Calcification-induced vascular disease:

insights from pseudoxanthoma elasticum

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CHAPTER 1

General introduction

Bone formation is not restricted to the bones.[1] This counterintuitive statement reflects an ongoing process that is actively prevented. Calcium and phosphate have a strong tendency to precipitate. This physiologically occurs in bone, teeth and certain parts of cartilage in the form of hydroxyapatite, a natural, stable mineral of calcium phosphate.[2] To prevent the whole human body to rapidly change into a calcium crystal, several inhibiting systems actively prevent ectopic mineralization, the calcification of other tissues.[3, 4] Despite these inhibiting systems it seems impossible to totally prevent ectopic mineralization, particularly given the increasing human life span due to ageing. Ectopic mineralization, such as arterial calcification, is common with ageing, and has a more rapid onset in prevalent conditions such as diabetes mellitus and renal failure.[5] This calcification eventually leads to bone formation. Arterial wall tissue can change into actual bone tissue after phenotypical change of vascular smooth muscle cells into osteoblasts. [6]

Calcification-induced vascular disease

Arterial calcification and vascular disease were already present in our ancient ancestors. Among ancient Egyptian mummies arterial calcification was common, particularly in those who died at older ages.[7] Figure 1 shows a computed tomography (CT) image of the upper legs of the Egyptian mummy Hatiay, a scribe during the 18th Dynasty (1550–1295 BC), who died at the age of 45. Extensive calcification in both femoral arteries are present.

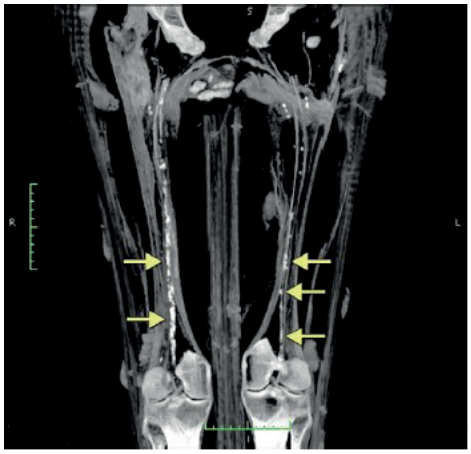

Figure 1 Computed tomography of the upper legs of an Egyptian mummy showing extensive calcification in the femoral arteries

Image taken from Allam AH, Thompson RC, Wann LS, et al. Atherosclerosis in ancient Egyptian mummies: the Horus study. JACC Cardiovascular imaging 2011; 4(4): 315-27

Over the last centuries the burden of vascular disease in the developed world strongly increased in concordance with changes in diet, lifestyle, and environmental risk factors.[8] Although this burden declined in recent years due to considerable improvement in treatment and prevention, vascular disease still results in an immense disease burden.[8, 9]

Epidemiological studies consistently show that arterial calcification relates to increased vascular risk.[10, 11] Since arterial calcification has an independent relation with vascular disease and improves vascular prediction scores on top of traditional vascular risk factors, it is no longer viewed as merely a marker of high vascular risk.[12] Calcification in itself induces vascular disease. Interestingly, arterial calcifications can be reversible, suggesting that interfering in calcification processes is possible.[13-15] Interfering in the process of arterial calcification may thus be an important target for further vascular risk reduction. However, at the moment, no available preventative treatment for vascular disease targets calcification processes. Also, when targeting arterial calcification it seems important to target the actual vascular disease inducing type of calcification.[5]

Based on the anatomical localization, two types of arterial calcification can be distinguished (figure 2).[16] Intimal arterial calcification (atherosclerotic calcification), calcification of the intimal layer of the arterial wall, occurs in the end-stage of the atherosclerotic plaque formation and may have a function in stabilizing the plaque. Medial arterial calcification (MAC, arteriosclerotic calcification), predominantly localized in the medial layer of the arterial wall, results in arterial stiffening and occurs even in the absence of atherosclerotic lesions.[16-20] In this rough distinction, all non-atherosclerotic calcification are included in the term MAC. This includes calcification of the internal elastic lamina, traditionally a part of the intimal layer, which also occurs in the absence of atherosclerotic lesions, either isolated or as continuum with medial layer calcification.[5, 21] In general it can be stated that calcification of the intimal layer merely represents high vascular risk through plaque formation and subsequent luminal narrowing, whereas MAC is the type of arterial calcification that results in arterial stiffening and in itself induces vascular disease.[5, 16, 22-26] MAC can thus be viewed as the vascular disease inducing type of arterial calcification.

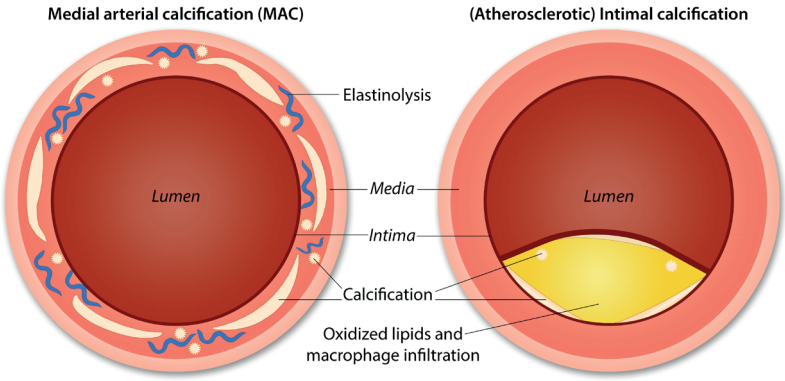

Figure 2 Distinction between intimal arterial calcification and MAC

This distinction between MAC and atherosclerotic calcification has been known for more than a century. In 1903 dr. Mönckeberg was the first to describe typical concentric calcifications in the tunica media of peripheral arteries.[27] Subsequently, in 1908 dr. Buerger showed that this phenomenon is associated with the formation of osteoblasts and osteoclasts and even bone marrow in the arterial wall.[1] During the Second World War Herman T. Blumenthal from the laboratory of the Jewish Hospital in Washington studied the prevalence of MAC in 60 human aortas. It was found that MAC was more prevalent than intimal changes and that its prevalence increased with age and was higher among hypertensive subjects.[28]

Since these early findings, MAC has long been viewed as innocent making it unsurprising that little research was performed into MAC and its effects.[5] However, the rise in arterial stiffness research during the last years automatically renewed interest in MAC.[5, 26] Novel evidence showed that MAC is not only more prevalent, but also more important in the pathogenesis of vascular disease, especially of heart failure, cerebrovascular disease, and peripheral artery disease, than previously thought.[5, 29-34] A study into the histopathology of peripheral artery disease in specimens of leg amputees, showed that 72% of the lower leg arteries contained MAC without atherosclerotic lesions, whereas <25% contained atherosclerotic lesions.[30] Another recent study in patients who were administered for cerebral autopsy showed that in the intracranial internal carotid artery MAC (in the form of internal elastic lamina calcification) is the predominant type of arterial calcification in 71% of the arteries.[34]

Controversy on the clinical importance of MAC remains. Although MAC is now an established contributor in the pathogenesis of vascular disease, little is known

about the causes of MAC or about the mechanisms through which MAC leads to vascular disease. Proposed mechanisms through which MAC induces vascular disease include high arterial stiffness induced pulse pressure damage (water hammer effects) and hemodynamic changes.[5, 35, 36] The fact that it is hard to study the clinical consequences of MAC in living subjects probably contributes to the controversy on the clinical importance of MAC. In clinical practice, intimal wall disease (atherosclerosis) and MAC are often seen simultaneously. Effects of MAC are frequently studied in common conditions associated with MAC, such as diabetes mellitus and renal failure, conditions typically known to have a mixed pattern of intimal and medial wall disease.[5, 37-40] Arterial calcifications located in the intracranial internal carotid, breast, and femoral arteries are generally used for this purpose since these arterial calcifications are assumed to predominantly consist of MAC.[5, 30, 34, 41] However, obviously, interference of intimal arterial wall disease cannot be excluded. Therefore, although radiological techniques nowadays may more efficiently differentiate between the two types of arterial calcifications,[34] it remains difficult to distinguish between intimal calcifications and MAC. This is further complicated by the fact that these two processes have a complex interplay and might amplify each other rather than have an isolated impact.[42] This complex interplay is only partly understood.[29]

Research in diseases with a more isolated type of MAC may provide novel insights in the causes and clinical consequences of MAC and calcification-induced vascular disease. In addition, etiological research into the effects of novel or established risk factors among patients at high risk of (calcification-induced) vascular disease, may enhance knowledge on the complex interplay between MAC and atherosclerosis, and how these processes induce vascular disease.

Calcification-induced vascular disease in pseudoxanthoma elasticum

A recent case. An 11-year-old girl visited the dermatologist because of yellowish papules on the side of her neck (figure 3A). The dermatologist recognized the yellowish papules as pseudoxanthomas, performed a skin biopsy and diagnosed her with pseudoxanthoma elasticum (PXE). Two years later she developed cramps, pain and heavy feelings in her legs, which especially occurred during her soccer training or soccer matches. As a result, she could not meet up with her teammates and had to be substituted more often during soccer matches. She was referred to the UMC Utrecht, where the vascular-internist suspected her of peripheral artery disease. A treadmill test and subsequent ankle-brachial index (ABI) measurements confirmed the diagnosis (figure 3B). An additional CT scan of the legs showed presence of some calcifications in the leg arteries. Her claudication diminished after initiation of walking therapy. Given the frequent ophthalmological involvement of PXE, she was referred to an ophthalmologist, even though no ophthalmological complaints were present. Retinal imaging showed a typical ophthalmological phenotype of PXE with peau d'orange of the retina and angioid streaks.[43]

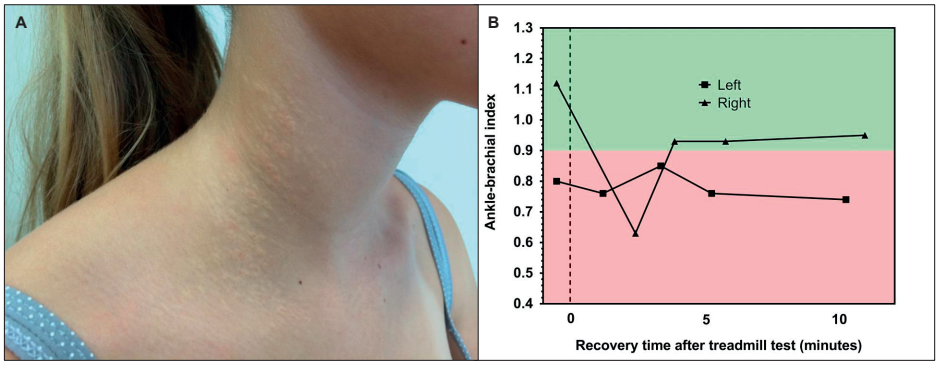

Figure 3 A: Pseudoxanthomas on the side of the neck of an 11-year-old girl with PXE. B: Results of treadmill test and ankle-brachial index measurements confirming the presence of peripheral artery disease. Ankle-brachial index <0.9 is considered abnormal. Printed with permission.

This clinical case illustrates that in PXE (OMIM #264800), a rare autosomal recessive systemic calcification disorder, arterial calcifications and vascular disease can occur at young age.[43-45] Arterial calcifications in PXE are thought to predominantly occur in the medial layers of medium and small sized arteries.[44-50]

Although it is clear that PXE is a rare disease, estimations of its prevalence vary widely (1:25,000 to 1:100,000).[51] PXE has a considerable morbidity, including severe visual impairment and blindness, peripheral artery disease, ischemic stroke, and vascular dementia.[51, 52] In general, it is a relatively late-onset disease with first symptoms mostly around the age of 40 years. However, as the clinical case illustrates, clinical expression of PXE varies widely.[44] Given the relatively young age in which MAC and vascular disease occur in PXE, little interference of atherosclerosis can be expected. PXE may therefore have an isolated type of MAC or arteriosclerotic arterial wall disease, making it a potential model disease for MAC and calcification-induced vascular disease.

Unfortunately, vascular involvement in PXE is somewhat unrecorded and poorly understood.[45, 50] The clinical characteristics of PXE follow a classical triad with skin involvement (e.g. yellowish papules or plaques), eye involvement (e.g. angioid streaks) and vascular involvement (arterial calcification).[51, 52] In 1940, the vascular component was the last component that was connected to PXE completing the triad.[53] Skin manifestations were first documented in 1881 by the French dermatologist Rigal[54], but it was dr. Darrier, who in 1896 first described the typical changes in elastin fibers in a skin biopsy. [55] It took until 1929 when the Swedish ophthalmologist Esther Grönblad and dermatologist Strandberg described a syndrome consisting of typical skin and eye manifestations.[56, 57] The term "Darriers disease" was substituted by the term "Grönblad-Strandberg syndrome" afterwards, which is now preferably replaced by the term "pseudoxanthoma elasticum" (PXE). The French dermatologist Touraine described the vascular manifestations of PXE for the first time.[53] Firm proof for PXE as multi-organ or systemic disorder was given in 1963 by dr. Goodman who made clinical and histopathological descriptions of 12 patients with the PXE triad.[58]

Relatively little has been added to the knowledge on the vascular phenotype of PXE ever since. The precise vascular beds involved in PXE or the precise physiological consequents of these arterial abnormalities remain unknown. Incidental descriptions report coronary arterial disease, cerebral arterial disease and especially peripheral artery disease (PAD).[59] Several case reports about brain abnormalities (mostly white cerebral matter abnormalities) in patients with PXE exist, but it is unclear to what extent this is part of the (vascular) phenotype of PXE.[60-64] Moreover, although arterial calcifications in the legs are prevalent in PXE and coincide with peripheral artery disease, the pathogenesis of peripheral artery disease in PXE remains not well understood.[45] Lastly, it remains unknown whether the treatment

of vascular disease in PXE should be different from treatment of vascular disease in non-PXE populations.

Pyrophosphate – an important inhibitor of calcification-induced vascular disease

Although little progress has been made in deciphering the vascular phenotype of PXE, major steps have been made in deciphering the etiology of PXE. First, in 2000, three research groups independently showed that mutations in the ATP-Binding Cassette Transporter C6 (*ABCC6)*-gene lead to mineralization of elastic fibers throughout the body and cause PXE.[65-67] PXE is inherited exclusively in an autosomal recessive manner. A dominant inheritance pattern was ruled out. [68, 69] This implies that PXE patients are either homozygous or compound heterozygous for mutations in the *ABCC6*-gene.

The second breakthrough came from a Dutch research group, who recently identified inorganic pyrophosphate (PPi) as the factor that normally prevents PXE.[70] *ABCC6*-mutations were shown to result in inefficient mediators of ATP secretion in the liver causing low levels of inorganic pyrophosphate (PPi).[70] PPi is, as part of the previously mentioned network of inhibiting systems, a strong inhibitor of ectopic mineralization.[3, 71, 72] The >60% decreased levels of PPi that are found in human PXE patients may very well cause the ectopic mineralization occurring in this disease.[73]

For some years, PXE has been viewed as part of a spectrum of several (rare) systemic calcification disorders that share a considerable overlap in phenotype.[74-77] With the finding of PPi as the causative factor of PXE it can be showed that these diseases also have an overlap in pathogenesis.[78] Table 1 summarizes associated genes and typical clinical features of several calcification disorders that all have been shown to have a deficiency in PPi or in the strictly regulated PPi/phosphate balance. Further studying these rare calcification disorders, of which PXE seems to be the most prevalent, could significantly enhance knowledge on the clinical expression of MAC.

Besides PPi, the inhibiting systems that prevent ectopic mineralization and thus prevent calcification-induced vascular disease include the vitamin K-dependent Matrix Gla Protein (MGP), the Klotho protein, osteoprotegerin, osteopontin, and fetuin-A.[3] Increasing the concentration of ectopic mineralization inhibitors may result in ectopic mineralization prevention and subsequent inhibition of disease progression, even in populations without a specific calcification disorder.[15, 73, 79-85] Given the fact that the absence of PPi is thought to be the main driver of ectopic calcification in PXE, supplementation of PPi seems to be an obvious treatment

of calcification-induced vascular disease. [86] Indeed, intraperitoneal supplementation of inorganic pyrophosphate was found to inhibit mineralization in rodent models.[87, 88] However, short plasma half-life and lack of a suitable dosage form make it an unattractive candidate for supplementation therapy in humans.[89]

Table 1

Disease	OMIM	Gene(s)	Phenotype
Pseudoxanthoma elasticum (PXE)	#264800	*ABCC6*	Calcification of skin, eyes, and vascular system
Generalized arterial calcification of infancy (GACI)	#208000	*ENPP1, ABCC6*	(Severe) arterial calcification, joint and spine ossification
Calcification of joints and arteries (CALJA)	#211800	*NT5E*	Vascular and joint calcification
Idiopathic basal ganglion calcification (IBGC1)	#213600	*SLC20A2, XPR1, PDGFRB, PDGFB*	Vascular and pericapillary calcifications in the brain
Hutchinson-Gilford progeria syndrome (HGPS)	#176670	*LMNA*	Premature aging with calcification of aorta and aortic valves
Hyperphosphatemic familial tumoral calcinosis (HFTC)	#211900	*KL, GALNT3, FGF23*	Calcification of skin, placental and femoral arteries, periarticular tissue
Singleton-Merten syndrome	#182250	*IFIH1, DDX58*	Arterial and aortic valve calcification, premature loss of secondary teeth, glaucoma, skeletal abnormalities
Keutel syndrome	#245150	*MGP*	Arterial and cartilage calcification
Gaucher disease, type IIIC	#231005	*GBA*	Arterial calcifications

Adapted from Nitschke Y, Rutsch F. Inherited Arterial Calcification Syndromes: Etiologies and Treatment Concepts. Current osteoporosis reports 2017; 15(4): 255-70. OMIM: Online Mendelian Inheritance in Man; an open-source online catalog of human genes and genetic disorders.

Bisphosphonates as potential treatment of calcification-induced disease

Bisphosphonates are well-established drugs for the treatment of osteoporosis and bone metastases. In the Netherlands, about 250,000 people use bisphosphonates and the most prescribed bisphosphonate alendronate is ranked 44 in the list of most provided medications in the Netherlands (Dutch pharmaceutical databank, GIPdatabank 2016). For more than 40 years, the anti-bone resorptive effects of bisphosphonates are used to treat osteoporosis and prevent fractures.[90] While frequently using their powerful osteoclast inhibiting effects, we seem to have forgotten their earlier documented effects on ectopic mineralization.[91] After their discovery

the first use of bisphosphonates was of non-medical nature. Between 1800 and 1900 bisphosphonates were used to prevent calcification of waterpipes and to soften water.[90] Subsequently, bisphosphonates have been shown to reduce soft tissue calcifications in rats even before their effect on bone resorption was known.[92] In fact this is not surprising looking at the chemical structure of bisphosphonates. Bisphosphonates are stable PPi analogues and could thus stimulate the inhibitory effects on ectopic mineralization, such as arterial calcification.[93, 94] Of the currently available bisphosphonates non-nitrogen containing bisphosphonates, such as etidronate, may have the largest potential to delay ectopic mineralization given their predominant inhibition of calcium precipitation and hydroxyapatite binding. This is different from newer, nitrogen-containing bisphosphonates, such as alendronate, which predominantly inhibit osteoclasts.[84, 95]

Some non-randomised and uncontrolled reports describe beneficial effects of etidronate in patients with calcification disorders mentioned in table 1. As illustrated in figure 4, in GACI etidronate treatment reduces arterial calcification.[15] Also, etidronate in GACI is associated with improved survival.[15, 85] In patients with basal ganglia calcifications or primary brain calcifications treatment with etidronate alleviates neurological symptoms.[83, 84] Also, an ongoing clinical trial (NCT01585402) is now investigating effects of etidronate treatment on ectopic mineralization in patients with CALJA.

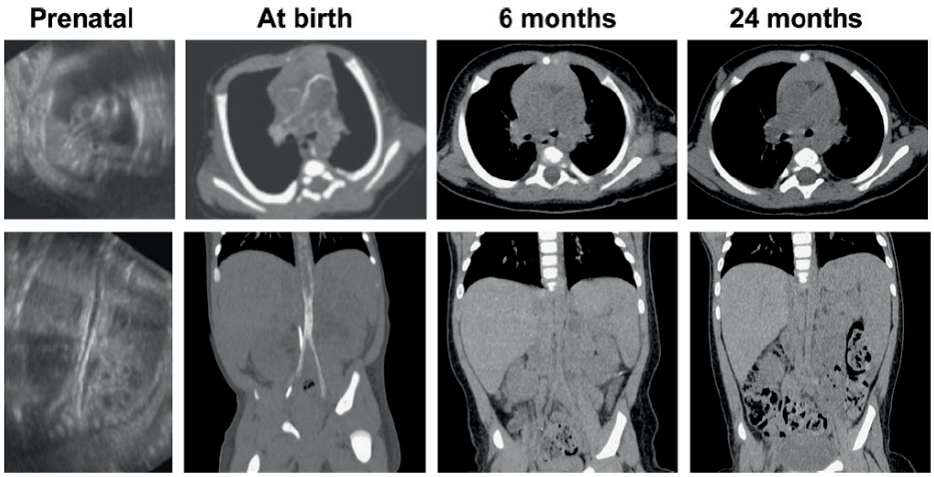

Figure 4 Resolution of arterial calcifications in aortic iliac vessels in a child with GACI treated with the etidronate. Illustration taken from Edouard et al. Eur J Pediatr 2011;170:1585-1590.

Some small randomised controlled trials have already established beneficial effects of etidronate on MAC and arterial wall disease in renal failure, diabetes mellitus and hypercholesterolemia patients.[14, 96-98] Also, previously performed large bisphosphonate trials may provide indications that bisphosphonates prevent vascular morbidity and mortality. For instance in the HORIZON trial, including 2,127 patients with a recent hip fracture, the bisphosphonate (zolendronic acid) group had an 11 % reduction of vascular events and 31% reduction of vascular mortality compared to placebo group.[99]

The finding of PPi as causative factor of ectopic mineralization and calcification-induced disease in PXE opened up the possibilities for treatment with etidronate, a stable PPi analogue. Therefore, the effects of bisphosphonates were studied in PXE mice models. In *ABCC6* knock-out mice etidronate but not alendronate significantly reduced mineralization suggesting a potential effect of etidronate in the treatment of PXE. Treatment with etidronate was also associated with alterations in bone micro-architecture.[80] Further investigations into the effects of etidronate in PXE mice models were performed in both young and older *ABCC6* knock-out mice. It was shown that etidronate prevents, but does not reverse ectopic mineralization.[79] The effectiveness of etidronate remains to be established in PXE patients in a randomised, placebo-controlled trial.

Hence, bisphosphonates, in particular etidronate, have a large potential to prevent calcification-induced vascular disease in humans. However, their effects on ectopic mineralization and on vascular morbidity and mortality remain to be established in randomised controlled trials, both in PXE and non-PXE populations. Probably in non-PXE populations, diabetes mellitus and renal failure patients may benefit most from the potential effects of bisphosphonates, since these populations have a higher risk of MAC and vascular disease.[5] Etidronate has been on the market for almost 40 years and has been frequently used. Etidronate has been shown to have an acceptable safety profile and is easily available at low cost.[90] Safety of bisphosphonates in specific populations as PXE populations remains to be established. Severe, but reversible, skeletal adverse events have been reported in patients with GACI after long-term treatment with etidronate.[100]

Objectives of this thesis

The general objectives of this thesis are to describe the clinical expression of calcification-induced vascular disease in patients with PXE, to investigate the effect of risk factors of (calcification-induced) vascular disease in high risk patients, and to

investigate the effects of bisphosphonates as potential treatment for calcification-induced vascular disease.

Outline of this thesis

The first part of this thesis describes calcification-induced vascular disease in PXE patients. First, in **chapter 2** we aimed to revise current estimations on the prevalence of PXE. In **chapter 3** the distribution and severity of arterial calcification in patients with PXE is described and compared to hospital controls using whole body CT imaging. In **chapter 4** we describe histopathological findings in two PXE patients and related these findings to CT findings. In **chapter 5** we describe the physiological effects of PXE on arterial thickening and stiffening by comprehensive arterial wall measurements in PXE patients and comparisons with general population and diabetes mellitus patients. **Chapter 6** describes the prevalence of different types of cerebral disease in a large Dutch PXE cohort. Also, to interpret and possibly generalize our findings outside the Dutch setting, we evaluated the literature by means of a systematic review. In **chapter 7** we aimed to give insights in the prevalence and pathogenesis of peripheral artery disease in PXE by establishing the prevalence of peripheral artery disease and investigating its relation with several determinants. In **chapter 8** we, in collaboration with our French colleagues from the PXE center in Angers, report the results of femoral angioplasties with stenting in four PXE patients in whom progressive peripheral artery disease was present.

The second part of this thesis focuses on the relation between risk factors and (calcification-induced) vascular disease among high risk patients. In **chapter 9** the relation between HbA1c and (recurrent) vascular events or mortality is studied in type 2 diabetes mellitus patients and it is investigated whether this relation is influenced by pre-existing vascular disease. In **chapter 10** the relation between inter-arm differences in systolic blood pressure and vascular events or mortality in high vascular risk patients and the influence of pre-existing vascular disease on this relation is studied.

The third part of this thesis investigates the effects of bisphosphonates on calcification-induced vascular disease. In **chapter 11** we performed a systematic review of previously performed randomized controlled bisphosphonates trials that reported on arterial calcification, arterial stiffness, vascular events, vascular mortality and all-cause mortality and summarized the results in a meta-analysis. Subsequently in **chapter 12** we report the results of the Treatment of Ectopic Mineralization in Pseudoxanthoma elasticum (TEMP) trial, a single-center, randomised, double-

blind, placebo-controlled trial we conducted in the Dutch National Expertise center for Pseudoxanthoma elasticum. The TEMP trial aimed to investigate effectiveness and safety of one year treatment with etidronate (cyclical 20 mg/kg for two weeks every 12 weeks) on ectopic mineralization among participants with PXE.

The main findings of this thesis are discussed in **chapter 13**, placing our findings in a broader perspective and identifying challenges and opportunities for clinical practice and future research. A summary can be found in **chapter 14**.

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PART ONE

Calcification-induced vascular disease in pseudoxanthoma elasticum

CHAPTER 2

The prevalence of Pseudoxanthoma Elasticum: Revised estimations based on genotyping in a high vascular risk cohort.

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Abstract

Background - Pseudoxanthoma elasticum (PXE), an autosomal recessive systemic calcification disorder, is caused by mutations in the *ABCC6*-gene and associated with severe visual impairment and peripheral arterial disease. Given the progress in development of a treatment for PXE, more precise estimations of its prevalence are warranted.

Methods - We genotyped the four most common *ABCC6* mutations (c.3421C>T, c.4182delG, c.3775delT, c.2787+1G>T) together responsible for half of the PXE cases in the Dutch population, in a Dutch high vascular risk cohort (n= 7,893). The obtained allele frequencies were used to estimate the prevalence of PXE using the Hardy-Weinberg equilibrium.

Results - The allele frequency of *ABCC6* mutations was 0.30% for c.3421C>T, 0.08% for c.4182delG, 0.03% for c.3775delT and 0.01% for c.2787+1G>T. The prevalence of PXE based upon the allele frequencies of these four mutations was estimated as 1 per 56,000 (95%CI 1 in 35,000 - 97,000).

Conclusion - The prevalence of PXE is at least 1 per 56,000 meaning that there would be at least 307 affected individuals in the Netherlands. Since this estimate is based on mutations responsible for half of the PXE cases, the actual prevalence will be higher.

Introduction

Pseudoxanthoma elasticum (PXE, OMIM #264800), an autosomal recessive systemic calcification disorder, is characterized by skin (yellowish papules or plaques), eye (angioid streaks) and vascular involvement (arterial calcification). PXE has a considerable morbidity, including severe visual impairment and blindness, peripheral arterial disease, and ischemic stroke.[1] Patients are homozygous or compound heterozygous for mutations in the ATP-Binding Cassette Transporter C6 (*ABCC6)*-gene.[2] A dominant inheritance pattern has been ruled out. [3 4] Although it is clear that PXE is a rare disease, estimations of its prevalence vary widely (between 1 per 25,000 and 1 per 100,000).[1 5 6]

Given the current scientific progress in developing a therapy for PXE,[7] more precise estimations of its prevalence are warranted in order to be able to trace all patients. It has been shown that there is no excess in carriers of the most common *ABCC6* mutation (R1141X, c.3421C>T) among Caucasians with vascular disease.[8] Thus, a cohort including patients with high vascular risk can be used to estimate the prevalence of PXE in the general population.

In this study, we aimed to revise previous estimations of the prevalence of PXE using genotyping of the four most common *ABCC6* mutations in the Dutch population in a large Dutch high vascular risk cohort

Material and methods

The four most frequent mutations in *ABCC6* in the Dutch population (c.3421C>T, c.4182delG, c.3775delT, c.2787+1G>T) were identified based on allele frequencies in our nationwide Dutch PXE cohort (table 1). Data from 170 consecutive PXE patients with available DNA information and a confirmed clinical diagnosis were used for this purpose.[9] These mutations are together responsible for about half of the PXE cases.

In order to estimate the prevalence in the general Dutch population, we genotyped these mutations in available blood samples of patients enrolled in a Dutch high vascular risk cohort (n=7,893), the Second Manifestations of ARTerial disease (SMART) study, a prospective single-center cohort study performed in the University Medical Center Utrecht. Patients eligible for inclusion had manifestations of vascular disease (coronary artery disease, cerebrovascular disease, peripheral artery disease, or abdominal aneurysm) or vascular risk factors (hypertension, diabetes and dyslipidemia). More detailed information about the design of the SMART cohort has been described previously.[10]

DNA was isolated from 10 mL of EDTA (citrate)-augmented blood stored at −80 °C, and amplified with polymerase chain reaction (PCR). Genotyping was performed for the four ABCC6-mutations, using the Kompetitive Allele Specific PCR (KASP™) genotyping system (Hoddesdon, England).

The Hardy-Weinberg equilibrium was used to estimate the prevalence of PXE using the allele frequencies for each of these four mutations in *ABCC6*. 95% CI were obtained using bootstrap methods with 5,000 repeats.

Results

The frequency of ABCC6 carriers was 0.60% (47/7,804) for c.3421C>T, 0.17% (13/7,798) for c.4182delG, 0.05% (4/7,798) for c.3775delT and 0.03% (2/7,832) for c.2787+1G>T (table 1). Using these frequencies, the prevalence of PXE was estimated as 1 per 56,000 (95%CI 1 per 35,000 - 97,000, supplemental table 1).

Table 1 Carrier frequencies ABCC6 mutations and estimated prevalence of PXE

***ABCC6* mutation**	**Type of mutation**	**Allele frequency PXE cohort (n=170)**	**Carrier frequency high vascular risk cohort (n=7,893)**
c.3421C>T	Stop-gain	26.47% (90 / 340)	0.60% (47 / 7,804)
c.4182delG	Frameshift	9.12% (31 / 340)	0.17% (13 / 7,798)
c.3775delT	Small deletion	7.35% (25 / 340)	0.05% (4 / 7,853)
c.2787+1G>T	Splice	6.18% (21 / 340)	0.03% (2 / 7,832)

Estimated PXE prevalence:
1:56,000 (95%CI 1:35,000 - 97,000)

Allele frequency was based on upon frequencies from PXE patients with available DNA information in our PXE cohort (n=170). Carrier frequency was based on frequencies in the SMART, high vascular risk cohort (n=8,063). PXE prevalence estimation was based on the Hardy-Weinberg equilibrium and all possible combinations of these four mutations.

Discussion

Based upon genotyping of the four most common mutations in *ABCC6* in a high vascular risk cohort, we estimate the prevalence of PXE to be 1 per 56,000. We assume that our estimation is applicable to the general population, since the frequency of the most common mutation in *ABCC6* among Caucasians (R1141X, c.3421C>T) was shown not to be different between patients with and without (a high risk of) vascular disease.[8]

The identified allele frequencies in this study are higher than allele frequencies in available data from non-Finnish European individuals in the Genome Aggregration Database (gnomAD, http://gnomad.broadinstitute.org/, supplemental table 2). The gnomAD database provides data on genomes of unrelated individuals sequenced as part of various disease-specific and population genetic studies.[11] However, the fact that this database consists of aggregated and primarily non-Dutch populations makes it an unattractive candidate to use in order to estimate the general population prevalence of PXE, particularly for the Dutch setting.

Also, some of the identified *ABCC6* mutations may be (more) specific to the Dutch population.[12 13] A founder effect has been shown to exist in the Dutch PXE population.[12] No sufficient data was available in Dutch general population databases as the Genome of the Netherlands (GoNL)[14] and the Rotterdam Study cohort.[15] Therefore, the allele frequencies of the most common *ABCC6* mutations determined in the SMART high vascular risk cohort currently provide the most valuable information to estimate the prevalence of PXE in the Dutch general population.

Our estimation underestimates the actual prevalence of PXE. We did not take into account all possible mutations in *ABCC6,* but genotyped the most common mutations among Caucasians which are responsible for half of all PXE cases (table 1).[16] Also, mutations in other genes as the *ENPP1* genes may cause PXE as well.[17] Thus, the actual prevalence of PXE will be higher.

In conclusion, the prevalence of PXE is at least 1 per 56,000 meaning that there would be at least 307 affected individuals in the Netherlands. Since this estimate is based on mutations responsible for half of the PXE cases, the actual prevalence will be higher.

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Appendix

Supplemental table 1 Calculation of the PXE prevalence

ABCC6 mutation	Allele	Unaffected	Carrier	Affected
c.3421C>T	a	AA	Aa or aA	aa
c.4182delG	b	BB	Bb or bB	bb
c.3775delT	c	CC	Cc or cC	cc
c.2787+1G>T	d	DD	Dd or dD	dd

Carrier	Frequency (SMART high vascular risk cohort)	Allele frequency*
Aa or aA	0.60% (47 / 7,804)	a=0.003011276
Bb or bB	0.17% (13 / 7,798)	b=0.000833547
Cc or cC	0.05% (4 / 7,853)	c=0.0002546797
Dd or dD	0.03% (2 / 7,832)	d=0.0001276813

* Calculated using the Hardy-Weinberg equilibrium. No homozygous mutations were found in the SMART high vascular risk cohort

Possible combinations	Frequency
aa	9.07*10^-06
bb	6.95*10^-07
cc	6.49*10^-08
dd	1.63*10^-08
ab	2.51*10^-06
ba	2.51*10^-06
ac	7.67*10^-07
ca	7.67*10^-07
ad	3.84*10^-07
da	3.84*10^-07
bc	2.12*10^-07
cb	2.12*10^-07
bd	1.06*10^-07
db	1.06*10^-07
Total affected	1.78*10^-05 **(1:56,000)**

Supplemental table 2 Allele frequencies in SMART and gnomAD

ABCC6 mutation	Allele frequency SMART	Allele frequency gnomAD (European, non-Finnish)
c.3421C>T	0.301128%	0.275300%
c.4182delG	0.083355%	0.010370%
c.3775delT	0.025468%	0.004176%
c.2787+1G>T	0.012768%	0.022920%

CHAPTER 3

Prevalence and severity of arterial calcifications in pseudoxanthoma elasticum (PXE) compared to hospital controls. Novel insights in the vascular phenotype of PXE

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Abstract

Background and aims – Pseudoxanthoma elasticum (PXE) is a monogenetic disorder with progressive calcifications of the skin, the Bruch's membrane in the eyes and the arterial wall. Vascular disease is considered to be very prevalent, but the whole-body distribution of arterial calcifications in PXE is unknown. We aimed to systematically investigate arterial calcifications in PXE.

Methods – We included 104 PXE patients from the Dutch PXE cohort and 93 hospital controls. All subjects underwent full-body low-dose CT scans without contrast. To investigate the prevalence and severity of arterial calcification per arterial location, the CT scans were scored using a reproducible semi-quantitative scale with four calcification categories (interobserver kappa 0.54-0.99).

Results – PXE patients (38/104 males) were 54 ± 13 years and controls (45/93 males) 54 ± 16 years old. Arterial calcifications were significantly more common in PXE patients in the intracranial internal carotid artery (75% vs. 44%), the arteries of the arms (20% vs. 3%), the femoral-popliteal arteries (74% vs. 44%) and the subpopliteal arteries (84% vs. 38%). In these arteries the calcification scores also indicated more severe calcification. No significant differences in prevalence of arterial calcification were observed in other arterial beds such as the coronary arteries (45% vs. 43%, p-value=0.776), the carotid arteries (52% vs 46%, p=0.476) and the abdominal aorta (71% vs. 63%, p-value=0.287). Analyses using patients younger than 55 years only, showed similar differences in prevalence of arterial calcifications between PXE patients and controls with most pronounced calcifications in the arteries of the lower legs (67% vs. 8%). Similar patterns were observed in those without concomitant diabetes or renal dysfunction.

Conclusions – In PXE a vascular phenotype can be identified with a distribution of arterial calcifications that is clearly distinct from hospital controls and involves arterial calcifications in the legs, the intracranial internal carotid artery and the arteries of the arms.

Introduction

The process of arterial calcification is actively regulated by several inhibitory pathways which suggests that the body is actively preventing the occurrence of arterial calcification.[1] Although much is known about the biology of arterial calcification as well as the prognostic value of arterial calcification, it is unclear whether prevention or treatment of arterial calcification is of any benefit. [2-4] Arterial calcification seems to be reversible and it has been shown that treatment can prevent or even reverse the arterial calcification process. [5-7] Monogenetic disorders might provide novel insights into the cause of arterial calcification and may reveal potential treatment targets.

Pseudoxanthoma elasticum (PXE, OMIM #264800) is a rare monogenetic disease caused by mutations in the *ABCC6* gene [8-10], which leads to calcification of the skin, the eyes and the arterial walls. [11-13] The prevalence of PXE is not precisely known but is estimated to be around 1:25,000-50,000. [11] Recently, major steps have been made in unraveling the etiology of PXE by the discovery that the ABCC6 mutations result in inefficient mediators of ATP secretion in the liver. This results in low levels of inorganic pyrophosphate leading to progressive ectopic calcification. [14]

Several incidental descriptions of PXE patients report prevalent cardiovascular manifestations including coronary arterial disease, cerebral arterial disease and, in particular, peripheral artery disease (PAD). [15] These vascular problems occur due to progressive arterial wall calcification. [16] However, descriptions of the arterial calcification phenotype in PXE are restricted to descriptions of arterial calcifications in the legs in relation to peripheral arterial disease in these patients. [17] It is unclear how arterial calcifications are distributed in the rest of the body in PXE patients and how this arterial calcification pattern differs from control patients.

Hence, the goal of the present study was to describe the distribution and severity of arterial calcification in patients with PXE in comparison with hospital controls using whole body computed tomography (CT) imaging.

Methods

PXE patients

For the present study, clinical data of 104 consecutive adult patients with confirmed PXE, who visited the University Medical Center Utrecht (UMCU), the Netherlands, were studied. For all patients a systematic diagnostic screening pro-

gram was performed which included a genetic, dermatological, ophthalmological and vascular screening. The diagnosis of PXE was based on the revised criteria of Plomp et al.[12] The vascular screening included a low-dose (<3 mSv) full-body CT scan without contrast, performed on CT scanner Brilliance 64 (Philips, Cleveland, Ohio) (for more detailed information on the CT settings see the supplement). Other variables, blood pressure, (current) smoking, renal function and the presence of diabetes were collected in the systematic screening and used for the present study. Renal function was estimated using glomerular renal filtration rate (eGFR) based upon the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) formula and renal failure was defined as an eGFR <30 mL/min/1.73m^2. Diabetes mellitus was defined as a history of diabetes mellitus in the medical files or the use of glucose-lowering agents.

Control patients

In order to compare the distribution and severity of arterial calcification, a hospital control group was used. To ensure comparability, only patients who underwent scans using a similar low-dose and full-body protocol as the PXE patients were selected (supplement). These low-dose CT images were available from fluorodeoxyglucose (FDG) positron emission tomography/computed tomography (PET-CT) scans. All patients who underwent these kinds of scans in our hospital between June 2011 and November 2015 were used as hospital controls. Only patients with a suspicion of vasculitis or endocarditis were excluded.26% of the hospital controls underwent this scan for melanoma staging, 68% because of fever with unknown origin and 6% for a suspicion of malignancy. The variables sex, age, blood pressure, (current) smoking, renal function and the presence of diabetes were derived from the medical files in all control patients as long as these variables were available within 6 months before or after the CT scan.

Systematic scoring of full-body CT scans

A semi-quantitative scoring system was designed to capture the distribution and severity of arterial calcification. The categories in this scoring system included no calcification, mild calcification, moderate calcification and severe calcification. The designed semi-quantitative severity scale was used for the scoring of the extent of vascular calcification per pre-specified arterial bed. These included: the intracranial internal carotid arteries (IICA), the extracranial carotid arteries (ECA), the vertebral arteries, the coronary arteries, the aortic and mitral valve, the thoracic and abdominal aorta, the three mesenteric arteries including side branches as the splenic artery, the arm arteries including brachial, ulnar and radial arteries, the internal iliac arteries, the external iliac arteries, the femoral-popliteal

arteries and the subpopliteal arteries including the arteries in the foot. For all pre-specified locations, the sum of the calcifications in the arterial bed was used to assess the severity of calcification. So, although the arterial calcification tends to occur fairly symmetrical, for paired right and left arteries the sum of right and left was used and for the coronary arteries the sum of the coronaries was used. The categories were defined as 'no calcification' in case of absence of calcification, 'mild calcification' in case of 1 or 2 small calcifications, 'moderate calcification' in case of 3-5 small calcifications and 'severe calcification' in case of more than 5 small calcifications or at least one large calcification on the CT slides of the artery. The origo of the brachiocepahlic arteries was excluded as it can be debated whether these are aortic or side branches.

Calcifications of the cardiac valves were subdivided in aortic valve calcifications and mitral valve calcifications. Mitral annular calcifications were not scored. Valve calcification could either be assessed as no calcification, mild calcification (one single affected leaflet with spotty calcification), moderate calcification (one leaflet with moderate/severe calcification)or severe calcification (multiple leaflets with calcification). The presence of a valve prosthesis was recorded and in case of a valve prosthesis no scoring of the extent of calcification of this valve was performed.

Inter-observer agreement of the designed arterial calcification severity scoring system was good or almost perfect for all arterial beds except for the mesenteric arteries, where the agreement between the observers was reasonable (supplemental table 1). The inter-observer agreement of our scoring system was based upon the systematic scoring of 25 random scans by two board certified radiologists and the inter-observer agreement was quantified by the weighted kappa per arterial bed. [18] One of the two radiologists scored the full number of CT scans in random order blinded for clinical data, including PXE diagnosis.

Data analyses

Descriptive statistics were used to present baseline characteristics (age, sex, renal function, BMI, systolic and diastolic blood pressure). Baseline characteristics were compared between PXE and controls using independent sample t-tests for normally distributed variables. For categorical variables Chi-square statistics were used to calculate p-values for differences between the PXE and control group.

Frequencies and percentages of the extent of calcification group for each arterial bed were calculated and compared between PXE and controls using Chi-square

statistics. Furthermore, stratified analyses were performed in youngest and oldest 50% of the participants of both groups.

To account for possible differences in arterial calcification patterns between PXE patients and control patients due to a potential misbalance between the two groups in the prevalence of diabetes and renal failure, sensitivity analyses were performed excluding participants with renal failure and/or diabetes mellitus from both groups. After exclusion of patients with concomitant diabetes or renal failure from both groups the differences in arterial calcification prevalence and severity per arterial bed were investigated. In addition, to adjust for potential confounders, multivariable adjustment was performed using logistic regression models for each arterial bed with presence of calcification as outcome. Adjustment took place for age, gender, eGFR, systolic blood pressure, BMI, current smoking and diabetes mellitus.

Statistical analyses were conducted using SPSS version 21 and R version 3.1.3.

Ethical committee approval

Given the retrospective use of routine care data no formal approval of this study was required as stated by the Medical Ethics Committee of the UMC Utrecht and informed consent was waived for anonymous analysis of the data (IRB number 15/446-C).

Results

Baseline characteristics PXE patients and control patients

The mean age of the PXE patients was 54 (±13) years and for controls this was 54 (±16) years (p=0.884) (Table 1). Diabetes mellitus was present in 4% of the PXE group versus 8% in the control group (p=0.261). None of the PXE patients had renal failure while six (7%) controls had renal failure (p=0.009). Body mass index (BMI) was similar between PXE patients and controls (p=0.865). Higher levels of blood pressure were observed in PXE patients compared to the hospital controls. Systolic blood pressure was 137 (±23) mmHg in PXE patients and 130 (±21) mmHg in control patients (p=0.062). Levels of diastolic blood pressure were 80 (±12) mmHg and 76 (±12) mmHg, respectively (p=0.009).

Table 1 Baseline characteristics of PXE patients compared to hospital controls

	PXE patients (n=104)	Control patients (n=93)	p-value
Sex (male)	39 (38%)	42 (45%)	0.275
Age	54 ± 13	54 ± 16	0.884
Systolic blood pressure - mmHg	137 ± 23	130 ± 21	0.062
Diastolic blood pressure - mmHg	80 ± 12	76 ± 12	0.009
BMI - kg/m²	25.9 ± 4.6	26.0 ± 6.4	0.865
Current smoking	16 (16%)	19 (20%)	0.220
Diabetes mellitus	4 (4%)	7 (8%)	0.261
eGFR <30 mL/min/1.73m²	0 (0%)	6 (7%)	0.009
Reason for full body CT			
PXE	103 (100%)	0 (0%)	NA
Fever of unknown origin	0 (0%)	63 (68%)	NA
Follow-up for melanoma	0 (0%)	24 (26%)	NA
Suspected malignity	0 (0%)	6 (6%)	NA

Data are reported as the mean ± SD or n (%). BMI, body mass index; eGFR, estimated glomerular filtration rate based on Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) formula. P-values were calculated using Chi-Square statistics for categorical variables. For continuous variables with a normal distribution indendent sample t-test were used.

Prevalence and severity of arterial calcification

In PXE patients calcification was significantly more prevalent in the IICA (75% vs. 44%, p<0.001), the arteries of the arms (20% vs. 3%, p<0.001), the femoral-popliteal arteries (74% vs. 44%, p<0.001) and the subpopliteal arteries (84% vs. 38%, p<0.001) compared to control patients. In control patients calcifications were significantly more prevalent in the external iliac arteries (30% vs 16%, p<0.001, Table 2) compared to PXE patients. No significant differences in prevalence of arterial calcification were observed in other arterial beds such as the coronary arteries (45% vs. 43%, p=0.776), the ECA (52% vs 46%, p=0.476) and the abdominal aorta (71% vs. 63%, p=0.287). After multivariable adjustment for potential confounders, significant differences in prevalence of calcification were still observed in the IICA (p<0.001), the arteries of the arms (p<0.001), the femoral-popliteal arteries (p<0.001) and the subpopliteal arteries (p<0.001). No significant differences were observed in the external iliac arteries (p=0.186) (supplemental table 2).

Table 2 Presence of arterial calcification in PXE patients compared to control patients

Presence of arterial calcification	PXE patients (n=104)	Control patients (n=93)	p-value
Intracranial internal carotid arteries	78 (75%)	41 (44%)	<0.001
Extracranial carotid arteries	47 (45%)	40 (43%)	0.776
Vertebral arteries	19 (17%)	9 (10%)	0.148
Coronary arteries	54 (52%)	43 (46%)	0.476
Aortic valve*	17 (16%)	16 (17%)	1.000
Mitral valve	1 (1%)	1 (1%)	0.102
Arm arteries	21 (20%)	3 (3%)	<0.001
Thoracic aorta	50 (48%)	52 (56%)	0.318
Abdominal aorta	74 (71%)	59 (63%)	0.287
Mesenteric arteries	28 (27%)	24 (26%)	0.873
Internal iliac arteries	58 (56%)	48 (52%)	0.570
External iliac arteries	17 (16%)	28 (30%)	0.027
Femoral-popliteal arteries	77 (74%)	41 (44%)	<0.001
Subpopliteal arteries	87 (84%)	35 (38%)	<0.001

p-values were calculated using Chi square tests for categorial variables *in the control patients 4 patients had an aortic valve prothesis.

Similar differences were observed in the severity of arterial calcification. Severe calcification was significantly more prevalent in PXE patients compared to controls in the IICA (36% vs 19%, p<0.001), femoral-popliteal arteries (51% vs 31%, p<0.001) and subpopliteal arteries (61% vs 24%, p<0.001). In the hospital controls, significant more severe calcification was observed for the thoracic aorta, the mesenteric arteries and the external iliac arteries (figure 1).

Severity of calcification in patients < 55 years of age

In the 51 youngest PXE patients included in the cohort (under 55 years) the mean age was 44 (±11), while the mean age of 48 older PXE patients was 64 (±6). For control patients this was 41 (±9) and 68 (±8), respectively. In PXE patients under 55 years arterial calcifications were also significantly more prevalent in the IICA (57% vs. 22%), the femoral-popliteal arteries (53% vs. 17%) and the subpopliteal arteries (67% vs. 8%) (figure 1). Also severe calcifications were more prevalent in younger PXE patients compared to younger control patients in the IICA (24% vs 0%), the femoral-popliteal arteries (27% vs 0%) and the subpopliteal arteries (37% vs 2%).

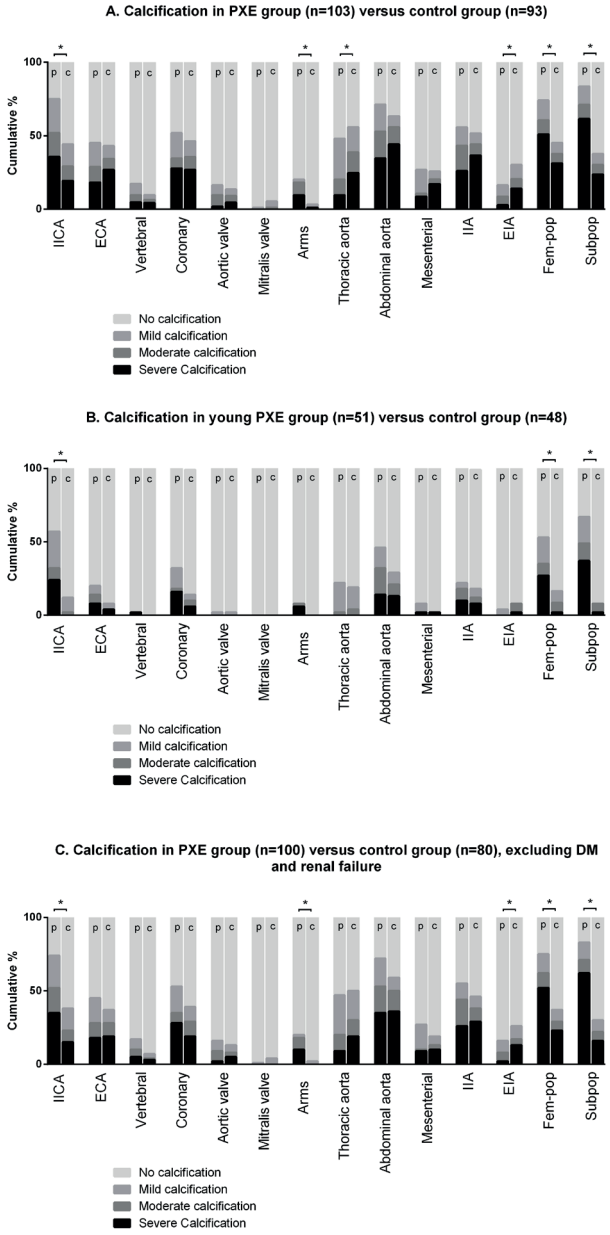

Figure 1 Severity of arterial calcifications in PXE patients compared to control patients.

p PXE group; c control group; * significant difference (Chi-square tests); DM Diabetes Mellitus; IICA intracranial internal carotid arteries; ECA extracranial internal carotid arteries; IIA internal iliac arteries; EIA external iliac arteries; Fem-pop femoral-popliteal arteries; Subpop subpopliteal arteries. Mild calcification 1or 2 small calcification; Moderate calcification 3-5 small calcifications or 1 big calcification accompanied with up to 2 little calcifications; Severe calcification Multiple calcifications or extensive calcification * in the control patients 4 patients had an aortic valve prosthesis.

Sensitivity analyses excluding patients with renal failure and/or diabetes mellitus

In sensitivity analyses excluding patients with renal failure and diabetes mellitus similar arterial calcification patterns for PXE patients (n=100), compared to control patients (n=80) were observed involving significantly more calcification compared to control patients in the IICA, the arm arteries, the femoral-popliteal arteries and the subpopliteal arteries of PXE patients. In control patients significantly more prevalent and more severe arterial calcification compared to control patients in the external iliac arteries was observed (Figure 1).

Discussion

In the present study we systematically investigated the differences in arterial calcification phenotype between PXE patients and hospital controls. We used full-body CT scans and a reproducible scoring system to score the presence and severity of arterial calcification in different arterial beds. In PXE patients, prevalence and severity of arterial calcifications were much higher in the intracranial internal carotid arteries, the arm arteries and the arteries of the legs even in younger PXE patients. These findings provide novel insights into the vascular phenotype of this disease and show that the intracranial internal carotid artery, the arm arteries and the arteries of the legs are the predominant places of arterial calcification in PXE.

It was previously known that calcifications are common and severe in leg arteries of PXE patients, but it was not known whether these calcifications were more common than in the general population.[17] One can assume that the higher prevalence of arterial calcification in the legs of PXE patients eventually leads to symptomatic peripheral arterial disease (PAD). The prevalence of PAD, defined by an ankle brachial index below 0.9, in PXE patients is approximately 44%. [17] This is remarkably high compared to the general population, where, even in patients between 85 and 89 years old, the prevalence of PAD is 18%.[19] The importance of arterial calcification in the pathogenesis of PAD is illustrated by the fact that in amputated legs from patients with PAD the majority of the arteries show medial wall calcification without atherosclerotic changes.[20] It can be assumed that the higher prevalence of arterial calcification in the legs of PXE patients eventually leads to symptomatic peripheral arterial disease (PAD), but the mechanisms behind this relation are not totally clarified yet. Arterial stiffening of the arterial walls in the legs due to the calcification might be an important mechanism that can explain the relation between leg calcifications and occurrence of PAD in PXE, but one would expect a high ABI instead of low ABI as described in PXE.[1721] It is also possible that the calcifications of the medial wall eventually progress to the intimal wall leading to stenosis and thrombotic changes contributing to the occurrence of

PAD which would be more consistent with the physiological measures. Although we did not systematically compare the PXE calcifications to those of patients with renal failure of diabetes mellitus, we are under the impression that in PXE the calcifications are less continuous and less often fully circular. This may also explain the low ABI as observed in PXE patients compared to diabetes patients where high ABI values have commonly been described.[22]

The observed calcification of the IICA may explain some of the suspected brain involvement in PXE. This brain involvement in PXE is characterized by frequent occurrence of cerebrovascular events and cerebral small vessel disease. [23, 24] In general, calcifications in the IICA are predominantly nonatherosclerotic and mostly located around the internal elastic lamina. [25] Calcification of the IICA is known to be an important predictor for cerebrovascular disease and is associated with small vessel disease.[26] Therefore, it is conceivable that the observed calcification of IICA contributes to the brain involvement in PXE. Intracranial carotid artery calcification may lead to brain disease through stiffening of the intracranial carotid arteries leading to abnormally high pulsatile stress in cerebral microvessels.[27]

This study describes the patterns of arterial calcification in PXE. In figure 2 examples of CT-images are shown which summarizes this typical arterial calcification pattern observed in PXE with involvement of arteries of the legs (2A), intracranial internal carotid arteries (2B) and arteries of the arm (2C). Analyses in the youngest patients indicated that the progressive arterial calcification seems to start in the leg arteries where the most distal arteries are affected first. On the opposite side of the arterial system, calcification of the intracranial carotid artery is also frequently observed, even in patients younger than 55 years. Based on these findings one should always consider a rare diagnosis as PXE when in the general population calcifications of the (distal) leg arteries or the IICA are observed at a young age. A striking but less frequent feature of arterial calcification in PXE seems to be the calcification of the arm arteries, which occur in 20% of the PXE patients in this study. Calcification of the arm arteries in patients without renal failure or diabetes mellitus is extremely rare and could be pathognomonic for PXE. The fact that calcification in the arm arteries is strongly related to risk of death, at least in patients with diabetes and renal failure, illustrates the potential prognostic importance of the observation of calcification of arm arteries.[28]

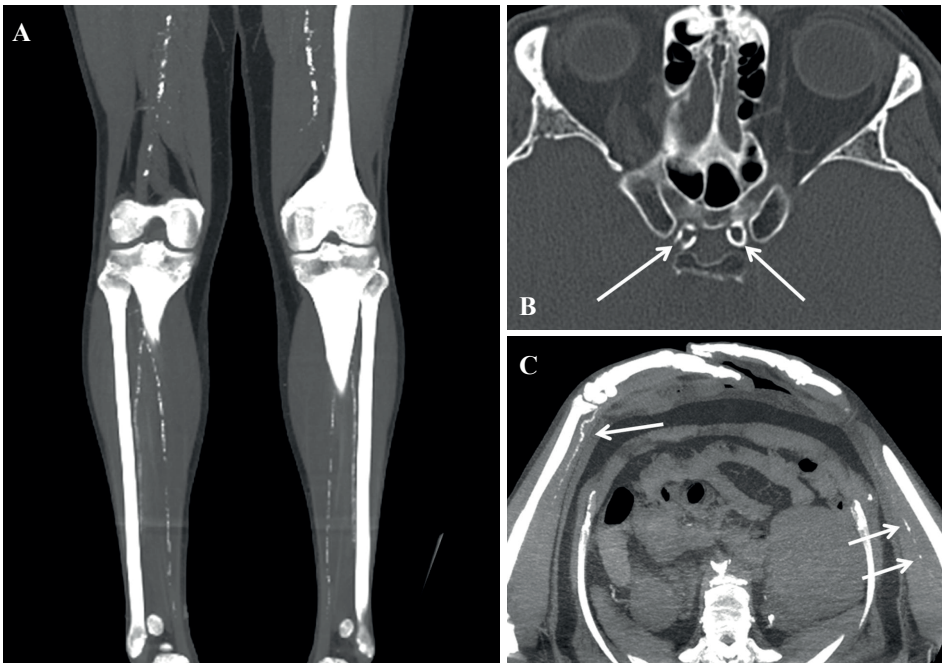

Figure 2 Arterial calcification phenotype of pseudoxanthoma elasticum.

A. Severe calcifications of the femoral and subpopliteal arteries as seen on a CT-scan in a PXE patient. B. Severe calcifications of the intracranial internal carotid arteries. C. Severe calcifications of the arm arteries.

PXE is part of a spectrum of disorders which are associated with progressive arterial calcification. [4] The diseases in this spectrum include generalized arterial calcification of infancy (GACI, OMIM #208000), the Arterial Calcifications due to Deficiency in CD73 (ACDC) syndrome (OMIM #211800), the Hutchinson-Gilford progeria syndrome (HGPS, OMIM #176670) and the Fahr's syndrome or idiopathic basal ganglia calcification (OMIM #213600). These diseases share a similar pathophysiological background which involves the phosphate and pyrophosphate homeostasis. Interestingly, although pyrophosphate has a systemic effect, arterial calcification patterns differ between these diseases.

Both in *ABCC6*-knockout mice as in human PXE patients lower levels of inorganic pyrophosphate have been found. [29, 30] The predominant arterial beds we identified in PXE are not similar to reports of arterial calcification in the generalized arterial calcification of infancy (GACI, OMIM #208000), while the pathophysiology of this disease is very similar to PXE. In fact, this disease, associated with low levels of pyrophosphate due to mutations in the *ENPP1* gene, is considered as a

severe entity of the same clinical spectrum as PXE. [31-33] The GACI syndrome is, in contrast to our findings in PXE patients, associated with progressive aortic calcification. [7] In PXE patients in our cohort aortic calcification did occur, however no differences were found when compared with the control group.

On the other hand, our findings seem to be similar to clinical observations in patients with the Arterial Calcifications due to Deficiency in CD73 (ACDC) syndrome (OMIM #211800), a monogenetic calcification disorder linked with phosphate and pyrophosphate homeostasis caused by mutations in the *NT5E* gene. In these patients severe progressive arterial calcifications in the lower extremities were observed, data on the arms and intracranial carotid arteries are lacking. [34]

In the Hutchinson-Gilford progeria syndrome (HGPS, OMIM #176670), caused by heterozygous mutations in the *LMNA*-gene, the pyrophosphate metabolism plays an important pathophysiological role. [35] Calcifications of the aorta and the aortic valves are described in HGPS, whereas aortic calcification was not more prevalent in PXE patients compared to control patients in our cohort. [36, 37]

Interestingly, in contrast to PXE and the aforementioned progressive arterial calcification diseases, the Fahr's syndrome or idiopathic basal ganglia calcification (OMIM #213600), caused by a heterozygous mutation in the *SLC20A2*-gene, seems to have a unique distinct distribution of arterial calcification. This monogenetic disease, also linked to phosphate metabolism, [38] is associated with dementia and Parkinsonism and extensive calcification of both the small and large vessels of the brain and calcium deposits in the basal ganglia, thalamus and cerebellum. [39, 40]

This study identified a distribution of arterial calcification in PXE, which was clearly distinct from hospital controls. The fact that the observed prevalent arterial calcifications in the legs and the intracranial carotid arteries in PXE coincide with a high prevalence of peripheral arterial disease and brain disorders illustrates the potential importance of arterial calcification in the pathogenesis of cardiovascular manifestations of this disease, but also contributes to the general hypothesis that arterial calcification increases cardiovascular risk. Future studies should describe the exact nature of arterial calcification in PXE, for instance by combining histological and radiological data. Arterial calcification in PXE starts at a young age, whereas in the general population arterial calcification and arterial stiffening occurs slowly with ageing.[41] Therefore, PXE might provide a vascular model for the ageing population. The use of new imaging techniques that are able to visualize the

process of arterial calcification at a very early stage in these rare diseases, such as PET-CT scan with radioactive sodium fluoride (NaF^{18}) could further contribute to the understanding and therapeutically target finding of cardiovascular burden caused by arterial calcification. [42] The NaF^{18} PET-CT can supply clinical trials with an early marker of the arterial calcification process.

The strengths of this study are that all patients underwent an extensive screening resulting in complete and extensive data. Limitations of this study include the use of hospital controls which obviously is a diseased control group with relatively high prevalence of disorders linked to arterial calcification such as vascular disease, renal failure and diabetes mellitus. In our opinion, sensitivity analyses excluding patients with renal disease and diabetes mellitus in both groups sufficiently accounted for this limitation, as the two groups were comparable in other aspects linked to arterial calcification such as age. In this study we compared calcification in pre-specified arterial beds only, whereas PXE is known to be associated with calcification of other tissues that cannot be visualized on the full-body CT scan. For instance, it is known that in PXE calcification of the testicles and testicular microlithiasis occur.[43] Another limitation is the fact that blinding for the presence of PXE of the radiologist who scored all CT images was not ensured for all patients as in some patients PXE related calcification of the skin was visible. This is probably of minor consequence for our conclusions, as the reproducibility of this scoring system was very good based upon the scoring of another independent radiologist who was not aware of the research question.

In conclusion, we identified a vascular phenotype of PXE with a distribution of arterial calcifications that was clearly distinct from hospital controls and involved arterial calcifications in the legs, the intracranial internal carotid artery and the arteries of the arms.

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Appendix

Detailed information on CT scanner

In this study the CT scanner Brilliance 64 of Philips (Cleveland, Ohio) was used.
The estimated effective dose for an average adult is <3 mSv
<80 kg 100 kVp, 40 mAs, iDOSE4
>80 kg 120 kVp, 40 mAs iDOSE4
Collimation 64*0.625
Recons 1 mm slices at 0.7 mm increment
Axial 5 mm slices at 4 mm increment
Coronal 3 mm slices at 3 mm increment
Sagital 3 mm slices at 3 mm increment

Supplemental table 1 Inter-observer agreement per vascular location

	Weighted Cohen's kappa	Interpretation
Carotis Siphon	0.70	Reasonable/Good agreement
Carotid arteries	0.74	Reasonable/Good agreement
Vertebral arteries	0.80	Almost perfect agreement
Coronary arteries	0.91	Almost perfect agreement
Aortic valve	0.54	Reasonable agreement
Mitralis valve	NA	NA
Thoracic aorta	0.68	Reasonable/Good agreement
Arm arteries	0.92	Almost perfect agreement
Mesenteric arteries	0.58	Reasonable agreement
Abdominal aorta	0.93	Almost perfect agreement
Internal iliac arteries	0.77	Reasonable/Good agreement
External iliac arteries	0.72	Reasonable/Good agreement
Femoral-popliteal arties	0.99	Almost perfect agreement
Subpoplitial arteries	0.92	Almost perfect agreement

Weighted Cohen's kappa 0 - 0.20 slight agreement; 0.21-0.40 moderate agreement; 0.41-0.60 reasonable agreement; 0.61-0.80 reasonable to good agreementt; 0.81-1.00 almost perfect agreement; NA not applicable, both observers scored no calcification in the mitral valve

Supplemental Table 2 Presence of vascular calcification in PXE patients compared to control patients multivariable adjusted

	PXE patients (n=104)	Control patients (n=93)	p-value
Presence of arterial calcification			
Intracranial internal carotid arteries	78 (75%)	41 (44%)	<0.001
Extracranial carotid arteries	47 (45%)	40 (43%)	0.701
Vertebral arteries	19 (17%)	9 (10%)	0.221
Coronary arteries	54 (52%)	43 (46%)	0.618
Aortic valve*	17 (16%)	16 (17%)	1.000
Mitral valve	1 (1%)	1 (1%)	0.107
Arm arteries	21 (20%)	3 (3%)	<0.001
Thoracic aorta	50 (48%)	52 (56%)	0.191
Abdominal aorta	74 (71%)	59 (63%)	0.490
Mesenteric arteries	28 (27%)	24 (26%)	0.475
Internal iliac arteries	58 (56%)	48 (52%)	0.861
External iliac arteries	17 (16%)	28 (30%)	0.186
Femoral-popliteal arties	77 (74%)	41 (44%)	<0.001
Subpopliteal arteries	87 (84%)	35 (38%)	<0.001

p-values are based upon the Wald test statistic from logistic regression model with multivariate adjustment for: eGFR, systolic blood pressure, BMI, current smoking diabetes mellitus, age and gender

CHAPTER 4

Pseudoxanthoma elasticum; a correlation between histologic and radiologic findings.

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Abstract

Background - Pseudoxanthoma elasticum (PXE) is a rare genetic disorder in which histologic alterations have been described in eyes, skin, and cardiovascular system. Most knowledge is obtained from limited autopsy studies. To study the clinical implications of tissue alterations in PXE patients, it would be useful to know what can be visualized using computed tomography (CT) imaging. The aims of this study were to expand the literature on histological alterations present in PXE patients and describe the correlation with CT.

Methods - During post mortem investigation tissue samples and total body CT-scans were obtained from two patients (69 year old male and 77 year old female), diagnosed with PXE ante mortem. The tissue samples were histologically extensively investigated and the findings were compared to both previous reports from histological studies in PXE and to the postmortem CT-scans of these patients.

Results - Degenerated and calcified elastic fibers and calcifications were histologically found in skin, subcutaneous fat, heart, arteries, pleura and around the esophagus. On CT-imaging only the intradermal alterations of the skin and the larger vascular calcifications were detected. The other PXE-related abnormalities were not visible on CT.

Conclusion - We extensively investigated two PXE patients and largely confirmed and extended previous histological observations. With CT-imaging we are able to detect vascular calcifications and skin alterations in PXE, but many of the other PXE-related abnormalities found during autopsy are not visualized by CT-scans.

Introduction

Pseudoxanthoma elasticum (PXE) or Grönblad-Strandberg syndrome is a rare autosomal recessive disorder characterized by ectopic calcifications of connective tissues.[1] The disease is, in majority of cases, caused by mutations in the *ABCC6* gene. [2-4] These ABCC6 gene mutations result in lower levels of inorganic pyrophosphate leading to progressive calcification throughout the body. [5]

Previous studies have shown that alterations can be found both histologically and radiologically in skin, testis and blood vessels.[6-10] Furthermore, histological alterations have been described in the eyes and the brain.[9-11] Based on imaging techniques, calcifications have been described juxta-articular, in the soft tissues of the extremities and in the breast.[6, 8]

Although some histological and radiological studies have been performed in PXE patients, a correlation study between the two is lacking. The combination of histology and radiology gives insights into the extent to which the imaging findings may be able to identify the PXE alterations in vivo; important knowledge for further diagnostics and research in living patients. The aim of this study is to describe the combined CT and autopsy findings in PXE patients.

Methods

Both tissue samples and CT images were obtained from two autopsy patients (69 year old male and 77 year old female), diagnosed with PXE ante mortem. One of the patients donated her body to science via the department of Anatomy of the University Medical Center Utrecht. From this patient, written informed consent regarding the use of her body for educational and research purposes was obtained during her life. For the other patient, relatives gave consent to the post mortem investigations. Collection of the material was approved by the local biobank review committee under protocol number 15-252.

Radiology

Subjects were scanned post mortem on a Philips Brilliance 256-slice Computed Tomography (CT)-scanner (Philips Healthcare, Best, The Netherlands). Tube voltage was 140 kV and tube current 200 mAs. Non-contrast enhanced CT-scans with slice thickness 0.9 mm were acquired.

Histology

Tissue samples were obtained during autopsy and fixed in 4% formaldehyde. (supplemental table 1) The macroscopically calcified samples were subsequently

decalcified using diaminoethylene tetraacetic acide solution (EDTA). Decalcification was necessary to maintain morphology of the tissue specimens. Since histologic evaluation of calcification is based on visualization of matrix previously altered by the calcification process, and not calcium ions itself, decalcification does not influence analysis.[12] Four micrometer slides were cut and stained with hematoxylin and eosin and, in anatomic locations where elastic fibers were expected, elastin van Gieson stain was used. In non-decalcified tissue with altered elastic fibers the Von Kossa stain was used to detect calcifications.

Results

Skin

Classical PXE skin alterations were found at the axillae of both patients, a localization typically known for skin changes in PXE. Microscopically dense clumps of degenerated and calcified elastic fibers were found in the mid- and lower dermis. On CT, these alterations were seen as thickened skin without obvious calcifications. The macroscopically normal skin of abdomen and extremities showed similar changes, but not located in the mid- and lower dermis but in the connective tissue septa between the subcutaneous fatty layer. (figure 1) On CT however, at these locations, no abnormalities were seen. (table 1)

Cardiovascular system

The hearts of both patients showed localized degenerated and calcified elastic fibers in the area underneath the endocardial layer, mainly present in both atria. Furthermore, some similar elastic fibers were present in fibrous tissue between the cardiomyocytes. (figure 2) On CT no calcifications were seen in the endo- or myocardial tissue. Calcification of the valves were not present in these patients. (table 1)

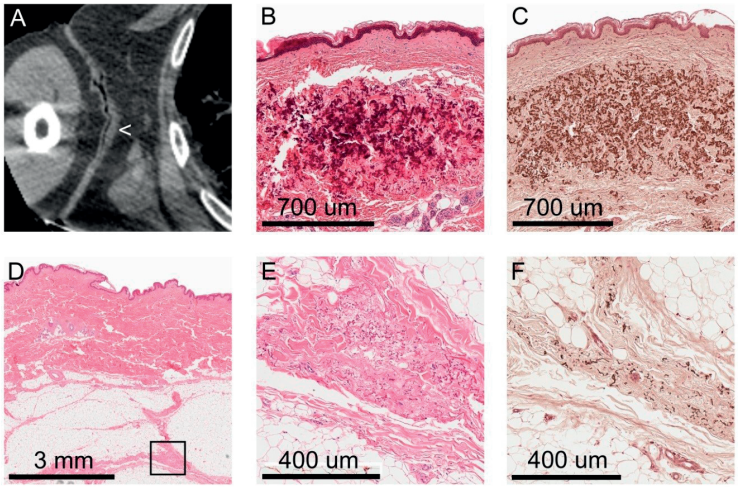

Figure 1 Skin alterations in pseudoxanthoma elasticum

A-C. In the axillae typical pseudoxanthoma elasticum lesions were found, consisting of clumps of degenerated elastic fibers in the mid- and lower dermis (B). Von Kossa stain showed calcifications of these elastic fibers (C). On CT-scan a thickened skin (<) was seen (A).

D-F. Other localizations of skin, macroscopically unremarkable, showed degenerated elastic fibers in the septa of the subcutaneous fatty layer. The marked area in D is shown in E (H&E stain) and F (Von Kossa stain).

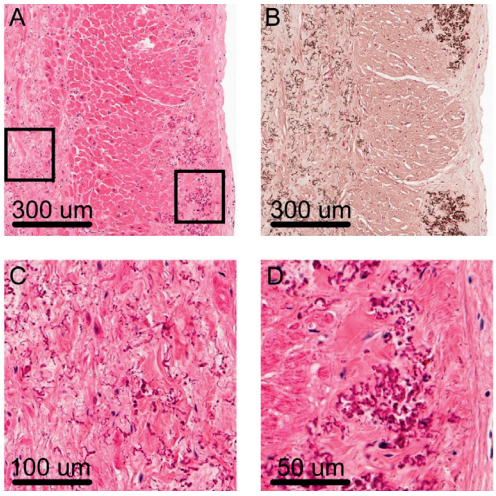

Figure 2 Elastic fiber alterations in the heart

A. Degenerated and calcified fibers were mainly found below the endocardial layer (right side of the picture). Furthermore, similar fibers were seen in the fibrous tissue in the myocardium (left side of the picture). B. Von Kossa stain shows the calcified elastic fibers. C. Enlarged picture of the abnormal elastic fibers within the fibrotic tissue in the myocardium. D. Enlarged picture of the abnormal elastic fibers in the subendocardial layer.

Table 1 Alterations found histologically and radiologically in the two PXE-patients

	Histology	Radiology
Macroscopically altered skin	Degeneration and calcification of the elastic fibers in the mid- and lower dermis	Thickened skin
Macroscopically normal skin	Degeneration and calcification of the elastic fibers in the septa between the subcutaneous fatty layer	-
Heart	Degeneration and calcification of elastic fibers mainly underneath the endocardial layer and to a lesser extent in fibrous tissue between the cardiomyocytes	-
Arteries (lower extremity, gastroepiploic artery)	Both atherosclerotic intimal lesions and calcifications in the medial layer	More or less circumferential calcifications in case of medial calcification, thick dots of calcification in case of intimal calcification
Arteries (other)	Small scattered calcified elastic fibers in the media and/or internal and external elastic lamina	Thick dots of calcification in case of intimal calcification
	Atherosclerotic intimal lesions	
Central nervous system	Lacunar infarction	-
	White matter abnormalities	Non-specific abnormalities in the white matter area
	Calcification of the small arteries in the area of the globus pallidus and hippocampal area	-
Kidney	Kidney stone	Kidney stone
Adrenal gland	Myelolipoma with calcifications and bony transformation	Calcified adrenal gland
Gallbladder	Gallstones	Gallstones
Lung	Some degenerated and calcified elastic fibers in the pleura	Thickened pleura
Esophagus	Some degenerated and calcified elastic fibers around the esophagus	-

The vascular system showed presence of both atherosclerotic intimal lesions with calcifications and calcifications in the medial layer and/or around the internal and external elastic lamina of the vascular wall. In the lower extremities large amounts of medial/elastic lamina calcification were present, accompanied by intimal lesions of variable severity. Also the gastroepiploic artery showed extensive calcification of

the medial layer and around the elastic lamina. On CT these medial calcifications were detected as more or less circumferential and present over a longer track of the vascular wall. The other large and middle sized arteries showed variable amounts of calcified elastic fibers in the media (in elastic arteries) and/or the internal and external elastic lamina. (figure 3) These small scattered calcified fibers were not detected on CT. Furthermore, in some of the small arteries in the organs (heart, lung, kidney, stomach, pancreas, thyroid) more or less circumferential calcification of the internal elastic lamina was present. These very small vessels were not detected on CT. Besides medial/elastic lamina calcification, also many calcifications were present in the atherosclerotic lesions found in both patients. These calcifications were much more clumped together and therefore, if large enough, detectable on CT. (table 1)

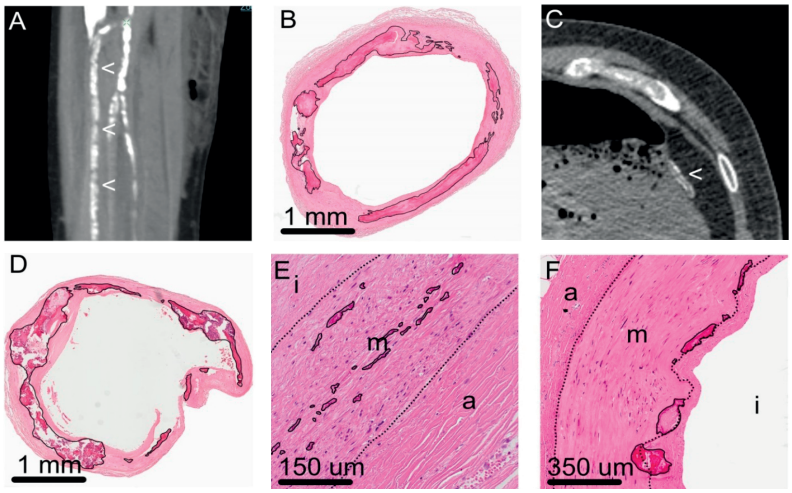

Figure 3 Vascular calcifications

A and B. Extensive calcifications, on CT scan seen as more or less circumferentially present calcifications in a longer segment of the vascular wall, were present around the internal elastic lamina and in the media of the vessels of the lower extremity (here anterior tibial artery (<)), calcifications are marked with a black line in B.

C and D. The same internal elastic lamina and medial calcifications were visible in the gastroepiploic artery, located along the greater curvature of the stomach, both in histology (calcifications are marked in D) and CT-scan (<).

E. In most of the other large and middle sized arteries variable amounts of calcified elastic fibers were seen in the media (in elastic arteries) and/or around the internal and external elastic lamina. (marked) These small calcifications could not be detected on CT-scan, on which also many atherosclerotic intimal calcifications were visible. (i=intima, m=media, a=adventitia, dotted lines indicate internal and external elastic lamina)

Central nervous system

In both patients a lacunar infarction was found (one in the left frontal lobe and one in the area of the basal nuclei). Furthermore, in one patient the central white matter showed dispersion of the fibrillary matrix with clear demyelination. The subcortical white matter was unremarkable. (figure 4) The cerebral microvasculature showed sclerosis of the vascular wall, with vascular calcifications in the area of the globus pallidus. In one of the patients some calcifications were present in the hippocampal region. On CT there were nonspecific abnormalities in the white matter area. The small vascular calcifications in the area of the globus pallidus, present in both patients, were not seen on CT. Also the vascular calcifications in the hippocampal area of one of the patients were not seen. (table 1)

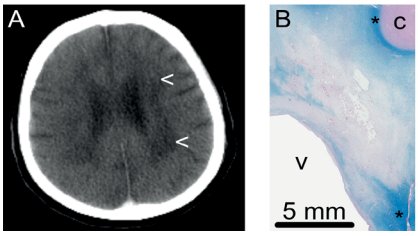

Figure 4 Cerebral white matter lesions

A. The CT-scan of the patient showed nonspecific white matter abnormalities (<). B. Histologic slides (Luxol fast blue-Pas stain) showed dispersion of the matrix with extensive demyelination in the central white matter. Normal myelination is seen subcortical and in the area around the basal nuclei (*). c=cortex, v=ventricle.

Other findings

In one of the patients a kidney stone in the right kidney was found, an observation also done via CT. The same patient also showed a calcified right adrenal gland, microscopically fitting with a myelolipoma with, or combined with, extreme calcifications and bony transformation. (figure 5) Furthermore, small gallstones were present in the gallbladder of this patient. These were seen on CT. In the other patient histologically some degenerated and calcified fibers were found in the pleura and around the esophagus. The CT showed a thickened pleura. However, since this patient also suffered from a malignancy in the lung, the cause of this thickening is uncertain, and possibly not due to the alterations caused by PXE. Around the esophagus no abnormalities were seen on CT. (table 1)

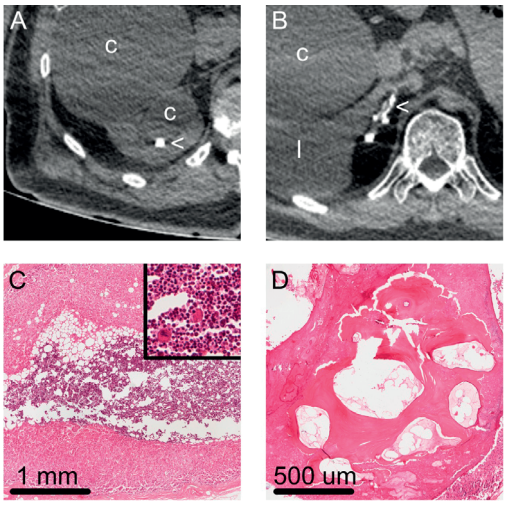

Figure 5 Kidney stone and calcified adrenal gland

A. In one of the patients a small kidney stone (<) was present in the right kidney. Furthermore a couple of (large) cortical renal cysts (c) were seen. B. The CT-scan of the same patient showed a calcified adrenal gland (<), adjacent to the cortical cyst (c) seen in A and the liver (l). C. Histologically the right adrenal gland showed presence of extramedullary hematopoiesis and adipocytes (inlet) fitting a myelolipoma. D. Besides the myelolipoma extensive calcifications with bony transformation were present.

Discussion

PXE is a systemic disease with abnormalities in eyes, skin, and the cardiovascular system. The present study combines radiologic and histologic findings in two PXE patients, to investigate which alterations can, and which cannot be seen, using CT. The study has two important results. First, it was shown that most of the abnormalities seen on histologic slides were not seen on CT, except for the intradermal skin alterations and part of the vascular calcifications. Second, this study adds to the existing knowledge regarding the abnormalities that can be seen in PXE patients. Degenerated and calcified elastic fibers were not only found in skin, arteries, heart and pleura, where they have been described before, but also in the fibrous bands in between the subcutaneous fat tissue and around the esophagus.

Skin

The skin alterations that were seen on CT were the intradermal lesions that were already macroscopically visible. These skin alterations represent the abnormalities in skin typically for PXE. We also found histologic alterations in macroscopically unaltered skin, most abundantly located in the subcutaneous fat. The presence of some abnormal elastic fibers in non-lesional skin has been described before.[13] However, the presence of subcutaneous lesions at these locations has not been

described before. A possible explanation could be that most knowledge about histological skin alterations is obtained by studies in skin biopsies, with only small amounts of subcutaneous fat.

Cardiovascular system

In the heart degenerated and calcified elastic fibers were present subendocardially and in fibrous tissue between the cardiomyocytes. This was not visible on CT-imaging. The presence of these altered elastic fibers in the cardiac tissue has been described before, and has been suggested as a cause of restrictive cardiomyopathy and congestive heart failure.[9, 14]

The vascular system showed presence of both atherosclerotic intimal lesions with calcifications and calcifications present in the medial layer and/or around the internal and external elastic lamina of the vascular wall. Larger vascular wall calcifications were visible on CT-scan. In case of extensive calcification of the medial layer or elastic lamina, present in the lower extremity and the gastroepiploic artery, on CT-scan a more or less circumferential pattern over a longer segment of the vessel was seen. This pattern is comparable to the pattern of medial calcification seen on X-ray described in scarce literature.[15] The smaller amounts of scattered elastic fibers present in most of the large, middle sized and small arteries were not visible on CT-scan. Our findings of vascular calcifications, and the combination of both atherosclerosis and medial calcification, are consistent with previous findings.[16]

Central nervous system

In both patients a lacunar infarction was found. The combination of PXE and lacunar infarctions of the brain has been described before as a complication of small vessel disease. (Pavlovic et al 2005) Also the white matter lesions, as seen in one of our patients, have been described before in association with PXE. [17-22] The combination of lacunar infarctions and white matter lesions have been described in association with cognitive deterioration, although reports also mention extensive white matter lesions in a patient with normal baseline cognitive status.[22] On CT-scan nonspecific abnormalities could be found in the white matter area. However, for diagnostic purposes and further research MRI probably is a better imaging technique.

Other findings

In one of the patients a kidney stone was found. A possible relation between PXE and nephrolithiasis has been suggested before.[23] However, in most of the patients described phosphocalcic abnormalities were present, which was not the case in

our patient. Since nephrolithiasis is not a rare condition, it is not unlikely that this is a coincidental finding. In the same patient also gallstones and a calcified adrenal gland were found. It is unknown to which extent this can be related to PXE. Furthermore, in the other patient some calcified fibers were found in the pleura and around the esophagus. To our knowledge degenerated and calcified elastic fibers have not been described on these locations before.

An important limitation of this study is the limited number of bodies studied, which can be explained by the low incidence of the disease and low autopsy rate in the Netherlands. Due to this small number of patients it is possible that by chance we selected two patients in which many abnormalities were not seen on CT-scans, while in larger series of patients this would not have been the case. Therefore, our findings need confirmation in a larger series of patients. Nevertheless, the findings in our patients during autopsy are comparable to those described in literature. Furthermore, we did not study the eyes of the patients. However, most of the ocular findings in PXE (peau d'orange, angioid streaks, chorioretinal atrophies) can already be diagnosed in living patients using a variety of diagnostic techniques.[24] It is doubtful whether CT, with a relatively low resolution for a small organ as the eye, can contribute in those diagnoses.

In conclusion, autopsy of two PXE patients revealed degenerated and calcified elastic fibers and calcifications in skin, heart, arteries and pleura, but also in between the subcutaneous fat tissue and around the esophagus; locations where they have not been described before. Only the intradermal and part of the vascular calcifications were seen on CT. Our results indicate that CT can be used to study vascular calcifications in this patient population. However, while doing so, one should keep in mind that small calcifications are not visible using this technique.

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Appendix – supplemental table List of organs and tissues examined

Organs	Case 1	Case 2
Skin	Macroscopically affected skin	Macroscopically affected skin
	Macroscopically unaffected skin	Macroscopically unaffected skin
Heart	Ventricular myocardium	Ventricular myocardium
	Atrial myocardium	
	Atrioventricular node	Atrioventricular node
	Pericardium	
Arteries	Abdominal aorta	Abdominal aorta
	Thoracic aorta	Thoracic aorta
	Common carotid artery	Common carotid artery
	Coronary artery	Coronary artery
	Celiac trunk	Celiac trunk
	Splenic artery	Splenic artery
	Superior mesenteric artery	Superior mesenteric artery
	Renal artery	Renal artery
		Inferior mesenteric artery
		Common iliac artery
		Internal iliac artery
	External iliac artery	External iliac artery
	Femoral artery	
		Superficial femoral artery
	Anterior tibial artery	Anterior tibial artery
	Intracranial internal carotid artery	Intracranial internal carotid artery
		Anterior cerebral artery
		Medial cerebral artery
		Posterior cerebral artery
	Vertebral artery	Vertebral artery
	Basilar artery	Basilar artery
Digestive system	Esophagus	Esophagus
	Stomach	Stomach
	Liver	Liver
		Pancreas
Respiratory system	Lungs with pleura	Lungs with pleura
	Trachea	

Organs	Case 1	Case 2
Genitourinary system		Prostate
		Testicle
	Bladder	Bladder
	Kidney	Kidney
Hematopoietic system	Spleen	Spleen
	Bone marrow	Bone marrow
	Lymph node	Lymph node
Endocrine system	Adrenal	Adrenal
	Thyroid	Thyroid
Central nervous system	Spinal cord	
	Cerebral cortex	Cerebral cortex
	Cerebellum	Cerebellum
	Basal nuclei	Basal nuclei
	Hippocampus	Hippocampus
	Brain stem	Brain stem

CHAPTER 5

Arterial stiffening and thickening in patients with pseudoxanthoma elasticum

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Submitted

Abstract

Background - Patients with pseudoxanthoma elasticum (PXE), a monogenetic calcification disease, are at high vascular risk. Although the precise arterial phenotype remains unestablished, it is hypothesized that PXE predominantly affects the medial arterial layer leading to arterial stiffening. We aimed to test this hypothesis by measuring arterial wall characteristics in PXE and comparisons with the general population and diabetes mellitus type 2 (DM2), a condition typically associated with mixed intimal and medial arterial disease.

Methods - Extensive arterial wall characterization was performed in 203 PXE patients involving intima-media thickness (IMT), pulse wave velocity (PWV) and pulse pressure (PP) measurements. IMT and PWV in PXE were compared with the general population using age, sex and mean arterial pressure corrected values for each PXE patient. IMT and PP were compared between PXE and DM2 independently of sex, age and systolic blood pressure, using data of DM2 patients (n=1033) from the Second Manifestations of ARTerial disease (SMART) cohort.

Results- PXE patients had significantly higher IMT (mean difference 0.09; 95%CI 0.07-0.12 mm) and PWV (mean difference 2.5; 95%CI 1.9-3.0 m/s) compared to the general population. IMT in PXE was lower compared to DM2 (0.72; 95%CI 0.68-0.75 mm vs. 0.85; 95%CI 0.83-0.87 mm, p-value<0.01), whereas PP in PXE was higher compared to DM2 (60; 95%CI 59-62 vs. 57; 95%CI 57-58 mmHg, p-value<0.01).

Conclusions PXE patients have thicker arterial walls than the general population, but thinner arterial walls than DM2 patients at similar age. Arterial stiffening is more pronounced in PXE patients compared to DM2 patients.

Introduction

Arteriosclerosis is a type of arterial wall disease distinct from the more generally known atherosclerosis or intimal arterial disease.[1] Atherosclerosis is an intimal arterial disease, caused by classical risk factors such as smoking, hypercholesterolemia and hypertension and typically results in narrowing or obstruction of arteries. Arteriosclerosis is a medial arterial disease which is seen in prevalent conditions such as ageing, vascular disease, diabetes mellitus and chronic kidney disease and results in the calcification and stiffening of the medial arterial wall.[2] The potential importance of arteriosclerosis in the pathophysiology of vascular disease is illustrated by a histopathology study showing that 72% of the arteries in leg amputees from patients suffering from severe peripheral arterial disease contained arteriosclerotic (medial layer) lesions, whereas only 23% of the arteries had atherosclerotic lesions.[3] Arteriosclerotic changes are related to a 3–4 fold higher risk for vascular events and (vascular) mortality.[4] Proposed mechanisms through which medial arterial disease results in vascular disease include high arterial stiffness induced pulse pressure damage and hemodynamic changes.[2, 5, 6]

Although this suggests that preventing or reducing arteriosclerosis might be an important target for further vascular risk reduction, the relative contribution of (intimal and) medial arterial disease to the development of clinical vascular events is unknown. Although arteriosclerosis and atherosclerosis represent distinct pathophysiological processes leading to vascular disease, these processes seem to have a complex interplay and may amplify each other rather than have an isolated impact.[7] In clinical practice, arteriosclerosis and atherosclerosis are often seen simultaneously. Effects of arteriosclerotic arterial wall changes are for instance frequently studied in type 2 diabetes mellitus (DM2), which is known to have a typical mixed pattern of both intimal and medial arterial disease.[2, 8]

Pseudoxanthoma elasticum (PXE, OMIM #264800) might be a model disease for arteriosclerosis as it thought to have an isolated arteriosclerotic type of arterial wall disease with relatively little interference of atherosclerotic arterial wall disease. PXE is caused by mutations in the *ABCC6*-gene resulting in low levels of inorganic pyrophosphate, a strong inhibitor of ectopic mineralization.[9, 10] This leads to a typical pattern of progressive calcification of elastic fibers in the skin, Bruch's membrane in the eye and the vasculature.[11] PXE patients are at high risk of vascular disease (in particular peripheral artery disease and cerebrovascular disease), which is thought to be the result of a proposed unique type of arterial wall disease.[12-15] Indeed, the PXE-related arterial abnormalities are comparable to arteriosclerotic changes in DM2 and CKD.[2, 12, 16] As this proposed medial arterial

wall phenotype would make PXE a particular interesting comparator for diseases with a more combined presence of medial and intimal changes and as it would imply that specific interventions are needed to address the vascular risk in PXE, we aimed to test whether PXE indeed has this unique arterial wall phenotype.

Hence, the goal of the present study was to compare the total arterial wall burden as measured with intima-media thickness (IMT) and specific arteriosclerotic changes as measured with pulse wave velocity (PWV) and pulse pressure (PP) between PXE patients, the general population, and DM2 patients.

Methods

PXE patients

Data of 203 consecutive PXE patients enrolled in the Dutch PXE cohort in the University Medical Center Utrecht (UMCU), The Netherlands, were used for the present study. PXE patients had a proven clinical diagnosis.[17] Extensive characterization of the arterial wall was performed in these patients. This included measurements of carotid-femoral pulse wave velocity (PWV), carotid intima-media thickness (IMT) and repeated blood pressure measurements from which the pulse pressure (PP) was extracted. Furthermore in all patients vascular risk factors (blood pressure, cholesterol, BMI, smoking status, history of cardiovascular disease) were assessed.

Expected general population values

To compare differences in arterial wall disease parameters between PXE patients and the general population, calculated expected values based upon age, sex and blood pressure were used. These estimates were calculated using validated formulas for carotid IMT [18] and carotid-femoral PWV [19], resulting in expected values of PWV and IMT. The formula for expected PWV is mean arterial pressure (MAP) dependent and is different for age groups, whereas the formula for expected IMT is age-dependent and different for men and women (formulas are included in the supplement).

DM2 patients

Data of 1033 patients with DM2 were derived from the Second Manifestations of ARTerial disease (SMART) cohort. The SMART study is an ongoing prospective single-center cohort study in patients with manifest vascular disease and/or vascular risk factors. Consecutive patients aged between 18 and 80 years, referred to the UMCU, with manifest vascular disease or a vascular risk factor underwent

a standardized vascular screening. The design of the SMART-cohort has been described in detail previously.[20] Baseline measurements of consecutive DM2 patients included in SMART after 2002 were used for the current study. DM2 was defined as a referral diagnosis of type 2 diabetes, self-reported type 2 diabetes, a fasting serum glucose ≥7.0 mmol/L at inclusion with initiation of glucose lowering treatment within one year, or the use of oral anti-hyperglycemic agents or insulin at baseline. Participants with known type 1 diabetes were excluded for this analysis.

Measurements of arterial wall burden and arterial stiffening

Measurement of total arterial wall burden: carotid IMT

The carotid IMT reflects both intimal wall and medial arterial wall disease and therefore can be seen as a measurement which entails the total burden of arterial wall disease (both the atherosclerotic and the arteriosclerotic burden).[21] In PXE and DM2 patients the carotid IMT was measured using ultrasound (Esaote, Florence, Italy) in the left and right carotid artery in the transversal, the posterolateral and the anterolateral direction. The average of these measurements in the three directions from both sides was used to obtain the IMT value.

Arteriosclerosis / arterial stiffness measurement: Carotid-femoral PWV

We used arterial stiffness values to estimate the burden of arteriosclerotic wall changes. In PXE patients, but not in DM2 patients, the carotid-femoral PWV was measured using applanation tonometry. Before these measurements, patients rested for 5-10 min in a supine position. Tonometry was performed with a micromanometer (Millar Instruments Inc. Houston, USA) in combination with Sphygmocor software (Atcore Medical Pty. Ltd., Australia) in order to record the waveforms. The PWV was determined as the difference in time between the foot of the wave in the carotid versus femoral artery, divided by the distance between the two points of measurements. The distance was defined as the path length between the sternal notch and the measuring point at the carotid/femoral artery. The velocity was given in meters per second.

Arteriosclerosis / arterial stiffness measurement: Brachial pulse pressure (PP)

In the absence of PWV measurements in DM2 patients we used we used brachial PP to compare arterial stiffening between PXE and DM2. Brachial PP is a generally accepted and valid measurement of arterial stiffening which relates to PWV.[22, 23] For this measurement, all PXE and DM2 patients underwent at least two blood pressure measurements of which the mean blood pressure was used.

To calculate the PP the diastolic blood pressure was subtracted from the systolic blood pressure.

Ethical committee approval

Given the retrospective use of routine care data no formal approval of this study was required as stated by the Medical Ethics Committee of the UMC Utrecht and informed consent for PXE patients was waived for anonymous analysis of the data (IRB number 15/446-C). The medical ethics review board at the University Medical Center Utrecht approved the SMART study, and all participants gave their written informed consent.

Data analyses

Descriptive statistics were used for baseline characteristics of PXE and DM2 patients. Data were presented as numbers and percentages for categorical variables, mean and standard deviation for normal distributed variables and median and interquartile range (IQR) for non-normally distributed variables.

In order to assess the differences in arterial wall disease between PXE patients and expected general population values, independent sample t-tests were used comparing the observed estimates of IMT and carotid-femoral PWV in PXE patients to their expected general population values. In addition, stratified analyses were performed for younger and older patients (< the median of 54 and ≥54 years). To further assess and visualize the differences in age-dependent arterial wall disease patterns, mean values and 95% confidence intervals of observed and expected IMT and PWV were calculated and plotted for the age groups >30 years, 30-39 years, 40-49 years, 50-59 years, 60-69 years and 70-80 years.

IMT is known to be strongly related to age and influenced by sex.[18] To be able to compare differences in IMT between PXE and DM2 independently of age and sex, a regression model was built. Linearity of the relation between age and IMT was tested and transformation was applied when this improved model fit based on Akaike's Information Criterion (AIC).[24] In addition, interaction terms were included when this improved model fit based on AIC. The final model included PXE, (a linear term of) age, sex and an interaction term between age and PXE. To visualize the age-dependent pattern of IMT in PXE and DM this model was plotted against age for PXE patients and DM2 patients separately.

PP is known to be strongly associated with and influenced by sex, age and systolic blood pressure.[22] To compare PP between PXE and DM2 independently of sex,

age and systolic blood pressure a regression model was built in a similar manner. The final model included PXE, a quadratic transformation of age, sex, systolic blood pressure and an interaction term between (transformed) age and PXE. To visualize the age-dependent pattern of PP in PXE and DM this model was plotted against age for PXE patients and DM2 patients separately.

In all analyses the level of significance was set at $p<0.05$. All data analyses were performed using R version 3.3.2.

Results

Baseline characteristics

PXE patients were 52±15 years and 35% was male, whereas DM2 patients were 61±10 and 71% was male. In terms of traditional vascular risk factors, PXE patients had a more favorable cardiovascular profile compared to DM2 patients based on current smoking (15% vs. 20%), systolic blood pressure (134±21 vs. 144±20), BMI (25.3±4.4 vs. 29.2±4.9), HDL-cholesterol (1.6±0.4 vs. 1.1±0.3) and triglyceride levels (median 1.1 IQR (0.8-1.5) vs. median 1.5 IQR (1.1-2.2)).

Blood pressure-lowering and lipid-lowering medication was less frequently used in PXE compared to DM2 patients (20% vs. 85% and 33% vs. 80%, respectively). A history of coronary disease and cerebral vascular disease was more prevalent in DM2 (54% and 17%, respectively) compared to PXE patients (6% and 10%, respectively), whereas peripheral arterial disease was more prevalent in the PXE patients (33%) compared to DM2 (10%).

Table 1 Baseline characteristics

	PXE n=203	**DM2 n=1033**
Age - years	52±15	61±10
Sex - male	71 (35%)	737 (71%)
Glucose - mmol/L	5.6±1.3	8.1±2.2
eGFR (CKD-EPI) – ml/min/1.73m^2	90 (76-90)	77 (64-90)
Systolic blood pressure - mmHg	134±21	144±20
Diastolic blood pressure - mmHg	78±11	82±12
BMI - kg/m^2	25.3±4.4	29.2±4.9
Current smoking	30 (15%)	210 (20%)
Total cholesterol - mmol/L	5.1±1.1	4.4±1.3
Triglycerides - mmol/L	1.1 (0.8-1.5)	1.5 (1.1-2.2)
LDL-cholesterol - mmol/L	3.0±1.0	2.5±1.0
HDL-cholesterol - mmol/L	1.6±0.4	1.1±0.3
Blood pressure-lowering medication	41 (20%)	883 (85%)
Lipid-lowering medication	66 (33%)	828 (80%)
Diabetes mellitus	11 (5%)	1033 (100%)
History of coronary disease	12 (6%)	561 (54%)
History of cerebral vascular disease	18 (9%)	177 (17%)
History of peripheral arterial disease	66 (33%)	102 (10%)

Data are reported as the mean (standard deviation for normal distributed parameters and median (interquartile range) for non-normally distributed parameters. Categorical parameters were presented as n (%).

Table 2 Parameters of arterial wall disease in PXE patients compared to expected general population values

	All PXE patients n=203	PXE patients <54 years n= 104	PXE patients ≥54 years n= 99
Carotid IMT - mm	0.69 ± 0.16	0.62 ± 0.12	0.76 ± 0.17
MD (95%CI) with general population	0.09 (0.07-0.12)	0.07 (0.04-0.09)	0.12 (0.09-0.16)
PWV - m/s	11.0 ± 3.8	9.5 ± 2.9	12.6 ± 3.8
MD (95%CI) with general population	2.5 (1.9-3.0)	1.7 (1.0-2.3)	3.3 (2.5-4.2)

Data are reported as the mean ± standard deviation. MD mean difference between observed and calculated age, sex and mean arterial pressure corrected general population values; CI confidence interval

Differences in arterial wall disease between PXE patients and general population values

Differences arterial wall disease (IMT)

The mean carotid IMT in PXE was 0.69±0.16 (table 2). IMT was significantly increased in all PXE patients compared to expected general population values (mean difference 0.09; 95%CI 0.07-0.12mm). The IMT in PXE patients younger than 54 years was significantly increased compared to expected general population values (mean difference 0.07; 95%CI 0.04-0.09). Also, in patients older than 53 years IMT-values were higher than expected general population values (mean difference 0.12; 95%CI 0.09-0.16). Stratified analyses per 10-year age group presented in figure 1a show that the IMT in PXE is higher than the expected general population values in all age groups.

Differences in arterial stiffening (femoral-carotid PWV)

The mean femoral-carotid PWV in PXE was 11.0 ± 3.8. PWV was significantly increased in PXE patients compared to expected general population values (mean difference 2.5; 95%CI 1.9-3.0 m/s). The PWV in PXE patients younger than 54 years was significantly increased compared to expected general population values (mean difference 1.7; 95%CI 1.0-2.3). Also, in patients older than 53 years PWV -values were higher than expected general population values (mean difference 3.3; 95%CI 2.5-4.2).Figure 1b shows that the observed PWV in PXE is higher than expected general population values for each 10-year age group.

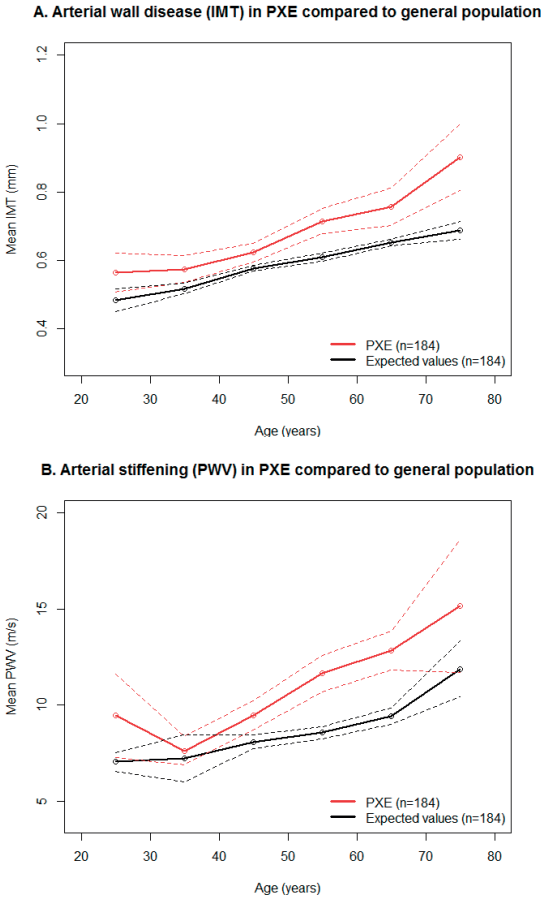

Figure 1 Patterns of arterial wall disease and arterial stiffening in PXE compared to the general population

Figure 1A Mean carotid IMT per age groups in PXE patients versus expected values (as estimate of general population)); Figure 1B Mean carotid-femoral PWV per age groups in PXE patients versus expected (as estimate of general population).

Differences in arterial wall disease between PXE patients and DM2 patients

Differences in arterial wall disease (IMT)

In general, IMT in PXE was lower compared to IMT in DM2 (0.72; 95%CI 0.68-0.75 mm vs. 0.85; 95%CI 0.83-0.87 mm, p-value<0.01). Figure 2a shows that IMT in PXE is lower compared to the IMT in DM2 in all ages. Also, the influence of age on IMT tends to differ between PXE and DM2 (interaction p-value = 0.07) with a less steep increase of IMT with age in PXE compared to DM2.

Differences in arterial stiffnening (PP)

In general, PP in PXE was higher compared to PP in DM2 (PP 61; 95%CI 59-62 mmHg vs. 58; 95%CI 57-58 mmHg). Figure 2b shows that PP in PXE is higher compared to the IMT in DM2 until 65 years and becomes similar in higher ages. Also, although non-significantly, the influence of age on PP seems to differ between PXE and DM2 (interaction p-value = 0.33), with little influence of age in PXE and an increasing PP with age in DM2.

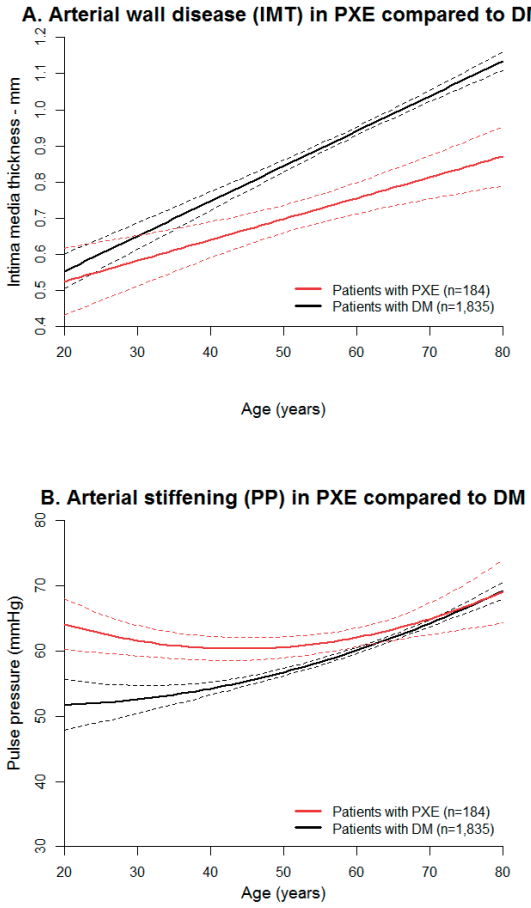

Figure 2 Patterns of arterial wall disease and arterial stiffening in PXE compared to DM2

IMT intima-media thickness; PXE pseudoxanthoma elasticum; DM2 diabetes mellitus type 2; PP pulse pressure

Figure 2A Sex corrected IMT against age in PXE versus DM2; Figure 2B Sex and systolic blood pressure corrected PP against age in PXE versus DM2

Discussion

We measured arterial wall characteristics in 203 PXE patients and found that PXE patients had thicker arterial walls (IMT) compared to expected general population values across all age groups, which is consistent with previous reports.[14, 15, 25] In fact, even carriers of one *ABCC6* mutation are shown to have high IMT values.[15] In addition, consistent with previously performed studies, arterial stiffness, measured using PWV, was found to be remarkably high in PXE when compared to calculated general population values.[14, 15] Furthermore, comparisons with DM2, a condition typically associated with mixed intimal and medial arterial disease, showed that IMT is thinner in PXE compared to DM2, whereas arterial stiffening, as measured using PP, is higher in PXE patients compared to DM2 patients. These findings support the hypothesis of a unique arterial wall phenotype in PXE with isolated arteriosclerotic changes and little interference of atherosclerotic arterial wall disease.

PXE has a high prevalence of vascular disease (in particular peripheral arterial disease and cerebrovascular disease), which is thought to be caused by the proposed specific arteriosclerotic arterial wall changes occurring in PXE. [12, 26, 27] Recently major steps have been made in deciphering the etiology of PXE. First, *ABCC6*-mutation were identified as the cause of PXE, [28-30] and more recently it was found that the double *ABCC6*-mutations result in inefficient mediators of ATP secretion in the liver causing low levels of inorganic pyrophosphate.[9] Inorganic pyrophosphate is, as a part of a complex interplay between promoting and inhibiting factors, [31] heavily involved in the body's prevention of ectopic mineralization. [9, 10] Besides calcification of elastic fibers in the skin and Bruch's membrane of the retina, the low levels of pyrophosphate in PXE are thought to cause the observed ill-understood mineralization and fragmentation of elastic fibers in the medial layers of arterial walls.[12] As arterial calcifications in PXE typically occur in the peripheral and intracranial arteries, while the vascular phenotype of PXE particularly involves peripheral artery disease and cerebrovascular disease, it seems conceivable that the vascular phenotype of PXE is caused by these specific arteriosclerotic lesions.[32-34] The observed arterial wall changes in PXE seem to be similar to arteriosclerotic lesions observed in DM2 and chronic kidney disease. [2, 12, 16]

Our study supports the hypothesis that a unique, predominantly arteriosclerotic arterial wall disease occurs in PXE. We found that, despite the fact that arterial wall thickness (IMT) was lower in PXE compared to DM2 patients, the medial arterial disease component (PP) was more pronounced in PXE compared to DM2. Although IMT increased with age both in PXE and DM2, lower values of IMT

for all age groups and a less steep increase were found in PXE. In contrast to PP, IMT not only reflects medial wall disease, but rather total arterial wall disease resulting from the combination of atherogenesis and arteriogenesis.[21] Probably the intimal wall component of the IMT in patients with DM2 was higher compared to PXE patients. Independently of sex and systolic blood pressure, age in PXE patients seemed to barely influence PP in PXE, whereas age influenced PP in DM2 patients. It is therefore conceivable that the arteriosclerotic changes in the arterial wall in PXE are present from a relatively young age, increasing the risk of actual vascular events at later age.

Thus, our findings support the hypothesis that PXE might be a model disease of isolated arteriosclerosis with little atherosclerosis interference. [2, 12, 16] This underlines the need for specific interventions to address the vascular risk in these patients. In addition, it suggests that further studying of PXE, for instance using long-term follow up of (young) PXE patients, might be helpful in understanding the mechanisms through which arteriosclerosis leads to vascular disease. Eventually this might contribute to the therapeutically target finding of cardiovascular burden caused by arteriosclerosis in the general population. Pathophysiological and phenotypically similar diseases such as the Calcification of joints and arteries (CALJA) syndrome (OMIM #211800), could serve as alternative model diseases of arteriosclerosis with little atherosclerotic interference.[35, 36]

Strengths of this study include the fact that a large group of PXE and DM2 patients underwent characterization of arterial walls resulting in extensive and complete data. Also, due to our design using comparisons against age, sex, and mean arterial blood pressure-based expected general population values and adjustment for age, sex and systolic blood pressure in the comparisons with DM2 patients, we expect little residual confounding of the chief confounders of arterial wall disease and arterial stiffness measurements. Some limitations need to be taken into account when interpreting the study results. First, we did not compare IMT and PWV in PXE patients to actual general population control patients but used a regression algorithm. However, since this algorithm is based on large general population samples, it may provide an even more valid control group, enabling us to calculate an expected value for each individual PXE patient. [18, 19] Second, to compare arterial stiffening between PXE and DM2 we used brachial PP, whereas PWV would have been a superior alternative. In absence of PWV measurements in the DM2 patients, brachial PP was used to compare arterial stiffening between PXE and DM2. As brachial PP has been shown to reflect stiffening of large arteries

and relates to PWV, this measurement provides a valid estimate for the difference in arterial stiffening between PXE and DM2. [22, 23]

In conclusion, PXE patients have thicker arterial walls than the general population, but thinner arterial walls than DM2 patients at similar age. Arterial stiffening is more pronounced in PXE compared to DM2 patients. These findings underline the need for specific interventions to address the vascular risk in these patients with a unique arterial phenotype. Further research of causes and effects of these arteriosclerotic changes in PXE might not only provide insights in the effects of arteriosclerosis in general, but might also provide potential therapeutic targets to reduce vascular risk.

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Appendix

Formula for expected carotid intima-media thickness

Gender	IMT (in mm) algorithm[18]
Male	0.3235+0.005201×age
Female	0.3217+0.004971×age

Formula for expected carotid-femoral pulse wave velocity

Age group (years)	PWV (in m/s) algorithm[19]
<30	0.0472 x Mean arterial pressure + 2.20
30-39	0.0423 x Mean arterial pressure + 2.20
40-49	0.0646 x Mean arterial pressure + 1.41
50-59	0.0731 x Mean arterial pressure + 1.35
60-69	0.0715 x Mean arterial pressure + 3.16
<70	0.0676 x Mean arterial pressure + 5.46

CHAPTER 6

Cerebral disease in a nationwide Dutch pseudoxanthoma elasticum cohort with a systematic review of the literature

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Abstract

Background - Pseudoxanthoma elasticum (PXE) is a monogenetic disease with progressive calcification of arteries and potential risk of stroke. To gain insights in the cerebral involvement in PXE, we evaluated prevalence and determinants of cerebral disease in our PXE cohort and performed a systematic review of literature.

Methods - Systematic history taking concerning cerebral disorders was performed in our PXE cohort. Cardiovascular risk factors were compared between PXE patients with and without cerebral disease. Additionally, Pubmed, Embase, the Cochrane Library and PsycINFO were systematically reviewed for studies published up to August 2016 about cerebral disease in PXE.

Results - Of the 178 PXE patients 31 (17.4%) had cerebral disease including ischemic stroke (n=15, 8.4%) or transient ischemic attack (n=13, 7.3%). The cerebral disease group was older (61 ± 12 vs. 52 ± 15 years, p-value 0.003) and had less favorable profiles of traditional cardiovascular risk factors regarding systolic blood pressure (137 ± 22 vs. 126 ± 21 mmHg, p-value 0.007) and HDL-cholesterol (1.4 ± 0.3 vs. 1.6 ± 0.4 mmol/L, p-value 0.011). One prospective cohort study reporting an incidence rate of ischemic stroke of 477/100,000/year and two cross-sectional studies with a reported prevalence of ischemic stroke of 14% and 0% were identified. Furthermore, 53 unique cases of cerebral disease in PXE including ischemic stroke (n=16) and transient ischemic attack (n=7) were reported.

Conclusions - Physicians and patients should be aware of the prevalent occurrence of cerebrovascular disease in PXE, which further stresses the importance of strict cardiovascular risk management in these patients.

Introduction

Intracranial arterial calcification has been independently associated with cerebrovascular disease.[1-3] This may be explained by calcification-induced stiffening of arteries, which can induce pulse pressure damage in the small cerebral vessels.[4, 5] Furthermore, this stiffening can lead to intimal damage although this mechanism is not well proven.

Two types of arterial wall calcification can be distinguished based on the affected arterial layer. Intima calcification has been described to be a part of the traditional atherosclerotic process. The other type of arterial wall calcification, medial calcification, also known as arteriosclerosis, has been associated with risk factors such as diabetes mellitus, renal failure and aging.[4, 6] Under normal conditions, medial calcifications are actively inhibited due to a balance between pro- and anticalcifying agents in the body.[7] Pyrophosphate is a strong inhibitor in this balance. Patients with pseudoxanthoma elasticum (PXE, OMIM #264800), a monogenetic disease caused by mutations in the *ABCC6* gene, have reduced plasma levels of inorganic pyrophosphate and subsequently an increased risk for progressive calcification of medium and small sized arteries.[8-12] Because of this progressive calcification of arterial walls throughout the body it is likely that patients with PXE are at increased risk for cerebral vessel calcification and for developing stroke. Contributive to this hypothesis is the fact that in familial idiopathic basal ganglia calcification (IBGC, OMIM #213600), a disease with a similar pathophysiology as PXE, mutations in genes expressing for the phosphate homeostasis lead to extensive calcification of cerebral arteries.[13] The association between PXE and stroke has been described in the literature, but the evidence is scarce.[14] Furthermore, little is known about cerebral complications besides cardiovascular disease in patients with this rare disease.

In order to be aware and possibly prevent or treat cerebral diseases in PXE patients, it is required to evaluate the effects of PXE on the brain. Therefore, we evaluated prevalence and determinants of any cerebral disease in a nationwide Dutch cohort of PXE patients. Furthermore, in order to be able to interpret and possibly generalize our findings outside the Dutch setting, we evaluated the literature by means of a systematic review.

Material and methods

Dutch PXE cohort

Dutch PXE patients are followed and treated in the Dutch National Expertise Center for PXE, which is situated in the University Medical Center Utrecht

(UMCU). The current cohort covers a large part of the Dutch PXE population. For the present study, data of 178 patients with a proven diagnosis of PXE enrolled in the Dutch PXE cohort between July 2008 and May 2016. The PXE diagnosis was based on the revised diagnostic criteria of Plomp et al. meaning that PXE is diagnosed if two of the following three aspects of the disease are present: skin involvement, eye involvement and genetically proven mutations of both alleles of the ABCC6 gene or a first-degree family member who meets the definitive diagnostic criteria for PXE.15 Cerebral disease was defined as any cerebral neurological disorder based on clinical signs, imaging, cerebrospinal fluid analysis or tissue examination. Given the retrospective use of routine care data no formal approval of this study was required as stated by the Medical Ethics Committee of the UMC Utrecht and informed consent for PXE patients was waived for anonymous analysis of the data (IRB number 15/446-C).

Dutch PXE cohort – Data collection

In the Dutch PXE cohort systematic history taking was performed by one of the investigators (WS). Also, determinants such as age, sex, cardiovascular risk factors and medication use were collected prospectively. Cardiovascular risk factors included blood pressure, glucose, lipids, estimated glomerular filtration rate based on the CKD-EPI formula, smoking status and cardiovascular disease in one or more first-degree family members before the age of 65.

Dutch PXE cohort – Data analyses

Differences in determinants between PXE patients with cerebral disease and without cerebral disease were compared using the Chi-square statistic for categorical variables. For continuous variables normality was assessed and the two sample t-test was used in case of normality and the Mann-Whitney U test in case of non-normality. P-values below 0.05 were considered significant. All analyses were performed using SPSS version 22.0.

Systematic review – Selection of studies

We performed a systematic search on the 22nd of August 2016 in Pubmed, Embase, the Cochrane Library and PsycINFO. We used broad search terms for the combination of PXE and cerebral or neuropsychological disease using terms as ‘brain’, ‘cognition’ and ‘mental’ (online supplemental table 1). After the removal of duplicates, two authors (FK and GK) independently screened the articles based on title and abstract. If there was discrepancy between the authors, consensus was achieved by means of discussion. Afterwards, articles were reviewed in more detail using the full-texts. Both human and animal studies were included, while

reviews and articles with language other than English, Dutch, German and French were excluded. Articles with an irrelevant publication type such as conference abstracts and editorials were excluded. If full-text was not available, we contacted the authors and requested the full-text versions of the articles. If the authors did not react on our request we excluded the article, because the full-text was not available. A manual reference check was performed of included articles and to pinpoint additional articles Web of Science was used. This systematic search was performed and reported following the PRISMA criteria.[16]

Systematic review – Evaluation of included studies and data extraction

Two authors (FK and GK) independently evaluated the risk of bias in the remaining studies based on the study design with the lowest risk of bias for cohort studies and the highest risk of bias for case reports. Also, to assess validity of the included studies, the diagnostic procedures used to diagnose PXE and the assessment of the described cerebral disease were collected. We deemed a study as less valid if the definitive diagnosis of PXE was incorrect according to the Plomp criteria.

From the included studies one author (FK) collected characteristics such as age, sex, country and sample size. Furthermore, we extracted data regarding the type of cerebral disease described and the diagnostic procedures used for the diagnosis of both PXE and cerebral disease.

Results

Dutch PXE cohort

Of the 178 PXE patients included in the Dutch PXE cohort 31 (17.4%) patients had a history of cerebral disease. Baseline characteristics of patients with and without cerebral complications are demonstrated in table 1. Patients with cerebral disease were older than the patients without cerebral disease (61 ± 12 vs. 52 ± 15 years, p=0.003). Furthermore, the group with cerebral complications had higher levels of systolic blood pressure (137 ± 22 vs. 126 ± 21 mmHg, p=0.007), lower levels of high-density lipoprotein (HDL) cholesterol (1.4 ± 0.3 vs. 1.6 ± 0.4 mmol/L, p=0.011) and estimated glomerular filtration rate (85, interquartile range (IQR) 76-90 vs. 90, IQR 82-90 ml/min/1.73m^2, p=0.012) and used more blood pressure lowering medication (41.9% vs. 19.7%, p=0.008) and lipid lowering medication (61.3% vs. 30.6%, p=0.001) compared to the patients without cerebral disease.

Table 1 Baseline characteristics

	Cerebral disease	No cerebral disease	p-value
	n=31	n=147	
Age - years, mean (SD)	61(12)	52 (15)	0.003
Male sex, n (%)	8 (26)	53 (36)	0.275
Coronary artery disease, n (%)	3 (10)	7 (5)	0.280
Peripheral artery disease, n (%)	17 (55)	56 (38)	0.085
Gastric bleeding, n (%)	3 (10)	4 (3)	0.070
Smoking, PY, median (IQR)	3 (0-20)	5 (0-13)	0.283
CVD in family*, n (%)	12 (39)	35 (24)	0.096
Blood pressure lowering medication, n (%)	13 (42)	29 (20)	0.008
Lipid lowering medication, n (%)	19 (61)	45 (31)	0.001
Glucose lowering treatment, n (%)	4 (13)	7 (5)	0.087
Systolic blood pressure – mmHg, mean (SD)	137 (22)	126 (21)	0.007
Diastolic blood pressure – mmHg, mean (SD)	73(9)	74 (11)	0.876
Fasting blood glucose - mmol/L, mean (SD)	6.2 (2.1)	5.5 (1.2)	0.089
Total cholesterol - mmol/L, mean (SD)	5.0 (1.1)	5.2 (1.1)	0.450
Triglycerides - mmol/L, mean (SD)	1.5 (0.7)	1.2 (0.9)	0.092
HDL-cholesterol - mmol/L, mean (SD)	1.4 (0.3)	1.6 (0.4)	0.011
LDL-cholesterol - mmol/L, mean (SD)	2.9 (1.0)	3.0 (1.0)	0.589
Non-HDL-cholesterol - mmol/L, mean (SD)	3.6 (1.1)	3.5 (1.1)	0.852
Estimated GFR†- ml/min/1.73m^2, median (IQR)	85 (76-90)	90 (82-90)	0.012

*: one or more first-degree family members under the age of 65 with cardiovascular disease

†: estimated glomerular filtration rate based on the CKD-EPI formula

SD: standard deviation, PY: packyears, IQR: interquartile range, CVD: cardiovascular disease, mmHg: millimeter of mercury, mmol/L: millimole per liter, HDL: high-density lipoprotein, LDL: low-density lipoprotein, GFR: glomerular filtration rate.

p-values were based on Chi-square statistic for categorical variables and for continuous variables normality was assessed and the two sample t-test was used in case of normality and the Mann-Whitney U test in case of non-normality.

Ischemic stroke occurred in 15 (8.4%) patients in our cohort (table 2). In 13 (7.3%) patients one or more transient ischemic attacks (TIA) occurred. Other cerebral diseases were Parkinson's disease (n=2, 1.1%), intracranial hemorrhage (n=1, 0.6%), aneurysm (n=1, 0.6%), vascular dementia (n=1, 0.6%), sinus thrombosis (n=1, 0.6%), bilateral carotid agenesis (n=1, 0.6%) and migraine (n=1, 0.6%).

Table 2 Cerebral diseases in the Dutch PXE cohort (n=178)

Cerebral disease n=31/178	**n**	**%**
Ischemic stroke	15	8
TIA	13	7
Parkinson's disease	2	1
Intracranial hemorrhage	1	1
Aneurysm	1	1
Vascular dementia	1	1
Sinus thrombosis	1	1
Bilateral carotid agenesis	1	1
Migraine	1	1

TIA: transient ischemic attack

Systematic review

The search strategy identified 331 unique articles and after screening 47 relevant articles remained (figure 1). We found one relevant prospective cohort study, three cross-sectional studies and 43 case series or case reports that described cerebral or neuropsychological complications in PXE (table 3). This prospective cohort study was the study with the highest validity and reported ischemic stroke in 7 of 94 patients with PXE (incidence rate 477/100,000/year) during a mean period of 17.1 years.[14] No intracranial aneurysm was found during the follow-up. In a group of 42 patients and 17 carriers of the *ABCC6* gene a history of ischemic stroke was found in 14% of the patients and in 12% of the carriers.[17] In a cross-sectional study of 100 patients with an average age of 29.5 years only one patient with a history of a ruptured cerebral aneurysm was found.[12] Neuropsychological tests were performed in 27 of these 100 PXE patients and showed neuropsychological deficits in two (7%) PXE patients. Furthermore, PXE patients scored significantly lower on the average impairment rating and the Halstead impairment index indicating deficits in cognitive processing speed, executive functioning, spatial memory and intelligence, but the means of the scores are not in the range of patients with known cerebral lesions.[18]

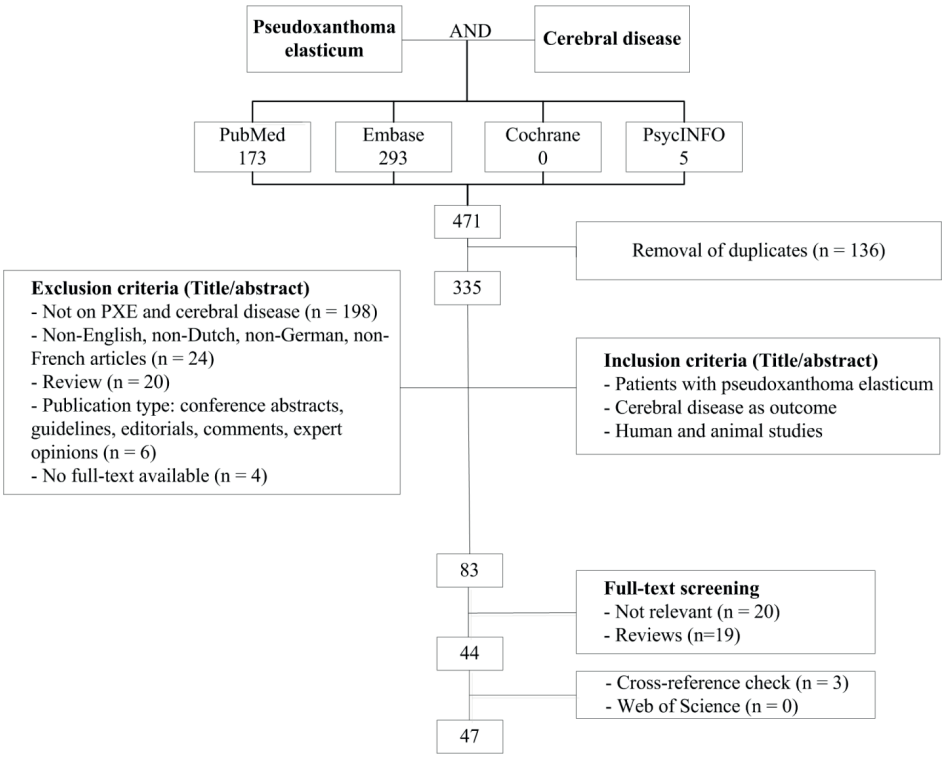

Figure 1. Flowchart of the systematic review of the literature on 22 August 2016

Table 3 Characteristics of included cohort studies and cross-sectional studies

Study	Age - Mean (SD)	Male %	Country	Study design	Cerebral disease	PXE patients - n	Cerebral disease - n (%)*
Van den Berg 2010	31.4 (NR)	37	Nether-lands	PFU	Ischemic stroke	94	477 / 100,000/ year
Vanakker 2008	52 (NR)	40	Belgium	Cross-sectional	Ischemic stroke	42	6 (14)
Neldner 1988	29.5 (NR)	30	USA/ Canada	Cross-sectional	Ruptured cerebral aneurysm	100	1 (1)
Heaton 1978	31.9 (11.8)	NR	USA	Cross-sectional	Neuro-psychological deficits	27	2 (7)

*: The reported incidence rate is shown in case of follow-up and the reported prevalence in case of a cross-sectional study design SD: standard deviation, FU: Follow-up, PXE: pseudoxanthoma elasticum, NR: not reported, PFU: prospective follow-up

From the remaining 43 articles, including several case series, we extracted 53 unique case reports, of which 44 met the criteria for the definitive diagnosis of PXE according to the Plomp criteria. For detailed information on all case reports and references see supplemental table 2. The mean age of the patients described in the cases was 38.9 years and 34% were male. The summaries of all case reports are shown in tables 4 and 5. Ischemic stroke and white matter lesions were reported most often (both in 16 cases of PXE). Other complications were transient ischemic attack (n=7), cerebral hemorrhage (n=5), aneurysm (n=5) and carotid rete mirabile (n=5).

Table 4 Baseline characteristics of case reports of PXE (n=53)

Characteristic	n
Age, years, mean (SD)	38.9 (16.5)
Male sex	18
PXE manifestations	
Skin	47
Eye	46
Vascular	19
Biopsy	35
Genetic	6
Country	
Australia	1
France	6
Germany	6
Hungary	1
India	3
Italy	3
Japan	4
Korea	1
Netherlands	1
Serbia	3
Sweden	12
Switzerland	1
UK	2
USA	9

SD: standard deviation, PXE: pseudoxanthoma elasticum, UK: United Kingdom, USA: United States of America

Table 5 Cerebral diseases in all case reports and selected case reports of PXE

	All case reports (n=53)	Selected case reports* (n=44)
	n (%)	n (%)
Ischemic stroke	20 (38)	16 (36)
White matter lesions	16 (30)	16 (36)
TIA	7 (13)	7 (16)
Cerebral hemorrhage	7 (13)	5 (11)
Aneurysm	8 (15)	5 (11)
Carotid rete mirabile	5 (0)	5 (11)
Neuropsychological deficits	3 (6)	3 (7)
Mental depression	2 (4)	2 (5)
Pituitary tumor	1 (2)	1 (2)
Glioblastoma multiforme	1 (2)	1 (2)
Pontine AVM	1 (2)	1 (2)
Seizures	3 (6)	1 (2)
Basilar dolichoectasia	2 (4)	1 (2)
Vascular cognitive impairment	1 (2)	1 (2)
Pseudobulbar paralysis	1 (2)	1 (2)
Moyamoya disease	1 (2)	-

*: Case reports with definitive diagnosis of PXE according to the Plomp criteria. TIA: transient ischemic attack, AVM: arteriovenous malformation

Discussion

PXE is characterized by progressive calcification in the medial layers of medium and small sized arteries. We hypothesized that this would result in a higher risk of cerebral disease and therefore determined the prevalence and determinants of any cerebral disease in the Dutch PXE cohort and systematically reviewed the literature. This is the first study that investigated the broad range of cerebral complications in a PXE cohort in combination with a systematic review of the literature to assess the generalizability of the findings. We found a remarkably high prevalence of cerebral complications in PXE patients, especially with respect to ischemic stroke or TIA. In the Dutch PXE cohort the prevalence of stroke was 8.4%, while the prevalence of stroke in the general population matched for age is estimated to be 2.6%, which implies an increased risk of stroke in patients with PXE compared to the general population.[19] TIAs, although often difficult to diagnose, were also more prevalent in the Dutch PXE cohort (7.3%) than in the general population (2.3%).[19]

A possible explanation for this observed remarkable high proportion of ischemic cerebrovascular disease might lay in higher cardiovascular risks due to more cardiovascular risk factors in the patients with cerebral involvement. Although cardiovascular risk factors indeed differed between patients with and without cerebral disease, the distribution of cardiovascular risk factors in PXE patients with a history of cerebral disease was not that different from the general population. For instance, the prevalence of medication use because of hypertension and increased cholesterol blood levels in the PXE patients with cerebral disease was similar to the prevalence of hypertension and increased total cholesterol blood levels in the general Dutch population (around 24% and 63%, respectively).[20, 21] Although HDL-cholesterol was significantly lower in the group of patients with cerebral disease compared to the group without cerebral disease, the values (1.4 ± 0.3 and 1.6 ± 0.4 mmol/L, respectively) are still within the normal range and therefore this finding is probably not associated with the increased prevalence of cerebral disease.[22] The difference in age between the two groups might have contributed to the observed difference in prevalence of cerebral disease as well. However, although the mean age of PXE patients with cerebral disease was only 60 years old, the observed prevalence of stroke in PXE is 1.5 times as high as the prevalence in the general population aged between 60 and 80.[19] Therefore, taken together, the large prevalence of cerebrovascular disease in PXE patients can not be totally explained by traditional cardiovascular risk factors or age.

The systematic search of the literature provided only one prospective cohort study.[14] This study based the diagnosis of PXE mostly on clinical symptoms according to the Berlin Nosology and, in some cases, on additional skin biopsy.[23] This is a less accurate way of diagnosing PXE compared to the Plomp criteria, in which skin biopsy and genetics are important aspects for the definitive diagnosis of PXE. Still, the reported incidence rate (477/100,000/year) of ischemic stroke in that study is higher than the incidence rate in the general population (84/100,000/year).[24]

The three included cross-sectional studies with a lower level of evidence compared to follow-up studies showed varying results. One study found an increased prevalence of ischemic stroke in patients with clinical signs of PXE and mutations of both *ABCC6* alleles similar to our findings.[17] In another cross-sectional study a relative young PXE population was included, in which only one ruptured cerebral aneurysm was found in 100 patients.[12] This inconsistency with our findings is probably due to the cross-sectional design and the very young population (mean age around 30 years) included in this study.

It remains difficult to provide a strong conclusion concerning the association between PXE and cerebral disease based on our cross-sectional study and only one prospective cohort study included in the systematic review. However, the combination of these findings with the findings of the three cross-sectional studies, of which one reported an increased prevalence of ischemic stroke and one found mild neuropsychological deficits in PXE patients, and the large amount of case reports on PXE and cerebral disease, especially ischemic cerebrovascular disease, included in the systematic review strongly indicates an association between PXE and ischemic cerebrovascular disease. The included case reports most frequently described ischemic stroke and white matter lesions. White matter lesions may be caused by ischemic microangiopathy.[25, 26] Probably, this cerebral small vessel disease also plays an important role in the pathophysiology of brain involvement in PXE.

Based on our study, we hypothesize that the progressive calcification of arterial walls caused by the reduced plasma levels of inorganic pyrophosphate in PXE patients might also occur in the medium and small intracerebral vessels.[3] The pathophysiology of PXE involves reduced levels of pyrophosphate and effects on the phosphate and calcium homeostasis and is similar to other rare diseases such as generalized arterial calcification of infancy (GACI, OMIM #208000) and idiopathic basal ganglia calcification (IBGC). GACI has also been associated with progressive arterial calcification in the brain, due to mutations of the *ABCC6* gene and the *ENPP1* gene.[27-29] A mutation in the *SLC20A2* gene leads to elevated cerebral phosphate levels and therefore to calcium phosphate depositions in cerebral arteries and in brain tissue causing neuropsychiatric symptoms in patients with IBGC.[13, 30, 31] These rare diseases with similar pathophysiology provide more insights in the process and effects of deficits in the calcium and phosphate homeostasis and these results suggest that subsequent cerebral arterial calcification is strongly related to cerebrovascular disease. Furthermore, these diseases could provide a potential therapeutic target in the calcium and phosphate homeostasis to prevent calcification in the brain. Therefore, further detailed studying of these monogenetic diseases might be very important.

Some limitations of this study need to be considered. The present study did not provide follow-up data after systematic history taking on cerebral outcome was performed in PXE patients. Future follow-up in these patients must demonstrate if more cerebral complications occur in PXE. It is likely that more patients with vascular signs of PXE were referred to the Dutch national expertise center for PXE than patients without vascular signs, as this was often the reason of referral to the

UMC Utrecht. Other reasons to refer a patient to the UMC Utrecht such as PXE related eye involvement or skin involvement may also be associated with risk of cardiovascular involvement and therefore possibly some selection of a high risk PXE group has taken place.[32] A limitation of this systematic review is the fact that we could not formally test whether publication bias was the case. It is likely that studies with positive findings on PXE and cerebral disease were published more often than studies without relevant findings. However, not all included studies we identified through systematic review of the literature found a high prevalence of cerebrovascular disease in PXE patients. In order to interpret our results it is important to take into account that the prospective cohort study included in the systematic review was conducted in the same country as the cohort in the UMC Utrecht we present in this article. Therefore, it is possible that some of the patients are used for both this study and the prospective cohort study included in the systematic review.

In conclusion, PXE, a disease with progressive calcification of medial layers of arteries, is associated with a remarkably high prevalence of cerebral disease, especially ischemic cerebrovascular disease. This high prevalence of cerebrovascular disease can not be explained by differences in cardiovascular risk factors and age only, which illustrates the role of arterial calcification in the pathophysiology of cerebrovascular disease. Physicians and patients should be aware of this prevalent occurrence of cerebrovascular disease in PXE, which further stresses the importance of strict cardiovascular risk management in these patients.

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Appendix

Database	Syntax
Pubmed	((((((((((((pseudoxanthoma*[Title/Abstract]) OR PXE*[Title/Abstract]) OR ABCC6[Title/Abstract]) OR “ectopic mineralization”[Title/Abstract]) OR “Grondblad Strandberg”[Title/Abstract]) OR Gronblad-Strandberg[Title/Abstract]) OR “Groenblad Strandberg”[Title/Abstract]) OR Groenblad-Strandberg[Title/Abstract]) OR elastor*[Title/Abstract]) OR “Pseudoxanthoma Elasticum”[MeSH Terms])) AND (((Hippocampus[Title/Abstract]) OR “small vessel disease”[Title/Abstract]) OR carotid[Title/Abstract]) OR “Hippocampus”[MeSH Terms]) OR “Cerebral Small Vessel Diseases”[MeSH Terms]) OR “Carotid Arteries”[MeSH Terms]) OR Stroke[Title/Abstract]) OR Strokes[Title/Abstract]) OR CVA[Title/Abstract]) OR CVAs[Title/Abstract]) OR Cerebrovascular[Title/Abstract]) OR apoplexy[Title/Abstract]) OR “transient ischemic attack”[Title/Abstract]) OR “transient ischaemic attack”[Title/Abstract]) OR TIA[Title/Abstract]) OR TIAs[Title/Abstract]) OR “Cerebrovascular Disorders”[MeSH Terms]) OR cerebral[Title/Abstract]) OR cerebrum[Title/Abstract]) OR cerebrums[Title/Abstract]) OR encephalon[Title/Abstract]) OR encephalons[Title/Abstract]) OR brain[Title/Abstract]) OR brains[Title/Abstract]) OR intracranial[Title/Abstract]) OR “Brain”[MeSH Terms]) OR “Brain Diseases”[MeSH Terms]) OR MMSE[Title/Abstract]) OR 3ms[Title/Abstract]) OR ADAS[Title/Abstract]) OR “clock test”[Title/Abstract]) OR “clock drawing”[Title/Abstract]) OR CDT[Title/Abstract]) OR mini-cog[Title/Abstract]) OR “mini cog”[Title/Abstract]) OR 6CIT[Title/Abstract]) OR “Six-item screener”[Title/Abstract]) OR “six item screener”[Title/Abstract]) OR SIS[Title/Abstract]) OR GPCOG[Title/Abstract]) OR AMT[Title/Abstract]) OR ACE[Title/Abstract]) OR MEAMS[Title/Abstract]) OR “severe impairment battery”[Title/Abstract]) OR SIB[Title/Abstract]) OR CDR[Title/Abstract]) OR dementia[Title/Abstract]) OR dementias[Title/Abstract]) OR dementive[Title/Abstract]) OR “Dementia”[MeSH Terms]) OR Alzheimer*[Title/Abstract]) OR cogniti*[Title/Abstract]) OR mental[Title/Abstract]) OR derangement[Title/Abstract]) OR “Lewy body”[Title/Abstract]) OR “Lewy bodies”[Title/Abstract]) OR amentia[Title/Abstract]) OR amentias[Title/Abstract]) OR “Mental Health”[MeSH Terms]) OR “Mild Cognitive Impairment”[MeSH Terms])))

Database	Syntax
Embase	((((((((((pseudoxanthoma*:ti,ab) OR PXE*:ti,ab) OR ABCC6:ti,ab) OR 'ectopic mineralization':ti,ab) OR 'Grondblad Strandberg':ti,ab) OR Gronblad-Strandberg:ti,ab) OR 'Groenblad Strandberg':ti,ab) OR Groenblad-Strandberg:ti,ab) OR elastor*:ti,ab) OR 'pseudoxanthoma elasticum'/exp)) AND ((Hippocampus:ti,ab) OR 'small vessel disease':ti,ab) OR carotid:ti,ab) OR Stroke:ti,ab) OR Strokes:ti,ab) OR CVA:ti,ab) OR CVAs:ti,ab) OR Cerebrovascular:ti,ab) OR apoplexy:ti,ab) OR 'transient ischemic attack':ti,ab) OR 'transient ischaemic attack':ti,ab) OR TIA:ti,ab) OR TIAs:ti,ab) OR cerebral:ti,ab) OR cerebrum:ti,ab) OR cerebrums:ti,ab) OR encephalon:ti,ab) OR encephalons:ti,ab) OR brain:ti,ab) OR brains:ti,ab) OR intracranial:ti,ab) OR MMSE:ti,ab) OR 3ms:ti,ab) OR ADAS:ti,ab) OR 'clock test':ti,ab) OR 'clock drawing':ti,ab) OR CDT:ti,ab) OR mini-cog:ti,ab) OR 'mini cog':ti,ab) OR 6CIT:ti,ab) OR 'Six-item screener':ti,ab) OR 'six item screener':ti,ab) OR SIS:ti,ab) OR GPCOG:ti,ab) OR AMT:ti,ab) OR ACE:ti,ab) OR MEAMS:ti,ab) OR 'severe impairment battery':ti,ab) OR SIB:ti,ab) OR CDR:ti,ab) OR dementia:ti,ab) OR dementias:ti,ab) OR dementive:ti,ab) OR Alzheimer*:ti,ab) OR cogniti*:ti,ab) OR mental:ti,ab) OR derangement:ti,ab) OR 'Lewy body':ti,ab) OR 'Lewy bodies':ti,ab) OR amentia:ti,ab) OR amentias:ti,ab) OR 'hippocampus'/exp) OR 'cerebrovascular disease'/exp) OR 'carotid artery'/exp) OR 'cerebrovascular accident'/exp) OR 'transient ischemic attack'/exp) OR 'brain'/exp) OR 'mental disease assessment'/exp) OR 'cognition assessment'/exp) OR 'cognition'/exp) OR 'mental disease'/exp)))
Cochrane	((((((((((('pseudoxanthoma*:ti,ab) OR PXE:ti,ab) OR ABCC6:ti,ab) OR 'ectopic mineralization':ti,ab) OR 'Grondblad Strandberg':ti,ab) OR Gronblad-Strandberg:ti,ab) OR 'Groenblad Strandberg':ti,ab) OR Groenblad-Strandberg:ti,ab) OR elastor*:ti,ab)) AND ((Hippocampus:ti,ab) OR 'small vessel disease':ti,ab) OR carotid:ti,ab) OR Stroke:ti,ab) OR Strokes:ti,ab) OR CVA:ti,ab) OR CVAs:ti,ab) OR Cerebrovascular:ti,ab) OR apoplexy:ti,ab) OR 'transient ischemic attack':ti,ab) OR 'transient ischaemic attack':ti,ab) OR TIA:ti,ab) OR TIAs:ti,ab) OR cerebral:ti,ab) OR cerebrum:ti,ab) OR cerebrums:ti,ab) OR encephalon:ti,ab) OR encephalons:ti,ab) OR brain:ti,ab) OR brains:ti,ab) OR intracranial:ti,ab) OR MMSE:ti,ab) OR 3ms:ti,ab) OR ADAS:ti,ab) OR 'clock test':ti,ab) OR 'clock drawing':ti,ab) OR CDT:ti,ab) OR mini-cog:ti,ab) OR 'mini cog':ti,ab) OR 6CIT:ti,ab) OR 'Six-item screener':ti,ab) OR 'six item screener':ti,ab) OR SIS:ti,ab) OR GPCOG:ti,ab) OR AMT:ti,ab) OR ACE:ti,ab) OR MEAMS:ti,ab) OR 'severe impairment battery':ti,ab) OR SIB:ti,ab) OR CDR:ti,ab) OR dementia:ti,ab) OR dementias:ti,ab) OR dementive:ti,ab) OR Alzheimer*:ti,ab) OR cogniti*:ti,ab) OR mental:ti,ab) OR derangement:ti,ab) OR 'Lewy body':ti,ab) OR 'Lewy bodies':ti,ab) OR amentia:ti,ab) OR amentias:ti,ab)))
PsycInfo	Pseudoxanthoma or PXE

Database	Syntax

PXE: pseudoxanthoma elasticum, ABCC6: ATP binding cassette subfamily C member 6, elastor: elastorrhexis, CVA: cerebrovascular accident, TIA: transient ischemic attack, MMSE: mini-mental state examination, 3ms: modified mini-mental state test, ADAS: Alzheimer's disease assessment scale, CDT: clock drawing test, 6CIT: six item cognitive impairment test, SIS: six item screener, GPCOG: general practitioner assessment of cognition, AMT: abbreviated mental test, ACE: Addenbrooke's cognitive examination, MEAMS: Middlesex elderly assessment of mental state, SIB: severe impairment battery, CDR: clinical dementia rating

Supplemental table Characteristics of case reports (n=53) of PXE patients with cerebral disease

	PXE manifestations					Cerebral disease					Determination of cerebral disease										
Study	Age	Sex	Skin[a]	Eye[b]	Vasc.[c]	Biopsy[d]	Gene[e]	iCVA	WML	CH	TIA	Aneurysm	CRM	Other	Clinical	LP	CT	MRI	Angio.	Autopsy	NR
Alinder 1972[1]	40	M	x	x						x											x
Alinder 1972	43	M	x	x				x												x	
Alinder 1972	41	F	x	x						x										x	
Alinder 1972	48	F	x	x					x											x	
Alinder 1972	49	F	x	x				x													
Alinder 1972	NR	F	x	x						x											x
Alinder 1972	25	M	x	x		x		x													
Alinder 1972	47	V	x	x										x							x
Alinder 1972	45	V	x	x										x							x
Araki 2001[2]	63	M		x	x	x		x							x		x				
Aralikatti 2002[3]	36	M	x	x				x	x						x			x			
Babu 2011[4f]	65	M	x			x		x						x	x		x	x	x		
Berger 1958[5]	Child	M	x	x		x								x							x
Berrouschot 2005[6]	64	F	x	x			X		x		x	x			x				x		
Bock 2008[7]	41	M	x	x		x	X	x	x	x					x		x	x	x		
Carlborg 1959[8]	61	F	x	x	x	x								x	x					x	
Carlborg 1959	39	F	x	x	x					x										x	
Carlborg 1959[f]	25	F	x		x					x											x
Cerrato 2005[9]	44	F	x	x		x	X		x									x			

Supplemental table Characteristics of case reports (n=53) of PXE patients with cerebral disease (*continued*)

Study	PXE manifestations					Cerebral disease							Determination of cerebral disease								
	Age	Sex	Skin[a]	Eye[b]	Vasc.[c]	Biopsy[d]	Gene[e]	iCVA	WML	CH	TIA	Aneurysm	CRM	Other	Clinical	LP	CT	MRI	Angio.	Autopsy	NR
Chalk 1989[10]	13	F	x	x	x	x								x	x	x	x		x		
Del Zotto 2012[11]	39	F	x	x		x	X		x				x					x	x		
Dixon 1951[12]	29	F		x		x						x			x				x		
Fasshauer 1984[13]	35	F	x	x	x	x		x							x		x			x	
Galle 1981[14]	Child	M	x	x	x	x		x	x					x	x		x		x		
Goto 1975[15]	12	F	x	x	x							x		x	x				x		
Grison 1976[16]	32	M	x	x	x	x		x							x				x		
Hemmati 1989[17]	40	M	x	x		x		x	x						x			x			
Iqbal 1978[18]	17	F	x	x	x	x		x							x		x				
Kumar 2007[19]	20	M	x	x		x		x						x	x		x				
Mayer 1994[20]	56	F	x	x		x		x	x		x			x	x		x	x			
Mayer 1994	56	F		x		x		x	x		x			x	x		x	x			
Messimy 1975[21]	46	F	x	x	x	x									x				x		
Messis 1970[22]	41	M		x	x	x														x	
Meyer 2005[23f]	2	F	x			x		x						x				x			
Mikol 1974[24]	38	F	x	x	x	x						x							x		
Munyer 1981[25f]	66	F	x									x							x		
Paonessa 2010[26f]	12	F						x							x			x			
Park 2013[27f]	28	M	x			x								x						x	

Supplemental table Characteristics of case reports (n=53) of PXE patients with cerebral disease (*continued*)

	PXE manifestations							Cerebral disease							Determination of cerebral disease						
Study	**Age**	**Sex**	**Skin[a]**	**Eye[b]**	**Vasc.[c]**	**Biopsy[d]**	**Gene[e]**	**iCVA**	**WML**	**CH**	**TIA**	**Aneurysm**	**CRM**	**Other**	**Clinical**	**LP**	**CT**	**MRI**	**Angio.**	**Autopsy**	**NR**
Pavlovic 2005[28]	42	M	x	x		x			x		x				x			x			
Pavlovic 2005	48	F	x	x		x		x	x					x	x			x			
Pavlovic 2005	47	F	x	x		x			x		x				x			x			
Postert 1995[29]	55	M	x	x		x		x	x						x		x	x	x		
Prick 1938[30]	48	F	x	x		x								x	x					x	
Renard 2008[31]	29	F	x	x		x			x						x			x			
Rios-Montenegro 1972 32	20	M	x	x		x							x		x				x		
Scheie 1957[33f]	56	F		x	x							x			x				x		
Schröder 2006[34f]	50	F	x		x	x		x							x						
Sharma 1974[35f]	35	M	x		x	x				x					x	x					
Takeshita 2003[36]	72	F	x	x		x			x		x				x			x			
Vasseur 2011[37]	12	F	x	x			X				x		x		x				x		
Yasuhara 2004[38]	14	F	x	x	x	x							x						x		
Zimmo 2013[39]	20	F	x	x	x	x						x									x
Zuily 2012[40f]	38	F		x	x							x									x

Reported characteristics of the case reports (n=53) of PXE patients with cerebral disease; a: yellowish papules and/or plaques on the lateral side of the neck and/or flexural areas of the body; b: peau d'orange of the retina or one or more angioid streaks, c: vascular disease based on clinical signs and/or imaging, d: skin biopsy with microscopically morphologically altered elastin with fragmentation, clumping and calcification of elastic fibers, e: a pathogenic mutation of both alleles of the ABCC6 gene or a first-degree relative who meets independently the diagnostic criteria for definitive PXE, f: the reported case does not meet the criteria for the definitive diagnosis of PXE according to the Plomp criteria PXE: pseudoxanthoma elasticum, Vasc.: vascular, iCVA: ischemic cerebrovascular accident, WML: white matter lesions, CH: cerebral hemorrhage, TIA: transient ischemic attack, CRM: carotid rete mirabile, LP: lumbar puncture, CT: computed tomography, MRI: magnetic resonance imaging, Angio.: angiography, NR: not reported, M: male, F: female

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CHAPTER 7

Peripheral artery disease in pseudoxanthoma elasticum (PXE): insights in its prevalence and determinants from a national PXE cohort.

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Abstract

Background and aims- Pseudoxanthoma elasticum (PXE) is a rare systemic calcification disorder with extensive leg arterial calcifications (LAC). Peripheral artery disease (PAD) seems prevalent in PXE, but its precise phenotype remains poorly understood. We aimed to evaluate the prevalence of PAD in PXE and to investigate the association between patient characteristics, including LAC, and PAD.

Methods – 203 PXE patients underwent vascular screening. The presence, extensiveness and annularity of LAC were assessed on computerized tomography scans without contrast. A treadmill test, ankle brachial index (ABI) measurements and a questionnaire to assess intermittent claudication and nocturnal leg pain were performed. The association between several patient characteristics and PAD (treadmill test ABI<0.9) were investigated with age and gender adjusted logistic regression models.

Results - 46% of the PXE patients (52±15 years, 71/203 male) had PAD, 39% limb claudication, and 22% nocturnal pain. Age (OR per 10 years 1.33; 95%CI 1.09 - 1.64) and carotid intima media thickness (OR per 0.1 mm 1.26; 95%CI 1.01-1.59) were associated with PAD. Neither traditional vascular risk factors, nor presence (OR 1.22; 95%CI 0.56-2.66), extensiveness (OR 1.04; 95%CI 0.81-1.33), or annularity (OR 1.13; 95%CI 0.54-2.34) of LAC were associated with PAD in PXE.

Conclusions - The prevalence of PAD among PXE patients is remarkably high. Traditional vascular risk factors are not associated with PAD, whereas age and arterial wall disease burden are. In this cross-sectional analysis leg arterial calcification characteristics are not associated with PAD in PXE patients.

Introduction

Peripheral artery disease (PAD) is characterized by insufficient blood flow in the limbs due to narrowing or occlusion of peripheral arteries. Patients suffering from PAD are at high risk for vascular morbidity and mortality.[1, 2] Despite the prognostic importance of PAD, little is known about the pathogenesis of PAD.[3] Generally it is thought that atherosclerosis, which mainly affects the intimal layer of arteries, drives the pathogenesis of PAD. However, recent evidence questions this general assumption and indicates that leg arterial calcification (LAC), specifically calcification localized in the medial arterial layer, in addition to atherosclerosis is a key player in PAD.[4-7] Diseases with an isolated type of medial arterial wall calcification and little atherosclerosis interference may provide novel insights on the role of medial arterial calcifications in the pathogenesis of PAD.

Pseudoxanthoma elasticum (PXE, OMIM #264800), a rare autosomal recessive systemic calcification disorder, may be such a model disease. Among PXE patients the prevalence of LAC (>80%), but also of PAD (>40%) seems remarkably high.[8, 9] PXE is caused by a double mutation in the *ABCC6*-gene.[10-12] PXE is characterized by mineralization and fragmentation of elastic fibers with a typical pattern of clinical manifestations in the skin, the eyes, and the arterial walls[13, 14] This ectopic calcification is caused by a misbalance in the complex interplay between calcification inhibitory and promoting pathways. [13, 15] *ABCC6*-mutations cause low levels of the calcification inhibitor inorganic pyrophosphate (PPi),[13] which normally inhibits hydroxyapatite crystal formation.[16, 17] The low levels of PPi in PXE result in progressive ectopic calcification and subsequent medial arterial calcification-like arterial abnormalities. It is proposed that these arterial abnormalities are the cause of the prevalent PAD and other types of vascular disease such as cerebrovascular disease.[9, 13, 18-21]

Clinical observations in PXE question general assumptions about clinical consequences of medial arterial calcification. Medial arterial calcification is believed to result in high ankle-brachial indexes (ABIs).[3] However, ABIs in PXE are rarely higher than 1.3. In fact, ABIs in PXE patients are generally low indicating that an obstructive type of PAD occurs in PXE.[8, 22] Also, the clinical manifestations of PAD in PXE seem to differ from the more general form of PAD. Critical limb ischemia, normally a typical complication of PAD, is hardly observed in PXE.[3, 8, 22] Detailed studying of clinical manifestations of PAD in a large group of PXE patients could improve our knowledge of the pathogenesis of PAD and elucidate the role of (medial layer) leg arterial calcifications.

Hence, we aimed to evaluate the prevalence of PAD in PXE and to investigate the association between patient characteristics, in particular leg arterial calcifications, and PAD.

Methods

PXE patients

In the Netherlands PXE patients are followed and treated in the Dutch National Expertise Center for PXE, situated in the University Medical Center (UMC) Utrecht. Clinical data of 203 consecutive PXE patients with a proven diagnosis based on the criteria of Plomp et al. were used for this study.[23] All patients underwent a systematic diagnostic program including a genetic, dermatological, ophthalmological and vascular screening. Given the retrospective use of routine care data no formal approval of this study was required as stated by the Medical Ethics Committee of the UMC Utrecht and informed consent for PXE patients was waived for anonymous analysis of the data (IRB number 15/446-C).

Vascular screening

A systematic history taking was performed in all patients in which determinants such as vascular risk factors were collected. Vascular risk factors included blood pressure, lipids, body mass index (BMI), estimated glomerular filtration rate based on the CKD-EPI formula, smoking status and history of diabetes mellitus and vascular disease. As a part of the vascular screening all patients underwent low-dose (<3 mSv) full-body CT scans without contrast (CT scanner Brilliance 64 Philips, Cleveland, Ohio). Also, extensive arterial wall measurements were performed in all patients. Carotid intima-media thickness (IMT) was measured using ultrasound (Esaote, Florence, Italy) and carotid femoral pulse wave velocity (PWV) was measured using applanation tonometry with a micromanometer (Millar Instruments Inc. Houston, USA) in combination with Sphygmocor software (Atcore Medical Pty. Ltd., Sydney, Australia).

Ankle-brachial index (ABI) measurements before and after a treadmill test were performed in all patients by experienced technicians. To determine the pre-treadmill test ABI patients were rested for 10 minutes in a supine position and the systolic blood pressure was measured in the left and right brachial arteries, the tibial posterior arteries and in the dorsal pedal arteries. The lowest value of the left and right side ABI was used. During the treadmill test patients were encouraged to walk on a treadmill with a 10% slope and a speed of 3.5 km/h for 6 minutes. Patients who stopped prematurely due to pain (claudication) were encouraged to

continue walking as soon as possible.[24] During recovery, the ABI was measured several times in a supine position using similar methods as in the pre-treadmill test ABI measurement. The lowest value of these ABI-measurements was used as the post treadmill test ABI.

PAD definition and assessment of PAD complaints

All patients underwent pre- and post-treadmill test ABI measurements. PAD was defined as a post-treadmill test ABI <0.9. In addition, all PXE patients received a standardized questionnaire on the presence of limb claudication and nocturnal leg pain. A total of 102 patients (50%) completed the questionnaire, after a second mail was send to non-responders in order to maximize the response rate. There were no substantial differences in characteristics between responders and non-responders. In accordance to the AHA/ACC guideline limb claudication was defined as fatigue, discomfort, cramping, or pain in the muscles of the lower extremities that is consistently induced by exercise and consistently relieved by rest.[25] Nocturnal leg pain was defined as any discomfort, cramping, or pain in the muscles of the lower extremities during the night that occurred at least once a week.

CT-based assessment of presence, extensiveness, and annularity of LAC

The CT images were used to assess the presence, extensiveness and annularity of LAC. For this assessment all arteries from femoral arteries to arteries in the foot were evaluated. A semi-quantitative scoring system with a good inter-observer agreement was designed to capture the extensiveness of arterial calcifications. This calcification score is described previously in more detail.[9] In short, four categories (no, mild, moderate or severe calcification) were used. Annularity of LAC was defined as the presence of complete (circular) closures of calcifications on at least one of the CT slides in the range between femoral arteries and arteries of the foot.

Data analyses

Prevalence of PAD, limb claudication, and nocturnal leg pain were determined in the Dutch PXE cohort. Also, descriptive statistics were performed for pre- and post-treadmill test ABI values and baseline characteristics stratified for the presence of PAD. In addition, to specifically evaluate differences between younger and older patients, the prevalence of PAD, limb claudication, and nocturnal leg pain was determined in patients younger and older than the median of 53 years. To visualize the age-dependent pattern of ABI in PXE, mean values and 95% confidence intervals of pre- and post-treadmill test ABIs were calculated and plot-

ted for the age groups <30 years, 30-39 years, 40-49 years, 50-59 years, 60-69 years and 70-80 years.

To investigate determinants of PAD in PXE, the association between different patient characteristics, such as LAC characteristics, with PAD was investigated. Separate logistic regression models were built for each patient characteristic and adjustment took place for age and gender, except in the models for age (only sex adjusted), sex (only age adjusted). Similar analyses were performed to investigate determinants of limb claudication and nocturnal leg pain.
In all analyses the level of significance was set at $p<0.05$. All data analyses were performed using R version 3.1.2.

Results

Baseline characteristics of patients with and without PAD are demonstrated in table 1.

Table 1 Baseline characteristics

	PAD **n=94**	**No PAD** **n=109**
Age	55 ± 13	49 ± 16
Gender (male)	34.0%	35.8%
Vascular risk factors		
Current smoking	16.1%	14.3%
Diabetes mellitus	5.3%	5.5%
Systolic blood pressure - mmHg	138 ± 20	132 ± 21
Diastolic blood pressure - mmHg	78 ± 12	78 ± 11
BMI - kg/m2	25.3 ± 4.1	25.2 ± 4.7
eGFR (CKD-EPI) - ml/min/1.73m^2	90 (79-90)	90 (72-90)
Non-HDL cholesterol - mmol/l	3.6 ± 1.1	3.5 ± 1.0
Arterial wall measurements		
Carotid IMT - mm	0.73 ± 0.16	0.65 ± 0.16
PWV- m/s	11.8 ± 3.9	10.2 ± 3.5
Manifest vascular disease		
Coronary arterial disease	8.5%	3.7%
History of cerebral vascular disease	10.6%	7.3%

Table 1 Baseline characteristics (*continued*)

	PAD n=94	No PAD n=109
CT-based LAC characteristics		
Presence of LAC	84.3%	82.0%
Severe LAC	67.4%	68.4%
Annular LAC	23.3%	20.0%
PAD measurements		
Claudication complaints	53.7%	22.9%
Nocturnal leg pain	29.6%	12.5%
ABI (pre treadmill test)	0.78 ± 0.18	1.04 ± 0.13
ABI (post treadmill test)	0.58 ± 0.21	1.04 ± 0.07

Data are reported as the mean (± standard deviation) for normal distributed parametrers and median (interquartile range) for non-normally distributed parameters. Categorical parameters were presented as %.ABI ankle-brachial index; eGFR estimated glomerular filtration ratio; IMT intima-media thickness; PWV Pulse Wave Velocity; LAC leg arterial calcifications

PAD in PXE

Of the 203 consecutive PXE patients 46% had PAD. The mean pre-treadmill test ABI was 0.92 ± 0.20 and the mean post-treadmill test ABI was 0.83 ± 0.28. Among younger patients (≤ the median of 53) the prevalence of PAD was 36%, whereas the prevalence of PAD among older PXE patients (>53 years) was 58% (figure 1). Pre- and post-treadmill test ABIs decrease with age as visualized in figure 2.

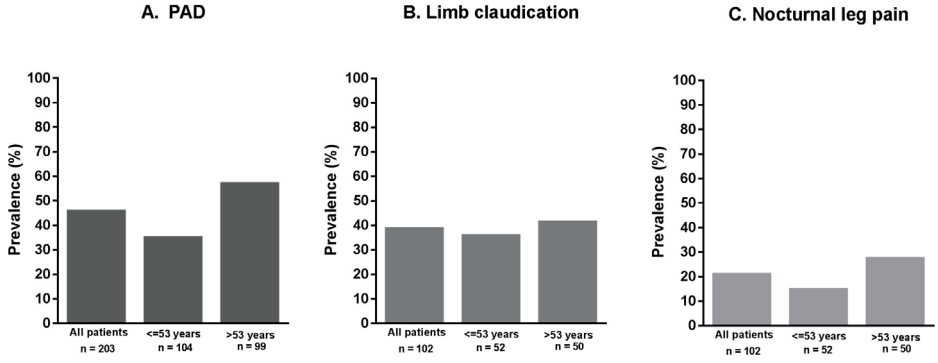

Figure 1 Prevalence of PAD, limb claudication, and nocturnal leg pain in PXE

Prevalence in % of PAD, limb claudication and nocturnal leg pain are given for all patients, patients ≤ or > the median of 53 years.

Of the 102 PXE patients who returned their questionnaire 39% had limb claudication. This was 37% among younger patients (≤53 years) and 42% among older patients (>53 years). 22% of the PXE patients who returned their questionnaire had nocturnal leg pain. This was 15% among younger patients (≤53 years) and 28% among older patients (>53 years). Critical limb ischemia did not occur in our PXE cohort.

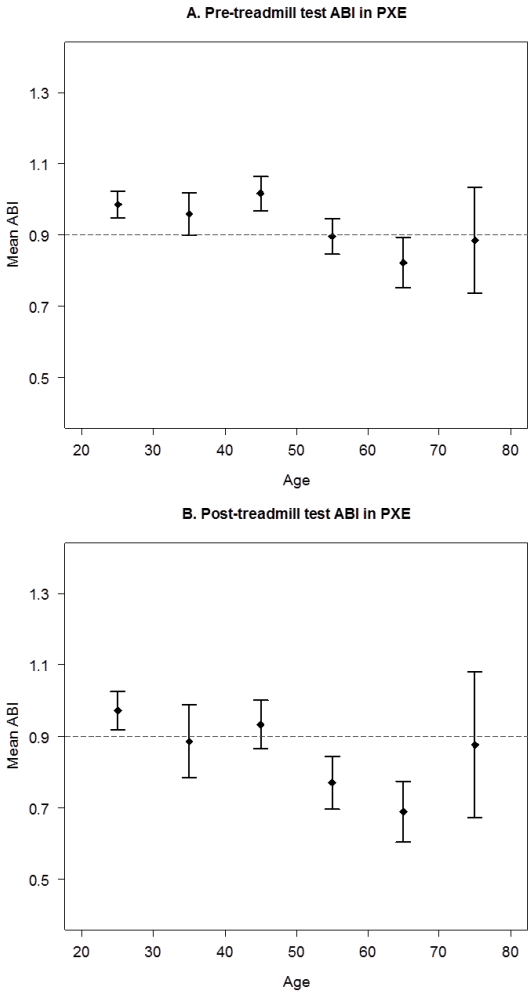

Figure 2 Age-dependent patterns of ABI in PXE

Means and 95%CI borders are depicted. ABI ankle brachial index

Determinants of PAD in PXE

In PXE patients, age was associated with PAD (OR per 10 years 1.33; 95%CI 1.09 - 1.64, table 2). Traditional vascular risk factors as smoking (OR per 10

packyears 0.72; 95%CI 0.38-1.34), diabetes mellitus (OR 1.19; 95%CI 0.34-4.18), systolic blood pressure (OR per 10 mmHg 1.06; 95%CI 0.87-1.19), BMI (OR per 1 kg/m^2 0.97; 0.91 – 1.04), and non-HDL cholesterol (OR per 1 mmol/L 1.00; 95%CI 0.75 – 1.32) were not associated with PAD. Higher levels of carotid IMT (OR per 0.1 mm 1.26; 95%CI 1.01 – 1.59) and carotid-femoral PWV (OR per 1 m/s 1.08; 95%CI 0.98 – 1.18) were associated with higher risks of PAD. The presence (OR 1.22; 95%CI 0.56-2.66), extensiveness (OR 1.04; 95%CI 0.81-1.33), or annularity of LAC (OR 1.13; 95%CI 0.54-2.34) were not associated with PAD.

In general, results were similar for associations with limb claudication and nocturnal leg pain. Smoking (OR per 10 packyears 1.69; 95%CI 1.10-2.58) and BMI (OR per 1 kg/m^2 1.14; 95%CI 1.00 - 1.29) were associated with limb claudication. Higher levels of carotid IMT (OR per 0.1 mm 1.23; 95%CI 0.94 – 1.76) and carotid-femoral PWV (OR per 1 m/s 1.28; 95%CI 1.09 – 1.51) were associated with higher risks of limb claudication.

Table 2 Determinants of PAD in PXE

	OR (95% CI)
Age - per 10 years	1.33 (1.09 - 1.64)
Gender (male)	1.17 (0.65 - 2.14)
Vascular risk factors	
Packyears – per 10 years	0.72 (0.38- 1.34)
Diabetes mellitus	1.19 (0.34 - 4.18)
Systolic blood pressure - per 10 mmHg	1.06 (0.87 - 1.19)
Diastolic blood pressure - per 10 mmHg	0.86 (0.65 - 1.13)
BMI - kg/m2	0.97 (0.91 - 1.04)
eGFR (CKD-EPI) - per 10 ml/min/1.73m^2	1.32 (0.85 - 2.06)
Non-HDL cholesterol - mmol/l	1.00 (0.75 -1.32)
Arterial wall measurements	
Carotid IMT - per 0.1 mm	1.26 (1.01 - 1.59)
PWV- m/s	1.08 (0.98 - 1.18)
Manifest vascular disease	
Coronary arterial disease	0.54 (0.15 - 1.95)
Cerebral vascular disease	0.90 (0.32 - 2.53)

Table 2 Determinants of PAD in PXE (*continued*)

	OR (95% CI)
CT-based LAC characteristics	
Presence of LAC	1.22 (0.56 - 2.66)
Severity of LAC*	1.04 (0.81 - 1.33)
Circular LAC	1.13 (0.54 - 2.34)

Adjustments took place for age and gender. Gender was adjusted only for age, age was adjusted only for gender; LAC leg arterial calcification; * per point increase in semi-quantitative calcification severity score

Discussion

Among PXE patients the prevalence of PAD was 46%, which is in agreement with previous studies.[8, 22, 26, 27] Also, 36% of the PXE patients had limb claudication and 22% had nocturnal pain. The prevalence of PAD in PXE patients is remarkably high, since the prevalence of PAD in the Dutch general population older than 55 years is estimated to be 19%.[28] Also, the prevalence of nocturnal leg pain (defined as nocturnal leg discomfort at least once a week) in PXE patients is high, since the prevalence of nocturnal leg pain (defined as nocturnal leg discomfort at least 5 times a month) in the general population is estimated to be 6%.[29] Increasing age and burden of arterial wall disease (higher carotid intima-media thickness) were associated to higher risks of PAD. Traditional vascular risk factors did not relate to PAD in PXE. In this cross-sectional analysis, neither the presence, nor the extensiveness, nor the annularity of LAC was associated with PAD.

In the present PXE cohort traditional (atherosclerotic) vascular risk factors were not associated with PAD, although smoking and BMI were associated to symptoms of limb claudication. This supports the hypothesis that the main driver of PAD in PXE is not atherosclerosis, but is likely another type of arterial wall disease. In the general population, traditional vascular risk factors as smoking, diabetes mellitus, systolic blood pressure, and (higher) HDL-cholesterol relate to PAD, supporting the general assumption of an atherosclerotic origin of PAD.[30] All currently available PAD therapies focus on the alleviation (or bypassing) of peripheral occlusions.[25] However, evidence suggesting a central role of medial wall arterial calcifications in the pathogenesis of PAD is emerging.[4] An illustrative example is a recent histopathology study in leg amputees from patients suffering from severe peripheral arterial disease in which it was shown that 72% of the arteries

contained medial calcifications, whereas only 23% of the arteries had atheromas.[5] Diseases from a spectrum of disorders associated with progressive calcification such as PXE might provide insights in the role of (medial) arterial calcifications in the pathogenesis of PAD.[18] Besides PXE, the Calcification of joints and arteries (CALJA) syndrome (OMIM #211800, caused by *NT5E*-gene mutations), is an example of such a disease. CALJA is also known to result in progressive arterial calcifications and PAD.[31]

Further supporting the hypothesis of a non-atherosclerotic driver of PAD in PXE is the fact that we found increased age and increased burden of arterial wall disease (as measured by carotid intima-media thickness) to be associated with PAD in PXE. We showed that a strong relation between age and ABI or PAD in PXE exists, which is in line with previous findings.[8, 27] In fact, the strong relation between age and PAD is also present in the general population, although the prevalence of PAD in PXE patients is much higher in across all age groups. [28] We also found that increased burden of arterial wall disease as measured by carotid intima-media thickness, representing the combination of intimal and medial arterial wall disease, was associated with PAD in PXE. Therefore, given the assumed ongoing progressive calcification in the medial layers of the vasculature in PXE, these findings add to the growing body of literature suggesting the central role of (medial) arterial calcifications, either instead of or on top of atherosclerosis, in the pathogenesis PAD.[5, 32]

The PAD phenotype of PXE observed in our cohort contradicts general assumptions on leg arterial calcifications indicating that PAD in PXE is not well understood. In non-PXE populations leg arterial calcifications increase the risk of critical limb ischemia.[6, 7, 33] However, in our PXE cohort critical limb ischemia did not occur, which is in line with previous findings in other cohorts.[8, 22] On the other hand, nocturnal pain was prevalent among PXE patients. Although it is generally believed that medial arterial calcification can result in high ABI values, the ABI values in our PXE cohort and previous PXE studies are low instead of high.[3, 8] This suggests that that the calcifications in PXE might progress to the intimal wall leading to stenosis and thrombotic changes contributing to PAD.[34, 35] Although we did not systematically compare the PXE calcifications to those of patients with diabetes mellitus, who also have progressive arterial calcification, [18] it might be that calcifications in PXE are less continuous and less often fully circular (22% in PXE). This may also explain the low ABIs in PXE patients compared to the high ABIs as commonly described in patients with diabetes mellitus.[36] Pathology studies in specimen of leg amputees show that medial arterial can occur in a circular

and in a localized form. [37, 38] This suggests that the medial arterial calcifications in PXE occur predominantly in a localized form and may explain the contradictions of our observations in PXE patients with general assumptions on clinical consequences of leg arterial calcifications.

The absence of associations between CT-based LAC characteristics and PAD in PXE patients further indicates that PAD in PXE is not well understood. We previously showed that leg arterial calcifications in PXE patients were significantly more prevalent compared to age-matched hospital controls (74% vs. 44% for femoral-popliteal arteries and 84% vs. 38% for subpopliteal arteries) and hypothesized that PAD in PXE was associated with LAC.[9] However, neither the presence, nor the extensiveness, nor the circularity of LAC was associated with PAD or symptoms. The cross-sectional design of this study and the fact that effects of walking therapy could not be taken into account could have caused the absence of these effects.

In the general population (supervised) walking therapy is the cornerstone of PAD treatment. Walking therapy leads to formation of collateral vessels which are able to bypass the arterial obstruction or occlusion.[25] In a recent study we showed that walking therapy is sufficiently effective in PXE patients with PAD and that surgical interventions, in particular angioplasty and stenting, should be delayed as long as possible.[39] PXE patients with first PAD symptoms are generally younger and relatively more mobile compared to general PAD patients, which might contribute to the remarkable effectiveness of walking therapy in PXE we observe in clinical practice. Also, traditional vascular risk factors are known to hinder collateral vessel formation which might explain the extensive collateral formation observed in PXE patients, who have relatively little traditional vascular risk factors.[40, 41] This is illustrated in figure 3 in which an image from a CT leg angiography from a 54-year-old non-smoking female PXE patient with PAD complaints undergoing walking therapy is shown. The extensiveness of collateral formation in this PXE patient is striking. Thus, the effectiveness of walking therapy and the formation of collateral vessels in PXE might have confounded the observed relations between CT-based LAC characteristics and PAD.

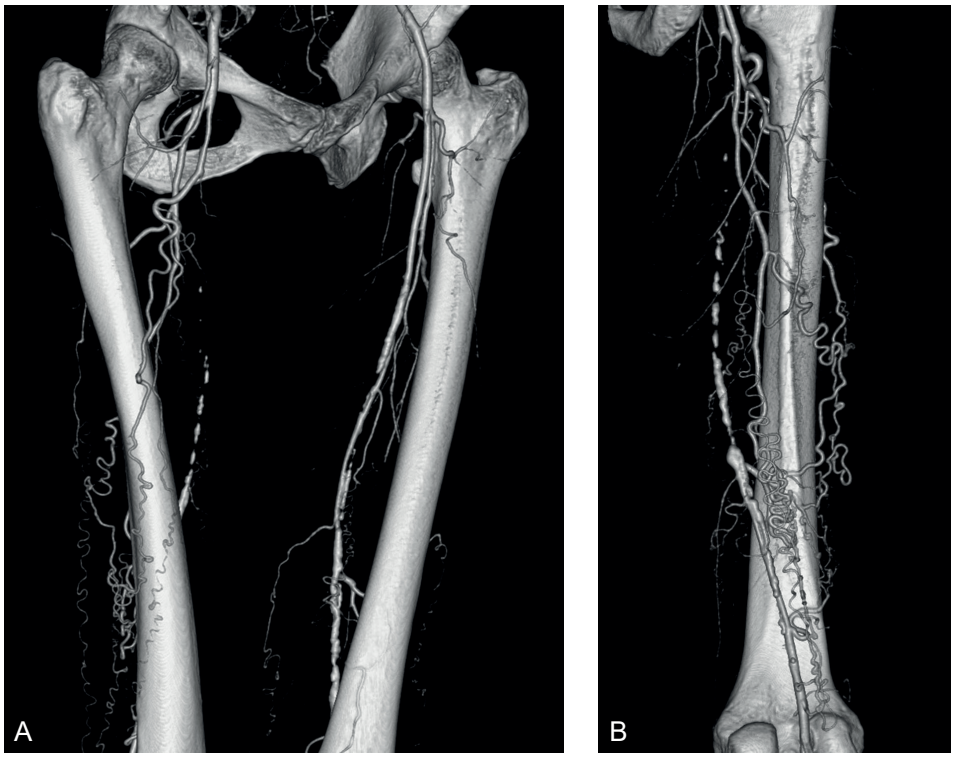

Figure 3 Collateral vessel formation in PXE

Images A and B are taken from a CT leg angiography from a 54-year-old non-smoking female PXE patient with PAD. Extensive collateral vessel formation is visualized. B dorsal view of the right upper leg.

Strengths of this study include the systematic vascular screening performed in all PXE patients resulting in complete and extensive data, enabling us to describe the PAD phenotype of PXE in detail. However, some limitations need to be considered as well. The main limitation is inherent to the cross-sectional design of our study, making it impossible to investigate the relation between characteristics as LAC and future PAD. Follow-up information is not yet available in our cohort and potential new onsets of PAD during follow-up could therefore not be taken into account in this study. Furthermore, systematic toe pressure measurements and angiographic measurements could have provided additional information. Lastly, information on limb claudication and nocturnal leg pain was only available in a subset of PXE patients (n=102). However, since there were no substantial differences in characteristics, including age, between responders and non-responders of the questionnaire, we think that the risk of selection bias is negligible.

In conclusion, the prevalence of PAD among patients with PXE, a systemic calcification disorder with progressive leg artery calcifications, is remarkably high. In PXE patients, traditional vascular risk factors are not associated with PAD, whereas age and arterial wall disease burden are associated with PAD. In this cross-sectional design leg arterial calcification characteristics are not associated with PAD in PXE patients. Further research using angiography and follow-up in a large cohort of PXE patients may enhance knowledge on PAD in PXE.

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CHAPTER 8

An abnormal high rate of failure of femoral angioplasty in patients with pseudoxanthoma elasticum

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Abstract

Pseudoxanthoma elasticum (PXE) is an inherited disease characterized by skin lesions, central blindness, and progressive peripheral occlusive disease. Severe claudication is a frequent symptom for which angioplasty represents a possible therapeutic avenue. We report the outcomes of four patients with PXE treated by angioplasty and stenting of the superficial femoral artery in two centers. These patients exhibited an abnormal failure rate for angioplasty and stenting of the superficial femoral artery, suggesting an as yet unknown susceptibility in such patients. In the absence of further evidence, we do not recommend arterial angioplasty with stenting as a primary surgical approach in PXE patients with femoral artery lesions.

Introduction

Three women and one man with clinically and genetically proven pseudoxanthoma elasticum (PXE), a rare inherited vascular disease, were referred for intravascular procedure for peripheral obstructive arterial disease (PAOD) with typical symptoms of intermittent claudication. The PXE disease had been diagnosed earlier on the basis of skin lesions, angioid streaks and skin biopsy[1] and further confirmed with genotyping in all patients. They suffered from severe intermittent claudication with a walking distance <250 m and in all medical attempt to restore PAOD had failed.

Case reports

Patient 1 (a woman, 68 years old) was diagnosed with PXE at 18 years of age. She was referred with a long history of lower limb claudication with an resting ankle-brachial pressure index (ABI) of 0.69 on the right and 0.85 on the left side. Duplex scan showed bilateral calcified short stenosis (>70%) of the superficial femoral arteries (SFAs). Primary angioplasty with stenting of the right SFA was performed (Fig 1), which was followed after 2 days by angioplasty using a nitinol stent (LifeStent; Bard, Tempe, Ariz) on the left side. The patient was discharged with combined oral antiplatelet medication (aspirin and clopidogrel). Four weeks later, intermittent claudication recurred, and duplex scanning disclosed bilateral thrombosis of the stented SFAs. The patient is currently stable and does not wish to have further treatment.

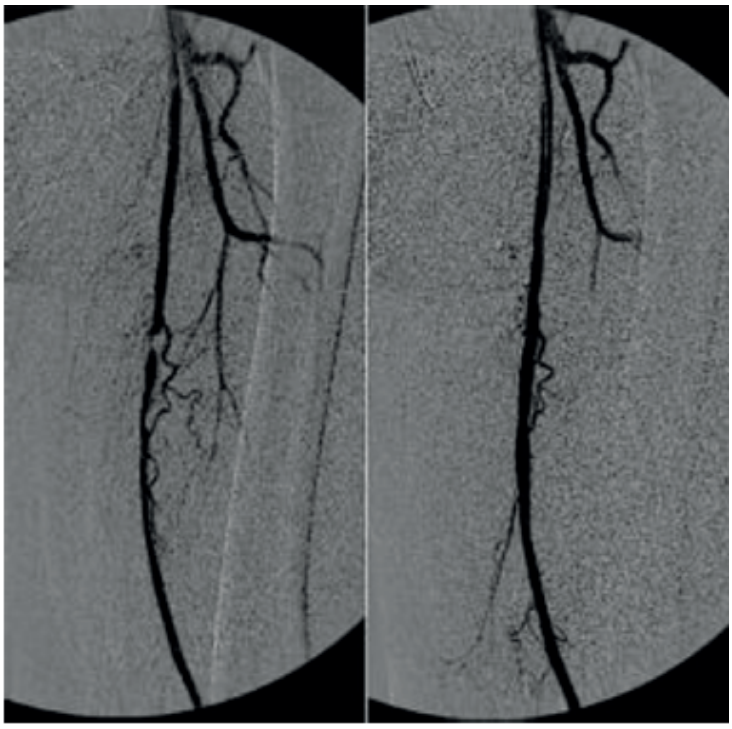

Figure 1 Intraoperative angiography demonstrating stenosis of the left superficial femoral artery (left image) and the result after angioplasty and stenting (right image).

Patient 2 (a woman, 65 years old) was diagnosed with PXE at 20 years of age. She was referred for severe claudication of the left leg (<150m) with an ABI of 0.51 and 0.68 (right side). Duplex scans disclosed significant stenosis (>70%) of the left SFA, and angioplasty of the left SFA with a nitinol stent (LifeStent; Bard) was performed. One day later, duplex scanning confirmed reocclusion (ABI 0.46) of the stented SFA. After patency was recovered by fibrinolysis (Fig2), a second stent was deployed, and the patient was discharged initially with low-molecular-weight heparin and clopidogrel. Later, she was switched to ticagrelor alone. A month later, the intermittent claudication recurred, and occlusion of the stented artery was shown on a duplex scan. A femoral-popliteal saphenous bypass was finally performed, and the patient has remained asymptomatic.

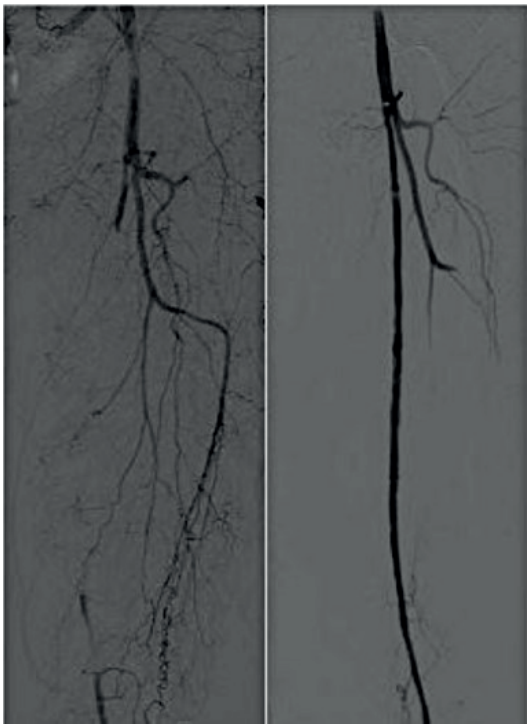

Figure 2 Angiography showing acute superficial femoral artery (SFA) thrombosis (left image) and result after intra-arterial in situ thrombolysis (right image).

Patient 3 (a woman) was diagnosed with PXE at 8 years of age. At 38 years, she suffered from severe claudication symptoms, in particular in her right leg and underwent her first angioplasty with stenting (S.M.A.R.T. stent; Cordis, Bridgewater, NJ) for stenosis of the right SFA. She was discharged with phenprocoumon (3 mg). However, one year later, a duplex scan showed a subtotal stenosis in the right SFA, which was treated by femoral-popliteal saphenous bypass. Nine years later, her claudication worsened on the left leg, and angioplasty of the SFA was performed. Twelve months later, in-stent stenosis of the left SFA was found, again treated by angioplasty. After 6 months, she was still suffering from claudication and was referred for a femoral-popliteal saphenous bypass.

Patient 4 (a man) was diagnosed with PXE at 41 years of age. At 44 years, he complained of severe claudication, and duplex scans showed severe stenosis of both SFAs. Bilateral angioplasty with stenting (S.M.A.R.T. stent; Cordis) was performed and he was discharged with aspirin (80 mg/d). Three years later, his maximum walking distance had decreased; duplex scans revealed restenosis on both sides, and bilateral angioplasty was repeated. Restenosis of the right SFA

was found and a third angioplasty was performed. Three years later, his maximum walking distance had again declined further (<150 m), and computed tomography angiography revealed significant restenosis of the left SFA. Since the patient had developed a dense network of collaterals in both legs, a watch and wait policy was decided.

Discussion

PXE is a rare autosomal recessive inherited disorder characterized by abnormal calcified elastic fibers in various connective tissues and mainly the internal elastic lamina of medium and small-sized arteries leading to a premature PAOD. The stenotic lesions develop preferentially in the femoral segments and start at young age with long lasting symptoms of intermittent claudication.[2]

To date, treatment of PAOD in PXE remains empirical and reports on the endovascular treatment in these patients is sparse. Donas et al successfully performed a balloon angioplasty for infra-renal aortic and iliac stenosis in one PXE patient.[3] Siskos and al reported an endovascular procedure, in a 58-year-old patient, suffering from PXE and PAD at the iliac level.[4]

Herein, we report the largest cohort of PXE patients treated with angioplasty. In all cases, claudication was related to an early development of SFA stenosis, for which endovascular angioplasty was legitimately considered as a first option after all medical treatment options had failed, including cardiovascular risk management and daily walking training. In all cases, endovascular procedures with stenting failed either due to thrombosis (2/4) or re-stenosis (2/4). In all cases, catheterisations of the lesions were performed according to conventional techniques and no specific technical problems were reported, the patency being confirmed with a final angiographic control immediately after stenting. Standardized peri-operative medical management was performed as recommended for this type of procedure after discharge (i.e. oral antiplatelet therapy).

Giving the very low number of published cases and the rarity of the disease, the reasons for this abnormal rate and repeated failures in patients from 2 different centres may reveal specific traits of the arterial remodelling in PXE. In accordance with our findings, Zimmo et al also experienced an unexplained failure in endovascular surgery in a PXE patient treated for a renal artery aneurysm.[5] The early thrombosis despite anti-platelet treatment in the first patient could advocate for a prothrombotic propensity more than for a technical error, although further investigations did not reveal any detectable abnormalities in the coagulability or

haemostasis. This is an unexpected observation since hypo-coagulability is rather reported in PXE due to a low vitamin K.[5]

Conclusion

Although PAOD is a common and under-treated manifestation of PXE, our experience combined with previous literature reveal an abnormal rate of failure of angioplasties and/orstenting on medium-sized arteries of these patients. Therefore, we do not recommend angioplasty as a primary choice for treating claudication in PXE. Further studies are needed to elucidate the cause for this abnormal rate of failure in this rare disease.

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PART TWO

Risk factors of (calcification-induced) vascular disease

CHAPTER 9

The relation between HbA1c and cardiovascular events in patients with type 2 diabetes mellitus with and without vascular disease

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Abstract

Objective – Poor glycemic control is related with vascular events in patients with type 2 diabetes, but the presence of vascular disease might influence this relation. We evaluated the relation between glycemic control (HbA1c) and new cardiovascular events and mortality in patients with type 2 diabetes, with and without vascular disease.

Research Design and Methods - In a cohort of 1687 patients with type 2 diabetes enrolled in the Second Manifestations of ARTerial disease (SMART) study, the continuous relation between HbA1c and vascular events (composite of myocardial infarction, stroke and vascular mortality) and all-cause mortality was evaluated with Cox proportional hazard analyses stratified for the presence of vascular disease.

Results – During a median follow-up of 6.1 years (IQR 3.1-9.5 years), 293 patients developed a new cardiovascular event and 340 patients died. In all patients the hazard ratio of the relation between HbA1c and cardiovascular events was 1.06 (95%CI: 0.97-1.17). A 1% point higher HbA1c was related to a 27% higher risk of a cardiovascular event in patients with type 2 diabetes without vascular disease (HR 1.27; 95%CI: 1.06-1.51) but not in patients with vascular disease (HR 1.03; 95%CI: 0.93-1.15, p for interaction 0.195). A 1% point higher HbA1c was related to a 16% higher risk of death (HR 1.16; 95%CI: 1.06-1.28) in patients with vascular disease and a non-significant 13% higher risk of all-cause mortality (HR 1.13; 95%CI 0.97-1.31) in patients without vascular disease.

Conclusions – In patients with type 2 diabetes there is a modest, but not statistically significant, relation between HbA1c and cardiovascular events and, as there was no statistical significant interaction, this relation was not different for patients with or without clinical manifest vascular disease.

Introduction

Strict glycemic control has been proposed as an important means to lower the risk of both microvascular and macrovascular complications of type 2 diabetes. A strong relation between glycemic control and microvascular complications (nephropathy, retinopathy and neuropathy) and macrovascular complications is observed in type 2 diabetes.[1-4] However, most of the cohort studies in patients with type 2 diabetes have investigated the relation between glycemic control and cardiovascular disease in patients without vascular disease at baseline.[3, 5]

As prolonged exposure to hyperglycemia results in vascular damage it seems feasible that strict glycemic control will be associated with a decrease in cardiovascular risk.[6] Although cohort studies indeed find a relation between glycemic control and incident cardiovascular disease, the cardiovascular risk in patients with type 2 diabetes does not seem to further decrease by intensive glycemic control beyond a HbA1c of 7%.[7-9] Identifying those patients with type 2 diabetes who would benefit from intensive glycemic control might be an opportunity for improving treatment. Existing guidelines on the treatment of diabetes are based on this principle and thus stress the importance of identifying characteristics for determining the optimal HbA1c target in individual patients. Guidelines do address which patient groups are more likely to profit or suffer from strict glycemic control. Unfortunately, as studies in specific patient groups are lacking, setting practical treatment goals in different patient groups is still difficult. [10, 11]

Post-hoc analyses from the Action to Control Cardiovascular Risk in Diabetes (ACCORD) [9], the Action in Diabetes and Vascular Disease: Preterax and Diamicron Modified Release Controlled Evaluation (ADVANCE) [8], the UK Prospective Diabetes Study (UKPDS) [12] and the Veterans Affairs Diabetes Trial (VADT) [7] suggested that the presence of vascular disease is an important patient characteristic to determine an individual glycemic goal since a different effect of glycemic control was found in patients with and without vascular disease.[13]

The goal of the present study is to investigate the relation between HbA1c and cardiovascular events and all-cause mortality in patients with type 2 diabetes with and without clinical manifestations of vascular disease.

Research design and methods

Study population

For this study, data of 1687 participants with diabetes mellitus type 2 who were enrolled in the Second Manifestations of ARTerial disease (SMART) study before March 1, 2013, were used. Diabetes mellitus was defined as a referral diagnosis of type 2 diabetes, self-reported type 2 diabetes, a fasting serum glucose ≥7.0 mmol/l at inclusion with initiation of glucose lowering treatment within one year, or the use of oral anti-hyperglycemic agents or insulin at baseline. Participants with known type 1 diabetes were excluded for this analysis. The SMART study is an ongoing prospective single-centre cohort study in patients with manifest vascular disease and/or cardiovascular risk factors. Starting from September 1996, consecutive patients aged 18–80, referred to the University Medical Center Utrecht (UMCU), The Netherlands, with manifest vascular disease or a cardiovascular risk factor underwent a vascular screening. Written informed consent was obtained from all participants. The study was approved by the Medical Ethics Committee of the UMCU.

Follow-up

Patients were biannually asked to fill in a questionnaire. Events of interest for the present study were the occurrence of vascular death, stroke, coronary artery disease, and the composite of these vascular events. In addition, we were interested in mortality and non-vascular death. Definitions have been described previously [14] and are added as web table (supplemental table 1). When a possible event was recorded by the participant, hospital discharge letters and results of relevant laboratory and radiology examinations were collected. With this information, all events were audited by three members of the SMART study Endpoint Committee, comprising physicians from different departments.

HbA1c measurement

HbA1c was measured at baseline in all patients enrolled in the SMART study after 2006. In patients who were enrolled in the SMART study before 2006, HbA1c was determined using available stored blood samples.

Data analyses

Missing data for HbA1c (n=128; 7.6 %) were singly imputed by weighted probability matching on the basis of multivariable regression using covariate and outcome data.

Baseline data are presented as mean ± standard deviation (SD) or median with interquartile range in the case of a skewed distribution.

Cox proportional hazards analyses were performed to estimate hazard ratios (HR) and 95% confidence intervals (CI) for the relation between HbA1c and the occurrence of cardiovascular events, defined as a composite of (non-)fatal myocardial infarction, (non-)fatal stroke or vascular mortality. If a patient had multiple events, the first event was used in the analyses. The proportional hazards assumption was satisfied based on a Schoenfeld residual plot.

To estimate the relation between HbA1c and cardiovascular events and mortality we built three models. First the unadjusted relation between HbA1c and cardiovascular events was estimated. In model II, age and sex were added. In model III this model was additionally adjusted for current smoking, systolic blood pressure, diabetes duration, non-HDL, and Modification of Diet in Renal Disease (MDRD). The mentioned variables in the models were a set of beforehand chosen confounders of the relation between HbA1c and cardiovascular events and mortality (age, sex, current smoking, diabetes duration) and a set of beforehand chosen traditional cardiovascular risk factors with a strong validity (systolic blood pressure, non-HDL cholesterol and MDRD).

To investigate the possible modifying effect of the presence of vascular disease at baseline, we stratified the population accordingly and performed separate analyses in the different strata. In addition, we performed standard multiplicative interaction analyses by adding the cross-product to the Cox proportional hazards models. Using a similar methodology we investigated whether vascular disease duration was an effect modifier in the relation between plasma HbA1c and new cardiovascular events since a differential effect with longer standing vascular disease might be plausible from a pathophysiological perspective. Also, the relation between HbA1c and all-cause mortality, non-vascular mortality and separate endpoints (vascular mortality, coronary ischemic disease and stroke) were studied in strata of patients with and without previous vascular disease. Finally, the relation between HbA1c and cardiovascular events and mortality was assessed in strata of HbA1c tertiles and on a continuous way using plots of restricted cubic splines. The p-values of nonlineair effect of baseline HbA1c on cardiovascular events and mortality was based on the χ^2 statistic.

Sensitivity analyses were performed after excluding patients with the 1% highest and 1% lowest HbA1c to eliminate the effect of outliers. As it is possible that the

relation of interest differed in the years of follow-up, for instance because of better risk management over time, year of inclusion was added to the Cox models. As differences in use of thrombocyte aggregation inhibitors and anticoagulants were expected between patients with and without vascular disease, it was investigated in the same matter whether the addition of usage of this medication differed the direction or magnitude of the relation. Also glucose lowering medication, blood pressure lowering treatment and statin use were included in the models as a sensitivity analysis. Finally sensitivity analyses were performed investigating whether the relation was similar when analyses were performed in patients with cerebrovascular, peripheral, coronary artery disease or vascular disease on various location at baseline separately. The level of significance was set at $P<0.05$ for all analyses. As the analyses were prespecified, no correction for multiple comparisons was performed. All statistical analyses were done using SPSS Statistics version 21 and R version 3.1.0.

Results

Baseline characteristics are presented in table 1. The mean age was 60.2 (SD 10.2) years and 30% of participants were female. During a median follow-up of 6.1 years (range: 3.1-9.5 years), 293 patients experienced a new cardiovascular event (event rate 17.3%). Of those 293 patients 189 patients died of a vascular cause while the all-cause mortality was 340 (event rate 20.1%). In total 6.9% of the patients were lost to follow-up (8.5% in patients without vascular disease and 6.2% in patients with vascular disease).

Table 1 Baseline characteristics of 1687 patients with type 2 diabetes

	DM2 and vascular disease present (n=1156)	DM2 and no vascular disease present (n= 531)
Age	62.7 ± 8.9	54.7 ± 11.0
Female sex - % (n)	25% (288)	41% (216)
Time since diagnosis of diabetes – yr	4 (1-10)	3 (0-7)
Oral glucose lowering treatment - % (n)	68% (745)	77% (411)
Use of insulin- % (n)	24% (273)	22% (119)
Both oral treatment and insulin - % (n)	10% (116)	9% (50)
Only lifestyle/diet treatment for DM - % (n)	22% (254)	20% (107)
HbA1c — %	7.5 ± 1.5	7.0 ± 1.2
HbA1c — mmol/mol	58 ± 16	52 ± 13
Fasting blood glucose — mmol/L	8.5 ± 2.7	9.3 ± 3.3

Table 1 Baseline characteristics of 1687 patients with type 2 diabetes (*continued*)

	DM2 and vascular disease present (n=1156)	DM2 and no vascular disease present (n= 531)
Total cholesterol – mmol/L	4.6 ± 1.2	5.3 ± 1.7
HDL cholesterol – mmol/L	1.1 ± 0.3	1.2 ± 0.4
LDL cholesterol– mmol/L	2.7 ± 1.0	3.1 ± 1.1
Triglycerides - mmol/l	1.6 (1.2-2.4)	1.8 (1.2-2.7)
nonHDL cholesterol – mmol/l	4.1 ± 1.7	3.5 ± 1.2
Creatinine - µmol/l	96 ± 43	84 ± 25
eGFR (MDRD)	75 ± 20	84 ± 22
Platelet inhibitor - % (n)	77% (886)	14% (75)
Oral anticoagulantics - % (n)	14% (160)	4% (23)
Statins	60% (695)	32% (171)
Blood pressure-lowering	83% (957)	63% (333)
Systolic blood pressure -mmHg	145 ± 20	146 ± 21
Diastolic blood pressure - mmHg	81 ± 11	86 ± 12
Weight – kg	86 ± 15	91 ± 20
BMI - kg/m^2	30.1 ± 6.1	28.4 ± 4.3
Waist circumference – cm	101 ± 12	101 ± 15
Current smoking - % (n)	26% (300)	23% (123)
Vascular disease_- % (n)	100%	
Coronary disease - % (n)	66% (766)	
Cerebral vascular disease - % (n)	29% (333)	
Peripheral arterial disease - % (n)	22% (253)	
Abdominal aortic aneurysm - % (n)	7% (82)	
Duration of vascular disease – yr	1 (0-9)	

Relation of HbA1c with cardiovascular events

In all patients with type 2 diabetes higher levels of HbA1c were non-significantly related to cardiovascular events (HR 1.06; 95%CI 0.97-1.17). In patients with type 2 diabetes and vascular disease at baseline, no relation between HbA1c and new cardiovascular events was found (HR 1.03; 95%CI 0.93-1.15) (table 2). Results were similar when performed in patients with cerebrovascular, peripheral, coronary artery disease or vascular disease on various location at baseline separately (supplemental table 2). On the other hand, in patients without vascular disease, a strong relation between HbA1c and cardiovascular events was observed (HR 1.27; 95%CI 1.06-1.51). Additional sensitivity analyses (exclusion of patients

with the 1% highest and lowest level of HbA1c and adjustment for year of inclusion or usage of platelet inhibitors ,anticoagulants, glucose lowering medication, blood pressure lowering treatment and statin use) did not change the direction and magnitude of the relation (data not shown). Analyses in tertiles of HbA1c (data not shown) and cubic splines describing the relation of HbA1c with new cardiovascular events and mortality did not indicate the presence of non-linearity both in patients with and without vascular disease (supplemental figure 1). The p-value for the cross product of HbA1c and the presence of vascular disease was 0.195 indicating no significant interaction.

The relation between HbA1c and separate endpoints (vascular mortality, the occurrence of coronary ischemic disease or ischemic stroke) in patients with and without vascular disease is shown in table 3. Though the numbers of events are small, HbA1c was significantly associated with the occurrence of ischemic stroke in patients without vascular disease (HR 1.40; 95%CI 1.01-1.94), while no relation was found in patients with vascular disease (HR 1.03; 95%CI 0.81-1.31).

Table 2 Relation between HbA1c and (new) cardiovascular events and all-cause mortality

		New cardiovascular events		All-cause mortality	
	Model	**HR (95%CI)†**	**p-value for interaction**	**HR (95%CI)†**	**p-value for interaction**
DM2 and vascular disease (n=1156)		New cardiovascular events (n=240)		All-cause mortality (n=264)	
	I	1.03 (0.93-1.14)		1.12 (1.02-1.22)	
	II	1.07 (0.96-1.18)		1.19 (1.09-1.31)	
	III	1.03 (0.93-1.15)		1.16 (1.06-1.28)	
			0.195		0.749
DM2 without vascular disease (n=531)		New cardiovascular events (n=53)		All-cause mortality (n=73)	
	I	1.16 (0.99-1.35)		1.11 (0.96-1.27)	
	II	1.24 (1.03-1.43)		1.15 (1.00-1.32)	
	III	1.27 (1.06-1.51)		1.13 (0.97-1.31)	

* Model I: crude; Model II: sex and age; Model III: Model II + current smoking, systolic blood pressure, diabetes duration, non-HDL, and MDRD. †Hazard ratio per 1% higher HbA1c. For example, in patients with type 2 diabetes without vascular disease a 1% higher HbA1c is associated with a 1.27 fold higher risk of vascular events. p-value for interaction between HbA1c and new cardiovascular events is 0.195 and for all-cause mortality 0.749

Table 3 Relation of HbA1c with different events

	DM2 and vascular disease (n= 1156)		**DM2 without vascular disease (n=531)**	
	HR (95% CI)*	events	HR (95% CI)*	events
Vascular mortality	1.11 (0.97-1.26)	n=161	1.25 (0.99-1.60)	n=28
Non-vascular mortality	1.16 (0.98-1.38)	n=87	1.01 (0.81-1.25)	n=39
Myocardial infarction	0.90 (0.75-1.09)	n=77	1.32 (0.98-1.78)	n=46
Ischemic stroke†	1.03 (0.81-1.31)	n=49	1.40 (1.01-1.94)	n=13
Peripheral arterial disease	1.10 (0.95-1.28)	n=119	1.13 (0.80-1.61)	n=18

* Hazard ratio per percentage point HbA1c for vascular events adjusted for sex, age, current smoking, systolic blood pressure, diabetes duration, non-HDL, and MDRD. For example in patients without vascular disease a 1% higher HbA1c is related to a 1.40 fold increased risk of ischemic stroke.
†Ischemic stroke does not include hemorrhagic stroke

The p-value for the cross product of vascular disease duration and new cardiovascular events was 0.490, indicating no statistical interaction by the duration of vascular disease. In all different groups of patients based on the duration of vascular disease no significant relations were found between HbA1c and new cardiovascular events. (table 4).

Table 4 Relation of HbA1c with vascular events and all-cause mortality in different groups of vascular disease duration

	Different duration Groups		New cardiovascular events		All-cause mortality	
		Model*	HR (95% CI)	p-value	HR (95% CI)	p-value
Time since clinical manifestation of vascular disease (n=1151)	0 years (n= 545, 93 events, 100 died)	I	0.98 (0.83-1.16)	0.800	1.04 (0.89-1.21)	0.634
		II	1.01 (0.85-1.21)	0.873	1.10 (0.94-1.29)	0.243
		III	0.97 (0.81-1.16)	0.723	1.02 (0.86-1.21)	0.787
	0 – 6 years (n= 220, 44 events, 57 died)	I	0.85 (0.65-1.12)	0.249	1.07 (0.88-1.30)	0.492
		II	0.88 (0.67-1.16)	0.351	1.13 (0.92-1.39)	0.257
		III	0.84 (0.62-1.13)	0.247	1.11 (0.88-1.41)	0.356
	6-51 years (n= 386, 99 events, 107 died)	I	1.11 (0.94-1.31)	0.207	1.18 (1.02-1.37)	0.029
		II	1.14 (0.97-1.34)	0.103	1.26 (1.08-1.46)	0.003
		III	1.12 (0.95-1.33)	0.179	1.25 (1.07-1.46)	0.006

* Model I: crude model; Model II: model I with sex and age; Model III: Model II with current smoking, systolic blood pressure, diabetes duration, non-HDL, and MDRD. †Hazard ratio per percentage point increase HbA1c.

Relation of HbA1c with mortality

Patients with type 2 diabetes and manifest vascular disease had a 16% higher risk of all-cause mortality per one percentage point increase in HbA1c (HR 1.16; 95%CI: 1.06-1.28), while a similar, albeit non-significant relation was found in patients without vascular disease (HR 1.13; 95%CI: 0.97-1.31). The p-value for the cross product of HbA1c and the presence of vascular disease in the relation with all-cause mortality was 0.749. The relation between HbA1c and allcause mortality in patients with vascular disease was found specifically in patients with coronary disease or cerebral vascular disease at baseline (web table 2).

When investigating the relation between HbA1c and all-cause mortality in tertiles of vascular disease duration, differential relations were found between the groups

(p-value of cross-product 0.044). Interestingly, a significant relation of HbA1c and all-cause mortality (HR 1.25; 95%CI: 1.07-1.46) was found in patients with the longest vascular disease duration (6-51 years). No significant relations were found between HbA1c and non-vascular mortality in all subgroups.

Discussion

The present study shows that in patients with type 2 diabetes there is a modest, but not statistically significant, relation between HbA1c and cardiovascular events and, as there was no statistical significant interaction, this relation was not different for patients with or without clinical manifest vascular disease.

To the best of our knowledge, this is the first prospective cohort study investigating the relation between HbA1c and new cardiovascular events in patients with and without vascular disease. Interestingly, a different relation between glycemic control and cardiovascular outcome was suggested in a meta-analysis of randomized controlled trials which investigated the effect of an intensive versus standard glycemic control (interaction p=0.04).[13] Although the point estimates and confidence intervals of the hazard ratio are different in direction between the groups, no significant interaction p-value (0.195) for multiplicative interaction was found. Since we observed no statistical interaction, we cannot conclude that the relation between HbA1c and cardiovascular events is really different depending on the presence or absence of vascular disease. However, the results of this study are mainly important for hypothesis generation. Further research is warranted to specifically investigate this potential difference in effect.

Our findings in patients with type 2 diabetes (with and without vascular disease) are in line with other cohort studies that studied the relation between HbA1c and macrovascular complications in patients with type 2 diabetes.[3, 5] Some cohort studies suggested the presence of an U-shaped relation between HbA1c and cardiovascular events.[15-17] In this study we did not find a U-shaped curve between HbA1c and macrovascular complications in patients with type 2 diabetes and thus proceeded to analyze the data in a linear fashion. The explanation for the difference between our findings and earlier studies is probably the different study population compromising younger patients and patients with diverse types of vascular disease versus only coronary artery disease in most other studies.

Findings of cohort studies investigating the relation between HbA1c and new cardiovascular events in patients with and without established vascular disease after cardiac interventions for coronary artery disease, are in line with our findings.[18-22]

In these studies no relation was found between HbA1c and cardiovascular events in patients after cardiac interventions with established type 2 diabetes.[19-21] In patients without established type 2 diabetes this relation did exist.[18, 21, 22] In the present study we expand on these findings by showing a consistent relation across different types of vascular disease.

Several explanations can be given for the different relation between HbA1c and new cardiovascular events in patients with type 2 diabetes with and without vascular disease. Although we could not support this hypothesis in this study, it is possible that the relation between HbA1c and cardiovascular events is more U-shaped in patients with vascular disease compared with the relation in patients without vascular disease. Such an U-shaped relation between HbA1c and vascular events and mortality in the total diabetic population was indeed suggested in several cohort studies.[15-17] As hypoglycemia is associated with severe cardiovascular events and arrhythmia[23] the left arm of this U-shape might be caused by the occurrence of hypoglycemia. Analyses of the data from the ACCORD-trial suggested that the increased all-cause mortality in the intensive treatment group could be associated with the occurrence of hypoglycemia, although the causality of this observation is uncertain [8]. An explanation for the different relation in patients with and without vascular disease could thus be that patients with established vascular disease might be more susceptible to the detrimental effects of hypoglycemia. However, analyses in the present study did not indicate the presence of an U-shaped relation between HbA1c and new cardiovascular events. Our findings probably differ from earlier cohort studies because of the inclusion of patients without vascular disease and with different types of vascular disease besides coronary artery disease.

Another explanation for the different relation between HbA1c and new cardiovascular events in patients with type 2 diabetes with and without vascular disease could be that in patients with established vascular disease hyperglycemia is not the key factor for progressive vascular damage. Factors as hypertension [24, 25] and dyslipidemia [26, 27] have been shown to be strongly related to new cardiovascular events in patients with type 2 diabetes and in patients with already established vascular disease. Thus, if hypertension and dyslipidemia are important modifiable risk factors contributing to the pathogenesis of cardiovascular events in patients with type 2 diabetes in general, these risk factors may be even more important in patients with type 2 diabetes who developed a cardiovascular event before inclusion in our study. In diabetes the pathogenesis of vascular disease is at least in part intrinsically different from vascular disease in patients without diabetes as medial vascular calcification or Mönckeberg's media sclerosis is often found in diabetes[28].

This difference in pathogenesis could therefore translate into a difference in the most important risk factors for new cardiovascular disease.

The interpretation of the strong relation between HbA1c and all-cause mortality in patients with type 2 diabetes with long-term vascular disease and the absence of this relation in patients with a short vascular disease duration is not obvious. An explanation might be that HbA1c, as a marker of glycemic regulation, is a proxy of overall condition or frailty and therefore singles out the patients with the poorest health status, especially in those patients with a longer vascular disease duration.

The chief strengths of this study include the prospective design and large number of participants, both with and without cardiovascular disease. Due to the substantial follow-up period and large cohort size, there was a relatively high number of events. Furthermore, the risk of bias in this study was reduced because of the completeness of data.

Several limitations of this study need to be addressed. As the SMART study is a single center cohort study in an Academic Hospital it may be questioned whether this cohort is a representation of the total population of patients with type 2 diabetes . It should be noted that the current cohort contains a broad scope of patients with type 2 diabetes with and without vascular disease representing clinical practice. Despite the relatively high number of participants in this cohort, a small number of endpoints in some groups could have resulted in insufficient power. This is most likely the case in the patients with type 2 diabetes without vascular disease at baseline because of the small number (n=531) in this group. Furthermore, only baseline HbA1c was used for the analyses in the present study, while the median follow up in this study was 6 years. While possible variation in HbA1c during follow-up could theoretically change the relations, this is not taken into account in the present study. The lack of statistical significant interaction of the presence of vascular disease at baseline needs to be taken into account when interpreting these results. Although the point estimates are clearly different between the two groups, no significant interaction was observed. The difference in points estimates may of course be due to chance, but we think that in light of the relatively wide confidence interval, the absence of statistical significant interaction could very well be due to due to insufficient power in this study. Further studies are needed to support our hypothesis of a differential effect of HbA1c on cardiovascular events between patients with and without vascular disease.

As we only studied patients with type 2 diabetes, our findings cannot be extrapolated to patients with type 1 diabetes, a population in which strict control has been shown to be associated with fewer cardiovascular events [2, 29]. Lastly, our study only takes into account macrovascular complications of type 2 diabetes. As HbA1c has been shown to be strongly related to microvascular complications [2, 12, 29, 30] the importance of strict glycemic control should not be devaluated completely in patients with type 2 diabetes and vascular disease. Microvascular complications should be taken into account when setting individualized therapeutic targets in patients with diabetes type 2, including those with vascular disease. Nevertheless, our findings are important for generating hypotheses which may eventually lead to more tailored treatment in this very high risk population. Further studies are therefore needed to establish whether the presence of vascular disease influences the relation between HbA1c and cardiovascular events.

In conclusion, HbA1c is related to cardiovascular events and no interaction of the presence or absence of vascular disease on this relation was observed. The effect of baseline HbA1c on all-cause mortality was of similar magnitude in patients with and without vascular disease, although the relation was not statistically significant in patients without vascular disease.

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Appendix

Supplemental table 1 Definitions in the SMART study

Vascular disease	
Coronary artery disease	Myocardial infarction, angina pectoris or coronary revascularization.
Cerebrovascular disease	Ischemic attack, cerebral infarction, cerebral ischemia, amaurosis fugax, retinal infarction or a history of carotid surgery
Peripheral arterial disease	Symptomatic and documented obstruction of distal arteries of the leg or interventions (Fontaine classification II-IV confirmed with ankle brachial index (ABI) ≤0.90 in rest and/or decrease of ABI >20% after exercise), percutaneous transluminal angioplasty, bypass or amputation
Abdominal aortic aneurysm	A supra- or infrarenal aneurysm of the aorta (distal aortic anteroposterior diameter ≥3 cm, measured with ultrasonography) or a history of AAA surgery
Diabetes mellitus type II	A referral diagnoses of type 2 diabetes, self-reported type 2 diabetes, a fasting serum glucose ≥7.0 mmol/l with initiation of glucose lowering treatment within a year, or the use of anti-hyperglycemic agents at baseline. Participants with diabetes mellitus type I were excluded for this analysis.
Outcome events SMART cohort	
Myocardial infarction	At least two of the following criteria (I) Chest pain for at least 20 minutes, not disappearing after administration of nitrates (II) ST-elevation > 1 mm in two following leads or a left bundle branch block on the electrocardiogram (III) Troponin elevation above clinical cut-off values or creatinine kinase (CK) elevation of at least two times the normal value of CK and a myocardial band-fraction > 5% of the total CK Sudden death: unexpected cardiac death occurring within one hour after onset of symptoms, or within 24 hours given convincing circumstantial evidence
Stroke	Relevant clinical features for at least 24 hours causing an increase in impairment of at least one grade on the modified Ranking scale, with a new cerebral *infarction* on CT or MRI Relevant clinical features for at least 24 hours causing an increase in impairment of at least one grade on the modified Ranking scale, *without a new (hemorrhage) cerebral infarction* on CT or MRI
Vascular mortality	Death from stroke, myocardial infarction, congestive heart failure, rupture of abdominal aortic aneurysm and vascular death of other causes

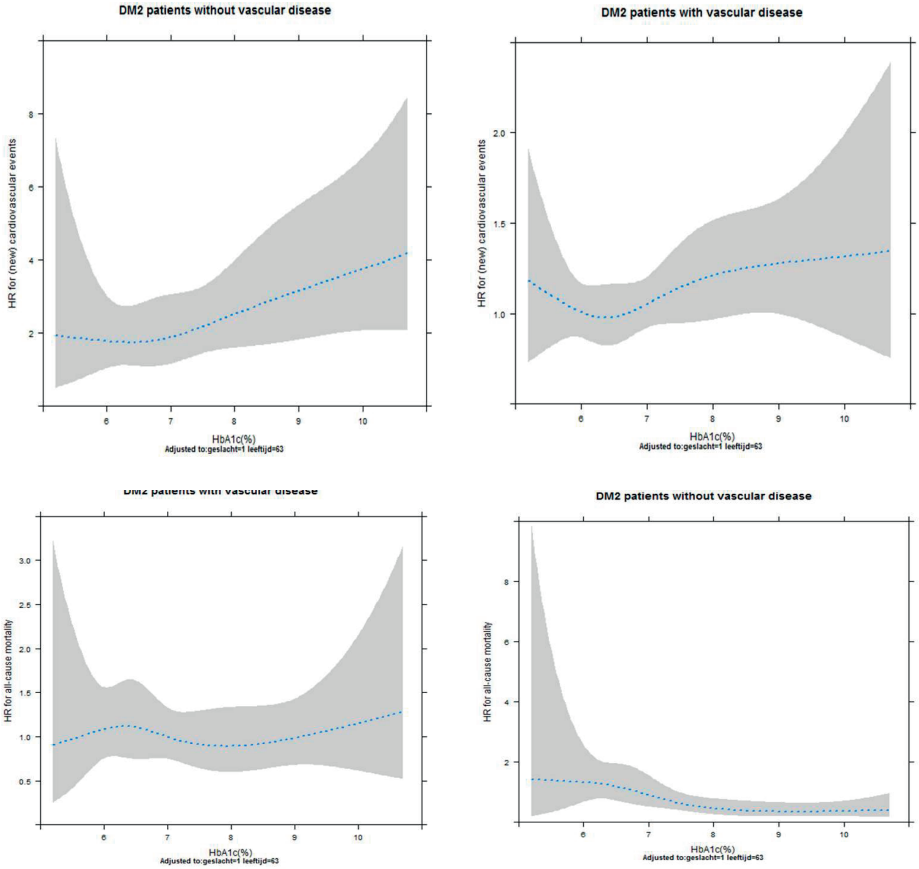
Supplemental table 1 Definitions in the SMART study (*continued*)

Vascular disease	
New cardiovascular events	Composite of myocardial infarction, stroke (infarction or hemorrhagic), retinal infarction, terminal heart failure, sudden death and vascular mortality
All-cause mortality	Death from any cause

Supplemental Table 2 Continuous relation of HbA1c with vascular events in patients with different types of vascular disease at baseline

Type of vascular disease	Model[a]	New vascular events		All-cause mortality	
		HR (95% CI) HbA1c[b]	p-value	HR (95%CI) HbA1c[b]	p-value
Peripheral artery disease					
N=253	I	0.99 (0.84-1.16)	0.883	1.07 (0.98-1.16)	0.954
Vascular events (n=76)	II	1.01 (0.86-1.20)	0.841	1.04 (0.90-1.21)	0.559
Mortality (n=99)	III	0.98 (0.82-1.17)	0.839	1.05 (0.90-1.22)	0.572
Coronary disease					
N=766					
Vascular events (n=144)	I	1.10 (0.96-1.26)	0.170	1.20 (1.06-1.36)	0.004
Mortality (n=140)	II	1.14 (1.00-1.30)	0.058	1.28 (1.13-1.46)	<0.001
	III	1.07 (0.94-1.21)	0.294	1.21 (1.06-1.38)	0.004
Cerebral vascular disease					
N=333	I	1.00 (0.84-1.20)	0.621	1.15 (0.97-1.15)	0.082
Vascular events (n=95)	II	1.05 (0.88-1.25)	0.624	1.22 (1.04-1.44)	0.017
Mortality (n=104)	III	1.03 (0.86-1.24)	0.732	1.21 (1.02-1.43)	0.027
Abdominal aortic aneurysm					
N=82	I	1.31 (0.97-1.77)	0.075	1.14 (0.83-1.57)	0.432
Vascular events (n=19)	II	1.26 (0.94-1.69)	0.118	1.12 (0.84-1.50)	0.448
Mortality (n=26)	III	1.33 (0.92-1.91)	0.131	1.08 (0.77-1.51)	0.671
Polyvascular disease (>1 vascular disease location)	I	1.12 (0.95-1.31)	0.169	1.14 (0.98-1.32)	0.093
	II	1.16 (0.98-1.36)	0.085	1.21 (1.03-1.41)	0.018
	III	1.14 (0.97-1.36)	0.121	1.20 (1.02-1.42)	0.028

[a] Model I: crude; Model II: model I with sex and age; Model III: Model II with current smoking, systolic blood pressure, diabetes duration, non-HDL, and MDRD.[b]Hazard ratio per percent point increase HbA1c



Supplemental figure 1 Spline interpolation for the relation between HbA1c (%) and hazard ratios for cardiovascular events and all-cause mortality

CHAPTER 10

Inter-arm systolic blood pressure differences, relations with future vascular events and mortality in patients with and without manifest vascular disease

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Abstract

Background– Inter-arm systolic blood pressure difference (SBPD) is an easily obtained patient characteristic which relates to vascular disease. We aimed to identify determinants of large inter-arm SBPD and to investigate the relation between inter-arm SBPD and vascular events in patients with and without manifest vascular disease.

Methods – In a cohort of 7344 patients with manifest vascular disease or vascular risk factors alone enrolled in the Second Manifestations of ARTerial disease (SMART) study, single bilateral non-simultaneous blood pressure measurements were performed. Logistic and Cox regression was used to identify determinants of large inter-arm SBPD (≥15 mmHg) and to investigate the relation between inter-arm SBPD and vascular events (composite of non-fatal myocardial infarction, stroke and vascular mortality) and all-cause mortality.

Results – In all patients the median inter-arm SBPD was 7 mmHg (IQR 3-11) and 1182 (16%) patients had inter-arm SBPD ≥15 mmHg. Higher age, higher systolic blood pressure, diabetes mellitus, peripheral artery disease, carotid artery stenosis, higher carotid intima-media thickness and lower ankle-brachial indices were related to large inter-arm SBPD (≥15 mmHg). Each 5 mmHg increase in inter-arm SBPD was related to a 12% higher risk of vascular events in patients without manifest vascular disease (HR1.12;95%CI 1.00-1.27), whereas no relation was apparent in patients with manifest vascular disease (HR0.98;95%CI 0.93-1.04, interaction p-value 0.036). Inter-arm SBPD was not related to all-cause mortality (HR1.05;95%CI 0.93-1.19).

Conclusions –Inter-arm SBPD relates to a higher risk of vascular events in patients without manifest vascular disease, whereas this relation is not apparent in patients with manifest vascular disease.

Introduction

Peripheral artery disease (PAD) significantly contributes to the total burden of vascular disease as it is related to high risks of vascular morbidity and mortality.[1, 2] Although PAD most often presents as an atherosclerotic disease of the leg arteries, atherosclerotic changes in the arteries of the upper limbs occur as well.[3] Inter-arm systolic blood pressure difference (SBPD) reflects subclavian stenosis which in most cases is caused by atherosclerosis in the upper limb arteries.[4] In rare cases there may be another origin such as arteritis, fibromuscular dysplasia, arterial injuries or arterial dissection.[5-7] International hypertension guidelines stress the importance of bilateral blood pressure measurement and state that large inter-arm SBPD is an indicator of elevated vascular risk.[8, 9] Indeed, high inter-arm SBPD (>15mmHg) relates to an approximately 1.6 fold risk of cardiovascular mortality.[4, 10] Although the relation of inter-arm SBPD with atherosclerotic stenosis of the upper arm arteries is generally acknowledged, little is known about the precise determinants of a large inter-arm SBPD.

Several epidemiological studies indicate that large inter-arm SBPD relates to the presence of cardiovascular risk factors, PAD of the lower extremities, cerebrovascular disease and confers a higher risk for (cardiovascular) mortality.[4, 10-14] Previous studies mostly use cut off values (≥10, ≥15 or ≥20 mmHg) to investigate the relation between inter-arm SBPD and prevalent vascular disease, while a continuous approach could provide more precise information. [10 11] A small prospective cohort study of 230 patients with hypertension with a median follow-up of 9.8 years shows that each 1 mmHg increase in inter-arm SBPD relates to future cardiovascular events (HR 1.04; 95%CI 1.02 -1.07).[15] Also, a larger community-based prospective cohort including 3390 participants shows that each standard deviation increase in inter-arm SBPD relates to cardiovascular events (HR 1.07; 95%CI 1.00-1.14).[14] Nevertheless, evidence on the relation in patients with manifestations of vascular disease is scarce.[10, 11]

The presence of clinical manifest vascular disease might influence the relation between inter-arm SBPD and vascular events. Patients with clinical manifest vascular disease are at higher risk for vascular events than patients without clinical manifestations of vascular disease. Therefore the relation between inter-arm SBPD and vascular risk might be different in patients with and without clinical manifest vascular disease.

The aim of the present study is to identify determinants of high inter-arm SBPD and to investigate the relation between inter-arm SBPD and vascular events and all-cause mortality in patients with and without clinical manifest vascular disease.

Material and methods

Study population

Data of 7344 participants enrolled in the Second Manifestations of ARTerial disease (SMART) study after the first of January 2002 and before the first of March 2014, were used for this study. The SMART study is an ongoing prospective single-center cohort study in the University Medical Center Utrecht (UMCU), The Netherlands. Patients with manifest vascular disease (coronary artery disease, cerebrovascular disease, peripheral artery disease or abdominal aortic aneurysm) and patients without clinical manifestations of vascular disease but with known cardiovascular risk factors such as hypertension, diabetes and dyslipidemia are included. As a part of the SMART study consecutive patients aged between 18 and 79 years of age undergo an extensive vascular screening as described previously.[16] The study is approved by the Medical Ethics Committee of the UMCU and written informed consent is obtained from all participants.

Follow-up

Twice a year participants of the SMART study are asked to fill out a questionnaire. Events of interest for this study were vascular death, stroke, myocardial infarction, and the composite of these vascular events. In addition, all-cause mortality was an event of interest in this study. Definitions of these events are added as supplemental table1 and have been described previously.[16] In case of a possible event, hospital discharge letters and results of relevant laboratory and radiology examinations are collected. Using the additional information three members of the SMART study Endpoint Committee, comprising physicians from different departments, audit all possible events.

Measurements of systolic blood pressure differences

At baseline all patients underwent a single bilateral non-simultaneous blood pressure measurement in both arms during ankle-brachial index (ABI) measurement. All measurements were performed by experienced professionals in the vascular lab of the UMC Utrecht. For these measurements patients were rested in a supine position and successively a single measurement of the blood pressure per arm was performed. In all patients the blood pressure in the right arm was measured before the measurement in the left arm. Inter-arm SBPD was calculated by subtract-

ing left arm systolic blood pressure (SBP) values from the right arm SBP values. Absolute values of this difference were used for further analyses.

Data analyses

Baseline data are presented as number and percentage for categorical variables, mean ± standard deviation (SD) for normally distributed variables or median with interquartile range in case of a skewed distribution. Data are presented stratified for the presence of manifest vascular disease. Missing data for left and right arm SBP (n=222; 3.0 %) were singly imputed by weighted probability matching based on multivariable regression using covariate and outcome data. We used absolute values of inter-arm SBPD for further analyses.

To investigate determinants of high inter-arm SBPD patient characteristics were related to an inter-arm SBPD ≥15mmHg using logistic regression models. Separate logistic regression models were built for each patient characteristic and adjustment took place for SBP, age and sex, except in the models for age (only sex adjusted), sex (only age adjusted) and SBP (only sex and age adjusted). To compare whether determinants for high inter-arm SBPD differed for patients with and without manifest vascular disease, similar analyses were performed stratified for the presence of manifest vascular disease.

To assess whether inter-arm SBPD relates to vascular morbidity and mortality we performed Cox proportional hazards analyses to estimate hazard ratios and 95% confidence intervals for the continuous relation between inter-arm SBPD and (composite of) vascular events and mortality. The proportional hazards assumption was checked visually by plotting Schoenfeld residuals and no violation of this assumption was observed.[17] The assumption of a linear relation was checked by visual inspection of restricted cubic splines plots of the relation between inter-arm SBPD and the composite of vascular events and all-cause mortality. No violation of the linearity assumption was observed (supplemental figure 1).[18]

We constructed two models to adjust for potential confounders. The first model only included sex and age. In the second model predefined traditional cardiovascular risk factors that were potential confounders of the relation of interest were included: current smoking, pack-years, non-HDL cholesterol, presence of diabetes mellitus, renal function (eGFR), ABI, the presence of manifest vascular disease at baseline (only in analyses with all patients) and the cross-product of pre-existing manifest vascular disease and inter-arm SBPD (only in analyses with all patients). Hazards ratios per 5 mmHg increase in inter-arm SBPD were re-

ported. To investigate whether the presence of manifest vascular disease at baseline influenced the relation of interest, we stratified the population accordingly and performed separate analyses in the different strata. Also, to assess the interaction on a multiplicative scale the p-value of the cross-product of pre-existing vascular disease and inter-arm SBPD in Cox proportional hazards models was used.

In order to assess whether the relation between inter-arm SBPD and vascular events and all-cause mortality was different for patients with higher SBP values in the (firstly measured) right arm and patients with higher SBP values in the left arm, interaction was evaluated on a multiplicative scale.

Sensitivity analyses were performed in patients with cerebrovascular disease, PAD or coronary artery disease separately. Also, to eliminate the effect of outliers sensitivity analyses were performed after excluding patients with the 1% highest SBPD. Finally, in line with previous studies we investigated the relation between cut-off values for inter-arm SBPD (≥10 mmHg, ≥15 mmHg and ≥20 mmHg) and vascular events and all-cause mortality.

In all analyses the level of significance was set at $p<0.05$. All data analyses were performed using R version 3.1.2.

Results

Table 1 summarizes the baseline characteristics. In patients with manifest vascular disease (n=5293) the mean age was 60 years (±10) and 73% of the participants were male. In patients without manifest vascular disease (n=2051) the mean age was 50 years (±13) and 52% of the participants were male.

Table 1 Patient characteristics of the study population at baseline

	Patients with manifest vascular disease n=5293	**Patients without manifest vascular disease n=2051**
Age (y)	60 ± 10	50 ± 13
Sex (Male)	73%	52%
Current smoking	29%	23%
Diabetes mellitus	18%	23%
Systolic blood pressure (mmHg)	139 ± 21	148 ± 23
Diastolic blood pressure (mmHg)	82 ± 11	90 ± 14
Body mass index	27.0 ± 4.1	27.3 ± 5.1
Total cholesterol (mmol/l)	4.6 ± 1.1	5.7 ± 1.6

Table 1 Patient characteristics of the study population at baseline (*continued*)

	Patients with manifest vascular disease **n=5293**	**Patients without manifest vascular disease** **n=2051**
Triglycerides (mmol/l)	1.3 (1.0-1.9)	1.4 (1.0-2.3)
HDL-cholesterol (mmol/l)	1.3 ± 0.4	1.4 ± 0.4
LDL-cholesterol (mmol/l)	2.6 ± 0.9	3.6 ± 1.3
Non-HDL-cholesterol (mmol/l)	3.3 ± 1.1	4.4 ± 1.6
Creatinine (µmol/l)	91 ± 31	83 ± 20
eGFR (MDRD)	77 ± 18	82 ± 19
Peripheral arterial disease	15%	0%
Coronary heart disease	64%	0%
Cerebrovascular disease	29%	0%
Abdominal aortic aneurysm	7%	0%
Blood pressure lowering therapy	77%	60%
Lipid lowering therapy	77%	34%
Antithrombotic therapy	86%	14%

All data are displayed as mean (± SD, median (interquartile range) or % HDL-C: High-density lipoprotein cholesterol, LDL-C: Low-density lipoprotein cholesterol, GFR: glomeral filtration rate estimated using MDRD formula

Distribution and prevalence of inter-arm SBPD.

The median absolute difference in SBP between arms was 7 mmHg (inter quartile range (IQR) 3-11 mmHg) for patients with manifest vascular disease and 6 mmHg (IQR 3-11 mmHg) for patients without vascular disease (table 2). Both in patients with and without manifest vascular disease 34% of the participants had an inter-arm difference ≥10 mmHg, 16% ≥15 mmHg, while 7% had an inter-arm difference ≥20 mmHg. In 52% of the patients the right arm SBP was higher than on the left arm while 42% of the patients had higher left arm SBP values. 6% had no differences in SBP between arms. The value of right minus left SBP was approximately normally distributed around the mean of 0.9 mmHg with a standard deviation of 11mmHg (supplemental figure 2).

Table 2 Distribution of SBPD and prevalence of large SBPD

	Patients with manifest vascular disease n=5293	Patients without manifest vascular disease n=2051
Inter-arm SBDP	7 (3-11)	6 (3-11)
Right SBP > left SBP	53%	48%
Left SBP > right SBP	41%	47%
Inter-arm SBPD ≥ 10 mmHg	34%	34%
inter-arm SBPD ≥ 15 mmHg	16%	15%
Inter-arm SBPD ≥ 20 mmHg	7%	7%

All data are displayed as median (interquartile rate) or %. SBPD systolic blood pressure differences; SBP systolic blood pressure

Distribution and prevalence of inter-arm SBPD.

SBP, age and sex adjusted logistic regression models indicated that large inter-arm SBPD (≥15 mmHg) were associated to age (OR per 10 years 1.16; 95%CI 1.10-1.23), diabetes mellitus (OR 1.20; 95%CI 1.02-1.39), SBP (OR per 10 mmHg 1.17; 95%CI 1.14-1.20), pack-years (OR per 10 pack-years 1.04; 95%CI 1.01-1.07), pre-existing PAD (OR 1.13; 95%CI 1.02-1.50), a stenosis in the carotid artery ≥70% (OR 1.52; 95%CI 1.17-1.97), carotid intima-media thickness (OR per 0.1 mm 1.07; 95%CI 1.04-1.10) and lower ABI (OR per 0.1 increase 0.80; 95%CI 0.77-0.82). No association was found between large inter-arm SBPD and pre-existing coronary artery disease (OR 0.98; 95%CI 0.86-1.13) or pre-existing cerebrovascular vascular disease (OR 1.06; 95%CI 0.91-1.24) (table 3). These associations were similar for patients with and without manifest vascular disease (supplemental table 2).

Table 3 Determinants of a large inter-arm SBPD (≥15 mmHg)

	OR (95% CI)
Age (per 10 years)	1.16 (1.10-1.23)
Male sex	1.05 (0.92-1.21)
Diabetes mellitus (present)	1.20 (1.02-1.39)
Diabetes duration (per year increase)	1.01 (0.99-1.02)
non-HDL cholesterol (per 1 mmol/L)	1.02 (0.97-1.07)
Systolic blood pressure (per 10 mmHg)	1.17 (1.14-1.20)
Renal insufficiency (eGFR <60 mL/min)	0.98 (0.82-1.18)
Smoking (current versus never)	1.17 (0.99-1.36)
Pack-years (per 10 pack-years increase)	1.04 (1.01-1.07)
Peripheral artery disease (present)	1.13 (1.02-1.50)
Coronary heart disease (present)	0.98 (0.86-1.13)
Cerebrovascular disease (present)	1.06 (0.91-1.24)
Abdominal aortic aneurysm (present)	1.02 (0.65-1.17)
Duration of vascular disease (per year increase)	1.00 (0.99-1.01)
Carotid stenosis 50-70% vs. <50%	1.47 (0.91-2.31)
Carotid stenosis ≥ 70% vs. <70%	1.52 (1.17-1.97)
Carotid IMT (per 0.1 mm increase)	1.07 (1.04-1.10)
Pulse pressure (per 10 mmHg)	1.02 (0.95-1.10)
ABI < 0.9 vs ≥0.9	1.51 (1.27-1.80)
ABI (per 0.1 increase)	0.80 (0.77-0.82)

Adjustments took place for sex, age and systolic blood pressure. Sex was adjusted only for age + systolic blood pressure, age was adjusted only for sex + systolic blood pressure, systolic blood pressure was adjusted for sex and age; eGFR estimated glomerular filtration ratio; IMT intima-media thickness; ABI ankle-brachial index

Relation between inter-arm SBPD and vascular events and all-cause mortality

During a median follow-up of 5.9 years (IQR 3.0 – 8.6) 652 patients had a new vascular event and 290 died of a vascular cause. In total 609 patients died from any cause and 3.9% of the patients were lost to follow-up.

In patients with manifest vascular disease, no relation between inter-arm SBPD and new vascular events was found (HR 1.05; 95%CI 0.93-1.19) (table 4). Results were similar when performed in patients with a history of coronary artery disease, cerebrovascular disease or PAD separately (data not shown). In patients without manifest vascular disease, a significant relation between inter-arm SBPD and vascular events was observed (HR 1.12; 95%CI 1.00-1.27, p-value for inter-

action 0.036). Cubic splines showed that each increase in inter-arm SBPD was related to vascular events and that there is not a clear threshold in inter-arm SBPD (supplemental figure 1). Inter-arm SBPD did not relate to vascular mortality (HR 0.98; 95%CI 0.86-1.12) or myocardial infarction (HR 0.97; 95%CI 0.77-1.22) in all patients (both patients with and without manifestations of vascular disease at baseline) and these relations were not influenced by the presence of manifest vascular disease (p-value for interaction 0.679 and 0.913 respectively). In patients without clinical manifest vascular disease inter-arm SBPD related to future stroke (HR 1.21; 95%CI 1.00-1.46), whereas no relation was apparent in patients with clinical manifest vascular disease (HR 1.00; 95%CI 0.90-1.10) (interaction p-value 0.095).

No relation between inter-arm SBPD and all-cause mortality was observed in patients with and without manifest vascular disease at baseline (HR 1.05; 95%CI 0.93-1.19) and the presence of manifest vascular disease at baseline did not alter this relation (p-value for interaction 0.524).

The relation between inter-arm SBPD and vascular events was not different for patients with higher right arm SBP values compared to patients with higher left arm SBP values (p-value for interaction 0.672).

Additional analyses

Sensitivity analyses excluding the 1% highest inter-arm SBPD in order to reduce the effects of outliers did not alter the magnitude or direction of the relations of interest (data not shown). No significant relations were found between an inter-arm SBPD ≥10 mmHg, ≥15 mmHg or ≥20 mmHg and vascular events (HR 1.10; 95%CI 0.71-1.72, HR 0.98; 95%CI 0.56-1.73 and HR 1.25; 95%CI 0.63-2.52 respectively). The presence of manifest vascular disease at baseline did not influence these relations (p-values for interaction 0.372, 0.854 and 0.284 respectively) (supplemental table 3).

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Table 4 Relation between inter-arm differences in systolic blood pressure and vascular events, all-cause mortality and separate endpoint

		Patients with manifest vascular disease (n=5293)	Patient without manifest vascular disease (n=2051)	p-value for interaction
	Model*	HR (95%CI)†	HR (95%CI)†	
Combined vascular endpoint	I	1.03 (0.98-1.09)	1.16 (1.04-1.30)	0.036
	II	0.98 (0.93-1.04)	1.12 (1.00-1.27)	
		565 events	87 events	
All-cause mortality	I	0.95 (0.88-1.03)	1.01 (0.89-1.15)	0.524
	II	0.96 (0.88-1.05)	1.01 (0.88-1.15)	
		538 events	71 events	
Vascular mortality	I	0.97 (0.89-1.05)	0.99 (0.87-1.13)	0.679
	II	0.96 (0.88-1.05)	0.98 (0.85-1.13)	
		258 events	32 events	
Ischemic stroke	I	1.06 (0.96-1.17)	1.23 (1.03-1.48)	0.095
	II	1.00 (0.90-1.10)	1.21 (1.00-1.46)	
		161 events	25 events	
Myocardial infarction	I	0.98 (0.90-1.08)	0.97 (0.77-1.24)	0.913
	II	0.95 (0.86-1.04)	0.96 (0.74-1.25)	
		230 events	36 events	

* Model I: sex and age; Model II: Model I + systolic blood pressure + current smoking, packyears, non-HDL cholesterol, presence of diabetes mellitus, renal function, ankle-brachial index, presence of manifest vascular disease (only in analyses with all patients), cross-product of manifest vascular disease and SBPD. †Hazard ratio per 5 mmHg higher difference in systolic blood pressure. For example in patients without vascular disease a 15 mmHg higher inter-arm SBPD is related to a 1.21 fold increased risk of ischemic stroke

Discussion

In our study in patients with and without clinical manifest vascular disease 16% had a large inter-arm SBPD (≥15 mmHg). Determinants of large inter-arm differences were age, SBP, diabetes mellitus, carotid stenosis ≥70%, carotid intima-media thickness and lower ankle-brachial indexes. In patients without clinical manifest vascular disease each 5 mmHg increase in inter-arm SBPD is significantly related to a 12% higher risk of vascular events, whereas in patients with manifest vascular disease no relation was found. Also, inter-arm SBPD relates to future stroke in patients without clinical manifest vascular disease, whereas no relation was observed in patients with vascular disease. No significant relations were found between inter-arm SBPD and future myocardial infarction, vascular mortality or all-cause mortality both in patients with and without manifest vascular disease.

With an observed prevalence of a large inter-arm SBPD (≥15 mmHg) of 16% this study confirms that upper limb PAD is prevalent in clinical practice.[3] Large inter-arm SBPD may reflect subclavian stenosis which in most cases is caused by atherosclerosis, but in rare cases may have another origin such as arteritis, fibromuscular dysplasia, arterial injuries or arterial dissection.[5-7] It is possible that a part of SBPD measured with successively measured bilateral blood pressure is not caused by stenoses but by blood pressure variability which in itself is a risk factor for cardiovascular morbidity and mortality.[19] In line with the most common etiology of vascular stenosis, atherosclerosis, we showed that traditional risk factors of atherosclerotic disease such as age, SBP, diabetes mellitus, pack-years and carotid intima-media thickness are determinants of large inter-arm SBPD which is supported by several epidemiological studies.[12-14, 20-22] Furthermore, we showed that inter-arm SBPD relates to pre-existing PAD and low ankle-brachial indexes, but not to other types of pre-existing vascular disease. These findings are in line with cross-sectional relations found in previous studies.[14, 22, 23] However, meta-analyses have suggested that inter-arm SBPD is additionally related to pre-existing cerebrovascular disease.[10, 11] These meta-analyses included only one study performed in a population of patients who underwent cardiac surgery in which a relation between large inter-arm SBPD and cerebrovascular disease was found.[24] Our study did not find a relation between inter-arm SBPD and pre-existing cerebrovascular disease.

Our study showed that a 5 mmHg increase in inter-arm SBPD relates to future vascular events (HR 1.12; 95%CI 1.00-1.27) in patients without manifestations of vascular disease. These results are very similar to previous findings from a small cohort study consisting of 230 patients from the primary care setting with

hypertension with a median follow-up of 9.8 years in which a 1 mmHg increase in inter-arm SBPD was related to a hazard ratio of 1.04 (95%CI 1.02 -1.07) for future vascular events or death.[15] In patients with and without manifestations of vascular disease, we could not confirm findings of previous performed studies suggesting that inter-arm SBPD relates to (vascular) mortality, probably due the shorter follow-up time (median follow-up 5.9 years) in our study.[4, 10, 12, 13]

To the best our knowledge, we are the first to show that the relation between inter-arm SBPD and future vascular events is different in patients with and without manifestations of vascular disease. Our findings of no relation in patients with manifest vascular disease are not in line with the significant relation between inter-arm SBPD and future vascular events found in a smaller cohort study consisting of 407 patients with stable coronary arterial disease.[25] Patients with manifest vascular disease represent a subpopulation at a higher risk for vascular events than patients without manifest vascular disease. Thus, the absolute extra risk conferred by inter-arm SBPD may be relatively smaller in high risk patients. The finding of an apparent relation between inter-arm SBPD and vascular events in these patients is not only in line with a previous cohort study in the primary setting that consisted for 80% of patients without manifest vascular disease, but also with findings from a larger community-based cohort.[14, 15]

Notably, a large part of the relation between inter-arm SBPD and future vascular events seems to be caused by the relation of SBPD with stroke. Our new finding that inter-arm SBPD relates to future stroke is plausible as previous studies have shown that inter-arm SBPD is associated with pre-existing cerebrovascular disease.[10, 11] We also showed that large inter-arm SBPD relates to carotid stenosis. Inter-arm SBPD might reflect arterial wall disease of the large vessels originating from the aorta and be a proxy of carotid stenosis, as suggested earlier.[26] A possible mechanism through which large SBPD leads to cardiovascular disease might thus be the associated carotid stenosis and subsequent higher risk for cerebrovascular disease.[27] As inter-arm SBPD has been related to pulse-wave velocity,[22, 28, 29] another mechanism might be arterial stiffening of large arteries with resulting pulse pressure damage and subsequent higher risks of vascular events and cerebrovascular disease.[30]

Inter-arm SBPD might be used in clinical practice to identify high risk individuals. This can be especially relevant for the primary care setting in patients without manifestations of vascular disease, as supported by previous research.[14, 15] However, our results might need further validation in community-based cohorts.

Also, although in a community-based cohort it has been shown that inter arm SBPD has additional prognostic value on top of traditional risk factors,[14] this finding might need validation before using inter-arm SBPD as a new marker of vascular disease in vascular risk stratification.

Strengths of this study include the prospective design with a substantial follow-up period, a standardized assessment of inter-arm SBPD and a standardized prospective outcome assessment. Due to this substantial follow-up period and the large numbers of participants we had sufficient power to assess the continuous relation of inter-arm SBPD with future vascular disease. Completeness of data with a low lost to follow-up resulted in limited risk of bias in this study.

Some limitations of this study need to be considered. Inter-arm SBPD was assessed using a single non-simultaneous bilateral measurement, which is related to overestimation and observer-bias.[31] Indeed, the prevalence of large inter-arm SBPD is higher in our study compared to previous studies suggesting that the inter-arm SBPD might have been overestimated in our study.[3, 14] A prospective design using repeated simultaneous measurements might have been a superior alternative to answer our research question,[32, 33] but these measurements were not available in our cohort. The use of simultaneous sphygmomanometers in clinical practice is however rare and therefore our approach might better reflect daily clinical practice. By showing that inter-arm SBPD, easily assessed in daily practice by bilateral non-simultaneous measured blood pressure, relates to future vascular events we have illustrated the potential relevance of this measurement. Another limitation involving this measurement is the fact that we were not able to investigate the relation between subclavian stenosis and future vascular events but only the relation between routinely measured inter-arm SBPD and future vascular events. Imaging of the subclavian arteries was not available. An alternative approach would have been to compare the SBP of the highest arm with the central blood pressure rather than with the SBP in the other arm, since subclavian stenosis can occur on both sides. However, central blood pressure measurements are not available in the SMART-study. Lastly, only baseline inter-arm SBPD was available. As the median follow-up was 5.9 years, possible variation in inter-arm SBPD during the follow-up period could have influenced the relations of interest.

In conclusion, inter-arm SBPD relates to a higher risk of future vascular events in patients without clinical manifest vascular disease, whereas this relation is not apparent in patients with manifest vascular disease.

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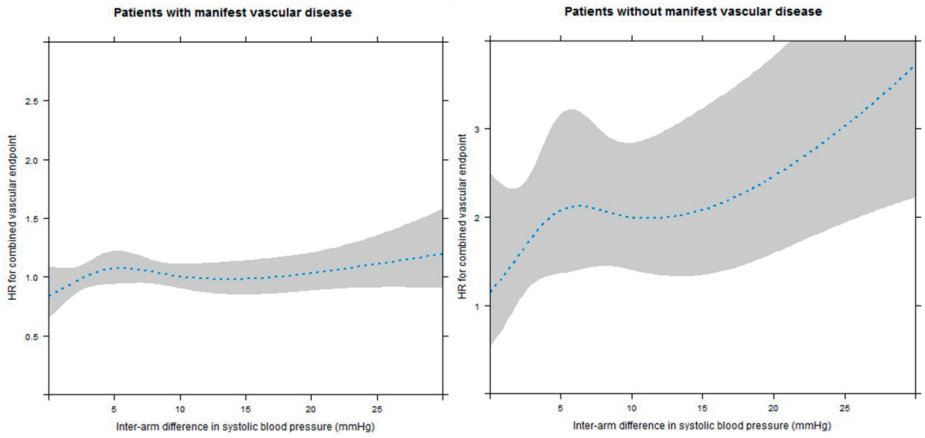
Appendix

Supplemental table 1 definitions of study outcomes

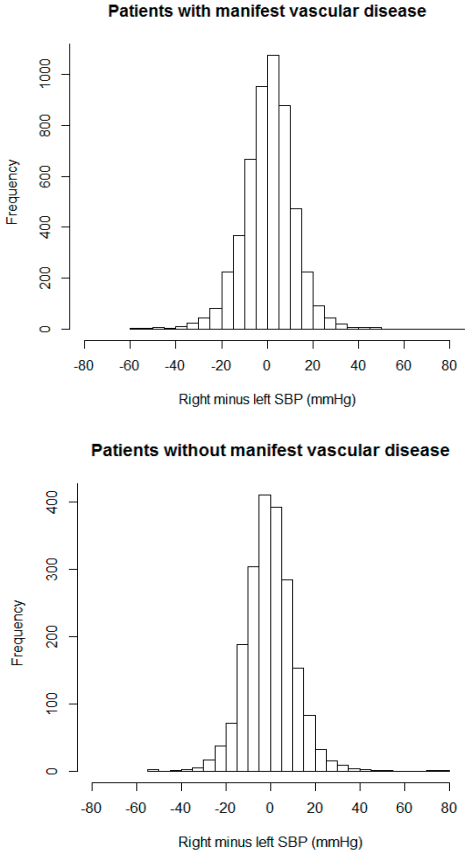
Manifest vascular disease at baseline	
Coronary artery disease	Myocardial infarction, angina pectoris or coronary revascularization.
Cerebrovascular disease	Ischemic attack, cerebral infarction, cerebral ischemia, amaurosis fugax, retinal infarction or a history of carotid surgery
Peripheral arterial disease	Symptomatic and documented obstruction of distal arteries of the leg or interventions (Fontaine classification II-IV confirmed with ankle brachial index (ABI) ≤0.90 in rest and/or decrease of ABI >20% after exercise), percutaneous transluminal angioplasty, bypass or amputation
Abdominal aortic aneurysm	A supra- or infrarenal aneurysm of the aorta (distal aortic anteroposterior diameter ≥3 cm, measured with ultrasonography) or a history of AAA surgery
Outcome events	
Myocardial infarction	At least two of the following criteria (I) Chest pain for at least 20 minutes, not disappearing after administration of nitrates (II) ST-elevation > 1 mm in two following leads or a left bundle branch block on the electrocardiogram (III) Troponin elevation above clinical cut-off values or creatinine kinase (CK) elevation of at least two times the normal value of CK and a myocardial band-fraction > 5% of the total CK Sudden death: unexpected cardiac death occurring within one hour after onset of symptoms, or within 24 hours given convincing circumstantial evidence
Stroke	Relevant clinical features for at least 24 hours causing an increase in impairment of at least one grade on the modified Ranking scale, with a new cerebral *infarction* on CT or MRI Relevant clinical features for at least 24 hours causing an increase in impairment of at least one grade on the modified Ranking scale, *without a new (hemorrhage) cerebral infarction* on CT or MRI
Vascular mortality	Death from stroke, myocardial infarction, congestive heart failure, rupture of abdominal aortic aneurysm and vascular death of other causes

Supplemental table 1 definitions of study outcomes (*continued*)

Manifest vascular disease at baseline	
Vascular events	Composite vascular outcome
	Composite of myocardial infarction, stroke (infarction or hemorrhagic), retinal infarction, terminal heart failure, sudden death and vascular mortality
All-cause mortality	Death from any cause



Supplemental figure 1 Cubic spline plots to assess linearity of the relation between SBPD and future vascular events



Supplemental figure 2 Histogram of right minus left systolic blood pressure

Supplemental table 2 Relations of several determinants with large (≥15 mmHg) inter-arm differences in systolic blood pressure, stratified for presence of manifest vascular disease

	Patients with manifest vascular disease (n=5293)	Patient without manifest vascular disease (n=2051)
Age (per 10 years)	1.12 (1.04-1.22)	1.17 (1.05-1.29)
Male sex	1.11 (0.93-1.31)	0.91 (0.71-1.17)
Diabetes mellitus	1.24 (1.03-1.49)	1.16 (0.86-1.55)
Diabetes duration (per year increase)	1.00 (0.98-1.02)	1.02 (0.99-1.05)
non-HDL cholesterol	1.05 (0.98-1.12)	0.99 (0.91-1.08)
Systolic blood pressure (per 10 mmHg)	1.18 (1.14-1.22)	1.24 (1.18-1.31)
Renal insufficiency (eGFR <60 mL/min)	0.98 (0.81-1.18)	1.14 (0.76-1.68)
Smoking (current versus never)	1.21 (0.97-1.52)	0.86 (0.61-1.21)
Packyears (per 10 packyears increase)	1.04 (1.01-1.08)	1.00 (0.92-1.08)
Peripheral artery disease	1.24 (1.01-1.51)	NA
Coronary heart disease	0.92 (0.77-1.08)	NA
Cerebrovascular disease	1.09 (0.92-1.28)	NA
Abdominal aortic aneurysm	0.87 (0.65-1.16)	NA
Duration of vascular disease (per year increase)	1.01 (0.99-1.02)	NA
Carotid stenosis 50-70% vs. <50%	1.47 (0.88-2.37)	1.50 (0.32-5.47)
Carotid stenosis > 70% vs. <70%	1.56 (1.19-2.03)	1.15 (0.31-3.46)
Carotid IMT (per 0.1 mm increase)	1.06 (1.03-1.10)	1.08 (1.01-1.15)
Pulse pressure (per 10 mmHg)	1.11 (1.02-1.21)	1.02 (0.88-1.13)
ABI < 0.9	1.70 (1.41-2.03)	1.94 (1.05-3.43)
ABI (per 0.1 increase)	0.80 (0.77-0.83)	0.65 (0.58-0.72)

Adjustments took place for sex, age and systolic blood pressure. Sex was adjusted only for age + systolic blood pressure, age was adjusted only for sex + systolic blood pressure, systolic blood pressure was adjusted for sex and age; eGFR estimated glomerular filtration ratio; IMT intima-media thickness; ABI ankle-brachial index

Supplemental table 3 Relation between inter-arm differences in systolic blood pressure and vascular events using several cut-off values

		Patients with manifest vascular disease (n=5293, events=565)	Patient without manifest vascular disease (n=2051, events=87)	p-value for interaction with manifest vascular disease
	Model*			
≥10 mmHg		(n=1762, events=194)	(n=689, events=33)	
	I	1.00 (0.84-1.19)	1.23 (0.79-1.91)	0.372
	II	0.89 (0.75-1.06)	1.07 (0.80-1.69)	
≥15 mmHg		(n=865, events=106)	(n=317, events=15)	
	I	1.10 (0.89-1.36)	1.12 (0.64-1.97)	0.854
	II	0.93 (0.75-1.16)	0.90 (0.50-1.62)	
≥20 mmHg		(n=379, events=44)	(n=141, events=9)	
	I	1.02 (0.75-1.39)	1.44 (0.71-2.91)	0.284
	II	0.83 (0.61-1.14)	1.22 (0.58-2.57)	

Estimates are given as hazard ratio's (95%CI intervals). * Model I: sex and age; Model II: Model I + systolic blood pressure + current smoking, pack years, non-HDL cholesterol, presence of diabetes mellitus, renal function, ankle-brachial index, presence of manifest vascular disease, cross-product of manifest vascular disease and SBPD

PART THREE

Bisphosphonates as treatment of calcification-induced vascular disease

CHAPTER 11

Bisphosphonates for cardiovascular risk reduction. A systematic review and meta-analysis.

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Abstract

Background and aims – Bisphosphonates might be effective in reducing cardiovascular events due to their ability to reduce calcification in arterial walls. We aimed to investigate the effects of treatment with bisphosphonates on the prevention of atherosclerotic processes and cardiovascular disease.

Methods – Pubmed, Embase and the Cochrane Library were systematically reviewed by two independent investigators for randomized controlled studies published up to January 2016 in which the effect of bisphosphonates on arterial wall disease, cardiovascular events, cardiovascular mortality or all-cause mortality were reported. There was no restriction for the type of population used in the trials. Random-effects model were used to calculate the pooled estimates.

Results – 61 trials reporting the effects of bisphosphonates on the outcomes of interest were included. Bisphosphonates had beneficial effects on arterial wall disease regarding arterial calcification (pooled mean percentage difference of 2 trials -11.52 (95%CI -16.51- -6.52, p<0.01, I^2 13%), but not on arterial stiffness (pooled mean percentage difference of 2 trials -2.82; 95%CI -10.71-5.07; p=0.48, I^2 59%). No effect of bisphosphonate treatment on cardiovascular events was found (pooled RR of 20 trials 1.03; 95%CI 0.91-1.17, I^2 16%), while a lower risk for cardiovascular mortality was observed in patients treated with bisphosphonates (pooled RR of 10 trials 0.81; 95%CI 0.64-1.02; I^2 0%) although not statistically significant. Patients treated with bisphosphonates had a reduced risk of all-cause mortality (pooled RR of 48 trials 0.90; 95%CI 0.84-0.98; I^2 53%).

Conclusions – In this systematic review and meta-analysis it is shown that bisphosphonates reduce arterial wall calcification but have no effect on arterial stiffness or on cardiovascular events. Bisphosphonates tend to reduce the risk of cardiovascular mortality, although no statistically significant effects were found in this meta-analysis and reduce all-cause mortality in various patient groups, including osteoporosis and cancer patients.

Introduction

Despite improvements in treatment of cardiovascular risk factors cardiovascular disease still results in an immense disease burden.[1] New treatment targets might further reduce the risk for first and subsequent cardiovascular events. As vascular calcifications are related to an increased cardiovascular risk, preventing or reducing arterial calcification might be an important target for further cardiovascular risk reduction.[2] Arterial calcifications are observed in several common conditions such as diabetes mellitus, renal failure and aging, all conditions known to be related to a high cardiovascular risk.[3]

Osteoporosis is related to a 2-fold increased risk of cardiovascular mortality, also known as the 'bone-vascular axis'.[4-8] The process of arterial calcification might play an important role in this relation.[9] Arterial calcification is regulated through a network of inhibitory (and promoting) pathways, such as vitamin K dependent pathways, the Klotho protein, Fetuin-A and pyrophosphate. [10] Pyrophosphate is a strong inhibitor of arterial calcification[11, 12] and bisphosphonates, well-established drugs for the treatment of bone diseases associated with excessive bone resorption including osteoporosis and bone metastasis, are pyrophosphate analogues and could thus stimulate the inhibitory effects of pyrophosphate on arterial calcification.[13, 14] In fact, bisphosphonates were first shown to reduce arterial calcification and soft tissues calcification in rats.[15]

Therefore it is conceivable that bisphosphonates interfere in the arterial calcification process and might be able to reduce the risk of cardiovascular disease [16]. Support for this hypothesis is growing as cohort studies show that the use of bisphosphonates in patients with maximum adherence is associated with a 20% lower risk of acute myocardial infarction [17] and randomized controlled trials such as the Health Outcomes and Reduced Incidence with Zolendronic Acid Once Yearly (HORIZON) trial show an 11 % reduction of risk of cardiovascular events and a 31% reduction of cardiovascular deaths was found after treatment with bisphosphonates compared to placebo.[18]

To investigate the effects of treatment with bisphosphonates on arterial wall calcification and stiffness, cardiovascular events, cardiovascular mortality and all-cause mortality, we performed a systematic review of randomized controlled trials with no restrictions on populations and summarized the results in a meta-analysis.

Methods

Search strategy

A systematic literature search of Medline, Embase and the Cochrane Library was performed reviewing articles published up to January 2016. A search filter was designed using synonyms for the determinant (bisphosphonates) and outcome (surrogate markers of cardiovascular disease such as arterial stiffness and arterial calcification, cardiovascular events, cardiovascular mortality, survival and mortality) and using synonyms for determinant (bisphosphonates) and randomized controlled trials. A full search string is provided in supplemental table 1. All articles were screened on title and abstract by two independent researchers (GK and JB) and subsequently the full text was independently evaluated on eligibility by both researchers. Consensus was achieved by discussion, if needed with another independent investigator (WS). Additionally, a manual search through the references of selected articles was performed to identify additional relevant studies. Authors were contacted when a publication was not available or when not all the required information could be retrieved from a study.

Study selection

Studies were considered eligible if they investigated the effects of bisphosphonates, if at least one outcome of interest was reported and if the study was a randomized controlled trial performed in human subjects. As we assumed that some duration of exposure to bisphosphonate treatment was needed for effects on the cardiovascular system, studies in which participants were treated less than one year were excluded for further analyses. Articles providing insufficient data for the analyses were excluded.

Data extraction and quality assessment

Two authors (GK and JB) extracted data from the included studies independently. Discrepancies between the authors were discussed and resolved. From each study the following information was extracted: surname of first author, year of publication, country, number of patients in the treatment and control group, type of bisphosphonate, dosage, duration of bisphosphonate treatment, treatment in control group, gender distribution, age distribution, outcome of interest and eventually absolute numbers of the dichotomous outcome and mean and standard deviation for continuous outcomes. In studies where cardiovascular mortality or cardiovascular events were reported the definition of these outcomes were extracted. For the HORIZON trial [18, 19] the total number of cardiovascular events was calculated by summing up the individual reported numbers of non-fatal

stroke, non-fatal myocardial infarction and death due to a vascular cause, as the follow-up was ended after a serious adverse event in these trials. For the continuous outcomes the mean percentage change in aortic calcification or pulse wave velocity and the standard deviation were calculated using the absolute numbers at baseline and after treatment.

The methodological quality of each included study was evaluated based on the Cochrane risk assessment tool for randomized controlled trials, using the following items: random sequence generation, allocation concealment, similarity of groups, blinding of outcome assessment, completeness of trial and intention to treat analysis.[20] A summary score can be calculated from 0 to 7 points with higher scores indicating a lower risk of bias.

Data analyses

The pooled relative risks of the effects of bisphosphonates on cardiovascular events, cardiovascular mortality and all-cause mortality were calculated using the inverse variance method. To unite the heterogeneous studies the random effects model of DerSimonian and Laird[21] was used. For percentage change in aortic calcification and pulse wave velocity the same method was used.

To explore heterogeneity, the I^2 statistic was used, reflecting the proportion of observed variance between the studies. A value of 25% was considered as low heterogeneity while a value of 75% was considered as high heterogeneity. To assess potential publication bias, visual inspection of funnel plots was performed.

Subgroup analyses were performed to assess whether the vascular effects of bisphosphonates differed per type of bisphosphonate. Further analyses in strata of population type were performed to assess the different effects of bisphosphonates in different populations. Finally, to analyze whether study type (placebo-controlled or not) and study quality (risk of bias category) the effects of bisphosphonates were studied in these strata.

Statistical analyses were conducted with Review Manager (RevMan), version 5.3., Copenhagen: The Nordic Cochrane Centre, The Cochrane Collaboration, 2012. To improve reporting in this systematic review and meta-analysis we followed the Quality of Reporting of Meta-analyses (QUORUM) checklist. [22]

Results

The search strategy identified 6573 unique articles. Initial screening on title and abstract decreased this number to 352 potentially relevant studies. After evaluation of these articles in more detail using the full-text, 292 articles were excluded for reasons shown in figure 1. No additional articles were identified through manual reference check, resulting in a total number of 61 articles for meta-analysis[18, 19, 23-81]. As from some of these articles multiple outcome measurements of interest could be extracted[18, 19, 31-33, 35, 40, 44, 48, 50, 52, 56, 63-65, 75, 76, 80] those articles were used for multiple analyses.

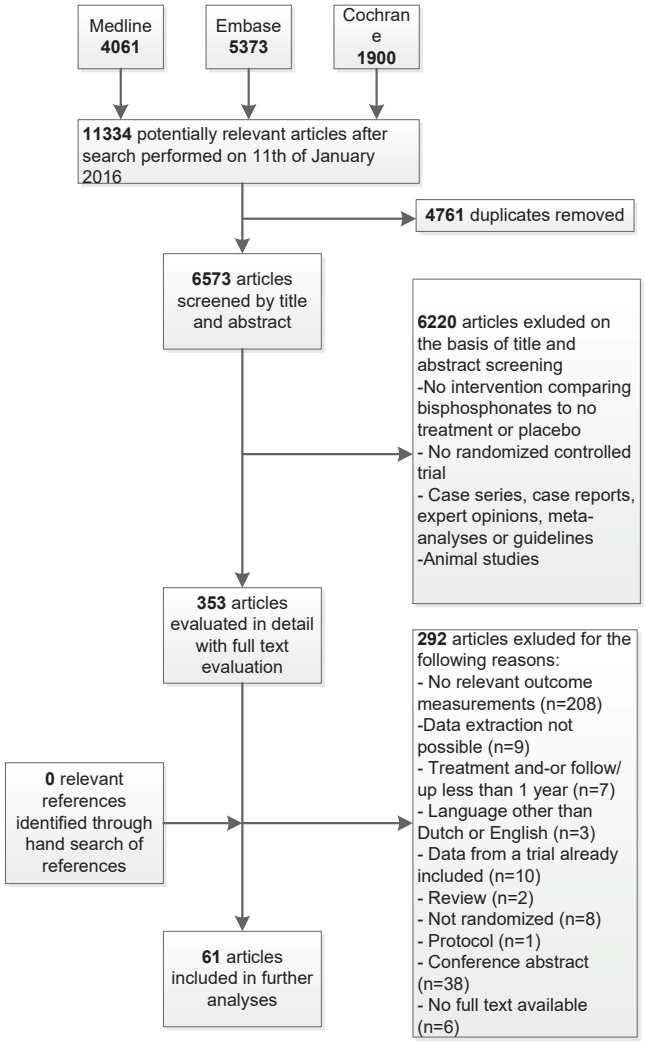


Figure 1 Flowchart of the systematic literature review

Description of included studies

All included studies were randomized controlled trials published in the period 1991-2014 investigating the effects of bisphosphonates in different populations using different research questions. The population differed across the included studies from cancer population to osteoporosis populations (table 1). A variety of bisphosphonates was used in the included studies. Also different dosages, administration methods and durations of bisphosphonate treatment were used in the included studies (Supplemental table 2 elaborate study characteristics). 45 studies were placebo-controlled [18, 19, 23, 25-33, 36, 39, 40, 42, 43, 45, 53-56, 58, 59, 61-81] and in the other 16 randomized studies[24, 34, 35, 37, 38, 41, 43, 46-52, 57, 60] the control group received standard of care without bisphosphonates.

Table 1 Study characteristics

	Total number of included studies
Population	
Multiple myeloma	6
Breast cancer without bone metastasis	11
Breast cancer with bone metastasis	6
Prostate cancer without bone metastasis	5
Prostate cancer with bone metastasis	3
Non-small cell long cancer	1
Chronic kidney disease	2
Osteoporosis	22
Rheumatic disorders	3
Osteogenesis imperfecta	1
Hypercholesterolemia	1
Type of bisphosphonate treatment	
Non-N-containing bisphosphonates	
Etidronate	5
Clodronate	11
N-containing bisphosphonates	
Pamidronate	10
Ibandronate	8
Alendronate	14
Zolendronate	9
Risedronate	4
Treatment in control group	
Placebo	45
No bisphosphonate	16

Table 1 Study characteristics (*continued*)

	Total number of included studies
Extracted outcome of interest	
Death	
All-cause mortality	48
Cardiovascular disease	
Cardiovascular mortality	10
Cardiovascular events	20
Cerebro vascular accident	2
Myocardial infarction	6
Atherosclerotic processes	
Arterial calcification	2
Pulse wave velocity	2

31 of these articles included only female participants[19, 24, 25, 27-32, 36, 37, 42, 45, 48, 49, 51, 55, 57, 58, 61, 63, 65-67, 70, 73, 75-77, 81, 82], while 10 studies were performed in only male subjects[33, 40, 41, 46, 47, 50, 56, 68, 71, 72] and the other 20 studies included both sexes.[18, 23, 26, 34, 35, 38, 43, 44, 52-54, 59, 60, 62, 64, 69, 74, 78-80] The included studies were all conducted in Europe, North America or Oceania, except for 3 trials that were conducted in Asia[52, 58, 80] and the HORIZON trial in which participants from South America were included as well[18, 19] (Supplemental table 2 elaborate study characteristics). In table 1 the main study characteristics are summarized. Two studies including 102 participants that reported on effects of bisphosphonates on arterial wall disease (arterial calcification and arterial stiffness) were selected. [44, 52] From 21,920 patients included in 20 trials the number of cardiovascular events could be derived[18, 19, 31-33, 55, 56, 59, 62, 64, 65, 67, 68, 70, 71, 76, 77, 80], 10 studies with a total of 12276 participants specifically reported cardiovascular mortality[18, 19, 32, 35, 40, 58, 63, 73, 75, 79] and from 48 of the included articles with a total of 43568 included patients absolute numbers of all-cause mortality could be extracted.[18, 19, 23-51, 53, 54, 56, 57, 60-62, 66, 69, 72, 74-76, 78, 79, 81], . The median follow-up duration in studies in which cardiovascular events were reported was between 2 and 3 years, for cardiovascular mortality this was around 4 years and for all-cause mortality median follow-up was between 3 and 4 years.

Quality varied across the different studies as summarized in supplemental table 3 with Cochrane risk of bias scores between 3 and 7. In general, high risk of bias was observed for the item blinding for outcome assessment. 7 studies were assessed as high risk of bias articles[41, 50, 57, 58, 69, 70, 80], 16 studies were scored as moderate risk of bias studies[24, 25, 29, 34, 35, 38, 43, 45, 47, 54, 55, 64, 71, 72, 74, 75, 77] and the other

38 studies[18, 19, 23, 26-28, 30-33, 36, 37, 39, 40, 42, 44, 46, 48, 49, 51-53, 56, 59, 60, 62, 63, 65-68] had high risk of bias scores (>5) and were thus seen as low risk of bias articles.

Effects of bisphosphonates on aortic arterial calcication

Pooling the 2 studies[44, 52] reporting on effects on arterial wall disease, showed that the aortic calcification in subjects treated with bisphosphonates decreased an absolute 11.2% more when compared to untreated participants (pooled mean difference -11.52%; 95%CI -16.51- -6.52; p<0.01, figure 2). Little heterogeneity was observed between the two studies (I^2 13%).

Effects of bisphosphonates on arterial stiffness (pulse wave velocity)

The summary difference percentage change in pulse wave velocity between participants treated and untreated with bisphosphonates was 2.8% (non-significant) with faster arterial stiffness progression in untreated participants (pooled mean difference -2.82%; 95%CI -10.71-5.07; p=0.48, figure 2).

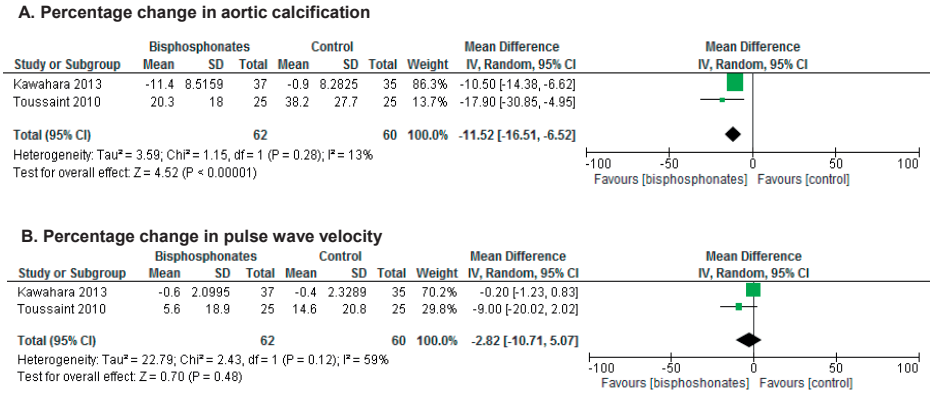


Figure 2 Forrest plot for the effects of bisphosphonates on atherosclerotic processes

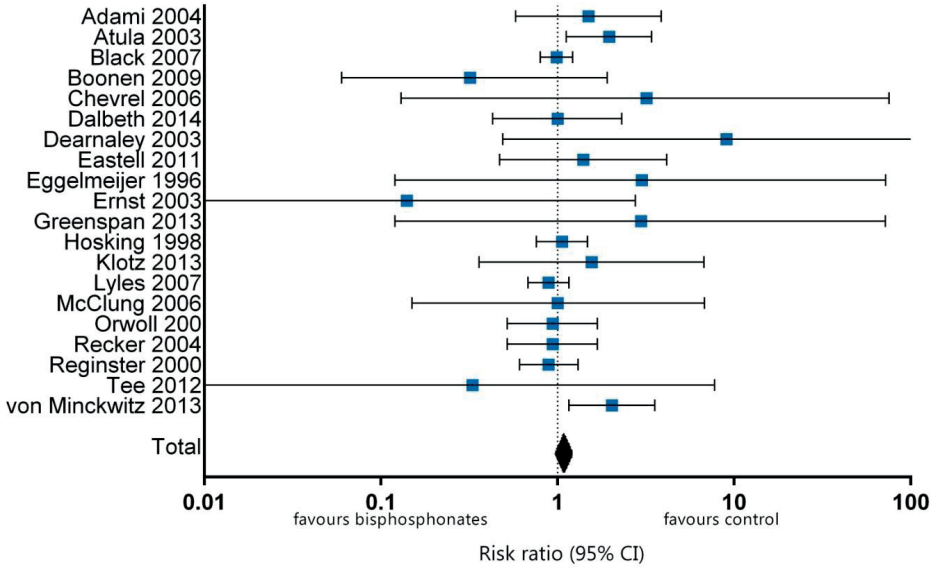
A: The mean difference in percentage change of aortic arterial calcification progression and the 95% confidence interval are depicted. To assess aortic arterial calcification the percentage change in maximum aortic vessel wall thickness from Kawahara et all. were used and Houndsfeld units from Toussaint et al. were used. B: The mean difference in percentage change of pulse wave velocity progression and the 95% confidence interval are depicted.

Effects of bisphosphonates on cardiovascular events

The summary risk of encountering cardiovascular events in patients treated with bisphosphonates in the 20 included trials was not different from the untreated group (pooled RR 1.03; 95%CI 0.91-1.17, figure 3A, table 2). Treatment with non-N-containing bisphosphonates was also not related to a lower risk of car-

diovascular events (pooled RR 0.98; 95%CI 0.89-1.07). Little heterogeneity was observed between the ten studies (I^2 16%). The funnel plot of the studies reporting cardiovascular events indicates no publication bias (supplemental figure 1). Stratification for population showed that treatment with zolendronate[48] and clodronate[31] increased the risk of cardiovascular events in patients with breast cancer (pooled RR 1.99; 95%CI 1.34-2.96), while in the other populations lower risk ratios of bisphosphonate treatment for cardiovascular events were observed (pooled RR 0.95; 95%CI 0.87-1.05, I^2 0%). Exclusion of articles with high (1 article) and moderate (4 articles) risk of bias did not change the magnitude or direction of the relation.

The pooled relative risks of bisphosphonate treatment for stroke and myocardial infarction were RR 1.06 (95%CI 0.82-1.35) and RR 0.82 (95%CI 0.57-1.17), respectively (supplemental figure 2).

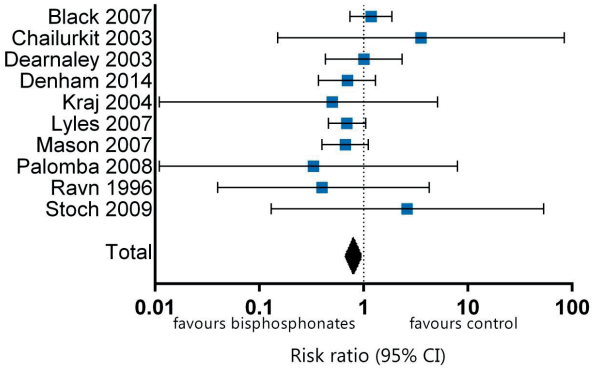


Effects of bisphosphonates on cardiovascular events	
# studies	20
Heterogeneity - I^2	16%
Events / #treated	1054 / 12582
Events / #control	678 / 9338
RR (95% CI)	**1.03 (0.91–1.17)**

Figure 3 Forrest plot for the effect of bisphosphonates on cardiovascular events

Effects of bisphosphonates on cardiovascular mortality

The summary relative risk for cardiovascular mortality from the 10 studies reporting cardiovascular mortality (definitions in supplemental table 5) was 0.81 (95%CI 0.64-1.02, I^2 0%, figure 4). The funnel plot showed little indication of publication bias (supplemental figure 2). Exclusion of 2 articles with high and 2 articles with moderate risk of bias did further attenuate the pooled relative risks and 95% confidence intervals for cardiovascular mortality (table 2). Similar effects on cardiovascular mortality were found for non-N-containing and N-containing bisphosphonates on cardiovascular mortality. Stratification for population did not reveal different effects of bisphosphonates on cardiovascular mortality.



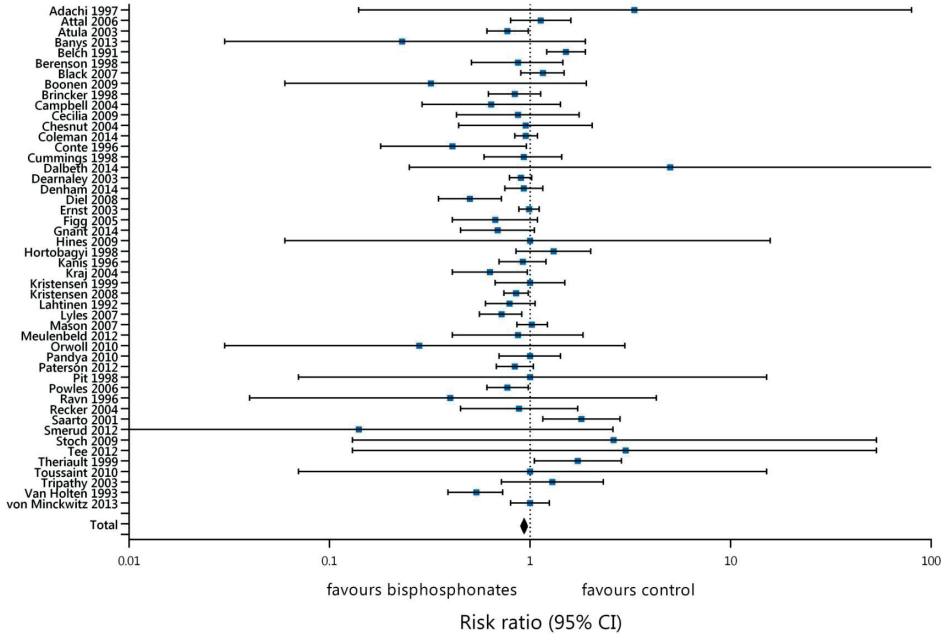
Effects of bisphosphonates on cardiovascular mortality	
# studies	10
Heterogeneity - I^2	0%
Events / #treated	129 / 6225
Events / #control	155 / 6051
RR (95% CI)	**0.81 (0.64-1.02)**

Figure 4 Forrest plot for the effect of bisphosphonates on cardiovascular mortality

Effects of bisphosphonates on all-cause mortality

Pooling the 48 trials reporting all-cause mortality showed that patients receiving bisphosphonate treatment were at lower risk for all-cause mortality (pooled RR 0.90; 95%CI 0.84-0.98; I^2 53%, figure 5). Visual inspection of the funnel plots gave no indication of publication bias (supplemental figure 2). After exclusion of 4 studies with a high risk of bias the magnitude and direction of the relative risk did change only slightly (table 2). Pooled relative risks were similar for studies using non-N-containing bisphosphonates (RR 0.92; 95%CI 0.81-1.05) and studies investigating N-containing bisphosphonates (RR 0.89; 95%CI 0.81-0.98).

Similar effects of treatment with bisphosphonates were found in patients from different populations (table 2), with the largest effect size on all-cause mortality in patients with osteoporosis (RR 0.88; 95%CI 0.75-1.03) and breast cancer (RR 0.86; 95%CI 0.76-0.98).



Effects of bisphosphonates on cardiovascular mortality	
# studies	48
Heterogeneity - I^2	53%
Events / #treated	2552 / 23507
Events / #control	2609 / 20061
RR (95% CI)	**0.90 (0.84-0.98)**

Figure 5 Forrest plot for the effect of bisphosphonates on all-cause mortality

Table 2 Pooled relative risk and 95% confidence intervals of effects of bisphosphonates on cardiovascular endpoints

Cardiovascular events				
	RR (95% CI)	**Nr of studies**	**Events treated/ Events control**	**Ntreated / Ncontrol**
Type of bisphosphonate				
Non-N-containing bisphosphonates	1.57 (0.29-8.41)	3	1015/657	11785/8536
N-containing bisphosphonates	0.98 (0.89-1.07)	17	39/21	797/802
Population				
Multiple myeloma	NA	0	NA	NA
Breast cancer	1.99 (1.34-2.96)	2	96/33	2534/1539
Prostate cancer	1.34 (0.20-8.98)	3	8/6	336/351
Non-small cell long cancer	NA	0	NA	NA
Chronic kidney disease	NA	0	NA	NA
Osteoporosis	0.95 (0.87-1.05)	12	939/630	9577/7311
Rheumatic disorders	1.07 (0.48-2.41)	2	10/9	104/104
Osteogenesis imperfecta	3.19 (0.13-75.43)	1	1/0	31/33
Treatment in control group				
Placebo	0.98 (0.89-1.07)	19	993/663	10586/8340
No bisphosphonate	2.03 (1.16-3.56)	1	61/15	1996/998
Risk of bias				
Only low or moderate risk of bias studies	1.04 (0.91-1.19)	19	1052/676	12536/9292
Only low risk of bias studies	1.08 (0.91-1.27)	15	937/617	11129/8608
Total cardiovascular events	**1.03 (0.91-1.17)**	20	1054/678	12582/9338

Cardiovascular mortality				
	RR (95% CI)	**Nr of studies**	**Events treated/ Events control**	**Ntreated / Ncontrol**
Type of bisphosphonate				
Non-N-containing bisphosphonates	0.74 (0.48-1.15)	2	32/42	409/410
N-containing bisphosphonates	0.84 (0.64-1.10)	8	97/112	5816/5641
Population				
Multiple myeloma	0.50 (0.05-5.14)	1	1/2	23/23
Breast cancer	NA	0	NA	NA
Prostate cancer	0.73 (0.51-1.04)	3	48/66	945/947
Non-small cell long cancer	NA	0	NA	NA
Chronic kidney disease	NA	0	NA	NA
Osteoporosis	0.88 (0.62-1.23)	5	78/87	5143/5022
Rheumatic disorders	2.61 (0.13-53.47)	1	2/0	114/59
Osteogenesis imperfecta	NA	0	NA	NA
Treatment in control group				
Placebo	0.83 (0.65-1.07)	8	112/130	5666/5491
No bisphosphonate	0.68 (0.37-1.25)	2	17/25	559/560
Risk of bias				
Only low or moderate risk of bias studies	0.82 (0.64-1.05)	8	112/132	5657/5476
Only low risk of bias studies	0.83 (0.65-1.07)	6	109/129	5484/5423
Total cardiovascular mortality	**0.81 (0.64-1.02)**	10	129/155	6225/6051

All-cause mortality				
	RR (95% CI)	**Nr of studies**	**Events treated/ Events control**	**Ntreated / Ncontrol**
Type of bisphosphonate				
Non-N-containing bisphosphonates	0.92 (0.81-1.05)	15	942/1041	4069/4086
N-containing bisphosphonates	0.89 (0.81-0.98)	33	160/1668	19438/15975
Population				
Multiple myeloma	0.95 (0.71-1.25)	6	269/258	829/792
Breast cancer	0.86 (0.76-0.98)	17	1397/1470	1663/1526
Prostate cancer	0.96 (0.89-1.03)	7	495/499	1663/1526
Non-small cell long cancer	1.00 (0.70-1.42)	1	47/25	98/52
Chronic kidney disease	1.54 (0.20-11.57)	2	3/1	139/84
Osteoporosis	0.88 (0.75-1.03)	14	339/356	11792/9613
Rheumatic disorders	5.00 (0.25-101.58)	0	2/0	50/50
Osteogenesis imperfecta	NA	0	NA	NA
Treatment in control group				
Placebo	0.96 (0.88-1.05)	33	1616/1671	17149/14722
No bisphosphonate	0.78 (0.67-0.91)	15	936/938	6358/5339
Risk of bias				
Only low or moderate risk of bias studies	0.92 (0.85-0.99)	44	2389/2409	22674/19238
Only low risk of bias studies	0.93 (0.85-1.02)	31	2147/2158	21530/18264
Total all-cause mortality	**0.90 (0.84-0.98)**	48	2552/2609	23507/20061

Discussion

This systematic review and meta-analysis included 61 randomized controlled trials using various patient groups including osteoporosis patients and cancer patients. 45 studies were placebo-controlled, while the other 16 trials used standard of care without bisphosphonates as the control group. Two studies reported on surrogate markers of cardiovascular disease and showed that bisphosphonates reduce arterial wall calcification, but have no effect on arterial stiffness or on cardiovascular events, based on 10 included studies reporting effects on cardiovascular events. Furthermore, based on 20 studies reporting effects on cardiovascular mortality, bisphosphonates reduce the risk of cardiovascular mortality with 19%, although this reduction is not statistically significant compared to placebo or no bisphosphonate treatment. Treatment with bisphosphonates is related to a significant pooled reduction of all-cause mortality of 10%, based on the 48 studies included in this meta-analysis reporting all-cause mortality.

Although not statistically significant this meta-analysis suggests potential beneficial effects of bisphosphonates on cardiovascular disease which is supported by animal models in which bisphosphonates were shown to be able to slow down atherosclerosis.[83-86] Different pathophysiological processes might play a role including the inhibition of arterial calcification by inhibiting the differentiation of vascular smooth muscle cells to bone forming cells (osteoblasts) and inhibition of instable plaque formation by inhibiting both cholesterol biosynthesis and formation of foam cells[87, 88]. Bisphosphonates are able reach a reduction in LDL-cholesterol level up to 7% in humans by these mechanisms and additional mechanisms that are not clarified yet.[89, 90]

The present meta-analysis supports the hypothesis of effects of bisphosphonates on arterial wall disease by showing beneficial effects on arterial calcification. However no beneficial effects on arterial stiffness progression were found in the present study, as was the case in other studies.[91] Other studies, which were excluded from the present meta-analysis for different reasons, support our findings by showing that bisphosphonates decrease the thickness of vessel walls[89, 92] and inhibit progression of arterial calcification[91, 93, 94].

We did not find effects of bisphosphonates on cardiovascular events. It is possible that both the duration of bisphosphonate exposure and the follow-up duration in the included studies were simply too short to observe beneficial cardiovascular effects of bisphosphonates. Interestingly, in sensitivity analyses pooling only the studies in patients with osteoporosis which had a longer follow-up duration the

effect estimate of the risk ratio on cardiovascular events was in favor of bisphosphonate treatment. Furthermore, methodological issues of the included studies could provide alternative contributing explanations for the fact that no beneficial effect on cardiovascular events was found. For instance the trial of von Minckwitz et al.[48] may have induced bias. Exclusion of this trial indeed decreased the pooled relative risks for cardiovascular events. Possible harmful cardiovascular effects, such as an increased risk of atrial fibrillation [95] of bisphosphonates in the included trials could have increased the risk ratios for cardiovascular events. Although many questions have been raised about the potential harmful effects of bisphosphonates, especially zolendronate, on atrial fibrillation [95], a recent meta-analysis showed that treatment with bisphosphonates did not result in higher risks for atrial fibrillation.[96]

Although not statistically significant, a strong reduction of cardiovascular mortality after treatment with bisphosphonates was observed in the present meta-analysis. A longer follow-up time in studies reporting cardiovascular mortality versus studies reporting cardiovascular events probably contributed to the discrepancy in observed effects of bisphosphonates. It is conceivable that a longer follow-up and treatment period is needed to detect potential cardiovascular effects of bisphosphonates.

While there were no statistically significant effects of bisphosphonates on cardiovascular mortality, we did find beneficial effects of bisphosphonate treatment on all-cause mortality. The fact that in the present meta-analysis a reduction of all-cause mortality was found even in the non-cancer populations suggests that the gain in survival might not only be due to reduction of cancer-related mortality. Although we did not find statistically significant effects of bisphosphonates on cardiovascular mortality in this meta-analysis, a potential reduction of cardiovascular mortality might also be contributive to the observed reduction of all-cause mortality. In addition to bone-protective mechanisms of bisphosphonates cardio-protective mechanisms and the bone-vascular axis might play an important contributive role in the explanation of these beneficial effects on survival of bisphosphonates. Interfering in the arterial calcification process by bisphosphonates might lead to the observed gain in survival.[9]

The findings of the present meta-analysis are supported by a recent meta-analysis on the effect of bisphosphonates on atrial fibrillation and cardiovascular events.[96] We expand the findings of this previously performed meta-analysis by showing effects of bisphosphonates on all-cause mortality and preliminary atherosclerotic

processes. Also we made some different methodological choices in conducting the meta-analysis. We chose not only to include search terms for bisphosphonates and randomized trial, but also for bisphosphonates and separate endpoints of interest. Furthermore, we did include clinical trials with cancer patients as we assumed that the presence of cancer did not change potential cardiovascular effects of bisphosphonates. Also, we decided only to include trials in this meta-analysis in which participants were treated for at least one year in order to be reasonably able to observe potential effects of bisphosphonates on cardiovascular and all-cause mortality.

It is important to take methodological quality of the individual included articles into account when interpreting the results of our meta-analysis. The methodological quality differed across the articles with a relatively smaller proportion of methodological flaws in articles reporting on cardiovascular outcomes. Usually methodological flaws lead to an overestimation of effect size. Indeed the effect sizes were attenuated in the present meta-analysis after exclusion of the articles with a high risk of bias. Nevertheless, the effect sizes remained in favor of bisphosphonate treatment for all-cause mortality and cardiovascular mortality, although the confidence intervals further widened.

In order to actually answer the question whether bisphosphonates are able to reduce the risk of cardiovascular disease randomized controlled trials designed to evaluate cardiovascular outcomes with a sufficient follow-up duration are needed. In the meantime the evidence for beneficial effects of bisphosphonates on arterial wall disease and cardiovascular disease is growing and future promising results might come from third generation (nitrogen-containing) bisphosphonates that have more beneficial effects on atherosclerotic processes.[97]

Strengths of this meta-analysis include the comprehensive search method and the large number of included studies. Randomization in all included trials strongly reduces the potential of confounding of the observed effects of bisphosphonates in this meta-analysis. Taking the large number of included studies into account the I^2 statistic for the amount of heterogeneity was low. Several limitations of the present meta-analysis need to be taken into account. Firstly, there was some heterogeneity across the included trials. Different bisphosphonates, administration methods, treatment durations, follow-up durations, control groups and populations were used in the included trials. Furthermore definitions used for cardiovascular events in the included studies differed slightly. In general the methodological quality of the included trials was high, yet some variation in risk of bias

scores was observed. To account for this expected heterogeneity several analytic methods were performed. We used a random effect model for all our analyses and performed subgroup analyses for type of bisphosphonate, population, treatment in the control group and risk of bias score. Secondly, adverse events are not always published in papers and therefore it is likely that not all randomized trials that had data for cardiovascular events and mortality after bisphosphonate treatment were included in the present meta-analysis.[98] However, this is of minor consequence for the interpretation of our results as the symmetric funnel plots indicate no publication bias and no differential underreporting of cardiovascular effects. Lastly, as most included studies were performed in patients with cancer or osteoporosis it is questionable whether these results could be translated to patients with diseases associated with arterial calcification such as chronic kidney disease, diabetes and vascular disease in which bisphosphonate treatment might be a potential treatment for further cardiovascular risk reduction. However those patients were also in the included trials and have contributed to the observed effects of bisphosphonates on cardiovascular endpoints. In trials including only patients with chronic kidney disease or hypercholesterolemia even stronger cardiovascular effects of bisphosphonates were found.

In conclusion, in this systematic review and meta-analysis it is shown that bisphosphonates reduce arterial wall calcification but have no effect on arterial stiffness or on cardiovascular events. Bisphosphonates tend to reduce the risk of cardiovascular mortality, although no statistically significant effects were found in this meta-analysis and reduce all-cause mortality in various patient groups, including osteoporosis patients and cancer patients.

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Appendix

Supplemental table 1 Search string

Pubmed	*(((((bisphosphonates[Title/Abstract] OR bisfosfonates[Title/Abstract] OR Etidronate[Title/Abstract] OR Clodronate[Title/Abstract] OR Tiludronate[Title/Abstract] OR Pamidronate[Title/Abstract] OR Neridronate[Title/Abstract] OR Olpadronate[Title/Abstract] OR Alendronate[Title/Abstract] OR Ibandronate[Title/Abstract] OR Risedronate[Title/Abstract] OR Zoledronate[Title/Abstract]) AND ((("cardiovascular disease"[Title/Abstract] OR "cardiovascular event"[Title/Abstract]) OR "coronary disease"[Title/Abstract] OR "coronary ischemia"[Title/Abstract] OR "coronary ischaemia"[Title/Abstract] OR "coronary artery disease"[Title/Abstract] OR "coronary heart disease"[Title/Abstract] OR "myocardial infarction"[Title/Abstract] OR "heart infarction"[Title/Abstract] OR "stroke"[Title/Abstract] OR "cerebrovascular disease"[Title/Abstract] OR "cerebrovascular event"[Title/Abstract] OR "cerebrovascular ischemia"[Title/Abstract] OR "cerebrovascular ischaemia"[Title/Abstract] OR "cerebrovascular accident"[Title/Abstract] OR "cerebrovascular hemorrhage"[Title/Abstract] OR "cerebrovascular infarction"[Title/Abstract] OR "cerebral disease"[Title/Abstract] OR "cerebral event"[Title/Abstract] OR "cerebral ischemia"[Title/Abstract] OR "cerebral ischaemia"[Title/Abstract] OR "cerebral accident"[Title/Abstract] OR "cerebral hemorrhage"[Title/Abstract] OR "cerebral haemorrhage"[Title/Abstract] OR "cerebral infarction"[Title/Abstract] OR "brain infarction"[Title/Abstract] OR "brain hemorrhage"[Title/Abstract] OR "brain haemorrhage"[Title/Abstract] OR "brain ischemia"[Title/Abstract] OR "brain ischaemia"[Title/Abstract] OR "transient ischemic attack"[Title/Abstract] OR "transient ischaemic attack"[Title/Abstract] OR "peripheral artery disease"[Title/Abstract] OR "peripheral arterial disease"[Title/Abstract] OR "peripheral artery occlusive disease"[Title/Abstract] OR "percutaneous coronary intervention"[Title/Abstract] OR "percutaneous transluminal angioplasty"[Title/Abstract] OR "percutaneous transluminal coronary angioplasty"[Title/Abstract] OR "coronary artery bypass graft"[Title/Abstract] OR "carotid endarterectomy"[Title/Abstract] OR "carotid artery stenting"[Title/Abstract] OR atherosclerosis[Title/Abstract] OR ("myocardial infarction"[MeSH Terms] OR "stroke"[MeSH Terms])) OR (((death[Title/Abstract]) OR mortality[Title/Abstract]) OR survival[Title/Abstract]) OR (((((((("ankle brachial index"[Title/Abstract]) OR "ankle brachial ratio"[Title/Abstract]) OR "arterial stiffness"[Title/Abstract]) OR "vascular stiffness"[Title/Abstract]) OR "pulse wave analysis"[Title/Abstract]) OR "intima media thickness"[Title/Abstract]) OR "pulse velocity"[Title/Abstract]) OR "pulse wave velocity"[Title/Abstract]) OR "augmentation index"[Title/Abstract])OR "aortic calcification"[Title/Abstract])).))))) OR ((((((bisphosphonates[Title/Abstract] OR bisfosfonates[Title/Abstract] OR Etidronate[Title/Abstract] OR Clodronate[Title/Abstract] OR Tiludronate[Title/Abstract] OR Pamidronate[Title/Abstract] OR Neridronate[Title/Abstract] OR Olpadronate[Title/Abstract] OR Alendronate[Title/Abstract] OR Ibandronate[Title/Abstract] OR Risedronate[Title/Abstract] OR Zoledronate[Title/Abstract])) AND ((randomized controlled trial [pt] OR controlled clinical trial [pt] OR randomized [tiab] OR placebo [tiab] OR clinical trials as topic [mesh: noexp] OR randomly [tiab] OR trial [ti]) NOT (animals [mh] NOT humans [mh])) AND adult [mh])) NOT ((Comment[ptyp] OR Editorial[ptyp] OR Guideline[ptyp] OR Letter[ptyp] OR Meta-Analysis[ptyp] OR Review[ptyp] OR systematic[sb])))))*

Embase	*bisphosphonates':ab,ti OR 'bisfosfonates':ab,ti OR 'etidronate':ab,ti OR 'clodronate':ab,ti OR 'tiludronate':ab,ti OR 'pamidronate':ab,ti OR 'neridronate':ab,ti OR 'olpadronate':ab,ti OR 'alendronate':ab,ti OR 'ibandronate':ab,ti OR 'risedronate':ab,ti OR 'zoledronate':ab,ti AND ('cardiovascular disease':ab,ti OR 'vascular disease':ab,ti OR 'cardiovascular event':ab,ti OR 'coronary disease':ab,ti OR 'coronary ischemia':ab,ti OR 'coronary ischaemia':ab,ti OR 'coronary artery disease':ab,ti OR 'coronary heart disease':ab,ti OR 'myocardial infarction':ab,ti OR 'heart infarction':ab,ti OR 'heart infarction'/exp OR 'heart infarction' OR stroke:ab,ti OR 'cerebrovascular disease':ab,ti OR 'cerebrovascular event':ab,ti OR 'cerebrovascular ischemia':ab,ti OR 'cerebrovascular ischaemia':ab,ti OR 'cerebrovascular accident':ab,ti OR 'cerebrovascular hemorrhage':ab,ti OR 'cerebrovascular haemorrhage':ab,ti OR 'cerebrovascular infarction':ab,ti OR 'cerebral disease':ab,ti OR 'cerebral event':ab,ti OR 'cerebral ischemia':ab,ti OR 'cerebral ischaemia':ab,ti OR 'cerebral accident':ab,ti OR 'cerebral hemorrhage':ab,ti OR 'cerebral haemorrhage':ab,ti OR 'cerebral infarction':ab,ti OR 'brain infarction'/exp OR 'brain infarction' OR 'brain infarction':ab,ti OR 'brain hemorrhage':ab,ti OR 'brain haemorrhage:ti:ab' OR 'brain ischemia':ab,ti OR 'brain ischaemia':ab,ti OR 'transient ischemic attack':ab,ti OR 'transient ischaemic attack':ab,ti OR 'abdominal aorta aneurysm'/exp OR 'abdominal aorta aneurysm' OR 'abdominal aortic aneurysm':ab,ti OR 'abdominal aorta aneurysm':ab,ti OR 'peripheral artery disease':ab,ti OR 'peripheral arterial disease':ab,ti OR 'peripheral artery occlusive disease':ab,ti OR 'peripheral occlusive artery disease'/exp OR 'peripheral occlusive artery disease' OR 'percutaneous coronary intervention':ab,ti OR 'percutaneous transluminal angioplasty':ab,ti OR 'percutaneous transluminal coronary angioplasty':ab,ti OR 'coronary artery bypass graft':ab,ti OR 'carotid endarterectomy':ab,ti OR 'carotid artery stenting':ab,ti OR atherosclerosis:ab,ti OR 'death'/exp OR 'death' OR 'mortality':ab,ti OR 'survival':ab,ti OR 'ankle brachial index':ab,ti OR 'ankle brachial ratio':ab,ti OR 'arterial stiffness':ab,ti OR 'vascular stiffness':ab,ti OR 'pulse wave analysis':ab,ti OR 'aortic calcification':ab,ti OR 'intima media thickness':ab,ti OR 'pulse velocity':ab,ti OR 'pulse wave velocity':ab,ti OR 'augmentation index':ab,ti) OR ('bisphosphonates':ab,ti OR 'bisfosfonates':ab,ti OR 'etidronate':ab,ti OR 'clodronate':ab,ti OR 'tiludronate':ab,ti OR 'pamidronate':ab,ti OR 'neridronate':ab,ti OR 'olpadronate':ab,ti OR 'alendronate':ab,ti OR 'ibandronate':ab,ti OR 'risedronate':ab,ti OR 'zoledronate':ab,ti AND ('randomized controlled trial':ab,ti OR 'controlled clinical trial':ab,ti OR 'randomized':ab,ti OR 'placebo':ab,ti OR 'randomly':ab,ti) NOT (animals NOT humans)) AND [embase]/lim*

Cochrane library	*(bisphosphonates:ti,ab,kw or Bisfosfonates:ti,ab,kw or "etidronate":ti,ab,kw or "clodronate":ti,ab,kw or "tiludronate":ti,ab,kw or "pamidronate":ti,ab,kw or `neridronate:ti,ab,kw or "olpadronate":ti,ab,kw or "alendronate":ti,ab,kw or "ibandronate":ti,ab,kw or "risedronate":ti,ab,kw or "zoledronate":ti,ab,kw) and ("cardiovascular disease":ti,ab,kw or "vascular disease":ti,ab,kw or "cardiovascular event":ti,ab,kw or "coronary disease":ti,ab,kw or "coronary ischemia":ti,ab,kw or "coronary ischaemia":ti,ab,kw or "coronary artery disease":ti,ab,kw or "coronary heart disease":ti,ab,kw or "myocardial infarction":ti,ab,kw or "heart infarction":ti,ab,kw or "stroke":ti,ab,kw or "cerebrovascular disease":ti,ab,kw or "cerebrovascular event":ti,ab,kw or "cerebrovascular ischemia":ti,ab,kw or "cerebrovascular ischaemia":ti,ab,kw or "cerebrovascular accident":ti,ab,kw or "cerebrovascular haemorrhage":ti,ab,kw or "cerebrovascular infarction":ti,ab,kw or "cerebral disease":ti,ab,kw or "cerebral event":ti,ab,kw or "cerebral ischemia":ti,ab,kw or "cerebral ischaemia":ti,ab,kw or "cerebral accident":ti,ab,kw or "cerebral haemorrhage":ti,ab,kw or "brain ischemia":ti,ab,kw or "peripheral arterial disease":ti,ab,kw or "peripheral artery disease":ti,ab,kw or "peripheral arterial occlusive disease":ti,ab,kw or "peripheral artery occlusive disease":ti,ab,kw or "percutaneous coronary intervention":ti,ab,kw or "percutaneous transluminal angioplasty":ti,ab,kw or "percutaneous transluminal coronary angioplasty":ti,ab,kw or "coronary artery bypass graft":ti,ab,kw or "carotid endarterectomy":ti,ab,kw or "carotid artery stenting":ti,ab,kw or atherosclerosis:ti,ab,kw or "death":ti,ab,kw or "mortality":ti,ab,kw or "survival":ti,ab,kw or "ankle brachial index":ti,ab,kw or "ankle brachial ratio":ti,ab,kw or "arterial stiffness":ti,ab,kw or "vascular stiffness":ti,ab,kw or "aortic calcification":ti,ab,kw or "pulse wave analysis":ti,ab,kw or "pulse velocity":ti,ab,kw or "pulse wave velocity":ti,ab,kw or "augmentation index":ti,ab,kw)*

Supplemental table 2 elaborate study characteristics

Author/ Year/ Country	N treated	N control	Population	(Additional) treatment in intervention group	Treatment in control group
Adachi / 1997 / Canada, Europe	67	74	Osteoporosis due to corticosteroid treatment	Cyclical etidronate 400 mg per day for 2 weeks, 10 weeks off	Placebo + in 10 weeks off 500 mg calcium
Adami / 2004 / Europe	392	128	Post menopausal osteoporosis	Ibandronate 1 or 2 mg iv every 3 months	Placebo + Daily calcium 500 mg and 400 IU vit D
Attal / 2006 / France	196	200	Multiple myeloma	Pamidronate 90mg iv 4wk interval	No bisphosphonate + chemotherapy/stem-cell therapy
Atula / 2003 / Canada, UK, Norway, Finland	538	541	Breast cancer without bone metastasis	Clodronate 1600mg	Placebo + standard therapy
Banys / 2013 / Germany	40	46	Breast cancer without bone metastas	Zoledronic acid iv every 4wks	No bisphosphonate + standard therapy
Belch / 1991 / Canada	92	74	Multiple myeloma	Etidronate 5mg/kg/day	Placebo + melphalan 9 mg/m2 and prednisone 100 mg 4 days every 4 weeks
Berenson / 1998 / North America, Australia, New Zealand	198	179	Multiple myeloma	Pamidronate 90mg iv	Placebo + standard therapy
Black/ 2007 / Europe, North and South America, Oceania, Asia	3875	3861	Postmenopausal women with a low bone mineral density	Zoledronic acid iv 5mg once yearly	Placebo
Boonen / 2009 / Europe, Lebanon, Australia, USA	191	93	Osteoporosis	Risedronate 35 mg/w	Placebo + Daily calcium 1000 mg and 400-500 IU vit D
Brincker / 1998 / Denmark, Sweden	152	148	Multiple myeloma	Pamidronate 75 mg	Placebo + melphalan 0.25 mg/kg/day and prednisolone 100 mg/day for 4 days
Campbell / 2004 / UK	81	95	Longterm corticosteroid users for asthma induced osteoporosis	Etidronate 400 mg	No bisphosphonate + continuous/intermittent oral prednisolone + inhaled glucocorticoid

	Duration of bisphosphonate treatment	Type of bisphosphonate	Men (%)	Mean age/age distribution	Follow-up years	Reported outcome of interest
	1 year	non-N-containing bisphosphonate	27	61 ± 15	1	All-cause Mortality
	1 year	N-containing bisphosphonate	0	66 ± 4	1	CV events
	Untill end of study or death	N-containing bisphosphonates	55	mean 59±8	median 3.3	All-cause Mortality
	2	Non-N-containing bisphosphonate	0	mean 53±11	median 5.5	All-cause Mortality, Cardiovascular adverse events
	2	N-containing bisphosphonates	0	median 54 (range: 27-77)	2	All-cause Mortality
	Untill end of study or death	Non-N-containing bisphosphonate	63	median 60-69	median 3.7	All-cause Mortality
	1.75	N-containing bisphosphonates	58	mean 63±10	median 2.4	All-cause Mortality
	Untill end of study or death	N-containing bisphosphonates	0	mean 73±5	3	All-cause Mortality, Cardiovascular Mortality, myocardial infarction, CVA
	2 years	N-containing bisphosphonate	100	61 ± 11	2	All-cause Mortality, CV events, myocardial infarction
	Untill end of study or death	N-containing bisphosphonates	53	median 69	4	All-cause Mortality
	Untill end of study or death	Non-N-containing bisphosphonate	52	mean 60±8	5	All-cause Mortality

Supplemental table 2 elaborate study characteristics (*continued*)

Author/ Year/ Country	N treated	N control	Population	(Additional) treatment in intervention group	Treatment in control group
Cecilia / 2009 / Spain	119	120	Osteoporosis	Alendronate 70 mg/ week	No bisphisphonate + Daily 500 mg calcium and 400 IU vit D
Chailurkit / 2003 / Thailand	32	38	Osteoporosis	Alendronate 10 mg/ day	Placebo + Daily 500 mg calcium
Chesnut / 2004 / North America, Europe	977 and 977	975	Postmenopausal women with a history of vertebral fractures	Ibandronate 2.5 mg or intermittent 20 mg	Placebo
Chevrel / 2006 / France	31	33	Osteogenesis imperfecta	Alendronate 10 mg daily	Placebo + Daily 1000 mg calcium and 1000 IU vit D
Coleman / 2014 / UK	1681	1678	Breast cancer without bone metastasis	Zoledronic acid iv 4mg 3-4wks	No bisphosphonate + standard therapy
Conte / 1996 / Europe, North and South America	143	152	Breast cancer with bone metastasis	Pamidronate 45 mg iv/3weeks	No bisphosphonate +chemotherapy
Cummings / 1998 / USA	2214	2218	Osteoporosis	Alendronate 10 mg/ day	Placebo
Dalbeth / 2014 / Australia and New Zealand	327	333	Tophaceous gout	Zolendronate 5 mg/ year iv	Placebo
Dearnaley / 2003 / UK	155	156	Prostate cancer without bone metastasis	Clodronate 4dd 520mg oral	Placebo + standard bisphosphonate
Denham / 2014 / Australia and New Zealand	268	268	Prostate cancer without bone metastasis	Zoledronic acid iv 4mg every 3 months	No bisphosphonate + standard endocrine therapy
Diel / 2008 / Germany	157	145	Breast cancer without bone metastasis	Clodronate 1000mg/ day	No bisphosphonate + standard therapy
Eastell / 2011 / Europe	57	57	Post menopausal osteoporosis	Alendronate 70 mg/ week	Placebo
Eggelmeijer / 1996 / The Netherlands	54	51	Rheumatoid athritis	Pamidronate 300 mg per day	Placebo

	Duration of bisphospho-nate treatment	Type of bisphos-phonate	Men (%)	Mean age/age distribution	Follow-up years	Reported outcome of interest
	1 year	N-containing bisphosphonate	20	81 ± 7	1	All-cause Mortality
	1 year	N-containing bisphosphonate	0	62 ± 6	1	CV Mortality
	3	N-containing bisphosphonates	0	mean 69±6	3	All-cause Mortality
	3 years	N-containing bisphosphonate	61	37 ± 12	3	CV events
	5	N-containing bisphosphonates	0	mean 51±10	5	All-cause Mortality
	Untill death or end of study	N-containing bisphosphonate	0	median 58	2	All-cause Mortality
	2 years	N-containing bisphosphonate	0	68 ± 6	4	All-cause Mortality
	3 years	N-containing bisphosphonate	47	56 ± 12	3	CV events
	3	Non-N-containing bisphosphonate	100	median 71 (range 47-88)	median 4.9	All-cause Mortality, Cardio-vascular Mortality, Cardio-vascular adverse events
	1,5	N-containing bisphosphonates	100	median 69 (IGR 64-73)	median 7.4	All-cause Mortality, Cardio-vascular Mortality
	2	Non-N-containing bisphosphonate	0	median 53 (range 24-78)	8.5	All-cause Mortality
	1 year	N-containing bisphosphonate	0	65 ± 4	1	All-cause Mortality, CV events
	3 years	N-containing bisphosphonate	32	50 ± 2	3	CV events, myocardial infarction

Supplemental table 2 elaborate study characteristics (*continued*)

Author/ Year/ Country	N treated	N control	Population	(Additional) treatment in intervention group	Treatment in control group
Ernst / 2003 / Canada	104	105	Prostate cancer with bone metastasis	Clodronate 1500mg iv every 4wks	Placebo + prednisone 5 mg 2dd and mitoxantrone 12 mg/m2
Figg / 2005 / The Netherlands	36	36	Prostate cancer with bone metastasis	Alendronate 40mg	No bisphosphonate + ketoconazol
Gnant / 2015 / Austria	900	903	Breast cancer without bone metastasis	Zolendronic acid iv 4 mg every 6 months	No bisphosphonate + STAS or ITAS
Greenspan / 2013 / USA	93	93	Post menopausal osteoporosis	Alendronate 10 mg /day	Placebo
Hines / 2009 / North America	106	106	Breast cancer without bone metastasis	Risedronate 35 mg	Placebo + Daily 600 mg calcium and 400 IU vit D
Hortobagyi / 1998 / North America, Australia	185	197	Breast cancer with bone metastasis	Pamidronate 90mg iv	Placebo + standard therapy
Hosking / 1998 / Europe, USA	997	502	Post menopausal osteoporosis	Alendronate 2.5-5 mg/day	Placebo
Kanis / 1996 / UK, Canada	66	67	Breast cancer without bone metastasis	Clodronate 4dd 400mg	Placebo + standard therapy
Kawahara / 2013 / Japan	37	35	Hypercholesterolemia	etidronate 400mg oral daily	No bisphosphonate + atorvastatin
Klotz / 2013 / Canada	84	102	Prostate cancer without bone metastasis	Alendronate 70 mg/ week	Placebo + Daily 500 mg calcium and 400 IU vit D
Kraj / 2004 / Poland	23	23	Multiple myeloma	Pamidronate 60mg iv monthly	No bisphosphonate + chemotherapy alone
Kristensen/ 1999 / Denmark	49	51	Breast cancer with bone metastasis	Clodronate 1600 mg	Placebo + standard therapy
Kristensen/ 2008 / Denmark	460	493	Breast cancer without bone metastasis	Pamidronate 300 mg	Placebo + standard therapy (CMF or CEF)
Lahtinen / 1992 / Finland	168	168	Multiple myeloma	Clodronate 800 mg/ day	Placebo + prednisolon-melphalan every 4th week

Duration of bisphosphonate treatment	Type of bisphosphonate	Men (%)	Mean age/age distribution	Follow-up years	Reported outcome of interest
Untill end of study or death	Non-N-containing bisphosphonate	100	median 70 (IQR 65-75)	2	All-cause Mortality, Cardiovascular adverse events
Untill end of study or death	N-containing bisphosphonates	100	median 71 (range 51-85)	median 2	All-cause Mortality
3	N-containing bisphosphonates	0	median 45 (range 26-56)	7	All-cause Mortality
2 years	N-containing bisphosphonate	0	72 ± 5	2	CV events, myocardial infarction
1 year	N-containing bisphosphonate	0	44 ± 6	1	All-cause Mortality
Untill end of study or death	N-containing bisphosphonates	0	NS	2	All-cause Mortality
2 years	N-containing bisphosphonate	0	53 ± 4	2	CV events
3	Non-N-containing bisphosphonate	0	median 58 (range 32-82)	3	All-cause Mortality
1	Non-N-containing bisphosphonate	58	mean 61±6	1	Aortic calcification, PWV
1 year	N-containing bisphosphonate	100	74 ± 8	1	CV events
Untill end of study or death	N-containing bisphosphonates	57	mean 63±10	3	All-cause Mortality, Cardiovascular Mortality
2	Non-N-containing bisphosphonate	0	median 53 (range 34-73)	2	All-cause Mortality
4	N-containing bisphosphonates	0	median 40-49	10	All-cause Mortality
2 years	non-N-containing bisphosphonate	49	median 65-74	2	All-cause Mortality

Supplemental table 2 elaborate study characteristics (*continued*)

Author/ Year/ Country	N treated	N control	Population	(Additional) treatment in intervention group	Treatment in control group
Lyles / 2007 / Europe, North and South America	1065	1062	Patients with a recent hip fracture	Zoledronic acid iv 5mg once yearly	Placebo
Mason / 2007 / UK, New Zealand	254	254	Prostate cancer without bone meta	Clodronate 2080 mg	Placebo + standard bisphosphonate
McClung / 2006 / USA	46	46	Post menopausal osteoporosis	Alendronate 70 mg/ week	Placebo
Meulenbeld / 2012 / The Netherlands / Norway	283	286	Prostate cancer with bone metastasis	Risedronate 30 mg	No bisphosphonate + docetaxel/prednisone
Orwoll / 2000 / USA, Canada, Europe	146	95	Osteoporosis	Alendronate 10 mg /day	Placebo + Daily calcium 500 mg and 400 IU vit D
Orwoll / 2010 / USA	87	48	Osteoporosis	Ibandrondate 150 mg/month	Placebo + Daily 1000 mg calcium and 400 IU vit D
Palomba / 2008 / Italy	45	45	Post menopausal osteoporosis	Risedronate 35 mg/ week	Placebo +1500 mg calcium and 800 IU vit D
Pandya / 2010 / USA	98	52	Non-small cell long cancer	Zoledronic acid 4 mg	No bisphosphonate + docetaxel/carboplatin
Paterson / 2012 / USA, Canada	1661	1662	Breast cancer without bone metastasis	Clodronate 1600 mg	Placebo + standard bisphosphonate
Pit / 1998 / UK	26	23	Osteoporosis due to corticosteroid treatment	Cyclical etidronate 400 mg per day for 2 weeks, 10 weeks off	Placebo + in 10 weeks off 500 mg calcium
Powles / 2006 / UK, Canada	530	539	Breast cancer without bone metastasis	Clodronate 1600 mg	Placebo + standard therapy
Ravn / 1996 / Denmark	150	30	Post menopausal osteoporosis	Ibandronate 0.25-5 mg/day	Placebo + Daily calcium 1000 mg
Recker / 2004 / Europe, USA	1911	949	Post menopausal osteoporosis	Ibandronate 0.5-1 mg iv every 3 months	Placebo + Daily calcium 500 mg and 400 IU vit D

	Duration of bisphosphonate treatment	Type of bisphosphonate	Men (%)	Mean age/age distribution	Follow-up years	Reported outcome of interest
	Untill end of study or death	N-containing bisphosphonates	24	mean 75±10	3	All-cause Mortality, Cardiovascular Mortality, myocardial infarction, CVA
	5	Non-N-containing bisphosphonate	100	median 70 (IQR 64-74)	15	All-cause Mortality, Cardiovascular Mortality
	1 year	N-containing bisphosphonate	0	63 ± 9	1	CV events
	2	N-containing bisphosphonates	100	median 69 (range 46-89)	2	All-cause Mortality
	2 years	N-containing bisphosphonate	100	63 ± 13	2	CV events
	1 year	N-containing bisphosphonate	100	63 ± 11	1	All-cause Mortality
	3 years	N-containing bisphosphonate	0	52 ± 3	3	CV Mortality
	1	N-containing bisphosphonates	64	median 64 (range 39-83)	1	All-cause Mortality
	3	Non-N-containing bisphosphonate	0	median >50	median 7.6	All-cause Mortality
	2 years	non-N-containing bisphosphonate	39	59 ± 12	2	All-cause Mortality
	2	Non-N-containing bisphosphonate	0	mean 53±11	10.5	All-cause Mortality
	1 years	N-containing bisphosphonate	0	65 ± 5	1	All-cause Mortality, CV Mortality
	3 years	N-containing bisphosphonate	0	67	3	All-cause Mortality, CV events

Supplemental table 2 elaborate study characteristics (*continued*)

Author/ Year/ Country	N treated	N control	Population	(Additional) treatment in intervention group	Treatment in control group
Reginster / 2000 / Europe, Australia, USA	815	407	Post menopausal osteoporosis	Risedronate 2.5-5mg/ day	Placebo + Daily 1000 mg calcium and 500 IU vit D
Saarto / 2001 / Sweden, Finland	149	150	Breast cancer without bone metastasis	Clodronate 1600 mg	Placebo + standard therapy
Smerud / 2012 / Norway	66	63	Renal transplantation	Ibandronate 3 mg iv/3 months	Placebo + calcium carbonate 1260 mg and calcitriol 0.25 mcg
Stoch / 2009 / USA	114	59	Rheumatid disorders	Alendronate 70 mg/ week	Placebo + Daily 1000 mg calcium and 400 IU vit D
Tee / 2012 / Singapore	22	22	Osteoporosis due to corticosteroid treatment	Alendronate 10 mg/ day	Placebo + calcium and vit D
Theriault / 1999 / North America, Australia, New Zealand	182	189	Breast cancer with bone metastasis	Pamidronate 90 mg iv every 4 weeks	Placebo + hormonal therapy
Toussaint / 2010 / Australia	25	25	Chronic kidney disease stage 3 and 4	Alendronate 70 mg	Placebo
Tripathy / 2003 / USA, Australia,New Zealand, East Europe and South Africa	292	143	Breast cancer with bone metastasis	Ibandronate 20-50 mg/day	Placebo
Van Holten / 1993 / The Netherlands	81	80	Breast cancer with bone metastasis	Pamidronate 600 mg 1dd, later 300 mg	No bisphosphonate + standard therapy
Von Minckwitz / 2013 / Germany	2015	1008	Breast cancer without bone metastasis (node positive)	Ibandronate 50 mg	No bisphosphonate + standard therapy

Duration of bisphosphonate treatment	Type of bisphosphonate	Men (%)	Mean age/age distribution	Follow-up years	Reported outcome of interest
2 years	N-containing bisphosphonate	0	71 ± 7	2	CV events
3	Non-N-containing bisphosphonate	0	mean 52	5	All-cause Mortality
1 year	N-containing bisphosphonate	77	51 ± 14	1	All-cause Mortality
1 year	N-containing bisphosphonate	42	53 ± 14	1	CV Mortality
1 year	N-containing bisphosphonate	57	59 ± 16	1	All-cause Mortality, CV events, myocardial infarction
Untill end of study or death	N-containing bisphosphonates	0	mean 61±12	median 3	All-cause Mortality
1,5	N-containing bisphosphonates	66	mean 63±11	1.5	All-cause Mortality, Aortic calcification, PWV
1.85 years	N-containing bisphosphonate	0	median: 57 (range 30-93)	1.85	All-cause Mortality
Untill end of study or death	N-containing bisphosphonates	0	mean 61	up to 5.5	All-cause Mortality
2	N-containing bisphosphonates	0	median 49 (range 20-72)	3,25	All-cause Mortality, Cardiovascular adverse events

Supplemental table 3 risk of bias per study quantified using the Cochrane risk of bias assessment scale

Randomized controlled trials	Randomization	Allocation concealmen	Similarity of groups	Blinding	Transparency	Completeness	Intention to treat analysis	Risk of bias score	Risk of bias
Adachi 1997	unclear	unclear	yes	yes	yes	yes	yes	5	moderate
Adami 2004	no	no	yes	yes	yes	yes	yes	5	moderate
Attal 2006	yes	unclear	yes	no	yes	yes	yes	5	moderate
Atula 2003	yes	yes	yes	yes	yes	yes	yes	7	low
Banys 2013	yes	yes	unclear	no	yes	yes	yes	5	moderate
Belch 1991	yes	yes	unclear	yes	yes	yes	yes	6	low
Berenson 1998	yes	yes	yes	yes	yes	yes	yes	7	low
Black 2007	yes	yes	yes	yes	yes	yes	yes	7	low
Boonen 2009	yes	yes	yes	yes	yes	yes	yes	7	low
Brincker 1998	yes	yes	yes	yes	yes	no	yes	6	low
Campbell 2004	yes	yes	yes	no	yes	no	yes	5	moderate
Cecilia 2009	unclear	unclear	yes	no	yes	yes	yes	4	high
Chailurkit 2003	unclear	unclear	yes	yes	no	yes	no	3	high
Chesnut 2004	yes	yes	yes	yes	no	yes	yes	6	low
Chevrel 2006	yes	yes	yes	yes	yes	yes	yes	7	low
Coleman 2014	yes	unclear	yes	yes	yes	yes	yes	6	low
Conte 1996	yes	yes	yes	no	yes	yes	yes	6	low
Cummings 1998	yes	yes	yes	yes	yes	yes	yes	7	low
Dalbeth 2014	yes	yes	yes	yes	yes	yes	yes	7	low
Dearnaley 2003	yes	yes	yes	yes	yes	yes	yes	7	low
Denham 2014	yes	yes	yes	no	no	no	yes	4	high
Diel 2008	unclear	yes	yes	no	yes	yes	unclear	4	high
Eastell 2011	yes	yes	yes	yes	yes	yes	yes	7	low
Eggelmeijer 1996	no	no	yes	yes	yes	yes	yes	5	moderate
Ernst 2003	unclear	yes	yes	yes	yes	yes	yes	6	low
Figg 2005	yes	yes	yes	no	yes	yes	yes	6	low
Gnant 2015	yes	yes	yes	no	yes	yes	yes	6	low
Greenspan 2013	yes	yes	yes	yes	yes	yes	yes	7	low
Hines 2009	yes	yes	yes	yes	yes	yes	yes	7	low
Hortobagyi 1998	yes	yes	yes	yes	yes	yes	yes	7	low
Hosking 1998	yes	yes	yes	yes	yes	yes	yes	7	low
Kanis 1996	yes	yes	yes	yes	yes	no	unclear	5	moderate
Kawahara 2013	yes	yes	yes	yes	yes	yes	yes	7	low
Klotz 2013	yes	yes	yes	yes	yes	yes	yes	7	low
Kraj 2004	yes	unclear	yes	no	yes	yes	yes	5	moderate
Kristensen 1999	yes	unclear	yes	no	yes	yes	yes	5	moderate
Kristensen 2008	yes	yes	yes	yes	yes	yes	yes	7	low
Lahtinen 1992	unclear	unclear	no	yes	yes	yes	yes	4	high
Lyles 2007	yes	yes	yes	yes	yes	yes	yes	7	low

Supplemental table 3 risk of bias per study quantified using the Cochrane risk of bias assessment scale (*continued*)

Randomized controlled trials	Randomization	Allocation concealmen	Similarity of groups	Blinding	Transparency	Completeness	Intention to treat analysis	Risk of bias score	Risk of bias
Mason 2007	yes	yes	yes	yes	yes	no	yes	6	low
McClung 2006	unclear	unclear	yes	no	yes	yes	yes	4	high
Meulenbeld 2012	yes	yes	yes	no	yes	yes	yes	6	low
Orwoll 2000	unclear	unclear	yes	yes	yes	yes	yes	5	moderate
Orwoll 2010	unclear	unclear	yes	yes	yes	yes	yes	5	moderate
Palomba 2008	yes	yes	yes	yes	yes	yes	yes	7	low
Pandya 2010	yes	yes	no	no	yes	yes	yes	5	moderate
Paterson 2012	yes	yes	yes	yes	yes	yes	yes	7	low
Pit 1998	unclear	unclear	yes	yes	yes	yes	yes	5	moderate
Powles 2006	yes	yes	yes	yes	yes	yes	yes	7	low
von Minckwitz 2013	yes	yes	yes	no	yes	yes	yes	6	low
Ravn 1996	unclear	unclear	yes	yes	yes	yes	yes	5	moderate
Recker 2004	yes	yes	yes	yes	yes	yes	yes	7	low
Reginster 2000	unclear	unclear	yes	yes	yes	yes	yes	5	moderate
Saarto 2001	yes	yes	yes	no	yes	yes	yes	6	low
Smerud 2012	yes	yes	yes	yes	yes	yes	yes	7	low
Stoch 2009	yes	yes	yes	yes	yes	yes	yes	7	low
Tee 2012	unclear	unclear	no	yes	yes	yes	yes	4	high
Theriault 1999	yes	yes	yes	yes	yes	yes	yes	7	low
Toussaint 2010	yes	yes	no	yes	yes	yes	yes	6	low
Tripathy 2003	yes	yes	yes	yes	yes	yes	yes	7	low
Van Holten 1993	yes	yes	yes	no	no	yes	yes	5	moderate

Randomization: Yes: random numbers, etc. No: eg. Patient number, day of week, etc. Unclear: method not stated

Allocation concealment: Yes: central. No: alternate. Unclear: not stated

Similarity of groups: were the participant characteristics at baseline similar in both groups regarding the most important prognostic factors? Yes, No, Unclear: no baseline characteristics

Blinding: was the treatment allocation masked?

Transparency: were withdrawals/ drop-outs / patients lost to follow up stated for each group?

Completeness: if transparent, drop-out rate <15%

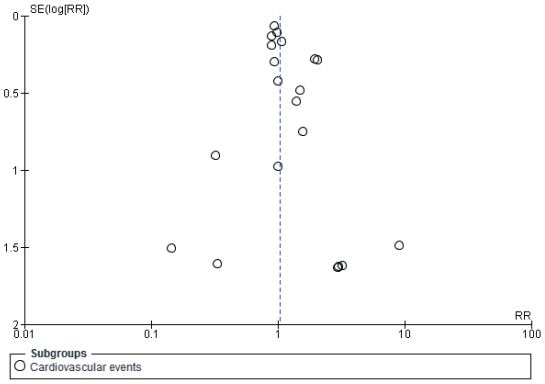
Intention to treat analysis: did the analysis include an ITT analysis and were there less than 10% of patients excluded in each group? Unclear: not stated

Risk of bias score: Higher scores indicate lower risks of bias. Sum of the ROB items per study where yes=1 point, no or unclear=0 points. Scores could vary between 0 (high risk of bias) and 7 (low risk of bias). Risk of bias score 0 untill 4 indicates a high risk of bias, scores of 5 indicate moderate risk of bias, scores higher than 5 indicate low risk of bias

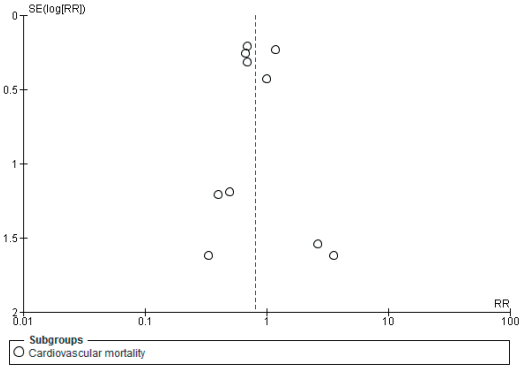
Supplemental table 4 Definitions of cardiovascular events and cardiovascular mortality in included studies

Study	Definition cardiovascular events used in the included study
Adami 2004	Adverse events cardiovascular system
Atula 2003	Adverse events: cardiovascular disorders general
Black 2007	Stroke + myocardial infarction + vascular death
Boonen 2009	Myocardial infarction
Chevrel 2006	Adverse events cardiovascular system
Dalbeth 2014	Cardiovascular adverse events
Dearnaley 2003	Cardiovascular reasons for stopping trial medication
Eastell 2011	Adverse events vascular disorders
Eggelmeijer 1996	Myocardial infarction
Ernst 2003	Cardiovascular serious adverse events
Greenspan 2012	Myocardial infarction
Hosking 1998	Adverse events cardiovascular system
Klotz 2013	Cardiac adverse events
Lyles 2007	Stroke + myocardial infarction + vascular death
McClung 2006	Adverse events cardiac and vascular disorders
Orwoll 2000	Adverse events cardiovascular system
Recker 2004	Adverse events cardiovascular system
Reginster 2000	Cardiovascular adverse events
Tee 2012	Myocardial infarction
Von Minckwitz	Cardiac and vascular adverse events
Black 2007	Death from cardiovascular causes
Chailurkit 2003	Death from myocardial infarction
Dearnaley 2003	Non-prostate cancer death
Denham 2014	Death from cardiac disease + death from cerebrovascular disease
Eastell 2011	Death from vascular disorders
Kraj 2004	Death from cardiac disease and cerebrovascular disease
Lyles 2007	Death from cardiovascular causes
Mason 2007	Non-prostate cancer death
Palomba 2008	Death from myocardial infarction
Ravn 1996	Death from myocardial infarction
Stoch 2009	Cardiac arrests

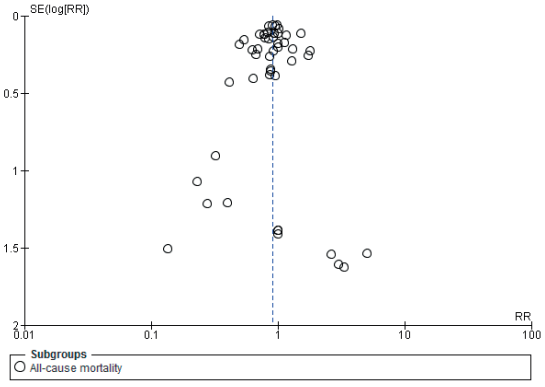
Cardiovascular events



Cardiovascular mortality



All-cause mortality



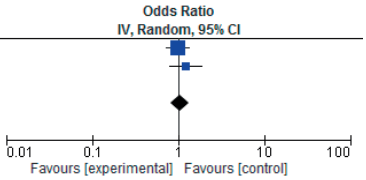
Supplemental figure 1 Funnel plots for cardiovascular events, cardiovascular mortality and all-cause mortality included studies

Supplemental figure 2 Forrest plot of effects of bisphosphonates on stroke and myocardial infarction

Stroke

Study or Subgroup	Bisphosphonates Events	Total	Control Events	Total	Weight	Odds Ratio IV, Random, 95% CI
Black 2007	87	3862	88	3852	68.1%	0.99 [0.73, 1.33]
Lyles 2007	46	1054	38	1057	31.9%	1.22 [0.79, 1.90]
Total (95% CI)		**4916**		**4909**	**100.0%**	**1.06 [0.82, 1.35]**
Total events	133		126			

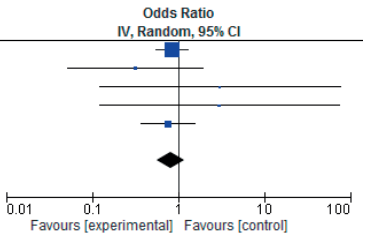
Heterogeneity: Tau² = 0.00; Chi² = 0.64, df = 1 (P = 0.42); I² = 0%
Test for overall effect: Z = 0.43 (P = 0.67)



Myocardial infarction

Study or Subgroup	Bisphosphonates Events	Total	Control Events	Total	Weight	Odds Ratio IV, Random, 95% CI
Black 2007	38	3862	45	3852	68.9%	0.84 [0.54, 1.30]
Boonen 2009	2	191	3	93	4.0%	0.32 [0.05, 1.93]
Eggelmeijer 1996	1	54	0	54	1.3%	3.06 [0.12, 76.70]
Greenspan 2013	1	94	0	93	1.3%	3.00 [0.12, 74.59]
Lyles 2007	13	1054	17	1057	24.6%	0.76 [0.37, 1.58]
Total (95% CI)		**5255**		**5149**	**100.0%**	**0.82 [0.57, 1.17]**
Total events	55		65			

Heterogeneity: Tau² = 0.00; Chi² = 2.37, df = 4 (P = 0.67); I² = 0%
Test for overall effect: Z = 1.11 (P = 0.27)



CHAPTER 12

The effect of etidronate on ectopic mineralization in pseudoxanthoma elasticum – a randomised, double-blind, placebo-controlled trial

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Abstract

Background - In pseudoxanthoma elasticum (PXE) low pyrophosphate levels may cause ectopic mineralization leading to skin changes, visual impairment, and peripheral arterial disease. We hypothesized that etidronate, a pyrophosphate analogue, might reduce ectopic mineralization in PXE.

Methods – In the TEMP trial (Dutch trial register number NTR5180) Aadults with PXE and leg arterial calcifications (n=74) were randomly assigned to etidronate or placebo (cyclical 20 mg/kg for 2 weeks every 12 weeks). The primary outcome was ectopic mineralization, quantified with ^{18}fluoride positron emission tomography scans as femoral arterial wall target-to-background ratios ($TBR_{femoral}$). Secondary outcomes were CT arterial calcification mass and ophthalmological changes. Safety outcomes were bone density, serum calcium and phosphate.

Results – During 12 months of follow-up the $TBR_{femoral}$ increased 6% (IQR -12% to 25%) in the etidronate group and 7% (IQR -9% to 32%) in the placebo group (p=0.465). Arterial calcification decreased 4% (IQR -11% to 7%) in the etidronate group and increased 8% (IQR -1% to 20%) in the placebo group (p=0.001). Etidronate treatment was associated with significantly fewer subretinal neovascularization events (1 versus 9, p=0.007). Bone density decreased with 4% (12%) in the etidronate and 6% (9%) in the placebo group (p=0.374). Hypocalcaemia (<2.20 mmol/L) occurred in three versus one patient (8.1% vs, 2.7%, p=0.304). Eighteen patients (48.6%) treated with etidronate, compared to zero patients treated with placebo (p-value<0.001) experienced hyperphosphatemia (>1.5 mmol/l) and recovered spontaneously.

Conclusion – In PXE patients, etidronate reduced arterial calcification and subretinal neovascularization events, while not lowering femoral ^{18}F-NaF PET activity, compared to placebo without important safety issues.

Introduction

Pseudoxanthoma elasticum (PXE, OMIM #264800) is an autosomal recessive systemic calcification disorder. PXE is characterized by skin involvement (e.g. yellowish papules or plaques), eye involvement (e.g. angioid streaks) and vascular involvement (arterial calcification) and has a considerable morbidity, including severe visual impairment and blindness, peripheral arterial disease, ischemic stroke, and vascular dementia.[1,2] The prevalence is approximately 1:25.000-100.000.[2] To prevent progression of visual impairment caused by choroidal neovascularization in patients with PXE, injections with anti-vascular endothelial growth factor (VEGF) are used.[3] However, there is no specific and preventive treatment available for PXE patients.

PXE is caused by mutations in the *ABCC6*-gene and associated with ectopic mineralization of elastic fibers in the skin, the Bruch's membrane beneath the retina and the medial layer of arteries.[4] Recently, major steps have been made in deciphering the etiology of PXE. *ABCC6*-mutations have been shown to result in inefficient mediators of ATP secretion in the liver causing low levels of inorganic pyrophosphate (PPi).[5] PPi is, as part of a network of inhibiting (and promoting) pathways, a strong inhibitor of ectopic mineralization.[6-8] The decreased levels of PPi in PXE may therefore cause the ectopic mineralization in PXE.[9]

Bisphosphonates, well-established drugs for the treatment of osteoporosis and bone metastases, are stable PPi analogues and could thus stimulate the inhibitory effects on ectopic mineralization.[10] In fact, bisphosphonates have been shown to reduce soft tissue calcifications in rats even before their effect on bone resorption was known.[11] Of the currently available bisphosphonates etidronate may have the largest potential to delay ectopic mineralization given its predominant inhibition of calcium precipitation and hydroxyapatite binding. This is different from newer bisphosphonates, such as alendronate, which predominantly inhibit osteoclasts.[12,13]

Several non-randomised and uncontrolled reports describe beneficial effects of etidronate in patients with rare diseases with ectopic mineralization due to a deficiency in the PPi homeostasis. In patients with basal ganglia calcifications or primary brain calcifications (OMIM#213600) treatment with etidronate alleviates neurological symptoms.[12,14] In generalized arterial calcification of infancy (GACI, OMIM #208000) etidronate treatment reduces arterial calcification and is associated with improved survival.[15,16] GACI can be seen as an aggressive form of PXE with a considerable overlap in genotype and phenotype.[17,18] Treatment with

etidronate in PXE mouse models results in prevention of ectopic mineralization and in alterations in bone micro-architecture.[19,20] The effectiveness of etidronate remains to be established in PXE patients in a randomised, placebo-controlled trial.

We thus hypothesized that synthetic PPi supplementation with etidronate treatment could reduce ectopic mineralization in PXE patients. To be able to investigate this hypothesis in a randomised, placebo-controlled trial we used femoral arterial wall ^{18}fluoride sodium positron emission tomography (^{18}F-NaF PET) activity and computerized tomography (CT) based femoral calcium scores as markers of ectopic mineralization.[21-25] Imaging with ^{18}F-NaF PET may be more sensitive to changes in ectopic mineralization compared to traditional CT, as it is thought to be able to visualize the active and ongoing calcification process and discriminate between the active and the more indolent calcifications.[22,26]

Here, we report the results of the Treatment of Ectopic Mineralization in Pseudoxanthoma elasticum (TEMP) trial in which we set out to investigate the effectiveness and safety of one year treatment with etidronate (cyclical 20 mg/kg for two weeks every 12 weeks) on ectopic mineralization among participants with PXE.

Methods

Trial design and study population

The TEMP trial was a single-center, randomized, double-blind, placebo-controlled trial conducted in the PXE expertise center at the University Medical Center Utrecht (UMCU), the Netherlands. Participants eligible for participation had a confirmed clinical diagnosis of PXE, were 18 years or older and had evidence of arterial calcification on a CT scan of the legs that was acquired in all patients during the first visit in our center. PXE was diagnosed if two of the following were present: skin involvement (e.g. yellowish papules/plaques), eye involvement (e.g. angioid streaks) and genetically confirmation (biallelic *ABCC6*-mutations).[1] Exclusion criteria were severe renal impairment, known abnormality of the esophagus, known sensitivity to etidronate, use of bisphosphonates during the last five years, osteomalacia, chronic diarrhea, pregnancy, claustrophobia, hypocalcaemia (calcium <2.20 mmol/L), and vitamin D deficiency (25-OH Vit D < 35 nmol/l). The TEMP trial was approved by the Institutional Review Board of the UMCU (IRB number 15/522). The trial is registered with Dutch trial register number NTR5180. All participants provided written informed consent.

Randomisation and intervention

Eligible participants were randomised in a 1:1 ratio to either a cyclical regime of 2 weeks 20 mg/kg etidronate with oral administration of 400 mg capsules every 12 weeks or to an identical treatment regime and capsules without the active pharmacological substance (i.e. placebo). Etidronate (OSTOPOR® Hard capsules, 400mg/cap) and placebo capsules were provided by UNI-PHARMA KLEON TSETIS PHARMACEUTICAL LABORATORIES SA (Greece). Randomisation with random permuted blocks for gender was performed using a random number generator at the pharmacy department, where the randomisation list was stored during the entire duration of the trial.

Procedures

Full body ^{18}F-NaF PET/CT scans were performed at baseline and after 12 months of follow-up on a Siemens Biograph 40 scanner (Siemens Healthcare, Erlangen, Germany). Images were reconstructed according to the European Association of Nuclear Medicine (EANM) Research Ltd (EARL) recommendations.[27] 90 minutes before imaging an intravenous injection of 2.0 MBq per kilogram ^{18}F-NaF (maximum dosage of 200 MBq) was administered. A conventional full-body CT scan was performed after 6 months of follow-up.

Measurements of carotid intima-media thickness (IMT) and carotid-femoral pulse wave velocity (PWV) were performed at baseline and after 12 months follow-up. IMT was measured with ultrasound (Esaote, Florence, Italy) in the left and right carotid artery. PWV was measured using applanation tonometry with a micromanometer (Millar Instruments Inc. Houston, USA) in combination with Sphygmocor software (Atcore Medical Pty. Ltd., Sydney, Australia).

Ophthalmological control took place at baseline and after three, six, nine and 12 months. The frequency of subretinal neovascularization events was monitored with optical coherence tomography scans and fundus photography during all visits. The frequency of anti-VEGF injections in the year before the baseline visit and during the trial was obtained. If needed medical information was retrieved from other hospitals. Best corrected visual acuity (BCVA) was measured at baseline and after 12 months follow-up using Early Treatment Diabetic Retinopathy Study (ETDRS) letter charts (supplemental information).[28]

Quality of life was assessed at baseline and after 12 months follow-up using the validated Dutch version of the 36-Item short form survey (SF-36) questionnaire.[29]

Laboratory measurements for serum calcium and phosphate were performed at baseline, and after three, six, nine and 12 months of follow-up. Estimated glomerular filtration rate (eGFR calculated using the CKD-EPI formula), aspartate transaminase (AST), and alanine transaminase (ALT) were measured at baseline, after six months, and after 12 months of follow-up.

Outcomes

The primary outcome was change in femoral ^{18}F-NaF PET activity. The femoral artery target-to-background ratio ($TBR_{femoral}$) as a measure of femoral ^{18}F-NaF PET activity was determined blinded for treatment status using previously described methods (supplemental information).[24,30]

Change in arterial calcification on conventional CT was a secondary outcome. Calcifications in both the left and right femoral artery were quantified blinded for treatment status using the femoral artery calcification mass (supplemental information).

Changes in IMT and PWV between month 12 and baseline were additional secondary outcomes that were assessed blinded for treatment status.

Ophthalmological changes were a secondary outcome. These changes were monitored blinded for treatment status by the occurrence of subretinal neovascularization events, the frequency of anti-VEGF administration and BCVA. A subretinal neovascularization event was defined as any of the following that provided an indication to start or intensify anti-VEGF injections to prevent (further) visual impairment: (1) retinal bleeding suspected to be caused by subretinal neovascularisation (if necessary confirmed by fluorescein angiography), (2) a significant increase in subretinal or intraretinal fluid, and/or (3) growth of a subretinal neovascular complex. During the TEMP trial, these events were scored by at least two investigators, including at least one board certified ophthalmologist, who were blinded for treatment status.

Transformed SF-36 scores between 0 and 100 on the nine quality of life domains were used: physical function, physical role functioning, pain, general health, mental health, social function, social role functioning, vitality, and health changes.

Safety monitoring

Safety assessments were changes in bone density, as measured with CT scans at baseline, month 6 and month 12 (supplemental information), changes in serum

calcium and phosphate, the occurrence of hypocalcaemia or hyperphosphatemia, and changes in eGFR, AST, and ALT, and recording of adverse events (supplemental information).

A DSMB, consisting of three independent members, monitored safety data (serious adverse events, bone mineral density, calcium levels, ophthalmological events, frequency of anti-VEGF injections) and performed interim analyses after the first 20 participants reached three and six months follow-up.

Statistical analysis

Under the assumption of a mean $TBR_{femoral}$ of 1.96 and a standard deviation of 0.58 and anticipating six drop-outs or loss to follow-up, 74 participants were required to detect a 20% change in $TBR_{femoral}$ with 80% power and a two-sided α of 5%.[24]

Descriptive data were presented as normally distributed continuous (mean (standard deviation)), non-normally distributed continuous (median, interquartile range (IQR)) or as categorical variables (n, %). Intention-to-treat analyses were performed comparing the intervention and placebo group using independent sample t-tests, Mann-Whitney U tests or Chi-squared tests when appropriate. In our primary analysis we compared the percentage difference in femoral TBR between the etidronate and the placebo group using a Mann-Whitney U test. All analyses were performed blinded, before definitive breaking of the blinding, hereby comparing treatment group A versus B. A p value of less than 0•05 was regarded as statistically significant. Analyses were performed with R version 3.3.2.

Role of the funding source

The funders of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report.

Results

Trial population

A total of 77 patients were screened between July 2015 and June 2016. Three patients were ineligible and the remaining 74 participants were enrolled between the 7th of October 2015 and the 8th of June 2016. One participant in the placebo group declined further participation at month three having experienced a severe uveitis following an anti-VEGF injection. One participant in the etidronate group discontinued treatment because of a hypersensitivity skin reaction at month six

but remained in the study (figure 1). Baseline characteristics are presented in table 1.

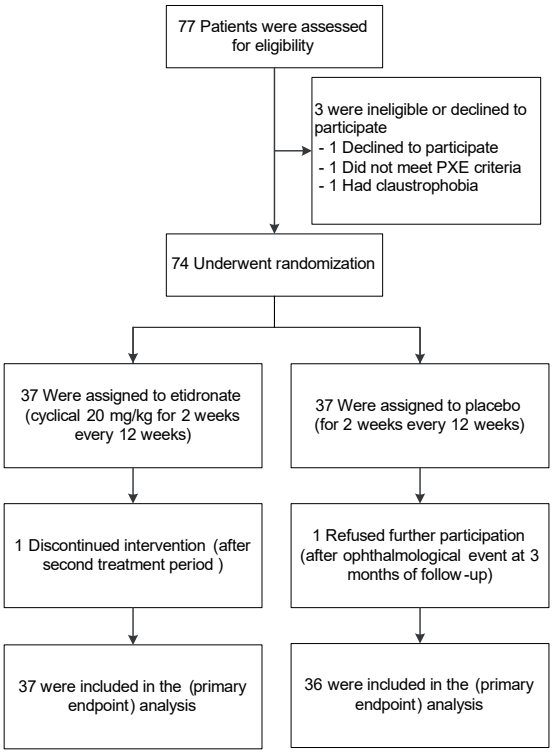

Figure 1 Flowchart eligibility, randomisation, and follow-up

Efficacy

The $TBR_{femoral}$ increased with 6% (IQR -12% to 25%) in the etidronate group and with 7% (-9% to 32%) in the placebo group in the 12 months of follow-up (p=0.465, table 2, figure 2).

During the 12 months of follow-up arterial calcification decreased 4% (IQR -11% to 7%) in the etidronate group and increased 8% (IQR -1% to 20%) in the placebo group (p=0.001, figure 2).

Carotid IMT increased 0.00 mm (0.09 mm) in the etidronate group and 0.02 mm (0.09 mm) in the placebo group (p=0.523). The PWV increased with 0.6 m/s (2.2 m/s) in the etidronate group and 0.6 m/s (2.1 m/s) in the placebo group (p=0.990).

Table 1 Patient characteristics of the study population at baseline

	Etidronate n=37	Placebo n=37
Clinical characteristics		
Age (years)	57 (9)	57 (8)
Gender (male)	19 (51%)	19 (51%)
Diabetes mellitus	3 (8%)	1 (3%)
Systolic blood pressure (mmHg)	142 (20)	138 (18)
Diastolic blood pressure (mmHg)	81 (9)	80 (11)
Body mass index (kg/m^2)	26.7 (4.6)	25.5 (3.5)
Non-HDL-cholesterol (mmol/L)	3.6 (1.1)	3.8 (1.2)
Femoral ^{18}F-NaF PET activity		
TBR	2.80 (1.04)	2.59 (0.90)
Arterial calcification		
Femoral calcium mass (miligram)	405 (35 to 1797)	1035 (282 to 1563)
Arterial wall characteristics		
Carotid IMT (mm)	0.72 (0.11)	0.79 (0.14)
Carotid-femoral PWV (m/s)	11.0 (3.1)	9.7 (1.9)
Ophthalmological status		
Total number of Anti-VEGF injections used last year	0 (0 to 8)	0 (0 to 9)
Best corrected visual acuity worse eye (ETDRS letters)	26 (5 to 79)	26 (10 to 79)
Best corrected visual acuity best eye (ETDRS letters)	78 (35 to 86)	72 (37 to 83)
Laboratory measurements		
25-OH vitamin D (nmol/L)	93 (45)	70 (45)
Calcium (mmol/L)	2.40 (0.09)	2.37 (0.07)
Phosphate (mmol/L)	1.05 (0.11)	1.05 (0.14)
eGFR (ml/min per 1.73 m^2)	90 (86 to 90)	90 (83 to 90)
AST (U/L)	29 (6)	28 (6)
ALT (U/L)	27 (13)	23 (10)
Bone density		
CT bone density (HU)	139 (50)	128 (34)

All data are displayed as mean (SD), median (interquartile range) or n (%); HDL: High-density lipoprotein; TBR: target-to-background-ratio; IMT: intima-media thickness; PWV: pulse wave velocity; VEGF: vascular endothelial growth factor; eGFR: estimated glomerular filtration rate using the CKD-EPI formula; AST: Aspartate transaminase; ALT: Alanine transaminase; HU: Houndsfield Units

During follow-up, one patient (2.7%) in the etidronate group had a subretinal neovascularization event compared to nine patients (24.3%) in the placebo group (p=0.007). The median number of anti-VEGF injections administered per year neither change in the etidronate group (0 (IQR -2 to 0), nor in the placebo group (0 (IQR -1 to 1) (p=0.137). The BCVA in LogMAR number of letters of the worse eye decreased with 1 (5) letters in the etidronate group and with 1 (7) letters in the placebo group (p=0.900). The BCVA of the best eye decreased with 2 (4) letters in the etidronate group and with 3 (7) letters in the placebo group (p=0.460).

No significant differences between the etidronate and placebo group were found in quality of life domains of the SF-36 questionnaire. (Supplemental table 1)

Table 2 Primary and secondary outcomes

	Etidronate	Placebo	p value
Femoral arterial wall ^{18}F-NaF PET activity			
Change in Target-to-background ratio	6% (-12% to 25%)	7% (-9% to 32%)	0.465
CT Arterial calcification			
Change in femoral calcification mass (month 12 vs. baseline)	-4% (-10% to 7%)	8% (-1% to 20%)	0.001
Arterial wall characteristics			
Change in carotid IMT	0.00 mm (0.09 mm)	0.02 mm (0.09 mm)	0.530
Change in carotid-femoral PWV	0.6 m/s (2.2 m/s)	0.6 m/s (2.1 m/s)	0.990
Ophthalmological status			
Subretinal neovascularization events during follow-up	1 (2.7%)	9 (24.3%)	0.007
Change in frequency of VEGF-inhibitors	0 (-2 to 0)	0 (-1 to 1)	0.137
Change in best corrected visual acuity worse eye (ETDRS letters)	-1 (5)	-1 (7)	0.900
Change in best corrected visual acuity best eye (ETDRS letters)	-2 (4)	-3 (7)	0.460

All data are displayed as mean (SD), median (interquartile range) or n (%); Treatment groups were compared using independent sample t-tests and Mann-Whitney U tests when appropriate. Change is displayed as % for femoral arterial wall ^{18}F-NaF PET activity and femoral calcium mass. ; IMT: intima-media thickness; PWV: pulse wave velocity; VEGF: vascular endothelial growth factor

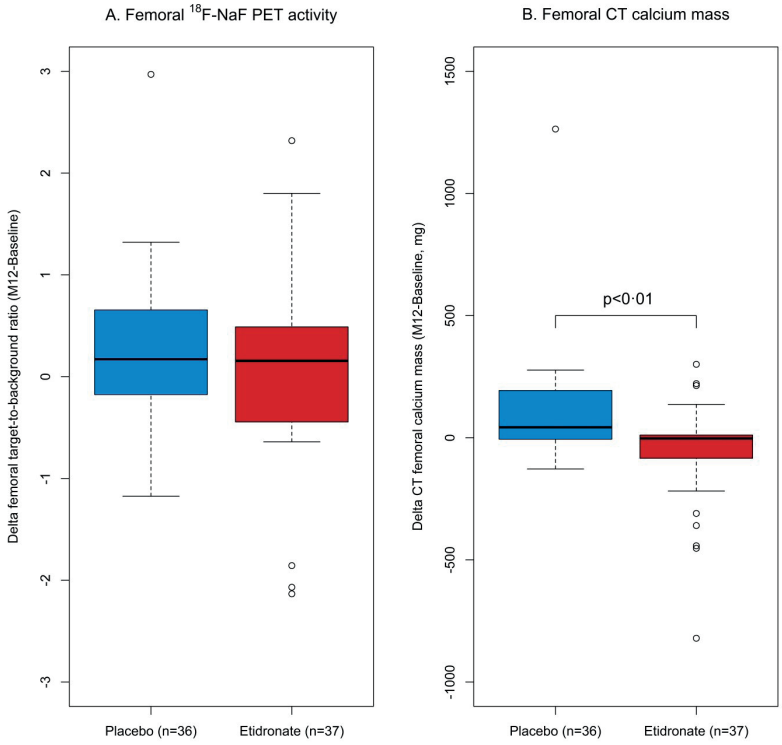

Figure 1 Effects on femoral mineralization

A: Differences in femoral target-to-background ratio ($TBR_{femoral}$) stratified for treatment group between baseline and 12 months of follow-up. B: Differences in femoral calcium mass stratified for treatment group between baseline and 12 months of follow-up.
The bottom and top of the boxes represent the first and third quartile. The end of the whiskers represent the lowest and highest value still within 1.5 times the inter-quartile range.

Safety

From baseline to 12 months of follow-up the bone mineral density changed with -4% (12%) (-7 HU (16 HU)) in the etidronate group compared to -6% (9%) (-9 HU (16 HU)) in the placebo group (p=0.374, table 3).

No significant differences were found between the etidronate and placebo group when comparing baseline to values after 12 months of follow-up of calcium, phosphate, eGFR, AST and ALT (table 3). During follow-up hypocalcaemia (calcium <2.20 mmol/L) occurred in three patients (8.1%) in the etidronate group and in one patient (2.7%) in the placebo group (p=0.304). Hyperphosphatemia (phosphate >1.5 mmol/L) occurred in 18 patients (48.6%) in the etidronate group, compared to zero patients (0%) in the placebo group (p<0.001). All cases were monitored and recovered without any medical intervention.

Table 3 Safety measurements and adverse events

	Etidronate	Placebo	p-value
Bone mineral density			
%Δ CT bone density	-4% (12%)	-6 (9%)	0.374
Laboratory measurements[a]			
Hypocalcaemia (calcium <2.20 mmol/L)	3 (8.1%)	1 (2.7%)	0.304
Hyperphosphatemia (phosphate >1.5mmol/L)[b]	18 (48.6%)	0	<0.001
Renal impairment (eGFR - 20%)	0	0	1.000
Liver impairment (AST/ALT + 50%)[b]	1 (2.7%)	0	0.304
Δ Calcium (mmol/l)	-0.03 (0.08)	-0.01 (0.07)	0.438
Δ Phosphate (mmol/l)	-0.01 (0.14)	-0.03 (0.14)	0.707
Δ eGFR (ml/min*1.73 m2)	0 (0 to 0)	0 (-1 to 0)	0.864
Δ AST (U/L)	0 (5)	0 (4)	0.841
Δ ALT (U/L)	-1 (9)	2 (8)	0.147
Adverse events			
Gastro-intestinal complaints	2 (5.4%)	2 (5.4%)	1.000
Hypersensitivity dermatological reaction[c]	1 (2.7%)	0	0.304
Posterior vitreous detachment	1 (2.7%)	0	0.304
Suspected uveitis after anti-VEGF injection	0	1 (2.7%)	0.304
Blurry sight after anti-VEGF injection, disappearing within a day	0	1 (2.7%)	0.304
Drop in visual acuity not related to neovascularization	0	1 (2.7%)	0.304
Hospital admission[d]	0	1 (2.7%)	0.304

All data are displayed as mean ± SD, median (interquartile range) or n (%); Treatment groups were compared using independent sample t-tests, Chi-squared tests and Mann-Whitney U tests when appropriate.

Δ Delta: Month 12 minus baseline value; CT bone density in Hounsfield Units; eGFR: estimated glomeral filtration rate estimated using the CKD-EPI formula; AST Aspartate transaminase; ALT Alanine transaminase;

a Calcium and phosphate were measured at baseline, after 3 months, 6 months, 9 months and 12 months of follow-up. Renal function and liver function were measured at baseline, after 6 months and 12 months follow-up.

b All cases were followed-up and recovered without any medical intervention.

c Skin rash arising during second treatment period after which the study medication was discontinued and the rash disappeared. Restart of study medication again resulted in rash after which this participant continued the study off study medication.

d Hospital admission due to (planned) hip operation.

Gastrointestinal complaints (reflux, stomach pain, diarrhoea) presented in two patients (5.4%) in the etidronate and in two patients in the placebo group (5.4%) (p=1.000). A hypersensitivity dermatological reaction occurred in one patient (2.7%) in the etidronate group compared to zero patients (0%) in the placebo group (p=0.304).

Discussion

The TEMP trial, a randomised double-blind, placebo-controlled trial including 74 PXE participants, showed that etidronate did not lower femoral arterial wall ^{18}F-NaF PET activity compared to placebo after 12 months follow-up. However, etidronate significantly decreased ectopic mineralization as quantified by arterial calcification compared to placebo. Also, etidronate treatment in PXE was associated with significantly lower rates of subretinal neovascularization events (one patient vs. nine patients). There were no important safety issues of etidronate treatment during the year of follow-up. Although in these dosages etidronate is known to increase the risk of bone-related adverse events and hypocalcaemia, no significant differences were observed between the etidronate and placebo group. Eighteen patients in the etidronate group compared to zero patients in the placebo group experienced hyperphosphatemia (>1.5 mmol/L), but all cases recovered without any medical intervention.

The body has several inhibiting pathways for ectopic mineralization including vitamin K-dependent pathways, the Klotho protein, Fetuin-A and inorganic pyrophosphate (PPi).[6] In PXE, *ABCC6*-mutations have been shown to result in low levels of PPi.[5] The decreased levels of PPi may cause the ectopic mineralization in PXE.[9] Increasing the concentration of ectopic mineralization inhibitors such as PPi may result in ectopic mineralization prevention and subsequent inhibition of disease progression.[9,12,14-16,19,20,31-33]

We hypothesized that etidronate could delay ectopic mineralization and subsequent disease progression in PXE patients. The TEMP trial confirmed this hypothesis with an observed decrease in ectopic mineralization, quantified with conventional CT femoral calcium scores in the etidronate group compared to the placebo group. The effects on CT arterial calcification in the TEMP trial are in line with findings from etidronate trials in chronic kidney disease, diabetes mellitus and hypercholesterolemia patients.[34-37]

The observed decrease in subretinal neovascularization events in the etidronate compared to the placebo group further confirmed our hypothesis. This finding is

in line with findings from intervention studies in non-PXE populations, showing preventive effects of bisphosphonates on subretinal neovascularization in age-related macular degeneration and pathological myopia.[38] Etidronate has been found to reduce the expression of several angiogenic factors in human retinal pigment epithelial cells, which likely play a key role in neovascularization.[39] Similar to the vascular effects, these ophthalmological effects of etidronate treatment in PXE may be attributed to inhibition of ectopic mineralization by etidronate. Bruch's membrane mineralization in PXE may impede the transport of oxygen and waste products across Bruch's membrane. Inhibition of this mineralization might thus prevent the growth or recurrence of activity of a choroidal neovascularization[3]

We assumed that ^{18}F-NaF PET activity would visualize the active calcification process and thus would be sensitive to small changes in arterial calcification providing an efficient marker of ectopic mineralization in the TEMP trial.[22] The TEMP trial is the first interventional trial that reports on femoral ^{18}F-NaF PET activity as outcome. The absence of an effect of etidronate treatment on femoral arterial wall ^{18}F-NaF PET activity is remarkably discrepant with the observed effects of etidronate on CT arterial calcification and subretinal neovascularization events. This discrepancy questions our assumption on the value of femoral ^{18}F-NaF PET activity as intermediate outcome in such an interventional trial. Although femoral ^{18}F-NaF PET activity relates to cardiovascular risk scores,[24] regional ^{18}F-NaF PET activity and regional conventional CT arterial calcification scores are not correlated, suggesting that the biological processes measured with ^{18}F-NaF PET scans are not exactly known.[40,41] Recent evidence shows that arterial wall ^{18}F-NaF PET activity co-localize closely and preferentially bind to pathological mineralization, but also that the increased surface area of microcalcifications relative to macrocalcifications resulted in increased tracer uptake and therefore does not necessarily represent active calcification.[41] Hence, our assumptions on the ^{18}F-NaF PET measurements as a reflection of disease activity proved to be incorrect. Another explanation for the discrepancy between effects of etidronate on ^{18}F-NaF activity and CT arterial calcification score may be that etidronate treatment increases dissolution of existing mineral deposits while not lowering new calcium deposition. However, our data provide no evidence for this explanation and potential biological mechanisms are unknown. All in all, taking the effects of etidronate treatment on conventional CT arterial calcification into account, even in the absence of an effect on the primary outcome, the TEMP trial showed that etidronate treatment can delay ectopic mineralization in PXE.

Based upon our findings, all PXE patients should be considered for etidronate treatment. Preferably, all PXE patients treated with etidronate should be carefully monitored in a research setting for long-term efficacy and safety. Being one of the oldest bisphosphonates, etidronate has been on the market for almost 40 years and is easily available at low cost. Given the large impact on quality of life of ophthalmological and vascular involvement in PXE, etidronate treatment may eventually enhance quality of life in PXE patients.[42] Other potential treatment strategies considered in PXE patients, should be weighed against the efficacy and safety profile of etidronate.

The TEMP trial provides proof of the concept that bisphosphonates can reduce arterial calcifications and may reduce subsequent vascular risk. Other populations than PXE patients may benefit from this potential of bisphosphonates. Medial arterial calcification is a prevalent process occurring in renal failure, diabetes mellitus and ageing, which relates to an increased vascular risk.[43,44] Investigations in the effects of bisphosphonates in these populations, associated with a high residual vascular risk, seem warranted.

Strengths of the TEMP trial include the relatively large number of PXE patients and its success to achieve a low drop-out rate. Limitations include the limited treatment and follow-up period. With this limited follow-up time we were unable to show effects of etidronate treatment on other outcomes besides CT arterial calcification and subretinal neovascularization events. Also, no safety information for etidronate treatment in PXE patients over more than one year is available. The lack of generalizability to PXE patients not eligible for the TEMP trial – such as patients without leg arterial calcification or with osteomalacia – is another limitation.

In PXE patients, etidronate treatment did not significantly change femoral arterial wall ^{18}F-NaF PET activity, compared to placebo after 12 months of follow-up. However, etidronate significantly reduced arterial calcification and the number of subretinal neovascularization events compared to placebo. No important safety issues of etidronate treatment occurred during the year of follow-up. Further research in the long-term effects and safety of etidronate seems warranted.

Contributors

WS was principal investigator. GK, PAdJ, HJV, JOvN, RvL, WPM, WS were involved in the trial design. GK was trial coordinator, prepared the study protocol and drafted the final manuscript. GK, JB, JOvN, SR, RvL, and WS contributed

to patient data collection. MGL, RHJAS, and GL were involved in the methodology of (PET) CT measurements. GK, PAdJ, JB, SJL, JdV, and AMdH performed measurements on (PET) CT images. All authors reviewed the final paper.

Declaration of interests

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Appendix

Measurement of best corrected visual acuity (BCVA)

Best corrected visual acuity (BCVA) was measured at baseline and after 12 months of follow-up using Early Treatment Diabetic Retinopathy Study (ETDRS) letter charts (supplemental information).[1] All BCVA measurements took place in the same room with the same lightning conditions. First, a subjective refraction was performed using chart "R". Then BCVA was measured unilaterally at a distance of four metres. If the participant was unable to read the largest letters, the measurement was repeated at one metre with an added +0.75 spherical correction. Different versions of the letter charts were used for the right and the left eye. The number of letters correctly read was scored and the corresponding visual acuity expressed in Logarithm of the Minimum Angle of Resolution (LogMAR) for statistical purposes.[2]

Quantification of 18F-NaF PET activity in the femoral artery

The intraclass correlation coefficient for inter-observer reliability of the femoral artery target-to-background ratio ($TBR_{femoral}$) measurement was 0.924 (95% CI 0.778 to 0.980) based upon the full scoring of 10 random scans by three independent investigators. One investigator, blinded for treatment status and clinical information, analyzed the full set of femoral PET/CT images in a random order and using dedicated software (IntelliSpace Portal v8.0., Philips Healthcare, Best, The Netherlands). Throughout the left and right femoral leg artery, starting a centimeter below the bifurcation of the communal femoral artery up till the femur condyles, the maximal standardized uptake value (SUV_{max}) was determined by manually drawing a circular region of interest (ROI) around the femoral artery. The ROI was drawn as large as possible with careful exclusion of ^{18}F-NaF activity originating from adjacent bone tissue. Slice thickness was was 5-mm with a 4-mm interval, and the measurements were performed every other slice. The $SUV_{femoral}$ was calculated by dividing the sum of all SUV_{max} values of the left and right femoral arteries by the number of measurements. Subsequently, the blood-pool ^{18}F-NaF activity was determined by manually drawing three circular ROIs in the superior vena cava on consecutive slices starting at the level of the aortic arch measuring the mean standardized uptake value (SUV_{mean}). The average of these three measurements was used as $SUV_{bloodpool}$. To calculate the primary outcome $TBR_{femoral}$, the $SUV_{femoral}$ was divided by the $SUV_{bloodpool}$.

Quantification of arterial calcification

The intraclass correlation coefficient for inter-observer reliability of the total femoral artery calcium mass measurement was 0.997 (95% CI 0.995 to 0.999) based upon the full scoring of 25 random scans by three independent investigators. One investigator, blinded for treatment status and clinical information, analyzed all femoral CT images in a random order using dedicated software (iX Viewer, ImageSciences Institute, Utrecht, the Netherlands) and applying a threshold of 130 Hounsfield Units for calcium.[3,4] Voxels above this threshold representing femoral artery calcifications were identified by the observer and quantified by a single mouse click on the calcification. Calcifications in both the left and right femoral artery were quantified using the calcification mass, starting a centimeter below the bifurcation of the communal femoral artery up till the femur condyles.

Measurement of bone density

CT scans (at baseline, month 6, and month 12) were used to measure bone density .Bone density was measured in Hounsfield units (HU) by placing a ROI in homogenous areas of trabecular bone in the first lumbar vertebra (L1) using a bone window (window level: 300, window width 1600). [5]

Adverse events

Adverse events were defined as any undesirable experience occurring to a participant during the study, whether or not considered related to treatment with etidronate. All adverse events reported by participants or observed by one of the investigators were reported. Serious adverse events were adverse events that resulted in death or that were life threatening, required hospitalisation, or resulted in significant disability or incapacity.

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Supplemental table 1 Effects on qulity of life

	Etidronate			Placebo			
SF-36 domain	**Baseline score**	**Follow-up score**	**Change**	**Baseline score**	**Follow-up score**	**Change**	**p value**
Physical health							
Physical function	10 (5 to 20)	15 (5 to 25)	0 (-5 to 5)	10 (5 to 30)	13 (5 to 29)	0 (-5 to 5)	0.698
Physical role functioning	100 (50 to 100)	100 (50 to 100)	0 (-19 to 0)	63 (25 to 100)	100 (32 to 100)	0 (0 to 25)	0.231
Pain	29 (22 to 39)	29 (22 to 57)	0 (-10 to 6)	39 (29 to 73)	43 (22 to 90)	0 (-15 to 32)	0.728
General health	50 (38 to 65)	53 (40 to 64)	0 (-10 to 10)	50 (45 to 60)	50 (40 to 55)	0 (-14 to 32)	0.790
Mental health							
Mental health	64 (49 to 76)	68 (40 to 80)	4 (-15 to 19)	72 (62 to 80)	70 (57 to 80)	0 (-11 to 8)	0.419
Social function	38 (38 to 38)	38 (25 to 38)	0 (-13 to 0)	38 (25 to 81)	38 (38 to 75)	0 (-22 to 13)	0.495
Social role functioning	100 (100 to 100)	100 (100 to 100)	0 (0 to 0)	100 (100 to 100)	100 (100 to 100)	0 (0 to 0)	0.555
Vitality	63 (22 to 39)	50 (35 to 55)	-15 (-25 to 6)	65 (45 to 70)	50 (35 to 60)	-10 (-20 to 0)	0.181
Health changes	75 (50 to 75)	75 (75 to 75)	0 (-10 to 10)	75 (75 to 88)	75 (75 to 75)	0 (-19 to 25)	0.496

All data are displayed as median (interquartile range); Mann-Whitney U tests were used for comparisons between the two treatment groups.

CHAPTER 13

General discussion

Over the last years, considerable progress has been made in treatment and prevention of vascular disease.[1] Nevertheless, vascular disease remains the number 1 cause of death.[2-4] Vascular disease and cancer are the main mortality contributors in the developed world and, despite efforts of patients, researchers, and doctors, this will probably remain so given the increase in life expectancy.[4] However, vascular disease not only results in mortality, but - maybe even more important- also in a substantial decrease in years in good quality of life. [2-4] In the prevention of vascular disease, our goal should not be to totally prevent vascular disease, but rather to delay vascular disease, enabling patients to maintain quality of life as long as possible.

Calcification-induced residual vascular risk

Among patients with manifestations of vascular disease, or with vascular risk factors only, the risk of vascular disease is high, even if all modifiable risk factors are at guideline-recommended targets.[5] This so-called residual risk exposes an area of unmet medical need. Effective life-style interventions, such as smoking cessation, diet, and exercise may reduce a large part of this residual risk. However, adherence to lifestyle recommendations is notably poor and even in case of full adherence a residual vascular risk remains that needs pharmaceutical intervention.[6]

The main strategy used to pharmaceutically reduce this residual risk has, since the landmark 4S trial, been cholesterol-lowering treatment.[7] Indeed the effect of cholesterol-lowering treatment on vascular risk reduction is impressive as has been shown in several statin trials, and more recently in trials with propretein covertase subtilisin kexin 9 (PCSK9) inhibitors, a novel, powerful – but expensive- family for cholesterol-lowering.[8,9] Recently, the CANTOS trial showed that anti-inflammatory therapy could reduce vascular risk independent of cholesterol-lowering.[10] After these findings reputable researchers once again stated that cholesterol-lowering and anti-inflammatory treatment represent *the* two sides of the prevention coin.[11] However, this statement is obviously oversimplified as even after adequate cholesterol-lowering and anti-inflammatory treatment, a large residual vascular risks remains.[5,11] Thus, other potential preventative strategies to further reduce the vascular risk and increase quality of life should be evaluated.

Arterial calcification, in particular medial arterial calcification (MAC), may open up a promising target for preventive pharmaceutical treatment on top of other preventative strategies. MAC is thought to induce vascular disease independent of cholesterol and inflammation.[12-18] Interference in the process of arterial calcification should thus be evaluated as a potential preventative treatment strategy

to further reduce vascular risk. This has a particular potential in patients with diabetes mellitus and renal failure, who are at high risk for MAC *and* vascular disease.[12]

However, so far little was known about the clinical consequences of MAC and calcification-induced disease, about the complex interplay between MAC and atherosclerosis in the induction of vascular disease, and about potential therapeutic strategies targeting MAC.[19] Pseudoxanthoma elasticum (PXE), a rare disease (estimated prevalence 1:56,000, **chapter 2**) with a progressive, non-atherosclerotic form of arterial calcification, may provide a model disease to study clinical consequences of MAC and calcification-induced disease and to study effects of therapeutic strategies to prevent calcification-induced disease.[20-26]

Importantly, in PXE patients, vascular and ocular involvement is associated with a considerable morbidity and results in a decreased quality of life.[27-29] A large unmet medical need therefore exists also in PXE patients, for which so far no proven effective treatment was available. Research in this rare disease may be of vital importance to increase and remain quality of life in PXE patients.

Hence, this thesis aimed to describe the clinical expression of calcification-induced vascular disease in patients with PXE, to investigate the effect of risk factors of (calcification-induced) vascular disease in high risk patients, and to investigate the effects of bisphosphonates as potential treatment for calcification-induced vascular disease both in non-PXE and PXE populations.

Key findings

The main findings of this thesis are the following:

Part one – Calcification-induced vascular disease in pseudoxanthoma elasticum

- PXE results in severe arterial calcifications predominantly in the intracranial internal carotid arteries and in the peripheral arteries (arms and legs). (**chapter 3**)
- Degenerated, calcified elastic fibers occur throughout the body in PXE patients. Vascular involvement is of non-atherosclerotic nature with alterations in the medial layer (and around the internal elastic lamina) of arterial walls and in the heart. (**chapter 4**)
- PXE patients are prone to arterial wall thickening and in particular to arterial wall stiffening. (**chapter 5**)

- Cerebrovascular disease is prevalent among PXE patients. The high prevalence of around 15% for TIA and stroke cannot be explained by traditional vascular risk factors. (**chapter 6**)
- Peripheral artery disease is prevalent in PXE patients. The high prevalence of around 45% can also not be explained by traditional vascular risk factors. (**chapter 7**)
- Preferably, angioplasty and stenting should not be used as treatment of peripheral artery disease in PXE. (**chapter 8**)

Part two –Risk factors of (calcification-induced) vascular disease
- HbA1c is modestly related to vascular events in patients with type 2 diabetes. (**chapter 9**)
- Inter-arm systolic blood pressure differences relate to vascular events in patients without clinical manifest vascular disease, whereas this relation is not apparent in patients with manifest vascular disease. (**chapter 10**)

Part three – Bisphosphonates as treatment of calcification-induced vascular disease
- Bisphosphonates reduce all-cause mortality in various patient groups, including osteoporosis patients and cancer patients. (**chapter 11**)
- In PXE, etidronate reduces arterial calcification and subretinal neovascularization compared to placebo without important safety issues. Etidronate is the first proven effective treatment to prevent disease progression and PXE-induced complications, and should be considered in all PXE patients. (**chapter 12**)

Calcification-induced vascular disease

In PXE a specific vascular phenotype can be identified (**chapters 3 to 8**).[30] Severe arterial calcifications occur with an increasing prevalence and increasing clinical consequences in older patients (**chapter 3, chapters 5-7**). Arterial calcifications in PXE are mainly of non-atherosclerotic nature (**chapter 4**) and result in arterial wall thickening, but predominantly in arterial wall stiffening (**chapter 5**). These findings support previous assumptions that PXE is a model disease of MAC and of calcification-induced vascular disease with little atherosclerotic interference.[12,21,31]
If we thus assume that PXE is a model disease of MAC and calcification-induced disease, the vascular manifestations of PXE *are* the clinical consequences of MAC. Using this assumption, knowledge on the vascular phenotype of PXE improves, but also questions, the current knowledge on clinical consequences of MAC on several points.

MAC results in specific types of vascular disease.[12] As already generally assumed, MAC seems to mainly result in arterial wall stiffening (**chapter 5**).[12] Peripheral artery disease and cerebrovascular disease seem to be the main manifestations of MAC-induced vascular disease (**chapters 3, 6, and 7**).[12] Histopathology studies in non-PXE populations confirm these findings. Both in peripheral arteries of patients with peripheral artery disease and in intracranial carotid arteries of patients who underwent a cerebral autopsy, a remarkable high prevalence of non-atherosclerotic calcification was observed (>70%).[32,33] In PXE sporadic reports about brain abnormalities (mainly with cerebral matter abnormalities) exist, but it has not yet been systematically assessed whether this is a part of the (vascular) phenotype and to what extent this is a clinical consequence of MAC (**chapter 6**).[34-38] It is questionable whether coronary artery disease, which strongly relates to atherosclerosis, is an actual clinical consequence of PXE and MAC (**chapter 3**).

It remains unknown how MAC, whether or not through arterial stiffening, results in this specific vascular disease phenotype. Proposed mechanisms through which MAC induces vascular disease include high arterial stiffness induced pulse pressure damage and hemodynamic changes.[12,39,40] Further research in PXE may elucidate mechanisms through which MAC leads to vascular disease.

The vascular phenotype of PXE also questions general assumptions on MAC and calcification-induced vascular disease. MAC is believed to result in high ankle-brachial indexes (ABIs).[41] However, ankle brachial indexes (ABIs) in PXE are rarely higher than 1.3. Thus, either the ABI is an insufficient marker of MAC and should not be used for this purpose, or the type of MAC in PXE differs from MAC in other populations, although we found no evidence for the latter (**chapter 4**). It may be that calcifications in PXE are less continuous and less often fully circular (22% in PXE, **chapter 7**). Pathology studies in specimen of leg amputees show that medial arterial can occur in a circular and in a localized form. [42, 43] This suggests that the medial arterial calcifications in PXE occur predominantly in a localized form.

In fact, ABIs in PXE patients are generally low indicating that an obstructive type of peripheral artery disease occurs in PXE (**chapter 7**).[25, 44] Conceptual drawbacks of the ABI measurement in the detection of peripheral obstructive artery disease may be particularly important in PXE. ABI measurements have been shown to be less reliable in patients with extensive arterial calcifications.[45, 46] First, the ABI test assumes that the distally measured pressure is representative of occlusive arterial disease more proximally. This assumption of the ABI test might hold for the arter-

ies above the knee. However, this assumption is questionable for the arteries below the knee since there are three parallel arteries distributing the pressure. As PXE is characterized by a high prevalence of below knee leg arterial calcifications (**chapter 3**), this assumption of the ABI measurement might be particularly questionable in PXE. Second, the arterial calcification phenotype of PXE involves calcification of arm arteries which in turn might cause inter-arm differences in systolic blood pressure and reduce the reliability of the ABI measurement (**chapter 10**).[47] Third, PXE is characterized by extensive arterial wall stiffening (**chapter 5**) [24] which might have disturbing influences on the pressure measurement used for the ABI.

The complex interplay between medial and intimal wall disease

The fact that low ABIs are found in PXE suggests that obstruction of the peripheral arteries occurs in PXE. This cannot be completely explained from the medial layer involvement in PXE (**chapter 5**). Future research in PXE including angiographic measurements and long-term follow-up may elucidate the pathogenesis of the obstructive type of peripheral artery disease in PXE (**chapter 7**). A possible explanation can be found in the interplay between medial arterial wall and intimal arterial wall disease. The medial layer involvement in PXE may induce intimal wall disease eventually leading to an obstructive type of vascular disease (e.g. obstructive peripheral artery disease). It is known that intimal wall disease and medial wall disease amplify other rather than have an isolated impact.[48] Elastin fragmentation in the medial layer of arteries, as occurs in PXE patients (**chapter 4**), has been shown to play a key role in plaque destabilization and rupture.[48, 49] This may eventually lead to obstructive vascular disease. However, this complex interplay between medial wall disease and intimal wall disease remains only partly understood.[19] Etiological research into the effects of novel or established risk factors among patients at high risk of (calcification-induced) vascular disease, may enhance knowledge on the complex interplay between MAC and atherosclerosis.

HbA1c as risk factor of vascular disease in patients with type 2 diabetes

Patients with type 2 diabetes mellitus have a typical mixed pattern of both intimal and medial wall disease (**chapter 5**) and are at high risk for (recurrent) vascular disease.[12, 50, 51] It seems conceivable that glycemic control is an important risk factor for vascular disease in these patients. However, vascular risk in patients with type 2 diabetes does not seem to further decrease by intensive glycemic control beyond a HbA1c of 7%, as is the case in patients with type 1 diabetes mellitus.[52-55] Also, in type 2 diabetes mellitus patients enrolled in a high vascular risk cohort we found a modest but not statistically significant, relation between HbA1c and vascular events. However, in patients with diabetes mellitus type 2 without manifestations

of vascular disease we did found a strong relation between HbA1c and vascular events (**chapter 9)**. Particularly in later stages of the disease, the typical mixture of medial and intimal wall disease in type 2 diabetes mellitus could translate into a difference in the most important risk factors for new vascular disease.[12] However, glycemic control may remain an important risk factor in early stages of the disease. In line with this hypothesis, a large recent cohort study showed that strong relations did exist between *early* achieved HbA1c reductions and vascular events.[56]

Inter-arm systolic blood pressure differences as risk factor for vascular disease

A difference in systolic blood pressure measured in the left and right arm is prevalent and these inter-arm systolic blood pressure differences (SBPD) may be a less established risk factor of vascular disease.[57-62] Inter-arm SBPD may result from asymmetric arterial wall disease, as a result of both medial and intimal wall disease.[59] We showed that in patients with manifestations of vascular disease no relation between inter-arm SBPD and vascular events is apparent, whereas each increase in inter-arm SBPD relates to a higher risk of vascular events patients without manifestations of vascular disease, independently of traditional vascular risk factors (**chapter 10**). Future studies should further elucidate the relation between inter-arm SBPD and vascular disease and investigate the additional contribution of inter-arm SBPD to prognostic risk estimation for vascular morbidity and mortality. An individual patient data (IPD) meta-analysis on this subject is to be expected soon.[63]

Bisphosphonates as treatment of calcification-induced vascular disease

Arterial calcification may provide a pharmaceutical target for further vascular risk reduction, in particular in diabetes mellitus and renal failure patients, who are at high risk for MAC and vascular disease.[12] MAC, rather than atherosclerotic calcification, may be viewed as the calcification-inducing type of arterial calcification and should thus be the target. (**chapter 1**).[12] The vascular phenotype of PXE indeed illustrates that MAC has important vascular consequences (**chapters 3 – 8**).

Bisphosphonates have a large potential to pharmaceutically target the residual vascular (calcification) risk (**chapter 1**). Recent findings in PXE reopened interest in this potential of bisphosphonates and formed the basis for this thesis. A Dutch research group identified inorganic pyrophosphate (PPi) as the factor that normally prevents PXE and showed that *ABCC6*-mutations were result in inefficient mediators of ATP secretion in the liver causing low levels of inorganic pyrophosphate (PPi).[64] PPi supplementation was thus proposed as treatment for

PXE. Bisphosphonates, well-established drugs for the treatment of osteoporosis and bone metastasis, are stable PPi analogues and could stimulate the inhibitory effects of PPi on ectopic mineralization.[65-67] Further support for the potential of bisphosphonates was available from previously performed bisphosphonate trials. In a systematic review and meta-analysis we showed that bisphosphonates reduce all-cause mortality and tend to reduce vascular mortality (**chapter 11**). Also, we showed that bisphosphonates reduce arterial wall calcification, although no effect on arterial stiffness or on cardiovascular events was found (**chapter 11**).

Based on the finding of PPi as causative factor of PXE, the bisphosphonate etidronate was tested as treatment of ectopic mineralization in PXE mouse models. It was found that etidronate resulted in prevention of ectopic mineralization and in alterations in bone micro-architecture.[68, 69] The effectiveness of etidronate remained to be established in PXE patients in a randomised, placebo-controlled trial. We thus performed the Treatment of ectopic mineralization in PXE (TEMP) trial, a single-center, randomised, double-blind, placebo-controlled trial conducted in the Dutch National Expertise center for Pseudoxanthoma elasticum in the University Medical Center Utrecht (**chapter 12**).

In the TEMP trial we found that in PXE patients, etidronate did not lower ectopic mineralization as measured by femoral ^{18}F-NaF PET activity, but reduced ectopic mineralization as measured by femoral artery calcification compared to placebo. Also etidronate treatment reduced subretinal neovascularization events compared to placebo without important safety issues (**chapter 12**).

^{18}F-NaF PET activity as intermediate endpoint and marker of ectopic mineralization

In order to test the hypothesis that etidronate could reduce ectopic mineralization, we used femoral arterial wall ^{18}fluoride sodium positron emission tomography (^{18}F-NaF PET) activity as marker of ectopic mineralization.[70-74] ^{18}F-NaF PET was thought to be able to visualize the active and ongoing calcification process and discriminate between the active and the more indolent calcifications.[71, 75] Thus we assumed that imaging with ^{18}F-NaF PET was more sensitive to changes in ectopic mineralization compared to traditional CT and used femoral ^{18}F-NaF PET activity as primary outcome in the TEMP trial.

However, this assumption is questioned by the results of the TEMP trial and recent evidence on the precise mechanisms of ^{18}F-NaF PET. The absence of an effect of etidronate treatment on femoral arterial wall ^{18}F-NaF PET activity in the

TEMP trial is remarkably discrepant with the observed effects of etidronate on CT femoral calcium mass and subretinal neovascularization events (**chapter 12**). Recent evidence shows that arterial wall ^{18}F-NaF PET activity indeed co-localizes closely and binds to pathological mineralization, but also that increased tracer uptake does not necessarily represent active calcification. An increased surface area of microcalcifications relative to macrocalcifications could result in an increased tracer uptake.[76] More research into the mechanisms of ^{18}F-NaF PET, for instance using TEMP data, seems warranted. All in all, we think that even in the absence of an effect on the primary outcome, the TEMP trial showed that etidronate treatment can delay ectopic mineralization in PXE. Etidronate is the first proven effective treatment to prevent disease progression and PXE-induced complications.

Future perspectives

Clinical implications for PXE patients

This thesis has several clinical implications for PXE patients. First, based upon our descriptions of the vascular phenotype of PXE, doctors and patients may become more aware of the natural course and prognosis of PXE. Nevertheless, *the* main clinical implication is that, based upon this thesis, etidronate should be considered in all PXE patients. Given the large impact on quality of life of ophthalmological and vascular involvement in PXE, etidronate treatment may eventually enhance quality of life in PXE patients.[29]

Preferably, etidronate should be initiated directly after the diagnosis PXE. Women with PXE of child-bearing potential, in particular those with a desire to have children, should be excluded for etidronate treatment given the potential hazards for mother and fetus of etidronate treatment and the limited clinical data.[77, 78] The current knowledge is mainly based on animal studies, showing that etidronate crosses the placenta and decreases fetal weight.[79] Also the effects of etidronate treatment on lactation are unknown. [80] A careful weighing of risks and benefits should be made before initiation of etidronate treatment in children. Treatment with non-nitrogen-containing bisphosphonates, such as etidronate, has a good safety profile in pediatric patients on the short term. However, there is no sufficient data available on long-term safety.[81]

Future research in calcification-induced vascular disease in PXE

This thesis contributed to the knowledge on the vascular phenotype of PXE. However, several additional questions remain. Addressing these questions could

improve the knowledge of the clinical consequences of PXE and of calcification-induced vascular disease in general. To gain more insights in the (vascular) phenotype of PXE a comparison with healthy controls without PXE, is needed. Therefore we started the Determinants of Ectopic Calcification in Pseudoxanthoma Elasticum and Healthy controls: Evaluation of their Relations (DECIPHER) study, a patient-control study in which we aim to decipher pathogenesis and consequences of PXE (IRB nr. 16/622). This study is still including. At the moment 75 PXE patients and 40 controls, mostly partners of PXE patients who are matched for age and lifestyle, have given their informed consent and participated. All participants underwent clinical screening including laboratory measurements and a large part additionally underwent ocular measurements and/or brain measurements.

The precise effects of PXE on the brain remain unknown. It is proposed that white matter lesions and a 'vascular-dementia-like disorder' are part of the PXE phenotype (**chapter 6**).[34-38] The arterial wall of the carotid siphon is more frequently affected in comparison to hospital controls with a full-body CT for other indications (75% in PXE patients versus 44% in control patients, **chapter 3**). It is most conceivable that brain abnormalities associated with PXE arise from the progressive calcification and stiffening of brain arteries, since it is known that arterial stiffness in the carotid artery is associated with structural brain changes.[82] The DECIPHER study uses modern radiological MRI techniques to more precisely measure flow and pulsatility of brain arteries and brain tissue perfusion in PXE patients and controls. Also, cognitive testing (tailored to visual impaired patients) are performed in patients and controls in the DECIPHER study. This enables us to gain more insights in physiological and clinical effects of PXE and of brain artery calcification.

Although inorganic pyrophosphate has been shown to be *the* causative factor of PXE (**chapter 12**) [64, 83], it is unknown why other calcification inhibitors apparently fail to take over the inhibiting effect on ectopic mineralization in PXE patients. Therefore, another aim of the DECIPHER study is to gain insights in the differences in serum levels of calcification-promoting and -inhibiting factors between PXE patients and healthy controls. This may eventually lead to additional pharmacological options to treat PXE.

Given the potential importance of (medial) arterial calcifications in the pathogenesis of peripheral artery disease,[12,19,32] more research into the role of leg arterial calcifications is needed (**chapter 7**). Therefore, further research among PXE patients into the prospective relation between characteristics as leg arterial calcifications

and future PAD using (long-term) follow-up is warranted. Given the striking effects of walking therapy in PXE, systematic angiographic measurements may provide further insights (**chapter 8**).

Future research in treatment of calcification-induced vascular disease

This thesis illustrates the importance of arterial calcification as pharmaceutical target to further reduce the residual vascular risk. Future research should focus on therapies interfering in the process of calcification-induced vascular disease and MAC. These therapies should be investigated in high risk patients such as diabetes mellitus and renal failure patients and evaluated on top of other preventive therapies. Several inhibitors and inhibiting systems for MAC including vitamin K-dependent pathways, the Klotho protein, Fetuin-A and inorganic pyrophosphate (PPi) could open up possibilities for a pharmaceutical treatment interfering in the process of calcification-induced vascular disease (e.g. bisphosphonate treatment to supplement inorganic pyrophosphate).[90] Among patients with type 2 diabetes mellitus, the effect of vitamin K2 (Menaquinone-7) on arterial calcification, quantified using femoral ^{18}F-NaF PET activity and CT-based femoral calcium scores, is currently evaluated in a randomised placebo-controlled trial in the UMC Utrecht (NCT02839044).

Bisphosphonates for vascular risk reduction

This thesis focused on bisphosphonate treatment and emphasized the potential of bisphosphonates for vascular risk reduction. The potential of bisphosphonates for vascular risk reduction needs further studying. Future research should focus on the effects of bisphosphonates on arterial calcification and vascular risk reduction in non-PXE populations. Small etidronate trials have already established beneficial effects on arterial wall disease in chronic kidney disease, diabetes mellitus and hypercholesterolemia patients.[91-94] Compared to cholesterol-lowering treatment the expected relative risk reductions for vascular disease of bisphosphonates may be quite small (**chapter 11**).[7-9] Therefore, it is important to evaluate the potential of bisphosphonates in patients with high (residual) risks of vascular disease and on top of other preventative therapies.

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CHAPTER 14

Appendix

- Summary
- Nederlandse samenvatting (voor niet ingewijden)
- Contributing authors
- List of publications
- Dankwoord
- Curriculum Vitae

Summary

Vascular disease has a large worldwide medical burden. Over the last years, considerable progress has been made in treatment and prevention of vascular disease. Even if all modifiable vascular risk factors are at recommended targets a large residual vascular risk remains, exposing an area of unmet medical need. Arterial calcification, in particular medial arterial calcification (MAC), may open up a promising target for preventive (pharmaceutical) treatment on top of other preventative strategies. However, since MAC and atherosclerosis (intimal wall disease often) occur simultaneously, little is known about the precise clinical consequences of MAC.

In the rare calcification disorder pseudoxanthoma elasticum MAC occurs even at young age and thus with little interference of atherosclerosis. PXE is caused by biallelic mutations in the *ABCC6* genes causing low levels of inorganic pyrophosphate and progressive extopic mineralization. PXE is characterized by mineralization of elastic fibers with skin involvement (e.g. yellowish papules or plaques), eye involvement (e.g. angioid streaks) and vascular involvement (arterial calcification). In **chapter 2** we show that the prevalence is at least 1 in 56,000.

To enhance knowledge of the clinical consequences of MAC, we described the calcification-induced vascular disease in PXE in the first part of this thesis. In **chapter 3** we performed full-body CT scans in 103 PXE patients and compared these to CT scans of hospital controls without PXE. We found that the peripheral arteries in the legs and arms and the intracranial carotid arteries are the predominant arteries that are calcified in PXE patients. Subsequently, in **chapter 4** we perfomed a histopathology study in two PXE patients and showed that in PXE degenerated, calcified elastic fibers occur throughout the body. Vascular involvement is of non-atherosclerotic nature with alterations in the medial layer (and around the internal elastic lamina) of arterial walls and in the heart. In **chapter 5** we investigated the physiological consequences of vascular involvement in PXE by performing extensive arterial wall characterization in 203 PXE patients and comparisons with the general population (expected values) and type 2 diabetes mellitus patients, who have a typical mixture of intimal and medial wall disease. We found that PXE patients have thicker arterial walls than the general population, but thinner arterial walls than DM2 patients at similar age. However, arterial stiffening is more pronounced in PXE patients compared to DM2 patients. In **chapter 6** we described the occurrence of cerebral disease in our PXE cohort and by means of a systematic review of the literature. It was found that cerebrovascular disease is very prevalent (15%) and that this high prevalence can not be

explained by traditional risk factors. In **chapter 7** we described peripheral artery disease (PAD) in PXE. PAD (ankle brachial index after treadmill test<0.9) has a prevalence of 46%, intermittent claudication has a prevalence of 39% in PXE. This remarkably high prevalence cannot be explained by tradional risk factors or (the extent of) arterial calcification. In **chapter 8** we, together with our French colleagues, described four PXE patients with PAD and intermittent claudication who underwent femoral angioplasties with stenting. In all cases there was an early failure of the stent suggesting that arterial angioplasty with stenting as a primary surgical approach in PXE patients with femoral artery lesions should not be recommended.

The second part of this thesis focused on risk factors of (calcification-induced) vascular disease. In **chapter 9** we showed that among type 2 diabetes mellitus patients HbA1c is modestly related to vascular events. In **chapter 10** we showed that inter-arm systolic blood pressure differences relate to vascular events in patients without clinical manifest vascular disease, whereas this relation is not apparent in patients with manifest vascular disease.

The last part of this thesis investigates the potential of bisphosphonates as treatment for calcification-induced vascular disease. Bisphosphonates are well-established drugs for the treatment of osteoporosis and bone metastases. While frequently using their powerful osteoclast inhibiting effects, we seem to have forgotten their earlier documented effects on ectopic mineralization.

In **chapter 11** we performed a systematic review and meta-analysis of randomized controlled bisphosphonate trials that reported on vascular disease. We showed that bisphosphonates reduce arterial wall calcification and tend to reduce the risk of cardiovascular mortality. Aslo we showed that bisphosphonates reduce all-cause mortality in various patient groups, including osteoporosis and cancer patients.

In **chapter 12** we report the results of the Treatment of Ectopic Mineralization in Pseudoxanthoma elasticum (TEMP) study, a randomized placebo-controlled trial in which we investigated the effectiveness and safety of one year treatment with etidronate (cyclical 20 mg/kg for two weeks every 12 weeks) on ectopic mineralization among participants with PXE. We showed that in PXE patients, etidronate reduced arterial calcification and subretinal neovascularization events, while not lowering femoral ^{18}F-NaF PET activity, compared to placebo without important safety issues. On the basis of these results all PXE patients should be considered for etidronate treatment.

In conclusion, this thesis confirmed that the clinical consequences of MAC are arterial stiffening and higher risks of vascular disease, in particular cerebrovascular disease and peripheral artery disease. Furthermore, we showed that bisphosphonates, in particular etidronate, are able to interfere in the process of arterial calcification and may inhibit calcification-induced vascular disease. Also, we showed that etidronate is an effective treatment to prevent disease progression in PXE, for which until now no treatment was available.

Nederlandse samenvatting (voor niet ingewijden)

Hart- en vaatziekten zijn een groot wereldwijd gezondheidsprobleem. Een hartaanval, beroerte of vernauwde slagaders in de benen zijn voorbeelden van hart- en vaatziekten. We weten steeds beter hoe we de kans op hart- en vaatziekten kunnen verlagen en daarmee het optreden van hart- en vaatziekte zo lang mogelijk kunnen uitstellen. Risicofactoren zoals roken, overgewicht, weinig lichaamsbeweging en een hoog cholesterol kunnen we effectief bestrijden met leefstijlveranderingen en medicijnen. Desondanks blijven hart- en vaatziekten een groot gezondheidsprobleem en zelfs wanneer alle risicofactoren worden aangepakt blijft er een grote kans op hart- en vaatziekte bestaan. Het is daarom belangrijk om op zoek te gaan naar nieuwe aanknopingspunten voor behandeling om het risico nog verder te kunnen verlagen. Verkalking van de vaatwand zou wel eens een belangrijke rol kunnen spelen in het optreden van hart- en vaatziekte en een aanknopingspunt voor verdere behandeling kunnen zijn.

Dit proefschrift gaat over de rol die verkalking speelt in het optreden van hart- en vaatziekte. In **hoofdstuk 1** wordt uitgelegd dat de vaten op twee manieren verkalken. Ten eerste is er de ziekte van de binnenste wand van het bloedvat waarbij cholesterol en vetten zich als plaque ophopen in de vaatwand. Hoewel dit proces in de volksmond (onterecht) “slagaderverkalking” wordt genoemd is de verkalking pas het laatste deel van dit proces. Waarschijnlijk is deze verkalking op zich gunstig omdat het de plaque inkapselt en daarmee scheuren voorkomt. Ten tweede is er de verkalking van de middelste laag van de vaatwand, de zogenaamde mediaverkalking. Deze verkalking gebeurt bij ons allemaal, met name wanneer we ouder worden, suikerziekte hebben of een slechtere nierfunctie hebben. Dit type vaatverkalking zorgt ervoor dat vaten “stijf” worden: met een nieuwe golf bloed kunnen de vaten niet meer op een goede manier uitrekken. Het lijkt erop dat dit type vaatverkalking leidt tot vaatziekte. Omdat het lastig is om deze ziekte van de middelste vaatwand te bestuderen los van de ziekte van de binnenste vaatwand is er veel onbekend over de precieze gevolgen.

Pseudoxanthoma elasticum (PXE) is een zeldzame ziekte waarbij al op jonge leeftijd deze mediaverkalking lijkt plaats te vinden. Onderzoek bij deze ziekte zou ons dus veel kunnen leren over de gevolgen van deze verkalking van de vaatwand. PXE wordt veroorzaakt door afwijkingen in het *ABCC6* gen waardoor verkalking van het lichaam niet goed wordt geremd en dus versneld optreedt. Naast de verkalking in de (middelste laag van de) vaatwand hebben patiënten typische huidafwijkingen (“pseudoxanthomen”) en oogafwijkingen die kunnen leiden tot ernstige slechtziendheid en blindheid. In **hoofdstuk 2** laten we zien dat er zeker

300 patiënten in Nederland zijn. Er is nog weinig onderzoek gedaan naar de gevolgen voor het vaatstelsel van de ziekte PXE.

Het eerste deel van het proefschrift gaat over de gevolgen van mediaverkalking bij PXE. In **hoofdstuk 3** hebben we onderzocht welke slagaders met name verkalken bij PXE. We maakten CT scans van het hele lichaam bij 104 PXE patiënten en vergeleken deze met CT scans van patiënten zonder PXE. Het werd duidelijk dat vooral de slagaders van de benen, de carotis slagaders die de hersenen ingaan en soms de slagaders van de armen verkalken bij PXE patiënten. In **hoofdstuk 4** hebben we twee lichamen van overleden patiënten met PXE precies en systematisch onderzocht. We vonden dat de elastische vezels door het lichaam verkalkt waren, ook op plekken waar dat met het blote oog of met een CT scan niet te zien was. De vaatwand was inderdaad vaak verkalkt in de middelste – en niet in de binnenste – laag. In **hoofdstuk 5** onderzochten we de effecten van PXE op de dikte en de elasticiteit van de vaatwand. Metingen bij 203 PXE patiënten lieten zien dat de vaatwand bij PXE veel dikker en stijver is dan bij mensen uit de algemene bevolking. In vergelijking met patiënten met suikerziekte, die zowel een binnenste als middelste vaatwandziekte hebben, waren de vaten dunner maar wel stijver. Dit past ook bij verkalking met name in de middelste vaatwand. In **hoofdstuk 6** onderzochten we het voorkomen van hersenziektes bij PXE. Dit deden we door 178 PXE patiënten te bestuderen en uitgebreid literatuuronderzoek te doen. We ontdekten dat met name beroertes en TIA's vaak voorkwamen bij PXE (15%) en dat dit niet te verklaren is door bekende risicofactoren. In **hoofdstuk 7** onderzochten we het voorkomen van perifeer vaatlijden, vernauwing van de slagaders in de benen, bij PXE. Dit deden we door metingen te doen bij 203 PXE patiënten en door een vragenlijst te versturen over de klachten. We ontdekten dat een groot deel van de patiënten (46%) een vernauwing in de beenvaten heeft en dat veel daarvan ook klachten zoals claudicatie ("etalagebenen") (39%) heeft. Ook dit kan niet verklaard worden door risicofactoren, maar ook niet door de verkalking in de slagaders van de benen. In **hoofdstuk 8** hebben we samen met Franse collega's vier PXE patiënten beschreven die een dotter of stent in de vernauwde slagaders van de benen hebben gekregen. In alle gevallen faalde deze behandeling sneller dan verwacht mag worden. We raden daarom een dotter of stent behandeling in de beenslagaders af bij patiënten met PXE. Looptraining, waardoor nieuwe, goede vaten gevormd worden in de benen, lijkt wel bijzonder goed te werken bij PXE.

In het tweede deel van dit proefschrift onderzochten we het effect van risicofactoren op het optreden van hart- en vaatziekten. In **hoofdstuk 9** lieten we zien dat bij patiënten met suikerziekte (type 2 diabetes) er niet zo'n sterke relatie is

tussen het suikergehalte (gemeten met het zogenaamde HbA1c, wat een beeld geeft van de langdurige suikercontrole) en hart – en vaatziekten. Bij patiënten met suikerziekte die al hart- en vaatziekte hadden vonden we helemaal geen relatie. In **hoofdstuk 10** hebben we verschillen in de bloeddruk tussen de linker en rechter arm als risicofactor voor hart- en vaatziekte onderzocht. We lieten zien dat dat bij patiënten met hart- en vaatziekte er geen relatie is tussen deze links-rechts verschillen en het optreden van (nieuwe) hart- en vaatziekte. Echter, bij mensen die nog geen hart- en vaatziekte hebben (maar wel een hoog risico hebben), zijn deze links-rechts verschillen wél een risicofactor voor het optreden van hart- en vaatziekte.

In het laatste deel van het proefschrift onderzochten we of vaatziekte door verkalking voorkomen kan worden door behandeling met de medicijnen bisfosfonaten. Bisfosfonaten worden op dit moment gebruikt om botontkalking tegen te gaan. Rond 1900, nog voordat deze medicijnen voor medische doeleinden werden gebruikt, werden deze medicijnen echter gebruikt om verkalking in waterleidingen tegen te gaan. Bovendien hebben muizenstudies laten zien dat bisfosfonaten, vooral het medicijn etidronaat, misschien als behandeling voor de ziekte PXE gebruikt kan worden.

Voor **hoofdstuk 11** hebben we alle onderzoeken die ooit zijn verschenen over de medicijnen bisfosfonaten nagekeken. Onderzoeken die voldeden aan onze kwaliteitscriteria en die rapporteerden over het voorkomen van hart- en vaatziekte werden nader bekeken. Door al deze onderzoeken te combineren vonden we dat behandeling met bisfosfonaten vaatverkalking tegen gaat en de kans op sterfte laat afnemen. Het lijkt erop dat bisfosfonaten ook sterfte door hart- en vaatziekten kunnen voorkomen. In **hoofdstuk 12** beschijven we de resultaten van een onderzoek dat we uitvoerden om het effect van behandeling met het bisfosfonaat etidronaat bij patiënten met PXE te onderzoeken. 74 PXE patiënten namen deel aan de zogenoemde TEMP studie. Door middel van loting werd bepaald of een patiënt een jaar lang met etidronaat (het echte medicijn) of een placebo (een nepmedicijn) behandeld werd. Zowel de patiënten als de onderzoekers waren niet op de hoogte van de groep waarin de patiënt zat. Tijdens het jaar van deelname werden alle deelnemers intensief gecontroleerd met bloedcontroles en oogheelkundige controles. Slechts één deelnemer (in de placebogroep) doorliep niet het gehele onderzoek. Voor en na de behandeling werd er onder andere gemeten hoeveel kalk er in de vaatwand aanwezig was. Ook maakten we voor en na behandeling een speciale scan waarmee we de activiteit van de vaatverkalking konden meten. Hoewel uit de metingen op deze speciale scan geen verschillen naar voren

kwamen lieten resultaten van andere metingen wel een effect van etidronaat behandeling zien. Etidronaat voorkomt ongewenste vaatnieuwvormingen in het netvlies en daarmee oogachteruitgang. Bij patiënten die het nepmedicijn kregen werden bij 9 van de 37 patiënten een dergelijke ongewenste vaatnieuwvorming waargenomen, waar dit maar bij 1 van de 37 patiënten behandeld met etidronaat voorkwam. Daarnaast remt etidronaat de vaatverkalking bij PXE. Gemiddeld nam de vaatverkalking met 8% toe bij patiënten die het nepmedicijn kregen, terwijl dit juist een afname van 4% was bij mensen die etidronaat behandeling kregen. Ook was behandeling in deze dosering veilig voor het jaar waarin het onderzoek plaatsvond. Deze resultaten laten zien dat behandeling met etidronaat de ziekte PXE kan afremmen.

Samenvattend laat dit proefschrift zien wat de gevolgen van verkalking van de (middelste wand van de) vaatwand zijn. Door deze zogenaamde mediaverkalking worden slagaders stijver en neemt het risico op hart- en vaatziekte, vooral op TIA's, beroerte en vernauwde beenslagaders, toe. Verder laten we zien dat de medicijnen bisfosfonaten dit proces van verkalking af kunnen remmen. Bisfosfonaten kunnen worden gebruikt om de ernstige ziekte PXE, waarvoor tot nu toe nog geen enkele behandeling beschikbaar was, te behandelen.

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Curriculum Vitae

Guido Kranenburg was born on the 26th of November 1990 in Amersfoort, The Netherlands. After secondary school at the "Stedelijk Gymnasium Johan van Oldebarnevelt", from which he graduated cum laude in 2008, he studied Medicine at the Utrecht University. During his bachelor and master degree, he took his first steps in medical research, while contributing to research on detection of frailty among elderly patients in the general practice. He completed a senior internship in Neurology at "St. Antonius Ziekenhuis" in Nieuwegein and a senior internship in Family Medicine at "Huisartsenpraktijk de Eglantier" in Hilversum. After his research internship at the department of Vascular Medicine, University Medical Center Utrecht (UMCU), he was given the opportunity to work as research physician at this department. In November 2014 he obtained his medical degree and started his PhD project at the department of Vascular Medicine under supervision of prof. F.L.J. Visseren, prof. P.A. de Jong, and dr. W. Spiering. In his PhD research he closely collaborated with different departments within the UMCU (vascular medicine, ophthalmology, radiology, and nuclear medicine) in order to set up and carry out the Treatment of Ectopic Mineralization in Pseudoxanthoma elasticum (TEMP) trial. He combined his work as study physician and PhD research with the postgraduate master Clinical Epidemiology at the University of Utrecht from which he graduated in August 2017. Guido starts training as a general practitioner in March 2018. In the future he aims to combine his clinical work as general practitioner with clinical research.