Advanced glycation end products in patients with peripheral artery disease

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Chapter 9

Summary, discussion and future perspectives
Summary

This thesis addresses the role of advanced glycation end products (AGEs) in patients with peripheral artery disease (PAD) and abdominal aortic aneurysm (AAA). These diseases share several risk factors, although the pathophysiology is different. Atherosclerosis develops in the tunica intima of the arterial wall, and is the main cause of PAD, resulting in stenosis or eventually occlusion of the arteries of the lower limb(s). Clinically, it may present with typical pain during exercise. An aneurysm of the aorta is caused by inflammation and proteolysis of the tunica media of the arterial wall and is most frequently an asymptomatic disease until rupture of the aneurysm. In this thesis, we evaluated the role of an emerging biomarker called AGEs in both these patient groups. AGEs are formed during healthy aging, but accelerated accumulation of the AGEs occurs during oxidative and glycemic stress. Elevated levels of AGEs are shown in patients with diabetes mellitus and renal failure. Furthermore, AGEs are also a predictive marker for cardiovascular end points in these patients. Skin AGEs can be assessed noninvasively by the AGE Reader™, due to fluorescent properties of several AGEs. The technique called skin autofluorescence (SAF) has been used to assess skin AGEs in several diseases. In this thesis, we aimed to evaluate the role of SAF as a marker of cardiovascular events and changes in renal function in patients with PAD and AAA.

In Chapter 2, a review provides detailed information about the formation of AGEs, on behaviour of AGEs in different tissues and which technique could be used for AGE measurement. In addition, all published clinical studies up to May 2016 about AGEs in PAD were reviewed, which includes Chapter 3-5.

The first publication of this research line on AGE in PAD is shown in Chapter 3. In this chapter, we showed a case-control study of 492 patients with PAD. From our initial cohort recruited from 2007 to 2010, we selected patients based on strict inclusion criteria. First, only patients with PAD were selected, based on the ankle-brachial index, combined with additional imaging techniques to confirm the diagnosis. Since age and diabetes mellitus are two major contributors of the formation of AGEs, we matched patients and controls for these risk factors. We excluded patients with end-stage renal disease (estimated glomerular filtration rate <15 mL/min per 1.73m²) since these patients suffer from severe long-term chronic inflammation. Also patients with an organ transplantation and recent cardiovascular events were excluded, since these events induces oxidative stress, and therefore may increase AGE formation. The first
result showed that patients with PAD had increased AGEs levels compared to diabetes- and age-matched controls. Multivariable backward linear regression analysis in patients with PAD only, showed that age, smoking, diabetes mellitus, renal function, cerebrovascular disease and abdominal aortic aneurysms were independent determinants of SAF. These risk factors are corresponding to other studies. To evaluate the independent role of SAF to detect patients with PAD, a logistic regression model was performed. This multivariable analysis showed that SAF was independently associated with PAD, also corrected for cardiovascular risk factors and comorbidity.

Patients included in 2007 and 2008 (n=252) were followed for five years. Chapter 4 and Chapter 5 describe systemic and local outcome of this cohort. In Chapter 4, we tested whether SAF was associated with all-cause mortality and major adverse cardiovascular events, defined as cardiovascular death, myocardial infarction and stroke. This study showed that SAF is independently associated with both end points, also after adjustment of cardiovascular risk factors. This study is the first evidence that SAF may identify patients at risk for cardiovascular end points in PAD.

In addition to systemic outcome, the end point amputation which is a severe outcome for patients with PAD, was evaluated in Chapter 5. This end point was defined as amputation due to critical limb ischemia. SAF was associated with amputation in patients with PAD. After correction for diabetes mellitus and Fontaine classification, a clinical method to categorize symptoms, SAF is still associated with amputation in these patients. In addition, a prediction model was performed in this study. Fontaine classification is well known as a predictor for amputation. Addition of SAF to Fontaine classification, resulted in a better prediction of amputation. A subgroup analysis was performed in patients with mild symptoms, defined as a Fontaine classification stage I and II. In patients with mild symptoms, SAF was the only predictor for amputation.

Chapter 6 shows the association between SAF and renal function decline. This was evaluated in the total cohort included between 2007 and 2011 (n=471). In a cross-sectional analysis of the cohort, increased SAF was associated with impaired kidney function (estimated glomerular filtration rate <60 mL/min), irrespective of diabetes mellitus. In the longitudinal analysis, the primary end point was renal function decline. Because of absence of annual renal function measurements, a mixed model analysis was performed to correct for different time between observations and different number of observations. This analysis showed no association between SAF and renal function decline. The secondary end points were estimated glomerular filtration rate
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<60 mL/min per 1.73m², <45 mL/min per 1.73m² and rapid renal function decline (>5 mL/min per 1.73m² per year). While in the crude model SAF was associated with decline in kidney function (<60 mL/min and <45 mL/min per 1.73m²), after correction for age, sex, current smoking and diabetes mellitus, this association disappeared. Rapid renal function decline was not associated with SAF at all.

In Chapter 7, we presented the first study which shows increased SAF in patients with an AAA. This case-control study included patients with an AAA (n=248) and age- and diabetes-matched controls. SAF was higher in patients compared to controls. A linear regression model showed that common risk factors were associated with SAF in the AAA patients, including age, smoking, renal function and PAD. SAF was also associated with AAA after correction for cardiovascular comorbidity. However, after correction for sex and smoking was SAF not associated with the presence of an AAA.

A first limitation of the above mentioned studies is that these studies are observational studies, performed in one cohort recruited from a single-center tertiary referral hospital. Possibly, we included patients with severe cardiovascular comorbidity and increased disease progression. Therefore, it would be interesting to evaluate whether our results correspond to another cohort of patients with PAD and AAA. We encourage other researchers to perform similar studies in patients with vascular disease. That would also provide a possibility for an external validation of SAF as a predictor for amputation.

Another limitation is that we only used SAF as a biomarker in addition to standard cardiovascular risk factors for the association with different end points. However, SAF reflects only a part of the AGE-RAGE axis. Therefore, instead of focusing on only SAF or AGE, preferably it would be more interesting to evaluate the association with other markers of the broader AGE-RAGE axis. This AGE-RAGE axis consists of AGEs and the receptor for AGE. Not only a large range of AGEs could be evaluated in different body compartments, also the receptors for AGEs can be measured. The best known receptor for AGE is called RAGE. RAGE is present on different cell surfaces including endothelial cells, and RAGE is present in the circulation, known as soluble RAGE (sRAGE). RAGE on cell surface can be measured in tissue biopsies, while sRAGE is easily obtainable from blood samples. RAGE and sRAGE have opposite effects. Binding of AGEs to RAGE causes several pro-atherosclerotic effects, including NF-κB activation and pro-inflammatory reactions, while sRAGE acts as a decoy for circulating AGEs, which prevents AGE to bind to RAGE on the cell surface. Therefore, sRAGE are thought to
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protect from the pro-atherosclerotic effects since they prevent binding of AGE to cellular RAGE. In our review (Chapter 2), we also showed four available studies which showed an association of (s)RAGE in patients with PAD.

One of the reasons that we did not investigate the total AGE-RAGE axis is that we did not collect blood, urine or tissue samples from these patients. Therefore, a new study was designed which is presented as a study protocol in this thesis (Chapter 8). The main focus of the ARTERY study (Advanced glycation end products in patients with peripheral artery disease and abdominal aortic aneurysms) is to explore the diverging effects of diabetes mellitus in PAD versus AAA. Diabetes mellitus is a risk factor in patients with PAD, while the incidence of diabetes mellitus is low in patients with an AAA and the growth rate of an aneurysm is retard in patients with diabetes mellitus. We hypothesize that AGEs may play a role in the diverging role of diabetes mellitus. Patients with diabetes mellitus are known to have increased AGE accumulation. Furthermore, AGEs are known to cause cross-links and therefore cause arterial stiffness. But perhaps, cross-linking of collagen and elastin fibers in the media may at the same time have a protective role against disruption of the tunica media. So, possibly, the AGE accumulation in the aorta of diabetic patients with an aneurysm causes stiffness, which prevents aneurysm growth. In this study, we will include 120 patients with PAD and AAA with and without diabetes mellitus, which are scheduled for surgery. Primary end point is to evaluate the different AGEs in arterial tissue of patients with and without diabetes mellitus. Secondary end points are the association between AGEs measured in the arterial tissue and AGEs measured in blood, urine and skin (SAF). Furthermore, AGEs of arterial tissue will be correlated to different mechanical properties of the arteries. Finally, AGEs in arterial tissue will be associated to serum, urine and tissue markers of inflammation and oxidative stress.

Discussion and future perspectives

The aim of this thesis was to evaluate whether in patients with vascular disease, including patients with PAD and with an AAA, a possible association with AGEs exists. The results of our studies indicate that increased levels of SAF, a noninvasive method to assess skin AGEs, were found in patients with PAD and AAA. It appeared that SAF was clearly associated with mortality and amputation, but not with renal function decline in patients with PAD. In this final part of the thesis, we will evaluate whether AGEs could be an emerging marker in atherosclerosis. Firstly, we will elaborate the association between AGEs and prognostic pathophysiological markers of
cardiovascular disease, as discussed in the introduction. Secondly, we will underscore the role of AGEs in cardiovascular disease, irrespective of the vascular disease presented in this thesis. In the last part of the discussion, we will present the role of AGEs as a predictor of cardiovascular disease. Finally, an evaluation will be made about the new insights of this thesis on vascular disease.

**AGEs and markers of cardiovascular disease**

In a clinical setting, AGEs are associated with several pathophysiological markers of cardiovascular disease, and therefore, AGEs are likely to have pathophysiological effects on the atherosclerotic process.

Endothelial dysfunction is known as an early marker of atherosclerosis and can be measured with flow-mediated dilatation and analysis of circulating endothelial progenitor cells (EPCs). In patients with chronic kidney disease, elevated values of SAF were associated with decreased flow-mediated dilatation, which represents endothelial dysfunction.⁸ Another marker of endothelial dysfunction, circulating endothelial progenitor cells, was associated with SAF and glyceraldehyde-derived AGEs in patients with end-stage renal disease and healthy volunteers, respectively.⁹,¹⁰ These studies indicate that increased AGE levels are associated with markers of endothelial dysfunction.

Arterial stiffness is associated with aging and results in sheer stress on vascular tissue. A noninvasive method to assess arterial stiffness is the technique pulse wave velocity (PWV). The association between arterial stiffness and AGEs is shown in several studies with conflicting results. Multiple studies showed a positive association between SAF and PWV in different diseases, including type 1 diabetes mellitus, adults and children with end-stage renal disease and coronary artery disease.⁹,¹¹-¹³ The association between serum AGEs and PWV is debatable, since different AGEs are measured with different techniques. Serum Nε-carboxymethyl-lysine (CML) was associated with PWV in relatively healthy subjects.¹⁴ Similar results were found between serum pentosidine and PWV in patients on hemodialysis and type 2 diabetes mellitus.¹⁵,¹⁶ However, a large study with Chinese subjects from the general public showed no association between serum AGEs and carotid-femoral PWV.¹⁷ Lack of evidence for this association was also shown in patients with coronary artery disease (serum AGEs) and in subjects without hypertension or diabetes mellitus (plasma CML).¹⁸,¹⁹ In conclusion, SAF seems
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to be a useful marker to detect arterial stiffness in several diseases, while no conclusion can be made for the association between arterial stiffness and serum AGEs.

A noninvasive technique to measure generalized atherosclerosis is with the use of the carotid intima-media thickness (cIMT). Serum methylglyoxal-derived hydroimidazolone (MG-H1) and pentosidine are independently associated with cIMT in children with type 1 diabetes mellitus and adults with type 2 diabetes mellitus, respectively.\textsuperscript{16,20} In a small sample of patients with type 1 diabetes mellitus, skin glucosepane is correlated with cIMT.\textsuperscript{21} SAF is correlated with cIMT in subjects with low cardiovascular risk, patients with type 1 diabetes mellitus and patients on hemodialysis, but as for independent associations between SAF and cIMT, these results varied between the studies.\textsuperscript{22-24} To summarize, AGEs level is correlated cIMT as a method to measure generalized atherosclerosis.

Calcification plays an important role in atherosclerosis. Calcification can be measured with computed tomography (CT). A CT of the heart allows determination of a coronary artery calcification (CAC) score which is associated with AGEs. SAF and skin intrinsic fluorescence, another noninvasive technique to assess skin AGEs, were associated with CAC score in patients with type 1 and type 2 diabetes mellitus and in patients with chronic kidney disease stage 3-5.\textsuperscript{25-27} However, only in patients with type 2 diabetes mellitus, this association was independent of cardiovascular risk factors.\textsuperscript{26} Furthermore, serum pentosidine was positively and independently associated with CAC score in patients with type 1 diabetes mellitus and patients on hemodialysis.\textsuperscript{28,29} On the other hand, plasma CML was not associated with CAC score in patients on hemodialysis.\textsuperscript{29} Finally, calcification was associated with tissue AGEs, measured with immunohistochemistry within a biopsy of the radial artery in patients with end-stage renal disease.\textsuperscript{30} Thus, different methods to measure AGEs are associated with arterial calcification, although not all of them are independently associated after correction for cardiovascular risk factors.

Vulnerable plaques are thought to be at risk for rupture, resulting in plaque migration with ischemic cardiovascular events as a consequence. Several techniques are available to identify high risk plaques \textit{in vivo}. Invasive techniques, including intravascular ultrasound (IVUS), may identify vulnerable plaques. Specifically, most research with the IVUS technique is performed by cardiologists in patients with coronary artery disease. This technique is only available and operational in very few centers. No studies have yet been performed to show the association between AGEs
and vulnerable plaques, measured with IVUS. In addition, the possible role of IVUS in PAD in the clinical setting is debatable, since experts in the field suggest the high risk of embolization induced by this method. Therefore, other methods to identify vulnerable plaques are preferable. \(^{18}\)F-fluorodeoxyglucose-positron emission tomography (FDG-PET) is a method which could detect vascular inflammatory plaques, which are considered as vulnerable plaques. An association was found between vascular inflammation measured with PET and serum AGEs, but not with CML in subjects from the general public of Japan.\(^{31}\)

In conclusion, several studies have been performed to identify the role of AGEs and markers of cardiovascular disease. All mentioned markers showed an association with either plasma AGEs or SAF, and, therefore, support the hypothesis that AGEs contribute to the pathophysiology of atherosclerosis. However, these associations were in most cases shown in patients with diabetes mellitus or renal insufficiency. Our observations showed increased SAF in patients PAD, as example for widespread atherosclerosis. Therefore, the question rises whether there is also an association between AGEs and cardiovascular markers in atherosclerotic disease. The ARTERY study, mentioned in Chapter 8, will measure SAF, serum AGEs, PWV as marker for arterial stiffness and IMT as marker for generalized atherosclerotic plaques. Therefore, this study will directly contribute to elucidate the unknown association between AGEs and markers of cardiovascular disease in PAD and AAA.

**AGEs in cardiovascular disease**

The association found between AGEs and PAD in this thesis is not surprising, since similar results are found in other forms of atherosclerotic disease. Most research on AGEs in atherosclerotic disease focused so far on two well-known cardiovascular diseases, coronary artery disease and cerebrovascular disease. And, one study performed an analysis of SAF and the degree of atherosclerosis.

The role of AGEs was investigated in several studies in patients with coronary artery disease. Two case-control studies were performed. SAF, as well as CML measured in heart tissue, was elevated in patients with coronary artery disease compared to controls.\(^{32,33}\) Whether serum AGEs differentiates between severe and less severe disease is debatable. Serum AGEs were not able to differentiate between patients with an STEMI and patients with an acute coronary syndrome without ST-segment elevation.\(^{34}\) On the contrary, serum level of CML was able to distinguish stable
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coronary artery disease and acute myocardial infarction. Plasma levels of AGEs and CML were also associated with multivessel disease and parameters of diastolic dysfunction, respectively. Tissue CML, however, was not associated with echocardiographic indices of diastolic dysfunction in the last mentioned study. Thus, AGEs are increased in coronary artery disease, and might have a role to identify patients with more severe characteristics.

Several studies showed also an association with AGEs and cerebrovascular disease, such as stroke and carotid artery disease. SAF was increased in patients with carotid artery disease and in patients with cerebral infarction. However, the independent role of SAF for these diseases remains unclear, since the association disappears after correction for cardiovascular risk factors and PAD. No multivariate analyses were performed in the study which included patients with cerebral infarction. In addition, serum pentosidine was associated with poor outcome and short-term deterioration in patients with acute ischemic stroke. The tissue AGEs MG-H1 and CML were associated with rupture-prone plaques in patients undergoing carotid endarterectomy for symptomatic and asymptomatic carotid artery disease. So, as a conclusion, AGEs are associated with cerebrovascular disease, but this association is not independent of other cardiovascular risk factors.

Den Dekker et al. showed an association between accumulation of SAF and the degree of atherosclerosis. Lowest SAF was found in patients without signs of atherosclerosis based on CAC score. Intermediate SAF was found in patients with subclinical atherosclerosis based on the CAC score. And highest SAF was found in a subgroup of patients with PAD recruited from the database described in Chapter 3. Also after correction for cardiovascular risk factors was SAF associated with the degree of atherosclerosis.

AGEs as predictive marker for outcome

Besides the association of AGEs with atherosclerotic markers and disease, several studies used AGEs as a predictor for cardiovascular events. The reason to search for new biomarkers is mainly based on two reasons. First reason is that most of the available risk scores, such as the Framingham risk score and Systematic Coronary Risk Evaluation (SCORE) chart, are built to predict cardiovascular events for primary prevention. For patients with existing cardiovascular disease, such as PAD, these risk scores are of no use. Second, patients have a longer survival after cardiovascular
events with all available medical treatment options. Therefore, the prevalence of patients surviving a cardiovascular event is high and secondary risk stratification is useful.

The role of AGEs to predict cardiovascular events is demonstrated in various cohort studies. Considering that AGEs in patients were first investigated in patients with diabetes mellitus and renal insufficiency, it is logical that most of the cohort studies have been performed in these patients.

Serum AGE score, including CML, Ne-carboxyethyl-lysine (CEL) and pentosidine, was predictive for cardiovascular events in two studies including patients with type 1 and type 2 diabetes mellitus, both after a long follow-up of 12 and 10 years respectively. Also SAF was an independent predictor for cardiovascular events in patients with type 2 diabetes mellitus.

In patients on hemodialysis, plasma levels of pentosidine and fluorescent serum AGEs were predictive for cardiovascular events. However, plasma CML was not predictive for cardiovascular events in patients with nephropathy and type 2 diabetes mellitus. Even more conflicting results were found for the use of SAF in patients with renal disease. Two studies showed that SAF was associated with cardiovascular disease in patients on hemodialysis, while one study which included both hemodialysis and peritoneal dialysis showed no predictive value. A larger study in patients with chronic kidney disease stage 3 showed that SAF was associated with cardiovascular events.

In patients with a myocardial infarction, serum AGEs as well as SAF were predictive for cardiovascular events.

Finally, in nondiabetic subjects from the general population of Finland, serum AGEs were not predictive for all-cause mortality, while these AGEs were predictive for this end point in the subgroup of women.

Interpretation of these studies with different outcomes is challenging. As mentioned above, it is difficult to compare the result because different AGEs are measured with different techniques. Another reason is that most studies do not specify whether the purpose of the research is to show an etiologic explanation or a prediction model. Most of these studies had the intention to show a causal association between AGEs
and cardiovascular event, but used a backward multivariate statistical model. This backward model has the possibility to exclude important confounders, and therefore, the causal interpretation may not be correct. Other studies use the terminology prediction in their study, but showed no prediction models.

One of the studies designed a prediction model. Lutgers et al. showed that SAF was associated with cardiovascular events.\textsuperscript{47} Additionally, C-statistic was performed and showed an increased area under the curve when SAF was added to the model. Unfortunately, the increase of the area under the curve was not significant.\textsuperscript{47} The median follow-up period for this publication was 3.1 years, unfortunately no results on longer follow-up periods of this study have been published. Our result, which showed a significant additional value of SAF for prediction of amputation (Chapter 5) is therefore exceptional.

Although the result that SAF may identify patients at risk for amputation is promising, SAF could not yet be used in clinical setting for this use. External validation in another centre in The Netherlands or in another country is necessary. In addition, the ultimate test to identify the predictive role of SAF for amputation would be to perform a randomized controlled trial. In this hypothetical randomized controlled trial, the control arm should get standard treatment, while the vascular surgeon of the patients in the intervention arm should be informed about the SAF value, and its possible value as predictor for amputation. Difficulties with this randomized controlled trial are the large number of patients needed, and the long follow-up is necessary, since the incidence of an amputation is low. Although our results in this thesis are promising, there is not yet enough evidence to use SAF in clinical setting for prediction of cardiovascular events.

**AGEs as therapeutic target**

In this thesis we focussed on AGEs as an emerging biomarker. Besides AGEs as a biomarker, it could also be a potential therapeutic target. In studies performed in humans, the AGE inhibitor aminoguanidine, the AGE breaker alagebrium, and the RAGE inhibitor PF-04494700 were not safe or efficient. As a consequence, these medicine are not available in clinical practice. In addition, it is unknown whether inhibition of the AGE/RAGE axis also reduces formation of atherosclerosis in humans and eventually reduces cardiovascular events.
In contrast to pharmacological treatment, lifestyle adjustments are currently of potential interest to interfere the AGE/RAGE axis. As stated in Chapter 2, smoking increases circulating AGEs levels and probably, smoking cessation reduces circulating AGEs. Furthermore, several studies focussed on the effect of low-AGE diets. A two-week low-AGE diet randomized clinical trial with cross-over design was performed in non-diabetic obese adults. As a result, these subjects had improved sensitivity for insulin. Meanwhile, questions have been raised whether long-term low-AGE diets could prevent development of type 2 diabetes mellitus. However, whether low-AGE diet could have beneficial effects for patients with already widespread atherosclerosis is doubtful.

**Vascular disease**

Available research on predictors in patients with PAD and AAA is limited, despite the serious consequences of these diseases. Most research on cardiovascular disease is focussed on myocardial infarction and stroke. These diseases have indeed more frequently a deathly outcome and myocardial infarction and stroke are more prevalent in the population. But, the prevalence of PAD is growing as well, with a 23% increase within a decade. Intermittent claudication decreases the quality of life for PAD patients. Furthermore, these patients have a markedly increased risk for other cardiovascular disease. Therefore, attention in research for cardiovascular risk assessment in PAD is desirable. In my opinion, this thesis contributed to this issue and I will challenge other researchers to perform more research in the field of secondary cardiovascular risk prediction.
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