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Oxidative stress-related factors in abdominal aortic aneurysm: potential clinical implications

SAKSHI VATS | CLINICAL SCIENCES, MALMÖ | FACULTY OF MEDICINE | LUND UNIVERSITY

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SAKSHI VATS joined the Center for Primary Healthcare Research (CPF) laboratory at Lund University as a PhD student in 2019. She has a background in pre-clinical research, holding a master's degree in molecular medicine from Imperial College London, United Kingdom. In her present thesis, she has investigated a vascular disease called abdominal aortic aneurysm (AAA) by examining certain oxidative stress-related factors associated with it. She is enthusiastic about employing high-throughput research techniques to explore how genetic-molecular factors interact with or modify the effects of clinical-environmental risk factors in multifactorial diseases like AAA. In addition to her research, Sakshi has actively participated in administrative committees at the faculty and has served as both chair and vice-chair of the Medical Doctoral Student Union (MDR) at Lund University.

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Oxidative stress-related factors in abdominal aortic aneurysm: potential clinical implications

Oxidative stress-related factors in abdominal aortic aneurysm: potential clinical implications

Sakshi Vats
साक्षी वत्स

DOCTORAL DISSERTATION

Doctoral dissertation for the degree of Doctor of Philosophy (PhD) at the Faculty of Medicine at Lund University to be publicly defended on 10th of November at 13.00 in **Agardh Hall, CRC, Malmö**

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Abstract:

Abdominal aortic aneurysm (AAA) is a mostly silent vascular disease, with a rupture-related mortality rate exceeding 80%. To aid in future diagnosis and prognosis efforts, the studies included in the present thesis have explored the role of specific oxidative stress-related factors in the context of AAA using two population-based cohorts: AAA screening cohort of 65-year-old males (n = 421) and the Malmö diet and cancer study (MDCS) (n = 25252). In **Paper I**, global DNA methylation (enzyme-linked immunosorbent assay (ELISA)) and homocysteine (diagnostic enzymatic assay) levels were investigated in the AAA screening cohort. Increased global DNA methylation and homocysteine levels were observed in AAA, with positive linear associations with baseline aortic diameter. However, no significant association was found with AAA growth, suggesting different mechanisms in initiation and progression. **Paper II** characterizes a novel mitochondrial genetic landscape in AAA from 48 cases and 48 matched controls from the AAA screening cohort. Whole mitochondrial genome sequencing revealed differential mutational landscapes in AAA as compared to the non-AAA group. MtDNA mutational load was significantly elevated in AAA, particularly in the regulatory and conserved region (MT-TAS2). Lower mitochondrial copy number exacerbated the effect of mtDNA mutation in AAA. A novel 24 bp mtDNA duplication was found exclusive to AAA cases, and the northern-European JTU mitochondrial haplogroup was associated with family history of AAA. **Paper III** explores genetic variations in oxidative stress-related genes and antioxidant vitamin intake in incident intact AAA and ruptured AAA (rAAA) in the MDCS cohort. A variant in *NOX3* gene was associated with higher rAAA risk, while antioxidant vitamins, riboflavin, and folate reduced intact AAA incidence. Both the studied genetic background and sex significantly modified the effect of specific vitamins' intake on intact AAA risk ,overall, suggesting a complex gene-sex-vitamin interplay in AAA. **Paper IV** investigates hemoglobin (Hb)/heme and their scavengers in the AAA screening cohort using colorimetric assays including ELISAs, droplet digital PCR (ddPCR), and allelic discrimination assay. Elevated plasma heme levels and reduced heme oxygenase-1 (HO-1) levels were associated with AAA, independent of potential confounders. The addition of heme to a previous diagnostic model improved it significantly. Whole blood Hb and plasma hemopexin (Hpx) levels were associated with AAA growth rate. Overall these findings indicate the disruption of heme homeostasis in AAA. These collective findings from the thesis underscore the multifaceted nature of oxidative stress-related factors in AAA pathogenesis, emphasizing their potential roles in diagnosis, prognosis, and potential therapeutic interventions. Future research endeavors are encouraged to continue investigating these complex factors and their interplay for reliable clinical translation.

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Oxidative stress-related factors in abdominal aortic aneurysm

Potential clinical implications

Sakshi Vats
साक्षी वत्स

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Cover photo by Jasper AI tool (concept by Sakshi Vats)
The larger red balloon soaring above a cluster of smaller white balloons symbolizes an abdominal aortic aneurysm. The precise factors leading to this deviation from the "norm" and the future trajectory of this red balloon remain shrouded in uncertainty.

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To my beautiful Baba

Clouds come floating into my life, no longer to carry rain
or to usher storm, but to add color to my sunset sky

Rabindranath Thakur

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Abstract

Abdominal aortic aneurysm (AAA) is a mostly silent vascular disease, with a rupture-related mortality rate exceeding 80%. To aid in future diagnosis and prognosis efforts, the studies included in the present thesis have explored the role of specific oxidative stress-related factors in the context of AAA using two population-based cohorts: AAA screening cohort of 65-year-old males (n = 421) and the Malmö diet and cancer study (MDCS) (n = 25252). In **Paper I**, global DNA methylation (enzyme-linked immunosorbent assay (ELISA)) and homocysteine (diagnostic enzymatic assay) levels were investigated in the AAA screening cohort. Increased global DNA methylation and homocysteine levels were observed in AAA, with positive linear associations with baseline aortic diameter. However, no significant association was found with AAA growth, suggesting different mechanisms in initiation and progression. **Paper II** characterizes a novel mitochondrial genetic landscape in AAA from 48 cases and 48 matched controls from the AAA screening cohort. Whole mitochondrial genome sequencing revealed differential mutational landscapes in AAA as compared to the non-AAA group. MtDNA mutational load was significantly elevated in AAA, particularly in the regulatory and conserved region (MT-TAS2). Lower mitochondrial copy number exacerbated the effect of mtDNA mutation in AAA. A novel 24 bp mtDNA duplication was found exclusive to AAA cases, and the northern-European JTU mitochondrial haplogroup was associated with family history of AAA. **Paper III** explores genetic variations in oxidative stress-related genes and antioxidant vitamin intake in incident intact AAA and ruptured AAA (rAAA) in the MDCS cohort. A variant in *NOX3* gene was associated with higher rAAA risk, while antioxidant vitamins, riboflavin, and folate reduced intact AAA incidence. Both the studied genetic background and sex significantly modified the effect of specific vitamins' intake on intact AAA risk, overall, suggesting a complex gene-sex-vitamin interplay in AAA. **Paper IV** investigates hemoglobin (Hb)/heme and their scavengers in the AAA screening cohort using colorimetric assays including ELISAs, droplet digital PCR (ddPCR), and allelic discrimination assay. Elevated plasma heme levels and reduced heme oxygenase-1 (HO-1) levels were associated with AAA, independent of potential confounders. The addition of heme to a previous diagnostic model improved it significantly. Whole blood Hb and plasma hemopexin (Hpx) levels were associated with AAA growth rate. Overall these findings indicate the disruption of heme homeostasis in AAA. These collective findings from the thesis underscore the multifaceted nature of oxidative stress-related factors in AAA pathogenesis, emphasizing their potential roles in diagnosis, prognosis, and potential therapeutic interventions. Future research endeavors are encouraged to continue investigating these complex factors and their interplay for reliable clinical translation.

Popular science summary

Imagine living with a ticking time bomb in your body, unaware. This is the reality for most patients with abdominal aortic aneurysm (AAA). In this condition, the aorta (largest blood vessel in the human body) passing through the abdomen, undergoes a progressive weakening and expands in size. AAA is often silent until it is too late, with a rupture-related mortality rate that exceeds a daunting 80%. The only treatment option available today is surgical, and that too for individuals with larger AAAs. Early detection through extensive screening programs is not universally available and it is hard to predict complications. We urgently require improved and more practical disease markers and treatment alternatives.

The papers included in the present thesis project have studied the role of specific oxidative stress-related factors in AAA. First, let's demystify "oxidative stress." Imagine your body as a bustling city, with cells working tirelessly to maintain harmony. Oxidative stress is like the city's air pollution, caused by harmful molecules called free radicals. Like the trees, certain defense mechanisms in our body help counter the free radicals. However, in cases of severe imbalance, these free radicals can wreak havoc within your cells, damaging DNA and proteins, and ultimately leading to or worsening diseases like AAA.

Our first paper looked at the level of methylation, a molecular switch that can turn genes on or off. In this study, we discovered that men with AAA have higher levels of DNA methylation in their blood compared to those without AAA. In addition, another molecule called homocysteine, previously associated with vascular diseases and oxidative stress, was found to be elevated in AAA cases. However, strangely, these factors did not seem to influence the growth of AAA, thus hinting at different causes for initiation and progression.

In our second paper, we delved deep into the tiny powerhouses of our cells, called mitochondria. Fun fact about mitochondria: they carry their own special DNA - many copies of them. Mitochondrial dysfunction has long been suspected in AAA development. Through advanced genetic analysis, we uncovered a novel landscape of genetic alterations in the mitochondrial DNA of AAA patients. These genetic changes, particularly in the regulatory parts of mitochondria, could be a critical missing piece of the AAA inheritance puzzle. Interestingly, a particular mitochondrial lineage marker, JTU, was linked to a family history of AAA. These findings offer unprecedented insights into the genetic landscape of AAA.

In the third paper, we investigated the intricate relationship between our oxidative stress-related genes and antioxidant vitamins in the development and rupture of

AAA. A particular genetic variation was found to increase the risk of AAA rupture, especially in males. On the other hand, a higher intake of antioxidant vitamins like riboflavin and folate was associated with a reduced risk of intact AAA. Interestingly, these vitamin-AAA associations were influenced by both genetic variations and sex, which hints at personalized treatment possibilities.

In our last paper, we studied the role of hemoglobin and heme, iron-rich molecules in our blood that promote oxidative stress if not kept in check by their scavengers. We found that AAA patients had altered levels of these molecules, with elevated heme and lowered levels of heme-oxygenase-1 (HO-1). These changes were linked to the presence of AAA, independent of other factors. Combining heme and HO-1 as diagnostic markers improved our ability to identify AAA cases. Additionally, hemoglobin and scavenger, hemopexin levels were associated with the expansion rate of AAA, opening new avenues for predicting disease progression.

In conclusion, this thesis project has unveiled the complex interplay of some conventional and unconventional oxidative stress-related factors in AAA. DNA methylation, mitochondrial genetics, antioxidant vitamins, and hemoglobin-related molecules all seem to play unique roles in the initiation, progression, and rupture of AAA, and all require further research. As research into personalized disease factors and therapy advances, AAA may evolve from being a mystery into a controllable challenge, ultimately improving the quality of life and prognosis for those affected.

Populärvetenskaplig sammanfattning

Föreställ dig att leva omedveten om en tickande bomb i kroppen. Detta är verkligheten för de flesta patienter med abdominellt aortaaneurysm (AAA). Tillståndet innebär att aortan (den stora kroppspulsådern) som passerar genom buken, genomgår en progressiv försvagning och expanderar i storlek. AAA är ofta utan symtom tills det är för sent. Rupturrelaterad dödlighet överstiger skrämmande 80%. Det enda behandlingsalternativet som finns tillgängligt idag är kirurgi, vilket enbart är tillgängligt för individer med större AAA. Tidig upptäckt genom omfattande screeningprogram är inte allmänt tillgänglig och det är svårt att förutse komplikationer. Det krävs akut förbättrade och mer praktiska sjukdomsmarkörer och behandlingsalternativ.

Arbeterna som ingår i denna avhandlinghar studerat rollen av specifika oxidativ stress-relaterade faktorer i AAA. Låt oss först avmystifiera "oxidativ stress". Föreställ dig din kropp som en livlig stad, med celler som arbetar outtröttligt för att upprätthålla harmoni. Oxidativ stress är som stadens luftföroreningar, orsakade av skadliga molekyler som kallas fria radikaler. Precis som träden i staden hjälper mot luftföroreningar, hjälper vissa försvarsmekanismer i vår kropp att motverka de fria radikalerna. Men i fall av allvarlig obalans kan dessa fria radikaler orsaka förödelse i celler, skada DNA och proteiner, vilket i slutändan leder till eller förvärrar sjukdomar som AAA.

Det första arbetet tittade på nivån av metylering, en molekylär switch som kan slå på eller stänga av gener. I den här studien upptäckte vi att män med AAA har högre nivåer av DNA-metylering i blodet jämfört med de utan AAA. Dessutom visade sig en annan molekyl som heter homocystein, som tidigare associerats med kärlsjukdomar och oxidativ stress, är förhöjd i fall med AAA. Konstigt nog verkade dessa faktorer inte påverka tillväxten av AAA, vilket antydde andra orsaker till initiering och progression.

I vårt andra arbete grävde vi djupt in i våra cellers små kraftverk, kallade mitokondrier. Intressant fakta om mitokondrier: de bär på sitt eget speciella DNA, många kopior av dem. Mitokondriell dysfunktion har länge misstänkts vid AAA-utveckling. Genom avancerad genetisk analys avslöjade vi ett nytt landskap av genetiska förändringar i mitokondriellt DNA hos AAA-patienter. Dessa genetiska förändringar, särskilt i de regulatoriska delarna av mitokondrierna, vilket kan vara en viktig saknad pusselbit i del av AAA-arvspusslet. Intressant nog var en speciell mitokondriell härstamningsmarkör, JTU, kopplad till en familjehistoria av AAA. Dessa fynd ger oöverträffade insikter i AAA:s genetiska landskap.

Det tredje arbetet undersökte vi intrikata förhållandet mellan våra gener som är relaterade till oxidativ stress och antioxidantvitaminer i utveckling och ruptur av AAA. En särskild genetisk variation visade sig öka risken för AAA-ruptur, särskilt hos män. Högre intag av antioxidantvitaminer som riboflavin och folat var associerat med en minskad risk för intakt AAA. Intressant nog påverkades samband mellan vitamin- och AAA av både genetiska variationer och kön, vilket tyder på personliga behandlingsmöjligheter.

I det sista arbetet studerade vi rollen av hemoglobin och hem, järnrika molekyler i vårt blod som främjar oxidativ stress om de inte hålls i schack av kroppens renhållningsystem. Vi fann att AAA-patienter hade förändrade nivåer av dessa järnrika molekyler, med förhöjt hem och sänkta nivåer av hem-oxygenas-1 (HO-1). Dessa förändringar var kopplade till närvaron av AAA oberoende av andra faktorer. Att kombinera hem och HO-1 som diagnostiska markörer förbättrade vår förmåga att identifiera AAA. Dessutom associerades hemoglobin- och scavenger-, hemopexinnivåer med expansionshastigheten för AAA, vilket öppnar nya vägar för att förutsäga sjukdomsprogression.

Sammanfattningsvis har denna avhandling avslöjat det komplexa samspelet mellan några konventionella och okonventionella oxidativ stress-relaterade faktoreri AAA. DNA-metylering, mitokondriell genetik, antioxidantvitaminer och hemoglobinrelaterade molekyler verkar alla spela unika roller i initieringen, progressionen och bristningen av AAA, vilket kräver ytterligare forskning. I takt med att forskningen om personliga sjukdomsfaktorer och terapi går framåt kan AAA utvecklas från att vara ett mysterium till en kontrollerbar utmaning, vilket i slutändan förbättrar livskvaliteten och prognosen för de drabbade.

List of papers

Paper I

Associations of global DNA methylation and homocysteine levels with abdominal aortic aneurysm: A cohort study from a population-based screening program in Sweden

Vats S**,** Sundquist K, Wang X, Zarrouk M, Ågren-Witteschus S, Sundquist J, Gottsäter A, Memon AA

International Journal of Cardiology 2020, 321:137-142

Paper II

Characterization of the Mitochondrial Genetic Landscape in Abdominal Aortic Aneurysm

Vats S, Sundquist K, Li Y, Wang X, Hong MG, Sundquist J, Zarrouk M, Gottsäter A, Memon AA

Journal of the American Heart Association 2023, 12(8):e029248

Paper III

Oxidative stress-related genetic variation and antioxidant vitamin intake in intact and ruptured abdominal aortic aneurysm: a Swedish population-based retrospective cohort study

Vats S, Sundquist K, Sundquist J, Zhang N, Wang X, Acosta S, Gottsäter A, Memon AA

European journal of preventive cardiology 2023,; doi: 10.1093/eurjpc/zwad271.

Paper IV

Hemoglobin Homeostasis in Abdominal Aortic Aneurysm: Diagnostic and Prognostic Potential of Hemoglobin/Heme and Scavenger Molecules

Vats S, Sundquist K, Grundberg A, Sundquist J, Wang X, Zarrouk M, Gottsäter A, Memon AA

Manuscript submitted.

List of papers not included in this thesis

Mitochondrial DNA Copy Number: Linking Diabetes and Cancer
Memon AA, Vats S, Sundquist J, Li Y, Sundquist K
Antioxidants & Redox Signal. 2022 Dec;37(16-18):1168-1190

Mitochondrial heteroplasmic shifts reveal a positive selection of breast cancer
Li Y, Sundquist K, Vats S, Hong M, Wang X, Chen Y, Hedelius A, Saal HL, Sundquist J, Memon AA
Manuscript in press. Journal of Translational Medicine.

Abbreviations

AAA	Abdominal aortic aneurysm
ABI	Ankle-brachial index
ATP	Adenosine triphosphate
AUC	Area under the curve
BMI	Body mass index
CAD	Coronary artery disease
CI	Confidence interval
CRP	C-reactive protein
CSTB	Cystatin B
CT	Computed tomography
CV	Coefficient of variation
CVD	Cardiovascular disease
DP	Depth (sequencing)
ECM	Extra cellular matrix
EDTA	Ethylenediaminetetraacetic acid
EIF2C1	Eukaryotic translation initiation factor 2c1
ELISA	Enzyme-linked immunosorbant assay
EVAR	Endovascular aneurysm repair
GDF15	Growth differentiation factor 15
GWAS	Genome-wide association study
Hb	Hemoglobin
HDL	High-density lipoprotein
HF	Heteroplasmic fraction
HO	Heme oxygenase-1
HP	Haptoglobin
Hpx	Hemopexin
HR	Hazards ratio
HRP	Horseradish peroxidase
ILT	Intraluminal thrombus

LDL	Low density lipoprotein
LELE	Leading edge to leading edge
MAF	Minor allele frequency
MDCS	Malmö diet and cancer cohort
MMP	Matrix metalloproteinase
MPO	Myeloperoxidase
MtDNA-CN	Mitochondrial DNA copy number
MTHFR	Methylenetetrahydrofolate reductase
MTL	Mitochondrial DNA amplicon
MT-TAS2	Mitochondrial extended termination-associated sequence
NA	Not applicable
NADPH	Nicotinamide adenine dinucleotide phosphate
NCBI	National Center for Biotechnology Information
ND1	Nadh dehydrogenase subunit 1
NOS	Nitric oxide synthase
NOX3	Nadph oxidase 3
OD	Optical density
OR	Odds ratio
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
QC	Quality control
rAAA	Ruptured AAA
RNS	Reactive nitrogen species
ROC	Receiver operating characteristic
ROS	Reactive oxygen species
SNP	Single nucleotide polymorphism
TG	Triglyceride
VCF	Variant call format
VSMC	Vascular smooth muscle cells

Introduction

In recent decades, healthcare and scientific research have reached new milestones, increasing overall survival in multiple pathologies. However, a lot depends on earlier detection and there are still challenges when it comes to targeted disease treatment. Identification and study of personalized disease-related factors can help overcome the present challenges in disease diagnosis, prognosis, and treatment. In the present thesis, we delve into such disease-related factors in the context of a vascular disease described below.

Abdominal aortic aneurysm: Definition

Cardiovascular diseases (CVDs) are a leading cause of mortality worldwide, with conditions like coronary artery disease (CAD) and stroke often taking center stage. However, there is another silent and potentially life-threatening condition that often goes unnoticed until it is too late - Abdominal aortic aneurysm (AAA). It is a vascular disorder characterized by a localized dilation of the abdominal aorta [1] (Figure 1). The aorta is the largest blood vessel in the human body, which originates from the heart, extends through the chest, and descends into the abdomen, where it supplies oxygenated blood to various organs and tissues. While the term "aneurysm" itself comes from the Greek word "aneurysma," meaning "a widening/an opening," it was only in more recent centuries that physicians began to differentiate between various types of aneurysms, including AAAs. Arterial aneurysms in general occur most commonly in the aorta, however, aneurysms can also occur in peripheral arteries.

The dilation in AAA most often occurs in the infra-renal (below the kidneys) part of the aorta [1]. There are other morphological and location-based types of aortic aneurysms but those are beyond the scope of the present thesis. When a segment of the abdominal aortic wall weakens and dilates to 30 mm (usually > 2 standard deviations over average diameter in both men and women) or at least 50% the size of its normal diameter (20 mm), it is classified as an AAA [1,2]. In European men, a threshold of 30 mm is suggested which may be lower for the Asian population and females [1]. Another suggested definition of AAA is when the maximum infra-renal aortic diameter is at least 1.5 times the expected normal diameter, mainly to account for inter-individual variation in the normal aortic diameter [3]. The expansion or

growth of an AAA is irreversible with progressive weakening of the aortic wall [4]. While it can remain asymptomatic for extended periods, ruptured AAA (rAAA) carries a high mortality rate, making AAA a matter of global health concern [5].

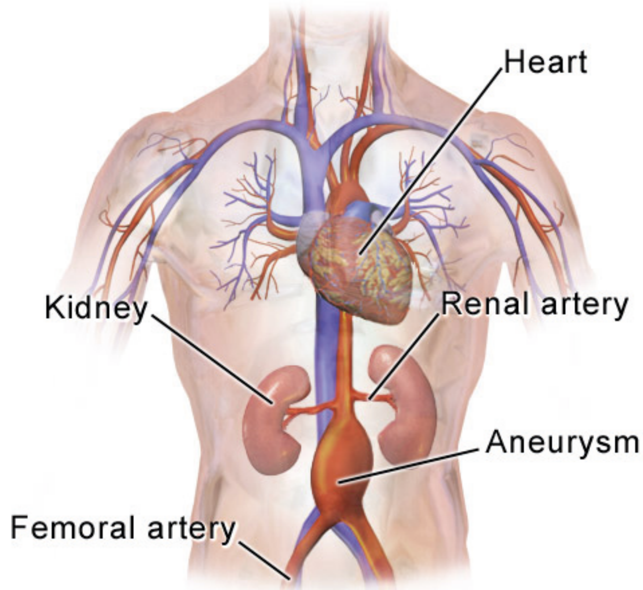

Figure 1. Depiction of an Infra-renal abdominal aortic aneurysm. "File:Abdominal Aortic Aneurysm Location.png" by Bruce Blaus is licensed under CC BY-SA 4.0.

Epidemiology

Abdominal Aortic Aneurysm (AAA) represents a significant public health concern due to its potentially life-threatening nature. The distribution and presentation of AAA and its complications differ considerably based on geographical location, age and sex. A brief overview of AAA burden and risk factors is provided below.

Disease burden

Disease prevalence is defined as the proportion of individuals (from total individuals at risk) affected by a disease at a specific time point and disease incidence is defined as the proportion of newly identified cases occurring within a defined population

over a specified period. Both measures are crucial for healthcare planning, resource allocation, understanding disease trends, and evaluating the effectiveness of public health interventions. A recent systematic review of cross-sectional studies from 2000 - 2021, with around 6.7 million participants (aged 30 - 79 years) from 19 different countries, reported a global AAA prevalence of 1.46% (95% confidence interval (CI) = 1.04 - 2.05) in males and 0.39% (95 % CI = 0.27 - 0.56) [6]. Population-based screening studies provide a good way to estimate the prevalence of AAA, however, these estimates are naturally limited to places with active screening programs and the targeted demographic group (mainly elderly males). A Swedish national screening program reported a screening-detected AAA prevalence of 1.5% in 65-year-old men (2006 - 2014) [7]. Before screening became popular, autopsy studies had provided valuable data on AAA prevalence, where one such Swedish study from the early 1990s reported an age-standardized 4.3% prevalence in males and 3% in females, which is much higher than the current trend [8]. Decrease in smoking, healthier lifestyle choices and efficient CVD treatment are often considered as the main reasons behind the declining AAA prevalence [9]. The majority of the AAA cases detected during screening have a diameter < 5.5 cm [7] which is the widely accepted threshold for consideration of prophylactic aortic aneurysm surgery in men [1]. The mean AAA growth rate for small AAAs has been described to be around 2 - 3 mm/year in most studies over the last decade [10].

Obtaining accurate epidemiological data on ruptured abdominal aortic aneurysm (rAAA) cases presents some challenges because of missed diagnoses and out-of-hospital deaths. However, the reported incidence of rAAA ranges from 5.6 to 17.5 per 100,000 person-years, with an overall mortality rate for rAAA over 80%. Even though intact AAAs occur at around four times greater frequency in males than in females, this difference is only 2:1 when it comes to rAAA, indicating that female intact AAAs might miss diagnosis and hence, not be repaired in time for prevention [11,12]. Females also have fast-growing AAAs, a greater risk of rupture of smaller AAAs and worse outcomes after surgical repair [13,14].

Risk factors

Several modifiable and non-modifiable risk factors contribute to the development of AAA and its complications. Non-modifiable risk factors include sex, age, ethnicity, family history, etc., whereas modifiable risk factors are smoking, hypertension, diet, and physical activity. The risk factors that can be modified could aid in disease prevention and treatment, while investigating non-modifiable risk factors can help with risk stratification and disease management strategies. It is important to note, however, that the modifiable and non-modifiable risk factors do not act in isolation, and often interact with each other. Therefore, a comprehensive understanding is important to clarify aspects of disease pathology and treatment.

AAA is more prevalent in individuals over the age of 65, with the risk increasing as people get older [15]. Moreover, AAA is more common in men than in women, with males being around four times more likely to develop the condition, but the difference is less prominent for rAAA, and the growth rate is elevated in females[12,13]. Men, especially when defined with a threshold of ≥ 30 mm, face a 3- to 16-fold increased risk of AAA, and suggestions have been made to lower the threshold in females and to account for body size [1,16]. Various studies, both clinical and in animal models, have explored sex disparities in AAA pathogenesis, with a focus on sex hormones and sex chromosomes [17]. This underscores the importance of considering gender-specific factors when assessing risk.

Smoking is a particularly significant risk factor for AAA with a meta-analysis estimated overall effect size of Odds ratio (OR) = 2.97 (1.20 - 7.30), $P = 0.018$ [18]. Smoking is associated consistently with AAA growth rate and rupture risk along with the presence of intact AAA [4,19-21]. Potential mechanisms by which smoking (tobacco-nicotine exposure) affects the initiation, growth and rupture of AAA, are proposed to be cellular senescence [22], genetic and epigenetic modifications [23-25] and induction of inflammatory pathways [26]. Cessation of smoking has been found to be one of the most effective ways to reduce the risk of intact AAA and rAAA, as well as slowing of AAA growth rate [27-29].

Studies have estimated, more than 70% hereditary component in AAAs, such that individuals with a positive family history of AAA are at higher risk [30,31]. Previous genetic studies have implicated polymorphisms in genes involved in extracellular matrix remodeling, inflammation, and oxidative stress in AAA [32,33]. However, despite great efforts through GWAS and genomics studies, a large chunk of the hereditary information is still missing; hence combining genetic and clinical-lifestyle information is recommended for reliable risk stratification [32,33].

High blood pressure (hypertension) is another established risk factor for AAA [21,34]. The constant stress exerted on the aorta's walls by high blood pressure can lead to weakening and the formation of an aneurysm [4]. However, pharmacological evidence on the benefit of anti-hypertensive medication on AAA risk has been scarce [35]. Dyslipidemia or elevated levels of cholesterol, triglycerides and lipoproteins have also been associated with AAA, like atherosclerosis [36]. During an atherosclerotic process, the buildup of fatty deposits in the arteries or plaque accumulation and inflammation can weaken and enlarge the vessel, thus increasing the risk of an aneurysm. Statins or lipid-lowering therapy has been associated with growth reduction in AAA [37], but the current evidence is contradictory. Statins, however, have been shown to confer mortality reduction after AAA repair [38]. Interestingly, diabetes has a negative association with AAA, which has lately partly been attributed to the action of anti-diabetic drug metformin [39,40]. Metformin has been shown to alleviate the AAA growth and rupture risk [40].

Certain racial and ethnic groups, particularly those of European descent, have a higher risk of developing AAA compared to others than the Asian or African population [41]. Disparities in racial access to AAA screening and treatment may partly explain these prevalence differences but require further research.

Apart from these traditional risk factors of AAA (depicted in Figure 2), studies have also shown associations with CVDs [42], obesity [43], physical activity [44], socioeconomic factors [45] and diet [46].

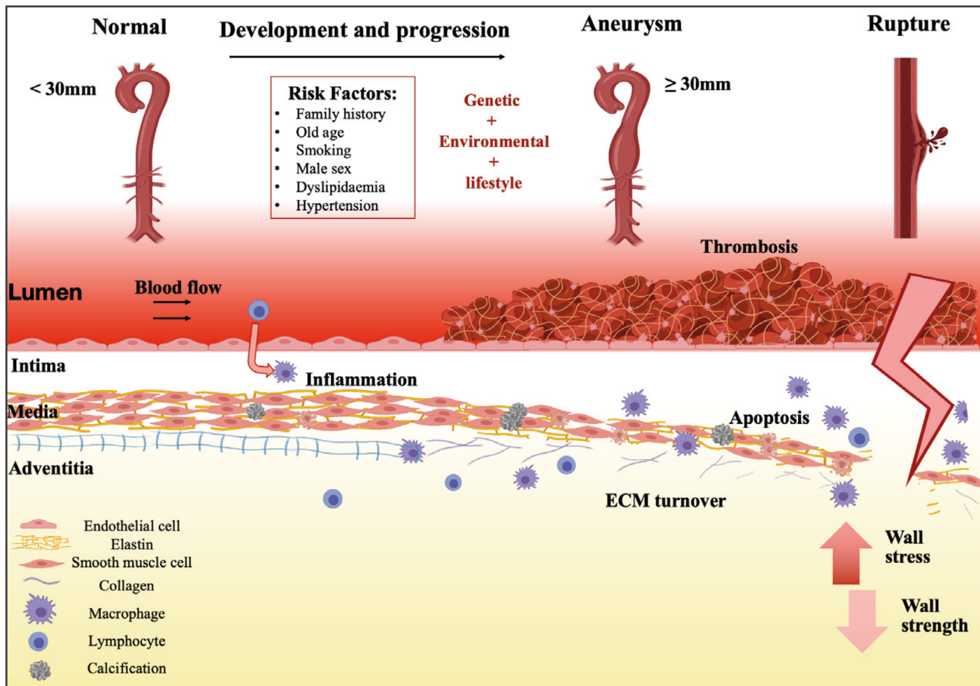

Figure 2. Risk factors and some pathophysiological hallmarks of AAA. ECM: Extra cellular matrix. Created with BioRender.com

Pathophysiology

The pathophysiology of AAA involves a complex interplay of various processes with hallmarks like chronic inflammation, oxidative stress, extracellular matrix (ECM) remodeling, increased vascular smooth muscle cell (VSMC) apoptosis, and thrombosis [47] (Figure 2). Different processes may take a central role depending on the disease stage and complications.

The exact mechanism of AAA initiation is not fully understood. However, previous research has shown that AAA initiation typically begins with the localized degradation of the extracellular matrix components like collagen and elastin in the aortic wall [48]. As the extracellular matrix weakens, the aortic wall becomes more susceptible to stress-induced damage and paves the way for inflammatory processes that play a pivotal role in AAA development. The infiltration of immune cells, such as macrophages and T cells, into the aortic wall triggers a cascade of events [49]. In response to various inflammatory stimuli, these immune cells release cytokines like tumor necrosis factor and angiotensin, proteases, inducing further aortic wall degradation and oxidative stress [50,51]. It is mainly the **Myeloperoxidase (MPO)** and **nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase)** complex proteins from the immune cells (or VSMCs) that contribute to the production of reactive oxygen and nitrogen species (ROS/RNS) molecules, leading to chronic oxidative stress in AAA [52-54]. Other potential sources of ROS/RNS in AAA are nitric oxide synthase, xanthine oxidase and mitochondria [55,56]. Chronic induction of ROS can ultimately overwhelm the physiological defense mechanisms of antioxidant enzymes, forming a continuous cycle [54] (Figure 3).

AAA often occurs at the infra-renal location [1,7]. AAAs experience continuous hemodynamic stress due to the high-pressure environment of the aorta, specifically the infra-renal location below the level where the renal arteries branch off [57]. This chronic mechanical stress exacerbates the expansion of the aneurysm [57]. The aortic wall attempts to compensate by remodeling itself, but this process often leads to further weakening [47]. Excessive production of matrix metalloproteinases (MMP), particularly MMP-2 and MMP-9, is a hallmark of AAA expansion [50,51,58]. These enzymes contribute to the continued degradation of elastin and collagen fibers in the aortic wall, leading to structural deterioration [47]. MMPs also enhance the inflammatory response within the aneurysm wall [50,51,58]. As AAA expands, the aneurysm wall progressively weakens [47]. This weakening or thinning is due to the loss of structural integrity, increased protease activity, and apoptosis of vascular smooth muscle cells [50,59,60]. The weakened wall is more susceptible to rupture [61]. The presence of chronic inflammation within the aneurysm wall intensifies the risk of rupture by disrupting the balance between tissue repair and degradation, further weakening the wall [47,62]. Finally, at a point when the hemodynamic stress on AAA exceeds the wall strength and capability, rupture may occur [61]. Although intraluminal thrombus (ILT) is found in the majority of large AAAs, but whether it perpetuates further harm or is a compensatory defense mechanism, or both, is still not elucidated [63].

It is important to note that the exact order and contribution of the described processes to AAA development, progression and rupture are not yet fully understood. Further research is needed to enhance our understanding of the pathophysiology of AAA and identify additional risk factors and biomarkers.

Specific oxidative stress-related factors

As introduced above, oxidative stress often takes a central role in the pathophysiological processes of AAA, in its growth and rupture (Figure 3). Therefore, in the present thesis, we focused on some factors related to oxidative stress in AAA. Some important concepts not introduced in the previous section are briefly introduced below.

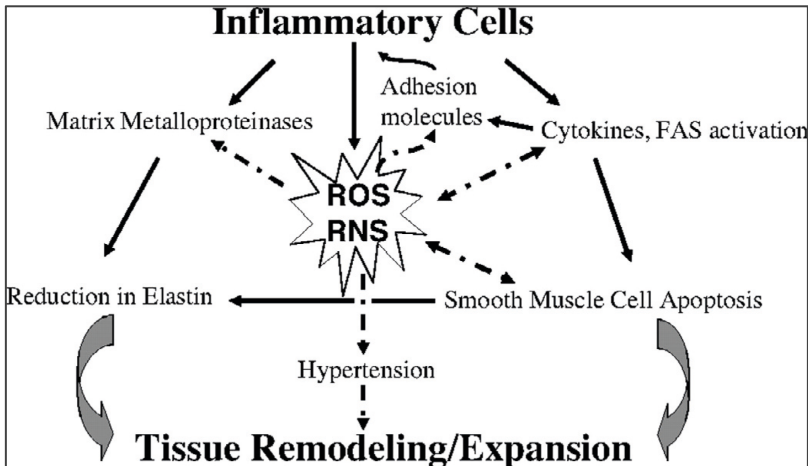

Figure 3. The central role of oxidative stress in AAA pathophysiology. ROS: reactive oxygen species; RNS: reactive nitrogen species. Reprinted from ATVB. 2007 Jan 11: 2007;27:461–469; Michael L. McCormick, Dan Gavrila and Neal L. Weintraub; Role of Oxidative Stress in the Pathogenesis of Abdominal Aortic Aneurysms Copyright © 2007, Wolters Kluwer Health.

DNA methylation and homocysteine

Epigenetic changes or acquired changes in the DNA are often seen as the manifestation of the surrounding environment and lifestyle in human physiology. DNA methylation is one such widely studied epigenetic phenomenon in the context of human health and physiology. DNA methylation is defined by the addition of methyl group on DNAs cytosine residue, which if present at specific, important loci, can lead to gene expression changes and possibly diseased phenotypes [64]. DNA methylation changes as a disease marker have been found to be associated with a variety of chronic human diseases and inflammation processes [65-67]. A next-generation-sequencing-based DNA methylation study has shown *SMYD2* promoter methylation in AAA potentially associated with adverse cardiovascular physiology and inflammation, providing strong evidence for the role of DNA methylation in AAA [68]. Often biologically related to DNA methylation, homocysteine is an amino acid produced during the metabolism of methionine, and elevated levels of homocysteine in the body have been greatly associated with oxidative stress and

CVDs including AAA [69-71]. However, there is still a lack of information on the exact interplay of DNA methylation and homocysteine concerning AAA initiation and growth, which requires further investigation.

Mitochondria

Mitochondria are double-membraned organelles found in eukaryotic cells, responsible for generating adenosine triphosphate (ATP) through a process called oxidative phosphorylation [72]. Beyond their central role in energy production, mitochondria are involved in various cellular processes, including calcium regulation, apoptosis, and the generation of reactive oxygen species (ROS) [72]. Dysfunctions in mitochondria are implicated in a variety of oxidative stress-related chronic diseases, including AAA [73-76]. Mitochondrial dysfunction can arise from errors in the mtDNA repair process, disruptions in mitochondrial biogenesis and antioxidant defenses, along with deregulated mitochondrial dynamic changes of fusion and fission [72,77,78]. These disruptions can lead to mitochondrial injury and malfunction, ultimately resulting in oxidative stress, inflammation, and cell death [54]. Recent evidence strongly suggests that the dysregulation in mitochondrial functioning or homeostasis is linked to the pathogenesis of AAA [79] (Figure 4).

In humans, the mitochondrion stands as the only cellular organelle, after the nucleus, that possesses its own genetic material (16.5 kilo bases (kb)), with 37 genes and a regulatory region called the displacement-loop (D-loop) [77]. In contrast to the nuclear genome, which typically comes in pairs, the circular mitochondrial genome is present in numerous copies, existing in a state of polyploidy within each cell [77]. This distinctive characteristic is frequently linked to the potential bacterial endosymbiotic ancestry of mitochondria [80]. Because of the multi-copy nature of mitochondria and its DNA, mutations in mitochondrial DNA (mtDNA) can either exist as heteroplasmy (mixture of wildtype and mutant DNA) or homoplasmy (either mutant or wildtype DNA) [72].

Mitochondria follow an exclusively maternal inheritance, such that a group of inherited homoplasmic mutations can be traced back to define lineages known as haplogroups [81]. Mitochondrial haplogroups have been associated with several diseases and phenotypes [82-84]. However, previous research emphasizes more on the dynamic heteroplasmic mtDNA mutations when it comes to chronic diseases like cancer and heart failure, where the level of heteroplasmy often correlates to disease severity [85-87]. A thorough examination of mitochondrial genetics holds the potential to address knowledge gaps in the hereditary factors contributing to AAA and may unveil fresh avenues for risk assessment and therapeutic interventions. The growing field of mitochondrial therapeutics presents an exciting opportunity for its application in the context of AAA [79,88].

Figure 4. Multi-faceted of mitochondrial dysfunction in AAA. Reprinted from THE FASEB JOURNAL. 2023; 37:e22969; Ding Wang, Longyuan Jia, Chengdong Zhao, Huitao Wang, Zhengnan Dai, Yuchen Jing, Bo Jiang, Shijie Xin; Mitochondrial quality control in abdominal aortic aneurysm: From molecular mechanisms to therapeutic strategies © 2023, John Wiley and Sons.

Heme-homeostasis

Heme homeostasis is a tightly regulated process crucial for maintaining cellular and systemic balance in the levels of hemoglobin, heme, hemopexin, haptoglobin, and heme oxygenase-1 (HO-1) [89]. Hemoglobin, a vital component of red blood cells, carries oxygen throughout the body. When hemoglobin breaks down, it releases heme, which can be toxic if not managed properly [90]. Haptoglobin (Hp) and hemopexin (Hpx) are proteins that bind to free heme, preventing its harmful effects [91]. Additionally, heme oxygenase-1 (HO-1) induced by Hb-Hp and heme-Hpx complexes, plays a pivotal role by breaking down excess heme into biliverdin, carbon monoxide, and iron, thereby mitigating oxidative stress and inflammation [92,93]. Disruptions in heme homeostasis can lead to the accumulation of free heme, contributing to oxidative stress [90]. Excess heme can generate reactive oxygen species (ROS), causing cellular damage and inflammation, through a process now termed as heme toxicity [89]. Therefore, maintaining a delicate balance in heme homeostasis is essential for averting oxidative stress and preserving cellular health. While some studies have individually examined Hp and Hemopexin (Hpx) in

relation to AAA, the findings have been inconsistent [94-98]. However, it is particularly relevant to explore heme toxicity in the context of AAA, which has a considerable oxidative stress component. Therapies through heme-scavenger molecules have been proposed for diseases in which heme toxicity is implicated [99]. For similar translational efforts in AAA, first a comprehensive understanding of potential factors involved in heme homeostasis is required.

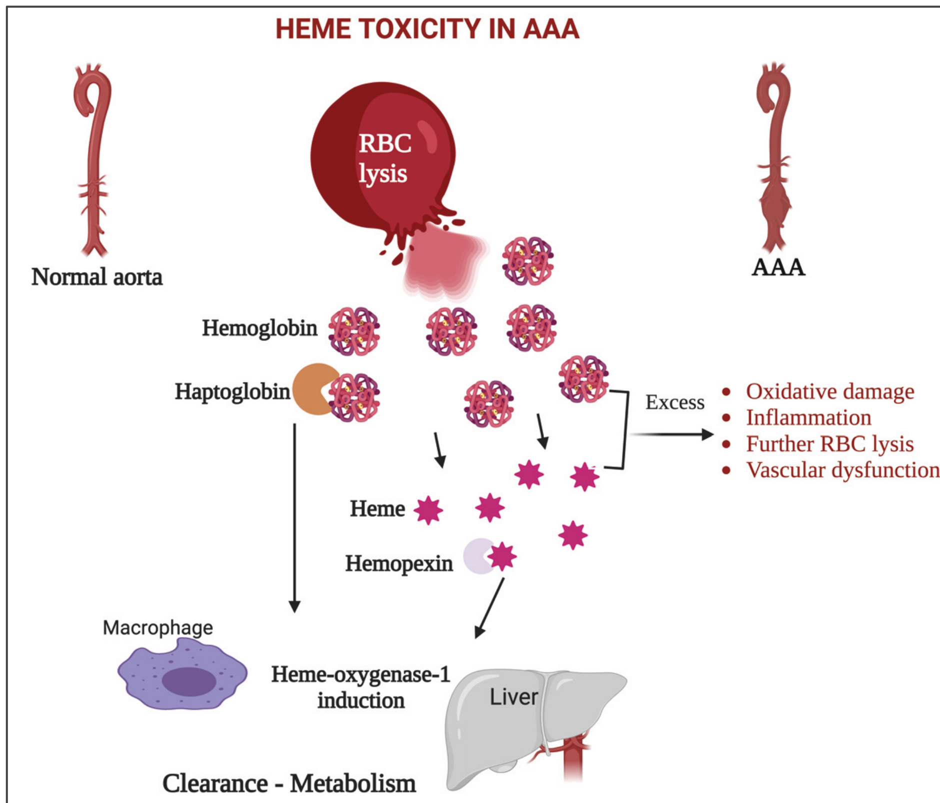

Figure 5. Potential mechanism of heme toxicity in AAA. Created with BioRender.com.

Current clinical outlook: Scope for improvement

Early diagnosis, accurate prognosis, and effective treatment are crucial for managing AAA and preventing catastrophic events. Nowadays, AAAs are either detected at screening, by chance (with some other diagnosis), or when symptoms or

complications arise [61]. The primary method for diagnosing AAA is through non-invasive imaging techniques [1]. Ultrasound is the most common and cost-effective initial screening tool, offering high sensitivity and specificity for AAA detection [1,100]. Computed Tomography (CT) scans provide detailed anatomical information and are especially valuable for assessing the size and extent of the aneurysm [1]. Screening programs, often targeting high-risk populations such as elderly males, have been effective in early detection [1]. Routine screening using ultrasound has significantly reduced the rate of rAAA and AAA - specific mortality [7,101]. However, screening programs are not available in all regions and do not target the younger population and females, which is a point of concern, as women have been shown to have worse prognosis than males [14,16,102,103]. Although the AAA size or diameter is the gold standard for AAA diagnosis and prognosis, it has limitations based on the discrepancies between sexes and ethnic groups [1]. Moreover, in some cases, the AAA diameter cannot completely predict the growth rate and risk of rupture [16,104].

There are some commonly used biomarkers like C-reactive protein (CRP), D-dimer and homocysteine, and experimental markers like MPO, interleukin - 8, tumor necrosis factor – alpha, and endothelin, associated with AAA and its growth [105]. However, no specific biomarker or combination is approved for AAA clinical diagnosis and prognosis. In addition, research on biomarkers of rAAA remains scarce. There is, therefore, a need to identify new and more efficient biomarkers and to develop specific and sensitive models for risk stratification, based on a diverse population.

When it comes to treatment, surgical options are recommended for individuals with large (≥ 5.5 mm for men and ≥ 5 mm for women) or symptomatic AAAs [1]. For small to medium AAAs and in patients with significant comorbidities, a conservative approach involving regular monitoring is recommended [1]. Lifestyle modifications, such as smoking cessation and blood pressure control, are also suggested for disease management [1,27]. Endovascular Aneurysm Repair (EVAR) is a minimally invasive procedure involving the placement of a stent graft within the aneurysm to redirect blood flow and relieve pressure on the weakened vessel wall [106,107]. It is suitable for many patients and is associated with lower mortality and morbidity compared to open surgery [107]. In cases where EVAR is not feasible or appropriate, open surgical repair involves replacing the weakened section of the aorta with a synthetic graft [106,107]. This procedure is more invasive and may require a longer recovery time but is necessary for certain anatomical configurations [106,107]. Interestingly, elective AAA repair mortality is reported to be higher in females as compared to males [108].

Some medications, such as statins, antihypertensive, and antiplatelet medication are recommended as secondary preventive measures [1], however, their effectiveness is still not confirmed due to contradictory findings [106,109]. Based on these shortcomings, future research efforts towards personalized AAA treatment approaches are encouraged.

Oxidative stress factors in AAA: clinical potential

As mentioned previously, oxidative stress plays a central role in the development and progression of AAA. Antioxidant vitamins offer a relatively practical and significant approach to treating diseases related to oxidative stress [110]. Micronutrients, comprising vital trace elements and vitamins, play essential roles in metabolism and function as both direct and indirect antioxidants [110]. Animal studies have explored the potential of antioxidant vitamin supplementation in preventing the formation and progression of AAA [111-113]. Additionally, high consumption of antioxidant-rich fruits and vegetables and adherence to the Mediterranean diet have shown associations with a reduced risk of AAA [46] . However, reports from various observational and interventional studies regarding the protective effects of these vitamins on CVD (including AAA) risk have been conflicting [114-117], which may be attributed to differences in study design (including dosage), genetic factors, and sex. Apart from dietary antioxidants, targets could also be found in models of heme toxicity and mitochondrial dysfunction, if established in AAA.

In order to enhance future risk assessment and develop effective disease management strategies, it could be advantageous to identify and understand particular sources of oxidative stress and how they might interact within the context of AAA.

Aims

The overall aim of the thesis is to investigate factors related to oxidative stress in AAA and its complications.

The specific aims of the studies were:

Paper I: To explore the association(s) between global DNA methylation, homocysteine levels, prevalent AAA, and its progression.

Paper II: To characterize the mitochondrial genomic landscape in AAA through a comprehensive next-generation sequencing analysis.

Paper III: To investigate the impact of genetic variations in genes associated with oxidative stress, the intake of antioxidant vitamins, and any potential interplay between these elements on the occurrence of intact AAA and ruptured AAA, while considering sex differences when applicable.

Paper IV: To investigate the association(s) and explore the diagnostic/prognostic potential of whole blood hemoglobin, plasma (cell-free) heme, Hpx, HO-1, and Hp phenotype-genotype, concerning both occurrence and growth of AAA.

The first three papers have been published with open access and are freely accessible. They have been reproduced in this doctoral thesis for non-commercial purposes. All rights belong to the authors.

Material and methods

The current thesis describes four studies investigating oxidative stress-related factors in AAA, potentially aiding future diagnosis, prognosis, or therapeutics. The research undertaken in this thesis draws upon a broad approach, encompassing clinical data analysis, experimental techniques, and bioinformatics methodologies described in detail below.

Study population and data collection

The four studies included in the thesis are based on data and/or biomaterial from two cohorts. Papers I, II, and IV are based on participants from the population-based AAA screening cohort and paper III is based on the Malmö diet and cancer study (MDCS) cohort.

AAA Screening cohort

The AAA screening cohort was established from an ultrasound (US) based screening program in the south of Sweden [118]. Since 2010, the Department of Vascular Diseases in the Skåne University hospital, Malmö, Sweden, invites all 65-year-old men from Malmö and 15 neighboring municipalities to participate in AAA screening. From 2010 to 2017, 24,589, 65-year-old men (born 1945-52) received physical invitations in Swedish including a website link for information in other languages. Invitations were sent out a second time if there was no attendance after the first one. The compliance rate for AAA screening reported until 2012 was 80.2% [118]. US examination of the aorta was performed by trained professionals using the LOGIQe US machine with 3.5-12 MHz probes [General Electric Healthcare Inc, Chalfont St. Giles, UK]. In the cases where US examination was inconclusive (1.1%), participants were referred for a CT scan. Finally, leading edge to leading edge (LELE) method was used to determine the maximum infrarenal anteroposterior aortic diameter. An aortic diameter of ≥ 30 mm was defined as AAA which had a prevalence of 1.7% (n = 415) [6], similar to previously published studies. With a compliance rate of 34%, 142 men with AAA agreed to physical examination,

give blood samples, and share medical history [62]. A possible reason for low compliance could be a slightly late initiation of systematic patient coordination. As a control group, 279 men from the same cohort with normal aortic diameter (<30 mm) at screening were randomly selected and invited. As per the Swedish screening principles [7] and the European recommendations [1], AAA patients were followed-up through US surveillance at time intervals depending on the baseline aortic diameter. Follow-up data pertaining to AAA growth rate (mm/year) were available for 113/142 men with AAA. According to standard clinical practice and guidelines in Sweden, men with ≥ 55 mm baseline aortic diameter, or extreme growth rate or symptomatic AAA, were referred to surgery.

Papers I and IV included the whole study population, except for individuals with missing (at random) main exposure data. Paper II included 96 individuals from the AAA screening cohort, with 48 AAA cases and 48 men without AAA, matched for CVDs and diabetes. Furthermore, all cases with a positive family history of AAA were included to investigate the hereditary component. Whole blood, serum, plasma, and post-operative snap-frozen AAA biopsies were included as biospecimens in different studies. The study population from the AAA screening cohort is described in the flow chart below (Figure 6.)

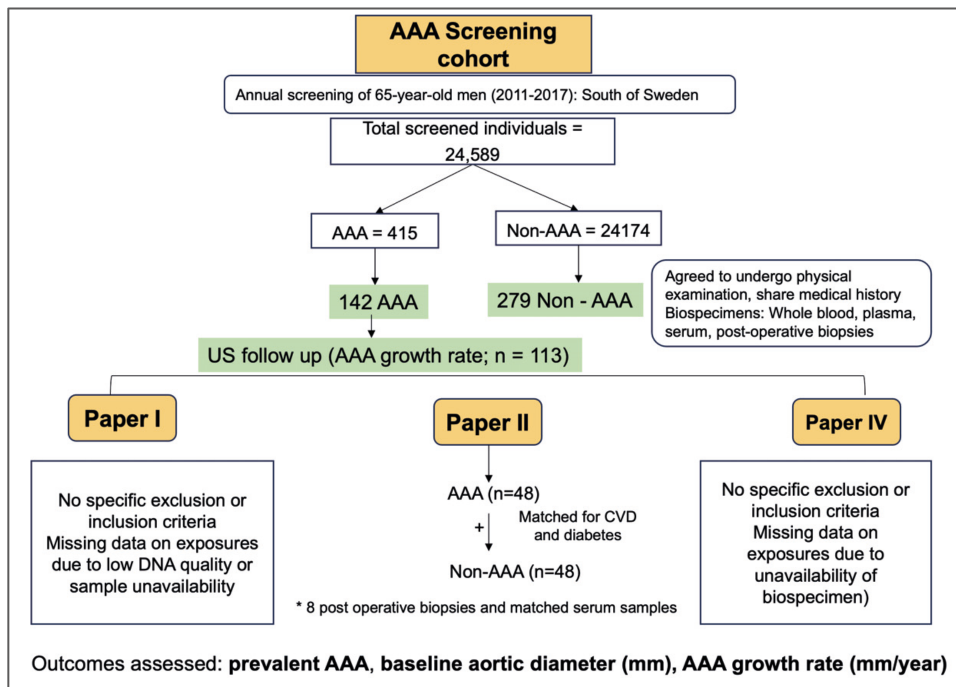

Figure 6. Study participants and information from the AAA screening cohort (Papers I, II and IV).

MDCS cohort

Paper III in the present thesis is based upon the MDCS cohort and follows a retrospective cohort study design [119]. This study cohort consists of 30,446 adults from the Malmö region in Sweden. After the fulfillment of Malmö city residency and Swedish language proficiency as the inclusion criteria, and signing of an informed consent form, a total of 12,120 men (born between 1923 to 1945) and 18,326 women (born between 1923 to 1950) were recruited. Baseline examination, which consisted of physical examination, medical history collection and laboratory sample assessment, was carried out between 1991 and 1996. Out of the total eligible population of 68,905 individuals, 30,446 were included as study participants as they completed at least one part of the baseline assessment. Out of the total 30,446 participants recruited, 27,213 individuals completed the diet assessment and were also genotyped. Upon exclusion of individuals with prevalent AAA, diabetes, and CVDs, the Paper III population consisted of 25,252 individuals including 15,717 women and 9535 men. Incident intact AAA and ruptured AAA (rAAA) events, updated until 31st December 2019, were included as study outcomes. The Paper III population along with inclusion/exclusion criteria is depicted in Figure 7.

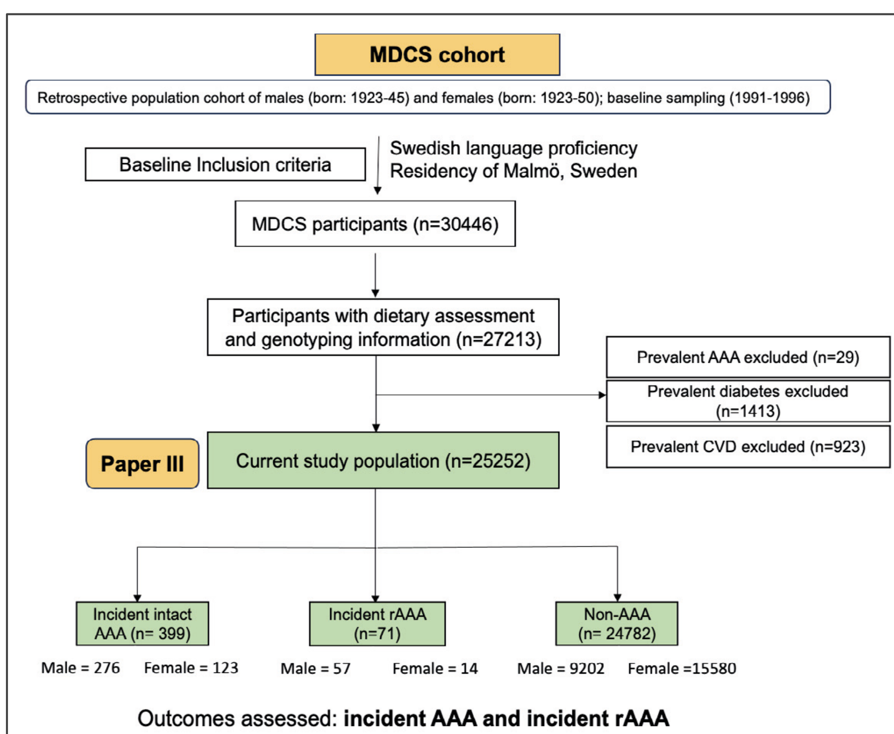

Figure 7. Study participants and information from the Malmö diet and cancer study (MDCS) cohort (Paper III).

Outcome assessment

AAA screening cohort

For the AAA screening cohort utilized in Papers I, II and IV, the assessment of intact AAA was made through objective ultrasound screening. As symptomatic/ruptured cases and cases with large AAA sizes, were directly referred to surgery at baseline, we did not have these as outcomes in the studies. With surveillance intervals decided by clinical AAA screening guidelines, the aortic diameter of men with AAA was followed up through US examinations. AAA growth rate (mm/year) was based upon the per year changes in the maximum aortic diameter, calculated as the: (diameter at the last follow-up - diameter at baseline)/number of years of follow-up.

MDCS cohort

For the MDCS cohort (Paper III) the incident intact AAA or rAAA cases were determined using Swedish national register data that included data until the 31st of December 2019. The Swedish national register data includes information from hospital admissions and discharge dates, with clinical diagnosis and intervention/surgery codes (inpatient and outpatient registers), along with cause of death information from death certificates (cause of death register). The unique Swedish personal identity number was used to extract information from these registers for the determination of incident AAA or rAAA events. The intact AAA and rAAA assessments through diagnosis and procedural codes have been validated in a previous study on MDCS cohort [120].

Laboratory methods

Biospecimen collection and routine assessment

Only at the time of baseline screening, 6 mL of fasting venous blood was collected in vacuum tubes containing Ethylenediaminetetraacetic acid (EDTA) [Becton-Dickinson, Franklin Lakes, USA]. Blood from EDTA tubes was centrifuged at 4°C at 1800g for 15 minutes and plasma samples were extracted and stored at -80°C until further use. Post-operative, snap-frozen biopsies and matched serum samples were also available for eight individuals and were utilized in Paper II.

DNA samples from various tissue sources were extracted using commercially available kits from Qiagen, USA, and manufacturer's guidelines (Table 1). DNA samples were kept at -20°C for long-term and at 4°C for short-term/routine use.

Table 1. DNA extraction kits for specific application.

DNA source	Commercial kit for extraction [Qiagen, USA]
Whole blood	QIAamp® DNA Blood Kit
Serum	QIAamp Circulating Nucleic Acid Kit
Snap-frozen AAA biopsy	DNeasy Blood and Tissue Kit

At the Department of clinical chemistry at Skåne University Hospital, routine laboratory markers were analyzed that included: plasma - cholesterol (mmol/L), creatinine (µmol/L), triglyceride (TG, mmol/L), high density lipoprotein (HDL, mmol/L), low-density lipoprotein (LDL, mmol/L), and glucose (mmol/L). Whole blood hemoglobin (Hb, g/L) was also assessed. Plasma homocysteine levels (µmol/L) were assessed using a photometric enzymatic assay approved for diagnostic use on the Cobas® modular platform [Roche, Switzerland]. All the analyses were performed according to standard SWEDAC (Swedish national accreditation body) practice and approval.

Colorimetric assays

<u>Global DNA methylation assessment</u>

The quantification of global DNA methylation (5-methyl cytosine (5-mc) levels) for Paper I was performed on DNA extracted from whole blood using the MethylFlash™ Global DNA methylation ELISA Easy assay [Epigentek, USA]. DNA concentration was measured using 1x double-stranded (ds) DNA high - sensitivity assay on Qubit 4.0 [Invitrogen, USA]. As per the assay guidelines, samples (n = 75) with concentration < 10 ng/µL were excluded. The assay was based on a direct colorimetric enzyme-linked immunosorbent assay (ELISA) method using standard assay guidelines (Figure 8). Reactions were performed in 96-well and duplicates and averaged during analysis. Six standard concentration points were prepared fresh and run as duplicates, by mixing 5% methylated positive control (PC) and 0% methylated negative control (NC), provided in the assay kit. All test samples were diluted to 10 ng/µL and a final amount of 100 ng was added per well. Absorbance was read as Optical density (OD) at a wavelength of 45 nm, using the Infinite® F200 multimode plate reader [Tecan Trading AG, Switzerland]. The percentage of global DNA methylation or 5-mc was interpolated using the generated

standard curves for each plate. The inter- and intra- assay coefficient of variations were < 10%.

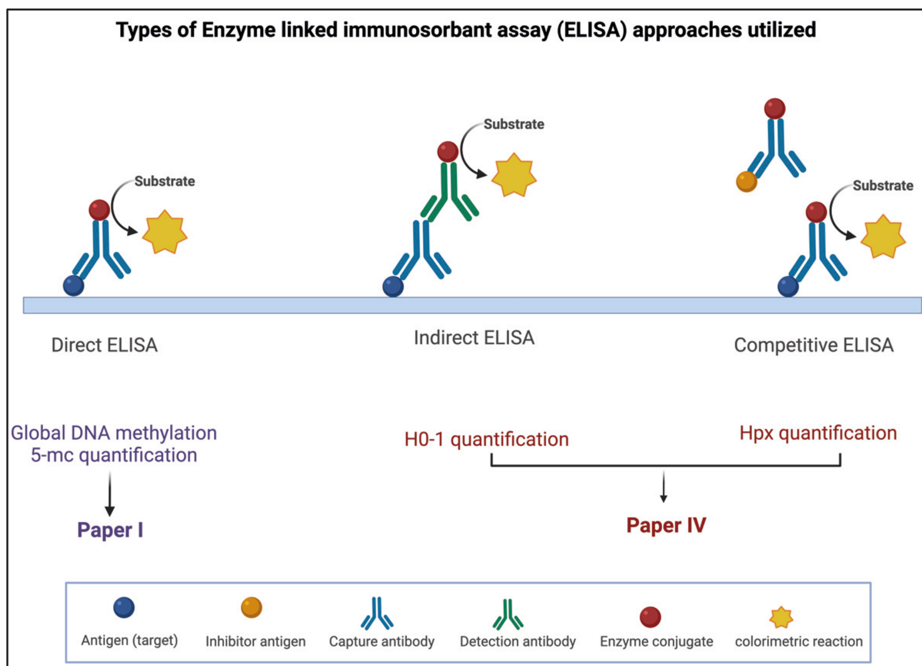

Figure 8. Depiction of different Enzyme linked immunosorbent assay (ELISA) methods used in the thesis studies. 5 mc: 5-methyl cytosine; HO-1: heme oxygenase-1; Hpx: Hemopexin; ELISA: enzyme-linked immunosorbent assay; 5-mc: 5-methyl cytosine; RT: room temperature. Created with BioRender.com.

Plasma HO-1 ELISA

For the quantification of plasma HO-1 (Paper IV), a commercial, indirect sandwich ELISA kit was used [ab207621, Abcam, USA]. The unique assay had the plate well (96-well) coated with a monoclonal antibody specific for an affinity tag conjugated to the capture antibody. Plasma samples were diluted by 1:11 and standards were prepared on the same day that the assay was run. The capture and horse radish peroxidase (HRP)-conjugated detection antibody cocktail were added in a single step, leading to the formation of an antibody-analyte sandwich. Colorimetric assay was finally finished with the addition of 3,3',5,5'-Tetramethylbenzidine (TMB) substrate followed by stop solution. Absorbance was read at a wavelength of 450 nm and test samples concentration was determined using standard curve interpolation from each plate. Inter- (11.3%) and intra- (3.9%) assay CVs were calculated using controls run in duplicate. The assay had a detection range of 23.44 pg/mL - 1500 pg/mL.

Plasma Hpx ELISA

For determining the concentration of Hpx (paper IV) in plasma samples, a commercially available competitive ELISA was used [ab108859, Abcam, USA]. Each well of the 96-well plate was pre-coated with primary capture antibody specific for human Hpx protein. Samples were diluted in a 1:400 ratio and standards were prepared on the day of running the assay. Equal volume of biotinylated Hpx was added to each well, immediately after adding standards and test samples and incubated. Colorimetric reaction was performed by the addition of a streptavidin-HRP conjugate, followed by HRP substrate, and stop solution. Absorbances were read at 450 nm wavelength and Hpx concentrations in the test samples were interpolated using the standard curve in each plate. The intra-assay CV was 7.8% and the inter-assay CV was 14.1% for duplicate control samples. The Hpx detection range of the assay was 0.078 µg/mL - 20 µg/mL.

Plasma Heme assay

For the quantification of plasma heme (Paper IV), a simple single-step colorimetric spectroscopic assay and guidelines were used [DIHM-250, BioAssay Systems, Hayward, CA, USA]. The assay uses an improved aqueous alkaline solution that reacts with hydrolyzed heme molecule and facilitates the conversion into a consistent colored form. The color intensity, detected at 400 nm by a spectrophotometer, is directly proportional to the concentration of heme (free heme or hemoglobin bound). The assay demonstrates high sensitivity, with a linear detection range of 0.6 - 125 µM. The plasma samples were diluted by a ratio of 1:2 on the day of the run and known control samples were run in duplicates. The intra- and inter- assay coefficients of variation were 7.5% and 9.6%, respectively.

Next generation sequencing

Firstly, a long-range PCR resulting in two large, overlapping mtDNA amplicons was performed for targeted amplification of the whole mitochondrial genome, using two sets of primers (Table 2).

Table 2. Primer sequences for long-range PCR targeting mtDNA

Primer	Sequence (5' to 3')	Amplicon size; name
MTL-FW-1	AAAGCACATACCAAGGCCAC	9.1 kb; MTL1
MTL-RV-1	TTGGCTCTCCTTGCAAAGTT	
MTL-FW-2	TATCCGCCATCCCATACATT	11.1 kb: MTL2
MTL-RV-2	AATGTTGAGCCGTAGATGCC	

MTL: mitochondrial DNA amplicon; FW: forward; RV: reverse; kb: kilobase

The sequencing library preparation was performed using the Nextera DNA flex kit [Illumina, CA, USA]. DNA samples (from whole blood, 8 pairs of matched biopsy and serum) were diluted to 0.2 ng/µL on the same day when long-range PCR was performed. The long-range PCR reactions were performed using TaKaRa LA TaqDNA Polymerase kit [TaKaRa, Shiga, Japan], with a 2 ng DNA input per reaction. All the reagents and master mixes were prepared on ice. The following thermal cycler conditions were used for the long-range PCR: 94°C for 5 minutes, followed by 30 cycles of (98°C for 15 seconds, 68°C for 10 seconds, 60°C for 15 seconds and 68°C for 11 minutes), finishing with 72°C for 10 minutes. For the quantification of the amplified product, Qubit 4.0 - 1X ds DNA High sensitivity assay and guidelines [Invitrogen, USA]. Amplicon sizes of the two long-range PCRs were determined using the DNA 12K kit, on the Experion automated electrophoresis system using the manufacturer's guidelines [Bio-Rad, Hercules, CA, USA]. Long-range PCR was repeated for samples that failed the quality and quantity check. Following the quality check, the PCR products were normalized to 10 ng/µL in Elution buffer buffer. MTL1 and MTL2 products for the same sample were pooled together before the tagmentation process with bead-linked transposome (BLT). At this stage, DNA was fragmented and Illumina adapters (unique i7 and i5 indexes) for each sample were attached, followed by amplification of tagmented DNA fragments. Final library clean-up was performed using Ampure XP beads (Beckman Coulter, MA, USA), in a double-sided bead purification process. After purification, libraries' concentrations, and sizes (500 bp - 1000 bp) were determined by Qubit 4.0 - 1X ds DNA High sensitivity assay [Invitrogen, USA] and Experion [Bio-Rad, Hercules, CA, USA], respectively. Twenty-four individual libraries were then diluted to a final concentration of 10 nM each in the resuspension buffer. To achieve optimal sequencing cluster density, equal volumes of the 10 nM libraries were pooled together. The library pool was normalized to 100 pM (with 2% phiX internal control) and was loaded into the iSeq100 sequencer system [Illumina, CA, USA]. Finally, with a read length of 151 bp, dual-index, paired-end sequencing run was performed. This workflow is based on the Human mtDNA Genome guide for the Illumina Sequencing Platform with optimizations (Figure 9).

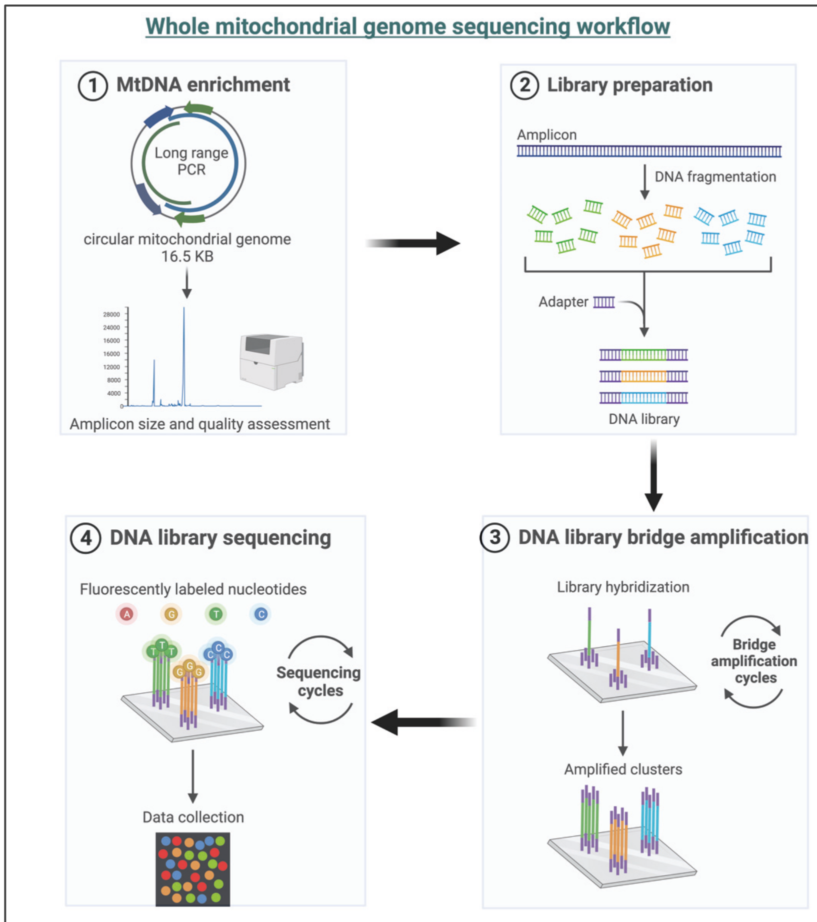

Figure 9. Depiction of next generation whole mitochondrial genome sequencing approach employed in Paper II. Created with BioRender.com.

Droplet digital PCR

MtDNA-CN quantification

The mtDNA-CN from whole blood DNA samples was determined using a well-optimized droplet digital PCR (ddPCR) method. The ddPCR method, used in Paper II, simultaneously quantifies the absolute copies of nuclear and mt-DNA. The method involves fractionating the sample into thousands of droplets (~ 20,000) and performing PCR amplification of the template DNA molecules in each droplet. Hence, it overcomes the limitations of traditional quantitative real-time PCR (qPCR) methods, which measure relative levels of mtDNA using an external

calibrator that may not be comparable across laboratories. Two primers and probes pair targeting one mtDNA-specific gene (*MT-ND1*) (assay ID: HsaCPE5029120, [Bio-Rad, Hercules, CA, USA and targeting nuclear DNA-specific gene (*EIF2C1*) (assay ID: HsaCP1000002, [Bio-Rad, Hercules, CA, USA]) were used for quantification. Nuclear DNA targeting probe was attached to a HEX fluorophore and mtDNA targeting probe was attached to FAM and all probes had an Iowa Black® FQ quencher [Bio-Rad (Hercules, CA, USA)]. A stringent quality control process was followed throughout, as described previously [121]. An input of 3 ng DNA from samples, positive and negative controls, was pooled separately in a 22 uL multiplex reaction containing: ddPCR Supermix for probes (no UTP, 2X), primers (900 nM), probes (250 nM), and 5 U/reaction restriction enzyme (HindIII), in 96-well plates. After a 30-minute incubation at room temperature to allow for optimal restriction digestion, the samples were divided into approximately 20,000 droplets, each theoretically containing one DNA molecule, using an automated droplet generator from the QX200 Droplet Digital PCR system followed by end-point PCR [Bio-Rad (Hercules, CA, USA)]. For maximum droplet recovery, the post-PCR, foil covered plate was kept overnight at 4°C. To calculate the numbers of positive and negative droplets in each sample, the plate was read on the droplet reader and analysis was done using QuantaSoft™ Software. The absolute number of copies (copies/µL) was determined by fitting the positive droplet fraction to the Poisson distribution. The mtDNA-CN was calculated as the ratio between mtDNA and nuclear DNA absolute copies. The inter- and intra-CVs for quantification of mtDNA-CN were 8.4% and 6.1%, respectively.

Hp-Typing

For Paper IV in the thesis, a new assay using Droplet Digital PCR (ddPCR) was developed to determine the three phenotypes of Hp in DNA samples extracted from whole blood (Figure 10). The concentration and quality of DNA extracted from whole blood samples were measured using the Thermo Scientific™ NanoDrop 2000 and normalized to 0.5 ng/µL before proceeding with the analysis. To quantify the duplicated *HP2* target, we used a primer pair and probe that specifically targeted the duplicated region in the Hp2 allele (assay ID: dHsaCNS626037518, [Bio-Rad, Hercules, CA, USA]). For reference quantification, we used a primer pair and probe targeting the stable (two copies in the diploid human genome) nuclear *EIF2C1* gene region. FAM fluorophore was used for probes targeting *HP2*, while HEX-conjugated probes were used for targeting the reference, *EIF2C1* (assay ID: HsaCP1000002, [Bio-Rad, Hercules, CA, USA]). The ddPCR amplification was carried out with a total multiplex reaction volume of 22 µl, including 3 ng of input DNA, 0.9X primer-probe concentrations, HaeIII enzyme (5 U/reaction) from Thermo Scientific, USA, and 1X of ddPCR Supermix for probes [Bio-Rad (Hercules, CA, USA)]. The choice of 3 ng input DNA was determined after testing various DNA concentrations for the *HP2* (target) and *EIF2C1* (reference) assays

individually and in combination. The droplet generation was performed in the same manner as the mtDNA-CN assay. The absolute copy numbers of the *HP2* and *EIF2C1* (copies/µL) were determined by fitting the fraction of respective positive droplets to a Poisson distribution using the QuantaSoft™ Software. To determine the three Hp types, we estimated the ratio of absolute copies of the target (*HP2*) to the reference (*EIF2C1*). Since *EIF2C1* always has two copies in the diploid human genome, a ratio of 1 represented two copies of the target Hp2 allele (Hp2-2), 0.5 represented a heterozygous state (Hp1-2), and 0 represented the absence of the Hp2 allele (Hp1-1). The intra and inter-assay coefficients of variation for the Hp typing ddPCR assay were 7.08% and 8.12%, respectively.

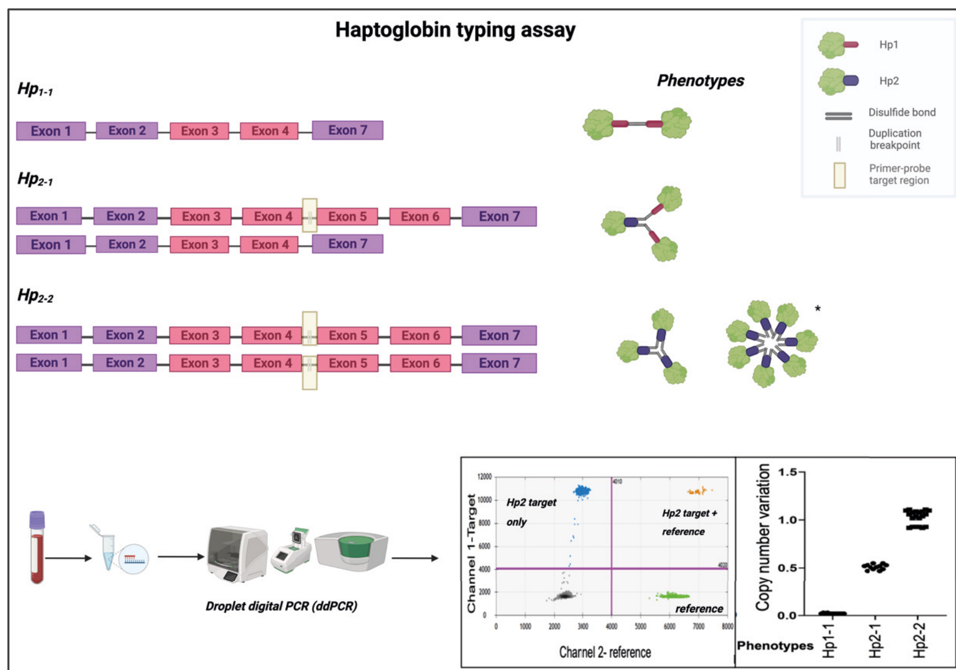

Figure 10. Depiction Hp-typing assay workflow in Paper IV. *Hp: Haptoglobin.* * Multimeric protein structures. Created with BioRender.com.

Allelic discrimination assay

An allelic discrimination assay was performed in the current thesis (Paper IV) to genotype a single nucleotide polymorphism (SNP) in the *Hp* gene (SNP ID: rs2000999) that has been strongly associated with circulating protein levels. This is a multiplex real-time PCR (qPCR)-based assay where multiple primer/probe pairs are used in a single reaction to allow for the detection of both the variant and

wildtype alleles in the DNA template. Each TaqMan assay consists of the following components: an unlabeled PCR primer pair and two TaqMan® probes from Applied Biosystems™ [Thermofisher Scientific, USA] featuring a fluorescent dye label (either FAM™ or VIC™) at the 5′ ends, along with a minor groove binder (MGB) and a nonfluorescent quencher (NFQ) at the 3′ ends. For the current *Hp* genotyping assay, FAM™ probe was a perfect match to the wildtype (allele 1) and the VIC™ probe was a perfect match to the variant (allele 2). Following the manufacturer's [Thermofisher Scientific, USA] guidelines, the allelic discrimination assay measured the change in fluorescence of the specific probe-dyes using a qPCR reaction performed on Bio-Rad CFX384 real-time PCR system [Bio-Rad, Hercules, CA]. Ultimately, using the Bio-Rad CFX manager software [Bio-Rad, Hercules, CA], the assay classified unknown samples as the following: homozygous wildtype (only allele 1), heterozygous (both allele 1 and allele 2), and homozygous variant (only allele 2). Figure 11 illustrates TaqMan® genotyping workflow chemistry, based on matches and mismatches between target and probe sequences. In Paper IV, TaqMan® pre-designed Genotyping Assay (SNP ID: rs2000999, assay ID: C_11439054_10) with VIC and FAM fluorescent probes targeting *Hp* gene polymorphism (NC_000016.10:g.72074194G>A) was used for genotyping on whole blood DNA samples.

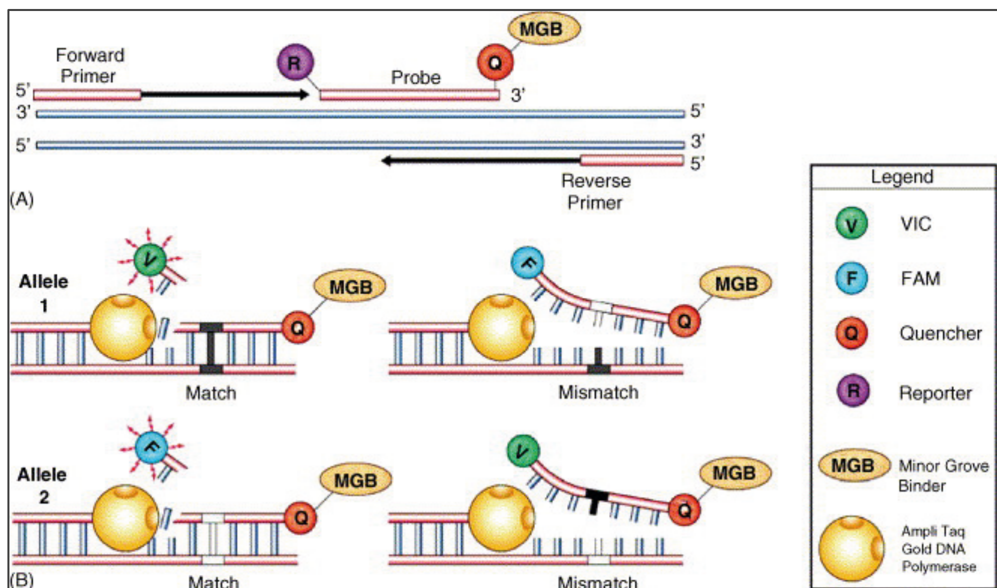

Figure 11: **TaqMan® Genotyping assay principle:** Reprinted from Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis Assessment of two flexible and compatible SNP genotyping platforms: TaqMan® SNP Genotyping Assays and the SNPlex™ Genotyping System, Mutat Res. 2005;573(1-2):111-135, Francisco M. De La Vega,Katherine D. Lazaruk,Michael D. Rhodes,Michael H. Wenz, © (2005) with permission from Elsevier.

Data from MDCS cohort

Vitamin intake assessment

In the MDCS cohort, the three-part modified diet history method used to estimate daily dietary intake, consisted of a 7-day food diary, a comprehensive food frequency questionnaire, and one-to-one interviews with trained personnel. Information on portion size, cooking methods, etc was well documented and accounted for. Using this comprehensive approach, where meals are reported individually, potentially minimized the possibility of recall bias, and improved the accuracy of vitamin intake measurements. Furthermore, this dietary assessment method has been validated against an 18-day weighted food records [122,123]. In Paper III, we focused on the daily mean intake of six vitamins: riboflavin (vitamin B2, mg/day), folate (vitamin B9, μg/day), beta-carotene (a precursor to vitamin A, mg/day), vitamin E (mg/day), vitamin D (μg/day), and vitamin B12 (μg/day). Additionally, we combined the mean daily vitamin intake from food and supplement sources to compute the total vitamin intake. The energy-adjusted Pearson coefficients for relative validity (men; women) were as follows: vitamin E (0.65; 0.83), beta-carotene (0.48; 0.70), and folate (0.75; 0.75) [122,123]. Moreover, the Pearson coefficients for reproducibility (men; women) were as follows: vitamin E (0.83; 0.74), beta-carotene (0.61; 0.53), and folate (0.33; 0.71) [124].

Genotyping and candidate SNPs

Illumina GSA v1 genotyping array was used to assess genotypes in the serum samples from the MDCS cohort [125]. As part of a rigorous quality control process, individuals with subpar samples, characterized by a SNP call rate of less than 90% and sample duplicates, as well as those displaying discrepancies between their reported and genetically determined sex, were systematically excluded. To address the issue of missing SNPs, imputation was carried out using a reference panel sourced from the Haplotype Reference Consortium [126]. Genetic variants that did not adhere to the Hardy-Weinberg equilibrium (with a significance threshold of $P < 1 \times 10^{-15}$) were further excluded. The genotype data were continuous, spanning a range from 0 to 2, signifying the count of alternative alleles as determined through either direct genotyping or imputation. The candidate SNP dataset underwent categorization into 0, 1, and 2 alleles, based on specific continuous value intervals (< 0.5), (0.5 - 1.5), and (≥ 1.5), respectively. Candidate loss of function (LoF) SNPs were selected from the SweGen database [127], in NADPH oxidase complex and MPO genes, with minor allele frequency (0.01 to 0.05), and functional predictions of:

'probably disease causing' or 'disease-causing/deleterious,' as corroborated by two or more independent prediction software tools [128-130]. To improve statistical power in Paper III, candidate SNPs were dichotomized into wild type (0), or variant (1) categories based on the dominant inheritance model, denoted as 0 (0 minor allele) or 1 (1 - 2 minor alleles).

Study covariates

For Paper I, II and Paper IV, information on study covariates (potential confounding) was obtained from the medical history data, through questionnaires, routine clinical test data, and physical examination that participants from the AAA study cohort consented to share during enrollment. The following variables were assessed and recorded: height (cm), weight (kg), blood pressure (systolic and diastolic, mmHg), ankle-brachial index (ABI) for both legs, medication use (antidiabetic and/or metformin, lipid-lowering, antihypertensive), information on AAA family history, and smoking status. Smoking pack year was calculated as: ((numbers of cigarettes smoked per day/20) × number of years smoked), to represent the extent of smoking/tobacco exposure. Smoking variable was further dichotomized by setting a cutoff at 15 pack years, based on previous literature [42,131]. Data on medication use, AAA family history, and comorbidities were coded: yes (1), no (2) or not available (NA) (3). Body mass index (BMI) was calculated as: weight (kg) divided by height (m^2). Routine clinical variables like blood cell composition (thrombocyte/leukocyte count), HDL, LDL, etc., were assessed by the clinical chemistry department at the hospital and used without categorization.

For Paper III based on the MDCS cohort, study participants' identity numbers were used to trace information on age and sex. Anthropometrical measurements for BMI (kg/m^2) and systolic-diastolic blood pressure were performed for study participants at baseline. For the assessment of educational level and medication use, answers from a detailed, self-administered questionnaire were used. Education level was categorized into six groups: elementary, primary, secondary, upper secondary, university (without degree), and university with degree level. Leisure time physical activity (hours per week) was estimated from physical activity intensity and time spent on performing 17 physical activity types) and was later grouped into quartiles for the Paper IV analyses. Alcohol habits were divided into two categories: drinkers and former drinkers or non-drinkers. Smoking status was dichotomized as current smoker and past or never smoker.

Covariates for adjustment analysis were selected based on known association(s) with both the outcome and exposure.

Bioinformatic analysis

Bioinformatic analysis was performed on Linux command line and Rstudio.

In Paper III, for mtDNA sequencing analysis, we first acquired raw sequencing data in Fastq format following a successful sequencing run. We performed the initial quality control (QC) process using FastQC [132], and generated a comprehensive report using MultiQC v1.8 [133]. Illumina sequencing adapters from the raw reads were removed using Trim Galore v0.6.5 [134].
For the specific analysis of mtDNA sequences, we employed the open-source bioinformatics pipeline MToolBox-v.1.0 [135]. This pipeline utilized the revised Cambridge Reference Sequence (NCBI reference: NC_012920.1) for sequence alignment and variant (homoplasmy and heteroplasmy) calling. Within this pipeline, we also performed sequence realignment around known insertion/deletion sites, flagged duplicate sequences, and recalibrated base quality scores. Subsequently, we generated variant call format (VCF) files, which contained all the identified variants in each sample, and contained heteroplasmic frequency (HF) and confidence interval (CI) data for each mtDNA variant. For extracting information on de novo trinucleotide mutational signature, SigProfilerExtractor was used [25]. Lineage-associated mtDNA haplogroups were determined using HaploGrep2 [136]. To investigate structural alterations in the mtDNA, such as deletions (mtDel) and duplications (mtDup), we employed the MitoSAlt package [137].

The VCF files produced by the MToolBox were subjected to parsing using the vcfR tool [138]. To ensure data quality, we applied the QC (quality control) criteria as outlined by Wei et al. in 2019, with slight adjustments, employing the R software version 4.1.1 [81,139]. Our QC efforts primarily aimed to enhance the precision of heteroplasmic variant identification. To achieve a list of refined heteroplasmic variants with good confidence, we instituted the following criteria: if the lower bound confidence interval (CI) of heteroplasmy fraction (HF) for a variant fell below 1%, that variant was excluded from subsequent analysis. MtDNA variants with an upper bound CI of HF less than 98% were classified as heteroplasmic, while those exceeding this threshold were categorized as homoplasmic. In instances where a genetic variant exhibited multiple alleles, we filtered out those heteroplasmic variants where the HFs of all alleles were less than 5%. Additionally, we removed any variants falling within the sequencing error-prone mitochondrial DNA regions specified previously, and those with a sequencing depth (DP) of less than 100. Furthermore, variants with DP below 250 along with HF less than 0.05 were also excluded. For variant annotation following final QC, AnnotationDbi (v.1.54.1) [140] and GenomicFeatures (v.1.44.2) [141], and SomaticSignatures [142], R packages were utilized.

Statistical analysis

In Paper I, statistical analyses were conducted using IBM® SPSS® statistics software version 25 and GraphPad Prism version 8.2.0 for Windows. The Mann-Whitney U test was employed for quantitative variables, while Fisher's exact test (2-sided) was used for nominal variables. Quantitative variables were presented as median and interquartile range, and nominal variables as frequencies and percentages. Pearson's correlation was utilized to assess the linear relationship between global DNA methylation and homocysteine levels with baseline aortic diameter and AAA growth. Univariate logistic regression analysis calculated odds ratios (ORs), and multivariate logistic regression was used to adjust for potential confounders.

Paper II employed statistical analyses with R studio [139] and IBM® SPSS® statistics software version 27. The Wilcoxon-Mann-Whitney test (R package for permutation tests: coin [143]) with exact distribution was used for continuous variables, and Fisher's exact test for categorical variables. Quantitative variables were presented as mean ± standard deviation (SD) or median (25th and 75th percentiles), while categorical variables as numbers and percentages. Fisher's exact test was employed to determine differences in mtDNA mutation frequency between groups. Univariate logistic regression analysis calculated ORs, and multivariate logistic regression adjusted for potential confounders using Firth's small size correction. Linear regression with permutation was used to analyze the association between quantitative traits and mtDNA variants test (R package for permutation linear models: lmPerm [144]). Two-way ANOVA following linear regression with permutation assessed interactions between continuous variables concerning factor variables (lmPerm). The choice of multiple testing correction (adjusted P value < 0.05) for the family-wise error or false discovery rate (Bonferroni or Benjamini-HochBerg) was noted for each analysis where used.

In Paper III, RStudio version 2022.02.3 [145] was utilized for statistical analyses. Baseline characteristics were presented for continuous variables as median (range) and for categorical variables as absolute count (percentage). To enhance the statistical power of the analysis, SNPs were dichotomized based on the dominant inheritance model (reference wildtype recessive and dominant variant). Cox regression analyses were then employed to calculate hazard ratios (HRs) and corresponding p-values, taking into account the years of follow-up as the time variable. Subgroup analyses stratified by sex were pre-planned, and interaction testing was performed. The main analysis involved categorizing total vitamin intake as high or low using median cut-offs, with supplementary analyses using vitamin intake quartiles and log-transformed continuous variables. Pearson correlation analysis was conducted to assess potential multicollinearity ($R^2 > 0.70$) among log-transformed vitamin intakes. Additionally, adjustments were made for various covariates, including age, sex, smoking status, and other relevant factors using two

models. Due to the exploratory nature of the study, multiple testing corrections were not applied.

In Paper IV, we employed RStudio version 2022.02.3 [145] for statistical analyses. Student's t-test and Pearson's chi-square test were used for continuous and categorical variables, respectively. Missing data were imputed using the MissForest R package [146]. Logistic regression analyses, both univariable and multivariable, were conducted to test associations with outcomes. Correlations were assessed using the Spearman's correlation coefficient test, and linear regression analysis was performed. Receiver-operating characteristic (ROC) curve analysis evaluated diagnostic and prognostic potential, including sensitivity, specificity, and area under the ROC curve (AUC). Delong's test for correlated ROCs compared discriminatory performance.

A significance level of $P < 0.05$ was considered statistically significant in all the studies.

Ethical considerations

For Paper I, II and Paper IV, data and patient material were derived from the AAA screening cohort. The studies were covered by ethical approval from the regional ethical committee at Lund University, Sweden (permit number: 2010/239 with later amendments). All participants gave written informed consent following a detailed and clear explanation of the study's aims). For Paper III based on the MDCS cohort, after initial ethical approval from the regional ethical review board in Lund of the study in 1990 (LU 51/90), informed written consent was obtained from study participants. Additional ethical approval for genetic evaluation was granted in 2013 (LU 2013/566). All research practices were in accordance with the declaration of Helsinki and based on well-informed participants' consent. Moreover, the studies presented in the thesis did not involve any animal work, and no research on vulnerable or dependent individuals was done.

Results and discussion

Paper I

This study aimed to examine the relationship between AAA, whole blood DNA methylation, and plasma homocysteine levels. Associations of DNA methylation and homocysteine were also investigated concerning AAA growth rate (mm/year) and baseline aortic diameter (mm). The cohort consisted exclusively of men (65 years old) recruited from a screening program.

The main findings from the study are as follows:

Positive association of global DNA methylation and homocysteine levels with AAA

Men with AAA had significantly higher levels of global DNA methylation (hypermethylation) and homocysteine (hyperhomocysteinemia) in their whole blood compared to those without AAA (Figure 12 A - B, $P < 0.001$, Mann Whitney U test). As depicted in Table 3, both global DNA methylation (OR: 1.8, 95% CI: 1.1 - 2.9) and homocysteine (OR:1.1, 95% CI:1.0 - 1.1) levels were found to be positively associated with the presence of AAA, independent of each other, as well as smoking, medication use (lipid-lowering, antihypertensive), major comorbidities (CVD and diabetes). Homocysteine and global DNA methylation did not show any significant correlation.

Linear association with baseline aortic diameter

Global DNA methylation (Pearson's $R^2 = 0.18$, $P = 0.001$) as well as plasma homocysteine showed linear relationship levels (Pearson's $R^2 = 0.27$, $P < 0.001$) with baseline aortic diameter (Figure 12 C - D), which is currently a gold standard measure for AAA diagnosis and prognosis.

No significant association with AAA growth rate

Global DNA methylation (Figure 12 E, Pearson's $R^2 = -0.02$, $P = 0.84$), did not show any significant association with AAA growth rate, whereas homocysteine showed a linear association with growth rate (Figure 12, Pearson's $R^2 = 0.21$, $P = 0.038$). However, upon adjusting with baseline aortic diameter in multivariable linear regression analysis, the significant relationship between homocysteine and

AAA growth rate was lost (Beta = 0.02, 95% CI: -0.003 - 0.042). The same findings could be recapitulated upon performing logistic regression analysis with AAA growth rate dichotomized as slow and fast-growing (cutoff at 2.5 mm/year).

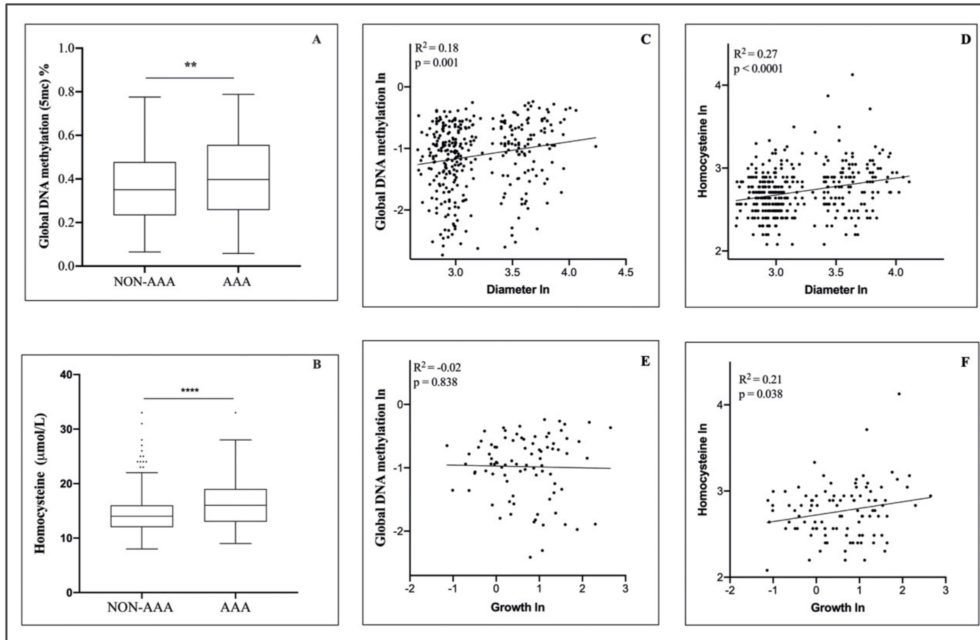

Figure 12. Associations of global DNA methylation and homocysteine with AAA prevalence, baseline aortic diameter and AAA growth rate. (A - B) Tukey box and whiskers plot depicting A) global DNA methylation and B) homocysteine levels in the non-AAA and AAA group of men. Boxes represent 25th, 50th, and 75th percentile and *P* values are estimated using Mann Whitney U test (*P* value: ** < 0.001, **** < 0.00001). (C - F) Linear associations of (C - D) Baseline Diameter and (E - F) AAA growth rate with (C & E) global DNA methylation and (D & F) homocysteine. Values were natural log (ln) transformed for the linear association Pearson's test. Values that resulted in infinity after undergoing a natural log transformation were not included in the analysis.

Table 3. Odds ratios and 95% confidence intervals for the association between global DNA methylation levels, homocysteine levels, and clinical variables with AAA.

Variables	Univariate			Multivariate*		
	OR	95% CI	*P*-value	OR	95% CI	*P*-value
DNA methylation	1.8	1.1 - 2.7	0.011	1.8	1.1 - 2.9	0.023
Homocysteine (μmol/L)	1.1	1.1 - 1.2	< 0.001	1.1	1.0 - 1.1	0.013
Lipid-lowering medication	1.6	1.2 - 2.1	< 0.001	1.1	0.6 - 1.9	0.76
Anti-hypertensive medication	1.7	1.3 - 2.3	< 0.001	1.4	0.8 - 2.5	0.31
Smoking pack year cut-off	3.0	2.2 - 4.1	< 0.001	2.9	1.9 - 4.1	< 0.001
CVD	3.6	2.2 - 5.8	< 0.001	3.2	1.7 - 5.8	< 0.001
Diabetes	2.5	1.3 - 5.2	0.013	1.4	0.5 - 3.8	0.56
Cancer	1.8	0.97 - 3.2	0.062	-	-	-
Metformin use	1.1	0.9 - 1.5	0.33	-	-	-
BMI	1.1	0.97 - 1.2	0.22	-	-	-

BMI: body mass index, CVD: cardiovascular disease, smoking pack year cut-off at 15 pack years.
* Adjusted using clinical variables significant in the univariate analysis.

Our study corroborates a prior study conducted on a UK-based white male cohort, confirming the presence of global hypermethylation in AAA cases and the linear association between hypermethylation and AAA size [68]. In the previous UK study, having a 10-year smoking history as an inclusion criterion might have resulted in selection bias, leading to overestimation of findings to some extent, as methylation has been highly correlated with smoking [24]. In the present study, we had a more diverse study population in terms of smoking history, to account for the differences in smoking exposure while testing associations. Moreover, unlike the UK study based on peripheral blood mononuclear cells (PBMCs) [68], we utilized DNA extracted from whole blood samples for our analysis on global methylation, which could have resulted in dilution of our results, due to the variation in methylation in various blood components [147]. Finally, even though we show a linear relationship between baseline aortic diameter and global DNA methylation, not having enough individuals with large AAAs (> 55mm/year) could be another factor associated with the underestimation of the current findings.

While we observed global hypermethylation and hyperhomocysteinemia in men with AAA at baseline, we did not see similar association trends concerning AAA growth rate. There could be two explanations: either the mechanisms of AAA growth/progression are distinct from the disease initiation as discussed previously [47,148], or the relation between DNA methylation and/or homocysteine could be more dynamic and require longitudinal data to base conclusions on.

Global DNA methylation and homocysteine levels have been implicated in several diseases and phenotypes, including inflammation, endothelial dysfunction, and oxidative stress induction [71,149-152]. In our study, global methylation homocysteine levels were not influenced by smoking, medication, or major comorbidities,

suggesting their role as independent AAA risk factors. However, the changes in global methylation and homocysteine levels could still be mediated by changes in diet [153,154], exposure to environmental toxins [155,156], biological aging [157], sex [158], etc., that we, unfortunately, could not account for in the present study. Interestingly, we did not find, in our study, a direct link between global methylation and plasma homocysteine levels, although the biological role of homocysteine in the regulation of DNA methylation through the one-carbon pathway is well-established [69,159]. Our findings add to the contradictory reports of association between homocysteine and global methylation [69,160,161]. It could be possible that we were not able to capture this due to the dynamic nature of the relationship, which requires repeated samples and sampling from the same tissue source. It is also a possibility that this regulation is not global but rather specific, and to test that more site-specific methylation studies are needed.

Our study had several strengths, such as investigating global DNA methylation and homocysteine in relation to both AAA occurrence and growth in a relatively large sample, considering the low prevalence of AAA. The cases and controls in the present study were recruited consecutively reducing selection bias, were anonymized, and run in a random order during experiments, to reduce investigator's bias. However, there were limitations to be considered too. A limited number of cases with large AAA diameters could result in an underestimation of the "true associations". The availability of samples from only one time point (baseline), reduced our chances of uncovering the complex dynamic and causal nature of the associations observed. Finally, although AAA is more prevalent in elderly men, females have been shown to have significantly worse outcomes [6,13,14], which could be due to late diagnosis, or biological differences. Therefore, further research, including mixed-sex studies, is required to refine our understanding.

Paper II

Here, we aimed to characterize the mitochondrial genetic landscape and its role in AAA. Next-generation whole mitochondrial genome sequencing and bioinformatics analyses were done in 48 non-AAA controls and 48 AAA cases, objectively diagnosed with imaging, and selected from the AAA screening cohort. Additionally, eight post-operative AAA biopsies and eight serum samples from the same individuals were also included.

General mutational characteristics between AAA and non-AAA

Upon sequencing, the mean mitochondrial genome coverage was ~1168×, making our mtDNA heteroplasmy assessment reliable at ≥ 1% minor allele frequency (MAF). Following stringent quality control criteria, our results elucidate differential mitochondrial DNA (mtDNA) mutational landscapes in individuals with and without AAA (Figure 13A). Individuals with AAA had more frequent insertions (exclusively heteroplasmic) in the mtDNA as compared to non-AAA controls (Figure 13B). Furthermore, AAA cases had higher homoplasmic mutational frequency in the gene loci, more frequent heteroplasmic mutations in the regulatory, D-loop region, as compared to individuals without AAA (Figure 13 C - D). In terms of the type of mutation, AAA cases had higher homoplasmic T>C transitions (*P* value = 0.022, Figure 13E) and significantly lower frequency of heteroplasmic T>A transversions (*P* value = 0.019, Figure 13F). The trinucleotide mutational signature test showed aging and DNA repair errors as potential sources of mutations in the current sample.

Structural changes in mtDNA

Interestingly, even though the gross count of mtDNA structural rearrangements was low in AAA cases (Figure 13G), the heteroplasmy levels of deletions and duplications were significantly elevated as compared to controls (Figure 13 H - I). In addition, we report a novel 24 bp mtDNA duplication (16401-16424) present in 4% AAA whole blood samples but in 75% (6 out of 8) of the AAA biopsies.

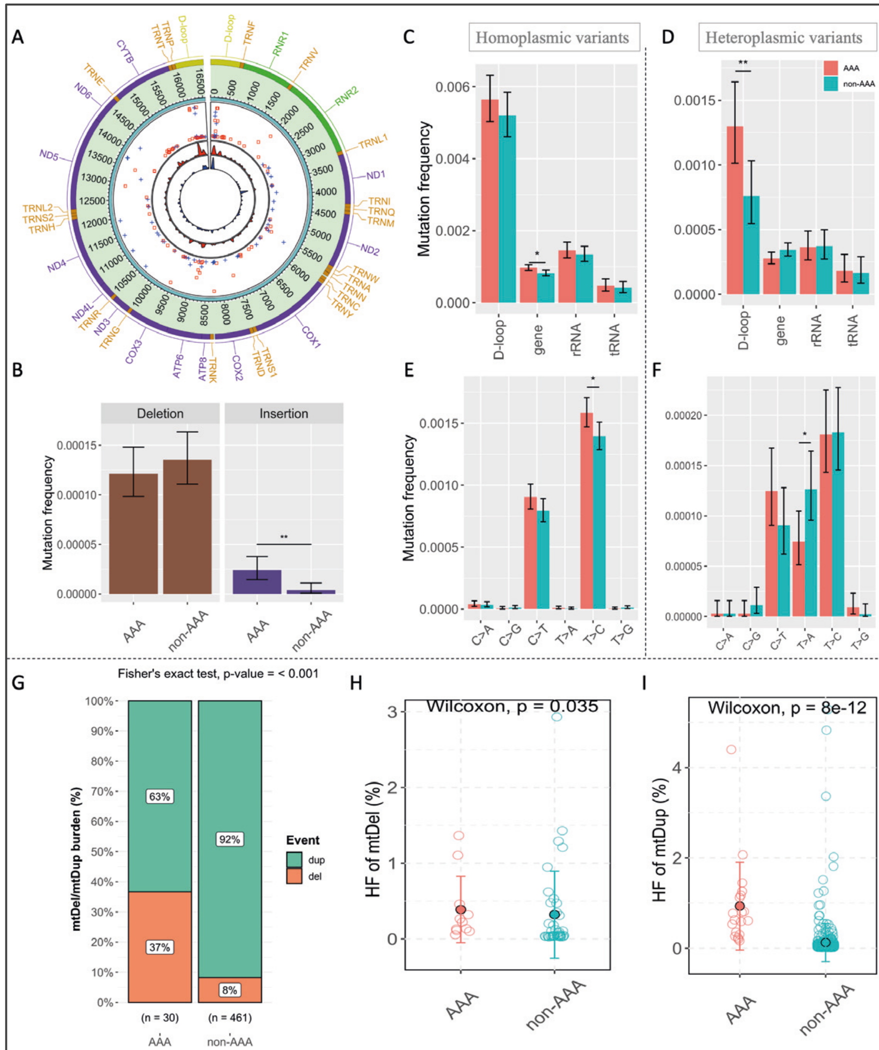

Figure 13. Differential mtDNA mutational landscape in AAA. A) mtDNA heteroplasmic variants in men with and without AAA. The circle represents whole mitochondrial genome (16.5 kb) with gene names depicted on the outermost 1st track. 2nd track marks the genomic location. 3rd track depicts the distribution of variants going from 100% to 0% on the inside. Red squares are individual variants in AAA while blue pluses represent non-AAA. The 4th and 5th tracks compare the density of variants. Red—AAA, Blue—non-AAA. **B** through **F**, Comparisons of mutation frequency between AAA and non-AAA group: (**B**) for INDELs, (**C** and **D**) for genomic regions: D-loop protein-coding, tRNA, and rRNA, and (**E** and **F**) for point mutation types. Mutation frequency = number of mutations (**B-D**) (in each mtDNA region/length of the region x number of subjects), (**E** and **F**) (at each site/number of reference alleles in mtDNA genome x the number of subjects). Error bars indicate the 95% CIs of the mutation frequency assuming Poisson distribution. Statistical significance for the comparison between AAA and non-AAA (***P* value < 0.01, **P* value < 0.05 by Fisher's exact test). **G**, mtDel-mtDup burden (%) in AAA vs non-AAA. HF of mtDel (H) and mtDup (I) in AAA cases and controls. Mean values are denoted by black outlined dots. Red: AAA; Green: non-AAA. *P* = 2-sided p value estimated by Wilcoxon-Mann-Whitney test (exact distribution). mtDNA, mitochondrial DNA; RNA, ribosomal RNA; and tRNA, transfer RNA; HF, heteroplasmic fraction; INDEL, insertion and deletion; mtDel, mtDNA deletion; mtDup, mtDNA duplication.

MtDNA mutations in the D-loop region

The regulatory mitochondrial D-loop region had significantly higher heteroplasmic mutational load in AAA cases (Figure 13D). Upon further investigation into the different D-loop subregions, we observed that AAA cases had high heteroplasmic mutational frequency in the functionally relevant and conserved replication fork barrier region corresponding to the mitochondrial extended termination-associated sequence (MT-TAS2) (Figure 13D & 14A).

As MT-TAS2 is a conserved region, most individuals had either one or rarely two mutations in the region. Upon categorizing the MT-TAS2 mutation status as individuals with and without MT-TAS2 mutation(s), we observed a significant difference in the distribution between AAA and non-AAA individuals (Figure 14B). The significant association between MT-TAS2 mutation status and AAA remained significant upon adjusting for potential confounders in multivariable logistic regression with Firth's small-sample size correction (OR: 4.94, 95% CI: 1.47 - 19.16, Table 4).

Table 4. Univariable and multivariable analysis of positive MT-TAS2 mutation status and clinical variables between 65-year-old men with and without AAA at ultrasound screening.

Variables	Univariate			Multivariate*		
	OR	95% CI	*P*-value	OR	95% CI	*P*-value
MT-TAS2 mutation status	3.27	1.45 - 7.65	0.004	4.94	1.47 - 19.16	0.009
Smoking pack year	1.06	1.03 - 1.10	1.9E-05	1.06	1.03 - 1.11	0.003
Diabetes	1.00	0.20 - 4.95	1.000	0.93	0.10 - 8.87	0.951
Cancer	0.41	0.04 - 2.61	0.351	0.03	0.00 - 0.48	0.01
HDL	0.15	0.04 - 0.46	2.9E-04	0.19	0.04 - 0.78	0.02
b-Leukocyte count (/L)	1.46	1.16 - 1.93	6.3E-04	1.07	0.75 - 1.60	0.71
b-Thrombocyte count (/L)	1.00	0.99 - 1.01	0.268	1.01	0.99 - 1.02	0.305

MT-TAS2: mitochondrial extended termination-associated sequence; HDL: high-density lipoprotein; b: blood.
* adjusted by mentioned covariates in a multivariable logistic regression model with Firth's small-sample size correction.

Although mitochondrial DNA copy number (mtDNA-CN) did not associate with AAA or mtDNA variations, we observed that individuals with MT-TAS2 mutation(s) had significantly higher baseline aortic diameter and oxidative stress marker, plasma MPO levels as compared to individuals without MT-TAS2 mutation, exclusively in the low mtDNA-CN (< median) group (*P* for interaction by 2-Way ANOVA: 0.035 & 0.081, respectively, Figure 14C & D).

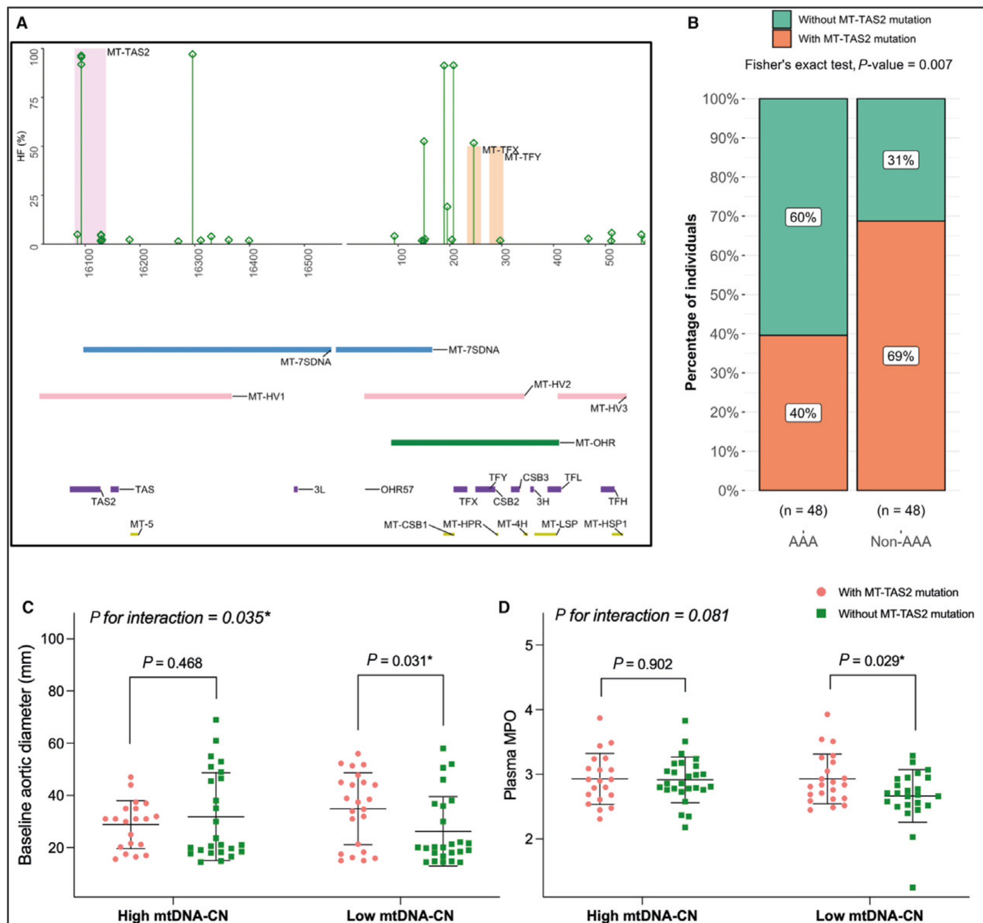

Figure 14. Distribution and context of mtDNA mutations in the D-loop region. (**A**) MtDNA D-loop mutations' HFs in AAA cases. Light pink shadow: MT-TAS2 region (OR, 4.7; adjusted *P* value=0.0003 by Fisher's exact test with Bonferroni correction). The bottom panel denotes subregions of the D-loop. (**B**) Distribution of individuals (%) with and without MT-TAS2 mutation(s), concerning AAA status. **C** and **D**, Comparisons (mean ± SD) of baseline aortic diameters (**C**) and plasma MPO plasma (**D**) between men with and without MT-TAS2 mutation(s), subgrouped according to low and high mtDNA-CN (median cutoff). *P* = p value estimated by linear model with permutation test, *P* for interaction (MT-TAS2 mutation status x mtDNA-CN) determined by 2-way ANOVA with permutation. HF, heteroplasmic fraction; D-loop, displacement loop; MPO, myeloperoxidase; mtDNA, mitochondrial DNA; mtDNA-CN, mitochondrial DNA copy number; MT-TAS2, mitochondrial extended termination-associated sequence.

MtDNA haplogroup

Finally, we reported that the Northern European haplogroup cluster JTU was overrepresented in AAA (non-significant association, Figure 18A) and significantly associated with a positive family history of AAA (P = 0.036, Fisher's exact test, Figure 15B).

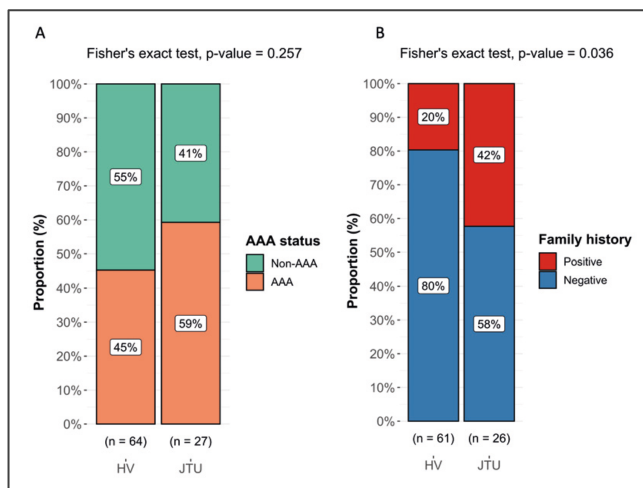

Figure 15. European-haplogroup clusters' distribution: The percentage of men (y-axis) belonging to respective (A) AAA status (AAA and non-AAA) and (B) AAA family history status (negative and positive), with respect to major haplogroup clusters, JTU and HV of Europe (x-axis). p-value estimated by Fisher's exact test.

In paper II we, for the first time, characterized a novel mitochondrial genetic landscape in AAA. Even though the sample size of this exploratory analysis was small, we were able to identify interesting mtDNA-related patterns in AAA.

Considering the important role of mitochondrial dysfunction in AAA pathophysiology, oxidative stress and a huge yet mostly unknown hereditary component of AAA [31,162], this was an important study. The trinucleotide mutational signature, in addition to the mutational types observed (more transitions) in AAA, supports previous studies challenging the free-radical theory of aging and old-age diseases [163], supporting rather a role of replication or DNA repair errors [164].

Despite a low count of structural, mtDel/mtDup mutations in AAA, their heteroplasmy levels were significantly higher than in controls. This suggests that it's not the presence of structural mitochondrial genetic defects but rather the load that might contribute to AAA pathophysiology. We identified a novel 24 bp mtDup in the majority of the AAA tissues sequenced, which corresponds to a disease-

associated 7S-DNA region of mtDNA [73]. However, this needs further methodological validation and replication in a larger cohort.

Our study further highlights the importance of heteroplasmic mutations in the regulatory D-loop region of mtDNA, in AAA. These mutations, especially in the conserved MT-TAS2 subregion could be linked to mitochondrial genomic instability [165] in AAA cases. Our results further suggest that the effects of MT-TAS2 mutations on AAA pathology are exacerbated in individuals with low mtDNA copy numbers (mtDNA-CN), a phenomenon that has been reported before in the context of pathogenic mtDNA mutations [166]. As suggested previously and supported by ongoing trials, increasing the abundance of healthy mitochondria (mtDNA-CN) could have a therapeutic potential in diseases where mitochondrial dysfunction is implicated [88,166].

Finally, it was very interesting to observe that the northern European mitochondrial haplogroup JTU was significantly associated with a positive family history of AAA but not AAA presence itself. It has been previously reported that the risk of AAA is significantly higher in individuals with a family history of AAA through a female relative [167]. As mitochondria are exclusively passed on from females, evolutionarily, the mtDNA will undergo natural selection in the context of females. Therefore, it is possible that the inherited homoplasmic mtDNA genetic aberrations that have no impact on female health might have a pathological effect in males, thus substantiating the “male disadvantage”. Unlike germline homoplasmic mutations, dynamic mtDNA heteroplasmy (mutation load), could play a greater role in AAA pathophysiology in the context of females.

While the study provides crucial insights into mitochondrial genetics in AAA, it has limitations, such as a relatively small sample size, residual confounding, exclusive focus on males, and the inability to establish causal relationships. Nonetheless, it opens new avenues for exploring the role of mtDNA in AAA and other chronic diseases. A comprehensive understanding the mitochondrial genetics in AAA (in both males and females) could have implications for risk stratification and therapeutics.

Paper III

In this retrospective Swedish cohort study with a median follow-up of 24.3 years, the cumulative incidences of intact AAA and rAAA were 1.6% (men: 2.9% and women: 0.8%) and 0.3% (men: 0.6% and women: 0.1%), respectively.

Genetic variation and AAA

We identified a novel association between a SNP in the NADPH oxidase, *NOX3* gene (rs3749930) and an increased risk of ruptured AAA (rAAA), in the overall study population, independent of potential confounders (Table 5, adjusted HR (aHR): 1.88; 95% CI: 1.05 - 3.37). Subgroup comparison and interaction analysis between male-females was not possible due to the overall low (especially in females) incidence of rAAA concerning the SNP. Another SNP (rs17849502) was weakly associated with AAA incidence in males only (aHR: 1.88, 95% CI:1.05 - 3.37), however, the interaction with sex was not significant (*P* = 0.25). There was no further evidence for interaction between SNPs and sex concerning intact AAA risk in the current study.

Table 5. Associations between genotypes and rAAA risk in the overall and male population.

Genotype	Non-AAA (N=24782)	Ruptured AAA (N=71)	OVERALL		MALE	
			CRUDE HR (95% CI)	ADJUSTED* aHR (95% CI)	CRUDE HR (95% CI)	ADJUSTED* aHR (95% CI)
NOX5 rs150003957						
AA (ref)	24685 (98.0%)	69 (97.2%)	1 (ref)	1 (ref)	1 (ref)	1 (ref)
AC + CC	496 (2.0%)	2 (2.8%)	1.43 (0.35-5.84)	1.57 (0.39-6.47)	2.05 (0.50-8.41)	2.15 (0.52-8.60)
NOX3 rs3749930						
GG (ref)	22267 (88.4%)	57 (80.3%)	1 (ref)	1 (ref)	1 (ref)	1 (ref)
GT+TT	2914 (11.6%)	14 (19.7%)	1.87 (1.04-3.35)	1.88 (1.05-3.37)	2.38 (1.30-4.35)	2.49 (1.36-4.55)
NCF2 rs17849502						
GG (ref)	23119 (91.8%)	68 (95.8%)	1 (ref)	1 (ref)	1 (ref)	1 (ref)
GT + TT	2062 (8.2%)	3 (4.2%)	0.50 (0.16-1.58)	0.52 (0.16-1.65)	0.62 (0.19-1.98)	0.66 (0.21-2.13)
CYBA rs9940427						
GG (ref)	23714 (94.2%)	68 (95.8%)	1 (ref)	1 (ref)	1 (ref)	1 (ref)
GT + TT	1467 (5.8%)	3 (4.2%)	0.72 (0.23-2.27)	0.75 (0.24-2.39)	0.90 (0.28-2.89)	0.98 (0.31-3.14)
MPO rs28730837						
CC	24099 (95.7%)	69 (97.2%)	1 (ref)	1 (ref)	1 (ref)	1 (ref)
CT + TT	1082 (4.3%)	2 (2.8%)	0.65 (0.16-2.64)	0.63 (0.15-2.57)	0.84 (0.21-3.46)	0.80 (0.19-3.26)

AAA, abdominal aortic aneurysm; aHR, adjusted hazard ratio; CI, confidence interval. Adjusted for age, sex (overall), education level, statin use, prevalent cancer, physical activity, BMI, alcohol, and smoking status (Model 1).

Table 6. Associations between vitamins intake and intact AAA risk in the overall population, stratified by sex.

Vitamins	Non-AAA	Intact AAA	OVERALL		MALE		FEMALE		P for interaction
	(N=24782)	(N=399)	CRUDE HR (95% CI)	ADJUSTED* aHR (95% CI)	CRUDE HR (95% CI)	ADJUSTED* aHR (95% CI)	CRUDE HR (95% CI)	ADJUSTED* aHR (95% CI)	
Riboflavin									
Low	12387 (50.0%)	206 (51.6%)	1 (ref)	1 (ref)	1 (ref)	1 (ref)	1 (ref)	1 (ref)	
High	12395 (50.0%)	193 (48.4%)	0.93 (0.77-1.10)	0.80 (0.65-0.98)	0.66 (0.52-0.83)	0.68 (0.54-0.87)	1.00 (0.72-1.50)	1.21 (0.84-1.74)	**0.038**
Folate									
Low	12365 (49.9%)	220 (55.1%)	1 (ref)	1 (ref)	1 (ref)	1 (ref)	1 (ref)	1 (ref)	
High	12417 (50.1%)	179 (44.9%)	0.78 (0.64-0.95)	0.81 (0.66-0-99)	0.67 (0.53-0.85)	0.78 (0.61-0.99)	0.67 (0.46-0.96)	0.92 (0.63-1.35)	0.99
Beta-carotene									
Low	12352 (49.8%)	235 (58.9%)	1 (ref)	1 (ref)	1 (ref)	1 (ref)	1 (ref)	1 (ref)	
High	12430 (50.2%)	164 (41.1%)	0.66 (0.54-0.80)	0.92 (0.75-1.13)	0.85 (0.67-1.10)	0.95 (0.74-1.21)	0.65 (0.45-0.92)	0.89 (0.62-1.29)	0.217
Vitamin E									
Low	12383 (50.0%)	207 (51.9%)	1 (ref)	1 (ref)	1 (ref)	1 (ref)	1 (ref)	1 (ref)	
High	12399 (50.0%)	192 (48.1%)	0.91 (0.75-1.10)	0.88 (0.72-1.07)	0.72 (0.57-0.91)	0.79 (0.62-0.99)	0.93 (0.65-1.30)	1.14 (0.79-1.65)	0.224
Vitamin D									
Low	12436 (50.2%)	160 (40.1%)	1 (ref)	1 (ref)	1 (ref)	1 (ref)	1 (ref)	1 (ref)	
High	12346 (49.8%)	239 (59.9%)	1.50 (1.30-1.90)	1.18 (0.96-1.46)	1.10 (0.86-1.40)	1.08 (0.84-1.38)	1.40 (0.97-2.00)	1.50 (1.05-2.16)	0.290
Vitamin B12									
Low	12418 (50.1%)	177 (44.4%)	1 (ref)	1 (ref)	1 (ref)	1 (ref)	1 (ref)	1 (ref)	
High	12364 (49.9%)	222 (55.6%)	1.30 (1.10-1.60)	0.98 (0.80-1.20)	0.82 (0.65-1.00)	0.81 (0.63-1.03)	1.40 (0.99-2.00)	1.55 (1.08-2.24)	**0.013**

AAA, abdominal aortic aneurysm; aHR, adjusted hazard ratio; CI, confidence interval.
Adjusted for age, sex (overall), education level, statin use, prevalent cancer, physical activity, BMI, alcohol, and smoking status (Model 1).

Vitamin intake and AAA

Higher daily intake of riboflavin was associated with a decreased incidence of intact AAA in both males (aHR: 0.68, 95% CI:0.54 - 0.87) and the overall study population (aHR: 0.80, 95% CI: 0.65 - 0.98). This relationship, however, was not observed in the female subgroup (aHR: 1.21, 95% CI: 0.84 - 1.74). The effect of riboflavin intake on intact AAA risk was modified by sex (*P* for interaction = 0.038). High folate intake also reduced hazards of intact AAA in the males (aHR: 0.78, 95% CI: 0.61 - 0.99) subgroup and the overall population (aHR : 0.81, 95% CI: 0.66 - 0.99) (*P* for sex interaction = 0.99). Vitamin E intake was associated with reduced AAA hazard only in the male subgroup (aHR: 0.79, 95% CI: 0.62 - 0.99), however, no significant interaction with sex was observed (*P* for interaction = 0.22) (Table 6).

A 3-category, vitamin score (by combining the low and high riboflavin and folate intakes), could stratify the current study's male population on intact AAA risk (Figure 16, log-rank *P* value < 0.001). Keeping a vitamin score of 0 as reference the hazards and 95 % CI for intact AAA incidence were: vitamin score 1 (HR: 0.70, 95% CI: 0.51 - 0.95), vitamin score 2 (HR: 0.58, 95% CI: 0.45 - 0.76).

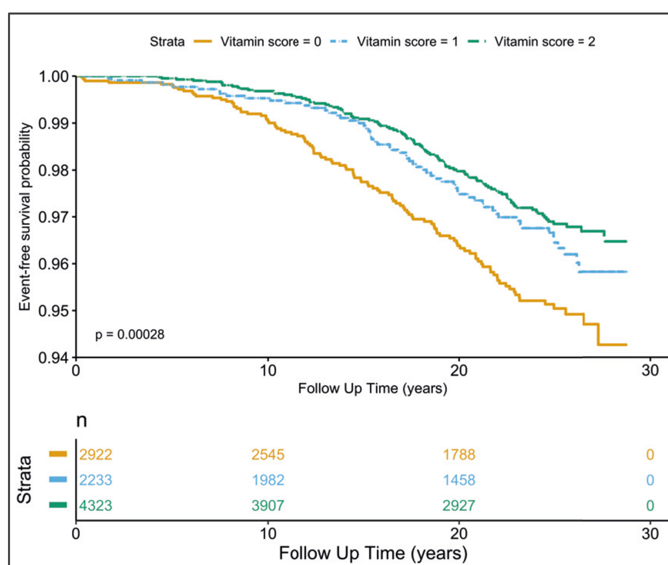

Figure 16. Intact AAA risk concerning antioxidant vitamin score in males: Kaplan-Meier plot with y-axis depicting intact AAA-free, survival probability, stratified by vitamin scores (0-2). x axis denotes the follow-up time in years. The bottom presents the number of individuals in color-coded vitamin score strata at the given times. *P* value (p) was estimated using log-rank test for differences in the survival curves.

Interestingly, higher intake of Vitamin B12 was associated with increased incidence of intact AAA in the female subgroup (aHR (95% CI): 1.55 (1.08 - 2.24)). There was evidence of effect modification by sex in the instance of vitamin B12 (*P* for interaction = 0.01) (Table 6).

<u>Gene-vitamin interaction</u>

Finally, we investigated whether the presence of oxidative stress-related SNPs modifies the effect of vitamin intake on future intact AAA risk. We observed that the inverse relationship of riboflavin and vitamin D intake with intact AAA risk was enhanced in individuals carrying a wildtype recessive *NOX3* (rs3749930) genotype by 60% and 66% respectively (Figure 17, *P* for interaction = 0.048 and 0.02, respectively).

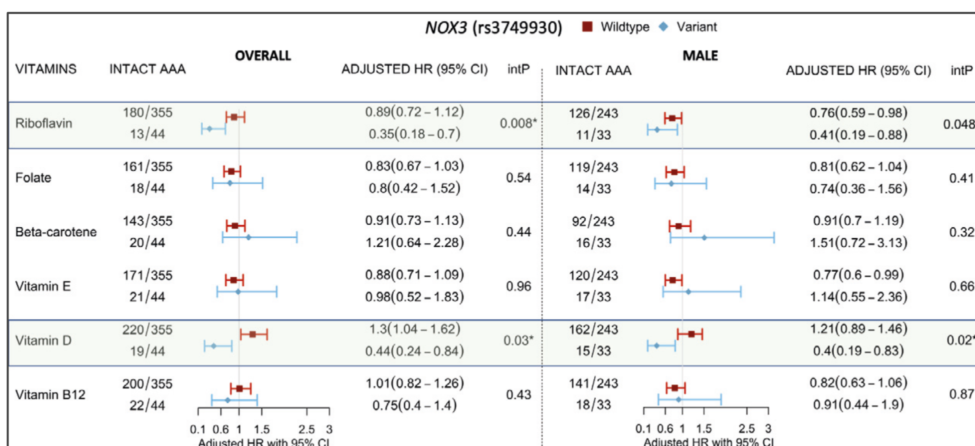

Figure 17. Adjusted hazard ratios (HRs) for antioxidant vitamin intakes and future intact AAA risk in the overall and male study population by *NOX3* genotype: The intact AAA column represents the number of individuals with high vitamin intake with intact AAA incidence by total number of intact AAA incidences. Adjusted HRs are estimated using multivariable cox regression analysis, adjusted for age, sex (overall), education level, statin use, prevalent cancer, physical activity, BMI, alcohol, and smoking status (model 1). The shaded boxes represent instances of significant multiplicative interaction (intP) between genotype and vitamin intake (intP < 0.05).

There is increasing evidence implicating oxidative stress-related genes: *MPO* and *NADPH oxidases* in the development and progression of AAA and its risk factors [52,56,168]. Despite oxidative stress being a central pathophysiological mechanism [52,169], the evidence on the beneficial effect of antioxidant diet and vitamin intake has been either scarce or contradictory. Hence, our study investigating the associations and/or

interactions between genetic variation in oxidative stress-related genes, antioxidant vitamin intakes, and sex in the context of AAA has relevance.

In the present study, we first investigated the association between SNPs in oxidative stress-related genes and AAA (intact and ruptured), identifying a novel SNP in the *NOX3* gene (rs3749930) associated with increased risk of rAAA in males and the overall population. The NOX3 protein has been associated with heart failure in an animal study [170] and the SNP has been previously associated with graft rejection [171], possibly mediated by oxidative and immunological processes that could also apply to AAA pathophysiology. We could not find any associations in the female subgroup, and evidence of sex-gene interaction in the present study, possibly, due to statistical limited power pertaining to the low AAA (intact and r-AAA) incidence in females.

Our findings showing the potential beneficial impact of riboflavin and folate intake in reducing the incidence of intact AAA are well supported by previous data on CVDs and *in vivo* AAA models [111,172-174]. The inverse association between riboflavin intake and AAA incidence was found to be significantly enhanced in males as compared to females. We speculated that this sex difference could be related to the well-established protection offered by riboflavin against AAA risk-factor, hyperhomocysteinemia, which affects males to a greater extent [175-177].

In another significant sex-vitamin interaction, we found that vitamin B12 intake was associated with intact AAA risk but only in females. Although the mechanisms are not clear, studies have reported sex differences in vitamin B12 mode of action and mechanism [178,179]. Furthermore, oxidative stress mechanisms and responses also seem to differ between males and females [180].

Given the well-studied sexual dimorphism in AAA (risk factors and outcomes) [14,17], we accounted for sex in our analyses wherever possible. However, the low AAA events in females limited some firm conclusions. A way to increase sample size and statistical power in the present cohort for further research on genetics and peripheral artery aneurysm would be to retrieve patients with thoracic aortic aneurysm [181] and popliteal artery aneurysm [182], and add them to the patients with AAA since these peripheral aneurysms have similar sex distribution and risk factor profile as AAA. Furthermore, even though we adjusted for BMI and physical activity in our analysis, there could have still been some overestimation of the vitamin intake effect by residual differences in total energy intake and other residual variability in bioavailability, confounding or mediating factors.

Touching on the emerging nutrigenetics concept, which had been mostly unexplored in AAA, we observed two significant gene-vitamin interactions in the present study. Although the exact mechanisms behind these interactions could not be elucidated, they indicate a way in which we can account for inter-individual or population-level differences between the effect of genotypes or vitamin intake.

The absence of repeated measures of dietary intake limited the analysis on dynamic associations or causation. In addition, the self-reported diet data could have introduced some information bias, thus affecting the accuracy of the findings. However, the dietary assessment method used is highly valid and reproducible [123,124]. Overall, the study underscores the complex interplay between genetic variations, antioxidant vitamins, and sex in AAA risk. It is also important to note that the findings are applicable to the European population, over the age of 44, and should be interpreted keeping the study limitations in mind.

Paper IV

Heme toxicity as a mechanism of pathophysiology is increasingly implicated in several diseases and phenotypes [90], and here, we aimed to study this phenomenon in the context of AAA. Our results, based on an exclusively older male population, demonstrate a widespread disruption in heme or heme-related proteins in both the prevalence and progression of AAA.

AAA prevalence and heme homeostasis

Elevated plasma levels of Hpx (OR: 1.42, 95% CI: 1.16 - 1.77) and heme (OR: 2.48, 95% CI: 1.92 - 3.28) and diminished HO-1 (OR: 0.51, 95% CI: 0.38 - 0.66) levels in baseline samples were associated with the presence of AAA, independent of potential confounders (Table 7). The combination of heme and HO-1 showed a good diagnostic potential (AUC: 0.76, sensitivity: 80%, specificity: 48%).

Table 7. Levels of heme-related markers in Non-AAA and AAA and their association with AAA prevalence (odds ratio (OR)) and diagnostic potential for AAA (area under curve (AUC)).

Marker	Non-AAA	AAA	*P*	Adjusted *P*[1]	OR[2]	95% CI[2]
Hpx (mg/mL), mean (SD)	1.56 (1.0)	1.94 (1.03)	< 0.001	< 0.001	1.42	1.16 - 1.77
HO-1 (ng/mL), mean (SD)	3.70 (1.53)	2.92 (1.39)	< 0.001	< 0.001	0.51	0.38 - 0.66
Heme (μM), mean (SD)	23.46 (12.2)	37.69 (23.4)	< 0.001	< 0.001	2.48	1.92 - 3.28
Hb (g/L), mean (SD)	148.1 (10.1)	147.0 (11.2)	0.31	0.45	0.90	0.73 - 1.10
Hp type, n (%)						
Hp1-1	50 (17.9)	23 (16.2)			0.92	0.51 - 1.62
Hp2-1	136 (48.8)	68 (47.9)	0.83	0.73	Ref	-
Hp2-2	93 (33.3)	50 (35.9)			1.10	0.70 - 1.72
Hp genotype, n (%)						
GG	177 (63.4)	85 (59.9)			Ref	-
GA	92 (33.0)	49 (34.5)	0.55	0.85	1.11	0.72 - 1.71
AA	10 (3.6)	8 (5.6)			1.67	0.62 - 4.37

Hpx: Hemopexin; HO-1: Heme-oxygenase-1; Hb: Hemoglobin; Hp:Haptoglobin
[1] Adjusted for smoking ≥ 15 packs/year, CVD, and medication (hypertension, lipid, metformin).[2] Odds ratios for standardized (continuous) biomarkers (mean = 0 and SD = 1).

AAA growth and heme homeostasis

Increase in whole blood Hb (Spearman's correlation coefficient (rho) = 0.32, *P* < 0.001) and decrease in plasma Hpx (Spearman's correlation coefficient (rho) = -0.38, *P* < 0.001) correlated significantly with AAA growth rate (Figure 18). The linear associations between Hb (adjusted beta = 0.13, *P* = 0.003) and Hpx (adjusted beta = -0.13, *P* = 0.008) levels at baseline with AAA growth rate (mm/year) were

independent of baseline aortic diameter and metformin usage. The combination of Hb and Hpx showed a good prognostic potential for discriminating fast and slow-growing AAA (AUC: 0.76, sensitivity, 80%, specificity, 62%).

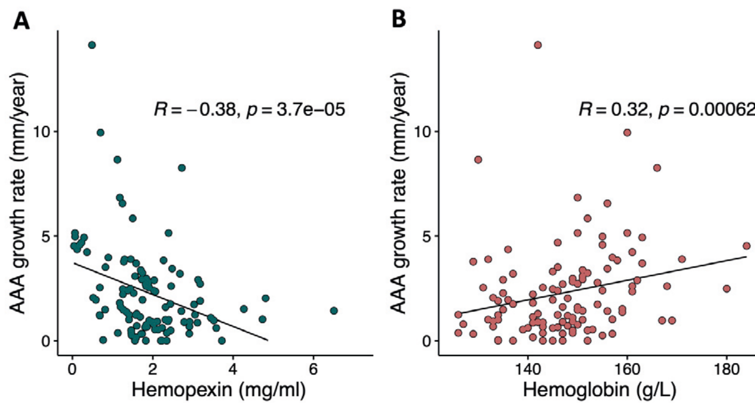

Figure 18. Heme-related markers for AAA growth: linear association of AAA growth rate (mm/year) in 65-year-old men with (A) Plasma hemopexin (Hpx, mg/mL) and (B) with whole blood hemoglobin (g/L). The plots display linear regression lines, Spearman's (rho) correlation coefficients (R) and p values from Spearman correlation analyses.

<u>Improvement in previously published biomarkers' model</u>

A previously published combination model of growth differentiation factor - 15 (GDF-15) and cystatin B (CSTB), on the same study population, showed the best diagnostic potential among ~ 90 proteins (AUC: 0.76, sensitivity, 80%, specificity, 52%)[169]. The addition of plasma heme to this model improved the diagnostic capability significantly (Figure 19, AUC: 0.82, sensitivity, 80%, specificity, 72%, *P* value = 0.004, Delong's test for correlated ROCs). No similar improvement in the previously published prognosis model(s) to discriminate between fast and slow-growing AAA was observed.

No association between Hp genotype/phenotype and AAA presence or growth was observed (Table 7).

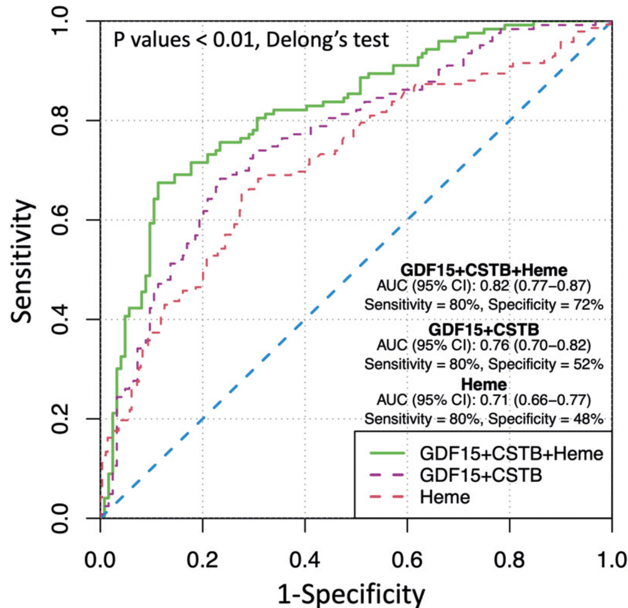

Figure 19. Receiver operating characteristic (ROC) curve analysis: for Heme (red curve), combination of GDF15-CSTB (pink curve), and combination of GDF15-CSTB-heme (green curve) for discrimination of individuals with and without AAA. X-axis presents sensitivity range and Y-axis denotes (1-Specificity). P: P value estimated by Delong's test for correlated ROCs (GDF15+CSTB+Heme vs GDF15+CSTB or Heme). GDF-15: growth differentiation factor-15; CSTB: cystatin B.

We found that men diagnosed with AAA had higher levels of plasma heme, suggesting a potential role for heme in AAA development or pathophysiology. Prior studies have linked elevated plasma heme with vascular conditions, such as atherosclerosis and preeclampsia [183,184]. However, this study was the first to investigate plasma heme's association with AAA. Heme could be involved in AAA pathophysiology through several mechanisms, including oxidative damage, hemolysis, and inflammation, following a "self-perpetrated cycle" [90].

Men with AAA displayed increased levels of Hpx, a protein involved in heme scavenging, in their plasma. However, we stumbled upon an intriguing contradiction – while plasma Hpx levels positively correlated with AAA prevalence, they exhibited a significant negative correlation with AAA growth rate. This paradoxical finding hints at the context-specific role of Hpx in different stages of AAA development. It's possible that Hpx undergoes upregulation during the early stages of AAA as a compensatory mechanism, with a higher magnitude of baseline levels

potentially indicating a slower growth rate. Based on previously published studies [94,95], we speculate that the role of Hpx in AAA might vary with the disease's stage or severity. However, further research is required to understand this discrepancy fully.

The observation of reduced plasma HO-1 levels in individuals with AAA at baseline aligns with the biological concept of HO-1 acting as a protective factor against oxidative stress and inflammation [93,185]. Animal models have corroborated this notion by demonstrating that HO-1 deficiency increases the incidence of AAA and AAA rupture, accompanied by increased levels of reactive oxygen species, vascular smooth muscle cell apoptosis, and inflammation [186]. However, the role of HO-1 in AAA appears to be multifaceted, with some studies suggesting that it might both protect against AAA development and exacerbate the severity of established AAA [187], more longitudinal data are needed to confirm this.

When combining heme and Hpx measurements, we achieved the highest diagnostic potential for distinguishing individuals with AAA from those without. Furthermore, adding heme to an already published model [169], further improved the diagnosis potential and specificity, significantly. These findings, if established in another independent cohort, could uncover the seemingly good potential of heme-markers in AAA diagnosis.

Unexpectedly, we found that higher whole blood Hb levels were associated with a more rapid AAA growth rate, independent of baseline aortic diameter. This observation aligns with previous studies demonstrating a U-shaped association (which we could not explore due to little, to no prevalence of anemic men in our study population) between Hb levels and cardiovascular disease (CVD) incidence and outcomes [188,189]. Biologically, high Hb levels can increase blood viscosity, impeding blood flow and tissue perfusion, while also triggering platelet activation, promoting thrombosis and oxidative stress [190,191], potentially contributing to AAA pathophysiology. Combining Hpx and Hb levels demonstrated potential for distinguishing between fast and slow-growing AAAs, showing promise for future prognosis applications, if validated.

We did not identify significant associations between Hp phenotype-genotype and either AAA prevalence or growth. These results add to the existing contradictory body of evidence regarding the role of Hp phenotype in AAA and cardiovascular diseases [96-98,192]. It is plausible that Hp's role in AAA is independent of phenotype and the currently studied functional genotype, indicating the need for further investigation.

The findings from this study are particularly relevant to older men (the most affected demographic group) and the European population. We have used well-validated and other precise and high-throughput techniques like ddPCR for quantification and

comprehensively covered some of the major markers related to heme-toxicity. In addition, our study design minimized chances of a selection bias, except for the inherent selection criterion for males. Our sensitivity and multivariable analyses, and alignment of results with prior studies and biological phenomena, strengthen the validity of the current findings. However, some issues that could not be addressed and need further work were optimization of pre-analytical factors, sex-specific effects, casual and dynamic effects, and replication in another independent cohort.

Conclusions and future perspectives

In conclusion, through the studies presented in this thesis, we have investigated and identified factors related to oxidative stress in AAA, its clinical aspects, and complication(s). Some newly identified markers show promise for future translation for diagnosis, prognosis, or treatment of AAA. Mentioned below are the specific conclusions and future perspectives from each study:

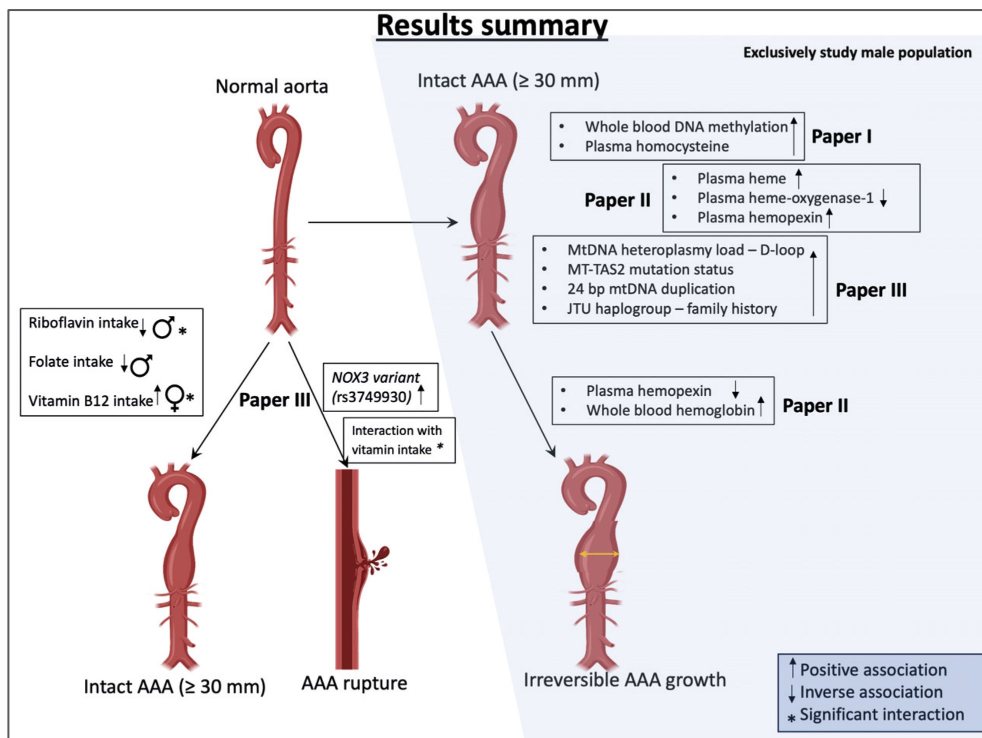

Figure 20. Visual thesis results summary. Created with BioRender.com and MS PowerPoint.

Paper I

We demonstrate that global DNA hypermethylation and hyperhomocysteinemia are independently associated with AAA and baseline aortic diameter, indicating a mechanism through which environmental or lifestyle factors manifest in AAA. Our results further support that the mechanisms or risk factor profiles of AAA initiation and expansion are distinct.

Future studies with repeated samples and site-specific methylation analysis are needed to establish the dynamic methylation-homocysteine relationship as well as the causal role in AAA. Enhancing the generalizability of these data by investigating in a more diverse age group and including female participants could prove useful. Finally, exploring the associations in another independent cohort and testing interaction(s) with diet and oxidative stress markers could aid future personalized and risk stratification strategies.

Paper II

In summary, this study breaks new ground in our understanding of AAA by exploring the novel mitochondrial genetics and uncovering distinct mutational patterns associated with AAA. These findings have the potential to reshape our approach to AAA diagnosis, risk assessment, and treatment, offering hope for improved patient outcomes in the future.

While providing significant insights, the study also highlights the need for further, more extensive investigations, including functional studies and studies on larger, mixed-sex populations. These studies could confirm and expand upon the current findings and provide a more comprehensive understanding of the role of mitochondrial genetics in AAA. Moreover, including mitochondrial genetic factors with known/unknown nuclear genetic markers could help fill some gaps in the missing heritability of AAA. This is something that we are currently investigating in a much larger population based-MDCS cohort.

Paper III

This study provides valuable insights into the complex relationship between genetics, antioxidant vitamin intake, and sex in the context of AAA. It suggests that

understanding an individual's genetic background and sex may be crucial in tailoring preventive strategies for AAA.

Further research is needed to confirm and expand upon these findings and to explore the mechanisms underlying these interactions in AAA pathogenesis. Using high-throughput whole genomic approaches, including data on the bioavailability of vitamins, and finally having repeated measures could aid in reliable understanding and translation of gene-vitamin-sex interaction(s) for personalized AAA prevention or management. Future clinical studies are encouraged to account for sex-based and genetic differences when investigating the benefits of antioxidant vitamins in AAA and similar pathologies.

Paper IV

Overall, this study reports the distinct disruption of heme and related proteins, associated with heme-toxicity in both the development and progression of AAA. Our findings underscore the potential clinical relevance of heme-related markers in diagnosing and prognosticating AAA.

Further investigations (including broader demographic groups) and longitudinal studies are essential to validate these findings and better understand the roles of these markers in AAA pathogenesis and progression. These findings, if established, warrant further exploration into comprehensive assessment and therapeutic strategies targeting heme toxicity in AAA management, potentially leading to improved diagnostic and treatment methods.

Oxidative stress and factors related to it are often modifiable. Therefore, the present thesis aimed to understand specific oxidative-stress-related factors, their interactions, and their associations with AAA. An in-depth understanding of specific oxidative stress markers can hold great promise for future personalized disease management and risk stratification strategies in AAA and similar diseases.

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If YOU are reading this, please know that I am grateful ☺