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Original Article

Screening of gestational diabetes mellitus in early pregnancy by oral glucose tolerance test and novel biomarkers: study protocol for an international, prospective, multi-centre cohort trial.

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Trial was registered under www.ClinicalTrials.gov under NCT02035059 on the 12th January 2014.

Conflict of interest

The authors declare that there are no further financial or personal relationships with other people or organizations that could inappropriately influence the work reported or the conclusions, implications, or opinions stated.

KEY WORDS: Gestational diabetes mellitus, diagnosis, screening, oral glucose tolerance test, glycosylated fibronectin, pregnancy.

Short title: Screening of gestational diabetes mellitus in early pregnancy by oral glucose tolerance test and biomarkers.

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ABSTRACT

INTRODUCTION: As the accurate diagnosis and treatment of gestational diabetes mellitus (GDM) is of increasing importance, new diagnostic approaches for the assessment of GDM in early pregnancy were recently suggested. We evaluate the diagnostic power of an "early" oral glucose tolerance test (OGTT) 75g and a promising new biomarker, glycosylated Fibronectin (glyFn), for GDM screening in a normal cohort.

METHODS AND ANALYSIS: In a prospective cohort study, 531 singleton pregnancies are recruited in six centres in Switzerland, Austria and Germany. Women are screened for pre-existing diabetes mellitus and GDM by an "early" OGTT 75g and/or the new biomarker, glyFn, at 12 to 15 weeks of gestation. Different screening strategies are compared to evaluate the impact on detection of GDM by an OGTT 75g at 24 to 28 weeks of gestation as recommended by the International Association of Diabetes and Pregnancy Study Groups (IADPSG). A new screening algorithm is created by using multivariable risk estimation based on "early" OGTT 75g and/or glyFn results, incorporating maternal risk factors. Recruitment began in May 2014.

ETHICS AND DISSEMINATION: This study received ethical approval from the ethics committees in Basel, Zurich, Vienna, Salzburg and Freiburg. It was registered under www.ClinicalTrials.gov (NCT02035059) on 12th January 2014. Data will be presented at international conferences and published in peer-reviewed journals.

STRENGTHS AND LIMITATION OF THIS STUDY

- This is an international, prospective, multi-centre cohort trial recruiting at six centres in Switzerland, Austria and Germany.
- It is the first study to assess an "early" OGTT 75g and novel biomarkers like glyFn for screening of gestational diabetes mellitus in early pregnancy.
- The recruitment of 531 pregnant women are planned and we have designed the study to be sufficiently powered to compare the different early screening approaches with the detection of gestational diabetes mellitus at 24 to 28 weeks of gestation.
- This study may be underpowered for the evaluation of neonatal outcomes like LGA infants, neonatal hypoglycaemia, shoulder dystocia or birth trauma (secondary outcomes).

INTRODUCTION

Gestational diabetes mellitus (GDM) is defined as "any degree of glucose intolerance that was first recognized during pregnancy"[1] regardless of whether or not the condition predated the pregnancy or persisted after pregnancy.[2] The increasing number of women with undiagnosed type 2 diabetes mellitus (T2DM) in pregnancy has led to the recommendation of screening women with risk factors for pre-existing diabetes at the first antenatal visit. GDM is still diagnosed in the late second or early third trimester, because accurate diagnostic approaches for GDM assessment in first trimester are still lacking.[3]

GDM is associated with adverse maternal and perinatal outcomes, such as fetal overgrowth, shoulder dystocia, operative delivery, birth injury, preeclampsia, haemorrhage and preterm delivery,[4–6] but also a seven fold higher risk of the mother developing T2DM after pregnancy.[7] In addition, the maternal metabolic milieu was also identified as a key determinant for the susceptibility to obesity, metabolic syndrome and T2DM in the offspring,[8] a phenomenon often described as "fetal programming".

The current – but still widely discussed – standard of care in GDM screening is the oral glucose tolerance test (OGTT) of 75g glucose performed late at 24 to 28 weeks of gestation as recommended by the International Association of Diabetes and Pregnancy Study Groups (IADPSG).[9] The new screening thresholds are based on the results of a large prospective cohort multicentre trial, the Hyperglycaemia and Adverse Pregnancy Outcome (HAPO) study.[5] The aim of the HAPO study

was to associate the degree of maternal glycaemia with adverse perinatal outcome, such as large for gestational age infants (LGA), neonatal hypoglycaemia and caesarean section rates. The results showed no obvious threshold, but rather a continuous increase of these adverse outcomes across the range of glucose concentrations. The IADPSG criteria resulted in a considerable increase in GDM prevalence of 17.8%, a detection rate of 83% for adverse outcome and a positive predictive value of 16%.[9]

An early and rapid diagnosis of GDM even before 24 weeks of gestation is desirable. By targeted early intervention including physical activity, moderate diet or insulin/drug therapy starting in the first trimester, rates of macrosomia (birth weight > 4000 g) or large for gestational age (LGA=birth weight > 90th percentile) infants, operative vaginal delivery and perinatal morbidity could be possibly reduced. Moreover, there could be a long term downstream effect on the offspring, thereby leading to considerable savings in healthcare costs by possibly decreased prevalence of generational transmission of metabolic diseases.

We propose that an "early" OGTT combined with maternal history, maternal condition and promising new biomarkers such as glycosylated fibronectin (glyFn) could diagnose similarly GDM, even in first trimester. Rasanen et al. published a study in September 2013 introducing glyFn as a new early GDM screening approach with an area under the curve (AUC) of 0.91 and a 95% confidence interval (CI) of 0.87-0.96, a positive predictive value of 63% and a negative predictive value of 95%. Although

some predictors of GDM have been studied retrospectively, no study to date has considered the use of promising new biomarkers combined with an "early" OGTT and maternal risk factors evaluation in first trimester of pregnancy.

STUDY OBJECTIVES

Primary objective

The use of the "early" OGTT 75g and/or the new biomarker, glyFn, as a new screening approach in late first/early second trimester will be evaluated and compared to GDM diagnosis by OGTT 75g at 24 to 28 weeks of gestation in a normal cohort.

Secondary objectives

1. A new screening algorithm will be created by using multivariable risk estimation based on "early" OGTT 75g and/or glyFn results, incorporating maternal risk factors.
2. The significance of the association between glyFn, "early" OGTT 75g and maternal body mass index and/or clinical conditions including chronic hypertension, pregnancy-induced hypertension or preeclampsia and fetal conditions such as intrauterine growth restriction will be evaluated.

METHODS

Study settings/design

This is an international, prospective, multi-centre cohort trial conducted at one secondary and five tertiary referral centres in Switzerland, Austria and Germany. Study recruitment commenced primarily at the coordination centre at University Hospital Basel on 1st May 2014. All other centres started recruitment consecutively until the end of March 2016. Recruitment is expected to last until December 2017. The aim is to enroll 531 women at 12 to 15 weeks of gestation with a minimum recruitment of 50 women planned for each centre.

Recruitment and informed consent

Participants are identified at their first antenatal visit between 6 to 15 weeks of gestation. The investigator or obstetrician in charge informs the women about all aspects pertaining to the trial. The informed consent includes permission for gathering data from medical records and the optional storage of blood for a maximum of 10 years for additional analyses related to the current study. Participants are informed that trial participation is voluntary and that they are free to withdraw without any effects on subsequent care. All members of the research team are aware of the guidelines for good clinical practice for obtaining consent.[10]

Eligibility criteria

Inclusion criteria are:

- women at least 18 years of age and not under guardianship

- healthy singleton pregnancy after spontaneous conception or after fertility treatment
- 6-15 weeks of gestation
- signed informed consent

Exclusion criteria are:

- previous bariatric surgery
- known pre-existing diabetes mellitus or under treatment with metformin
- known chronic infection like hepatitis or human immunodeficiency virus or chronic kidney, liver or heart disease
- known maternal history of hypertensive diseases in a previous pregnancy and now under prophylactic acetylsalicylate treatment
- fetal genetic, chromosomal or intervention-requiring morphologic abnormalities
- the inability to read and/or understand the participant`s information sheet

Study procedure

All healthy pregnant patients with regular care at the participating hospitals are counselled and asked at 6 to 15 weeks of gestation to participate. At 11+0 to 13+6 weeks of gestation, all women have a first trimester ultrasound scan which is standard care at participating sites. The ultrasound scan is used to confirm gestational age, diagnose any major foetal abnormalities, and optionally measure foetal nuchal translucency

thickness, which together with maternal free beta-chorionic gonadotropin and pregnancy-associated plasma protein A, is used for screening for chromosomal abnormalities. In addition, if informed consent had been given during the first antenatal visit or at the time of the first trimester scan, the maternal history and condition are assessed, and blood for biomarker analysis and for the "early" OGTT 75g is drawn at the study visit at 12 to 15 weeks of gestation. The "early" OGTT 75g is compared to plasma glucose results obtained at 24 to 28 weeks of gestation after the OGTT 75g. No additional visit is necessary beyond the further standard routine antenatal care visits.

GlyFn and "early" OGTT 75g

All participants are instructed to fast for at least 10 hours. Two fasting glucose samples are taken. One sample is collected for storage of two aliquots (2x 1ml) at -80 °C for later analysis of glyFn and another sample for the fasting glucose value. After intake of the 75g glucose load, blood samples are drawn 60 and 120 minutes later for determination of glucose levels. Plasma glucose is measured by an automated colorimetric-enzymatic method (hexokinase/glucose-6-phosphate-dehydrogenase) on a Hitachi/Roche-Modular P analyser. GlyFn will be analysed as previously reported by Rasanen et al.[11] by DiabetOmics Inc., Beaverton, Oregon, USA. The maternal glyFn and "early" OGTT 75g results are blinded to the investigators.

Un-blinding

Values will be un-blinded if fasting glucose levels are ≥ 7.0mmol/L or 2-hour plasma glucose levels are ≥ 11.1mmol/L, suggesting pre-existing diabetes mellitus. The diagnosis of pre-existing diabetes mellitus needs to be confirmed by an elevated glycosylated haemoglobin A1c (HbA1c) value ≥ 6.5%. Plasma glucose levels of ≤ 2.5mmol/L are also abnormal and requires further clarification. Women with confirmed pre-existing diabetes mellitus are treated according to a standardised protocol in line with current recommendations.

Study outcomes

Diagnosis of GDM

All women who screen positive with OGTT 75 g at 24 to 28 weeks of gestation are followed up by a nutritionist and a diabetic nurse in contact with a diabetologist, and have frequent regular appointments in our obstetrical outpatient clinic in 2 to 4 week intervals depending on clinical condition, glucose values and ultrasound findings. Women who fail to meet the target glucose values after 1 to 2 weeks of diet management are treated with insulin according to the guidelines of the Swiss Society for Endocrinology and Diabetology (SGED), the Austrian Diabetes Association (ÖDG), the German Diabetes Association (DDG) and the German Association of Gynaecology and Obstetrics (DGGG).[12–14] The glycaemic targets, insulin therapy, dose adjustments, concomitant medication and/or supplements are recorded.

Pregnancy, delivery and neonatal outcome data

Maternal data such as preeclampsia, pregnancy induced hypertension, rate of sonographic estimated polyhydramnios or macrosomia, delivery outcome including delivery mode and indication, and neonatal outcome data such as birth weight, preterm birth, 5 and 10 minute Apgar scores, arterial umbilical cord pH $\leq$ 7.0, shoulder dystocia, birth trauma, hypoglycaemia, jaundice, respiratory distress syndrome, congenital anomalies and admission to intensive care unit are prospectively collected.

Statistics

Sample size justification

We aim to demonstrate that the recently reported biomarker, glyFn and/or the "early" OGTT 75g at 12 to 15 weeks of gestation has sufficient diagnostic power to evaluate women at risk of developing GDM compared to the OGTT 75g at 24 to 28 weeks of gestation. The sample size calculation is based on a test for the ROC (receiver operator characteristic) curve of glyFn by Rasanen et al. 2013.[11] The OGTT 75g screening test using the IADPSG criteria has not been tested in early pregnancy so far. We assume that 0.5-2% of recruited women will be diagnosed with pre-existing diabetes mellitus by the "early" OGTT 75g. The prevalence of GDM is assumed to be 17.8% according to the HAPO study.[5] A proposed true area under the curve of 0.9 with a lower boundary of 0.8 would lead with a power of 90% and an α-level of 5% to an

estimated sample size of 462 (66 women with GDM, 396 women without GDM). Offsetting a dropout of 15%, this leads to a total sample size of 531. The power calculation was performed using MedCalc version 12.7 2013.[15]

Data analysis plan

Descriptive statistics and graphical examination will be performed for all primary and secondary study variables.

Primary objective: In order to predict GDM, ROC curves with corresponding AUCs will be calculated separately for glyFn and the "early" OGTT 75g. GDM diagnosis by OGTT 75g at 24 to 28 weeks of gestation is considered as routine method. AUCs will be estimated with a 95% confidence interval (CI). It will be hypothesized that a 95% CI of the AUC of glyFn > 0.8.

Secondary objectives: Logistic regression will allow combining glyFn and the "early" OGTT in a multivariable risk model. Subsequent AUC will be calculated with 95% CI. Results will be internally cross-validated to prevent overoptimistic results. Optimal cut-off points to predict GDM will be determined based on these ROC curve. Sensitivity, specificity, positive and negative predictive value will also be estimated with 95% CI. However other "machine learning" algorithms could be better than logistic regression. Therefore, other popular procedures will be additionally tested: Random Forest, penalized logistic regression (Lasso). Details are described in Hastie et al..[16] Predictive performance will be internally cross-

validated and explored in examining AUC of the ROC and predicted vs. observed probabilities. Random forest and penalized logistic regression avoid overfitting (to certain extent) whereas logistic regression does not. Random forest will be chosen because of its popularity and good benchmark results. Lasso is known for its good interpretability. Logistic regression will probably show its inferiority compared to the other methods. Internal cross-validation will be done using the package "mlr" within R. Internal cross-validation (e.g. 10-fold) is a good possibility to estimate the fitting on a potential future dataset. Based on these results it will be decided whether a new prediction model for GDM will be proposed. In order to potentially improve the primary study variables, secondary study parameters such as changes in maternal body mass index and/or clinical conditions like chronic hypertension and pregnancy induced hypertension or preeclampsia and fetal conditions including intrauterine growth restriction will be added to the machine learning models.

Statistical analyses and graphs will be performed using the current version of the statistical computation program R.[17]

Data recording

Each participant receives an identification number to ensure confidentiality and the collected data is exchanged between centres using only the identification number. The name and birth date of each participant are stored with a different identification number in order to preserve the possibility to look for inconsistencies during the study.

Reporting of adverse events

This study is a low risk trial. Any (serious) adverse events (AE/SAE) related to the first additional "early" OGTT 75g or the blood sampling are recorded by the investigator using the specific AE/SAE sheet of the clinical report form (CRF). All SAE are reported to the responsible ethics committee within an appropriate time frame.

ETHICS AND DISSEMINATION

The study is conducted in accordance with the "Helsinki Declaration" 1996. It was approved by each local institutional ethical board in Basel, Zurich, Freiburg, Salzburg and Vienna. Furthermore, written informed consent is obtained from each participant. All findings will be disseminated through presentations at national and international conferences and publications in peer-reviewed journals. The trial was registered under www.ClinicalTrials.gov under NCT02035059 on 12th January 2014.

OTHER STUDY MEASUREMENTS

Other biomarkers

An extensively studied biomarker for early GDM screening is adiponectin.[18–20] Adiponectin is an adipocyte-derived hormone and reflects whole body insulin sensitivity.[21] A recently published meta-analysis calculated a summary sensitivity of 60.3% and a specificity of 81.3% with an AUC of 0.79.[19] Maternal serum adiponectin concentration is measured

by a quantitative sandwich enzyme-linked immunosorbent assay (ELISA) technique. Another potential biomarker is pregnancy-specific glycoprotein-1 (PSG-1).[22] PSG-1 had a detection rate of 74% with a false positive rate of 6% and an AUC of 0.81. PSG-1 is analysed as previously reported by Nagalla et al.[22] by DiabetOmics Inc., Beaverton, Oregon, USA.

Evaluation of insulin and HbA1c

At the early study visit at 12 to 15 weeks of gestation, insulin, c-peptide and HbA1c are additionally measured from the fasting blood sample in all women. HbA1c is measured by reversed-phase cation exchange chromatography (ADAMS HA-8160, Menarini Diagnostics Benelux, Zaventem, Belgium) or high performance liquid chromatography (HPLC) with Variant II, Bio-Rad (IFCC standardized and DCCT aligned with CV 1.8% for HbA1c=5.6%). Insulin is measured by chemiluminescence immunoassay CLIA (Roche Modular E170, Basel, Switzerland). The homeostasis model assessment of insulin resistance (HOMA-IR) or the Quantitative Insulin Sensitivity Check Index (QUICKI) is used as an approximate of fasting (i.e. hepatic) insulin resistance.[23,24] Beta-cell function is assessed from fasting glucose and insulin levels according to Wallace et al. 2004.[23]

Measurement of Vitamin D

25 OH Vitamin D seems to have an association with glucose metabolism and influences insulin secretion and sensitivity in type 1 diabetes.[25] It

additionally leads to an immunologic reaction resulting into type 2 diabetes.[25] We would like to have actual values for 25 OH Vitamin D and it will be evaluated in this cohort by CLIA (Liaison, DiaSorin, Saluggia, Italy).

Influence of stress in early pregnancy on development of GDM

Psychological factors in early pregnancy might contribute to adverse obstetric outcome.[26] This trial investigates the influence of perceived stress, stressful life events and depression on the development of GDM. The participants recruited in Basel are therefore asked to collect salivary samples for cortisol directly at time of awakening, at 30 and at 60 minutes after awakening. The saliva samples are stored at -20 °C until analysis. After thawing, saliva samples are centrifuged. Cortisol levels are determined employing a competitive solid phase time-resolved fluorescence immunoassay with fluorometric end point detection (DELFIA®).[27] Copeptin is a more stable precursor hormone of arginine-vasopressin and is found to be elevated in many diseases.[28–31] This effect can be attributed to the response of the hypothalamic-pituitary-adrenal axis to psychological stress. Copeptin will be measured in the fasting blood sample with time-resolved amplified cryptate emission technology (TRACE™) by BRAHMS Kryptor Compact immunoanalyzer by Thermo Scientific Brahms GmbH, Henningsdorf, Germany.

Three self-administered questionnaires are obtained at the study visit at 12 to 15 weeks of gestation:

Questionnaire on perceived stress

The perceived stress scale is a 10-items inventory for measuring the perception of stress.[32] It is widely used and has been validated in pregnancy.[33]

Questionnaire on stressful life events

The Holmes and Rahe Stressful Life Events Scale (SLE)[34] is an 43-items instrument for evaluation of stressful experiences in the past 12 months prior to the answering the questionnaire.

Questionnaire on depression

The ten-items Edinburgh Postnatal Depression Screen (EPDS)[35] is used to assess symptoms of depression during the past seven days. It has been widely validated in pregnancy[36,37] and it is part of the standard evaluation of pregnant women attending routine prenatal visits at the University Hospital in Basel. Scores ≥13 indicate at least probable minor depression and scores ≥15 indicated probable major depression. Pregnant women with scores ≥13 are routinely offered psychological or psychiatric counselling during pregnancy.

Histologic examination of the placenta

The fetal nutrients supply is regulated by maternal-foetal glucose and lipid concentration, placental blood flow and trophoblastic nutrient transporters.[38] The placenta reacts with adaptive changes in structure and function to a hyperglycaemic milieu.[39] These changes of the placenta will be assessed depending on the maternal glycaemic control by standard pathology examination.[40]

DISCUSSION

To our knowledge, this is the first large European cohort study that prospectively evaluates the promising new biomarker, glyFn, and the "early" OGTT 75g by comparing the impact of different GDM screening strategies with IADPSG criteria at 24 to 28 weeks of gestation. Our hypothesis is that glyFn and/or the "early" OGTT 75g should at least result in an AUC of 0.8 compared to the OGTT 75g in later pregnancy. A multivariable prediction model incorporating risk factors, glyFn and/or "early" OGTT values might have a good predictive accuracy for the development of GDM and could facilitate early universal screening or help improve risk stratification for GDM. Rasanen et al.[11] could show that glyFn was independent of maternal age, parity, gestational age, time of sample collection and the administration of the OGTT at 24 to 28 weeks of gestation. Our trial might clarify whether glyFn is depending on maternal body mass index or clinical conditions such as chronic hypertension, pregnancy induced hypertension or the development of preeclampsia or intrauterine growth restriction. GlyFn can be analyzed out of a dried blood

stain and the resulting test is affordable, which would help especially underdeveloped countries that suffer disparities in diabetes care, to benefit from a cheap screening tool for GDM.[41]

One limitation of our trial is that it is not powered for the evaluation of neonatal outcomes like LGA infants, neonatal hypoglycaemia, shoulder dystocia or birth trauma (these are planned secondary outcomes). The study would need to be significantly larger to detect effects on these neonatal outcomes. If this trial were to show positive diagnostic power for the new screening algorithm, a large multi-centre study may be required to be sufficiently powered to determine the algorithm`s effect on rate of LGA infants or neonatal hypoglycaemia as proposed in the HAPO study.[5]

The results of our study may have a major impact on future screening approaches for GDM by development of a simple, cost effective and for the pregnant women comfortable screening method for GDM in first trimester.

List of abbreviations

AE, adverse events; AUC, area under the curve; BMI, body mass index; CRF, clinical report form; GDM, gestational diabetes mellitus; GlyFn, glycosylated Fibronectin; HOMA-IR, homeostasis model assessment of insulin resistance; IADPSG, International Association of Diabetes and Pregnancy Study Groups; LGA, large for gestational age; OGTT, oral glucose tolerance test; QUICKI, quantitative insulin sensitivity check index; ROC, receiver operator characteristic; SAE, serious adverse events.

Disclosure of interests

The authors declare that there are no further financial or personal relationships with other people or organizations that could inappropriately influence the work reported or the conclusions, implications, or opinions stated.

Contribution to authorship

EH is principal investigator, study protocol author, obtained ethical approval and drafted this manuscript. IH is the sponsor, assisted with the original study protocol and revised the manuscript. SH and EBR designed the study together with EAH and revised the manuscript. AS performed the power calculation and statistical planning. TF, CSG, WE, PH, MK, NO, EBAE, HP, MT, MK, RZ made important contributions and critically reviewed the content. All authors have given final approval of the version to be published.

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the data acquisition. Special thanks to all families who participate in this study.

Details of ethics approval

The study was approved by each local institutional review board in Basel (Ethikkommission Nordostschweiz (EKNZ)), Zurich, Freiburg, Salzburg and Vienna. The trial was registered under www.ClinicalTrials.gov under NCT02035059 on 12th January 2014.

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SPIRIT 2013 Checklist: Recommended items to address in a clinical trial protocol and related documents*

Section/item	Item No	Description
Administrative information		
Title	1	Descriptive title identifying the study design, population, interventions, and, if applicable, trial acronym
Trial registration	2a	Trial identifier and registry name. If not yet registered, name of intended registry
	2b	All items from the World Health Organization Trial Registration Data Set
Protocol version	3	Date and version identifier
Funding	4	Sources and types of financial, material, and other support
Roles and responsibilities	5a	Names, affiliations, and roles of protocol contributors
	5b	Name and contact information for the trial sponsor
	5c	Role of study sponsor and funders, if any, in study design; collection, management, analysis, and interpretation of data; writing of the report; and the decision to submit the report for publication, including whether they will have ultimate authority over any of these activities
	5d	Composition, roles, and responsibilities of the coordinating centre, steering committee, endpoint adjudication committee, data management team, and other individuals or groups overseeing the trial, if applicable (see Item 21a for data monitoring committee)
Introduction		
Background and rationale	6a	Description of research question and justification for undertaking the trial, including summary of relevant studies (published and unpublished) examining benefits and harms for each intervention
	6b	Explanation for choice of comparators
Objectives	7	Specific objectives or hypotheses
Trial design	8	Description of trial design including type of trial (eg, parallel group, crossover, factorial, single group), allocation ratio, and framework (eg, superiority, equivalence, noninferiority, exploratory)

Methods: Participants, interventions, and outcomes

Study setting	9	Description of study settings (eg, community clinic, academic hospital) and list of countries where data will be collected. Reference to where list of study sites can be obtained
Eligibility criteria	10	Inclusion and exclusion criteria for participants. If applicable, eligibility criteria for study centres and individuals who will perform the interventions (eg, surgeons, psychotherapists)
Interventions	11a	Interventions for each group with sufficient detail to allow replication, including how and when they will be administered
	11b	Criteria for discontinuing or modifying allocated interventions for a given trial participant (eg, drug dose change in response to harms, participant request, or improving/worsening disease)
	11c	Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (eg, drug tablet return, laboratory tests)
	11d	Relevant concomitant care and interventions that are permitted or prohibited during the trial
Outcomes	12	Primary, secondary, and other outcomes, including the specific measurement variable (eg, systolic blood pressure), analysis metric (eg, change from baseline, final value, time to event), method of aggregation (eg, median, proportion), and time point for each outcome. Explanation of the clinical relevance of chosen efficacy and harm outcomes is strongly recommended
Participant timeline	13	Time schedule of enrolment, interventions (including any run-ins and washouts), assessments, and visits for participants. A schematic diagram is highly recommended (see Figure)
Sample size	14	Estimated number of participants needed to achieve study objectives and how it was determined, including clinical and statistical assumptions supporting any sample size calculations
Recruitment	15	Strategies for achieving adequate participant enrolment to reach target sample size

Methods: Assignment of interventions (for controlled trials)

Allocation:

Sequence generation	16a	Method of generating the allocation sequence (eg, computer-generated random numbers), and list of any factors for stratification. To reduce predictability of a random sequence, details of any planned restriction (eg, blocking) should be provided in a separate document that is unavailable to those who enrol participants or assign interventions

Allocation concealment mechanism	16b	Mechanism of implementing the allocation sequence (eg, central telephone; sequentially numbered, opaque, sealed envelopes), describing any steps to conceal the sequence until interventions are assigned
Implementation	16c	Who will generate the allocation sequence, who will enrol participants, and who will assign participants to interventions
Blinding (masking)	17a	Who will be blinded after assignment to interventions (eg, trial participants, care providers, outcome assessors, data analysts), and how
	17b	If blinded, circumstances under which unblinding is permissible, and procedure for revealing a participant's allocated intervention during the trial
Methods: Data collection, management, and analysis		
Data collection methods	18a	Plans for assessment and collection of outcome, baseline, and other trial data, including any related processes to promote data quality (eg, duplicate measurements, training of assessors) and a description of study instruments (eg, questionnaires, laboratory tests) along with their reliability and validity, if known. Reference to where data collection forms can be found, if not in the protocol
	18b	Plans to promote participant retention and complete follow-up, including list of any outcome data to be collected for participants who discontinue or deviate from intervention protocols
Data management	19	Plans for data entry, coding, security, and storage, including any related processes to promote data quality (eg, double data entry; range checks for data values). Reference to where details of data management procedures can be found, if not in the protocol
Statistical methods	20a	Statistical methods for analysing primary and secondary outcomes. Reference to where other details of the statistical analysis plan can be found, if not in the protocol
	20b	Methods for any additional analyses (eg, subgroup and adjusted analyses)
	20c	Definition of analysis population relating to protocol non-adherence (eg, as randomised analysis), and any statistical methods to handle missing data (eg, multiple imputation)
Methods: Monitoring		
Data monitoring	21a	Composition of data monitoring committee (DMC); summary of its role and reporting structure; statement of whether it is independent from the sponsor and competing interests; and reference to where further details about its charter can be found, if not in the protocol. Alternatively, an explanation of why a DMC is not needed

	21b	Description of any interim analyses and stopping guidelines, including who will have access to these interim results and make the final decision to terminate the trial
Harms	22	Plans for collecting, assessing, reporting, and managing solicited and spontaneously reported adverse events and other unintended effects of trial interventions or trial conduct
Auditing	23	Frequency and procedures for auditing trial conduct, if any, and whether the process will be independent from investigators and the sponsor
Ethics and dissemination		
Research ethics approval	24	Plans for seeking research ethics committee/institutional review board (REC/IRB) approval
Protocol amendments	25	Plans for communicating important protocol modifications (eg, changes to eligibility criteria, outcomes, analyses) to relevant parties (eg, investigators, REC/IRBs, trial participants, trial registries, journals, regulators)
Consent or assent	26a	Who will obtain informed consent or assent from potential trial participants or authorised surrogates, and how (see Item 32)
	26b	Additional consent provisions for collection and use of participant data and biological specimens in ancillary studies, if applicable
Confidentiality	27	How personal information about potential and enrolled participants will be collected, shared, and maintained in order to protect confidentiality before, during, and after the trial
Declaration of interests	28	Financial and other competing interests for principal investigators for the overall trial and each study site
Access to data	29	Statement of who will have access to the final trial dataset, and disclosure of contractual agreements that limit such access for investigators
Ancillary and post-trial care	30	Provisions, if any, for ancillary and post-trial care, and for compensation to those who suffer harm from trial participation
Dissemination policy	31a	Plans for investigators and sponsor to communicate trial results to participants, healthcare professionals, the public, and other relevant groups (eg, via publication, reporting in results databases, or other data sharing arrangements), including any publication restrictions
	31b	Authorship eligibility guidelines and any intended use of professional writers
	31c	Plans, if any, for granting public access to the full protocol, participant-level dataset, and statistical code

Appendices

Informed consent materials	32	Model consent form and other related documentation given to participants and authorised surrogates
Biological specimens	33	Plans for collection, laboratory evaluation, and storage of biological specimens for genetic or molecular analysis in the current trial and for future use in ancillary studies, if applicable

*It is strongly recommended that this checklist be read in conjunction with the SPIRIT 2013 Explanation & Elaboration for important clarification on the items. Amendments to the protocol should be tracked and dated. The SPIRIT checklist is copyrighted by the SPIRIT Group under the Creative Commons "Attribution-NonCommercial-NoDerivs 3.0 Unported" license.

BMJ Open

Screening of gestational diabetes mellitus in early pregnancy by oral glucose tolerance test and glycosylated fibronectin: study protocol for an international, prospective, multi-centre cohort trial.

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Keywords:	gestational diabetes mellitus, diagnosis, screening, oral glucose tolerance test, glycosylated fibronectin, pregnancy

Original Article

Screening of gestational diabetes mellitus in early pregnancy by oral glucose tolerance test and glycosylated fibronectin: study protocol for an international, prospective, multi-centre cohort trial.

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Trial was registered under www.ClinicalTrials.gov under NCT02035059 on the 12th January 2014.

Conflict of interest

The authors declare that there are no further financial or personal relationships with other people or organizations that could inappropriately influence the work reported or the conclusions, implications, or opinions stated.

KEY WORDS: Gestational diabetes mellitus, diagnosis, screening, oral glucose tolerance test, glycosylated fibronectin, pregnancy.

Short title: Screening of gestational diabetes mellitus in early pregnancy by oral glucose tolerance test and glycosylated fibronectin.

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ABSTRACT

INTRODUCTION: As the accurate diagnosis and treatment of gestational diabetes mellitus (GDM) is of increasing importance, new diagnostic approaches for the assessment of GDM in early pregnancy were recently suggested. We evaluate the diagnostic power of an "early" oral glucose tolerance test (OGTT) 75g and glycosylated Fibronectin (glyFn) for GDM screening in a normal cohort.

METHODS AND ANALYSIS: In a prospective cohort study, 748 singleton pregnancies are recruited in six centres in Switzerland, Austria and Germany. Women are screened for pre-existing diabetes mellitus and GDM by an "early" OGTT 75g and/or the new biomarker, glyFn, at 12 to 15 weeks of gestation. Different screening strategies are compared to evaluate the impact on detection of GDM by an OGTT 75g at 24 to 28 weeks of gestation as recommended by the International Association of Diabetes and Pregnancy Study Groups (IADPSG). A new screening algorithm is created by using multivariable risk estimation based on "early" OGTT 75g and/or glyFn results, incorporating maternal risk factors. Recruitment began in May 2014.

ETHICS AND DISSEMINATION: This study received ethical approval from the ethics committees in Basel, Zurich, Vienna, Salzburg and Freiburg. It was registered under www.ClinicalTrials.gov (NCT02035059) on 12th January 2014. Data will be presented at international conferences and published in peer-reviewed journals.

STRENGTHS AND LIMITATION OF THIS STUDY

- This is an international, prospective, multi-centre cohort trial recruiting at six centres in Switzerland, Austria and Germany.
- It is the first study to assess an "early" OGTT 75g and novel biomarkers like glyFn for screening of gestational diabetes mellitus in early pregnancy.
- The recruitment of 748 pregnant women is planned. We have designed the study to be sufficiently powered to compare the different early screening approaches with the detection of gestational diabetes mellitus at 24 to 28 weeks of gestation.
- This study may be underpowered for the evaluation of neonatal outcomes like LGA infants, neonatal hypoglycaemia, shoulder dystocia or birth trauma (secondary outcomes).

INTRODUCTION

Gestational diabetes mellitus (GDM) is defined as diabetes diagnosed during pregnancy that is not clearly overt diabetes.[1] The increasing number of women with undiagnosed type 2 diabetes mellitus (T2DM) in pregnancy has led to the recommendation of screening women with risk factors for pre-existing diabetes at the first antenatal visit. GDM is still diagnosed in the late second or early third trimester, because accurate diagnostic approaches for GDM assessment in first trimester are still lacking.[2]

GDM is associated with adverse maternal and perinatal outcomes, such as fetal overgrowth, shoulder dystocia, operative delivery, birth injury, preeclampsia, haemorrhage and preterm delivery,[3–5] but also a seven fold higher risk of the mother developing T2DM after pregnancy.[6] In addition, the maternal metabolic milieu was also identified as a key determinant for the susceptibility to obesity, metabolic syndrome and T2DM in the offspring,[7] a phenomenon often described as "fetal programming".

The current – but still widely discussed – standard of care in GDM screening is the oral glucose tolerance test (OGTT) of 75g glucose performed late at 24 to 28 weeks of gestation as recommended by the International Association of Diabetes and Pregnancy Study Groups (IADPSG).[8] The new screening thresholds are based on the results of a large prospective cohort multicentre trial, the Hyperglycaemia and Adverse Pregnancy Outcome (HAPO) study.[4] The aim of the HAPO study was to associate the degree of maternal glycaemia with adverse perinatal

outcome, such as large for gestational age infants (LGA), neonatal hypoglycaemia and caesarean section rates. The results showed no obvious threshold, but rather a continuous increase of these adverse outcomes across the range of glucose concentrations. The IADPSG criteria resulted in a considerable increase in GDM prevalence of 17.8%, a detection rate of 83% for adverse outcome and a positive predictive value of 16%.[8]

An early and rapid diagnosis of GDM even before 24 weeks of gestation is desirable. By targeted early intervention including physical activity, moderate diet or insulin/drug therapy starting in the first trimester, rates of macrosomia (birth weight > 4000 g) or large for gestational age (LGA=birth weight > 90th percentile) infants, operative vaginal delivery and perinatal morbidity could be possibly reduced. Moreover, there could be a long term downstream effect on the offspring, thereby leading to considerable savings in healthcare costs by possibly decreased prevalence of generational transmission of metabolic diseases. But further research is necessary to evaluate the effects of an early intervention on short and long term outcomes for mother and child.

We propose that an "early" OGTT combined with maternal history, maternal condition and promising new biomarkers such as glycosylated fibronectin (glyFn) could diagnose similarly GDM, even in first trimester. Rasanen et al. published a study in September 2013 introducing glyFn as a new early GDM screening approach with an area under the curve (AUC) of 0.91 and a 95% confidence interval (CI) of 0.87-0.96, a positive

predictive value of 63% and a negative predictive value of 95%. Although some predictors of GDM have been studied retrospectively, no study to date has considered the use of promising new biomarkers combined with an "early" OGTT and maternal risk factors evaluation in first trimester of pregnancy.

STUDY OBJECTIVES

Primary objective

The use of the "early" OGTT 75g and/or the new biomarker, glyFn, as a new screening approach in late first/early second trimester will be evaluated and compared to GDM diagnosis by OGTT 75g at 24 to 28 weeks of gestation.

Secondary objectives

1. A new screening algorithm will be created by using multivariable risk estimation based on "early" OGTT 75g and/or glyFn results, incorporating maternal risk factors.
2. The significance of the association between glyFn, "early" OGTT 75g and maternal body mass index and/or clinical conditions including chronic hypertension, pregnancy-induced hypertension or preeclampsia and fetal conditions such as intrauterine growth restriction will be evaluated.

METHODS

This study protocol was developed on the basis of Standard Protocol Items: Recommendations for Interventional Trials (SPIRIT) (see the supplementary "SPIRIT checklist" for further details).

Study settings/design

This is an international, prospective, multi-centre cohort trial conducted at one secondary and five tertiary referral centres in Switzerland, Austria and Germany. Study recruitment commenced primarily at the coordination centre at University Hospital Basel on 1st May 2014. All other centres started recruitment consecutively until the end of March 2016. Recruitment is expected to last until December 2017 (see Figure 1 for details about expected time frame). The aim is to enrol 748 women at 12 to 15 weeks of gestation with a minimum recruitment of 50 women planned for each centre dependent on size and time of recruitment (Aarau: n=50, Basel: n=358, Freiburg: n=60, Salzburg: n=100, Vienna: n=100, Zurich: n=80). Fifty percent of eligible women are expected to accept participation. The study was approved by each local institutional ethical board of Basel, Zurich, Freiburg, Salzburg and Vienna. The trial was registered under www.ClinicalTrials.gov under NCT02035059 on 12th January 2014.

Recruitment and informed consent

Participants are identified at their first antenatal visit between 6 to 15 weeks of gestation. The investigator or obstetrician in charge informs the

women about all aspects pertaining to the trial. The informed consent includes permission for gathering data from medical records and the optional storage of blood for a maximum of 10 years for additional analyses related to the current study. Participants are informed that trial participation is voluntary and that they are free to withdraw without any effects on subsequent care. All members of the research team are aware of the guidelines for good clinical practice for obtaining consent.[9]

Eligibility criteria

Inclusion criteria are:

- women at least 18 years of age and not under guardianship
- healthy singleton pregnancy after spontaneous conception or after fertility treatment
- 6-15 weeks of gestation
- signed informed consent

Exclusion criteria are:

- previous bariatric surgery
- known pre-existing diabetes mellitus or under treatment with metformin
- known chronic infection like hepatitis or human immunodeficiency virus or chronic kidney, liver or heart disease
- known maternal history of hypertensive diseases in a previous pregnancy and now under prophylactic acetylsalicylate treatment

- fetal genetic, chromosomal or intervention-requiring morphologic abnormalities
- the inability to read and/or understand the participant`s information sheet

Study procedure

All healthy pregnant patients with regular care at the participating hospitals are counselled and asked at 6 to 15 weeks of gestation to participate. At 11+0 to 13+6 weeks of gestation, all women have a first trimester ultrasound scan which is standard care at participating sites. The ultrasound scan is used to confirm gestational age, diagnose any major fetal abnormalities, and optionally measure fetal nuchal translucency thickness, which together with maternal free beta-chorionic gonadotropin and pregnancy-associated plasma protein A, is used for screening for chromosomal abnormalities. In addition, if informed consent had been given during the first antenatal visit or at the time of the first trimester scan, the maternal history and condition are assessed, and blood for biomarker analysis and for the "early" OGTT 75g is drawn at the study visit at 12 to 15 weeks of gestation. The "early" OGTT 75g is compared to plasma glucose results obtained at 24 to 28 weeks of gestation after the OGTT 75g. No additional visit is necessary beyond the further standard routine antenatal care visits.

GlyFn and "early" OGTT 75g

All participants are instructed to fast for at least 10 hours. Two fasting glucose samples are taken. One sample is collected for storage of two aliquots (2x 1ml) at -80 °C for later analysis of glyFn and another sample for the fasting plasma glucose (FPG) value. After intake of the 75g glucose load, blood samples are drawn 60 and 120 minutes later for determination of glucose levels. Plasma glucose is measured by an automated colorimetric-enzymatic method (hexokinase/glucose-6-phosphate-dehydrogenase) on a Hitachi/Roche-Modular P analyser. GlyFn will be analysed as previously reported by Rasanen et al.[10] (monoclonal glyFn antibody) by DiabetOmics Inc., Beaverton, Oregon, USA. The maternal glyFn and "early" OGTT 75g results are blinded to the investigators.

Un-blinding

Values will be un-blinded if FPG levels are $\geq$ 7.0mmol/L or 2-hour plasma glucose levels are $\geq$ 11.1mmol/L, suggesting pre-existing diabetes mellitus.[11] The diagnosis of pre-existing diabetes mellitus needs to be confirmed by an elevated glycosylated haemoglobin A1c (HbA1c) value $\geq$ 6.5%. Plasma glucose levels of $\leq$ 2.5mmol/L are also abnormal and requires further clarification. Women with confirmed pre-existing diabetes mellitus are treated according to a standardised protocol in line with current recommendations.

Study outcomes

Diagnosis of GDM

GDM is diagnosed if at least one value of the 75g OGTT at 24 to 28 weeks of gestation exceeds the recommended IADPSG threshold: FPG of ≥ 5.1 mmol/L (92 mg/dL), 1-hour glucose of ≥ 10.0 mmol/L (180 mg/dL) and 2-hour glucose of ≥ 8.5 mmol/L (153 mg/dL). All women who screen positive are followed up by a nutritionist and a diabetic nurse in contact with a diabetologist, and have frequent regular appointments in our obstetrical outpatient clinic in 2 to 4 week intervals depending on clinical condition, glucose values and ultrasound findings. Women who fail to meet the target glucose values after 1 to 2 weeks of diet management are treated with insulin according to the guidelines of the Swiss Society for Endocrinology and Diabetology (SGED), the Austrian Diabetes Association (ÖDG), the German Diabetes Association (DDG) and the German Association of Gynaecology and Obstetrics (DGGG).[12–14] The glycaemic targets, insulin therapy, dose adjustments, concomitant medication and/or supplements are recorded.

Pregnancy, delivery and neonatal outcome data

Maternal data such as preeclampsia (blood pressure BP ≥ 140/90mmHg > 20 weeks of gestation with proteinuria), pregnancy induced hypertension (BP ≥ 140/90 mmHg > 20 weeks of gestation, rate of sonographic estimated polyhydramnios (amniotic fluid index ≥ 25cm) or macrosomia (estimated birth weight ≥ 90. Percentile), delivery outcome including delivery mode (spontaneous vaginal, forceps, vacuum, planned caesarean section or during labour) and indication, and neonatal outcome data such

as birth weight, rate of LGA (birth weight ≥ 90. Percentile), preterm birth ≤ 37 completed weeks of gestation, 5 and 10 minute Apgar scores, arterial umbilical cord pH ≤ 7.0, shoulder dystocia, birth trauma, neonatal hypoglycaemia (glucose value of < 2.5 mmol/L in infants born ≥ 34 weeks of gestation), jaundice (transcutaneous bilirubin > 95. Percentile or need of phototherapy at any time after delivery), respiratory distress syndrome, congenital anomalies and admission to intensive care unit are prospectively collected.

Statistics

Sample size justification

We aim to demonstrate that the recently reported biomarker, glyFn and/or the "early" OGTT 75g at 12 to 15 weeks of gestation has sufficient diagnostic power to evaluate women at risk of developing GDM compared to the OGTT 75g at 24 to 28 weeks of gestation. The ROC (receiver operator characteristic) curve of glyFn has a reported area under the curve (AUC) of 0.91 (95% confidence interval (CI) 0.87-0.96).[15] The OGTT 75g screening test using the IADPSG criteria has not been tested in early pregnancy so far. The FPG value in early pregnancy has an AUC of 0.61 (95% CI: 0.54-0.68) compared to IADPSG criteria in later pregnancy in a retrospective study.[16] We assume – according to unpublished data from the centre in Basel – that around 0.6% of recruited women will be diagnosed with pre-existing diabetes mellitus by the "early" OGTT 75g. The prevalence of GDM is assumed to be around 10.9% according to a

current IADPSG screening study from various Swiss laboratories.[17] The new screening approach should have a proposed true AUC of 0.9 with a lower boundary of 0.8 (95% CI>0.8) which would lead with a power of 90% and an α-level of 5% to an estimated sample size of 650 (65 women with GDM, 585 women without GDM). This power calculation is valid for OGTT, glyFn or combined markers. It ensures that the AUC is estimated with a good precision regardless of the chosen biomarker or any combination. Offsetting a dropout of 15%, this leads to a total sample size of 748. The dropout rate is expected to be equally distributed between centres. A sample size review will be performed after the first 300 recruitments. The power calculation was performed using MedCalc version 15.11 2015.[18]

Statistical analysis plan

Descriptive statistics and graphical examination will be performed for all primary and secondary study variables.

Primary objective: In order to predict GDM, ROC curves with corresponding AUCs will be calculated separately for glyFn and the "early" OGTT 75g. GDM diagnosis by OGTT 75g at 24 to 28 weeks of gestation is considered as routine method. AUCs will be estimated with a 95% confidence interval (CI). It will be hypothesized that a 95% CI of the AUC of glyFn alone or in combination with FPG, post-load glucose values is > 0.8.

Secondary objectives: Logistic regression will allow combining glyFn, the "early" OGTT and maternal risk factors in a multivariable risk model. Subsequent AUC will be calculated with 95% CI. Results will be internally cross-validated to prevent overoptimistic results. Optimal cut-off points to predict GDM will be determined based on these ROC curve. Sensitivity, specificity, positive and negative predictive value will also be estimated with 95% CI. However other "machine learning" algorithms could be better than logistic regression. Therefore, other popular procedures will be additionally tested: Random Forest, penalized logistic regression (Lasso). Details are described in Hastie et al..[19] Predictive performance will be internally cross-validated and explored in examining AUC of the ROC and predicted vs. observed probabilities. Random forest and penalized logistic regression avoid overfitting (to certain extent) whereas logistic regression does not. Random forest will be chosen because of its popularity and good benchmark results. Lasso is known for its good interpretability. Logistic regression will probably show its inferiority compared to the other methods. Internal cross-validation will be done using the package "mlr" within R. Internal cross-validation (e.g. 10-fold) is a good possibility to estimate the fitting on a potential future dataset. Based on these results it will be decided whether a new prediction model for GDM will be proposed. In order to potentially improve the primary study variables, secondary study parameters such as changes in maternal body mass index and/or clinical conditions like chronic hypertension and pregnancy induced hypertension or preeclampsia and fetal conditions including intrauterine

growth restriction will be added to the machine learning models. Statistical analyses and graphs will be performed using the current version of the statistical computation program R.[20]

Data recording

Each participant receives an identification number to ensure confidentiality and the collected data is exchanged between centres using only the identification number. The name and birth date of each participant are stored with a different identification number in order to preserve the possibility to look for inconsistencies during the study.

Reporting of adverse events

Any (serious) adverse events (AE/SAE) are recorded by the investigator using the specific AE/SAE sheet of the clinical report form (CRF). All SAE are reported to the responsible ethics committee within an appropriate time frame.

OTHER STUDY MEASUREMENTS

Other biomarkers

An extensively studied biomarker for early GDM screening is adiponectin.[21–23] Adiponectin is an adipocyte-derived hormone and reflects whole body insulin sensitivity.[24] A recently published metaanalysis calculated a summary sensitivity of 60.3% and a specificity of 81.3% with an AUC of 0.79.[22] Maternal serum adiponectin concentration is measured

by a quantitative sandwich enzyme-linked immunosorbent assay (ELISA) technique. Another potential biomarker is pregnancy-specific glycoprotein-1 (PSG-1).[25] PSG-1 had a detection rate of 74% with a false positive rate of 6% and an AUC of 0.81. PSG-1 is analysed as previously reported by Nagalla et al.[25] by DiabetOmics Inc., Beaverton, Oregon, USA.

Evaluation of insulin and HbA1c

At the early study visit at 12 to 15 weeks of gestation, insulin, c-peptide and HbA1c are additionally measured from the fasting blood sample in all women. HbA1c is measured by reversed-phase cation exchange chromatography (ADAMS HA-8160, Menarini Diagnostics Benelux, Zaventem, Belgium) or high performance liquid chromatography (HPLC) with Variant II, Bio-Rad (IFCC standardized and DCCT aligned with CV 1.8% for HbA1c=5.6%). Insulin is measured by chemiluminescence immunoassay CLIA (Roche Modular E170, Basel, Switzerland). The homeostasis model assessment of insulin resistance (HOMA-IR) or the Quantitative Insulin Sensitivity Check Index (QUICKI) is used as an approximate of fasting (i.e. hepatic) insulin resistance.[26,27] Beta-cell function is assessed from fasting glucose and insulin levels according to Wallace et al. 2004.[26]

Measurement of Vitamin D

Vitamin D deficiency is associated with inhibited insulin secretion, insulin resistance, and β-cell dysfunction in the pancreas in T2DM.[28,29]

Additionally, Vitamin D has immunomodulatory properties which protects against the development of T1DM. Supplementation of 1,25-OH Vitamin D seems to alter T cells composition and reduces cytokine-induced apoptosis of pancreatic islet cells.[30,31] We would like to have actual values for 25-OH Vitamin D and it will be evaluated in this cohort by CLIA (Liaison, DiaSorin, Saluggia, Italy).

Influence of stress in early pregnancy on development of GDM

Psychological factors in early pregnancy might contribute to adverse obstetric outcome.[32] This trial investigates the influence of perceived stress, stressful life events and depression on the development of GDM. The participants recruited in Basel are therefore asked to collect salivary samples for cortisol directly at time of awakening, at 30 and at 60 minutes after awakening. The saliva samples are stored at -20 °C until analysis. After thawing, saliva samples are centrifuged. Cortisol levels are determined employing a competitive solid phase time-resolved fluorescence immunoassay with fluorometric end point detection (DELFIA®).[33] Copeptin is a more stable precursor hormone of arginine-vasopressin and is found to be elevated in many diseases.[34–37] This effect can be attributed to the response of the hypothalamic-pituitary-adrenal axis to psychological stress. Copeptin will be measured in the fasting blood sample with time-resolved amplified cryptate emission technology (TRACE™) by BRAHMS Kryptor Compact immunoanalyzer by Thermo Scientific Brahms GmbH, Henningsdorf, Germany.

Three self-administered questionnaires are obtained at the study visit at 12 to 15 weeks of gestation:

Questionnaire on perceived stress

The perceived stress scale is a 10-items inventory for measuring the perception of stress.[38] It is widely used and has been validated in pregnancy.[39]

Questionnaire on stressful life events

The Holmes and Rahe Stressful Life Events Scale (SLE)[40] is an 43-items instrument for evaluation of stressful experiences in the past 12 months prior to the answering the questionnaire.

Questionnaire on depression

The ten-items Edinburgh Postnatal Depression Screen (EPDS)[41] is used to assess symptoms of depression during the past seven days. It has been widely validated in pregnancy[42,43] and it is part of the standard evaluation of pregnant women attending routine prenatal visits at the University Hospital in Basel. Scores ≥13 indicate at least probable minor depression and scores ≥15 indicated probable major depression. Pregnant women with scores ≥13 are routinely offered psychological or psychiatric counselling during pregnancy.

Histologic examination of the placenta

The fetal nutrients supply is regulated by maternal-fetal glucose and lipid concentration, placental blood flow and trophoblastic nutrient transporters.[44] The placenta reacts with adaptive changes in structure and function to a hyperglycaemic milieu.[45] These changes of the placenta will be assessed depending on the maternal glycaemic control by standard pathology examination.[46]

DISCUSSION

To our knowledge, this is the first large European cohort study that prospectively evaluates the promising new biomarker, glyFn, and the "early" OGTT 75g by comparing the impact of different GDM screening strategies with IADPSG criteria at 24 to 28 weeks of gestation. The IADPSG criteria have not been tested prospectively in early pregnancy, despite the suggestion of the IADPSG consensus panel in 2010[8] to take a FPG value $\geq$ 5.1 mmol/L as a cut-off value for GDM. This recommendation was based on a retrospective study observing that high first-trimester FPG in early pregnancy was associated with adverse pregnancy outcome.[47] We propose that glyFn with FPG alone or in combination with post-load glucose values should at least result in an AUC of 0.8 compared to the OGTT 75g in later pregnancy. Rasanen et al.[15] could show that glyFn was independent of maternal age, parity, gestational age, time of sample collection and the administration of the OGTT at 24 to 28 weeks of gestation. The current trial might clarify whether glyFn is depending on

maternal BMI or clinical conditions such as chronic hypertension, pregnancy induced hypertension or the development of preeclampsia or intrauterine growth restriction.

The screening approach combining glyFn +/- FPG could overcome some problems of the OGTT. Firstly, an OGTT is time consuming. GlyFn and FPG alone can be drawn in a fasting state of the women in early morning and the women do not have to wait further. Secondly, OGTT is inconvenient to administer and some women suffer intolerance to the glucose load resulting in nausea and vomiting. No glucose administration would be necessary with a screening method combining glyFn and FPG alone. Thirdly, we propose that glyFn might overcome the problem of low reproducibility as the OGTT. Like HbA1c, glyFn might also assess long term serum glucose concentration. But this is hypothetical and needs to be proven. Additionally, we suppose that a multivariable prediction model incorporating risk factors i.e. maternal age and/or BMI, together with glyFn and/or FPG, post-load glucose values might improve risk stratification in early pregnancy and could possibly decrease the required OGTTs later in pregnancy.

In the current study, the diagnostic power of glyFn will be evaluated using serum samples. But glyFn can be analysed additionally out of a dried blood stain. The resulting test is affordable (ie. estimated costs in India are 2-3 USD/dried blood spot, in Europe 20-30 USD/serum sample), which would help especially developing countries that suffer particularly from problems with the implementation of the IADPSG recommendations,[48] to

benefit from a possibly simple and cheap screening tool for GDM. The analysis of glyFn in dried blood is not part of this trial and needs to be validated separately in future studies.

Cost effectiveness analyses of IADPSG criteria using decision analysis models showed that the one step screening with OGTT 75g might be cost effective when post-delivery care would reduce the development of T2DM in the mothers.[49,50] Another study reported cost effectiveness if preeclampsia would decrease > 0.55% and caesarean delivery rate would fall > 2.7%.[51] A new screening in first trimester could be cost-effective if the method would reduce firstly the 1- and -2h blood sampling and/or secondly would decrease laboratory workload by avoiding a second screening in 24 to 28 weeks of gestation. Additionally, the new found screening approach could result in the identification of women with overt diabetes or/and GDM in first trimester. Aim of an early GDM diagnosis is the start of a timely intervention with diet, exercise or – if necessary – insulin therapy in early second trimester. Earlier treatment potentially should result in reduction of neonatal and maternal morbidities i.e. physical exercise reduces total maternal weight gain and the rate of GDM.[52] But still there is a paucity of randomized-controlled interventional trials that diagnosis and treatment of GDM < 24 weeks of gestation improve pregnancy outcomes. Until the efficacy of early treatment is not studied and verified thoroughly, a cost effectiveness analysis will be of restricted value, but a cost analysis could be performed assuming different outcome scenarios.

One limitation of our trial is that it is not powered for the evaluation of neonatal outcomes like LGA infants, neonatal hypoglycaemia, shoulder dystocia or birth trauma (these are planned secondary outcomes). The study would need to be significantly larger to detect effects on these neonatal outcomes. If this trial were to show positive diagnostic power for the new screening algorithm, a large multi-centre study may be required to be sufficiently powered to determine the algorithm`s effect on rate of LGA infants or neonatal hypoglycaemia as proposed in the HAPO study.[4]

Finally, the glyFn assay is not yet commercially available and will be perfomed by DiabetOmics Inc., Beaverton, Oregon, USA. For a widespread implementation of the finally proposed screening approach, a standardisation between laboratories will be necessary first.

The results of our study may have a major impact on future screening approaches for GDM by development of a potentially simple, cost effective and for the pregnant women comfortable screening method for GDM in first trimester.

List of abbreviations

AE, adverse events; AUC, area under the curve; BMI, body mass index; CI, confidence interval; CRF, clinical report form; GDM, gestational diabetes mellitus; GlyFn, glycosylated Fibronectin; HbA1c, glycosylated haemoglobin A1c; HOMA-IR, homeostasis model assessment of insulin resistance; IADPSG, International Association of Diabetes and Pregnancy Study Groups; LGA, large for gestational age; OGTT, oral glucose

tolerance test; T1DM, type 1 diabetes mellitus; T2DM, type 2 diabetes mellitus; QUICKI, quantitative insulin sensitivity check index; ROC, receiver operator characteristic; SAE, serious adverse events.

Disclosure of interests

The authors declare that there are no further financial or personal relationships with other people or organizations that could inappropriately influence the work reported or the conclusions, implications, or opinions stated.

Contribution to authorship

EH is principal investigator, study protocol author, obtained ethical approval and drafted this manuscript. IH is the sponsor, assisted with the original study protocol and revised the manuscript. SH and EBR designed the study together with EAH and revised the manuscript. AS performed the power calculation and statistical planning. TF, CSG, WE, PH, MK, ED, NO, EBAE, HP, MT, MK, RZ made important contributions and critically reviewed the content. All authors have given final approval of the version to be published.

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Details of ethics approval

The study was approved by each local institutional review board in Basel (Ethikkommission Nordostschweiz (EKNZ)), Zurich, Freiburg, Salzburg and Vienna. The trial was registered under www.ClinicalTrials.gov under NCT02035059 on 12th January 2014.

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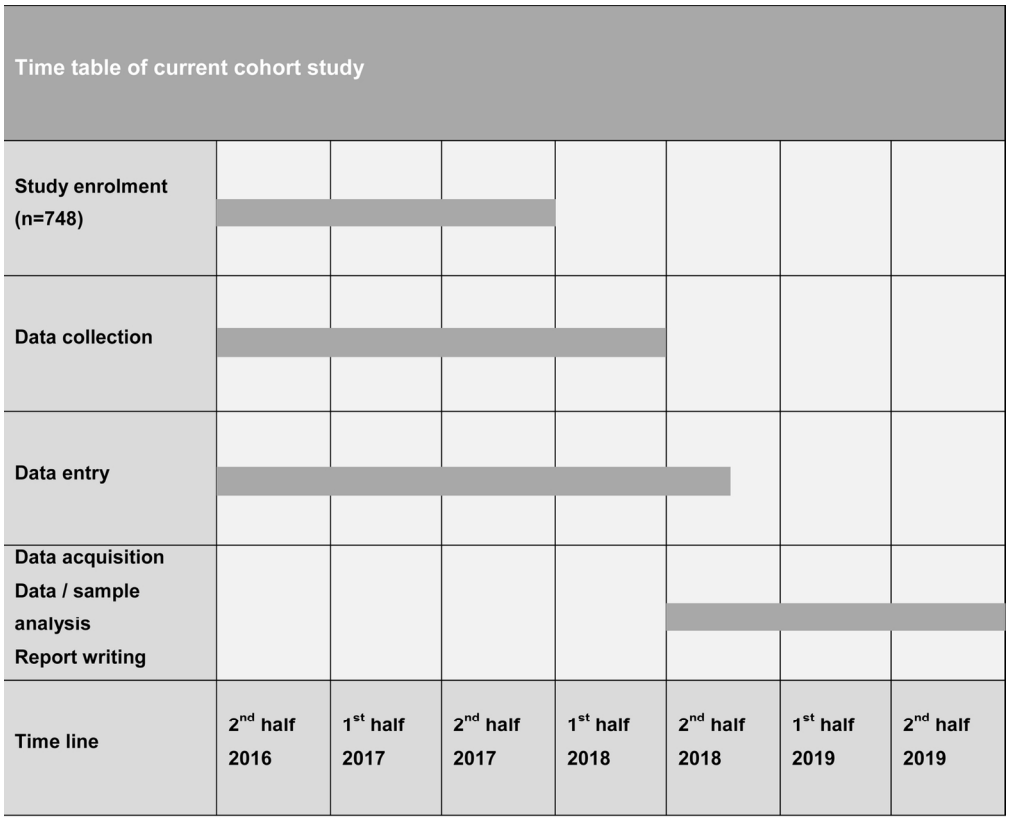

Figure 1: Expected time frame

141x115mm (300 x 300 DPI)

SPIRIT
STANDARD PROTOCOL ITEMS: RECOMMENDATIONS FOR INTERVENTIONAL TRIALS

SPIRIT 2013 Checklist: Recommended items to address in a clinical trial protocol and related documents*

Section/item	Item No	Description
Administrative information		
Title	1	Descriptive title identifying the study design, population, interventions, and, if applicable, trial acronym → title page
Trial registration	2a	Trial identifier and registry name. If not yet registered, name of intended registry → page 2
	2b	All items from the World Health Organization Trial Registration Data Set
Protocol version	3	Date and version identifier → only in original protocol
Funding	4	Sources and types of financial, material, and other support → page 2
Roles and responsibilities	5a	Names, affiliations, and roles of protocol contributors → page 2
	5b	Name and contact information for the trial sponsor → page 2/21
	5c	Role of study sponsor and funders, if any, in study design; collection, management, analysis, and interpretation of data; writing of the report; and the decision to submit the report for publication, including whether they will have ultimate authority over any of these activities
	5d	Composition, roles, and responsibilities of the coordinating centre, steering committee, endpoint adjudication committee, data management team, and other individuals or groups overseeing the trial, if applicable (see Item 21a for data monitoring committee)
Introduction		
Background and rationale	6a	Description of research question and justification for undertaking the trial, including summary of relevant studies (published and unpublished) examining benefits and harms for each intervention → page 5-7 (Introduction)
	6b	Explanation for choice of comparators
Objectives	7	Specific objectives or hypotheses → page 7 (Study objectives)

Trial design	8	Description of trial design including type of trial (eg, parallel group, crossover, factorial, single group), allocation ratio, and framework (eg, superiority, equivalence, noninferiority, exploratory) → see Title
Methods: Participants, interventions, and outcomes		
Study setting	9	Description of study settings (eg, community clinic, academic hospital) and list of countries where data will be collected. Reference to where list of study sites can be obtained → page 7/8 (Study settings/design)
Eligibility criteria	10	Inclusion and exclusion criteria for participants. If applicable, eligibility criteria for study centres and individuals who will perform the interventions (eg, surgeons, psychotherapists) → page 8/9 (Eligibility criteria)
Interventions	11a	Interventions for each group with sufficient detail to allow replication, including how and when they will be administered → page 9/10 (Study procedure)
	11b	Criteria for discontinuing or modifying allocated interventions for a given trial participant (eg, drug dose change in response to harms, participant request, or improving/worsening disease) → not applicable
	11c	Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (eg, drug tablet return, laboratory tests) → not applicable
	11d	Relevant concomitant care and interventions that are permitted or prohibited during the trial → not applicable
Outcomes	12	Primary, secondary, and other outcomes, including the specific measurement variable (eg, systolic blood pressure), analysis metric (eg, change from baseline, final value, time to event), method of aggregation (eg, median, proportion), and time point for each outcome. Explanation of the clinical relevance of chosen efficacy and harm outcomes is strongly recommended → page 11/12 (Study outcomes)
Participant timeline	13	Time schedule of enrolment, interventions (including any run-ins and washouts), assessments, and visits for participants. A schematic diagram is highly recommended (see Figure) → Figure 1
Sample size	14	Estimated number of participants needed to achieve study objectives and how it was determined, including clinical and statistical assumptions supporting any sample size calculations →page 12/13 (Sample size justification)
Recruitment	15	Strategies for achieving adequate participant enrolment to reach target sample size
Methods: Assignment of interventions (for controlled trials) → not applicable		

Allocation:		
Sequence generation	16a	Method of generating the allocation sequence (eg, computer-generated random numbers), and list of any factors for stratification. To reduce predictability of a random sequence, details of any planned restriction (eg, blocking) should be provided in a separate document that is unavailable to those who enrol participants or assign interventions
Allocation concealment mechanism	16b	Mechanism of implementing the allocation sequence (eg, central telephone; sequentially numbered, opaque, sealed envelopes), describing any steps to conceal the sequence until interventions are assigned
Implementation	16c	Who will generate the allocation sequence, who will enrol participants, and who will assign participants to interventions
Blinding (masking)	17a	Who will be blinded after assignment to interventions (eg, trial participants, care providers, outcome assessors, data analysts), and how –> not applicable
	17b	If blinded, circumstances under which unblinding is permissible, and procedure for revealing a participant's allocated intervention during the trial → see section "Un-blinding"
Methods: Data collection, management, and analysis		
Data collection methods	18a	Plans for assessment and collection of outcome, baseline, and other trial data, including any related processes to promote data quality (eg, duplicate measurements, training of assessors) and a description of study instruments (eg, questionnaires, laboratory tests) along with their reliability and validity, if known. Reference to where data collection forms can be found, if not in the protocol
	18b	Plans to promote participant retention and complete follow-up, including list of any outcome data to be collected for participants who discontinue or deviate from intervention protocols
Data management	19	Plans for data entry, coding, security, and storage, including any related processes to promote data quality (eg, double data entry; range checks for data values). Reference to where details of data management procedures can be found, if not in the protocol
Statistical methods	20a	Statistical methods for analysing primary and secondary outcomes. Reference to where other details of the statistical analysis plan can be found, if not in the protocol → page 13-15 (Study analysis plan)
	20b	Methods for any additional analyses (eg, subgroup and adjusted analyses) → page 15-19 (Other study measurements)

	20c	Definition of analysis population relating to protocol non-adherence (eg, as randomised analysis), and any statistical methods to handle missing data (eg, multiple imputation)
Methods: Monitoring		
Data monitoring	21a	Composition of data monitoring committee (DMC); summary of its role and reporting structure; statement of whether it is independent from the sponsor and competing interests; and reference to where further details about its charter can be found, if not in the protocol. Alternatively, an explanation of why a DMC is not needed
	21b	Description of any interim analyses and stopping guidelines, including who will have access to these interim results and make the final decision to terminate the trial
Harms	22	Plans for collecting, assessing, reporting, and managing solicited and spontaneously reported adverse events and other unintended effects of trial interventions or trial conduct → see Reporting of adverse events
Auditing	23	Frequency and procedures for auditing trial conduct, if any, and whether the process will be independent from investigators and the sponsor
Ethics and dissemination		
Research ethics approval	24	Plans for seeking research ethics committee/institutional review board (REC/IRB) approval → page 8 (Study settings/design)
Protocol amendments	25	Plans for communicating important protocol modifications (eg, changes to eligibility criteria, outcomes, analyses) to relevant parties (eg, investigators, REC/IRBs, trial participants, trial registries, journals, regulators)
Consent or assent	26a	Who will obtain informed consent or assent from potential trial participants or authorised surrogates, and how (see Item 32)
	26b	Additional consent provisions for collection and use of participant data and biological specimens in ancillary studies, if applicable
Confidentiality	27	How personal information about potential and enrolled participants will be collected, shared, and maintained in order to protect confidentiality before, during, and after the trial → page 15 (Data recording)
Declaration of interests	28	Financial and other competing interests for principal investigators for the overall trial and each study site → (page 2 (Conflict of interest)
Access to data	29	Statement of who will have access to the final trial dataset, and disclosure of contractual agreements that limit such access for investigators

Ancillary and post-trial care	30	Provisions, if any, for ancillary and post-trial care, and for compensation to those who suffer harm from trial participation
Dissemination policy	31a	Plans for investigators and sponsor to communicate trial results to participants, healthcare professionals, the public, and other relevant groups (eg, via publication, reporting in results databases, or other data sharing arrangements), including any publication restrictions → not applicable
	31b	Authorship eligibility guidelines and any intended use of professional writers
	31c	Plans, if any, for granting public access to the full protocol, participant-level dataset, and statistical code
Appendices		
Informed consent materials	32	Model consent form and other related documentation given to participants and authorised surrogates
Biological specimens	33	Plans for collection, laboratory evaluation, and storage of biological specimens for genetic or molecular analysis in the current trial and for future use in ancillary studies, if applicable

*It is strongly recommended that this checklist be read in conjunction with the SPIRIT 2013 Explanation & Elaboration for important clarification on the items. Amendments to the protocol should be tracked and dated. The SPIRIT checklist is copyrighted by the SPIRIT Group under the Creative Commons "Attribution-NonCommercial-NoDerivs 3.0 Unported" license.