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Identification of manifest diabetes and complication development in gestational diabetes mellitus. The pursuit for biomarkers.

Dereke, Jonatan

2018

Document Version: Publisher's PDF, also known as Version of record

Link to publication

Citation for published version (APA):

Dereke, J. (2018). Identification of manifest diabetes and complication development in gestational diabetes mellitus. The pursuit for biomarkers. [Doctoral Thesis (compilation), Department of Clinical Sciences, Lund]. Lund University: Faculty of Medicine.

Total number of authors: 1

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Identification of manifest diabetes and complication development in gestational diabetes mellitus

The pursuit for biomarkers

JONATAN DEREKE DEPARTMENT OF CLINICAL SCIENCES | FACULTY OF MEDICINE | LUND UNIVERSITY



Identification of manifest diabetes and complication development in gestational diabetes mellitus

The pursuit for biomarkers

Jonatan Dereke



DOCTORAL DISSERTATION by due permission of the Faculty of Medicine, Lund University, Sweden. To be defended at Belfragesalen, BMC D15, Sölvegatan 19, Lund. June 7th at 09:00

Faculty opponent Professor Peter Rossing, University of Copenhagen, Copenhagen, Denmark

Organization LUND UNIVERSITY Faculty of Medicine Department of Clinical Sciences Diabetes Research Laboratory	Document name Doctoral thesis		
	Date of issue 2018-05-16		
Author: Jonatan Dereke	Sponsoring organization		
Title and subtitle Identification of manifest diabetes and complication development in gestational diabetes mellitus – The pursui for biomarkers			
Abstract Gestational diabetes mellitus (GDM) is a type of diabetes which is first recognised during pregnancy. In the county of Skåne in Southern Sweden, over 2% of all pregnancies are complicated by GDM, a number that is generally several times higher worldwide. Women with GDM during pregnancy are at risk of developing GDM anew in subsequent pregnancies, but also have an increased risk of developing postpartum type 1 or type 2 diabetes. It is not only the mother who is at risk of developing complications due to the increased blood sugar levels in GDM. The foetus responds to the elevated levels of blood sugar by producing more insulin. Aside from lowering the blood glucose levels, insulin also acts as a growth hormone resulting in a larger foetus which in tum can cause birth trauma. Offspring to women with GDM during pregnancy are also at increased risk of developing future obesity and diabetes. GDM is today most commonly diagnosed in late pregnancy. Thus there is a possibility that both the mother and child are exposed to elevated blood sugar levels for a long time before it is recognised which may increase the risk of adverse outcomes. Providing pregnant women with the possibility of earlier screening for hyperglycaemia could help decrease the risk of such complications. The aim with this thesis was to identify proteins soluble in blood in patients with GDM which could improve the diagnosis of GDM in early pregnancy. We also aimed to identify gene variants and soluble proteins capable of indicating women with GDM at increased risk of developing type 1 and type 2 diabetes after delivery. The patients included in our studies presented in this thesis have received the GDM diagnosis following an oral glucose tolerance test at Lund University Hospital, Lund, Sweden 1996-2015. We have also certuide pregnant women without diabetes from the same geographical region in paper II . IV and V to act as controls. Genetic variants of the gene encoding zinc transporter 8 (ZnT8) was compared in women with			
Gestational diabetes mellitus, pregn	ancy, hyperglycaemia, biomarkers, infl	ammation, autoantibodies, prediction	
Classification system and/or index terms (if any)			
Supplementary bibliographical information		Language	
ISSN and key title 1652-8220		ISBN 978-91-7619-647-2	
Recipient's notes	bient's notes Number of pages Price 82		
Security classification			

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Identification of manifest diabetes and complication development in gestational diabetes mellitus

The pursuit for biomarkers

Jonatan Dereke



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Faculty of Medicine Department of Clinical Sciences

Lund University, Faculty of Medicine Doctoral Dissertation Series 2018:81

ISBN 978-91-7619-647-2 ISSN 1652-8220

Printed in Sweden by Media-Tryck, Lund University Lund 2018



Media-Tryck is an environmental-ly certified and ISO 14001 certified provider of printed material. MADE IN SWEDEN III work at www.mediatryck.lu.se

To Marita and Johnny, my wonderful parents

"Tell me and I forget. Teach me and I may remember. Involve me and I learn."

Chinese proverb

"Research is what I am doing when I don't know what I am doing."

Wernher von Braun

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Paper II - Circulating IL-1Ra are lower in patients with GDM and associated with postpartum type 2 diabetes
Paper III - The SLC30A8 R325W polymorphism and ZnT8A in GDM and postpartum diabetes development
Paper IV - Increased circulating relaxin-2 in early pregnancy complicated by gestational diabetes
Paper V - Soluble CD163 and TWEAK as biomarkers of GDM in early and late pregnancy
Discussion and concluding remarks
Future research
Acknowledgements
References

Original papers

This thesis is based on the following papers which will be referred to by their roman numerals in the text.

- I. Dereke, J., Nilsson, C., Strevens, H., Landin-Olsson, M. & Hillman, M. (2016). IgG4 subclass glutamic acid decarboxylase antibodies (GADA) are associated with a reduced risk of developing type 1 diabetes as well as increased C-peptide levels in GADA positive gestational diabetes. *Clinical Immunology*, 162, 45-48.
- II. Katra, P., Dereke, J., Nilsson, C. & Hillman, M. (2016). Plasma Levels of the Interleukin-1-Receptor Antagonist Are Lower in Women with Gestational Diabetes Mellitus and Are Particularly Associated with Postpartum Development of Type 2 Diabetes. *PLoS One*, 11, e0155701.
- III. Dereke, J., Palmqvist, S., Nilsson, C., Landin-Olsson, M. & Hillman, M. (2016). The prevalence and predictive value of the SLC30A8 R325W polymorphism and zinc transporter 8 autoantibodies in the development of GDM and postpartum type 1 diabetes. *Endocrine*, 53, 740-6.
- IV. Alonso Lopez, Y., Dereke, J., Landin-Olsson, M., Strevens, H., Nilsson, C. & Hillman, M. (2017). Plasma levels of relaxin-2 are higher and correlated to C-peptide levels in early gestational diabetes mellitus. *Endocrine*, 57, 545-547.
- V. **Dereke, J.**, Nilsson, J., Nilsson, C., Strevens, H., Landin-Olsson, M. & Hillman, M. Soluble CD163 and TWEAK as biomarkers of gestational diabetes development in early and late pregnancy and their association to postpartum glucose intolerance (*Manuscript*)

Papers I, III and IV have been reprinted with permission from the respective publishers.

Peer-reviewed papers outside of this thesis

- 1. **Dereke, J.**, Nilsson, C., Landin-Olsson, M. & Hillman, M. (2012). Prevalence of zinc transporter 8 antibodies in gestational diabetes mellitus. *Diabetic medicine*, 29, e436-9.
- Samuelsson, M., Dereke, J., Svensson, M. K., Landin-Olsson, M., Hillman, M. & on the behalf of the DISS Study Group. (2017). Soluble plasma proteins ST2 and CD163 as early biomarkers of nephropathy in Swedish patients with diabetes, 15-34 years of age: a prospective cohort study. *Diabetology & Metabolic Syndrome*, 9, 41.
- 3. **Dereke, J.** & Hillman, M. (2017). The significance of biomarkers such as FABP4 in gestational diabetes mellitus and the importance of rigorous peer review. *Acta Diabetologia*.

Scientific summary

Diabetes mellitus can be defined as a group of metabolic diseases with hyperglycaemia as common feature. The global prevalence of diabetes is estimated to be 422 (8.5%) million people. In Sweden, the prevalence of diabetes mellitus is approximately 450,000 (4.5%), with type 2 diabetes constituting 85-90% of all cases while type 1 diabetes accounts for 5-10%. There is however an estimated number of unknown cases with every third patient with type 2 diabetes yet to be diagnosed.

Gestational diabetes mellitus (GDM) is a type of diabetes which is recognised during pregnancy. In the county of Skåne in Southern Sweden, over 2% of all pregnancies are complicated by GDM, a number that is generally several times higher worldwide. Risk factors for developing this disease include advanced age, over-weight and obesity, a family history of diabetes, previous GDM, Asian or Middle-eastern ethnicity, and macrosomia in previous pregnancies. Women with GDM during pregnancy are at risk of developing GDM anew in subsequent pregnancies, but they also have an increased risk of developing type 1 or type 2 diabetes later in life. It is not only the mother who is at risk of developing complications due to the increased blood glucose levels in GDM. The foetus responds to the elevated levels of blood sugar by producing more insulin. Aside from lowering the blood glucose levels, insulin also acts as an anabolic hormone resulting in a larger foetus which in turn can cause birth trauma. Offspring to women with GDM during pregnancy are also at increased risk of developing future obesity and diabetes.

GDM is most commonly diagnosed in late pregnancy. Thus, there is a possibility that both the mother and child are exposed to elevated blood glucose levels for a long time before it is recognised, which may increase the risk of adverse outcomes. Providing pregnant women with the possibility of earlier screening for hyperglycaemia could help decrease the risk of such complications. An oral glucose tolerance test is commonly used to screen for hyperglycaemia, a procedure which is time-consuming and onerous for the patient.

The aim with this thesis was to identify proteins soluble in blood which could improve the diagnosis of GDM in early pregnancy. We also aimed to identify gene variants and soluble proteins capable of indicating women with GDM at increased risk of developing type 1 and type 2 diabetes after delivery.

The patients included in our studies presented in this thesis have received the GDM diagnosis following an oral glucose tolerance test at Lund University Hospital, Lund, Sweden 1996-2015. We have also recruited pregnant women without diabetes from the same geographical region in **paper II**, **IV** and **V** to act as controls. Genetic variants of the gene encoding zinc transporter 8 (ZnT8) was compared in women with GDM and female blood donors in **paper III**.

Our results from **paper I and III** show that women with GDM positive for antibodies against glutamic acid decarboxylase (GAD) during pregnancy have an increased risk of developing type 1 diabetes after delivery. We can also conclude that patients positive for a specific subclass of these antibodies (GADA IgG₄) have a reduced risk of later type 1 diabetes development compared to patients with only GADA IgG₁. We can also for the first time conclude that antibodies against ZnT8 are a less good predictor of type 1 diabetes development compared to antibodies against GAD.

In **paper II**, **IV** and **V** soluble proteins were analysed with the aim to improve the diagnosis of GDM. In **paper IV** only patients who received the GDM diagnosis in early pregnancy were studied while in **paper V** patients were divided depending on in what stage during pregnancy they received the diagnosis. Despite finding interesting results in these studies, more studies are required to identify soluble proteins with high sensitivity in order to improve the pre-screening of GDM.

The papers included in this thesis have collectively shown that testing for GADA positivity in patients with GDM will define women at an increased risk of developing type 1 diabetes later in life. We have also emphasised the need of finding novel biomarkers capable of improving the diagnosis of GDM during pregnancy, and especially already in early pregnancy. Identifying these women could potentially decrease the risk of developing later complications. Although getting interesting results from these studies, future studies are necessary in order to find biomarkers capable of identifying women at increased risk of developing type 2 diabetes later in life.

Populärvetenskaplig sammanfattning

Diabetes mellitus är samlingsnamnet för en rad sjukdomar som påverkar blodsocker och ämnesomsättning. Man brukar dela in diabetes mellitus i två huvudsakliga grupper: Typ 1 och typ 2 diabetes. Typ 1 diabetes, som man oftast drabbas av när man är barn och som innebär att kroppens eget immunförsvar har förstört cellerna som producerar insulin i bukspottskörteln. Vid typ 1 diabetes så finns det i blodet mätbara nivåer av så kallade antikroppar som är specifika för just den här sjukdomen. För patienter med typ 1 diabetes är det livsviktigt att man får insulinbehandling. Typ 2 diabetes drabbar främst äldre, men det blir allt vanligare att unga människor får diagnosen. Levnadsvanor, kost och motion är betydande för uppkomsten av typ 2 diabetes som uppkommer då kroppens förmåga att producera tillräckligt med insulin för att hålla blodsockret på normala nivåer inte längre räcker till samtidigt som insulinets verkningsgrad minskar.

Ungefär 450 000 (4,5%) personer har diabetes mellitus i Sverige idag. Cirka 85-90% av dem har typ 2 diabetes medan ungefär 5-10% har typ 1 diabetes. Dock finns det ett stort mörkertal och man räknar med att var tredje person med typ 2 diabetes inte är diagnosticerad.

Graviditetsdiabetes (GDM) är en form av diabetes som uppkommer under graviditeten. I Skåne drabbas strax över 2% av alla graviditeter av GDM, men den siffran är generellt många gånger högre utomlands. Risken att drabbas av GDM ökar om man är äldre, överviktig, om man har ärftlighet för diabetes, om man kommer från länder med hög frekvens av GDM eller om man i tidigare graviditeter har haft GDM eller fött ett stort barn. Om man har haft GDM under sin graviditet så ökar risken för att få framtida GDM i senare graviditeter, men man löper också ökad risk för att utveckla diabetes senare i livet. Även fostret kan ta skada av de förhöjda glukosnivåerna i blodet vid GDM. Fostret kommer att försöka minska glukosnivån genom att producera insulin. Förutom att det sänker blodsockernivån så är insulin också en tillväxtfaktor som leder till att barnet blir större, vilket kan bidra till skador vid förlossningen. Barn till mammor med GDM under graviditeten löper dessutom ökad risk för framtida övervikt och diabetes.

Diagnos av GDM sker idag i de flesta fall sent under graviditeten. Det finns då en risk att mamman och fostret utsätts för förhöjda blodglukosnivåer under lång tid innan detta upptäcks vilket kan öka risken för komplikationer. Genom att erbjuda gravida kvinnor möjligheten att testas för GDM tidigt under graviditet så kan man

minska risken för komplikationer. GDM testas för med en så kallad glukosbelastning som innebär att man dricker ett glas sockervatten på fastande mage och sedan två timmar efteråt mäter mängden socker i blodet. Glukosbelastningen är en tidskrävande, kostsam och jobbig procedur för patienten.

Syftet med den här avhandlingen var dels att hitta lösliga proteiner i blodet hos patienter med GDM som skulle kunna förbättra och underlätta diagnos av GDM i tidig graviditet. Vi ville också se om vi kunde identifiera genvarianter och lösliga proteiner i blod som skulle kunna förutspå vilka patienter med GDM som efter sin graviditet löper ökad risk att utveckla både typ 1 och typ 2 diabetes.

De patienter som har medverkat i våra studier har fått diagnosen GDM efter en glukosbelastning i Lunds universitetssjukhus upptagningsområde 1996-2015. I **delstudie II, IV och V** har även gravida kvinnor utan diabetes rekryterats från mödravårdscentraler i Dalby, Staffanstorp och Malmö för att agera kontroller. Genvarianter i genen för zink transportör 8 (ZnT8) har jämförts mellan patienter med GDM och kvinnliga blodgivare i **delstudie III**.

Resultaten från **delarbete I och III** tyder på att patienter med GDM som har antikroppar mot proteinet glutaminsyradekarboxylas (GAD) under sin graviditet löper en ökad risk för att utveckla typ 1 diabetes efter förlossningen. Vi har även sett att patienter som har en variant av dessa antikroppar (GADA IgG₄) har en minskad risk gentemot patienter som hade en annan variant (GADA IgG₁). Vi kan också för första gången fastställa att antikroppar mot proteinet ZnT8 inte visar lika tydligt vilka som kommer utveckla typ 1 diabetes efter graviditeten i jämförelse med antikroppar mot GAD.

I **delarbete II, III och V** så har vi studerat om ett flertal genvarianter och lösliga proteiner i blodet kan förutspå vilka patienter med GDM som löper ökad risk för att drabbas av typ 2 diabetes efter förlossningen. Även om vi i dessa studier kunde observera vissa skillnader mellan de patienter som senare utvecklade och inte utvecklade typ 2 diabetes så kan vi inte fastställa att någon av dessa genvarianter eller proteiner är specifika nog för att vara praktiskt användbara. Fler studier krävs för att hitta bättre indikatorer för utveckling av typ 2 diabetes efter GDM.

I **delarbete II, IV och V** så har vi analyserat lösliga proteiner för att kunna förbättra diagnosen av GDM. I **delarbete IV** studerades enbart patienter med GDM i tidig graviditet, medan i **delarbete V** så delades patienterna upp med avseende på när under graviditeten de fått diagnosen. Våra resultat i dessa studier visade dock att fler studier kommer behövas för att identifiera mätbara lösliga proteiner som kan förbättra och underlätta diagnosen av GDM.

Tillsammans har de arbeten som ingår i den här avhandlingen visat att vi bättre kan identifiera vilka patienter med GDM som löper ökad risk för typ 1 diabetes efter förlossningen. Vi har även belyst behovet av att hitta markörer som kan förutspå vilka kvinnor som kommer att drabbas av diabetes under sin graviditet. Detta för att förbättra diagnosen och minska risken för framtida komplikationer hos dessa kvinnor. Trots att vi har fått intressanta resultat i våra studier så är GDM och typ 2 diabetes komplexa sjukdomar och det kommer krävas fler studier för att hitta genvarianter och lösliga proteiner som kan identifiera vilka kvinnor som löper ökad risk att drabbas.

List of abbreviations

ADA	American Diabetes Association
ANOVA	Analysis of variance
AUC	Area under the curve
BMI	Body mass index
BSA	Bovine serum albumin
CI	Confidence interval
DIP	Diabetes in pregnancy
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
FGP	Fasting plasma glucose
GAD	Glutamic acid decarboxylase
GADA	Autoantibodies against GAD
GCT	Glucose challenge test
GDM	Gestational diabetes mellitus
HAPO	Hyperglycemia and Adverse Pregnancy Outcome
HLA	Human leukocyte antigen
IA-2	Tyrosine phosphatase-like protein
IA-2A	Autoantibodies against IA-2
IADPSG	International Association of Diabetes and Pregnancy Study Groups
ICA	Islet cell antibodies
Ig	Immunoglobulin
IGF	Insulin growth factor
IGFBP	Insulin growth factor binding protein

IGT	Impaired glucose tolerance
IL-1	Interleukin-1
IL-1Ra	IL-1 receptor antagonist
IL-1RI	IL-1 receptor I
IL-1RII	IL-1 receptor II
IQR	Interquartile range
LADA	Latent autoimmune diabetes in adults
LGA	Large for gestational age
LPBA	Liquid phase binding assay
MAPK	Mitogen-activated protein kinase
MMP	Matrix metalloproteinases
MODY	Maturity-onset diabetes in adults
OD	Optical density
OGTT	Oral glucose tolerance test
OR	Odds-ratio
PBS	Phosphate buffered saline
PCOS	Polycystic ovary syndrome
PCR	Polymerase chain reaction
RCT	Randomised controlled trial
ROC	Receiver operating characteristic
RT	Room temperature
sCD163	Soluble CD163
SD	Standard deviation
SGA	Small for gestational age
SHBG	Sex hormone-binding globulin
SNP	Single nucleotide polymorphism
sTWEAK	Soluble tumour factor-like weak inducer of apoptosis
TLR	Toll-like receptor
TMB	Tetramethylbenzidine

- TNF-α Tumour necrosis factor alpha
- WHO World Health Organization
- ZnT8 Zinc transporter 8
- ZnT8A Autoantibodies against ZnT8

Background

The history of diabetes mellitus

Diabetes mellitus and its symptoms have been recognised as far back as ancient times with descriptions found in Egyptian papyri as well as Greek, Arab and Chinese literature. The term Diabetes (from the Greek word diabaino, meaning "to pass through) was first coined in the second century AD by Aretaeus of Cappadocia who wrote what is considered to be the first accurate description of the clinical symptoms of diabetes (Karamanou et al., 2016). Indian texts dating back to the fifth century describe urine from patients with diabetes as being sweet and tasting of honey. However, it was the English physician Thomas Willis who in 1674 reported the evaporated urine from patients with diabetes to be sweet as honey which later led to the addition of the word mellitus (meaning sweet as honey), in order to differentiate diabetes from other types of polyuria (Eknoyan and Nagy, 2005).

Diabetes mellitus should not be confused with the less common diabetes insipidus, a type of polyuria first described in 1794 by Johann Peter Frank as having an abnormally increased secretion of non-saccharine urine (Valenti and Tamma, 2016). Diabetes insipidus is today known to be a disease caused by a disrupted production of antidiuretic hormone from the pituitary gland or a reduced sensitivity to antidiuretic hormone in the kidneys (Robertson, 2016).

Another scientist of great importance to medicine was the French physiologist Claude Bernard who, among other valuable findings, discovered the glycogenic function of the liver. Barnard observed that animal blood contained sugar not only after feeding, but also in the fasting state. By feeding dogs a carbohydrate diet just before sacrificing them, he found the hepatic veins to contain large amounts of sugar, a result also confirmed in a study with a strict meat diet (Breathnach, 2014). By studying liver tissue samples, Barnard found not only increased amounts of sugar compared to other organs and tissues, but also the presence of a water insoluble polysaccharide which could be converted into sugar and secreted into the blood stream. In 1855 Barnard published these results, however it was not until 1857 that he could isolate this substance which he named "*la matière glycogène*", today known as glycogen (Karamanou et al., 2016, Young, 1957).

The pancreas had since its discovery been referred to as a non-vital organ and was rather seen as a "fleshy cushion to on which the surrounding visceral organs rested". Later anatomical studies showed that the pancreas had a role in the digestive process. Experiments performed in the late 17th century by the Swiss anatomist Johann Conrad Brunner in which he removed the pancreas in dogs after which they survived confirmed the belief that the pancreas was non-vital. The first mention of a relationship between the pancreas and diabetes was made by Thomas Cawley in 1788 when he described a case of pancreatic calcification to be linked to diabetes (Eknoyan and Nagy, 2005).

In 1889, Oskar Minkowski and Joseph von Mering performed a pancreatectomy in a dog which soon after surgery developed polyuria with elevated levels of sugar in its urine. Repeated surgeries on more dogs confirmed the initial results and it was concluded that the dogs had indeed developed diabetes following this procedure (Karamanou et al., 2016). In 1869, a couple of decades before Minkowski and von Mering performed their experiments, the German medical student, Paul Langerhans, described the presence of small cells lying in groups scattered across the rabbit's pancreas. Langerhans admitted to not knowing himself the function of these groups of cells. It was not until 1893 that the French histology professor Édouard Laguesse proposed that these cells could be involved in internal secretion, naming them the Islets of Langerhans in honour of their discoverer (Sakula, 1988).

In November 1920, Fredrick Banting, a young Canadian surgeon, approached John James Rickard Macleod, a professor of physiology and head at the University of Toronto, with a proposal of a way to obtaining pancreatic extracts via ligation of pancreatic ducts of dogs and allowing the pancreas to degenerate. In spring 1921, Macleod agreed to give Banting laboratory space and a student assistant who would help him out during the summer named Charles Best (Rosenfeld, 2002). After months of experiments, Banting and Best managed to extract, crush and ground atrophied pancreas from dogs. Upon injecting this extract in pancreatectomised dogs, they could observe a significant decrease in blood sugar. Once the procedure had been improved, Macleod got actively involved in the research himself and agreed to Banting's request to have the experienced biochemist James Collip join the team. Collip set to work on increasing the purity of the pancreatic extracts Banting and Best had been using. Using alcohol for precipitation, he managed to isolate a powder containing the active agent which was pure enough to be used in clinical patients (Karamanou et al., 2016, Rosenfeld, 2002).

On the 2nd of December 1921, a 14-year old boy was admitted to the Medical Wards, Toronto General Hospital. His name was Leonard Thompson and he had severe diabetes which, despite dietary regulation, was only getting worse as time went by. On the 11th of January 1922 he received his first injections of the dissolved extract Collip had purified. The low concentration given in this first treatment showed only a moderate decrease in blood glucose levels. The next two weeks Thompson received daily injections of higher concentrations of the extract to which an improvement was evident. Another six patients received similar treatment and all improved clinically. Thus, the efforts made by Banting, Best, Collip and Macleod had resulted in an efficient treatment for diabetes mellitus (Banting et al., 1922). They named this extract insulin, a name already proposed for a hypothetical substance of the pancreas independently by Jean de Mayer in 1909 and Sir Edward Sharpey-Schäfer in 1913. The name insulin referring to its origin being the Islets of Langerhans. With the introduction of insulin, a world of diabetes patients could now receive efficacious treatment. The only major obstacle left was to start manufacturing insulin on a commercial scale. The team accepted an offer to collaborate with the pharmaceutical company Eli Lilly and by the start of 1923, the first large scale commercial insulin product was available (Rosenfeld, 2002).

The 1923 Nobel prize in medicine was awarded to Banting and Macleod for the discovery of insulin. Banting was furious when he heard the news. He and Macleod had not been on good terms for a long time and Banting believed that it would be more appropriate if the prize would have been awarded to Best rather than Macleod. They however accepted the nomination and Banting decided to share his part of the cash award with Best, whereupon Macleod did the same with Collip (Karamanou et al., 2016, Rosenfeld, 2002).

Classification of diabetes mellitus

In 1880, the French physician Étienne Lancereaux made the distinction between diabetes in obese (*diabète gras*) and lean (*diabète maigre*) people which called for different treatment regimens (Lancereaux, 1880). From experiments performed and published 1936 by Harold Himsworth a difference in insulin sensitivity between patients with diabetes could be observed. His patients were given a dose of insulin based on their body surface area and directly after drank a glucose solution. From blood samples drawn at regular intervals, Himsworth could clearly see a difference in blood glucose levels between patients whom he later divided into an insulinsensitive and insulin-insensitive group. Himsworth concluded that insulin in some patients had little or no effect in suppressing hyperglycaemia. He also noted that patients in the insulin-sensitive group required less insulin to stay normoglycaemic, whereas patients who were insulin-insensitive required much higher doses to reach their glycaemic goals. Furthermore, Himsworth describes a relationship between the type of diabetes and the onset of the disease in which the insulin-sensitive patients presents severe symptoms, whereas the insulin-insensitive patients, diabetes present few or none of the classical symptoms of thirst or polyuria (Himsworth, 2011).

Continuing the work of Himsworth and others, John Lister and colleagues studied insulin sensitivity in relation to clinical features in 100 patients with diabetes. Lister et al. introduced a new nomenclature, type 1 and type 2 diabetes mellitus to distinguish the two clinically different groups of patients with diabetes. Besides confirming the difference in insulin sensitivity previously shown, they also described typical clinical differences between patients in the type 1 and type 2 group as well as a partial inverse correlation in obesity and insulin sensitivity (Lister et al., 1951).

The modern classification of diabetes mellitus can be divided into four major categories as described by the American Diabetes Association (ADA) (American Diabetes Association, 2018):

- Type 1 diabetes, characterised by autoimmune β-cell destruction
- Type 2 diabetes, characterised by a progressive loss of β -cell insulin secretion in combination with insulin resistance
- Gestational diabetes mellitus (GDM), diabetes diagnosed in the second or third trimester of pregnancy that was not clearly overt diabetes prior to gestation¹
- Specific types of diabetes due to other causes, e.g. maturity-onset diabetes of the young or neonatal diabetes

Diabetes in pregnancy

Approximately 16% of live births in the world is affected by hyperglycaemia. An estimated 86.4% is due to GDM, 7.4% due to other types of diabetes first detected in pregnancy, and 6.2% due to pre-gestational diabetes. The prevalence of hyperglycaemia in pregnancy vary depending on ethnicity and region, with the highest prevalence observed in South East Asia (24%) and the Middle-east and North Africa (21%). The estimated prevalence in Europe is 16.2% (International Diabetes Federation, 2017). Pregnancy leads to extensive changes in the metabolism. Initially there is a fall in plasma glucose and amino acid levels with concomitant rise in free fatty acids. In the latter part of pregnancy there is an increase in postprandial glucose levels together with increased insulin secretion and a decreased insulin sensitivity which reverses with delivery. Deficiencies in insulin

¹ This is the exact definition of gestational diabetes mellitus presented by the American Diabetes Association 2018. Other definitions exist.

production and/or insulin resistance as seen in diabetes can lead to abnormal metabolism of carbohydrates, proteins, and fat (McCance, 2015).

Insulin secretion and sensitivity in pregnancy is closely linked to hormonal regulation. Maternal cortisol levels are doubled in pregnancy compared to the nonpregnant state, and have a diabetogenic effect by leading to increased pancreatic insulin secretion (Mouzon and Lassance, 2015). The pregnancy hormone relaxin-2, which peaks during the first trimester, has been shown to correlate with insulin sensitivity and lower levels of relaxin-2 have been reported in women with type 2 diabetes (Zhang et al., 2013). The versatile lactogenic polypeptide prolactin increases concomitant to insulin in the latter half of pregnancy and stimulates insulin production and secretion (Wang et al., 2013). Human placental growth hormone has been shown to increase insulin resistance in mice overexpressing the hormone leading to hyperinsulinemia (Barbour et al., 2002).

In the UK and US, GDM accounts for the majority of diabetes in pregnancy (DIP) (~87%), followed by type 1 diabetes (7%) and type 2 diabetes (5%) (Fraser and Lawlor, 2014). Even though the frequency of type 1 diabetes in the general population is much lower (5-10%) than type 2 diabetes (90-95%), it still represents a slightly higher fraction of diabetes in pregnancy due to the relatively earlier onset of disease. However, as obesity increases worldwide, so does also the number of women who are obese prior to pregnancy, resulting in a growing number of pregnant women with type 2 diabetes (both undiagnosed and diagnosed) going into pregnancy. This increase in obesity is evident and well-studied in developed countries (Guelinckx et al., 2008), but can also be observed worldwide in developing countries (Popkin et al., 2012).

Pre-gestational diabetes in pregnancy results in an overall increased risk of maternal and foetal complications (table 1). Compared to normoglycaemic pregnancies there is an increased risk of caesarean section, foetal hyperglycaemia, shoulder dystocia, perinatal death, pre-term delivery and large for gestational age (LGA). The risk of giving birth to a baby being small for gestational age (SGA) is however decreased (Eidem et al., 2011, Persson et al., 2009). Most of the adverse outcomes have been reported to be similar in both maternal type 1 or type 2 diabetes present during pregnancy. A 2009 meta-analysis (Balsells et al., 2009) comparing 33 studies on maternal and foetal outcomes in women with either pre-gestational type 1 or type 2 diabetes concluded an increased rate of perinatal mortality in the offspring to women with type 2 diabetes. There was however no difference in congenital malformations, neonatal mortality or stillbirth depending on type of diabetes. Further, the study by Balsells et al. confirmed that women with type 1 diabetes displayed a slightly increased risk of caesarean section. An infrequent, yet life threatening complication for both mother and child is diabetic ketoacidosis, mainly seen in pregnancies complicated by type 1 diabetes. Ketoacids readily crosses the placenta and foetal

mortality rate associated with diabetic ketoacidosis has been reported to be 9-36% (Sibai and Viteri, 2014). A Danish study investigated the association between hyperglycaemia during pregnancy in women with either GDM or type 1 diabetes and glucose tolerance in adult offspring. The results showed an increased prevalence of pre-diabetes and type 2 diabetes in 22 years old offspring to mothers with GDM (21%) or type 1 diabetes (12%) during pregnancy compared to the background population (4%) (Clausen et al., 2008).

Table 1. Risk of complication development in type 1 diabetic pregnancies.

Summary of the risk of complication development in pregnancies complicated by type 1 diabetes with an odds-ratio showing the increased risk of developing these complications with an exception for SGA where the risk is decreased.

Complication	OR (95% CI) of complication development in diabetic pregnancies	Reference
Stillbirth	3.6 (2.5-5.3)	(Eidem et al., 2011)
Perinatal death	2.9 (2.0-4.1)	(Eidem et al., 2011)
Infant death	1.9 (1.1-3.2)	(Eidem et al., 2011)
Preterm delivery	4.9 (4.3-5.5)	(Eidem et al., 2011)
Pre-eclampsia	4.5 (3.8-5.3)	(Persson et al., 2009)
Caesarean section	5.3 (5.0-5.7)	(Persson et al., 2009)
SGA	0.7 (0.6-0.9)	(Persson et al., 2009)
LGA	11.4 (10.6-12.4)	(Persson et al., 2009)
Major malformations	2.5 (2.1-2.9)	(Persson et al., 2009)

Abbreviations: OR: Odds-ratio, CI: Confidence interval, SGA: Small for gestational age, LGA: Large for gestational age

History of gestational diabetes

The term gestational diabetes was introduced by Elsie Reed Carrington in 1957 (Carrington et al., 1957). The first case of diabetes during pregnancy in history was however recorded in Berlin by Dr H.G. Bennewitz already in 1823. Bennewitz described a young woman who, in her fourth and fifth pregnancy, suffered from polydipsia. The fifth birth was premature and stillborn, the baby itself had been unusually large. He also noted that the symptoms in the mother had disappeared postpartum (Hadden and Hillebrand, 1989). In 1952 Jorgen Pedersen proposed what has later been known as the Pedersen Hypothesis, stating that maternal hyperglycaemia leads to foetal hyperglycaemia which in turn results in foetal hyperinsulinism and ultimately macrosomia (Catalano and Hauguel-De Mouzon, 2011). Data from the Hyperglycemia and Adverse Pregnancy Outcome (HAPO) study published in 2008 could confirm the Pedersen hypothesis by showing a strong linear association of risk for >90th percentiles of birth weight, cord C-peptide levels, percent body fat, and maternal glucose (Hapo Study Cooperative Research Group

et al., 2008, International Association of Diabetes Pregnancy Study Groups Consensus Panel et al., 2010).

In the 1960s, the need for a standardised method of diagnosing diabetes led to the development of the oral glucose tolerance test (OGTT) with different recommendations based on the glucose load, ranging from 50-100g (Bartoli et al., 2011). In 1964 O'Sullivan and Mahan performed extensive evaluations of the OGTT and its cut-off values for GDM in pregnant women. They introduced the need for two separate abnormal glucose values for diagnosis with the cut-off of two standard deviations (SD) from the mean, resulting in a 2.5% prevalence of GDM. Applying these new diagnostic criteria, 23% of women diagnosed with GDM during their pregnancy could be observed to develop overt diabetes up to 8 years postpartum. With better laboratory methods later being developed, O'Sullivan's recommendations were adjusted and turned into the Carpenter and Coustan criteria still used for diagnosis (Carpenter and Coustan, 1982, Coustan, 2013, O'Sullivan and Mahan, 1964).

Pathogenesis and risk factors in gestational diabetes

The mechanisms underlying the development of GDM can vary, but as with other types of diabetes, GDM is a result of dysfunctional β -cells in combination with insulin resistance. Patients with GDM in some cases present with β -cell specific autoantibodies, as seen in type 1 diabetes, targeting antigen such as glutamic acid decarboxylase (GAD) (Nilsson et al., 2007), tyrosine phosphatase-like protein (IA-2) (Fuchtenbusch et al., 1997) or zinc transporter 8 (ZnT8) (Dereke et al., 2012). Approximately 10% of patients with GDM present with these and other β -cell specific autoantibodies (Dereke et al., 2012, Buchanan et al., 2012). Circa 1-5% of women diagnosed with GDM have the genetic variants seen in monogenic diabetes mellitus such as maturity-onset diabetes in adults (MODY). A recent Danish study found possibly pathogenic MODY genes to be present in almost 6% of women with GDM and recommends screening for these variants in (Gjesing et al., 2017). However, the vast majority of women diagnosed with GDM however, have an increased insulin resistance in combination with obesity comparable to patients with type 2 diabetes (Buchanan et al., 2012).

Advancing age has repeatedly been confirmed to be a risk factor for developing GDM. In an Australian cohort study including almost 12,000 women with GDM, women above 35 years of age had a 7.0% risk of GDM development as compared to a 2.1% risk in women below 25 (Abouzeid et al., 2015). Another study concluded that the risk of developing GDM in women over 40 compared to women between 20-30 was increased by almost four times (Favilli et al., 2012). Another well-known

risk factor for the development of GDM is overweight or obesity. An extensive meta-review focusing on maternal obesity and the risk of GDM development published in 2007 included 20 studies with over 800,000 pregnant women. The women were divided into four groups according to their body mass index (BMI): normal, overweight, obese and severely obese (BMI cut-off values varied depending on study criteria). Based on the results of the meta-review, the authors estimated a 2-fold, 4-fold and 8-fold increase in the risk of developing GDM for women in the overweight, obese, and severely obese groups respectively when compared to the normal-weight group (Chu et al., 2007). Women who have undergone a pregnancy complicated by GDM are at an increased risk of developing GDM in subsequent pregnancies with studies reporting rates varying between 30-84% with an average of 48% (Schwartz et al., 2015, Kruse et al., 2015). When comparing the risk of GDM in the second pregnancy in women with and without previous GDM, the risk is increased from 4.2% to 41.3% in an American study (Getahun et al., 2010). Also, when comparing women in their third pregnancy who had both their previous pregnancies complicated by GDM to women with two previous normoglycaemic pregnancies, the risk of developing GDM increased further to 56.9% compared to 4.7% respectively (Getahun et al., 2010).

Maternal inflammation during pregnancy

In pregnancy, the innate immune system undergoes a regulatory shift to prevent rejection of the foetus. In healthy pregnancies the Th1/Th2 balance shifts as the pregnancy progresses (figure 1). The first and early second trimester of pregnancy requires a strong inflammatory response for proper implantation and placentation and here the Th1 activity is increased. After implantation however, the immune system balance is shifted to an anti-inflammatory Th2 response which acts to protect the foetus from rejection. Following completed foetal development, the immune system needs to adapt once again, this time to enable proper parturition. During this phase, a shift toward Th1 takes place and the pro-inflammatory environment promotes the contraction of the uterus, expulsion of the foetus and removal of the placenta (Mor et al., 2011). Infections and inflammatory processes may tilt the balance toward a Th1 dominant activity during pregnancy, which causes an increase in pro-inflammatory cytokine production leading to an increased risk of spontaneous abortion or pre-eclampsia (Challis et al., 2009).

FIRST TRIMESTER

- Pro-inflammatory
- Th1 dominant
- Implantation & placentation

SECOND TRIMESTER

- Anti-inflammatory
- Th2 dominant
 - Foetal growth

•

THIRD TRIMESTER

- Pro-inflammatory
- Th1 dominant
- Parturition & rejection of the placenta

Figure 1. Inflammation throughout pregnancy

The innate immune system undergoes important changes as pregnancy progresses. In the first trimester and the end of the third trimester the shift is towards a pro-inflammatory Th1 dominant environment, while the period in between is focused on foetal growth and development requiring an anti-inflammatory milieu.

In pregnancies complicated by GDM there is an evident dysregulation of inflammation already in the first trimester (Richardson and Carpenter, 2007). An enhanced T-cell activation in combination with a decreased number of CD45RA+ regulatory T-cells can be seen in GDM when compared to healthy pregnancy, suggesting a less effective immune suppression. The elevated glucose levels seen in GDM may have a direct effect on inflammation. Increased levels of glucose have been shown to upregulate Toll-like receptors (TLRs) TLR2 and TLR4 in human monocytes, resulting in NF-KB activation and subsequent production of proinflammatory cytokines. TLR4 also activates the mitogen-activated protein kinase (MAPK) signalling pathway leading to transcription of genes involved in inflammation (Lekva et al., 2016). Tumour necrosis factor alpha (TNF- α) is a Th1 cytokine mainly produced by immune cells, particularly in macrophages, and is decreased during normal pregnancy compared to the non-pregnant state. Levels of TNF- α are correlated to BMI and have been shown to be increased during the second and third trimester in women with GDM and is possibly involved in propagating insulin resistance (Noureldeen et al., 2014, Pantham et al., 2015).

Diagnosing gestational diabetes

Diagnosis worldwide and the 2010 IADPSG criteria

The O'Sullivan diagnostic criteria introduced 1964 and subsequent adaptations were validated on their ability to predict future diabetes in the mother. Another set of criteria previously used is the World Health Organization (WHO) criteria based on fasting plasma glucose (FGP) or a 75-g OGTT, distinguishes DIP from GDM with DIP being diagnosed in patients with higher FPG or blood plasma glucose following

an OGTT compared to in GDM (WHO, 2013). Similar to the WHO, ADA recommends differentiating pre-existing diabetes from GDM. The rationale behind this decision is based on the ongoing epidemic of obesity which in turn leads to an increased number of overweight and obese women at childbearing age. This eventually results in a larger number of undiagnosed type 2 diabetes going into pregnancy. Thus, ADA endorses screening for diabetes already at the first prenatal visit using the standard criteria for the non-pregnant state in women with risk factors for diabetes (American Diabetes Association, 2018). While the WHO states that GDM can be diagnosed in both early and late pregnancy, ADA classifies GDM as being a form of diabetes first recognised during second or third trimester of pregnancy. Their conclusion is based on the data of the International Association of Diabetes and Pregnancy Study Groups (IADPSG) being derived from late pregnancy, claiming that there is a lack of evidence supporting early pregnancy GDM diagnosis (American Diabetes Association, 2018, McIntyre et al., 2016, WHO, 2013).

Today, the most common diagnostic criteria for GDM used is the IADPSG recommendation based on the HAPO study. Prior to the HAPO study, most diagnostic criteria used were validated solely on their ability to predict later diabetes development in the mother (Coustan, 2013). The HAPO study, including roughly 25,000 participating pregnant women performing OGTTs at 15 international centres, aimed to evaluate maternal hyperglycaemia and the risk of adverse pregnancy outcomes. The primary outcomes considered were birth weight above the 90th percentile for gestational age, primary caesarean delivery, clinically diagnosed neonatal hyperglycaemia, and cord blood C-peptide levels above the 90th percentile. Secondary outcomes considered were pre-term delivery, shoulder dystocia and pre-eclampsia among others (Hapo Study Cooperative Research Group et al., 2008). The results from this extensive study showed associations between increased glucose levels in the fasting state, 1-hour and 2-hours following an OGTT to all of the primary outcomes. The IADPSG criteria recommend a mean venous blood glucose value cut off corresponding to an odds ratio (OR) of 1.75 for the development of any of the HAPO primary outcomes (International Association of Diabetes Pregnancy Study Groups Consensus Panel et al., 2010). These and other international diagnostic criteria are presented in table 2.

Table 2. Diagnostic criteria for GDM

Most frequently used guidelines with recommended glucose load and corresponding cut-off values in mmol/L for diagnosing gestational diabetes worldwide

	Fasting plasma glucose	Recommen ded glucose load (OGTT)	1-hour plasma glucose	2-hour plasma glucose	3-hour plasma glucose
C&C*	≥5.3	100g	≥10.0	≥8.6	≥7.8
WHO	5.1-6.9	75g	≥10.0	8.5-11.0	N/A
IADPSG	≥5.1	75g	≥10.0	≥8.5	N/A
EASD	≥7.0	75g	≥11.0	≥9.0	N/A

References: (Carpenter and Coustan, 1982, Lind and Phillips, 1991, International Association of Diabetes Pregnancy Study Groups Consensus Panel et al., 2010, WHO, 2013). * The ACOG recommends performing an 100g OGTT with the C&C criteria following a non-fasting 1-hour, 50g glucose challenge test (GCT) with positive outcome.

The implementation of the IADPSG guidelines resulted in a substantial increase in GDM prevalence worldwide (Brown and Wyckoff, 2017, Duran et al., 2014, Huhn et al., 2017, O'Sullivan et al., 2011) (figure 2). A Swiss retrospective cohort study published in 2017 compared the GDM prevalence using a two-step 1-hour 50g GCT followed by a 75g OGTT when the GCT was positive for diagnosis. This diagnostic method was used from 2008 up until 2010, followed by the IADPSG diagnostic criteria being used between 2010 and 2013 (Huhn et al., 2017). The prevalence of GDM using the original criteria reached a total of 3.3%, while the prevalence using the IADPSG criteria amounted to a total of 11.8%, an almost four-fold increase. Interestingly, 10% and 22% respectively of the women diagnosed with GDM using the different diagnostic criteria received the diagnosis already in early pregnancy.



Reported Prevalence of GDM by Country Using IADPSG criteria

Figure 2. International increase in the prevalence of GDM

Visualisation of the increased prevalence of GDM in different countries and regions using respective their previous diagnostic criteria (if applicable) and HAPO results or IADSG criteria. Adapted from Brown and Wyckoff, 2017 with permission from the authors.

Several studies have investigated the impact on clinical care, outcomes, and eventual rise in costs associated with this substantial increase in GDM prevalence as comprehensively reviewed by Brown and Wyckoff in 2017 (Brown and Wyckoff, 2017). When comparing the two-step C&C, and IADPSG criteria, the costs for testing has been concluded to increase, while laboratory work load is decreased for the IADPSG criteria. Also the IADPSG criteria was estimated to decrease the costs related to adverse pregnancy outcomes. To the best knowledge of the author, all studies discussing the economic pros and cons of the IADPSG criteria compare these to the two-step approach using C&C criteria still recommended by e.g. the American College of Obstetrics and Gynecology (ACOG). A cost-benefit analysis of the IADPSG criteria in relation to the previously used WHO criteria or the European Association for the Study of Diabetes (EASD) criteria would be beneficial in regions where the two-step approach is not in use.

Diagnosis in southern Sweden and the 1991 EASD criteria

There are huge discrepancies in GDM screening between regions in Sweden with one of the underlying reasons being the lack of national consensus regarding screening guidelines (Lindqvist et al., 2014). The prevalence of GDM in Sweden has, in observational studies, been estimated to be approximately 2% (Berg et al., 2007, Ignell et al., 2014), which from a global perspective is low.

In the region of southern Sweden, pregnant women have been routinely screened for GDM using an OGTT since 1991 for the catchment area of the university hospital of Lund, and 1995 for all of Skåne county (Anderberg et al., 2007). Here, all pregnant women regardless of risk factors are offered to perform a 75g OGTT. following overnight fast, around the 28th week of gestation, when the insulin requirement is at its highest. For women with risk factors for GDM (previous GDM, previous macrosomia, BMI over 30kg/m² and/or family history of diabetes), an OGTT is offered already in the 12th week of gestation with a subsequent OGTT being performed in the 28th week of gestation if the first is negative (Nilsson et al., 2015). The diagnostic criteria used is based on the 1991 EASD guidelines which was recommended in Sweden at the start of the Lund routine GDM screening (Åberg et al., 2001, Lind and Phillips, 1991). In contrast to the universally measured venous whole-blood glucose levels, in Skåne the rationale is to measure capillary plasma glucose using a HemoCue® Glucose System (HemoCue AB, Ängelholm, Sweden). When measuring capillary plasma glucose, a conversion factor of 1.11 must be applied to be able to compare the levels with venous whole-blood glucose. The 1991 EASD guidelines adopted in Sweden recommends a 2-hour blood glucose concentration of >9mmol/L for the diagnosis of GDM. That, when converted to capillary plasma glucose, roughly equals >10.0mmol/L (Åberg et al., 2001, Ignell and Berntorp, 2011). The diagnostic criteria for women diagnosed with GDM in Skåne is thus a 2-hour capillary plasma glucose of >10.0mmol/L for a 75-g OGTT following overnight fast. FPG and 1-hour capillary plasma glucose values have not been used for diagnosis in Skåne.

In 2015 the Swedish National Board of Health and Welfare presented new national directives for clinical care of GDM (The National Board of Health and Welfare, 2015). These directives were released as a response to the lack of national and international consensus regarding diagnosis and treatment of women with GDM. The recommendation regarding diagnosis is to endorse the IADPSG criteria nationally in Sweden. This recommendation was presented in 2015 and these diagnostic criteria have recently been evaluated and decided to be adopted nationally in Sweden in 2018. In Lund, the new criteria will be used starting on the 1st of June.

Diagnosing gestational diabetes in early pregnancy

Whether or not you should classify hyperglycaemia in early pregnancy as GDM is controversial. For example, the ADA have chosen to classify early pregnancy hyperglycaemia as overt diabetes rather than GDM based on the IADPSG diagnostic criteria for which an early pregnancy OGTT was not recommended (American Diabetes Association, 2018). The WHO on the other side, in their 2013 report (WHO, 2013), states that GDM should be diagnosed at any time in pregnancy when the diagnostic criteria is met. The guidelines currently used in southern Sweden are in agreement with the WHO.

Despite not recommending using an OGTT to diagnose GDM in early pregnancy, the IADPSG proposed a FPG \geq 5.1mmol/L as the diagnostic criteria (International Association of Diabetes Pregnancy Study Groups Consensus Panel et al., 2010). A retrospective Chinese study evaluating these criteria concluded that pregnant women with a FPG between 6.1-7.0mmol/L during their first prenatal visit should be diagnosed as GDM and that the IADPSG recommendation of \geq 5.1mmol/L should not be used (Zhu et al., 2013). A similar conclusion was reached in an Italian study where no correspondence between first trimester FPG and later OGTT testing could be found. The authors concluded that the proposed IADPSG early GDM criteria should not be used for GDM diagnosis, but rather as a possible predictor of late pregnancy GDM development (Corrado et al., 2012). In 2016 the IADPSG themselves, based on these studies, concluded that their previous recommendation of using an FPG \geq 5.1mmol/L for early GDM diagnosis could not be justified and chose to retract this recommendation. The IADPSG however significate the need for better detection of early pregnancy hyperglycaemia and increased risk of adverse outcomes (McIntyre et al., 2016).

Identifying women with hyperglycaemia already in early pregnancy provide the possibility of earlier interventions, reducing the risk of metabolic disturbances which in turn could improve pregnancy outcomes. An Australian study evaluated the experience of changing the screening policy of GDM to include early pregnancy GCT and OGTT testing for six months in a large tertiary hospital. During this study period, the prevalence of GDM was 7.9% with 1.6% receiving the diagnosis in early pregnancy and 6.3% in late pregnancy (Ng et al., 2015). Other studies have investigated whether testing for early pregnancy glucose levels could be used to identify women at increased risk of developing hyperglycaemia later in pregnancy. High levels of early pregnancy random glucose or FPG has been found to be associated to the development of later overt diabetes (Church et al., 2011) and GDM development, as well as adverse outcomes such as LGA and caesarean delivery (Riskin-Mashiah et al., 2009, Zhu et al., 2013). Some studies on the other hand do not recommend using FGP measurements for the prediction of later GDM and
advice against universal early pregnancy glucose screening due to lack of evidence regarding pregnancy outcomes (Agarwal and Dhatt, 2006, Yeral et al., 2014).

Screening for hyperglycaemia using oral glucose challenge tests is both time consuming and demanding for the health care providers and patients. Thus, identifying non-glycaemic biomarkers for GDM development which ideally remain stable regardless of fasting status are of great interest (Powe, 2017). Preferably, a combination of clinical and biochemical biomarkers could be used already in early pregnancy to predict women at high risk of developing hyperglycaemia. Using a biomarker panel with high specificity and sensitivity would eliminate the need for unnecessary GDM screening in women at low risk while it would act as an incentive for prevention and intervention of hyperglycaemia in women at high risk of GDM (Correa et al., 2014, Kennelly and McAuliffe, 2016, Powe, 2017).

Clinical risk factors for GDM used in southern Sweden as incentives for early pregnancy GDM screening includes previous GDM, BMI >30kg/m², previous macrosomia and a family history of diabetes. Combinations of these risk factors and others such as age, ethnicity, previous infant stillbirth, multiparity or polycystic ovary syndrome (PCOS) are commonly used to identify women with increased risk of glucose intolerance in early pregnancy (American Diabetes Association, 2018, Kennelly and McAuliffe, 2016, Powe, 2017).

Novel biomarkers of GDM development

A number of potential soluble biomarkers have previously been studied during early pregnancy. One promising biomarker is sex hormone-binding globulin (SHBG), a glycoprotein produced by the liver, regulated by insulin, and associated with insulin resistance and type 2 diabetes (Katsuki et al., 1996). SHBG was identified as a potential early pregnancy biomarker for later GDM development already in 2003 (Thadhani et al., 2003). Fascinatingly, one study has shown decreased levels of SHBG as early as up to six years before pregnancy in women who developed GDM. Women in the lowest quartile of SHBG concentrations experienced a four-fold increased OR of GDM development compared to women in the highest SHBG quartile (Hedderson et al., 2014). The adipocyte-produced adipokine adiponectin increases insulin sensitivity and have been inversely associated with obesity and type 2 diabetes. Adiponectin also stimulates insulin production and insulin secretion. Adiponectin levels have been comprehensively investigated in pregnancies complicated by GDM with the majority of studies reporting decreased adiponectin levels (Fasshauer et al., 2014). A meta-analysis published in 2016 summarised results from 13 studies on adiponectin as an early pregnancy predictor for GDM. This analysis presented a pooled OR for GDM prediction of 6.4 for serum adiponectin. A receiver operating characteristic (ROC) curve of the adiponectin

levels resulted in an area under the curve (AUC) of 0.78 with a sensitivity of 64.7% and specificity of 77.8% (Iliodromiti et al., 2016). Another biomarker which have been showing promise as an early GDM predictor is glycosylated fibronectin. Glycosylated fibronectin has been shown to be increased in patients diagnosed with GDM in early pregnancy with one study reporting a sensitivity of 81% and specificity of 90% for a cut-off level of 107mg/L, the ROC curve reaching an AUC of 0.91 (Rasanen et al., 2013). A European multi-national prospective cohort study including 748 singleton pregnancies is currently ongoing aimed at evaluating the use of early pregnancy OGTT and/or glycosylated fibronectin for the diagnosis of GDM (Huhn et al., 2016). The study is estimated to be finished in the second half of 2019.

Inflammation is regulated by anti- and pro-inflammatory cytokines. One important family of cytokines is the interleukin-1 (IL-1) superfamily including the proinflammatory ligands IL-1 α and IL-1 β , the IL-1 receptor type I (IL-1RI) and II (IL-1RII) and the anti-inflammatory IL-1 receptor antagonist (IL-1Ra) among others (Banerjee and Saxena, 2012, Gabay et al., 2010). The role of IL-1ß has been investigated in the inflammatory process seen in both type 1 diabetes (Eizirik and Mandrup-Poulsen, 2001), type 2 diabetes (Feve and Bastard, 2009) and GDM (Lappas, 2014) via decreased insulin secretion and increased β -cell apoptosis (Banerjee and Saxena, 2012). IL-1Ra structurally resembles IL-1a and IL-1B, but has an important anti-inflammatory role by blocking the binding of the IL-1 receptor accessory protein to IL-1RI, inhibiting signal transduction and reducing apoptosis of β-cells (Arend, 2002, Larsen et al., 2007). A decreased expression of IL-1Ra can be observed in β-cells from patients with type 2 diabetes, suggesting an increased biological effect of IL-1 signalling, ultimately leading to increased inflammation and β -cell destruction (Maedler et al., 2004). The role of IL-1 mediated signalling during pregnancy has also been studied. A previous study could show increased IL-1Ra in response to microbial invasion of the amniotic cavity in pre-term labour (Rizzo et al., 1996). Other studies have found IL-1Ra to be increased in pregnancies complicated by pre-eclampsia (Amash et al., 2012, Greer et al., 1994). It is relevant to further study the potential impact of the IL-1 superfamily on the pathogenesis leading to the development of GDM.

Structurally similar to the insulin family is the relaxin family of peptides consisting of relaxin-1, -2 and -3. Relaxin-2 is a pregnancy hormone involved in the regulation of matrix metalloproteinases (MMPs) in the endometrium (Goldsmith et al., 2004), promoting growth for the uterus and cervix (Vasilenko and Mead, 1987), and preparing for parturition (Conrad, 2011). Studies have suggested that relaxin peptides affect the binding of insulin to its receptor, hence having an impact on glucose homeostasis (Jarrett et al., 1984, Olefsky et al., 1982). Relaxin-2 levels have been shown to correlate to insulin sensitivity with decreased levels observed in women with type 2 diabetes (Zhang et al., 2013). In contrast, plasma levels of

relaxin-2 has been shown to be elevated in pregnant women with type 1 diabetes (Whittaker et al., 2003). Considering that relaxin-2 has an effect on glucose homeostasis, while being an important pregnancy hormone, studies should focus on its potential role in the pathogenesis of GDM.

The haptoglobin-haemoglobin scavenger receptor CD163 is abundantly expressed on macrophages resident in adipose tissue. The extracellular part of CD163 is shed upon inflammatory macrophage activation resulting in soluble CD163 (sCD163) (Møller, 2011). Increased concentrations of sCD163 can be observed in obesity and has been implicated as a predictor of type 2 diabetes development in the general population (Møller et al., 2011). A clear correlation between sCD163 and insulin resistance has also been shown (Zanni et al., 2012). High levels of sCD163 in combination with increased relaxin and other mediators has been shown to predict pre-term delivery (Vogel et al., 2005), a known complication to GDM.

The cytokine tumour necrosis factor-like weak inducer of apoptosis (TWEAK) is an important inflammatory regulator and a proposed ligand for CD163 (Bover et al., 2007, Vendrell and Chacón, 2013). TWEAK is a member of the TNF superfamily which has been proposed to protect the implanting embryo against harmful effects of TNF, interferon- γ and IL-12 signalling (Mas et al., 2008) and has been shown to be expressed on placental macrophages (Phillips et al., 2001). A soluble version of TWEAK (sTWEAK) is created through cleavage by the furin endopeptidase. Previous studies on circulating sTWEAK reports decreased serum levels in patients with both type 1 and type 2 diabetes (Kralisch et al., 2008, Llaurado et al., 2012). A previous study has found decreased levels of sTWEAK, but no difference in sCD163 levels in women with GDM (Simon-Muela et al., 2015), while another study could observe increased sCD163 in GDM (Bari et al., 2014). Thus, sTWEAK could be an important mediator in the pathogenesis of GDM and related complications and its potential role as a biomarker should gain more focus.

Diabetes development following gestational diabetes

Despite most women returning to the normoglycaemic state following pregnancy, and aside from the increased risk of recurring GDM, women diagnosed with GDM during their pregnancy are at increased risk of developing manifest type 1 or type 2 diabetes postpartum (Bellamy et al., 2009, Järvelä et al., 2006). Women with GDM that are positive for autoantibodies targeting β -cell antigens during pregnancy are at high risk at developing type 1 diabetes (Fuchtenbusch et al., 1997). Fifty percent of autoantibody-positive women with GDM will receive the diagnosis type 1 diabetes up to 10 years postpartum, with the majority being diagnosed already during the first year after delivery (Nilsson et al., 2007). One study has shown that positivity

for an increasing number of autoantibodies (GADA, IA-2A and islet cell antibodies (ICA)) at delivery conferred a progressive risk of developing postpartum type 1 diabetes. The two-year risk increased from 2.2% in women without autoantibodies to 17% when one of the three was present, 61% when two were present, and 84% when positive for all three (Fuchtenbusch et al., 1997). Positivity for β -cell specific autoantibodies in patients with GDM is highly indicative of postpartum type 1 diabetes development, and these patients should be tested for postpartum glucose intolerance early on after delivery and continually assessed with annual intervals (Fuchtenbusch et al., 1997, Järvelä et al., 2006, Nilsson et al., 2007).

GDM and type 2 diabetes have a closely related pathogenesis with similar risk factors such as increasing age and BMI, family history of diabetes, and an increasing prevalence in women of high risk ethnicity. Progression to type 2 diabetes postpartum is thus more common in women with GDM with these risk factors (Kim et al., 2002). The rate of women progressing to type 2 diabetes after GDM varies a great deal between studies depending on tests used, diagnostic criteria, selection bias, and follow-up time with studies reporting rates between 3-70% (Feig et al., 2008, Järvelä et al., 2006). An extensive systematic review and meta-analysis including over 600,000 women from multinational studies have done a thorough estimation of the risk of postpartum type 2 diabetes risk (figure 3). This study showed that women who develop GDM during their pregnancy has an over seven times increased risk of developing manifest type 2 diabetes compared to women with normoglycaemic pregnancies (Bellamy et al., 2009).



Figure 3. The risk of developing postpartum type 2 diabetes in patients with GDM

The risk of type 2 diabetes development after GDM have been extensively studied in various populations. This metaanalysis of 20 studies including almost 11.000 type 2 diabetes events and over 600.000 women found a cumulative increased relative risk of 7.43. Adapted from Bellamy et al., 2009 with permission from Elsevier and the authors. While autoantibodies in GDM have a high predictive value for postpartum type 1 diabetes, biomarkers yielding high precision in predicting the development of type 2 diabetes are needed. Clinical risk factors for developing type 2 diabetes include maternal age, ethnicity, parity, pre-pregnancy weight, weight gain, previous GDM, family history of diabetes and insulin treatment in GDM. Adjusting for confounders however diminishes the statistical significance for the majority of these risk factors and there are discrepancies between studies (Kim et al., 2002, Leuridan et al., 2015). FPG, 1- and 2-h glucose levels seem to be better predictors of type 2 diabetes after GDM with some studies reporting the OGTT AUC to be predictive (Kjos et al., 1995, Steinhart et al., 1997), while other find no association (Persson et al., 1991).

Several novel biomarkers for the prediction of postpartum type 2 diabetes have been investigated in women with GDM. Increased C-peptide and decreased ghrelin levels when measured 12 weeks postpartum have been reported to be significant risk factors. Interestingly, fasting insulin, despite correlated to C-peptide levels, was not found to be associated to the development of type 2 diabetes (Lappas et al., 2015a). The same research team have also found that several lipid species improve the net reclassification of type 2 diabetes by over 20% up to 8.5 years postpartum when added to a clinical prediction model (Lappas et al., 2015b). They also report members of the insulin growth factor (IGF) family and IGF-binding proteins (IGFBP) IGF-I and IGFBP-2 to be increased and decreased respectively in women developing postpartum type 2 diabetes (Lappas et al., 2016). Studies on novel non-glycaemic biomarkers for postpartum type 2 diabetes have thus far mainly been published by this Australian research team and only in a limited number of patients. More studies are needed to confirm these findings in other populations and to investigate other potential biomarkers attaining high precision.

Lifestyle changes have been shown to reduce the risk of developing type 2 diabetes. One randomised controlled trial (RCT) showed a 58% risk reduction of type 2 diabetes development in men and women with impaired glucose tolerance (IGT) with dietary changes and increased exercise (Tuomilehto et al., 2001). A RCT published in 2008 studied the effects of metformin treatment and intensive lifestyle interventions in women with previous GDM (Ratner et al., 2008). This multicentre study found that both metformin and lifestyle interventions decreases the rate of progression to manifest type 2 diabetes within a 3-year follow-up period. While metformin and lifestyle interventions proved most efficient in lowering the risk for women with out GDM during their previous pregnancy. With this in regard, lifestyle changes after delivery should be encouraged in women at risk of developing manifest type 2 diabetes.

Aims of the thesis

Paper I

The primary aim of this study was to investigate if the distribution of GADA immunoglobulin (Ig) subclasses IgG_1 and IgG_4 in women with GDM was associated to endogenous insulin secretion and postpartum type 1 diabetes development. We also sought to identify if human leukocyte antigen (HLA) risk types DQ2 and DQ8 could be associated with GADA subclass distribution or could predict the development of manifest type 1 diabetes.

Paper II

This study aimed to investigate if circulating levels of IL-1Ra could be used as a complementary biomarker for the diagnosis of GDM and if IL-1Ra was able to predict the development of postpartum overt diabetes.

Paper III

The primary aims of this study were to: evaluate possible associations between the ZnT8 gene SLC30A8 R325W polymorphism, GDM and postpartum type 2 diabetes, to confirm the prevalence of autoantibodies against ZnT8 (ZnT8A) in a large GDM population, and to investigate the predictive value of ZnT8A in the development of postpartum type 1 diabetes.

Paper IV

The aim of this study was to investigate circulating relaxin-2 levels in patients diagnosed with GDM in early pregnancy compared to pregnant women without diabetes recruited in the same gestational age.

Paper V

In this study the aim was to investigate the potential role of sCD163 and sTWEAK as biomarkers of GDM development in early and late pregnancy as well as their association to postpartum development of glucose intolerance.

Methodology

Participants

Pregnant women in the catchment area of the Department of Obstetrics and Gynaecology at Lund University Hospital, Lund, Sweden, have been offered an OGTT (*see Diagnosis in southern Sweden and the 1991 EASD criteria section for the diagnostic criteria used*) as part of a general screening program for GDM since 1995 and venous blood samples have been collected and sent to the Diabetes Research Laboratory since 1996 (**paper I-V**) (figure 4). Patients are checked with a FPG the day after delivery to establish that the elevated blood glucose levels are normalised. If not, their diagnosis is reclassified as manifest diabetes rather than GDM. Patients presenting with autoantibodies during their pregnancy are offered first follow-up three months postpartum while patients without autoantibodies are followed up first one year after delivery. Pregnant women without diabetes or a history of diabetes were recruited in their 12th week of gestation 2014-2015 from three maternal healthcare centres in the same geographical region to act as controls (**paper II, IV and V**).

		GDM 1996-2015 (n=1753)		
Paper I	Paper ll	Paper III	Paper IV	Paper V
1996-2013	2004-2013	2004-2013	2006-2014	2011-2015
(n=1225)	(n=227)	(n=776)	(n=137)	(n=227)
	Controls	Controls	Controls	Controls
	2014-2015	2000-2001	2014-2015	2014-2015
	(n=156)	(n=511)	(n=114)	(n=227)

Figure 4. Women with GDM and controls included in the thesis

The patients included in this thesis have received their diagnoses as part of the general GDM screening in the catchment area of the Department of Obstetrics and Gynaecology at Lund University Hospital. Pregnanct women without diabetes included as controls in study II, IV and V were recruited from maternal healthcare centres in the same geographical region 2014-2015. Non-pregnant women without diabetes were included as controls in paper III for comparison of genetic variants only.

Blood samples from women with GDM and pregnant controls are sent to the Diabetes Research Laboratory via post. Serum and ethylenediaminetetraacetic acid (EDTA)-plasma are separated from blood cells at 2000 x g and stored at -70°C until analysed. DNA is extracted from human leukocytes where available and stored at 70°C until analysed (**paper I and III**).

All studies have been approved by the Regional Ethical Review Board in Lund (Regionala etikprövningsnämnden i Lund: LU526/00, 849/2005, 244/2007, 2009/307, 2014/78, 2014/744, 2014/744 and 2017/416) and performed in accordance with the 1964 Declaration of Helsinki. Women with GDM (**paper I-V**) and pregnant controls (**paper I, II, IV and V**) received oral and written information regarding the study before giving written informed consent to participate. The samples collected from non-pregnant women and included in paper III for DNA analysis are from female blood donors and no information regarding the study and their samples were made anonymous. As such informed consent is not required from these participants.

Analysis of soluble biomarkers

Circulating proteins or autoantibodies have been analysed in all papers included in this thesis using either enzyme linked immunosorbent assay (ELISA) or liquid phase binding assay (LPBA) as described below.

Enzyme linked immunosorbent assay

Autoantibodies

Commercially available ELISA kits have been used to study GADA (**paper I and III**), IA-2A (**paper III**) and ZnT8A (**paper III**) (RSR ltd, Cardiff, UK) according to the manufacturer's instructions.

Briefly, serum was added to wells pre-coated with GAD, IA-2 or ZnT8 in duplicates with a reaction enhancer added for the IA-2A assay. The GADA assay incubated for 1-h at room temperature (RT), while the IA-2A and ZnT8A incubated overnight at 4°C. Biotin conjugated to antigen was added to the wells after washing and incubated for 1-h at RT. Streptavidin conjugated peroxidase was added after washing and incubated 20 min on a 96-well plate shaker. Tetramethylbenzidine (TMB) was added after washing and incubated in darkness at RT. The reaction was stopped by the addition of sulphuric acid and the optical density (OD) was measured at 450nm in a FLOUstar Optima ELISA plate reader (BMG Labtech Gmbh,

Ortenberg, Germany). A four-parametric logistic regression standard curve was used to calculate the autoantibody concentration of unknown samples.

C-peptide

C-peptide levels have been analysed in duplicates using commercially available ELISA kits (**paper I, II, IV and V**) according to the manufacturer's instructions (Mercodia AB, Uppsala, Sweden). The lower detection limit of the C-peptide assay was reported to be 25pmol/L and the intra-coefficient of variation ranged between 2.9-4.8%.

Novel biomarkers

Circulating IL-1Ra, relaxin-2, adiponectin, sCD163 and sTWEAK have been analysed in plasma using commercial DuoSet ELISA kits and supplementary component ancillary kits (table 3) from R&D Systems (R&D Systems, Minneapolis, MN, USA). The DuoSet ELISA kits require some optimisation for optimal precision when running plasma samples. A dilution series of control and patient samples were run in order to find a sample dilution which reached the highest precision on the 7point recombinant standard curve for each individual analyte. Once identified, all samples were run with the same dilution. For participants whose samples reached values above the standard curve when analysed, further dilutions were performed. All dilutions were made using 1% bovine serum albumin (BSA) in phosphate buffered saline (PSB). In order to minimise bias caused by intra-assay variation, patient and control samples were alternated on each plate. Samples, controls and standards were run as duplicates in according to the manufacturer's instructions for all DuoSet ELISA analyses. The intra-coefficient of variation for IL-1Ra, relaxin-2, adiponectin, sCD163 and sTWEAK was 20.0%, 3.2%, 2.2%, 8.7% and 7.8% respectively.

Table 3. Commercial kits used for detection of circulating proteins in plasma

Commercial human DuoSet ELISA kits and complementary ancillary kits from R&D Systems have been used to detect levels of circulating proteins in EDTA-plasma from women with and without GDM. In order to achieve optimal precision, participant samples were diluted in PBS with 1% BSA with a higher dilution factor if samples were above the standard curve when analysed.

Analyte	Human DuoSet ELISA	Ancillary kit	Optimal sample dilution	Paper
IL-1Ra	DY280	DY008	1:5	Paper II
Relaxin-2	DY2804-05	DY008	1:5	Paper IV
Adiponectin	DY1065	DY008	1:2000	Paper IV
sCD163	DY1607	DY008	1:200	Paper V
sTWEAK	DY1090	DY008	1:5	Paper V

Liquid phase binding assay

A LPBA was used to detect GADA IgG subclasses in women with GDM positive for GADA when analysed with the commercial ELISA explained above (**paper I**). The LPBA used to detect GADA IgG_1 and IgG_4 subclasses was developed and first presented by my supervisors in 2007 (Hillman et al., 2007).

Briefly, patient serum or plasma was incubated with recombinant ³⁵S-methionine (PerkinElmer, Waltham, MA, USA) labelled GAD₆₅, followed by incubation with mouse anti-human GADA IgG1 (15µg/mL, BD Pharmingen, Franklin Lakes, NJ, USA) or IgG4 (25µg/mL, BD Pharmingen, Franklin Lakes, NJ, USA) antibodies overnight at 4°C allowing the formation of immune complexed of GADA and GAD₆₅, but also between IgG subclasses and anti-human antibodies. The immune complexes were precipitated onto 96-well filter plates (Merck Millipore, Burlington MA, USA) coated with streptavidin sepharose (40% dilution, GE Healthcare, Little Chalfront, UK) pre-coated with 1% BSA to reduce unspecific binding and punched out into vials containing Ultima Gold liquid scintillation cocktail (PerkinElmer, Waltham, MA, USA). Counts per minute were measured in a Tri-Carb® 2100TR liquid scintillation counter (PerkinElmer, Waltham, MA, USA) for two minutes per sample. In-house standards in quadruplicates were used and indexes were calculated to estimate IgG subclass titres. The cut-off level for GADA IgG₁ and IgG₄ positivity was set to an index of 0.03 and 0.04 respectively as previously described by our research group (Hillman et al., 2007).

Identification of genetic risk variants

DNA was extracted from leukocytes using a standard salting-out precipitation method (Miller et al., 1988). DNA concentration and purity was spectrophotometrically determined with the absorbance ratio at $\lambda = 260/280$ nm (Biowave DNA, Biochrom, Cambridge, UK).

HLA genotyping

The HLA-DQ β 1 risk alleles DQ2 and DQ8 were determined using a commercially available MutaGEL[®] HLA-DQ2+8 kit (**paper I**) (Immundiagnostik AG, Bensheim, Germany) with specific primers for HLA DQ2 (A1*05/B1*02) and DQ8 (A1*03/B1*0302). The HLA genotyping was performed according to the manufacturer's instructions.

SLC30A8 R325W genotyping

The coding single nucleotide polymorphism (SNP) rs13266634 (C > T) affecting the amino acid at location 325 (R325W) in the ZnT8 was studied using polymerase chain reaction (PCR) and specific endonucleases (**paper III**). Primers described by Huang et al. was used when running the PCR (Huang et al., 2010). The PCR was run with denaturation (94°C, 30s), annealing (57°C, 30s) and elongation (72°C, 30s) for 30 cycles and a final extension at 72°C for 7min in a GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA, USA). The PCR product fragment size was 429bp.

In order to detect the R325W SNP, the PCR products were incubated at 37°C for 1h with the specific endonucleases BcnI and PvuII (Thermo Fisher Scientific Inc. Waltham, MA, USA). BcnI generated two fragments when the R325W C-allele was present (235 + 194bp), while PvuII generated two fragments when the T-allele was present (233 + 196bp) (figure 5). All fragments were visualized with the use of 3% agarose gel electrophoresis and GelStar[®] Nucleic Acid Stain (Lonza, Rockland, ME, USA).



Figure 5. Endonuclease cleavage of the R325W PCR fragment When the R325W SNP C-allele was present in the PCR product fragment, the Bcnl endonoclease generated two fragments, while if the T-allele was present, the endonuclease Pvull generated two fragments.

Statistical methods

Distribution of continuous data was estimated using the D'Agostino-Pearson test for normality. Mean \pm SD was used to present normally distributed data and median followed by interquartile range (IQR) was used when normality was rejected. Depending on data distribution, student's t-test or the Mann-Whitney *U*-test was used to test for differences in continuous variables between groups (**paper I, II, IV**)

and V). The Wilcoxon signed rank test was used to compare IgG subclass titres between the first and second pregnancy in women with GDM (paper I). When comparing differences in continuous variables in more than two groups, analysis of variance (ANOVA) or the Kruskal-Wallis test with post hoc pairwise comparison using Sheffé or Conover's method was used for normal and non-normal data respectively (paper II, III and V). Correlations between continuous variables were estimated using Spearman's rho (paper I-V). Fisher's exact test (paper I) or the γ^2 test (paper II, III and V) or was used to test for differences in frequencies between groups. A power calculation was used to estimate the sample size necessary for a statistical power of 0.8 and a significance level of 0.05 regarding R325W allelic The power calculation was frequencies (paper III). performed with http://osse.bii.a-star.edu.sg/calculation1.php. Stepwise logistic regression analyses were used to identify continuous variables as possible confounders and determining the role of circulating biomarkers as independent predictors of GDM development (paper III and IV). Cox proportional-hazards regression was performed to analyse the effects of the R325W polymorphisms on the development of postpartum type 2 diabetes (paper III). The precision of potential novel biomarkers for the development of GDM and/or postpartum diabetes development was evaluated using ROC curve analyses (paper II and V). A p-value less than 0.05 was considered statistically significant for all analyses and all presented p-values are two-sided. All statistical analyses were performed in MedCalc Statistical Software v12-18 for Windows (MedCalc Software, Ostend, Belgium).

Results

Paper I - IgG₄ subclass GADA is associated with a reduced risk of developing type 1 diabetes after GDM

Positivity of GADA could be observed in 53 (4.3%) out of the 1225 women with GDM included in this study. Serum or plasma samples were available for 51 of 53 GADA positive women, enabling IgG subclass LPBA analysis. All women with GDM positive for GADA were followed-up with their first visit three months postpartum followed by annual visits. Thirteen of the 51 women with GDM included in this study developed postpartum type 1 diabetes within 5 years postpartum with a mean time to development of 1.4 ± 1.1 years (table 4). Fifteen of the women with GDM returned with a second pregnancy complicated by GDM 1-9 (4.5 ± 2.8) years postpartum. Two also had a third pregnancy complicated by GDM within two years from the second GDM pregnancy.

Table 4. Clinical and laboratory data in women with GDM with or without later type 1 diabetes development Thirteen of a total of 51 patients with GDM developed type 1 diabetes within 5 years postpartum. Statistically significant differences between patients with and without postpartum type 1 diabetes could be observed in total GADA (measured with commercial ELISA) and GADA IgG₄ (measured with LPBA). The precence of HLA-DQ2/8 is given in total. Adapted from paper I.

	No postpartum type 1 diabetes (n=38)	Type 1 diabetes postpartum (n=13)	P-value
Age (years)	31.8 ± 5.3	31.4 ± 5.0	0.80
Total GADA (u/mL)	74.2 [30.1-227.8]	142.1 [61.6-821.5]	0.04
GADA IgG ₁ (index)	0.16 [0.04-0.41]	0.41 [0.12-0.54]	0.19
GADA IgG₄ (index)	0.04 [0.02-0.06]	0.01 [0.00-0.03]	0.03
C-peptide (nmol/L)	1.35 [0.64-1.90]	1.09 [0.42-1.40]	0.23
HLA DQ2/8 (yes/no)	10/28 (26.3%)	5/8 (38.5%)	0.48

Titres of total GADA were significantly higher in patients developing postpartum type 1 diabetes (142.1 [61.6-821.5] u/mL) compared to those who did not (74.2 [30.1-227.8] u/mL; p=0.04). The titres of GADA IgG₄ on the other hand were decreased in patients with postpartum type 1 diabetes development (p=0.03) and the frequency of GADA IgG₄ positivity was lower in this group (p=0.04) (figure 6). No

difference in GADA IgG_1 subclass titres could be observed between these groups (p=0.19).

All patients positive for GADA IgG₄ were also positive for GADA IgG₁. Positivity for GADA IgG₁ was twice as common as GADA IgG₄ (42/51 (82%) and 21/51 (41%) respectively). Total GADA was also found to be correlated to IgG₁ (r_s =0.65; p<0.001), but not with IgG₄ (r_s =0.11; p=0.47).



Figure 6. GADA IgG4 titres in women with or without postpartum type 1 diabetes development A lower frequency of GADA IgG4 positivity could be observed in women with GDM developing later type 1 diabetes. Adapted from paper I.

A correlation could be observed between C-peptide and GADA IgG₄ titres (r_s =0.32; p=0.04) (figure 7), C-peptide and IgG₁ however did not correlate (p=0.86). Women with repeated pregnancies complicated by GDM retained their GADA levels as well as their IgG subclass profile.

GADA IgG₄ positive women had a significantly lower frequency of HLA DQ2/8 compared to women negative for GADA IgG₄ (2/21 (11%) and 13/30 (43%) respectively; p=0.012).



Figure 7. GADA IgG4 was correlated to C-peptide

A statistically significant correlation between GADA IgG₄ and C-peptide could be observed in this study (r_s=0.32; p=0.04). As C-peptide is released in equimolar levels and in combination with insulin, this result could be suggesting a protective role of GADA IgG₄. Adapted from paper I.

Paper II - Circulating IL-1Ra are decreased in GDM and associated with postpartum type 2 diabetes

Women with pregnancies complicated by GDM had lower plasma levels of IL-1Ra (1964 [306-5276] pg/mL) compared to pregnant controls (2902 [1074-6030] pg/mL; p=0.012). Additionally, women with GDM who developed postpartum IGT or type 2 diabetes had lower levels of IL-1Ra when compared to women with GDM who did not develop postpartum glucose intolerance (p=0.023) (table 5). Performing ROC curve analyses on the possible role of IL-1Ra as a diagnostic tool for GDM resulted in an optimal sensitivity of 37.9% and specificity of 79.5%. Performing a ROC curve for the development of postpartum glucose intolerance in patients with GDM resulted in an optimal sensitivity of 52.2% and specificity of 67.1%.

An inverse correlation could be observed between C-peptide levels and IL-1Ra for the control group (r_s =-0.31; p<0.001). This correlation was however lost when analysed in the GDM group (p=0.49). Circulating IL-1Ra was also found not to correlate to neither BMI nor age in any of the groups.

Table 5. Clinical and laboratory data for controls and women with GDM with and without later glucose intolerance

Patients with GDM had decreased IL-1Ra levels compared to pregnant women without diabetes (p=0.012). In addition, women with GDM with postpartum IGT or type 2 diabetes development had significantly lower IL-1Ra levels when compared to women with GDM without postpartum glucose intolerance (p=0.023). Adapted from paper II.

	Controls (n=156)	GDM without postpartum diabetes (n=158)	GDM with postpartum IGT/T2DM (n=69)	p-value
Age (years)	29.8 ± 5.3	32.1 ± 5.3	32.0 ± 6.0	<0.001
Body mass index (kg/m²)	25.4 [23.1-28.9]	26.3 [23.5-31.6]	28.3 [23.1-32.9]	0.053
Family history of diabetes (yes/no)	47/109	60/98	34/35	0.021
C-peptide (nmol/L)	0.47 [0.31-0.74]	0.93 [0.53-1.61]	1.30 [0.97-1.93]	<0.001
IL-1Ra (pg/mL)	2902 [1074- 6030]	2285 [491-5826]	883 [0-4047]	0.0033

Paper III - The SLC30A8 R325W polymorphism and ZnT8A in GDM and diabetes development later in life

The SLC30A8 R325W SNP was analysed in women with GDM with or without positivity for ZnT8A. The majority of women positive for ZnT8A (11/18 (61%)) were heterozygous for C/T while the most frequent genotype in women without ZnT8A was heterozygosity for the risk C-allele (285/518 (55%)), although no significant association between presence of ZnT8A and R325W genotypes or allele frequencies was observed.

A higher level of association to homozygosity for the R325W C-allele was found in women with GDM when compared to women without diabetes (OR: 1.47, 95% CI: 1.16–1.88; p = 0.0018) (table 6). Homozygosity for the R325W T-allele on the other hand was decreased in GDM (OR: 0.58, 95% CI: 0.38-0.88; p=0.0110). Also, in patients with GDM, the allele frequency of the C-allele (789/1072 (74%)) was significantly increased and the T-allele (283/1072 (26%)) was decreased compared to controls (679/1022 (66%) and 343/1022 (34%) respectively; p=0.0004).

Table 6. Distribution of the R325W polymorphism

Homozygosity for the SLC30A8 R325W risk C-allele was found to be significantly increased in women with GDM compared to controls while homozygosity for the T-allele was decreased in the patient group. Adapted from paper III.

	GDM (n=536)	Controls (n=511)	OR (95% CI)	p-value
C/C	292 (54%)	229 (45%)	1.47 (1.16-1.88)	0.0018
C/T	205 (38%)	221 (43%)	0.81 (0.63-1.04)	0.0997
T/T	39 (7%)	61 (12%)	0.58 (0.38-0.88)	0.0110

A number of women with GDM in this study developed type 2 diabetes postpartum (66/776 (9%)) with a median follow-up time of 2 [1-4] years and the R325W SNP was studied in these patients when DNA was available (n=49). The majority of these patients were homozygous for the C-allele (30/49 (61%)) (table 7). When calculating the predictive value of the R3252W genotypes in the development of postpartum type 2 diabetes, none of the genotypes were retained in the model (p=0.4374).

Table 7. Distribution of the R325W polymorphism in relation to postpartum diabetes development A number of women with GDM developed postpartum type 1 diabetes (n=8) or type 2 diabetes (n=49). The majority of patients developing postpartum type 2 diabetes were homozygous for the R325W C-allele. Adapted from paper III.

	No postartum diabetes (n=479)	Postpartum type 1 diabetes (n=8)	Postpartum type 2 diabetes (n=49)
C/C	257	5	30
C/T	188	3	14
T/T	34	0	5

Autoantibodies against GAD, IA-2 and ZnT8 were detected in 53/776 (6.8%) of women with GDM diagnosed 2004-2013 (figure 8). The majority of autoantibodypositive women were positive for only one of the antibodies. The most frequent single autoantibody was GADA (21/53 (40%)), followed by ZnT8A (16/53 (30%)) and IA-2A (4/53 (8%)). The total number of women with GDM positive for ZnT8A was 25/776 (3.2%). Ten of the 53 (19%) autoantibody-positive patients developed postpartum type 1 diabetes with a median follow-up time of 1 [1-2] years. While 4/10 (40%) of these women were positive for ZnT8A and IA-2A, all were positive for GADA.



Figure 8. Autoantibodies in women with GDM

Venn diagram visualising the prevalence and combinations of autoantibodies in women with GDM. A total of 53 patients was positive for GADA, IA-2A and/or ZnT8A during their pregnancy. Adapted from paper III.

Paper IV - Increased circulating relaxin-2 in early pregnancy complicated by gestational diabetes

Plasma levels of relaxin-2 was found to be significantly increased in women with GDM in the 12th gestational week (0.83 [0.40-1.53] ng/mL) compared to controls recruited at the same gestational age (0.47 [0.3-1.0] ng/mL; p=0.001) (table 8). C-peptide levels were higher in women with GDM (1.12 [0.85-2.17] nmol/L) compared to controls (0.60 [0.40-0.83] nmol/L; p<0.001). Circulating relaxin-2 was positively correlated with C-peptide (r_s =0.198; p=0.002).

Plasma levels of adiponectin were lower in women with GDM in the 12th gestational week (2.8 [0.9-4.4] μ m/mL) compared to controls (5.2 [3.5-6.8] μ g/mL; p<0.001). Adiponectin was found to inversely correlate to C-peptide (r_s=-0.365; p<0.001), but not to relaxin-2 (p=0.167). Also, relaxin-2 was found not to correlate to BMI (p=0.151) nor age (p=0.173). In a logistic regression model with GDM as the dependent variable and relaxin-2, adiponectin, BMI, C-peptide and maternal age as independent variables, relaxin-2 and maternal age lost their statistical significance, while retained by the other variables.

Table 8. Clinical and laboratory data of women included in the study

Women diagnosed with GDM in early pregnancy and pregnant women without diabetes recruited at the same gestational age were included in this study. Adapted from paper IV.

	Pregnant women without gestational diabetes (n=114)	Women diagnosed with gestational diabetes (n=137)	Ρ
Relaxin-2 (ng/mL)	0.47 [0.33 – 1.00]	0.83 [0.40 – 1.53]	0.002
Adiponectin (µg/mL)	5.2 [3.5 - 6.8]	2.8 [0.9 - 4.4]	<0.001
C-peptide (nmol/L)	0.60 [0.40 - 0.83]	1.12 [0.85 – 2.17]	<0.001
Age (years)	30.4 ± 5.1	32.1 ± 5.7	0.019
BMI (kg/m ²)	24.7 [22.1 – 27.6]	29.0 [23.4 – 32.4]	<0.001

Paper V - Soluble CD163 and TWEAK as biomarkers of GDM in early and late pregnancy

Plasma levels of sCD163 were higher in women diagnosed with GDM in early (322 [253-437] ng/mL) and late pregnancy (395 [303-513] ng/mL) compared to controls (287 [228-364] ng/mL; p=0.004 and p<0.001 respectively) (table 9). Although not statistically significant, sCD163 showed a tendency to correlate to BMI (p=0.08), but was correlated to C-peptide (r_s =0.250; p<0.001). No difference in plasma levels of sCD163 could be observed between patients who developed postpartum glucose intolerance (n=34; 386 [299-540] ng/mL) and those who did not (n=193; 367 [281-499] ng/mL; p=0.50).

Circulating sTWEAK was lower in women diagnosed with GDM in early (0.7 [0.4-2.1] ng/mL) and late pregnancy (0.8 [0.4-3.3] ng/mL) compared to controls (1.4 [0.7-5.1] ng/mL; p<0.001 for both comparisons). Plasma levels of sTWEAK was not correlated to neither BMI (p=0.28) nor C-peptide (p=0.20). Plasma levels of sTWEAK did not differ between women with GDM who developed postpartum glucose intolerance (0.6 [0.4-1.8] ng/mL) and those who did not (0.8 [0.4-3.4] ng/mL; p=0.24). sCD163 and sTWEAK was not found to be correlated in this study (p=0.12).

	Controls (n=227)	Early pregnancy GDM (n=63)	Late pregnancy GDM (n=164)	p-value
Age (years)	31.3 ± 4.7	32.3 ± 4.9	31.0 ± 4.6	p=0.18
BMI (kg/m ²)	24.9 [22.5-28.1]	29.0 [25.1-33.1]	24.2 [22.2-26.8]	p<0.001 [†]
Scandinavian (Yes/no)	158/69 (70%)	31/23 (57%)	93/53 (64%)	p=0.20
Family history of diabetes (Yes/no)	51/176 (23%)	32/22 (59%)	50/96 (34%)	p<0.001
C-peptide (nmol/l)	0.5 [0.3-0.8]	0.7 [0.4-1.0]	0.9 [0.5-1.5]	p<0.001
sCD163 (ng/ml)	287 [228-364]	322 [253-437]	395 [303-513]	p<0.001
sTWEAK (ng/ml)	1.4 [0.7-5.1]	0.7 [0.4-2.1]	0.8 [0.4-3.3]	p<0.001 [‡]

Table 9. Clinical and biochemical variables in pregnant controls and women with GDM Women included as controls were recruited in the 12th week of gestation. Patients with GDM included were diagnosed in early or late pregnancy. The control and patient group as a whole were matched for age (p=0.84) and BMI (p=0.77).

† Even though the controls and women with GDM as a whole are matched for BMI (p=0.77), a BMI >30kg/m2 is considered a risk factor for GDM, warranting early pregnancy GDM screening, thus a significant difference between the groups when performing the Kruskal Wallis test can be observed.

‡ Plasma levels of sTWEAK was significantly different between controls and patients with GDM, but no significant difference could be observed between the two patient groups.

The level of association of sCD163 and sTWEAK to GDM development in early and late pregnancy was estimated with a stepwise logistic regression analysis. Included independent variables was age, BMI, C-peptide, sCD163 and sTWEAK. All variables apart from age retained their significant association to GDM in both early and late pregnancy.

A ROC curve analysis of sCD163 in the development of GDM in early and late pregnancy (figure 9, panel A and B) resulted in a sensitivity and specificity of 57% and 63% in early pregnancy and 69% and 68% in late pregnancy. The AUC for the ROC curves were 0.619 and 0.717 respectively. A ROC curve analysis of sTWEAK in the development of GDM in early and late pregnancy (figure 9, panel C and D) resulted in a sensitivity and specificity of 62% and 67% in early pregnancy and 28% and 93% in late pregnancy. The AUC for the ROC curves were 0.663 and 0.597 respectively.



Figure 9. ROC curves for sCD163 and sTWEAK as biomarkers of early and late pregnancy GDM A: sCD163 in early pregnancy generates an AUC of 0.619 with a sensitivity of 57% and specificity of 63%. B: sCD163 in late pregnancy generates an AUC of 0.717 with a sensitivity of 69% and specificity of 68%. C: sTWEAK in early pregnancy generates an AUC of 0.663 with a sensitivity of 62% and specificity of 67%. D: sTWEAK in late pregnancy generates an AUC of 0.597 with a sensitivity of 24% and specificity of 93%.

Discussion and concluding remarks

Hyperglycaemia during pregnancy increases the risk of adverse outcomes for both the mother and the child. Blood glucose lowering interventions in women with pregnancies complicated by GDM have been shown to reduce the risk of complications (American Diabetes Association, 2017). GDM is today universally screened for in late pregnancy, however development of new, non-fasting, methods of identifying women at risk of GDM in early pregnancy could improve the screening procedures while also helping to decrease the unnecessary cost and burden of screening women at low risk of developing GDM (Powe, 2017).

Women with pregnancies complicated by GDM are at an increased risk of developing GDM in subsequent pregnancies and postpartum manifest type 1 or type 2 diabetes (Järvelä et al., 2006). Identifying women with GDM at increased risk of postpartum glucose intolerance could act as an incentive for necessary lifestyle changes and pharmacological interventions in these women, possibly reducing the risk of later diabetes development.

In **paper I** we investigated if the distribution of GADA IgG subclasses IgG_1 and IgG_4 could influence the risk of developing postpartum type 1 diabetes. We found that women with GDM positive for GADA with higher titres of GADA IgG_4 had a reduced risk of postpartum type 1 diabetes, regardless of total GADA titres. We could also observe a positive correlation between IgG_4 and C-peptide. The C-peptide is released through proteolytic cleavage of proinsulin upon transit from the Golgi apparatus into secretory vesicles, resulting in equimolar levels of insulin and C-peptide upon release (Weiss, 2009). C-peptide, being more stable than insulin, can thus be used as a useful way of estimating insulin secretion for when immediate analysis of insulin is unavailable. The correlation between IgG_4 and C-peptide levels can therefore be proposed to reflect a better insulin secretion in women with GDM and increased IgG₄.

Further we could not observe any significant difference in IgG_1 titres between patients with and without postpartum type 1 diabetes development. Women with GDM who developed type 1 diabetes had a mean follow-up time of 1.4 years postpartum. The follow up time for the only GADA IgG_4 positive patient who later developed type 1 diabetes was however 5 years, suggesting a slower β -cell destruction. Although previously not studied in GDM, GADA titres have been reported to be of prognostic value for β -cell function in patients with latent autoimmune diabetes in adults (LADA) (Buzzetti et al., 2007, Liu et al., 2015, Lohmann et al., 2001). A previous study on HLA distribution in women with GDM concluded that HLA alleles associated with type 1 diabetes could add to the prediction of postpartum type 1 diabetes development (Ferber et al., 1999). In our study, no difference in HLA-DQ2/8 distributions with regard to postpartum type 1 diabetes development could be observed, although GADA IgG₄ positive women had a lower frequency of HLA-DQ2/8.

In **paper II** we studied circulating IL-1Ra in women with GDM compared to healthy pregnant controls and aimed to elucidate the predictive value of IL-1Ra in postpartum diabetes development. This was to our knowledge the first study where IL-1Ra was investigated in GDM. IL-1Ra has however previously been studied in metabolic syndrome and type 2 diabetes. A Finnish study found IL-1Ra to be an independent predictor of diabetes development with increased levels in both metabolic syndrome and participants who developed diabetes (Luotola et al., 2011). These findings are supported by a 2013 review concluded that there is a clear and well-studied association between circulating IL-1Ra and incident type 2 diabetes (Herder et al., 2013). One study found IL-1Ra to be increased in offspring to type 2 diabetes patients in the pre-diabetic state when compared to normoglycaemic offspring (Ruotsalainen et al., 2006). Another study however found significantly lower levels of IL-1Ra in patients with type 2 diabetes compared to individuals without diabetes (Marculescu et al., 2002).

We could in this study observe significantly decreased levels of IL-1Ra in women with GDM when compared to healthy controls. Interestingly, women with GDM and later postpartum IGT or type 2 diabetes had significantly lower levels of IL-1Ra than patients who did not develop postpartum glucose intolerance. We hypothesised that we would find the opposite trend as previous studies have shown increased IL-1Ra levels to be associated to type 2 diabetes. Few studies have been made in pregnant patients with diabetes, but IL-1Ra has been shown to be increased in pregnancies complicated by pre-eclampsia while being lower in the pregnant state compared to the non-pregnant state (Molvarec et al., 2011). This was the first study published in women with GDM and more studies are necessary to further elucidate the role of IL-1Ra in pregnancies complicated by GDM. Performing a ROC curve analysis on IL-1Ra for the diagnosis of GDM or for the development of postpartum IGT or type 2 diabetes did not result in sufficient precision. We however suggest that IL-1Ra could be included in a panel of biomarkers which together would be able to reach satisfactory precision.

In **paper III** we studied the coding R325W polymorphism and ZnT8A in GDM and postpartum diabetes development. R325W C-allele has been identified as a risk allele in the development of type 2 diabetes by several studies (Chauhan et al., 2010, Gamboa-Melendez et al., 2012, Scott et al., 2007, Tabara et al., 2009), although

studies investigating the association between R325W and type 1 diabetes development concluded that no such association could be observed (Brorsson et al., 2008, Raj et al., 2009). A previous study reported ZnT8A to be a good predictor of type 1 diabetes development in patients with a family history of type 1 diabetes (Gorus et al., 2013). Our study is the first to investigate the predictive value of ZnT8A in the postpartum development of type 1 diabetes following GDM.

Here we found the R325W C-allele to be significantly associated with an increased risk of GDM development, while the R325W T-allele was associated with a decreased risk. We could however not observe a significant association between R325W and the development of postpartum type 2 diabetes in patients with GDM. This lack of association may be explained by the limited number of patients with GDM who developed postpartum type 2 diabetes in this study. Additional studies with longer follow-up time are required to further investigate this association. In this study we identified a number of women with GDM progressing to type 1 diabetes postpartum. While ZnT8A or IA-2A were present in almost half of these patients, all were positive for GADA. Thus we could conclude that positivity for GADA is a better predictor for postpartum type 1 diabetes development in women with GDM, which is in accordance with a previous study performed by our research group on patients diagnosed 1995-2005 (Nilsson et al., 2007).

In **paper IV** we investigated circulating relaxin-2 in women with GDM diagnosed in early pregnancy compared to healthy pregnant women in the same gestational age. Relaxin-2 is an important pregnancy hormone in shaping the endometrium in early pregnancy (Goldsmith et al., 2004). In a previous study, relaxin-2 was found to correlate to insulin sensitivity in women with type 2 diabetes, although in the same study an inverse correlation to β -cell function was observed (Szepietowska et al., 2008). Previous to our study, no reports have been published on relaxin-2 in GDM.

In this study we found higher plasma levels of relaxin-2 in women with GDM diagnosed in early pregnancy compared to controls. Relaxin-2 was also found to correlate to C-peptide levels. Previous studies have observed relaxin-2 levels to be decreased in patients with type 2 diabetes (Szepietowska et al., 2008, Zhang et al., 2013), which is in contrast to our findings. Relaxin-2 is however an important pregnancy hormone and pregnancy itself might explain this discrepancy. In a 2014 poster presentation for the 16th European Congress of Endocrinology, relaxin-2 in late pregnancy GDM was shown to be elevated which is in accordance with our findings in early pregnancy (Zaman et al., 2014).

In **paper V** the role of sCD163 and sTWEAK as biomarkers of GDM development in early and late pregnancy, as well as postpartum glucose intolerance was investigated. An association between insulin resistance and the pro-inflammatory soluble scavenger receptor sCD163 has previously been described (Zanni et al., 2012) and sCD163 has been shown to be independently predictive of type 2 diabetes development in a Danish study (Møller et al., 2011). sTWEAK, an antiinflammatory proposed ligand to CD163, has been shown to be decreased in both type 1 and type 2 diabetes (Kralisch et al., 2008, Llaurado et al., 2012). One study has found elevated levels of sCD163 and decreased levels of sTWEAK in patients with GDM (Simon-Muela et al., 2015). Our study is however the first to investigate sCD163 and sTWEAK in both early and late pregnancy in women with GDM.

We found that plasma levels of sCD163 were higher in patients with GDM with the highest levels being observed in late pregnancy independent of BMI and C-peptide. sTWEAK on the other hand was found to be decreased in patients with GDM with slightly lower levels in early pregnancy. GDM is characterised by chronic inflammation which is supported by our findings of increased sCD163 and decreased sTWEAK levels when compared to pregnant women without diabetes. These findings are also in accordance with a previous study in women with GDM (Simon-Muela et al., 2015). Further, we performed ROC curve analyses on sCD163 and sTWEAK as independent indicators of GDM development in early and late pregnancy (figure 9). Circulating sCD163 exhibited highest precision as a late pregnancy GDM biomarker, while sTWEAK had highest precision in early pregnancy. The precision yielded was not high enough for these biomarkers to be used for GDM prediction A larger number of women recruited in early pregnancy could however further determine the predictive value of sTWEAK in the development of early pregnancy GDM. No difference in neither sCD163 nor sTWEAK could be observed for women with GDM who did or did not develop later glucose intolerance in this study.

The general aims of this thesis was to identify biomarkers capable of improving the prediction of postpartum diabetes development following pregnancies complicated by GDM as well as identifying early pregnancy biomarkers of GDM development. In paper I and III we can conclude that GADA positivity during pregnancies complicated by GDM is a reliable biomarker of postpartum type 1 diabetes development while IA-2A and ZnT8A only have limited predictive value. We can further conclude that GADA positive women with increased GADA IgG₄ have a reduced risk of type 1 diabetes development when compared to IgG₁. In **paper II**, **III and V** we investigated genetic and soluble biomarkers for the development of postpartum type 2 diabetes. We can observe significant differences in the novel biomarkers between patients with or without postpartum diabetes development as presented in these studies. When estimating the predictive value however, the biomarkers studied did not reach satisfactory precision. Type 2 diabetes is a complex disease and in order to identify women with GDM at risk of postpartum development, a combination of clinical risk factors and biochemical biomarkers is most likely required. In paper II, IV and V we aimed to identify soluble biomarkers of GDM development. In paper IV we only studied patients diagnosed with GDM

in early pregnancy and in **paper V** patients diagnosed in early and late pregnancy were subdivided. We found significant differences in the biomarkers studied between women with GDM and pregnant controls in these papers. However, the level of association was not high enough to reach satisfactory precision for these biomarkers to be used independently. There is a need for biomarkers to improve prediction of postpartum type 2 diabetes and to develop better non-fasting diagnostic tools for GDM in early pregnancy.

Future research

Future studies should continue focusing on identifying novel biomarkers reaching high precision for early pregnancy GDM. Preferably a combination of clinical and non-fasting biochemical biomarkers could be used to assemble a cost-efficient panel which could recognise women at increased risk of hyperglycaemia in pregnancy. Thus encouraging early pregnancy OGTT screening for hyperglycaemia in women at increased risk while decreasing the unnecessary cost and burden of screening women at low risk of developing GDM.

A biomarker panel with high precision identifying women with GDM at increased risk of developing postpartum type 2 diabetes should also be the subject for future research. Identifying these patients already during pregnancy could act as an important incentive for necessary lifestyle changes and, if needed, pharmacological interventions. In our studies, the follow-up time has been somewhat limited and since type 2 diabetes is a disease with slow progression until discovery it would be highly interesting to follow a greater number of women with GDM over an extended period of time, identifying an increasing number of women developing glucose intolerance later in life. Maybe the biomarkers we have studied thus far still could be indicative of diabetes development if the timeframe for follow-up would be extended?

We would also like to study adverse outcomes associated with the children to our patients with GDM. It would be of interest to observe if there are associations between maternal inflammation during GDM in both early and late pregnancy, and obesity, metabolic syndrome and type 2 diabetes development. A subsequent study of interest would be to investigate if early pregnancy interventions in women with GDM affect the risk of future metabolic disorders in the offspring.

Acknowledgements

I would like to thank everyone who have contributed to this thesis and supported me during my PhD studies.

First of all, I would like to thank my main supervisor **Mona Landin-Olsson** for all your help, guidance and support since I joined your research group. You have always provided valuable input whenever I needed it. I would also like to thank you for helping me better understand science from a clinical perspective.

There is one person in particular without whom this thesis would not have existed. **Magnus Hillman** (insert fist bump here). You have been my co-supervisor during my PhD studies, but you are first and foremost one of my best friends. You are truly an inspiration and a great scientist. You have really helped me develop my scientific, statistical and critical ways of thinking, enabling me to become a better scientist. Crammed together into our tiny office we have not only managed to performed great science, but we have also had endless discussion about whisky and wine whenever possible. We make a great team and hopefully we can continue doing what we enjoy most in the future. Slàinte mhath!

Birgitte Ekholm! You bring tons of energy and happiness to the lab. Your technical expertise and assistance when performing laboratory analyses is essential in all of our studies. It has been a pleasure working with you.

A big thank you to **Charlotta Nilsson**. Your ambition and enthusiasm is contagious! We have so many ideas of what we want to study if we only had the time and possibility. If I ever need help with anything related to science or the clinic, I can always rely on you to help me out. Through our combined supervising we have helped many students perform brilliant thesis work and it has been great fun.

Thank you **Helena Strevens** for helpful scientific input, interesting discussions and your assistance in the recruitment of patients with gestational diabetes.

I would also like to thank **Yoatzin Alonso Lopez**, **Pernilla Katra**, **Jacob Nilsson** and **Sanna Palmqvist** whose project work in our laboratory has led to the papers included in this thesis.

Everyone at B11 who helps create an intellectually stimulating work environment while also inviting to have tons of fun. Everything from Friday breakfasts to board game nights and summer/winter parties really makes B11 a great place to be

working in. I would in particular like to thank **Emelie Andersson** for your support, help and discussions regarding everything between heaven and earth (and just to make it clear, I won our dart competition O). I would also like to give a special thanks to **Nadja**, **Alex**, **Ulrikke**, **Isak**, **Katarina**, **Oscar**, **Marina**, **Chris**, **Tomas**, **Antonio** and **Martina**. B11 would not be as much fun without you.

Thank you to our previous next-door colleagues from the appetite regulation unit: **Caroline**, **Eva-Lena** and **Emil** for making everyday life in the laboratory great fun and sharing exiting ideas about diet and obesity. I hope we get to share more exiting ideas in the future!

Since I started in our research group, I have had the great opportunity to supervise a lot of excellent students. I thank you all for also teaching me a lot and helping me develop as a supervisor. It has been great to see you grow as scientists and critical thinkers during your time in our laboratory. Apart from the students already mentioned above I would like to thank **Emma**, **My**, **Louise**, **Evelina**, **Oscar**, **Gabriella**, **Tobias**, **Stina**, **Amra** and **all upper secondary school students** who have visited our lab over the years.

I would also like to take this opportunity to thank the mentorship programme MentLife and in particular my mentor **Olof Böök** who has shown me a company's approach to life science and given me valuable feedback on my own ideas and thoughts. We have had a lot of good talks and I have learned a lot from you Olof.

My studies included in this thesis have all included clinical patients and pregnant volunteers. I would like to thank all **clinicians** and **nurses** involved in our studies for your contributions in the recruitment and sample collection. I also want to thank all **participants** in our studies for making them possible through their participation.

I would like to give a huge thanks to my **family**. Special thanks to my wonderful parents, **Marita** and **Johnny** for always being there for me, believing in me, and not ever growing tired of me rambling on about pregnant women and diabetes. Big thanks to **Joakim**, **Josefine** and **Julia** for both your support, but also all the fun stuff we do whenever I am home. I also want to give **Neo** and **Fred**, the cute and crazy kids, a big hug for being so much fun to play with whenever I get the opportunity O. A big hug and a kiss to you **Mary Farrell**. Thank you for your help, support and understanding during my time writing this thesis.

A special thanks to my previous biomedicine class mates and dear friends **Erik Tenland** and **Henrik Löfvall**. Our so-called PhD after work group where we have discussed life as PhD students from the very start of our PhD studies has been invaluable. I hope we can continue with our after works also after we are done studying.

I also want to give a big hug to my study buddy **Frida Peterson** for your help during our biomedicine studies and continued support (and nice lunches together). It was

thanks to you I contacted Magnus from the start and see how great that turned out O.

I also would like to extend a big thanks to two of my best friends: **Michael Arvidsson**, you have always been there whenever I have needed your support and no matter what we are up to, it is always crazy fun! And my uncle **Robert Molin**. Everything from building apartments to long nights spent in your cinema room have been great fun.

Lastly I want to thank the founding sources making our studies possible. Thanks to Sydvästra Skånes Diabetesförening, Skåne University Hospital Funding and Donations, the Swedish Medical Research Council, the Swedish Diabetes Foundation and the Gorthon Foundation.
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Paper I

Clinical Immunology 162 (2016) 45-48



Contents lists available at ScienceDirect

Clinical Immunology

journal homepage: www.elsevier.com/locate/yclim

IgG4 subclass glutamic acid decarboxylase antibodies (GADA) are associated with a reduced risk of developing type 1 diabetes as well as increased C-peptide levels in GADA positive gestational diabetes





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ARTICLE INFO

Article history: Received 1 September 2015 Received in revised form 3 November 2015 accepted with revision 4 November 2015 Available online 5 November 2015

Keywords: Gestational diabetes mellitus Glutamic acid decarboxylase antibodies Type 1 diabetes IgG subclasses

1. Introduction

Gestational diabetes mellitus (GDM) is a heterogeneous disorder affecting approximately 2.5% of pregnant women in southern Sweden. A few percent of these women in turn present with antibodies targeting islet specific antigens, glutamic acid decarboxylase antibodies (GADA) in particular [1–3]. The presence of GADA at onset of GDM [4] or at delivery [1] has been suggested to predict future development of type 1 diabetes.

Type 1 diabetes is considered to be a cellular Th1/Th17 mediated disease. The exact mechanisms are debated but the topic concerning T cell subsets, tolerance interference and post-translational modifications of antigens is comprehensively reviewed by Haskins and Cook [5]. The adaptive immune system during pregnancy, however, is polarized towards a humoral Th2 response [6]. In settings dominated by Th1/Th17 cells, the local cytokine profile will induce an isotype switch towards the human IgG₁ subclass [7.8], while Th2 cells will induce an isotype switch towards the human IgG₄ subclass [9]. Whether this actually may prevent type 1 diabetes or not will need to be further investigated in future studies, but promising results have been shown in NOD mice [10,11].

ABSTRACT

Some women with gestational diabetes (GDM) present with autoantibodies associated with type 1 diabetes. These are usually directed against glutamic acid decarboxylase (GADA) and suggested to predict development of type 1 diabetes. The primary aim of this study was to investigate if GADA lgG subclasses at onset of GDM could assist in predicting postpartum development. Of 1225 women diagnosed with first-time GDM only 51 were GADA-positive. Total GADA was determined using ELISA. GADA subclasses were determined with radioimmunoassay. Approximately 25% of GADA-positive women developed type 1 diabetes postpartum. Titers of total GADA women that developed type 1 diabetes (142.1 vs 74.2 u/ml; p = 0.04) and they also had lower titers of GADA lgG4 (index = 0.01 vs 0.04; p = 0.03). In conclusion we found that that women with high titers of total GADA but low titers of GADA lgG4, were more prone to develop type 1 diabete spostpartum. \mathbb{C} 2015 Elsevier Inc. All rights reserved.

We have previously shown that patients with latent autoimmune diabetes in adults (LADA) have a higher frequency of GADA IgG4 antibodies compared to patients with adult onset type 1 diabetes [12]. We have suggested that the Th1 and Th2 balance within pancreatic beta cells may be different between patients with type 1 diabetes and LADA since the IgG₄ isotype switch is mediated mainly by Th2 cytokines. Thus it seems reasonable to hypothesize that also GADA positive women with GDM could have higher titers of GADA IgG4 antibodies. Only one study has previously reported GADA reactivity, including GADA IgG1 and IgG4 as well as epitope binding during GDM [13]. The GADA IgG4 positivity was reported to be 44%, while GADA IgG1 positivity was as high as 76% in the 34 women with GDM. The authors could not find a difference in circulating GADA IgG subclasses between patients with type 1 diabetes and GDM, although a reduced and more restricted antibody response was suggested due to lesser epitope spreading in patients with GDM.

The association between the HLA-region at chromosome 6 and autoimmune diabetes has been known for more than three decades [14] and while certain variants of the DQ beta chain encodes susceptibility motifs (DQB1*0201 and DQB1*0302 and also known as DQ2 and DQ8 respectively), others are protective (DQB1*0602) [15]. Both DQB1*0201 and DQB1*0302 are reported in one study to be slightly more common in Scandinavian women with GDM compared to in pregnant controls [16]. The presence of GADA was especially associated with DQB1 risk genotypes in that study. Another study suggested the protective genotype to be less frequent in GDM patients but without being able to

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confirm an increased frequency of high risk genotypes [17]. A third study investigated HLA risk alleles in autoantibody positive women with GDM and concluded that there was an association with autoantibodies and the development of type 1 diabetes postpartum [18].

Our aim with this study was to investigate if GADA $\lg G_1$ and $\lg G_4$ subclass distribution in women with GDM is associated with the endogenous insulin secretion and development of type 1 diabetes postpartum. We were also interested in whether DQB1 risk types (DQ2 and DQ8) could be associated with the GADA subclass distribution or could predict the development of manifest type 1 diabetes postpartum in our material.

2. Material and methods

2.1. Participants

Women diagnosed with new-onset GDM at Skåne University Hospital in Lund, Sweden, 1996–2013 (n = 1225) were asked to participate in these studies. All women consented and were screened for autoantibodies. Criteria for GDM diagnosis were blood glucose values of ≥ 10 mmol/L after a 2 hour 75 g oral glucose tolerance test (OGTT). Patients were followed up postpartum to make sure that the pathological blood glucose value was normalized or a classification of manifest diabetes was used instead of GDM. All GDM women are offered followup at 3-month to 1-year intervals postpartum.

The Study was conducted in accordance with the Helsinki declaration and approved by the Regional Ethical Review Board at Lund University (LU526/00; 849/2005; 244/2007, 2009/307 and 2014/78).

2.2. Blood tests

Blood was drawn in serum and EDTA plasma vials and sent to the laboratory by hospital post. Plasma and serum was separated by centrifugation at $2000 \times g$ and stored at -70 °C until use.

2.3. Detection of GADA positivity

GADA was identified using commercially available enzyme linked immunosorbent assay's (ELISA) from RSR Ltd. with a reported specificity and sensitivity of 100% and 90% respectively [19]. Absorbance was measured in a FLOUstar Optima (BMG Labtech). The cut-off level for positivity was set at 10 u/mL. A total of 53 women were positive for GADA (4.3%) and were included in the study. Serum or EDTA plasma was available from 51 of the GADA positive patients.

2.4. GADA IgG1 and IgG4 subclasses

A liquid phase binding radioimmunoprecipitation assay (LPBA) was used to detect GADA IgG1 and IgG4 subclasses in GADA positive women as described in detail elsewhere [20]. Briefly, serum or plasma was incubated in duplicates with recombinant 35 S (Perkin-Elmer)-labeled GAD65, followed by incubation with anti-human IgG1 antibodies (15 µg/mL, BD Pharmingen) or anti-human IgG₄ antibodies (25 µg/mL, BD Pharmingen) overnight at 4 °C, forming immune complexes between GADA and GAD65, as well as between human IgG-subclasses and mouse anti-human subclass antibodies. The immune complexes were precipitated onto streptavidin sepharose (40% dilution, GE healthcare) in 96-well filter plates (Millipore, France) pre-coated with 1% BSA to avoid unspecific binding and punched out into vials with 4 mL scintillation fluid (Ultima Gold, Perkin-Elmer). Counts per minute (cpm) were measured in a beta-counter (Packard Tri-carb 2100 TR) for 2 min per sample. Quadruplicates of in-house standards were used and indexes were calculated to estimate IgG-subclass titers. An index of 0.04 was used as cut-off level for GADA IgG1 positivity and 0.03 for GADA IgG₄ positivity [20].

2.5. C-peptide

C-peptide levels were measured with commercially available readyto-use ELISA kits (Mercodia) according to manufacturers' instructions. Internal controls were also purchased from the same manufacturer. The reported detection limit was 15 pmol/L and absorbance was measured at 450 nm in a FLOIStar Optima (BMG Labtech).

2.6. HLA-genotyping

DNA was extracted from leukocytes with a salt-out precipitation method [21]. Concentration and purity was determined by measuring the ratio of absorbance at 260/280 nm. HLA-DQB1 risk alleles (DQ2/ DQ8) were determined using a commercially available MutaGEL® HLA-DQ2 + 8 kit (immundiagnostik AG) with specific primers for HLA DQ2 (allele combination DQx1*05/DQB1*0201) and HLA-DQ8 (allele combination DQx1*03/DQB1*0302). All genotyping was performed according to the manufacturers' instructions.

2.7. Statistical analyses

Distribution of data was estimated using D'Agostino–Pearson test [22]. Results are reported as mean \pm standard deviation for data that followed a normal distribution and median followed by interquartile range (IQR) in brackets when normal distribution was rejected. The two-tailed Mann–Whitney U-test was used to analyze differences in IgG-subclass titers between the two groups and the Wilcoxon signed rank test to compare IgG-subclass titers between the first and second pregnancy with GDM. The Spearman rank correlation was used to detect associations between antibodies and/or C-peptide levels. Fischer's exact test was used to test for differences in frequency of GADA IgGsubclass positivity in the groups. P < OJS was considered to be statistically significant. The statistical software MedCalc for Windows® v.14.12.0 was used to analyze the data.

3. Results

Clinical and laboratory data are presented in Table 1. A total of 53 of the 1225 GDM women were positive for GADA (4.3%) and serum or EDTA plasma was available from 51 of the GADA positive GDM women for IgG subclass analyses. The two women without available serum or EDTA plasma samples were excluded from the study. Neither one of these two women were reported to have developed manifest diabetes during follow-up. All GADA positive GDM women participated in follow-up at regular 3-month to 1-year intervals. Twelve of the 51 GDM women with GADA positive to GDM (1.4 \pm 1.1 years). Some of the GADA positive women (n = 15) returned, during their second pregnancy with GDM, 1–9 years after postpartum (4.5 \pm 2.8 years). Two of them also had a third pregnancy with GDM within two years after the second.

Total GADA titers were significantly higher in patients that developed type 1 diabetes; 142.1 (61.6-821.5) u/mL, compared to in patients that did not; 74.2 (30.1-227.8) u/mL (p = 0.04).

The GADA lgG₄ titers, to the contrary, were significantly lower in women that developed type 1 diabetes (p = 0.03) and the frequency of GADA lgG₄ positivity was significantly decreased in this group (Fig. 1, p = 0.04). No significant differences of the GADA lgG₁ levels were found between the groups.

All patients positive for GADA IgG_4 were also positive for GADA IgG_1 . However, positivity for GADA IgG_1 was twice as frequent as GADA IgG_4 in our material with frequencies of 82% (42/51) and 41% (21/51) respectively. Total GADA correlated significantly with GADA IgG_1 ($r_s=0.65;$ p<0.0001) but not with GADA IgG_4 ($r_s=0.11;$ p=0.47).

C-peptide levels were not significantly different between women who developed type 1 diabetes; 1.09 (0.42–1.40) nmol/L and women

Table 1

Serum or plasma was available for 51 GADA positive women at clinical onset of GDM. Thirteen women developed type 1 diabetes within 5 years postpartum. Age is reported as mean ± standar deviation. Antibody titres and C-peptide levels are reported as median followed by interquartile range. The presence of HLA-DQ2 (DQB1*0201) and/or HLA-DQ8 (DQB1*03201) is given in total.

	No postpartum type 1 diabetes (n = 38)	Type 1 diabetes postpartum (n = 13)	P-value
Age (years) Total GADA (u/mL) GADA IgG ₁ (index) GADA IgG ₄ (index) C-peptide (nmol/L) HLA DQ2/8 (yes/no)	31.8 ± 5.3 74.2 (30.1-227.8) 0.16 (0.04-0.41) 0.04 (0.02-0.06) 1.35 (0.64-1.90) 10/28 (26.3%)	31.4 ± 5.0 142.1 (61.6-821.5) 0.41 (0.12-0.54) 0.01 (0.00-0.03) 1.09 (0.42-1.40) 5/8 (38.5%)	0.80 0.04 0.19 0.03 0.23 0.48

who did not; 1.35 (0.64–1.90) nmol/L. However, a positive correlation was found between C-peptide levels and GADA IgC₄ (Fig. 2; $r_s = 0.32$; p = 0.04). There was no such correlation between C-peptide and GADA IgC₄ ($r_s = -0.03$; p = 0.86).

Women with GDM during their second pregnancy (n = 15), and in a few cases also their third pregnancy (n = 2), retained their GADA levels as well as their IgG subclass profile. Only one of the women, with very low levels of total GADA, IgG₁ and IgG₄ at clinical onset, in her first pregnancy, turned out to be GADA negative during her second pregnancy with GDM.

In our material 15 women were positive for the investigated HLA risk alleles DQ2 (n = 7), DQ8 (n = 6) or both (n = 2). No statistically significant increase of the analyzed HLA risk alleles was found in women who developed type 1 diabetes. However, GADA IgG₄ positive women had a significantly lower frequency of DQ2 or 8 (2/21; 11%, both being DQ8) compared to GADA IgG₄ negative patients (13/30; 43%; p = 0.012). Also, women with the DQ2 genotype (n = 9) had higher levels of GADA; 240.6 (132.1–816.7) u/mL compared to in women without DQ2 (n = 42); 74.3 (48.7–140.5) u/mL, although this was not statistically significant (p = 0.07).

4. Discussion

Our main finding was that not only GADA positivity in itself, but also GADA subclass distribution could influence the risk of developing type 1 diabetes postpartum. GADA positive women with GDM and higher titers of GADA IgG₄ were less prone to develop type 1 diabetes, regardless of GADA titers. We also found a positive correlation between GADA IgG₄ and endogenous insulin secretion, as reflected by the C-peptide levels, which seemed to be higher in IgG₄ positive women.

One strength of this study is that it was conducted in a region of Sweden, where a screening program for GDM includes all pregnant women. An OGTT is offered to all pregnant women at 28 weeks of



Fig. 1. GADA IgG_4 titers in women that developed type 1 diabetes (n = 13) compared to in women without manifest type 1 diabetes (n = 38; p = 0.03).

gestation and also as early as the 12th week of gestation in the case of known risk factors. These include a previous GDM, 1st degree relative with diabetes or BMI > 30.

Virtually all women in the region accept the offer of OGTT. Therefore practically all pregnancies with GDM are detected. A limitation of the study is the relatively small number of participants due to the low amount of GADA positive women with GDM. The association between C-peptide and GADA IgG₄ antibodies must be interpreted with caution due to the small number of patients.

In our material GADA $\lg G_1$ positivity was twice as frequent as GADA $\lg G_4$ positivity with frequencies of 82% and 41% respectively. These figures are well in accordance with another GDM study reporting frequencies of 76% and 44% respectively [13]. However, the authors of that study also reported presence of GADA $\lg G_4$ in patients with type 1 diabetes, which we have not yet ourselves observed in two previous GADA studies in adult patients [12,23]. This discrepancy could be due to differences in methodology and determination of the cut-off levels for positivity in each $\lg G_{-}$ subclass.

There was no difference in GADA IgG₁ levels between patients that developed type 1 diabetes postpartum and those who did not. This suggests that the cytotoxic T cell response in Th1/Th17 cells are similar in both groups of women, but might be better regulated with Th2 cells present in the patients without development of type 1 diabetes. Most of the women who developed type 1 diabetes presented within the first two years postpartum. One GADA IgG₄ positive woman with GDM did actually develop type 1 diabetes (Fig. 2). Although, not until five years postpartum, which suggests that the presence of Th2 cell cytokines - as reflected by the IgG₄ subclass—in pancreatic lymphoid tissues might slow down the beta cell destruction.

Autoantibody positivity, especially GADA positivity, has been implied as a risk factor for the development of type 1 diabetes postpartum in several studies [1–4]. GADA titers in women with GDM have not yet assembled such interest. However, in LADA patients GADA titers have been reported to be of prognostic value for beta cell function [24–26], which makes it reasonable to assume that this would be the case also in GADA positive women with GDM. In most cases there was a good correlation between total GADA and GADA Igc1 levels, suggesting that IgG_1 is the most abundant heavy chain in the circulation in GADA positive women. In some women with high total GADA both GADA IgG1 and IgG4 were lower than expected. This could be due to higher GADA IgM levels in these subjects or that the epitopes recognized by the assays are different.

When comparing titers of total GADA between women with and without DQ2 or DQ8 no differences were found. However, we did find a trend towards higher levels of GADA in women with the DQ2 allele alone, which also is in accordance with the NIRAD study group's report on LADA patients. DQ2 was reported to be more prevalent in the hightiter GADA group while DQ8 was equally distributed between high-



Fig. 2. A significant correlation was found between endogenous insulin secretion and GADA IgG₄ subclass of antibodies ($r_s = 0.32$, p = 0.04) which suggests a protective role of GADA IgG₄.

and low-titer GADA patients with LADA [25]. In our material only one woman with DQ2 and only two women with DQ8 were positive for GADA IgG_4 , Similarly, only 11% (2/21) of GADA IgG_4 positive women had risk alleles compared to 43% (13/30) of the GADA IgG_4 negative women.

Previous studies on HLA risk alleles in GDM have reported the presence of GADA to be associated with DQ risk genotypes in Scandinavian women [16]. A further study found no statistically significant association between DQ risk types and GDM but instead that the protective allele DQB1*0602 (DQ6) was less frequent in Swedish women with GDM [17]. An explanation to these different findings could be the varying inclusion criteria in the studies. In our study only GADA positive GDM women were included, which would explain why DQ risk types were more common in the material.

GADA positive women with more than one GDM pregnancy retained their GADA positive and IgG subclass profile during their second and third pregnancy, suggesting a remaining risk for development of type 1 diabetes postpartum, often within two years after clinical onset of their first GDM.

Future studies should be conducted in larger populations of GADA positive women with GDM to further investigate the association between C-peptide and IgG subclasses. More investigation is required before this information may be used for prognostic evaluation of residual beta-cell function.

In conclusion we found that women with GDM that had high titers of GADA were more prone to develop type 1 diabetes postpartum. However, presence of GADA IgG₄ was positively correlated with C-peptide levels in our study, associated with a lower frequency of DQ β 1 risk alleles and reduced risk of type 1 diabetes.

Conflict of interest

The authors declare no conflict of interest.

Author contributions

JD: Laboratory analyses, compilation of data, statistical analyses and writing parts of the manuscript.

CN: Review of medical records for follow-up of manifest diabetes mellitus postpartum, ethical application, critically review of the manuscript.

HS: Critical review of the manuscript, proofreading, language and collection of blood samples.

MLO: Parts of the study design, ethical application and collection of blood samples.

MH: Study design, ethical application, method development, compilation and interpretation of data, statistical analyses and writing parts of the manuscript.

Acknowledgments

Funding from Sydvästra Skånes Diabetesförening (registration number 1040390) for Dr. Magnus Hillman and from Skåne University Hospital Funding and Donations (registration number 1400741) for Dr. Mona Landin-Olsson were used to support the study. Mrs Birgitte Ekholm is thanked for her excellent technical assistance in the research laboratory.

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Paper II





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Citation: Katra P, Dereke J, Nilsson C, Hillman M (2016) Plasma Levels of the Interleukin-1-Receptor Antagonist Are Lower in Women with Gestational Diabetes Mellitus and Are Particularly Associated with Postpartum Development of Type 2 Diabetes. PLoS ONE 11(5): e0155701. doi:10.1371/journal. pone.0155701

Editor: Victor Sanchez-Margalet, Virgen Macarena University Hospital, School of Medicine, University of Seville, SPAIN

Received: August 28, 2015

Accepted: March 28, 2016

Published: May 25, 2016

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Data Availability Statement: Due to ethical restrictions, the authors must ensure that the integrity of the participants in this study is preserved. Since it is clearly stated in the paper from which health care centers and hospitals the authors' have recruited the controls and patients, the authors cannot provide any clinical data (age, BMI, family history of diabetes) that enables possible identification of the participants. The authors can, however, provide a minimal data set of IL-1Ra and C-peptide levels in each group, which RESEARCH ARTICLE

Plasma Levels of the Interleukin-1-Receptor Antagonist Are Lower in Women with Gestational Diabetes Mellitus and Are Particularly Associated with Postpartum Development of Type 2 Diabetes

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Abstract

Diabetes mellitus is a group of diseases characterized by chronic hyperglycemia. Women who develops hyperglycemia for the first time during pregnancy receive the diagnosis gestational diabetes mellitus (GDM). Presently, there is no consensus about the diagnostic criteria for GDM. A majority of these women subsequently develop postpartum overt diabetes making it important to identify these patients as early as possible. In this study we investigated if plasma levels of the interleukin-1 receptor antagonist (IL-1Ra), an endogenous inhibitor of IL-1 signaling, can be used as a complementary biomarker for diagnosing GDM and predicting postpartum development of overt diabetes mellitus. Patients participating in this study (n = 227) were diagnosed with their first GDM 2004-2013 at Lund University Hospital, Lund, Sweden. Healthy pregnant volunteers (n = 156) were recruited from women's welfare centers in the same region 2014-2015. Levels of IL-1Ra and C-peptide were analyzed in ethylenediaminetetraacetic acid (EDTA)-plasma or serum using enzyme linked immunosorbent assay (ELISA). GDM patients had significantly lower levels of IL-1Ra than the control group (p = 0.012). In addition, GDM patients that had developed impaired glucose tolerance (IGT) or type 2 diabetes mellitus postpartum had significantly lower levels of IL-1 Ra, and significantly higher levels of C-peptide than GDM patients that had not developed diabetes mellitus postpartum (p = 0.023) and (p = 0.0011) respectively. An inverse correlation was found between IL-1Ra and serum C-peptide levels in the control group (rs = -0.31 p = 0.0001). Our results show that IL-1Ra might be included in a future panel of biomarkers, both for diagnosing GDM to complement blood glucose, and also identifying GDM patients that are at risk of developing type 2 diabetes mellitus postpartum. However, the ROC curve analysis provided a sensitivity of 52.2% and specificity of 67.1%, which nonetheless may not be sufficient enough to use IL-1Ra as a sole biomarker.



could be of use to research laboratories trying to confirm the results from this study. The authors also believe that IL-IRa and C-peptide are the only data required to replicate the results found in this study. The corresponding author of this paper is available for inquiries regarding all the data if required. The minimal data set supporting this study is provided as a Supporting Information file.

Funding: MH received funding for this study from Sydvästra Skånes Diabetesförening (grant number 1040390) (web page: http://www.diabetes.se/ Foreningar/Skane-lan/Lokalforeningar/Sydvastra-Skanes-Diabetesforening/). The funder had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

Introduction

Diabetes mellitus is a group of diseases characterized by hyperglycemia due to lack of insulin or disturbances in insulin signaling. The most common forms of diabetes are type 1 and type 2 diabetes mellitus. Type 1 diabetes mellitus is an autoimmune disease that results in an insulin deficiency, whereas type 2 diabetes mellitus is characterized by peripheral insulin resistance frequently in combination with a dysfunctional insulin production. [1]

During pregnancy, the metabolic state undergoes a substantial change, which also affects insulin action and sensitivity. During the second half of pregnancy this affect is increased with resulting insulin resistance and subsequent hyperglycemia. In most cases the body is able to compensate for this with increased insulin secretion and most cases resolves with delivery. [2, 3]

Gestational diabetes mellitus (GDM) is defined by the American diabetes association as glucose intolerance first diagnosed during pregnancy. GDM affects approximately 1-14% of all pregnancies depending on the ethnicity of the patient group studied and diagnostic criteria used [1]. In southern Sweden the prevalence of GDM is 2.2% [4]. Patients with GDM are hyperglycemic and suffer from increased insulin resistance, similar to patients with type 2 diabetes mellitus [5]. Many patients with GDM develop impaired glucose tolerance (IGT) or type 2 diabetes mellitus postpartum [6]. The reported incidence of type 2 diabetes postpartum varies between 2.6–70% [7].

Measuring plasma glucose has long been the gold standard for diagnosing GDM, commonly determined with fasting plasma glucose (FPG) and oral glucose tolerance tests (OGTT). However, the diagnostic criteria for GDM varies in different countries, and there is a lack of consensus concerning the plasma glucose threshold level. The Hyperglycemia and Adverse Pregnancy Outcome (HAPO) study was designed to evaluate hyperglycemia during pregnancy in relation to the risk of adverse perinatal outcomes [8]. The HAPO study found a linear increase in risk of adverse outcomes with increasing plasma glucose, without a clear cut-off level [9]. Based on the results of the HAPO study the International Association of Diabetes and Pregnancy Study Group (IADPSG) delivered new diagnostic guidelines for GDM [10]. The new guidelines resulted in an overall prevalence of GDM of 17.8% in the HAPO patient material [11], an increase by almost 50% [12]. However, the new guidelines have been criticized by others [12, 13], and consensus is yet to be established.

Despite that GDM is acknowledged by researchers to be a complex disorder, focus for establishing diagnostic criteria has solely been on hyperglycemia, while other factors that promote the pathogenesis of the disease have received less attention. Soluble biomarkers are successfully used in the diagnosis of many diseases including type 1 diabetes mellitus [14, 15], and could together with blood glucose improve the diagnosis of GDM.

In addition, since GDM during pregnancy greatly increases the risk of postpartum development of overt diabetes mellitus [16] it is important to find biomarkers or clinical parameters that can predict postpartum development of diabetes mellitus already during pregnancy, in order to be able to provide early treatment.

The interleukin-1 (IL-1) receptor antagonist, IL-1Ra, is an endogenous IL-1 inhibitor and binds to the IL-1 receptor type 1 (IL-1RI), but it fails to induce intracellular signaling, and thus serves as a competitive inhibitor of IL-1 [17].

The recombinant IL-1Ra drug Anakinra has been shown to improve plasma glucose levels and β -cell function but not insulin resistance in patients with type 2 diabetes mellitus [18]. Subsequently it seems that IL-1Ra has positive effects on β -cell function and insulin secretion, but its effect in other tissues appears to be less beneficial. Interestingly, a knock-down study of IL-1Ra in obese insulin resistant mice showed reduced insulin resistance in the liver, and also reduced body weight and blood glucose levels [19]. The aims of this study were to investigate if levels of IL-1Ra can be used as a complementary biomarker for diagnosing GDM and predicting the development of overt diabetes mellitus postpartum.

Materials and Methods

Participants

First-time GDM patients (n = 227) diagnosed at Lund University Hospital, Lund, Sweden, between 2004 and 2013 were included in the study. GDM was diagnosed with a 2 hour 75g OGTT following overnight fast. The diagnostic criteria for GDM was a plasma glucose value exceeding 10 mmol/L. Some of the women had developed IGT (n = 28) or type 2 diabetes mellitus (n = 34) within 6 years (in median 3 years) after clinical onset while the majority remained normoglycemic (n = 165). A control group (n = 156) of pregnant volunteers without a family history of diabetes was recruited at women's welfare centers in the same region (Malmö (Lindängen), Dalby and Staffanstorp) in 2014-2015. Body mass index (BMI) was available for the majority of GDM patients (n = 215) and controls (n = 147). Blood samples were drawn into ethylenediaminetetraacetic acid (EDTA)-plasma and serum tubes in the 12th week of gestation from healthy controls (n = 156) and from women with a family history of diabetes mellitus or a BMI>30 (n = 139). Samples from patients without a family history of diabetes mellitus and BMI < 30 were taken in the 28^{th} week of gestation (n = 88). Samples were sent to the laboratory by ordinary mail and stored at -70°C until time of analysis except for C-peptide which was analyzed immediately. This study was approved by the Regional Ethical Review Board in Lund (Regionala etikprövningsnämnden i Lund; 2014/383, 2014/744), and performed in accordance with the Declaration of Helsinki. All participants were given oral and written information about the study before giving written informed consent.

IL-1Ra analysis

IL-1Ra was analyzed in EDTA-plasma using a commercially available enzyme linked immunosorbent assay (ELISA) kit (R&D systems, Minneapolis, MN, USA) according to the manufacturers' instructions, optimized for human plasma. Samples were diluted 1:5, or 1:20 if the concentration at dilution 1:5 was found to exceed the highest standard concentration, and analyzed in duplicates. The absorbance was measured at 450 nm and 405 nm in a FLOUstar Optima ELISA plate reader (BMG Labtech Gmbh, Ortenberg, Germany). The highest concentration in the 7-point standard dilution series was changed from 2500 pg/mL to 5000 pg/mL, since the lowest concentration was undetectable by the plate reader. The inter- and intra-coefficient of variation were 18.9% and 20.0%, respectively. Control and patient samples were alternated on each ELISA plate in order to minimize the effect of inter-variation.

C-peptide analysis

C-peptide was analyzed in serum using a commercially available ELISA kit (Mercodia AB, Uppsala, Sweden) according to the manufacturers' instructions. The detection limit of the assay was 25 pmol/L. The intra-assay and inter-assay coefficient of variation were 2.9–4.8% and 0.6–4.8%, respectively.

Statistical analyses

Normal distribution was estimated using the D'Agostino-Pearson test for normality. Normally distributed data is presented as mean ± standard deviation (SD), and non-normally distributed data as median [interquartile range]. Depending on the distribution, t-test or the Mann-

Whitney U test were performed to test for differences in mean or mean rank respectively between two groups. In order to test for differences in more than two groups, analysis of variance (ANOVA) or the Kruskal-Wallis H test was performed depending on the distribution of the parameters analyzed. The χ^2 -test was used to determine differences in family history of diabetes mellitus between GDM patients and controls. The Spearman rank-correlation test was performed to investigate correlations in continuous variables. The precision of the L-1Ra ELISA as a diagnostic and prognostic tool was evaluated using a receiver operating characteristic (ROC) curve analysis. A p-value <0.05 was considered statistically significant. All statistical analyses were performed using MedCalc (MedCalc Software, Ostend, Belgium) for Windows \Re v12.7.0.0.

Results

Clinical and biochemical data for controls and women with GDM and/or postpartum development of IGT or type 2 diabetes mellitus are presented in <u>Table 1</u>. The p-values given are calculated with ANOVA, the Kruskal-Wallis H test or χ^2 -test depending on the variable analyzed.

Lower levels of IL-1Ra in GDM patients

Women with GDM had significantly lower plasma levels of IL-1Ra (1964 [306–5276] pg/mL) compared to pregnant controls (2902 [1074-6030] pg/mL; p = 0.012). Performing a ROC curve analysis on plasma levels of IL-1Ra as a possible diagnostic tool for GDM generated a criterion of \leq 820 pg/ml. Applying this criterion resulted in a sensitivity of 37.9% and a specificity of 79.5%.

In addition, patients with postpartum development of IGT or type 2 diabetes mellitus had significantly lower levels of IL-1Ra than GDM patients that had not developed any glucose intolerance postpartum (883 [0–4047] pg/mL and 2285 [491–5826] pg/mL respectively; p = 0.023). A ROC curve analysis of IL-1Ra as a possible prognostic tool for postpartum development yielded a criterion of \leq 889pg/ml with a sensitivity and specificity of 52.2% and 67.1%, respectively. There was no statistically significant difference in IL-1Ra or C-peptide levels between samples taken from patients in the 12th or 28th week of gestation (p = 0.21 and p = 0.99).

Increased levels of C-peptide in GDM patients

The controls had significantly lower levels of C-peptide (0.47 [0.31–0.74] nmol/L) than both GDM patients without postpartum glucose intolerance 0.93 [0.53–1.61] nmol/L) and GDM patients with postpartum development 1.30 [0.97–1.93] nmol/L; p<0.000001). There was also

Table 1. Clinical and biochemical data for controls and GDM patients with and without postpartum IGT or type 2 diabetes mellitus.

	Controls (n = 156)	GDM without postpartum diabetes (n = 158)	GDM with postpartum IGT/T2DM (n = 69)	p-value
Age (years)	29.8 ± 5.3	32.1 ± 5.3	32.0 ± 6.0	<0.001
Body mass index (kg/m ²)	25.4 [23.1–28.9]	26.3 [23.5–31.6]	28.3 [23.1–32.9]	0.053
Family history of diabetes (yes/ no)	47/109	60/98	34/35	0.021
C-peptide (nmol/L)	0.47 [0.31–0.74]	0.93 [0.53–1.61]	1.30 [0.97–1.93]	< 0.000001
IL-1Ra (pg/mL)	2902 [1074-6030]	2285 [491–5826]	883 [0–4047]	0.0033

Values are presented as mean ± SD or median [interquartile range]

doi:10.1371/journal.pone.0155701.t001

a statistically significant difference between women with and without postpartum development (p = 0.0011).

Correlation between C-peptide and IL-1Ra

An inverse correlation between C-peptide and IL-1Ra levels was found the control group ($r_s = -0.31$, p = 0.0001), but not in the group of GDM patients ($r_s = -0.05$, p = 0.49). No significant correlation was found when subdividing patients at week 12 (rs = -0.04, p = 0.61) and at week 28 (rs = -0.06, p = 0.59). We could not observe any correlation between levels of IL-1Ra and age or BMI in any of the groups.

The data set containing raw data of IL-1Ra, C-peptide, gestational age and postpartum development is provided as Supporting Information (<u>S1 Data set</u>).

Discussion

In this study we showed that GDM patients have significantly lower levels of IL-1Ra in plasma than healthy pregnant controls. This suggest that since patients with GDM have lower levels of the anti-inflammatory IL-1Ra, the biological effect of IL-1 is enhanced and thus promote the inflammatory process associated with GDM. As mentioned above, there is a need for better diagnostic markers for GDM. One possibility is to put together a panel of biomarkers used to screen for GDM. However, the ROC curve analysis of the IL-1Ra ELISA as a diagnostic tool resulted in a relatively low sensitivity and specificity. Therefore, IL-1Ra might not be a fully satisfactory biomarker on its own, but it may however play an important role in a future panel of biomarkers.

We also showed that women with GDM that developed IGT or type 2 diabetes mellitus postpartum had significantly lower levels of IL-1Ra compared to GDM patients that did not develop any postpartum glucose intolerance. The ROC curve analysis provided a sensitivity of 52.2% and specificity of 67.1%, which nonetheless may not be sufficient enough to use IL-1Ra as a sole biomarker. Therefore, we suggest that IL-1Ra could be included in a panel of biomarkers for the prediction of postpartum development of IGT or type 2 diabetes mellitus in GDM patients already during pregnancy. It is important to identify the GDM patients that will go on to develop IGT or type 2 diabetes mellitus postpartum as early as possible to be able to provide better treatment.

Levels of C-peptide were higher in patients with postpartum development of IGT or type 2 diabetes mellitus than in patients without postpartum glucose intolerance, indicative of increased insulin resistance and increased insulin secretion in the former group. In a previous study no difference in C-peptide levels was found between patients with or without postpartum development of diabetes mellitus [20]. However, that study included patients with both type 1 and type 2 diabetes mellitus.

An inverse correlation between C-peptide and IL-1Ra levels was also found, but only in the control group. Since all controls were collected in week 12 we wanted to exclude the possibility of bias due to gestational age. But no correlations were found in patients at week 12 and at week 28 which suggests that in normal pregnancy IL-1Ra promotes normal β -cell function and maintaining normal C-peptide levels. It is well known that high glucose levels are toxic for β -cells, via the induction of IL-1 β . One study has reported that this glucotoxicity can be prevented by IL-1Ra thus restoring β -cell function [21]. This is well in accordance with our findings, where the controls have higher levels of IL-1Ra and also normal β -cell function as estimated with C-peptide values, whereas GDM patients have lower levels of IL-1Ra in combination with a β -cell dysfunction and generally high C-peptide values.

A strength with the study is that it is conducted in a region of Sweden where there is a screening program for GDM that includes all pregnant women performing an OGTT. Thus, all patients with GDM in the region are represented. In addition, the controls in this study are also pregnant women, which increases the reliability of our findings. Limitations of the study is that some C-peptide levels were taken fasting and some were taken non-fasting at the different women's welfare centers. Nevertheless, this includes both GDM patients and controls. Also, the samples were taken at different gestational age, all samples from controls were taken in week 12, whereas samples from GDM patients were taken in week 12 and most importantly, there was no difference in levels of IL-1Ra or C-peptide between samples that from patients in week 12 or 28. Furthermore, the GDM patients and control group were not matched for age. However, the difference in mean age was only 2.3 years, there are no studies that have shown that this age difference affects levels of IL-1Ra.

To our knowledge no other reports have been published concerning IL-1Ra in GDM patients, but studies have been made in patients suffering from other diseases, including metabolic syndrome and type 2 diabetes mellitus. In one of these studies IL-1Ra was analyzed in 12,885 controls and patients with metabolic syndrome or diabetes mellitus in Finland [22]. The reported values of IL-1Ra in all groups were much lower than levels found in our study even though the same ELISA kit from R&D Systems is used. However, it is not stated in the study if the samples of use were serum or plasma. It is unclear if this reflects that levels of IL-1Ra are increased in pregnancy.

In future studies plasma levels of soluble IL-1 receptor type II (IL-1RII) may be analyzed, to further investigate the role of IL-1 and inhibitors of IL-1 signaling in GDM patients. IL-1RII is a decoy receptor for IL-1, as it lacks the intracellular TIR domain that mediates signaling [23]. In addition, the correlation between IL-1Ra and C-peptide levels in the control group could be further investigated in a larger group of participants to be able to decipher the role of IL-1Ra in β -cell function.

In conclusion, this is the first study to show that GDM patients have significantly lower plasma levels of IL-1Ra than healthy pregnant women. Further, we show that GDM patients with postpartum development of IGT or type 2 diabetes mellitus have ever lewer levels of IL-1Ra. In addition, we showed that GDM patients have significantly higher C-peptide levels than healthy controls and that GDM patients with postpartum development of IGT or type 2 diabetes mellitus have in turn even higher C-peptide levels. Our results show that IL-1Ra might be included in a future panel of biomarkers, both for diagnosing GDM to complement blood glucose, and also identifying GDM patients that are at risk of developing type 2 diabetes mellitus postpartum. However, the ROC curve analysis provided a sensitivity of 52.2% and specificity of 67.1%, which nonetheless may not be sufficient enough to use IL-1Ra as a sole biomarker.

Supporting Information

S1 Data set. Raw data of IL-1Ra, C-peptide, gestational age and postpartum development. (XLS)

Acknowledgments

We would like to thank Mrs. Birgitte Ekholm for excellent technical assistance and nurses and midwifes at the women's welfare centers in Lindängen, Dalby and Paletten in Staffanstorp for assisting in the collection of healthy pregnant controls.

Author Contributions

Conceived and designed the experiments: JD CN MH. Performed the experiments: PK JD. Analyzed the data: PK JD MH. Contributed reagents/materials/analysis tools: CN MH. Wrote the paper: PK JD MH. Ethical approval: CN MH. Critical appraisal of the manuscript: CN.

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Paper III

ORIGINAL ARTICLE



The prevalence and predictive value of the SLC30A8 R325W polymorphism and zinc transporter 8 autoantibodies in the development of GDM and postpartum type 1 diabetes

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Received: 13 January 2016/Accepted: 17 March 2016 © Springer Science+Business Media New York 2016

Abstract The objectives were to evaluate possible associations between the SLC30A8 R325W polymorphism and gestational diabetes mellitus (GDM) as well as postpartum development of type 2 diabetes. Furthermore, we wanted to confirm the prevalence of zinc transporter 8 autoantibodies (ZnT8A), as previously reported, in a larger population and study its predictive value in relation to other β cell specific autoantibodies in postpartum development of type 1 diabetes. Women diagnosed with GDM (n = 776) and women without diabetes (n = 511) were included in the study. Autoantibodies were analyzed in all women using enzymelinked immunosorbent assay. DNA was extracted when possible from women with GDM (n = 536) and all of the controls. R325W was detected through polymerase chain reaction and specific restriction digestion. The R325W Callele were more frequent in women with GDM compared to in controls (OR 1.47, 95 % CI 1.16–1.88, p = 0.0018) but not significantly increased in women with GDM and postpartum development of type 2 diabetes. Autoantibodies were found in 6.8 % (53/776) of the women with GDM and approximately 3.2 % (25/776) were ZnT8A positive. Approximately 19 % (10/53) of the autoantibody positive women with GDM developed postpartum type 1 diabetes. In conclusion, this is the first study to report a significant

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³ Department of Endocrinology, Skåne University Hospital, Lund, Sweden association between the R325W C-allele and increased risk of developing GDM. All of the autoantibody positive women with GDM who developed postpartum type 1 diabetes were positive for autoantibodies against glutamic acid decarboxylase (GADA). Thus ZnT8A did not have any additional predictive value in postpartum development of type 1 diabetes.

Keywords Gestational diabetes mellitus · Autoantibodies · SNP · Prediction of type 1 diabetes

Introduction

Zinc cations are essential for stabilizing proinsulin and insulin hexamers in pancreatic β cells [1]. The zinc homeostasis in β cells is partly regulated by the zinc transporter 8 (ZnT8) protein [2], a member of the SLC30 family, which transports Zn²⁺ from the cytoplasm into organelles or to the extracellular space [3]. The gene encoding ZnT8 (SLC30A8) is located on chromosome 8q24.11 [2]. A single nucleotide polymorphism (SNP; rs13266634, C > T) affects the amino acid residue 325 in the ZnT8 *C*-terminal [4]. The codon CGG generates an arginine (*R*) while TGG instead generates a tryptophan (*W*) (R325W). ZnT8 is an important component of insulin secretion, and there are some reports of associations between polymorphisms in SLC30A8 and insulin secretion or proinsulin to insulin conversion [5–7].

The R325W C-allele was identified as a risk allele for type 2 diabetes [4] which has been confirmed in studies of several different populations [8–12]. Interestingly, the SNP does not seem to associate with increased risk of type 1 diabetes [13, 14]. A previous study has concluded that R325W lacked predictive value for the postpartum

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development of type 2 diabetes in women with gestational diabetes mellitus (GDM) [15]. ZnT8 as an antigen in diabetes was first identified through the presence of autoantibodies (ZnT8A) [16], and more recently findings of ZnT8 reactive T cells have been reported [17, 18]. The presence of ZnT8A is a good independent marker of type 1 diabetes in children [16, 19, 20] and could also assist to identify adults with autoimmune diabetes [21, 22]. In addition, ZnT8A has been suggested to be a reliable predictor for rapidly progressive type 1 diabetes in first-degree relatives [23].

GDM affects approximately 2 % of pregnant women in southern Sweden [24]. In some cases, autoantibodies against glutamic acid decarboxylase (GADA) and the tyrosine phosphatase like protein (IA–2A) appear, and progression toward manifest type 1 diabetes postpartum increases [25]. Previously we demonstrated that women with GDM could be positive for ZnT8A even in absence of other β cell autoantibodies [26]. However, whether the finding has an impact on the development of manifest type 1 diabetes or not was unclear.

The aims with this study were to

- 1) Study if the R325W SNP contribute to the development of ZnT8A in women with GDM.
- Evaluate the association of R235W allele frequencies and genotypes to GDM.
- Study the predictive value of R325W in the development of postpartum type 2 diabetes.
- Confirm the prevalence of ZnT8A in GDM from our previous study in a larger population.
- 5) Investigate the additional predictive value of ZnT8A in relation to other β cell specific autoantibodies in the development of type 1 diabetes postpartum.

Materials and methods

Participants and sample preparation

As part of a universal screening process, all pregnant women in the district of Lund are offered to be tested for GDM. The test is performed, following overnight fast, as a 2-h 75 g oral glucose tolerance test (OGTT) in the 28th week of gestation except for women with a family history of diabetes or body mass index (BMI) above 30 kg/ m² who are tested already in the 12th week. A 2-h capillary plasma glucose value ≥ 10 mmol/L (≥ 180 mg/dL) was used as the diagnostic criterion for GDM as recommended by the European Association for the Study of Diabetes (EASD) [27]. Women tested in the 12th week of gestation are offered to take the OGTT again at the 28th week if the first test is negative. Participants included in this study were diagnosed with their first GDM at Skåne University Hospital, Lund, Sweden, 2004–2013 (n = 776). Women without diabetes were included as controls for investigating R325W genotypes and allele frequencies (n = 511). The median age in women with GDM was 33 [29-36] years and none of the women were treated with insulin at the time of sample collection. Approximately half of the women with GDM received insulin treatment prior to delivery [28]. Information regarding postpartum development of manifest diabetes was retrieved from the patients primary and/or secondary records both by studying journals and ICD-10 codes. The median age in controls without diabetes was 31 [26-37] years. Blood was drawn into serum and ethylenediaminetetraacetic acid (EDTA) plasma tubes and centrifuged at 2000×g for 10 min at 20 °C. Serum and plasma were separated from blood cells and stored in -70 °C until use. Blood cells from the EDTA plasma samples were stored in -70 °C and used for DNA extraction.

This study was approved by the Regional Ethical Review Board in Lund (Regionala etikprövningsnämnden i Lund; 2014/383) and performed in accordance with the Declaration of Helsinki. All participants were given oral and written information about the study before giving written informed consent.

Identification of rs13266634 C > T

DNA was extracted from human leukocytes when available in women with GDM (n = 536) and controls (n = 511) using a standard salting-out technique [29]. Concentration and purity were determined with absorbance at $\lambda = 260/$ 280 nm. Specific restriction digestion was used to study the polymorphism of interest. Polymerase chain reaction (PCR) was performed using primers described by Huang et al. [30] with 30 cycles at 94 °C (30 s), 57 °C (30 s), and 72 °C (30 s), and a final extension of 7 min in a GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA, USA). The fragment size of the PCR product was 429 bp.

The endonuclease BcnI (Thermo Fisher Scientific Inc. Waltham, MA, USA) generated two fragments (235 + 194 bp), only if the R325W *C*-allele was present. If undigested products were found after BcnI treatment, the PvuII endonuclease (Thermo Fisher Scientific Inc. Waltham, MA, USA) was used to produce two fragments (233 + 196 bp) if the R325W *T*-allele was present.

All fragments were visualized by the use of 3 % agarose gel electrophoresis and GelStar[®] Nucleic Acid Gel Stain (Lonza, Rockland, ME, USA).

Antibody assays

Serum autoantibodies directed against GADA, IA-2A, and ZnT8A were analyzed using commercially available enzyme-linked immunosorbent assay (ELISA) (RSR Ltd, Cardiff, UK), according to manufacturer's instructions. Briefly, serum was added to wells coated with GAD₆₅, IA– 2A, or ZnT8 in duplicates. A reaction enhancer was added to the wells of the IA–2A assay. The GADA assay was incubated for 1 h at RT and the other two assays incubated overnight at 4 °C. Biotin-conjugated antigen was added after washing and incubated for 1 h at RT. Streptavidin peroxidase was added after washing and incubated for 20 min on an ELISA plate shaker. Tetramethylbenzidine was added after washing and incubated for 20 min in a dark cabinet at RT. The reaction was stopped with H_2SO_4 and the optical density was measured at 450 nm. A fourparametric logistic regression standard curve was used to calculate the concentrations.

The cut-off levels for positivity were 10 U/ml for GADA and 15 U/ml for IA-2A and ZnT8A. The reported specificities were 99, 100, and 99 % and the sensitivities 82, 64, and 76 % for GADA, IA–2A, and ZnT8A, respectively, in the 2015 Islet Autoantibody Standardization Program (IASP).

Statistical analyzes

Normal distribution of data was investigated using the D'Agostino-Pearson test. Mean and standard deviations were used to report data when normality was accepted and median and interquartile range [Q₃-Q₁] when normality was rejected. The Kruskal-Wallis test was used to test for differences in multiple groups. The χ^2 -test was used to test for differences in observed and expected frequencies. Odds ratio (OR) was used to quantify levels of association. Spearman rank correlation was used to test for correlations. Cox proportional hazards regression was used to estimate the predictive value of R325W in the development of type 2 diabetes postpartum. A power calculation of the sample size required for a statistical power of 0.8 and a significance level of 0.05 regarding R325W allelic frequencies resulted in a sample size of n = 514 for both controls and patients. The power calculation was performed with http://osse.bii.a-star.edu.sg/calculation1.php. p values less than 0.05 were considered to be statistically significant. All statistical analyzes were performed in MedCalc Statistical Software version 15.6.1 (MedCalc Software bvba, Ostend, Belgium; https://www.medcalc. org; 2015).

Results

R325W genotypes and alleles were analyzed in women with GDM with or without positivity for ZnT8A. The majority of women positive for ZnT8A (61 %; 11/18) were heterozygous for C/T, while the prominent genotype in women without ZnT8A were C/C (55 %; 285/518). The relationship between genotypes and the presence of ZnT8A, however, was not statistically significant. Further, no statistically significant relationship in allelic frequencies between GDM patients with or without ZnT8A could be observed.

An increased level of association to homozygosity for the R325W C-allele in women with GDM compared to controls (OR 1.47, 95 % CI 1.16–1.88, p = 0.0018) was observed (Table 1). Homozygosity for the *T*-allele on the other hand was found to have a significantly decreased level of association with GDM (OR 0.58, 95 % CI 0.38–0.88, p = 0.0110). Additionally, an increased *C*allele frequency and decreased *T*-allele frequency in women with GDM (74 %; 789/1072 and 26 %; 283/1072, respectively) compared to in controls (66 %; 679/1022 and 34 %; 343/1022, respectively) (p = 0.0004) were found.

Approximately 9 % (66/776) of the women with GDM developed type 2 diabetes postpartum with a median follow-up time of 2 [1–4] years (Fig. 1). R325W was studied in these patients when DNA was available (n = 49). The majority of these patients were homozygous for C/C (61 %; 30/49) (Table 2). This was also the most frequent genotype in women without postpartum development of type 2 diabetes (54 %; 262/487; p = 0.2682). The predictive value of R325W was estimated using the Cox proportional Hazards model. None of the genotypes were, however, retained in the model (p = 0.4374). Thus no predictive value was observed.

GADA, IA–2A, and/or ZnT8A were detected in 6.8 % (53/776) of women with GDM diagnosed 2004–2013 (Fig. 2). Most of the women were positive for one single autoantibody and almost 40 % (21/53) of these were positive for GADA, 30 % (16/53) were positive for ZnT8A, and 8 % (4/53) were positive for IA–2A. A number of women were positive for a combination of two or more autoantibodies (22.6 %; 12/53). The total number of women with GDM positive for ZnT8A was 3.2 % (25/776).

Approximately, 19 % (10/53) of autoantibody positive women with GDM developed type 1 diabetes postpartum

Table 1 Distribution of the SLC30A8 R325W polymorphism in women with GDM and controls		GDM $(n = 536)$	Controls $(n = 511)$	OR (95 % CI)	p value
	C/C	292 (54 %)	229 (45 %)	1.47 (1.16-1.88)	0.0018
	C/T	205 (38 %)	221 (43 %)	0.81 (0.63-1.04)	0.0997
	T/T	39 (7 %)	61 (12 %)	0.58 (0.38-0.88)	0.0110

Fig. 1 Kaplan–Meier graph of the development of type 2 diabetes postpartum for women with GDM. The median time until development was 2 years. A total of 66 women developed postpartum type 2 diabetes



 Table 2
 Distribution of the SLC30A8
 R325W
 polymorphism in women with GDM with regard to postpartum diabetes development

	No postpartum diabetes ($n = 479$)	Postpartum type 1 diabetes $(n = 8)$	Postpartum type 2 diabetes $(n = 49)$
C/C	257	5	30
C/T	188	3	14
T/T	34	0	5

with a median follow-up time of 1 [1–2] years (Fig. 3). All of these women were GADA positive, while 40 % (4/10) were also positive for ZnT8A and 40 % (4/10) positive for IA–2A.

Discussion

In this study, we found that the R325W *C*-allele was associated with an increased risk of developing GDM, while the R325W *T*-allele was associated with a decreased risk. Further we were able to confirm the prevalence of ZnT8A in GDM from our previous study [26].

The primary aims with this study were to investigate the impact and predictive value of the SLC30A8 R325W SNP in both the development of GDM per se and ZnT8A in women with GDM. This study is the first to investigate the predictive value of ZnT8A in the development of manifest type 1 diabetes postpartum in women with GDM. One strength with this study is that due to our screening process for GDM, we have been able to include all pregnant



Fig. 2 Venn diagram visualizing the prevalence and combinations of autoantibodies in participants with GDM. A total of 53 women with GDM were positive for autoantibodies. Abbreviations: *GADA* autoantibodies against glutamic acid decarboxylase, *ZnT8A* zinc transporter 8, *IA*–2A tyrosine phosphatase like protein

women in our medical district that developed their first GDM 2004–2013. Another strength of this study is that we use autoantibody assays that have reached excellent specificity in IASP 2015. Fig. 3 Kaplan–Meier graph of the development of type 1 diabetes postpartum for autoantibody positive women with GDM. The median time until development was 1 year. A total of ten women developed postpartum type 1 diabetes



A limitation with this study is the low number of women with GDM which later developed type 1 diabetes. In this study, 6.8 % of all women with GDM had either GADA, IA-2A, ZnT8A, or a combination of these. In order to be able to further study the postpartum prevalence of type 1 diabetes in GDM, it would be necessary to either study a more extensive dataset or extend the follow-up period to up to 10 years as done in an earlier study [25]. The majority of the women which developed type 1 diabetes postpartum in this study had a median follow-up time of 1 year. Even though the minimum time from sample collection to information retrieval regarding postpartum diabetes development was 2 years, it is probable that more patients may develop type 1 or type 2 diabetes after study cessation due to the variations in follow-up time.

A previous study reports the presence of ZnT8A to be a good predictor for the development of type 1 diabetes in patients with a family history of type 1 diabetes [23]. Thus, we hypothesized that the presence of ZnT8A would be a good predictor for postpartum development of type 1 diabetes in women with GDM as well. Our results however showed that GADA was a superior predictor compared to ZnT8A or IA–2A. In our material 19 % or 10 of 53 women with GDM positive for autoantibodies developed type 1 diabetes postpartum and all of them were positive for GADA, while several were positive for ZnT8A or IA–2A. This is in accordance with a previous study [25] where the authors established that GADA was present in all women with GDM that developed type 1 diabetes within 10 years postpartum. We therefore suggest the use of GADA, rather than ZnT8A, as a marker for predicting the development of type 1 diabetes postpartum in women with GDM.

An extensive case-control study compared R325W genotypes in participants with type 1 diabetes and concluded that no association could be observed [13]. A similar conclusion was reached in a British study by Raj et al. [14]. Several studies have identified the R325W C-allele as a risk allele for type 2 diabetes in different populations [8-12]. One study which focused on the postpartum development of type 2 diabetes in participants with GDM did, however, not find any association with R325W and type 2 diabetes development [15]. In 2009, a Danish group aimed to examine a number of risk loci in type 2 diabetes which was associated with GDM, one of them being the rs13266634 SNP in SLC30A8. The authors of that study did not, however, detect a significant association between the R325W C-allele and GDM, even though a tendency was observed [31]. We have in our study found the R325W C-allele to be significantly associated to the development of GDM which is in line with both the studies made in type 2 diabetes and the Danish GDM study. The fact that our study showed a significant association between the R325W C-allele where the Danish study only found a tendency might be attributed to the fact that we included almost twice the number of women with GDM despite having a lower number of controls. Additionally, we also found the R325W T-allele to be significantly associated to a reduced risk of developing GDM.

To our knowledge, only one previous study has investigated the risk of postpartum type 2 diabetes mellitus development in women with GDM [15]. That study, however, found no such association. The predictive value of R325W in the development of type 2 diabetes was also investigated in this study, and we could, however, not observe any statistical significance in this matter. One explanation for this may be the limited number of women with GDM which developed postpartum manifest type 2 diabetes in our study. Additional and more extensive studies made with a longer follow-up period should be made on the subject in order to truly evaluate the impact of genetic variants such as R325W in the postpartum development of type 2 diabetes.

In conclusion, this is, to our knowledge, the first study to report a significant association between the R325W *C*allele and increase risk of developing GDM. We can also report a protective role for the R325W *T*-allele, resulting in a reduced risk of developing GDM for patients homozygous for the *T*-allele. We could not observe any predictive value for R325W in the development of postpartum type 2 diabetes in women with GDM. Furthermore, approximately 19 % of the autoantibody positive women developed type 1 diabetes and they were all GADA positive. Thus, ZnT8A did not have any additional predictive value in postpartum development of type 1 diabetes.

Acknowledgments The authors would like to thank Mrs. Birgitte Ekholm for her excellent technical assistance in the research laboratory. The authors would also like to thank Carl and Erik Olsson for their invaluable help with the extraction of DNA. This study was supported by grants from the Swedish Medical Research Council and the Swedish Diabetes Foundation.

Compliance of ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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Paper IV

RESEARCH LETTER



Plasma levels of relaxin-2 are higher and correlated to C-peptide levels in early gestational diabetes mellitus

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Received: 9 June 2017 / Accepted: 12 June 2017 © Springer Science+Business Media, LLC 2017

Introduction

Relaxin-2 is an important gestational hormone in shaping the endometrium in early pregnancy and its secretion peaks during the first trimester [1]. The relaxin family of peptides are structurally found to be similar to insulin and insulinlike growth factors with A-chains and B-chains making up the bioactive molecule [2, 3] and a C-peptide connecting the pre-molecule [4].

A study investigating relaxin levels in women with type 2 diabetes found a correlation between relaxin and insulin sensitivity. Contrarily, a negative correlation between relaxin and beta cell function has been suggested [5]. Another study reported lower levels of relaxin-2 but not of relaxin-3 in patients with recent onset type 2 diabetes. This data combined would suggest that relaxin-2 could be involved in the pathophysiology of type 2 diabetes and beneficial in increasing insulin sensitivity in patients with insulin resistance [6].

Pregnancy is a normal state of insulin resistance which ensures continuous glucose transport to the fetus. Women are however not normally hyperglycemic during pregnancy.

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If hyperglycemic, the women are diagnosed with gestational diabetes mellitus [7].

There are currently no reports on levels of relaxin-2 in gestational diabetes mellitus. The aim of this study was to investigate plasma levels of relaxin-2 in patients with gestational diabetes mellitus, in comparison to pregnant controls without diabetes.

Materials and methods

Women included in this study were diagnosed with gestational diabetes mellitus (GDM) at Skåne University Hospital in Lund as part of a general screening program offered to all pregnant women in the region. The diagnostic criterion was a capillary plasma glucose value $\geq 10 \text{ mmol/L}$ ($\geq 180 \text{ mg/dL}$) after a 2-h 75 g oral glucose tolerance test (OGTT) following overnight fasting [8]. When a family history of diabetes was present or the woman's body mass index (BMI) was above 30 kg/m², the OGTT was offered at the 12th week of gestation, otherwise the OGTT was offered at the 28th week of gestation. Pregnant women without diabetes were included as controls and recruited from nearby maternity care centers (Lindängen Malmö, Staffanstorp and Dalby). Venous blood samples were drawn into ethylenediaminetetraacetic acid-plasma tubes and arrived at the laboratory the next day. Plasma was separated from blood cells by centrifugation at 2000 x g and was stored at -70 °C until use. Since relaxin levels peak during the first trimester we included only women with GDM (n = 137)diagnosed already at 12 weeks of gestation and controls (n = 114) recruited at the same gestational age.

Plasma samples were analyzed for human relaxin-2 and adiponectin with DuoSet enzyme linked immunosorbent assay (ELISA) (R&D Systems, Minnesota, MN, USA)

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according to the manufacturer's instructions. Plasma samples were diluted 1:5 for relaxin-2 and 1:2000 for adiponectin and run in duplicates. The intra-coefficient of variation for relaxin-2 and adiponectin was 3.2 and 2.2%, respectively. The relaxin-2 antibody was reported not to cross-react with recombinant human insulin, pro-insulin or C-peptide.

C-peptide levels were measured in duplicates with ELISA (Mercodia, Uppsala, Sweden) according to manufacturer's instructions. The intra-coefficient of variation ranged between 2.9-4.8%. The detection limit of the assay was reported to be 25 pmol/L.

Data following a normal distribution were reported as mean ± standard deviation (SD). Non-parametric data were ranked and reported as median followed by the interquartile range. Student's t-test or the Mann-Whitney U-test was used to compare differences between groups. Spearman's rank correlation coefficient was used to test for associations. Logistic regression was used to analyze the effect of several independent variables on GDM. A p-value < 0.05 was considered statistically significant and MedCalc Statistical Software version 16.4.3 (MedCalc Software bvba, Ostend, Belgium) was used to perform all the statistical analyses.

Results

Clinical and laboratory data are presented in Table 1.

Plasma levels of relaxin-2 were significantly higher in women with GDM at 12 weeks of gestation (0.83 [0.40-1.53] ng/mL) compared to controls (0.47 [0.3-1.0] ng/mL; p = 0.001). Also the C-peptide levels were higher in women with GDM (1.12 [0.85-2.17] nmol/L) compared to controls (0.60 [0.40-0.83] nmol/L; p < 0.001). Relaxin-2 was positively correlated to C-peptide ($r_s = 0.198$; p =0.002).

Plasma levels of adiponectin were significantly lower in women with GDM at 12 weeks of gestation (2.8 [0.9-4.4] µg/mL) compared to controls (5.2 [3.5-6.8] µg/mL; p < 0.001). Adiponectin was negatively correlated with C-peptide ($r_s = -0.365$; p < 0.001) but not with relaxin-2.

BMI was higher in women with GDM (29.0 [23.4-32.4] kg/m²) compared to controls (24.7 [22.1–27.6] kg/m²; p <0.001). There was no significant correlation between BMI and relaxin-2, but a positive correlation with C-peptide $(r_s = 0.319; p < 0.001)$ and a negative correlation with adiponectin ($r_s = -0.216$; p < 0.001).

Women with GDM were older $(32.1 \pm 5.7 \text{ years})$ compared to controls $(30.4 \pm 5.1 \text{ years}; p = 0.019).$ Maternal age was correlated with BMI ($r_s = 0.180$; p =0.002) and C-peptide levels ($r_s = 0.128$; p = 0.024) but not relaxin-2 or adiponectin.

In a logistic regression model with GDM as the dependent variable and relaxin-2, adiponectin, BMI, C-peptide, and maternal age as independent variables, relaxin-2 and maternal age lost their statistical significance while it was retained by the other variables.

Discussion

In this study, we report higher levels of relaxin-2 in the first trimester of pregnancy complicated by GDM than in pregnant women without diabetes. Relaxin-2 levels were also correlated with plasma C-peptide levels but not with adiponectin, BMI or age.

A strength in this study is that both women with GDM and controls were analyzed in the first trimester to avoid the normal pregnancy variation of relaxin-2. Also, all samples were received and stored within 1 day.

Limitations of the study include the lack of insulin resistance measurements. There was also an age difference between the GDM and control group, age did however not correlate to neither relaxin-2 nor adiponectin.

Relaxin-2 did not retain statistical significance as an independent variable explaining presence of GDM in the multivariate analysis. The role of relaxin-2 as an independent predictor for GDM must still be considered to be uncertain, and addressed in future studies.

Women diagnosed with GDM already at 12 weeks of gestation will have an inherent increased risk of developing type 2 diabetes in the future [9]. Thus, it would seem reasonable to expect findings regarding relaxin-2 levels similar to those previously reported in type 2 diabetes [5, 6].

Table 1 Clinical and laboratory data of women included in the study. study. Spearman's rank correlation coefficient was applied to test for associations		Pregnant women without gestational diabetes ($n = 114$)	Women diagnosed with gestational diabetes $(n = 137)$	Р
	Relaxin-2 (ng/mL)	0.47 [0.33-1.00]	0.83 [0.40-1.53]	0.002
	Adiponectin (µg/mL)	5.2 [3.5-6.8]	2.8 [0.9-4.4]	< 0.001
	C-peptide (nmol/L)	0.60 [0.40-0.83]	1.12 [0.85-2.17]	< 0.001
	Age (years)	30.4 ± 5.1	32.1 ± 5.7	0.019
	BMI (kg/m ²)	24.7 [22.1–27.6]	29.0 [23.4-32.4]	< 0.001

In contrast, we found higher plasma levels of relaxin-2 in women with GDM in our study. Pregnancy itself, since relaxin-2 is an important reproductive hormone, might explain the difference in results. Currently, there is a lack of knowledge regarding plasma relaxin-2 levels in pregnant women with type 2 diabetes. However, a few reports of pregnant patients with type 1 diabetes [10, 11] showed markedly elevated levels of relaxin. The authors suggest a possible connection to the diabetic pregnancy but call for further studies.

Elevated levels of relaxin-2 in the third trimester of 26 women with GDM has been reported, though not reaching statistical significance [12]. The study suggested that increased levels of relaxin-2 might be to compensate for the increased insulin resistance.

Plasma levels of adiponectin were significantly lower in women with GDM in this study, which was expected and well in agreement with other reports [13–15]. To our knowledge, there are no reports on the relationship between relaxin-2 and adiponectin, neither in pregnancy nor in diabetes.

In conclusion, this is the first study to report significantly higher levels of relaxin-2 in patients with GDM. Relaxin-2 may be a possible confounder to the known parameters influencing more severe insulin resistance in these pregnant women, as reflected by higher C-peptide levels and a higher BMI, as well as lower adiponectin values. We suggest a possible role for increased plasma levels of relaxin-2 in compensating for increased insulin resistance in GDM.

Acknowledgements The authors would like to thank Birgitte Ekholm, the Diabetes Research Laboratory, Lund University, for excellent technical assistance with sample preparation and some of the laboratory analyses. The authors would also like to thank midwives and nurses at the maternal health care centers of Lindängen in Malmö, Paletten in Staffanstorp and in Dalby for valuable assistance in the recruitment of healthy pregnant women serving as controls. The study was funded by grants from Sydvästra Skånes Diabetesförening (reg no 1040390) to Dr. Magnus Hillman, Gorthon Foundation (reg no 2015-12-27) to Dr. Charlotta Nilsson and Skåne University Hospital Funding and Donations (reg no 1040741) to Dr. Mona Landin-Olsson. The funders were not included in performing any parts of the study.

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

Ethical approval All procedures performed were in accordance with the ethical standards and approved by the Regional Ethical Review Board in Lund (2014/78 and 2014/383). The study was performed in agreement with the 1964 Declaration of Helsinki and its later amendments. This study does not contain any studies with animals performed by any of the authors.

Informed consent All participants were given written and oral information about the study before signing the informed consent.

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Paper V

Soluble CD163 and TWEAK as biomarkers of gestational diabetes development in early and late pregnancy and their association to postpartum glucose intolerance

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Department of Clinical Sciences

Lund University, Faculty of Medicine Doctoral Dissertation Series 2018:81 ISBN 978-91-7619-647-2 ISSN 1652-8220

