SAF study

Advanced Glycation End products, measured as Skin Autofluorescence, during pregnancy in patients with pre-existing diabetes, gestational diabetes and normoglycemic controls and its relationship with adverse outcomes

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Abstract - English

Introduction Advanced glycation end products (AGEs) are irreversibly modified proteins that accumulate during normal ageing and are increased in conditions of oxidative or glycemic stress, like diabetes mellitus. Little is known about AGEs in hyperglycemic pregnancy. Therefore we measured AGE levels as Skin Autofluorescence (SAF, in arbitrary units (AU)) with the AGE reader CU™ in pregnant women with pre-existing diabetes (DM), gestational diabetes (GDM) and normoglycemic controls. We investigated SAF levels and its relationship with adverse pregnancy outcomes as well as the one year postpartum development of type 2 diabetes (T2DM) after GDM.

Patients/methods In this monocenter observational study, SAF was measured in 79 GDM, 24 DM and 58 controls 3-5 times during pregnancy and about 8 weeks postpartum. Primary outcomes were: SAF levels, macrosomia and T2DM one year postpartum. Secondary outcomes were: preeclampsia, caesarean section, prematurity, foetal death and neonatal hypoglycemia, jaundice and birth trauma.

Results SAF decreases significantly in controls in the second half of pregnancy (0.018 AU per week, p=0.003) and rises back to levels earlier in pregnancy 8 weeks postpartum in GDM and controls. SAF course in DM is significantly higher than in controls (p=0.024). No association is found between SAF and adverse pregnancy outcomes. Too few GDM patients were screened one year postpartum for further analysis.

Conclusion A physiological decrease in SAF in the second half of pregnancy is seen in normoglycemic pregnancy, followed by a postpartum increase back to levels earlier in pregnancy in GDM and controls. These results in controls can serve as reference values for non diabetic pregnancy. SAF during pregnancy is higher in DM than in controls, probably due to long term hyperglycemia exposure in DM. In these small study groups, no relationship was found between SAF during pregnancy and adverse pregnancy outcomes. No conclusions can be drawn on the relationship between SAF levels during GDM pregnancy and the risk of T2DM one year postpartum.

Samenvatting – Nederlands

Introductie “Advanced glycation end products” (AGEs) zijn irreversibel gemodificeerde eiwitten die stapelen in weefsels tijdens normale veroudering en verhoogd zijn bij aandoeningen die gepaard gaan met oxidatieve of glycemische stress, zoals diabetes mellitus (DM). Er is weinig bekend over AGE waarden bij hyperglycemische zwangeren. Daarom hebben wij weefsel AGE waarden gemeten in de vorm van huid autofluorescentie (“Skin Autofluorescence”, SAF in “Arbitrary Units”, AU) met de AGE reader CU™ tijdens zwangerschap bij vrouwen met DM, diabetes gravidarum (GDM) en normoglycemische controles. Wij onderzochten SAF en de relatie tussen SAF en zowel zwangerschapscomplicaties als het ontwikkelen van type 2 diabetes (T2DM) één jaar na GDM.

Patiënten/methode In deze monocenter observationele studie werd SAF gemeten bij 79 GDM, 24 DM en 58 controles 3-5 keer tijdens de zwangerschap en ongeveer 8 weken postpartum. Primaire uitkomsten waren: SAF waarden, macrosomie, T2DM een jaar postpartum. Secundaire uitkomsten: preeclampsie, sectio caesaria, prematuriteit, foetale dood en neonatale hypoglycemie, icterus en geboortetrauma.

Resultaten Bij controles daalt SAF significant in de tweede helft van de zwangerschap (0.018 AU per week, p=0.003), en stijgt 8 weken postpartum terug naar waarden vroeger in de zwangerschap. Het beloop van SAF bij DM is significant hoger dan bij controles (p=0.024). Geen associatie werd gevonden tussen SAF waarden en zwangerschapscomplicaties. Er werden te weinig GDM patiënten gescreend één jaar postpartum voor verdere analyse.

Conclusie In de normoglycemische zwangerschap is sprake van een fysiologische daling van SAF in de tweede helft van de zwangerschap, gevolgd door een stijging postpartum bij GDM en controles terug naar waarden eerder in de zwangerschap. Onze resultaten bij controles kunnen dienen als referentiewaarden voor een niet diabetische zwangerschap. SAF is tijdens de zwangerschap hoger bij DM dan bij controles, waarschijnlijk als gevolg van lange termijn blootstelling aan hyperglycemie bij DM. In deze kleine studiegroepen werd geen relatie gevonden tussen SAF tijdens de zwangerschap en zwangerschapscomplicaties. Er kunnen geen conclusies worden getrokken over de relatie tussen SAF tijdens GDM zwangerschap en het risico op T2DM een jaar postpartum.
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Introduction

Advanced glycation end products – a ‘metabolic memory’

Advanced glycation end products (AGEs) are irreversibly modified proteins that accumulate throughout the body during aging.(1) AGEs are formed by several glycation and oxidation pathways.(2) The glycation-based pathway is known as the ‘Maillard reaction’(Figure 1): AGEs form when a reducing sugar like glucose, reacts nonenzymatically with free aminogroups on polypeptides, lipids or nucleic acids. So called ‘Schiff bases’ and ‘Amadori-products’ are formed. These are reversible early and intermediate glycation products of which HbA1c and fructosamine are common known examples.(3) Further biochemical rearrangements of these Amadori-products result in irreversible and stable AGEs.(4;5) Another acknowledged pathway is AGE formation driven by oxidative stress. Proteins are modified either directly by oxidation of amino acids or indirectly generated by the autoxidation of carbohydrates, lipids or amino acid.(6) Because of both glycation as well as oxidation-based formation of AGEs, they are also referred to as advanced glycoxidation end products.

Due to the irreversible and stable character of AGEs, AGE levels depend on the half life of the protein the AGE is formed on.(7) AGE accumulation in long-lived proteins, like skin collagen, is thought to be a reflection of long-term glycemic and oxidative states. Therefore it is often called the “metabolic memory” or “hyperglycemic memory”.(8)

![Figure 1. Simplified schematic presentation of the “Maillard reaction”](image)

The clinical relevance of measuring AGE levels is still being investigated. Currently, no consensus or standards exist on how to determine AGE levels or on the implementation in clinical practice. One limitative factor in investigating AGE accumulation is the invasive techniques necessary to measure AGE levels, like biopsies and blood samples. A recently developed, non-invasive measurement of AGE accumulation makes use of intrinsic fluorescent properties of some AGEs which can be measured in skin collagen.(4) This skin autofluorescence (SAF) is measured by the AGE-Reader CU™ (DiagnOptics B.V., Groningen, the Netherlands, formerly known as the AFR (Autofluorescence Reader)). The AGE reader calculates SAF using measurements of detected light from the skin after illumination. This method is validated with AGE levels measured in skin biopsies of diabetic and control patients.(9;10) SAF level appears to be correlated with a few specific AGEs(10), which makes the AGE reader a useful tool for measuring skin AGE accumulation. The AGE reader is described in more detail later.

The formation of AGEs continually takes place in every human being and is therefore part of the normal ageing process. Koetsier et al(1) investigated SAF in healthy subjects to provide reference values for SAF at different age. This shows a linear increase in SAF level with rising age up to 70 years.(1)

Compared to AGE formation in the normal ageing process, accelerated AGE accumulation is
found in patients with conditions of glycemic and oxidative stress. This includes patients with diabetes mellitus or autoimmune disease, patients admitted to intensive care, smoking individuals and patients with renal failure.(4;7;11) Besides that, diet is a source of AGEs. AGE levels in food are related to heat treatment and nutrient composition.(12) No difference in SAF is seen in relation to glycemic variation, for example during an oral glucose tolerance test (OGTT).(13;14) AGEs and its precursors are cleared by the kidneys. Together with increased AGE formation driven by oxidative stress in renal failure, this explains why increased AGE levels are found in renal failure.(7;15)

Apart from being a metabolic memory, AGEs are thought to play an important causative role in the pathophysiology of diabetic and cardiovascular complications. Tissue damage by AGE-formation can result from two pathways. First, AGE-formation causes cross-linking between proteins, which damages the structure and function of these proteins. Second, AGE can bind to AGE receptors on cells, which activation results in intracellular damage.(7;16)

**Diabetes, its complications and AGEs**

Diabetes mellitus is a group of metabolic disorders characterised by hyperglycemia as a result of impaired insulin secretion, insulin action or a combination of both.(17) The relationship between diabetes mellitus and the development of microvascular complications (retinopathy, nephropathy and neuropathy) as well as macrovascular complications is widely acknowledged. Between 1983 and 1993, the large clinical Diabetes Control and Complications Trial (DCCT)(18) demonstrated that intensive diabetes therapy delays onset and slows the progression of diabetic microvascular complications in patients with insulin-dependent diabetes mellitus.(18) In a follow-up study of this trial, after all patients received intensive treatment, also a beneficial effect of strict glucose control on cardiovascular disease was found.(19) Intensive glucose control in type 2 diabetes patients was investigated in the United Kingdom Prospective Diabetes Study (UKPDS), in which intensive therapy resulted in a reduction in risk of microvascular complications. Also here, a post-trial follow-up of 10 years showed a long-term reduction in microvascular as well as macrovascular complications in the intensive treated group compared to conventional treated patients.(20) This long-term continuing effect of conventional poor glucose control on the development of diabetic complications, even after more recent intensive glucose control, supports the aforementioned term “metabolic memory” and its association with complications.

Nowadays, the relationship between diabetes and its complications is well-established and so is the importance of tight glucose control. Further research is focusing on improvement of prevention and treatment of diabetes and its complications. As mentioned, there is increasing evidence and attention for the key role of AGEs in the development of diabetic complications.

Associations between AGE levels and diabetic micro- and macrovascular complications have been demonstrated.(4;21) One year before the end of the DCCT, patients underwent a skin biopsy in which AGE levels were measured.(21) The intensive treated group showed significantly lower skin AGE levels than the conventional treated group. Furthermore, a 10-year follow-up of these patients showed that the incidence of retinopathy and nephropathy was significantly associated with these AGE levels, also after adjustment for HbA1c(21). This makes AGE level an independent and stronger predictor of microvascular complications than HbA1c. In patients with T2DM, Gerrits et al. showed that SAF is an independent predictor of development of neuropathy and (micro)albuminuria with a mean follow-up of 3.1 years after SAF measurement.(22)

In a clinical review, Goh et al(4) describe that also on biochemical level associations between AGE accumulation and diabetic complications are found. For example, renal AGE
accumulation is associated with several structural aspects of diabetic nephropathy. And a correlation is found between AGE localisation in retinal blood vessels and degree of retinopathy. Also increased AGE levels are found in peripheral nerves of diabetes patients, causing neuropathy. (4)

In the context of macrovascular diabetic complications, protein cross-linking is known to cause a decrease in arterial and myocardial compliance and an increase in vascular stiffness. (4) AGEs are found in atherosclerotic lesions and are considered to contribute to the formation of atherosclerosis through several pathways, like inhibiting vascular repair after injury. (4;5;23)

Moreover, in animal studies, agents that interfere with AGE formation, so-called “AGE-inhibitors”, have shown to be effective in inhibiting cardiovascular damage. (23) This suggests a pathogenic role of AGE accumulation in the development of complications even more.

After the introduction of the AGE reader, several studies showed a relationship between SAF and diabetes-related complications. Bos et al. concluded in a systematic review, that SAF level in diabetes patients is related to neuropathy and nephropathy, but not retinopathy. (16) Also associations with macrovascular complications are described: in diabetes patients, SAF is a strong predictor of cardiac mortality and a marker of vascular damage. (24;25)

Measuring AGE levels might play a role in the detection of diabetes: Maynard et al. (26) measured SAF, HbA1c and fasting plasma glucose in a group of subjects that were not previously diagnosed with diabetes and performed an OGTT as gold standard. SAF showed a higher sensitivity in diagnosing diabetes and pre-diabetes than HbA1c and fasting plasma glucose. (26)

Diabetes & pregnancy – is there a role for measuring AGES?

Patients with diabetes during pregnancy can roughly be divided in two groups: patients with pre-existing diabetes mellitus and patients with gestational diabetes (GDM). Traditionally, GDM is defined as glucose intolerance with onset or first recognition during pregnancy. (17) GDM is known to be a major risk factor for the development of T2DM (27) and is therefore thought to be a precursor or a first expression of subsequent or underlying T2DM. During pregnancy, insulin resistance evolves as a consequence of normal physiologic changes. Only when compensatory increased insulin release fails, GDM becomes apparent. (28) Based on laboratory results, a woman diagnosed with diabetes during pregnancy can be suspected to have undiagnosed T2DM, sub-clinically present before pregnancy. Therefore, the American Diabetes Association recently recommended that ‘high-risk women found to have diabetes at their initial prenatal visit, using standard criteria for T2DM diagnosis, receive a diagnosis of overt, not gestational, diabetes’. (17) In the same article, a GDM prevalence of approximately 7% of pregnancies is reported, ranging from 1-14%. This prevalence has increased along with rising obesity and diabetes prevalence worldwide. (17) In the Netherlands, a GDM prevalence of 2-5% is estimated. (28)

Diabetes mellitus during pregnancy is associated with an increased risk of a range of adverse pregnancy outcomes like macrosomia, caesarean delivery, neonatal hypoglycemia and neonatal trauma. The recent Hyperglycemia and Adverse Pregnancy Outcomes Study (HAPO study) (29) showed that even hyperglycemia less severe then that in diagnosed diabetes mellitus during pregnancy is associated with an increased risk of maternal and perinatal complications. (29) Studies on the relationship between glucose control and pregnancy complications show that treatment of diabetes during pregnancy with tight control of blood glucose levels can improve pregnancy outcome. (30;31) Whether new diabetes-related measurements like AGE levels can help improve diagnosis, monitoring and risk assessment of
pregnancies complicated by diabetes, in order to improve pregnancy outcomes, needs to be investigated.

Literature on AGE levels in the context of pregnancy is limited. One study found increased serum AGE levels in preeclamptic compared to non-preeclamptic women(32) and higher SAF levels are described in women with a history of preeclampsia 3-13 months and 4 years after pregnancy.(33;34) Higher serum AGE levels were found during pregnancy in DM compared to non-diabetic pregnant women in some(35-37) but not all studies(38). Also higher serum AGE levels were found in GDM women compared to pregnant controls.(36;38) One study describes a relationship between serum AGE levels and adverse foetal outcome.(35)

Recently, results of this SAF-study(13) showed no difference in SAF between GDM at diagnosis and non-diabetic pregnant controls. This is probably explained by the relatively short term hyperglycemia exposure in GDM women. However, this study does not describe SAF level later in pregnancy, so whether differences in SAF between GDM and controls develop later in pregnancy remains unknown. Besides that, to our knowledge no previous study describes SAF in patients with pre-existent DM during pregnancy. Since DM patients have a longer history of exposure to hyperglycemia, one would expect SAF levels to be higher than in non-diabetic pregnant controls. It would be interesting to know whether there is a relationship between SAF and adverse pregnancy outcomes and whether SAF can be useful as a clinical tool for the rapid risk assessment of pregnancy complications.

GDM is not only associated with adverse pregnancy outcomes, but also with an increased risk for the mother of developing T2DM in later life. Early detection of this development with the aim to start intensive glucose control as early as possible is, as described above, important in the prevention of diabetic complications. Kim et al(27) concluded in a meta-analysis that 50%-70% of the women with previous GDM develop T2DM within 10 years, the majority within 5 years after delivery. Therefore, GDM in general could be seen and used as a predictor for the development of T2DM.(27;39) Since measuring SAF appears to be a more sensitive tool in detecting (pre-) diabetes than fasting plasma glucose and HbA1c(26), we investigated whether SAF during pregnancy could be a predictor of a woman’s risk of developing T2DM after GDM.

Research questions
Since SAF might be a better tool for detecting diabetes and its complications than HbA1c and glucose levels, plus the fact that little is known about SAF during pregnancy, we investigated SAF levels during pregnancy and its relationship with adverse pregnancy outcomes as well as the development of T2DM one year postpartum. This leads to the following research questions:

- Are SAF levels increased in type 1 and type 2 diabetes patients compared to controls during pregnancy?
- If SAF levels are increased in type 1 and type 2 diabetes patients, how do SAF levels change during pregnancy compared to GDM and controls? Is there a relationship between these changes and adverse pregnancy outcomes?
- Is there a relationship between SAF levels during pregnancy complicated by GDM and the development of type 2 diabetes one year after delivery?

Patients and Methods
Study design
In this monocenter observational prospective study, we included patients visiting the outpatient obstetric clinic of the University Medical Center Utrecht. Patients were treated in
first, second, or third line obstetric care by midwife and if indicated by gynaecologist and/or endocrinologist and diabetes nurse educator. Patients were included from April 2010 until November 2011. The study protocol was approved by the local ethics committee and informed consent was obtained from all subjects before measurements started. Patients could leave the study at any time for any reason without consequences.

**Patients**

The inclusion criteria included pregnant patients diagnosed with GDM, pre-existent DM (T1DM or T2DM) or control subjects, written informed consent and knowledge of Dutch. In the DM group we included patients with T1DM, diagnosed before pregnancy, and T2DM patients that were either diagnosed before pregnancy or during OGTT: on the basis of an expert’s opinion and according to the most recent American Diabetes Association recommendations(17), patients with laboratory findings suggestive for T2DM were also included in the T2DM group. In the rare case in which T2DM did not persist after pregnancy, the patient was excluded later.

GDM patients were diagnosed by a positive 75 or 100 g OGTT during pregnancy. During an OGTT, blood glucose levels are measured in fasting state and after glucose intake. From the beginning of this study until mid-August 2011, a 100 g OGTT was used, with four measurements: fasting glucose, 1, 2 and 3 hours after 100 g glucose intake. GDM was diagnosed if two or more of the following blood glucose levels, measured in mmol per litre in capillary blood, were met or exceeded: fasting glucose 5.3; 1 hours 10.0; 2 hours 8.7; 3 hours 7.8. Starting mid-August 2011, a 75 g OGTT was used. Cut-off points in this test are, measured in mmol per litre in venous plasma: fasting glucose 7.0; 2 hours 7.8. Controls were recruited from patients that had a 50 g glucose challenge test or an OGTT with all values below the cut-off points and from patients visiting the outpatient obstetric clinic without risk factors for GDM. These patients are considered normal glucose tolerant. Glucose challenge tests and OGTTs were performed in patients with high risk for GDM, including a family history of diabetes, obesity, polycystic ovary syndrome, previous GDM or foetal growth acceleration.

Exclusion criteria included situations that could already have elevated AGEs levels prior to the study, such as renal failure (<30 ml/min); preeclampsia at inclusion or in the past; recent (< 6 months) serious infection or infarction or hospital admission, active autoimmune disease or active use of prednisone, smoking during pregnancy, proteinuria (>300 mg/24hrs) and skin reflectance <6% (a situation in which SAF measurement is not possible, the AGE-reader will give an alarm when reflection is too low for reliable measurement). Gestational age less than 20 weeks was an exclusion criterion for GDM and controls, because of possible uncertainty about the diagnosis or of later development of GDM, respectively. Patients with a gestational age more than 32 weeks were also excluded because in those patients too few measurements were possible.

All patients were treated following standard obstetric care. This consists of monitoring, diet and if necessary insulin therapy for DM and GDM patients.

After inclusion, a questionnaire was completed with self-reported data: age, ethnicity (both parents white European, Moroccan or other), pre-pregnancy weight, length, previous pregnancies, medical history, first degree family history of diabetes mellitus and medication use. After fulfilling the questionnaire, SAF levels were measured about every four weeks during normal visits of the patients to the outpatient obstetric clinic in the following periods of gestational age in weeks: 18-21, 22-25, 26-29, 30-33, 34-37, and 38. If the patient visited the hospital postpartum, SAF measurement was repeated about 6 weeks after delivery. Altogether this resulted in a total maximum of about 6 measurements per patient.
GDM patients were contacted by phone one year after delivery for a one year postpartum diabetes evaluation. Patients visited either the hospital or their general practitioner for diabetes screening including fasting glucose (FBG) and HbA1c. If possible, SAF measurement was performed. Development of T2DM postpartum was defined as an HbA1c ≥ 48 mmol/mol (6.5%) or a FBG ≥ 7.0 mmol/L (126 mg/dL), according to the most recent American Diabetes Association guidelines.(17) If a woman was pregnant again, SAF was not measured and the regular GDM-screening was awaited. If this showed no GDM, this was noted as no T2DM one year postpartum. If a woman had GDM again, a missing value was noted in the one year postpartum diabetes evaluation.

**Study outcomes**

Primary outcomes of this study are: SAF during pregnancy, macrosomia and the presence of T2DM one year postpartum. Macrosomia is defined as birth weight by gestational age at P90 or more, according to the macrosomia tables for the Dutch population by Visser et al(40).

Secondary outcomes are: maternal endpoints: preeclampsia, premature delivery (< 37 weeks), primary or secondary caesarean section (CS); foetal endpoints: foetal death (> 20 weeks), premature birth; and neonatal endpoints: hypoglycemia (< 2.0 in the first 24 hours or glucose infusion), neonatal jaundice which requires phototherapy and birth trauma. Outcomes were obtained from searching patient’s and their children’s files. T2DM one year postpartum was either determined with a diabetes screening as described above or derived from a patient’s file.

**Skin autofluorescence measurement**

Skin auto fluorescence is non-invasively measured by the AGE-reader CU™ (DiagnOptics Technologies BV, Groningen, The Netherlands) (Figure 2). This measurement makes use of the principle of autofluorescence: when proteins with fluorescent characteristics are illuminated, these will emit light with another wavelength than that of the excited light. When measurement starts, first calibration measurements are performed: one dark measurement and one white measurement (assumed to give a 100% reflection), which are used as reference values. A skin surface of about 4 cm² is illuminated with an excitation ultraviolet blacklight source within the 350-420 nm wavelength range. Then, light emitted from the skin is detected in the range between 300-600 nm by a spectrometer. Since excited light is within the 350-420 nm range, detected light in this range is considered skin reflectance and detected light in the 420-600 nm range is considered to be fluorescence.

SAF level is calculated by dividing the average light intensity detected in the range between 420-600 nm, by the average light intensity excited per nm within the 300-420 nm range. In other words, SAF level is the ratio of the fluorescent light emitted by the skin, and the light

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Figure 2. SAF measurement with the AGE reader. Left: the patient is asked to put the volar side of the forearm on the machine. The average of three measurements is shown on the display in SAF and skin reflectance (R%). Right (copied from Mulder et al.(2)): Schematic view on the AGE reader. A UV black light source illuminates a skin surface of about 4 cm². A spectrometer detects reflected and fluorescent light from the skin.
excited by the AGE Reader. Multiplied by 100, this outcome is expressed in arbitrary units (AU). Also skin reflectance is calculated: the detected light in the 350-420 nm range is divided by the reflectance light intensity found in the white calibration measurement, given as R%. (2) SAF levels are validated against AGE levels measured in skin biopsies of controls, DM patients and patients on hemodialysis. (2;9;10)

During one measurement, SAF level is determined three times at the volar side of the lower right arm (Figure 2). In between two measurements the patient is asked to replace the arm on the machine to ensure every measurement to be on a slightly different part of the skin. The average of the three measurements is given as the outcome on the screen of the AGE-reader.

SAF-measurement is affected by skin pigmentation. This is corrected by a validation method developed by Koetsier et al., based on SAF measurements in healthy subjects with various skin types. (41) If measured skin reflectance is below 6%, which is the case in patients with dark skin, measurement is not possible and a warning sign is given on the display. If reflectance is between 6% and 12%, automatically a correction is made using an extra measurement with a LED light.

Variation in measured SAF levels within individuals daily and throughout the year is limited: in reproducibility studies an overall mean relative error of about 5% is found intra-individually, both over a single day and throughout the seasons. (2;10) De Ranitz et al. report similar results: they show a coefficient of variation of 4.9% in four consecutive measurements of 37 patients during an OGTT. (13)

Since body lotion and creams interfere with SAF measurement (42), patients were asked to wash their arm with soap if they had used any kind of cream on their arms on the day of measurement.

The UV-radiation exposure to the patient is far below the maximum allowed values. Therefore, no adverse side effects are expected from the use of the AGE-reader for the woman or her child. Measurement takes only a few minutes and is not uncomfortable to the patient.

Statistical analysis

Results are presented as mean and standard deviation for normally-distributed continuous parameters and as median and percentiles for continuous parameters with a skewed distribution. Normal distribution of continuous variables was tested by visual inspection of different characteristics of Q-Q plots. Categorical parameters are presented as percentages. Differences in baseline characteristics between DM, GDM and control groups for continuous, normally distributed variables were tested using a Student’s T-test or an Analysis of Variance (ANOVA), if significant followed by Bonferroni post hoc analysis. If not normally distributed, a Kruskall-Wallis test or Mann Whitney U test was performed. For categorical variables a Chi Square or Fisher’s Exact test was used.

Differences in SAF between DM and controls were tested using a Student’s T-test for normally distributed parameters or a Mann Whitney U Test if not normally distributed. SAF difference is tested at two different time points: at the end of the second trimester (SAF at 26-29 weeks) and at the end of pregnancy (SAF at 34-37 weeks). Differences in SAF were adjusted for pre-specified factors associated with higher SAF levels (age and ethnicity) (1;13) using linear regression.

Differences in course of SAF between groups during pregnancy are tested using a one-way ANOVA (Analysis Of Variance) for repeated measurements, if significant followed by a post-hoc Bonferroni analysis. We used 4 time points of SAF measurement: SAF at 26-29 weeks,
30-33 weeks, 34-37 weeks and 38 weeks. Since a repeated measures analysis only includes cases in which all four measurement points are completed, we selected all cases in which 3 of 4 measurement points were completed. Missing values imputation was carried out as follows: when the first or the fourth value was missing, the data were copied from the second or the third value, respectively. When the second or the third measurement was missing, the average of the first and third, or the second and fourth was taken respectively. Also analysis on only completed data as well as another way of imputation was performed: a “last observation carried forward” imputation in which we copied the last measured value to the missing value. When there is no measured value before a missing value, the following value was taken.

The relationship between time (gestational age) and SAF value was tested using a linear regression. In this analysis all SAF measurements and accompanying weeks of gestation were taken into account. Linear regression was performed in all three groups separately. Differences in SAF postpartum and SAF during pregnancy were analysed with paired sample T-tests for normally distributed data and Wilcoxon tests for skewed distributions.

Associations between ‘SAF last’ (last measured SAF of SAF 34-37 and SAF 38) and adverse pregnancy outcomes were tested using logistic regression and corrected for factors of significant influence on pregnancy outcome (age, BMI and HbA1c were tested). ‘SAF last’ was used for practical reasons: measurement at the end of pregnancy is easiest since most patients are in care at this gestational age and it precedes gestational age of delivery.

An association between SAF in GDM during pregnancy and development of T2DM within one year post partum was tested with a logistic regression model. A p-value < 0.05 was considered significant.

Sample size calculation

The sample size calculation for this study was based on the primary and secondary outcomes of the large SAF-study. The expected prevalences of outcomes were obtained from a database of GDM patients in the UMCU obstetric clinic of the past 10 years. The primary outcome of the SAF-study is macrosomia, which has an expected prevalence of 26%. Expected prevalences of secondary outcomes are: preeclampsia (6%), pre-term delivery (13%), caesarean section (31%), neonatal hypoglycemia (14%), hyperbilirubinemia (13%), and foetal death (0.9%), development of T2DM 6 weeks after delivery (10%). In previous studies, odds ratios were found ranging from 3-4 for adverse pregnancy outcome in patients with hyperglycemia during pregnancy.(29;43) Assuming that the risk of adverse pregnancy outcomes is of the same magnitude with increased SAF, a sample size of 100 GDM patients is sufficient to detect a relative risk of 2.15 for macrosomia (power 80%, alpha=0.05), which is a smaller effect size than reported for hyperglycemia. For secondary endpoints a sample size of 100 GDM patients is sufficient to detect relative risks ranging from 4.0 (preeclampsia) to 1.5 (insulin use). Because the incidence of foetal death is very low, we did not expect to find a significant relationship with SAF here.

We intended to include 100 GDM patients and 100 controls. Since the expected incidence of adverse pregnancy outcome is estimated to be twofold higher in pregnant patients with pre-existent diabetes mellitus than patients with GDM we intended to include 50 T1DM and 50 T2DM patients.

Results

Patient characteristics

From April 2010 to November 2011 a total of 197 patients signed informed consent. We included 161 pregnant women: 79 GDM patients, 24 T1DM and T2DM patients and 58 controls. We excluded 36 women with one or more exclusion criteria: skin reflectance <6% (n=3), preeclampsia at inclusion or in the past (n=3), recent serious infection or infarction or
hospital admission (n=1), active autoimmune disease (n=1) or active use of prednisone (n=1), smoking during pregnancy (n=17), or were less than 20 weeks pregnant (if GDM or control) or more than 32 weeks pregnant (n=7). Two women withdrew informed consent and one patient included as T2DM appeared to be misdiagnosed and was excluded. All GDM patients were confirmed by an OGTT. In the control group, 38 were confirmed normoglycemic by a negative OGTT (n=29) or challenge test (n=9). The rest of the control group had no risk factors for diabetes. The DM group consisted of 13 T1DM and 11 T2DM patients. 7 T2DM were diagnosed before pregnancy and 4 during pregnancy. Because of the small groups sizes of pre-existent diabetes patients, these T1DM and T2DM patients are analysed as one DM group.

Baseline characteristics are shown in Table 1. BMI was significantly higher in both GDM and DM patients compared to controls. The percentage of a positive family history was lower in controls compared to GDM (significantly lower) and DM (not significant). GDM patients were diagnosed at a gestational age average of 26.9 weeks. This is in accordance with the policy of testing for GDM in week 24-28 in high risk women. A mean HbA1c of 41.91 mmol/mol in DM patients is significantly higher than 33.90 mmol/mol in GDM patients. All DM patients used insulin during pregnancy, whereas insulin use was significantly lower in GDM patients (25%). As expected, glucose levels during OGTT (fasting and 2 hours after glucose intake) were significantly higher in the GDM group than in the control group. SAF was measured significantly more often in the DM group (mean of 5.08 times) compared to GDM (mean 3.95) and controls (mean 3.19). Also the difference in total SAF measurements between GDM and controls is significant. No significant differences are found between the three groups in age, ethnicity and nulliparity.

Table 1 Baseline characteristics of pregnant patients with DM and GDM and pregnant controls without diabetes

<table>
<thead>
<tr>
<th></th>
<th>DM patients</th>
<th>GDM patients</th>
<th>Control patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>32.9 (4.3)</td>
<td>32.7 (4.9)</td>
<td>32.9 (5.2)</td>
</tr>
<tr>
<td>BMI before pregnancy</td>
<td>29.5 (8.2)*</td>
<td>28.2 (6.4)*</td>
<td>25.4 (6.4)**</td>
</tr>
<tr>
<td>Ethnicity (white)</td>
<td>66.7%/20.8%/12.5%</td>
<td>63.3%/19%/17.7%</td>
<td>82.8%/10.3%/6.9%</td>
</tr>
<tr>
<td>Positive family history</td>
<td>33.3%</td>
<td>53.2%**</td>
<td>21.1%**</td>
</tr>
<tr>
<td>Nulliparous</td>
<td>45.8%</td>
<td>45.6%</td>
<td>46.6%</td>
</tr>
<tr>
<td>GDM in previous pregnancy</td>
<td>-</td>
<td>16.5%*</td>
<td>0%</td>
</tr>
<tr>
<td>Gestational age (weeks)</td>
<td>26.9 (2.6)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HbA1c (mmol/mol) in 2nd trimester</td>
<td>41.9 (8.5)**</td>
<td>33.9 (3.8)**</td>
<td>-</td>
</tr>
<tr>
<td>Insulin therapy during pregnancy</td>
<td>100%**</td>
<td>25.3%**</td>
<td>-</td>
</tr>
<tr>
<td>OGTT - fasting glucose (mmol/L)</td>
<td>-</td>
<td>5.6 (0.7)**</td>
<td>4.8 (0.4)**▲</td>
</tr>
<tr>
<td>OGTT - 2h glucose (mmol/L)</td>
<td>-</td>
<td>9.3 (1.7)**</td>
<td>7.4 (0.8)** ▲</td>
</tr>
<tr>
<td>Total number of SAF measurements</td>
<td>5.1 (1.9)**<em>#</em>#*</td>
<td>4.0 (1.4)**#<em>#</em></td>
<td>3.2 (1.6)**#<em>#</em></td>
</tr>
</tbody>
</table>

Continuous data are expressed as mean (standard deviation). Categorical data are presented as percentages. Significant difference between groups is presented with asterisks (* = P<0.05; ** = P<0.001) after the mean/median or percentage, followed by a number corresponding with the mean/median or percentage with which the difference is significant. ▲: of 58 control patients, 29 underwent an OGTT

Skin autofluorescence in pregnant DM and control patients
As illustrated in Figure 3, SAF during pregnancy was significantly higher in DM patients compared to controls. The difference was largest at the end of pregnancy (34-37 weeks) with
a mean SAF of 1.90 AU (SD 0.23) in DM and 1.66 AU (SD 0.27) in controls (p=0.001). Earlier in pregnancy (26-29 weeks) a mean SAF of 1.89 AU (SD 0.20) is seen in DM and 1.74 AU (SD 0.33) in controls (p=0.048). The difference in SAF between DM and controls at the end of pregnancy remains significant after correction for age and ethnicity (p=0.005). Significance was not reached anymore at SAF 26-29 weeks when corrected for these factors. Age and ethnicity were significantly associated with SAF levels (Table 2): compared to white European women, non white European show a 0.317 AU (p=0.001) higher SAF at 26-29 weeks and a 0.258 AU (p=0.001) higher SAF at 34-37 weeks. With every year increase in age, SAF rises with 0.026 AU at 26-29 weeks (p<0.001) and 0.016 AU in SAF 34-37 (p=0.024).

Changes in SAF levels during pregnancy in DM, GDM and controls
Figure 4 shows the results of a repeated measures analysis on SAF changes during pregnancy with separate lines for each study group. There is a significant difference between the three courses (p=0.027). SAF course in DM patients was significantly higher than in control patients (p=0.024). Higher SAF course in DM compared to GDM is not significant (p=0.086). No difference is found between SAF course in GDM versus controls. When correcting for age and ethnicity, difference between the groups remains significant with the same p-value (p=0.027). Repeated measures analysis of the smaller groups with only cases that were measured in all four time points shows, as presented in Appendix Figure A, similar results and significances. Also analysis of data with a "last carried forward" imputation showed similar results.

Changes in SAF over time during pregnancy shows a significant relationship (p=0.003) between gestational age and SAF level in the control group, with a decline in SAF of 0.018

| Table 2 Differences in SAF level in DM versus controls, ethnicity and increase in age |
|-----------------------------------|----------------------------------|-----------------|---|
| SAF 26-29                          | DM vs Co                         | NS              | 0.317                      | 0.001 |
|                                   | Non white European vs white European | -               | <0.001 |
|                                   | Increase in age                   | 0.026           | <0.001 |
| SAF 34-37                          | DM vs Co                         | 0.192           | 0.005 |
|                                   | Non white European vs white European | 0.258           | 0.001 |
|                                   | Increase in age                   | 0.016           | 0.024 |

Beta represents the difference in SAF (AU) in DM versus controls, in non white European women versus white European women and increase in SAF per year increase in age at 26-29 and 34-37 weeks pregnancy.
AU per week. After correction for age and ethnicity this relationship remains significant (p<0.001). In the GDM and DM group, no significant linear change in SAF over time is found.

SAF was measured postpartum with a mean follow-up of 7.9 weeks (mean follow-up GDM 7.8, controls 7.3 and DM 9.1 weeks). As presented in Figure 5, the outcomes show a rise in SAF postpartum in GDM and controls: mean SAF postpartum in GDM is 1.71 AU, which is significantly higher than SAF at 34-37 weeks (p=0.015) and SAF at 38 weeks (p=0.014).

In controls mean SAF postpartum is 1.69 AU, which is significantly higher compared to SAF at 30-33 weeks (p=0.044), SAF at 34-37 weeks (p=0.009) and SAF at 38 weeks (p=0.001). No significant difference in SAF postpartum compared to levels during pregnancy is seen in the DM group.

Figure 4. SAF level during pregnancy after imputation of missing values
Difference in SAF during pregnancy is significantly higher in DM versus controls (p=0.024)
Note: SAF on the Y-axis ranges from 1.0 AU to 2.5 AU

Figure 5. SAF level during pregnancy and about 8 weeks postpartum
Compared to Figure 4, only cases with also a postpartum measurement were taken into analysis
Note: SAF on the Y-axis ranges from 1.0 AU to 2.5 AU
SAF and adverse pregnancy outcomes

Adverse pregnancy outcomes in each group are presented in Table 3. Birth weight did not differ between the groups, but this is corrected for gestational age at delivery in the percentage of macrosomia, which shows a significant higher amount in the DM group compared to GDM and controls. The DM groups shows a significantly higher percentage of neonatal hypoglycemia compared to both GDM and control patients. No significant differences were found in percentage of preeclampsia, premature delivery, caesarean section, macrosomia and any adverse outcome.

Table 3 Adverse pregnancy outcomes in GDM, DM and control patients

<table>
<thead>
<tr>
<th></th>
<th>DM patients (n=18)</th>
<th>GDM patients (n=71)</th>
<th>Control patients (n=38)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Birth weight;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% macrosomia (&gt;P90)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Preeclampsia</td>
<td>0%</td>
<td>1.4%</td>
<td>4.9%</td>
</tr>
<tr>
<td>Premature delivery</td>
<td>16.7%</td>
<td>2.4%</td>
<td>4.1%</td>
</tr>
<tr>
<td>Caesarean section</td>
<td>44.4%</td>
<td>30.1%</td>
<td>31%</td>
</tr>
<tr>
<td>Foetal death</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>Neonatal</td>
<td>41.2%*</td>
<td>9.6%*</td>
<td>4.8%**</td>
</tr>
<tr>
<td>hypoglycemia</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neonatal jaundice</td>
<td>11.8%</td>
<td>2.8%</td>
<td>0%</td>
</tr>
<tr>
<td>Neonatal trauma</td>
<td>11.8%</td>
<td>4.2%</td>
<td>0%</td>
</tr>
<tr>
<td>Any complication</td>
<td>82.4%*</td>
<td>42.3%*</td>
<td>42.5%**</td>
</tr>
</tbody>
</table>

Data are presented as percentages. Significant difference between groups is presented with asterisks (* = P<0.05; ** = P<0.001) after the percentage, followed by a number corresponding with the percentage with which the difference is significant.

neonatal jaundice and neonatal trauma. No foetal death occurred in the entire group. The presence of one or more complications versus no complications, shows significantly more adverse pregnancy outcomes in the DM group compared to both GDM as well as control patients.

We investigated the relationship between SAF levels and three adverse pregnancy outcomes that were most seen (caesarean section, macrosomia and any complication). In Table 4, mean SAF at 34-37 weeks is presented per group per adverse pregnancy outcome. No significant difference is found in SAF per adverse pregnancy outcome in each group, with the exception of the control group in which a significantly higher SAF is found in women with a macrosome child (p=0.037).

Table 4 SAF at 34-37 weeks in GDM, DM and control patients, presented for caesarean section, macrosomia and any adverse outcome

<table>
<thead>
<tr>
<th></th>
<th>Co</th>
<th>GDM</th>
<th>DM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caesarean section</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>1.67 (0.28)</td>
<td>1.65 (0.48)</td>
<td>2.00 (0.15)</td>
</tr>
<tr>
<td>No</td>
<td>1.61 (0.26)</td>
<td>1.73 (0.27)</td>
<td>1.84 (0.29)</td>
</tr>
<tr>
<td>Macrosomia</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>2.00* (0.28)</td>
<td>1.54 (0.53)</td>
<td>1.84 (0.17)</td>
</tr>
<tr>
<td>No</td>
<td>1.62* (0.24)</td>
<td>1.72 (0.33)</td>
<td>1.95 (0.29)</td>
</tr>
<tr>
<td>Any adverse outcome</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 or more</td>
<td>1.70 (0.29)</td>
<td>1.69 (0.44)</td>
<td>1.86 (0.24)</td>
</tr>
<tr>
<td>No</td>
<td>1.59 (0.20)</td>
<td>1.71 (0.26)</td>
<td>1.93 (0.15)</td>
</tr>
</tbody>
</table>

SAF at 34-37 weeks, presented as mean (standard deviation), per group and adverse pregnancy outcome; * represents significance set at p<0.05

In a logistic regression analysis per group, no relationship is found between last measured SAF of SAF at 34-37 and 38 weeks (‘SAF last’) and caesarean section, macrosomia or any complication in each group. Results of analysis of the entire group (n=161) are presented in Table 5, which shows the relationship between adverse pregnancy outcomes and ‘SAF last’ as
well as other factors that might influence adverse outcomes. Also here, no relationship between HbA1c and adverse pregnancy outcomes is found. A significant relationship is seen between HbA1c and each outcome and also between the presence of DM versus no DM and macrosomia and any complication.

Table 5 Relationship between SAF last and adverse pregnancy outcomes

<table>
<thead>
<tr>
<th>Adverse pregnancy outcome</th>
<th>SAF last</th>
<th>Univariate</th>
<th>P-value</th>
<th>Multivariate</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sectio caesaria</td>
<td>NS</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HbA1c</td>
<td>OR 1.106</td>
<td>0.009</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Age</td>
<td>NS</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>BMI</td>
<td>NS</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>DM yes/no</td>
<td>NS</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Macrocomia</td>
<td>SAF last</td>
<td>NS</td>
<td>-</td>
<td>NS</td>
<td>-</td>
</tr>
<tr>
<td>HbA1c</td>
<td>OR 1.171</td>
<td>0.001</td>
<td>-</td>
<td>NS</td>
<td>-</td>
</tr>
<tr>
<td>Age</td>
<td>NS</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>BMI</td>
<td>NS</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>DM yes/no</td>
<td>OR 11.657</td>
<td>&lt;0.001</td>
<td>NS</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Any complication</td>
<td>SAF last</td>
<td>NS</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HbA1c</td>
<td>OR 1.210</td>
<td>&lt;0.001</td>
<td>1.179</td>
<td>0.008</td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>NS</td>
<td>-</td>
<td>NS</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>BMI</td>
<td>OR 1.107</td>
<td>0.003</td>
<td>NS</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>DM yes/no</td>
<td>OR 6.355</td>
<td>0.005</td>
<td>NS</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Univariate as well as multivariate analysis of relationship between several factors and adverse pregnancy outcomes. Significant factors in the univariate analysis are taken into the multivariate analysis, if significant odds ratio (OR) is presented NS = not significant

**SAF level during pregnancy and the postpartum development of type 2 diabetes**

For the analysis of T2DM postpartum, 47 patients that were already one year postpartum were contacted and 17 could be included (16 could not be reached, 13 refused participation and 1 developed T1DM instead of T2DM). Of the 17 GDM patients, 2 developed T2DM one year postpartum. One was diagnosed a couple of weeks after pregnancy, this information was derived from the patient’s file. One was diagnosed during our postpartum analysis. Table 6 presents ‘SAF last’ and mean SAF during entire GDM pregnancy of patients with T2DM one year postpartum versus no T2DM. No trend can be suggested of a relationship between SAF value during GDM pregnancy and a one year postpartum development of T2DM. Because of the small group sizes, we did not perform any analyses on these data.

Table 6 SAF during pregnancy in women with or without T2DM

<table>
<thead>
<tr>
<th></th>
<th>SAF last</th>
<th>SAF mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>GDM (n=17)</td>
<td>T2DM (n=2)</td>
<td>1.6 (n=1)</td>
</tr>
<tr>
<td>No T2DM (n=15)</td>
<td>1.78</td>
<td>1.75</td>
</tr>
</tbody>
</table>

SAF last = last measured SAF, either SAF 34-37 or SAF 38
SAF mean = mean SAF of all SAF measurements during pregnancy

**Discussion**

This is the first study in which the course of SAF levels during pregnancy is reported in women with and without diabetes. A physiological decrease in SAF in the second half of pregnancy is seen in non-diabetic pregnancy followed by a postpartum increase in GDM and controls. No change in SAF is seen during GDM or DM pregnancy. SAF levels in DM during pregnancy are higher than SAF in normoglycemic pregnancy.

In non-diabetic pregnancy, a decrease in SAF in the second half of pregnancy was observed with a sharp increase after pregnancy back to levels earlier in pregnancy in GDM and
controls. A different pattern was seen in DM pregnancy in which SAF did not change and was significantly higher than in control patients.

To our knowledge, no data are reported on AGEs during normoglycemic pregnancy. Apparently, normal pregnancy is associated with a temporary fall in SAF in the second half of pregnancy, supported by the observation that SAF levels about 8 weeks postpartum are comparable with SAF earlier in pregnancy. A number of mechanisms may contribute to this phenomenon. Firstly, AGEs are cleared by the kidneys(7) and glomerular filtration rate increases about 40-50% until the end of pregnancy(44). Secondly, in a previous study little influence on SAF is found of local skin blood flow, with lower SAF levels measured in situations of higher blood flow through most superficial capillaries(42) and during pregnancy peripheral vasodilatation is seen(44). Also, a physiological increase in protein turnover is seen during pregnancy(45), which might be of temporary influence on new AGE formation or on the half-life of skin collagen, on which half-life of skin AGEs depends.(7) Since normal pregnancy physiology appears to be a condition of oxidative stress(46), a fall in SAF can not be explained by a change in oxidative state. The described phenomena disappear after delivery, leading to an increase in SAF postpartum, probably a return to normal values.

In contrast to the situation in normal pregnancy, SAF does not exhibit any significant change during and after pregnancy complicated by DM. Since the abovementioned physiological changes also apply to hyperglycemic pregnancy, the fact that no change in SAF is seen in GDM and DM indicates an additional mechanism is operative in the diabetic pregnancy. Most likely, the hyperglycemic episodes result in sufficient generation of AGEs to modify the physiological change in SAF. Unchanged SAF levels in pregnancy are therefore an indication of increased glycemic exposure.

Buongiorno et al. compared serum AGE levels in pre-existent diabetic pregnant women between trimester 1 and 3 and report a trend towards increasing serum AGE levels during pregnancy in T1DM and T2DM pregnant women, which did not reach significance.(38) Guosheng et al. compared serum AGE in midgestation GDM with late-gestation GDM which did not show any difference.(35) Although these studies investigated serum AGE instead of tissue AGE levels, these support our findings that in pregnant hyperglycemic women no decrease in AGE levels is found during pregnancy.

Our results leave GDM in a vague area between DM and controls when it comes to SAF levels during pregnancy. In our study SAF course in GDM is similar to controls throughout pregnancy, which is an addition to the study by de Ranitz et al., in which no significant difference in baseline SAF between GDM and controls is found.(13) When visually inspecting Figure 4, SAF in GDM shows levels lower than in DM, but this was not significant. We expect this to be due to small group size. Probably in larger study groups significantly higher SAF course will be found in DM compared to GDM throughout pregnancy. Like controls, GDM shows a rise of SAF postpartum, which is not seen in DM patients. But the fact that SAF in both GDM and DM group shows no significant decline and SAF in controls does, suggests that also short term hyperglycemia in GDM affects SAF levels during pregnancy, making SAF levels steadier and less influenced by pregnancy physiology than SAF in controls.

Higher SAF levels in DM compared to controls are an expected outcome, since DM patients have a long history of exposure to hyperglycemia, which is accompanied by higher SAF levels.(10) Reference values for SAF levels, investigated by Koetsier et al.(1), show that normal SAF values for our study groups range approximately between 1.53 AU (SD 0.30, age 20-30) and 1.73 AU (SD 0.42, age 30-40). In our study groups, mean SAF levels of about 1.7 AU in controls and 1.9 AU in DM patients were found during pregnancy. With a mean age of 33 years, SAF levels of our control group fall within the normal range. Therefore, SAF course
as presented in Figure 4 can serve as reference values for non-diabetic women during pregnancy.

An important question in our study was the association of SAF with the incidence of adverse pregnancy outcome. We did not find an association between SAF levels during pregnancy and adverse pregnancy outcomes, but our groups might be too small to detect such a relationship. Higher HbA1c levels were associated with increased risk of caesarean section, macrosomia and any complication in the entire group. This is probably due to higher hyperglycemia levels, of which HbA1c is a reflection, that are associated with increased risk of adverse pregnancy outcomes.(29) A major problem in this study was the much lower incidence of various adverse outcomes than expected on the basis of results in previous years in the same clinic. As a consequence, the required group size to evaluate many single outcomes was basically higher than originally calculated.

The relationship between serum AGE levels during pregnancy and adverse pregnancy outcomes was investigated in GDM and pregnant control patients by Guosheng et al.(35) They found significantly higher AGE levels in GDM women with the abnormal foetal outcomes foetal distress, foetal malformation and stillbirth compared to GDM women with normal foetal outcome. However, this study is performed in Asian women and it investigates serum AGE levels instead of tissue AGEs, which makes it less comparable to our study on tissue AGE levels in a white European and Moroccan population. Since SAF appears to be a predictor of diabetes complications(21;22), we wondered whether SAF levels could serve as a predictor in the risk of adverse pregnancy outcomes as well. With our data we can not exclude such a relationship, so this is an interesting field for further research.

The high prevalence of T2DM after pregnancy complicated by GDM(27), makes it important to identify high risk women as early as possible. Current guidelines on postpartum follow-up of GDM women vary greatly worldwide and so do the non-attendance rates, ranging between 38 and 100%. (27) In our hospital, the policy is screening for diabetes six weeks after delivery and then every year. Increasing attention is paid for the development of prediction models based on measurable factors during pregnancy to estimate a woman’s risk of developing T2DM postpartum, in order to achieve more effective follow-up with higher attendance rates. Several articles report risk factors that might be used in such a prediction model, like fasting glucose levels on OGTT, pre-pregnancy BMI, need of insulin use during pregnancy and gestational age at diagnosis of GDM. (27) Our aim was to investigate whether SAF level could be a predictor as well. To our knowledge, such a relationship has not been investigated before. High HbA1c levels, even within the normal range, show predictive value for the development of T2DM(17;47), which is in accordance with T2DM being preceded by a state of mild hyperglycemia, resulting in increased HbA1c levels before reaching diagnostic criteria. Since HbA1c is an intermediate glycation product and a precursor in the formation of AGEs, and since SAF appears to be a sensitive detector of (pre-)diabetes(26), the question is whether tissue AGE levels might also be increased in the early stages of T2DM development. Unfortunately, because of small group sizes in our study, analysis of such a relationship is not feasible. Exploration of the data did not reveal any trend towards a relationship, which means that this question remains unanswered.

**Limitations and recommendations for future research**

One limitation of this study is the small size of the investigated DM group. Too few patients were eligible for inclusion, which resulted in a total of 24 DM patients instead of the aim of 50 T1DM and 50 T2DM. This resulted in limited possibilities for analysis of the data. In addition, the percentages of adverse pregnancy outcomes turned out to be less than expected.
Since our sample size calculation was based on these expected percentages, the analysed groups are probably too small to demonstrate an existing significant difference. Future research on this subject should take into account the possibility of lower than previously reported complications rates in GDM patients, possibly as a result of a continuously improving quality of care.

Besides that, because of missing values we made use of 13.5% imputed data in the repeated measures analysis of changes in SAF levels during pregnancy, which is not the ideal situation. However, this turned out to be of little influence when compared to analyses of only real data and to another way of imputation.

Knowing that SAF levels are higher during pregnancy in DM patients compared to controls, larger studies are necessary to evaluate the role of SAF measurement during pregnancy in order to predict the risk of adverse pregnancy outcomes in GDM and DM patients. The same applies to a relationship between SAF levels during GDM and the development of T2DM postpartum.

It should also be noted that the DM group is a combination of T1DM and T2DM patients, which are conditions different in pathophysiology and patients characteristics. Since in both disorders hyperglycemia exists over a period of time before pregnancy and increased AGE levels are found in both conditions(4), we expect limited influence of this heterogeneity within the DM group on SAF measurements. In future research, it would be interesting to investigate differences in SAF during pregnancy between T1DM and T2DM patients.

In the control group we recruited part of our subjects from negative OGTT’s and glucose challenge tests. This probably resulted in a control group with more risk factors for diabetes than the normal population, but since these patients were confirmed normoglycemic, we do not expect this of large influence on measured SAF levels. Therefore, SAF course as presented in Figure 4 in this control group is, to our opinion, generalisable to the normal population and can serve as reference values for non-diabetic women during pregnancy. Explanation for the decline in SAF during normoglycemic pregnancy accompanied by a postpartum rise constitutes an interesting subject for further research.

Conclusion
SAF shows a decrease in the second half of pregnancy in non-diabetic pregnant controls but not in GDM and DM patients, followed by a rise back to SAF levels earlier in pregnancy about 8 weeks postpartum in GDM and controls. Apparently, pregnancy physiology makes SAF temporarily lower in normal, non-diabetic pregnancy. These results can serve as reference values for SAF levels during normoglycemic pregnancy. SAF levels during pregnancy are higher in DM compared to non diabetic controls, which is probably due to the long term hyperglycaemia exposure in DM patients. In these small study groups, no relationship was found between SAF levels during pregnancy and adverse pregnancy outcomes. No conclusions can be drawn on the predictive value of measuring SAF levels during pregnancy for the risk of type 2 diabetes one year after GDM pregnancy.

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Appendix

Appendix Figure A Results of a repeated measures analysis of all cases in which SAF was measured at all four measurement points: SAF 26-29, 30-33, 34-37, 38. The figure shows SAF course during pregnancy with separate lines for GDM, control and DM. Difference in SAF course between the three groups is significant (p=0.038). A post hoc Bonferroni analysis shows only a significant difference in SAF course between DM and control (p=0.037)


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