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First trimester biomarkers for prediction of gestational diabetes mellitus

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ABSTRACT

Purpose: To develop a first trimester prediction model for gestational diabetes mellitus (GDM) using obesity, placental, and inflammatory biomarkers.
Methods: We used a first trimester dataset of the ASPRE study to evaluate clinical and biochemical biomarkers. All biomarkers levels (except insulin) were transformed to gestational week-specific medians (MoMs), adjusted for maternal body mass index (BMI), maternal age, and parity. The MoM values of each biomarker in the GDM and normal groups were compared and used for the development of a prediction model assessed by area under the curve (AUC).
Results: The study included 185 normal and 20 GDM cases. In the GDM group, compared to the normal group BMI and insulin ($P = 0.003$) were higher (both $P < 0.003$). The MoM values of uterine artery pulsatility index (UtA-PI) and soluble (s)CD163 were higher (both $P < 0.01$) while pregnancy associated plasma protein A (PAPP-A), placental protein 13 (PP13), and tumor-necrosis factor alpha (TNFα) were lower (all $P < 0.005$). There was no significant difference between the groups in placental growth factor, interleukin 6, leptin, peptide YY, or soluble mannose receptor (sMR/CD206). In screening for GDM in obese women the combination of high BMI, insulin, sCD163, and TNFα yielded an AUC of 0.95, with detection rate of 89% at 10% false positive rate (FPR). In non-obese women, the combination of sCD163, TNFα, PP13 and PAPP-A yielded an AUC of 0.94 with detection rate of 83% at 10% FPR.
Conclusion: A new model for first trimester prediction of the risk to develop GDM was developed that warrants further validation.

1. Introduction

Gestational Diabetes Mellitus (GDM) is a major pregnancy complication associated with increased morbidity and mortality for the mother and fetus baby. GDM is associated with increased risks for fetal macrosomia - often requiring cesarean delivery, shoulder dystocia preterm birth and perinatal mortality; as well as adjunct comorbidities, sharing common placental pathophysiology, such as preeclampsia [1,2]. A large international study estimated the prevalence of GDM to be 18% [2]. After pregnancy GDM is also associated with increased maternal morbidity due to frequent post pregnancy obesity, the development of type 1 and 2 diabetes mellitus (T_1DM and T_2DM) and of cardiovascular diseases (CVDs) [1–6]. Further, newborns with macrosomia are also at risk of developing obesity, T_1DM and T_2DM, and CVDs [7]; and there are additional health complications for both mothers and infants [8].

At present, GDM is typically diagnosed at the end of the second trimester by elevated glucose challenge test (GCT) and/or oral glucose

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Abbreviations

AIH	Artificial Insemination Homologous
APGAR	**A**ppearance, **P**ulse, **G**rimace, **A**ctivity, **R**espiratio
AUC	Area Under the Curve
BMI	Body Mass Index
BP	Blood Pressure
CD163	Cluster of Differentiation 163
sCD163	soluble CD163
CI	Confidence Interval
95% CI	95% Confidence Interval
CRL	Crown Rump Length
CVDs	Cardiovascular Diseases
dBP	Diastolic Blood Pressure
DR	Detection Rate
FPR	False Positive Rate
GCT	Glucose Challenge Test
GDM	Gestational Diabetes Mellitus
HBC	Hofbauer cells
IL-6	Interleukin 6
IUGR	Intrauterine Growth Restriction
IVF	In Vitro Fertilization
IVFed	In Vitro Fertilization with Egg Donation
MAP	Mean Arterial Blood Pressure
MoM	Multiple of the median
MR/CD206	Macrophage related Mannose Receptor Cluster of Differentiation 206
NT	Nuchal Translucency
OGTT	Oral Glucose Tolerance Test
PAPP-A	Pregnancy Associated Plasma Protein A
PlGF	Placental Growth Factor
PP13	Placental Protein 13
PTD	Preterm Delivery
PYY	Peptide YY (known as peptide tyrosine tyrosine)
ROC	Receiver Operating Characteristic
sBP	Systolic Blood Pressure
sCD163	Soluble Cluster of Differentiation 163
T_1DM and T_2DM	Diabetes type 1 and type 2
TNFα	Tumor necrosis factor alpha
T_1DM and T_2DM	Type 1 Diabetes Mellitus and Type 2 Diabetes Mellitus
TNFα	Tumor Necrosis Factor Alpha
UtA-PI	Uterine artery Pulsatility Index

tolerance test (OGTT) [1]. In our center at the time of this study, these women are treated either by diet or anti-diabetic medications (Glyburide, and more recently, also by Metformin) and insulin [8,9]. Meta-analyses and single randomized trials showed that lifestyle changes (e.g. physical exercise, dietary regimens, pharmacological and psychological interventions) early in pregnancy can improve both maternal and neonatal outcomes in singleton pregnancies with and without maternal obesity [10]. Hence, earlier interventions in pregnancy may be more efficient to reduce GDM prevalence without any harm to the mother or the fetus [11,12]. This may also be more cost effective. However, at present there is no method for first trimester identification of women at risk at developing GDM that could benefit from early intervention.

Rabin Medical Center participated in the ASPRE study [13] where biophysical and biochemical markers were measured in gestational week 11^{+0} to 13^{+6} weeks to identify pregnancies at risk of developing pre-eclampsia, followed by risk prevention by the ingestion of 150 mg aspirin daily from the time of screening until 36 weeks of gestation [13,14]. The study proved the important value of first trimester risk prediction for pre-eclampsia and the introduction of early administration of aspirin to prevent this major pregnancy complication.

We have conducted this study to evaluate whether the ASPRE dataset could be further used to establish a first trimester prediction model for GDM and thereby to form the foundation for early intervention and prevention of GDM. We hypothesized that specific markers from the ASPRE trial in combination with novel inflammatory and placenta markers either alone or in combination will provide a specific score for first trimester risk prediction of GDM.

2. Material and methods

2.1. The cohort

2.1.1. Enrollment

The ASPRE dataset included biomarker levels tested prospectively in women attending their routine first trimester pregnancy evaluation at 11^{+0} to 13^{+6} weeks' gestation (GA) at the Helen Schneider Women's Hospital, at the Rabin Medical Center in Petach Tikva, Israel [13,14]. The women were enrolled between October 2014 and March 2016 and delivered between May 2017 to December 2016. Each woman gave written informed consent to participate in the study, which was part of the ASPRE study of first trimester screening for preterm pre-eclampsia. Blood samples for biomarker analysis were handled as previously described [13,14]. Ethical approval was obtained from the Rabin Medical Center Institutional Review Board (#0066-14-RMC of March 02, 2014) and from the National Ethics Committee (20140059 of May 01, 2014).

2.1.2. The inclusion and exclusion criteria

The inclusion criteria were women carrying a singleton viable gestation when undergoing combined first trimester screening for aneuploidy. The exclusion criteria were the detection of fetal aneuploidies or major fetal anomalies, increased nuchal translucency thickness >3.5 mm or treatment with aspirin prior to enrollment. Patients with placentation support hormonal treatment for in vitro fertilization (IVF) were only included after discontinuing the treatment. Pregnancies were also excluded if they ended in termination, miscarriage, or fetal death before 24 weeks' gestation [14]. Additionally, we excluded women who developed pre-eclampsia [13,14], those who delivered neonates with birthweight below the 5th percentile for gestational age [small for gestational age - SGA, 14] and those who delivered before 37 weeks' gestation for reasons other than GDM, pre-eclampsia, or SGA.

2.1.3. Outcome measures

Data on pregnancy outcomes were collected from the hospital maternity records or from the delivery records of neighboring hospitals (Sheba and Meir Medical Centers) where some of the women delivered.

2.2. Definition of GDM

Diagnosis of GDM was made in the second trimester using the standard two-step protocol [15]: At 24–28 weeks' gestation, a 1-h GCT (50 g) was performed and if the glucose level exceeded 140 mg/dL, a 3-h OGTT (100 g) was carried out. GDM was diagnosed in women who had two or more abnormal OGTT results according to Carpenter and Costan [16]: fasting ≥95 mg/dL (5.2 mM, 1-h ≥180 mg/dL (10 mM), 2-h ≥155 mg/dL (8.6 mM), 3-h ≥140 mg/dL (7.8 mM).

The GDM group was subsequently managed either by diet ($GDMA_1$) or treated by glyburide or insulin ($GDMA_2$) in accordance with the guidelines of the Israel Society of Obstetrics and Gynecology. Note that

at the time of this study this routine was followed the world consensus. The new WHO and FIGO recommendations were introduced later according to modified glucose range limits as identified by the HAPO study [2].

2.3. Procedures at enrollment

As mentioned above, the first trimester visit included all ASPRE procedures [13,14]: (1) recording of maternal characteristics and medical history; (2) determination and validation of gestational age from measurement of the fetal crown-rump length (CRL); (3) measurement of the left and right uterine artery pulsatility index (UtA-PI) by transabdominal color Doppler ultrasound scanning and calculation of the mean UtA-PI; (4) measurements of MAP by validated automated devices and standardized protocols, and (5) measurements of the serum concentration of PAPP-A and PlGF.

2.4. Examined biomarkers

Our dataset included four groups of biomarkers.

First, existing clinical and biochemical biomarkers from the ASPRE study: BMI, blood pressure (systolic, diastolic and mean arterial blood pressure (MAP)), uterine artery pulsatility index (UtA-PI), pregnancy associated placenta protein A (PAPP-A), and placental growth factor (PlGF) [14]. We added placental protein 13 (PP13), another pre-eclampsia predicting biomarker [17,18]. The information on all these markers was extracted from the ASPRE database for evaluation of their potential inclusion in first trimester GDM prediction. Previous studies indicated the potential benefit of ASPRE biomarkers in early prediction of GDM [30]. These mainly included higher MAP [27,29,30] and reduced levels of maternal serum PAPP-A [19–30], PLGF [23,26, 28], and PP13 [28,29].

Second, obesity biomarkers, including insulin [22], leptin [31,32], and PYY [33], which were previously shown to be elevated in GDM in the second and third trimester.

Third, novel macrophage specific inflammation markers, including soluble CD163 (sCD163) and mannose receptor (sMR/CD206), were analysed [34–37]. Previous studies showed that sCD163, a lineage specific monocyte/macrophage marker, is increased at the time of GDM, associated with insulin resistance and later risk of developing T_2DM [34–36]. Blood sMR/CD206, a C-type lectin, is increased in critical illness and inflammatory liver disease [37]. The sMR/CD206 is primarily expressed on the surface of macrophages, and also on dendritic cells, liver sinusoidal endothelial cells, human dermal fibroblasts, and keratinocytes, all associated with the risk to develop GDM [37].

Fourth, known inflammation markers included tumor necrosis factor alpha (TNFα) [38] and interleukin 6 (IL6) [39]. These have previously been identified as second and third trimester biomarkers of GDM but were tested here in the first trimester.

The testing of the second, third and fourth biomarker sets was performed on stored first trimester samples of the ASPRE study.

2.5. Stored samples analysis

Aliquots of serum samples collected at the enrollment visit were transferred to −70 °C storage. At the time of this study, coded aliquots of all patients were retrieved from the sample bank. One aliquot was then shipped on dry ice without defrizzing to the Department of Clinical Biochemistry, Aarhus University Hospital, Denmark, for testing sCD163 and sMR/CD206 and another aliquot was sent on dry ice to the Microbiology Research group of the Azrieli Faculty of Medicine, Safed, Israel for testing of the remaining biomarkers. Note that it is important to avoid sample thaw/freeze cycles for keeping biomarker content stable.

sCD163 and sMR/CD206: sCD163 and sMR/CD206 were determined in duplicates using in-house sandwich enzyme-linked immunosorbent assays (ELISA) with a BEP-2000 ELISA-analyzer (Dade Behring, Siemens, Erlangen, Germany) as previously described [40,41]. sCD163 and sMR/CD206 are resistant to repeated freezing and thawing. Control samples and serum standards were included in each run.

Insulin, leptin, PYY, TNFα, and IL_6: The Human Metabolic Hormone Magnetic Bead Panel kit (HMHEMAG-34K, Merck Millipore, MA, USA) was used for the parallel testing of all five biomarkers. The kit was employed according to the manufacturer's instructions. The results were read using a Bio-Plex MAGPIX reader and analysed with Bio-Plex manager 6.1 software (Bio-Rad, CA, USA).

2.6. Multiple of the medians (MoM)

All markers were converted into gestational week specific multiples of the Medians (MoMs) using weighted linear regression and were further adjusted for maternal body mass index (BMI) at enrollment and maternal age (both continuous variables were divided into 5 equal size groups), and parity (nulliparous and parous) [13,14]. The ASPRE equation included biomarker adjustment to ethnic groups but in our dataset, there were only two non-Caucasian women. It also included adjustment to smoking but we had only three smokers. Thus, adjustments to these confounders did not change MoM calculation.

2.7. Descriptive statistics

SPSS version 24 (SPSS Inc., Chicago, IL, USA) was used. Statistical significance was defined as $P \leq 0.05$. The frequencies of means or medians for the continuous variables were calculated with a 95% Confidence Interval [95% CI]. Pearson's chi-square tests were applied to test for correlations between the study groups for the categorical parameters. The non-parametric Mann-Whitney (for two independent samples) or Kruskal-Wallis (for more groups) tests were applied to probe for significant differences. Area under the curve (AUC) of the receiver operation characteristic (ROC) graphs were used to extract the observed detection rate (DR) at fixed false positive rates (FPR).

3. Results

There were 256 pregnancies in the dataset and we excluded 50 cases from analysis (8 withdrew consent or were lost to follow up, 12 miscarried before 23 weeks' gestation, in 6 there was a fetal trisomy, 3 cases were treated with aspirin, 5 cases developed pre-eclampsia, 3 that had T_1DM_1, 5 delivered small for gestational age neonates, and 10 delivered preterm). We examined 205 cases, including 20 that developed GDM and 185 normal pregnancies delivering a healthy baby at term.

Baseline maternal, pregnancy characteristics and delivery data are presented in Table 1. At enrollment there were no differences in most baseline characteristics, except that the GDM group had significantly higher BMI ($P < 0.001$), systolic, diastolic and mean arterial blood pressure (MAP) (although neither diastolic blood pressure was ≥90 mm Hg nor systolic blood pressure was ≥140 Hg) ($P < 0.01$).

At delivery, the gestational week tended to be shorter in the GDM group ($P = 0.076$) and both systolic, diastolic and MAP were higher in the GDM group ($P < 0.05$, all) (Table 1).

3.1. Clinical and biochemical biomarkers

Median marker levels are presented in Table 2 A&B as raw values and MoMs. The MAP was higher in GDM women. As raw values, the serum blood level of sCD163, sMR/CD206, insulin, leptin, PYY and MAP were significantly higher in the GDM group ($P = 0.002$, $P = 0.037$, $P = 0.003$, $P < 0.001$, $P = 0.008$, respectively), while TNFα and PP13 were significantly lower ($P = 0.037$, $P < 0.001$, respectively) (Table 2A, left side). The differences were larger for the obese group (BMI>30 kg/m^2, $N = 8$) compared to the non-obese group (BMI<30 kg/m^2, $N = 12$) (Table 2B) except for PP13 and sCD163.

After conversion to gestational week specific MoM and sequential

Table 1
Baseline maternal and pregnancy characteristics in the normal and the GDM groups.

Variable	Normal (n = 185)	GDM (n = 20)	P
Enrolment			
Gestational age (w, mean [95% CI])	12.6 [12.5–12.7]	12.7 [12.3–13.1]	0.492
Crown rump length (mm, mean [95% CI])	63 [62–64]	62 [58–65]	0.569
Maternal age (y, mean [95% CI])	31.0 [30.3–31.6]	33.4 [30.7–36.1]	0.079
Body mass index (Kg/m^2, mean [95% CI])	23.3 [22.8–23.9]	30.0 [27.0–33.0]	**<0.001**
Parity (mean [95% CI])	1.1 [0.9–1.3]	0.9 [0.3–1.4]	0.211
Systolic BP (mmHg, mean [95% CI])	106 [105–108]	116 [110–123]	**0.002**
Diastolic BP (mmHg, mean [95% CI])	65 [64–66]	69 [65–73]	**0.045**
Mean arterial pressure (mmHg, mean [95% CI])	79 [77–80]	85 [80–89]	**0.008**
Previous preeclampsia (n, %)	1 [0.5]	0	0.742
Previous small for gestational age (n, %)	2 [1.1]	0	0.640
Previous preterm birth (n, %)	11 [5.9]	0	0.393
Conception by IVF (n, %)	20 [10.8]	3 [15.0]	0.706
Chronic hypertension (n, %)	0	0	-
Smoking (n, %)	3 [1.6]	1 [5.0]	0.339
Delivery			
Gestational age (w, mean [95% CI])	39.6 [39.4–39.8]	39.0 [38.3–39.6]	0.076
Systolic BP (mmHg, mean [95% CI])	105 [104–107]	115 [109–120]	**<0.001**
Diastolic BP (mmHg, mean [95% CI])	64 [63–66]	68 [64–73]	**0.049**
Mean arterial pressure (mmHg, mean [95% CI])	78 [77–79]	84 [79–88]	**0.006**
Mode of Delivery (%)			
Vaginal	150 [81.1]	14 [70.0]	0.422
Caesarean section	35 [18.9]	6 [30.0]	
Delivery <37 weeks (n, %)	5 [2.7]	2, 10.0	0.141
Baby's weight (g, mean [95% CI])	3260 [3199–3321]	3242 [3032–3452]	0.855
Live birth (n, %)	185 [100]	20, 100	-
Baby's gender (male, %)	105 [56.8]	42.1	0.221
5min Apgar < 7 (n, %)	0	0	-

Categorical values frequencies (Pearson Chi square test) and arithmetic means for continuous variables (Mann-Whitney test) are presented as the mean with a 95% Confidence Interval [95% CI].
BP-blood pressure, GDM-gestational diabetes mellitus.
APGAR- Appearance, **P**ulse, **G**rimace, **A**ctivity, **R**espiration.

adjustment to BMI, maternal age, and parity, the MoM value in GDM of sCD163 was significantly higher (P = 0.015) whereas TNFα, PP13, and PAPP-A were lower (P = 0.002, P = 0.005, and P = 0.002, respectively). After conversion to MoM sMR/CD206, Leptin, PYY, lL6, PLGF, and MAP were no longer significantly different in GDM versus normal women.

Uterine artery pulsatility index was similar in GDM and normal as raw values, but MoMs values were significantly increased in the GDM group.

Of note insulin was not suitable for MoM conversion since the values in the GDM group did not follow a Gaussian distribution even after log transformation (not shown).

For the MoMs of PP13 and PAPP-A the comparison to GDM was not different for the obese and non-obese GDM patients, but for UtA-PI, sCD163, and TNFα the differences were larger for the obese women (BMI>30 kg/m^2) compared to the non-obese GDM women (Table 2B).

The interquartile differences is depicted in the Box Plot (Fig. 1). It shows that for sCD163, PP13, and PAPP-A, the values are tightly packed around the medians in the GDM group compared to the normal cases, indicating a homogeneous effect of the pathology on the marker level. For insulin (presented as raw values) the upper quartile of the GDM group had a much wider distribution, with the top 5% emerging as quite different from the rest, which is consistent with the non-linear distribution.

3.2. AUC- single markers

ROC curves were prepared from MoM values except for insulin where raw values were used. BMI was the best predictor for GDM as a single marker with AUC = 0.87 and with a DR at 63% for 10% FPR (Table 3A, Fig. 2A). Insulin, TNFα, and PAPP-A (AUC = 0.73 for each) came next with DR of 45%, 40%, and 27%, respectively, at 10% FPR (Table 3A, Fig. 2A). The AUC for sCD163 and UtA-PI was 0.68 and 0.63, with DR of 28% for each at 10% FPR, (Table 3A, Fig. 2A). MAP had AUC of 0.64 with a DR = 10% only at 10% FPR, and both PYY and PLGF were poor markers (AUC = 0.59, DR = 0) (Table 3A, Fig. 2A). AUC was 0.51, and DR of 12% at 10% FPR while no further analysis was performed (not shown).

For most of the above mentioned biomarkers, the prediction accuracy increased for the GDM subgroup of obese women (BMI >30 kg/m^2) compared to the non-obese GDM subgroup (Fig. 2B and C, Table 3A middle and lower panels), except PP13 and UtA-PI, where level of prediction accuracy remained similar and unaffected by the BMI of the GDM patients.

3.3. AUC- multiple marker analysis

Curve smoothing for combined analysis used polynomial regression of $y = ax^3 + bx^2 + cx + d$ or a lower polynomial level (selected according to the highest regression coefficient) except for the case of CD163 in model 2, where a moving average was used.

In Model 1 the AUC was 0.85 [95% CI = 0.75–0.95, P = 0.006) for BMI as a single marker, and DR of 64% at 10% FPR. After combining BMI with sCD163, the AUC increased to 0.92 [95% CI = 0.82–0.97] and the DR increased to 75% and adding insulin to BMI and sCD163 further improved prediction with an AUC of 0.94 [95% CI = 0.822–0.97], and the DR increased to 89%. Combining BMI, sCD163, insulin and TNFα brought the AUC to 0.95 [95% CI = 0.89–0.99] without any further increase in DR. Adding PP13, PAPP-A or UtA-PI did not further increase the AUC or the DR (Table 3B, Fig. 3A). Note that in starting from two and increasing to four biomarkers, the combined analysis provided a better and more accurate prediction compared to obesity alone (larger AUCs, DRs, and P values). A change in the order of the markers or other combinations did not further increase the DRs (data not shown). Except BMI, other prior risk factors, such as age >40 years, history of previous GDM, and obesity- BMI >30Kg/h2, that together provided some level of accuracy (Table 3A), were not found useful for multiple marker prediction, potentially due to their rare occurrence in our ASPRE cohort.

In model 2 BMI as a marker was excluded. The best multi-marker prediction accuracy was obtained with a combination of sCD163, insulin, TNFα, PP13, and PAPP-A, yielding an AUC of 0.94 [95% CI = 0.85–0.99], and DR of 83%, all at 10% FPR. Adding UtA-PI did not increase the DR (Fig. 3B, Table 3B). A change in the order of the markers or other combinations did not further increase the AUCs or the DRs (not shown).

4. Discussion

4.1. Principal findings of the study

This study showed that pregnancies at risk for GDM can be identified already in the first trimester by combining selected clinical risk factors and biomarkers. It may thus provide a time window for investigating potential prevention therapies. In pregnancies that subsequently develop GDM, BMI and the MoMs levels of first trimester sCD163, insulin, and UtA-PI were significantly higher than in normal pregnancies, whereas TNFα, PP13, and PAPP-A were significantly lower. In obese

Table 2
Marker Level
A) Median Marker levels (95% CI) in normal and GDM women presented as raw values and multiple of the medians (MoMs). B) Median raw Biomarker data (A) and their MoMs in patients with higher/lower BMI.

2A) Median Marker levels (95% CI) in normal and GDM women presented as raw values and multiple of the medians (MoMs).

Biomarker	Raw values			Multiple of the Medians		
	Normal (n = 185)	GDM (n = 20)	p	Normal (n = 185)	GDM (n = 20)	p
Soluble CD163 (sCD163)	1.36 [1.30–1.41]	1.73 [1.62–2.17]	**0.002**	1.00 [0.95–1.04]	1.16 [1.12–1.43]	**0.015**
sMR/CD206	0.24 [0.22–0.25]	0.28 [0.23–0.31]	**0.037**	1.00 [0.95–1.05]	1.06 [0.98–1.17]	0.315
Insulin	40.3 [35.0–50.0]	70.0 [50.0–147.0]	**0.003**			
Leptin	1842 [1535–2425]	3562 [3007–4977]	**<0.001**	1.00 [0.91–1.06]	1.32 [1.24–1.57]	0.206
Peptide YY (PYY)	59.5 [57.5–67.5]	85.0 [53.0–125.0]	0.061	1.00 [0.95–1.12]	1.24 [0.81–2.13]	0.286
Tumor-necrosis factor α (TNF α)	75.5 [72.0–83.5]	67.0 [58.5–75.3]	**0.037**	1.00 [0.94–1.11]	0.77 [0.67–1.01]	**0.002**
Interleukin 6 (LI6)	12.5] 12.0–13.5]	13.5 [12.5–15.0]	0.524	1.00 [0.96–1.08]	1.02 [0.91–1.13]	0.467
Placental protein 13 (PP13)	414 [385–451]	229 [180–335]	**<0.001**	1.00 [0.88–1.12]	0.60 [0.49–0.84]	**0.005**
Pregnancy associated plasma protein A (PAPP-A)	-	-	-	1.01 [0.88–1.15]	0.63 [0.49–0.77]	**0.002**
Placental growth factor	33.4 [31.5–35.2]	35.1 [27.6–52.3]	0.605	1.00 [0.95–1.06]	1.09 [0.86–1.33]	0.451
Mean arterial blood pressure (MAP)	79 [77–80]	85 [79–91]	**0.008**	1.00 [0.98–1.01]	1.04 [0.94–1.10]	0.524
Uterine Artery Pulsatlity Index (UtA-PI)	1.63 [1.54–1.76]	1.72 [1.51–2.24]	0.165	1.00 [0.95–1.04]	1.13 [1.02–1.44]	**0.015**

2B: Median raw Biomarker data (A) and their MoMs in patients with higher/lower BMI

Parameter	Normal (n = 185)	GDM All (n = 20)	GDM - BMI<30 (n = 12)	GDM - BMI≥30 (n = 8)	p*
Raw values					
sCD163	1.36 [1.30–1.41]	1.73** [1.62–2.17]	1.61* [1.43–1.80]	1.81**[1.73–2.82]	**<0.001**
sMR/CD206	0.24 [0.22–0.25]	0.28* [0.23–0.31]	0.28* [0.21–0.32]	0.27* [0.23–0.36]	0.042
Insulin	40.3 [35.0–50.0]	70.0** [50.0–147.0]	58.5 [42.0–116.5]	111.8**[50.0–232.7]	**0.001**
Leptin	1842 [1535–2425]	3562** [3007–4977]	3245* [2550–3890]	4910**[2592–6540]	**<0.001**
PYY	59.5 [57.5–67.5]	85.0 [52.5–125.0]	102.3 [50.0–150.0]	65.0 [53.0–146.0]	0.108
TNFα	75.5 [72.0–83.5]	67.0 [58.5–75.3]	68.0 [60.0–85.5]	62.8 [49.5–86.8]	0.052
IL-6	12.5 [12.0–13.5]	13.5 [12.5–15.0]	12.5 [10.5–20.0]	14.3 [12.5–18.5]	0.260
PP13	414 [385–451]	229**[180−335]	229** [180−339]	228*[152−590]	**<0.001**
PAPP A	1.01 [0.88–1.15]	0.63** [0.49–0.77]	0.72* [0.55–1.04]	0.42**[0.15–0.77]	**<0.001**
PlGF-1	33.4 [31.5–35.2]	35.1 [27.6–52.3]	34.6 [27.1–52.9]	38.6 [25.0–60.2]	0.849
UtA-PI	1.63 [1.54–1.76]	1.72 [1.51–2.24]	1.86 [1.49–2.40]	1.63 [1.45–1.90]	0.171
MoM values					
sCD163	1.00 [0.95–1.04]	1.16* [1.12–1.43]	1.14 [1.03–1.25]	1.23* [1.12–1.199]	0.011
sMR/CD206	1.00 [0.95–1.05]	1.06 [0.98–1.17]	1.06 [0.90–1.17]	1.02 [0.91–1.185]	0.603
Insulin	1.00 [0.91–1.12]	1.21 [0.99–2.90]	1.00 [0.80–1.79]	2.10 [0.99–3.99]	0.111
Leptin	1.00 [0.91–1.06]	1.32 [1.24–1.57]	1.32 [0.81–1.61]	1.42 [1.24–1.86]	0.277
PYY	1.00 [0.95–1.12]	1.24 [0.81–2.13]	1.57 [0.79–2.29]	0.95 [0.81–2.48]	0.570
TNFα	1.00 [0.94–1.11]	0.77** [0.67–1.01]	0.83* [0.67–1.06]	0.73** [0.54–1.11]	**0.001**
IL-6	1.00 [0.96–1.08]	1.02 [0.91–1.13]	0.91 [0.77–1.13]	1.05 [0.92–1.44]	0.302
PP13	1.00 [0.88–1.12]	0.60* [0.49–0.84]	0.55* [0.49–0.92]	0.66 [0.42–1.84]	**0.002**
PAPP A	1.01 [0.88–1.15]	0.62** [0.49–0.77]	0.72* [0.62–1.04]	0.46** [0.30–0.54]	**<0.001**
PlGF-1	1.00 [0.95–1.06]	1.09 [0.86–1.33]	1.03 [0.83–1.33]	1.17 [0.86–1.72]	0.593
MAP	1.00 [0.98–1.01]	1.04 [0.94–1.10]	1.01 [0.91–1.10]	1.05 [0.94–1.12]	0.603
UtA-PI	1.00 [0.95–1.04]	1.13* [1.02–1.44]	1.26* [0.93–1.54]	1.09 [0.97–1.23]	**0.008**

PAPP-A is only presented as MoM since no raw values were available.
Raw values for sCD163, sMR/CD206 are presented as ng/mL, Interleukin 6, Tumor-necrosis factor α, Placental protein 13, Placental growth factor, leptin, and Peptide YY are presented as pg/mL, Insulin as mcUn/ml, and Mean arterial blood pressure as mmHg.
Of note insulin was not suitable for MoM conversion since the values in the GDM group did not follow a Gaussian distribution even after log transformation.
Median multiple of the medians (MoMs) calculated by raw value conversion to gestational week specific medians further adjusted for BMI, Maternal age and Parity. There were too few smokers or non-Caucasians to adjust for these parameters. PAPP-A is only presented as MoMs since no raw data were available.

women with BMI >30 kg/m^2 these differences were even larger. Even as single biomarkers, BMI, sCD163, insulin, TNFα, PP13, and PAPP-A yielded acceptable GDM prediction (AUC≥0.65); however, the prediction accuracy increased significantly by combining these in a multi-marker model of BMI with sCD163, insulin and TNFα. In addition, the multi-marker approach was also effective for women with BMI <30 kg/m^2 using the combination of sCD163, insulin, TNFα, PP13, and PAPP-A.

The link between high BMI and GDM is well established and pre-pregnancy obesity is a known prior risk factor to develop GDM [1, 42–44]. During pregnancy there is an increase in anabolic hormones, adipose tissues, inflammation, and blood vessels remodeling, and therefore obesity may serve as the most potent risk factor of diabetes in pregnancy [42] and its use in a GDM risk prediction algorithm appears crucial. Further, high BMI has been linked to the gut microbiome and to elevated liver enzymes, both contributing to insulin resistance and inflammation during pregnancy [45]. Interestingly, baby weights were similar between healthy and GDM patients, when frequently GDM babies are born larger or smaller. As Yamamoto et al. previously said [46] prevention of overweight gain is linked to strict diet regiment, that is careful controlled by our clinical team for GDM patients.

Elevated serum insulin levels on its own provided good prediction of GDM with AUC = 0.73 and DR of 45%, at 10% FPR, which was to be expected. In obese women, combining BMI >30 kg/m^2 with sCD163, insulin and TNFα yielded the highest AUC (0.95) and the highest DR (89%) at a 10% FPR. Insulin was, however, also especially important for predicting GDM in the non-obese GDM women where the prediction by combining insulin with sCD163, TNFα, PAPP-A and PP13 reached AUC of 0.94 and a detection rate of 83%.

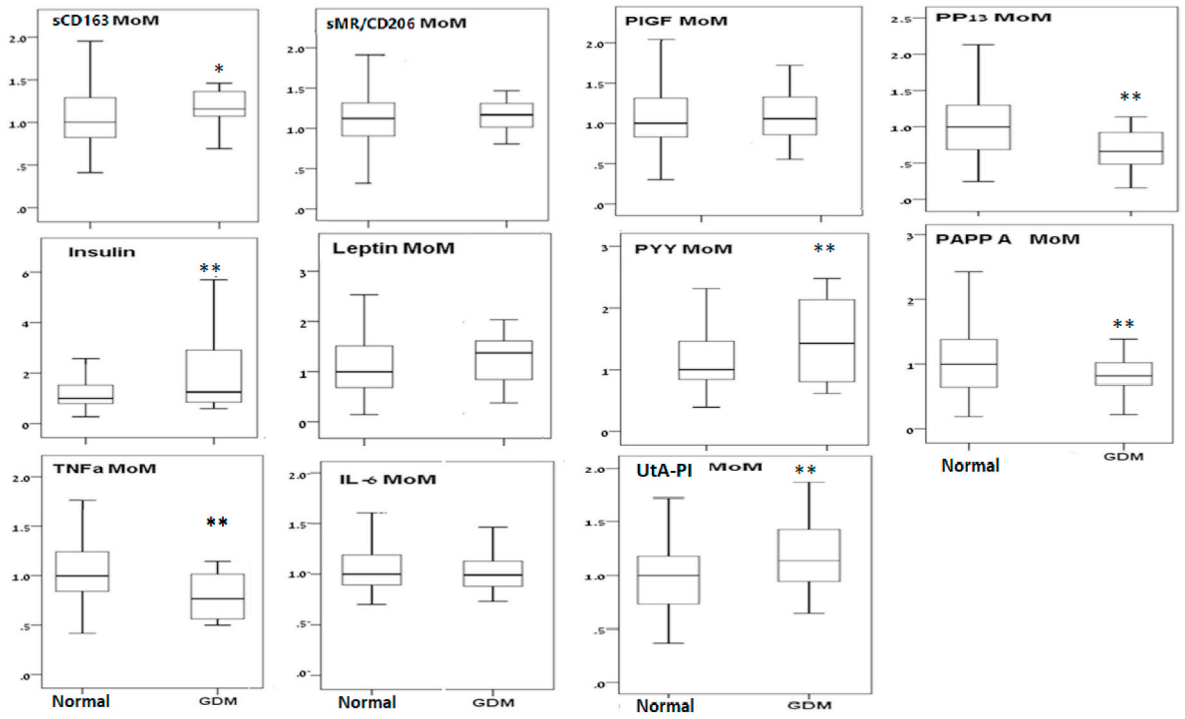

Fig. 1. Box plot presentation of each biomarkers after conversion to MoM. All markers are compared between the group of cases who developed GDM and the group of normal pregnancies. Insulin is shown in raw data. *
P ≤ 0.02, **P ≤ 0.005. Of note insulin was not suitable for MoM conversion since the values in the GDM group did not follow a Gaussian distribution even after log transformation. Thus, insulin is presented over raw value scale.

The macrophage activation marker sCD163 was a good single biomarker of GDM prediction. In previous studies elevated sCD163 levels were associated with obesity, insulin resistance and diabetes and high sCD163 levels predicted later development of type2 diabetes [35]. Further, elevated sCD163 was demonstrated at the time of GDM and also predicted post pregnancy development of Type 2 Diabetes [35,36]. Here we describe for the first time, that sCD163 can serve as a biomarker of GDM already in the first trimester. While the liver is considered a major source of sCD163 release, adipose tissue and other macrophage rich organs may also contribute to the serum levels [47,48]. This is consistent with our finding of higher sCD163 level in the obese (BMI>30 kg/m^2) compared to the non-obese GDM patients. Further, during early stages of pregnancy there is a massive infiltration of macrophages into the placenta, particularly by the Hofbauer cells (HBC) [48,49]. These cells express CD163 on their surface and the molecule may subsequently be shed into the maternal circulation, contributing to the serum level of sCD163 [47].

Previous studies reported that in pregnancies that develop GDM serum levels of TNFα are increased during the second and third trimesters of pregnancy [38,50], and we have no obvious explanation for our finding that during the first trimester this inflammatory marker is reduced instead of elevated, and that low levels can serve for the prediction of GDM.

Reduced serum PP13 levels in the first and the second trimester of singleton and twin pregnancies that develop GDM has been reported previously [29]. However, we found that prediction of GDM by first trimester PP13 alone was poor (18% at 10% FPR), but it was improved among the non-obese GDM cases (42%). Low PP13 is linked to narrower uterine arteries and veins, thereby limiting the blood flow to and from the placenta [51], as reflected by the increased UtA-PI. Low PP13 was also linked to decreased placental immune tolerance [17,52]. Hence, it may be linked to increased placenta macrophage HBCs and HSC cell infiltration as mentioned above [48,49]. This is consistent with improved GDM prediction combining low PP13 with high sCD163, lower TNFα, and elevated insulin in obese and non-obese women.

Previously, reduced serum PAPP-A was found both in the first and second trimesters in women that subsequently developed GDM [19–30]. PAPP-A encodes a secreted metalloproteinase which cleaves an insulin-like growth factor binding protein, and activates the insulin growth factor. As a single marker PAPP-A was a poor predictor of GDM but among the obese women (BMI >30 kg/m^2) it performed better. Reduced PAPP-A is widely used for the prediction of trisomy 21 as well as pre-eclampsia. It may be suggested that low first trimester levels may be used as an indication for testing for sCD163, insulin TNFα in the early prediction of GDM.

FIGO's guidelines recommend routine first trimester screening to predict pre-eclampsia [53] and we further showed that decreased placental perfusion reflected by increased UtA-PI was a good predictor of GDM. We suggest that higher UtA-PI may be used as an indication for testing sCD163, insulin and TNFα for the early prediction of GDM. Note that these new FIGO guidelines would theoretically add more GDM and cases, that may increase the accuracy of the prediction model that a large scale study will verify.

Previous studies have reported that serum IL6 is increased during the second trimester in pregnancies with GDM [39], but in our first trimester study there was no significant differences between the GDM and the normal group. IL6 may thus be important for inflammation and potentially for GDM pathogenesis but are not useful as a first trimester predictor of GDM.

4.2. *Clinical implications of the study*

First trimester prediction of the risk for GDM development could promote earlier lifestyle changes and initiate pharmacological interventions at an earlier pregnancy stage to improve outcomes [1]. Syngelaki et al. [54] have shown that maternal factors but not inflammatory marker can assist in developing first trimester screening for GDM. Here we used the ASPRE dataset, including the maternal factor

Table 3
Area under the receiver operating characteristics curve (AUROC), detection rate and false positive rate for the analysis of single markers (A) and for multiple markers (B).

3A. Single Markers

Variable	AUC Mean (95%CI)	p	Detection rate %		
			5% FPR	10% FPR	15% FPR
All patients					
BMI	0.87 (0.79–0.95)	**<0.001**	50	53	74
sCD163 MoM	0.68 (0.56–0.80)	**0.016**	12	28	54
Insulin (raw)	0.73 (0.62–0.84)	**0.003**	28	45	51
Leptin MoM	0.70 (0.44–0.96)	0.142	7	14	70
PYY MoM	0.59 (0.30–0.88)	0.514	0	0	0
TNFα MoM	0.73 (0.61–0.85)	**0.002**	35	40	42
PP13 MoM	0.69 (0.55–0.83)	**0.013**	7	8	44
PAPP A MoM	0.73 (0.61–0.86)	**0.002**	17	27	53
PlGF MoM	0.59 (0.31–0.87)	0.514	0	0	10
UtA-PI MoM	0.63 (0.49–0.76)	*0.091*	13	28	30
MAP MoM	0.64 (0.38–0.91)	0.288	10	10	30
Male baby	0.58 (0.31–0.84)	0.568	7	14	20
Maternal risk factors for GDM	0.63 (0.42–0.83)	0.227	7	13	18
BMI < 30 kg/m^2					
sCD163 MoM	0.65 (0.51–0.78)	0.148	28	28	32
Insulin (raw)	0.65 (0.62–0.75)	**0.02**	14	37	46
TNFα MoM	0.70 (0.55–0.85)	**0.044**	45	45	45
PP13 MoM	0.71 (0.58–0.84)	**0.038**	42	42	53
PAPP A MoM	0.62 (0.46–0.79)	0.228	17	17	29
UtA-PI MoM	0.65 (0.47–0.84)	0.134	28	28	48
BMI ≥ 30 kg/m^2					
sCD163 MoM	0.73 (0.54–0.92)	**0.042**	15	35	48
Insulin (raw)	0.83 (0.71–0.96)	**0.003**	55	56	66
TNFα MoM	0.77 (0.61–0.96)	**0.017**	33	33	38
PP13 MoM	0.68 (0.43–0.93)	0.111	8	8	12
PAPP A MoM	0.86 (0.74–0.98)	**0.001**	56	56	72
UtA-PI MoM	0.59 (0.40–0.78)	0.440	10	10	48

3B. **Multiple Markers**

Method of screening	AUC Mean (95% CI)	P	DR at 10% FPR	P
Model 1				
BMI	0.85 [0.75–0.95]	0.006	64%	0.04
BMI + sCD163	0.92 [0.79–0.96]	0.003	75%	0.008
BMI + sCD163+insulin	0.94 [0.82–0.97]	0.002	89%	0.004
BMI + sCD163+insulin + TNFα	0.95 [0.88–0.99]	<0.001	89%	0.003
BMI + sCD163+insulin + TNFα+PP13	0.95 [0.89–0.99]	<0.001	89%	<0.001
BMI + sCD163+insulin + TNFα+PP13+PAPP-A	0.95 [0.90–0.99]	<0.001	89%	<0.001
BMI + sCD163+insulin + TNFα+PP13+PAPP-A + UtA-PI	0.95 [0.91–1.00]	<0.001	89%	<0.001
Model 2				
sCD163	0.79 [0.61–0.83]	0.03	28%	0.045
sCD163+ insulin	0.87 [0.69–0.90]	0.009	47%	0.005
sCD163+ insulin + TNFα	0.90 [0.8–0.98]	0.004	64%	0.003
sCD163+ insulin + TNFα+ PP13	0.92 [0.82–0.98]	<0.001	82%	<0.001
sCD163+ insulin + TNFα+ PP13+PAPP-A	0.94 [0.85–0.99]	<0.001	83%	<0.001
sCD163+ insulin + TNFα+ PP13+PAPP-A + UtAPI	0.94 [0.88–0.99]	<0.001	83%	<0.001

CI - confidence interval, FPR-false positive rate.
Maternal risk factors (RF) for GDM (0–3): age >40 years, family history of diabetes (y/n), obesity- BMI >30 kg/h^2. Insulin was calculated as raw data. P values for AUC was calculated compared to the AUC = 0.5 of a random area (ROC curve drawn between the zero sensitivity and 100% specificity to 100% sensitivity and zero specificity. Markers are color coded in Fig. 2.
AUC values that were below 0.55 were not depicted in this table. Thus, the table neglect including values for sMR/CD206 and IL6 in the all GDM group analysis, sMR/CD206, leptin, IL6, MAP, maternal risk factors and baby gender in the subgroups, and UtA-PI for the non-obese subgroup.
AUC- area under the curve of Receiver Operating Characteristic (ROC) curves, DR-detection rate, FPR-false positive rate.
Combined analysis used curve smoothing and polynomial regression of $y = ax^3 + bx^2 + cx + d$ etc. Polynomial level was determined according to the highest regression coefficient. For the case of CD163 in model 2, the smoothing used moving average. Detection rates (DRs) were extracted from the ROC curves of Fig. 3 according to 10% FPR.
For obtaining the AUC for the ROC curves, values were compared to AUC = 0.5 (arbitrary).

and first trimester UtA-PI, MAP, PAPP-A, PLGF, and PP13 as effective biomarkers for predicting the risk to develop preterm pre-eclampsia. We have found that elevated UtA-PI, and reduced PAPP-A, and PP13 could be also useful in the early prediction of GDM. Hence, we suggest a contingency approach that when such changes are detected in the first trimester, the patients will be referred for prediction of GDM development using elevated sCD163 and insulin, and reduced TNFα, paving the way to the improved prediction of an additional major pregnancy complication.

4.3. Strength and weaknesses

The major strength of the study is the very well characterized cohort of patients included in the ASPRE trial and with prospective follow-up at delivery. Novel biomarkers were analysed using state of the art validated assays. A weakness is the small number of women developing GDM; however, we observed significant differences in specific biomarkers of insulin resistance and inflammation between women that subsequently develop GDM, which may provide the basis for validation in larger cohorts leading to early prediction of GDM and potential prevention by earlier life-style or anti-diabetic drug intervention [4,8,11]. We see this paper as a first report that warrant further studies, and we hope that co-authors who have accesses to larger patient clinics will embark on the

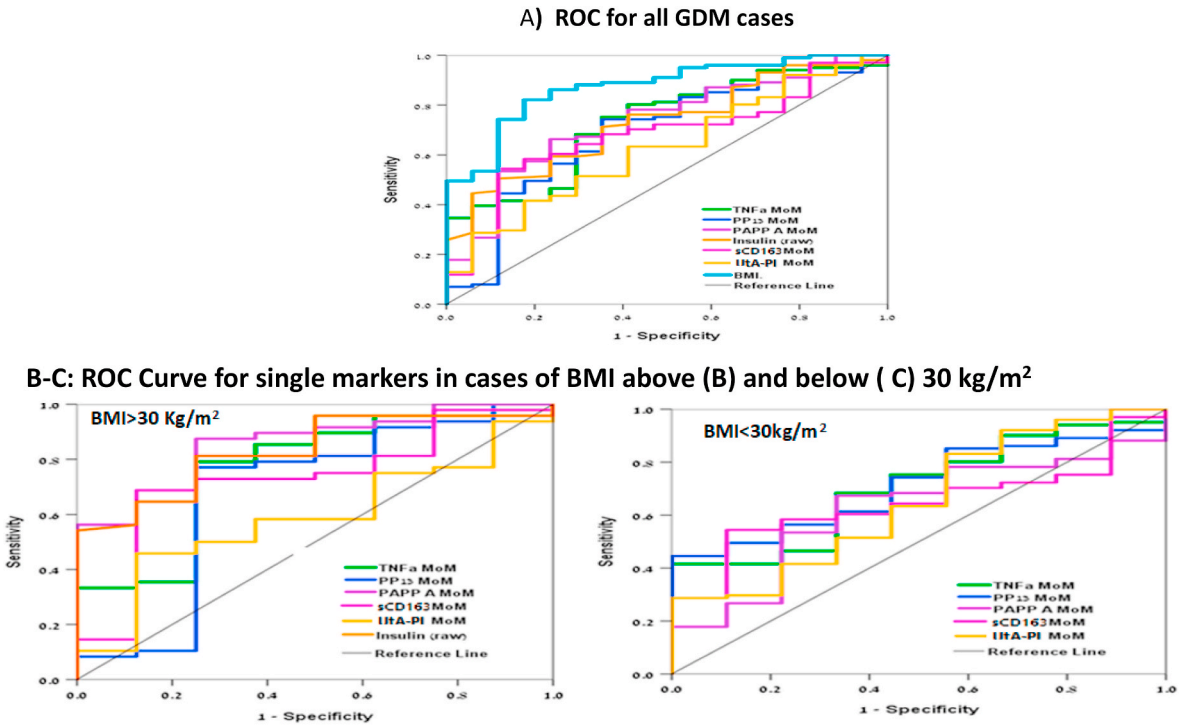

Fig. 2. Single Markers ROC Curves. The receiver operating characteristic (ROC) curves are shown for all cases (top), and separately for obese (BMI>30 kg/m^2) (bottom left) and non-obese (bottom right) GDM patients. Curves are depicted without smoothing. The 50% random area is drawn between the zero sensitivity and 100% specificity to 100% sensitivity and zero specificity. Markers are color coded presented in the figure. Only markers with AUC>0.55 are presented, which excluded sMR/CD206 and IL6 (Fig. 2) and sMR/CD206, leptin, IL6, MAP, UtA-PI, maternal risk factors and baby gender (Fig. 2B and C). . (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

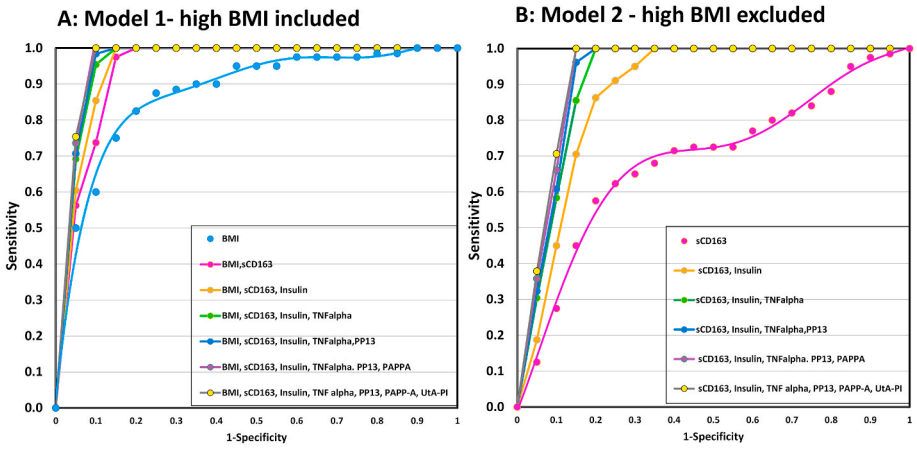

Fig. 3. Multiple Marker ROC curves. The ROC curves are shown for all cases after smoothing. Curve smoothing for combined analysis used polynomial regression of $y = ax^3 + bx^2 + cx + d$ or a lower polynomial level (selected according to the highest regression coefficient) except for the case of CD163 in model 2, where a moving average was used. Left – Model 1 that included high BMI. Right- Model 2 that excluded BMI. Figures are shown for the best prediction accuracy after testing any combination at any order. Combinations are color coded as presented in the figures. Detection rate (DR) and false positive rate (FPR) were extracted from the figures. The 50% random area is shown below the line connecting between the zero sensitivity and 100% specificity to 100% sensitivity and zero specificity. . (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

challenge. The possibility of introducing additional markers was suggested by Gabby-Benziv et al. [30] who used metabolic parameters (triglycerides, cholesterol, hemoglobin A1C, etc.) which might be further added to improve prediction accuracy. Sweeting et al. [55] published a multivariate model of first-trimester risk prediction for GDM development incorporating high BMI with novel maternal lipid markers, South/East Asian ethnicity, and history of previous GDM. Here leptin and PYY, which are other lipid markers, became insignificant when converted to MoM, possibly reflecting different nutrition habits that may influence the efficacy of using obesity markers for GDM prediction [43,44].

This study further emphasizes that there is a differential marker profile for different pregnancy complications. For example, PLGF, that is so powerful in first trimester prediction of preterm pE is not useful in first trimester prediction of GDM.

5. Conclusion

GDM is a major pregnancy complication, and its incidence is increasing [1,56]. This has numerous implications for adult chronic illnesses and poses a risk to the offspring. The current study suggests efficient prediction of GDM development already in the first trimester and identifies a multi-marker approach consisted of BMI and markers of insulin resistance and inflammation, that warrants validation in a larger independent cohort. If confirmed, we suggest an expansion of the first trimester inverted pyramid model [57] to also include the prediction of GDM – a major pregnancy complication, thereby expanding the personalized approach to first trimester screening and prevention.

Authors' contribution

This study is an extension of the ASPRE project (FP7 # 601852 grant) initiated by Kypros Nicolaides and Hamutal Meiri, who were engaged in the fundraising for this project, preparing the clinical study protocol and project development, data management, data analysis, manuscript writing, and editing. Dr. Meiri further coordinated the PP13 testing performed at Hy-Laboratories, Israel during the ASPRE project.

Kinneret Tenenbaum Gavish was the Clinical Director of Rabin Medical Center clinical site in the ASPRE study. Together with Eran Hadar, David Danon, Ana Idelson and Lihi Rothman, she and her partners adapted the protocol to the clinical study according to the ethical requirements in Israel, obtained the ethical approval of the Rabin Medical Center IRB and the national committee, obtained informed consent from the enrolled pregnant women, built the database, collected all the clinical and demographic data at enrollment and after delivery, evaluated the biophysical parameters (CRL, MAP, UtA-PI, etc.), and drew the patient blood samples, that were processed and measured locally for the biomarkers during ASPRE or stored in the Bio Bank and retrieved for this study.

Henning Gronbaek initiated the introduction and testing of sCD163 and sMR/CD206 as candidate novel biomarkers for first trimester prediction of GDM. Together with Holger Jon Møller they conducted the marker testing on coded samples. Subsequently, Dr. Gronbaek participated in all stages of building the database, analysis and manuscript writing.

Omry Koren and Dana Benyamin initiated and designed the testing of the other serum markers including Insulin, TNFα, IL6, PYY and leptin as potential markers of GDM. Testing was conducted by Dana Benyamin. They were further involved in the data analysis, manuscript writing and editing.

Ida Vogel initiated the statistical data analysis, and the use of SPSS software that was further expanded by Adi Sharhabi-Nov, who performed the data cleaning, organization, further evaluated the MoMs, AU, etc., and conducted the remaining analyses.

All authors approved the final version of the manuscript.

Compliance with ethical standards

All the procedures performed here with human specimens were conducted in accordance with the ethical standards of the institutional and/or national research committees of the countries involved and complied with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Upon recruitment to the study, all women signed an informed consent and the study received approval from the Rabin Medical Center Institutional Review Board (#0066-14-RMC of March 02, 2014) and the National Ethics Committee (20140059 of May 01, 2014).

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Declaration of competing interest

Hamutal Meiri holds partial patent rights for using placental protein 13 as a drug to fight pre-eclampsia. All the other co-authors declare no conflict of interest.

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