Diverging effects of diabetes mellitus in patients with peripheral artery disease and abdominal aortic aneurysm and the role of advanced glycation end-products: ARTERY study — protocol for a multicentre cross-sectional study

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ABSTRACT

Introduction: Diabetes mellitus is a well-defined risk factor for peripheral artery disease (PAD), but protects against the development and growth of abdominal aortic aneurysm (AAA). Diabetes mellitus is associated with arterial stiffening and peripheral arterial media sclerosis. Advanced glycation end-products (AGEs) are increased in diabetes mellitus and cardiovascular disease. AGEs are known to form cross-links between proteins and are associated with arterial stiffness. Whether AGEs contribute to the protective effects of diabetes mellitus in AAA is unknown. Therefore, the ARTERY (Advanced glycation end-pProducts in patients with peripheral arterY disEase and abdominal aortiC aneurYsm) study is designed to evaluate the role of AGEs in the diverging effects of diabetes mellitus on AAA and PAD.

Methods and analysis: This cross-sectional multicentre study will compare the amount, type and location of AGEs in the arterial wall in a total of 120 patients with AAA or PAD with and without diabetes mellitus (n=30 per subgroup). Also, local and systemic vascular parameters, including pulse wave velocity, will be measured to evaluate the association between arterial stiffness and AGEs. Finally, AGEs will be measured in serum, urine, and assessed in skin with skin autofluorescence using the AGE Reader.

Ethics and dissemination: This study is approved by the Medical Ethics committees of University Medical Center Groningen, Martini Hospital and Medisch Spectrum Twente, the Netherlands. Study results will be disseminated through peer-reviewed journals and scientific events.

Trial registration number: trialregister.nl NTR 5363.

BACKGROUND

Diabetes mellitus is a risk factor for various diseases, including retinopathy, renal insufficiency and atherosclerosis. Remarkably, diabetes mellitus is a risk factor for peripheral artery disease (PAD), but protects against the development of abdominal aortic aneurysm (AAA). Evidence for an inverse association between diabetes mellitus and AAA was shown in a large case-control study with veterans (n=73 451) in which an AAA was detected in 1031 participants. This study demonstrated an OR of 0.54 for the presence of diabetes mellitus and AAA. This inverse association was confirmed by a meta-analysis consisting of 17 large studies. In addition, AAA growth was shown to be decreased substantially in patients with diabetes mellitus (n=49) as compared to those without diabetes mellitus (n=311) after 36 months of follow-up. The explanation for the diverging effects of diabetes mellitus...
on occlusive versus dilating arterial disease remains unclear.

Differences in effects of diabetes mellitus on aorta versus large peripheral arteries may contribute to these diverging effects. Advanced glycation end-products (AGEs) might also play a role in these different effects of diabetes mellitus on vascular tissues. AGEs are formed by non-enzymatic glycaemic and oxidative stress reactions. Diseases with increased glycaemic or oxidative stress are associated with increased AGE levels. Indeed, increased serum and skin AGEs are found in patients with diabetes mellitus compared to controls. Oxidative stress plays a role in the pathogenesis of AAA and PAD. In line with this reasoning, increased serum AGEs and skin AGEs were found in patients with PAD as compared to controls. Furthermore, our data show increased skin AGEs, non-invasively assessed, in 252 patients with AAA compared to controls (Boersema J et al, unpublished data, 2017). Two effects of AGEs have been described, namely formation of cross-links on long-lived proteins and induction of cellular stress responses by engagement of the receptor for AGEs. Cross-linking causes stiffness, but may also protect against mechanical structural loss of the affected tissues. The association between AGEs and arterial stiffness is shown in several studies. In patients with end-stage renal disease and in type 1 diabetes mellitus, skin AGE level was associated with pulse wave velocity, a measure of arterial stiffness. Increased arterial stiffness is associated with atherosclerotic disease. However, in a mouse model, homogenous stiffening reduced aneurysm growth, whereas segmental aortic stiffening caused aneurysm growth.

Therefore, the aim of the ARTERY study (Advanced glycation end-products (AGEs) might also play a role in these different effects of diabetes mellitus on vascular tissues. AGEs are formed by non-enzymatic glycaemic and oxidative stress reactions. Diseases with increased glycaemic or oxidative stress are associated with increased AGE levels. Indeed, increased serum and skin AGEs are found in patients with diabetes mellitus compared to controls. Oxidative stress plays a role in the pathogenesis of AAA and PAD. In line with this reasoning, increased serum AGEs and skin AGEs were found in patients with PAD as compared to controls. Furthermore, our data show increased skin AGEs, non-invasively assessed, in 252 patients with AAA compared to controls (Boersema J et al, unpublished data, 2017). Two effects of AGEs have been described, namely formation of cross-links on long-lived proteins and induction of cellular stress responses by engagement of the receptor for AGEs. Cross-linking causes stiffness, but may also protect against mechanical structural loss of the affected tissues. The association between AGEs and arterial stiffness is shown in several studies. In patients with end-stage renal disease and in type 1 diabetes mellitus, skin AGE level was associated with pulse wave velocity, a measure of arterial stiffness. Increased arterial stiffness is associated with atherosclerotic disease. However, in a mouse model, homogenous stiffening reduced aneurysm growth, whereas segmental aortic stiffening caused aneurysm growth.22

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skin AGEs in patients with and without diabetes mellitus. This study reported a mean skin AGE level of 2.79±0.8 arbitrary units (AU) in 973 patients with diabetes mellitus and 2.14±0.6 AU in 231 patients without diabetes mellitus. To show a similar difference between patients with and without diabetes mellitus of skin AGEs, 25 patients per group are required. Taking a failure plus dropout rate of 20% into account together with the possibility of subgroup analysis, we should include 30 patients per subgroup and a total of 120 patients.

**Recruitment**

The patients will be recruited from the outpatient clinic of vascular surgery located in one of the three following Dutch hospitals: University Medical Center Groningen (UMCG), Martini Hospital Groningen and Medisch Spectrum Twente (MST).

Patients will be informed about the study by their treating vascular surgeon. Detailed written and oral information will be given by the investigator or research coordinator.

**Study procedures**

After signing the informed consent form, patients are asked to fill in a questionnaire and have an appointment in the vascular laboratory. Blood will be drawn and 24-hour urine will be collected before surgery. Several biopsies will be obtained during surgery, depending on the type of surgery and of which more details are shown below. Collected tissue biopsies will be stored until analysis.

**Blood and urine sampling**

Blood will be sampled for routine analysis and stored to determine AGEs. For direct analysis, blood will be drawn in an EDTA tube, lithium heparin tube and a sodium fluoride tube. Citrate plasma, EDTA plasma and serum will be stored at −80°C. For the analysis of AGEs, EDTA tubes will be used for blood collection, which will be transported on ice, centrifuged on 4°C and stored at −80°C until batch analysis.

Patients will be asked to collect urine for 24 hours and to keep the container cool until the urine is returned to the laboratory. The urine will be centrifuged, routine
analysis will be performed and samples will be stored at −80°C.

**Biopsy**

During surgery, full-thickness biopsies from the arterial wall will be taken at predefined locations. These biopsies will be marked with sutures to assure cranio-caudal directions.

- In case of an open aneurysm repair, a biopsy will be obtained 5 cm below the lowest renal artery on the left ventral side with a size of 3 (length)×1 (width) cm.
- In case of bypass surgery for PAD, a biopsy will be obtained from the proximal anastomosis in the groin of 1 (length)×0.5 (width) cm.
- In case of an endarterectomy for PAD, usually the common femoral artery, a biopsy will be obtained from the proximal part of the artery with a size of 1 (length)×0.5 (width) cm.

In addition, skin tissue, venous tissue and fat tissue will be obtained and stored as described below. Skin tissue will be obtained from the incision with a size of 1 (length)×0.5 (width)×1 (depth) cm. Venous tissue is only available in case of an autologous venous bypass. In that case, a circular segment from the venous graft will be obtained at the level of the distal anastomosis with a size of 0.25–0.50 cm. Peri-aortic adipose tissue will be obtained during open aneurysm repair and perivascular adipose tissue will be obtained from the groin when available during bypass surgery or endarterectomy for PAD.

**Storage**

After surgery, biopsy material of the arterial tissue will be divided into three equal parts. The first two parts, together with skin, venous and adipose tissue, will be snap frozen and stored at −80°C. The third part, marked with a suture on the most distal part of the biopsy, will be stored in formaldehyde for 24–72 hours. Afterwards, tissue will be dehydrated with ethanol and embedded in paraffin. Materials of MST will be fixed with formaldehyde, stored in 96% ethanol and transported to the UMCG per batch of 10 samples where these tissues will be embedded in paraffin. Additional biopsy material, which will not be used for primary or secondary research questions, will be stored for future research. Information about the use of additional tissue for future research is included in the patient information and in the informed consent form.

**Outcome measures**

Table 2 presents an overview of the outcome measures and measurement methods, which will be described in detail in the following paragraphs.

**Demographics**

The patients’ characteristics such as date of birth, sex, cardiovascular risk factors, cardiovascular disease, cardiovascular family history, duration of diabetes mellitus, glycaemic control and drug use will be assessed by evaluating medical records and will be confirmed or complemented by a questionnaire consisting of 25 questions.

| Table 2 | Measures and methods |
|----------|-----------------------|
| **Domain** | **Outcome measure** | **Methods/equipment** |
| Demographics | Cardiovascular risk factors | Evaluation medical records |
| | Cardiovascular disease | Questionnaire |
| | Cardiovascular family history | Routine blood analysis, including random glucose, HbA1c, creatinine, total cholesterol, LDL, HDL, triglycerides, ALAT, ASAT, gamma-GT, LDH, TSH, T4 and hsCRP. EDTA, il-heparine tube, NaF tube. |
| | Drug use | |
| | Diabetes mellitus | |
| | Renal function | |
| | Lipid profile | |
| | Liver function | |
| | Thyroid function | |
| | Weight | Seca 877 scale |
| AGEs in arterial wall | Quantitative measurement | High-performance liquid chromatography |
| | Types and localisation | Immunohistochemistry |
| AGEs in fluids | Serum AGEs | High-performance liquid chromatography |
| | Urine AGES | High-performance liquid chromatography |
| Skin AGES | Skin autofluorescence | AGE Reader |
| Vascular parameters | Blood pressure | Microlife WatchBP Office |
| | Ankle-brachial index | Vicorder |
| | Diameter aorta | Siemens Acuson S2000 |
| | Intima-media thickness | Esaote MyLabOne |
| | Pulse wave velocity | Sphygmocor EM3 |

AGEs, advanced glycation end-products; ALAT, alanine aminotransferase; ASAT, aspartate aminotransferase; GT, glutamyltransferase; HDL, high-density lipoprotein; hsCRP, high sensitivity C-reactive protein; LDH, lactate dehydrogenase; LDL, low-density lipoprotein; TSH, thyroid-stimulating hormone.
Weight, length, waist and hip diameter will be recorded during visit at the vascular laboratory. Routine blood and urine analysis will be performed to evaluate the presence of diabetes mellitus and to determine several cardiovascular risk factors, including renal function.

**AGEs in arterial wall**

Analysis of AGEs in the arterial wall, serum and urine will be performed by the laboratory for Metabolism and Vascular Medicine, Maastricht University Medical Center, the Netherlands. Protein-bound carboxymethyllysine (CML), Nε-(carboxyethyl)lysine (CEL) and 5-hydro-5-methylimidazolone (MG-H1) will be measured in the first part of the arterial biopsy material with ultra-performance liquid chromatography tandem mass spectrometry (UPLC–MS/MS), as described previously.24

To localise different types of AGEs, 4 mm slices will be made from the paraffin-embedded arterial tissue. Consecutive slices will be stained with several antibodies, including polyclonal anti-AGE antibody (Abcam, ab23722), monoclonal anti-AGE antibody for lysine derivatives (Biorbyt, orb27490) and monoclonal anti-pentosidine (Biorbyt, orb27502).

**AGEs in fluids**

Specific protein-bound plasma and urine AGEs, CML, CEL and MG-H1, will be measured with UPLC–MS/MS, as described previously.25 Protein-bound plasma and urine pentosidine will be measured using high-performance liquid chromatography, as described previously.26

**Skin AGEs**

AGEs in the skin will be assessed non-invasively with the AGE Reader (DiagnOptics Technologies BV, Groningen, the Netherlands). This method uses ultraviolet light to excite specific AGEs. Reflection and emission light are detected and converted into a skin autofluorescence (SAF) level expressed in AU’s. Detailed information about this method is described elsewhere.27 Both inner forearms will be scanned three times. Patients will be asked not to use sun blockers, skin tanners or skin creams 2 weeks before the measurement, since these creams influence the SAF measurement.28

**Vascular parameters**

Several vascular parameters will be obtained for the ARTERY study. These parameters include blood pressures, toe pressures and duplex ultrasound of the aorta. In addition, carotid artery intima-media thickness and carotid-femoral pulse wave velocity will be measured in the vascular laboratory of the UMCG only. Measurements will be performed by trained vascular technicians.

Brachial artery blood pressures will be measured three times on both arms in sitting position after 10 min of rest.

For the ankle-brachial index measurement, patients should be in supine position. For the ankle-brachial index, the systolic pressure will be measured of the brachial artery, and the posterior tibial artery and the dorsalis pedis artery bilaterally. This will be measured during cuff release using a Doppler probe. Toe-brachial index will be measured using toe photo-electric plethysmography during gradual pressure release of a cuff placed at the base of the first toe, the toe pressure being defined by reappearance of the plethysmographic signal.

Screening to exclude concomitant AAA and PAD will be performed with duplex ultrasound in supine position. An AAA will only be reassessed in case the last imaging procedure was more than 2 months ago.

Carotid intima-media thickness will be measured as a marker of generalised atherosclerotic burden. The far wall of the left and right common carotid artery will be measured with the MyLabOne (Esaote Europe B.V., Maastricht, the Netherlands). Three measurements will be performed with an average of six heart beats of the wall segment of 10 mm before the transition to the carotid bulb.

For the measurement of the carotid-femoral pulse wave velocity, patients are asked not to drink coffee or alcohol and not to smoke 3 hours before the measurement. Patients are allowed to have a light meal. Arterial waveforms will be obtained from the carotid and femoral artery and the time delay will be measured between the feet of the two waveforms. The distance covered by the waves will be established as 80% of the distance between the two recording sites. Pulse wave velocity will be calculated as distance/time delay in m/s with the use of the Sphygmocor EM3 (AtCor Medical Pty, West Ryde, Australia).

**Data handling and statistical analysis**

Informed consent forms will be collected and stored at the UMCG. Biomaterials from patients will be coded. All generated data will be stored and coded in an electronic Case Report File with the software OpenClinica. Data will be exported to the SPSS software (IBM Corp., Armonk, New York, USA) for statistical analysis.

Described data will be shown as mean with SD for normal distributed variables, mean with IQR for skewed data and number with percentages for categorical data. Difference between groups will be tested with the independent Student’s t-test or non-parametric tests for skewed data. For categorical variables, χ² test will be performed. After comparing differences with a univariate analysis, multivariable analyses will be performed to adjust for important confounders of AGEs. A value of p<0.05 will be considered significant.

**Ethics consideration**

All investigators performed and passed the examination of the Dutch Basic course on Regulations and Organisation for clinical investigators.
DISCUSSION

The ARTERY study is designed to define the possible role of AGEs in the protection against AAA formation in diabetes mellitus. The protocol for this cross-sectional multicentre study addresses this issue by combining local and systemic AGE assessments with vascular functional and imaging methods.

Several research groups have evaluated the localization and content of AGEs in human tissue before. Most studies have been performed assessing skin AGE levels. Furthermore, Hofman et al described the measurement of AGEs in right atrial appendages and vein graft material from patients with coronary artery disease. However, to the best of our knowledge, this is the first study that will compare AGE accumulation in human vascular tissue and will investigate the effects of diabetes mellitus on AGE accumulation and distribution in different vascular diseases. Furthermore, the current study has been designed to provide answers on several additional research questions, including the association between tissue AGEs and vascular stiffness. In addition, this study will show the association between skin AGEs measured non-invasively and AGEs measured in vascular tissue, urine, and blood.

The AGE Reader uses fluorescence for assessing skin AGEs. SAF is strongly associated with AGEs from skin biopsies. Several studies have shown that SAF, measured with the use of the AGE Reader, is related to arterial parameters such as pulse wave velocity and intima-media thickness, and predicts cardiovascular events. However, the correlation between AGEs measured in the arterial wall compared to SAF is unknown. It is likely that there is a correlation between both, since Hofmann et al showed that skin AGE levels were strongly correlated to AGEs in cardiac tissue from patients with coronary artery disease. A strong association between AGEs of the arterial wall and AGEs measured with SAF would underscore the role of the AGE Reader as an easy and non-invasive technique as a marker of vascular tissue accumulation.

Although we designed our study carefully, there are some limitations. We do not exclude patients suffering from renal insufficiency. These patients also have increased AGE accumulation. We have chosen not to exclude these patients, since patients with vascular disease frequently have renal insufficiency. Exclusion of patients with concurrent renal failure and vascular disease would result in a selection bias. Therefore, we have decided to increase our sample size to allow subgroup analysis. In addition, renal function will be a variable which will be included into multivariate analyses. In addition, biopsy material obtained at the location of disease may not be representative for vascular tissue in general. We standardise the biopsies procedure to minimise location-specific differences. However, it is technically and ethically not possible to obtain additional biopsies from different vascular sites to determine whether AGEs are distributed equally.

In conclusion, the ARTERY study will investigate the diverging effects of diabetes mellitus on AAA and PAD and the role of AGEs as a possible explanatory factor. The study will be conducted in a multicentre setting. Primary objective is to analyse AGEs in the arterial wall. As secondary objectives, AGEs of the arterial wall will be compared to AGE measurements in blood, urine and skin, and with vascular wall parameters.

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Contributors LCdV, CJZ and JDL conceived the ARTERY study. LCdV drafted the first version of this manuscript. LCdV and JB are responsible for the management of the study. All authors read, revised and approved the final manuscript and also contributed to the design of the ARTERY study.

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Competing interests AJS is a founder and shareholder of DiagnOptics BV, the Netherlands, manufacturing autofluorescence readers (http://www.diaognoptics.com). All other authors have nothing to disclose related to this paper.

Ethics approval Ethical approval was obtained from the Medical Ethics committee, University Medical Center Groningen, Groningen, the Netherlands (METc 2014/269). Local approval for the ARTERY study was given by the Medical Ethics committee and the board of directors of the Martini Hospital (METc 2014/269) and MST (METc H15-076).

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