Aneurysms at multiple levels

To get a better picture

Bastiaan G. L. Nelissen

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Thesis, Utrecht University, The Netherlands

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Aneurysmata op verscheidene niveaus

Voor een beter beeld

(met een samenvatting in het Nederlands)

Proefschrift

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Bastiaan Gijsbert Leo Nelissen

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Promotoren:

Prof.dr. F.L. Moll
Prof.dr. G. Pasterkamp

Copromotor:

Dr. J.A. van Herwaarden

Voor Hankie, Guust, Sophie en Pim

Contents

Chapter 1

General introduction

Introduction

What is an aneurysm?

Arterial diseases are the most common cause of death worldwide, and its incidence is still increasing.[7] Arterial diseases can roughly manifest in two ways, narrowing or a widening of the artery. A permanent focal widening of an artery is called an aneurysm. Aneurysms can be saccular (as a pouch), or fusiform (spindle-shaped). Some arteries are more prone to develop an aneurysm than others. The most common location is the abdominal aorta. Other common sites are brain, popliteal, iliac and the carotid arteries. The definition of 'an aneurysm' is if the diameter of the artery is 1.5 times larger than the expected diameter. For the abdominal aorta a diameter of 2.9 cm is still considered normal, whereas a diameter of 3.0 cm or larger is assumed to be aneurysmal.[8] Other guidelines suggest that a local increase of 50% of the diameter is considered to be an aneurysm. The latter method can be used for any artery. The diameter of the abdominal aorta has a large inter-individual variation, and increases with presence of risk actors.[9]

Risk factors for aneurysm formation

The most important risk factor for the development abdominal aneurysms is smoking. Smokers have an increased risk of 6 percent of developing an aneurysm. Other known risk factors are a positive family history, increased age, male gender coronary artery disease, high cholesterol, and COPD.[10,11] Interestingly, diabetes is associated with a lower risk of aneurysm formation.12 A family history of aneurysms gives a doubling of the risk for of AAA. In women, this increase is even higher, whereas in identical twin siblings, the risk is 24%. The familial influence is probably not entirely genetic; the environment still plays an important role.[10,13] In some cases an AAA is the direct result of a specific cause such as an infection (Q-fever), trauma,

immune-mediated vasculitides such as Behçet's disease and Takayasu disease, or a connective tissue disorder such as Ehlers-Danlos or Marfan syndrome.[11]

Pathology

The emergence of an aneurysm is a dynamic and complex process. In the past it was thought that the development of an AAA was a direct result of atherosclerosis. Nowadays atherosclerosis is seen only as a part of the compound process that also leads to aneurysm formation. The phenomena which lead to a reduced vessel wall strength and result in dilation of the aorta are two-fold: both degradation of the extracellular matrix (a layer outside the cells that provides strength) as well as inflammation play a major role. A healthy aorta can absorb cardiac pressure waves by accurate composition of smooth muscle cells and extracellular proteins, such as elastin and collagen.[13] Degradation of the extracellular matrix in the vascular wall of an AAA is manifested by a decrease in elastin, by poorly structured collagen and by apoptosis of the smooth muscle cells. As a result, the vessel wall becomes weak. In addition, inflammation plays a major role in reducing the strength of the vessel wall. It has been shown that aneurysm tissue contains an increased concentration of inflammatory cytokines. Also, increased plasma levels of inflammatory mediators are detectable. However it still remains unclear whether inflammation plays a causal role or is just the result of the degenerative process.[14]

Aneurysm-Express Biobank

“The tissue is the issue”. The goal of the Aneurysm-Express Biobank is to unravel the etiology of the disease. The Aneurysm-Express Biobank study was started in 2004 to investigate aneurysmatic artery walls. Presently, the biobank contains a collection of hundreds of aneurysm wall samples, mainly AAA tissue samples, but also specimens from carotid, femoral and popliteal aneurysms. Each sample is cut into multiple slices and immunohistochemically stained, followed by the time consuming and labor intensive process of manual slide analysis and quantification. Patient characteristics and medication use are obtained using the combination of

clinical records and questionnaires at the time of recruitment. These questionnaires include cardiovascular risk factors (age, gender, smoking, hypertension, diabetes).[15]

This thesis

In this thesis we discuss multiple levels of aneurysm formation, from nationwide aneurysm data we zoom all the way into individual cell characteristics.

In chapter 2 mortality trends from AAA in the Netherlands are described. Interestingly, a convincing descent in the number of AAA-related deaths in males was discovered. Presently there is hardly any difference between men and women. As described earlier, it is widely known that smoking is the most important risk factor for aneurysm formation of the abdominal aorta. During the last decades a rapid decline has been seen in male smokers whereas female smokers have not shown any habitual change at all. Following this trend a paralell trend in AAA-related deaths has been observed. Is this trend the explanation for the shift in mortality rates? There appears to be a different clue for the shift in AAA-related deaths which can be found in the aneurysm tissue itself.[1]

Chapter 3 contains an important focus of this thesis, the slideToolkit. In general histological analysis is essential for the understanding of diseases. The aneurysm biobank provides us with a large amount of histological data. The classic manual histological analysis is a delicate and time-consuming process. There is an increasing demand for accurate and reproducible histological characterization, especially for subsequent analysis and interpretation of data in association studies (for example DNA/SNP comparison studies). Computerized histological analysis is still a poorly developed research area. In this chapter the development of software for automating whole digital slide analysis is discussed. Detailed analysis of histological samples is a powerful way of phenotyping tissue. Nonetheless, purely manual visual assessment of histological slides is time-consuming and liable to observer variation. Automated quantification of digitized histological slides is often preferred as a more precise reproducible, and even a more sensitive approach. Relatively few functional software packages are available for fully digitized whole microscopic slides. To comply with this need we developed the slideToolkit as a fast method to handle large quantities of whole slides images using advanced cell detecting algorithms. The slideToolkit provides a free, powerful and versatile

collection of tools for automated feature analysis of whole slide images to create reproducible and meaningful phenotypic data sets.[2]

In chapter 4 the slideToolkit is tested and compared with manual and semi-automatic methods. Compared to these methods the slideToolkit shows to be a rigid method with the highest precision and agreement in repeated measurements, specificity and reproducability for whole digital slide quantification.[3]

Inflammation is one of the most prominent causative factors for aneurysm formation. In chapter 5 the validated slideToolkit is used to describe the histological inflammatory characteristics between aortic, popliteal and femoral aneurysms. We mainly looked at the different types of inflammatory cells fin the arterial wall. No difference was found between aorta versus femoral and popliteal in innate immune response. In contrast, a difference was detected in the adaptive immune response within these arteries. Why do we find this difference? Is it the same process at a different point in time?[4]

In chapter 6 the risk factors and histological characteristics of carotid artery aneurysms of 13 patients are described. The tissue was manually analysed. The findings showed two distinct histological types originating from the carotid artery; dissection, and degeneration of the arterial wall. The different histological findings might imply a difference in pathology of disease. The relevance of the findings with regards to to the treatment of extracranial artery aneurysms are discussed.[5]

Chapter 7 continues on this topic and discusses the management of extracranial carotid artery aneurysms. We summarize the data on the largest available series in the literature on ECAA management. We found the most favorable treatment for ECAA is invasive surgery. However, invasive surgery is frequently complicated by cranial nerve damage. Could it be possible that the less invasive endovascular surgery will show better results in the future?[6]

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Chapter 2

Shifting abdominal aortic aneurysm mortality trends in The Netherlands

Journal of Vascular Surgery - 2015;61:642-7

Bastiaan G. L. Nelissen,a MD, Joost A. Herwaarden,[a] MD, PhD, Gerard Pasterkamp, MD, PhD,[b] Frans L. Moll, MD, PhD,[a] and Ilonca Vaartjes, PhD,[c] Utrecht

a *Department of Vascular Surgery, University Medical Center Utrecht, Utrecht, the Netherlands*

b *Department of Experimental Cardiology, University Medical Center Utrecht, Utrecht, the Netherlands*

c *Julius Center for Health Sciences and Primary Care, University Medical Center Utrecht, Utrecht, the Netherlands*

Abstract

Objective: Mortality from abdominal aortic aneurysm (AAA) in developed countries has decreased since the late 1990s. Our objective was to get an insight of mortality trends in The Netherlands for AAA disease.

Methods: Data of all AAA deaths (1980 to 2010) were collected from the Dutch cause of death register. Cause of death was divided in two groups: with the mention of rupture and without the mention of rupture. Data were standardized and divided into three age groups (55-69, 70-84, and ≥85 years). Mortality rates per 100,000 were analyzed for both sexes and for each age group. Significant points of change were identified using joinpoint regression analysis.

Results: Total standardized AAA mortality increased from 1980 (1062 deaths) until 1995 (1728 deaths), followed by a decline until 2010 (930 deaths). This decline was most prominent in men. Deaths without mention of rupture showed an increase from 1980 until 2010. The age of AAA death was higher in women (79.2 in 1980 and 82.1 in 2010) than in men. This difference declined as the age of death from AAA increased from 72.1 in 1980 to 77.9 years in 2010 in men. Decline in AAA mortality was first seen in the young age group (55-69 years) and then seen consecutively older age groups.

Conclusions: Mortality from AAA is declining due to a reduction in deaths from ruptured AAAs. This was first observed in the young age groups. Men died more often and at a lower age.

Introduction

Developed countries have shown a decrease in abdominal aortic aneurysm (AAA) deaths since the late 90s.[1-3] This may be due to improved treatment and favorable changes in risk factors, such as a reduction in smoking, and to increased use of cardiovascular drugs[4-6] and increased elective repair.[1-4] In The Netherlands, we see similar changes in treatment and risk factors. Unfortunately, there is no active AAA screening program in The Netherlands, and we have no insight in the prevalence of AAA. Our objective was to obtain insight in AAA mortality trends in The Netherlands; therefore, we determined mortality rates from AAA deaths with and without the mention of rupture. We evaluated all mortality data from 1980 until 2010 and compared trends for three different age groups for both sexes.

Methods

Mortality from AAA in The Netherlands was investigated from 1980 to 2010. For this period, the cause of death was registered using the World Health Organization International Classification of Diseases International Classification of Diseases and Related Health Problems (ICD), Ninth Revision codes until 1996 and ICD-10 since. Included were abdominal aneurysm, ruptured (441.3/I71.3); abdominal aneurysm, without mention of rupture (441.4/I71.4); aortic aneurysm of unspecified site, ruptured (441.5/I71.8); and thoracoabdominal aneurysm, without mention of rupture (441.7/I71.9). Aortic aneurysms of unspecified site were included because these are most likely to be abdominal. A complete distribution of the different groups for 1980 and 2010 is shown in Supplementary Table I (online only).

Anonymous data regarding cause of death and the discrimination between deaths in and out of the hospital were acquired from the cause of death register the Statistics Netherlands. Statistics Netherlands obtains the data through an obligatory reporting system that obligates the treating doctor or the municipal coroner of a deceased person to document one underlying and up to three secondary causes of death on a death certificate (B statement). The “secondary” and “tertiary causes of death” were originally introduced to enable analyses of multidisease patterns. The location of death (in or out of the hospital) and the time span between different causes are also noted on the B statement. The B statement is sent directly to Statistics Netherlands and is collected for statistical purposes only. The causes of death recorded in the B statement are analyzed and translated by a professional of the Statistics Netherlands into one cause of death and coded according to the ICD.

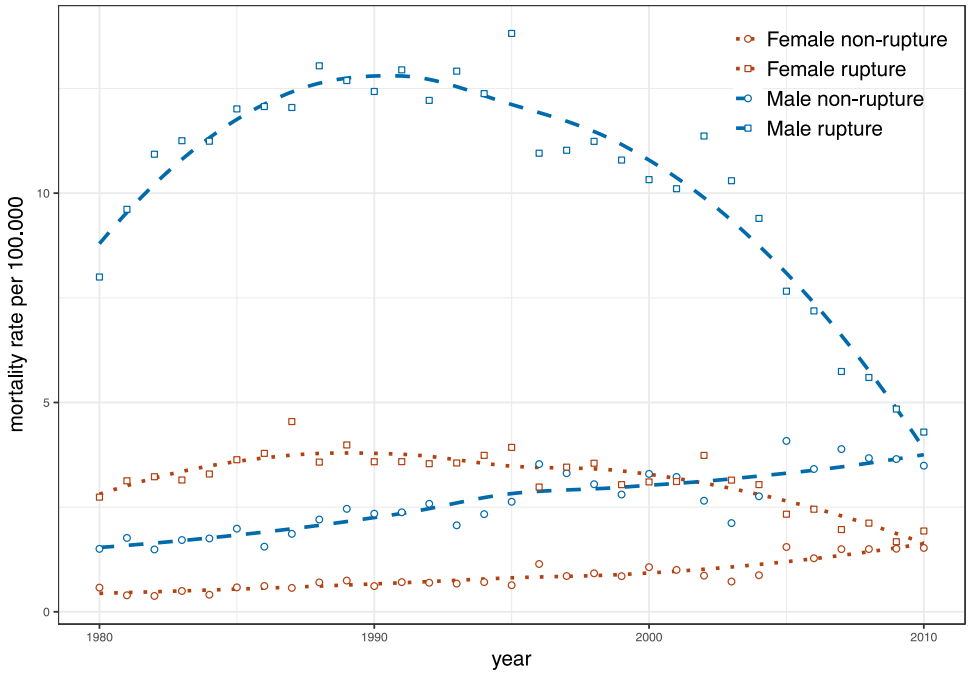

Fig 1. *Abdominal aortic aneurysm (AAA) standardized mortality rates in The Netherlands per 100,000 from 1980 to 2010. The year of standardization is 2010. Subgroups are made for rupture and nonrupture in men and women. For each group, a locally weighted regression curve was added for smoothing the variability in measurements.*

The primary cause of death is the disease that starts the chain of events that eventually leads to the patient's death. When an AAA rupture is mentioned, the coder will choose "abdominal aneurysm, ruptured" as the cause of death. When an AAA rupture is not specifically mentioned, the coder will choose "abdominal aneurysm without mention of rupture."

Data were stratified by year, sex, and 5-year age bands. The AAA mortality rate for each year, defined as the total number of AAA deaths in each age group of patients aged ≥55 years per 100,000, was standardized to the Dutch population of 2010 as provided Statistics Netherlands.

To identify points in time where a significnt change of the ageand sex-specific trend in mortality occurred, joinpoint regression analysis[4-7] was performed with Joinpoint 3.5.2 software provided by the U.S. National Cancer Institute Surveillance Research Program (2011). For every period, the linear slope of the trend and probability value of the final model of the joinpoint regression analysis, the absolute minimum and maximum observed number of deaths, and the minimum and maximum observed

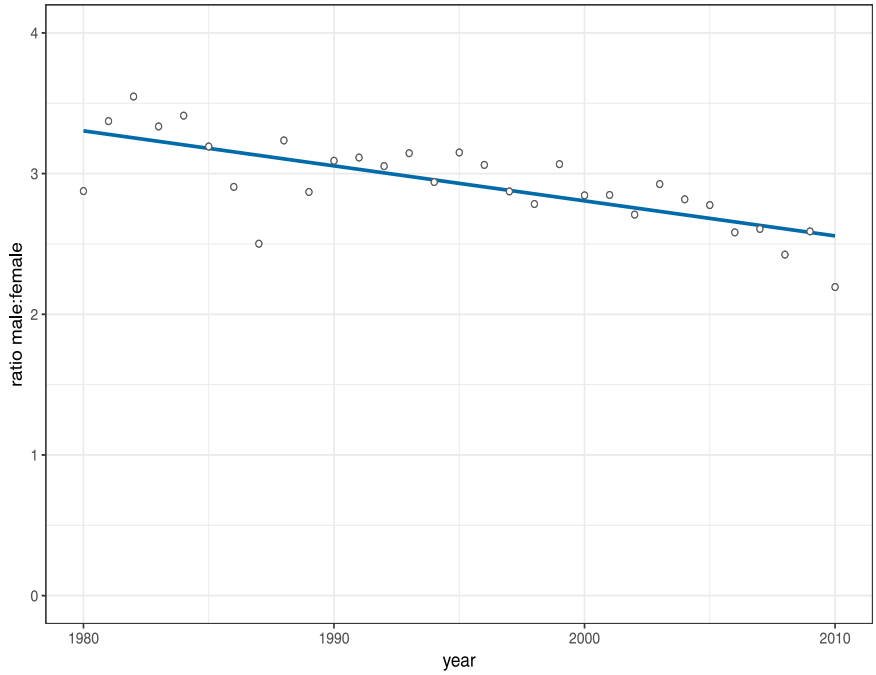

Fig 2. *Ratio of male-to-female abdominal aortic aneurysm (AAA) deaths in The Netherlands from 1980 to 2010 (points), with a linear regression line (r = -0.74, R2 = 0.54, P < .01).*

AAA mortality rates per 100,000, were as tabulated. Furthermore, the change in observed AAA mortality rate per 100,000 per period [(AAA mortality rate last year of period AAA mortality rate first year of period)/AAA mortality rate first year of period] was calculated. Finally, we present three age-specific graphs showing the observed and modeled AAA mortality rates per 100,000 from 1980 to 2010; age groups were 55 to 69 years, 70 to 84 years, and ≥85 years. The dots in the age-specific graphs represent the observed number of deaths per 100,000 from 1980 to 2010. The line in each ageand sex-specific graph represents the final model from the joinpoint regression analysis.

Smoking was analyzed for both sexes in two age groups, 50 to 64 years and ≥60 years, as provided by Stivoro, the Dutch expert center for tobacco use prevention. Commissioned by Stivoro, a shifting and distributed sample was taken weekly with ~20,000 participants annually. Until 1989, interviews were per household with a paper questionnaire, from 1990 until 2001 interviews were conducted per person using a laptop (Computer-Assisted Assisted Personal Interviewing), and from 2001

and onwards, questionnaires were completed online (source www.trimbos.nl, accessed August 12, 2014).

Approval for the use of the anonymized patient data was covered by a general agreement with Statistics Netherlands. No informed consent was needed frvom the patients, and no separate ethical approval was necessary for the use of these data. All analysis were performed according to the privacy legislation in The Netherlands.[8]

The default settings in R 3.0.2 software (www.r-project.org) were used to analyze the data and model the locally weighted regression curves[9] in Fig 1 and in the Supplementary Fig (online only).

Results

Between 1980 and 2010, 31,917 patients died of an AAA, of which 31,447 (98.5%) were aged ≥55 years. For every period, the absolute number of AAA deaths by sex, age group, and AAA type are presented in the Table.

Total age-adjusted AAA mortality increased between 1980 (1062 deaths [12.8 deaths/100,000]) and 1995 (1728 deaths [21.0 deaths/100,000]), followed by a decline until 2010 (930 deaths [11.2 deaths/100.000]). From 2000 to 2010, AAA deaths declined 43% in men and 17% in women. The AAA male-to-female mortality ratio is changing. The ratio in 1980 was >3:1 and steadily declined to >2:1 in 2010 (Fig 2).

Joinpoint regression on standardized mortality rates (Fig 3) showed that the decline started in 1988 in young AAA patients (55-69 years) and was followed in later years (2002) in older patients (70-84 years). The oldest group (≥85 years), however, did not show a decline in mortality. The average age at which people died from AAA increased. From 1980 until 2010, women died at an older age (79.2 [standard deviation {SD}, 9.7] years in 1980, 82.1 [SD, 8.8] years in 2010) compared with men. However, this difference decreased as the age of death from AAA in men increased from 72.1 (SD, 9.7) years in 1980 to 77.9 (SD, 9.0) years in 2010.

AAA with mention of rupture. Death due to ruptured AAA is more common than AAA deaths without the mention of rupture. After a steady increase since 1980, mortality due to ruptured AAA decreased since the millennium (Fig 1). Standardized rates showed that deaths due to rupture decreased 46.4%, from 13.42 per 100,000 in 2000 to 6.22 per 100,000 in 2010. This trend was observed in men

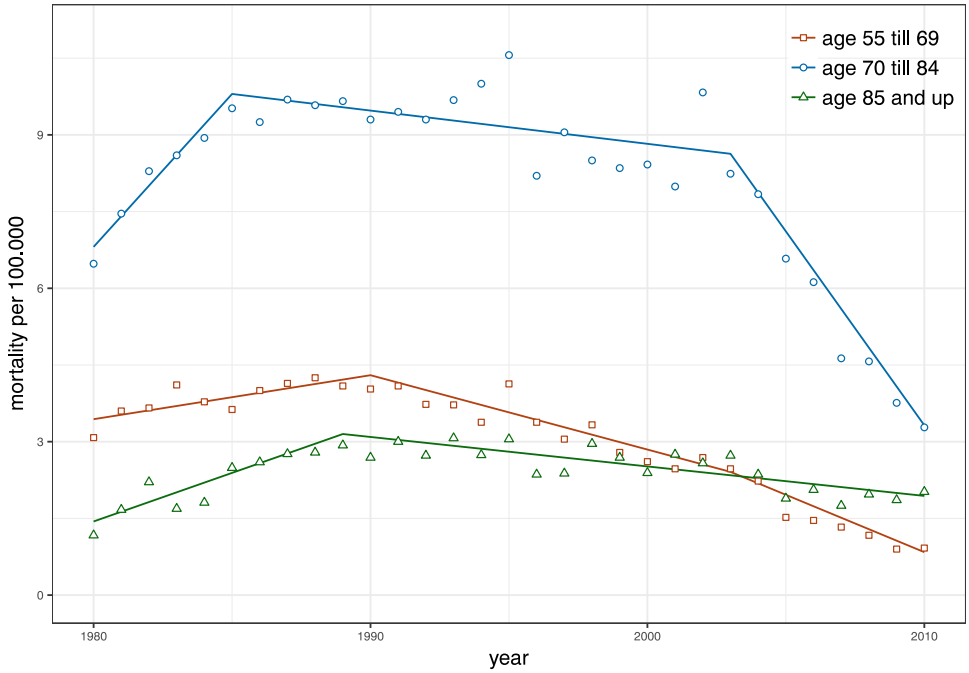

Fig 3a. *Joinpoint regression analysis of ruptured abdominal aortic aneurysm (AAA) mortality rates in The Netherlands per 100,000. Mortality rates are divided in three age groups (55 to 69 years, 70 to 84 years, and ≥85 years). Changes in trend as observed by the Joinpoint software are for age 55 to 69, found in year 1990 and 2003; for age 70 to 84, joinpoints are found in year 1985 and 2003; and for age ≥85, a joinpoint was found in 1989.*

(41.6%) and in women (62.2%). The share of deaths in (60%) and out (40%) of the hospital remained equal between 1980 and 2010 for all ruptured AAAs. Joinpoint regression (Fig 3, A; Supplementary Table II, A, online only) showed that the decline in mortality due to ruptured AAA started in the youngest age group in ~1990 and was evidently followed in later years (2003) in older patients (70-84 years). In contrast, an evident decline was not present in the oldest group (≥85 years).

AAA without mention of rupture. Between 1980 and 2010, standardized mortality rates showed that AAA deaths without mention of rupture increased from 2.08 per 100,000 in 1980 to 5.02 per 100,000 in 2010 (Fig 1). This steady increase was observed in men (69.6%) and women (30.4%). Similar to the trend in ruptured AAAs, for all AAA deaths without the mention of rupture the share of in-hospital deaths (60%) and out-of-hospital deaths (40%) remained equal between 1980 and 2010. Joinpoint regression (Fig 3, B; Supplementary Table II, B, online only) shows that an increase in AAA deaths without mention of rupture is absent in the youngest

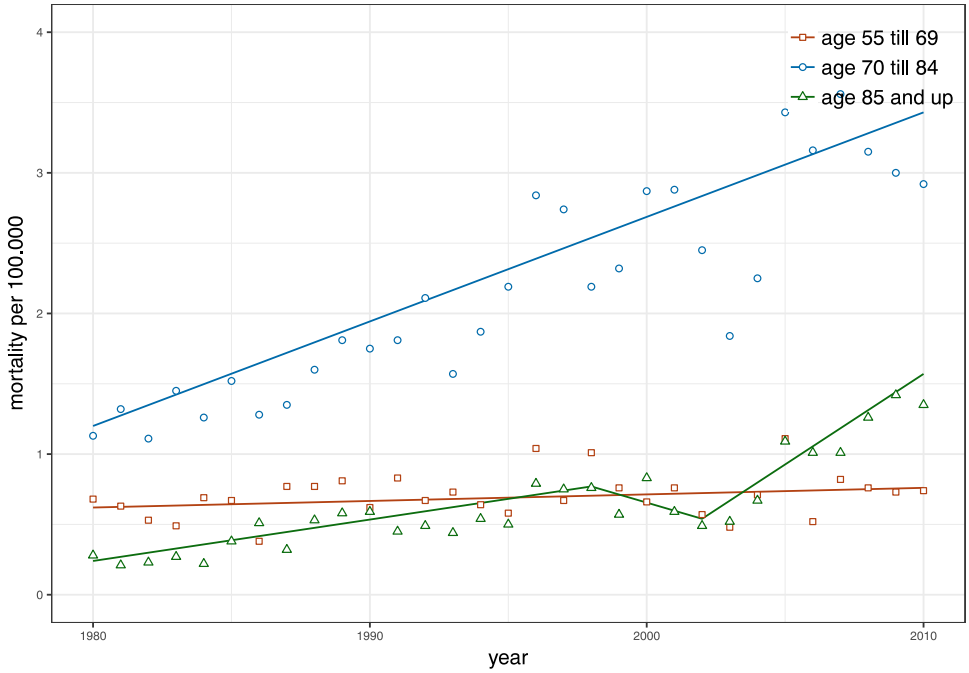

Fig 3b. *Joinpoint regression analysis of nonruptured AAA mortality rates in The Netherlands. Mortality rates are divided in three age groups (55 to 69 years, 70 to 84 years, and ≥85 years). Changes in trend as observed by the Joinpoint software are for age 55 to 69 and 70 to 84 were not found, and for age ≥85 years, joinpoints were found in 1998 and 2002.*

group (55-69 years), but in contrast is markedly visible in patients of older age groups (70-84 years and ≥85 years).

Discussion

This study covers 30 years of nationwide AAA mortal- ity data in The Netherlands, from 1980 to 2010. Multiple changes in AAA mortality in this period came to our atten- tion. At first, after an increasing mortality from 1980, a decrease in AAA deaths was observed since the mid-90s, which accelerated after the millennium. The decline in AAA mortality was first observed in the young age groups and in later

	1980		1995		2010	
	Males (n=440) No. (%)	**Females (n=153) No. (%)**	**Males (n=945) No. (%)**	**Females (n=300) No. (%)**	**Males (n=634) No. (%)**	**Females (n=289) No. (%)**
Age 55-69	156 (35)	18 (12)	236 (25)	43 (14)	113 (18)	24 (8)
Age 70-84	254 (58)	98 (64)	594 (63)	162 (54)	366 (58)	143 (49)
Age ≥85	30 (7)	37 (24)	115 (12)	95 (32)	155 (24)	122 (42)
Rupture	368 (84)	127 (83)	791 (84)	257 (86)	351 (55)	161 (56)
Nonrupture	72 (16)	26 (17)	154 (16)	43 (14)	283 (45)	128 (44)

Table. *Absolute, unadjusted, number of abdominal aortic aneurysm (AAA) deaths in The Netherlands, subdivided by sex, three different age groups in years, and "ruptured" and "without mention of rupture".*

years in the elderly as well. This trend was observed in both sexes and was mainly driven by the trends in mortality due to ruptured AAA.

Second, our data showed a sustained waning of the male-to-female mortality ratio, which is now <3:1.

Third, women die of AAA at an older age than men, but this difference is declining as the age of death from AAA increases in men.

In past decades, AAA patients were subject to multiple changes in treatment, risk factors, and prevention that might have affected these changes in AAA mortality. Mortality trend analyses over a large (30-year) time frame using joinpoint regression were performed, thereby avoiding the need to specify time periods, which might introduce bias in analyzing the trends.

The continuing decrease in AAA deaths observed since the mid-90s is evident and was likely driven by the decreased exposure of risk factors, increased availability of detection and imaging, and improved treatment modalities. Nevertheless, from our data it is hard to prove that improved detection, prevention, and treatment were indeed responsible for the reduced AAA mortality. Multiple regional AAA screening studies have been performed in The Netherlands,[10,11] but there is currently no active nationwide screening program. Owing to the public awareness of AAA, it might be

that more men proactively asked for screening. However, we have no data to give insight in the diagnostic ratio of AAA through time.

Smoking is the most important risk factor for the development of AAA. A correlation between smoking and AAA formation has evidently been demonstrated.[12] Anjum at al[2] estimated that approximately one-fifth of all AAA deaths could be avoided by reduction of smoking. Smoking is also known to increase the rate of aneurysm rupture.[13] These findings are likely to be true for The Netherlands as well. In The Netherlands in 1970, >75% of adult men and <25% of women were smokers (Supplementary Fig, online only). The ratio of smoking between sexes shifted dramatically in the last 40 years. The decrease in tobacco use in men runs parallel with the decline in AAA mortality. A similar resemblance is seen in women, where tobacco use and AAA mortality remained unchanged in the last 40 years (Fig 1; Supplementary Fig, online only).

In addition, women likely received suboptimal primary and secondary prevention.[14] AAA is mainly seen in men, whereas women have a five-times smaller chance of developing n AAA.[15] Our data show a sustained waning of the male-to-female mortality ratio, which is now <3:1. This finding is in line with the decline of male smokers.

The role of cardioprotective drugs in the prevention of AAA growth is uncertain.[16,17] One of the first guidelines that suggested the use of lipid-lowering drugs, among others, for secondary prevention in AAA was the American College of Cardiology/American Heart Association 2005 Practice Guidelines for the Management of Patients With Peripheral Arterial Disease.[5,6,18] Statin use in The Netherlands has increased substantially in the last 15 years,[19] but unfortunately, women receive suboptimal primary cardiovascular protective treatment.[20] Our data show the biggest decline in AAA mortality in those aged <85 years, pointing toward changes in management and risk factors of AAA.

One important change since the early 90s is the shift toward endovascular aneurysm repair (EVAR) as this treatment modality keeps continuously improving, with lower migration rates, type I endoleaks, and ruptures after implant. The less invasive EVAR makes aneurysm repair in elderly more accessible. EVAR, and the increased rates of advanced abdominal imaging, may be responsible for the rapid increase in AAA repairs.[21-23] Within the United States Medicare population, a reduction in mortality was seen in patients aged >80 years receiving an elective repair with the use of EVAR, from 9.6% in 1995 to 3.3% in 2008, whereas the mortality rate of open aneurysm repair remained constant at 5%. Besides the

increase in elective AAA repairs and the increased use of EVAR, that study also showed an evident decline in AAA ruptures.[22]

The decline in mortality could also be the result AAA treatment guidelines. Treatment guidelines for AAA did not exist and were proposed in 1990.[5,6,24] The first AAA treatment guideline was created by Hollier et al25 in 1992, and multiple other guidelines have followed since.[4,18,26,27] Noteworthy is the decrease in AAA mortality, just after the introduction of these guidelines, in the mid-90s (Fig 1). Yearly updated treatment guidelines for AAA as used in The Netherlands were first composed by The Dutch Society for Surgery in 2009[2,28] and were based on the 2003 "Guidelines for the treatment of abdominal aortic aneurysms"[1,2,26,29] and the previously mentioned 2005 American College of Cardiology/American Heart Association guidelines.[18,24]

In addition, the average age of AAA mortality has risen. Overall, women die at an older age than men, but this difference is continuously declining. Others have noted a similar decrease in AAA mortality since the late 90s.[1,2,16,30] The Rotterdam screening study, which ran from 1989 until 1993, showed that women have a later onset of AAA growth.[11] Our mortality data support this finding. Age per se is greatly associated with surgical mortality risk.[26] With a 50% decrease in short-term mortality, EVAR is a preferred treatment for octogenarians compared with open aneurysm repair. The use of EVAR may be responsible for an increasing number of AAA repairs and for lower mortality from AAA rupture.[22,31] The shift toward less invasive treatment (eg, EVAR) could have contributed to the decline of AAA mortality after 2000 in elderly individuals.[22]

This study has some limitations that should be acknowledged. Any study that uses mortality data across multiple revisions of the ICD will suffer attribution bias owing to the change between ICD versions and the procedures used to code deaths. However, that the findings of this study are the result of such bias is very unlikely because the observed time periods of decline in mortality are very different from the year of revision (1996). In addition, quality of coding was similar for all age groups and for men and women as well, and therefore, this cannot be responsible for the reported trends.

As we explained in the Methods, the treating doctor of the deceased person or the municipal coroner will document one underlying and up to three secondary causes of death. In practice in most deaths outside the hospital, this is the general practitioner, who is aware of the patient's medical history. The doctor documents the cause of death on the death certificate (B statement). The statement is sent directly to Statistics Netherlands, where professional coders recode it into ICD-10

(before 1996 the ICD-9 coding was used). If the treating doctor or municipal coroner does not mention the word "rupture," the case is coded as death due to "abdominal aneurysm without mentioning of rupture." This protocol is according to the World Health Organization guidelines and has not changed since 1956. But certainly, some miscoding will occur. The patients who died of AAA without rupture being mentioned will probably have died of complications of elective aneurysm repair, rarely due to embolic complications, and maybe of a rupture that was not specifically mentioned by the general practitioner or municipal coder. Nevertheless, the mortality trends described in this report are clearly seen.

Strengths of the study are that we analyzed mortality trend analyses over a large (30-year) time frame using joinpoint regression, thereby avoiding the need to specify time periods, which might introduce bias in analyzing the trends.

Furthermore, the overall quality of Dutch registries has been shown to be adequate. Even though the autopsy rate in The Netherlands is 8%, which is relatively low compared with other countries in Europe,[32] Mackenbach et al[33] reported that the validity of the Dutch national cause of death register was higher than the average validity of a considerable number of other countries in the European Community.

A recent study by Klijs et al[34] showed that at the population level, causes of death represent reasonably well the diseases that are most common at the end of life. Although various studies found that cause of death is not registered perfectly and that absolute mortality levels may be somewhat overestimated or underestimated (depending on the underlying cause), the cause of death register has been shown to validly describe mortality trends.[35,36]

Conclusions

After an increasing mortality from 1980, a decrease in AAA deaths was observed since the mid-90s, which accelerated after the millennium. This decline was first observed in the young age groups and in later years in the older age groups as well, occurred in both sexes, and was mainly driven by the trends in mortality due to ruptured AAA. The prevalence of death from AAA is higher and the age of death is lower in men than in women. However, the male-to-female ratio and the difference in age of death declined in the past decades.

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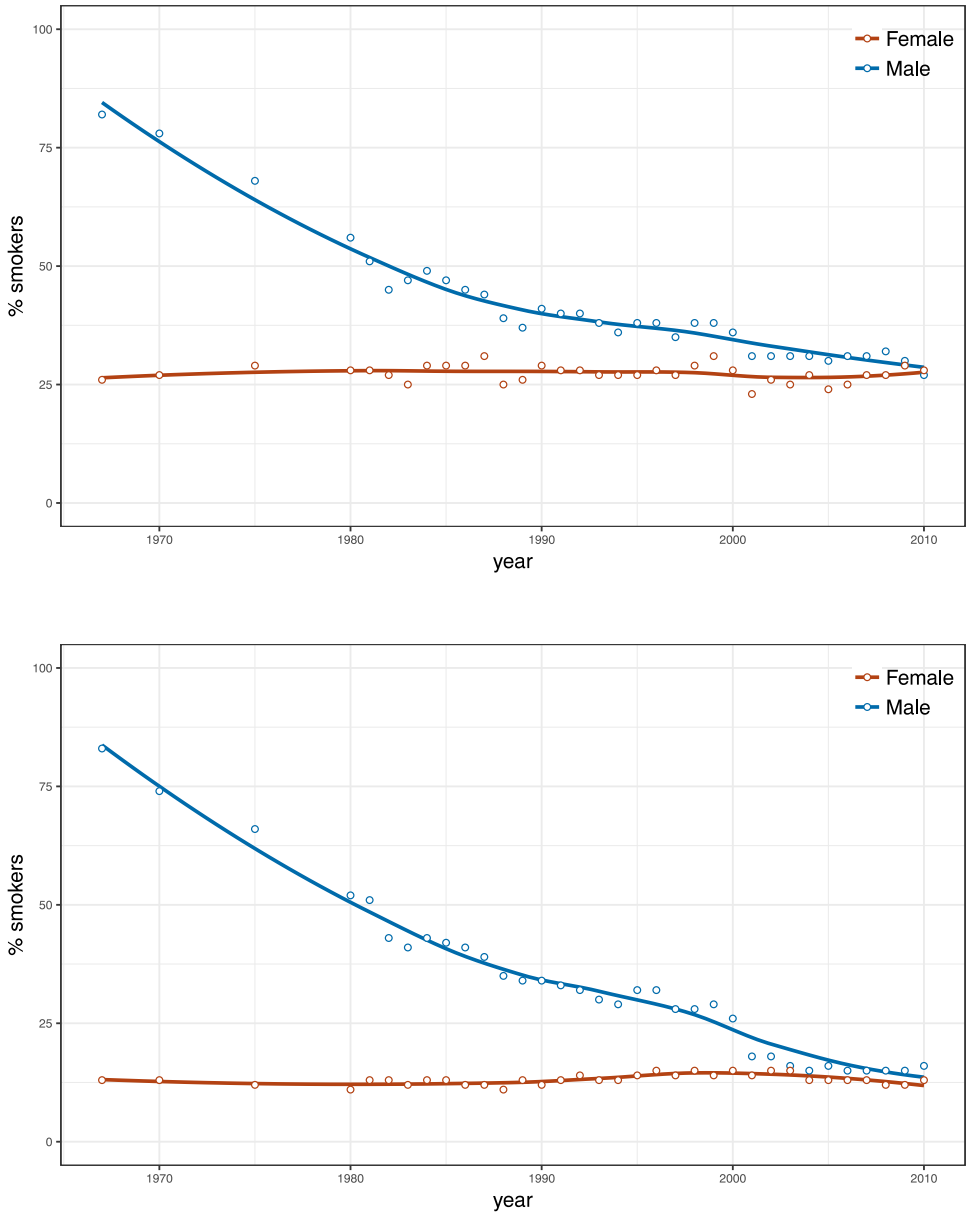
Supplemental



Supplementary Fig (online only). *Smoking was analyzed for men and women in two age groups: (top) 50 to 64 years and (bottom) ≥60 years. Data were provided by Stivoro, the Dutch expert center for tobacco prevention in The Netherlands. For both groups, a locally weighted regression curve was added for smoothing the variability in measurements.*

ICD-code	n (%)
AAA, ruptured (I71.3)	435 (46.8%)
AAA, without mention of rupture (I71.4)	333 (35.8%)
Ruptured aortic aneurysm of unspecified site (I71.8)	80 (8.6%)
Aortic aneurysm of unspecified site, without mention of rupture (I71.9)	82 (8.8%)
	930

Online table 1. *Abdominal aortic aneurysm (AAA) mortality in 2010, subdivided by International Classification of Diseases (ICD) codes, as absolute numbers.*

Age group	Identified periods[a]	Linear slope	Absolute number of deaths (min-max)	AAA Mortality rates per 100.000 (min-max)	Change in AAA mortality rate[b]
55-69	1980 - 1990	3.956[c]	142 - 223	3.08 - 4.25	0.308
	1990 - 2003	2.079[c]	161 - 244	2.47 - 4.13	-0.387
	2003 - 2010	0.496[c]	72 - 167	0.9 - 2.47	-0.628
70-84	1980 - 1985	8.438[c]	229 - 477	6.48 - 9.52	0.469
	1985 - 2003	8.086[c]	467 - 658	7.99 - 10.56	-0.134
	2003 - 2010	2.087[c]	270 - 556	3.28 - 8.24	-0.602
85+	1980 - 1989	2.990[c]	54 - 158	1.17 - 2.93	1.504
	1989 - 2010	1.790[c]	130 - 184	1.75 - 3.07	-0.311

Online table 2a. *a Periods were identified by joinpiont regression analysis. b (rate last year of period segment-rate first year of period segment)/rate first year of period segment. c Linear slope of the identified period is significantly different from previous or next identified period (p<0.05).*

Age group	Identified periods[a]	Linear slope	Absolute number of deaths (min-max)	AAA Mortality rates per 100.000 (min-max)	Change in AAA mortality rate[b]
55-69	1980 - 2010	0.739	19 - 79	0.38 - 1.11	0.088
70-84	1980 - 2010	3.39[c]	53 - 269	1.11 - 3.56	1.433
85+	1980 - 1998	0.757[c]	10 - 48	0.21 - 0.79	1.714
	1998 - 2002	0.348	33 - 54	0.49 - 0.83	-0.355
	2002 - 2010	1.503[c]	33 - 112	0.49 - 1.42	1.755

Online table 2b. *a Periods were identified by joinpiont regression analysis. b (rate last year of period segment-rate first year of period segment)/rate first year of period segment. c Linear slope of the identified period is significantly different from previous or next identified period (p<0.05).*

Chapter 3

SlideToolkit: An Assistive Toolset for the Histological Quantification of Whole Slide Images

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Bastiaan G. L. Nelissen[1], Joost A. van Herwaarden[1], Frans L. Moll[1], Paul J. van Diest[2], Gerard Pasterkamp[3]

1 *Department of Vascular Surgery, University Medical Center Utrecht, Utrecht, The Netherlands*

2 *Department of Pathology, University Medical Center Utrecht, Utrecht, The Netherlands*

3 *Laboratory of Experimental Cardiology, University Medical Center Utrecht, Utrecht, The Netherlands*

Abstract

The demand for accurate and reproducible phenotyping of a disease trait increases with the rising number of biobanks and genome wide association studies. Detailed analysis of histology is a powerful way of phenotyping human tissues. Nonetheless, purely visual assessment of histological slides is time-consuming and liable to sampling variation and optical illusions and thereby observer variation, and external validation may be cumbersome. Therefore, within our own biobank, computerized quantification of digitized histological slides is often preferred as a more precise and reproducible, and sometimes more sensitive approach. Relatively few free toolkits are, however, available for fully digitized microscopic slides, usually known as whole slides images. In order to comply with this need, we developed the slideToolkit as a fast method to handle large quantities of low contrast whole slides images using advanced cell detecting algorithms. The slideToolkit has been developed for modern personal computers and high-performance clusters (HPCs) and is available as an open-source project on github.com. We here illustrate the power of slideToolkit by a repeated measurement of 303 digital slides containing CD3 stained (DAB) abdominal aortic aneurysm tissue from a tissue biobank. Our workflow consists of four consecutive steps. In the first step (acquisition), whole slide images are collected and converted to TIFF files. In the second step (preparation), files are organized. The third step (tiles), creates multiple manageable tiles to count. In the fourth step (analysis), tissue is analyzed and results are stored in a data set. Using this method, two consecutive measurements of 303 slides showed an intraclass correlation of 0.99. In conclusion, slideToolkit provides a free, powerful and versatile collection of tools for automated feature analysis of whole slide images to create reproducible and meaningful phenotypic data sets.

Introduction

Biobanking has become a significant corner stone in pathogenetic studies of multiple diseases and is an important resource for identifying mechanisms of many complex diseases.[1] It is evident that adequate and reproducible histological characterization of large amounts of collected of tissue is key, especially when used for association studies, such as genome wide association studies. In our Athero-Express biobank study, for instance, over 3000 patients have been included, which has resulted in >20.000 immunohistochemically stained cross-sectional slides using different types of antibodies that call for sufficient and consistent phenotyping. For immunohistochemical staining, antibodies are visualized by a chromogenic substrate, such as DAB (brown), 3-AEC (red) or a fluorescent dye.[2] To visualize the remaining tissue a hematoxylin (blue) counter stain is often applied. Until recently, we applied manual semi-quantitative scoring methods or case-by-case quantitative scoring of immunohistochemically stained cross-sections. However, manual or case-by-case phenotyping of histological slides is a time-consuming process liable to observer variability, and therefore, fast, unbiased and reproducible computerized phenotyping is indispensable. For many years, interactive morphometric techniques on live video images[3] and image analysis on sampled digital[4] have been applied, which has improved reproducibility, but this was still time consuming. A computeraided method (analySIS FIVE, Olympos soft imaging solutions) to score inflammatory cells and smooth muscle cells quantitatively was previously implemented to improve reproducibility that indeed performed well.[5] However, this method requires the user to manually set color thresholds for the positively stained areas within subjectively selected regions of interest. However, slides can now be completely scanned at high magnification in minutes with currently available slide scanners and stored as digital images, also known as whole slide images (WSI) or digital slides. After scanning, these slides can be viewed and analyzed on a computer. Automated quantification methods for analyzing WSIs are available. Generally, two methods exist: measuring surface area of a staining pattern, or identifying specific stained structures, like a cell, using advanced image analysis software. The first method, measuring surface area using a specific stain, is a fast approach that allows the measurement of stained areas in WSIs. Unfortunately, this method has a tendency of measuring 'false positive' or 'false negative' areas since only a range of colors are measured independent of morphological structures, such as individual cells. The other method of quantifying WSIs is by identifying each individual cell or blob using image analysis software. Unfortunately, the latter method can only handle relative small images (due to current computer hardware limitations) and works best with uncluttered cells and

high-contrast stains (preferably fluorescence). We here describe the validation of the slideToolkit, which is a collection of open-source libraries and scripts that handle each step of WSI analysis. Our goal was to create a fast method to handle large quantities of low contrast stained WSIs using advanced cell detecting algorithms. In this paper, we illustrate the power of slideToolkit for quantitation of CD3 stained cells using samples of our vascular biobanks.

Methods

Aneurysm-Express biobank

The Aneurysm-Express study (which is a derivative of the previously mentioned Athero-Express study) is a longitudinal biobank study that includes aneurysm tissue from all patients undergoing open surgical abdominal aortic aneurysm (AAA) repair in two Dutch hospitals.[6] The indications for open AAA repair were based on current guidelines.[7] The medical ethics committees of both hospitals approved the study, and all participants provided written informed consent. In short, during elective open AAA repair a full-thickness specimen of the ventral aneurysm wall was collected and is then transported to the lab where 5 mm segments were cut. The middle segment was fixed in 4% formaldehyde and embedded in paraffin for histological analyses; other segments were snap-frozen using liquid nitrogen and stored in -80°C freezers for future use. For histological analysis, consecutive sections were stained following standard procedures with hematoxylin and eosin (H&E), elastin Von Giessen (EvG), Sirius red, Von Willebrand factor, as well as immunohistochemistry (developed with diaminobenzidine (DAB)) for macrophages (CD68), T-lymphocytes (CD3) and B-lymphocytes (CD20) antigens. All slides were then archived in slidecabinets. To obtain a representative sample of our biobank, we retrieved the (arbitrarily chosen) CD3 stained AAA slides from our archive. These slides were obtained from 303 patients and routinely stained between 2005 and 2013 The selected samples were then retrieved from our archive, manually cleaned with alcohol and prepared for scanning.

Ethics Statement

The Aneurysm-Express study was approved by the institutional review boards of both participating hospitals (University Medical Center Utrecht, Utrecht, The

Netherlands, and St. Antonius Hospital, Nieuwegein, The Netherlands) and patients gave written informed consent.

slideToolkit

The slideToolkit is a collection of open-source scripts to handle each step from digital slide to the storage of your results. For a complete overview of all the tools, and there function in the slideToolkit, see table 1. An overview of the dependent libraries are listed in table 2. The toolkit is developed for modern (2014) personal computers (running *nix system [Linux, OS X, Unix]) and high-performance computing (HPC) systems. A common slideToolkit workflow consists of four consecutive steps. In the first step, "acquisition", WSIs are collected and converted to TIFF files. In the second step, "preparation", all the required files are created and organized. The third step, "tiles", creates multiple manageable tiles to count. The fourth step, "analysis", is the actual tissue analysis and saves the results in a meaningful data set. These steps are schematically depicted in figure 1. We present the slideToolkit as an open-source github repository (github.com/ bglnelissen/ slideToolkit).

Slide scanning

All slides were scanned using a Roche iScanHT whole slide scanner at 40x and digitally stored as a multi-page pyramid TIFF file (example in figure 2) containing separate layers (i.e. scanned tissue at magnifications of 40x, 20x and 1.25x, and a thumbnail image of each slide). The slideInfo command revealed that they consisted of 10 layers of different magnifications (ranging from 00.078x until 40x). Each layer was stored in JPEG format with a 90% compression level. Just before archiving, each digital slide was renamed manually using the 'slideRename' tool from the slideToolset as 'studynumber.stain.tif' (e.g. AAA100.CD3.tif)

Step 1 – Acquisition. Most slide scanners are, in addition to their own proprietary format, capable of storing the digital slides in pyramid TIFF files. The slideToolkit uses the Bio-Formats library to convert other microscopy formats (Bio-Formats supports over 120 different file formats, www.openmicroscopy.org) into the compatible pyramid TIFF format if needed. TIFF is a tag-based file format for raster images. A TIFF file can hold multiple images in a single file, this is known as a multi-layered TIFF. The term "Pyramid TIFF" is used to describe a multi-layered TIFF file that wraps a sequence of raster images that each represents the same image at increasing resolutions (figure 2). The different layers contain, among

others, the slide label and multiple enlargements of the tissue on the slide. To read WSIs, the open-source libTIFF libraries and the OpenSlide libraries are used. These libraries are also applied to extract metadata (e.g. scan time, magnification and image compression) of the scanned slides. For image processing we use ImageMagick (ImageMagick 6.8.7-0 2013-10-16 Q16 http://www.imagemagick.org). ImageMagick is a command-line image manipulation tool that is fast, highly adjustable and capable of handling big pyramid TIFF files. Descriptive information about the slide is stored as metadata and contains, for example, pixels per micrometer, presence of different layers, and scan date.

Step 2 – Preparation. In the following steps multiple output files for each slide are created. For each digital slide, a staging directory is constructed in which the slide, and all output data concerning the slide are stored. In digital image manipulation, a mask defines what part of the image will be analyzed and what part will be hidden. Usually a mask can be defined as black (hidden) or white (not hidden). The slideToolkit creates a mask using convert (ImageMagick) and a miniature version of the WSI (in our example this is layer 6 of the multi layered TIFF). At first, the image is blurred, this will remove dust and speckles. Now, the white background is identified using a fuzzy, non-stringent selection and then background is replaced with black. Settings for blur and fuzziness can be found and changed in the slideMask tool. Generated masks can be adjusted manually in an image editor of choice (such as the freely available GNU Image Manipulation Program; GIMP (http://www.gimp.org). Sometimes this is necessary to remove unwanted areas on the WSI (like marker stripes or air bubbles under the coverslip). For our analysis, only non-tissue parts of the WSIs were masked by BN (figure 1 step 2).

Step 3 – Tiles. Image analysis of memory intensive, whole 20x representations of the digitized slides is currently impossible due to hardware and software limitations. The goal of this step is to create multiple smaller images (i.e. tiles) from the 20x WSI. An upscaled version of the mask is placed over the 20x WSI (in our example this is layer 3 of the multi layered TIFF). Image manipulation on 20x sized WSIs requires large amounts of computer RAM. To make it possible for computers without sufficient RAM to handle these files, the slideToolkit uses a memory-mapped disk file of the program memory. Using disk mapped memory files

Tool	Step	Function	Dependencies
slideConvert	Acquisition	Converts a whole slide image file to TIFF format	bfconvert, convert
slideRename	Acquisition	Batch rename slides	convert, display, identify, parallel, perl, tiffinfo
slideInfo	Acquisition	Fetch slide metadata (resolution, dates, etc)	awk, identify, openslide-show-properties, perl, tiffinfo
slideDirectory	Preparation	Create a staging diretory per slide	parallel
slideThumb	Preparation	Create slide thumbnail, including label	convert, parallel, perl
slideMask	Preparation	Create scaled mask and marco from a virtual slide	convert, parallel
slide2Tiles	Tiles	Cut virtual slide into tiles	convert, identify, openslide-write-png, parallel, perl
slideJobsCellProfiler	Analysis	Create a `qsub` joblist for the HPC to analyse all tiles	cellprofiler, convert, parallel
slideSQLheader	Analysis	Copy column headers from SQL file to CSV file	perl

Table 1, Tools available in the slideToolkit. *For each tool a accompanying help text can be found by running the tool followed by the --help flag (e.g. slideMask --help). Different tools are used in different steps. Most tools depend on other libraries and software packages.*

(ImageMagick.mpc files), the slideToolkit can efficiently extract all tiles. Without a mask, a faster and more memory efficient method is used.

Step 4 – Analysis. At this step, multiple tiles containing tissue data have been made, and the different objects in this tissue will be identified. CellProfiler is designed to quantitatively measure phenotypes from thousands of images automatically without training in computer vision or programming. CellProfiler can run using a graphical user interface (GUI) or a command-line interface (CLI). Using the CellProfiler's GUI, different algorithms for image analysis are available as individual modules that can be modified and placed in sequential order to form a pipeline. Such a pipeline can be used to identify and measure biological objects and features in images. Pipelines can be stored and reused in future projects. A pipeline for CD3 analysis using the CellProfiler GUI was created. The CLI was used to run the pipeline for actual image analysis. An illustrated example on how to create pipelines in CellProfiler is described by Vokes and Carpenter.[8]

Pipeline

We created a pipeline to assess the tissue surface area and the number of positively stained nuclei. Tissue was defined using the gray-scale version of the image, applying a Gaussian blur with a 20 pixels diameter, and inverting the resulting image (using the 'ColorToGray', 'Smooth' and 'ImageMath' modules respectively). Tissue was then identified as objects using the 'IdentifyPrimairyObjects'. We selected only objects larger than 40 pixels and with an absolute manual threshold of 0.03. Positively stained nuclei were identified as follows. The 'UnmixColors' module extracted the brown DAB color as a separate grayscale channel. In this DAB channel, nuclei were identified using the 'IdentifyPrimaryObjects' module. Positive nuclei were defined as objects with diameters between 8 and 26 pixels. Objects outside this range were discarded. An 'Otsu Global' with a 'three classes' threshold was used with pixels in the middle intensity class assigned to the foreground. Entropy was minimized. The threshold correction factor for pixels was entered as '1.3', the lower and upper bounds on the threshold were '0.5' and '1.0', respectively. Clumped objects were distinguished by intensity. Occupied area for the 'Tissue' and the 'DAB' objects were measured using the 'MeasureImageAreaOccupied' module and exported to a .csv data file. To verify identified objects, an image was saved with an overlay of each object using the 'OverlayOutlines' and the 'SaveImages' modules (blue outline for tissue, green outline for DAB positive nuclei, figure 3). We store CellProfiler measurements, like cell count, cell position, tissue surface area and

Software	License	URL
bftools	GNU Copyleft [a]	gnu.org/copyleft
CellProfiler	GNU GPL v2 [a]	cellprofiler.org
GNU Bash	GNU GPL v3 [a]	gnu.org/s/bash
GNU Parallel	GNU GPL v3 [a]	gnu.org/s/parallel
ImageMagick	ImageMagick license [c]	imagemagick.org
Libtiff	BSD-like license [b]	remotesensing.org/libtif
Openslide	GNU LGPL v2.1 [a]	openslide.org
Perl	GNU GPL v1 a	perl.org

Table 2, slideToolkit software dependencies, licenses and project websites. *a www.gnu.org/licenses. b www.simplesystems.org/libtiff. C www.imagemagick.org/script/license.php. Copies of the different licenses can be found on the associated websites.*

other information in a database file (e.g. MySQL or .csv). Measurements can then be gathered and further analyzed using preferred statistical software, like R.

Processing

For some steps, we ran the slideToolkit on local hardware (MacMini with 2 GHz i7 and 16 GB RAM). Other steps ran on a high-performance cluster (HPC) (8x Intel® Xeon® CPU E5-2630 0 @ 2.30 GHz, 38x Intel® Xeon® CPU E5-2640 0 @ 2.50 GHz, 11x Intel® Xeon® CPU E5-2630 v2 @ 2.60 GHz, all with 12 cores and 128 GB RAM per node). Tools, wall-clock times and instructions can be found in table 3.

Outcome

The 303 slides were scanned in two runs at 40x, using the Roche iScanHT slide scanner. From these 606 digital slides, masks were created, reviewed and adjusted. The number of CD3 positive cells per tissue area for each digital slide were compared between two runs. With the exception of the CellProfiler pipeline, all steps were unique for each 606 slides. Measurements were stored in .csv files. We used R (CRAN R version 3.0.2) to collect, concatenate and analyze the .csv files. Baseline characteristics of these measurements can be found in table 4. For each slide, measurements per tile were combined. Nuclei per area were defined as the number of CD3 positive cells per tissue area. Variability between measurements was

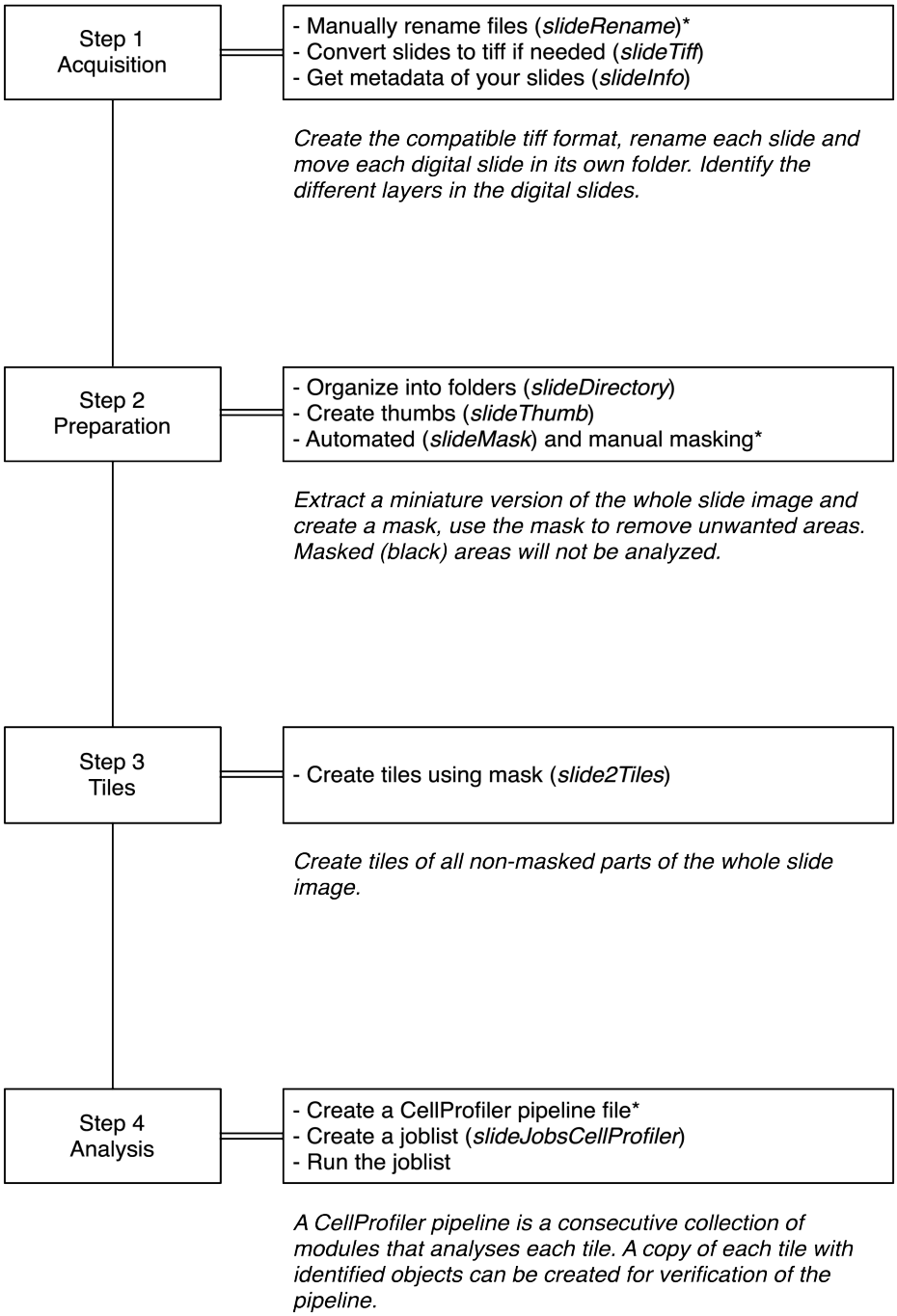
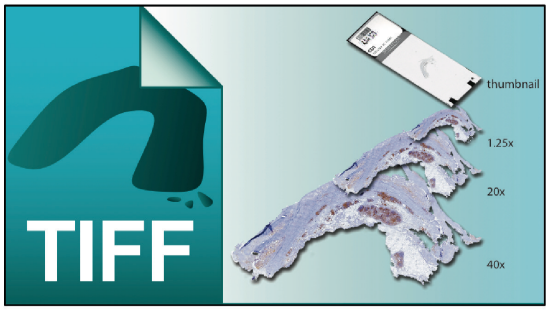
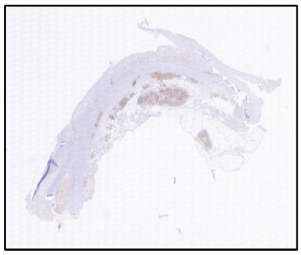
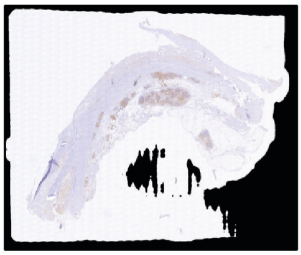


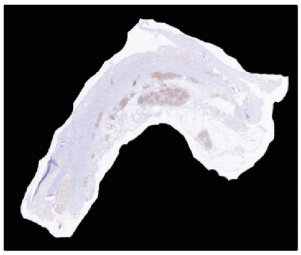
Figure 1 (both pages). *The four step slideToolkit workflow. An overview of the slideToolkit workflow with a summary and illustration for each step. * By hand.*

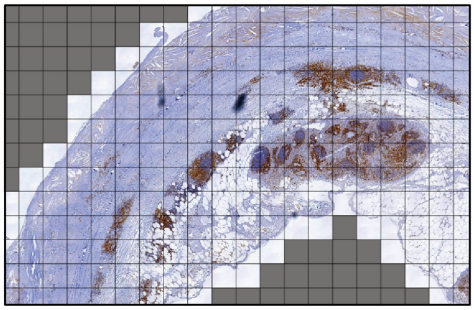

CD3
Starfrost

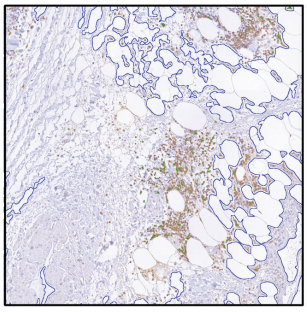

TIFF
thumbnail
1.25x
20x
40x

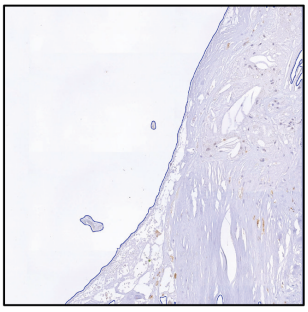












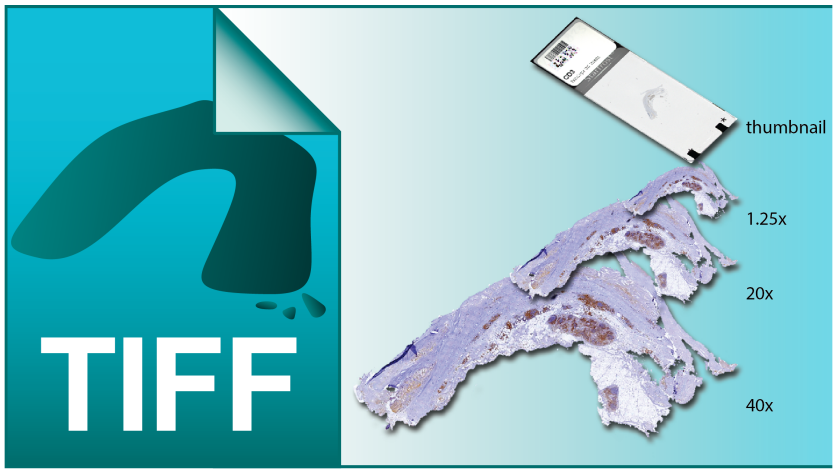


Figure 2. *A visualisation of a multi-page pyramid TIFF file. This illustration shows a TIFF file with 4 layers (thumbnail, 1.25x, 20x, 40x), digital slides stored as TIFF files often contain up to 11 or more layers.*

determined by the difference in mean nuclei density per digital slide. The variability was analyzed by calculating intraclass correlation coefficient (ICC) using a two-way mixed single measure's model. In addition to ICC, Bland-Altman plots[9] were made to visualize the amount of agreements between the continuous scorings using the ggplot2 (version 0.9.3.1)[10] and psych (version 1.4.5)[11] packages in R statistics. Results of the raw CellProfiler measurements and companion R script (in which the ICC and Bland and Altman plots are calculated) can be found as supplemental data.

Results

Acquisition, Preparation & Tiles

We collected a total of 303 unique digital slides containing CD3 stained AAA tissue. File characteristics can be found in table 3. Each mask file was then visually checked using Adobe Photoshop CS6 (version 13.1.2) and adjusted if non-tissue was in the image (like the shade of coverslip-border or markings on the slide). Using these masks, tiles were created (using slide2Tiles) from the 20x layer of each digital slide

Tool	Step	N. slides	Wall-clock time[1]	Command
slideRename[m]	Acquisition	303	2 hours[a]	find . -iname "*tif" -exec slideRename --file="{}" \;
slideInfo[m]	Acquisition	1	50 ms[a]	slideInfo --file="${HOME}/dir/DigitalSlide.tif"
slideDirectory[m]	Preparation	303	10 s[a]	find. -iname "*tif" -exec slideDirectory --file="{}" \;
slideThumb[m]	Preparation	303	4 m 23 s[a]	find. -iname "*tif" | parallel slideThumb --file="{}"
slideMask[m]	Preparation	303	5 m 39 s[a]	find. -iname "*tif" | parallel slideMask --file="{}"
slideMark[m]	Preparation	303	9.88 s [7.53-12.23][b]	qsub -pe threaded 1 slideMask --file="/dir/DigitalSlide/DigitalSlide.tif"
slide2Tiles[h]	Tiles	303	6m 36 s [322 s-1193 s][b]	qsub -pe threaded 3 slide2Tiles --file="/dir/DigitalSlide/DigitalSlide.tif" —mask="/dir/DigitalSlide/DigitalSlide.mask.png"
slideJobsCellProfiler[h]	Analysis	302 [2]	2 m 57 s [97 s-1934 s][b]	qsub -pe threaded 3 cellprofiler -i "/dir/DigitalSlide/DigitalSlide/tiles" -p "/dir/pipeline.cp"

Table 3, tools used, wall-clock times and instructions. *a: wall-clock time is calculated in total. b: wall-clock time is calculated per digital slide (median, 1st–3rd quartile). m: Mac mini, 2.0 GHz i7, 16 GB RAM (capable of 8 threads). h: High performance cluster (HPC), (8x Intel(R) Xeon(R) CPU E5-2630 0 @ 2.30 GHz, 38x Intel(R) Xeon(R) CPU E5-2640 0 @ 2.50 GHz, 11x Intel(R) Xeon(R) CPU E5-2630 v2 @ 2.60 GHz, all with 12 cores and 128 GB RAM per node), '-pe threaded 3' represents three threads and 45 GB RAM (for each thread an additional 15 GB becomes available). 1: Total elapsed wall-clock times are obtained using the GNU-time utility. 2: One sample failed in the 'Tiles' step.*

File characteristics	
Number of digital slides	303
Digital slide file size (median [IQR])	774.00 [441.60-1297.00] MB
Whole slide image x-axis dimension (median [IQR])	33250 [25120-42520] pixels
Whole slide image y-axis dimension (median [IQR])	44880 [31110-62300] pixels
Tiles per whole slide image (mean)	208
Tile file size (median [IQR])	3.05 [1.72-5.27] MB

Table 4. *Run 1 digital slide file characteristics. 1 MB = 1024*1024 bytes*

and stored in the slide staging directory and analyzed by CellProfiler. A visualization of identified layers can be found in figure 2.

Analyse & Data

One digital slide failed the conversion to tiles in both runs for unknown reasons. Tile analysis of the remaining 302 slides was done using CellProfiler (2.1.0.Beta_2a.linux/20131205152921). A CellProfiler pipeline was created by BN using multiple random tiles. Modules used in this pipeline are, among other, UnmixColors (to extract the DAB and the background stain), IdentifyPrimaryObject (to find nuclei within the positive DAB figureareas), OverlayOutlines (to visually check if object detection was correct) and ExportToSpreadsheet (to save the measurements data to file) (Code S2). Baseline characteristics of the two replicate measurements of 302 digital slides can be found in table 4. Wall-clock times of measurements done on the HPC are per slide, as the cumulative times would greatly depend on the availability of threats on the HPC. The collected 303 digital slides used 290.63 GB of disk space. 1.22 TB of disk space was needed to store every file for our analysis. In total, 134976 tiles were analyzed. More runtime and file characteristics can be found in tables 3 and 4 respectively. Variability between measurements was determined by the difference in mean nuclei density. The intraclass correlation coefficient (ICC) using two-way mixed single measures was 0.99. Bland and Altman plots to visualize the variability are depicted in figure 4. Outliers in this plot (differences of both measurements >0.50 or <0.50) are manually checked. This revealed that a discrepancy in masks caused these outliers.

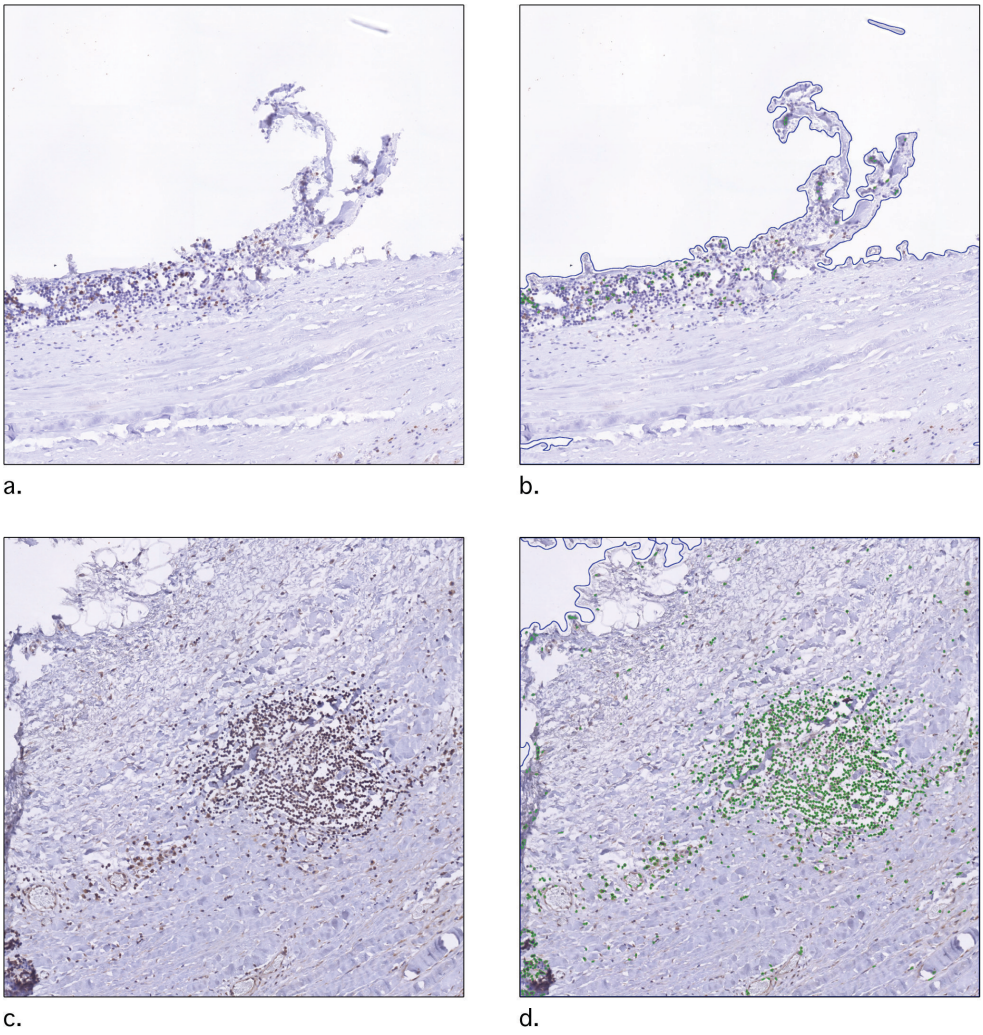


Figure 3. *An example of object identification using the pipeline. a. and c. are original tiles, b. and d. are the same tiles with oulines of the identified objects. Blue lines outline the areas identified as tissue, green lines outline areas identified as DAB positive nuclei. These images are created using the 'SaveImages' module in CellProfiler.*

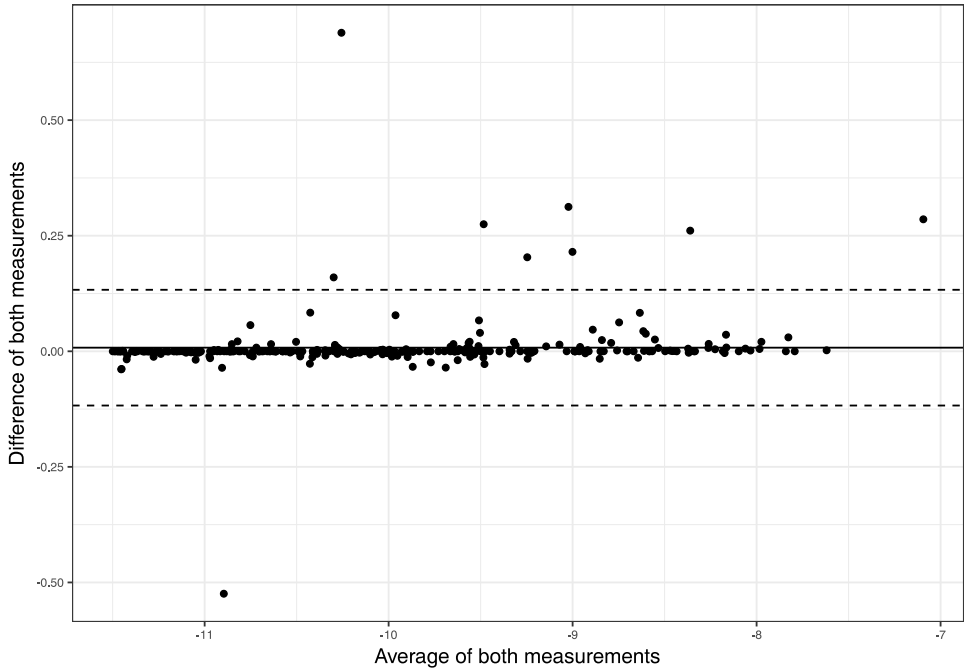


Figure 4. *Bland and Altman plot of both measurements of number identified cells per area (Run1 and Run2). Measurements were log transformed, log(0.0001+ identified cells per area). The intraclass correlation coefficient (ICC) using two-way mixed single measures was 0.99.*

Figure 5 illustrates this difference; one mask contained air bubbles, and one mask contained a shadow artifact from the coverslip.

Discussion

Biobanks become increasingly popular and the demand for adequate and reproducible histological characterization of large amounts of WSIs is increasing. For research purposes, manual assessment of a determinant in histologic slides is time-consuming and the reproducibility is not optimal, which is why computerized quantification of whole histological slides is preferred.

This study introduces the slideToolkit; a collection of opensource libraries and scripts which was developed to analyze WSIs. A slideToolkit workflow consists of four consecutive steps. In the first step, acquisition, digital slides are collected and converted to TIFF files. In the second step, preparation, files are organized in folders. In the third step, tiles, WSIs are divided into easy to handle tiles. The fourth

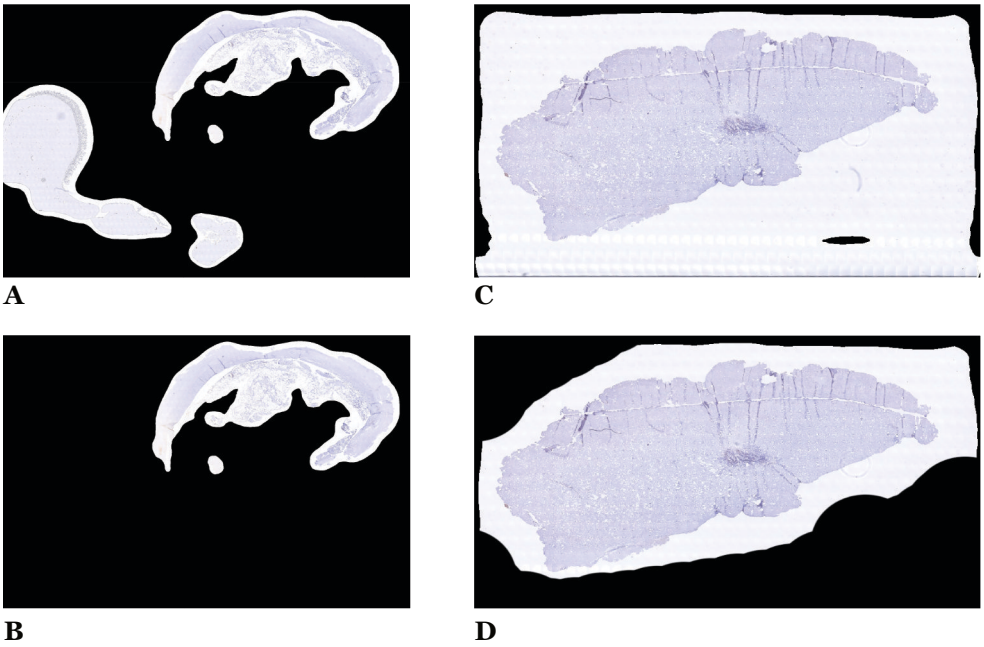


Figure 5. *An example of discrepancy in mask formation. An air bubble was not masked in a., this same bubble was masked in b. The shadow of the coverslip (on the left side of the image) was not masked in c., this same shadow was masked in d. Automatically created masks should be checked manually to avoid unexpected results.*

step, (analysis), previously created tiles are analyzed and results are stored using CellProfiler. CellProfiler makes it easy to use advanced image analysis algorithms, and we demonstrate that we can reliably handle common DAB stains. A modern computer can do the process of slide analysis using our toolkit, but large quantities of WSIs are analyzed much faster using an HPC. An intraobserver ICC of 0.99 on 303 analyzed slides shows that WSIs can be accurately and reproducibly analyzed using the slideToolkit.

Image quality

Bad images yield bad data. The quality of digital slides depends on many things. First, the histological slides need to be of sufficient quality. During fabrication, histologic slides must be produced using a uniform and strict protocol. Artifacts can occur that influence the sample quality due to improper processing, sectioning, staining and mounting of the tissue.[8,12] Second, tissue on the slide is never completely flat. Most slide scanners, such as the one used in this study, automatically determine multiple focus points before scanning. Poorly selected

focus points can result in blurred images and make it harder for the analysis software to detect objects.[13] Also, the slide scanner and its setup should be constant for every sample, including parameters like zoom levels, exposure times, shutter speed, focus, lighting conditions, and sensor sensitivity.[8]

Furthermore, image compression is often used when storing large images. Image quality can be stored as lossy and lossless. Using a lossy compression, 'unnecessary' bits of information are discarded to reduce file size but this can cause artifacts in the image. In contrast, lossless compression does not discard visual information. Commonly used lossy image compression is JPEG and an example of a lossless compression is PNG. High-quality lossy compression can be sufficient for image processing,[14] but lossless compression is recommended.[8] We used 90% lossy compression (JPEG format) for the 20x WSIs which was sufficient for our analysis; lower quality settings were not tested. The slideToolkit uses lossless PNG compression when it extracts image components, so there is no further loss of image quality.

Masking can aid to exclude parts of the image from being processed. However, bad masks can result in unexpected measurements. Our results show a difference in measurements greater than 0.50 in two samples (figure 4). Retrospectively we learned that bad masking caused these outliers; one mask contained air bubbles, and one mask contained a shadow artifact from the coverslip (figure 5). A better manual check of these masks could have prevented such difference in measurements.

Large quantities of slides versus speed

Adequate and reproducible histological characterization of large amounts of collected of tissue is key for, for example, the association with genetic data (e.g. large genome-wide association studies). Manually quantifying histologic slides is a difficult and time-consuming process, and the reproducibility is moderate to good.[5,15] Computer aided analysis seems insurmountable when processing huge quantities of digital slides, but speed of the analysis depends on the architecture of the software. Various software tools have been developed for serial WSI processing, which can therefore only run as one thread (or task) at a time. Roughly speaking, using software built for serial processing, a faster computer would result in a more rapid analysis. However, there are other efficient methods to speed up analysis. The slideToolkit has been developed for parallel processing, which makes it possible to spread threads simultaneously over multiple computers. A professional computer cluster is not necessary. Using GNU parallel, a cluster of computers in a laboratory

or an office will already result in enormous speed improvements.[16] The help menu within slideToolkit gives instructions on how to facilitate this.

Software considerations

Open-source. We created the slideToolkit as open-source software following common development rules.[17,18] These rules provide a fast way to build and improve software. It is promoted to release often and in early stages of development. Users will identify problems and new requirements in an early stage of development, and developers can fix them more quickly. Furthermore, providing the source code enables for more insight into what is happening. It also prevents other developers from reinventing the wheel when encountering a similar problem.[18] SlideToolkit is released under the terms of the MIT license (wikipedia.org/wiki/MIT_License). In short, it implies that one can freely use the code, and that places almost no restrictions on what you can do with its source code. The software used by the slideToolkit is distributed under similar licenses (table 2).

Scripting. Most of the code is written in Bash (www.gnu.org/software/bash). Bash is a widely known scripting language and is a powerful way to streamline batch processes. Bash scripting makes it easy to exchange work flows between different computer systems.

Image manipulation

To read digital slides, we use the open-source libTIFF libraries and the OpenSlide libraries. These libraries are also used to extract metadata (e.g. scan date, pixels per micrometer, magnification and image compression) of the scanned slides. For image processing, we use ImageMagick (ImageMagick 6.8.7-0 2013-1016 Q16 http://www.imagemagick.org). ImageMagick is a command-line image manipulation tool that is fast, highly adjustable and capable of handling big pyramid TIFF files. When a mask is available, ImageMagick is used for the creation of tiles. The Openslide library is used when no mask is present.

Image analysis

CellProfiler (www.cellprofiler.org) is used for the actual image analysis. CellProfiler makes use of modules that each performs an image-processing function, for example: unmixing colors into DAB and HE channels, object identification and measurement, storing measurements into a database. Most modules are highly

configurable. A pipeline is a consecutive collection of modules to analyze images. The combination of different modules makes it possible to analyze even challenging images. Developing a working CellProfiler pipeline can be time consuming since it involves a lot of testing, and requires a certain homogeneity within the images you analyze. Variation of color intensities within WSIs can result in a consistent deviation of the outcomes. Pipelines can be reused and shared between computer systems and projects. The accuracy of the pipeline will determine the validity of the measurements.

In this paper we show an ICC of 0.99. This high correlation is found by looking at the number of identified cells per area. We have chosen for a specific cell type (nuclei of T-lymphocytes). One could argue that a small measurement error of a more present object (all nuclei) would result in a lower ICC. We have unpublished research were we find high ICCs in other tissue and for other measurements as well (ICC >0.90).

Dependencies

The modular approach of the slideToolkit allows each tool to update independently without losing functionality of the toolkit.

Future applications

Cloud based high-performance computing has become a popular way for scientists to do big analyses. The slideToolkit is primary developed for HPCs and is a powerful tool to take image analysis into the cloud.

CellProfiler creates the possibility to store the location of each identified object (e.g. a cell). When consecutive histological slides from tissue are stained for different antibodies, a virtual stack can be created and spatial information of the cells relative to each other can be analyzed.

Shortcomings

During development we found that some slides contained undesired artifacts, like air-bubbles, pen markings or positive antibody control tissue. These artifacts can result in false measurements. To exclude these parts of the WSI we created the option to mask parts of the slide. Masking requires loading an uncompressed version of the WSI into the computer's memory and can require 60 GB or more. Masking is no issue for most HPCs, but can cause extremely slow performance on

computers with insufficient RAM memory. Often, automatically created masks need to be altered to remove unwanted artifacts and this will influence reproducibility. Without the use of masks, undesired tiles can always be manually deleted before analysis.

Code Availability

The slideToolkit is free software; you can redistribute it and/or modify it under the terms of the MIT license. The license and the source code for the slideToolkit can be found at: https://github.com/bglnelissen/slideToolkit. The present original paper is requested to be cited when using the slideToolkit to process data for publication.

Conclusion

We present our open-source slideToolkit for manipulating and analysis of whole digital slides. The slideToolkit provides a free, powerful and versatile collection of tools for automated feature analysis of whole slide images to create reproducible and meaningful phenotypic data sets.

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Supplemental

Code S1

http://journals.plos.org/ plosone/ article?id=10.1371/ journal.pone.0110289#s6, this is the image of the slideToolkit source code. When using the code, you are encouraged to download the code from https://github.com/ bglnelissen/ slideToolkit.

Code S2

pipeline.cp.zip. The CellProfiler pipeline used in this project. (ZIP)

http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0110289#s6

RawData S1

ICC and BlandAltman plot - measurements and r-script.zip. Measurements of Run1 and Run2, including the code for the calculation of the intraclass correlation coefficient (ICC) and the code for the Bland and Altman plot. (ZIP)

http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0110289#s6

Chapter 4

High Reproducibility of Histological Characterization by Whole Virtual Slide Quantification; An Example Using Carotid Plaque Specimens

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Bastiaan G. L. Nelissen[1,3], Joyce E. P. Vrijenhoek[1,2,3], Evelyn Velema[1], Kristy Vons[1], Jean-Paul P. M. de Vries[4], Marinus J. C. Eijkemans[5], Hester M. den Ruijter[1], Gert Jan de Borst[3], Frans L. Moll[3], Gerard Pasterkamp[1]

1 *Experimental Cardiology Laboratory, University Medical Center Utrecht, Utrecht, the Netherlands*

2 *Interuniversity Cardiology Institute of the Netherlands, Utrecht, the Netherlands*

3 *Department of Vascular Surgery, University Medical Center Utrecht, Utrecht, the Netherlands*

4 *Department of Vascular Surgery, St. Antonius Hospital Nieuwegein, Nieuwegein, the Netherlands*

5 *Julius Center of Health Sciences and Primary Care, University Medical Center Utrecht, Utrecht, the Netherlands*

☯ *These authors contributed equally to this work.*

Abstract

Objective: Tissue biobanks are an important source for discovery and validation studies aiming for new proteins that are causally related with disease development. There is an increasing demand for accurate and reproducible histological characterization, especially for subsequent analysis and interpretation of data in association studies. We assessed reproducibility of one semiquantative and two quantitative methods for histological tissue characterization. We introduce a new automated method for whole digital slide quantification. Carotid atherosclerotic plaques were used to test reproducibility.

Methods: 50 atherosclerotic plaques that were obtained during carotid endarterectomy were analysed. For the semiquantitative analysis, 6 different plaque characteristics were scored in categories by two independent observers, and Cohen's κ was used to test intra- and interobserver reproducibility. The computer-aided method (assessed by two independent observers) and automated method were tested on CD68 (for macrophages) and a smooth muscle actin (for smooth muscle cells) stainings. Agreement for these two methods (done on a continuous scale) was assessed by intraclass correlation coefficients (ICCs).

Results: For the semiquantitative analysis, k values ranged from 0.55 to 0.69 for interobserver variability, and were slightly higher for intraobserver reproducibility in both observers. The computer-aided method yielded intra- and interobserver ICCs between 0.6 and 0.9. The new automated method performed most optimal regarding reproducibility, with ICCs ranging from 0.92 to 0.97.

Conclusions: The analysis of performance of three methods for histological slide characterization on carotid atherosclerotic plaques showed high precision and agreement in repeated measurements for the automated method for whole digital slide quantification. We suggest that this method can fulfill the need for reproducible histological quantification.

Introduction

Biobanking of human tissues is an important cornerstone in the discovery and validation of causally related determinants of life-threatening diseases. Genotyping studies of human DNA are characterized by stringent quality controls. However, accurate phenotyping of a diseased patient and biosamples is becoming a major hurdle for the assessment of genophenotypic associations. Dissected tissues collected in biobanks are a great asset for phenotyping diseases and for prognostic studies, in which histological characterization is widely applied. It is evident that accuracy and reproducibility of histological characterization is key for optimal phenotyping of human tissues and subsequent interpretation of data for association studies. An example of a research field where phenotyping by histological characterization is commonly executed is the characterization of atherosclerotic plaques, where stable and unstable lesions are differentiated based on inflammatory, lipid, and fibrous components. Previously, assessment of semiquantitative scoring (SQ) of different carotid plaque characteristics indicated moderate to good reproducibility as indicated by Cohen's kappa (κ) values.[1, 2] Still, further improvement regarding intra- and interobserver variability is required. Also, in ongoing longitudinal studies, a continuing check of reproducibility is essential. Furthermore, though variability of semiquantitative scoring based on subjective scoring within a laboratory could be acceptable, it may be difficult to extrapolate this other external studies. In atherosclerotic plaques, a computeraided method (further annotated as method Q1) to score inflammatory cells and smooth muscle cells quantitatively was previously implemented to improve reproducibility that indeed performed well[2] However, this method requires the user to manually set color thresholds for the positively stained areas within subjectively selected regions of interest. Therefore, a targeted method to quantify characteristics in total tissue specimen is needed. Whole slide virtual imaging can be used for this purpose.[3, 4] To quantify characteristics on these whole slide images, we have developed the slideToolkit software (further annotated as method Q2). This is a new method using free open-source software, targeted to overcome the aforementioned limitations. We tested the precision (not the accuracy) of this automated method in the Athero-Express biobank, comprising carotid artery plaque specimens. The aim of this study was 1) to reassess intra- and interobserver variability of carotid plaque scoring

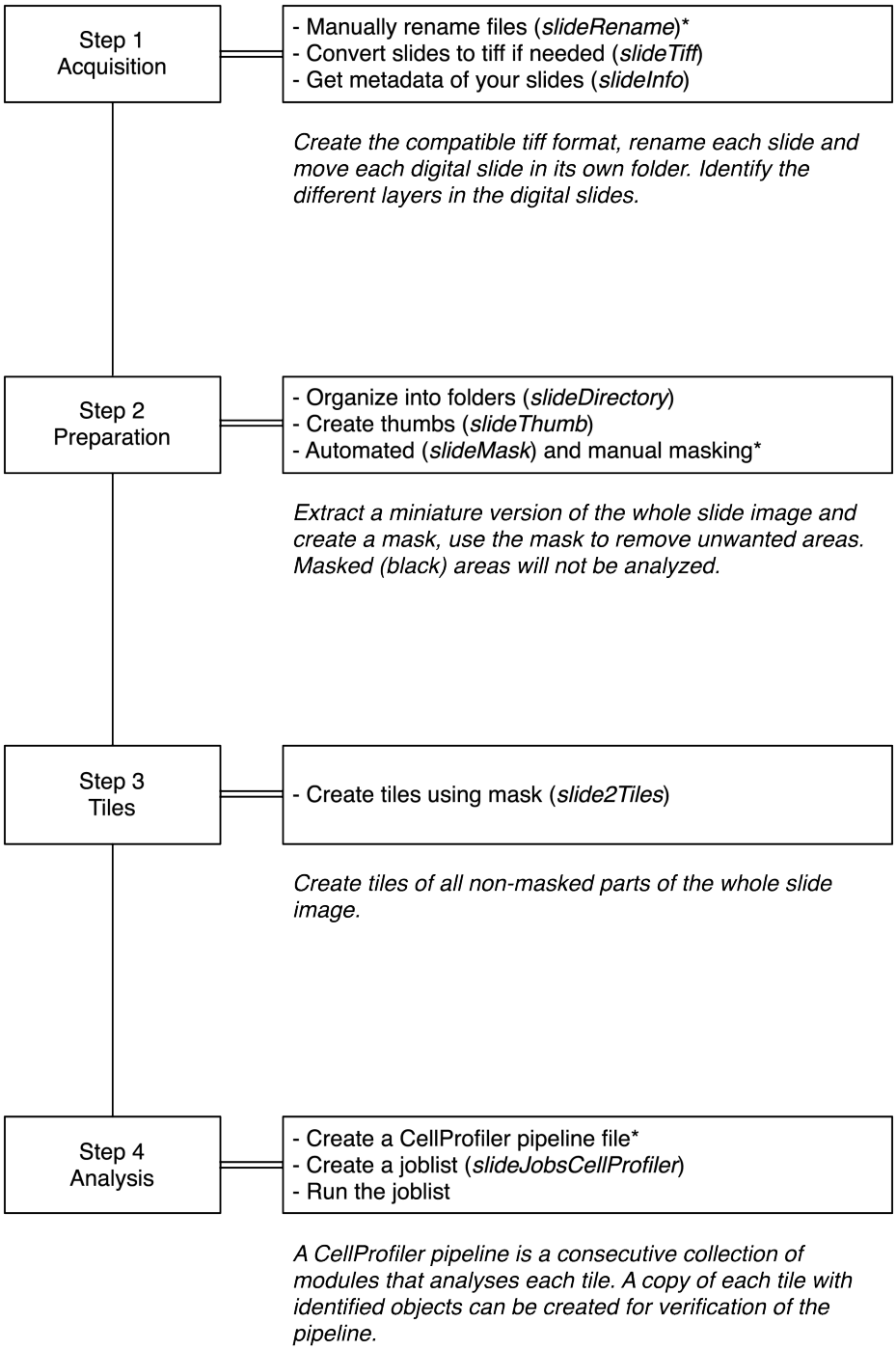

Figure 1 (both pages). *A common slideToolkit workflow consists of 'Acquisition', 'Preparation', 'Tiles' and 'Analysis'.*


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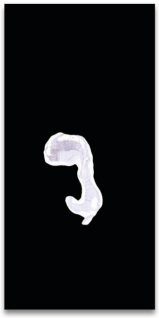

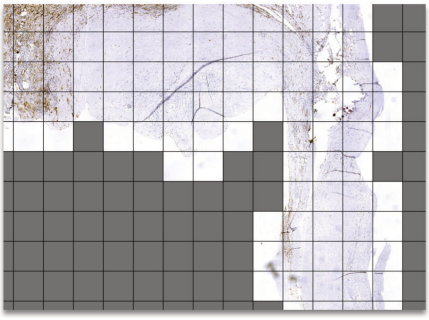

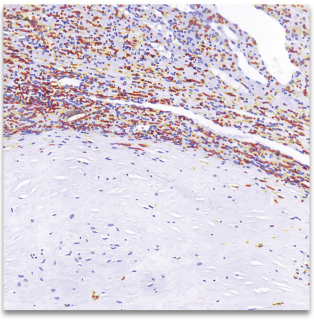

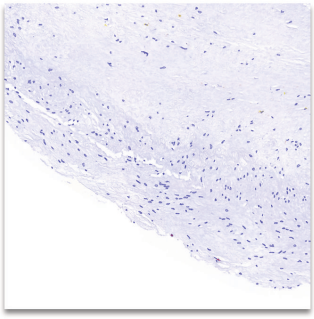

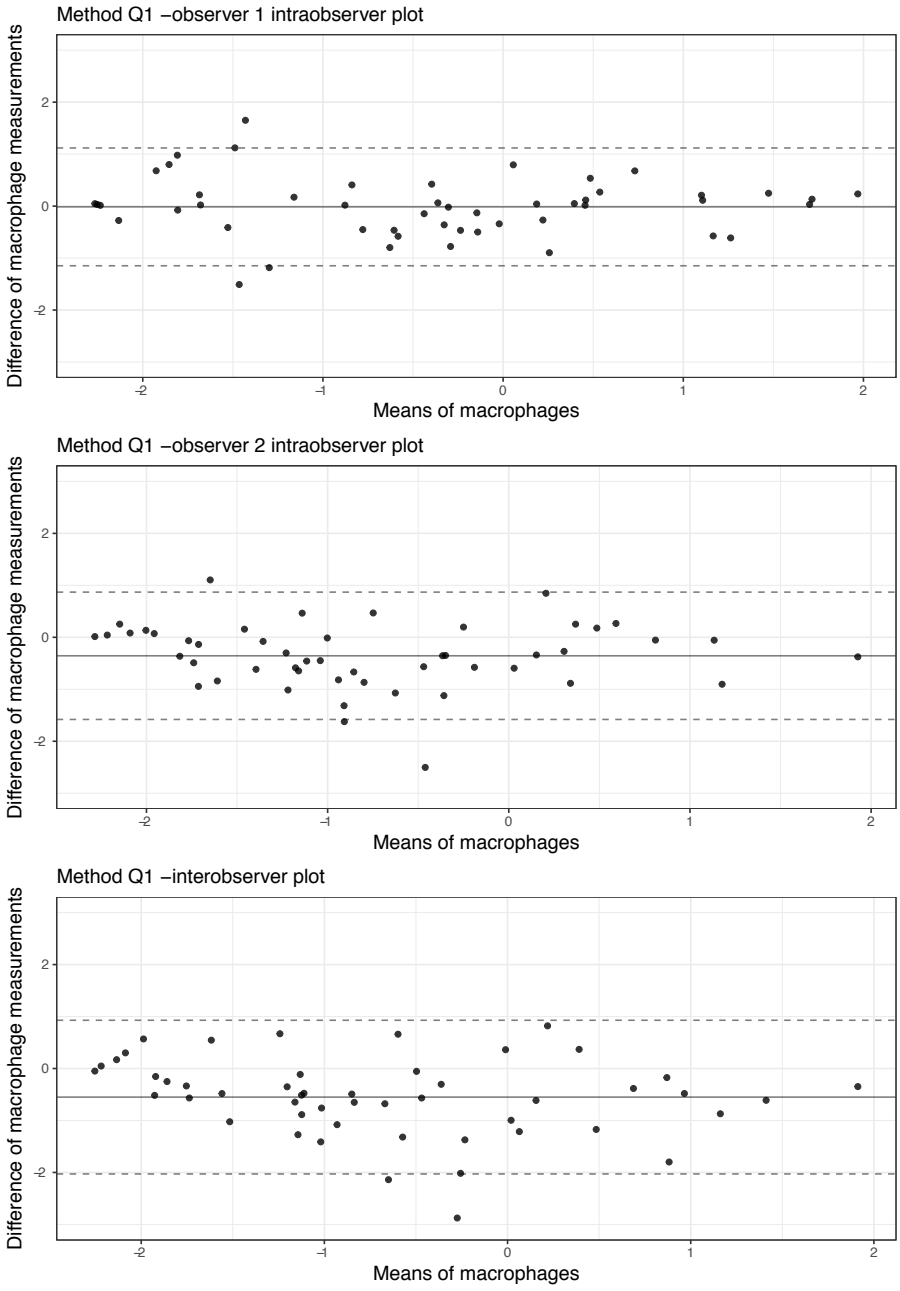

Figure 2. *Bland-Altman plots of intra- (upper two panels) and interobserver (lower panel) reproducibility of method Q1 for measurement of macrophages. Measurements were logarithmically transformed after adding 0.1. The continuous line shows the mean difference of measurements, the dotted line indicates 95% limits of agreement.*

within our biobank with the methods used before and 2) to evaluate the performance and precision of the new method Q2.

Methods

Sample selection

50 carotid plaque specimens were randomly selected from carotid endarterectomy (CEA) patients who were included in the Athero-Express study between 2002 and 2012.5 68% of these patients was male with a mean age of 69 years. 78% was symptomatic with median time from event to CEA of 38 days. All patient characteristics are shown in table 1.

Ethics Statement

The Athero-Express study was approved by the institutional review boards of both participating hospitals (University Medical Center Utrecht, Utrecht, The Netherlands, and St. Antonius Hospital, Nieuwegein, The Netherlands) and patients gave written informed consent.

Plaque processing

Plaques were removed during CEA and immediately processed in the laboratory, were the culprit lesion with a length of 5 mm was fixed in 4% formaldehyde, subsequently followed by decalcification and embedding in paraffin. 5 mm crosssections were sliced and routinely stained for different characteristics: lipid core size (hematoxylin and eosin (HE) and Picrosirius Red with polarized light when appropriate), macrophages (CD68, Clone KP1, Novacastra reagents, Leica Biosystems, Rijswijk, the Netherlands), smooth muscle cells (α smooth muscle actin antibody, Clone 1A4, Sigma-Aldrich, Zwijndrecht, the Netherlands), collagen (picrosirius red), calcification (assessed using HE), and thrombus (HE and fibrin).[5] CD68 and SMA were visualized with DAB (3,39-diaminobenzi- dine), and were used

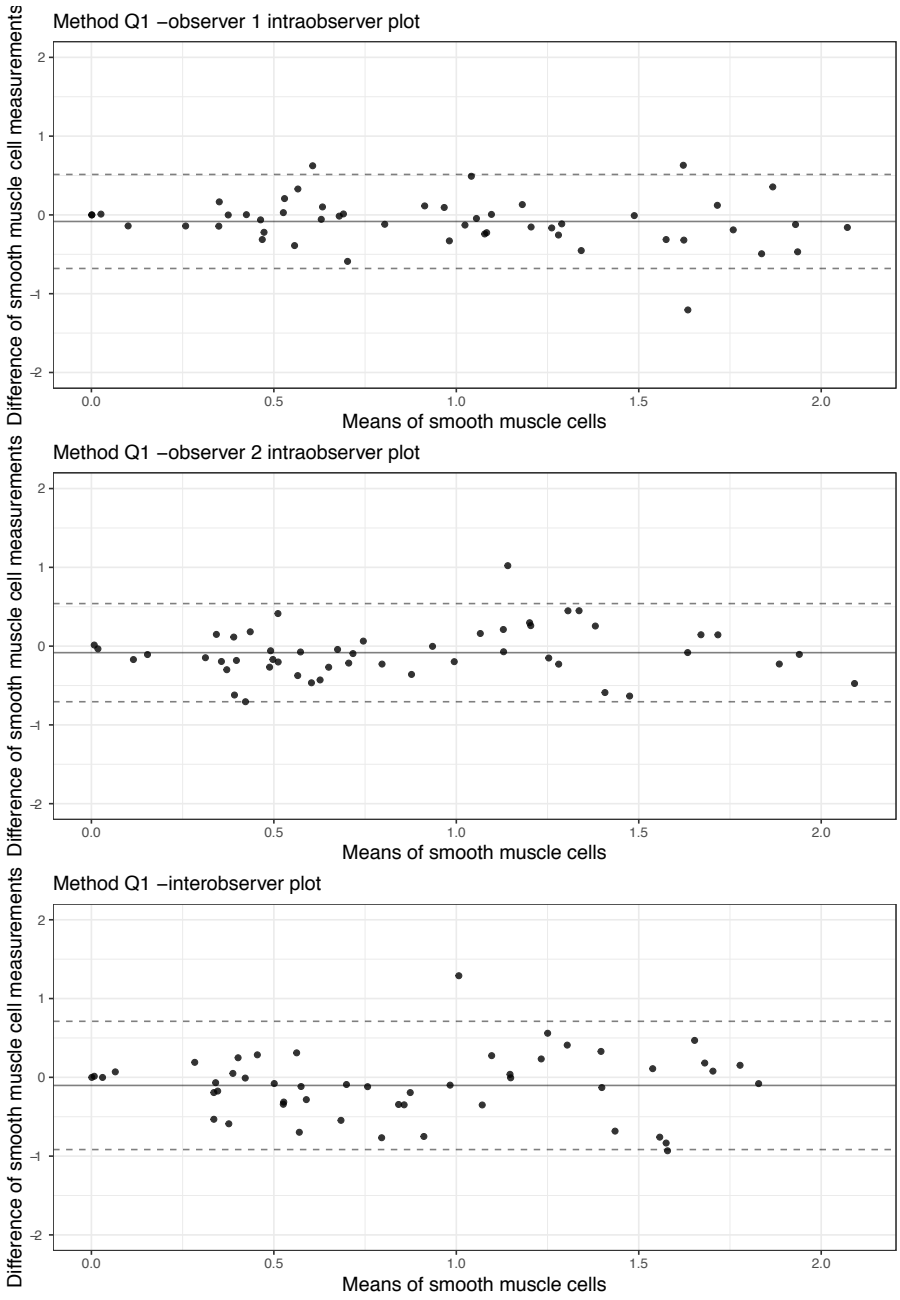

Figure 3. *Bland-Altman plots of intra- (upper two panels) and interobserver (lower panel) reproducibility of method Q1 for measurement of smooth muscle cells. Measurements were logarithmically transformed after adding 1. The continuous line shows the mean difference of measurements, the dotted line indicates 95% limits of agreement.*

in quantitative analyses in this study (methods Q1 and Q2). Other characteristics were not included in the quantitative analyses in this study.

Semiquantitative analysis (method SQ)

Plaques were categorised in no, minor, moderate and heavy staining for most characteristics, except for lipid core (no, <40%, >40%) and overall phenotype (fibrous, fibroatheromatous and atheromatous). Scorings were done by two observers (EV and GP), both at 2 timepoints with at least a one-month interval. They were blinded for each other's scoring, their previous scoring (at the second observation), and for patient characteristics.

Quantitative analysis, computer-aided with visual interpretation (method Q1)

In the CD68 three representative regions of interest of the plaque (excluding lumen and media) were selected at x40 magnification according to decision of the same experienced technician over time (observer EV). The area occupied by DAB staining was determined by manually selecting colour thresholds by visual interpretation, for each of the three regions of interest separately. Thereafter, the total field occupied by tissue was calculated as a percentage of total area occupied by plaque on each region of interest (analySIS FIVE, Olympos soft imaging solutions). The mean percentage of three fields was taken as the final value. Scorings were done by observer 1 (KV) and observer 2 (TB), both at two timepoints with a one-month interval. They were blinded similarly as noted above, and also for the results of semiquantitative scorings.

Automated quantitative analysis (method Q2)

For this analysis, histological slides were scanned in total using a Roche VENTANA iScan HT slide scanner. Each virtual slide was stored as a multi-page pyramid tiff using 90% JPEG compression. In short, a Q2 workflow consists of four consecutive steps. In the first step (acquisition), whole slide images are collected and converted to TIFF files. In the second step (preparation), files are organized. The third step (tiles), creates multiple manageable tiles to count. In the fourth step (analysis), tissue is analyzed and results are stored in a data set (Fig. 1 in S1 Methods and pipelines in S1 and S2 Data for details). As a part of this method, actual image analysis was done using CellProfiler (version 2.1.0.Beta_2a.linux, Cellprofiler Cell Image Analysis Software, Broad Institute, Cambridge, Massachusetts, USA).

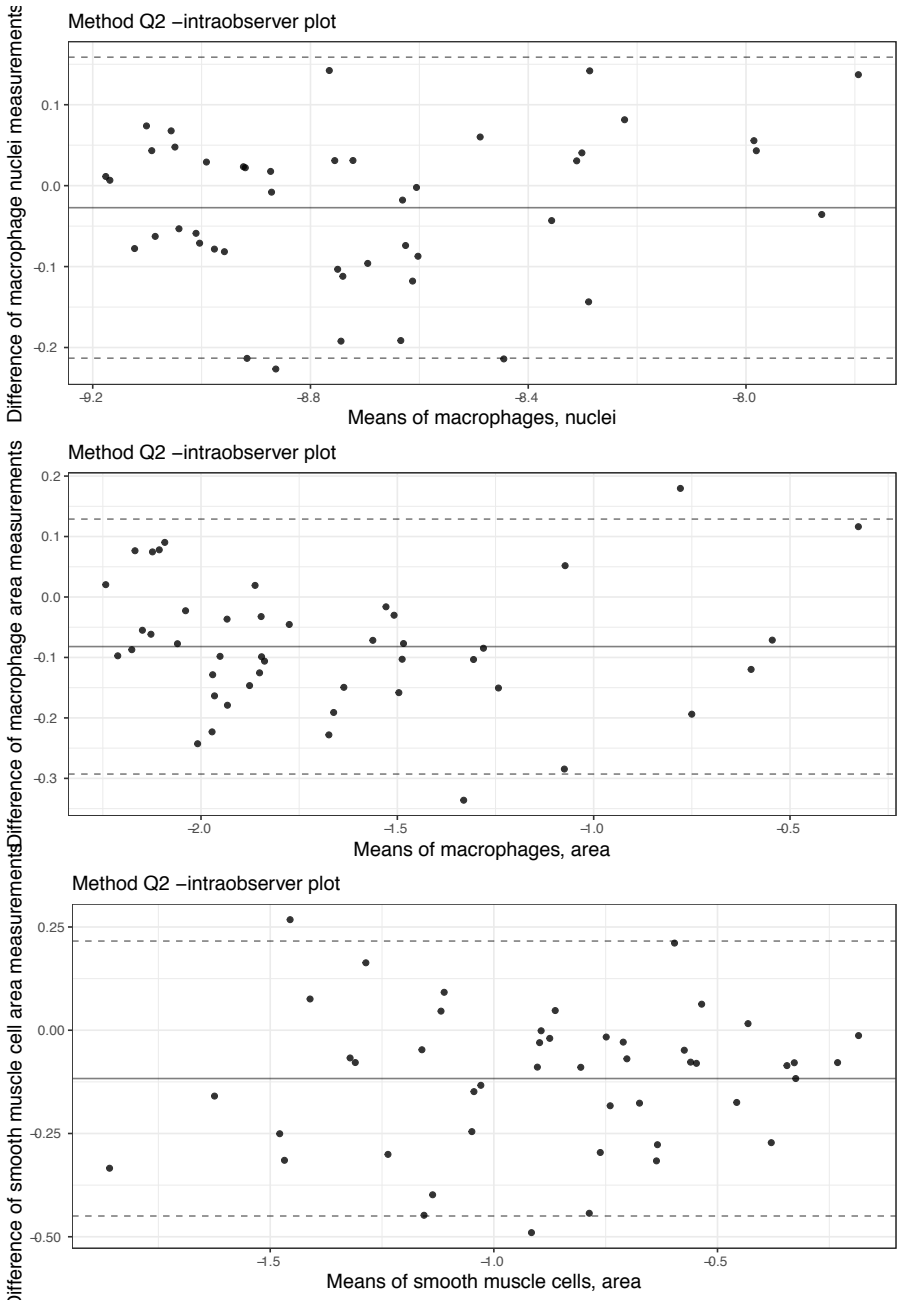

Figure 4. *Bland-Altman plots of intraobserver reproducibility of method Q2 for measurement of macrophage nuclei (upper panel), macrophage area (middle panel), and smooth muscle cell area (lower panel). Measurements were logarithmically transformed after adding 0.0001, 0.1, and 0.1, respectively. The continuous line shows the mean difference of measurements, the dotted line indicates 95% limits of agreement.*

CellProfiler uses predefined pipelines to analyze and measure histological images. A pipeline is a sequential series of modules that each performs an image processing function. For example, to measure the DAB positive cells, the following was done during the analysis step. First we used a module to convert the image into two separate gray scale images. One gray scale image was analyzed for HE, and only HE was white (positive), the rest was black (negative). We did the same for DAB, where DAB was white (positive) and the rest was negative (black). To find all nuclei within DAB positive areas, we used a module to use the DAB gray scale image as a mask for the HE gray scaled image. In this way it is possible to only analyze the HE positive nuclei within DAB positive areas. Finally, to identify these nuclei within DAB positive areas, a module to identify objects was used to find HE nuclei sized between 8 and 40 pixels. Hence, method Q2 differs from Q1 in that Q2 analysis the whole slide, so it has no selected regions of interest, and thresholds and measurements

Patient characteristics	
Male sex	68%
Age (mean) (SD)	69 (10)
Current smoking	39%
Diabetic mellitus	22%
Hypertension	72%
Body Mass Index (mean) (SD)	26 (3.6)
Hypercholesterolemia	54%
History of coronary artery disease	32%
History of peripheral intervention	24%
Preoperative acetylsalicyl acid use	96%
Preoperative statin use	69%
Total cholesterol (mean) (SD)	4.7 (1.3)
HDL (mean) (SD)	1.1 (0.35)
Clinical presentation:	
- Asymptomatic	22%
- Ocular symptoms	27%
- Transient ischemic attack	33%
- Stroke	18%
Event to operation time (median) (IQR)	37.5 (18.5–70.3)

Table 1, patient characteristics. *SD: standard deviation. HDL: high density lipoprotein. IQR: interquartile range.*

settings are set before analysis. More details on how method Q2 works and the accompanying pipelines can be found in S1 Methods and S2 Data. For CD68 and SMA stained slides, separate CellProfiler pipelines were created. For macrophages, two parameters were calculated: total DAB positive nuclei as a ratio of total plaque area (macrophage nuclei), and total DAB positive area as a ratio of total plaque area (macrophage area). For smooth muscle cells, only the area was calculated (smooth muscle cell area).

Statistical analysis

Reproducibility of different scoring methods was assessed by different measures of agreement, as shown in table 2. Intra- and interobserver variability was assessed within the same methods, except for method Q2 that only includes intraobserver variability. Variability of scorings done on a categorical scale (method SQ) was analyzed by Cohen's κ, with linear and quadratic weightings.[6] Values between 0 and 0.2 are generally indicated as slight agreement, 0.21-0.4 as fair, 0.41-0.6 as moderate, 0.61-0.8 as substantial, and 0.81-1 as almost perfect agreement. Variability of scorings done on a continuous scale (method Q1 and Q2) was analyzed by calculating intraclass correlation coefficients (ICCs), by a two-way mixed, single measures model. Interobserver ICCs of method Q1 were calculated by using only the first measurements of both observers (otherwise we would calculate a composite of intra- and interobserver variability). To check if this approach was valid, a random effects model was fit using the 'lme4' package in R statistics[7] with slide number, rater, and repetition as random effects. A fixed effect was not included, because there was only 1 method to be assessed. For both macrophages and smooth muscle cells, variance induced by repetition was less than 1% of the total variance. For method Q2, there was only 1 observer (the computer). Therefore, only intraobserver ICC and no interobserver variability, was calculated. We did not calculate ICCs

between measurements of different methods, because these were measured on different scales and had different ranges. Therefore, it was not possible to accurately define cutoffs or ranks within different ranges of measured values to compare the different methods. The different scales (categorical for SQ and continuous for Q1 and Q2) were also the reason why we could not use the same statistical methods for all scoring methods. For comparison between κ and ICC, quadratic weighted κ can be used, as this is expected to be equal to the ICC; quadratic weighted κ can be written as a ratio of the difference between expected and observed disagreement, divided by the expected disagreement. Since for quadratic weighted κ all disagreement terms contain quadratic differences between categorical numbers, after some mathematical manipulations as shown by Fleiss and Cohen, this is the same as the ratio of variances that defines the intraclass correlation coefficient.[8] In addition to ICCs, Bland-Altman plots were made to visualize the amount of (dis)agreement between the continuous scorings.[9] using the ggplot2 and gridExtra packages in R statistics[7] Because the data were right-skewed and the spread of differences increased with increasing mean of the observations, natural logarithmic transformed values were used in these plots. SPSS version 20.0 (IBM Corp, IBM SPSS Statistics for Windows, Armonk, NY) was used to calculate all ICCs.

Results

Semiquantitative analysis (SQ)

For all plaque characteristics under study, κ values were calculated with linear and quadratic weights, as shown in table 3. In this results section we only report the quadratic weighted κ. Quadratic weighted κ can also be compared to the ICC, as

	SQ	**Q1**	**Q2**
SQ	Cohen's κ	NC	NC
Q1	NC	ICC +Bland-Altman	NC
Q2	NC	NC	ICC + Bland-Altman

Table 2, statistical tests used for analyzing agreement of different plaque scoring methods. *SQ: semiquantitative analysis; method Q1: quantitative analysis, computer-aided; method Q2: automated quantitative analysis; NA: not applicable; NC: not comparable (measurements are on different scales).*

			Intraobserver x EV (95% CI)			Intraobserver x GP (95% CI)		Interobserver x GP (95% CI)
	Cat.	**Agr.**	**Linear weighted**	**Quadratic weighted**	**Linear weighted**	**Quadratic weighted**	**Linear weighted**	**Quadratic weighted**
Lipid core	3	58%	0.58 (0.37-0.79)	0.65 (0.32-0.98)	0.65 (0.48-0.83)	0.68 (0.44-0.93)	0.44 (0.25-0.63)	0.55 (0.28-0.81)
Macrophages	4	57%	0.65 (0.48-0.82)	0.77 (0.51-1)	0.68 (0.56-0.81)	0.84 (nc)	0.56 (0.41-0.71)	0.69 (0.47-0.90)
Smooth muscle cells	4	46%	0.65 (0.45-0.85)	0.72 (0.20-1)	0.57 (0.38-0.76)	0.63 (0.27-0.99)	0.40 (0.23-0.57)	0.59 (0.31-0.87)
Collagen	4	79%	0.75 (0.59-0.91)	0.80 (0.33-1)	0.67 (0.50-0.84)	0.75 (0.35-1)	0.72 (0.56-0.88)	0.79 (0.41-1)
Calcification	4	40%	0.75 (0.63-0.88)	0.84 (0.66-1)	0.67 (0.53-0.80)	0.81 (nc)	0.42 (0.28-0.56)	0.59 (0.41-0.77)
Thrombus	4	42%	0.71 (0.54-0.880	0.76 (0.36-1)	0.54 (0.39-0.69)	0.72 (0.65-0.80)	0.38 (0.23-0.54)	0.55 (0.33-0.77)

Table 3, intra- and interobserver variability of semiquantitative scoring of plaque histology. *Cat.: Categories, Agr.: Agreement, nc: not calculated due to a substantial proportion of zeros in the crosstable. CI: confidence interval. EV and GP indicate different observers.*

	Macrophages, nuclei	Macrophages, area or %*	Smooth muscle cells
Q1, interobserver ICC	NA	0.71 (0.53–0.82)	0.62 (0.42–0.77)
Q1, intraobserver ICC observer 1	NA	0.92 (0.86–0.95)	0.76 (0.61–0.85)
Q1, intraobserver ICC observer 2	NA	0.84 (0.73–0.91)	0.86 (0.80–0.88)

Table 4, intra- and interobserver variability of quantitative methods scoring of plaque histology. *Method Q1: quantitative computer-aided technique of selected areas with visual interpretation of color threshold; method Q2: automated quantitative analysis: whole slide imaging and automated quantitative analysis software; NA: not applicable (macrophage nuclei were not assessed in method Q1). *For method Q1: percentage stained area of total plaque; for method Q2: total percentage stained area per total area in hematoxylin staining..*

explained previously. Inter- and intraobserver variability was moderate to substantial for all characteristics; intraobserver κ's were between 0.65 to 0.84 and 0.63 to 0.84 for observers EV and GP respectively, for interobserver comparisons κ's ranged between 0.55 and 0.69.

Quantitative analysis, computer-aided with visual interpretation (method Q1)

For macrophage staining, median value of percentage of stained area of all observations (n=200) was 0.46% (interquartile range (IQR): 0.11-1.1%). Intraobserver ICC for observer 1 was 0.92 (95% confidence interval (CI): 0.86- 0.95) and for observer 2 0.84 (95% CI: 0.73-0.91). For smooth muscle cells, median value of percentage of stained area of all observations was 1.3% (IQR: 0.54-2.6%). Intraobserver ICC was 0.76 (95% CI: 0.61-0.85) and 0.86 (95% CI: 0.80-0.88), respectively. Interobserver ICCs were lower, with 0.71 for macrophages and 0.62 for smooth muscle cells (table 4).

Bland-Altman plots show that the variation in log transformed macrophage and smooth muscle cell measurements remains quite constant with increasing percentage staining, both for intra- and interobserver measurements (Figs. 2 and 3).

Automated quantitative analysis (method Q2)

For the ratio of macrophage nuclei and area per total plaque area, median level of all observations (n=91, remaining 9 scorings were missing) was 6.0×10^5 (IQR: 2.3

$\times 10^5$-9.8 $\times 10^5$), and 0.065 (interquartile range (IQR): 0.030-0.14), respectively. Intraobserver ICC for macrophage nuclei was 0.97 (95% CI: 0.94-0.98) and for macrophage area 0.97 (95% CI: 0.95-0.98).

Median level of smooth muscle cell area of all observations (n595) was 0.32 (IQR 0.21-0.47). ICC of smooth muscle cell scoring was 0.92 (95% CI: 0.86-0.95) (table 4). Again, Bland-Altman plots suggest that the variation in log-transformed macrophage and smooth muscle cell measurements remains constant across the range of all measurements (Fig. 4).

Discussion

Here we report that a method to quantify tissue histology fully automatically can be executed with high reproducibility. We used macrophage and smooth muscle cell infiltration in carotid atherosclerotic plaques as markers to test this method. If we compare this method to other (semi)quantitative techniques that have been applied previously, this new quantitative method seems to perform better regarding ICC values of two repeated measurements, with narrower 95% confidence interval ranges. The high precision suggests that quantification by this technique allows for more reliable association studies, compared to traditional methods using semiquantitative analysis that showed modest to substantial reproducibility in this study, as shown before.[2] This indicates that characteristics that require visual interpretation may suffer from variability among researchers and research centers, and should be interpreted with caution.

Applications in research

There are different research applications in which reproducibility of atherosclerotic plaque histology is important. First, plaque imaging (by MRI) is increasingly used as a technology to detect vulnerable plaque characteristics noninvasively, for example for associations with symptoms and/or future cardiovascular events.[10-13] For validation of imaging techniques, comparison with atherosclerotic plaque histology is necessary. Measured variability between plaque histology and plaque imaging for example, is a result of the added variability of both methods. Second, human genotyping studies are emerging and applied to study causality of disease and for discovery of potential therapeutic targets. Therefore, accurate and reproducible association of phenotypic tissue characteristics with genetic data is relevant, which requires improved phenotyping of tissues and disease. This

objective applies for all diseases where histological phenotyping is an issue and goes beyond the scope of atherosclerotic disease. Phenotypic differences in disease presentation between subjects (such as differences between macrophage infiltration in atherosclerotic plaques) are a result of real variation, and 'noise': variability within and between observers using a certain technique. Studies on genetic variability are characterized by high qualitative output and reproducibility and the large variability in phenotypic outcomes is a major challenge before we can optimally assess and interpret genotypic-phenotypic associations.

Applications in clinical practice

Another application, in which similar digital imaging techniques are already in use in some clinical laboratories, is assessing Her2 receptor status in breast cancer by automated cellular imaging system III (ACIS III, Dako). Standardized scoring protocols using this digital image analysis system have been tested and showed better concordance with fluorescence in situ hybridization, compared to manual semiquantitative interpretation of immunohistochemical slides.[14] Drawbacks of the ACIS III system is that the algorithm for the color threshold is fixed, so that changes in staining quality will influence scoring, and that this software only works on specific staining kits from the same company (like HercepTest).

Strengths and limitations

This study comprised a random sample of carotid plaques, and a comprehensive analysis of two prior techniques and a new method to indicate performance of these different techniques. The new method (method Q2) uses whole slide imaging and thus contains all the information that is available in the tissue, in contrast to only a selection of tissue (method Q1) that may not reflect the total plaque. Other advantages of method Q2 are that it can be used on regular slides, and is fast and efficient due to parallel processing, which makes it possible to handle large numbers of slides simultaneously. While we show high precision of method Q2, accuracy could not be assessed, as there is no gold standard to quantify plaque histology. Measuring accuracy is a challenge, because it remains unknown what the 'real' quantity of a certain marker is. We can only try to approach this 'true' quantity as good as possible by assessing reproducibility of repeated measurements, which is a measure of precision. Furthermore, precision is also dependent on which marker is used to visualize cells in tissue specimens, and measurements may accordingly be different. We tested only CD68 as a marker for macrophages, and α smooth muscle actin as a marker for smooth muscle cells. There are several reasons why

reproducibility remains an issue in an automated system such as Q2. First, we found that some slides contained undesired artefacts, like airbubbles, pen markings or positive antibody control tissue. When analyzing slides semiquantitatively or when manually selecting areas of interest (Q1), these artefacts are non-concerning. However, during whole slide analysis (Q2), these artefacts can result in false measurements. We created masks to exclude these parts of the whole slide images. Masks can be created automatically, but often these masks need to be altered by a human to remove unwanted artifacts (pen markings etc.) and this can theoretically influence reproducibility. In addition, different circumstances could occur while creating the digital image bij the scanner (dust, lack of focus, increased lighting, etc.), which could also result in different measurements. Q1 and Q2 were not applied for quantifying other plaque characteristics. This is because Q1 was only found to be feasible on DAB-stained sections, and the application of Q2 has started on stainings visualized by DAB, with pipelines for the other characteristics still in development. Other stainings and/or antibodies may also show different results, and our antibodies may not always be the most optimal. However, as discussed before, this study did not aim to study accuracy of the antibodies, but focuses on reproducibility and application of a new scoring method on currently used stainings. This means that we do not yet present a standardized technique, however in the future consensus may be reached on what the most optimal method for tissue quantification should be, and good precision of this quantification method is a good starting point. In addition, we cannot yet generalize the applicability of our new quantitative method to other laboratories, and tissue types. Therefore, with every new application, reproducibility of scoring methods used on specific tissue specimens should be tested first. Pipelines for other stains are currently under development. Finally, we only analyzed reproducibility of the culprit lesion and cannot make inferences regarding reproducibility of other plaque segments. However, previous research by our group has shown that spatial differences in the plaque are minimal.[2] In the future, histological tissue quantification has to remain subject to ongoing reproducibility assessment and necessary updating, as computer-aided technology is in continuous development, and variability in tissue examination may change over time. The use of open source software makes frequent releases and updates possible.[15] It is to be expected that digitizing slides will become

increasingly common, making automated quantification such as method Q2 cheaper and more accessible.

Conclusion

In conclusion, the (semi)quantitative methods currently studied to analyze plaque histology perform well, according to intra- and interobserver variability. The new automated method (Q2) presented here, using whole slide imaging and open source software, showed high precision and agreement in repeated measurements. This suggests that this technique can be used to reliably quantify tissue histology for research purposes.

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Supporting Information - The slideToolkit method

The slideToolkit is a collection of open source libraries and scripts to handle each step from virtual slides to the storage of your results. The toolkit is developed for modern (2014) personal computers (running *nix system[Linux, OS X, Unix]) and high-performance computing (HPC) systems. A common slideToolkit workflow consists of four consecutive steps. In the first step, “acquisition”, virtual slides are collected converted to .TIFF files. In the second step, “preparation”, files are organized for future steps. The third step, “tiles”, creates multiple manageable tiles to count. The fourth step, “analysis”, is the actual tissue analysis and saves the results in a meaningful dataset. These steps are schematically depicted in figure 1 of the main article.

Step 1 - Acquisition

Most slide scanners are, in addition to their own proprietary format, capable of storing the virtual slides in pyramid TIFF files. The slideToolkit uses the Bio-Formats library to convert other microscopy formats into the compatible pyramid TIFF format if needed. TIFF is a tag-based file format for raster images. A TIFF file can hold multiple images in a single file, this is known as a multi-layered TIFF. The term "Pyramid TIFF" is used to describe a multi-layered TIFF file that wraps a sequence of raster images that each represents the same image at increasing resolutions (figure 1). The different layers contain, among others, the slide label and multiple enlargements of the tissue on the slide.

To read whole slide images we use the opensource libTIFF libraries and the OpenSlide libraries. These libraries are also used to extract metadata (e.g. scan time, magnification and image compression) of the scanned slides. For image processing we use ImageMagick (ImageMagick 6.8.7-0 2013-10-16 Q16 http://www.imagemagick.org). ImageMagick is a command line image manipulation tool that is fast, highly adjustable and capable to handle big pyramid TIFF files. Descriptive information about the slide is stored as metadata and

contains, for example, pixels per micrometer, presence of different layers, and scan date.

Step 2 - Preparation

In the following steps we create multiple output files for each slide. For each virtual slide a staging directory is created in which the virtual slide and all output data concerning the slide will be stored. In digital image manipulation, a mask defines what part of the image will be analyzed and what part will be hidden. Usually a mask can be defined as black (hidden) or white (not hidden). We will use this technique to mask unwanted areas. The slideToolkit automatically creates a mask using a miniature version of the virtual slide (in our example this is layer 6 of the multi layered TIFF). Generated masks can be adjusted manually in an image editor of choice (such as the freely available GNU Image Manipulation Program; GIMP (http://www.gimp.org)), sometimes this is necessary to remove unwanted areas on the virtual slide (like marker stripes or air bubbles under the coverslip). Only non tissue parts of the virtual slides were masked.

Step 3 - Tiles

Working with whole 20x representations of the digitized slides is currently not possible due to hardware limitations. The goal of this step is to create multiple smaller images (i.e. tiles) from a whole slide. An upscaled version of the mask is placed over the 20x image of the slide (in our example this is layer 3 of the multi layered TIFF). Image manipulation on 20x sized virtual slides requires large amounts of computer RAM. To make it possible for computers without sufficient RAM to handle these files, the slideToolkit uses a memory-mapped disk file of the program memory. Using generated disk mapped memory files, the slideToolkit can efficiently extract all tiles. When no mask is used the slideToolkit uses a faster and more efficient way to create tiles without the use of a memory-mapped disk file.

Step 4 - Analysis

At this step, multiple tiles containing tissue data have been made, and the different objects in this tissue will be identified. CellProfiler is designed to quantitatively measure phenotypes from thousands of images automatically without training in computer vision or programming. CellProfiler can run using a graphical user interface (GUI) or a command line interface (CLI). Using the CellProfiler's GUI, different algorithms for image analysis are available as individual modules that can

be modified and placed in sequential order to form a pipeline. Such a pipeline can be used to identify and measure biological objects and features in images. Pipelines can be stored and reused in future projects. We created two pipelines for CD68 and SMA using the CellProfiler GUI and used CLI to run these. Both pipelines can be downloaded as .cp files, added as Supplementary data ('CD68pipeline.cp' and 'SMApipeline.cp'). An illustrated example on how to create pipelines in CellProfiler is described by Vokes and Carpenter.[1]

CellProfiler CD68 pipeline

This pipeline analyses the surface area and amount of CD68-DAB-positive cells compared to hematoxylin (HE) surface area. Each image was processed using the 'UnmixColors' module allowing the DAB stain and HE stain to be extracted into two channels. In the DAB channel, surface area was defined using the 'ApplyThreshold' module and included the 'Otsu Global' and the 'three classes' options. Weighted variance was minimized and pixels in the middle intensity class assigned to the foreground. The threshold correction factor was entered as '1.3' and the lower and upper bounds on the threshold were '0.1' and '1.0' respectively. In the HE channel, surface area was defined using the 'ApplyThreshold' module and included the 'Otsu Global' and the 'two classes' options. Entropy was minimized and threshold correction factor for pixels was entered as '0.8', and the lower and upper bounds on the threshold were '0.1' and '1.0', respectively. The 'MeasureImageAreaOccupied' module was used to measure surface areas of the defined CD68 and HE objects. DAB positive cells were identified using the 'IdentifyPrimaryObjects' module. The cells to be identified were defined within 8 and 40 pixels, objects outside this range were discarded. A three-class thresholding method was used with the method set to 'Otsu Global'. Entropy was minimized and the pixels in the middle intensity were set to the foreground. The threshold correction factor was set to 1 and the lower and upper bounds were set to '0.3' and '1' respectively. The method to distinguish clumped objects was set to 'Shape'.

CellProfiler SMA pipeline

This pipeline was used analyses the surface area and amount of SMA positive cells compared to HE surface area. Each image was processed using the 'UnmixColors' module allowing the DAB stain and hematoxylin stain to be extracted into two channels. DAB surface area and the HE surface area used similar 'IdentifyPrimaryObjects' module settings. Both included the 'Otsu Global' and the 'three classes' options. Entropy was minimized and pixels in the middle intensity class assigned to the foreground. The threshold correction factor was entered as '1.3' and the lower and upper bounds on the threshold were '0.1' and '1.0' respectively.

The method to distinquish clumped objects was set to 'none' and holes were filled in identified objects. The 'MeasureImageAreaOccupied' module was used to measure surface areas of identified SMA and HE objects.

We store CellProfiler measurements, like cell count, cell position, tissue surface area and other information in a database file (e.g. MySQL database or csv). Measurements can than be gathered and further analyzed using preferred statistical software, like R.

Supporting Information

References

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Files

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Chapter 5

Quantitative histological comparison of inflammatory cells in femoral, popliteal and aortic aneurysms

In preparation

Bastiaan G. L. Nelissen[1], Joost A. van Herwaarden[1], Gerard Pasterkamp[2], Saskia de Jager[2], Frans L. Moll[1]

1 *Department of Vascular Surgery, University Medical Center Utrecht, Utrecht, The Netherlands*

2 *Laboratory of Experimental Cardiology, University Medical Center Utrecht, Utrecht, The Netherlands*

Abstract

Objective: This study wants to assess the difference between the three groups of intravascular inflammatory cells (T-cells, B-cells, macrophages) in femoral, popliteal and aortic aneurysm biopsies. We hypothesized that despite the aneurysm site (abdominal aorta, femoral and popliteal), the type and amount of inflammation (characterized by T-cells, B-cells, and macrophages) within these aneurysms is similar.

Design of study and setting: The Aneurysm-Express is an ongoing longitudinal vascular biobank study were all patients undergoing an open surgical aneurysm repair in two Dutch hospitals are included. We included aneurysm biopsies from patients who underwent elective surgical aneurysm repair from April 2003 until April 2014. All fusiform AAA's were included, and all popliteal and femoral specimens were included that were not 'false aneurysms'. The slideToolkit was used for automated analysis and quantification of the digitized histological slides. Measurements were statistically compared.

Subjects, interventions and main outcome measure: From 362 different patients we collected aorta (n=312), femoral (n=18) and popliteal (n=27) aneurysm wall tissue samples. In total 252 slides were analyzed and quantified for T-lymphocytes (CD3), 333 slides were analyzed and quantified for B-lymphocytes (CD20) and there were 301 slides analyzed and quantified for macrophages (CD68). Four patients are included more than once. Before the initial analysis, all samples were manually checked for quality. Using the slideToolkit we found great diversity in tissue size per sample.

Results: We did find a difference in the type and amount of inflammation within the different aneurysms. Significantly more lymphocytes in the aorta samples compared to femoral and popliteal samples. No significant difference was found in the number of macrophages.

Conclusions: These findings could suggest a difference in pathology of disease between aorta, popliteal and femoral aneurysm formation.

Introduction

After the abdominal aorta, the popliteal and femoral arteries are the most common locations for aneurysm formation in the arterial system. This permanent arterial dilatation has several known causes including heritable connective tissue disorders and wall weakness after injury. However, in the vast majority of patients, the etiology is not understood.

In general, the incidence of peripheral aneurysms appears to be increasing. Prevalence varies considerably with the definition of an aneurysm at a given location, detection modality, and population studied. Abdominal aortic aneurysms (AAAs) are the most common (Singh et al.[1]) reported with an incidence of 8.9% in males, and 2.2% in females, followed by popliteal aneurysms (7.39% of men and 1% of women), and then by aneurysms of the femoral artery (6.58% of men and 0.26% of women).[2,3]

Patients with an aneurysm in one peripheral vascular territory have an increased risk for aneurysm development in another region. One can only hypothesize why some individuals are more susceptible to peripheral aneurysm formation than others.[4]

The clinical presentation of aortic and peripherally located aneurysms differs. Unlike abdominal aortic aneurysms (AAAs) which are prone to rupture, popliteal artery aneurysms (PAA) and femoral artery aneurysms (FAA) are limbs threatening because of potential to cause embolisms and thrombosis, but most aneurysm discoveries are mostly by coincidence.[5]

Degeneration of the medial layer of the arterial wall is how most aortic and peripheral aneurysms manifest. The pathological degenerative processes involved in aneurysm formation are complex and include up-regulation of proteolytic pathways, inflammation, and loss of arterial wall matrix. Risk factors for aortic and peripheral aneurysm formation include advanced age, cardiovascular disease, family history, hypertension, smoking and male sex. Although environmental and genetic factors may play a major role in artery wall inflammation and determine which individuals develop an aneurysm, regional anatomical factors are a likely determinant of local aneurysm formation.[3]

Inflammation, and, with that, the innate immune system, plays a critical role in vascular disease. In atherosclerosis, there is macrophage (or monocyte) infiltration in the intimal layer. In aneurysm formation, this is seen as an abundance of macrophages and lymphocytes in the adventitia and medial layer. Inflammatory

	Aorta (n=312)	Femoral (n=18)	Popliteal (n=37)	P-value
Male	267 (86%)	17 (94%)	35 (95%)	0.271
Age	69 ±8	68 ±10	66 ±10	0.096
Diameter	64 ±13	35 ±10	33 ±14	FA < .0000, PA < .0000, PF 0.4697
Diabetes	44 (14%)	0 (0%)	6 (16%)	0.215
Statin use	232 (74%)	15 (83%)	25 (68%)	0.482
Hypertension	272 (87%)	16 (89%)	31 (84%)	0.843
Smoker	259 (83%)	15 (83%)	27 (73%)	0.307

Table 1, baseline characteristics per sample per artery. *Data are presented as mean with standard deviation (SD), or as frequency (percentages). FA is femoral versus aorta, PA is popliteal versus aorta, and PF is popliteal versus femoral artery aneurysm. P-values <0.05 are considered significant.*

infiltrates of AAA are composed of B cells (CD20), but predominantly T cells (CD3) and macrophages (CD68).[6,7] Histological analysis of AAA tissue contain features of a chronic inflammatory disorder. A typical histological characteristic is a breakdown of the medial layer (the loss of elastin and loss of organized smooth muscle cells) and an increase in the extracellular matrix, causing vessel wall to weaken, expand, and eventually rupture.[8-10]

The cause of femoral aneurysms might be degenerations (true aneurysms) or might be related to an arterial injury caused by vascular reconstructions or, for example, injuries due to punctures (false aneurysms).[3]

This study wants to assess the difference between the three groups of intravascular inflammatory cells (T-cells, B-cells, macrophages) in femoral, popliteal and aortic aneurysm biopsies. We hypothesized that despite the aneurysm site (abdominal

	Aorta (n=312)	Femoral (n=18)	Popliteal (n=37)	P-value
CD3 (T-lymfocytes)	216	7	29	0.447
CD20 (B-lymfocytes)	292	10	31	0.422
CD68 (Macrophages)	255	15	31	0.992

Table 2, number of analyzed slides per stain per artery. *Data are presented as frequency. P-values <0.05 are considered significant.*

aorta, femoral and popliteal), the type and amount of inflammation (characterized by T-cells, B-cells, and macrophages), is similar.

Methods

Tissue collection

We used aneurysm biopsy samples from the Aneurysm-Express biobank. The Aneurysm-Express is an ongoing longitudinal vascular biobank study. All patients undergoing an open surgical aneurysm repair in two Dutch hospitals are asked to participate in the study.[11] During surgical repair, vascular aneurysm wall is collected following surgical approach. The Medical Ethics Committees of both hospitals approved this study, and participants provided written informed consent. For the current study, we included aneurysm biopsies from patients who underwent elective surgical aneurysm repair from April 2003 until April 2014. We included all fusiform AAA's, and all popliteal and femoral specimens that were not 'false aneurysms'. Indications for intervention are based on current guidelines and include: AAA diameter exceeding 50 - 55mm (for both sexes), femoral and popliteal aneurysms exceeding 30mm or 20mm respectively or symptomatic aneurysms.[12]

Baseline characteristics

Baseline characteristics and medication use were obtained using the combination of clinical records and questionnaires at the time of recruitment. These questionnaires included cardiovascular risk factors (age, gender, smoking, hypertension, diabetes). We defined hypertension as systolic blood pressure >140 mmHg or use of blood pressure–lowering drugs. We defined hypercholesterolemia by statin use and clinical records or questionnaires. The use of insulin or oral hypoglycemic agents defined diabetes. We defined a patient positive for smoking when he was a current smoker or had smoked for at least 10 years. Aneurysm diameter and morphology were assessed via computed tomography angiography and obtained from patients records.

Immunohistochemistry

During open repair, aneurysm wall biopsies were collected at the site of maximal diameter and dissected into 5mm segments. The specimen was fixed in 4%

formaldehyde, decalcified for 1 week in ethylenediaminetetraacetic acid and then embedded in paraffin. Of the paraffin segments, 4-µm-thick sections were cut for histological analyses. These slides were stained for T-lymphocytes (CD3), B-lymphocytes (CD 20) and macrophages (CD68). Isotype antibodies were used as negative control. We inspected all stained slides manually and excluded histological specimens that proved unsuitable for automated examination (mainly due to damage to the tissue or because of time induced fainting of the histological stain).

Histological slide preparation and analysis

The stained slides were scanned using a Roche VENTANA iScan HT slide scanner. We used the slideToolkit for quantification of the slides. The slideToolkit is a method used for whole slide analysis, its exact workings and its reproducibility is described elsewhere.[13,14] In short, digital whole slides images are spit into smaller, receptive images and optimized for computer analysis. As a part of the slideToolkit, histological slide analysis was done automatically with CellProfiler (version 2.1.0 Beta_2a Linux, Cellprofiler Cell Image Analysis Software, Broad Institute, Cambridge, Massachusetts, USA). CellProfiler makes use of pipelines. A pipeline is a sequential series of modules that each performs image processing functions such as unmixing colors, object identification and object area measurement. In this project, we used the pipelines to quantify the number positively stained cells per tissue area (as an arbitrary unit). We created different pipelines for T-cell, B-cell and macrophage stained slides. For each of the samples, we calculated the number of positively identified cells per surface area. Because we used this quantification method, statistical comparisons can be performed within arteries (aorta vs. femoral artery vs. popliteal artery), per stain, but not within stains (CD3 vs. CD20 vs. CD68).

Statistical analysis

Continuous variables were given median with interquartile range (IQR). Discrete variables were reported as number and percentages. We performed a natural logarithmic transformation to normalize the distributions of the number of positively identified cells per surface. We conducted a Fisher's Exact test, Students T-test, and Tukey's Honestly Significant Difference test when appropriate. Ordinal regression was used to assess independent associations for non-normally distributed data. P-values less than 0.05 were considered to be statistically significant, and we corrected for multiple testing when needed. We used R version

	Aorta	Femoral	Poplitea	P-value
CD3 (T-lymfocytes)	-8.179 ±0.7548	-8.413 ±0.7224	-8.699 ±0.3622	PA 0.00097
CD20 (B-lymfocytes)	-6.079 ±1.7407	-7.491 ±0.9742	-7.209 ±1.3361	FA 0.0227, PA 0.0017
CD68 (Macrophages)	-5.29 ±1.3287	-5.368 ±1.3866	-4.86 ±1.2123	NS

Table 3, means per stain per artery. *Number of positively identified cells per surface area, per stain per artery. Data are presented as mean with standard deviation (SD). FA is femoral versus aorta, PA is popliteal versus aorta, and PF is popliteal versus femoral artery aneurysm. NS is not significant. P-values <0.05 are considered significant. The means for CD3 and CD20 measurements in aorta, femoral and politeal aneurysm tissue both showed a significant difference between aorta and popliteal samples.*

3.2 for the statistical analysis, provided with the 'ggplot' and 'plyr' package R-project.org.

Results

Baseline

Baseline characteristics can be found in table 1. The majority of the patients was male, in his late sixties, smoked, and used statins and antihypertensive drugs. In total 367 tissue samples were analyzed that were collected from 362 different patients. We collected 312 aorta (of which 54 were symptomatic), 18 femoral (of which 1 was symptomatic) and 37 popliteal (of which 11 were symptomatic) aneurysms biopsies.

Four patients are included more than once. One patient was operated on his left and his right femoral artery. One patient was operated on his left and his right popliteal artery. Another male patient was operated on his right femoral artery and on his aorta. The fourth patient was operated three times, once on his lift femoral artery, once on his right femoral artery and once on his aorta.

Illustrative samples for aorta, femoral and popliteal tissue for T-lymphocytes (CD3), B-lymphocytes (CD 20) and macrophages (CD68), are depicted in figure 1. We used the slideToolkit to quantitatively analyze these three groups of inflammatory cells in the different aneurysm groups. The number of slides analyzed per stain per aneurysm type can be found in table 2. An overview of the measurement results can be found in figure 2. For each of the samples we calculated the number of positively identified cells per surface area. All measurements were transformed using the

natural logarithm (ln). In total, 252 slides were analyzed and quantified for T-lymphocytes, there were 333 slides analyzed and quantified for B-lymphocytes and there were 301 slides analyzed and quantified for macrophages. The number of positively identified cells per surface area, per stain per artery can be found in table 3.

The means for T-lymphocytes measurements show a significant difference between aorta and popliteal samples, likely due to the low number of samples this difference was not significant for femoral tissue. The means for B-lymphocytes measurements show a significant difference between aorta and popliteal, and aorta and femoral tissue. These differences could not be explained by any of the baseline characteristics, such as smoking. The measurements for macrophages showed no significant between aorta, femoral and popliteal slides.

Before the initial analysis, all samples were manually checked for tissue quality and fainting of the stain. There was a great diversity in tissue size per sample. No correlation is found between the area of the analyzed tissue and the number of infiltrative cells.

Discussion

We assessed the difference between T-lymphocytes (CD3), B-lymphocytes (CD20) and macrophages (CD68) in aortic, femoral and popliteal aneurysm biopsies. We hypothesized that the type of inflammatory cells and amount of inflammation, is similar despite the aneurysm site. Our data suggests that the role of inflammation in the mechanisms for aneurysm formation between aorta, femoral and popliteal arteries might differ. T-lymphocytes, B-lymphocytes and macrophages are prominently present and confirm the role of inflammation in aneurysm formation. We found significantly more T-lymfocytes in the aorta samples compared to femoral and popliteal samples, we found more B-lymfocytes in aorta samples compared to popliteal samples and no significant difference was found in the number of macrophages.

In aortic aneurysm tissue we observe cells that are recognized for both the faster, innate immune response (represented by macrophages) but also the cells responsible for a slower, adaptive immune response (represented by lymphocytes).

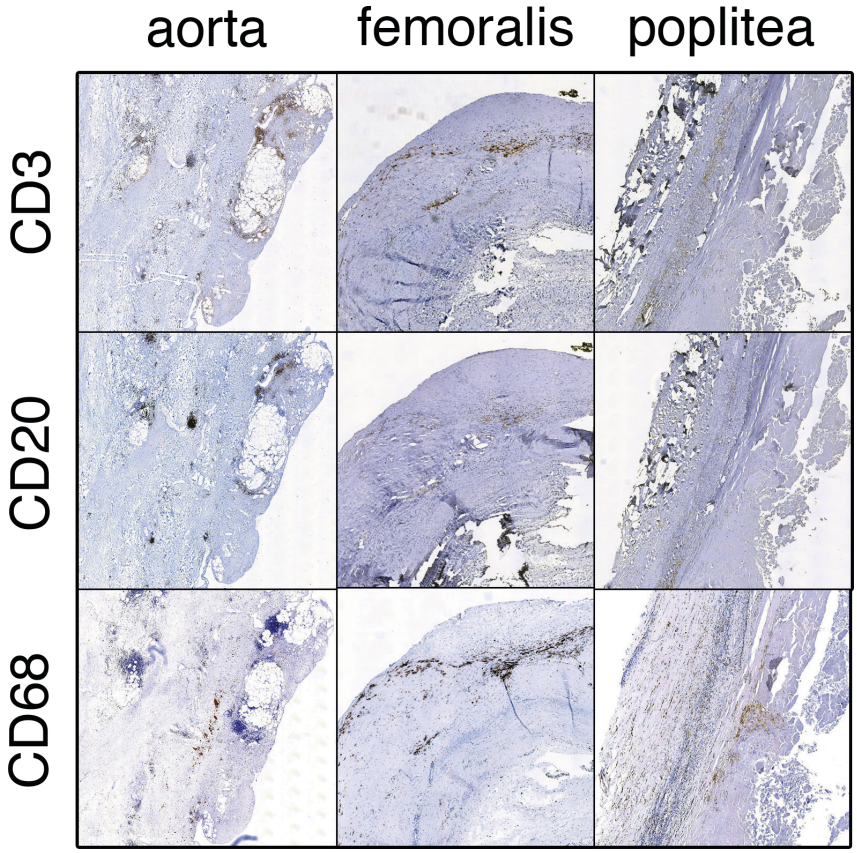

Figure 1. *An illustrative overview of the different arteries and the different types of stains. After resection the arterial wall curls outward, in all images the adventitial layer is on the right side.*

In the peripheral aneurysms (femoral and popliteal) we see a dominance of the slower, adaptive immune response.

The method of quantification prevented us to look the location of the cellular infiltrate and prevented us to compare the amount of infiltrate between the different kinds of inflammatory cells. In aorta it is known that the bulk of inflammatory cells are present in the adventitia and media layer of the vascular wall and that T-lymphocytes composite the most (70%-80%) of the inflammatory infiltrate, compared to peripheral aneurysms were the infiltrate is mainly located in the intima.[6,7]

A typical histological characteristic of aneurysm formation due to inflammation is the breakdown of the media layer (characterized by the loss of elastin and loss of

organized smooth muscle cells) and an increase in the extracellular matrix, causing vessel wall to weaken and dilate.[6,15]

Pathophysiology

Macrophages are part of the less specific innate immune response and an important component of the inflammatory infiltrate in arterial aneurysm wall. Macrophages are a source of proteases and pro-inflammatory mediators and can promote or sustain T-cell proliferation. This role of macrophages in aneurysm formation is known in AAA, but is less described in peripheral aneurysms. Our data suggest that the T-cell proliferation is less prominent in popliteal aneurysms, and probably also in femoral aneurysms. T-cells and B-cells play an important role in the slower, adaptive immune response, which is antigen-specific and based on the recognition of specific "non-self" antigens. This might suggest that the pathophysiology differs between AAA and peripheral (femoral and popliteal) aneurysms.[8,10,16]

In this study inflammatory properties of the femoral artery aneurysms tempt to approach these popliteal aneurysms. These results show a trend were lymphocytes, and not macrophages, are dominant in peripheral aneurysms (femoral and popliteal). AAA shows a dominance of both, lymphocytes and macrophages.

Riskfactors and inflammatoiry infiltrates

Smoking, hypertension and male sex are known risk factors for the development of aorta and peripheral aneurysms. We see no difference between risk factors and artery type. Our baseline characteristics, including the subdivision of aneurysms per artery, and the number of patients with multiple aneurysms are conform literature.[2,3,17]

The number of smokers in vascular patients is always worth mentioning, as smoking remains the most important and avoidable risk factor. Smoking is strongly linked to abdominal aortic aneurysm formation but is likely to be the cause for peripheral aneurysms as well. Smoking is known lead to activation of the less specific innate immune system that causes macrophages to occupy the vascular wall.[4]

Four patients are operated on more than one aneurysm. All are men. Other studies show a recognized association between the prevalence of femoral, popliteal and aortic aneurysms. For example, a patient with an aneurysm found in his popliteal artery is likely to have an aneurysm on the contralateral side or in the abdominal aorta as well. One can only hypothesize why some arteries are more susceptible to

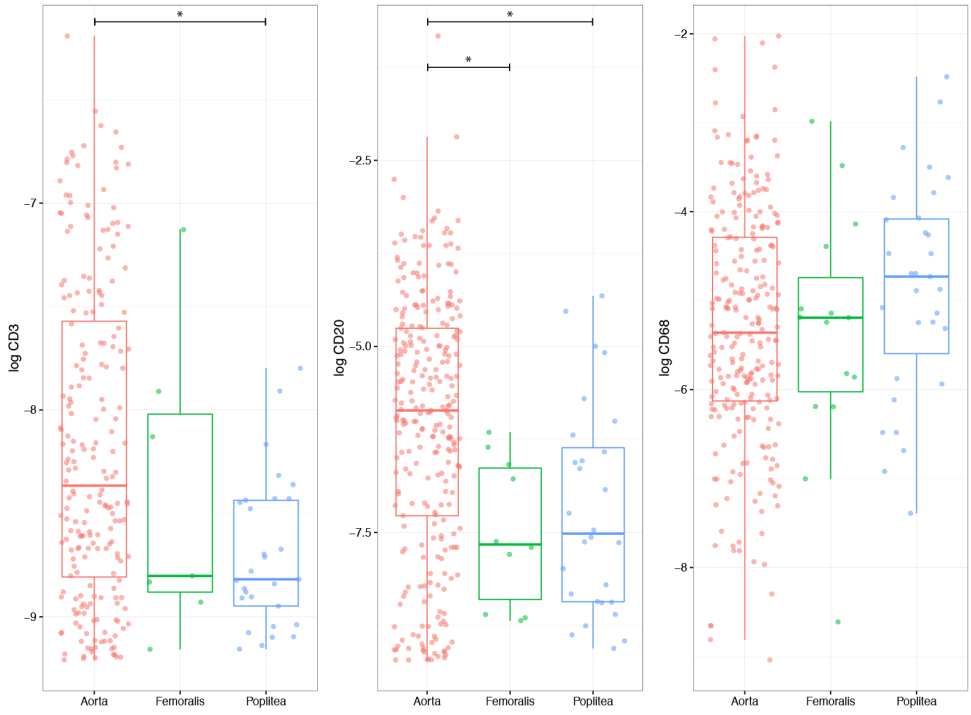

Figure 2. *An overview of the measurements results for CD3 (T-lymphocytes), CD20 (B-lymfocytes) and CD68 (macrophages) for the different aneurysm locations (aorta, femoral and poplitea). Significant different groups are marked with an asterisk (*). Note that axis per stain are not similar and log transformed.*

aneurysm formation than others. It is hypothesized that estrogens might protect premenopausal women for degenerative vascular disease, like aneurysm formation, but this has been refuted in multiple large studies.[4,5,18]

Computer analysis of the tissue

We used an automated and high precision method to reliably quantify aneurysm histology. The accuracy of this method depends on the pipeline used and which marker is used to visualize cells in tissue specimens. Measuring accuracy is a challenge, because it remains unknown what the 'real' quantity of a certain marker is. This method does not gives us any in depth information about the inflammation

like a pathologist does, nevertheless, with a valid pipeline and with its high reproducibility it can trustworthy be used for research purposes.

Limitations

Several limitations in our study need to be addressed. The sample size for femoral artery aneurysms is small; this is mainly due to the lower incidence of aneurysm formation in this artery. Besides, no non-aneurysmatic control tissue was used as a baseline comparison. We have only quantified the amount of inflammatory infiltrate for specific cell types; we have not analyzed the composition of the arterial layers (to detect media generation, for example) or registered the length of time that an aneurysm was present in the body. Therefor we cannot detect a stage of the disease with its corresponding inflammatory cell types.

Conclusion

We found significantly more lymphocytes in the aorta samples compared to femoral and popliteal samples. No significant difference was found in the number of macrophages. This could suggest a different pathology of disease between aorta, popliteal and femoral aneurysm formation.

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Chapter 6

Histological Analysis of Extracranial Carotid Artery Aneurysms

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Bastiaan G. L. Nelissen[☯1,2], *Janna C. Welleweerd*[☯1], *Dave Koole*[1,2], *Jean-Paul P. M. de Vries*[3], *Frans L. Moll*[1], *Gerard Pasterkamp*[2], *Aryan Vink*[4], *Gert Jan de Borst*[1]

1 *Department of Vascular Surgery, University Medical Center Utrecht, Utrecht, The Netherlands*

2 *Laboratory of Experimental Cardiology, University Medical Center Utrecht, Utrecht, The Netherlands*

3 *Vascular Surgery, St Antonius Hospital, Nieuwegein, the Netherlands*

4 *Department of Pathology, University Medical Center Utrecht, Utrecht, the Netherlands*

☯ *These authors contributed equally to this work.*

Abstract

Introduction: Extracranial carotid artery aneurysms (ECAA) are rare but may be accompanied with significant morbidity. Previous studies mostly focused on diagnostic imaging and treatment. In contrast, the pathophysiological mechanisms and natural course of ECAA are largely unknown. Understanding the pathophysiological background may add to prediction of risk for adverse outcome and need for surgical exclusion. The aim of this study was to investigate the histopathological characteristics of ECAA in patients who underwent complete surgical ECAA resection.

Material and Methods: From March 2004 till June 2013, 13 patients were treated with open ECAA repair. During surgery the aneurysm sac was resected and processed for standardized histological analysis. Sections were stained with routine hematoxylin and eosin and special stains to detect elastin, collagen, different types of inflammatory cells, vascular smooth muscle cells and endothelial cells.

Results: Histopathological characterization revealed two distinct categories: dissection (abrupt interruption of the media; n = 3) and degeneration (general loss of elastin fibers in the media; n = 10). In the degenerative samples the elastin fibers in the media were fragmented and were partly absent. Inflammatory cells were observed in the vessel wall of the aneurysms.

Conclusions: Histological analysis in this small sample size revealed dissection and degeneration as the two distinct underlying mechanisms in ECAA formation.

Introduction

Extracranial carotid artery aneurysms (ECAA) are rare with an incidence varying from 0.09 to 2.0% of all carotid surgical procedures.[1,2] Aneurysms of the extracranial carotid artery are de- fined as a dilatation of 50% or more of the diameter of the expected healthy carotid artery.[3] Studies on ECAA mostly comprise case reports or small case series focusing on diagnostic imaging and treatment outcome.[2,4–6] Although the natural course of ECAA is largely unknown, the clinical presentation of ECAA may be accompanied with significant morbidity. Previous studies reported a stroke prevalence of 50% and a mortality of 60–70% when ECAA is left untreated.[7]

Some authors suggest that small asymptomatic ECAA could be treated conservatively with strict follow-up, but surgery is generally the accepted treatment for symptomatic ECAA.[7–10] The etiology of ECAA is heterogeneous and includes atherosclerosis, post-dissection, trauma and infection.[2,11] The exact pathophysiological mechanisms however remain unclear, and prognostic factors for clinical outcome are largely unknown.[12] Detailed understanding of the mechanisms of ECAA and general aneurysm formation could be the start of improving diagnostics and treatment.

Accordingly, the present study was conducted to investigate the histopathological character- istics of ECAA in patients who underwent complete surgical ECAA resection.

Methods

Subjects

From March 2004 till June 2013, 38 patients were treated for ECAA in the two participating hospitals (University Medical Center Utrecht, Utrecht, and St Antonius hospital Nieuwegein, the Netherlands). In this study, all patients (n = 15) that underwent open ECAA repair with complete aneurysm sac resection (Fig. 1) were included. Histological analysis was not possible in two patients due to incomplete preserved samples. Therefore, these two cases were excluded, leaving 13 cases for analysis. Operation indication was decided on after multidisciplinary deliberation and based on presenting symptoms, location, and ECAA size. The medical ethics committees of both participating hospitals (Verenigde Commissies

Mensgebonden Onderzoek, St Antonius hospital Nieuwegein and Medisch Ethische Toetsings commissie University Medical Center Utrecht) approved the study and all study participants provided written informed consent.

Imaging

Morphological characteristics of the aneurysms were assessed by preoperative imaging diagnostics. Computed tomography angiogram (CTA) was used in ten patients, magnetic resonance angiography (MRA) was used in two, and conventional angiography in one patient.

Result interpretation

Elastin was graded as an estimation of the percentage of media containing elastin fibers. Collagen was graded as an estimation of the percentage present in the vessel wall. The presence of inflammatory cells in aneurysm wall was semi-quantitatively scored as minor or heavy staining. Dissection was defined as an abrupt interruption of the media with signs of organized thrombus in the tear of the vessel wall. Degeneration was defined as decrease of elastin in the media. Histological examination was retrospectively performed collectively by three independent observers (BN, DK and AV) unaware of clinical data. In case of discrepancies in judgment, sections were reanalyzed until consensus was reached.

Controls

Post-mortem non-aneurysmal carotid specimens from five patients with a median age of 63 (range 51–90) without relevant medical history were used as controls. Two bodies were donated for education and research to University Medical Center Utrecht. Written and witnessed consent for body donation was given prior to death by both controls. Three more specimens were collected in patients for whom the carotid artery was investigated for diagnostic reasons in an autopsy procedure. The use of these specimens is described in the code of proper use of human tissue that is used in the Netherlands.[13]

Statistical analysis

Discrete variables are shown as frequencies and percentages of the total. Continuous variables are shown as median and interquartile range. Categorical variables were investigated using the chi-square test or the Fisher's exact test.

Continuous variables were compared using the Student's t-test. P value ≤.05 was considered statistically significant.

Results

Clinical patients characteristics

Thirteen patients with a median age of 55 years (IQR: 35–75, six males) were included. Baseline characteristics are presented in (Table 1. All treated patients were symptomatic and the most common symptom was cerebral ischemia (N = 6) (see Table 2). All but one of these patients had ischemia due to (temporary) occlusion of the ipsilateral medial cerebral artery. One patient presented with ischemia of the contralateral medial cerebral artery and a contralateral Horner syndrome.

Imaging findings

ECAAs were mostly located in the Internal carotid artery (ICA) (n = 12) and one ECAA was located in the common carotid artery (CCA). Eight aneurysms were

	Total (n = 13)
Gender male	6 (46%)
Age, years [a]	55 (35–75)
PAD	1 (8%)
Hypertension	7 (54%)
MI	1 (8%)
COPD	1 (8%)
Hypercholesterolemia	2 (15%)
Connective tissue disorder	0 (0%)
Smoking	4 (33%)
DM	0 (0%)
Statin use	6 (46%)

Table 1, patient characteristics. *Data are presented as No. (%) unless otherwise indicated. a Median and interquartile range (IQR). Abbreviations. PAD, peripheral artery disease; MI, myocardial infarction; COPD, chronic obstructive pulmonary disease; DM, diabetes mellitus.*

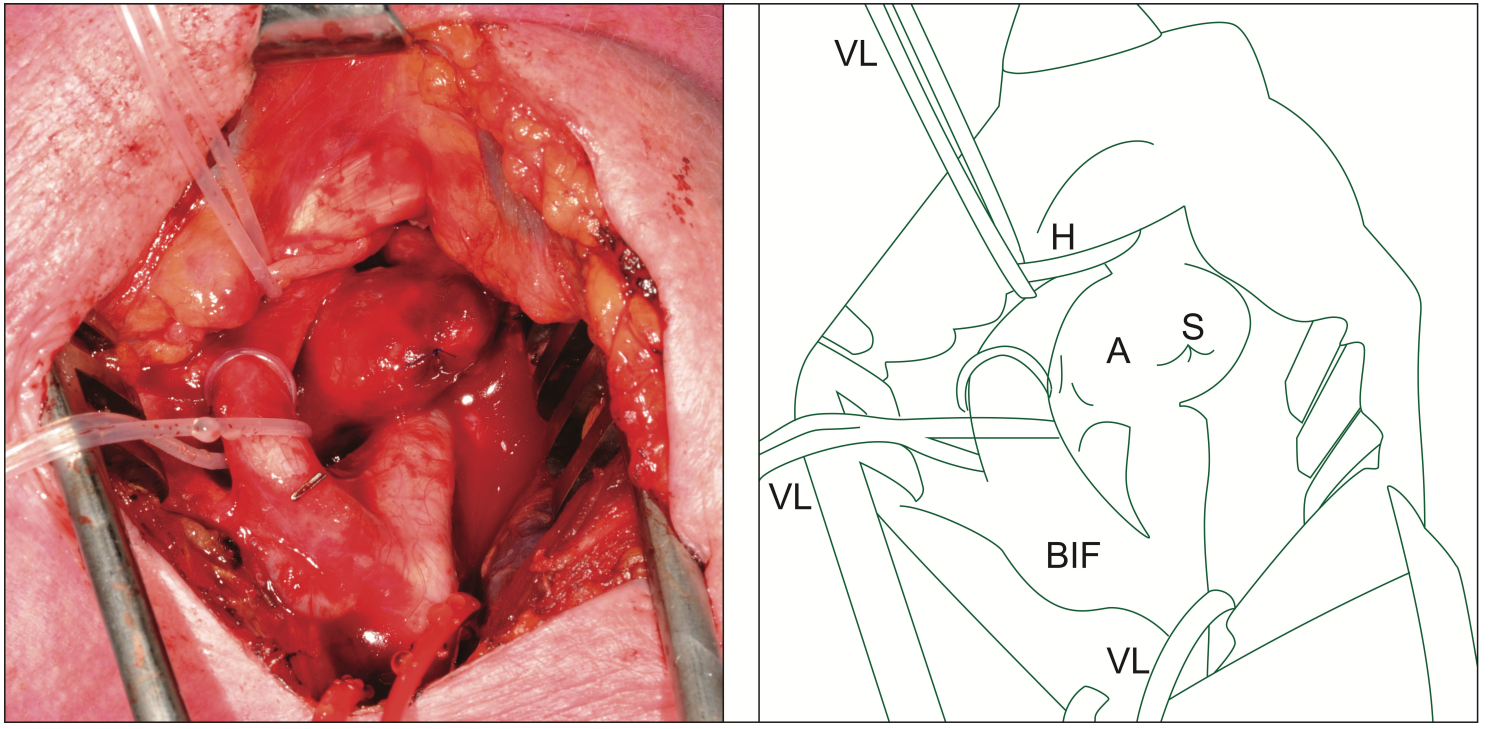

Figure 1, in vivo aneurysm. *Aneurysm of a left saccular carotid artery visible between the internal carotid artery (ICA) and the external carotid artery (ECA) and originating from a dorsal loop in the ICA. The common carotid artery is ligatured in red, the ECA is identified with transparent ligatures. A, aneurysm of the ICA; BIF, carotid bifurcation; H, nervus hypoglossus; S, suture; VL, vessel loop.*

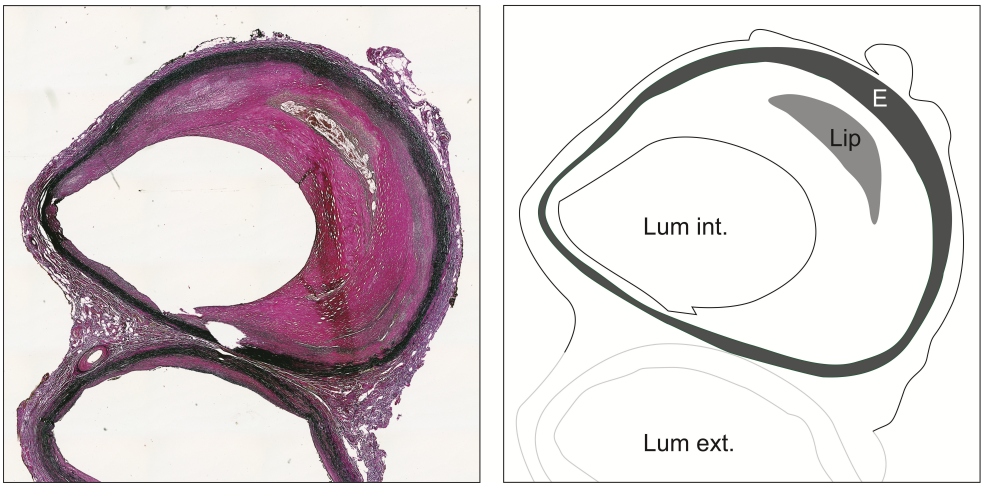

Figure 2, histology of control sample: fibrous cap atheroma. *Histology of control sample. Sample taken just distal from the bifurcation. Elastin-van Giesson (EvG) stain. In black the elastic fibers are clearly present and well organized. Atherosclerotic changes, atheroma with a lipid core. E, Elastin; Lip, Lipid core; Lum ext., lumen of the external carotid artery; Lum int., lumen of the internal carotid artery.*

Gender/age	Location	Size (mm)	Morphology	Symptoms	Pathology
M/76	LICA	30	Fusiform	Stroke	Degenerative
F/65	RICA	NR	Fusiform	Pain, mass	Degenerative
F/41	LICA	4	Saccular	TIA/Stroke	Dissection
M/53	RICA	34	Saccular	Pulsatile mass	Degenerative
M/46	RCCA	13	Fusiform	Pain, mass	Degenerative
M/55	LICA	NR	Fusiform	TIA	Dissection
M/50	LICA	12	Fusiform	TIA	Degenerative
M/26	LICA	46	Fusiform	Pain, mass	Degenerative
M/47	RICA	5	Saccular	TIA	Dissection
F/66	RICA	15	Saccular	Pain	Degenerative
F/75	LICA	27	Fusiform	CL TIA, Horner	Degenerative
F/62	LICA	12	Saccular	Hoarseness	Degenerative
F/67	LICA	27	Saccular	Mass	Degenerative

Table 2, aneurysm characteristics. *Size is in millimeters. Abbreviations. M male; F female; LICA left internal carotid artery; RICA right internal carotid artery; RCCA right common carotid artery; NR not reported; TIA transient ischemic attack; CL contralateral.*

located on the left side. Seven aneurysms were fusiform and six were saccular. The aneurysms varied in size; the median length was 30mm (range: 10–100) and the mean outer diameter 13mm (range: 4–46). There were no radiological signs of dissection pre-operative in any of the aneurysms. Thrombus was present in two aneurysms and six aneurysms had calcifications in the vascular wall. Presence of thrombus or calcifications was not significantly different in degenerative aneurysms or aneurysms after dissection ($p = .164$ and $p = .577$).

Controls

Our five control ICA samples showed no media degeneration or dissection. In all samples the elastin, smooth muscle cells, and collagen fibers were present and well organized (see Table 3). Inflammatory cells were absent in all but one control specimens. Two samples did show atherosclerotic lesions (sample 3 and 5) that could be classified as a fibrous cap atheroma and pathological intimal thickening (Modified American Heart Association Classification) (Fig. 2).[14] The atheroma was surrounded by CD68 positive macrophages.

Samples

Histological examination was retrospectively performed, four sections were reanalyzed because of discrepancies in judgment between the independent observers, in all sections consensus was reached. Histological examination revealed two distinct categories of ECAA (Table 3). The majority (n = 10; 77%) of the samples showed a distinct degenerative pattern of the vessel wall without signs of a dissection (Fig. 3). In the degenerative samples the elastin fibers of the media were fragmented and were partly absent (Fig. 3E-3F). Inflammatory cells were present in each sample. In most degenerative samples inflammatory cells were clearly present, both lymphocytes and macrophages were seen; only three samples scored ‘minor’ for all observed types of inflammatory cells.

The remaining three samples (23%) showed a dissection with an abrupt interruption of the medial layer (Fig. 3A-3D). The gap in the arterial wall was filled with an organized thrombus with groups of myofibroblasts. All dissective samples showed marks of degeneration in the media and scored ‘minor’ for the different inflammatory cells in the vessel wall. There were no significant differences in any type of inflammatory cell between samples with dissection or samples with degeneration (Table 3). We did find significant differences in SMC and collagen.

		Control (n = 5)	Dissection (n = 3)	Degeneration (n = 10)	P value
Elastin [a]		95% (30–100%)	25% (2–50%)	30% (0–95%)	.063
Smooth muscle [a]		88% (15–100%)	95% (60–100%)	65% (5–95%)	.004
Vasa vasorum	minor	4 (80%)	1 (33%)	3 (30%)	1.000
	heavy	1 (20%)	2 (67%)	7 (70%)	
Collagen	minor	3 (60%)	2 (100%)	1 (10%)	.014
	heavy	2 (40%)	0 (0%)	9 (90%)	
Lymphocytes	minor	5 (100%)	3 (100%)	6 (60%)	.497
	heavy	0 (0%)	0 (0%)	4 (40%)	
Plasma cells	minor	5 (100%)	3 (100%)	10 (100%)	NA
	heavy	0 (0%)	0 (0%)	0 (0%)	
B-Lymphocyte	minor	5 (100%)	3 (100%)	9 (90%)	1.000
	heavy	0 (0%)	0 (0%)	1 (10%)	
T-lymphocytes	minor	5 (100%)	3 (100%)	6 (60%)	.497
	heavy	0 (0%)	0 (0%)	4 (40%)	
Macrophages	minor	4 (80%)	3 (100%)	4 (40%)	.192
	heavy	1 (20%)	0 (0%)	6 (60%)	

Table 3, histological characteristics. *Continuous data are presented as median (range). Categoric data are presented as number (%) of heavy staining as opposed to minor staining unless otherwise indicated. a Percentage of fibers present in a non-diseased vessel wall.*

SMC was higher in the dissection group and collagen was higher in the degenerative samples.

Discussion

In the current study, histological examination of ECAA showed two distinctive categories: aneurysms after dissection versus degenerative aneurysms. Retrospectively, there were no radiological signs of dissection pre-operative in any of the aneurysms.

Clinically and radiological diagnosed aneurysmal formation after previous dissection has been described in literature.[15] In the aorta and its branches, degeneration is a well-recognized cause for aneurysm formation.[16,17] Non-dissective causes of peripheral aneurysms, such as carotid or popliteal aneurysms, are believed to exist but had not been histologically confirmed yet.[18] We found degenerative aneurysms in 10/13 (73%) patients. The fact that we observed inflammatory cells in the aneurysms suggest that inflammation might also play a role.

It must be pointed out that the possibility exists that these two distinctive categories could be different stages of the same disease. Although decrease of SMC was higher in the degenerative samples, degeneration of the medial layer was also observed in all dissection cases and could eventually weaken the vascular wall and facilitate dissection of the intimal and medial layer. Increase of collagen in aneurysms has been demonstrated in analysis of other aneurysmal vessels.[19] Theoretically, while collagen is load-bearing at large dimensions and elastin being load-bearing at small dimensions the collagen-elastin ratio changes in aneurysms.[20]

The result of this study could be the start of understanding the mechanism or mechanisms of ECAA development. This could eventually lead to improved treatment strategies. For example, in most cases of dissecting aneurysms invasive treatment is not indicated, as a major part of these aneurysms remains asymptomatic and does not increase in size while some even spontaneously resolve.[21] On the contrary, atherosclerotic origin of ECAA may need more aggressive intervention, although there is a clear need for further natural follow-up in patients with ECAA in general.[12]

There are some study limitations. Most important, it must be noticed that this study was performed on a small group of patients, consequently our findings have little power and definitive conclusions are difficult to draw. This might also explain why

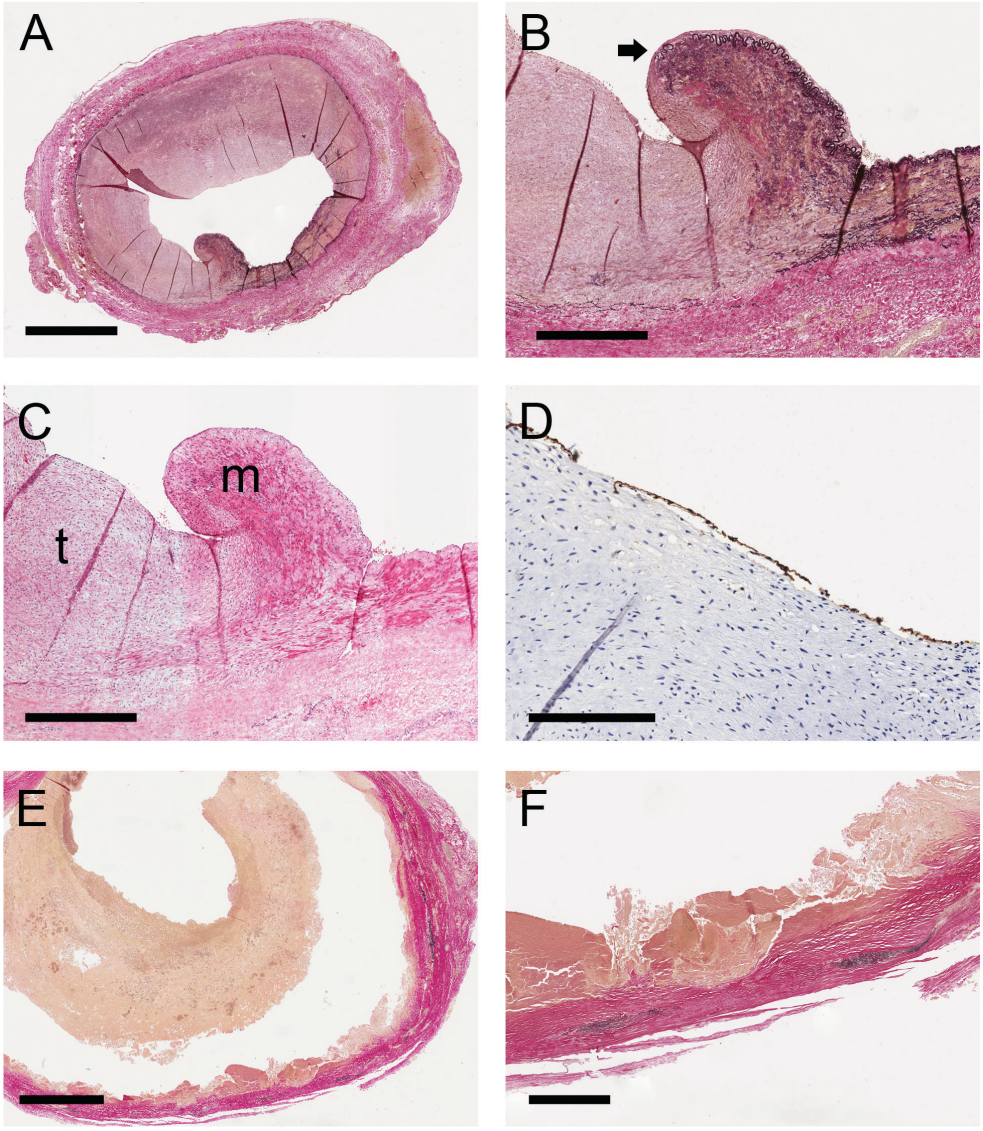

Figure 3, histology of carotid aneurysms. *A-D, dissection; E and F, degeneration. A, overview of aneurysm due to dissection. Elastin-van Giesson (EvG) stain. Bar = 1.5 mm. B, higher magnification of the same staining as A. Arrow indicates the disrupted internal elastic lamina. Bar = 500 µm. C, Hematoxylin and eosin staining of the same panel as B. m, media; t, organized thrombus that replaces the absent media. Bar = 500 µm. D, CD34 immunostain showing endothelial coverage of the thrombus (in brown). Bar = 250 µm. E, overview of an aneurysm due to degeneration. Elastin-van Giesson (EvG) stain. Bar = 4 mm. F, higher magnification of the same staining as E. In black remnants of the elastic fibers of the media. Bar = 1 mm.*

we did not find any aneurysms with another etiology, for example mycotic aneurysms, in our analysis. However, ECAA is a rare disease and only around 1000 cases have been reported in the international literature so far.[12] Furthermore, only a selected subgroup of patients presenting with ECAA will undergo surgical resection of the aneurysm. As a consequence, also in our tertiary referral center, the number of patients operated on is limited and therefore it is hard to study larger patient groups. Second, in this study patients were selected based on intervention. The result of this study and the conclusions drawn from them only apply to patients that underwent surgical repair for their ECAA.

Conclusion

In conclusion, this is the first histological study of samples taken from ECAA. Histopathological ECAA characterization in this study revealed two distinct categories: dissection and degeneration. The result of this study could be used as a basis for understanding the mechanism of ECAA development. Further study in resected ECAA is needed to explore the clinical relevance of this mechanism.

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Chapter 7

Management of Extracranial Carotid Artery Aneurysm

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J.C. Welleweerd [a], *H.M. den Ruijter* [b], *B.G.L. Nelissen* [a], *M.L. Bots* [b], *L.J. Kappelle* [c], *G.J.E. Rinkel* [c], *F.L. Moll* [a], *G.J. de Borst* [a]

a *Department of Vascular Surgery, University Medical Center Utrecht, Utrecht, The Netherlands*

b *Julius Center for Health Sciences and Primary Care, University Medical Center Utrecht, Utrecht, The Netherlands*

c *Department of Neurology and Neurosurgery, University Medical Center Utrecht, Utrecht, The Netherlands*

Abstract

Introduction: Aneurysms of the extracranial carotid artery (ECAA) are rare. Several treatments have been developed over the last 20 years, yet the preferred method to treat ECAA remains unknown. This paper is a review of all available literature on the risk of complications and long-term outcome after conservative or invasive treatment of patients with ECAA.

Methods: Reports on ECAA treatment until July 2014 were searched in PubMed and Embase using the key words aneurysm, carotid, extracranial, and therapy.

Results: A total of 281 articles were identified. Selected articles were case reports (n = 179) or case series (n = 102). Papers with fewer than 10 patients were excluded, resulting in the final selection of 39 articles covering a total of 1239 patients. Treatment consisted of either conservative treatment in 11% of the cases or invasive treatment in 89% of the cases. Invasive treatment comprised surgery in 94%, endovascular approach in 5%, and a hybrid approach in 1% of the patients. The most common complication described after invasive therapy was cranial nerve damage, which occurred in 11.8% of patients after surgery. The 30 day mortality rate and stroke rate in conservatively treated patients was 4.67% and 6.67%, after surgery 1.91% and 5.16%. Information on confounders in the present study was incomplete. Therefore, adjustments to correct for confounding by indication could not be done.

Conclusions: This review summarizes the largest available series in the literature on ECAA management. The number of ECAAs reported in current literature is scarce. The early and long-term outcome of invasive treatment in ECAA is favorable; however, cranial nerve damage after surgery occurs frequently. Unfortunately, due to limitations in reporting of results and confounding by indication in the available literature, it was not possible to determine the optimal treatment strategy. There is a need for a multicenter international registry to reveal the optimal treatment for ECAA.

Introduction

Invasive treatment for extracranial carotid artery aneurysms (ECAAs) pertains to only 0.6-3.8% of all extracranial carotid interventions,[1-13] 0.6-2% of all carotid endarterectomies,[3,5,7,10] and 0.4-2% of all extracranial arterial aneurysm repairs.[14-16] A substantial portion of the ECAAs will probably remain clinically silent. However, ECAAs may lead to neurologic symptoms including transient ischemic attacks (TIAs) or ischemic stroke.[17-19] Other symptoms include pulsating mass and related cranial nerve dysfunction (CND). Information regarding the natural history, indications, and the best treatment in patients with ECAAs is scarce and guidelines are lacking. Both medical, surgical, and endovascular treatment of the aneurysm have been recommended.[14,15,18,20] The best medical treatment comprises antithrombotic treatment and regular follow up and may have a place in the treatment of asymptomatic patients. Traditional surgical treatment, which is the current treatment of choice of symptomatic or growing ECAAs, consists of open resection of the entire aneurysm with or without arterial replacement with an interposition graft.[14,20-22] However, this approach has been associated with the risk of stroke and cranial nerve damage.23 Endovascular ECAA repair has only been described in small case series.[23]

For a proper assessment about which treatment should be preferred, a better insight into natural history and risk of complications of the different treatments is needed. This paper is a review of all available literature on the risk of complications and long-term outcome after conservative or invasive treatment of patients with ECAA.

Methods

Search strategy

In July 2014 a search was performed of all literature since 1900 in Medline (with Pubmed as interface) and Embase combining the following search terms: aneurysm, carotid, extracranial, therapy (and all synonyms for all treatment options). The search was performed according to the search strategy and data collection

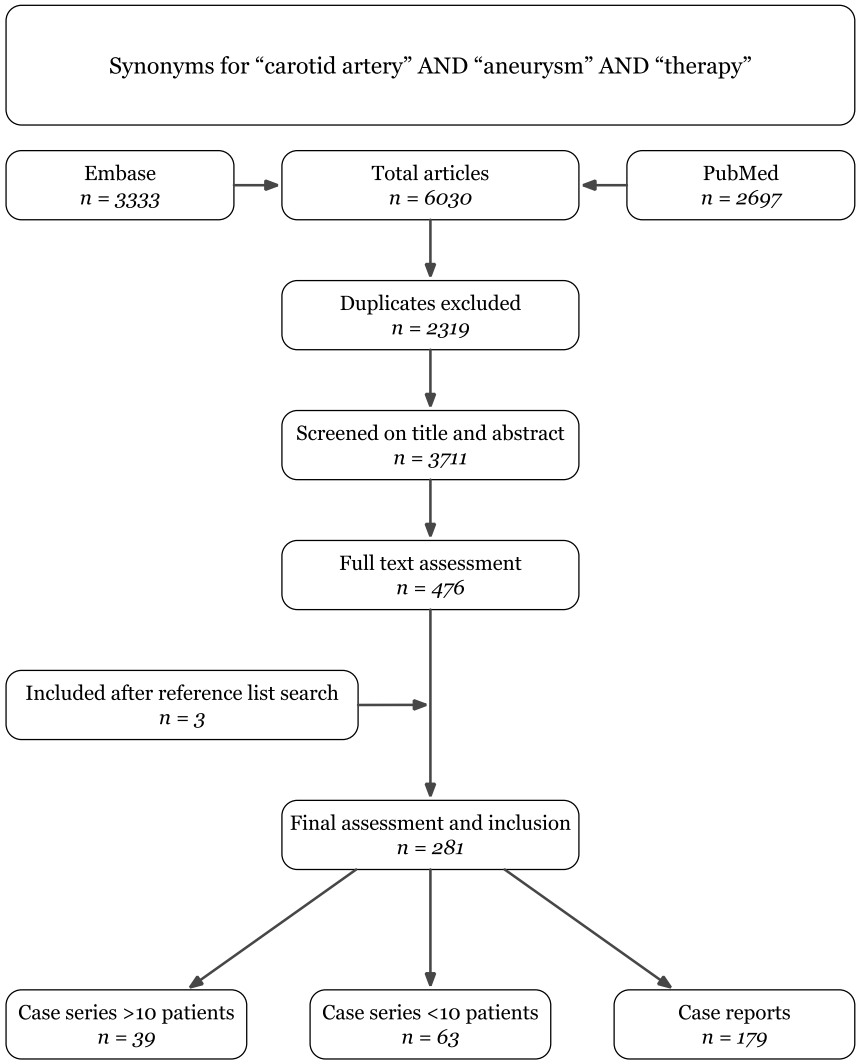

Figure 1, literature search flow chart.

guidelines of the Meta-analysis of Observational Studies in Epidemiology (MOOSE) Group.[24]

Definition of ECAA

Because no generally accepted definition of ECAA exists, all aneurysms defined as such by the authors of the parent paper, regardless of the definitions used, located in the internal carotid artery (ICA) or in the common carotid artery (CCA) were

included. Only aneurysms located between the CCA origin at the aortic arch and base of the skull were included.

Selection of studies

Retrieved records were independently screened by two authors (J.W., G.B.) on title, abstract, and full text. All discrepancies (3%) were discussed until final agreement was reached. If necessary, a third opinion could be obtained, but agreement between authors was reached in all papers. Inclusion criteria were (a) adult patients with an ECAA; (b) description of the type of intervention (conservative treatment, surgery, endovascular treatment, or any combination); (c) report of data on outcome during follow up (case fatality, fatal or non-fatal stroke, or local cervical symptoms); and (d) series describing 10 patients or more.

Language of publications was restricted to Dutch and English. Studies regarding aneurysms located at the level of the skull base or above, aneurysms located in the external carotid artery (ECA), non-human data, and unavailable full text papers were excluded. Studies presenting data at a group level containing the ECA were included because of the low number of ECAs in these series and the relevance to present these large series.[12,19,25-27] The reference list of all selected articles was hand searched to retrieve additional studies. Selected studies were critically appraised based on study design, study quality, consistency, and directness using the GRADE system.[28] Subsequently, the level of evidence of the studies was graded by one author (J.W.). The level was graded high, moderate, low, or very low.

Data extraction

Three authors (J.W., B.N., G.B.) independently extracted data by means of predefined parameters. Individual patient data were obtained when available. The following data were retrieved: publication year, country of origin, number of patients, study design, patient characteristics (age, gender, history of smoking, diabetes, hypertension, and hyperlipidemia), aneurysm characteristics (affected vessel, exact location, aneurysm shape, affected side, and aneurysm size), etiology, and detailed method of treatment.

Outcome measurements included case fatality, stroke, and local cervical symptoms. Local cervical symptoms are defined as any symptom, most likely related to the aneurysm, in the cervical region on the ipsilateral side of the aneurysm. Local cervical symptoms were scored as reported by authors. Furthermore, any neurological deficit with an acute onset persisting for at least 24 hours for which no

Variables	n	(%)
Reports included	39	
Patients	1239	
Aneurysms	1322	
Etiology		
- Atherosclerosis	509	38
- Traumatic	144	11
- Mycotic	65	5
- Other	329	25
- Not reported	275	21
Symptoms [a]		
- Cerebral ischemia	476	36
- Mass	442	33
- Asymptomatic	172	13
- Compression	119	9
- Local pain	39	3
- Other	185	14
Location		
- ICA	608	46
- Bifurcation	261	20
- CCA	108	8
- ECA	9	1
- Not reported	336	25

Table 1, aneurysm characteristics. *Note. Other etiology includes granulomatous diseases, connective tissue disorders, iatrogenic aneurysms, post carotid endarterectomy cystic medial necrosis, and arteritis. ICA = internal carotid artery; CCA = common carotid artery; ECA = external carotid artery.* [a] *Some patients experienced multiple symptoms from one aneurysm.*

other cause could be found was considered a stroke. Early complications included all events that occurred within 30 days after intervention, or after detection of the ECAA in patients who received conservative treatment. Late outcome consisted of death from any cause and any stroke that occurred after at least 30 days.

Statistical analyses

A pooled or summary estimate of the risk of all cause mortality and of all and non-fatal strokes across all studies was calculated together with a 95% confidence

interval using a random effects model. The heterogeneity in results among studies was evaluated by I2 statistics and by prediction intervals. A 95% prediction interval shows the likely range of values for the risks than can be expected if a new and large study would be performed similar to those included in this review. The prediction interval provides insight into the variability or consistency between the results of individual studies whereas a 95% confidence interval around the pooled estimates provides insight into how certain we are about the significance of the pooled estimate. The amount of between study variation (tausquared value of a random effects model) is a key factor determining the width of a 95% prediction interval: large values of between study variation will result in a large prediction interval, even if a large number of studies is included in a review.[29]

Results

After removing duplicates, the search identified 3,711 articles (Fig. 1). Following screening of title, abstract, and full text, 278 articles were selected. Hand searching the reference lists of selected articles revealed three more articles, resulting in a final selection of 281 articles. Selected articles were case reports (n = 179) or case series (n = 102). Exclusion of papers with fewer than 10 patients resulted in the final selection of 39 articles on a total of 1,239 patients (a complete list of all included articles can be found in the Appendix I).[1-4,6-8,11,12,14-16,18,19,25-27,30-51] The level of evidence from 23 studies was graded low and 16 records very low.

Patient and aneurysm characteristics

At presentation, 1,150 aneurysms (87%) were symptomatic, 476 (36%) presented with cerebral ischemia (120 strokes, 291 TIAs, and in 56 patients cerebral ischemia was not further specified). Other symptoms at presentation were a cervical mass, a hematoma or rupture, pain or CND (Table 1, Fig. 2).

Aneurysm size was defined, by only two reports, as a localized increase of the caliber in the carotid artery of more than 50% compared to reference values or to the expected vessel diameter.[2,8] The main cause of the ECAA was atherosclerotic disease, followed by trauma, but for 275 aneurysms (21%) no specific cause was given (Table 1, Fig. 2). Most aneurysms, 608 (46%), were located in the ICA (Table

Intervention	n
Conservative therapy	
- Anticoagulant therapy	24
- No therapy	6
- Medical management n.s.	2
- Antiplatelet therapy	1
- Conservative therapy n.s.	112
Endvascular treatment	
- Stent placement	22
- Balloon exclusion[a]	9
- Stent placement with coil embolization	6
- Embolization	1
- Endovascular n.s.	19
Surgery	
- Resection with interposition graft	376
- Resection with direct anastomosis	264
- Partial resection with reconstruction	107
- Ligation	61
- Aneurysmorraphy	34
- ECA to ICA transposition	11
- Bypass	39
- Surgery n.s.	210
Combined approach	
- Combined approach n.s.	16
- ECIC bypass with balloon occlusion[a]	2

Table 2, treatment details. *n.s. = not specified; ECA = extracranial carotid artery; ICA = internal carotid artery; ECIC = extracranialeintracranial. [a]In this procedure a balloon is placed (and left behind) in the aneurysm or parent vessel. Balloon occlusion may also be accompanied with concomitant ECIC bypass.*

	n	30 day mortality, % (95% CI)	30 day stroke, % (95% CI)	30 day non-fatal stroke, % (95% CI)
Conservative	39	4.67 (0.60-28.42)	6.67 (2.17e18.73)	2.22 (0.31e14.16)
Surgery	934	1.91 (1.01-3.57)	5.16 (3.94e6.73)	4.15 (3.07e5.59)
Overall	988	2.05% (1.14-3.63)	5.04 (3.81e6.62)	3.05 (1.97e4.70)

Table 3, treatment outcome. *The data of the following articles was not included in this analysis because the data could not be split for the different interventions: Higashida et al.,[38] Aleksic et al.,[30] De Jong et al.,[35] McCollum et al.,[15] Padayachy and Robbs,[41] Frankhauser et al.[27]*

1, Fig. 2). Since information on aneurysm shape was missing in over 50% of cases this item was not reported in this paper.

Treatment

A minority of aneurysms, 145 (11%), was treated without an invasive intervention (Table 2). Invasive treatment consisted of surgery in 1,102 (94%) aneurysms, endovascular treatment in 57 (5%) aneurysms, or a combined approach in 18 (1%) (Table 2).

Early outcome

In total 24 (2.05%, 95% CI 1.14-3.57) patients died, of whom 13 had a stroke (5.04%, 95% CI 3.81-6.62), and 34 (3.05%, 95% CI 1.97-4.70) had a non-fatal stroke within 30 days (Table 3). The pooled estimate of the risk of all cause mortality and of all and non-fatal strokes across all studies describing conservative and surgical treatment are reported in Table 3. The pooled estimates could not be calculated for the endovascular and combined approach due to low study numbers. CND occurred in 110 (12%) patients after surgery and was never reported after endovascular treatment or combined surgical and endovascular treatment.

Long-term outcome

Follow up duration was most often presented as a mean follow up of the study population and patients lost to follow up was not reported in the majority. Therefore, it is not possible to present the number of patients available for follow up or the exact duration. During follow up 81 patients died, of whom eight had a fatal stroke, and 11 had a non-fatal stroke. Most deaths (n = 68, 83%) were reported as not aneurysm related.

Confounding by indication

An important issue considered in the evaluation of which treatment option is superior using observational data is confounding by indication. Patients with certain characteristics receive a certain treatment, but these specific characteristics could be associated with a worse or beneficial outcome. For correcting confounding by indication, completeness of potential confounders is needed. However, information on confounders in the present study is far from complete and could not be retrieved from the papers. Therefore, adjustments could not be made and

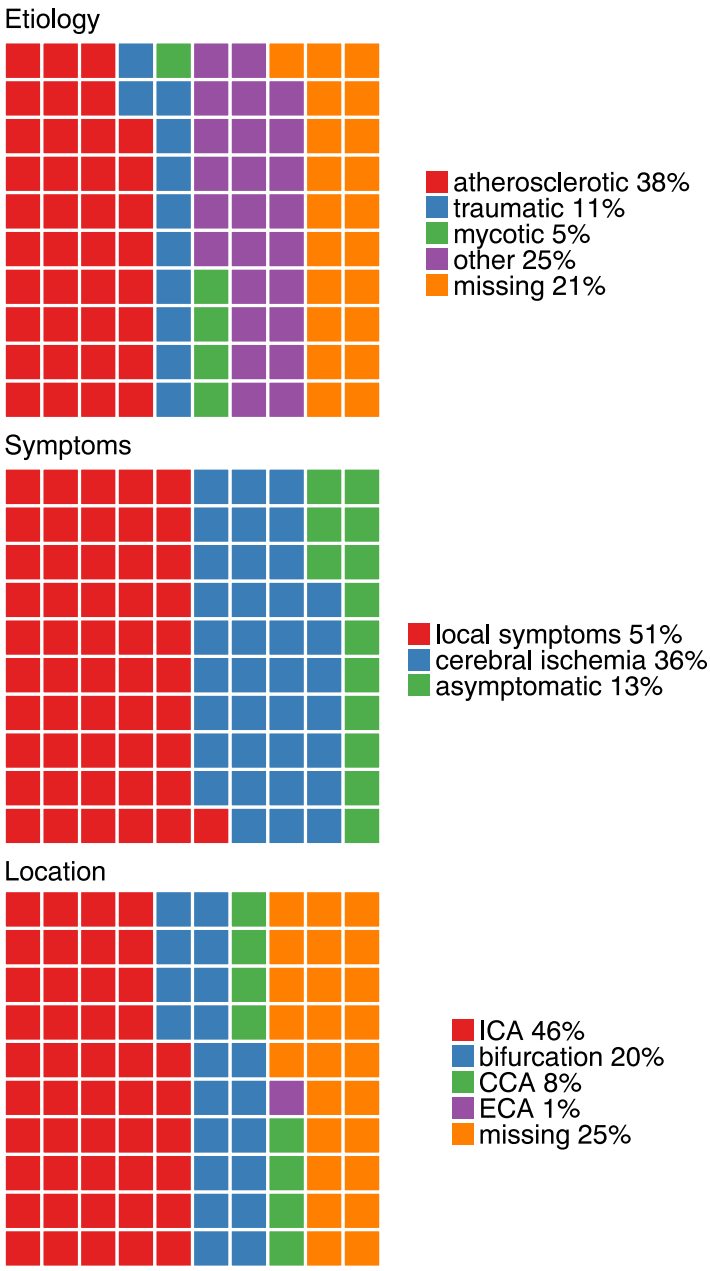

Figure 2, symptoms at presentation, etiology and location of the extracranial carotid artery aneurysms. *ICA = internal carotid artery; CCA = common carotid artery; ECA = external carotid artery.*

therefore, no valid comparison of outcome for the four different treatments was possible.

Discussion

ECAAs are rare, leading to retrieval of only 39 case series containing 10 or more patients. Early mortality and number of strokes is low in surgical and endovascular treatment. Furthermore, the long-term follow up demonstrates low stroke numbers in both intervention groups, which supports the assumption that invasive treatment could prevent stroke. These findings are in line with other publications.[2,14,23] The high CND in surgically treated patients is probably related to the distal location of aneurysms in the ICA and to the extensive dissection needed to perform complete aneurysm resection. The available information on ECAA treatment in the literature suffers greatly because of its rarity, from small case series, missing data, publication bias, and confounding by indication. Furthermore, the level of evidence in the available literature is low to very low; therefore, any estimate of effect based on these records is very uncertain. Therefore, no evidence based recommendation can be given for an individual patient with an ECAA.

The natural course of ECAAs is still hardly understood. Since knowledge of the natural course is required to balance the benefit of any type of intervention, thus far no treatment guideline or expert consensus for the management of ECAA has been developed. Probably, the main goal in the management of ECAAs is to prevent thromboembolic complications of the aneurysm. In aneurysms in other vascular territories, size is often used as an indication for intervention. Aneurysm size may probably be most related to aneurysm rupture. However, rupture is considered very unusual in ECAAs, and most surgeons may only intervene in patients with thrombo-embolic symptoms or proven progressive ECAA growth.

Medical therapy in ECAAs, including medication choice, remains unexplored and needs to be further investigated. However, ECAAs can occur after dissection in the carotid artery or in patients with generalized atherosclerotic disease. In these diseases, medical therapy has long been used and is scientifically substantiated. Following the medical treatment guidelines for the most probable underlying disease is recommended until more evidence is available regarding medical treatment in ECAA patients.

Operative therapy has been advocated for any ECAA because of the high mortality risk in non-operated cases.[15,18,20,22] Nowadays, small case series advocating an

endovascular approach to treat ECAA have reported favorable procedural results but with a limited number of cases and no midor long-term follow up.[23,52] Endovascular treatment with a stent may be the most favored option of the invasive treatments, mainly because of a high prevalence of CND associated with surgical treatment.

Although the approach of this study allowed presentation of early and late outcomes of ECAA treatment, the present study has several limitations. First of all, no randomized controlled trials have been published on ECAA treatment, and data for the present analysis were obtained from case series only. Inherently, the assessment was limited to information provided in these articles resulting in a high rate of missing data. The reported rates for neurological complications may be biased because none of the studies performed independent and structured confirmation of these events. Furthermore, publication bias may have occurred while case series regularly present only striking and/or invasively treated cases. Another disadvantage is that physicians may tend to publish successful rather than unsuccessful cases. However, by excluding small case series the risk of publication bias was reduced. Because of this publication bias, the results of this study may not be generalizable to the ECAA population. In addition, since this was an observational study, the baseline characteristics of the patients undergoing different treatments were not completely identical. In this study there was confounding by indication because treatment choice was based on patient and aneurysm characteristics. Some of these characteristics may give rise to a different prognosis. Unfortunately, due to incomplete data and low patient numbers, this confounding by indication could not be corrected and comparison between treatment outcomes for the different treatment groups was not possible. Because in 31 of 39 studies the data were presented at a group level, performing any sub-analyses was not possible.

The present study does represent the largest evaluation of treatment in patients with an ECAA. This review for the first time clearly shows the lack of knowledge on the natural course of ECAAs, and the fact that population based reporting makes it impossible to perform an individualized patient data analysis. To gain more accurate information regarding the prognosis and results of both conservatively and invasively treated ECAAs more research is needed. Initiating a randomized controlled trial is not feasible because of the low incidence of ECAAs and the low case fatality rates, but more knowledge regarding natural history and treatment indications might be obtained from a well designed prospective study.

Consequently, the authors have designed a prospective web-based international registry to collect data on ECAA (www.carotidaneurysmregistry.com).[53]

Conclusion

This review summarizes the largest available series in the literature on ECAA treatment. The few data consist of a mix of conservative, open surgical, and a growing number of endovascular interventions. There is no consensus in ECAA treatment. The early and long-term outcome of invasive treatment in ECAA is favorable; however, cranial nerve damage after surgery occurs frequently. This review sup- ports the need for an international multicenter registry to reveal the optimal treatment for ECAA.

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First Author	Level of evidence	Patients (n)	Aneurysms (n)	Age (mean/range)	Male (%)
Nordanstig 2014[1]	Low	48	48	64 and 67	56/58
Radak 2014[2]	Low	84	84	65	71
Angiletta 2014[3]	Low	25	26	55	76
Pulli 2013[4]	Low	50	50	66 and 73	53/87
Frankhauser 2013[5]	Very low	132	141	61	52
Garg 2012[6]	Low	15	15	63	40
Padayachy 2012[7]	Very low	22	22	37	82
Malikov 2010[8]	Very low	13	13	43	85
Srivastava 2010[9]	Low	19	19	68	79
Sayed 2010[10]	Very low	12	12	30	92
Attigah 2009[11]	Low	57	64	62	75
Donas 2009[12]	Low	55	61	65	85
Radak 2007[13]	Low	74	91	61	20
Zhou 2006[14]	Low	42	42	56	86
Aleksic 2005[15]	Very low	14	14	60	57
Szopinski 2005[16]	Low	15	15	51	80
Davidovic 2004[17]	Low	16	17	65	94
El-Sabrout 2000[18]	Low	65	67	63	66
Rosset 2000[19]	Low	25	25	54	88
Nair 2000[20]	Low	29	32	35	83
Coldwell 2000[21]	Low	14	16	NR	50
Zhang 1999[22]	Very low	63	66	49	78
Guillon 1999[23]	Very low	16	18	47	81
Coffin 1997[24]	Very low	14	17	52	71
Pulli 1997[25]	Low	21	21	63	52
Faggioli 1996[26]	Low	20	24	55	35
Moreau 1994[27]	Low	35	38	6 to 73	63
Schievink 1994[28]	Very low	22	22	39	68
Bower 1991[29]	Very Low	25	26	47	64
De Jong 1989[30]	Very low	14	18	51	86
Higashida 1986[31]	Very low	10	10	36 to 69	80
Sundt 1986[32]	Very low	19	20	NR	NR
Zwolak 1984[33]	Low	21	24	61	71
Krupski 1983[34]	Very low	21	22	NR	NR
Pratschke 1980[35]	Low	27	28	51	67
Friedman 1980[36]	Very low	13	15	43	54
Busuttil 1980[37]	Low	19	19	57	NR
McCollum 1979[38]	Very low	34	37	59	68
Rhodes 1976[39]	Low	19	23	18 to 78	74

Appendix Table 1 (both pages), summary of all studies included in the review. *CNS central neurological symptoms, ICA internal carotid artery, CCA common carotid artery, ECA external carotid artery, CND cranial nerve dysfunction, ECAA extracranial carotid artery aneurysm(s), NR not reported. This is part of a multipage table.*

First Author	Etiology (N)	Symptoms (N)
Nordanstig 2014[1]	34 atherosclerotic, 2 mycotic, 12 other	18 asx, 10 CNS, 32 local
Radak 2014[2]	77 atherosclerotic, 3 other	16 asx, 32 CNS
Angiletta 2014[3]	18 atherosclerotic, 4 traumatic, 1 mycotic, 3 other	13 asx, 10 CNS, 2 local
Pulli 2013[4]	1 traumatic, 49 other	13 CNS, 6 local
Frankhauser 2013[5]	49 other	69 asx, 18 CNS, 54 local
Garg 2012[6]	4 atherosclerotic, 5 traumatic, 5 other	13 asx, 1 CNS, 2 local
Padayachy 2012[7]	22 mycotic	1 asx, 1 CNS, 20 local
Malikov 2010[8]	4 atherosclerotic, 2 traumatic, 7 other	1 asx, 6 CNS, 4 local
Srivastava 2010[9]	7 atherosclerotic, 7 mycotic	8 CNS, 7 local
Sayed 2010[10]	12 other	12 local
Attigah 2009[11]	42 atherosclerotic, 1 mycotic, 14 other	3 asx, 29 CNS, 15 local
Donas 2009[12]	29 atherosclerotic, 32 other	4 asx, 29 CNS, 20 local
Radak 2007[13]	73 atherosclerotic, 6 traumatic, 12 other	5 asx, 44 CNS, 47 local
Zhou 2006[14]	22 atherosclerotic, 5 traumatic, 15 other	7 asx, 5 CNS, 40 local
Aleksic 2005[15]	10 atherosclerotic, 1 traumatic, 3 mycotic	3 asx, 11 local
Szopinski 2005[16]	5 atherosclerotic, 6 traumatic, 1 mycotic, 3 other	12 CNS, 10 local
Davidovic 2004[17]	17 atherosclerotic	10 CNS, 7 local
El-Sabrout 2000[18]	23 atherosclerotic, 6 traumatic, 38 other	28 CNS, 48 local
Rosset 2000[19]	9 atherosclerotic, 3 traumatic, 13 other	6 asx, 18 CNS, 3 local
Nair 2000[20]	3 atherosclerotic, 10 mycotic, 10 other	4 asx, 1 CNS, 49 local
Coldwell 2000[21]	16 traumatic	NR
Zhang 1999[22]	28 atherosclerotic, 22 traumatic, 7 mycotic, 9 other	16 CNS, 55 local
Guillon 1999[23]	7 traumatic, 1 mycotic, 8 other	8 CNS, 10 local
Coffin 1997[24]	3 atherosclerotic, 3 traumatic, 9 other	9 CNS, 3 local
Pulli 1997[25]	10 atherosclerotic, 11 other	7 asx, 10 CNS, 5 local
Faggioli 1996[26]	9 atherosclerotic, 1 traumatic, 14 other	7 CNS, 19 local
Moreau 1994[27]	12 atherosclerotic, 6 traumatic, 1 mycotic, 19 other	26 CNS, 12 local
Schievink 1994[28]	11 traumatic, 11 other	10 asx, 4 CNS
Bower 1991[29]	3 atherosclerotic, 8 traumatic, 2 mycotic, 9 other	17 CNS, 23 local
De Jong 1989[30]	NR	11 CNS, 2 local
Higashida 1986[31]	5 traumatic, 5 other	1 asx, 1 CNS, 1 local
Sundt 1986[32]	7 traumatic, 1 mycotic, 11 other	14 CNS, 5 local
Zwolak 1984[33]	24 atherosclerotic	15 CNS, 13 local
Krupski 1983[34]	8 atherosclerotic, 5 traumatic, 5 mycotic, 3 other	7 CNS, 27 local
Pratschke 1980[35]	13 atherosclerotic, 9 traumatic, 2 mycotic, 4 other	12 CNS, 30 local
Friedman 1980[36]	1 traumatic, 12 other	2 asx, 13 CNS, 3 local
Busuttil 1980[37]	NR	2 asx, 12 CNS, 5 local
McCollum 1979[38]	6 atherosclerotic	5 CNS
Rhodes 1976[39]	16 atherosclerotic, 4 traumatic, 3 other	13 CNS, 20 local

First Author	Location (N)	Outcome
Nordanstig 2014[1]	5 CCA, 12 bifurcation, 31 ICA	5 Deaths, 4 strokes, 2 Ipsilateral stroke, CND 12.5 %
Radak 2014[2]	22 CCA, 4 bifurcation, 54 ICA	2 deaths, 4 strokes, 2 CND
Angiletta 2014[3]	3 CCA, 23 bifurcation	1 stroke, 2 CND
Pulli 2013[4]	50 ICA	19 deaths, 3 strokes, CND 10.5%, 13%
Frankhauser 2013[5]	11 CCA, 15 bifurcation, 114 ICA, 1 ECA	1 death, 1 stroke
Garg 2012[6]	6 CCA, 3 bifurcation, 5 ICA	1 death , 1 CND
Padayachy 2012[7]	NR	3 deaths, 1 stroke, 3 CND
Malikov 2010[8]	13 ICA	1 death, 14 CND
Srivastava 2010[9]	1 CCA, 10 bifurcation, 8 ICA	2 strokes, 1 CND
Sayed 2010[10]	2 CCA, 3 bifurcation, 7 ICA	2 deaths, 1 stroke, 1 CND
Attigah 2009[11]	6 CCA, 25 bifurcation, 33 ICA	11 deaths, 3 stroke, 17 CND
Donas 2009[12]	9 CCA, 26 bifurcation, 26 ICA	2 deaths, 4 strokes, 6 CND
Radak 2007[13]	29 bifurcation, 61 ICA, 1 ECA	5 deaths, 5 strokes, 2 CND
Zhou 2006[14]	NR	19 deaths, 1 stroke, 4 CND
Aleksic 2005[15]	10 bifurcation, 4 ICA	1 death, 2 strokes, 3 CND
Szopinski 2005[16]	1 CCA, 3 bifurcation, 11 ICA	4 deaths, 2 stroke, 1 CND
Davidovic 2004[17]	2 CCA, 15 ICA	2 deaths, 3 strokes, 2 CND
El-Sabrout 2000[18]	NR	23 deaths, 5 strokes, 4 CND
Rosset 2000[19]	NR	2 deaths, 2 ipsilateral strokes, 11 CND
Nair 2000[20]	13 CCA, 12 bifurcation, 7 ICA	2 deaths, 1 stroke
Coldwell 2000[21]	NR	none
Zhang 1999[22]	10 CCA, 41 bifurcation, 13 ICA, 2 ECA	4 deaths, 4 strokes
Guillon 1999[23]	20 ICA	1 death
Coffin 1997[24]	15 ICA	2 Deaths, 4 CND
Pulli 1997[25]	1 CCA, 8 bifurcation, 12 ICA	3 strokes, 2 CND
Faggioli 1996[26]	24 ICA	1 death, 2 stroke, 5 CND
Moreau 1994[27]	NR	4 death, 1 stroke, 29 CND
Schievink 1994[28]	22 ICA	2 strokes, 17 CND
Bower 1991[29]	NR	1 death, 1 stroke, 7 CND
De Jong 1989[30]	2 CCA, 12 ICA	1 death , 1 CND
Higashida 1986[31]	NR	2 strokes
Sundt 1986[32]	20 ICA	1 stroke, 6 CND
Zwolak 1984[33]	2 CCA, 12 bifurcation, 10 ICA	7 deaths, 4 stroke, 6 CND
Krupski 1983[34]	1 CCA, 8 bifurcation, 3 ICA	2 death, 3 stroke, 6 CND
Pratschke 1980[35]	9 CCA, 4 bifurcation, 10 ICA, 5 ECA	10 deaths
Friedman 1980[36]	NR	1 death , 1 stroke
Busuttil 1980[37]	NR	3 deaths, 2 stroke
McCollum 1979[38]	NR	13 deaths, 3 strokes
Rhodes 1976[39]	2 CCA, 13 bifurcation, 8 ECA	4 deaths, 2 strokes, 3 CND

Appendix Table 1 (both pages), summary of all studies included in the review. *CNS central neurological symptoms, ICA internal carotid artery, CCA common carotid artery, ECA external carotid artery, CND cranial nerve dysfunction, ECAA extracranial carotid artery aneurysm(s), NR not reported. This is part of a multipage table.*

First Author	Study Details
Nordanstig 2014[1]	Review of all ECAA patients registered in the Swedish National Registry 'Swedvasc'.
Radak 2014[2]	Retrospective analysis of an operative approach for ECAA in two vascular surgery clinics.
Angiletta 2014[3]	Single center retrospective study of surgical and endovascular treated ECAA.
Pulli 2013[4]	Retrospective analysis of surgical management in true (group 1) and false ECAA (group 2).
Frankhauser 2013[5]	Single center retrospective review of patients with true (group 1) and false ECAA (group 2).
Garg 2012[6]	Single center retrospective review of ECAA.
Padayachy 2012[7]	Single center retrospective review on all patients with HIV carotid aneurysms.
Malikov 2010[8]	Retrospective study reporting on cervical-to-petrous carotid artery bypass in ECAA.
Srivastava 2010[9]	Single center retrospective review of ECAA treatment.
Sayed 2010[10]	Single center retrospective review of Behcet ECAAs.
Attigah 2009[11]	Retrospective review of surgically treated ECAA.
Donas 2009[12]	Single center retrospective review of surgically treated ECAA.
Radak 2007[13]	Retrospective, multicenter review of treatment of ECAA.
Zhou 2006[14]	Retrospective review, ECAA patients in three hospitals. Groups 1985-1994 & 1995-2004.
Aleksic 2005[15]	Retrospective review of ECAA.
Szopinski 2005[16]	Single center retrospective review of ECA treated surgically and endoluminally.
Davidovic 2004[17]	Single center retrospective review of ECAA treatment.
El-Sabrout 2000[18]	Retrospective review of surgically treated ECAA in two hospitals.
Rosset 2000[19]	Single center retrospective review of surgically treated ECAA.
Nair 2000[20]	Single center retrospective review of spontaneous ECAA.
Coldwell 2000[21]	Single center retrospective review of posttraumatic ECAA treated with endovascular stent.
Zhang 1999[22]	Single center retrospective review of management of ECAA.
Guillon 1999[23]	Single center retrospective review of ECAA after ICA dissection.
Coffin 1997[24]	Single center retrospective review of surgically treated ECAA.
Pulli 1997[25]	Retrospective review of surgically treated ECAA.
Faggioli 1996[26]	Single center retrospective review of ECAA treatment.
Moreau 1994[27]	Single center retrospective review of surgically treated ECAA.
Schievink 1994[28]	Single center retrospective review of surgically treated dissecting aneurysms.
Bower 1991[29]	Single center retrospective review of surgically treated brachiocephalic aneurysms.
De Jong 1989[30]	Single center retrospective review of ECAA.
Higashida 1986[31]	Single center retrospective review of ECAA.
Sundt 1986[32]	Single center retrospective review of surgically treated ECAA.
Zwolak 1984[33]	Retrospective review of atherosclerotic ECAA encountered in three affiliated hospitals.
Krupski 1983[34]	Single center retrospective review of ECAA.
Pratschke 1980[35]	Single center retrosprective review of ECAA.
Friedman 1980[36]	Single center retrospective review of cervical carotid dissecting aneurysm.
Busuttil 1980[37]	Single center retrospective review of surgically treated ECAA.
McCollum 1979[38]	Single center retrospective review of ECAA.
Rhodes 1976[39]	Single center retrospective review of ECAA.

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Chapter 8

Summary and general discussion

Tripple A's and other aneurysms

Arterial diseases are the most common cause of death worldwide, and its incidence is still increasing.[1] Arterial diseases can roughly manifest in two way, narrowing or widening of the artery. A permanent focal widening of an artery is called an aneurysm. The definition 'aneurysm' is often used is when the diameter of the artery is exceeding a certain threshold. For the abdominal aorta a diameter of 2.9cm is still within normal range, and a diameter of 3.0cm or larger is considered to be aneurysmal.[2] There are also guidelines that persist that a local increase of 50% of the diameter is considered to be an aneurysm.[3] The latter method implies a more reasonable approach for any artery as the standard abdominal aorta diameter varies.

Some arteries are more prone to develop aneurysms than others. The most common location for aneurysm development is the abdominal aorta. Other sites are the brain, the popliteal arteries, the iliac arteries and the carotid arteries. Aneurysms can be saccular in shape (as a pouch), or fusiform (spindle-shaped). When speaking of aneurysms, we usually refer to fusiform shaped Abdominal Aorta Aneurysms (AAA's). Aneurysms are ticking time bombs and can rupture there is a high probability for significant morbidity. AAA's have a high rate of mortality and in 2010 this dissease was responsible for 1% of all deaths of people aged sixty and up in the Netherlands.[4]

Known risk factors for AAA include a positive family history, high age, male sex, coronary artery disease, high cholesterol and COPD and the strongest known risk factors for the development of AAA is smoking. Interestingly, diabetes is known to prevent aneurysm formation.[5] Sometimes an AAA is the direct result of a specific cause, such as an infection (Q-fever), trauma, immune-mediated vasculitis (like Behçet's disease and Takayasu), or a connective tissue disorder (like Ehlers-Danlos or Marfan syndrome).[6]

A family history of aneurysms gives a doubling of the risk for of AAA, in women this is higher, and in identical twin siblings this is even 24%. The familial influence is probably not entirely genetic, and environment still plays an important role. Nevertheless, people see AAA as a male disease and not without reason, the prevalence of AAA is 3–5 times greater than females of the same age. Oestrogen is

considered to protect females from developing AAA, and this could explain the delay in AAA development of 10-15y compared to males.[6-8]

There is a shift of AAA mortality from the 'younger' population towards the octogenarians pointing towards changes in AAA management and risk factors. One important change is explained by the shift towards more available diagnostic imaging and endovascular aneurysm repair since the early '90's. Looking at the Netherlands, we found that in 1980 >75% of adult men and <25% of women were smokers. This ratio of smokers between sexes shifted dramatically in the last 40 years, with a male female ratio of 2:1 in 2010. Striking is that the decrease in tobacco use in men which runs parallel with the decline in AAA mortality and that this trend is seen in women as well, where tobacco use and AAA mortality remained unchanged in the last 40 years. Note that smoking doubles the risk of rupture, and female sex almost quadruples it.[9] Abdominal aorta aneurysm is now a mortal disease that dominantly comes to expression in both sexes.

Aneurysm-Express Biobank

The Aneurysm-Express is the largest aneurysm tissue bank in the world and was initiated to get a better understanding of this mortal disease. The Aneurysm-Express biobank is a spinoff of its bigger sister the Athero-Express biobank. One of the main goals of the Aneurysm-Express is to link certain clinical traits with pathological changes in the vessel wall. This biobank started in 2003. This is how the tissue collection works, during open surgical aneurysm repair, a small tissue specimen of the aneurysm wall is collected. The sample is then fixed in 4% formaldehyde, decalcified for one week in EDTA (ethylenediaminetetraacetic acid). Then 4µm-thick sections are cut for histological analyses. Sections are stained for markers like elastin, collagen, and markers for different inflammatory cells (lymphocytes, macrophages). We ask informed patients to give consent to enroll in the study, we create histological samples from harvested tissue and in some cases collect blood. Patients enrolled in the Aneurysm-Express biobank have a three-year followup with a registration of cardiovascular morbidity and mortality. We based this thesis on tissue collected by the Aneurysm-Express biobank. The Aneurysm-Express predominantly banks aorta tissue but also contains a substantial amount of popliteal, femoral and carotid artery wall. From some patients, we have collected

aneurysms from different arteries. The variety of vessel types makes comparison interesting.

The emergence of an aneurysm is a dynamic and complex process. Once was thought that AAA development was a direct result of atherosclerosis. Today atherosclerosis is seen as part of the process that also leads to aneurysm formation. The phenomena which lead to a reduced vessel wall strength and its effect in dilation of the aorta are two-fold: both degradations of the extracellular matrix and inflammation play a major role. A healthy aorta is consists of a well-structured composition of smooth muscle cells and lengthy extracellular proteins, such as elastin and collagen. Degradation of the extracellular matrix in the vascular wall of an AAA is manifested by a decrease in elastin, by poorly structured collagen and by apoptosis of the smooth muscle cells, this causes a weak vessel wall.

Inflammation also has a major role in reducing the wall strength. Aneurysm tissue has shown an increase in inflammatory cytokines also increased levels of inflammatory mediators are measurable in the blood. Although, it is still unclear whether the inflammation has a causal role or just the result of the degenerative process.[7,10]

To learn more about the pathology of disease correlation with other high-resolution measurements (e.g., other vessels, DNA profiles, SNP data, blood markers) are needed. For our research, we mainly looked at histological tissue characteristics and a presence of inflammatory cells. Formally histological characteristics were manually estimated and classified using a low-resolution ordinal scale, this makes a statistical comparison with other precise measurements less valuable. Therefore a sound method of precise measurement of histological characteristics is needed.

Carotid artery

Although extracranial carotid artery aneurysms (ECAA) are very uncommon, they can have substantial morbidity with a stroke rate of 50%.[11] Just like AAA's, the pathophysiological mechanisms and natural course of ECAA are largely unknown. Understanding these mechanisms may prevent an adverse outcome. In chapter 6 we manually investigate the histopathological characteristics of ECAA tissue samples collected during surgery using a light microscope.[12]

During our histological examination of ECAA, we found two distinctive categories: aneurysms with signs of dissection versus degenerative aneurysms. Aneurysms

formation after dissection is known and described before using radiology imaging.[13] Also, in the aorta and its branches, degeneration is a well-recognized cause of aneurysm formation.[6,7] Non-dissective causes of peripheral aneurysms (e.g., carotid or popliteal aneurysms) are believed to exist but had not been histologically confirmed yet.[14] We find degenerative aneurysms in most of the patients (ten of thirteen). The possibility exists that these two distinctive categories (dissection and degeneration) can be different stages of the same disease. We observe inflammatory cells in both types of aneurysms, and this suggests that an inflammatory process plays a role in the development of these aneurysms. All the thirteen patients with ECAA in our study underwent surgical repair. But surgery is not the only treatment available.

ECAAs are rare, around 1000 cases of ECAA have been reported in the international literature of which 39 case series contain 10 or more patients. Short and long-term mortality and stroke rates are low after surgical and endovascular management. This supports the assumption that invasive treatment could prevent stroke. However, the available information on ECAA treatment suffers greatly because of its rarity due to small case series, missing data, publication bias, and confounding by indication. Additionally, the level of evidence in the available literature is low, and therefore any estimate of the effect based on these records is very uncertain.

We reviewed all available literature on the risk of complications and long-term outcome after conservative or invasive treatment of patients with ECAA. We found conservative management is justified in an ECAA that is stable, small and asymptomatic. Despite cranial nerve damage which frequently occurs (as it did in our study as well), the short and long-term outcome of surgical management for ECAA is favorable. Endovascular therapy is still under development and might be in favor of surgical repair due to the much lower incidence of cranial nerve injury. Grouping all found carotid artery aneurysms in one study could help us to provide better treatment for these patients. An international registry could provide a better insight on the optimal treatment for ECAA. On the other hand, the rareness of the disease and the variety of patients characteristics is more in favor of personalized and customised care.

Tissue is the issue

The redoubtable professor of pathology, Prof. dr. J.G. van den Tweel used to say: "The tissue is the issue." Biobanking of human tissue is an important cornerstone in

the discovery and validation of causally related determinants of diseases. The AAA biobank is a collection of hundreds of aneurysm wall samples, which makes precise analysis and quantification of different tissue characteristics possible. The goal of this biobank is to use the tissue to unravel the etiology of the disease. It is evident that accuracy and reproducibility of histological characterization are key for optimal phenotyping of human tissues and subsequent interpretation of data for association studies.

There is an increasing demand for accurate and reproducible histological characterization, especially for subsequent analysis and interpretation of data in association studies. Slide quantification makes it possible to correlate slide characteristics with other measurements, like patient characteristics (age, gender, smoking, hypertension, diabetes), DNA variations or single nucleotide polymorphisms (SNPs). More accurate aggregation of data leads to a better understanding of the pathology of the disease. There are a number of scientific applications in which reproducibility of atherosclerotic plaque histology is important.

First, plaque imaging (by MRI) is increasingly frequently used as a technology to detect vulnerable plaque characteristics in a noninvasive manner to detect, for example, an association between symptoms and future cardiovascular events. For validation of imaging techniques comparison, knowledge of plaque histology is necessary. Obviously, the total variability is the sum of the plaque histology measurement variability and plaque imaging measurement variability.

Second, human genotyping studies are emerging and applied to study causality of disease as well as for the discovery of potential therapeutic targets. Therefore, an accurate and reproducible association of phenotypic tissue characteristics with genetic data is relevant, which requires improved phenotyping of tissue and disease. Computerized quantification is often preferred over manual quantification as a more precise and reproducible, and in many instances more sensitive, approach. Moreover, when it comes to labor-intensive repetitive tasks, automated procedures are preferable.

Traditional manual analysis of histological samples is time-consuming and a massive burden on skilled labor. Furthermore, it is an unwanted variation in accuracy and reproducibility of histological characteristics.[15] A traditional microscope functions by sending light through stained tissue and by projecting a small part of that slide,at a magnified scale, on our retina. The digital slide scanner works almost similar, however, instead of a human eye, it uses a digital camera and creates a digital version of the small fraction of that slide at a magnified scale.

Concatenation of each digital fraction creates one big image, a digital virtual slide. For today's measurements, these digital slides are massive in size and can easily be around 50 Gigabytes (like the concatenation of 20.000 iPhone photos).

Digital images can be automatically analyzed. Relatively few software packages are available for analyzing fully digitized microscopic slides (also known as whole slides images). These software packages all have considerable downsides and do not meet our needs. They almost all are expensive, slow, the proprietary software has an inferior performance and there is no possibility to upscale and do parallel processing.

An important focus of this thesis is the development of software for automating whole digital slide analysis, the slideToolkit. The slideToolkit is an original new method to accuraterly and efficiently handle the whole slide analysis and large quantities of whole slide images using advanced cell detecting algorithms. The slideToolkit makes it possible to handle large-scale analysis of tissue samples in an accurate fashion. The high precision suggests that quantification by this technique allows for more reliable association studies, compared to traditional manual methods. The slideToolkit does not suffer from visual interpretation that occurs between researchers and research centers. The slideToolkit shows a reliable reproducibility with a Cohen kappa value ranging from 0.92 to 0.97 compared to a reproducibility of 0.55 to 0.69 for manual tissue analysis.

While this new method has a very high reproducibility, its accuracy could not be assessed since no 'valid' reference standard is available to quantify histological tissue. Measuring accuracy is a challenge because it remains a subjective value of what the 'real' quantity of a certain marker/characteristic is. We can only try to approach this 'true' quantity as good as possible by assessing reproducibility of repeated measurements, which is a measure of precision. Furthermore, precision is also dependent on the marker used to visualize characteristics in the tissue. Because of these features, the slideToolkit can reliably quantify tissue histology for research purposes.

The slideToolkit is a solution for a long-standing problem. The slideToolkit provides a free, powerful and versatile collection of tools for automated feature analysis of whole slide images to create reproducible and meaningful phenotypic data sets. The slideToolkit is freely available (MIT license) on GitHub. It uses an easy scripting

language and software libraries that have proven themselves. Scalability is simple, and it can run as easy on old hardware as on a high-performance cluster.

Big data

Before the year 2000, data were stored mainly as an analog signal on a carrier, for example, newspapers, books, records, VHS tape, and cassette. Since the year 2000 there is an exponential growth in cheaper digital storage possibilities. Gorden Moore predicted that the number of transistors in a dense integrated circuit would double approximately every two years. Until now, Moore's law is still applicable for digital storage and computing power.

The more data we collect, the harder it is to make sense of that data. However, it is believed that more data is likely to store more useful information. Researchers are looking for ways to explore that valuable information to get a deeper understanding of their subject. Swiftly the data sets become so large and so complex that traditional ways of dealing with data are becoming inadequate. There is a point in time that we no longer talk about data, but we start using the term 'big data.' Big data requires nontraditional methods for analysis. Due to its complexity, it can be hard to determine if the data does or does not contain a certain property.

During this research, we noticed a hiatus between the science, data-handling (including writing software), and clinical work. A researcher is required to develop knowledge and understanding of different skills, methods, and tools. Although it might look a bit trite, often a mental paradigm shift is needed to enforce change. Look at each obstacle differently. There is a need for the development of skills, tools, and methods. Untrained and unskilled researchers are expected to work with this data and have sufficient knowledge of software, hardware, and statistics. When the only available tool is a hammer, everything looks like a nail. At a magnified scale I think we underestimate the skills needed to handle these kinds of data.

How reliable are the results we get from our data? In the end we must not forget our goal, we want the patient to receive the best care.

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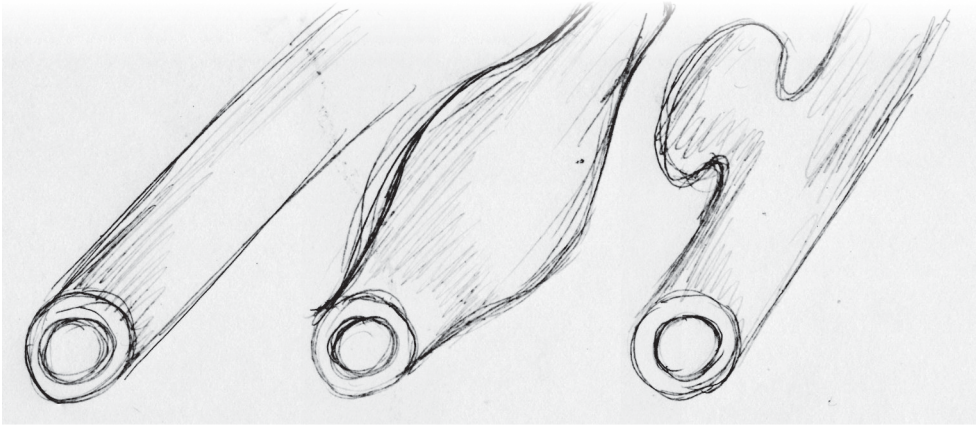
Chapter 9

Nederlandse samenvatting

Het wijder worden van de slagaders

De meest voorkomende doodsoorzaak wereldwijd komt door een slagader aandoening. Deze aantallen blijft stijgen. Om het simpel te houden bestaan er grofweg twee vormen van aandoeningen aan de slagader, namelijk; een vernauwing van de slagader (plaque) of een verwijding van de slagader. Een permanent verwijde slagader wordt 'aneurysma' genoemd. De term 'aneurysma' wordt vaak gebruikt wanneer de diameter van de slagader een bepaalde drempel overschrijdt. Voor de aorta (lichaamsslagader) vinden we een diameter van 2,9 cm nog normaal en een diameter van 3,0 cm of meer vinden we te groot en wordt als aneurysma beschouwd. Er zijn ook richtlijnen die aanhouden dat een lokale toename van 50% van de diameter wordt beschouwd als een aneurysma. Mijn inziens impliceert deze laatste methode een meer redelijke benadering omdat de diameter van een gezonde aorta per persoon varieert.

Sommige slagaders zijn gevoeliger voor het ontwikkelen van aneurysmata dan andere. De meest voorkomende locatie voor ontwikkeling van het aneurysma is de aorta. Andere plekken waar aneurysmata ontstaan in zijn de hersenen, de knie, de liezen en de hals. Aneurysmata kunnen sacculair van vorm zijn (een uitstulping op het bloedvat, als een champignon) of fusiform (spoelvorming, het gehele bloedvat is dan breder in het midden en smaller richting de uiteinden). Wanneer we spreken



Op volgorde van links naar rechts. *Een normaal bloedvat, een fusiform aneurysma, een sacculair aneurysma.*

van aneurysma bedoelen we meestal een fusiform aneurysma in de aorta, ook wel AAA of abdominale aorta aneurysma genoemd. AAA's zijn tijdbommen die plots kunnen openscheuren. Een scheur in de aorta is zeer ernstig is en vaak lijdt tot overlijden. In hoofdstuk 2 kan je lezen dat in 2010 deze ziekte verantwoordelijk voor 1% van alle sterfgevallen onder mensen van zestig jaar en ouder in Nederland.

Risicofactoren dragen bij aan de kans dat je aneurysma ontwikkeld. Bekende risicofactoren voor AAA's zijn onder andere; een familielid met deze ziekte, een hoge leeftijd, het mannelijk geslacht, vernauwing van de kransslagaders van het hart, een hoog cholesterol en COPD. De sterkste risicofactor voor het krijgen van een aneurysma van de aorta is roken. Het interessante is dat suikerziekte de vorming van een aneurysma kan voorkomen. Soms is een aneurysma het directe gevolg van een specifieke oorzaak. Directe oorzaken voor het ontstaan van een aneurysma zijn bijvoorbeeld een infectie (Q-koorts), trauma, immuun-gemedëeerde vasculitis (zoals de ziekte van Behçet en Takayasu) of een bindweefsel aandoening (zoals het syndroom Ehlers-Danlos of Marfan).

Als er familieleden zijn met een aneurysma geeft dit een verdubbeling van het risico op het ontwikkelen van een AAA, bij vrouwen is dit nog hoger dan bij mannen en bij een ééneiige tweeling is dit zelfs 24%. De familiale invloed is waarschijnlijk niet volledig genetisch, ook risicofactoren die voorkomen in het gezin spelen een belangrijke rol. AAA's komen 3 tot 5 keer meer voor bij mannen. Oestrogeen wordt geacht vrouwen te beschermen tegen ontwikkeling van AAA. De aanmaak van oestrogeen valt weg na de overgang en dit zou kunnen verklaren waarom het voorkomen van aneurysmata ook bij vrouwen op oudere leeftijd toeneemt maar dat deze toename wel 10-15 jaar achterloopt in vergelijking met mannen.

Er is een verschuiving van het aantal mensen dat overlijd aan een AAA van de 'jongere' bevolking naar de tachtig-plussers. Deze verschuiving wijst op veranderingen in de behandeling en de risicofactoren van abdomiale aorta aneurysmata. Een belangrijke verandering wordt verklaard door beeldvorming die steeds toegankelijker wordt (bijvoorbeeld door scans) en een nieuwe behandelmethode die is opgekomen in jaren '90 waarbij iemand via de slagader in de lies geopereerd kan worden aan het aneurysma in de buik (EVAR, of endovascular aneurysm repair). Wij laten in ons onderzoek zien dat in 1980 meer dan 75% van de volwassen mannen en minder dan 25% van de vrouwen rookten. Deze verhouding van rokers tussen mannen en vrouwen verschoof in de afgelopen 40 jaar en heeft in 2010 een man:vrouw ratio van 2:1. Opvallend is dat de daling van het aantal rokers bij mannen parallel loopt met de afname van het aantal mannen dat overlijd aan een AAA. Bij vrouwen zien we dezelfde trend, maar hier is het

tabaksgebruik en de AAA-sterfte de afgelopen 40 jaar onveranderd bleven. Roken zorgt niet alleen voor een grotere kans op het ontstaan van een AAA, het zorgt ook voor meer kans op een scheur. Bij mannen verdubbelt roken het risico op een scheur, bij vrouwen is dit risico verviervoudigt. Abdominale aorta aneurysmata moet niet meer gezien worden als een ziektebeeld dat veel voorkomt bij mannen. Het is een ziekte geworden die veel voorkomt bij zowel mannen als bij vrouwen.

Aneurysma-Express Biobank

Dit proefschrift gebruikt weefsel dat is verzameld door de Aneurysma-Express biobank. De Aneurysma-Express is de grootste aneurysma-weefselbank ter wereld en is gestart om deze dodelijke ziekte beter te begrijpen. De Aneurysma-Express biobank is een 'spin-off' van zijn grotere zus de Athero-Express biobank. Eén van de hoofddoelen van de Aneurysma-Express biobank is om bepaalde kenmerken van patiënten te koppelen aan bepaalde veranderingen in de wand van de slagader. De biobank is in 2003 opgezet. Ik leg hier kort uit hoe we alle gegevens verzamelen. Tijdens de operatie aan het aneurysma wordt een klein stukje weefsel van de slagader bewaard (anders zou het worden weggegooid). Het weefsel wordt vervolgens gefixeerd in 4% formaldehyde en gedurende één week ontkalkt in EDTA (ethyleendiaminetetra-azijnzuur). Daarna worden 4 micrometer dikke coupes gesneden en zo klaargemaakt voor weefsel onderzoek. Deze coupes worden gekleurd voor bepaalde eiwitten die op in de vaatwand zitten zoals elastine, collageen en verschillende ontstekingscellen (lymfocyten, macrofagen). Per coupe kunnen we maar één eigenschap aankleuren, om meerdere eigenschappen te herkennen moet je dus meerdere coupes aankleuren. Wij gebruiken vrijwel altijd een bruine kleur om aan te kleuren, de achtergrond wordt blauw gekleurd. Alle patiënten die meedoen hebben toestemming gegeven voor deelname en worden drie jaar vervolgd. De Aneurysma-Express heeft voornamelijk betrekking op het aortaweefsel, maar bevat ook weefsel van slagaders uit de knie, lies en hals. Van sommige patiënten hebben we meerdere aneurysmata verzameld uit verschillende slagaders. De verscheidenheid aan slagaders maakt vergelijken interessant.

Het ontstaan van een aneurysma is een dynamisch en complex proces. Ooit werd gedacht dat het ontstaan van een AAA een direct gevolg was van aderverkalking. Tegenwoordig wordt aderverkalking gezien als onderdeel van het proces dat ook leidt tot aneurysma-vorming. De verschijnselen die leiden tot een verminderde vaatwandsterkte en het effect daarvan bij dilatatie van de aorta zijn tweeledig: zowel degradaties van de extracellulaire matrix (dat is een netwerk van eiwitten die voor

de stevigheid van het bloedvat zorgen) als ontsteking spelen een grote rol. Een gezonde aorta bestaat uit een goed gestructureerde samenstelling van gladde spiercellen en lange eiwitten zoals elastine en collageen. Afbraak van de extracellulaire matrix in de vaatwand van een AAA manifesteert zich door een afname van elastine, door slecht gestructureerd collageen en door celdood van de gladde spiercellen, dit bij elkaar veroorzaakt een zwakke vaatwand.

Ontsteking in de vaatwand heeft ook een belangrijke rol bij het verminderen van die sterke structuur. Aneurysmaweefsel heeft veel ontstekingseiwitten in de vaatwand. Het is niet duidelijk of het ontstekingsproces een oorzaak of juist het gevolg is van de zwakker wordende vaatwand.

Om meer te weten te komen over hoe deze ziekte ontstaat zijn andere metingen belangrijk (door bijvoorbeeld te kijken naar andere bloedvaten, DNA-profielen en markers/eiwitten in het bloed). Voor ons onderzoek hebben we vooral gekeken naar weefselkenmerken. Weefselkenmerken kunnen worden beoordeeld door het gebruik van een microscoop. Voordat ik met dit onderzoek begon werden deze eigenschappen handmatig met de microscoop bepaald. Vaak werd er met een grove schaal een schatting gemaakt hoeveel een bepaalde eigenschap aanwezig was (bijv. veel, beetje veel, weinig of geen ontstekingscellen zichtbaar). Deze grove schatting maakt het lastig om nauwkeurige statistische vergelijking met andere metingen te doen. Er was behoefte aan een methode die veel nauwkeuriger de verschillende eigenschappen kon vastleggen.

‘The tissue is the issue’

Hoogleraar pathologie, prof.dr. J.G. van den Tweel zei altijd: "The tissue is the issue". Biobanken van menselijk weefsel zijn essentieel om meer te leren van verschillende ziektebeelden. De Aneurysma-Express biobank is een verzameling van honderden stukjes aneurysma vaatwand. Deze collectie maakt het mogelijk om nauwkeurige analyse en kwantificering van verschillende weefselkenmerken te doen (fenotypering). Het doel van de biobank is om het weefsel te gebruiken om er achter te komen hoe aneurysmata ontstaan. Het is duidelijk dat de nauwkeurigheid en reproduceerbaarheid van weefseleigenschappen belangrijk is als je dit wilt vergelijken met andere eigenschappen die worden gevonden bij ander onderzoek (zoals DNA-onderzoek of MRI onderzoek).

Er is dus steeds meer behoefte naar een nauwkeurige en reproduceerbare methode voor het vastleggen van weefseleigenschappen. Je kan weefseleigenschappen

vergelijken met andere metingen, zoals patiëntkenmerken (leeftijd, geslacht, roken, hypertensie, diabetes), DNA-variaties of SNP's (single nucleotiden polymorfismen). Het nauwkeuriger verzamelen van gegevens zorgt ervoor dat je meer waardevolle informatie uit je gegevens kan halen. Onnauwkeurige meetgegevens zijn 'garbage' en zoals elke onderzoeker weet: 'garbage in is garbage out'. Er zijn steeds meer wetenschappelijke toepassingen waarbij reproduceerbaarheid van weefsel-eigenschappen essentieel is.

Een lokale vernauwing van een slagader wordt ook plaque genoemd. Beeldvorming door MRI wordt steeds vaker gebruikt om kwetsbare kenmerken van de plaque te detecteren zonder dat je de patiënt hoeft te opereren. Dit gebeurd bijvoorbeeld om een verband te ontdekken tussen klachten van de patiënt nu en een mogelijke beroerte in de toekomst. Om met MRI te kunnen voorspellen hoe een de ziekte zich gaat gedragen in de toekomst is veel kennis nodig van de opbouw van de plaque. Je wil dus de opbouw van de plaque kunnen herkennen op de MRI.

Ten tweede zijn DNA studies in opkomst waarbij wordt gekeken naar het oorzakelijke verband van ziekten om op deze manier nieuwe behandelmethodes te ontdekken. Voor DNA studies is het erg belangrijk dat er nauwkeurig en reproduceerbare associaties van weefselkenmerken gemaakt kunnen worden met genetische gegevens. Dit betekend dat er een verbeterde analyse van weefselkenmerken vereist is. Geautomatiseerde kwantificatie (beoordelen en tellen van eigenschappen van het weefsel) verdient vaak de voorkeur boven handmatige kwantificering omdat het vaak preciezer, meer reproduceerbaar en gevoeliger is. Bovendien zijn geautomatiseerde procedures veel minder arbeidsintensief.

Handmatige analyse van weefseleigenschappen is tijdrovend en kost erg veel tijd. Verder is er bij handmatige analyse snel sprake van variatie in de nauwkeurigheid en reproduceerbaarheid van de metingen. Wij maakten vroeger gebruik van een lichtmicroscoop, tegenwoordig maken wij gebruik van een digitale coupe scanner. Bij een lichtmicroscoop wordt er licht door het weefsel op de coupe gestuurd en dan wordt een klein deel van de coupe op vergrote schaal op ons netvlies geprojecteerd. De digitale coupe scanner werkt vrijwel hetzelfde, maar in plaats van een menselijk oog maakt hij gebruik van een digitale camera en maakt hij een digitale versie van de coupe. De digitale versie van een volledig gedigitaliseerde coupe is dan makkelijk 50 gigabytes groot, om een vergelijking te geven, dit is zo groot als een collage van 20.000 iPhone foto's.

Digitale afbeeldingen kunnen automatisch worden geanalyseerd. Het analyseren van kleine afbeeldingen kan goed met software. Relatief weinig softwarepakketten zijn er beschikbaar voor het analyseren van volledig gedigitaliseerde coupes (die dus

groot zijn). De softwarepakketten die het wel kunnen hebben allemaal aanzienlijke nadelen en voldoen niet aan onze behoeften. Ze zijn duur, traag en meestal zijn de resultaten van de gepatenteerde software maar matig met ons biobank weefsel. Er is met de huidige softwarepakketten geen mogelijkheid om op grote schaal of door meerdere computers tegelijkertijd de analyses uit te laten voeren.

Hoofdstuk 3 bevat een belangrijk onderwerp van dit proefschrift, namelijk de ontwikkeling van de software voor het automatiseren van de analyse van volledig gedigitaliseerde coupes. We noemen het pakket dat we hebben ontwikkeld de slideToolkit. De slideToolkit is een nieuwe methode om accuraat en efficiënt de volledig gedigitaliseerde coupes in grote hoeveelheden te analyseren. De slideToolkit maakt het mogelijk om op grote schaal nauwkeurig weefsel te analyseren en de gegenereerde data te verwerken. De hoge precisie maakt kwantificering met bijvoorbeeld DNA onderzoeken mogelijk en dit was met traditionele handmatige methoden niet haalbaar. In hoofdstuk 4 laten we zien dat de slideToolkit een betrouwbare reproduceerbaarheid toont (dat je het zelfde resultaat krijgt als je meerdere keren de zelfde meting doet) met een Cohen-kappa-waarde van 0,92 tot 0,97 (vrijwel perfecte overeenkomst tussen de metingen) vergeleken met een reproduceerbaarheid van 0,55 tot 0,69 (matige overeenkomst tussen de metingen) voor handmatige weefselanalyse.

Deze nieuwe methode heeft een zeer hoge reproduceerbaarheid. De nauwkeurigheid van deze methode kan niet goed worden beoordeeld. Telt de slideToolkit echt wat hij moet tellen?. Er is namelijk geen 'echte' vergelijking beschikbaar om weefsel te kwantificeren. Het meten van 'nauwkeurigheid' is een uitdaging omdat het een subjectieve waarde blijft van wat de 'echte' hoeveelheid van een bepaald kenmerk is (wat is eigenlijk een ontstekingscel?). We kunnen alleen proberen deze 'ware' hoeveelheid zo goed mogelijk te benaderen door de reproduceerbaarheid van herhaalde metingen te beoordelen, wat een maat voor precisie is. Bovendien is precisie ook afhankelijk van de marker die wordt gebruikt om kenmerken in het weefsel zichtbaar te maken (de bruine kleur met de blauw achtergrond). Vanwege de reproduceerbare eigenschappen kan de slideToolkit op betrouwbare wijze weefselhistologie kwantificeren voor onderzoeksdoeleinden.

De slideToolkit is een oplossing voor een al lang bestaand probleem. De slideToolkit biedt een opensource verzameling van krachtige en veelzijdige software tools voor geautomatiseerde analyse van volledig gedigitaliseerde coupes. De slideToolkit is gratis beschikbaar (MIT-licentie) op GitHub. Het maakt gebruik van een eenvoudige programmeertaal en betrouwbare softwarebibliotheken. Je kan

projecten makkelijk opschalen en het werkt net zo gemakkelijk op een simpele RaspberryPi als op een supercomputer.

Voor hoofdstuk 5 gebruiken we de slideToolkit om ontstekingscellen te analyseren in aneurysmata van de aorta, de knie slagader en de slagader van de lies. Wij vinden een verschil in ontstekingsreactie waarbij de aorta een ander beeld laat zien dan slagaders van de knie en de lies. Nu is de vraag of we naar het zelfde ziektebeeld kijken op een ander tijdstip in het ziekteproces of naar twee verschillende ziektebeelden.

Halsslagader

Hoewel aneurysmata van de halsslagader zeldzaam zijn kunnen ze een aanzienlijke morbiditeit hebben, namelijk 50% van de patiënten krijgt een beroerte. Net zoals AAA's is het ziekteproces en het natuurlijk beloop van aneurysmata van de halsslagader grotendeels onbekend. Als we begrijpen hoe deze ziekte zich ontwikkeld kunnen we de morbiditeit verbeteren. In hoofdstuk 6 onderzoeken we met de handmatige methode de weefsel kenmerken van aneurysmata van de halsslagader.

Een slagader bestaat uit drie verschillende lagen die strak tegen elkaar aanliggen (de intima, de media en de adventitia). Tijdens het onderzoek naar aneurysmata van de halsslagader vonden we twee verschillende type aneurysmata; aneurysmata met tekenen van dissectie (bij een dissectie zijn er lagen in de vaatwand van elkaar losgescheurd) versus degeneratieve aneurysmata (waarbij de sterke structurele eiwitten in de lagen in de vaatwand worden afgebroken). Degeneratie is een algemeen erkende oorzaak van aneurysma-vorming. Niet-dissectieve oorzaken van aneurysmata zijn nog nooit eerder aangetoond buiten de lichaamsslagader (bijv. halsslagader of knie slagader) en waren nog nooit eerder bevestigd met weefsel onderzoek. In de halsslagader zien we degeneratieve aneurysmata bij de meeste patiënten (tien van de dertien). Het kan zijn dat de twee verschillende categorieën (dissectie en degeneratie) verschillende stadia van hetzelfde ziektebeeld. We zien ontstekingscellen in beide typen en dit suggereert dat een ontstekingsproces een rol speelt. Alle dertien patiënten met aneurysmata van de halsslagader zijn chirurgisch behandeld. Chirurgie is niet de enige beschikbare behandeling.

Nogmaals, aneurysmata van de halsslagader zijn zeldzaam, in totaal zijn er ongeveer 1000 gevallen zijn er gemeld in de internationale literatuur. Op de korte en lange termijn laat de chirurgische behandeling en de endovasculaire behandeling via het

bloedvat goed resultaat zien met een laag sterfte cijfer en ook het aantal beroertes na dit soort operaties is laag. Dit ondersteunt de aanname dat deze invasieve behandeling (zoals chirurgie en via het bloedvat) een beroerte zou kunnen voorkomen. Er is weinig informatie beschikbaar over de juiste behandeling van deze aneurysmata. Vaak hebben onderzoeken weinig patiënten geincludeerd en ontbrekende gegevens en verder speelt publicatie bias een rol (onderzoeken met een mooi resultaat worden eerder geplubliceerd dan onderzoeken met een minder mooi resultaat). Bovendien is er in de beschikbare literatuur weinig hard bewijs en daarom is elke aanname van het effect van operatie weinig betrouwbaar.

In hoofdstuk 7 hebben we alle beschikbare literatuur over het risico van complicaties en lange termijn uitkomsten onderzocht. We hebben gekeken naar conservatieve (met alleen medicatie) of invasieve (chirurgie of behandeling via het bloedvat) behandeling van patiënten met aneurysmata van de halsslagader. We vonden een afwachtend beleid (dus niet opereren, conservatief) gerechtvaardigd in een aneurysmata van de halsslagader die stabiel, klein en asymptomatisch (zonder ziekte verschijnselen) is. Ondanks de vaak optredende hersenzenuw beschadiging (zoals ook in onze studie), is de korte en lange termijn uitkomst van een chirurgische behandeling voor aneurysmata van de halsslagader gunstig. De endovasculaire benadering (via het bloedvat) is nog in ontwikkeling en zou in het voordeel van chirurgische reparatie kunnen zijn vanwege de veel lagere incidentie van zenuwbeschadiging. Het groeperen van alle gevonden hals slagader aneurysmata in één studie zou ons kunnen helpen om een betere behandeling voor deze patiënten te bieden. Een internationaal register zou een beter inzicht kunnen geven in de optimale behandeling van aneurysmata van de halsslagader. Aan de andere kant maakt de zeldzaamheid en de wisselende presentatie van deze ziekte het logisch dat voor elke patient persoonlijke en aangepaste zorg krijgt.

'Big data'

Vóór het jaar 2000 werden gegevens voornamelijk opgeslagen als een analoog signaal op een medium, bijvoorbeeld kranten, boeken, platen, VHS-tapes en cassettes. Sinds het millennium is er een exponentiële groei van goedkope digitale opslag. Gorden Moore voorspelde dat de capaciteit van elektronische circuits (zoals chips die gebruikt worden voor digitale opslag) ongeveer elke twee jaar zou verdubbelen. Tot nu toe is de wet van Moore nog steeds van toepassing voor digitale

opslag en voor computer rekenkracht. Het verzamelen en analyseren van data wordt steeds toegankelijker.

Hoe meer gegevens we verzamelen, hoe moeilijker het is om waardevolle informatie uit die gegevens te halen. Er wordt aangenomen dat meer data waarschijnlijk ook meer nuttige informatie bevat. Als onderzoeker ben je op zoek naar manieren om die waardevolle informatie te vinden. Tegenwoordig worden de datasets zo groot en zo complex dat de oude traditionele manieren om met data om te gaan ontoereikend worden. Er is een moment dat we niet langer over 'data' praten, maar we de term 'big data' gebruiken. Big data vereist vaak nieuwe en onbekende analysemethoden, methoden die alleen van toepassing zijn op die unieke dataset. Vanwege de complexiteit van de enorme hoeveelheden data kan je vaak door de bomen het bos niet meer zien.

Tijdens dit onderzoek viel mij op dat er hiaat was tussen de wetenschap, dataverwerking (en schrijven van software) en het klinische werk. Elke onderzoeker wordt verwacht om zich te ontwikkelen in deze verschillende vaardigheden. Ik denk dat dit voor de lange termijn niet haalbaar is en dat er een verandering in denkpatroon of opvattingen nodig is. Elk obstakel moet als een uniek obstakel benaderd worden, elk probleem vergt een eigen oplossing. In het huidige klimaat is er voor de onderzoekers een sterke behoefte aan de ontwikkeling van vaardigheden om hier goed mee om te gaan. Van onervaren onderzoekers wordt verwacht voldoende kennis hebben van software, hardware en statistiek, maar als je enige stuk gereedschap een hamer is behandel je elk probleem als een spijker. Ik denk dat we op grote schaal onderschatten welke vaardigheden nodig zijn om met dit soort gegevens om te gaan. Met dit in het achterhoofd, hoe betrouwbaar zijn de resultaten die we uit de 'big data' halen? Uiteindelijk willen we dat de patiënt de beste zorg krijgt, want daar gaat uiteindelijk het om.

Appendix

Review committee

Dankwoord

Curriculum Vitae

Review Committee

Prof. Dr. R.L.A.W. Bleys
Department of Anatomy
University Medical Center Utrecht
Utrecht, The Netherlands

Prof. Dr. G.J. de Borst
Department of Surgery
University Medical Center Utrecht
Utrecht, The Netherlands

Prof. Dr. P.J. van Diest
Department of Pathology
University Medical Center Utrecht
Utrecht, The Netherlands

Prof. Dr. D.P.V. de Kleijn
Department of Surgery
University Medical Center Utrecht
Utrecht, The Netherlands

Prof. Dr. W. Wisselink
Department of Surgery
VU University Medical Center Amsterdam
Amsterdam, The Netherlands

Dankwoord

Zonder de bijdrage van velen zou dit proefschrift niet tot stand zijn gekomen. Daarom wil ik op deze plaats iedereen bedanken die direct en indirect een bijdrage heeft geleverd aan dit proefschrift. Naast alle patiënten die hun bijdrage hebben geleverd aan dit onderzoek wil ik nog een aantal personen in het bijzonder bedanken.

Prof. dr. F.L. Moll, beste professor, tijdens een wetenschappelijke stage heeft u aan mij gevraagd of ik dit promotie onderzoek wilde doen en daar ben ik u zeer dankbaar voor. Ik waardeer u ontzettend voor uw geduld, uw sympathie en uw toegankelijkheid. Dank alle kansen die u voor mij heeft gecreëerd en voor alle keren dat u met Rob 'een eitje heeft geklutst'. Mede uw vermogen om mij te laten focussen en uw charismatische vermogen om ideeën te creëren hebben geleid tot dit boekje. Hartelijk dank.

Prof. dr. G. Pasterkamp, beste Gerard, wat heb jij mij veel geleerd in tijdens mijn onderzoek. De energie waarmee jij wetenschap benaderd en drive om goed onderzoek te doen is aanstekelijk. Zolang het plan goed is mag alles, niets is te gek. Jij denkt in mogelijkheden. Toen ik voor het eerst succesvol 10 weefsel coupes met de computer had geanalyseerd verbaasde het mij niet dat je de gehele biobank wilde scannen (20.000 coupes). Met erg veel respect heb ik altijd gekeken met het gemak hoe je de juiste mensen bij elkaar weet te zetten en zo de zaadjes plant voor nieuwe ideeën. Mede door jou als promotor is dit voor mij een zeer leerzaam promotietraject geweest.

Beste J.A. van Herwaarden, beste Joost, dank voor alle kopjes koffie die we samen hebben gedronken. Ik heb jou klinische blik op het vrij basale onderzoek erg gewaardeerd. "Bas, is dit klinisch relevant?". Verder kon ik de discussie over de problemen van het echte leven erg waarderen; welke Mac moet er gekocht worden en hoeveel RAM geheugen moet er in? Hoe krijg je drie autostoeltjes naast elkaar op de achterbank? Joost bedankt, en zoals jij vrijwel alle WhatsApp berichten beantwoord:👍

Leden van de promotiecommissie: prof. dr. R.L.A.W. Bleys, prof. dr. G.J. de Borst, prof. dr. P.J. van Diest, prof. dr. D.P.V. de Kleijn en prof. dr. W. Wisselink, dank voor het toetsen van mijn proefschrift en het plaatsnemen in de promotiecommissie.

Jantien, wat hebben wij veel geleerd, zeker in het begin was alles nieuw. Ik vond het leuk om samen te werken en ik waardeer jouw drive voor perfectie. Ik denk dat we samen twee mooie artikelen hebben geschreven.

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Beste Torenbewoners: Amir, Crystel, Dave, Guus, Herman, Joyce, Sander, Saskia, Quirina en Vincent. Wat fijn dat ik met jullie onderzoek heb mogen doen en dat jullie altijd in waren voor een potje Ticket to Ride of een potje basketbal op één van de lege verdiepingen van van Geuns-gebouw. Ik heb stuk voor stuk waardering gehad voor jullie, ik denk dat we daar met een unieke onderzoeksgroep zaten en dat we mooie dingen hebben gedaan. Natuurlijk ben ik jullie dankbaar voor het aanhoren van al mijn ongevraagde Linux en Apple adviezen ;)

Nikolas Stathonikos, thanks for all your help with the scanner. You were one of the few guys that understood my frustrations when things went wrong and than helped me with good advice, thanks! Beste ICT-dienst, ik wil mijn excuses aanbieden voor het ongeautoriseerd gebruik maken van het ziekenhuis netwerk tijdens het eerste jaar van mijn onderzoek, is de beveiliging nu beter op orde?

Paranimfen! Beste Jurjen en Maarten. Ik ken jullie nu zeker 25 jaar en heb altijd op een onvoorwaardelijke vriendschap kunnen rekenen. Wat geweldig dat jullie vandaag naast mij staan.

Lieve papa en mama, ik ben enorm dankbaar voor de onbezorgde jeugd die ik heb gehad en voor de eindeloze mogelijkheden die jullie mij hebben gegeven. Dankzij

jullie steun heb ik altijd kunnen doen wat ik graag wilde en ben ik geworden wie ik nu ben. Ik heb fantastische ouders en het geeft mij veel voldoening dat we zoveel met elkaar hebben kunnen mee maken.

Lieve Hankie, voor ons is dit boekje meer dan alleen een proefschrift. In deze periode hebben wij veel voor onze kiezen gehad en, jij en ik samen, zijn hier ijzersterk doorheen gekomen. Het is een periode waarin wij het ultieme geluk hebben meegemaakt met de geboorte van Guust, Sophie en Pim, en natuurlijk ons trouwen. Jou bijdrage aan dit boekje is enorm, zonder jou was ik waarschijnlijk nog steeds bezig. Jij geeft mij meer geluk dan ik kan wensen. Doeshi, ik zou niet weten wat ik zonder jou zou moeten.

Serre - Zwarte humor & mannen in het wit. *'Open AAA repair'*

Curriculum Vitae

Bastiaan Gijsbert Leo Nelissen is op 19 februari 1981 in Den Ham geboren als tweede kind van Wilma en Bruno Nelissen (1987†). Tijdens de lagere school is Wilma met Aart van Rheineck Leyssius hertrouwd en niet veel later verhuizen ze naar Nijverdal. Hij is de middelbare school begonnen in Almelo en heeft de mavo (1995), de havo (1997) en het vwo (1999) afgerond in Zwolle.

In Utrecht is Bas de studie biologie gestart. Bij de derde keer is hij ingeloot, en gaat dan geneeskunde studeren (2002-2012). Naast zijn studie heeft hij uitgebreide interesse voor programmeren en dit resulteerde in een eigen software bedrijf. In het laatste jaar van zijn studie kreeg Bas een unieke mogelijkheid om zich te ontwikkelen als onderzoeker onder de begeleiding van dr J. van Herwaarden, professor G. Pasterkamp en professor F. Moll. Deze uitdaging waarin vertrouwen en samenwerking voorop stonden resulteerde in dit proefschrift. Het artikel over de histiologische kwantificering van cellen is een uitstekend voorbeeld over de kracht van het combineren van geneeskunde en informatietechnologie.

Na deze onderzoeksperiode heeft hij een brede ervaring opgedaan als arts-assistant (chirurgie Erasmus Rotterdam 2014-2015, psychiatrie Meander Amersfoort 2015-2016, huisartsopleiding Utrecht 2016-2017). Deze unieke combinatie komt samen in zijn huidige baan als arts-assistent op de spoedeisende hulp in het Gelderse Vallei ziekenhuis in Ede. De brede belangstelling in het menselijk lichaam en zijn autodicatische achtergrond op het gebied van informatica en programmeren zullen, net als dit proefschrift, een verrassende bijdrage leveren aan de huidige gezondheidszorg.

Bas is getrouwd met Hankie en zij wonen met hun kinderen Guust, Sophie en Pim in de Vogelenbuurt in Utrecht.