C and A3399T mutations in *ND1*, were also found in Singaporean women but were not significantly increased in women with GDM [275].

Insulin receptor (INSR) and insulin-like growth factor 2 (IGF2)

The insulin receptor (*INSR*) is a heterotetramer composed of two extracellular α-subunits that bind insulin and two β-subunits that span the plasma membrane and have an intracellular tyrosine kinase domain [278, 279]. Mutations in *INSR* have been described in individuals with extreme insulin resistance [280]. Insulin-like growth factor 2 (IGF2) is a single chain polypeptide that share amino acid sequence homology with insulin [281]. It mediates growth hormone action and stimulates insulin action as well as it is involved in development and growth [281-283].

In 1989, Ober et al studied 96 women with GDM and 164 pregnant control women with different ethnic backgrounds for a potential association with variants (RFLPs) in or near *INS*, *INSR*, glucose transporter (*GLUT1*) and *IGF2* genes. Using logistic regression analysis, the authors showed an association between the *INSR* variant and GDM as well as an interaction with BMI in Black

and Caucasian subjects. In addition, Caucasian carriers of variants in both *INSR* and *IGF2* had an increased risk of GDM. However, none of the studied variants conferred risk for GDM in Hispanic subjects [284].

Insulin receptor substrate 1 (*IRS1*)

Insulin receptor substrate 1 (IRS1) is a major substrate for the insulin receptor and regulates insulin signalling in skeletal muscle and adipose tissues [285, 286]. It links tyrosine-phosphorylated insulin receptor to the downstream part of the insulin-signalling pathway [287]. Genetic regulation of IRS1 has been suggested; for example, the common Gly972Arg variant of *IRS1*, which is located between two potential tyrosine phosphorylation sites, has been associated with impaired insulin-stimulated signalling along the PI3-kinase pathway [288]. In addition, a rare mutation in *IRS1*, found in a patient with extreme insulin resistance, markedly reduced the phosphorylation of IRS1 by the insulin receptor [289].

IRS1 protein level was decreased by 43% in adipose tissue of GDM women as compared to pregnant control women [290], but no significant difference was observed in skeletal muscle [291]. However, in skeletal muscle from $Lepr^{db/+}$ mice, an animal model of spontaneous GDM, a 35% reduction in IRS1 protein levels was found compared with control mice and this contributed partially to the decreased IRS1 tyrosine phosphorylation [292]. Thus, IRS1 might play an important role in the cellular mechanism of insulin resistance in GDM pregnancy. Interestingly, the Gly972Arg variant has been associated with obesity, as well as higher fasting insulin and glucose levels in women with GDM [293].

GLUT4 (*SLC2A4*)

GLUT4 is the main insulin-responsive glucose transporter isoform [294]. It is encoded by the *SLC2A4* [solute carrier family 2 (facilitated glucose transporter), member 4] located on 17p13 [295].

Shao et al. found that basal and insulin-stimulated plasma membrane GLUT4 levels in skeletal muscle were lower in $Lepr^{db/+}$ mice with GDM than in control mice [292]. In adipose tissue from women with GDM, the cellular content of GLUT4 was decreased by 44% compared to pregnant control women [296]. Furthermore, insulin stimulation induced translocation of GLUT4 to plasma membranes in control subjects but not in women with GDM [296]. Thus, defective insulin-stimulated GLUT4 translocation might contribute to the insulin resistance during GDM pregnancy. Importantly, over-expression of GLUT4 in $Lepr^{db/+}$ mice with GDM improved insulin-signalling, increased insulin secretion and improved glycaemic control [297].

Adiponectin (APM1)

Adiponectin is a circulating protein secreted by adipocytes, potentially modulating insulin sensitivity [298]. It is encoded by *APM1*, which is exclusively expressed in human adipose tissue [299] and is found abundantly in human plasma [300]. Adiponectin is inversely correlated with body weight, intra-abdominal fat and measures of insulin resistance [300-302].

Several recent studies have demonstrated decreased plasma adiponectin in GDM compared to normal pregnancy [302-304]. In addition, women with NGT and a history of GDM had lower plasma adiponectin levels after pregnancy as compared to control women [305]. Consistent with these studies, Ranheim et al. have shown that adiponectin mRNA levels were lower in abdominal subcutaneous adipose tissue of women with GDM compared to pregnant control women [306].

Leptin (LEP)

Leptin, the protein encoded by the *LEP* (also called *ob*), is mainly produced by adipocytes. It is also expressed in other tissues including skeletal muscle, stomach and placenta [307]. Leptin inhibits food intake, reduces body weight and stimulates energy expenditure [308, 309]. In humans, there are four leptin receptor isoforms, which can be divided into three classes: long, short, and soluble isoforms [309, 310].

A crucial role of leptin in the development of GDM has been suggested since mice heterozygous for the leptin receptor ($Lepr^{db/+}$) develop spontaneous GDM during pregnancy [311]. In addition, leptin administration during late gestation has been shown to reduce adiposity as well as improve insulin sensitivity and glucose tolerance in $Lepr^{db/+}$ mice with GDM [312].

In humans, studies have demonstrated that placental leptin mRNA expression is increased in women with GDM compared with pregnant controls using *in situ* hybridisation [313], microarray analysis [314] and RT-PCR [315]. Furthermore, mRNA of a short isoform of the leptin receptor was increased in placentas from women with GDM compared to pregnant controls [314]. Also, leptin release was higher from adipose tissue and skeletal muscle of women with GDM compared to pregnant control women [316]. These results are in line with the finding that hyperleptinaemia in early pregnancy predicts the development of GDM later in pregnancy [317]. However, other studies reported no differences in all isoforms of placental leptin receptor mRNA [315] or protein expression [313].

Visfatin (PBEF1)

Visfatin is a novel adipocytokine predominantly expressed in and secreted from visceral adipose tissue in both humans and mice [318]. The pre-B-cell colony enhancing factor 1 (*PBEF1*) gene located on 7q22.2 encodes visfatin [319]. It has insulin-like properties as administration of recombinant visfatin to mice leads to a reduction in plasma glucose independent of changes in insulin levels [318]. In addition, mice heterozygous for a targeted mutation in the *visfatin* gene had higher plasma glucose levels than wild-type littermates [318]. It has been shown that plasma visfatin concentration and visceral visfatin mRNA expression correlate positively with BMI and percent body fat [320]. Further, patients with T2D had elevated plasma visfatin compared to non-diabetic subjects [321].

In a recent study from Austria, women with GDM had elevated visfatin concentrations compared to healthy pregnant controls even after adjustment for BMI [322]. Interestingly, in women with GDM, visfatin increased during the course of pregnancy and 2 weeks after delivery [322]. Of note, visfatin is also expressed in human placental tissue [323].

Interleukins and inflammatory markers

Interleukin-1 (IL-1) and IL-8 are cytokines that play a central role in inflammatory and immune response [324, 325]. IL-1 acts through several receptors including IL-1 receptor-type I (IL-1RI), IL-1 receptor accessory protein (IL-1RAcP), IL-1 receptor-related protein (IL-1Rrp), and IL-1 receptor-like 1 (IL1RL1) [326], whereas IL-8 acts through the interleukin-8 receptor, alpha (IL-8RA) and beta (IL-8RB) [327].

Radaelli et al. found, using microarray analysis, that placental mRNA expression of IL1RL1 and IL8RB receptors was increased in women with GDM compared to pregnant control women [314]. This supports the concept that inflammation might contribute to the pathogenesis of GDM. Other studies have also shown that concentrations of several inflammatory markers such as tumour necrosis factor-alpha (TNF-α) and its soluble receptors [328, 329], C-reactive protein (CRP) [330-332], plasminogen activator inhibitor-1 (PAI-1) [333], IL-6 [333], fibrinogen [330], Sialic acid [334] as well as leukocyte count [332] were higher in women with GDM than in healthy control women.

AIMS

The overall aim of this thesis was to study genetic and immunological risk factors that increase susceptibility to GDM.

The specific aims were:

1. To investigate whether autoimmunity and genetic variations affecting insulin secretion and action, or both, contribute to the development of GDM and whether GDM pathogenesis differs between women with Scandinavian and Arabian background.

2. To study whether GDM has a similar genetic predisposition as type 2 diabetes by studying common genetic polymorphisms that have previously been associated with type 2 diabetes.

3. To investigate whether common variations in MODY [Glucokinase (*GCK*), hepatocyte nuclear factor 1-alpha (*HNF1A*), and *HNF4A*] genes increase the risk of GDM.

4. To study whether common genetic variations that have been associated with the metabolic syndrome or related traits would also confer risk for GDM.

SUBJECTS AND METHODS

Screening and diagnosis of GDM

Since 1995, all pregnant women in southern Sweden (Skåne) are routinely offered a 75 g OGTT at 27–28 weeks of pregnancy. Women with a family history of diabetes or previous GDM are also offered a 75 g OGTT at 12–13 weeks. The tests are performed in the local maternity health-care clinics, using HemoCue devices (HemoCue, Ängelholm, Sweden) for capillary whole-blood analysis. According to the proposal by the European Diabetic Pregnancy Study Group of the EASD [67] and the local experience [46, 335], GDM is defined as a 2-h capillary glucose concentration (double test) of at least 9 mmol/l (162 mg/dl).

Subjects

The study subjects were recruited from two different sources: 1) two hundred and ninety seven women with GDM (227 Scandinavian and 70 Arabian) were recruited among women who were referred to Malmö or Lund University Hospitals during the period from March 1996 until December 2003, and 2) four hundred and fifty three women with GDM (423 Scandinavian and 30 Arabian) were ascertained among women participating in the Diabetes Prediction in Skåne (DiPiS) Study, which is a prospective, longitudinal study of the prediction of T1D in all newborns in southern Sweden [336, 337]. All non-diabetic pregnant controls (1354; 1232 Scandinavian and 122 Arabian) were ascertained from the DiPiS study. Of note, different combinations of these women were included in the studies presented in this thesis (see the description of each study).

Phenotypic characterization

A detailed phenotypic characterization during pregnancy, including OGTT with measurements of fasting and post-load insulin and glucose concentrations, was carried out in a subset of GDM women who lived in the city of Malmö and participated in a 5-year follow-up study with repeated OGTTs at 1, 2 and 5 years postpartum (Table 4). At delivery (for DiPiS subjects) and after oral consent, a blood sample was drawn and information obtained about possible GDM or diabetes status. When the child was 2 months old and had been entered into the population registry, the parents were invited by letter to participate with their child in the DiPiS study. If the parents agreed to do so, they gave their written consent and filled out a psychosocial and hereditary questionnaire including information about diabetes status in the family and their country of birth. Ethnicity was also determined using both surname and given name. Since the DiPiS and "Malmö-Lund" studies were not restricted to Swedish subjects but included immigrants as well, we chose only women with a Scandinavian (Study I-IV) or Arabian (Study I) background. Most of the Scandinavian women were of Swedish origin and a few were of Danish, Norwegian or

Finnish origin. Arabian women were immigrants from most of the Arab countries (Iraq, Lebanon, Morocco, Palestine, Syria etc.). The phenotypic characteristics of Scandinavian women are presented in Table 5.

Metabolic measurements

Blood glucose was measured using a HemoCue device or with a glucose oxidase method on a Beckman Glucose Analyzer II (Beckman Instruments, Fullerton, CA, USA; coefficient of variation (CV) < 1%). Serum insulin concentrations were measured using an enzyme immunoassay from DAKO (DAKO, Cambridgeshire, UK; interassay CV of 7%). C-peptide concentrations were measured with radioimmunoassay (Linco Research, St. Charles, MO, USA; interassay CV of 9 %) Glycated haemoglobin (HbA1c) was analyzed using high-pressure liquid chromatography (HPLC).
Body mass index (BMI) was calculated as weight/height^2 (kg/m^2). Homeostasis model assessment (HOMA-IR) [fasting serum insulin x fasting plasma glucose / 22.5] was used to estimate the degree of insulin resistance [338]. 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) [339]. 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 [340].

GAD65Ab

GAD65Ab were measured using radio-immunoprecipitation assay employing ^{35}S-labelled recombinant human GAD65 produced by *in vitro* transcription-translation as described previously [341]. GAD65Ab were measured either in serum or in punch-outs from dried blood spots (DBS), which were incubated in assay buffer over night to elute antibodies. 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) x 100. The cut-off limit for positivity was 5 RU. According to standardized international units, 5 RU is equal to 32 IU/ml. At the Combined Autoantibody Workshop [342], the specificity and sensitivity of the GAD65Ab assay were 99 and 75%, respectively.

DNA extraction

Genomic DNA was extracted from peripheral blood lymphocytes using standard methods [343]. Briefly, white blood cells were separated from blood by centrifugation in high sucrose. The cells were lysed with proteinase K and sodium dodecyl sulphate (SDS). Proteins were salt-precipitated and separated together with other cell debris by centrifugation. Genomic and mitochondrial DNA from the supernatant was precipitated with isopropanol, washed with ethanol and stored at 20^{o}C.

Table 4. Phenotypic 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 2hr (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 was not available from all study subjects, the number of individuals with data available is given in parenthesis. [a]After adjustment for BMI (ANCOVA).

Table 5. Phenotypic characteristics of Scandinavian women with and without GDM.

Variable	GDM % (n)	Controls % (n)	*P* value
Age (year)	32.3±0.2 (649)	30.5±0.1 (1232)	<0.0001
Weight gain during pregnancy			
• < 5 kg	11.0 (41/ 374)	4.1 (34/ 833)	<0.0001
• 5-10 kg	30.5 (114/ 374)	19.2 (160/ 833)	<0.0001
•11-15 kg	31.8 (119/ 374)	39.5 (329/ 833)	0.011
• > 15 kg	26.7 (100/ 374)	37.2 (310/ 833)	0.0004
Smoking	10.1 (38/ 377)	9.4 (79/ 841)	0.71
At least one pregnancy before index pregnancy	59.2 (232/ 392)	53 (453/ 854)	0.043
Twin or triple pregnancies	2.8 (15/ 535)	1.4 (17/ 1232)	0.051
Insulin treatment during pregnancy	4.8 (14/ 290)	0.0 (0/ 452)	<0.0001

Data are means ± SEM. As all data was not available from all study subjects, the number (n) of individuals is given in parenthesis (i.e. positive data on certain variable/ total available data on the same variable).

Dried blood spots

Dried blood spot (DBS) were collected on Schleicher and Schuell Grade 2992 filters (Schleicher and Schuell, Dassel, Germany).

Genotyping

HLA DQB1

We used biotinylated PCR primers to amplify the second exon of the *DQB1* as described previously with modification of the forward primer (5'- CA TGT GCT ACT TCA CCA ACG G) [344]. PCR was carried out with 25 ng of DNA or 3 mm of DBS. After amplification, PCR product was captured onto DELFIA streptavidin-coated microtitration plates (Perkin Elmer Life Sciences, Boston, MA, USA) and denatured using mild alkaline solution. Hybridization was performed with a panel of lanthanide labelled probes specific for *HLA DQB1* alleles and with a probe controlling DNA-amplification (Perkin Elmer Life Sciences). Five probes were used to distinguish *DQB1* alleles. Of them, four (Eu-*DQB1**0602/3, Sm-*DQB1**0301, Tb-*DQB1**0201 and Eu-*DQB1**0302) have been described previously [344] in addition to (Sm-*DQB1**0603/4; 5'-TTG TTA CCA GAC ACA). After washing and addition of DELFIA enhancement solution, the Eu and Sm signals were counted in a Victor2 MultiLabel Counter (Perkin Elmer Life Sciences). The Tb signal-to-noise ratio was calculated with MultiCalc (Perkin Elmer Life Sciences).

SNP genotyping

Genotyping using DNA

Genotyping of all SNPs was carried out using *TaqMan allelic discrimination assay* apart from *PPARG* Pro12Ala and *INS* -23 HphI polymorphisms, which were genotyped by RFLP. The assay was carried out using an ABI Prism 7900 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) in a reaction volume of 2 µl, according to the manufacturer's instructions. Primers and probes were designed using Assays-by-Design (Applied Biosystems) except for the *FOXC2* −512C>T polymorphism, which was ordered from MWG Biotech Scandinavia A/S (Risskov, Denmark).

Genotyping using DBS

For DBS samples, a template PCR was initially carried out to amplify the region of interest. The template PCR was followed by RFLP, SNaPshot or TaqMan allelic discrimination assay.

Template PCR

The template PCR was carried out with an initial two cycles at 4°C for 30 s followed by 98°C for 3 min, followed by holding at 80°C while the PCR mix was added. Then the PCR programme was continued with an initial

denaturation (94 or 96°C for 5 min), followed by 35-45 cycles of denaturation (94 or 96°C for 30 s), annealing (30 s) and extension (72°C for 30-60s), followed by final extension (72°C for 10 min). PCR amplification was carried out with 3 mm of *DBS* in a total volume of 40 µl containing 1x Pharmacia Amersham buffer (Amersham Pharmacia Biotech, Uppsala, Sweden) or 1x $(NH_4)_2SO_4$-buffer (16 mmol/l $(NH4)_2SO_4$; 67mmol/l Tris (pH 8.8); 0.01% Tween 20), 4-8 nmol each dNTP (MBI Fermentas, St Leon-Rot, Germany), 20 pmol of each primer and 1.5-2.5 U *Taq* polymerase (New England Biolabs, Beverly, MA, USA). When needed, 10-30 µmol Betaine (Sigma-Aldrich, Stockholm, Sweden), 30-120 nmol $MgCl_2$, 1.5% Formamide or 5% DMSO were added to the PCR mix.

RFLP

After template PCR, the following restriction enzymes were used to digest the PCR products, with name, origin, incubation conditions and gel concentrations in parentheses: *KCNJ11* E23K (*Ban*II; New England Biolabs; 37°C for 4 h; 3.5% agarose gel), *UCP2* −866G>A (*Mlu*I; MBI Fermentas; 37°C for 4 h; 3% agarose gel), *IRS1* G972R (*Bst*NI; New England Biolabs; 60°C for 2 h; 4.5% agarose gel), *GCK* –30G>A (*Alw21I*; MBI Fermentas; 37°C for 4h; 2% agarose gel), *APM1* +276G>T (*Mva 1269I*; MBI Fermentas; 37°C for 4h; 3% agarose gel), *PPARG* Pro112Ala (*BstUI*; New England Biolabs; 60ºC for 2h; 4.5% agarose gel), *INS –23 HphI* (*HphI;* New England Biolabs; 37°C overnight; 4.5% agarose gel), and mitochondrial $tRNA^{leu\,(UUR)}$ (*ApaI*; New England Biolabs; 37ºC overnight; 5% polyacrylamide gel). PCR products were separated on gel electrophoresis and stained with ethidium bromide to visualise the fragments. Ultraviolet (UV) light was used to detect fragments on agarose gel, whereas GELSCAN2000 analyser (Applied Biosystems) detected the fragments on polyacrylamide gel.

Single-base extension (SNaPshot assay)

SNaPshot assay was carried out using 1µl of the template PCR (see description of template PCR above) on an ABI Prism 3100 Sequence Detection System (Applied Biosystems), according to the manufacturer's instructions. Figure 4 shows the output from GeneMapper software (Applied Biosystems), which was used to analyze genotyping data.

TaqMan allelic discrimination assay

TaqMan allelic discrimination assay was carried out using 2µl of the template PCR (see description of template PCR above) on an ABI Prism 7900 Sequence Detection System (Applied Biosystems), according to the manufacturer's instructions. Figure 5 shows the output from SDS software (Applied Biosystems), which was used to analyze genotyping data.

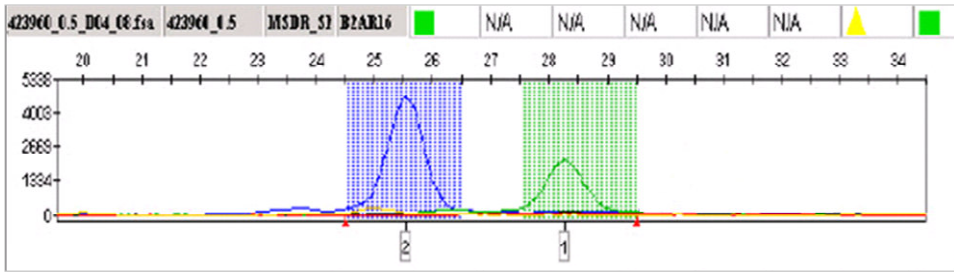


Figure 4. SNaPshot assay. Output from GeneMapper software showing both alleles of *CAPN10* SNP44 polymorphism. The Y-axis shows the relative signal intensity of each allele while the X-axis shows the size of each allele (bp).

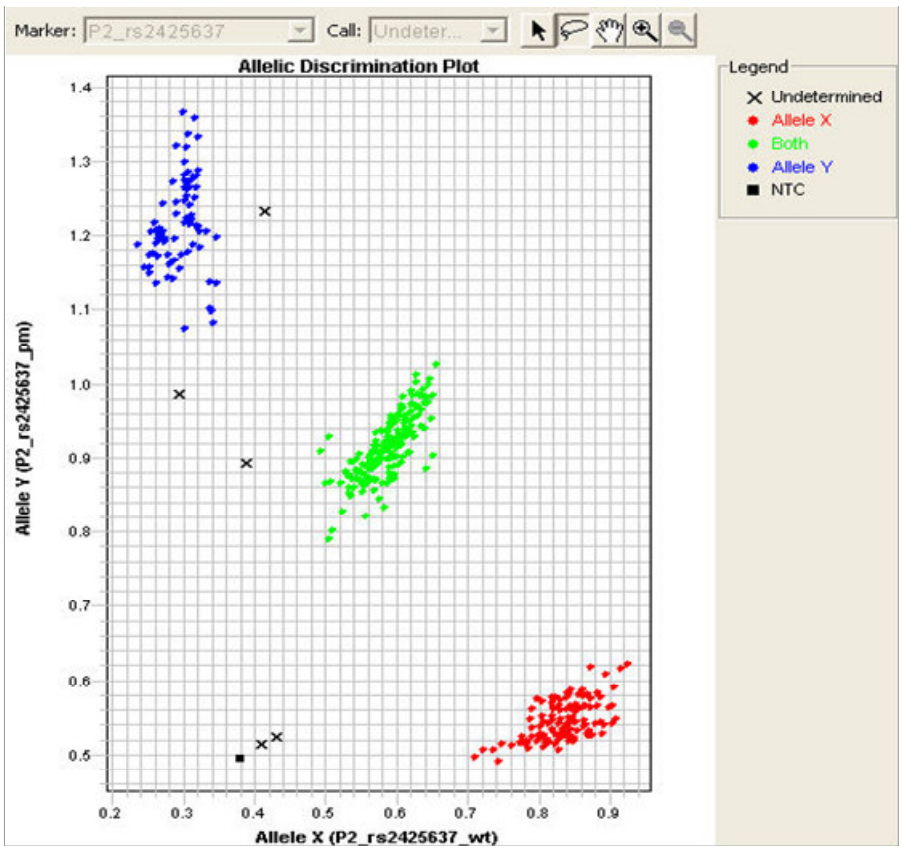


Figure 5. TaqMan allelic discrimination assay. The output signals are plotted in a scatter-plot with one fluorescent colour signal intensity on the y-axis (*FAM*) and the other on the x-axis (*VIC*). Accordingly, three clusters that represent the three genotypes (wt/wt, wt/pm and pm/pm) could be determined. Signals that fall outside of these clusters are "not determined".

Statistical analyses

Clinical data are presented as mean ± SEM. Significance of differences between group means was tested by an ANOVA or a Mann-Whitney test. Logarithmic transformation was used for data with right-skewed distribution. HOMA-IR index was adjusted for BMI or *PPARG* genotype using ANCOVA (Study I). Group frequencies were compared by Chi-square or Fisher's exact test. Odds ratios (ORs) and 95% CIs were obtained from logistic regression analysis. The significance of difference in allele frequencies between GDM and controls was also tested by 1000 or 10000 permutations (Studies II-IV). In addition, 10,000 permutations as implemented in Haploview version 3.2 were used to correct for multiple testing in *Study III* [345]. The statistical analyses were carried out with the Number Cruncher Statistical Systems (NCSS, Kaysville, UT, USA) or BMDP Statistical Software, Version 1.12 (BMDP statistical software, Los Angeles, CA, USA). Two-sided *p*-values less than 0.05 were considered statistically significant.

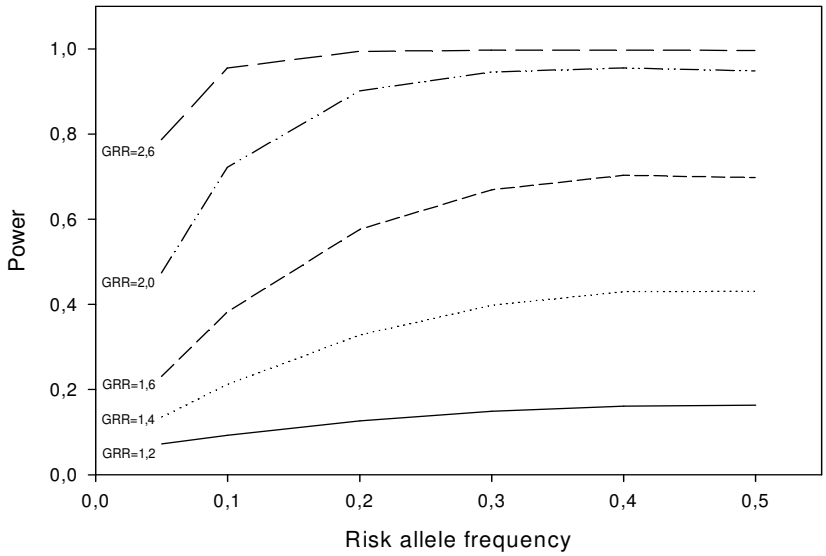
Power calculations

Power calculations were carried out using the Genetic Power Calculator (available at http://ibgwww.colorado.edu/~pshaun/gpc/) [346]. Figure 6 shows the power calculations for studies (I-IV) -under a multiplicative model- in Arabian and Scandinavian women. The effect size was measured as the genotypic relative risk with a 5% type 1 error rate.

A

Power Calculations for Study I (*Arabians*)

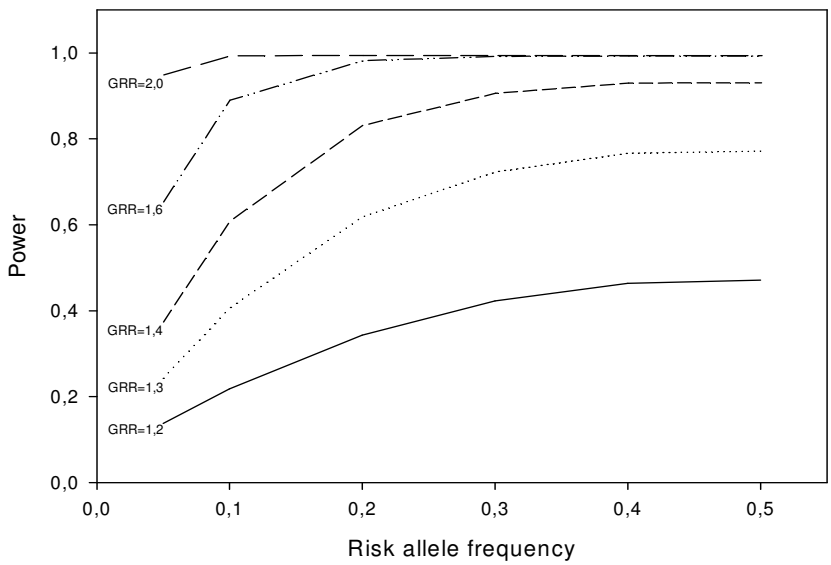
[*GDM (n=100) & controls (n=122)*]



B

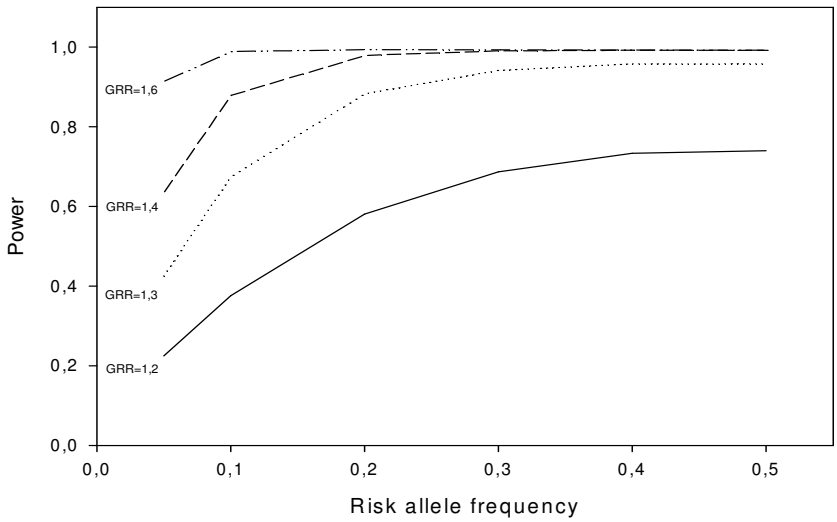
Power Calculations for Study I (*Scandinavians*)

[GDM (n=400) & controls (n=428)]



C

Power Calculations for Study II (*Scandinavians*)
[*GDM* (*n=588*) *& controls* (*n=1189*)]



D

Power Calculations for Study III/IV (*Scandinavians*)
[*GDM* (*n=648/649*) *& controls* (*n=1232*)]

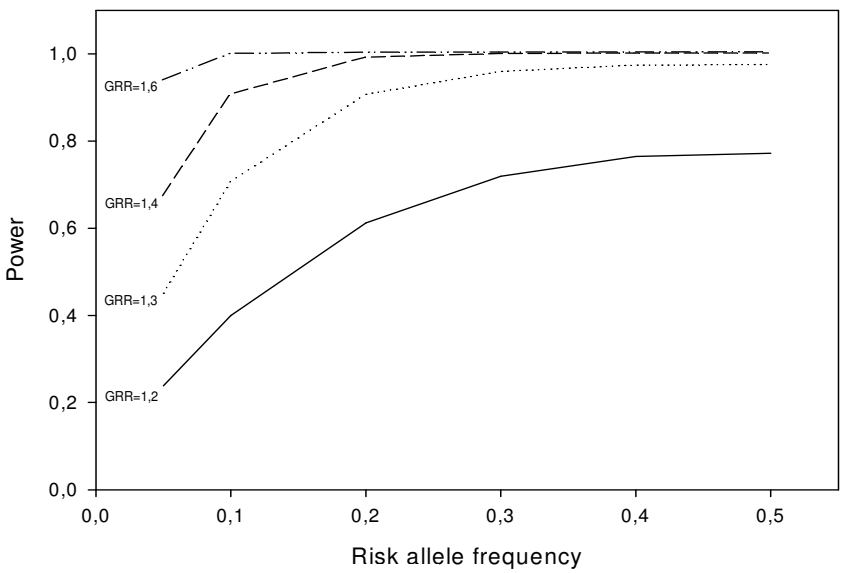


Figure 6. Power calculations for study I in Arabians (A) and in Scandinavians (B), study II (C), and study III/IV (D). Power was calculated using a multiplicative model with (α=0.05).

RESULTS

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

We studied 500 unrelated GDM women (400 Scandinavian and 100 Arabian) and 550 unrelated pregnant non-diabetic controls (428 Scandinavian and 122 Arabian) matched for ethnicity. All subjects were genotyped for *HLA DQB1* risk genotypes, GAD65Ab, insulin gene variable number of tandem repeat (*INS* VNTR), mitochondrial $tRNA^{leu}$ A3243G mutation, and peroxisome proliferator-activated receptor, gamma 2 (*PPARG* Pro12Ala) polymorphism.

Phenotypic characteristics of women with GDM

Arabian women with GDM were approximately 50% more insulin resistant as compared to Scandinavian women with GDM for the same BMI (HOMA-IR; 3.2±0.3 vs. 2.2±0.2, p=0.02). In addition, in Arabian women with GDM, beta-cell compensation for the degree of insulin resistance was impaired by 42% compared with Scandinavian women with GDM after adjustment for BMI (Disposition index; 3.3±0.6 vs. 5.7±0.6, p=0.01).

GAD65Ab

GAD65Ab were more frequent in women with GDM compared to control women 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 controls. The same was observed in Arabians where 4/87 (4.6%) of women with GDM were positive for GAD65Ab compared with 0/122 (0%, p=0.03) in controls (Figure 7).

HLA DQB1

In Scandinavian women with GDM, the frequency of *HLA DQB1**0201/0302 or *0201/X or *0302/X (X excludes 0602/3) risk genotypes was slightly higher than in Scandinavian controls (OR 1.36, [95% CI 1.03−1.79], p=0.03; corrected p-value for multiple comparisons p > 0.1). However, no significant difference was seen between Arabian women with GDM and Arabian controls (0.83 [0.49−1.41], p=0.47) (Figure 8). The presence of GAD65Ab was associated with *HLA DQB1* risk genotypes (*p*=0.04) in Scandinavian women with GDM.

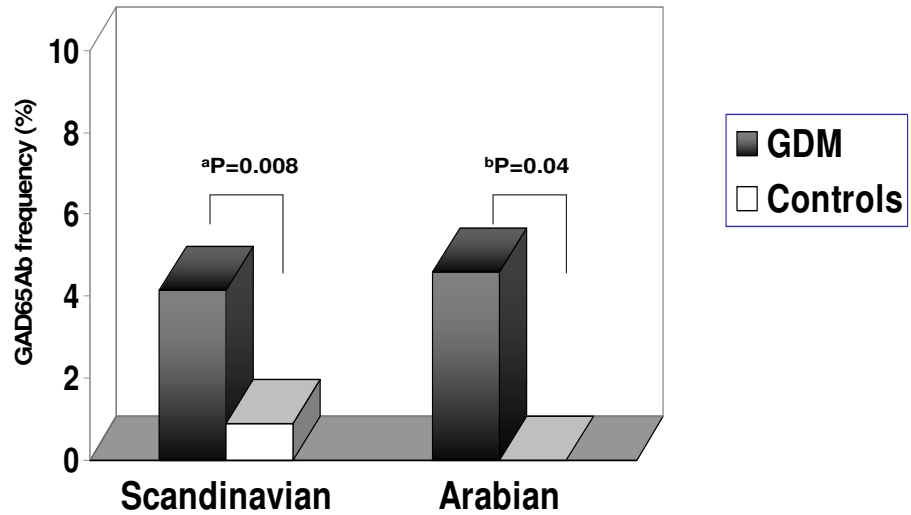


Figure 7. GAD65Ab frequency in Arabian and Scandinavian women with and without GDM. [a] p=0.008, Scandinavian women with GDM (n=289) compared to Scandinavian pregnant controls (n=428). [b] p=0.04, Arabian women with GDM (n=87) compared to Arabian pregnant controls (n=122).

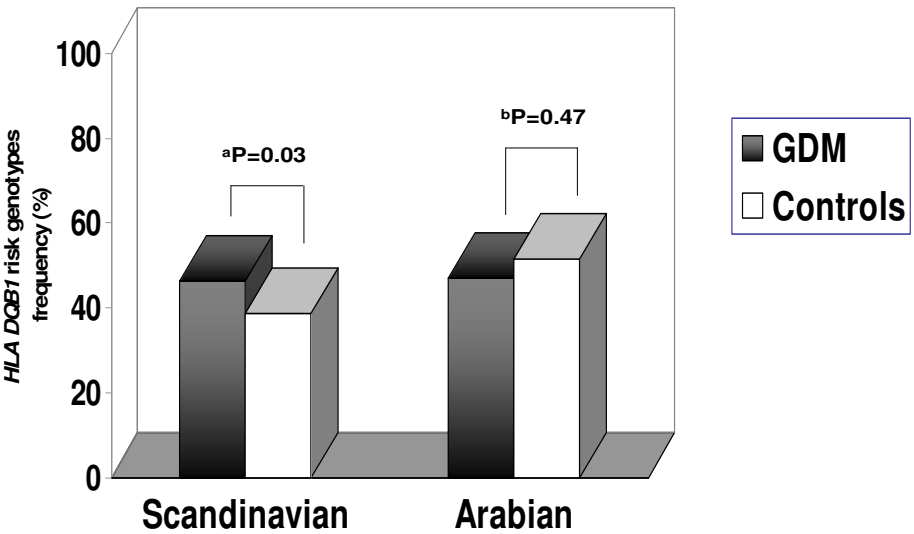


Figure 8. *HLA DQB1* risk genotypes frequency in Arabian and Scandinavian women with and without GDM. *HLA DQB1* risk genotypes include (02/X or 0302/X or 02/0302) where X means either a homozygous allele or any allele other than 02, 0302 or 0602(3). [a] p=0.03, for differences between Scandinavian women with GDM (n=400) and Scandinavian pregnant controls (n=428). [b] p=0.47, for differences between Arabian women with GDM (n=100) and Arabian pregnant controls (n=122).

PPARG Pro12Ala

There were no significant differences in the Pro/Pro, Pro/Ala and Ala/Ala genotypes between GDM and matched control women neither in Scandinavians (71.5, 27.7 and 0.8% vs. 74.1, 24.5 and 1.4%, p=0.40) nor in Arabians (91.0, 9.0 and 0.0% vs. 86.9, 12.3 and 0.8%, p=0.48). Similar allele frequencies were also observed in GDM and matched controls in Scandinavian (14.6 vs. 13.7%, p=0.58) and Arabian (4.5 vs. 7.0%, p=0.31) women. Carriers of the Ala-allele had lower HOMA-IR index compared to Pro/Pro-genotype carriers (1.9±0.1 vs. 2.5±0.2, p=0.11; one-tailed p-value < 0.05). However, the observed difference in HOMA-IR index between Arabian and Scandinavian GDM women remained significant after adjusting for the *PPARG* Pro12Ala genotype (p=0.02).

INS VNTR

The frequency of the I/I, I/III and III/III genotypes of the *INS* VNTR was similar in GDM and control women in Scandinavians (50.5, 42.3 and 7.2% vs. 50, 43.2 and 6.8%, p=0.94) and in Arabians (61.0, 34.0 and 5.0% vs. 65.6, 31.1 and 3.3, p=0.70). Neither was there any significant difference in the frequency of class III VNTR between GDM and controls in Scandinavians (28.4% vs. 28.4%, p=0.99) or in Arabians (22.0% vs. 18.9%, p=0.41).

Mitochondrial *tRNA^{leu}* A3243G

The A3243G mutation was found only in 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. She had a fasting C-peptide concentration of 0.28 nmol/l and was GAD65Ab-negative. The Scandinavian woman had no family history of diabetes. She was 34 years old at the diagnosis and also GAD65Ab-negative. Neither woman had hearing loss.

Study II. Association of the E23K polymorphism in the *KCNJ11* gene with gestational diabetes mellitus

Here, we studied polymorphisms in the genes encoding for the potassium inwardly-rectifying channel, subfamily J, member 11 (*KCNJ11* E23K), insulin receptor substrate 1 (*IRS1* G972R), uncoupling protein 2 (*UCP2* –866G>A) and Calpain 10 (*CAPN10* SNP43 & -SNP44) in 1777 unrelated Scandinavian women (588 GDM and 1189 pregnant non-diabetic controls).

KCNJ11 E23K

The frequency of the EE, EK and KK genotypes of the *KCNJ11* E23K polymorphism was significantly different between GDM and control women (31.5, 52.7 and 15.8% vs. 37.3, 48.8 and 13.9%, respectively, p=0.050).

Furthermore, the frequency of the K-allele was increased in women with GDM compared to controls (1.17 [1.02−1.35], p=0.027). Under a dominant model [KK+EK vs. EE], the K-allele was associated with a greater effect (1.3 [1.05−1.60], p=0.016) (Figure 9). The association was almost the same when women, who were positive for GAD65Ab and/or IA-2Ab, or who had low fasting C-peptide levels (<0.3 nmol/l) were excluded (data was not available for all subjects).

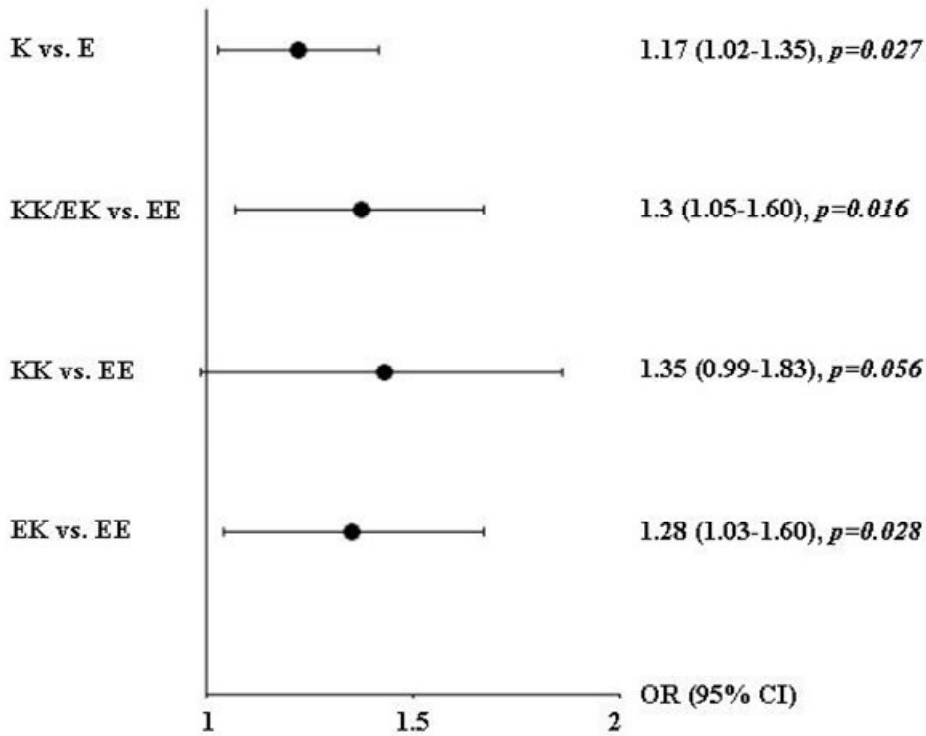


Figure 9. Odds ratios and 95% CI for *KCNJ11* E23K polymorphism in women with GDM. The E/E genotype or the E allele is defined as the reference (i.e. OR=1.0).

IRS1 G972R

The RR genotype of the *IRS1* G972R polymorphism was found only in women with GDM (91.0, 8.3 and 0.7% vs. 90.7, 9.3 and 0.0% for GG, GR and RR genotypes respectively, p=0.014), and this difference was statistically significant under a recessive model [RR vs. GR+GG] (0.7 vs. 0.0%, p=0.011). However, the R972-allele frequency was similar in both groups (1.04 [0.75–1.44], p=0.80).

There were no significant differences in the allele frequencies between GDM and controls for the other polymorphisms studies [*UCP2* –866G>A (1.07 [0.92–1.23], p=0.38); *CAPN10* SNP43 (0.96 [0.82–1.13], p=0.65) and *CAPN10* SNP44 (0.97 [0.80–1.16], p=0.71)].

Study III. Common variants in MODY genes increase the risk for gestational diabetes mellitus

In this study, we genotyped 5 common variants in *GCK*, *HNF1A* and *HNF4A* genes in 1880 Scandinavian women (648 women with GDM and 1232 pregnant non-diabetic controls).

GCK –30G>A

The frequency of the GG, GA and AA genotypes of the *GCK* −30G>A polymorphism differed significantly between GDM and control women (67.8, 28.2 and 4.0% vs. 72.3, 25.7 and 2.0%, respectively, p=0.010). Furthermore, the A-allele frequency was more common in GDM women compared to controls (1.28 [1.06−1.53], p=0.008, corrected p-value, p=0.035). Under a recessive model [AA vs. GA+GG], the OR increased further to 2.12 ([1.21−3.72], p=0.009). Using a dominant model [AA+GA vs. GG], the OR for GDM was 1.24 ([1.01–1.53], p=0.039) (Figure 10).

HNF1A I27L

The genotype frequencies of the *HNF1A* I27L polymorphism were significantly different between GDM and control women (39.4, 48.5 and 12.1% vs. 46.1, 41.8 and 12.1% for II, IL and LL genotypes respectively, p=0.016). The L-allele was slightly more common in women with GDM compared with controls (1.16 [1.001−1.34], p=0.048, corrected p-value, p=0.17). However, the IL-genotype was more frequent in GDM women than in controls as compared with the II-genotype (1.36 [1.10−1.67], p=0.004). Under a dominant model [IL+LL vs. II], the L-allele was also associated with an increased risk of GDM (1.31 ([1.08−1.60], p=0.007) (Figure 11).

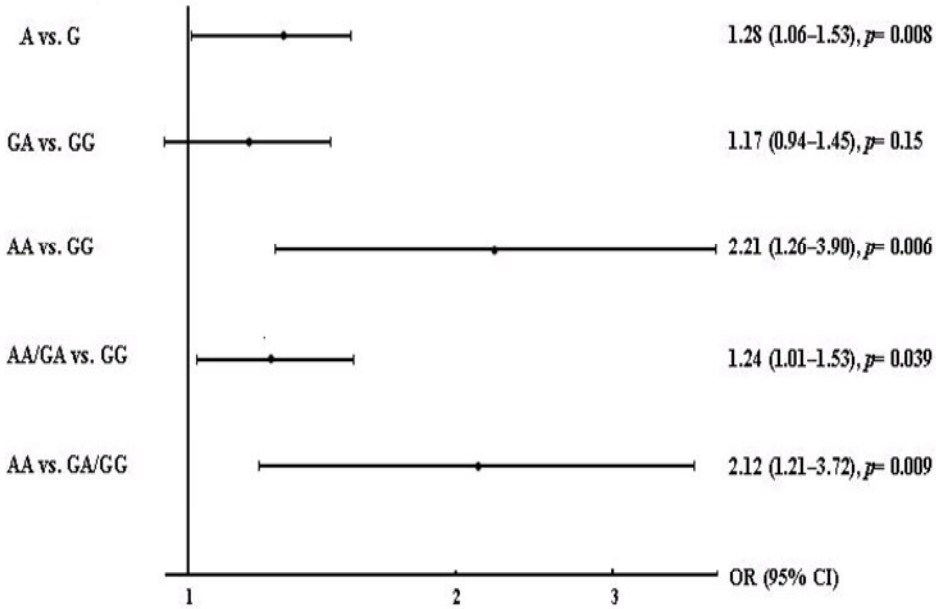


Figure 10. Odds ratios and 95% CI for *GCK* −30G>A polymorphism in women with GDM. The GG-genotype or the G-allele is defined as the reference (i.e., OR=1.0)

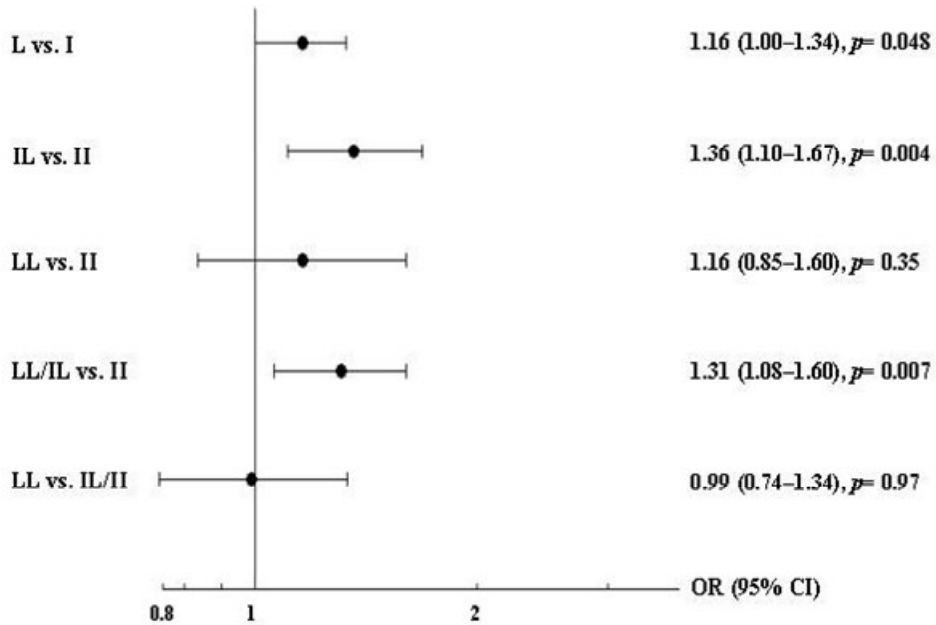


Figure 11. Odds ratios and 95% CI for *HNF1A* I27L polymorphism in women with GDM. The II-genotype or the I-allele is defined as the reference (i.e., OR=1.0)

HNF4A variants

There was no evidence for linkage disequilibrium between studied variants located downstream of the beta-cell-specific (P2) promoter of the *HNF4A* (rs2144908, rs2425637 and rs1885088). D′ values were between 0.01 and 0.5 and r^2 values were between 0.0 and 0.01. The allele frequencies of these variants did not differ significantly between GDM women and controls [rs2144908 (1.14 [0.96–1.37], p=0.14); rs2425637 (1.09 [0.95–1.24], p=0.23) and rs1885088 (0.96 [0.81–1.14], p=0.66)].

Study IV. Association testing of common genetic variants predisposing to the metabolic syndrome or related traits with gestational diabetes mellitus

Here, we studied 1881 unrelated Scandinavian women (649 women with GDM and 1232 pregnant non-diabetic controls) for polymorphisms in the adiponectin (*APM1* +276G>T), *PPARG* (Pro12Ala), *PPARG*-coactivator 1, alpha (*PPARGC1A* Gly482Ser), forkhead box C2 (*FOXC2* −512C>T), and β3-adrenergic receptor (*ADRB3* Trp64Arg) genes.

APM1 +276G>T

The frequency of the T-allele of the *APM1* +276G>T polymorphism was higher in GDM than in control women (1.17 [1.01–1.36], p=0.039). In addition, the GT-genotype carriers had an increased risk of GDM (1.27 [1.04–1.55], p=0.020) as compared to GG-genotype carriers. The effect was similar (1.26 [1.04–1.53], p=0.018) under a dominant model (TT+GT vs. GG) (Figure 12).

The differences in allele frequency between GDM women and controls did not reach significance for the other polymorphisms studied [*PPARG* Pro12Ala (1.06 [0.87–1.29], p=0.53); *PPARGC1A* Gly482Ser (0.96 [0.83–1.10], p=0.54); *FOXC2* −512C>T (1.01 [0.87–1.16], p=0.94) and *ADRB3* Trp64Arg (1.22 [0.95–1.56], p=0.12)].

Gene-gene interaction

In a post hoc analysis of data from the four papers, we also looked for a potential gene-gene interaction between variants, which either have shown association with GDM (*KCNJ11* E23K, *GCK* -30G>A, *HNF1A* I27L, and *APM1* +276G>T) or with T2D in large meta-analyses (*PPARG* Pro12Ala, *CAPN10* SNP43 and -SNP44). Evidence for interaction was found only between *HNF1A* I27L and *CAPN10* SNP44 (1.78 [1.11–2.86], p=0.02).

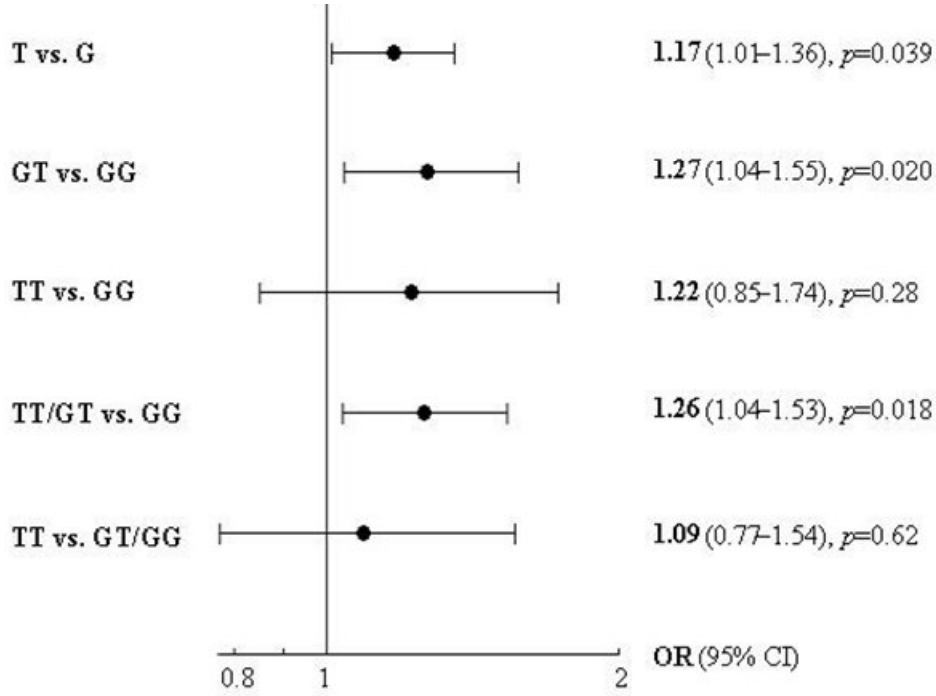


Figure 12. Odds ratios and 95% CI for *APM1* +276G>T polymorphism in women with GDM. The GG-genotype or the G-allele is defined as the reference (i.e., OR=1.0)

Combination of susceptibility variants

We assessed the combined effect of the alleles of the susceptibility variants for GDM (*KCNJ11* E23K, *GCK* -30G>A, *HNF1A* I27L, and *APM1* +276G>T). We found that the combination of all "non-risk" alleles was associated with reduced risk for GDM (OR=0.75 [95% CI 0.64–0.89], p=0.0009). Table 6 shows the frequency of all combinations in GDM and control women and corresponding ORs for risk of GDM.

Table 6. Frequencies of combinations of susceptibility or protective alleles (*KCNJ11* E23K, *GCK* -30G>A, *HNF1A* I27L, and *APM1* +276G>T) in GDM and control women and corresponding ORs for risk of GDM.

GCK **30G>A**	***HNF1A*** **I27L**	***APM1*** **+276G>T**	***KCNJ11*** **E23K**	**Frequency (%)**		**OR**	**p-value**
				GDM	**Controls**		
G	A	G	G	**0.210**	**0.261**	**0.75**	**0.0009***
G	A	G	A	0.160	0.166	1.07	0.65
G	C	G	G	0.111	0.119	0.93	0.51
G	A	T	G	0.097	0.095	1.02	0.83
G	C	G	A	0.084	0.074	1.15	0.28
G	A	T	A	0.062	0.056	1.12	0.42
G	C	T	G	0.049	0.047	1.04	0.83
A	A	G	G	0.044	0.044	1.00	0.99
G	C	T	A	0.045	0.035	1.28	0.17
A	C	G	G	0.034	0.026	1.34	0.17
A	A	G	A	0.028	0.022	1.26	0.28
A	C	G	A	0.024	0.015	1.57	0.08
A	A	T	G	0.019	0.014	1.36	0.27
A	A	T	A	0.015	0.013	1.23	0.53
A	C	T	G	0.014	0.012	1.16	0.59
A	C	T	A	0.005	0.003	1.88	0.32

Risk alleles are shaded. *The combination of all "non-risk" alleles was associated with reduced risk for GDM (OR=**0.75** [95% CI 0.64–0.89], p=0.0009).

DISCUSSION

Association Studies (Studies I-IV)

So far, few genetic factors predisposing to the development of GDM have been identified. In the papers presented in this thesis, we used an *association study* design to identify genetic variants contributing to GDM. Association studies are powerful tools to examine common genetic variants with a relatively weak genetic effect in complex disorders [218, 347]. The allele frequencies of several polymorphisms in "candidate" genes are compared between unrelated individuals with the disease and matched healthy controls.

Selection of control samples is crucial as it is more difficult than choosing individuals with the disease. Controls must be chosen from the same population and during the same period as cases. A strength of our studies was that we ascertained GDM and control women from the same population in southern Sweden and during the same period to minimize the effect of heterogeneity.

Another important aspect is that the study must be sufficiently powered. Thus to detect common variant(s) with low relative risk, one must genotype a large number of cases and controls. In this thesis, we had enough power to detect a modest effect (OR between 1.25-1.5) in Scandinavian women (*Study I-IV*); however, the study in Arabian women (*Study I*) was underpowered to detect such an effect (Figure 6). Therefore, we restricted the studies of further genetic variants to the larger Scandinavian population.

The impact of ethnicity on GDM (*Study I*)

There have been reports of a higher prevalence of GDM among Arabian (5-7%) [20, 60] as compared to Scandinavian (~2%) women [46, 348, 349]. In this study, we found that Arabian women with GDM were about 50% more insulin resistant as compared to Scandinavian women with GDM and with the BMI (Figure 13). This is in line with the observation that T2D is more common in Arabs [350] than in Scandinavians [172]. Also, the recent finding that Caucasian women with prior GDM were more insulin sensitive than Afro-Caribbean women with prior GDM supports our finding [100]. This might suggest that the relative contribution of insulin resistance in GDM differs between Arabian and Scandinavian women. In fact, Asian ethnicity has independently been associated with increased insulin resistance in late pregnancy compared with Caucasian heritage. Moreover, pre-pregnancy BMI had a greater effect on insulin resistance in Asian than in Caucasian pregnant women [351].

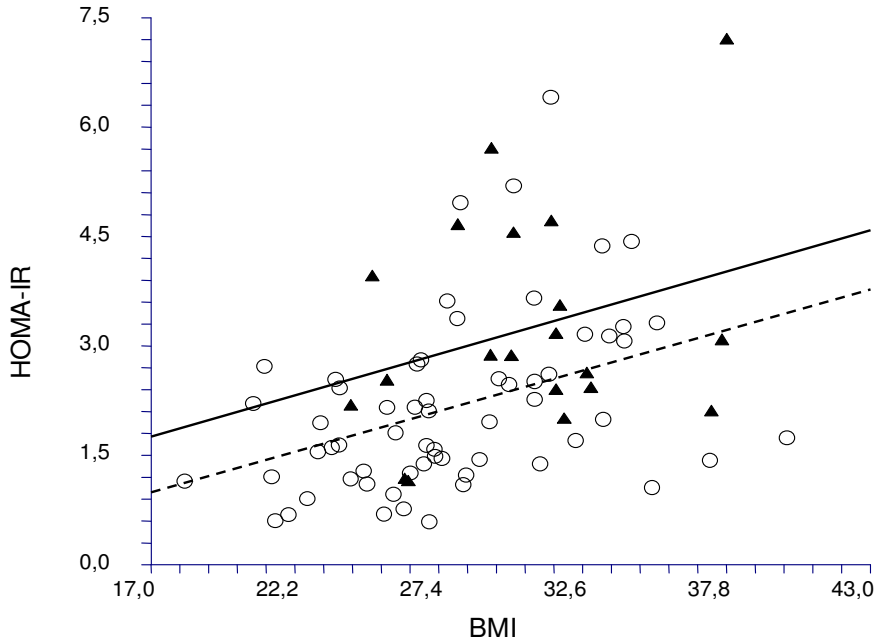


Figure 13. Relation between HOMA-IR and BMI in Arabian (*solid triangles and solid line*) and Scandinavian (*empty circles and dashed line*) women with GDM

GDM and genetic and immunological markers associated with type 1 diabetes (*Study I*)

GAD65Ab have the highest sensitivity and predictive value for T1D [170]. In addition, *HLA DQB1* alleles have consistently been associated with T1D [170]. Since GDM develops in an immunologic milieu, we hypothesized that autoimmunity might be responsible for development of GDM in at least a subset of these women. Thus, we studied GAD65Ab and *HLA DQB1* risk genotypes in Arabian and Scandinavian women with and without GDM. Given the fact that the prevalence of T1D in Scandinavians is among the highest in the world, whereas it is significantly lower in Arabians [171], we also tested the hypothesis that the relative contribution of these genetic and autoimmune markers in GDM might differ between Arabian and Scandinavian women.

The overall frequency of GAD65Ab positivity was low in both GDM and control women although both Arabian and Scandinavian women with GDM had a significantly higher frequency of GAD65Ab (> 4%) as compared to matched pregnant control women (< 1%) (Figure 7). This prevalence falls midway between the 0% reported from northern Italy [207] and the 9.5% found in a German multi-centre study [192]. The prevalence in Scandinavian women with GDM was consistent with the prevalence found in Finland [188, 211].

However, lower frequency of GAD65Ab (2.2%) has recently been reported in Arabian women with GDM from Saudi Arabia [212]. This might be due to true heterogeneity of the study populations since Arabian women in this study were ascertained among immigrants living in Sweden. Hypothetically, additional environmental factors might trigger an autoimmune process in Arabian women living in Sweden as compared to women living in Saudi Arabia. It is more likely, though, that differences in diagnostic criteria and age at diagnosis of GDM may explain these small differences.

We also found a modest effect of *HLA DQB1* risk genotypes on the predisposition to GDM in Scandinavians (OR=1.36), but not in Arabians (OR=0.83). This might suggest that *HLA DQB1* alleles do not contribute to GDM pathogenesis in Arabians, as *HLA DQB1* *0201 and *0302 alleles also confer an increased risk for T1D in Arabs [352-354]. Given that the study was underpowered to detect ethnic differences, larger studies are needed to investigate the impact of *HLA* alleles in Arabian women with GDM.

The *INS* VNTR is a polymorphic minisatellite located 596 bp upstream in the promoter region and is comprised of 3 classes depending on the number of repeats: class I (26–63 repeats), II (64–140 repeats) and III (141–209 repeats) [355, 356]. Class I has been associated with an increased risk of T1D, while class III protected against T1D [356-358]. However, the role of *INS* VNTR in the predisposition to other metabolic disorders, such as T2D and polycystic ovary syndrome (PCOS), is still controversial [359-363]. We were unable to detect any significant impact of *INS* VNTR on the risk of GDM, neither in Arabian nor in Scandinavian women. In fact, the allele and genotype frequencies in Scandinavian women are comparable with those found in Danish Caucasians [360], but unfortunately there is no prior data available for Arabs.

GDM and genetic markers associated with type 2 diabetes (*Study II*)

The aim of this study was to test whether common variants known to influence beta-cell function and thereby increase risk for T2D also confer risk for GDM.

Our key finding was that the E23K polymorphism in *KCNJ11* was associated with a modest increased risk for GDM with an OR of 1.17 and the effect was greater under a dominant model (OR=1.3). In fact, the effect was the same (OR=1.18) with overlapping 95%CI after excluding women at risk for T1D (i.e. women positive for autoantibodies or who had low C-peptide levels during pregnancy). This was consistent with the findings in T2D, particularly in Caucasians [110, 234, 364, 365]. The present finding supports the concept that GDM and T2D share a common genetic background. In addition, the previously reported deleterious effect of E23K polymorphism on insulin secretion [234, 366] also supports the concept of a detrimental role of beta-cell dysfunction in

the pathogenesis to GDM [139]. This variant has also been associated with diminished suppression of glucagon secretion in response to hyperglycaemia [367]. In vitro, the E23K variant leads to reduced ATP sensitivity of the K_{ATP} channels, which in turn leads to overactive channels and thereby decreased insulin release from beta cells [368] as well as impaired glucose uptake in muscles during exercise [369].

The other polymorphisms studied (*CAPN10* SNP43 & -SNP44, *IRS1* G972R, and *UCP2* -866G>A) showed no significant impact on GDM status. This might indicate that these variants do not have a major role in the pathogenesis of GDM or that their role is too small to be detected in the study. We cannot exclude the possibility that the variants might have an effect on metabolic parameters during pregnancy as *IRS1* G972R polymorphism has recently been associated with obesity, and high insulin and glucose levels in women with GDM [293]. Also, SNP63 in *CAPN10* and a haplotype combination of SNP43, SNP19 and SNP63 increased the risk of GDM in a small study in Austrian Caucasians [226].

GDM and common variants in MODY genes (*Study III*)

Since impaired beta-cell function is the hallmark of both GDM and MODY, we hypothesized that common polymorphisms in MODY genes might contribute to the risk of GDM. Indeed, we found that the –30G>A polymorphism in the beta-cell-specific promoter of *GCK* moderately increased the risk for GDM (OR=1.28). This supports the hypothesis and extends the knowledge about the deleterious effect of this variant on glucose metabolism during pregnancy [146]. Our finding and the previously reported association of this variant with impaired beta-cell function and impaired glucose regulation (IGR) might suggest that the A-allele or another variant in strong linkage disequilibrium with it, reduces the activity of *GCK* and/or alters its expression [145, 370]. Surprisingly, Shelton et al. have shown that deletion of a 10-bp sequence of the *GCK* promoter, including the -30G>A variant, had no effect on transcriptional activity of *GCK* in insulinoma cell line [371].

Mutations in *HNF1A* are commonly seen in women with GDM [133, 144] (also unpublished own observations). Another variant (*HNF1A* I27L) was associated with a modestly increased risk for GDM (OR=1.16). This supported our recent study, which demonstrated reduced transcriptional activity of the I27L polymorphism in vitro [372]. Other studies have also shown that the polymorphism influence beta-cell function [373]. Thus, this polymorphism might predispose women with a slight impairment of their beta-cell function to be affected by a deteriorated glucose tolerance when becoming insulin resistant during pregnancy. We also found a nominal association of the V-allele of the

HNF1A A98V variant with GDM (3.5% vs. 1.3%, p=0.03) in 226 women with GDM and 229 NGT women (unpublished observations). However, the limited power of this rare polymorphism forced us to exclude it from further studies.
Although GDM and T2D may share common pathogenic pathways, they might differ on essential points as GDM does not progress to T2D in all cases and as many women with T2D never had GDM. This might partially explain the discrepancy between this study and two recent large studies in T2D that could not find an association of variants in *HNF1A* (including the I27L polymorphism) with T2D [374, 375]. However, a recent study from our laboratory indeed showed an association between the *HNF1A* gene polymorphisms and T2D as well as intermediate traits [372].

The expression of *HNF4A* in beta cells is primarly mediated by a distant upstream promoter (P2) [376, 377]. Mutations in the *HNF1A* and *IPF1* binding sites of the P2 promoter have been associated with MODY1 [376, 377]. Recent studies suggest that variants near the *HNF4A* P2 have a modest effect on the risk for T2D [378-381]. None of the three tested SNPs in *HNF4A* (rs2144908, rs2425637 and rs1885088) was associated with an increased risk of GDM. The data on these SNPs in T2D are somewhat contradictory. This might be due to the fact that our study was not powered enough to detect such a small OR (1.14-1.15) as reported in Caucasians from UK or Denmark [380, 381]. Interestingly, the rs2144908 variant was associated with reduced beta-cell function in unaffected Finnish offspring of parents with T2D [378]. In a subset of 52 women with GDM, no effect of the variant was observed on measures of insulin secretion (data not shown).

GDM and a mutation in mitochondrial *tRNA^{leu}* gene (*Study I*)

MIDD is a maternally inherited monogenic type of diabetes with an age of onset around 35 [382]. Impaired insulin secretion is the main feature of MIDD. This is mainly caused by the A3243G mutation in the mitochondrial *tRNA^{leu}* [272]. Thus, we hypothesized that this mutation might predispose to GDM by affecting beta-cell function. We only found two GDM women carrying this mutation. Thus, we exclude it as a major cause of GDM both in Arabians and in Scandinavians. This is consistent with reports in other populations [274, 275].

GDM and common genetic variants associated with the metabolic syndrome or related traits (*Study I and IV*)

In *Study IV*, we demonstrated that the T-allele of the *APM1* +276G>T polymorphism was associated with an increased risk for GDM (OR=1.17). This is not surprising since the T-allele has been associated with insulin resistance and an increased risk for T2D [383, 384]. This is also consistent with the fact

that the prevalence of MetS is higher in women with prior GDM than in women with normoglycaemia during pregnancy [98, 99, 167]. In addition, the T-allele has been associated with decreased serum adiponectin levels [385], a trait that was associated with GDM *per se* [302-304, 306] and insulin resistance in women with GDM [302]. However, other studies reported association of the G-allele with lower adiponectin concentrations [160, 386-389] and decreased mRNA levels in visceral adipose tissue of obese individuals [390].

The *PPARG* Pro12Ala is one of the most reproducible variants for association with T2D and insulin resistance. Surprisingly we found no effect of this polymorphism on the risk of GDM neither in Arabians (*Study I*) nor in Scandinavians (*Study I and IV*). Lack of appropriate power might partially explain this negative association (Figure 6). However, it could also be that the modest effect on insulin sensitivity imposed by this variant could not break the massive insulin resistance characteristic of pregnancy. In fact, PPARG mRNA and protein levels were reduced in subcutaneous adipose tissue of pregnant women regardless of GDM as compared to non-pregnant women [290]. This might suggest that *PPARG* has an effect on pregnancy-induced insulin resistance [290]. In vitro, the Ala allele was associated with decreased binding affinity to PPARG response element and lower transactivation capacity of responsive promoters in mouse adipocyte cell lines. This may lead to less efficient stimulation of PPARG target genes and a predisposition to lower levels of adiposity, which in turn improves insulin sensitivity [161]. This view is supported by findings of increased mRNA expression of *PPARG* in adipose tissue of obese compared to lean subjects [391]. This should lead to insulin resistance.

Arabian women had a significantly lower frequency of the Ala-allele compared to Scandinavian women (5.9 vs. 14.1%, p=0.0002). Our finding and the previously reported association of the Ala-allele with insulin sensitivity suggest that this polymorphism may partly explain the observed difference seen in HOMA-IR between the Arabian and Scandinavian women with GDM. Interestingly, a recent study in the Saudi population also demonstrated a low frequency of the Ala-allele [392].

SUMMARY

- Arabian women with GDM are more insulin resistant compared to Scandinavian women with GDM and with the same BMI.

- Arabian and Scandinavian women with GDM have a higher frequency of GAD65Ab (> 4%) compared to matched pregnant control women (< 1%).

- Scandinavian women with GDM share some genetic features with type 1 diabetes such as *HLA DQB1* risk genotypes (OR=1.36).

- The E23K polymorphism in *KCNJ11* is associated with a modest increased risk for GDM (OR =1.17).

- The –30G>A polymorphism in the beta-cell-specific promoter of *GCK* increases the risk for GDM (OR=1.28).

- The I27L polymorphism in *HNF1A* is associated with an increased risk for GDM (OR=1.16).

- The +276G>T polymorphism in *APM1* modestly increases the risk for GDM (OR=1.17).

- A combination of the "protective" alleles of *KCNJ11* E23K, *GCK* -30G>A, *HNF1A* I27L, and *APM1* +276G>T variants is associated with reduced risk for GDM (OR=0.75).

CONCLUSIONS

- ✓ GDM shares features with both type 1 and type 2 diabetes.
- ✓ Common variants in several type 2 diabetes candidate genes increase susceptibility to heterogeneous GDM.
- ✓ Many of these variants influence beta-cell function.
- ✓ Genetic variants may also aggravate insulin resistance during pregnancy in women with GDM. A likely consequence of this is that Arabian women are more insulin resistant than Scandinavian women for the same BMI.

SWEDISH SUMMARY (POPULÄRVETENSKAPLIG SAMMANFATTNING)

Graviditetsdiabetes (GDM) definieras som nedsatt glukostolerans av varierande svårighetsgrad som upptäcks under graviditet och som oftast försvinner efter förlossning. GDM innebär ökad risk för såväl moder som barn. Kvinnor med GDM föder ofta stora barn och har ökad risk för komplikationer i samband med förlossningen. GDM-prevalensen varierar avsevärt mellan olika populationer. Medan den i Sverige ligger kring 2 % har siffror kring 5-7 % rapporterats från Arabvärlden och 5-10 % från Asien. Cirka 90 % av alla kvinnor med diabetes under graviditeten har GDM, medan typ 1 diabetes och typ 2 diabetes svarar för resterande 10 %. Upp till 50 % av kvinnorna med GDM bedöms utveckla diabetes inom en 10-årsperiod efter förlossningen, de flesta typ 2 diabetes och endast en ringa andel typ 1. Även om glukostoleransen normaliseras efter förlossningen återfår 26-70 % av kvinnorna GDM vid en eventuell efterföljande graviditet.

GDM karakteriseras av en otillräcklig förmåga att insöndra insulin i takt med den ökade insulinresistens som inträder under en graviditet. Orsaken till GDM är sannolikt en interaktion mellan riskgener och diabetogena faktorer uppkomna under graviditeten. Familjär anhopning av GDM talar för att genetiska faktorer kan spela en avgörande roll. Olika genetiska variationer i ”kandidat”-gener som *SUR1*, *CAPN10*, *MBL2, ADRB3* och *HFE* har associerats med GDM. Andra predisponerande faktorer för GDM är övervikt, ytterligare viktökning efter förlossningen samt upprepade graviditeter.

Den övergripande målsättningen i den här avhandlingen var att identifiera genetiska och immunologiska riskfaktorer som bidrar till utveckling av GDM hos kvinnor av olika etnisk bakgrund.

I delarbete I undersöktes 400 skandinaviska och 100 arabiska kvinnor med GDM samt 428 skandinaviska och 122 arabiska gravida kvinnor med normal glukostolerans avseende autoimmunitet (GAD-antikroppar); typ 1 diabetesrelaterade HLA-genotyper och polymorfismer i generna för insulin (*INS* VNTR) och ”peroxisome-proliferative activated receptor-gamma 2” (*PPARG* Pro12Ala) samt en mutation i mitokondriellt DNA ($tRNA^{leu}$ A3243G). GDM var förenat med GAD-antikroppar hos såväl skandinaviska som arabiska kvinnor med GDM men endast med HLA-riskgenotyper hos skandinaviska kvinnor. Ingen signifikant skillnad i prevalens av *PPARG* Pro12Ala och *INS* VNTR polymorfismer kunde ses. Däremot var de arabiska kvinnorna mer insulinresistenta än de skandinaviska kvinnorna med samma viktindex (BMI).

Både GDM och typ 2 diabetes karakteriseras av insulinbrist och insulinresistens. I delarbete 2 undersöktes om polymorfismer i gener (*KCNJ11* E23K, *IRS1* G972R, *CAPN10 SNP43 & -SNP44*, och *UCP2* –866G>A), som

tidigare visat sig vara associerade med typ 2 diabetes, också är associerade med GDM hos 588 skandinaviska kvinnor med GDM och 1189 skandinaviska gravida kvinnor med normal glukostolerans. En E23K polymorfism i genen för *KCNJ11* var signifikant associerad med 1,17 gånger ökad risk att drabbas av GDM, vilket är förenligt med dess kända effekt på insulinsekretionen.

”Maturity Onset Diabetes of the Young” (MODY) är en speciell ärftlig form av typ 2 diabetes med sjukdomsdebut ofta före 25 års ålder. Ärftlighetsgången är autosomalt dominant, d.v.s. sjukdomen förekommer i alla generationer. MODY karaktäriseras vidare av insulinbrist, en central faktor i patogenesen av GDM. I delarbete 3 undersöktes om vanligt förekommande polymorfismer i tre MODY-gener (*glukokinas* = MODY 2, *HNF4A* = MODY1 och *HNF1A* = MODY 3) ökar risken för GDM genom att studera 648 skandinaviska kvinnor med GDM och 1232 gravida kvinnor med normal glukostolerans. En –30G>A polymorfism i glukokinas och I27L i *HNF1A* var signifikant associerade med respektive 1,28 och 1,16 gånger ökad risk för GDM. Alla dessa variationer kan förmodligen kopplas till en försämrad betacellfunktion och insulinsekretion.

Det metabola syndromet är en grupp av riskfaktorer associerade med en ökad risk att drabbas av hjärt-kärlsjukdom. Det omfattar förhöjt blodsocker, fetma, förhöjt blodtryck, och förhöjda blodfetter. Insulinresistens har en central roll i patofysiologin av både GDM och metabolt syndrom. Delarbete 4 bygger på samma patientmaterial som delarbete 3 och delvis delarbete 2. Här har vi undersökt om polymorfismer i ett antal gener, som associerats med insulinresistens eller metabolt syndrom också är associerade med GDM. Av de undersökta genvarianterna (*APM1* +276G>T, *PPARG* Pro12Ala, *PPARGC1* Gly482Ser, *FOXC2* −512C>T, och *ADRB3* Trp64Arg) visade sig en variation i genen för adiponektin (*APM1* +276G>T) vara associerad med 1,17 gånger ökad risk för GDM.

Sammantaget tyder resultaten på att vanligt förekommande variationer i ett antal gener, som är kopplade till insulinsekretion och insulinresistens i samspel med immunologiska faktorer ökar risken för GDM. Denna kunskap kan i förlängningen leda till att rätt individer bättre kan identifieras för medicinskt omhändertagande under graviditet samt på sikt en minskad morbiditet för mor och barn.

ARABIC SUMMARY

تجدر الإشارة أنه تم إكتشاف أن السيدات العربيات المصابات بداء سكر الحمل كن أكثر مقاومة لتأثير للأنسولين بنسبة تصل إلى 50% بالمقارنة مع السيدات الإسكندنافيات المصابات بهذا الداء عند تطابق معدل كتلة الجسم (BMI).

دراسة رقم 2:

استقر من خلال الدراسات أن هناك تشابه بين مرض السكري من النوع الثاني وداء سكر الحمل حيث أن كلاهما يحدث نتيجة نقص الأنسولين بالأضافة الى زيادة مقاومة الأنسولين.
في هذا البحث تم دراسة ما إذا كان هناك علاقه بين 5 مرفيات في 4 مورثات (والتي سبق وأن أظهرت الدراسات وجود علاقه بينها وبين مرض السكري من النوع الثاني) وخطر الإصابة بسكر الحمل عند 588 سيدة اسكندنافية مصابة بسكر الحمل و1189 من غير المصابات .
لقد تم اكتشاف أن المورفية (E23K) في المورث (*KCNJ11*) تزيد من خطر الاصابة بداء سكر الحمل بمعدل يساوي 1.17 ضعف، وهذا يتفق تماما مع ما تم اكتشافه سابقا من تاثير هذه المورفية على خفض معدل افراز الانسولين.

دراسة رقم 3:

يتميز كل من سكر الحمل والـ (MODY) (مرض السكر للبالغين الذي يصيب الشباب) بوجود نقص في إنتاج الأنسولين من خلايا "بيتا" في البنكرياس. في هذا البحث تم دراسة ما إذا كانت بعض المورفيات في 3 من المورثات التي تسبب (MODY) قد تزيد من خطر الإصابة بسكر الحمل عند 648 سيدة اسكندنافية مصابة بسكر الحمل و1232 سيدة اسكندنافية غير مصابة .
وجدنا أن مورفيات (*HNF1A* I27L *& GCK* –30G>A) تزيد من خطر الإصابة بسكر الحمل بمعدل يساوي 1,28 و 1,16 ضعف على التوالي وهذا يتفق مع الدراسات السابقة التي أظهرت وجود علاقة وثيقة بين هذة المورفيات وبين إختلال خلايا "بيتا" المنتجة للأنسولين.

دراسة رقم 4:

المُتلازمة الأيضية هى عباره عن مجموعة من الأمراض التي تحدث نتيجة خلل في أيض الجسم و تتكون من السمنة، إرتفاع سكر الدم، إرتفاع ضغط الدم و إرتفاع مستوى الشحوم في الدم.
قمنا بدراسة ما إذا كانت خمسة مورفيات، والتى سبق وأن وجد علأقة بينها وبين مقاومة الأنسولين أو المتلازمة الأيضة، تزيد من خطر حدوث داء سكر الحمل عند نفس السيدات الإسكندنافيات اللواتى تم دراستهن في الدراسة رقم 3.
بين كل المورفيات التى تم دراستها وجد أن المورفية (*APM1* +276G>T) تزيد من خطر الإصابة بداء سكر الحمل بمعدل 1,17 ضعف.

مما تقدم نستخلص أن بعض المورفيات في المورثات المهمة والتى لها علاقة وثيقة بالاختلال الوظيفي في خلايا "بيتا" أو مقاومة الأنسولين بالأضافة الى بعض العوامل المناعية تزيد من خطر الإصابة بسكر الحمل. هذه المعرفة قد تساعدنا في المستقبل على أمكانية تحسين نظم العلاج أو الوقاية من داء سكر الحمل مع إمكانية تقليل تأثيره على الأم وطفلها أثناء الحمل وبعده.

سكر الحمل هو عبارة عن ارتفاع مستوى السكر بالدم أثناء فترة الحمل فقط وعودته للمعدل الطبيعي بعد الولادة. تتعرض السيدة المصابه بداء سكر الحمل وجنينها لعدد من المضاعفات منها : زيادة وزن الجنين، الولادة القيصرية، التشوهات الخلقيه للجنين، تسمم الحمل وغير ذالك. تختلف نسبة الاصابه بداء سكر الحمل كثيرا بين الامم و الاعراق. فيما يصيب سكر الحمل 2% من مجموع السيدات الحوامل فى السويد فإن معدل الإصابة بهذا المرض تتراوح بين5-7% في البلدان العربية أو عند ذوات الأصول العربية وحوالي 5-10% عند الأسياويات. يشكل سكر الحمل حوالي 90% من مرض السكري أثناء الحمل بينما تشكل النسبة الباقية السيدات الحوامل المصابات أصلا بمرض السكري (النوع الأول أو الثاني).
بالرغم من أن السكر يعود إلى مستواه الطبيعي غالبا بعد الولادة إلا أن نسبة 50% من السيدات معرض للإصابة بسكر الحمل مرة أخري في الحمل التالي. نحو 50% من السيدات اللواتي يصبن بداء سكر الحمل عرضه للإصابة بمرض السكري (النوع الأول أو الثاني) خلال السنوات العشر التي تلي الحمل، غالبيتهم يصبن بالنوع الثاني وقليل يصبن بالنوع الأول.
يحصل سكر الحمل نتيجة عدم قدرة خلايا "بيتا" في غدة البنكرياس على إفراز كميات كافية من الأنسولين (الهرمون الذي ينقل السكر الى الخلايا لإنتاج الطاقه) بالإضافة الى قصور فى فاعليته (ما يسمى بمقاومة الأنسولين) والتى تتفاقم أثناء الحمل.
الدراسات الحديثة تؤكد أن أسباب حدوث سكر الحمل ليست وراثية بحتة وإنما هي غالبا نتيجة تفاعل عوامل وراثية وبيئية مع بعضها البعض. إن زيادة نسبة الإصابة بسكر الحمل في بعض العائلات إضافة إلى إكتشاف العديد من الطفرات في بعض المورثات (الجينات) والتي تسبب بعض حالات سكر الحمل هو مؤشر يدعم مقولة أن العوامل الوراثية تلعب دورا هاما في حدوث المرض. بالإضافة الى ذالك تم إكتشاف أن وجود بعض المورفيات " polymorphisms" {نوع خاص وشائع (<5%) من الطفرات والذي يسمى أيضا بمتعددة الصور} في بعض المورثات تزيد من إحتمالية حدوث سكر الحمل. العوامل البيئية كالبدانه (السمنة) وغيرها تزيد أيضا من احتمالية حدوث سكر الحمل.

الهدف الإجمالي لهذه الرسالة هو تحديد العوامل الوراثية والمناعية التى قد تسهم في الإصابة بداء سكر الحمل عند السيدات العربيات والإسكندنافيات .

دراسة رقم 1:

في هذا البحث تم دراسة 400 سيدة اسكندنافية و100 سيدة عربية مصابات بسكر الحمل و 428 سيدة اسكندنافية و 122 سيدة عربية من غير المصابات به. لقد تم تحليل أحد أنواع الأضداد الذاتية (GAD65Ab) وبعض المورفيات في أحد المورثات (*HIA DQB1*) والتي لها علاقة قوية للإصابة بداء السكري ذو النوع الأول، هذا بالإضافة الى دراسة بعض "المورفيات" في عدد من المورثات (*PPARG* Pro12Ala *& INS* VNTR) وطفرة في أحد مورثات الميتوكوندريا ($tRNA^{leu}$ A3243G) والتى لها علاقة بنقص نسبي في الأنسولين أو تأثيرة على الخلايا.
لقد تم إكتشاف أن (GAD65Ab) لها علاقة بالإصابة بداء سكر الحمل عند كل من السيدات العربيات والإسكندنافيات. من ناحية أخري فإن بعض المورفيات في المورث (*HIA DQB1*) وجد أنها تزيد من خطر الإصابة بداء سكر الحمل عند السيدات الإسكندنافيات فقط.

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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

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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].

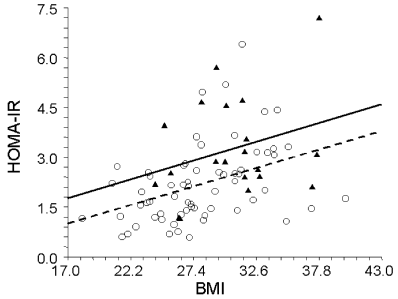

Fig. 1. Relation between HOMA-IR and BMI in Arabian (*solid triangles and solid line*) and Scandinavian (*empty circles and dashed line*) women with GDM

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.

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ARTICLE

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Association of the E23K polymorphism in the *KCNJ11* gene with gestational diabetes mellitus

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Abstract *Aims/hypothesis:* Gestational diabetes mellitus (GDM) and type 2 diabetes share a common pathophysiological background, including beta cell dysfunction and insulin resistance. In addition, women with GDM are at increased risk of developing type 2 diabetes later in life. Our aim was to investigate whether, like type 2 diabetes, GDM has a genetic predisposition by studying five common polymorphisms in four candidate genes that have previously been associated with type 2 diabetes. *Materials and methods:* We studied 1,777 unrelated Scandinavian women (588 with GDM and 1,189 pregnant non-diabetic controls) for polymorphisms in the genes encoding potassium inwardly rectifying channel subfamily J, member 11 (*KCNJ11* E23K), insulin receptor substrate 1 (*IRS1* G972R), uncoupling protein 2 (*UCP2* −866G→A) and calpain 10 (*CAPN10* SNP43 and SNP44). *Results:* The EE, EK and KK genotype frequencies of the *KCNJ11* E23K polymorphism differed significantly between GDM and control women (31.5, 52.7 and 15.8% vs 37.3, 48.8 and 13.9%, respectively; p=0.050). In addition, the frequency of the K allele was increased in women with GDM (odds ratio [OR]=1.17, 95% CI 1.02−1.35; p=0.027), and this effect was greater under a dominant model (KK/EK vs EE) (OR=1.3, 95% CI 1.05−1.60; p=0.016). Analysis of the *IRS1* G972R polymorphism showed that RR homozygosity was found exclusively in women with GDM (91.0, 8.3 and 0.7% vs 90.7, 9.3 and 0.0% for GG, GR and RR genotypes, respectively; p=0.014). The genotype and allele frequencies of the other polymorphisms studied were not statistically different between the GDM and control women. *Conclusions/interpretation:* The E23K polymorphism of *KCNJ11* seems to predispose to GDM in Scandinavian women.

Keywords Association · *CAPN10* · E23K · Gene · Gestational diabetes mellitus · GDM · *IRS1* · *KCNJ11* · Polymorphism · Scandinavian · Type 2 diabetes · *UCP2*

Abbreviations *CAPN10*: gene encoding calpain 10 · DBS: dried blood spots · ESM: electronic supplementary material · GDM: gestational diabetes mellitus · *IRS1*: gene encoding insulin receptor substrate 1 · *KCNJ11*: gene encoding potassium inwardly-rectifying channel, subfamily J, member 11 · OR: odds ratio · SNP: single-nucleotide polymorphism · *UCP2*: gene encoding uncoupling protein 2

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Introduction

Gestational diabetes mellitus (GDM) is defined as glucose intolerance that is first diagnosed during pregnancy [1]. It complicates about 2% of pregnancies in Sweden [2]. However, the prevalence varies between populations [3]. GDM results when pancreatic beta cells fail to compensate for the increased insulin resistance during pregnancy [4, 5]. However, the degree of beta cell dysfunction seems to be the predominant determinant of who will develop GDM [6]. In support of this, several studies have demonstrated that insulin secretion was substantially reduced in women with NGT and a history of GDM compared with controls after pregnancy [7, 8]. In addition, impaired insulin secretion as well as a history of GDM have been shown to predict future type 2 diabetes [9, 10]. Epidemiological

studies have shown that beta cell dysfunction and insulin resistance are the main determinants of type 2 diabetes [11, 12]. Furthermore, both type 2 diabetes and GDM may share other risk factors and the same genetic susceptibility [13]. Also, women with a family history of type 2 diabetes are at increased risk of developing GDM [14].

Type 2 diabetes is considered a paradigm for a multifactorial polygenic disease where common variants in several genes interact with environmental factors to cause the disease [11, 15]. We have originally shown that the Ala allele of the peroxisome proliferator activated receptor gamma (*PPARG* Pro12Ala) polymorphism has been associated with reduced risk of type 2 diabetes [16]. In addition, we and others have reported association between the E23K polymorphism of the potassium inwardly rectifying channel subfamily J, member 11 (*KCNJ11*) gene and increased risk of type 2 diabetes [15, 17]. Although the G972R polymorphism of the insulin receptor substrate 1 (*IRS1*) gene has been associated with type 2 diabetes in several studies [15], no association was found in a recent large study [18]. Variations in the calpain 10 (*CAPN10*) gene have also been associated with type 2 diabetes [15]. A promoter polymorphism (–866G→A) in the uncoupling protein 2 (*UCP2*) gene was originally associated with reduced risk of obesity [19] as well as with reduced [20, 21] or increased [22] risk of type 2 diabetes.

Genetic predisposition to GDM has been reported for variations in the insulin receptor (*INSR*), insulin-like growth factor 2 (*IGF2*), β_3-adrenergic receptor (*ADRB3*), sulphonylurea receptor 1 (*ABCC8*), *CAPN10* and mannose-binding lectin (*MBL2*) genes [23–27], whereas no associations were found for the *PPARG* Pro12Ala polymorphism or insulin gene variable number of tandem repeats (*INS* VNTR) [28]. Also, an association with the *ADRB3* W64R variant could not be replicated in subsequent studies [29, 30]. However, this might be due to lack of power, given the small effect size of most common variants, or due to ethnic heterogeneity between different populations.

There are few data on the role of the *KCNJ11* E23K, *IRS1* G972R, *UCP2* −866G→A and *CAPN10* (SNP43 and SNP44) variants in the risk of GDM. Therefore, in the present study we investigated whether GDM has a genetic predisposition similar to that of type 2 diabetes by genotyping these variants in a case–control study of 1,777 pregnant Scandinavian women, 33.1% of whom had GDM.

Subjects and methods

Study population

In southern Sweden (Skåne), all pregnant women are routinely offered a 75-g OGTT at 27–28 weeks of pregnancy. Women at high risk (previous GDM or a family history of diabetes) are also offered a 75-g OGTT at 12–13 weeks. The tests are performed in the local antenatal care clinics, using HemoCue devices (HemoCue, Ängelholm, Sweden) for capillary whole-blood analysis. GDM is defined as a 2-h capillary glucose concentration (double test) of at least 9 mmol/l according to the proposal by the European Diabetic Pregnancy Study Group [31].

We studied 1,777 unrelated Scandinavian women (588 women with GDM and 1,189 non-diabetic pregnant controls). Women were recruited from two different resources. Two hundred and twenty seven women with GDM were recruited from women referred to Malmö or Lund University Hospitals during the period from March 1996 until December 2003. The other group of women with GDM (n=361) and all non-diabetic pregnant controls (n=1,189) were ascertained among women participating in the Diabetes Prediction in Skåne (DiPiS) study, which is a prospective, longitudinal study of the prediction of type 1 diabetes in all newborns in southern Sweden [32]. At delivery (for DiPiS subjects) and after oral consent, a blood sample was drawn and information obtained about possible GDM or diabetes status. When the child was 2 months old and had been entered into the population registry, the parents were invited by letter to participate with their child in the DiPiS study. If the parents agreed to do so, they gave their written consent and filled out a psychosocial and hereditary questionnaire including information about diabetes status in the family and their country of birth. Ethnicity was also determined using both surname and given name. Since the DiPiS study was not restricted to Swedish subjects but included immigrants as well, we chose only women with a Scandinavian background for the present study. Most of the Scandinavian women were of Swedish origin and a few were of Danish, Norwegian or Finnish origin. Informed oral and/or written voluntary consent was obtained from all study subjects. The study was approved by the ethics committee of Lund University.

Genetic analyses

DNA extraction

Total DNA was isolated from peripheral blood lymphocytes or blood samples were collected as dried blood spots (DBS) on Schleicher and Schuell Grade 2992 filters (Schleicher and Schuell, Dassel, Germany) and punch-outs in 96-well plates were soaked in PCR amplification buffer.

Genotyping using DNA

When peripheral blood DNA was available from the subjects, genotyping of all single nucleotide polymorphisms (SNPs) was carried out using a TaqMan allelic discrimination assay. The assay was carried out using an ABI Prism 7900 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) in a reaction volume of 5 μl, according to the manufacturer's instructions. Primers and probes were designed using Assays-by-Design (Applied Biosystems). The primers and probes used are listed in electronic supplementary material (ESM) Table S1.

Genotyping using DBS

When DBS were available from the subjects, SNP genotyping was carried out using PCR-RFLP, SNaPshot or TaqMan allelic discrimination assay.

The polymorphisms *KCNJ11* E23K (rs5219), *UCP2* −866G→A (rs659366) and *IRS1* G972R (rs1801278) were genotyped using PCR-RFLP or TaqMan allelic discrimination assay (see below). The primers used for template PCR amplification are listed in ESM Table S2. The template PCR was performed with an initial two cycles at 4°C for 30 s followed by 98°C for 3 min, followed by holding at 80°C while the PCR mix was added. Then the PCR programme was continued with an initial denaturation (94°C for 5 min), followed by 45 cycles of denaturation (94°C for 30 s), annealing (30 s) and extension (72°C for 30 s), followed by final extension (72°C for 10 min). PCR amplification was carried out with 3×3 mm of DBS in a total volume of 40 μl containing 1× Pharmacia Amersham buffer (Amersham Pharmacia Biotech, Uppsala, Sweden), 4 nmol each dNTP (MBI Fermentas, St Leon-Rot, Germany), 20 pmol of each primer, 20 μmol Betaine (Sigma-Aldrich, Stockholm, Sweden) and 1.5 U *Taq* polymerase (New England Biolabs, Beverly, MA, USA). The following restriction enzymes were used to digest the PCR products, with name, origin, incubation conditions and agarose gel concentrations in parentheses: *KCNJ11* E23K (*Ban*II; New England Biolabs; 37°C for 4 h; 3.5% agarose gel), *UCP2* −866G→A (*Mlu*I; MBI Fermentas; 37°C for 4 h; 3% agarose gel) and *IRS1* G972R (*Bst*NI; New England Biolabs; 60°C for 2 h; 4.5% agarose gel). PCR products were separated on agarose gel (SeaKem, Rockland, ME, USA) and stained with ethidium bromide to visualise the fragments.

CAPN10 SNP43 (rs3792267) and SNP44 (rs2975760) were genotyped using the SNaPshot assay with an ABI Prism 3100 Sequence Detection System according to the manufacturer's instructions or using the TaqMan allelic discrimination assay (see below). The template PCR was carried out as described above (see description of PCR-RFLP above) and followed by primer extension. The template PCR primers are listed in ESM Table S2. The primers used for primer extension were: *CAPN10* SNP43 5'-GGCTTAGCCTCACCTTCAAA and SNP44 5'-GACT GAGGGCGCTCACGCTTGCTG.

The majority of the samples (n=949) were genotyped using TaqMan allelic discrimination assay for all the SNPs. Initially, a template PCR was carried out as described above (see description of PCR-RFLP above) using primers listed in ESM Table S2. The template PCR was followed by a TaqMan allelic discrimination assay, which was carried out with 2 μl of the PCR product according to the manufacturer's instructions.

Genotyping and quality control

The genotyping success rate was 99.2% for cases (*KCNJ11* E23K, 100%; *UCP2* −866G→A, 98.3%; *IRS1* G972R, 99.8%; *CAPN10* SNP43, 98.1%; SNP44, 99.6%) and 99.3% for controls (*KCNJ11* E23K, 99.2%; *UCP2* −866G→A, 98.7%; *IRS1* G972R, 100%; *CAPN10* SNP43, 99.3%; SNP44, 99.3%). Genotyping accuracy, as determined by regenotyping a random 1124 (12.6%) duplicates for all SNPs [*KCNJ11* E23K, 170 (9.6%); *UCP2* −866G→A, 212 (11.9%); *IRS1* G972R, 176 (9.9%); *CAPN10* SNP43, 297 (16.7%); SNP44, 269 (15.1%)], was 99.82%. In addition, 38 (6.5%) of women with GDM had both peripheral blood DNA and DBS and their genotype results were compared to assess the concordance between the different genotyping methods; we found no discrepancies. For all SNPs, both GDM and control groups were in Hardy–Weinberg equilibrium (χ^2 test, p>0.05), apart from the control group for the *UCP2* −866G→A polymorphism, which showed mild deviation from equilibrium (p=0.029). Our quality control measures suggest that the deviation is due to chance variation rather than genotyping error.

Table 1 Characteristics of Scandinavian women with and without GDM

Variable	GDM % (n)	Controls % (n)	p value
Age (years)	32.2±0.2 (588)	30.5±0.1 (1189)	<0.0001
Weight gain during pregnancy			
<5 kg	11.8 (38/323)	4.0 (32/794)	<0.0001
5–10 kg	31 (100/323)	19.1 (152/794)	<0.0001
11–15 kg	30.6 (99/323)	39.6 (314/794)	0.005
>15 kg	26.6 (86/323)	37.3 (296/794)	0.0007
Smoking	10.7 (35/327)	9.5 (76/802)	0.53
At least one pregnancy before index pregnancy	59.1 (202/342)	52.9 (431/815)	0.053
Twin or triple pregnancies	2.7 (13/474)	1.4 (17/1189)	<0.0001
Insulin treatment during pregnancy	4.9 (13/263)	0.0 (0/429)	<0.0001

Data are mean±SEM

As all data were not available from all study subjects, the number (n) of individuals is given in parentheses (i.e. positive data on variable/ total available data on the same variable)

Statistical analyses

Significance of the difference in age (mean±SEM) between GDM and control groups was tested by ANOVA using the Number Cruncher Statistical Systems (NCSS, Kaysville, UT, USA). The χ^2 or Fisher's exact test was used to compare group frequencies. Odds ratios (ORs) and 95% CIs were obtained from logistic regression analysis. The significance of difference in allele frequencies of the *KCNJ11* E23K polymorphism between GDM and controls was also tested by 1,000 permutations. Two-sided *p* values equal to or less than 0.05 were considered statistically significant.

Power calculations were performed using the Genetic Power Calculator (available at http://ibgwww.colorado.edu/~pshaun/gpc/) [33]. Our power estimates have shown that, under a multiplicative model, the present study with a sample size of 588 cases and 1,189 controls has 80% power to detect an effect size of 1.23 (as measured in terms of genotypic relative risk) when the frequency of the predisposing allele equals to 30%, with a 5% type 1 error rate.

Results

Table 1 shows some phenotypic characteristics of the study subjects. Women with GDM were slightly older than non-diabetic control women (32.2±0.2 vs 30.5±0.1 years, $p<0.0001$) and gained more weight (5–10 kg) during pregnancy (31 vs 19.1%, $p=0.0001$). The genotype and allele frequency distributions of all polymorphisms are presented in Table 2.

KCNJ11 E23K

The EE, EK and KK genotype frequencies of the *KCNJ11* E23K polymorphism differed significantly between GDM

Table 2 Genotype and allele distributions and corresponding odds ratios for GDM

SNP (rs number)	Genotype or allele	GDM *n* (%)	Controls *n* (%)	OR (95% CI) for GDM	OR (95% CI) for GDM, recessive model	OR (95% CI) for GDM, dominant model
KCNJ11 E23K (rs5219)	EE	185 (31.5)	440 (37.3)			
	EK	310 (52.7)	576 (48.8)	1.28 (1.03–1.60)[b]		
	KK	93 (15.8)	164 (13.9)[a]	1.35 (0.99–1.83)[c]	1.16 (0.88–1.53)	1.3 (1.05–1.60)[e]
	K	496 (42.2)	904 (38.3)	1.17 (1.02–1.35)[d]		
IRS1 G972R (rs1801278)	GG	534 (91)	1078 (90.7)			
	GR	49 (8.3)	111 (9.3)	0.89 (0.63–1.27)		
	RR	4 (0.7)	0 (0.0)[f]	Not applicable	Not applicable	0.96 (0.68–1.36)
	R	57 (4.8)	111 (4.7)	1.04 (0.75–1.44)		
UCP2 −866G→A (rs659366)	AA	87 (15.0)	164 (13.9)			
	GA	268 (46.4)	607 (51.7)	0.83 (0.62–1.12)		
	GG	223 (38.6)	404 (34.4)	1.04 (0.77–1.41)	1.2 (0.98–1.47)	0.92 (0.69–1.21)
	G	714 (61.8)	1415 (60.2)	1.07 (0.92–1.23)		
CAPN10 SNP43 (rs3792267)	AA	52 (9.0)	85 (7.2)			
	GA	220 (38.1)	476 (40.3)	0.76 (0.52–1.11)		
	GG	305 (52.9)	620 (52.5)	0.80 (0.55–1.17)	1.01 (0.83–1.24)	0.78 (0.55–1.12)
	G	830 (71.9)	1716 (72.6)	0.96 (0.82–1.13)		
CAPN10 SNP44 (rs2975760)	TT	32 (66.9)	787 (66.7)			
	TC	177 (30.2)	351 (29.7)	1.01 (0.81–1.26)		
	CC	17 (2.9)	43 (3.6)	0.79 (0.45–1.41)	0.79 (0.45–1.40)	0.99 (0.80–1.22)
	C	211 (18.0)	437 (18.5)	0.97 (0.81–1.16)		

[a] $p=0.050$ for difference in genotype frequencies between women with and without GDM
[b] $p=0.028$ for comparison of EK vs EE between women with and without GDM
[c] $p=0.056$ for comparison of KK vs EE between women with and without GDM
[d] $p=0.027$ for difference in allele frequencies between women with and without GDM
[e] $p=0.016$ for comparison of KK + EK vs EE between women with and without GDM
[f] $p=0.014$ for difference in genotype frequencies between women with and without GDM

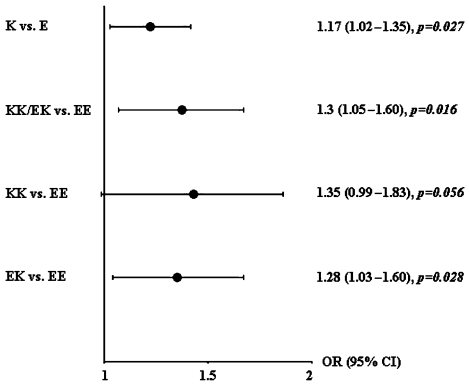

Fig. 1 Odds ratios and 95% CI for *KCNJ11* E23K polymorphism in women with GDM. The E/E genotype or the E allele is defined as the reference (i.e. OR=1.0)

and control women (31.5, 52.7 and 15.8% vs 37.3, 48.8 and 13.9%, respectively; p=0.050). In addition, the K allele was increased in women with GDM (OR 1.17, 95% CI 1.02−1.35; p=0.027) and the effect was greater under a dominant model (KK/EK vs EE) (OR 1.3, 95% CI 1.05 −1.60; p=0.016) (Fig. 1). The association became slightly stronger when women who were positive for GAD65Ab, IA–2Ab or both (n=21; data were not available for all subjects) or when women with GDM who had low fasting C-peptide levels (<0.3 nmol/l) (n=15; data were not available for all subjects) were excluded (Table 3). To verify the results obtained with the χ^2 test (in an exact manner), we further analysed differences in allele frequency between cases and controls using 1,000 permutations and the empirical two-tailed p value was the same as that found with the χ^2 test.

IRS1 G972R

RR homozygosity of the *IRS1* G972R polymorphism was found exclusively in women with GDM (91.0, 8.3 and 0.7% vs 90.7, 9.3 and 0.0% for GG, GR and RR genotypes, respectively; p=0.014), and this was statistically significant under a recessive model (RR vs GR/GG) (0.7 vs 0.0%; p=0.011). However, the R972 allele frequency was similar in the two groups (OR 1.04, 95% CI 0.75–1.44; p=0.80).

UCP2 −866G→A

There was no significant difference in genotype frequencies of the AA, GA and GG genotypes of the *UCP2* −866G→A polymorphism between GDM and control women (15.0, 46.4 and 38.6% vs 13.9, 51.7 and 34.4% respectively; p=0.11). Also, the allele frequencies were similar in the two groups (OR 1.07, 95% CI 0.92−1.23; p=0.38).

CAPN10 SNP43 and SNP44

To test for linkage disequilibrium between SNP43 and SNP44, we calculated both pairwise linkage disequilibrium measures (D' and r^2). The D' was 1.0 with high LOD (log of the odds) score values in cases (D'=1.0; CI 0.9–1.0; LOD=17.4) and controls (D'=1.0; CI 0.94–1.0; LOD=33.3), while the r^2 was 0.09 in both groups. Both SNPs were in Hardy–Weinberg equilibrium (p>0.4) for GDM and controls. There was no significant difference in the frequencies of the GG, GA and AA genotypes of SNP43 between GDM and controls (52.9, 38.1 and 9.0% vs 52.5, 40.3 and 7.2%, respectively; p=0.34) or in the allele frequencies of this SNP (OR 0.96, 95% CI 0.82–1.13; p=0.65). Neither was there any significant difference in the CC, TC and TT genotypes of SNP44 between women with GDM and control women (2.9, 30.2 and 66.9% vs 3.6, 29.7

Table 3 Genotype and allele distributions of the *KCNJ11* E23K polymorphism and corresponding odds ratios for GDM in women without islet autoantibodies or low C-peptide (<0.3 nmol/l)

SNP (rs number)	Genotype or allele	GDM n (%)	Controls n (%)	OR (95% CI) for GDM	OR (95% CI) for GDM, recessive model	OR (95% CI) for GDM, dominant model
KCNJ11 E23K (rs5219)	EE	171 (30.8)	439 (37.3)			
	EK	299 (53.9)	574 (48.8)	1.34 (1.07–1.68)[b]		
	KK	85 (15.3)	164 (13.9)[a]	1.33 (0.97–1.83)[c]	1.12 (0.84–1.48)	1.34 (1.08–1.66)[e]
	K	469 (42.2)	902 (38.3)	1.18 (1.02–1.36)[d]		

[a] p=0.030 for difference in genotype frequencies between women with and without GDM
[b] p=0.011 for comparison of EK vs EE between women with and without GDM
[c] p=0.076 for comparison of KK vs EE between women with and without GDM
[d] p=0.027 for difference in allele frequencies between women with and without GDM
[e] p=0.008 for comparison of KK+EK vs EE between women with and without GDM

and 66.7%, respectively; p=0.71) or in the allele frequencies (OR 0.97, 95% CI 0.81–1.16; p=0.71).

Discussion

To our knowledge, this is the largest study evaluating the role of common variants in genes predisposing for type 2 diabetes for their putative role in GDM.

KCNJ11 E23K

The key finding of the present study is the modest association between the K allele of the E23K polymorphism in *KCNJ11* and GDM. This is in line with the dominating role of beta cell dysfunction in GDM [5–9]. In vitro, the E23K variant leads to a modestly overactive pancreatic beta cell ATP-sensitive K^+ (K_{ATP}) channel subunit (Kir6.2) with decreased sensitivity to ATP, resulting in decreased insulin release [34]. We have previously shown that the E23K variant in *KCNJ11* is associated with decreased insulin secretion in glucose-tolerant subjects [17]. Some caution is still warranted in the interpretation of the data. We did not correct for multiple comparisons, as we primarily tested the hypothesis that a polymorphism increasing susceptibility to type 2 diabetes would also increase susceptibility to GDM.

IRS1 G972R

IRS1 is a major substrate for the insulin receptor and is present in insulin-sensitive tissues [35]. The G972R polymorphism of *IRS1*, which is located between two potential tyrosine phosphorylation sites involved in binding of the p85 subunit of PI-3 kinase, has previously been associated with type 2 diabetes [15], although we could not replicate this finding in our recent large study of 9,000 individuals [18], which is a common problem in genetic association studies [36]. The G972R polymorphism has also been associated with impaired beta cell function in NGT subjects as well as with reduced insulin content and impaired insulin secretion in isolated human islets [37, 38]. Our finding that homozygosity for the G972R polymorphism was found only in women with GDM might indicate an increased risk for GDM in Scandinavian women. This is consistent with a report on a healthy man homozygous for the R allele, who showed 22% reduction of fasting insulin and 48% reduction of C-peptide values as well as ~25% reduction in acute responses of insulin and C-peptide to intravenous glucose compared with carriers of the wild-type allele [39]. Of note, the IRS1 protein level is reduced in adipose tissue of obese women with GDM [40].

UCP2 −866G→A

UCP2 is a member of the mitochondrial inner membrane carrier family that is expressed in a number of tissues and cell types, including the pancreatic islets [41]. Increased expression of *UCP2* in pancreatic islets is associated with increased uncoupling, decreased formation of ATP and reduced insulin secretion [42]. The A allele of the common (−866G→A) polymorphism in the promoter of *UCP2* has originally been associated with reduced risk of obesity [19]. Subsequently, a study by Wang et al. has shown association of the G allele with increased risk of type 2 diabetes (OR=1.43) in individuals of Northern European ancestry [20]. This was supported in the same study by the finding that the G allele was associated with decreased insulin secretion adjusted for the degree of insulin resistance (i.e. the disposition index) in non-diabetic individuals [20]. Another study has also shown association of the A allele with decreased risk of type 2 diabetes in Caucasians from Italy [21]. On the contrary, the AA genotype conferred an increased risk of type 2 diabetes (OR=1.84) in Italian women [22]. In line with that study, Sesti et al. found that the A allele was associated with decreased glucose-stimulated insulin secretion in subjects with NGT as well as in human islets [43]. Here, we could not find any association between the −866G→A polymorphism and GDM in Scandinavian women despite the fact that our study had 99% power to detect the OR reported for the AA genotype in Italian women with type 2 diabetes [22], or for the G allele reported by Wang et al. [20], as well as for the AA genotype reported in Caucasians [21].

CAPN10 SNP43 and SNP44

In keeping with previous results from our laboratory, SNP43 and SNP44 were in linkage disequilibrium [44]. Whereas D' reflects recombination events between two SNPs, r^2 reflects the absolute redundancy between them. The difference we observed between D' and r^2 occurs mainly because SNP44 arose on the same haplotype more rarely than SNP43. CAPN10 is a cysteine protease with the gene located on chromosome 2q37 [45]. It is widely expressed in different tissues, including the pancreatic islets [45, 46]. Calpain inhibitors have been shown to increase insulin secretion by accelerating exocytosis of insulin granules in mouse pancreatic islets [47]. In addition, an isoform of CAPN10 that is a Ca^{2+} sensor has recently been shown to trigger exocytosis in pancreatic beta cells [46]. The GG genotype of the SNP43 has been associated with reduced *CAPN10* mRNA expression in skeletal muscle and subcutaneous adipose tissue [48, 49]. Moreover, it has been associated with increased insulin secretion [50], insulin resistance [44] and a decreased rate of glucose oxidation [48]. Consistent with the findings in the small study by Leipold et al. for SNP43, we did not

observe any significant differences in allele or genotype frequencies between GDM and controls [26]. However, these authors reported association with SNP63 as well as a haplotype combination of SNP43, 19 and 63 (121/221) [26], but no data were available on the degree of linkage disequilibrium between these SNPs. Of note, SNP63 has been shown to be in tight linkage disequilibrium with SNP43 and SNP44 in Scandinavians [44].

Given the fact that GDM and type 2 diabetes have beta cell dysfunction in common, we tested the hypothesis that common variants in candidate genes that have been associated with type 2 diabetes, particularly with beta cell dysfunction, might also be operative in GDM. We conclude that the K allele of the E23K polymorphism in *KCNJ11* seems to predispose to GDM in Scandinavian women. This is compatible with its effect on insulin secretion and the crucial role of impaired beta cell function in the pathogenesis of GDM.

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III

Common variants in MODY genes increase the risk of gestational diabetes mellitus

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Abbreviations

DBS	dried blood spots
ESM	Electronic Supplementary Material
GDM	gestational diabetes mellitus
GCK	glucokinase gene
HNF1A	hepatocyte nuclear factor-1 α gene
HNF4A	hepatocyte nuclear factor-4 α gene
OR	odds ratio

Abstract

Aims/hypothesis Impaired beta cell function is the hallmark of gestational diabetes mellitus (GDM) and MODY. In addition, women with MODY gene mutations often present with GDM, but it is not known whether common variants in MODY genes contribute to GDM.

Methods We genotyped five common variants in the glucokinase (*GCK*, commonly known as *MODY2*), hepatocyte nuclear factor 1-α (*HNF1A*, commonly known as *MODY3*) and 4 α (*HNF4A* commonly known as *MODY1*) genes in 1880 Scandinavian women (648 women with GDM and 1232 pregnant non-diabetic control women).

Results The A allele of the *GCK* −30G→A polymorphism was more common in GDM women than in control subjects (odds ratio [OR] 1.28 [95% CI 1.06−1.53], p=0.008, corrected p-value, p=0.035). Under a recessive model [AA vs GA+GG], the OR increased further to 2.12 (95% CI 1.21−3.72, p=0.009). The frequency of the L allele of the *HNF1A* I27L polymorphism was slightly higher in GDM than in controls (1.16 [1.001−1.34], p=0.048, corrected p-value, p=0.17). However, the OR increased under a dominant model (LL+IL vs II; 1.31 [1.08−1.60], p=0.007). The rs2144908, rs2425637 and rs1885088 variants, which are located downstream of the primary beta cell promoter (P2) of *HNF4A*, were not associated with GDM.

Conclusions/interpretation The −30G→A polymorphism of the beta-cell-specific promoter of the *GCK* gene and the I27L polymorphism of the *HNF1A* gene seem to increase the risk of GDM in Scandinavian women.

Introduction

Gestational diabetes mellitus (GDM) is the most common metabolic disorder during pregnancy, and is defined as glucose intolerance with onset or first recognition during pregnancy [1]. The prevalence of GDM ranges from 0.6 up to 15% [2, 3], and the frequency has increased in several populations during the last decade [4, 5]. Impaired beta cell function and insulin resistance characterise pregnancy complicated by GDM [6]. However, when insulin secretion is adjusted for the degree of insulin resistance, women with GDM have a severe reduction in beta cell function compared with normal pregnant women [7]. This beta-cell dysfunction seems to persist in women with a history of GDM post partum [6, 8].

MODY is a clinically and genetically heterogeneous monogenic disease characterised by an autosomal dominant mode of inheritance, early onset (usually before the age of 25 years) and pancreatic beta cell dysfunction [9]. Mutations in the genes encoding the glycolytic enzyme glucokinase (*GCK*, commonly known as *MODY2*) and the transcription factors hepatocyte nuclear factor 4-α (*HNF4A* commonly known as *MODY1*) and 1-α (*HNF1A*, commonly known as *MODY3*), insulin promoter factor 1 (*IPF1*, commonly *MODY4*), transcription factor 2 (*TCF2*, commonly: *MODY5*) and neurogenic differentiation factor 1 (*NEUROD1*, commonly: *MODY6*) have been shown to cause MODY [9]. The most common forms of the disease are MODY2 and MODY3, which account for 20–65% of all MODY subtypes in Europe [10, 11]. Mutations of genes involved in MODY1 are less frequent and may account for 5% of subjects with MODY [10, 11], while MODY4–6 are very rare [9, 10].

Women with mutations in *GCK* [12–17] or *HNF1A* [16, 18] often present with GDM. In addition, mutations in *IPF1* have been reported in women with GDM [16, 19]. Common variants in MODY genes, including *GCK* –30G→A [20, 21] and *HNF1A* I27L [22] variants as well as the rs2144908, rs2425637 and rs1885088 variants in *HNF4A* [23–25], have been associated with beta cell dysfunction, diabetes or related traits.

Since rare mutations in MODY genes are associated with GDM and beta cell dysfunction is the hallmark of GDM and MODY, we hypothesised that common variants in MODY genes would also increase the risk of GDM.

Since a comprehensive screening of MODY genes has already been performed in Caucasian patients with type 2 diabetes [26–28] (Winckler et al., unpublished data), we did not perform such screening of these genes and regulatory regions in our study subjects. Instead, we selected five variants in the MODY1–3 genes (i.e. the most common MODY subtypes in Europe) that fulfilled the following criteria: (1) the allele frequency of at least ~15% in order to have sufficient power to detect a relatively modest odds ratio (OR ~1.3); (2) evidence of association with beta cell dysfunction and/or type 2 diabetes or related traits; and (3) for *HNF4A* variants, to represent distinct haplotype

blocks as measured by linkage disequilibrium in Caucasians [23, 28]. We genotyped the *GCK* –30G→A, *HNF1A* I27L and *HNF4A* (rs2144908, rs2425637 and rs1885088) variants in a case–control study of 648 unrelated Scandinavian women with GDM and 1232 unrelated Scandinavian pregnant non-diabetic controls.

Subjects and methods

Study population

All pregnant women are routinely offered a 75-g OGTT at 27–28 weeks of pregnancy in southern Sweden (Skåne). Women at high risk (previous GDM or a family history of diabetes) are also offered a 75 g OGTT at 12–13 weeks. The tests are performed in the local maternity health-care clinics, using HemoCue® devices (HemoCue, Ängelholm, Sweden) for capillary whole blood analysis. GDM is defined as a 2 h capillary glucose concentration (double test) of at least 9 mmol/l according to the proposal by the Diabetic Pregnancy Study Group of the European Association for the Study of Diabetes [29].

The characteristics of the majority of the participants in the present study have been reported earlier [30]. Detailed OGTT data during pregnancy were available only for a small subset of GDM women who were prospectively followed with repeated OGTTs [31]. Briefly, we selected 1880 unrelated Scandinavian women (648 women with GDM and 1232 pregnant non-diabetic controls). Women with GDM were recruited from Malmö or Lund University Hospitals during the period from March 1996 until December 2003 (n=226) as well as among women participating in the Diabetes Prediction in Skåne (DiPiS) study, which is a prospective, longitudinal study for the prediction of type 1 diabetes in all newborn infants in southern Sweden during the period from September 2000 to August 2004 (n=422) [32]. All pregnant non-diabetic controls (n=1232) were ascertained from the DiPiS study. Both GDM groups and the control group are considered to be homogeneous since the GDM women who were recruited from the Malmö or Lund hospital were referred from maternity health-care clinics and underwent the same screening procedure as the DiPiS subjects. In addition, the study groups were recruited during a similar period, and the population in the southern Sweden is very homogeneous. All women were Scandinavians. Informed voluntary consent was obtained from all study subjects. The study was approved by the ethics committee of Lund University.

Genetic analyses

DNA extraction and template preparation

Total DNA was isolated from peripheral blood lymphocytes or blood samples were collected as dried blood spots (DBS) on filters (Grade 2992 filters; Schleicher and Schuell, Dassel, Germany).

For DBS samples, initially a template PCR was carried out to amplify the region of interest using the primers listed in Electronic Supplementary Material (ESM), Table 1.

The template PCR was performed with an initial two cycles at 4°C for 30 s followed by 98°C for 3 min, followed by holding at 80°C while the PCR mix was added. Then the PCR was continued with an initial denaturation (94ºC for 5 min), followed by 45 cycles of denaturation (94°C for 30 s), annealing (30 s) and extension (72°C for 30–60 s), followed by final extension (72°C for 10 min). PCR amplification was carried out with a 3 mm DBS in a total volume of 40 µl containing 1 × Pharmacia Amersham buffer (Amersham Pharmacia Biotech, Uppsala, Sweden) (*GCK* –30G→A [rs1799884] and *HNF4A* [rs2425637 and rs1885088]) or 1 × $(NH_4)_2SO_4$ buffer (16 mmol/l $(NH4)_2SO_4$; 67 mmol/l Tris [pH 8.8]; 0.01% Tween 20) (*HNF1A* I27L [rs1169288] and *HNF4A* [rs2144908]), 4–8 nmol of each dNTP (MBI Fermentas, St Leon-Rot, Germany), 20 pmol of each primer, 60 nmol $MgCl_2$ (*GCK* –30G→A, *HNF1A* I27L and *HNF4A* [rs2144908 and rs2425637]), betaine (Sigma-Aldrich Sweden, Stockholm, Sweden) (20 µmol: *GCK* –30G→A and *HNF4A* [rs1885088]; 30 µmol: *HNF4A* [rs2425637]) and 1–1.5 U *Taq* polymerase (New England Biolabs, Beverly, MA, USA).

Genotyping

SNP genotyping was carried out using the TaqMan allelic discrimination assay or RFLP.

For the TaqMan allelic discrimination assay on an ABI Prism 7900 Sequence Detection System (Applied Biosystems, Foster City, CA, USA), we used 2 µl (5–10 ng) of DNA or 2 µl of template PCR (for DBS samples as described above) according to the manufacturer's instructions. Primers and probes were designed using Assays-by-Design (Applied Biosystems). The primers and probes used are listed in ESM Table 2.

Since TaqMan assay did not work out properly for the *GCK* –30G→A variant on DBS samples, genotyping was carried out using RFLP. The template PCR (see description of template PCR above) product was digested with the enzyme *Alw21*I (MBI Fermentas, St Leon-Rot, Germany) at 37°C for 4 h. PCR products were separated on 2% agarose gel (SeaKem, Rockland, ME, USA) and stained with ethidium bromide to visualise the fragments using UV light.

Genotyping and quality control

The genotyping success rate was 97.5% for cases (*GCK* –30G→A, 99.1%; *HNF1A* I27L, 94.8%; rs2144908, 96.8%; rs2425637, 99.2%; rs1885088, 97.5%) and 99.0% for controls (*GCK* –30G→A, 99.8%; *HNF1A* I27L, 98.5%; rs2144908, 98.9%; rs2425637, 99.8%; rs1885088, 97.8%). The genotyping error rate was determined to be <0.3% using 943 (10%) duplicate genotypes and 89 double samples (i.e. GDM women who had both peripheral blood DNA and DBS or two DBS spotted at different deliveries). In the control group, all SNPs conformed to Hardy–Weinberg equilibrium (χ^2 test, $p>0.05$), apart from *HNF4A* rs2144908, which deviated mildly ($p=0.027$). Since the measures described above rule out possible genotyping errors, this deviation might be due to chance variation.

Statistical analyses

We used χ^2 analysis to test for differences in allele and genotype frequencies between GDM and control groups. Logistic regression analysis was used to calculate the OR and 95% CI. ANOVA was used to test the significance of differences in continuous variables, such as age, between GDM and control groups using the Number Cruncher Statistical Systems (NCSS, Kaysville, UT, USA). Age was expressed as mean ± SEM. Haplotype analysis was carried out using Haploview software 3.2 [33]. To correct for multiple testing, we permuted the data as implemented in Haploview version 3.2 [33]. We used 10 000 permutations, but using more permutations gave the same results. This study was not designed to detect differences between genetic models. However, since we did not have a predefined genetic model of the potential effect of these variants, we chose to present the data for additive, recessive and dominant models. Two-sided *p*-values less than 0.05 were considered statistically significant.

Power calculation

By studying a sample of 648 cases and 1232 controls, the present study had more than 80% power, under a multiplicative model, to detect an effect size of 1.3 (as measured in terms of genotypic relative risk) when the frequency of the predisposing allele equalled 15% (for $\alpha=0.05$). When the predisposing allele frequency was >30%, the study had at least 80% power to detect an OR of 1.22 under a multiplicative model (for $\alpha=0.05$). Power calculations were performed using the Genetic Power Calculator (available at http://ibgwww.colorado.edu/~pshaun/gpc/) [34].

Results

Subject characteristics

Women with GDM were slightly older than pregnant non-diabetic controls (32.3±0.2 vs 30.5±0.1, $p<0.0001$). The genotype and allele frequency distributions of all polymorphisms studied are presented in Table 1.

GCK –30G→A

The GG, GA and AA genotype frequencies of the *GCK* −30G→A polymorphism differed significantly between GDM and control women (67.8, 28.2 and 4.0% vs 72.3, 25.7 and 2.0% respectively, $p=0.010$). In addition, the A allele was found to be more common in GDM women than among control subjects (OR 1.28, 95% CI 1.06−1.53, $p=0.008$, corrected p-value, $p=0.035$). Under a recessive model (AA vs GA+GG), the OR increased further to 2.12 (95% CI 1.21−3.72, $p=0.009$). Using a dominant model, the OR for GDM in carriers of the GA or AA genotypes compared with carriers of the GG genotype was 1.24 (95% CI 1.01−1.53, $p=0.039$). Of note, the ORs were almost the same, with overlapping 95% CIs, when women who were positive for GAD65Ab, IA–2Ab or both (antibody measurements were not available for all subjects) were removed from the analyses (data not shown).

HNF1A I27L

The II, IL and LL genotype frequencies of the *HNF1A* I27L polymorphism differed significantly between GDM and control women (39.4, 48.5 and 12.1% vs 46.1, 41.8 and 12.1% respectively, $p=0.016$). The L allele was slightly more frequent in GDM women than in controls (OR 1.16, 95% CI 1.001−1.34, $p=0.048$, corrected p-value, $p=0.17$). However, the IL genotype was more frequent in GDM women than in controls, compared with the wild-type II genotype (OR 1.36, 95% CI 1.10−1.67, $p=0.004$). In addition, under a dominant model [IL+LL vs II], the L allele conferred an increased risk of GDM (OR 1.31, 95% CI 1.08−1.60, $p=0.007$). As for the *GCK* −30G→A polymorphism, the ORs and 95% CIs remained almost the same when women who were positive for GAD65Ab, IA–2Ab or both were excluded from the analyses (data not shown).

HNF4A variants

The degree of linkage disequilibrium between *HNF4A* variants (rs2144908, rs2425637 and rs1885088) was estimated using D′ and r^2 values. There was no evidence of linkage disequilibrium between these variants; D′ values were between 0.01 and 0.5 and r^2 values were between 0.0 and 0.01.

The frequency of the A allele of the rs2144908 variant, which is located 1272 bp downstream of the primary beta cell promoter (P2) of *HNF4A*,

did not differ significantly between GDM and controls (OR 1.14, 95% CI 0.96–1.37, p=0.14).

Neither was there any difference in the frequency of the T allele of the rs2425637 variant, which is located 39 604 bp downstream of P2, between GDM and control women (OR 1.09, 95% CI 0.95–1.24, p=0.23).

The intronic variant (rs1885088) is located 54 595 bp downstream of the P2. Similar frequencies of the A allele were observed in women with GDM and control women (OR 0.96, 95% CI 0.81–1.14, p=0.66).

Discussion

The key finding of the present study was that common variants in two MODY genes, *GCK* and *HNF1A*, increase the risk of GDM.

***GCK* –30G→A**

In the pancreatic islets, glucokinase plays a critical role in the regulation of insulin secretion by acting as a glucose sensor [35]. The –30G→A variant in the beta-cell-specific promoter of the *GCK* was shown to co-segregate with diabetes in a French family in which the proband was a woman with GDM [15]. Subsequently, it has been associated with reduced beta cell function in middle-aged Japanese-American men [36]. In addition, in women in the third trimester of pregnancy, the AA genotype led to a reduction in early-phase insulin secretion [37]. In a recent study of 755 pregnant women, the A allele was associated with increased fasting plasma glucose measured at 28 weeks of gestation in healthy Caucasian women from the UK [38]. In support of this, another recent study reported association of this polymorphism with elevated fasting and post-OGTT glucose levels as well as with impaired glucose regulation (i.e. type 2 diabetes, IGT and IFG) and features of the metabolic syndrome in Caucasians [21]. However, no association of the –30G→A variant with GDM was observed in two small studies that included women of Caucasian, black and oriental origin [39] or in American black women [40]. Interestingly, it has been demonstrated that the A allele increases the risk of coronary artery disease in individuals with and without type 2 diabetes and it was also associated with an increased prevalence of type 2 diabetes in subjects with coronary artery disease [20].

In the present study, the A allele was associated with a modestly increased risk of GDM and this effect was more pronounced using a recessive mode of inheritance. The previously demonstrated deleterious effect of this polymorphism on beta cell function during pregnancy [37] might be a plausible explanation for the observed association, which is consistent with the key role of impaired beta cell function in the pathogenesis of GDM [6–8].

***HNF1A* I27L**

Defective insulin secretion is the hallmark of patients with *MODY3* mutations [18]. The I27L polymorphism is located within the dimerisation domain of the *HNF1A* gene [41], and the amino acid isoleucine is conserved among several species, suggesting a potential functional importance of this residue [22]. Chiu et al. have reported association of the I27L polymorphism with lower first- and second-phase insulin secretion in glucose-tolerant subjects [22]. In line with this, we found a nominal association of the L allele of the I27L polymorphism with type 2 diabetes in Scandinavian/Canadian subjects, but this was not the case in the larger sample including also subjects from US and Poland [27] or in a recent large study in UK Caucasian population [26]. Moreover, we have recently observed an association of the I27L polymorphism with increased risk of type 2 diabetes in a large new Swedish case–control study [42]. This was supported by in vitro findings that the L allele was associated with decreased transcriptional activity in HeLa and INS-1 cells [42].

In keeping with the findings for the *GCK* −30G→A variant, we observed a modest effect of the L allele of the *HNF1A* I27L polymorphism on the risk of GDM, which might be mediated by its effect on beta cell function [22]. It may be expected that individuals with a slight impairment in their beta cell function are more prone to deteriorated glucose tolerance when becoming insulin-resistant during pregnancy. It was, however, not possible to address a potential effect on beta cell function in the present study, as this would have required assessment of beta cell function prior to and during pregnancy. Unfortunately, we did not have this information. However, this finding should be interpreted with some caution since the difference in allele frequencies between GDM and controls was not statistically significant after correction for multiple comparisons.

***HNF4A* variants**

Somewhat surprisingly, variants in the *HNF4A* gene, which have repeatedly been associated with a modestly increased risk of type 2 diabetes [23–25], were not associated with GDM in the present study. *HNF4A* is a member of the nuclear receptor family of transcription factors, which is expressed in several tissues, including the liver, gut, kidney and pancreas [43]. Whereas the expression of *HNF4A* in the liver is mediated by a proximal promoter (P1), its expression in beta cells is driven by an alternative beta cell promoter (P2) located 46 kb upstream of P1 [44, 45]. Mutations in the *HNF1A* and *IPF1* binding sites of the P2 promoter have been associated with MODY1 [44, 45]. The rs2144908, rs2425637 and rs1885088 variants, which are located downstream of the P2 promoter, were originally associated with type 2 diabetes in Finns [23]. In addition, the rs2144908 variant has been associated with type 2 diabetes in Ashkenazi Jewish [24] as well as in Caucasians from the UK [25]. Interestingly, the rs2144908 variant was also associated with reduced beta cell function (i.e. decreased acute insulin response to glucose and decreased disposition index) in unaffected Finnish offspring of parents with type 2

diabetes [23]. In the present study, there was no evidence for association of these variants with GDM. This may suggest that the studied variants in the *HNF4A* gene have no major impact on predisposition to GDM. However, it should be stressed that a smaller effect (OR < 1.22–1.27 depending on the allele frequency) of these variants on the risk of GDM could have been missed. Indeed, the present study had adequate power to detect the ORs (1.23–1.46) reported for type 2 diabetes in the original studies [23, 24], but not the ORs (1.14–1.15) reported in Caucasians from the UK and Denmark in recent large studies [25, 46]. Consistent with the other studies, these three variants were not in linkage disequilibrium in our study and the frequency of the minor alleles in controls was comparable to that reported in other populations [23–25, 46].

In conclusion, the −30G→A polymorphism of the beta-cell-specific promoter of the *GCK* gene and the I27L polymorphism of the *HNF1A* gene seem to increase the risk of GDM in Scandinavian women, suggesting a role of common variants that are known to affect beta cell function in the aetiology of GDM. However, to demonstrate a direct effect on beta cell function more studies are required, with assessment of beta cell function prior to and during pregnancy in carriers of these polymorphisms.

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Tables

Table 1. Genotype and allele distributions and corresponding odds ratios for GDM.

SNP (rs number)	**Genotype or allele**	**GDM** *n (%)*	**Controls** *n (%)*	**OR (95% CI) for GDM**	**OR (95% CI) for GDM, recessive model**	**OR (95% CI) for GDM, dominant model**
GCK -30G>A (rs1799884)	GG	435 (67.8)	889 (72.3)			
	GA	181 (28.2)	316 (25.7)	1.17 (0.94–1.45)		
	AA	26 (4.0)	24 (2.0)[a]	2.21 (1.26–3.90)[b]	2.12 (1.21–3.72)[b]	1.24 (1.01–1.53)[c]
	A	233 (18.1)	364 (14.8)	1.28 (1.06–1.53)[b]		
HNF1A I27L (rs1169288)	II	242 (39.4)	559 (46.1)			
	IL	298 (48.5)	508 (41.8)	1.36 (1.10–1.67)[b]		
	LL	74 (12.1)	147 (12.1)[c]	1.16 (0.85–1.60)	0.99 (0.74–1.34)	1.31 (1.08–1.60)[b]
	L	446 (36.3)	802 (33.0)	1.16 (1.001–1.34)[c]		
HNF4A (rs2144908)	GG	425 (67.8)	854 (70.1)			
	GA	167 (26.6)	316 (25.9)	1.06 (0.85–1.32)		
	AA	35 (5.6)	48 (4.0)	1.47 (0.93–2.30)	1.44 (0.92–2.25)	1.12 (0.91–1.37)
	A	237 (18.9)	412 (16.9)	1.14 (0.96–1.37)		
HNF4A (rs2425637)	GG	159 (24.7)	317 (25.8)			
	GT	310 (48.2)	617 (50.2)	1.00 (0.79–1.27)		
	TT	174 (27.1)	295 (24.0)	1.18 (0.90–1.54)	1.17 (0.94–1.46)	1.06 (0.85–1.32)
	T	658 (51.2)	1207 (49.1)	1.09 (0.95–1.24)		
HNF4A (rs1885088)	GG	412 (65.2)	791 (65.6)			
	GA	199 (31.5)	354 (29.4)	1.08 (0.87–1.33)		
	AA	21 (3.3)	60 (5.0)	0.67 (0.40–1.12)	0.66 (0.40–1.09)	1.02 (0.83–1.25)
	A	241 (19.1)	474 (19.7)	0.96 (0.81–1.14)		

[a] p-value = 0.01
[b] p-value < 0.01
[c] p-value < 0.05

ESM Table 1. Primers used for template PCR

Polymorphism (rs number)	**Forward primer (5'→3')**	**Reverse primer (5'→3')**	**Fragment Size (bp)**	**Annealing temp. (°C)**
GCK –30G>A (rs1799884)	ATCTGAACAGGTGGC-AAAGGC	CCAACGAGTCGGC-AAGCAT	523	63
HNF1A I27L (rs1169288)	TGGCAGCCGAGCCATG-GTTTC	GAAGGGGGGCTC-GTTAGGAGC	415	64
HNF4A (rs 2144908)	GGAACAAGGATGTAAA-GCCC	AGGTCCTGTTGTTAT-CTTCATTTT	314	57
HNF4A (rs 2425637)	GCCCCAAGTCTATGG-TTCAGT	ACCCCTGCCTCCCA-TCTGA	298	61
HNF4A (rs 1885088)	AGGATAGGAGAGTTG-GCTGATG	AGACTTTCTTTTGG-CTTTGGGAG	257	61

ESM Table 2. Primers and probes used for TaqMan allelic discrimination assay

Polymorphism (rs number)	**Forward primer (5'→3')**	**Reverse primer (5'→3')**	**Probe (5'FAM)**	**Probe (5'VIC)**
GCK –30G>A (rs1799884)	GCCACTCCTGGTC-ACCAT	GATTCTCCTGCCA-GGGCTT	CCTCTCAGGAA-CACAGT	CCTCTCAGGAG-CACAGT
HNF1A I27L (rs1169288)	CCTTCTCCAGCCAG-GAGGTA	CTGGCGGCCCTGCT	AAGAGGCACT-GCTCCA	AAAGAGGCACT-GATCCA
HNF4A (rs2144908)	GCATTGCAAAGAC-ACAATCAACATTT	GATCAGGCCCTG-ATTCTGTCAT	TCCCTGGCTCTC-TGT	TCCCTGGCCCT-TGT
HNF4A (rs2425637)	GCTTTGTGGGTG-CCTGATTTG	CCCTCCCTTTCTC-TTCCTTGAG	CTAAAATGCCA-ATCATAC	CCTAAAATGC-CACTCATAC
HNF4A (rs1885088)	GGTTACCTGGAA-GATCATGACACAT	GCTTGACCACAG-TGGCAACT	TTTTGAGAACA-GGCCAGAG	TTGAGAACGG-GCCAGAG

IV

Association Testing of Common Variants Predisposing to the Metabolic Syndrome or Related Traits with Gestational Diabetes Mellitus

***Short title*:** Genetics of GDM and Metabolic syndrome.

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Abbreviations: Adiponectin (*APM1*), dried blood spots (DBS), gestational diabetes mellitus (GDM), forkhead box C2 (*FOXC2*), metabolic syndrome (MetS), peroxisome-proliferative activated receptor, gamma 2 (*PPARG*), *PPARG*-co-activator 1 alpha (*PPARGC1A*), β3-adrenergic receptor (*ADRB3*), and odds ratio (OR).

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ABSTRACT

Context. Insulin resistance is a key player in the pathophysiology of gestational diabetes mellitus (GDM) and the metabolic syndrome (MetS). In addition, women with GDM share some features with MetS and are at increased risk of developing the syndrome later in life.
Objective. To investigate whether common variants in genes that have previously been associated with MetS or its components would also confer risk for GDM.
Design. We genotyped the Adiponectin (*APM1* +276G>T), peroxisome-proliferative activated receptor, gamma 2 (*PPARG* Pro12Ala), *PPARG*-coactivator, 1 alpha (*PPARGC1A* Gly482Ser), forkhead box C2 (*FOXC2* −512C>T), and β3-adrenergic receptor (*ADRB3* Trp64Arg) polymorphisms in a case-control study.
Participants. We studied 1881 unrelated pregnant Scandinavian women (649 women with GDM and 1232 non-diabetic controls).
Results. The T-allele of the *APM1* +276G>T polymorphism was more common in GDM as compared to control women (odds ratio [OR] 1.17 [95% CI 1.01−1.36], p=0.039). In addition, the risk increased under a dominant model [TT+GT vs. GG] (1.26 [1.04−1.53], p=0.018). A similar effect was observed when comparing GT-genotype carriers with GG-genotype carriers (1.27 [1.04−1.55], p=0.020). The difference in allele frequencies between GDM and control women did not reach significance for the other polymorphisms studied (*PPARG* Pro12Ala: 1.06 [0.87−1.29], p=0.53; *PPARGC1A* Gly482Ser: 0.96 [0.83−1.10], p=0.54; *FOXC2* −512C>T: 1.01 [0.87−1.16], p=0.94 and *ADRB3* Trp64Arg: 1.22 [0.95–1.56], p=0.12).
Conclusion. The *APM1* +276G>T polymorphism was associated with increased risk of GDM, but this finding need to be replicated in other populations.

INTRODUCTION

Insulin resistance is a common metabolic abnormality characterized by impaired ability of cells to respond to the normal action of insulin [1]. It has been considered as the main underlying pathophysiological feature of a constellation of cardiovascular risk factors known as the metabolic syndrome (MetS) [2, 3]. Although there is no broad agreement on the definition of the MetS, the definitions developed by World Health Organization, National Cholesterol Education Program and International Diabetes Federation, overlap on the major components including impaired glucose tolerance, obesity, hypertension and dyslipidemia [3].

There is accumulating evidence of common genetic variants contributing to the risk of the MetS or its components. Among many variants, associations with the Adiponectin (*APM1* +276G>T) [4], peroxisome-proliferative activated receptor, gamma 2 (*PPARG* Pro12Ala) [5], *PPARG* coactivator 1, alpha (*PPARGC1A* Gly482Ser) [6], forkhead box C2 (*FOXC2* −512C>T) [7], and β3-adrenergic receptor (*ADRB3* Trp64Arg) [8] polymorphisms have been reported.

Gestational diabetes mellitus (GDM) is a heterogeneous disorder characterized by impaired β-cell function and insulin resistance [9]. It has been demonstrated that insulin resistance persists in glucose-tolerant women with a history of GDM postpartum [10]. A possible link between GDM and MetS has been suggested since many components of MetS predict GDM [11]. For more than two decades, O'Sullivan et al have shown that women with a history of GDM were at increased risk for hypertension, hyperlipidemia, electrocardiographic abnormalities and mortality [12]. In agreement with that observation, it has recently been shown that women with history of GDM are at increased risk of developing MetS later in life [13]. Furthermore, the offspring of women with GDM during pregnancy are at high risk of developing MetS in childhood [14].

Since MetS is common in women with GDM and insulin resistance is a pathophysiological link between the two disorders, we hypothesized that common variants in genes that have previously been associated with features of MetS would also confer risk for GDM. Thus, we genotyped the *APM1* +276G>T, *PPARG* Pro12Ala, *PPARGC1A* Gly482Ser, *FOXC2* −512C>T and *ADRB3* Trp64Arg polymorphisms in a case-control study of 649 unrelated Scandinavian women with GDM and 1232 pregnant control women.

MATERIALS AND METHODS

Study population

In southern Sweden (Skåne), all pregnant women are routinely offered a 75-g OGTT at 27-28 weeks of pregnancy. Women with previous GDM or a family history of diabetes are also offered a 75-g OGTT at 12-13 weeks. The tests are performed in the local Maternity Health Care clinics, using HemoCue devices (HemoCue, Ängelholm, Sweden) for capillary whole blood analysis. GDM is defined as a 2-hour capillary glucose concentration (double test) of at least 9 mmol/l according to the proposal by the Diabetic Pregnancy Study Group of the European Association for the Study of Diabetes [15].
The characteristics of the majority of the participants in the present study have been reported earlier [16]. Briefly, we selected 1881 unrelated Scandinavian women (649 women with GDM and 1232 pregnant non-diabetic controls). GDM women were recruited from Malmö or Lund University Hospitals during the period from March 1996 until December 2003 (n=226) as well as among women participating in the Diabetes Prediction in Skåne (DiPiS) study, which is a prospective, longitudinal study for the prediction of type 1 diabetes in all newborns in southern Sweden during the period from September 2000 until August 2004 (n=423) [17]. All pregnant non-diabetic controls (n=1232) were ascertained from the DiPiS study. Both GDM groups and the control group are considered to be homogeneous since GDM women who recruited from Malmö or Lund Hospitals were referred from Maternity Health Care clinics, and underwent the same screening procedure as the DiPiS subjects. In addition, the study groups were recruited during the same period, and the population in Southern Sweden is very homogeneous. All women were Scandinavians. Detailed phenotypic characteristics including OGTT data were available only for a small part of GDM women [18]. An informed voluntary consent was obtained from all study subjects. The study was approved by the ethics committee of Lund University.

Genetic analyses

Samples' collection

Total DNA was isolated from peripheral blood lymphocytes or blood samples were collected as dried blood spots (DBS) on filters (Schleicher & Schuell, Grade 2992 filters; Schleicher and Schuell, Dassel, Germany).

Genotyping using DNA

For DNA samples, genotyping of *APM1* +276G>T (rs1501299), *PPARGC1A* Gly482Ser (rs8192678), *FOXC2* −512C>T and *ADRB3* Trp64Arg (rs4994) was carried out using TaqMan allelic discrimination assay, whereas *PPARG*

Pro12Ala (rs1801282) polymorphism was genotyped by RFLP as previously described [18]. TaqMan assay was carried out on an ABI PRISM 7900 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) using 2 µl of DNA (5-10 ng) according to the manufacturer's instructions. Primers and probes were designed using Assays-by-Design (Applied Biosystems, Foster City, CA, USA), except for the *FOXC2* −512C>T, which was ordered from MWG Biotech Scandinavia A/S (Risskov, Denmark). The primers and probes used are listed in Supplement 1.

Genotyping using DBS

For DBS samples, initially a template PCR was carried out to amplify the region of interest using the primers listed in Supplement 2. The template PCR was followed by TaqMan allelic discrimination assay or RFLP.

The template PCR was performed with initial 2 cycles at 4°C for 30 s, followed by 98°C for 3 min, followed by holding at 80°C while the PCR mix was added. Then the PCR was continued with an initial denaturation (94ºC for 5 min, except for *APM1* +276G>T at 96ºC), followed by 45 cycles of denaturation (94°C for 30 s, except for *APM1* +276G>T at 96ºC), annealing (30 s) and extension (72°C for 30 - 60 s), followed by final extension (72°C for 10 min). PCR amplification was carried out with 3 mm of DBS in a total volume of 40 µl containing 1 x Pharmacia Amersham buffer (Amersham Pharmacia Biotech, Uppsala, Sweden) [*PPARG* Pro12Ala and *PPARGC1A* Gly482Ser] or 1 x $(NH_4)_2SO_4$-buffer (16 mmol/l $(NH_4)_2SO_4$; 67mmol/l Tris (pH 8.8); 0.01% Tween 20) [*APM1* +276G>T, *FOXC2* −512C>T and *ADRB3* Trp64Arg], 4-8 nmol each dNTP (MBI Fermentas, St Leon-Rot, Germany), 20 pmol of each primer, $MgCl_2$ [30 nmol: *PPARGC1A* Gly482Ser; 60 nmol: *APM1* +276G>T and *FOXC2* −512C>T; 120 nmol: *ADRB3* Trp64Arg], Betaine (Sigma-Aldrich Sweden AB, Stockholm, Sweden) [10 µmol: *PPARGC1A* Gly482Ser; 20 µmol: *APM1* +276G>T, *PPARG* Pro12Ala, *FOXC2* −512C>T and *ADRB3* Trp64Arg], 5% DMSO [*PPARGC1A* Gly482Ser and *FOXC2* −512C>T] and 1.5-2.5 U Taq polymerase (New England Biolabs, Beverly, MA, USA).

-RFLP

For a part of the DBS samples, *APM1* +276G>T polymorphism was genotyped by RFLP. The template PCR (see description of template PCR above) product was digested with Mva 1269I (Fermentas, St Leon-Rot, Germany) at 37°C for 4h. PCR products were separated on 3% agarose gel (SeaKem, Rockland, ME, USA) and stained with ethidium bromide to visualize the fragments using UV light. The *PPARG* Pro12Ala polymorphism was also genotyped by RFLP for some of the DBS samples as described previously [18].

- TaqMan allelic discrimination assay

For all DBS samples, *PPARGC1A* Gly482Ser, *FOXC2* −512C>T and *ADRB3* Trp64Arg variants as well as a part of the DBS samples for *APM1* +276G>T and *PPARG* Pro12Ala variants were genotyped using TaqMan allelic discrimination assay, which was carried out with 2 µl of the template PCR product (see description of template PCR above) according to the manufacturer's instructions.

Genotyping and quality control

The genotyping success rate was 98.5% for cases (*APM1* +276G>T; 98.8%, *PPARG* Pro12Ala; 98.1%, *PPARGC1A* Gly482Ser; 99.2%, *FOXC2* −512C>T; 97.8%, and *ADRB3* Trp64Arg; 98.5%) and 99.2% for controls (*APM1* +276G>T; 99.4%, *PPARG* Pro12Ala; 100%, *PPARGC1A* Gly482Ser; 99.4%, *FOXC2* −512C>T; 97.7%, and *ADRB3* Trp64Arg; 99.6%). Genotyping error rate was determined to be 0.3% using 1515 (16.1%) duplicate genotypes as well as 89 double samples (i.e. women with GDM who had both peripheral blood DNA and DBS or two DBS spotted at different deliveries). All polymorphisms conformed to Hardy-Weinberg equilibrium (x^2 $p > 0.05$) in both GDM and control groups.

Statistical analyses

Analysis of variance (ANOVA) was used to test the significance of difference in continuous variables as age between GDM and control groups. The age was presented as mean ± SEM. Chi-square analysis was used to test for differences in genotype frequency between GDM and control groups. Logistic regression analysis was used to test for differences in allele frequency and other genetic models between GDM and controls as well as to calculate the odds ratios (ORs) and 95% confidence intervals (95% CIs) using the Number Cruncher Statistical Systems (NCSS, Kaysville, UT, USA). The significance of difference in allele frequencies of the studied polymorphisms between GDM and controls was also tested by 10,000 permutations. Two-sided *p*-values less than 0.05 were considered statistically significant.

Power calculation

The present study has more than 80% power - under a multiplicative model- to detect an effect size of 1.3 (as measured in terms of genotypic relative risk) when the frequency of the predisposing allele equals to 15% (for α=0.05). In case when the predisposing allele frequency >30%, the study had at least 80% power to detect an OR of 1.22 (for α=0.05). Power calculations were performed using the Genetic Power Calculator (available at http://ibgwww.colorado.edu/~pshaun/gpc/) [19].

RESULTS

Women with GDM were slightly older than pregnant non-diabetic controls (32.3±0.2 vs. 30.5±0.1, p<0.0001). The genotype and allele frequency distributions of all polymorphisms studied are presented in Table 1.

***APM1* +276G>T.** The T-allele of the *APM1* +276G>T polymorphism was more common in GDM as compared to control women (odds ratio [OR] 1.17 [95% CI 1.01−1.36], p=0.039) [Figure 1]. In addition, the GT-genotype carriers had an increased risk of GDM with an OR of 1.27 ([1.04−1.55], p=0.020) as compared to GG-genotype carriers [Figure 1]. A similar OR (1.26 [1.04−1.53], p=0.018) was also observed under a dominant model (TT+GT vs. GG) [Figure 1]. The p-value for difference in allele frequencies between GDM and controls remained the same when we permuted the data 10,000 times. Also, the ORs were almost the same with overlapping 95% CIs when women who were positive for GAD65Ab, IA–2Ab or both [antibody measurements were not available for all subjects] were removed from analyses (data not shown).

***PPARG* Pro12Ala.** There was no significant difference in the Pro/Pro, Pro/Ala and Ala/Ala genotype frequencies between GDM and control women (73.5, 24.8 and 1.7% vs. 74.5, 24.2 and 1.3%, respectively, p=0.72). Neither was there any significant difference in the Ala-allele frequency between GDM and control women (1.06, [0.87−1.29], p=0.53).

***PPARGC1A* Gly482Ser.** The frequency of the Gly/Gly, Gly/Ser and Ser/Ser genotypes of the *PPARGC1A* Gly482Ser polymorphism was similar in GDM and control women (44.1, 45.7 and 10.2% vs. 43.5, 44.8 and 11.7%, respectively, p=0.64). Also, similar allele frequency was observed in both groups (0.96 [0.83–1.10], p=0.54).

***FOXC2* −512C>T.** The CC, CT and TT genotype frequencies of the *FOXC2* −512C>T polymorphism did not differ significantly between GDM and control women (15.8, 45.8 and 38.4% vs. 14.9, 47.2 and 37.9%, p=0.83). The same was observed for difference in the C-allele frequency between GDM and control women (1.01 [0.87–1.16], p=0.94).

***ADRB3* Trp64Arg.** There was no significant difference in the Trp/Trp, Trp/Arg and Arg/Arg genotypes of the *ADRB3* Trp64Arg polymorphism between GDM and control women (83.6, 15.6 and 0.8% vs. 86.4, 12.9 and 0.7% respectively, p=0.52). Neither was there any significant difference in Arg-allele frequency between GDM and controls (1.22 [0.95–1.56], p=0.12).

DISCUSSION

The key finding of the present study is that the *APM1* +276G>T polymorphism, which has previously been associated with features of MetS is associated with GDM in Scandinavian women.

***APM1* +276G>T.** Adiponectin is a physiologically active polypeptide hormone derived from adipose tissue with insulin-sensitising properties [20]. A genetic regulation of adiponectin has been suggested since serum adiponectin concentrations have a heritability of about 30% [21]. Decreased plasma adiponectin during pregnancy has been associated with GDM [22-24]. In addition, serum concentrations of adiponectin correlate negatively with measures of insulin resistance in women with GDM [22]. Notably, reduced adiopnectin mRNA levels in adipose tissue in women with GDM have also been reported [23]. The common +276G>T variant of the *APM1* is one of the most extensively studied variants within the adiponectin gene. It has been associated with type 2 diabetes [25, 26] and features of MetS [4]. Interestingly, the G-allele has been found to be the risk in some studies [4, 25], while protective in others [26, 27]. In the present study, we found a modest effect of the T-allele of the *APM1* +276G>T variant on the predisposition for GDM. Our finding that the T-allele is the risk allele is in agreement with studies in women from USA [26] and in Caucasian subjects from Italy [27], as well as in our recent study in which the TT-genotype was associated with increased total and LDL-C levels in children with juvenile obesity [28]. The mechanism by which this intronic variant would increase susceptibility to GDM might be mediated, as previously shown, by its effect on insulin sensitivity [27]. It is possible that another variant in linkage disequilibrium with the *APM1* +276G>T variant could confer the risk, especially as studies have not been consistent in terms of the risk allele. This finding should be interpreted with some caution. We did not correct for multiple testing since our primary hypothesis was that a variant previously shown to increase the risk for MetS or related traits would also predispose to GDM.

***PPARG* Pro12Ala**. *PPARG* is a transcription factor with a pivotal role in adipocyte differentiation and function, in which a heterozygous mutations in the ligand-binding domain of this gene results in severe insulin resistance, type 2 diabetes and hypertension in humans [29]. The Ala-allele of the Pro12Ala polymorphism has been consistently associated with reduced risk for type 2 diabetes [30]. *In vivo*, the Ala allele leads to decreased PPARG activity and thereby to decreased transcription of a number of target genes, which result in increased insulin sensitivity [5]. In the present study, we could not find association of this variant with GDM, which is consistent with our previous finding in a smaller study of women with GDM [18]. This might indicate that this variant has no role in the predisposition to GDM. However, we could not role out a smaller effect size (OR<1.3) of this variant on risk of GDM.

***PPARGC1A* Gly482Ser.** *PPARGC1A* is a transcriptional co-activator of several nuclear receptors including *PPARG* and PPARα, which plays a role in the transcriptional control of mitochondrial fatty acid beta-oxidation enzymes [31]. The gene encoding *PPARGC1A* is located on chromosome 4p15.1. In humans, *PPARGC1A* is expressed in various tissues including adipose tissue, skeletal muscle and pancreas [32]. A common (Gly482Ser) polymorphism in the *PPARGC1A* has been associated with type 2 diabetes and insulin resistance [6, 33]. In addition, we have recently shown that this variant was associated with reduced VO2max in a twin study [34]. Also, a possible role in the predisposition to MetS has been suggested as it was associated with HDL-C concentrations in obese non-diabetic French-Canadian subjects [35]. Again, we could not find an effect of this variant on GDM in Scandinavian women, which is in agreement with a recent small study in Austrian Caucasians [36].

***FOXC2* -512T>C.** *FOXC2* is a key regulator of adipocyte metabolism [37]. Transgenic mice over-expressing the *FOXC2* in adipose tissue have been shown to be protected from diet-induced obesity and insulin resistance [37]. Subsequently, we have reported an association between whole-body insulin sensitivity and expression of mRNA of *FOXC2* in human subjects [7]. In addition, the common polymorphism (-512C>T) located in the 5'UTR of the *FOXC2* has been associated with enhanced insulin sensitivity and lower plasma triglycerides in female sib-pairs discordant for the variant [7] as well as with features of the metabolic syndrome in Swedish population [38]. Interestingly, it has also been associated with increased basal glucose turnover and lower plasma triglyceride in Pima Indian women [39]. This sex-specific association with insulin resistance urged us to investigate whether this polymorphism influence the risk of GDM as well. However, our results suggest no role of this polymorphism in the development of GDM in Scandinavian women. Of note, the C-allele frequency in control women was comparable to what has been reported in healthy individuals in our previous studies [7, 38].

***ADRB3* Trp64Arg.** We have previously reported a polymorphism in the first intracellular loop of the receptor (Trp64Arg) that was associated with abdominal obesity and features of MetS [8]. In addition, analysis of subsequent studies have shown association of this polymorphism with features of MetS [30]. The role of Trp64Arg polymorphism in GDM has been investigated in three populations. In Caucasian women from Austria, it has been associated with mild GDM defined by 60-min postload glucose during OGTT [40]. In addition, it was associated with increased weight gain during pregnancy and increased postload glucose, insulin, and C-peptide values during OGTT [40]. Conversely, this polymorphism was not associated with GDM in Greek women [41]. However, Arg-allele carriers had higher HOMA index compared to Trp/Trp-genotype carriers in GDM women, but the difference was no longer significant after adjustment for prepregnancy BMI [41]. Though no association of the Trp64Arg polymorphism with GDM was observed in Taiwanese women,

the Arg-allele was associated with increased fasting and 2h insulin levels in GDM women [42]. Consistent with the studies in Greek and Taiwanese women [41, 42], the present study report no effect of this variant on the risk of GDM in Scandinavian women.

In conclusion, of the studied variants, the *APM1* +276G>T polymorphism was associated with an increased risk of GDM, but the finding must be considered tentative until replicated in an independent study.

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TABLES

Table 1. Genotype and allele distributions and corresponding odds ratios for GDM.

Polymorphism (rs number)	Genotype or allele	GDM n (%)	Controls n (%)	OR (95% CI) for GDM	OR (95% CI) for GDM, recessive model	OR (95% CI) for GDM, dominant model
APM1	GG	301 (46.9)	646 (52.7)			
+276G>T	GT	285 (44.5)	482 (39.4)	1.27 (1.04–1.55)[b]		
(rs1501299)	TT	55 (8.6)	97 (7.9)[a]	1.22 (0.85–1.74)[c]	1.09 (0.77–1.54)	1.26 (1.04–1.53)[e]
	T	395 (30.8)	676 (27.6)	1.17 (1.01–1.36)[d]		
PPARG	Pro/Pro	468 (73.5)	918 (74.5)			
Pro12Ala	Pro/Ala	158 (24.8)	298 (24.2)	1.04 (0.83–1.30)		
(rs1801282)	Ala/Ala	11 (1.7)	16 (1.3)	1.35 (0.62–2.93)	1.34 (0.62–2.89)	1.06 (0.85–1.31)
	Ala	180 (14.1)	330 (13.4)	1.06 (0.87–1.29)		
PPARGC1A	Gly/Gly	284 (44.1)	533 (43.5)			
Gly482Ser	Gly/Ser	294 (45.7)	548 (44.8)	1.01 (0.82–1.23)		
(rs8192678)	Ser/Ser	66 (10.2)	143 (11.7)	0.87 (0.63–1.20)	0.86 (0.63–1.18)	0.98 (0.81–1.19)
	Ser	426 (33.1)	834 (34.1)	0.96 (0.83–1.10)		
FOXC2	TT	244 (38.4)	456 (37.9)			
-512C>T	CT	291 (45.8)	568 (47.2)	0.96 (0.78–1.18)		
	CC	100 (15.8)	180 (14.9)	1.04 (0.78–1.39)	1.06 (0.82–1.39)	0.98 (0.80–1.19)
	C	491 (38.7)	928 (38.5)	1.01 (0.87–1.16)		
ADRB3	Trp/Trp	534 (83.6)	1060 (86.4)			
Trp64Arg	Trp/Arg	100 (15.6)	158 (12.9)	1.26 (0.96–1.65)		
(rs4994)	Arg/Arg	5 (0.8)	9 (0.7)	1.10 (0.37–3.31)	1.07 (0.36–3.20)	1.25 (0.96–1.63)
	Arg	110 (8.6)	176 (7.2)	1.22 (0.95–1.56)		

[a] P-value for difference in genotype frequencies between women with and without GDM, p=0.059
[b] P-value for comparison of GT vs. GG between women with and without GDM, p=0.020
[c] P-value for comparison of TT vs. GG between women with and without GDM, p=0.28
[d] P-value for difference in T-allele frequency between women with and without GDM, p=0.039
[e] P-value for comparison of TT/GT vs. GG between women with and without GDM, p=0.018

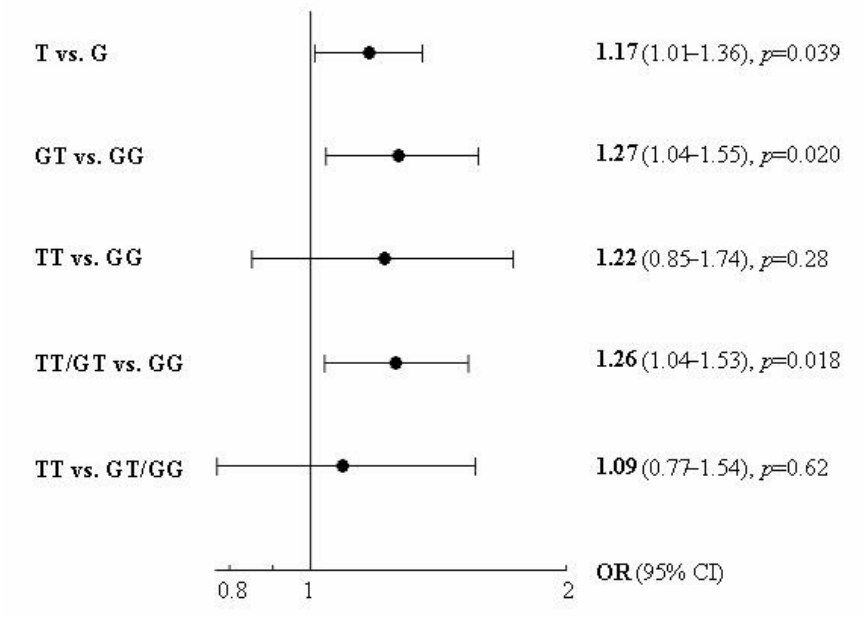

Figure 1. Odds ratios and 95%CI for *APM1* +276G>T polymorphism in women with GDM. The GG-genotype or the G-allele are defined as the reference (i.e., OR=1.0).

Supplemental Data

Supplement 1. Primers and probes used for TaqMan allelic discrimination assay

Polymorphism (rs number)	**Forward primer (5'→3')**	**Reverse primer (5'→3')**	**Probe (5'FAM)**	**Probe (5'VIC)**
APM1 **+276G>T (rs1501299)**	TTCATCACAGACCT-CCTACACTGA	TCCCTGTGTCTAG-GCCTTAGTTAAT	AAACTATATGAAG-TCATTCAT	ACTATATGAAGGCA-TTCAT
PPARG **Pro12Ala (rs1801282)**	*GTTATGGGTGAAA-CTCTGGGAGATT*	GCAGACAGTGTA-TCAGTGAAGGAAT	*CTATTGACGCAGA-AAG*	*CTCCTATTGACCCAG-AAAG*
PPARGC1A **Gly482Ser (rs8192678)**	TGGAGAATTGTTCAT-TACTGAAATCACTGT	GGTCATCCCAGTC-AAGCTGTTTT	ACAAGACCAGTGA-ACTG	CAAGACCGGTGAA-CTG
FOXC2 **-512C>T**	CGGGTGATTGGC-TCAAAGTT	GCCAAGTCCCTT-TTAGGGATTG	TCGCTTTCAGCAAG-AAGATTTTTGAAA-CT-**(BHQ1)**	**(TAM)-**TCGCTTTCAG-CAAGAAGACTTTTGA-AACT-**(BHQ2)**
ADRB3 **Trp64Arg (rs4994)**	GTTGGTCATGGT-CTGGAGTCT	GCAACCTGCTGG-TCATCGT	ATCGCCCGGACTC	CATCGCCTGGACTC

Supplement 2. Primers used for template PCR

Polymorphism (rs number)	**Forward primer (5'→3')**	**Reverse primer (5'→3')**	**Fragment size (bp)**	**Annealing temp. (°C)**
***APM1* +276G>T (rs1501299)**	AGAAAGCAGCT-CCTAGAAGT	GGCACCATCTAC-ACTCATCC	518	58
***PPARG* Pro12Ala (rs1801282)**	CAAACCCCTAT-TCCATGCTG	CCTTACATAAAT-GCCCCCAC	157	59
***PPARGC1A* Gly482Ser (rs8192678)**	GGGGTCTTTGAG-AAAATAAGG	CAAGTCCTCAG-TCCTCAC	611	58
***FOXC2* -512C>T**	GTCTTAGAGCC-GACGGATTCCTG	TGGGGACCAAG-GTGGACCCTCG	306	63
***ADRB3* Trp64Arg (rs4994)**	CGCCCAATACC-GCCAACACC	CCACCAGGAGT-CCCATCACC	210	63