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Director ATHANASIOS GIANNOUKAS

PhD Thesis

«The role of metalloproteinases 13 and 9 in abdominal aortic aneurysm development»

by

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Awards

Best e-Poster Award:

The association of MMP9 and MMP13 functional genetic polymorphisms and abdominal aortic aneurysm in a Greek population.

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«Risk factor assessment in patients with large abdominal aortic aneurysms»

Georgios Makrygiannis

University of Thessaly, Faculty of Medicine, 2020

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Abstract

Abdominal aortic aneurysm (AAA) is a complex multifactorial disease with multiple environmental and genetic risk factors. Matrix metalloproteinases (MMPs) are known to be related with the pathobiology of AAAs. The subject of this thesis concerns the study of environmental and genetic risk factors, and their role in large AAAs. Thereafter, a prospective, nonrandomized case-control study was conducted to explore the risk factors for large AAAs with a maximal diameter equal or superior to 5.5 cm. The cohort of patients included 175 men with AAA and the control cohort included 166 men without aortic dilatation. The control subjects had their abdominal aortas measured by ultrasound on the basis of a AAA screening programme. Differences of AAA risk factors in both groups were assessed and analyzed statistically. We likewise surveyed the relationship between two functional single nucleotide polymorphisms (SNPs) in the genes MMP9 (-1561C/T; rs3918242) and MMP13 (-77A/G; rs2252070), and their distribution in both cohorts. Statistical analysis found family history of AAA ($P = .028$), hypercholesterolemia ($P < .001$), and current smoking ($P < .001$) as risk factors for AAA. The genotypic ($P = .047$) and allelic ($P = .037$) distribution of the SNP MMP13 (-77A/G; rs2252070) was statistically different in both groups, but not after multiple testing. The genotypic and allelic distribution of the SNP MMP9 (-1561C/T; rs3918242) was not deviated statistically in both groups.

Περίληψη

Περιβαλλοντικοί και γενετικοί παράγοντες κινδύνου συνεισφέρουν στην αιτιολογία του ανευρύσματος της κοιλιακής αορτής (ΑΚΑ). Μεταλλοπρωτεϊνάσες της θεμέλιας ουσίας (MMPs) έχουν επίσης συσχετιστεί με την παθοφυσιολογία του ΑΚΑ. Το θέμα της διατριβής αφορά τη μελέτη περιβαλλοντικών και γενετικών παραγόντων ως προς το ΑΚΑ. Για αυτό το λόγο, πραγματοποίθηκε μία προοπτική, μη τυχαιοποιημένη μελέτη ασθενών και μαρτύρων με σκοπό να διερευνηθούν οι παράγοντες κινδύνου σε μεγάλα ΑΚΑ (≥5.5 cm) σε 175 άνδρες ασθενείς με ΑΚΑ, οι παράγοντες των οποίων συγκρίθηκαν με 166 άνδρες μάρτυρες. Οι μάρτυρες δεν παρουσίαζαν διάταση της κοιλιακής αορτής όπως επιβεβαιώθηκε υπερηχογραφικά από το υπάρχον προληπτικό πρόγραμμα για ανεύρεση ΑΚΑ που πραγατοποιείται στην περιοχή της Θεσσαλίας υπό την αιγίδα της αγγειοχειρουργικής κλινικής του Πανεπιστημιακού Νοσοκομείου της Λάρισας. Επίσης μελετήθηκε η πιθανή συσχέτιση δύο λειτουργικών μονήρων νουκλεοτιδικών πολυμορφισμών των γονιδίων MMP9 (-1561C/T; rs3918242) και MMP13 (-77A/G; rs2252070) με την παρουσία μεγάλων ΑΚΑ (≥5.5 cm). Η στατιστική ανάλυση ανέδειξε το οικογενειακό ιστορικό για ΑΚΑ (P = .028), την υπερχολεστερολαιμία (P < .001) και το τρέχων κάπνισμα (P < .001) ως παράγοντες κινδύνου για ΑΚΑ. Στατιστική διαφορά βρέθηκε στη συχνότητα των γονοτύπων (P = .047) και των αλληλίων (P = .037) για τον πολυμορφισμό rs2252070, χωρίς ωστόσο τα αποτελέσματα να παραμένουν σημαντικά μετά από πολλαπλή στατιστική ανάλυση. Επίσης δεν βρέθηκαν διαφορές στη συχνότητα των γονοτύπων και των αλληλίων για τον πολυμορφισμό rs3918242.

TABLE OF ABREVIATIONS

AAA abdominal aortic aneurysm

AGE Advanced Glycation End-products

AOD atherosclerotic occlusive disease

AR androgen receptor

BCE before current era

CAD coronary artery disease

CDKN2BAS1/ ANRIL CDKN2B antisense RNA 1 also known ANRIL

CE current era

CI confidence interval

COPD chronic obstructive pulmonary disease

DZ dizygotic

ECM extracellular matrix

ERG member of the ETS (erythroblast transformation-specific) family of transcription factors

ESR estrogen receptor

ESVS European Society for Vascular Surgery

GWAS genome-wide association studies

HR hazard ratio

HRT hormone replacement therapy

ISCVS International Society for Cardiovascular Surgery

IL-6 interleukin-6

IL6R interleukin-6 receptor

IL-8 interleukin-8

LDLR low density lipoprotein receptor

LINC00540

MASS Multicenter aneurysm screening study

MMPs matrix metalloproteinases

MZ monozygotic

MRI magnetic resonance imaging

OR odds ratio

PAD peripheral artery disease

PCIF1 Phosphorylated CTD Interacting Factor 1

SMCs smooth muscle cells

SMYD2 MYND domain-containing protein 2

SNP single nucleotide polymorphism

SORT1 Sortilin

SVS Society of Vascular Surgery

Th1 T helper 1

Th2 T helper 2

VSMCs vascular smooth muscle cells

ZNF335 Zinc Finger Protein 335

General Introduction

Generalities

The abdominal aorta is the terminal part of the aorta before its bifurcation to both iliac arteries. It begins at the diaphragm as a continuation of the thoracic aorta (**Fig 1., Fig 2.**).

The aortic wall is composed by three different layers:

- The intima layer, which is the most internal layer in contact with blood circulation. It is composed by one layer of endothelial cells and a basic membrane.
- The media layer, which represents around 80% of the aortic wall. It is composed by smooth muscle cells (SMCs), elastic fibers, collagen fibers, glycoproteins, and proteoglycans organized in lamellar units. The units are orientated in concentric way, enabling the elastic and mechanic properties of the aortic wall.
- The adventitia layer, which is the most external part of the aortic wall. It is composed by connective tissue rich in collagen. The principal cells of the adventitia are the fibroblasts, but adipocytes and stem cells can also be found. The adventitia is vascularized by vasa vasorum and lymphatic vessels. Apart from its nutritional role, adventitia also participates at the compliance and resistance of the aorta.

Finally, these three layers are separated by the internal and external elastic laminae, with nearly 50 μm of width. They are composed almost exclusively by elastic fibers.

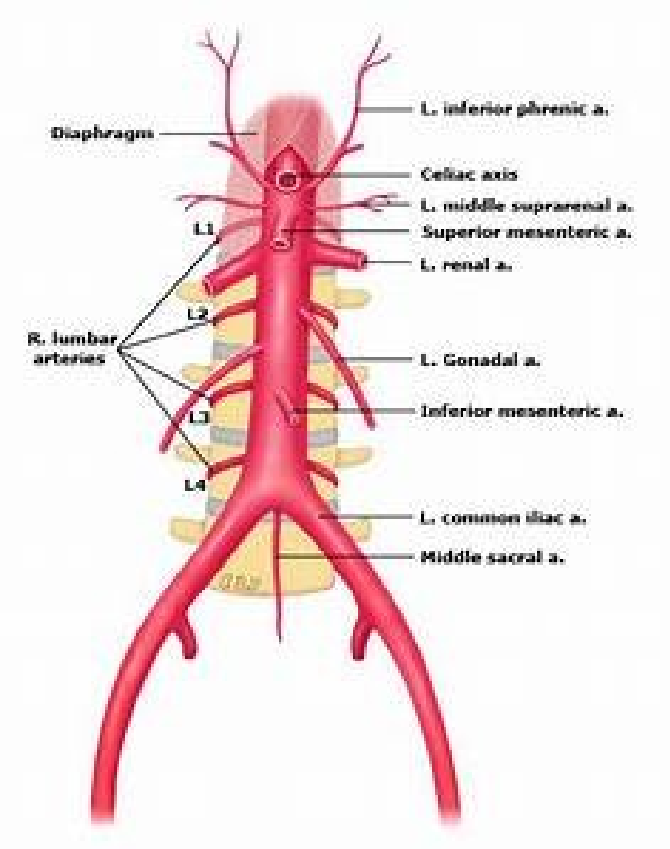

Fig 1. Normal aorta.

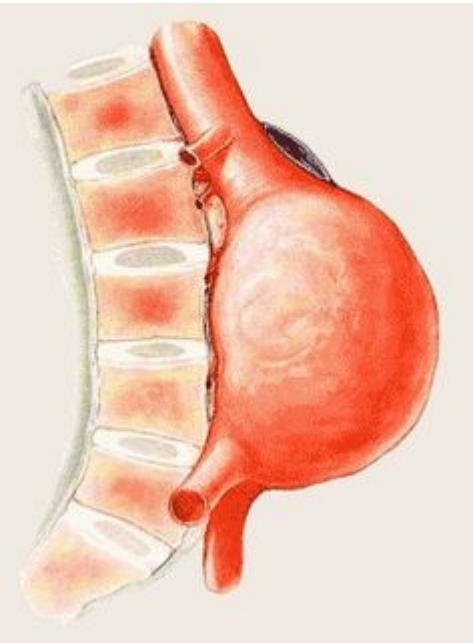

Fig 2. Aneurysmal abdominal aorta.

Etymology

The word aneurysm derives from the Greek word ανεύρυσμα which means ‘’widening’’.[1] It is a compound word which is consisted from the word άνω which means ‘’up’’ and the word ευρύς which means ‘’wide’’. The word aorta has the same etymological origin as the word artery. The word aorta derives from the ancient Greek verb aorteo which is the lengthened form of the verb aeiro which means ‘’to rise’’, “to lift’’, ‘’to hungup’’ (**Fig 3.**). In Ancient Greece there was a notion that the heart is lifted by the aorta. Aorta had also common origin with aorthr which means “shoulder strap’’ and was used by ancient Greek soldiers as case for their sword.

Moreover, the verb aeiro derives from the word aer = ‘’air’’. Therefore, aorta has relative origin with the word artery which derives from the word aer and terein = ‘’keep’’. The arteries thought to be pipelines that conducted not blood but air.

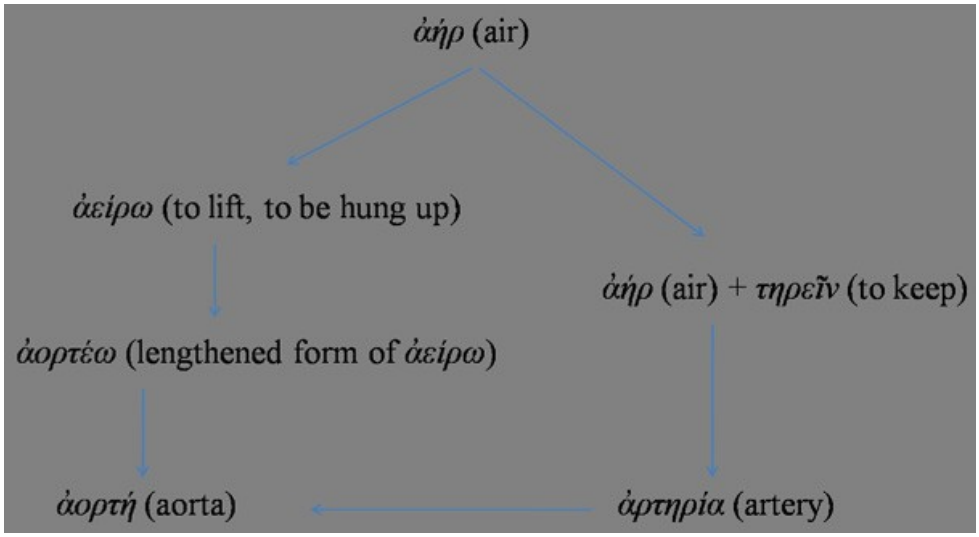

Fig 3. Etymology of word aorta and artery. (From Antoniou et al.)

Brief history of aneurysms

The first possible written proof of aneurysm was found in the Ebers papyrus back in 1,500 BCE. In the text of Ebers papyrus was written that a swelling (metu) of a conduit has to be ‘’treated with the knife and burned with fire so that it bleeds not too much’’. However, it is not certain what conduit exactly refers to. In the 6th century BCE in Ancient India, Sushruta which was a famous surgeon, distinguished post-traumatic from spontaneous arterial

swellings. The first time that the word aneurysm is written is probably by Rufus of Ephesus in the first century CE. Galen in the second century among others distinguished true from false aneurysms. In the second century Antyllos also described surgical treatment of traumatic and spontaneous aneurysms by ligating the proximal and distal parts of the aneurysm and extracting the material of the sac. Oribasios, a byzantine surgeon of the fourth century described also the treatment of peripheral aneurysms. After the fall of roman empire, the barber-surgeons treated traumatic aneurysms at the bend of the elbow. Many centuries afterwards, in the 16th century, Ambroise Paré and Andreas Vesalius described and introduced the medical terminology of aortic aneurysms. Until 19th century the majority of aortic and peripheral aneurysms were treated conservatively by rest, starvation and sedation, and by long-term compression of arterial aneuryms of the extremities. The first resection of a AAA and treatment by homograft replacement was performed by Dubost in 1951. The first replacement of a TAA by homograft was performed by DeBakey in 1953. The first endo-vascular stent-graft was performed by Volodos in 1986 who treated a saccular aneurysm of the thoracic aorta. In 1991 came the first publication by Parodi and colleagues concerning the endovascular treatment of AAA.

Definition

There are several definitions of AAA. In current clinical practice the most common is that of McGregor et al. which defined an AAA in 1975 as a segmental dilatation of the infrarenal aorta of equal or superior of 30mm. This definition was based on the work of Steinberg and Stein who in 1965 examined arteriographic analyses and found that 3 cm was 2SD more than the mean diameter. Other definitions of AAA are from Sterpetti et al. who define AAA as a dilatation of ≥ 1.5 times of the diameter of the suprarenal aorta. Collin et al. defines AAA as a diameter of the infrarenal aorta ≥ 40 mm or 5 mm more than the suprarenal aortic diameter. The ISCVS/SVS define as a diameter ≥ 1.5 times from the expected normal diameter. An ectasia of the infrarenal aorta is a dilatation of the infrarenal aorta between 25 mm and 29 mm. Wanhainen et al. found based on a population-based MRI study conducted in Uppsala, Sweden in 70-years old men and women that the dividing line (mean diameter + 2SD) between a normal and an aneurysmal aorta was 30 mm for men and 27 mm for women. They calculated also the threshold ratio (mean ratio + 2SD) between the infrarenal and suprarenal aorta, which was 1.1 for men and 1.0 for women. The diameter of the aorta depends on

different factors such as the age, the sex, the body size, and the imaging technique. Less regular types of AAAs that include the renal arteries are called pararenal AAAs, though infrarenal AAAs that firmly lie beneath the renal arteries without including them are classified as juxtarenal AAAs (**Fig 4.**). These types of AAAs, as well as suprarenal AAAs pose particular technical challenges for endovascular repair due to their complex anatomy. Aneurysm should be differentiated from arteriomegaly, the latter being a non-segmental expansion of the aorta. A true aneurysm involves a dilatation of all the three layers of the aortic wall. It should also be differentiated from a pseudo-aneurysm which does not involve all the three layers. AAAs may also less commonly involve only a portion of the circumference of the vessel, so called saccular AAAs (**Fig 5.**).

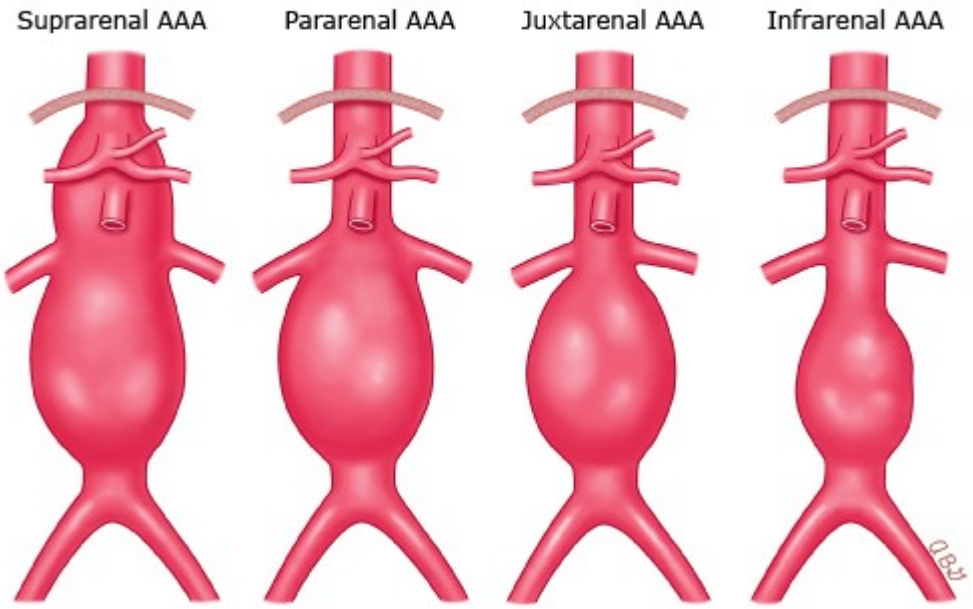

Fig 4. Classification of AAA according to their relationship with renal arteries

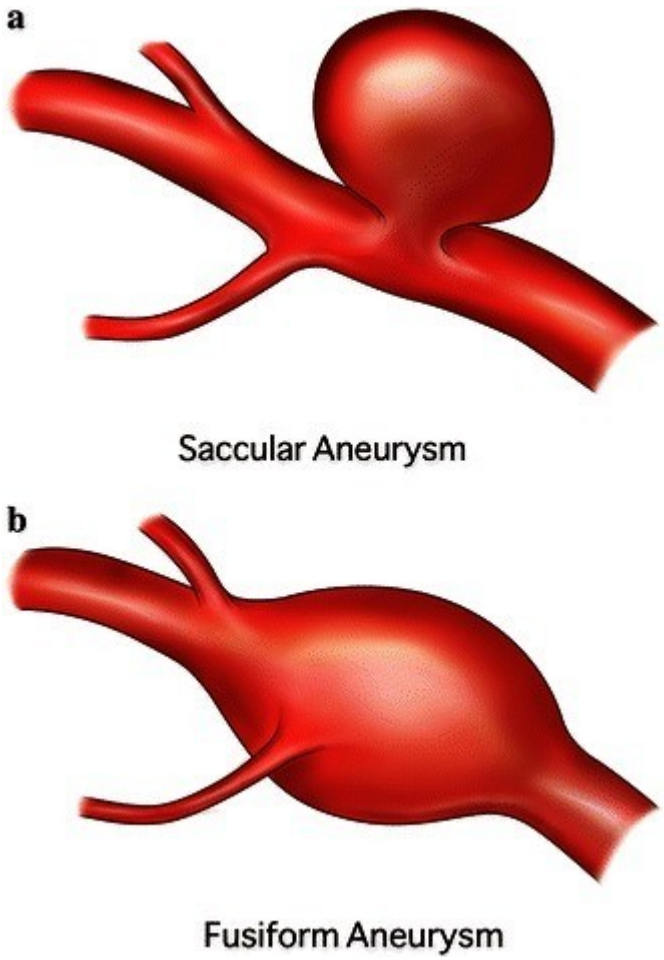

Fig 5. a. fusiform AAAs are concentric, involve all the aortic wall. b. saccular AAAs are eccentric, involve only a portion of the circumference of the vessel wall.

Epidemiology of AAA

The prevalence of AAA depends on different variables including advanced age, sex, AAA family history, and smoking habit. The prevalence of AAAs that are 29-49 mm varies from 1.3% in men from 45 to 54 years old to 12.5% in men from 75 to 84 years old. In women, the prevalence varies from 0% to 5.2%, with a prevalence increasing by age. In addition, AAA is found approximately 10 years later in females than in males. Worldwide, around 1% of men more than 65 years old die from AAA.[2] The prevalence of AAA is higher in Australia (6.7% (6.5-7.0%) than North America 2.2% and Europe 2.5%. The prevalence in Asia 0.5% is lower than in western countries. The large proportion of AAAs are asymptomatic. The primary risk of AAA is rupture with a mortality of 75-90% after rupture. In USA there are

about 16,000 deaths per year from AAA rupture, being the 15th cause of death and the 10th cause of death in men more than 55 years. The rupture risk raises with increased dimeter, with a rupture risk of 30-33% in AAAs with diameter more than 70mm.[3]

During the second half of the 20th century, the incidence and mortality from AAA has been steadily increased.[4] Concerning the incidence of intact AAA repair a nationwide analysis in Sweden showed an increase of 46%, from 33 repairs per 100,000 male population >50 years in 1994-1999 to 48 in 2010-2014. In the female population, the increase was 42%, from 5.7 to 8.1 repairs per 100,000 female population >50 years during the same period. However, in recent years, the incidence of intact AAA repair remained stable at approximately 27 repairs of intact AAAs per 100,000 inhabitants ≥50 years per year.[5] There is evidence also that the death from ruptured AAA are declining, as well as the incidence of repair of ruptured AAAs.

Four large population-based randomized screening trials has been conducted in late 80s and 90s answering different questions in AAA epidemiology, including why to screen for AAA. These trials were performed in UK, Denmark and western Australia.[6–9] All the trials were conducted in men except the Chichester trial which also included women. The trials found that the screening reduced long-term AAA-related mortality and reduced AAA rupture rate. Moreover, the longer the program is implemented the more cost-effective it is. The benefit begins three years after screening and persists up to 15 years. None of the studies showed benefit in terms of all-cause mortality. The UK began AAA screening programme offering one-time ultrasound scan to men 65 years old in 2009 and covered all the country in 2013. In Sweden, screening has gradually been introduced since 2006 and covered all the country in 2015. In USA, screening started by Medicare insurance in 2007.

As concern the screening recommendations, in 2019 the European Society for Vascular Surgery (ESVS) recommended screening with ultrasonography of every men aged 65 (class I, level A).[10] Men with an initial AAA diameter of 2.5-2.9 cm may be regarded for another screening after five to ten years (class IIb, level C). ESVS does not recommend screening for AAA in women (class III, level B). ESVS also recommends that screening at 5-10 years interval may be considered for both sexes with a true peripheral arterial aneurysm, as well as in both sexed 50 years of age and more with a first-degree member of family with aneurysm. The Society of Vascular Surgery (SVS) in 2018 recommended a scan with ultrasonography

for AAAs in both sexes 65 to 75 years old with a history of smoking (1,A).[11] One-time screening is also suggested in both sexes older than 75 years of good health with no previous screening control (2,C). Ultrasound is the preferable modality for screening because it is safe, easy, non-invasive, reproducible, low cost, has no radiation, and effective to detect AAA with a sensitivity of 94%-100% and specificity of 98-100%.[12]

Screening may have other benefits. Collecting data on risk factors can deepen our knowledge about AAA etiology and high risk patients to develop AAA could be screened preferentially.[13] Screening may also offer a better understanding of what influences AAA to expand. A finding such as an ectatic aorta (2.5-2.9cm), which may also be considered as incipient AAA, could also signify increased cardiovascular risk.

In the last ten years the prevalence of AAA measured in population screening programs has been decreased. In England the prevalence has been decreased from 4.9% in the Multicenter aneurysm screening study (MASS) in 1997-1999 to 1.1% in 2016. In Sweden the prevalence has decreased from 3.5% in 1980 to 1.7% in 2010. The reasons why that happens are not fully elucidated, but a possible reason could be the reduced prevalence of smoking, and improved treatment of hyperlipidemia and hypertension.[5] Lederle showed diachronically the rise and fall of cigarette consumption followed by the rise and fall of AAA mortality in a parallel trend.[14]

AAA and risk factors

AAA is a multifactorial disease with several risk factors. The risk factors vary from being genetic to being environmental. Large population-based studies have revealed the association of different risk factors such as smoking, advanced age, male sex, hypertension, hyperlipidemia, coronary artery disease, family history of AAA, height, alcohol consumption, and obesity with the development, growth, and rupture of AAA.[15,16]

Smoking is the greatest modifiable risk factor for AAA, and this association is greater than the linkage between smoking and peripheral athero-occlusive disease. Different components of cigarette smoking, alone or in combination, can affect the formation of AAA.[17] Cigarette

smoking extract stimulates the activation of macrophages by induction of proinflammatory cytokines and proteases.[17] The macrophages release MMPs and provoke a degradation of the elastin and collagen of the aortic tunica media. The production of MMPs by macrophages is also regulated by tissue plasminogen activator which may be activated by smoking.[18] A meta-analysis of 22 prospective studies about the role of smoking on AAA confirmed this strong association.[19] The risk increases 5-fold, 2-fold and 3.3-fold among current, former and ever smokers respectively. Moreover, another finding is the strong dose-response positive relations between cigarette number per day and pack per years with the hazard of AAA. Another outcome of this meta-analysis was the inverse relation between total years of smoking quit and risk of AAA. The risk of AAA was similar with people who never smoked and those who ceased for 25 years.[19]

AAA share many common risk factors with atherosclerotic occlusive disease (AOD), but they are distinct disease entities.[20] The ECM modulation and the role of VSMCs are different between patients with AAA and AOD.[21] Moreover, there is a different gene expression profile and significant pathway alteration, particularly in the upregulation of distinct inflammation pathways.[21] Activation of different pathways may result towards lumen dilatation or stenosis. Aneurysmal formation may be predisposed when the atheromatic plaque is associated with local dilatation, weakening of tunica media, and degradation of elastin, whereas stenotic formation may be predisposed when there is weakening of tunica media without local dilatation, and without elastin degradation.[22] Inflammatory responses that are dominated by IL-6 and IL-8 are abound in AAA rather than in atherosclerotic aortas. Moreover both Th1 and Th2 inflammatory responses prevail in AAA than in AOD.[23]

Diabetes mellitus is a known risk factor for AOD but has paradoxically a protective association with AAA. Diabetic patients have a lower risk of presenting a AAA, and a slower AAA growth rate.[20] This protection shown in large epidemiologic studies has been investigated by studies who examined interactions diabetes-AAA in terms of aortic wall stress, MMPs, and Advanced Glycation End-products (AGE).[24] Aortic wall in diabetes is thicker and according to LaPlace's Law, a thicker wall reduces the aortic wall stress, thus protecting from AAA.[24] MMP2 and MMP9 are also decreased in patients with diabetes mellitus resulting in less destruction of ECM and less collagen loss.[25,26] AGEs are augmented in diabetes and cardiovascular disease. These end-products can provoke cross-links between

proteins, especially between elastin and collagen, and are related with arterial stiffening.[24] That stiffening of the aortic wall results in proteolytic resistance, and thus protection from aneurysmal formation and growth. However there are studies showing an opposite phenomenon by up-regulation of inflammatory mediators by AGE, but this was demonstrated in thoracic aortic aneurysms.[27] Furthermore antidiabetic agents seem to protect independently from AAA.[24]

Men are in greater risk than women to develop AAA, with a prevalence of 4 to 1 in men compared to women, and women seem to be protected from estrogens.[28] Women have a diagnosis of AAA usually 10 years after men, however they have a risk of rupture 3 to 4 times greater than men, but this may reflect an advanced AAA in women because of a smaller aortic diameter baseline.[29] The remodeling of aortic ECM in men is more pronounced than that of women by age, and that may be due to anti-inflammatory, anti-proliferative and anti-oxidative effects of female sex hormones.[30] There are some contradictory results about the role of postmenopausal hormone therapy, and the protective effect of sex female hormones may be less important than the negative associations of smoking, hypertensions, and CAD with AAA.[31] Furthermore the problem is that most of the studies about the effect of female sex hormones in AAA formation, development and rupture, has been studied in animal models, mostly rodent models, which is difficult to translate in the complexity of woman's menstrual cycle and transition to menopause.[30]

AAA and genetic risk factors

Several genetic factors have been described to have an association with AAA and family history of AAA is a well-known risk factor for AAA. In twin populations, it is estimated that the genetic contribution is responsible for 70% of AAA development.[32] Different approaches to establish genetic risk factors is used for such a complex genetic disease, with no mendelian pattern, and with a genetic variant that usually elevates less than two folds the risk for AAA, whereas in classic Mendelian disorders the risk is elevated by hundreds of times. The approaches to study this complex genetic disease are 1) candidate gene association studies, 2) genome-wide association studies (GWAS), 3) genome-wide linkage studies, and 4) genome-wide expression profiling studies using microarrays.

Bradley et al. reviewed nearly 100 candidate gene association studies that investigated 263 genes, and associations were reported in 87 SNPs.[33] Among them, correlations with CDKN2BAS, SORT1, LRP1, IL6R, MMP3, AGTR1, ACE, APOA1 and AAA were found by meta-analyses. In family studies, Shibamura et al and examined 119 families and found two loci, 4q31 and 19q13, to be associated with AAA after correction for sex and the number of subjects with AAA in a family.[34]

In the era of GWAS large volume of AAA and controls are being tested and independently validated. A meta-analysis by jones et al. of all GWAS and a validation study including 10204 cases and 107766 controls found four new AAA specific risk loci.[35] The new identified loci were 1q32.3 (SMYD2), 13q12.11 (LINC00540), 20q13.12 (near PCIF1/MMP9/ZNF335), and 21q22.2 (ERG). A network analysis of this study showed that MMP9 had a key role. This study confirmed the association with five previously described loci: IL6R and CDKN2BAS1/ ANRIL (inflammation, immune function) and SORT1 and LDLR (low-density lipoprotein metabolism), but the heritability of AAA is explained only partially by these mediators. The lead SNPS of the four new genetic loci do not show a cross phenotype with other cardiometabolic phenotypes, adding further evidence of the distinct entity of AAA vs other cardiovascular diseases. AAA is not a mono-genic disorder and the interpretation of the functional effects of these SNPs should be careful, although an extensive series of bioinformatics and lookups were performed.[36] In different network analysis tools, genes such as matrix MMP9, IL6R, and LDLR were central features in the pathway networks.[35] As MMP activity, tissue inflammation, and lipoprotein accumulation are documented features of AAA pathobiology, these findings are promising (**Fig 6., Fig 7.**).[36]

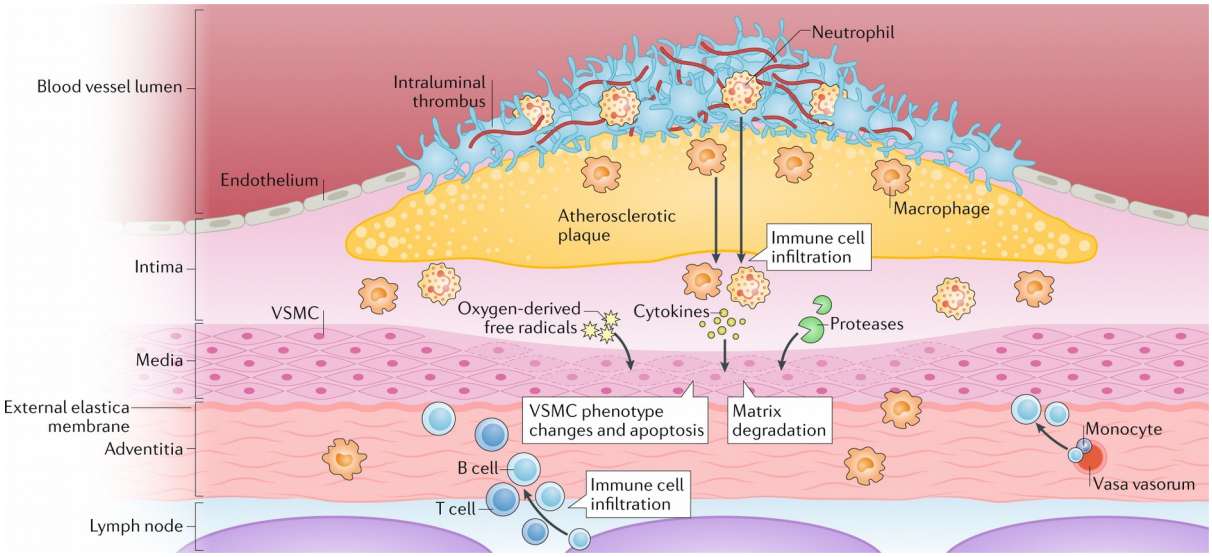

Fig 6. Summary of pathobiology of AAA development (From Golledge et al., Nat Rev Cardiol. 2019)

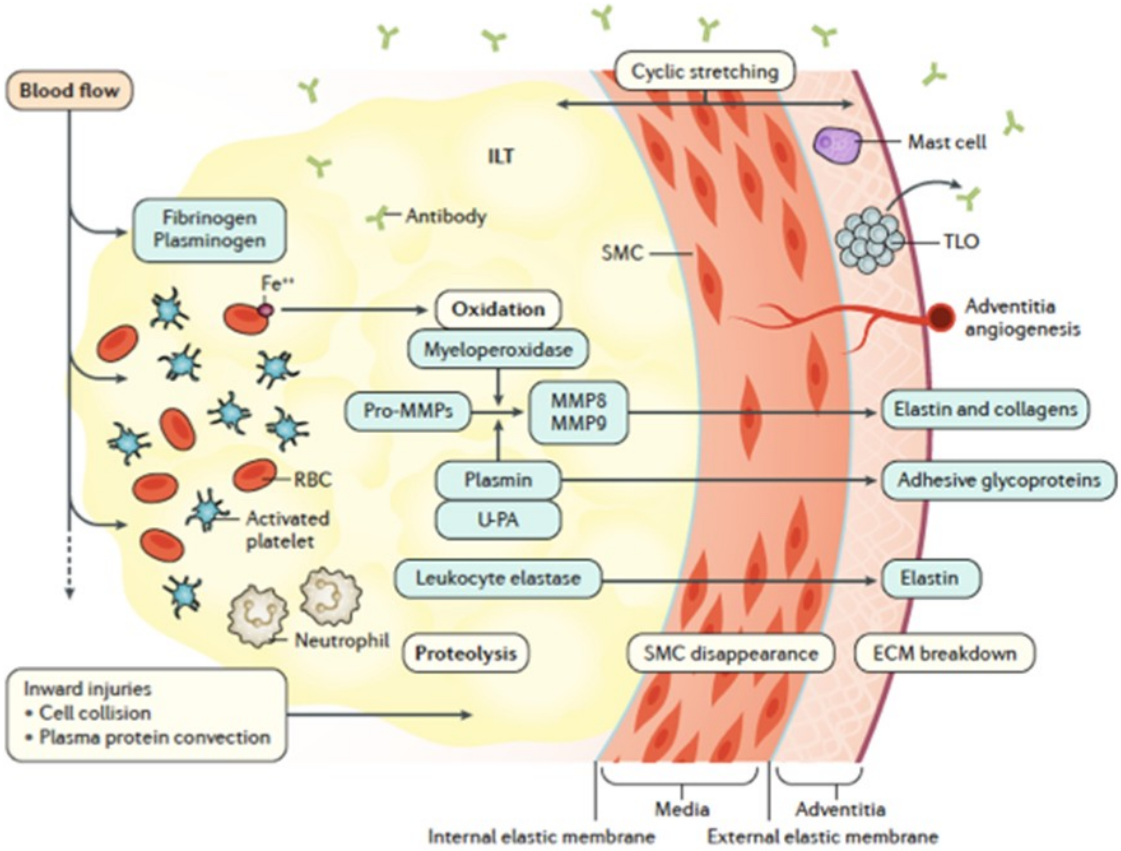

Fig. 7. Summary of pathobiology of AAA development (From Sakalihasan et al, Nat Rev Dis Primers. 2018)

Aim of the Thesis

The aim of the thesis was to assess risk factors for AAAs in our local population. For that reason, we performed a prospective non-randomized case control study. The patient group included 175 men with AAA who were hospitalized at the University Hospital of Larissa and the control group included 166 control men from the local AAA screening programme. Apart from the assessment of the classic risk factors for AAA we investigated the possible association of two genetic markers with AAA. Therefore, we investigated the genotypic and allelic distribution of the MMP9 (-1561C/T) and MMP13 (-77A/G) SNPs between AAA patients and controls. Furthermore, we performed a meta-analysis including all the other genetic studies that examined the association of the SNP MMP13 (-77A/G) with AAA in order to strengthen our results. The aim of the thesis was also to make a systematic review of population-based screening programs, in order to find the prevalence rates in different countries.

Risk factor assessment in a cohort of patients with large abdominal aortic aneurysms

Introduction

Various lesions are provoked by the AAA such as aortic wall inflammation, ECM degradation, apoptosis of SMCs, oxidative damage, and complex atherosclerosis.[37] The precise etiology of AAA is unknown, but it known that different environmental and genetic risk components play a central role at the etiology of AAA.[38] AAAs occur more often in the same family if one member has already the disease, approximately 13% of patients have a first-degree member with AAA,[39] and the estimation of heritability of AAA are 70-80%.[32,39,40] Few genetic susceptibility loci have been so far recognized by family-based DNA linkages studies[34] and GWAS[35].

A central key in the etiology of AAA, which is already mentioned to be complex, is the degradation of ECM. The main enzymes in this process are the MMPs. The MMPs are zinc-containing endopeptidases. These enzymes are found to be rich at the aneurysmal aortic wall. MMPs are implicated on the degradation of numerous components of the ECM, possess different affinities with different substrates, and therefore can be classified based on their principal target. For example, MMP1, MMP8, and MMP13 are called collagenases because their preferential substrate is fibrillar collagens. MMP12 degrades preferentially the elastin, MMP2 and MMP9 can be qualified as elastases but they also degrade different substrates. Furthermore, other MMPs are implicated in the degradation of proteoglycans and matrix glycoproteins. The MMPs are not only responsible for ECM degradation, but they also possess other functions. They modulate, especially, inflammation by regulating the activity of cytokines by the cleavage of their latent pro-domain.

It is recognized that SNPs can impact the *MMPs* transcription and the function of their proteins.[41] This has been shown to be exerted by variations in the promoter region of *MMP*s that alter the tying of transcriptional factors, and thus alter the transcription rate.[42] The *MMP9* and *MMP13* is principally controlled at the transcriptional level. At that level the promoter reacts to various cotrollers.[43,44] In that manner, an SNP which change the level of the

transcription of *MMPs* may influence ECM degradation, and therefore be related with AAA formation.

A prospective case-control study was conducted to research the risk factors for large AAAs in men AAA patients in comparison with control men without aneurysmal or ectatic aortas. We additionally surveyed the relationship between two functional SNPs, *MMP9* (-1561C/T; rs3918242) and *MMP13* (-77A/G; rs2252070), and AAA. Furthermore, we performed a meta-analysis including all the other genetic studies that examined the association of the SNP MMP13 (-77A/G) with AAA. We also took a systematic review of population-based screening programs, in order to find the prevalence rates in different countries and the tendencies of prevalence rates the last twenty years.

Materials and Methods

Our study had the approval of the Committee of Ethics of the University of Thessaly, Medical School of Larissa, Greece. All subjects gave signed informed consent before starting the experiments and the blood sample collections.

We undertook a prospective, non-randomized case-control study with 175 AAA men patients from the Department of Vascular Surgery, University Hospital of Larissa, in central Greece, who presented a large AAA with a maximal diameter of equal or superior to 5.5 cm. These patients undertook open surgical or endovascular repair of their aneurysm. The control group included 166 men who did not present aneurysmal dilatation of their abdominal aorta. The control group was recruited from the local AAA screening programme in the periphery of Thessaly (first screening programme performed in Greece), and the aneurysmal dilatation was excluded during the ultrasound screening. The enrollment of the patients and controls was occurred between January 2010 and May 2013. In the patient group all the aneurysms were detected by computed tomography scans. In case of inflammatory, thoraco-abdominal aneurysms or aortic dissections, these were exclusion criteria for a patient not to participate in the study. The ultrasound screening as well as the collection of the questionnaires were performed by medical doctors from the same Department. The screening was performed in

polyclinics, and the screening subjects were invited by the local communities. The diameter of the aorta was measured by a portable ultrasound equipment (GE Logiq e Ultrasound, Transducer: curved array 2-5 MHz; Wisconsin, USA) using the outer-to-outer method.[45] Controls that had their maximum aortic diameter measured inferior to 2.5 cm were participated.

The characteristics that registered were sex, age, height, weight, body mass index (BMI), family history of AAA, history of inguinal hernia, smoking habits, hypertension, coronary artery disease (CAD), diabetes, hypercholesterolemia, chronic obstructive pulmonary disease (COPD) and stroke. The participants questioned for the presence or not of the aforementioned risk factors or these factors were detected from previous visits to general doctors or other medical specialties.

The work in the laboratory included isolation of the genomic DNA from blood samples. This procedure was performed by a kit (iPrep Pure Linkg DNA Blood Kit, Life Technologies) based on the instruction of the manufacturer. For genotyping the rs3918242 and rs2252070 SNPs of *MMP9* and *MMP13* genes, respectively, these sets of primers were used: rs3918242: forward 5'-GCCTGGCACATAGTAGGCCC-3' and reverse: 5'-CTTCCTAGCCAGCCGGCATC-3'. rs2252070: forward 5'-GATACGTTCTTACAGAAGGC-3' and reverse: 5'-GACAAATCATCTTCATCACC-3'. Thermal cycling conditions were: 40 cycles of denaturing at 95^{o}C for 15 s, annealing at 64^{o}C for 15 s (rs3918242) or 54^{o}C for 15 s (rs2252070) and extension at 72^{o}C for 10 s. An aliquot of every PCR product was digested with restriction enzyme (*SphI* for rs3918242 and *XspI* for rs2252070) at 37^{o}C overnight. 2% agarose gel was used in order to visualize the restriction fragments after being electrophorized.

Statistical analysis

Chi square or Fisher's exact tests were used to evaluate correlations between categorical variables and AAA. The independent samples t-test was utilized to assess the effect of age. The independent prognostic factors for AAA were assessed by a logistic regression model. Significance was reached when two-sided *P* was found inferior of 0.05. Bonferroni correction was used for multiple testing. We combined the results from this study with the results from

two other previous genetic studies and we conducted a meta-analysis with 964 AAA cases and 1004 controls using the dominant and recessive model of inheritance.[42,46] Forest plots were created by this internet instrument: https://www.evidencepartners.com/resources/forest-plot-generator/.

Power Calculations

Power calculations for the *MMP13* SNP were conducted by the Genetic Power Calculator: http://zzz.bwh.harvard.edu/gpc/. An assumption was made that the SNP and the disease locus were in complete linkage disequilibrium and that they present the same allelic frequency, that is the SNP was the disease locus. We assumed a prevalence of AAA to be 0.02. With the dominant inheritance model and α=0.05, we found 28% power for our sample of 175 AAAs and 166 controls. The power increased at 92% in the meta-analysis of 964 AAAs and 1004 controls.

Results

All baseline variables are depicted in **Table 1.** The patients and the controls had similar mean age. Higher height and family history of AAA were more common in patients than in controls. (**Table 1**). Other risk factors more common in patients than in controls were hypertension, CAD, hypercholesterolemia, COPD, and current smoking.

There was not found a deviation of genotype frequencies from the Hardy-Weinberg equilibrium. The genotype (P = .047) and allelic frequencies for SNP rs2252070 of the *MMP13* gene was different in both groups. The G allelic frequency was more common in patients when compared with controls [nominal P = .037; OR (95%CI) 1.40 (1.02–1.93)]. The genotypic and allelic frequencies for the rs3918242 SNP of the *MMP9* gene were not different (**Table 2**). The genetic findings did not stay significantly different when multiple testing was implemented. Furthermore, we performed a meta-analysis by combining the results of this study with two other studies on the rs2252070 of *MMP13* gene with 964 AAAs and 1004 controls. The G allelic frequency was correlated with the disease when using

dominant [(nominal *P* = .013; OR (95%CI) 1.26 (1.05-1.50)] or recessive [(nominal *P* = .013; OR (95%CI) 1.38 (1.07-1.78)] inheritance modes (**Table 3, Fig 7**).

Finally, we performed a multiple logistic regression analysis with AAA as outcome variable. Risk factors that remained significant were family history of AAA (OR =5,00; 95%CI = 1.12-21.05; *P* = .028), hypercholesterolemia (OR = 2.55; 95%CI = 1.51-4.31; *P* <0.001) and smoking (OR = 10.41; 95%CI = 5.57-19.45 *P* <0.001.)-

In order to have a better view of the trends of AAA prevalence we performed, in parallel to this study, a search of the Pubmed database for screening programs that took place from 1998 to 2019 using ‘’abdominal aortic aneurysm’’ and ‘’screening programme’’ as key words. In **Table 4** are summarized the prevalence and attendance rates of these screening programs.

Discussion

We conducted a prospective case-control study in order to research the distribution of risk factors between men AAA patients with aneurysms superior or equal to 5.5cm and men control subjects of similar age. We found that the risk factors associated with AAA were height, AAA family history, hypertension, hypercholesterolemia, CAD, current smoking and COPD. Risk factors that had statistical significance after logistic regression analysis were family history of AAA (OR =5,00; 95%CI = 1.12-21.05; *P* = .028), hypercholesterolemia (OR = 2.55; 95%CI = 1.51-4.31; *P* <0.001) and current smoking (OR = 10.41; 95%CI = 5.57-19.45 *P* <0.001.)

In 1977, first Clifton, described three male siblings with AAA suggesting an autosomal dominant with incomplete penetrance pattern of inheritance.[47] 13% of patients with AAA present in their family a first-degree relative with AAA. [48] There is 25% prevalence among brothers found in the Liège AAA Family Study.[39] In a Danish study that identified 414 twins with AAA from the Danish Twin Regisrty the proband concordance rate in monozygotic twins was 30% (95% CI 20.3–43.3%) and 12% (95% CI 7.0–20.1%) in dizygotic twins.[49] Additive genetic factors can explain nearly 80% and non-shared environmental factors the rest. Akai et al. showed, in a Japanese study, that family history of AAA was an independent

risk factor for rapid growth of small AAAs.[50] They showed that the rapid AAA expansion was more frequent in patients with family history AAA than in those without (4.2mm/y vs 2.0 mm/y, p=0.009). As part of the results of the Danish population-based cross-sectional VIVA-trial, Joergensen et al., found that when AAA family history was present the subjects had larger aortas (20.5mm vs 19.07mm, p<0.0001).[51] First-degree men relatives of AAA subjects had also two-fold higher prevalence than those without positive family history. When the relatives were females the odds ratio was even bigger, with a value of 2.65. The trial included 18614 men.

The association between AAA and high cholesterol has been manifested in different studies.[52] The ARIC study (Atherosclerosis Risk in Community study) found that the OR for AAA was three times elevated in the group of 25% higher cholesterol in comparison with the lowest 25%.[53] AAA share many common risk factors with AOD, but they are distinct diseases.[20] The ECM modulation and the role of VSMCs are different between patients with AAA and AOD.[21] CAD patients have greater prevalence of AAA than those without, and CAD is a risk factor of AAA. Nonetheless hypercholesterolemia is more important in atherosclerosis than in AAA.[54]

Among all the risk factors for AAA current smoking has the higher OR for AAA, with a value of 13.7 between current smokers who smoke twenty cigarettes per day or more with those who have never smoked.[55] The association between tabagism and AAA is more important than the association between tabagism and PAD.[56] A systematic review of large studies (>3 million subjects in total) of outcomes associated to smoking found that the RR for aortic aneurysm in current smokers was three to six, compared with one to two for CAD and cerebrovascular disease.[56] Tabagism provokes a different burden of toxicity at different diseases and the high association between tabagism and AAA may be related in terms of pathobiology at the induction of macrophages who produce MMPs, and this phenomenon is stimulated by smoking.[56]

A prospective population-based cohort study showed that during the 284,969 person-years of follow-up obstructive type spirometry was related with augmented AAA risk. This result was not related to smoking, implicating that COPD may raise independently the AAA risk.[57]

Moreover, a meta-analysis showed that COPD is not related with AAA growth, despite that COPD was independently and positively related with the presence of AAA.[58]

In our study we failed to confirm the negative correlation of diabetes mellitus with AAA as it was demonstrated by other screening programs,[59] likely because of the little size of our study. Diabetes is a known risk factor for PAD, but it acts in a protective manner against AAA. Diabetic patients have a diminished risk of presenting a AAA, and when they present a AAA they have a slower growth rate.[20] The protection has been confirmed by large epidemiological studies, and diabetes-AAA interactions in terms of aortic wall stress, MMPs, and Advanced Glycation End-products (AGE) have been studied.[24]

After thorough literature review, we identified MMP13 and MMP9 as two potential genetic markers of AAA development. Surprisingly, our findings demonstrated that AAA was not correlated with neither of the two studied SNPs, MMP13 or MMP9 ($p>0.5$). These findings contradict the current knowledge on the potential implications of these two markers in triple A formation and development. MMP13 is an enzyme that participates in the degradation of the ECM. It acts via activation of other latent pro-MMPs or by targeting substrates such as gelatin, proteoglycan, type I-III collagens, and others.[60] Studies identified the localization of immunoreactive MMP13 in the medial SMCs in aortic aneurysmal tissue.[61] *MMP13* mRNA expression found to be increased in aneurysmal walls of AAA open repair patients (n=36) compared to non-aneurysmal control autopsies (n=20).[62] On the gene level, it has been found that MMP13 gene expression was significantly increased in AAAs with active F-FDG-positive inflammation sites on the aneurysmal wall as assessed by PET/CT.[63] The SNP -77A/G (rs2252070) of the MMP13 gene is a functional promoter which has been related with abdominal aortic atherosclerosis in young black men.[64] Yoon et al. showed in six independent *in vitro* transfection experiments that the transcriptional activity of A allele constructs was approximately the double compared to G allele constructs on the same position. Two other case control studies investigating the influence of this SNP on AAA development were identified after reviewing the English literature (**Table 4**). [42,46] These studies showed contradictory results. However, we showed that the allele frequency was nominally different between AAA patients and controls. A meta-analysis of the total obtained results combining the three studies together, with almost 1,000 AAAs and 1.000 controls, revealed a significant association between the G allele and AAA under dominant and recessive models of

inheritance. (**Table 4, Fig 7**).

MMP9 also degrades the ECM by targeting type IV collagen and elastin, and in interaction with other MMPs.[60] The rs3918242 is a functional promoter SNP in the *MMP9* gene. Jones et al.[65] showed an association with rs3918242 and AAA. However, all the other studies found no association.[42,44,46,66–68] A meta-analysis on 2,191 cases and 2,013 controls confirmed that there was no association.[41]

In terms of limitations only large AAAs were analyzed and therefore the results may not correspond to the beginning of AAA formation. Moreover, correlations with a small effect size were difficult to be found due to the relatively small size of the study. Furthermore, in our genetic study, significant *P*-values were nominal. Finally, after testing two specific SNPs it is difficult to make conclusions about MMPs in AAA etiology, since epigenetic or other parameters may alter the activity of MMPs.[69]

Table 1. Demographics and Risk factors in AAAs vs no AAAs

Characteristic	AAA Group n=175	Control Group n=166	*P*
Male, %	100	100	
Age, mean± SD, years	72.7 ± 7.6	71.5 ± 7.1	.122
Height, mean ± SD, cm	173 ± 5.6	170 ± 6.6	.001
Weight, mean ± SD, kg	82.3 ± 9.8	80.7 ± 10.5	.153
BMI, mean ± SD	27.5 ± 3.1	27.8 ± 3.4	.479
Fam AAA, %	6.3	1.8	.037
Fam inguinal hernia, %	12.0	13.9	.609
Hypertension, %	76.6	63.9	.010
CAD, %	37.7	26.5	.027
Diabetes, %	12.6	12.7	.982
Hypercholesterolemia, %	60.0	35.5	<.001
COPD, %	18.9	8.4	.005
Stroke, %	10.9	6.6	.163
Current smoker, %	52.0	11.4	<.001
Inguinal hernia, %	19.4	22.9	.434

Abbreviations: BMI, body mass index; CAD, coronary artery disease; COPD, chronic obstructive pulmonary disease; Fam AAA, positive family history of AAA; Fam inguinal hernia, positive family history of inguinal hernia; SD, standard deviation.

Table 2. Genotypic and Allelic frequencies for rs3918242 *MMP9* and rs2252070 *MMP13* SNPs in AAAs and Controls.

Genotype/Allele	AAA n=175 for *MMP9* n=164 for *MMP13*[a]	Controls n=166 for *MMP9* n=161 for *MMP13*[a]	*P*
MMP9			
CC, count (frequency)	133 (.76)	133 (.80)	.68[b]
CT, count (frequency)	40 (.23)	31 (.19)	
TT, count (frequency)	2 (.01)	2 (.01)	
Allele C, count (frequency)	306 (.87)	297 (.90)	
Allele T, count (frequency)	44 (.13)	35 (.11)	.41[c]
MMP13			
AA, count (frequency)	55 (.34)	75 (.47)	.047[b]
AG, count (frequency)	82 (.50)	64 (.40)	
GG, count (frequency)	27 (.17)	22 (.14)	
Allele A, count (frequency)	192 (.59)	214 (.67)	
Allele G, count (frequency)	136 (.42)	108 (.34)	.037[c]

[a]Smaller sample due to technical problems during genotyping; [b]2x3 contingency table analysis with chi-squared distribution, [c]2x2 contingency table analysis with chi-squared distribution.

Table 3. Genotypic and Allelic Distributions from all studies on rs2252070 *MMP13*

Study	MA	Total AAA Cases /Controls	Genotype	n AAA Cases	n Controls	AAAs MAF	Controls MAF	Dominant Model (AA/ AG+GG) OR (95%CI) *P*	Recessive Model (AA+AG/GG) OR (95%CI) *P*
Saracini	G	423/423	AA AG GG	147 186 90	169 194 60	.433	.371	1.25 (0.95–1.65) .118	1.64 (1.14–2.34) .007
Ogata Belgium	G	177/265	AA AG GG	92 69 16	136 103 26	.285	.292	.97 (.67–1.43) .892	.91 (.48–1.76) .786
Ogata Canada	G	200/155	AA AG GG	94 81 25	80 58 17	.328	.297	1.20 (.79–1.83) .389	1.16 (.60–2.23) .658
Current study	G	164/161	AA AG GG	55 82 27	75 64 22	.415	.335	1.73 (1.10–2.71) .016	1.25 (.68–2.29) .480
Combined data	G	964/1,004	AA AG GG	388 418 158	460 419 125	.381	.333	1.26 (1,05–1,50) .013	1.38 (1.07–1.78) .013

Abbreviations: AAA, abdominal aortic aneurysm; MA, minor allele; MAF, minor allele frequency; OR, odds ratio.

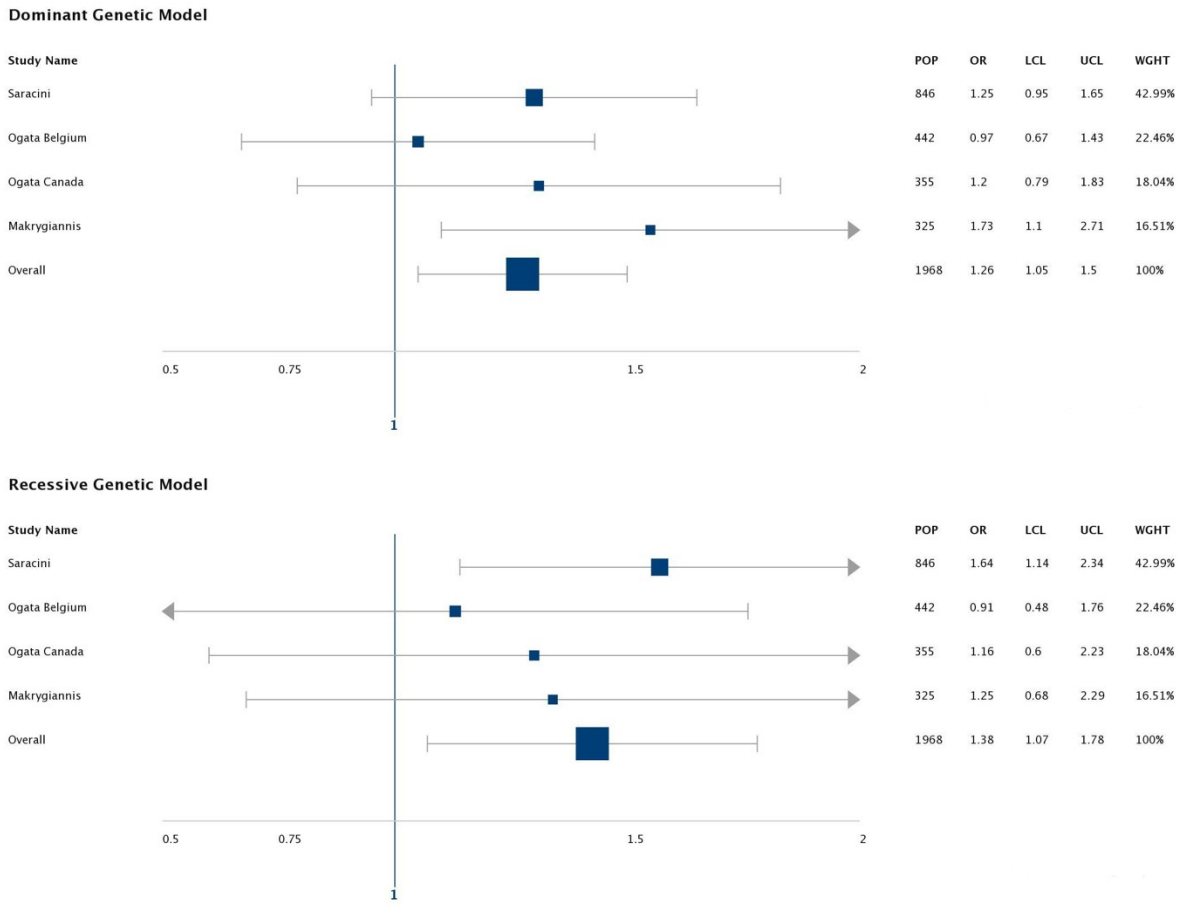

Figure 7. Forest plot illustrating unadjusted ORs and 95% CIs for the correlation between rs2252070 *MMP13* SNP and AAA.
Upper panel shows unadjusted ORs using dominant model of inheritance and lower panel shows results with recessive model of inheritance. The size of the box is proportional to the weight of the study. Abbreviations: LCL, lower control limit; OR, odds ratio; POP, number of cases and controls; UCL, upper control Limit; Wght, weight. Results were generated using online tool https://www.evidencepartners.com/resources/forest-plot-generator/. See Table III for details of the different studies.

Table 4. Review of population-based screening programs for AAA from 1998 to 2019

Author	Year	Country	Age	Sex	Invited	Screened	Acceptance rate	Screened prevalence	AAA diameter
Sandiford et al.	2019	Nea Zealand (Maori)	M: 54-74, F: 65-74	M/F	M: 2467, F: 1526	2507	62.8%	M: 3.6% F: 1.7 % (30mm tresh, F: 2.3% (27mm tresh)	M: ≥30mm F: ≥30mm or ≥27mm
Lindholt et al.	2019	Denmark	65-74	M	16768	10471	62.4%	5.1%	≥30mm (low dose CT scan)
Chun et al. (10 y results)	2019	USA	65-75	M ever smokers (>100 cig)	nr	19649	nr	6.3% (13.5% decrease from the 5y results)	≥30mm
Castro-Ferreira et al.	2019	Portugal	≥65	M	933	715	76.6%	2.1%	≥30mm
Li et al.	2018	China	≥40	M/F (at risk population)	Sample of 12550	5402	nr	0.33% M: 0.55% F: 0.14%	>30mm or ≥50% more than mean diameter
Gianfagna et al.	2018	Italy	M: 50-75, F: 60-75	M/F	5918	3777	63.8%	0.9% M: 1.3% F: 0.3%	≥30mm
Oliver-Williams et al.	2018	UK	65	M	100574	81150	80.7%	In 1991: 5% In 2015: 1.3%	≥30mm
Dahl et al.	2018	Denmark	F: 61, 66, 71, 76 (median)	F	1984	1474	74.3	Age group 1: 0 Age group 1: 0.6 Age group 1: 0.9 Age group 1: 2.1	≥30mm
Lindholt et al.	2017	Denmark	65-74	M	25078	18748	74.7	3.3%	≥30mm
Siso-Almirall et al.	2017	Spain	≥60	M	1367	1024	74.9%	1.5%	≥30mm
Engelberger et al.	2017	Switzerland	65-80	M	1634	745	45.6%	4.2%	≥30mm
Wanhainen et al.	2016	Sweden	65	M	302957	253896	84%	1.5%	≥30mm
Jacomelli et al.	2016	UK	65	M	896287	70000	78.1%	1.34%	≥30mm
Benson et al.	2016	UK	65	M	32119	24851	77%	1.18%	≥30mm
Janwien et al.	2014	Poland	>60	M	nr	1559	nr	6%	≥30mm
Cho et al.	2014	Korea	≥65	M	nr	1609	nr	3.2	≥30mm
Svensjo et al.	2013	Sweden	70	F	6925	5140	74.2%	0.4	≥30mm
Svensjo et al.	2013	Sweden	70	M	2811	2247	79.9%	2.4 (1.5% at 65)	≥30mm
Hager et al.	2013	Sweden	70	M	5623	4721	84.0	2.3	≥30mm
Barba et al.	2013	Spain	65	M	1413	781	70.8	4.7	≥30mm
Chun et al. (5y results)	2013	USA	65-75	M ever smokers (>100 cig)		9751		7.1	≥30mm
Conway et al.	2011	UK	65	M	6091	4216	69.2	1.6	≥30mm
Badger et al.	2011	UK	65-75	M	13316	5931	44.5	5.4	≥30mm
Svensjo et al.	2011	Sweden	65	M	26256	22139	85	1.7	≥30mm
Palombo et	2010	Italy	65-92	M/F	15151	8243	54.3	6.2 (10.8M, 1.1F)	≥30mm

al.									
Schermerhorn et al.	2008	USA	≥65	M/F	30000	2005	6.7	2.8M, 0.2F	≥30mm
Laws et al.	2006	UK	65-80	M	4000	2870	71.7	4.1	≥30mm
Duncan et al	2005	UK	65-74	M	9323	8355	89.6	5.1	≥30mm
Lindholt et al.	2005	Denmark	64-73	M	6333	4860	76.6	4.0	≥30mm
Norman et al.	2004	Australia	65-83	M	17516	12203	70	7.2	≥30mm
Ashton et al.	2002	UK	65-74	M	33839	27147	80	4.9	≥30mm
Scott et all	2002	UK	65-80	F	4682 (7887T)	3052 (5394T)	65.2 (68.4T)	1.3F 7.6M (male branch of the study)	≥30mm
Vasquez et al.	1998	Begium	65 and 75	M	1773	727	41	3.8	>30mm

Conclusions and Perspectives

We investigated the risk factors for large abdominal aortic aneurysms in a Greek cohort of patients**.** Family history of AAA, hypercholesterolemia, and current smoking were correlated with large AAA in men. The meta-analysis with nearly 1,000 AAA cases and 1,000 controls

revealed that the G allele of rs2252070 in *MMP13* was correlated with AAA in both dominant and recessive models of inheritance.

International consortia with large data sets have found susceptibility genetic loci. Jones et al. did a meta-analysis of GWAS and found 5 previews and 4 new loci. Relationship found with immune system, LDL metabolism, and aneurysm biology. SNPs are the tools to identify genetic loci regions in human genome. The genes with functional variants could be in distance from the markers. Genetic loci contain many genes. It will require a lot follow-up work and further investigation to establish which proteins are involved.

AAA is an age-related and deadly disease with a chronic natural history. The main risk of AAA without treatment is progression, expansion, and eventual rupture. Not all AAAs have similar diameters when rupture occurs, there are large AAAs that do not rupture and small AAAs that rupture. Thorough understanding of environmental and genetic risk factors may help to create targeted screening programs of AAA assesement. It could also help to understand the mechanisms behind more susceptible AAA. That could offer a possibility to generate drugs that stabilize and may even decrease the progression of AAAs.

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