Angiotensin Converting Enzyme Insertion/Deletion (ACE I/D) and Methylene Tetrahydrofolate Reductase C677T (MTHFR C677T) genetic polymorphisms in the pathogenesis of abdominal aortic aneurysms (AAA)

MD THESIS

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Abstract

Title: Angiotensin Converting Enzyme Insertion/Deletion (ACE I/D) and Methylene Tetrahydrofolate Reductase C677T (MTHFR C677T) genetic polymorphisms in the pathogenesis of Abdominal Aortic Aneurysms (AAA).

Objectives: The aims of the study were to identify associations between ACE I/D and MTHFR C677T and AAA.

Methods: A retrospective case-control study in which polymerase chain reaction (PCR) methodology was employed to identify associations between ACE I/D and MTHFR C677T polymorphisms and AAA. DNA was extracted from reasonably matched cases and controls after suitable screening for group assignment. There were a total of 1352 subjects genotyped for the MTHFR C677T polymorphism comprising 674 controls and 678 cases. Comparative figures for ACE I/D polymorphism genotyping were 812 and 1107, respectively. All statistical analyses were conducted using R programming software with user-written codes.

Results: The ACE II, ID and DD genotype distributions in controls (177, 410 and 225) and cases (218, 529 and 270) were in Hardy-Weinberg Equilibrium (HWE), P=0.21.There was no difference in allele ("I" and "D") distributions between cases and controls (odds ratio(OR),1.001; 95% CI, 0.88-1.14; P =0.98). There was no difference between cases and controls in terms of the II, ID and DD distributions irrespective of the genetic model adopted. Similarly, the MTHFR CC, CT and TT genotype distributions for controls (358, 257 and 59) and cases (321, 292, and 65) were in HWE (P = 0.39) but the allele ("C" and "T") distributions were not significantly different between groups (OR, 1.172; 95%CI, 0.99 -1.38; P=0.057). However, MTHFR C677T polymorphism was significantly associated with AAA under a heterozygote co-dominant (OR, 1.27; 95% CI, 1.01-1.59) and dominant (CT+TT vs. CC) (OR, 1.26; 95% CI, 1.02-1.56; P= 0.034) genetic inheritance models, respectively. However, there was no association under the over-dominant (CT vs. CC +TT) model (OR, 1.23; 95% CI, 0.99-1.53; P=0.06). Similarly, the trend test was not significant (OR, 1.14; P=0.06) and when corrected for confounders.

Conclusion: The ACE I/D and MTHFR C677T genetic polymorphisms were not independently associated with AAA in this study.

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Dedication

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> “But he said to me, "My grace is sufficient for you, for my power is made perfect in weakness." Therefore I will boast all the more gladly about my weaknesses, so that Christ's power may rest on me.”
>
> 2 Corinthians 12:9 (NIV)

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Chapter 1. Abdominal Aortic Aneurysms

1.1 Introduction

Abdominal aortic aneurysm (AAA) is a localized bulge in the wall of the infra-diaphragmatic abdominal aorta (AA). It is a pathological degenerative condition of the AA with a propensity to grow and rupture if left untreated. It is distinguished from pseudo-aneurysm which does not involve the whole layers (intima, media and adventitia) of the vessel. It can be termed saccular if the bulge is asymmetric as an out-pouching from a part of the circumference of the aorta or fusiform if it is symmetric involving the whole circumference at the site of the involvement.[1] AAA can be Suprarenal which is localized to the segment of the AA from below the aortic hiatus at the level of the 12th thoracic vertebra to just proximal to the origin of the renal arteries incorporating the visceral (celiac and superior mesenteric) arteries or involve the renal segment of the AA to within 1 cm of the lowest border of the most distal renal artery origin (Juxtarenal or Pararenal);[2] or Infrarenal which extend distally from 1 cm distal to the renal artery to the bifurcation of the AA into common iliac arteries at the lower border of the 4th lumbar vertebra. The Suprarenal, Juxtarenal and Infrarenal AAA account for ~2%, ~15% and ~80%, respectively of all AAA.[3, 4] Consequently, except otherwise stated, AAA used in this thesis refers to the infrarenal type.

AAA accounts for 9510 deaths per year in England and Wales with men accounting for a disproportionate 70% of these deaths and with rupture-related mortality rate (MR) as high as 90%.[5]

The annual incidence (new cases in the population) of AAA is less than 1 per 1000 in those younger than 60 years of age but peaks at about 7/1000 among those in their mid sixties and subsequently diminishes to 3/1000.[6] The increase incidence of AAA with age is due to the fact that the AA grows progressively with age and thus more AAA would be expected in people in their 7th decade of life but an increased comorbid burden resulting in death could result in fewer detected cases in the aged population.[7] However, the incidence of AAA also varies depending on the adopted definition of AAA. The normal infrarenal aortic diameter (AD) varies as a function of age and sex. For example, in men the AD is about 1.75cm at age 25 years increasing to 2.25cm at age 55years.[8] According to another study,[9] the AD in a 75-year old person ranges from 12.4mm in a small female to 27.6mm in a large male. A diameter of 2.7cm in the anterior-posterior axis represents the 95th percentile for the non – aneurysmal aorta in men 65 - 83 years of age, whereas 2.9 cm exceeds the upper limit of normal given age, sex and body size and is consistent with McGregor's[10] definition of AAA as the maximum infra renal aortic diameter exceeding 30mm (3.0cm) and this is the commonly accepted definition of AAA. In any case others have proffered different thresholds for AAA diagnosis. For example, The Society for Vascular Surgery and the International Society of Cardiovascular Surgery (SVS/ISCVS) defined an aneurysm as a 50% dilatation of the AD adjusted for gender and imaging method.[11] Similarly, others have suggested a diagnosis of AAA when the ratio of the suprarenal abdominal aorta exceeds 1.5.[12]

1.1.1 Natural History of the Abdominal Aorta

Growth of the normal AA is directly correlated with age and body surface area (BSA) in both males and females and the AA shows a progressive increase in diameter with age thus accounting for larger diameter AA in the elderly as compared to younger persons. It has been

estimated that the infra-renal AA grows by ~ 26% and 24% in males and females respectively between the ages of 25 and 70 years of age.[13] In a larger population screening study of ~10,000 persons, the threshold diameter for "normal AA" progressively increased from 25mm to 35.5mm in males aged 60 years to 75years respectively.[14] In this population, the mean prevalence of AAA (40mm in diameter) was 2.3% reflecting a progressive increase from 0.5% at age 60 years to 5.7% at age 75 years.[14] Interestingly, correlation between AD (mm) and BSA (m^2) shows that for every 1 m^2 increase in BSA, the AAD increase by ~ 9mm ($r = 0.83$, $P< 0.001$). One can therefore speculate that AA"growth" is a function of age and BSA.[13]

There is evidence which suggests that AD is correlated with body length (BL) in men (but not in women) with significantly higher AD for men ≥1.7m in height compared to <1.7m across all age groups albeit within a context of increasing AD with age.[15] Nevertheless, a formula that allometrically scales the AA to the body length suggests that body length has ~ 2-fold effect on AD in males compared to females:

Female AD (cm) = 0.00816*(Age, years) + BL (m)*0.32 + 0.63 **Equation 1**

Male, AD (cm) = 0.0093* (Age, years) + BL (m)*0.75 - 0.05 **Equation 2**

The relationship between AD and age has also been confirmed by another study which also showed an interesting relationship between AD and sex, body mass index (BMI) and vessel wall calcification.[16] This study showed that on average the AD at the suprarenal and mid infrarenal levels was ~ 3mm larger in males compared to females: 22.6mm vs.19.3mm and 20.5mm vs. 17.5 mm, respectively ($P<0.01$).[16] Interestingly, a 1-unit increase in calcium score in the AA and iliac arteries was associated with 0.13mm ($P<0.01$) and 0.09 mm ($P=0.02$) increases, respectively in suprarenal AAD and 0.19mm ($P<0.01$) and 0.12mm ($P=0.01$) respectively at mid infrarenal AAD.[16] Additionally, there are differences between the supra-coeliac and infrarenal

aortic diameters (19.3 ± 2.2mm vs.15.0 ± 1.4mm) and growth rates (0.14mm/year vs. 0.03mm/year), respectively.[17]

These findings are similar to those of a screening study for AAA in a population of 3068 adult males with an initially screened normal AA.[18] Significant AA expansion (>5mm) in this cohort was 2% (71/3068) with a cumulative incidence of 3.5/100-person years after 5.5 years. The highest incidence of 5.2 /100-person years (95%CI, 3.7-7.0 / 100-person years) was found in the 60-69 year group which was ~3-fold higher than in the 50-59 year group (1.8; 95%CI, 1.1 - 2.9) but similar to those > 70 years of age. It was demonstrated that 1 mm increase in the initial diameter increases the relative risk of expansion by 20% (95%CI, 18 - 23%) with a threshold effect at 2.5cm AD. That is, AD greater than 2.5cm has a 4-fold increased risk (OR 4.8; 95%CI, 2.5- 9.4) of significant expansion as compared to AD less than 2.5cm (OR, 1.2; 95%CI, 0.6-2.2). Interestingly, for initial AD <3.0cm, there is a 3-fold risk of significant expansion (>5mm) as compared to 50-59 and >70 year groups (OR, 3.0; 95%CI, 1.3 - 4.3; P=0.002).

1.1.2 Prevalence of AAA

The implication of the foregoing is that an AD >2.5cm in males between 60-69 years of age will most likely develop into AAA. Accordingly, from autopsy studies,[19] AAA is uncommon in men less than 55 years of age, increasing to about 6% between the ages of 80 to 85 years and decreasing thereafter perhaps due to the decreased survival in the after 85 years of age. AAA appears 10 to 15 years later in women and peaking at 5% after the age of 85 years. Compared to women, the prevalence rate from autopsy studies is higher in men (4.3% vs.2.1%). These figures are almost similar to those from population – based ultrasound screening programmes like the ADAM (Aneurysm Detection And Management) study which showed an AAA prevalence rate of 4.3% in males and 1% in females.[20] The Chichester study from the UK, reported a prevalence

of 5.1% in men and 0.4% in women at age 65 years when a threshold aortic diameter of 30mm (3.0cm) was used as their criteria but in all age groups (65-80years) the prevalence was 7.6% in men and 1.3% in females, an obvious increase in prevalence with age.[21] Most other screening programmes used different threshold diameters, but in general the prevalence rate of AAA between 2.9cm and 4.9cm ranges from 1.35% for men aged 45 to 54 years to 12.5% for men aged 75 to 84 years of age, but for women , the comparative figures were 0% to 5%, respectively.[22, 23]

A study from Brazil[24] which screened 2281 (all males over the age of 54 years) with ultrasound showed a prevalence rate of 3.7% and a strong association between cigarette smoking and AAA (relative risk of 4.67, 95% CI: 1.66-13.2). In this study, there was a consistent increase in prevalence with increasing age at 0.64% for 55-64 year group, 1.97% for the 65-74years group and 3.2% for 75 years and older. There are 920 new cases/ year of AAA in Hong Kong consistent with an incidence of 13.7 per 100 000.[25] The male: female ratio was approximately 2:1 with subjects mostly older than 65 years (mean age 73years). The prevalence rate of AAA from autopsy studies in Hong Kong is 1.5%.[26] This corroborates the low prevalence rate of AAA amongst Asians. There was no AAA found in a predominantly Asian community in the UK.[27]

This has also been corroborated by the Huntingdon study which showed an annual incidence of AAA of 3.5 /1000 person-years (95%CI; 2.8 - 4.4).

1.1.3 Natural History of AAA

The natural history of AAA is that of progressive growth and eventual rupture if left untreated.

AAA rupture accounts for 1.7% of all male deaths over the age of 65 in England and Wales.[28] The population – based reported incidence of ruptured AAA varies from 2.9 to 14.1 per 100,000 persons per year.[29, 30] In another (retrospective) population study of 14,138 patients over a median period of 6 years, the annual incidence of AAA was estimated to be 2.6/10,000 and the rate of ruptured AAA was 10% which translated into an annual incidence of rupture of 0.37/10,000 with an associated mortality rate of ~55%.[31] Interestingly, mortality following AAA rupture in this population was higher than 50% for patients older than 65 years of age and lower than 50% for those younger than 65 years with a logistic regression modelling showing that mortality increased by 0.6% per year over 65 years of age (P=0.001).[31] Data from a single centre which followed 111 patients (mean age 80 years) over a median time of 14.7 months (range, 0.3 months - 47months) showed that for AAA diameter ≥ 5cm, the cumulative risk of rupture at 1-year, 2- and 3-years were 13%, 24% and 44% respectively.[32] From the Kaplan-Meier analysis, 1-, 2- and 3-year survival rates were 72%, 48% and 23% respectively. This is a relatively aged population and death from other causes accounted for 60% of all deaths.[32]

Interestingly, in a cohort of 106 patients with AAA diameter ≥ 5.5cm (mean age 78.4 years) turned down for surgery, the 1-,2- and 3-year survival rates were 54%, 40% and 17% respectively.[33] Of the 76 deaths (71.1%) at the end of the study period (10years), 49% were due to ruptured AAA. Patients with AAA diameter > 7cm had a median survival time of 9 months as compared to 19 months for those with AAA diameters of 5.5-5.9cm (n=23) and 6.0-7.0cm (n=62) respectively. The foregoing survival rates are in sharp contrast to a cumulative 5-year survival rate of 52% from another study which followed 67 non-operated patients, mean age 72 years (range 50 to 91) for a mean of 36 months (range 3 to 99).[34] In this study, the 5-year AAA rupture rate from actuarial analysis was 26% and the attendant mortality rate of 20% as opposed to 28% from non-aneurysm related causes. Of significance in this study is the mean AAA

diameter of 3.7cm ±0.1cm implying a correlation between survival and AAA diameter in patients with AAA.[34]

The risk of rupture is directly correlated with the diameter of the AAA. In one population-based study which followed-up 218 AAA patients (4% of screened 5394) for a period of 7 years, the rupture rate for AAA with diameters between 3.0cm and 4.4cm was 0.7%/year as opposed to 1.7%/year for AAA diameters of 4.5cm - 5.9cm diameter.[35] This shows that small AAA can rupture.

The relationship of risk of rupture with AAA diameter is exponential. It has been shown that the risk of rupture in a 3cm AAA is 1-2%; for a 4cm AAA it is 5-13%, and for a 5cm AAA the risk is 25-38%.[36] Figures from a meta-analysis were however conservative (n=13 studies):[37] 0% for AAA < 3.0cm, 0.4% for 3-3.9cm, 1.1% for 4-4.9cm, 3.3% for 5-5.9cm, 9.4% for 6-6.9cm and 24% for 7-7.9cm. It is noteworthy, that AAA diameter interacts with other co-morbid conditions to accentuate the risk of rupture. For example, COPD, diastolic HT and initial AAA diameter have been shown to predict AAA rupture from one study. Depending on the severity of the diastolic HT and COPD and initial AAA diameters of 3cm, 4cm and 5cm, the 1-, 3- and 5-year predicted rupture rates from a Cox proportional hazard model varied from 0 to 84%; 0 to 98% and 2% to 100% respectively.[34]

The dependence of rupture on the initial AAA diameter was confirmed in a prospective study which followed up 198 patients with AAA of at least 5.5cm in diameter over a period of 1.52 years.[38] During follow-up, there were 112 deaths (57%) with an all-cause mortality rate at 1-year, 2-years and 3-years of 29.8%, 55.5% and 75.4% respectively. With an autopsy rate of 46%, there were 35 confirmed definite ruptures (17.7%). The cumulative incidence of probable

ruptures (definite ruptures and deaths following symptoms consistent with ruptures) for AAA in 5.5-5.9cm group at 1 year, 2 years and 3years of follow-up were 9.4%, 22.1% and 27.6% respectively. Meanwhile, for AAA diameter in 6.0 – 6.9cm group, the corresponding figures were 10.2%, 18.9% and 32.1% respectively. For AAA with initial diameters ≥7cm, the 1 year and 2 years cumulative incidence of probable ruptures were 32.5% and 43.4% respectively and for AAA which attained 8cm during follow-up, these rates were 36.4% and 54.7% respectively.

In a different study consisting of 735 patients, 63 of whom died following treated conservative treatment with a median follow-up of 8months (range,1day – 94 months), 30% (16/63) of deaths were due to AAA rupture with a 5-year cumulative hazard rate (HR, (±SD)) of 0.36 ± 0.10 corresponding to an annual incidence risk of rupture of 7%.[39] In this study, the cumulative 5-year hazard rate of rupture of AAAs < 6cm was 21% ± 9% corresponding to an annual risk of ≤ 5%. Conversely, the 5-year cumulative HR for AAA ≥ 6cm was 61% ± 22% which translated into an annual risk of rupture of 10-15%. The initial AAA diameter was found to be significantly associated with rupture.

In a single centre analysis which prospectively followed 267 patients with small AAA (4-5.5cm diameter) for a period of approximately 5 years, the cumulative risk of death secondary to rupture at 5 years was 15%.[40] Interestingly, for AAA< 4 cm in diameter this was 4% and for those between 4.0cm -5.5cm, the analogous figure was 21% thus underscoring the dependence of aneurysm-related survival on initial AAA diameter. Thus, the actuarial survival at 1, 3 and 5 years for this population of patients were 100%, 93.9% and 84.9% and for AAA< 4cm and 4-5.5cm cohorts, the corresponding figures were 100%, 95.6% and 95.6% and 100%, 90.2% and 79% respectively.[40]

In another study which retrospectively analyzed prospectively diagnosed AAA in a cohort of 187 patients in a 9-year period, there were 11 ruptures in AAA of 4.8 – 8.0 cm translating into a cumulative rupture risk of 4.3%, 11.6% and 16.4% at 1, 5 and 8 years respectively.[41] When these AAA are stratified into small (<5cm) and large AAA (≥ 5cm), the rupture risks for the former was 2.5% at 1 year with no increase at 7 years. Conversely, for the latter, the rupture risks at 1 and 3 years were 8.7% and 28% respectively (P = 0.0001). There was a significant difference between the rate of rupture in small AAA versus large AAA (5.5% vs. 21%; P = 0.0001). Reflecting, the association between AAA diameter and survival, the 1, 5 and 8 year survival for the small AAA were 85%, 72% and 37% respectively. The analogous figures for large AAA were 84% and 54% for 1 and 3 years respectively with no difference in survival between the two curves. Interestingly, the actuarial survival probabilities for the whole cohort were 85%, 51% and 33% (± 6.7%) for 1, 5 and 8 years respectively.

A recent systematic review of the AAA rupture literature showed that the rupture rates in AAA smaller than 5.5cm (N=7 studies) was 0- 1.61/100 person-years whilst for AAA > 5.5 cm (N=14 studies) the corresponding rates were 0 – 2.51/100 person-years although the study was confounded by significant heterogeneity and rupture diagnosis.[42]

1.1.4 Epidemiology of AAA from screening studies

There is evidence of clinical and economic benefits from AAA screening programmes.[43] In the Multicentre Aneurysm Screening Study (MASS)[44] which recruited 67,800 men between the ages of 65 to 74 years with a 53% acceptance rate, there was an estimated 42% reduction in AAA– related mortality (hazard ratio (HR),0.58; 95% CI,0.42-0.78) and 41% reduction in nonfatal rupture (HR,0.59;95% CI,0.45 - 0.77) but at a cost of 6% post operative mortality.

The Chichester trial[21] was a randomized controlled trial in which 15,775 men and women between the ages of 65 to 80 years were randomized to ultrasound screen (USS) or no intervention. The response rate was 68.4% (7887) and in whom the prevalence rate of AAA was 7.6% in males and 1.3% in females. Those in whom an aneurysm was identified and is between 3.0 and 4.4cm were rescanned annually, and those with an aneurysm between 4.5cm and 5.9cm were rescanned every three months. Surgery was offered to those with annual growth rate greater than 1cm/year, larger than 6cm or with symptoms related to the aneurysm. In a 5 year follow up there were 9 ruptures (0.28%) in the 3205 men USS group compared to 20 (0.62%) ruptures in the 3228 in the control group accounting for a 55% reduction in risk of rupture (RR, 0.45; 95%CI, 0.21- 0.97). After 10 years of follow-up, there was a 21 % reduction in AAA related mortality (RR, 0.79; 95% CI, 0.53 -1.40). There was no difference in mortality in women between the two groups (RR, 1.49; 95%CI, 0.25 - 9.93).

In the Viborg County study (Denmark),[45] of 6339 men invited for screening, the response rate was 76% (4843) and aged between 65 and 73years. In this randomized trial with a mean follow-up of 5.1years the aneurysm detection rate at a threshold of 30mm (3.0cm) was 3.9% of which 24 (0.5%) were greater than 5cm. There was a 79% reduction in the number of ruptured AAAs (relative risk (RR), 0.46; 95%CI, 0.23 - 0.93), 74% reduction in the number of emergency operations for AAAs (RR, 0.63; 95%CI, 0.53 - 0.75) and a 68% reduction in overall AAA-related hospital (RR, 0.48; 95%CI, 0.24 - 0.86) although at a cost of a 51% increase in operations. There were no sex-biased differences in all cause mortality.

The fourth randomized screening trial was the Western Australian AAA program. [46,47, 48] Of 19,352 men invited , the response rate was 69.7% (12,203). With an acceptable AD threshold of 3cm, the AAA detection rate of 7.2% of which aneurysm greater than 5.5cm constituted 7%.

There were no statistically outcomes between the screening and control groups (RR = 0.61;95%CI, 0.33-1.11).

Furthermore, a recent Cochrane review and meta-analysis suggested a 36% reduction in AAA-related mortality (OR=0.64; 95% CI, 0.50- 0.81),[49] attesting to the benefit of screening programmes (but no significant difference between both arms of the studies in all-cause mortality (OR=0.95: 95% CI,0.87-1.07)). There are also long term benefits of screening even after screening has ceased. In the Huntingdon Aneurysm Screening Programme, there were obvious benefits in outcomes 5 years after screening has stopped or 13 years since the initiation of the screening programme.[50] The reduction in the incidence of ruptured AAA at 5 years was 49% (95% CI,3 - 74%) and at 13 years, 73% (95%CI, 58 - 82%). Similarly, the reduction in the ruptured AAA-related mortality at 5 years was 45% (95%CI,15% - 74%) as compared to a comparative reduction at 13 years of 75% (95% CI: 58-85%). The incidence of new AAA in men aged 55 years who were initially AAA-free on previous screening was 2%.

The Aneurysm Detection And Management (ADAM)[51] study screened 73 451 participants, all aged between 50 and 79 years, of whom 97% were males .The AAA prevalence rate from this study was 4.6% in keeping with most of the population – based studies. A repeat screening programme (after 4 years)[52] from this cohort of participants (2622, 50.9%) showed new cases (incidence) of 2.2% (95% CI, 1.6%-2.8%) bringing the total to 2.6% when added to 0.5% who developed AAA. These results are consistent with findings from the Huntingdon study (*supra*). Cigarette smoking was also, strongly associated with AAA (OR, 3.09; 95%CI, 1.74 - 5.50).

There were also quantifiable cost-effectiveness benefits from the screening programmes. In the MASS study, there were 47 fewer deaths in screened group than in the control group resulting

in an additional £63.39 per patient and over 4 years this amounted to an incremental cost-effectiveness ratio (ICER of £28,000 or approximately £36,000 per quality – adjusted life year QALY).[53] In long term (7 years) follow up, the ICER approximated £10,000 or £12,300/ QALY.[54] It is noteworthy that the NICE (National Institute of Clinical Effectiveness) threshold figure for acceptable programmes is £30 000 per QALY.

1.1.4.1 Clinical Epidemiology of AAA

Given the public health interest of AAA, it is not surprising that past and current efforts were directed at identifying the risk factors for this condition. In a recent systematic review of 14 published articles,[55] most of which defined AAA as aortic diameter ≥ 30mm, the risks of AAA were significantly increased with male sex (OR, 5.69; 95%CI, 3.36-9.64; n=6 studies), previous myocardial infarction (OR, 2.28; 95%CI, 1.90-2.74; n=6 studies), peripheral vascular disease (OR, 2.50; 95%CI, 2.1-2.95; n=8 studies), smoking (OR, 2.41; 95%CI, 1.94-3.01; n=11 studies) and hypertension (OR, 1.33; 95%CI, 1.14-1.55; n=9 studies) using a random effects meta-analysis methodology. Conversely, diabetes mellitus was not significantly associated with AAA from the results of 6 studies combined (OR, 1.02; 95%CI, 0.81-1.29).

1.2 Pathomechanics of AAA

Current evidence seems to suggest that the site-specificity of AAA in the IR segment of the AA may be due to the combination of anatomical,[56] hemodynamic,[57,58] biomechanical,[59] and wall material properties of the aorta[60,61] in addition to segmental properties of the infra-renal (IR) abdominal aorta.[62]

The human IR suffers from the same disadvantage as the supra-renal murine abdominal aorta: adverse pulsatile strain.[63] In addition, an unfavourable anatomy[64] (anterior bowing in line with lumbar lordosis) coupled with the presence of the renal arteries[65] subjects this segment to adverse haemodynamic stresses ranging from low wall shear stresses,[66] oscillatory shear stress, increased reflectance, flow separation, vortices and transition to turbulence.[67,68, 69] See Figure 1

The human IR abdominal aorta is a structure designed by nature to fail.

Blood flow within the IR aorta is not laminar as commonly depicted in textbooks. The lordotic lumbar curvature facilitates the generation of vortices in the IR aorta[70] which cause circumferential recirculation flows similar to what happens in the aortic arch.[71] Furthermore, this abnormal haemodynamics progressively worsens with aging and contribute to the pathogenesis of AAA.[72] This is because of progressive histological and gross anatomical changes of aging within the aorta . Age – related stiffness is associated with reduced compliance and this translates into elevated haemodynamic stress on the aortic wall.[73] Furthermore, as the individual ages, the IR aorta also elongates becoming more tortuous and eventually buckles. Tortuosity is an exaggerated feature of arteries from patients with HT and old age (> 40 years).[74] Interestingly, tortuosity resulting in AAA asymmetry could increase the peak wall stress by ~ 14% (23.8N/cm^2 vs.27.7N/cm^2) implying its contribution to aneurysm pathophysiology.[75] In a study involving 19 patients with AAA with ILT (intraluminal thrombus), the maximal AAA diameter (7.5cm ± 2.4cm) was found to correlate with internal tortuosity (0.12 ± 0.10), r=0.72, p=0.01).[76] Similarly, the peak wall stress in the presence of ILT also correlated with internal tortuosity (r=0.65, p=0.003) and maximal diameter (r=0.88, p<0.0001). In this study, PWS was found to be dependent on maximal AAA diameter and tortuosity [PWS=8.791 + 2.4*diameter + 25* tortuosity].[76]

1.2.1 Flow-induced vibration and aortic wall fatigue failure

Tortuosity could also predispose to turbulent blood flow conditions which in the presence of blood vessel stiffening characteristic of aging cause the aorta to vibrate.[77,78,79,80] Accumulating evidence suggests that this vibration leads to degradation of the aortic wall structural components.[81, 82] Aortic wall damage can further progress if the vibration leads to resonance.[83] Resonance occurs if a structure is forced to vibrate at its natural frequency or one of the fundamental frequencies. A completely tethered artery is unlikely to resonate because of damping and buffering by surrounding tissues but this is not the case with the abdominal aorta.[84] It is virtual free of surrounding solid structures everywhere except at back where it is covered by the lumbar vertebra. Vessel vibration ultimately leads to dilatation, accentuated by age and HT. In a classical study, it was observed that exposing a segment of an external iliac artery to 100mmHg pressure and vibrated between 50 and 400Hz resulted in progressive dilatation of the vessel radius at a rate of ~7% per day.[85] There is also evidence that the accumulated pulsatility with aging also has adverse effects on the elastin content of the aorta, a consequence of material fatigue from repeated cyclic strain. It has been shown that after 10^9 cycles of 8% stretch over 40years , the elastin in the aorta will fracture but fracture may not occur if less than <5% stretch was applied.[86]

Areas of recirculation within the circulation are referred to as vortices (singular-vortex). They are capable of generating vibrating forces in vivo. Vortices have been identified in the tortuous and kinked non-aneurysmal aorta and also in experimental murine Angiotensin II infusion AAA models.[87 , 88] These were characterized by unsteady flow patterns, recirculation and distal impingement. The latter were co-localized with sites of intimal tears of AAA dissection which characterized these aneurysms.[88] Furthermore, these vortices can be translated distally (vortex

shedding) which may account for the co-localization of common and external iliac aneurysms with AAA.[89] Shedding of vortices can set the vessel wall into vibration, a phenomenon called lock-in, which occurs when the shedding frequency equals the resonance frequency of the vessel wall resulting in damaging synchronized vibration (vortex-induced vibration, (VIV)) of the vessel wall.[90] The phenomenon of VIV has been documented in a stenosed infra-inguinal venous bypass graft using an ultrasonic pulse-echo multigated system which strongly correlated (in-phase) blood stream coherent vortices with vessel wall vibrations.[91] Venous wall vibration with ~1μm displacements and intensities of $0.08mW/cm^2$ - $0.09mW/cm^2$ occurred at a fundamental frequency of 145Hz and its harmonics (290Hz, 435Hz) suggesting resonance in the venous wall in association with vortex structures.[92] It is noteworthy that the cumulative residual strain under cyclic loading-unloading (vibration) is inversely correlated with extent of displacement (amplitude) rate and directly with frequency, applied load and number of cycles.[93] Thus a smaller displacement rate (e.g., 1mm/min) will accumulate more residual strain (plastic deformation) compared to a 5mm/min displacement rate. In addition, a 100Hz frequency will accumulate more residual strain than a 50Hz frequency.[93] Furthermore, after certain cycles, the residual strain cannot be recovered completely a phenomenon referred to as visco-plastic deformation which is accompanied by collagen microfiber fracture.[93]

1.2.1.1 Vortices and intraluminal thrombus (ILT)

A consequence of vortex dynamics within the evolving AAA is the formation of ILT.[94] The study further showed that larger AAA are more likely to form ILT compared to smaller AAA and this is ascribed to the positive correlation of vorticity with aneurysm diameter. Vortices activate the endothelium and platelets readily resulting in the formation of ILT.[94]

The ILT far from being an inert structure also contributes to the evolution of AAA growth. Interestingly, a recent study has estimated that a 31% increase in thrombus volume was associated with a 3mm/year growth in AAA diameter ($p<0.01$); 13% reduction in 99 percentile wall stress ($p<0.01$) and 1.6 % reduction in average wall strain ($p=.03$) suggesting that ILT contributes to ongoing AAA pathophysiology.[95] Furthermore, there is evidence of aneurysm progression with ILT; being comparatively larger in ruptured AAA compared to intact AAA ($148.9 \pm 90.4 cm^3$ vs. $92.1 \pm 75.6 cm^3$, $P=0.003$).[96]

Thrombus is a rich source of proteolytic enzymes which continue to degrade the underlying AAA matrix. The process of intraluminal thrombus (ILT) formation has been implicated in the pathogenesis of AAA through the generation of thrombin and plasmin, two activities associated with progressive AAA.[97, 98] The functional role of these proteolytic peptides in the pathogenesis is further underscored by a study which showed that there is more degradation in the wall adjacent to thrombus as compared to thrombus-free adjacent areas.[99] In addition, elements of the fibrinolytic system and blood-borne serine-proteases like urokinase type plasminogen activator (uPA) and tissue plasminogen-activator (tPA) and Plasminogen Activator Inhibitor-Type 1(PAI-1) have all been localized to the mural thrombus in AAA.[100] Furthermore, it has been demonstrated that relative to the aneurysmal wall, the ILT in elastase-infusion AAA mice model is a significantly richer source of MMP-9, urokinase-type Plasmin (uPA) and Elastase with the former two being positively correlated with AAA diameter.[101]

The thrombus is a rich source of neutrophils which are trapped in the fibrin meshwork of the ILT. A recent study evaluating the concordance of the pathophysiologic processes in AAA and popliteal arterial aneurysms (PAA) demonstrated that neutrophils and their protease products are essential to the aneurysmal process[102]. Neutrophils are a rich source of MMP-8 and MMP-9,

Cathepsins K, L and S which have been found to be consistently elevated in AAA and PAA. The MMP-9 which may also be released by the underlying activated endothelium or macrophages is trapped in complexes with neutrophil gelatinase associated lipocalin (NGAL) complexes (NGAL: MMP-9)[103]. It has been suggested that MMP-9 in these complexes exist in the activated form. This study underlies the importance of neutrophils in the AAA pathogenesis. Furthermore, systemic depletion of neutrophils with antibodies is associated with a significant reduction in AAA formation in these elastase infusion mice AAA models with ~ 8-fold increase in AAA formation as compared to controls in one study.[104]

1.2.1.2 Shear Stresses

Vortices and turbulence induce viscous drag on the endothelium causing increased wall shear stress gradients (WSSG) which stimulates the endothelium (Figure 1). The impingement of the blood on the wall also induces high wall shear stress in the immediate surroundings resulting in high spatial wall shear stress gradients (SWSSG) which from in vitro studies were shown to be associated with morphological and phenotypic changes in the endothelial cells ("activated endothelium") with an accompanying pressure change of ~ 2mmHg.[105] A recent study showed that low wall shear stress (SS) (0.0 -2.2Pa) was associated with elastin degradation, collagen turnover and aneurysm progression.[106] Furthermore, oscillatory SS (OSS) is more likely to induce endothelial inflammation than laminar SS.[107, 108] This is probably reflective of the endothelial ability to discriminate between, and respond differentially to these forms of shear stresses.[109, 110, 111] Moreover, low laminar SS was more likely to induce adverse aortic wall changes compared to high laminar SS like macrophage infiltration and endothelial activation to an inflammatory phenotype associated with MMP secretion.[112, 113] Interestingly, high laminar SS associated with high flow states were associated with regression of AAA in experimental murine AAA models.[114,115]

Obviously, a combination of VIV and low SS can not only cause endothelial dysfunction but also increased VSMC migration into the intima due to the induced pathological events within the aortic wall.[116]

1.2.2 Pressure wave reflection

It has been speculated that factors like pulsatility (PP) and maximum rate of upstroke of the initial blood pressure (dP/dt) waves in the aorta may contribute to material wall failure which can possibly underlie the patho-physiological mechanism of hypertension in AAA.[117] The abdominal aorta is subjected to higher pulsatile stresses because of a tapering cross-sectional area, reflected waves from atherosclerotic periphery and increasing stiffness (see Figure 1). There is evidence implicating PP in the pathogenesis of AAA. In a case (n=25) control (n=25) study, increased pulse pressure (PP) was found to be significantly associated with AAA [odds ratio (OR), 1.4 (95%, 1.0 - 1.8) for every 10mmHg increase in PP, p=0.0032].[118] There is also evidence implicating high peripheral vascular resistance in the pathogenesis of AAA. In fact a study has shown significant association between lower limb amputation and AAA formation[119] and peripheral vascular disease.[120] The reason for this association may be attributed to the increased peripheral resistance with attendant increased wave reflection from distal sites. These demographic groups have increased pulse wave reflection form distal sites to the aorta and this was associated with increased central aortic systolic BP. Interestingly, high central pulsatile pressure (PP) predisposed to aortic wall oxidative stress through the generation of reactive oxygen species (ROS).[121] Current evidence seem to suggest that shear stress or cyclic strain was transmitted via intermediate fibres/ microfilaments to the attached mitochondria and focal adhesion complexes leading to the generation of ROS and subsequent pro-inflammatory cascades.[122, 123]

It was also noteworthy that high PP was correlated with a high mean arterial pressure (MAP)[124] and the latter could predispose to arterial wall remodelling through the activation of the VSMC focal adhesion kinases to increase the generation of matrix metalloproteinases -2 and -9 (MMP-2 and -9) in arteries.[125 126] Furthermore, high MAP is associated with apoptosis of VSMC[127]

Understandably, pulsatile wall stress (cyclic strain) energy is transferred to aortic wall elastin (stored) during systole and this stored energy is returned during diastole to "pump" the blood (windkessel phenomenon). Elastin storage capacity (95 Jkg^{-1}) is less than that of collagen (1000 Jkg^{-1}) implying a requirement for repetitive cyclic loading to cause to elastin.[128] However, some of the energy is absorbed as viscous energy by the VSMC. Approximately 15-20% of total strain energy is dissipated in the vessel wall in this manner.[129] This lost energy by visco-elasticity helps to attenuate travelling pressure pulse which propagates along arteries as waves of circumferential distension of the vessel wall and they also help in preventing peripherally reflected pressure waves from resonating in the arterial system. Increasing the visco-elasticity of the vessel wall is associated with damping of the peak wall stress and a reduction in the stress wave amplitude.[130] It has been shown from constitutive numerical studies that visco-elasticity decreases maximal radial velocity and acceleration, wall stress and wall strain of the vessel wall thus reducing wall fatigue failure.[131] In addition, numerical studies also show that VSMC in the activated state significantly reduces and homogenizes transmural wall stress.[132] Reduced wall movement may help in attenuating arterial wall fatigue failure. There is also evidence to show that at normal heart rate (70 beats/min), the arterial wall experiences ~37 million pulsatile loading cycles annually in an individual which at about 25 years of age would have accumulated ~800 million cycles which is sufficient to cause elastin fatigue fracture at 10% stretch in the aorta.[133] Furthermore, higher static stretching as would occur with high mean BP will lead to elastin fatigue at fewer pulsations and at an earlier age.[134]

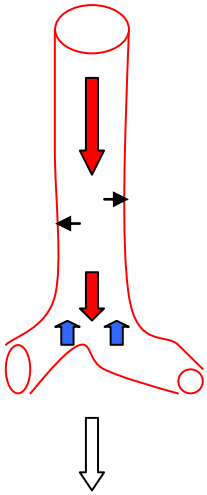
1.3 AAA Pathogenesis

1.3.1 Inflammation

There is an established relationship between AAA and systemic inflammation as evidenced by the results of the Kaiser Multiphasic Checkup Cohort Study which demonstrated in a cohort of 104, 813 subjects followed over a 13 year period that the risk for AAA was significantly associated with raised white cell counts (WBC).[135] Quartiles of multivariate adjusted WBC were progressively associated with increasing risks of developing AAA. With the lowest WBC as a reference category (Quartile I, QI), the hazard ratios (HR) for QII, QIII, QIV were 1.32 (95%CI, 1.02-1.71), 1.38(95%CI, 1.07-1.79) and 1.59(95%CI, 1.24-2.05) respectively. In addition, a case-control study suggested that highly sensitive CRP (hsCRP) was significantly associated with the pathogenesis of AAA in a 12-year longitudinal study.[136] . Furthermore, the mean hsCRP in AAA (n=35) vs. controls (n=140), was 4.8mg/L (95%CI, 2.2-7.3) vs. 2.4mg/L (95%CI, 1.8-3.0mg/L), respectively, p=0.003. However, the change in mean hsCRP levels was significant (p=0.039) for AAA patients relative to the controls over the 12 year [2.5mg/L (95%CI, -0.3 to 5.3mg/L) vs. -0.1mg/L (95%CI, -1.3 to 1.1) respectively].

1.3.2 Cytokines

Histological analysis of the AAA wall is characterized by an upregulation of proinflammatory cytokines, chemokines and the inducing signalling pathways. When compared to non-aneurysmal aorta (n=9), the AAA tissue (n=10) significantly upregulates cytokines like IL-6 (p=0.001), IL-1α (p=0.001), IL-1β (p<0.001) and TNFα (p=0.002) in addition to IL-8 (p=0.001), MCP-1 (p=0.003) and growth related oncogene (Gro)(p<0.001) using a microarray (“Chips”) technology.[137] Interestingly, the study also showed a significantly elevated anti-inflammatory IL-10 (p=0.002) in the AAA wall tissue compared to control non-aneurysmal aorta as well.



Vascular aging;

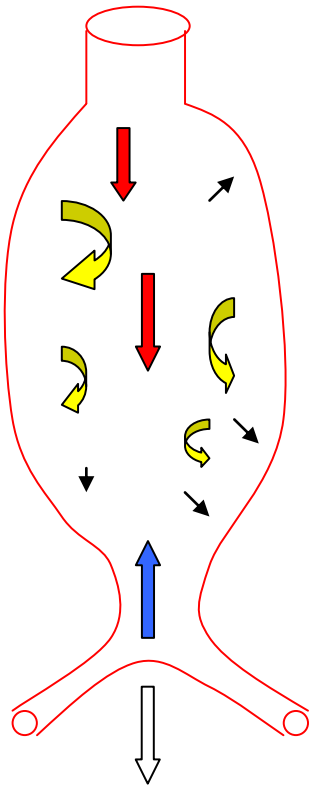
Smoking;

Hypertension;

Male gender;

Arterial stiffening- Impedance,Reflectance, PWV/AIx;

Pulsatile stress/cyclic strain



Proteases:

MMPs, Cathepsins ;

Oxidative stress;

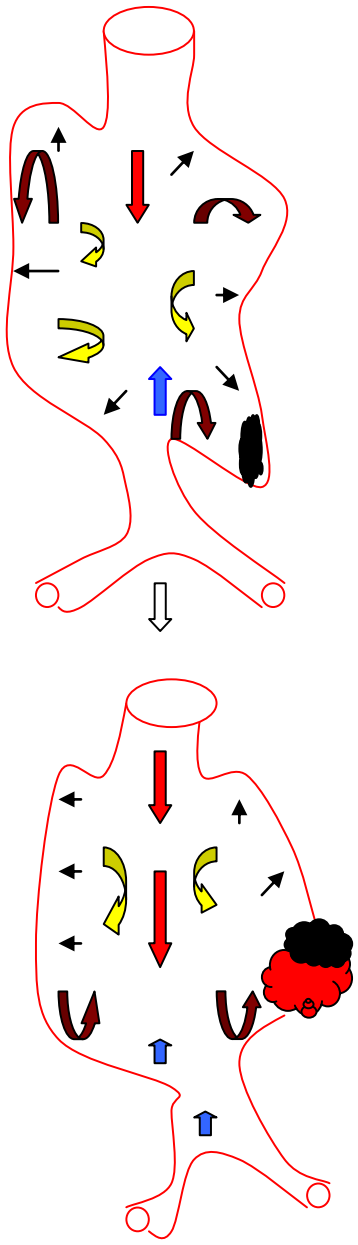
Elastolysis, Collagenolysis;

VSMC apoptosis;

Elastokines & Matrikines;

Recruitment of pro-inflammatory cells

Legend:
Arrows: red=blood flow; blue=reflected pressure waves; yellow=cyclic strain; black=summed peak wall stress



Wall asymmetry -> increased peak wall stress

Shear stress -> endothelial activation-> thrombus formation->
- **protease activity,**
- **elastolysis,**
- **collagenolysis,**
- **elastokines,**
- **matrikines;**

Recruitment of pro-inflammatory cells

Adventitial collagenolysis -> rupture

Legend
Brown arrows=shear stress; black mass=thrombus; red mass= AAA rupture; Others as before.

Figure 1: Pathomechanics of AAA formation (see text for description and explanation)

Accumulating evidence also suggests that the predominant cells in histological wall specimen of AAA are the CD3+ T cells which account for 67-80% of cells with preponderance of CD4+ cells as evidenced by increased CD8/CD4 ratio from 1:3 to 1:20.[138] There were also B cell predominant lymphoid follicles with dendritic cells and macrophages in germinal centres. The adventitia and media also showed marked neovascularisation with perivascular macrophages and lymphocytes. The density of neovessels correlated with AAA size and MMP-9 signal intensity. Pro-inflammatory cytokines like IL-1β, IL-6, IL-8 and TNFα were also markedly increased. Whilst the endothelial cells expressed ICAM-1, the scattered VSMC expressed MMP-1 and HLA-DR on their surfaces suggesting an adaptive immunologic response.

In a microarray comparative study between AAA tissues (n=4) and normal aorta (n=4),[139] pro-inflammatory markers were significantly upregulated in the former compared to the latter and these included myeloid cell nuclear differentiation antigen (MNDA), platelet-derived growth factor-A (PDGF-A), IL-8, RANTES (Regulated on Activation Normal T cell Expressed and Secreted; CCL5), ICAM-1 and CD11a/CD18, Protein Kinase C delta (PKCδ), CXCR4 (chemokine receptor), Interleukin-2 receptor-γ, early growth response -1 (Egr-1), MMP-9 and Cathepsin H. Conversely, myosin light chain kinase (MLCK) and β-1 integrin were downregulated.

The same pattern of gene expression was shown by the same authors in an elastase infusion murine AAA model[140] in addition to IL-1β, monocyte chemotactic protein 3 (MCP-3), monocyte inflammatory protein (MIP-1 α),Osteopontin, hypoxia –inducible factor 1α (HIF-1 α), CD14, Interleukin -6 and monokine induced by gamma interferon (MIG). A similar profile of pro-inflammatory mediators were significantly expressed in an Angiotensin II infusion murine AAA model[141] and included IL-1β, IL-6, C-C chemokines like CCL2, CCL4, CCL7, CCL8, CCL19,

CCL21, CCR5, C-X-C chemokines like CXC3R1, CXCL5, CXCL9, CXCL10, CXCL12 and proteases like MMP-2, MMP12, MMP13, MMP14 out of >1000 genes that were differentially expressed in this AAA model. Interestingly, the pattern of chemokine release highlights the inflammatory cells recruited: CCL2, CCL7 (monocytes, MC), CCL5, CCL19 and CCL21 (T cells and dendritic cells), CXC9 (T cells), CXCL5 (neutrophils). A similar study[142] identified >6,000 differential expressed genes with the Angiotensin II infusion model involving chemokines (CCL2, CCL3, CCL5, CX3CL1 and CXCL16), cell cycle pathway, apoptosis, Toll-like receptor (TLR) signalling (TLR-1, -2,-6,and -7), Mitogen Activated Protein Kinase (MAPK) signalling, p53, leukocyte transendothelial migration, fibroblasts proliferation and extracellular matrix remodelling. There was a progressive reduction of the upregulated inflammatory genes with time, replaced by matrix remodelling genes including collagen genes. Interestingly, this model identified activated molecular networks of Osteopontin, Lysyl Oxidase (Lox), MyD88 and TACE (TNFα-Converting Enzyme) with a downregulation of the TGF/BMP signalling network in contained AAA rupture.

Current evidence suggests that serum IL-6 level is positively correlated($r = 0.3$, $p=0.003$) with AA diameter in non-aneurysmal patients (n=113) in whom IL-6 (pg/dl; $\beta=3 \times 10^{-3}$,$p=0.04$), Serum Amyloid A (mg/dl; $\beta=5 \times 10^{-3}$,$p=0.001$) and plasma homocysteine (μmol/L; $\beta=11 \times 10^{-3}$,$p<0.001$) were significantly associated with indexed aortic diameter in a linear adjusted model.[143]

1.3.3 Oxidative Stress

Accumulating evidence is consistent with a pathogenic role for reactive oxygen species (ROS) in

AAA pathology.[144] These ROS include O^{2-}, H_2O_2 and HOCl which are generated by membrane associated NAD(P)H Oxidase system and mitochondrial redox systems. Oxidative stress results from tissue damage secondary from unchecked or excess ROS. Some of the implicated roles in AAA pathogenesis include, increased production of cytokines like chemokines, MCP-1, IL-8 and adhesion molecules like P-selectin.[144] They also upregulate the pro-inflammatory NF-κB pathway, generate matrix metalloproteinases (MMPs) and cause VSMC apoptosis.

There is evidence to suggest that oxidative stress plays a pivotal role in the pathogenesis of AAA. In a histological study of AAA wall tissue (n=7) compared to adjacent normal aortic wall, it was shown that there was significantly more O2- production by AAA tissue compared to controls as assessed qualitatively by fluorescent dye, dihydroethidium (DHE).[145] Further quantitative assessment by lucigenin - chemiluminiscence showed ~ 2.5 fold increase in O2- production in AAA relative to adjacent normal aorta. There were areas of inflammatory infiltrates with disruption of medial SMC architecture in AAA tissue and these were associated with the foci of increased O2- production. Furthermore, compared to normal AAA tissue (n=6) there was > 10 fold increase in O2- levels. It is also worth mentioning that lipid peroxidation was also increased ~ 3.0 fold in AAA tissue compare to normal aorta and the increased O2- was attenuated by ~ 60% by NAD(P)H Oxidase inhibition. Conversely, 0.1mmol/L NAD(P)H oxidase increased O2-generation by ~ 3.0 fold compared to normal aorta. Interestingly, immunostaining showed increased expression of p47phox and p22phox throughout the AAA tissue consistent with a functional role for this enzyme complex in the pathogenesis of AAA by an oxidative stress mechanism.

In an Angiotensin II infusion AAA model in $ApoE^{-/-}$ mice compared to $ApoE^{-/-}p47phox^{-/-}$, there were more AAA in the former compared to the latter (90% vs. 17%; $p<0.05$).[146] Further, there

were more advanced AAA lesions (class III + IV) in the former compared to the latter (40% vs. 17%; $p<0.05$). The study also showed that there was less quantifiable oxidative stress as measured with gluthathionine reduction assay (GSH nmol/mg protein) in the double knock-out (KO) mice compared to the apoE$^{-/-}$ mice, [18.75 ± 0.41 (n=3) vs. 15.60 ± 1.32 (n-6); $p<0.05$). Unsurprisingly, there was more macrophage infiltration and MMP-2 formation in the aortic tissue of the apoE$^{-/-}$ compared to the double KO mice.

Considering that Aortic medial dissection precedes the formation of AAA in these experimental Angiotensin II infusion mice models, another study showed that the NOX1 enzyme was necessary for maintaining the integrity of the aortic media.[147] In this study, NOX1 deficient C57BL/6J mice were compared to their wild type littermates under Angiotensin II (3mg/kg/day) and norepinephrine (12mg/kg/day) infusion for 7 days. Interestingly, there was a blunted BP rise in the Nox-1 deficient mice infused with Angiotensin II compared to the wild type or the NE infusion groups suggesting that BP elevation by Angiotensin II is in part mediated by the NOX1 system. However, there were significantly ($p<0.05$) fewer dissections in the NOX1 deficient group (1/25) compared to the WT (6/26) which was associated with ~10 fold and ~ 70 fold increase in TIMP-1 mRNA and protein respectively in Nox-1 deficient group compared to ~6 fold and 0-fold comparative changes in the WT respectively. There were no changes in the MMP-2 and MMP-9 proteins in both groups suggesting that and imbalance between MMPs and their inhibitors may be pathological in acute aortic conditions.. A concomitant microarray analysis showed that there were 185 genes upregulated and > 576 genes downregulated in the NOX1 deficient mice compared to the WT.

Inducible NOS (iNOS) also plays a significant role in the generation of ROS by producing NO under inflammatory conditions and current evidence seems to suggest that iNOS plays a

significant role in the pathogenesis of AAA. In a recent study which employed $CaCl_2$ mice AAA model, it was shown that the frequency of AAA formation in C57BL/6 $iNOS^{-/-}$ compared to wild type C57BL/6 wild type mice was significantly attenuated (63% vs. 20%; $p<0.05$) when AAA was defined as $\geq$ 50% in diameter.[148] Experimental and human AAA histological studies showed that iNOS was upregulated in macrophages, T and B lymphocytes and VSMCs in AAA specimen as compared to normal aorta.[149] These findings are corroborated by others an in elastase infusion mice AAA model in which elastase induced ~32% increase in AA diameter in $iNOS^{-/-}$ mice compared to 140% increase in WT ($ApoE^{-/-}$ or C57BL/6) littermates.[150] AAA formation in both groups was accompanied by increased expression of MMP-2, MMP-9 and MMP-12 but MMP-13 was only expressed in the WT mice. The study further showed that induced NO upregulated EMMPRIN (extracellular matrix inducer) which then increased the expression and activation of MMP-13 in aortic endothelial and VSMC cells. EMMPRIN and MMP-13 were also expressed in human AAA tissues.

Oxidative stress activates the both inflammatory and apoptotic pathways through a key enzyme, apoptosis signal regulated kinase -1 (ASK-1)[151] which is a proximal activator of the pro-survival pro-inflammatory NF-κB pathway and the pro-apoptotic c-Jun N-terminal kinase (JNK) and p38 MAPK signalling cascades.[152] There is evidence implicating JNK in upregulating both pro-apoptotic pathways by activating the mitochondrial (intrinsic) pathway.[153] It also upregulates p53 and stimulates c-Myc to mediate apoptosis. JNK can also induce the surface expression of Fas to mediate Fas-dependent extrinsic apoptosis. However, JNK is inhibited by NF-B which stimulates the gene expression of Mn-SOD and inhibitors of apoptosis like cIAP and x-linked IAP and JNK proximal acting sequences.[154]

1.3.3.1 Osteopontin

This oxidative stress mediated activation of NF-κB activation has been implicated in the induced expression of Osteopontin (OPN) , an acidic extracellular matrix glycoprotein, which is expressed in vascular cells and inflammatory cells like monocytes-macrophages. It has been demonstrated that the 5'-UTR of the promoter of OPN contains recognition elements for NF-κB and TGFβ inducible early gene-1 (TIEG-1) both of which are essential for the oxidative stress mediated induction of OPN.[155] Redox sensitive NF-κB activated OPN secretion is associated with the induced expression of pro-MMP-9 and urokinase type plasminogen activator (uPA).[156, 157] Osteopontin binds to surface integrin receptor, $\alpha_v\beta_3$ to activate NF-κB-inducing kinase (NIK) a proximal activator of the MAPK and NF-κB pathways to induce the formation of uPA and proMMP-9 respectively. uPA is a recognized activator of proMMP-9 to MMP-9 and both enzymes are implicated in the pathogenesis of AAA. Interestingly, OPN also induces the production of proMMP-9 through a pathway involving the upregulation of NAD(P)H oxidase system in aortic mesenchymal cells. There is also evidence that OPN acting through the NADPH Oxidase (NOX2) upregulated the production of pro-MMP9 5-fold in aortic A7r5 VSMC and myofibroblasts (AMF) in vitro.[158] In this study it was also shown that TNFα enhanced pro-MMP-9 activity in wild type OPN+/+ cells but not in $OPN^{-/-}$ AMF (adventitial myofibroblasts). In addition, mechanistic studies have implicated OPN in the formation of AAA in Angiotensin II infused mouse model with a concomitant decrease in the activity of MMP-2 and -9.[159] Serum OPN levels have been associated with the presence of AAA in a recent study which compare AAA patients (n=233) to controls (n=233) and which showed that the mean (± SD) serum levels of Osteopontin was significantly elevated in AAA patients as compared to controls (77.19 ± 31.36 vs. 63.37 ± 29.14pg/mL, $p<0.001$).[160] In this same cohort it was found that OPN was

independently associated with AAA as evidenced from the adjusted odds ratio between the highest tertiles and the lowest tertiles of 2.23 (95%CI, 1.29-3.85, p=0.004).

1.3.3.2 Genotoxic Stress

In another study, increased evidence of oxidative DNA damage was associated with AAA compared to controls as evidenced by the presence of the marker 7,8-dihydro-8-oxo-2'-deoxyguanosine (**8-oxo-dG**) which was ~ 4-fold increased (lymphocytes), ~5-fold increased (endothelial cells), ~5.5-fold increased in VSMC and ~2.0 increased in epidermal cells.[161] Interestingly, blood lymphocyte telomere lengths were significantly shorter in AAA compared to controls (0.099 telomere fluorescence units (IQR, 0.092-0.101) vs. 0.106TFU (IQR, 0.103-0.109), respectively, P<0.0001) and inversely correlated with oxidative DNA damage (r=-0.57, P<0.0001) in the former. The presence of **γ-H2AX**, a histone marker of the DNA damage response (DDR) was significantly increased in AAA vs. controls [EC (38%; range 22%-64%) vs. (2%; range, 0.1%-6%); P=0.006) and VSMC (35%; range, 28%-59%) vs. (1.7%; range, 0.15%-5.7%; P=0.010)].

1.3.3.2.1 Senescence

The presence of shortened telomeres and marks of oxidative DNA damage in AAA tissues suggests that senescence and aging may be causal in the pathogenesis if AAA. One study showed 74% decrease in the density of VSMC in AAA as compared to normal aortic tissue [50.9 ± 6.1 SMC/HPF (n=10) vs. 199.5 ± 14.9 SMC/HPF (n=5); p<0.01]. Up to 30% of residual VSMC exhibited DNA fragmentation and p53 protein, a mediator of senescence and apoptosis was elevated ~ 4 fold in AAA tissue and produced predominantly by lymphocytes and VSMC.[162]

The telomere lengths below a certain length is sensed by the proteins of the PML bodies as a double strand break and thus initiate the DNA damage response (DDR). The DDR can also be initiated by oxidative stress mediated genotoxic stress in which the reactive oxygen products (ROS) damage DNA resulting in double – strand break. The DDR can thus occur in stem/progenitor cells as well as adult somatic cells. The final common pathway is the activation of retinoblastoma protein (Rb) and recruitment of repressive chromatin marks to form a complex, known as senescent associated heterochromatic focus (SAHF).[163] The SAHF releases p53 which then up-regulates p21, a cell cycle specific protein that function as a cell-cycle inhibitor at the G1-S phase . Interestingly, telomere length shortening has been documented in the wall tissue of AAA.[164] In this latter study comparison of site-specific telomere DNA content between AAA (n=20) and biopsy specimen from normal aorta (n=12) showed a decreased content in the former (p=0.03), a difference which remained significant after adjusting for confounding factors. Similarly, replicative senescence of VSMCs has been implicated in the pathogenesis of AAA.[165] In this study tissue specimen from the AAA wall (n=15) were compared to those from adjacent inferior mesenteric artery (IMA) wall as controls. It was shown that AAA tissue VSMCs exhibited morphology of senescent cells as compared to those from IMA by being larger and flatter with a corresponding 55% decrease in mean DNA synthesis, 44% decrease in maximal proliferation, reduced [^{3}H] thymidine incorporation and replicative senescence in serial subcultures. Telomere shortening below a certain size (Hayflick limit) triggers cellular senescence via the DDR. However, there is current evidence to suggest the loss of telomerase within the aortic wall. Recent evidence showed a 55% decrease in telomerase expression in human AAA histological tissues compared to normal aorta (22.4% vs. 79.2%; $P < 0.001$) which translated in a significantly reduced telomerase after adjusting for other factors (odds ratio (OR), 0.47; 95%CI, 0.14-0.58; $P< 0.01$).166 The DDR is effectively a p53- mediated process either through the formation of the telomere dysfunction foci (TIF) from telomere

attrition due to loss of telomerase or replicative attrition of the telomere or from genotoxic stress from the effects of reactive oxygen species on the chromosomes.[167]

1.3.3.2.2 Nuclear Factor-kappa Beta (NF-κB)

Oxidative stress activated poly-(ADP)- ribose 6-phosphate 1 (PARP-1) is implicated in the cleavage activation of the apoptotic pro-Caspase-3 to Caspase-3 resulting in apoptosis and inhibition of sirtuin 1 (SIRT1) a histone deacetylase. Caspase-3 also upregulates p53 which activates the DNA-damage response (DDR) characterized by the variant histone (γ-H2AX) dependent senescence - associated heterochromatic focus (SAHF)[168]. Similarly, PAPR-1 suppresses sirtuin 6 (SIRT6) by competitive sequestration of NAD^+ leading to the de-repression of NF-κB because SIRT6 participates in the ageing process through the repressive deacetylation of H3K16 on the promoter of NF-κB.[169] Senescence - associated NF-κB upregulation is associated with increased expression of pro-inflammatory cytokines like IL-6 and its receptor IL-6r, IFNγ, and chemokine ligands like IL-8, CXCL-1,-5,-7 and their common receptor CXCR2.[170] Similarly, there is increased expression of MMP1 and MMP2 and p38MAPK dependent activation of C/EBP_β-mediated upregulation of IL-6,-8, CXCL-1 and -7. This global proinflammatory changes associated with senescence is referred to as senescence-messaging secretome (SMS) and it is dependent on the p53 and NF-κB pathways.[171] A role for the involvement of the NF-κB pathway in the pathogenesis was demonstrated in an Angiotensin II infusion model of experimental AAA formation in ApoE deficient mice. In this model , AAA formation was positively associated with increased production of NF-κB and its downstream pro-inflammatory gene products like ICAM-1,VCAM-1, MCP-1 and inducible nitric oxide[172]. Furthermore, the importance of NF-κB in the pathogenesis of AAA is demonstrated in an elastase mouse model in which the administration of a NF-κB inhibitor is associated with a 40%

reduction in AAA formation and a 70% reduction in aortic diameter of AAA[173]. This was accompanied by resolution of inflammatory mediators. These findings have been extended by another study in which administration of decoy oligonucleotides (ODN) for NF-κB and Ets transcription factors in AAA elastase rabbit models resulted in reduced AAA formation and regression of formed AAA[174]. This salutary effect is associated with reduction in the production of MMP-2 and -9. These findings have been demonstrated in the clinical setting where it was shown that activated form of NF-κB (p65 NF-κB-phosphorylated) is significantly increased in AAA wall specimen as compared to atherosclerotic aortic wall specimen ($p<0.001$).[175]

1.3.4 Aortic Medial Hypodensity and AAA formation

Current evidence suggests that the highest structural constituent of the aortic media is collagen at 47% followed by elastin (29%) and VSMC (24%).[176] However, 71% of the elastin is in the elastic lamellae and 27% in interlamellar elastin fibres (IEF) which abut on the VSMC and the remainder 2% constitutes radially oriented thick elastin struts which link upper and lower elastin lamellae. VSMC link these thick struts at fenestrae which are gusseted and reinforced by these IEF.

The VSMC is an important functional unit of the aortic wall. As part of the medial elastic lamellar unit,[177] it is connected in-series with 75% of medial elastin[178] and also requires collagen for optimal function.[179] The disruption of this functional unit in the primary transmedial dissection of the Angiotensin II infusion model triggers the transition to aneurysmal dilatation.[180] Current evidence from Angiotensin II infusion murine models suggests that medial dissection precedes inflammatory mononuclear accumulation and AAA can form in the absence of macrophage infiltration with this model.[181, 182] Intriguingly, despite immediate loss of elastin in

the elastase infusion murine model, AAA does not form until the accumulation of inflammatory cells and adventitial remodelling.[183, 184] In addition, extracellular calcium in the $CaCl_2$ model is associated with disruption of medial lamellar structure comprising 13% reduction in breaking stress, 3% reduction in breaking strain but 167% increased elastin specific breaking stress. In addition, extracellular calcium is associated with VSMC apoptosis via the calcium sensing receptor-dependent pathway which activates ceramidase with consequent activation of pro-apoptotic Caspase-3. The foregoing taken as a whole suggests that loss of the functional MLU is a necessary and sufficient condition for aneurysm formation. Conversely, endovascular seeding of an already formed AAA with VSMC is associated with suppression of AAA.[185]

A functional MLU functions as a hydraulic buffer to dampen hemodynamic wall stresses which are injurious to the aortic wall. [186, 187,188] Attrition of the aortic tunica media consequent upon loss of integrity of the MLU results in a disproportionate stress on the other cells of the aortic wall resulting in endothelial activation and dysfunction and fibroblasts phenotypic switch and apoptosis.[189] These will manifest as increased inflammatory cell recruitment into the aortic wall and adventitial wall thickening which are the hall marks of early aneurysm formation and consequent expansion respectively.

1.3.4.1 VSMC Migration

A previously unrecognized cause of paucity of medial VSMC is increased proliferation and migration out of the tunica media via the IEL fenestrae into the intima.[190] This is may perhaps be an apparently evolutionarily conserved response to vascular injury. The cells undergo a phenotypic switch to a more invasive, synthetic form from the resident tensile cell under

chemotactic gradient from the endothelium.[191,192,193] The migration is under a chemotactic gradient induced by the injured endothelium which secretes mediators like Angiotensin II, VEGF and PDGF, all of which are chemotactic for the VSMC.[194] The synthetic VSMC secretes OPN, MMP-1, MMP-2 and MMP-9 which facilitate its egress from the tunica media. Of interest though is the fact that the injured / activated endothelium is a rich source of cytokines, chemokines and growth factors like MCP-1, CCL11 (eotaxin), stromal derived factor (SDF-1α), IL-1β, TNFα, IFNγ and fractalkine (CX3CL1) which set up a chemotactic gradient for VSMC.[195] Interestingly, injured VSMC and endothelium express OPN and αvβ3 thus establishing a mechanism for aortic wall remodelling characteristic of AAA. This is because, the OPN receptors CD44 and αvβ3 are required for a FAK-dependent migration of VSMC and Myofibroblasts[196] thus contributing to medial hypodensity and reduced adventitial collagen synthesis. The OPN-induced HMGB1 is an intensely pro-inflammatory cytokine which is also expressed by injured endothelium. Furthermore, activated MC/MØ is also known to induce the expression of NF-κB dependent inflammatory cytokines like TNFα, IL-1, IL-6, IL-8, MIP, ICAM-1, VCAM-1 and RAGE.[197]

It is noteworthy that VSMC is also present in atherosclerosis but what differentiates this pathology from AAA is the VSMC loss in the latter to apoptosis. Histological analysis of TA compared with AA in males (n=67) with age range from 41-95 years of age (mean, 66 years), increasing AA lumen diameter directly correlated with atherosclerotic plaque area ($r=0.61$; $p<0.05$) in a cohort with TA/AA ratio <1.3 (group III).[198] Plaque sizes (mm2) in TA/AA ratio >1.3 (group I) or 1.2-1.3 (II) and group III were 29.2 ± 14.6, 44.6 ±19.4 and 74.9 ± 39.3, respectively with corresponding lumen diameters of 12.0± 2.0, 12.6±1.9 and 16.4 ±4.2, respectively. The same authors previously showed that plaque areas were inversely correlated with media thickness ($r=-0.75$; $p<0.01$) in AA.[199] The implication is that the progression of

atherosclerotic AA to AAA is accompanied by aortic media attrition. In a clinical study, patients with severe atherosclerosis with disrupted plaques (n=119) had 20%, 50% and 79% increased medial inflammation, medial atrophy and adventitial inflammation respectively, relative to non disrupted plaque atherosclerosis (n=306; p=0.003).[200] Medial inflammation correlated with plaque area (p=0.0001) and ruptured IEL (p=0.0001).

1.3.4.2 VSMC Apoptosis

Apoptosis refers to programmed cell death meaning that it is a regulated process involving gene transcription and activation of specific signalling pathways with the consequence for an orderly cell demise.[201] This is characterized by nuclear fragmentation, "blebbing" of cellular membranes and the accumulation of cytoplasmic inclusion bodies.[202] It is a highly regulated energy requiring process that involves proteases of all known classes (serine, cysteine, threonine, aspartyl, and metalloproteinases) and characterized by proteolytic cleaving of cytoplasmic, nuclear and membrane structural components.[203]

There is evidence to suggest that the initial step in arterial aneurysm formation is contingent on the loss of VSMC. In one such study,[204] which consisted of AAA (n=6), Iliac artery aneurysms (IAA, n=10), Femoral Artery Aneurysm (FAA, n=6) and Popliteal Artery Aneurysms (PAA, n=18) all males with a mean age of 71 years (range, 48-93 years) it was shown that there were disproportionately fewer VSMC in aneurysms relative to controls for AAA, PAA, FAA and IAA (2.2 ± 0.4% vs. 14.6 ± 1.8, n=1; p=0.0001), (1.5 ± 0.4% vs. 11.4 ± 1.3%, n=6; p=0.0001), (0.8 ± 0.1 vs. 12.5 ± 1.7%, n=4; p=0.001) and (1.2 ± 0.3% vs. 10.8 ± 1.5%, n=4; p=0.0001) respectively. With all aneurysms combined compared with all controls, it was shown that there were significantly ($p<0.05$) more apoptotic markers in the former for BAX, caspase-3, Fas and

p53 (82% vs. 48%; 58.28 ± 4.33% vs. 4.55± 1.2%; 14.70 ± 1.5% vs. 3.5 ± 0.55% and 14.5 ± 2.1% vs.1.0 ± 0.2 %, respectively). Another study also demonstrated the presence of apoptotic VSMC in the aortic media with associated paucity of these cells and may account for loss of up to 80% of the aortic media in AAA.[205] The tissue specimen also exhibited marked infiltration of inflammatory leukocytes and marked elevation of Fas and FasL in VSMC and T cells respectively.[206]

1.3.4.3 Elastolysis

Degradation of the aortic wall elastin is mediated by a class of proteases known as cysteine cathepsins (Cathepsins), matrix metalloproteinases (MMPs) and serine proteases (urokinase-type Plasminogen Activator (uPA) and the tissue-type PA (tPA) of which the former two are the most important. These proteases also collectively know as matrixines for their ability to degrade the ECM are regulated by their endogenous inhibitors like Cystatin C(for Cathepsins) and tissue inhibitors of metalloproteinases (TIMP) for MMPs and Plasminogen Activator Inhibitor type 1 (PAI-1) for the serine proteases.

The important cysteine cathepsins implicated in AAA pathogenesis include Cathepsins K, L, S and V. They are secreted by VSMCs, endothelial cells, neutrophils and macrophages and have also been implicated in atherosclerosis. Cathepsin K has the highest affinity (68%) for aortic elastin being 1.5-fold higher than cathepsins S (45%) and ~ 4-fold higher than Cathepsin L (15%) in vitro.[207] The cooperation amongst the Cathepsins is also evident in a recent study of an elastase-infusion AAA model with a Cathepsin K knockout (Cts $K^{-/-}$).[208] Elastase infusion in these mice was accompanied by a reduced incidence of AAA formation associated with reduced $CD4^+$ T cell infiltrate and absent Mac-3^+ MØ despite the presence of VSMC apoptosis. Characteristically, there was an associated reduction in Cathepsins L and S in the aortic tissue.

Similarly, Cathepsin L deficiency was associated similar features except for VSMC apoptosis which was absent in the Cts $L^{-/-}$ genotype.[209] Interestingly, Cathepsin K deficiency did not affect AAA formation in $ApoE^{-/-}$ mice infused with Angiotensin II for 28 days.[210] Interestingly, the Cts $K^{-/-}$ Apo $E^{-/-}$ mice demonstrated increased circulating granulocytes and activated T cells and their aortic tissue showed marked adventitial infiltration with proinflammatory cells in addition to upregulated Cathepsin S and Cathepsin C compared to Apo $E^{-/-}$ mice under similar experimental conditions.

Current evidence suggests that Cathepsins may be active in the pathogenesis of AAA because of their biological properties in vivo which include elastolysis, collagenolysis of collagen I (intima and adventitia) and III (media), VSMC apoptosis and neovascularisation which are the hallmarks of AAA pathology.[211] Another feature of the cysteine cathepsins is the reciprocal relationship with their endogenous inhibitor, Cystatin C which is significantly reduced in AAA tissues

A recent study[212] which compared AAA (n=82) wall histological specimen to aortic wall specimen from atherosclerotic occlusive disease (AOD, n=14) showed that protein levels of Cathepsin H (ng/mg sol protein) was significantly increased compared to the latter, 8.92 (IQR, 5.2-15.8) vs. 2.7(IQR, 1.5- 8.9), p=0.007. Conversely, the Cystatin C levels (ng/mg sol protein) was significantly decreased in AAA tissues compared to aortic specimen from AOD subjects, 30.9 (IQR, 17.5-44.9) vs. 43.3 (IQR, 26-53.6), p =0.03. Interestingly, the activity of Cathepsin B,[31.6 (13.0-66.3) vs. 8.4 (3.7 – 16.4), p<0.001], Cathepsin H ,[275 (IQR, 152- 570) vs. 144(IQR, 78-331), p=0.019],Cathepsin L [207 (IQR,85-629) vs. 93(IQR, 36-141), p=0.002)]and Cathepsin S[246 (QR,117 – 500) vs. 203 (IQR, 37-269), p=0.045] in AAA wall tissues were significantly elevated relative to AOD aortic wall tissues underscoring the relative importance of the cysteine cathepsins in the pathogenesis of AAA despite sharing a spectrum of risk factors.

The study also underscored the importance of post-translational modification in the activity profile of the cysteine cathepsins.

In a histological analysis of Cathepsins (their inhibitors) and MMPs (and their inhibitors), it was shown that relative to controls (n=11), there was significantly more increased expression of MMP-9 ($p<0.01$) and MMP-14 ($P<0.05$) in stable AAA (n=17) and ruptured AAA (n=15).[213] Similarly, the mRNA of Cathepsins K, L and S were significantly elevated in growing and ruptured AAA relative to controls ($p<0.04$). Interestingly, western blot showed significantly ($p<0.05$) increased MMP-8, Cathepsin -L,-S,-K and v-H^+-ATPase (proton pump) in growing and ruptured AAA compared to controls. In contrast there was no difference ($p<0.1$) in TIMP-1 and -3 but there was reduced Cystatin C levels ($p<0.05$) in both growing and ruptured AAA with an inverse correlation between Cystatin C and MMP-8 ($r=-0.78$; $p<0.05$). The authors further showed evidence of in vitro Cystatin C degradation by neutrophil elastase (89%), MMP-8 (64%) and MMP-9 (37%).

The matrix metalloproteinases (MMPs) constitute the other major elastolytic and collagenolytic protease implicated in AAA pathogenesis. The MMPs are secreted as inactive latent forms and require a catalytic cleaving by another protease to expose the active site in a process referred to as "cysteine switch" by virtue of removal of the cysteine containing latency pro-peptide.[214] MMPs have been phenomenologically classified into subgroups on the basis of their abilities to cleave intact fibrillar collagen (Interstitial collagenases; MMP-1,-8,-13 and MT1-MMP), type IV collage in basement, laminin and elastin (gelatinases; MMP-2,-9); proteoglycans, fibronectin, laminin and collagen IV,V,XI,X and elastin (stromelysisn; MMP-3,-7,-10,11) and membrane type-MMP [MT1-MMP (MMP-14), MT2-MMP (MMP-15), MT3-MMP (MMP-16) and MT4-MMP (MMP-17)].[214] Within the aortic wall, inflammatory cells actively express MMPs in a sequential step from an initial contact/injury stage in which MMP-9 are produced by MC on the

acquisition of the MØ phenotypes. However, under inflammatory or cytokine stimulation, macrophages (MØs) secrete MMP-12 their hallmark MMP in addition to MMP-14 which activates MMP-2.[214] It is further suggested that MØ express MMP-1,-3,-8 and -11 on CD40/ CD40L stimulation. The foregoing underscores the fact that vascular pathological remodelling requires a plethora of MMPs. In contrast, VSMC which constitutively express MMP-2 are stimulated by injury/inflammation to upregulate MMP-9 and depending on persistence, type (PDGF, IL-1β or TNFα) or strength of the stimulus can progressively express other MMPs like MMP-1, -3 , -8 or -9.[214] Interestingly, a similar pattern may be operative in EC which constitutively express MMP-2 and under CD40/CD40L stimulation express MMP-1,-3 and -9. It is noteworthy, that consistent with a constitutive expression of MMP-2, EC express MT1-MMP under cyclic strain.[214] The other inflammatory cell which has recently been shown to play a significant role in AAA formation is Mast Cell which constitutively secrete MMP-2 and under stimulation upregulate MMP-9.

MMPs have different affinity for the different structural protein components of the aortic wall. For example, MMP-2 and MMP-9 are known as the gelatinases because they can degrade denatured collagen. They also cleave basement membrane collagen IV and collagen degradation products resulting from the activities of other proteases like cathepsins and the collagenases like MMP-1, -8 and 13 which have been shown to cleave native collagen. Furthermore, MMP-2 and -9 cleave elastin into elastin degradation products (EDP) which also function as pro-inflammatory chemokines functional in the recruitment and activation of inflammatory cells in the AAA pathology.

In one early study,[215] the activity of MMP-9 in AAA of 5.0-6.9cm diameter was 98.06 ± 15.19 and was significantly different from that in controls (13.57 ± 1.13, $p<0.003$), AAA of 3.0-4.9cm

(20.87 ± 5.15, p<0.03) and large AAA >7.0cm (27.16 ± 4.56, p<0.01). There is also evidence to suggest that MMP-9 but not MMP-2 is significantly correlated with human AAA size (p<0.05) although MMP-2 and MMP-9 were also significantly correlated (p<0.001)in the study (N=46).[216] In a comparative analysis, plasma MMP-9 in AAA (n=22), atherosclerotic occlusive disease (AOD, n=9) and controls (n=8) were 85.66 ± 66ng/ml, 25.75 ± 4.16ng/ml and 13.16 ng/ml ± 1.94 respectively, p<0.001 although there was no difference between tissue levels of MMP-9 between AAA and AOD.[217] Conversely, one study did not find any correlation (r=0.09, p=0.19) between AAA (3.5-4.9cm diameter) and plasma levels of MMP-9.[218] In this study (n=213), plasma MMP-9 levels were unchanged in 2 (1%), decreased in 112 (57%) and increased in 82 (42%) patients. There is evidence also implicating the macrophage elastase MMP-12 in the pathogenesis of AAA. In one study,[219] the immunoreactivity MMP activity in normal donor aorta (n=12), atherosclerotic occlusive aorta (AOD; n=12) and AAA (n=15) were 91.02 ± 42.77 mean density units (MDU), 538.43 ± 32.73 MDU and 631.80 ± 69.3 MDU, respectively indicating a 6-7 fold increase in the diseased aortae. Notwithstanding the presence of MMP-2,-7,-9 and -12 in the amorphous media and MMP-2 in inflammatory outer media, MMP-12 was localized to active transitional media. In vitro analysis demonstrated that TIMP-1 was only marginally (~10%) active against MMP-12. Mechanistic studies have suggested that MMP-9, Cathepsin C (activates neutrophil elastase, Cathepsin G and chymase), Cathepsin S, TNFα, IL-1β and IL-6 are important molecular mediators in the elastase-infusion AAA model.[220]

1.3.5 Non-coding RNA in AAA pathogenesis

The recent association of variants in non-coding DNA sequences (intergenic and intronic) in significant association with AAA is compelling evidence for a causal role for non-coding RNAs (ncRNAs) in the pathogenesis of AAA. This assertion is underscored by recent molecular

genetics advances which suggest that these non-coding RNAs have regulatory functions and are not "Junk" DNA as was previously thought. There is emerging evidence that these ncRNAs (long ncRNA and short microRNA) play a role in the pathogenesis of AAA.

1.3.5.1 MicroRNAs

MicroRNAs are a group of 20-22 nucleotide RNA processed from non-coding genomic transcripts (Figure 2). It has been suggested that each miRNA has the capacity to regulate up to 300 mRNA in this manner. Furthermore, miRNAs regulate ~ 60% of the human genome thus making the study of miRNA in AAA pathology relevant.[221] Each miRNA is capable of regulating up to 300 mRNA by binding to the 3'-UTR of the latter and directing it for cleavage. This ability for a single miRNA molecule to regulate up to 300 mRNA makes them ideal candidate for study in AAA pathogenesis.

A recent study suggested that dysregulation of miRNA may be of pathogenic importance in AAA disease.[222] The miRNAs that were upregulated included miR-19a, miR-19b, miR-34b, miR-132, miR-146a and miR-221. Interestingly, the cluster of miR-143/145 was recently found to be downregulated in human thoracic aortic aneurysm histology specimen compared to controls.[223] Furthermore, a murine miR-143/145 knockout model was associated with a de-differentiated VSMC phenotype with increased migratory/invasive capabilities and a blunted response to hypertension.[223] Furthermore, the de-differentiated VSMC demonstrated a decrease marker of the differentiated VSMC like myosin heavy chain and α-smooth muscle actin (α-SMA). The authors further showed that pathological injury to the arterial wall was associated with a downregulation of miR-143/145 and the emergence of a de-differentiated VSMC phenotype.

1.3.5.1.1 Micro-RNA 21

MiR-21 was recently associated with the pathogenesis of AAA in the elastase infusion and Angiotensin II infusion murine models (augmented with nicotine) where it was found to temporally increase with aneurysm progression.[224] Interestingly, miR-21 was only expressed at "injured" site (AAA) and was augmented by nicotine in these two models. A lentivirus pre-miR-21 plasmid vector increased miR-21 attenuation of AAA diameter. Conversely, locked nucleic acid (LNA)-anti-miR-21 (antagomir-21) or silencing RNA (siRNA) was associated with increased frequency of AAA formation and severity of aneurysmal lesions. Nevertheless, tissue miR-21 correlated with MCP-1 and IL-6 and downregulated PTEN (Phosphatase and Tensin Homolog), SPRY1 (Sprouty 1) and PCD4 (Programmed Cell Death 4).

Co-culture of human aortic VSMC greatly increased miR-21 with nicotine, IL-6 and Angiotensin II in a NF-κB dependent mechanism. Human AAA tissues from non-smokers and smokers compared with normal aorta from organ donors showed that miR-21 was elevated ~7-fold in AAA of non-smokers and ~13-fold in smokers ($p<0.05$ all). Furthermore, the target protein PTEN was decreased 2.4 fold in non-smokers and 4.3-fold in smokers with AAA. MicroRNA-21 is transcribed from the same locus as ACE, 17q23 and has come to become one of the most important miRNAs in human pathology. As an oncomir, it activates the PI3K-Akt survival pathway by suppressing PTEN a naturally occurring endogenous inhibitor of PI3K-Akt pathway.MiR-21 other target proteins include pro-apoptotic p53 homologues (p63, p73), co-activators (heterogeneous nuclear ribonucleoprotein K; HNRPK) and targets (IGFBP3, GADD45A, p21, NOXA), TGFβ pathway proteins and mitochondrial apoptotic proteins (APAF1, BAX and Caspase-3).[225] There is also evidence implicating miR-21 in the increased expression of MMP-2 in human fibroblasts in a PTEN- dependent pathway possibly via the PI3K-Akt signalling cascade.[226] In addition to the foregoing, miR-21 also targets RECK

(reversion-inducing cysteine-rich protein with kazal motifs), sprouty (SPRY1 and SPRY2) in haematological cells.[227] It is noteworthy that RECK is a natural inhibitor of multiple MMP expression and its inhibition by miR-21 de-represses MMP expression leading to increased MMP expression by miR-21. In addition, SPRY1 and SPRY2 are involved in collagen deposition and vascular remodelling by negatively regulating receptor tyrosine kinase (RTK) signalling including fibroblasts growth factor (FGF) and TGFβ.

It is noteworthy that the oestrogen receptor, ERα, regulates the transcription of miRNA-21 from the Alu element of the ACE gene ("I" allele) at 17q23.2.[228] However, since the miR-21 gene (17q23.1) has no promoter proximal oestrogen response element (ERE) it means that ERα can bind to its RE in the Alu sequence at intron 16 to regulate this gene and other genes from a distance.

1.3.5.1.2 Micro-RNA 26

In contrast, miR-26 (miR-26a-1, miR-26a-2 and miR-26b) is decreased during AAA pathogenesis in the elastase and Angiotensin II infusion murine AAA models.[229] In these models, its levels were decreased 2.3-fold at day 3 of infusion and 3.8-fold at day 7. Its forced expression with a lentiviral delivery system decreases VSMC differentiation markers, suppress VSMC proliferation and migration and also the TGFβ signalling intermediates. However, these cells become more prone to H_2O_2-dependent apoptosis. The latter may not be unconnected with the paradoxical property of the AKT-signalling pathway to increase susceptibility to ROS-dependent apoptosis because of the suppression of Mn-SOD which is expressed by FOXO3. In any case,

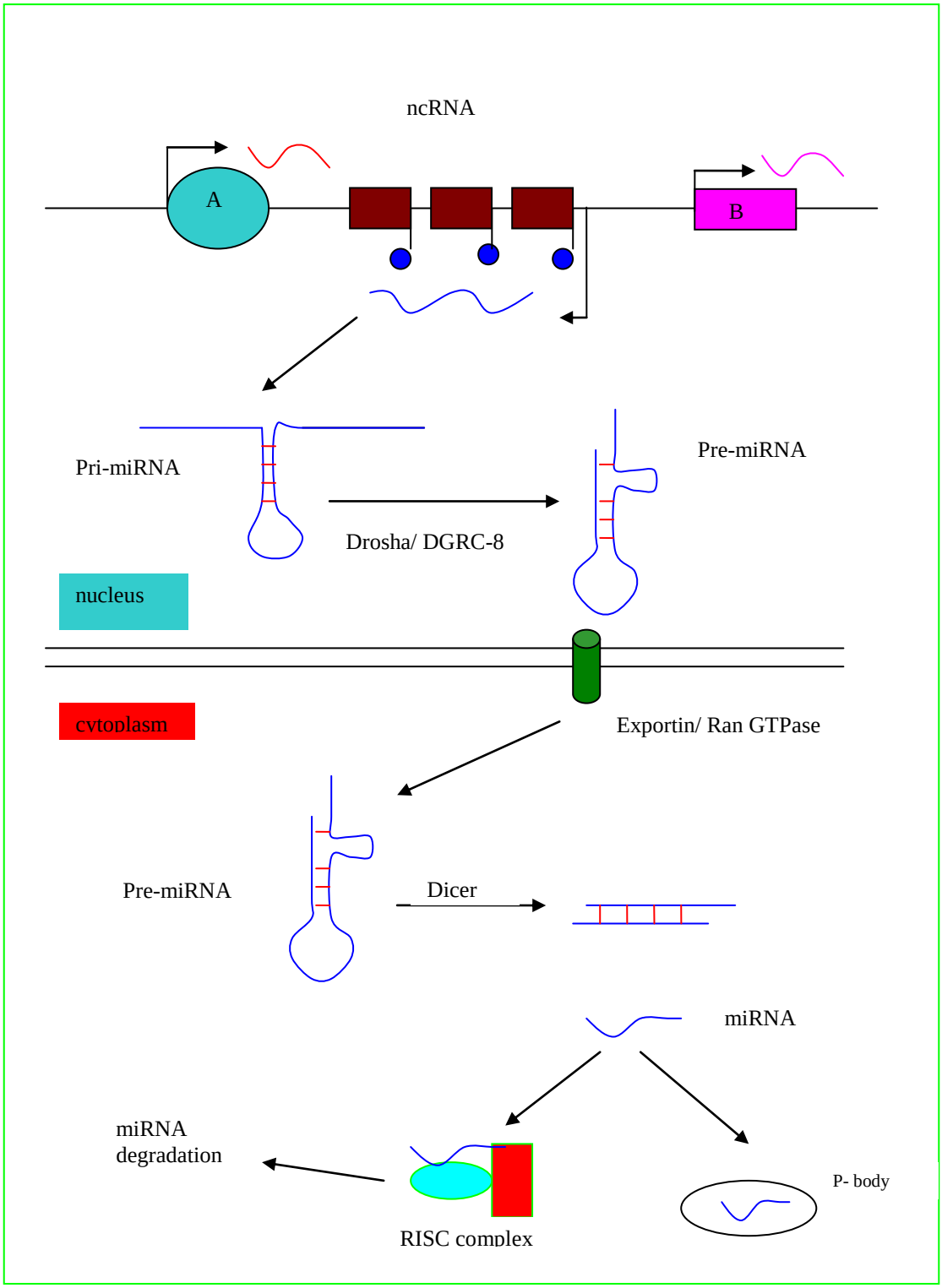


Figure 2: MicroRNA biogenesis pathway. MicroRNAs are non-coding RNAs. (see text).
MicroRNAs are a group of 20-22 nucleotide RNA processed from non-coding genomic transcripts.

These non-coding regions are transcribed into long ncRNA and short ncRNA. The latter transcript of 60-70 nucleotide length is referred to as primary RNA (Pri-miRNA) and it is further cleaved by an initial intra-nuclear RNAse III enzyme, Drosha and its partner pasha (DGRC-8), into the pre-microRNA. The latter is thereafter exported from the nucleus into the cytoplasm by the transnuclear membrane protein channel, exportin, in association with a Ran GTPase In the cytoplasm , Pre-miRNA is processed into the 20-22 nucleotide single stranded micro-RNA (miRNA) by Dicer a cytoplasmic RNAse III. The mature miRNA is further complexed in a microprocessor complex with Argonaute-2 (P-body). This complex inhibits mRNA through a RISC (RNA-Induced Silencing Complex) by binding to the 3'-UTR (untranslated region) of the mRNA by low-fidelity covalent binding. The RISC complex is subsequently degraded in the ubiquitin proteasome pathway

antagonism of miR-26a is accompanied by increased expression of VSMC differentiation markers like, myosin heavy chain (MHYII) and smooth muscle α-actin (ACTA2). Its antagonism was associated with increased AAA formation and severity of lesions and like miR-21 its over-expression results in fewer and less severe aneurysms in the same murine models. However, its target mRNA are members of the TGFβ signalling pathway like SMAD-2 and SMAD-3 which it inhibits to mediate phenotypic switch of VSMC from a contractile cell to a synthetic one. Furthermore, it also inhibits the apoptotic properties of TGFβ (via death – associated protein 6; DAXX) in these cells. It is noteworthy that some of the TGFβ pathway (DAXX, TGFβR2, TGFβR3, SMAD, PDCD4 and CDKN1A) are also targets of miR-21.[225] Furthermore, it also targets PTEN, retinoblastoma protein (Rb1) and proximal JNK MAPK Kinase (MEKK2) further confirming is anti-apoptotic properties like miR-21.[230] In this regard, it also activates the AKT signalling pathway, inhibits JNK MAPK – mediated apoptosis and facilitates transit through the G1-S cell cycle checkpoint.

These are unusual "protective" features since earliest manifestations of AAA pathogenesis are phenotypic switch of VSMC to the synthetic type which in this state secrete proinflammatory cytokines, chemokines like MCP-1 and secrete MMP-2. Possibly, there are yet undiscovered properties of miR-26. It is noteworthy that as AAA progresses, the mesenchymal cells undergo apoptosis thus accounting for the decrease of this oncomir with AAA progression.

Similar to miR-26a, MiR-29b is another micro-RNA that is decreased with AAA progression with the two murine models but more noticeably at days 14 and 28 of the Angiotensin II infusion model.**Error! Bookmark not defined.** Concomitantly there was increased expression of its target genes COL1A1, COL3A1, COL5A1 and ELN (elastin) and their respective proteins. Over expression of miR-29b is associated with decreased COL1A1, COL3A1 (in adventitial

fibroblasts and human VSMC) and ELN (human VSMC) in vitro. Interestingly, TGFβ inhibit miR-29b in adventitial fibroblasts but not in VSMC.

1.3.5.1.3 Micro-RNA 29b

In another study, it was shown that enhanced miR-29b expression with a lentiviral system in the elastase infusion model was accompanied by exaggerated increase in AA diameter (AAD) whereas its inhibition with an antagomir caused a decreased AAD from day7 to day 28.[231] Compared to scrambled miR-29b, anti-miR-29b increased the expression of collagen and elastin mRNA at days 7, 14 and 28 following elastase infusion. MiR-29b upregulated MMP-2 and MMP-9 and its over-expression in the Angiotensin II infusion model was associated with more aneurysm-related ruptures compared to scr-miR-29b (63% vs. 33%, P <0.01) and antagomir-29b (20%; P<0.01). Perhaps consistent with its cellular origin, miR-29b was decreased (~2.3-fold) in human AAA tissue specimen in which COL1A1, COL3A1, COL5A1 and ELN were increased. Predicted targets of miR-29b include interstitial (COL1A1) and basement membrane (COL4A2) collagens, TGFβ3 and the wingless (Wnt) pathway inhibitor (CNNBIP1) which binds to β-catenin preventing binding to TCF/LEF transcription factors.[232] Further targets of miR-29b include proteins associated with apoptosis (TCL1 an MCL1), cell proliferation [(AKT p85α and Cdc 42 leading to de-repression of p53), CDK6, B-Myb], immune function (IFNγ) and ECM proteins (COL4A1, 7A1, 8A1, MMP2, ITGB1(β1-integrin), Laminin γ1, Fibrillin 1).[233]

1.3.5.2 Long non-coding RNAs and GWAS

Long non-coding RNAs (lncRNA) may also participate in AAA pathology. Recent genome-wide studies have shown significant associations between non-coding intronic and intergenic polymorphisms with AAA. One of such loci is the 9p21.3 locus which contains genes for the

cyclin-dependent kinase inhibitors $p16^{INK4A}$, $p15^{INK4B}$ and $p19^{ARF}$. Gene modification at this locus is mediated by a combination of chromatin modification, polycomb group (PcG) proteins and DNA methylation. The ncRNA, ANRIL, present at this locus undergoes differential expression depending on the particular polymorphism (Figure 3). ANRIL presumably recruits polycomb proteins like EZH2 and DNA methyltransferases 3 (DNMT3) and histone deacetylases to suppress this locus. ANRIL when transcribed antisense to p15 silences the nearby through the recruitment of polycomb group proteins and histone modification.[234] Increased expression of ANRIL from this locus (detected in peripheral blood monocytes) has been shown to be associated with increased risk of atherosclerosis unlike p15, p16 and p19 (which suppress atherosclerosis).[235] However, conjoint reduction in expression of ANRIL, p15, p16 and p19 ARF in purified peripheral blood T cells induced a positive association between a 9p21.3 locus polymorphism, rs10757278, and stroke, AAA and coronary artery disease.[236] It is notable that this locus is very polymorphic and as yet unidentified splice variants in different tissues of studies may contribute to these contradictory findings.[237] Ras-mediated ERK MAPK signalling, NF-KB and p53 have all been associated with de-repressing this locus by upregulating the specific H3K27 demethylase, JMJD3 and subsequently expressing p16, p15 and p19 to mediate cell cycle inhibition with G1-S arrest.[238] It is noteworthy that G1-S inhibition cause cellular senescence, an emerging molecular pathophysiological mechanism in AAA pathogenesis.

Another recently identified DAB2IP polymorphism (97906 C>A; rs7025486) in the first intron of the DAB2IP gene at 9q33 locus is a common polymorphism of the gene that is common in males but has no impact on the mRNA/protein levels of DAB2IP thus making it an evolutionarily conserved genetic marker.[239] DAB2IP is not a ncRNA but the polymorphism is within an intron which may likely affect the expression of a putative transcription site-associated (TSS) ncRNA thus affecting the expression of DAB2IP (see (4) in Figure 3)

Importantly, DAP2IP also known as apoptosis-regulated signalling kinase -1 interacting protein -1 (AIP-1) effectively sequesters TRAF2 from activating NF-κB and instead activates the ASK-1-p38MAPK pathway to induce apoptosis particularly following endoplasmic reticulum stress. This is because IRE-1, an ER-stress sensor, binds to AIP-1 during ER stress linking it to ASK-1-p38 signalling cascade. It was recently shown that AIP1 binds to a protein phosphatise (PP2A) and directs the latter to apoptosis inhibitory kinase -1 (ASK-1) to activate it.[240] There is evidence suggesting that this pathway inhibits the PI3-K-Akt signalling cascade leading to cell cycle arrest and apoptosis.[241]

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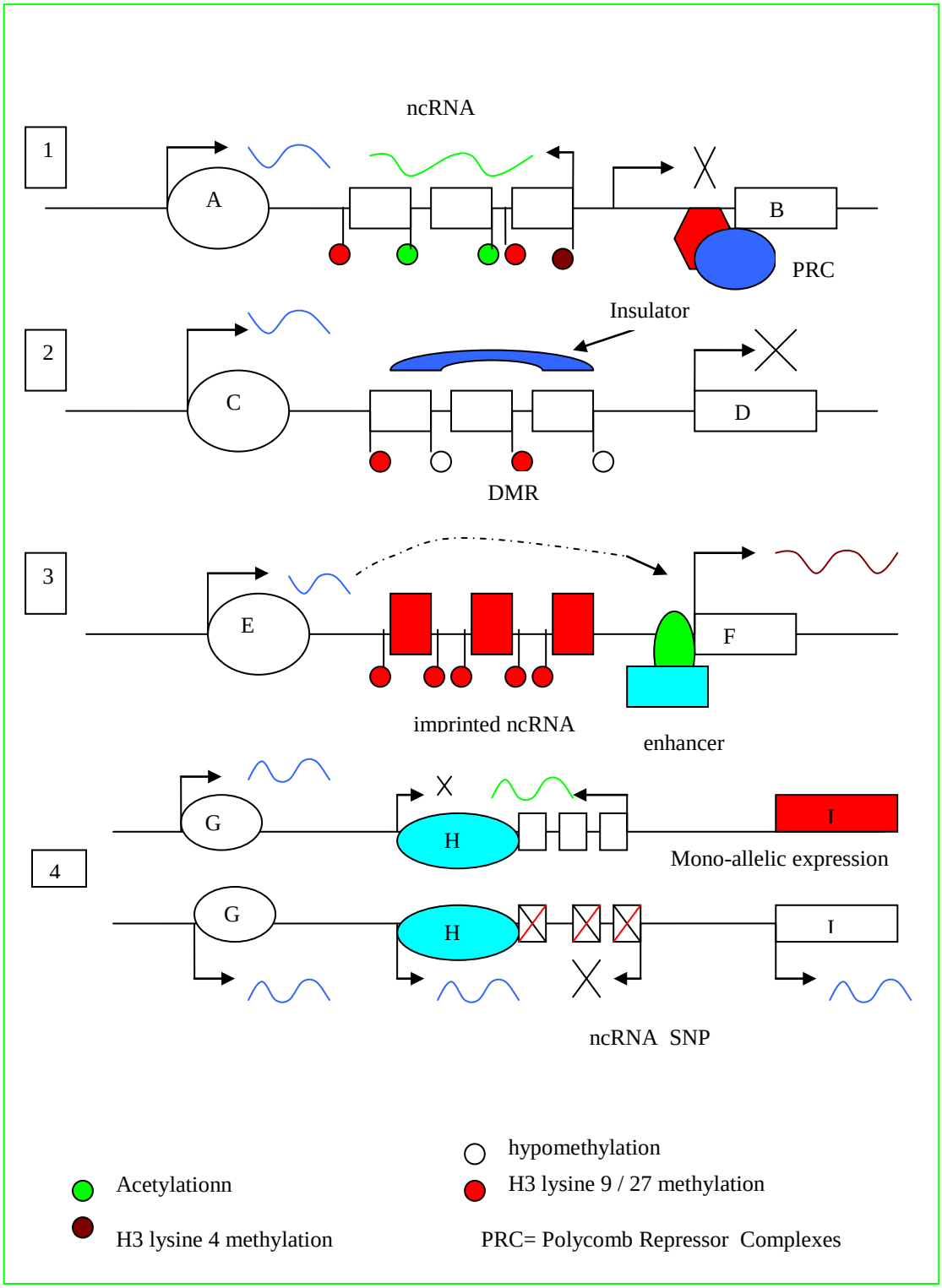

Figure 3: Non-coding RNA in genomic regulation. Non-coding RNAs (ncRNA) are transcribed from introns and intergenic regions. Their transcription is also subject to the same regulatory mechanisms as coding

regions. In (1), the ncRNA is the green transcript transcribed anti-sense to gene A whilst gene B is silenced by methylation. Expression of ncRNA is repressed by an insulator in (2) and methylation in (3). The insulator can also be a ncRNA (e.g., H9) which recruits silencing complexes to the adjacent gene promoter. Insulators normally separate transcriptionally active regions from silent ones in the genome. Differentially methylated regions (DMR) within ncRNA promoters implies transcriptionally equipoised loci which can be expressed or silenced under certain conditions unlike imprinted regions in 3 which are transcritptionally silenced. The special case of monoallelic expression due to genomic hypermethylation is known as imprinting . This usually applies to certain inherited diseases but current evidence implicates this mechanism in suppression of pervasive expression of ncRNA from the human genome (3). With ncRNA imprinting, the nearby gene (F) is protected from RITS (RNA-Induced Transcriptional Silencing) (4; gene I). But under conditions of global hypomethylation, these previously imprinted loci become transcribed due to loss of the methyl groups from DNA and histones. Transcribed ncRNA then mediate cis-suppression by mobilizing Polycomb Repressive Complexes (PRC) to the local gene promoter to methylate histone 3 at lysine 27 (H3K27) resulting in gene silencing (1; gene B). This is the explanation for the paradox of global genomic hypomethylation with promoter hypermethylation as seen in ageing and hyperhomocysteinaemia. Polymorphisms in ncRNA may suppress its expression allowing its regulated gene to be expressed (de-repressed) (4; gene T). A good example of 4 is ANRIL at 9p21.3 chromosomal locus. Reduced expression of ANRIL due to certain polymorphisms lead to increased expression of the tumour suppressor proteins p16 and p15 and consequent induction of senescence as in AAA. Increased expression of ANRIL on the other hand leads to suppression of p16 and p15 and consequent atherosclerosis and coronary artery disease. This may explain the association of this locus with diverse cardiovascular diseases.

Chapter 2. Candidate gene polymorphisms in the pathogenesis of Abdominal Aortic Aneurysms (AAA)

A recent population based study[242] showed a prevalence of AAA in a cohort of 172, 890 twins to be 0.16% (265 / 172, 890) of which the distribution in monozygotic (MZ) and dizygotic (DZ) twins were 1.2% and 1.4% respectively with probandwise concordance rates of 24% and 4.8% in the former and latter respectively. Furthermore, odds ratio (OR) of 71.1 (95%CI, 27.5-183.4) and 7.6 (95%CI, 3.0-19.2) were shown for MZ and DZ respectively. The ORs showed a decrease with age > 55years to 36.2 (05%CI, 13.3-98.3) and 4.5 (95%CI, 1.8-11.4) for the former and latter respectively. Notably, the proportion of variance explained by genetic factors using an additive genetic model was 0.70 implying that 70% of AAA could be due to independent genetic factors and 30% to unshared environmental factors. However, another study[243] which examined 313 pedigrees of 357 patients with 81 familial AA suggested that the proportion of the phenotypic variance explained by genetic factors (Heritability) was 0.79 in the full sample but 0.84 in the familial subsample assuming a multifactorial model of inheritance. Consequently, the foregoing constitute sufficient reasons for genetic association studies in which genetic polymorphisms defined as naturally occurring DNA sequence variants in at least 1% of the population that are in causal relationship with are identified as candidate gene polymorphisms.

2.1 Genetic Epidemiology

Some of these genetic polymorphisms which have been studied in the pasts include Chemokine CCR2 V641 polymorphism,[244] Heme Oxygenase-1 (HO-1) promoter dinucleotide (GT)n repeats,[245] CCR5 Δ32 deletion polymorphism,[246] IL-6 -572GC promoter polymorphism,[247] COX-2 -756G->C (rs20417).[248]

Recent GWAS efforts have led to the identification of some polymorphic loci in significant association with AAA. For example, certain polymorphisms at the 9p21.3 locus like rs1333049 (G>C),[249] 9p21.3 rs10757278 (A>G),[250, 251] 9q33 rs7025486 (97906 C>A),[252] 3p12.3 rs7635818 G>C,[253] 12.q13.3 rs1466535, [254] Furthermore, there are putative associations with polymorphisms at chromosomal loci 4q31 and 19q13.[255] A further extension of this study showed a significant linkage of AAA to chromosome 19 (19q13.3) in three Dutch families with AAA 19q13.4[256]. It is noteworthy that another study to investigate the shared genetic risk factors for intracranial (IA), thoracic aortic (TAA) and abdominal aortic (AAA) aneurysms identified further loci on chromosomes 6 and 11[257]. A recent review also showed that the loci on chromosome 4 (4q32-34) and 11 (11q24) contains genes that are shared between IA and AAA.[258]

2.1.1 Mendelian Randomization

The presence of genetic polymorphism is comparable to nature's own way of perturbing a causal variable (gene) in one group relative to another in a manner similar to a randomized controlled trial (RCT) in which nature does the randomization and allocate the groups The foregoing has given rise to the concept of MR which derives from Mendel's 2nd law of genetics (Figure 4):[259]

"the behaviour of each pair of differentiating characteristics in hybrid union is independent of the other differences between the two original plants, and, further, the hybrid produces just so many kinds of egg and pollen cells as there are possible constant combination forms"

(Gregor Mendel, 1865)

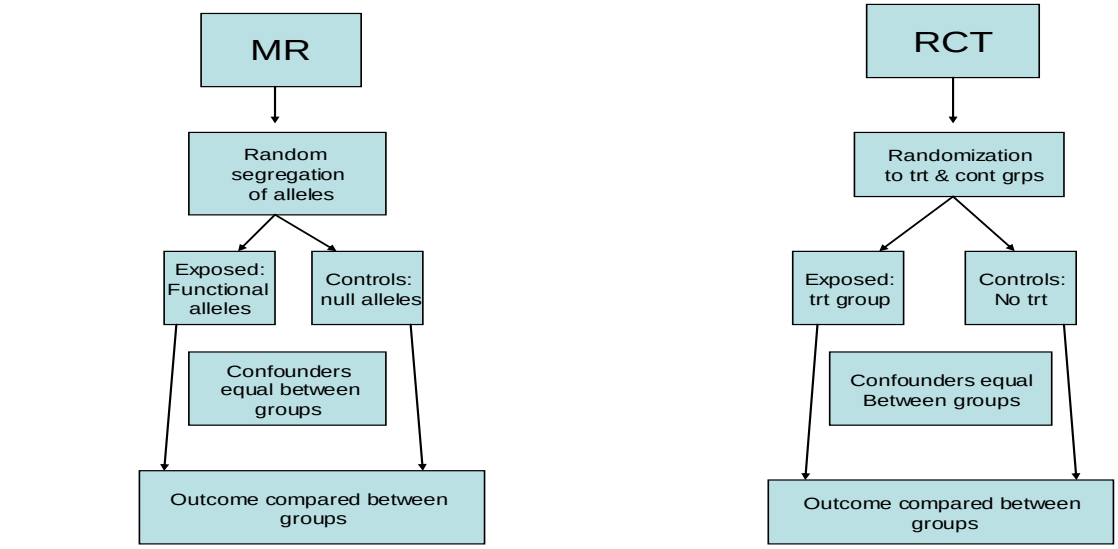

Figure 4: Comparison between MR and RCT. The similarities between a RCT and MR are depicted above. In MR, random segregation of alleles during meiosis is likened to the randomization done in RCT. Comparatively, the presence of a polymorphism of interest in a particular allele makes that allele functionally equivalent to a treatment group in the RCT whilst the allele without the polymorphism is likened to the control arm of an RCT. Further development of this concept follows in the text.

It exploit's the idea that the genotype only affects the disease indirectly and is assigned randomly (given parental genes) at meiosis independent of confounding factors.[260] These properties also define what is commonly referred to as an instrumental variable in the statistical literature.

2.1.1.1 Instrumental Variable Approach

An instrumental variable (IV) is a variable related with the intermediate (endo)-phenotype, X, but only relates to outcome Y through X.[261] Core conditions under the IV approach have been advocated based on conditional independencies, where $A \perp B|C$ implies that A is conditionally independent of B given that we know C already exists.[262]

Suppose we have a graph G with vertices V, and edges E and a probability density P(q) over the natural state of V parameterized by q;[263] then (G,q) is a causal pair if and only if (G,P) satisfies the causal markov condition (of conditional independencies) for the whole graph below (Figure 5):

The intermediate (endo-) phenotype, X, is characterized by the following:[264]

- Relevance to the disease
- Inheritable
- Pathway specific (typically involving in vitro experiments)
- In vitro response should accurately reflect in vivo response
- Is reproducible (rank preserving)

Certain principles need to be established for the application of the MR and IV approach to the present study. The first principle is that of the Front – Door Principle with reference to Figure 5

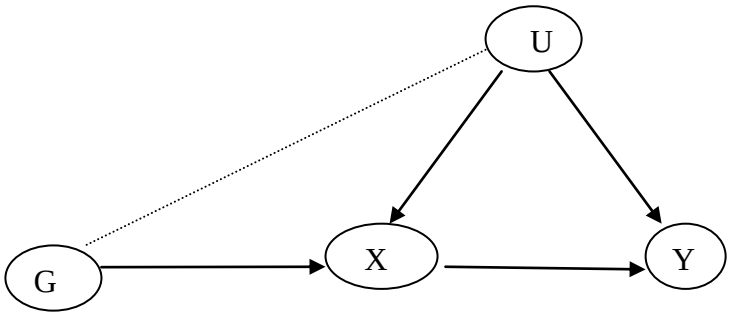

Figure 5: Directed acyclic graph, G, represents gene(s), X represents an intermediate phenotype (e.g., homocysteine) and Y is the phenotype of interest, for example AAA. It must be emphasized that X must be in the causal pathway from the gene (G) to the phenotype (Y) for the MR concept to apply. U is a confounder in the causal relationship X to Y.

There are conditions in which the MR concept can be violated. These are comprehensively discussed in Appendix 1

Confounders originate backdoor paths that need to be blocked by conditioning.[265, 266]

$Y \not\perp G|X$: Y is conditionally independent of G given X

$$P(Y,U,G) = P(Y|X)P(Y|U)P(G) \qquad \text{Equation 3}$$

But,

$$P(Y|U) = \frac{P(Y,U)}{P(U)}$$

If there is a link from U to G then P (Y|G) becomes identifiable because it is now easy to condition through X which is in the causal pathway. This is known as the front-door principle.

$$P(U|G) = P(U|X,G)$$

$$P(Y|X,U) = P(Y|G,X,U)$$

$$\begin{aligned}\sum_U P(Y|X,U)P(U) &= \sum_G \sum_U P(Y|G,X,U)P(U|G)P(G) \qquad \textbf{Equation 4}\\ &= \sum_G \sum_U P(Y|G,X,U)P(U|G,X)P(G)\\ &= \sum_G P(Y|G,X)P(G)\end{aligned}$$

The second principle is the Back-Door Principle which is more relevant to the genetic case-control study.

With further reference to the DAG previously (Figure 5), the following are the core conditions:

$G \perp U$, this means that G must be marginally independent of U, the confounder between X and Y;

$G \not\perp X$, G must not be independent of X

$Y \perp G|(X,U)$, conditional on X and U, the instrument (G) is independent of Y.

With the foregoing the graph can be factorized as:

$$P(Y,X,G,U) = P(Y|U,X)P(X|U,G)P(U)P(G)$$

In the case control study when we condition only on the outcome Y, the properties of the distribution

$$p(X,U,G|Y) = \frac{P(Y|U,X)P(X|U,G)P(U)P(G)}{\sum_u P(Y|U,X)P(X|U)P(U)} \qquad \textbf{Equation 5}$$

This will not factorize because there are no independencies among X, G, U conditional on Y. But if assumed as one would in a prospective case that $Y \perp X|U$ means no "causal effect" then

$$P(X,U,G|Y) = \frac{P(Y|U)P(X|U,G)P(U)P(G)}{\sum_u P(Y|U)P(U)}$$ If there is no causal effect (null hypothesis)

$$P(G|Y) = P(G) \qquad \textbf{Equation 6}$$

Equation 6 implies the null hypothesis of no genotype distribution differences between cases and controls. This is the basis of genetic case-control studies. With this approach, two candidate genes, Angiotensin Converting Enzyme (ACE) Insertion-Deletion (I/D) and Methylene Tetrahydrofolate reductase (MTHFR) C677T polymorphisms will be analyzed as possible causal genetic factors in AAA pathogenesis.

2.2 Methylene Tetrahydrofolate Reductase (MTHFR) C677T Single Nucleotide Polymorphism

MTHFR gene is located on the short arm of chromosome1 (1p36.3).[267] It carries a polymorphism at position 677 of the gene in which thymidine is substituted for cytosine (C677T) with consequent substitution of alanine for valine in the protein. The common (major) 'C' allele is protective whilst the risk allele 'T' is associated with two copies of both protective and risk alleles corresponding to the homozygous "CC" and "TT" genotypes and the presence of both (individual copies) corresponding to the heterozygote (intermediate) "CT" genotype. The presence of the T allele is associated with thermolability (instable at 37^{o}C) which means a reduction in enzyme activity: the CT and TT genotypes exhibit 25-30% and 54-65% of CC enzyme activity, respectively.[268, 269] The biological significance of this polymorphism derives from the fact that it plays a central role in the metabolism of homocysteine (Hcy). Hcy is a sulphydryl containing intermediate metabolite in the metabolism of methionine an essential amino acid. It is predominantly intracellular but also present in the circulation either as free Hcy or conjugated with plasma protein carriers. However, under conditions of impaired metabolic enzyme activity, raised plasma levels referred to as hyperhomocysteinaemia (HHcy) occurs. The normal plasma levels of homocysteine is less than 14μmol/L and pathological concentrations (HHcy) can be sub-classified into mild (15-30 μmol/L), moderate (30-100 μmol/L) and severe (> 100 μmol/L) HHcy.[270] These levels of HHcy are correlated with cardiovascular risk profile and adverse events. For example, HHcy >14.5 μmol/L increases the risk of myocardial infarction by a factor of 3.4 and 5 μmol/L increase in plasma homocysteine levels is associated with risk of 1.6-1.8 fold of cardiovascular disease.[271] The current evidence suggests that HHcy causes cardiovascular disease by causing endothelial injury from endothelial cell (EC) oxidant stress and inflammation, EC apoptosis from endoplasmic reticulum-dependent stress, EC senescence,

VSMC proliferation, oxidative modification of LDL and platelet activation causing atherothrombosis.[272] In addition, HHcy induces collagen synthesis by VSMC.[273] It does this by affecting several genes and several important pathways. Recent evidence from literature mining suggests that Homocysteine may affect the expression of 112 genes which participate in lipid metabolism, oxidative stress and / or endoplasmic reticulum stress and the bioavailability of NO.[274]

It was established in one study of 1006 twin pairs that the heritability (genetic variance explained) of plasma Hcy levels was 57% (95%CI, 51-63%) with the MTHFR C677T polymorphism contributing <1% to this plasma variability.[275] In another study of 603 adult twin pairs, the heritability was found to vary with age being 63% (95%CI, 53%-71%) in 18-39 age group and 27% (95%CI, 10-41%) at age 40-65years.[276] In this latter study, the MTHFR locus was found to account for 53% (95%CI, 7-67%) in the younger age group as compared to 24% (95%CI, 0-39%) in the older age group using an additive genetic model. However, in a multiplicative multivariate- adjusted model, the CC, CT and TT genotypes accounted for 15.4%, 28.4% and 76.1% of the model variance for plasma Hcy levels whereas the model without the MTHFR C677T polymorphism could only account for 28.8% of plasma Hcy variance.[277]

It is noteworthy that MTHFR C677T alleles ("C" and "T") and corresponding genotypes (CC, CT and TT) are uniquely distributed in the population being different for European, Asian and African populations.[278] The distribution of the TT genotypes in Africans, Caucasians, South Americans and Asians were 6.6%, 18.6%, 32.2% and 20.8%, respectively. The allele distributions also differ according to the different methods of curation and archiving. For example according to the CEPH database, the allele distributions amongst white European

populations is ~50% for C and 50% for the T alleles, respectively with a distribution of genotypes given in the following proportions CC (16%), CT(67%) and TT(16%)

Many studies have implicated this polymorphism in the pathogenesis of AAA. In one such study,[279] AAA patients (n= 428) and patients with peripheral vascular disease (PVD; n=226) and CAD patients (n=271) were compared with healthy controls (n= 282).In this study, C677T genotyping was done by PCR with primers designed to hybridize exon 4 and partial sequences of introns 3 and 4 to generate amplicons of 151 bp in size with *HinfI* restriction fragment endonuclease activity generating 51 bp and 100bp for the GATTC restriction site but not for the GATCC sequence. The C677 and 677T allele frequencies in the controls and cases were 69.2% vs. 68.8% and 30.8% vs. 31.2% respectively and not statistically different between groups. Similarly, the CC, CT and TT genotype frequencies in controls and cases were 47.5 % vs. 49.1%, 43.3% vs. 39.5%, and 9.2% vs. 11.4%, respectively with no statistically different distributions between groups. Interestingly, in this study, the 677T allele was most likely to be associated with a larger baseline AAA diameter relative to C677 allele, 6.6 ± 1.9 cm vs. 6.0 ± 1.6 cm, p=0.037.

In another study[280] comparing patients with AAA (n=63) to controls (n=75) the C677 and 677T alleles frequency distributions in controls and cases were 118 (79%) versus 80 (63%) and 32 (21%) versus 46 (37%) respectively with a statistically significant difference between the 677T allele in cases and controls ($p<0.007$). Similarly, the frequencies for the CC, CT and TT genotypes in controls and cases were 49 (65%), 20 (27%), 6 (8%) and 21(33%), 38 (60%), 4 (6%), respectively with a significant difference in the CT genotype between cases and controls ($p<0.001$) as reflected in an odds ratio (OR) of 4.43 (95%CI, 2.11- 9.34). Similarly a dominant inheritance model (CT +TT vs. CC) was significantly different between cases and controls [42

(67%) vs. 26 (35%), p<0. 0003] with an associated OR of 3.77 (95%CI, 1.86-7.65). The same trend in genotype frequencies and inheritance model was observed in males in the study.

In the study by Brunelli et al [281] they compared 58 male patients (age range, 49-78 years, mean 69.5 ± 6.6) with AAA to 60 age and sex-matched healthy controls (age range mean age 67.6 ± 7.0). The distributions of the CC, CT and TT genotypes in cases and controls were 14 (24%), 32 (55%), 12 (21%) and 19 (32%), 35 (58%), 6 (10%), respectively with no statistical differences in genotype frequencies between groups. However, in patients there was a significantly higher plasma level of homocysteine in the TT genotype (22.0 ± 8.6 μmol/L) compared to the CT genotype (14.8 ± 4.8 μmol/L, p<0.05) and the CC genotype (12.2 ± 4.0 μmol/L), respectively. The same trend was also observed in controls but this did not reach statistical significance: 9.3 ± 2.9 μmol/L, 9.4 ± 4.4 μmol/L and 11.6 ± 2.6 μmol/L respectively for CC, CT and TT genotype in controls. Interestingly, a higher mean AAA diameter was found in patients with plasma Hcy > 95th of controls (n=26) versus < 95th percentile of controls (n=32), 5.79 ± 1.5 versus 5.09 ± 0.84, respectively (p<0.05).

Interestingly, another study[282] compared the MTHFR C677T genotype distribution in those <60 years (n=46; age, 49-59 years; mean (±SD), 54.13 ± 3.93 years) and mean AAA diameter of 5.9 ± 1.5cm with those > 60 years (n=42; age, 65-75 years; mean 70.61 ± 2.69) with mean AAA diameter of 6.0 ± 1.6cm with a healthy control population (n=44; 49-75 years, mean 62.95 ± 6.61) using a combined polymerase chain reaction and nuclear laser (there is no mention of a restriction enzyme step). The C677 and 677T allele frequencies for controls, >60 years and <60 years were 68 (77%), 36 (43%), 20 (22%) and 20 (23%), 48 (57%), 72 (78%) respectively showing a decreased C677 allele distribution in the < 60 years cohort relative to controls (p=0.000003) and >60years group (p=0.0135) whilst the distribution of the same allele is

significantly different in the > 60 years relative to controls (p=0.000107). Similarly, the corresponding CC, CT and TT genotypes in controls, > 60 years and < 60 years were 34 (75%), 18 (43%), 10 (22%); 10 (23%), 23 (55%), 33(72%) and 1 (2%), 1 (2%), 3 (6%), respectively indicating a reduced frequency of the CC genotype in > 60 years relative to controls (p=0.0147) and in patients <60 years relative to controls (p=0.000039) but not statistically from CC genotype frequency in patients > 60 years (p=0.174). However, in a dominant genetic model (TT+CT vs. CC), the frequency of the TT +CT combined genotype was 11 (24%), 24 (57%) and 36 (78%) in controls, > 60 years and <60 years, respectively, but not statistically different (p=0.174). The authors concluded that the presence of the C677T substitution was associated with a higher risk of AAA in the < 60 years group relative to those > 60 years (OR, 4.803; 95%CI, 1.895-12.172).

In another study from Netherlands,[283] consisting of 85 cases and 86 controls, there was no difference between the former and the latter in terms of MTHFR C677T genotype distributions [CC (40), CT (30) and TT(15) vs. CC (43), CT (36) and TT (7); OR, 2.1; 95%CI, 0.9-5.3].Intriguingly, low plasma vitamin B6 but not an adjusted plasma homocysteine concentration was associated with AAA (OR, 6.92; 95%CI, 1.63- 29.28).

Like the Brunelli study, the study by Warsi[284] also showed an association between AAA and HHcy. In a case control study comparing AAA patients (n=38; age 53-79 years, mean 70 years) to healthy controls (n=36; age 48-79 years, mean 66 years), it was observed that 26 (68%) of cases and 2(6%) of controls had HHcy resulting in a mean Hcy level of 19.4 μmol/L (95%CI, 17.17 – 21.65) in cases compared to a mean Hcy level of 10.9 μmol/L (95%CI, 9.95-11.88) in controls which was a statistically significant difference (p<0.001). However, this study did not show any correlation between HHcy and AAA diameter.

In a more recent study[285] consisting of 423 cases and 423 controls matched for age, the distribution of the MTHFR C677T genotypes in the former were not significantly differently from the latter [CC (32.8%), CT (48.7%) and TT (18.5%) vs. CC (25.7%), CT (50.6%) and TT (23.7%); P=0.061 for dominant model (CT+TT vs. CC)]. Interestingly, the T allele was associated with significantly higher Hcy in AAA compared to controls in a recessive model, TT vs. CT +CC [(17.3(8.3-144.3) μmol/L) vs. (14.5(6.7-93.6) μmol/L); P<0.0001].

Interestingly, Sofi et al,[286] in their case control study observed that the T allele proportion in AAA patients (n=438; age 40-94 years , median 73.4 years) was 0.43 as compared to 0.38 (p=0.03) in age-matched healthy controls (n=438) corresponding to a TT odds ratio of 1.7 (95%CI, 1.2 -2.6; p=0.006). However, the following an electronic microchip PCR, the C677T MTHFR CC, CT and TT genotypes in cases and controls were 141(32.2%), 217 (49.5%), 80 (18.3%) and 166 (37.9%), 211 (48.2%), 61 (13.9%) respectively with no statistically different genotype frequencies between groups. It is noteworthy that with the given values, an odds ratio of 1.28 (95%CI, 0.97-1.69; P=0.07) will be obtained for a dominant (CT+TT vs. CC) in cases relative to control. Another interesting feature of this study was the positive correlation between HHcy, PAI-1 and AAA. HHcy defined as > 95th percentile in controls (> 19μM in males and >15 μM in females) was observed in 148 (33.8%) of cases compared to 24 (5.5%) of controls and was statistically significant (adjusted (adj.) OR, 8.2; 95%CI, 4.7 -14.3; p<0.0001). Similarly elevated PAI-1 (> 42.8mg/dL) was observed in 90 (20.5%) cases compared to 22 (5.0%) of controls corresponding to an adjusted OR of 3.05 (95%CI, 1.6 – 5.91; p=0.001). A similar trend was observed for lipoprotein a (Lp(a)). Like the Brunelli study there was a mild but significant correlation between the AAA diameter and Hcy levels (r=0.13; p=0.005).

The MTHFR C677T-AAA studies combined in Meta-Analysis (M-A) using a Random Effects

(RE) approach (cases=1583 and controls=1408) did not confirm any association (OR, 1.32; 95%CI, 0.998 – 1.761; P=0.051) (Table 1; Figure 6) and additional Cumulative RE M-A Table 2; Figure 7) also showed that the evidence for an association between this polymorphism and AAA was at best equivocal. Furthermore, RE M-A Sensitivity Analysis showed that the contributions to the pooled effects by the large studies were negative unlike the small studies which may suggest the presence of type 1 error or spurious associations (Table 6; Figure 8)

Furthermore, a RE M-A of homocysteine from the foregoing studies suggested that homocysteine is associated with AAA (Figure 10). One standard deviation (SD) increase in plasma Homocysteine levels (μM/L) is associated with AAA [Relative Risk (RR), 1.94; 95%CI, and 1.04 – 2.83].

Table 1: Random Effects (RE) Meta-analysis of MTHFR C677T-AAA

Study	Fixed-effects ORs	95% Conf. Intervals		Study weights
Brunelli, 2000	1.4496	0.8646	2.4303	14.3887
Strauss, 2003	2.1203	1.2442	3.6132	13.5201
Sofi, 2005	1.2319	1.0177	1.4913	105.2504
Jones, 2005	1.0161	0.8073	1.2786	72.7043
Peeters, 2007	1.3309	0.8441	2.0982	18.5342
Ferrara, 2007	3.5238	1.7801	6.9757	8.2372
Giusti, 2008	0.7804	0.6443	0.9452	104.6151

Fixed effects OR=1.0877 (95%CI, 0.9776 -1.2102; P=0.1225)
Q statistics (Chi-sq)=32.8452; P=1.00E-05

Random effects OR=1.3255 (95%CI,0.9979 -1.7607; P=0.0517)

[The upper part of table 1 shows the various studies that were pooled in the meta-analysis (M-A) of the association of MTHFR C677T SNP and AAA. Note worthily are the studies contributing the highest weights to the overall effect. These are the studies by Sofi and Giusti by far the largest studies of over 800 study participants. The overall effect shows lack of association. Interestingly, the Fixed effects (FE) M-A implies that all studies are drawn from a common population whilst the Random Effects (RE) is consistent with different populations and methodologies. Another difference between FE and RE M-A is the smaller point estimate in the former compared to the latter but longer 95%CI in the latter compared to the former. This is because in RE M-A there is an implied heterogeneity and this is adjusted for in the analysis. Consequently, all further meta-analyses in this thesis are conducted as RE M-A]

Table 2: Cumulative RE M-A of MTHFR C677T-AAA

Study Added	Random Effects	95% Conf. Intervals		RE Chi-square	P value
Brunelli, 2000	1.4496	0.8646	2.4303	1.9838	0.1589
Strauss, 2003	1.7429	1.2009	2.5295	8.5472	0.0034
Sofi, 2005	1.4443	1.0634	1.9617	5.5402	0.0185
Jones, 2005	1.2802	1.0046	1.6316	3.9881	0.0458
Peeters, 2007	1.2712	1.0408	1.5526	5.5321	0.0186
Ferrara, 2007	1.4582	1.1108	1.9142	7.3826	0.0065
Giusti, 2008	1.3255	0.9979	1.7607	3.7848	0.0517

[With a Cumulative Meta-Analysis, the first study is usually not taken into consideration. Subsequent studies according to year of study are sequentially added and a pooled estimate conducted. The interest is usually at the point where the association of interest occurs and the evidence is said to have changed at that stage. As a rule of thumb a minimum of three studies are needed for a M-A. As seen from the table above, addition of the second study shows a positive association with a p=0.0034.This association was maintained until 2008 when it became at best equivocal (p=0.0517). The explanation could be that there is publication bias or the associations are spurious since some of these studies (Strauss et al, 2000 and Ferrara et al, 2007) were not in Hardy-Weinberg Equilibrium (HWE)]

Table 3: Sensitivity analysis of MTHFR –AAA meta analysis

Study removed	Random Effects	95% Conf. Intervals		RE Chi-square	P value
Brunelli, 2000	1.3149	0.9643	1.7929	2.9941	0.0835
Strauss, 2003	1.2348	0.9287	1.6418	2.1067	0.1466
Sofi, 2005	1.3846	0.9586	1.9998	3.0103	0.0827
Jones, 2005	1.4349	1.0006	2.0577	3.8555	0.0495
Peeters, 2007	1.3338	0.9717	1.8307	3.1781	0.0746
Ferrara, 2007	1.1789	0.9185	1.5133	1.6709	0.1961
Giusti, 2008	1.4582	1.1108	1.9142	7.3826	0.0065

[Within a meta-analytic framework, a sensitivity analysis is usually conducted to check the individual study contribution to the overall effect size. This is likened to a backward elimination in a variable selection in regression analysis. Starting with all the studies in the pool, a study is removed one at a time and the RE M-A conducted to see the impact of that removal. If a previously positive association becomes negative, it implies a strong contribution to the overall effect size by that study. Similarly, if the removal of that study is associated with a positive effect from a previously negative effect, it implies a strong contribution of that study in the opposite direction. These contributions are usually picked up from the 95%CI because the point estimates hardly change with RE M-A. From the table, the studies by Jones et al (2005) and Giusti et al (2008) showed strong negative contribution to the overall effect unlike the other studies which all showed positive contributions since their removal push the overall effect size into negative territory.]

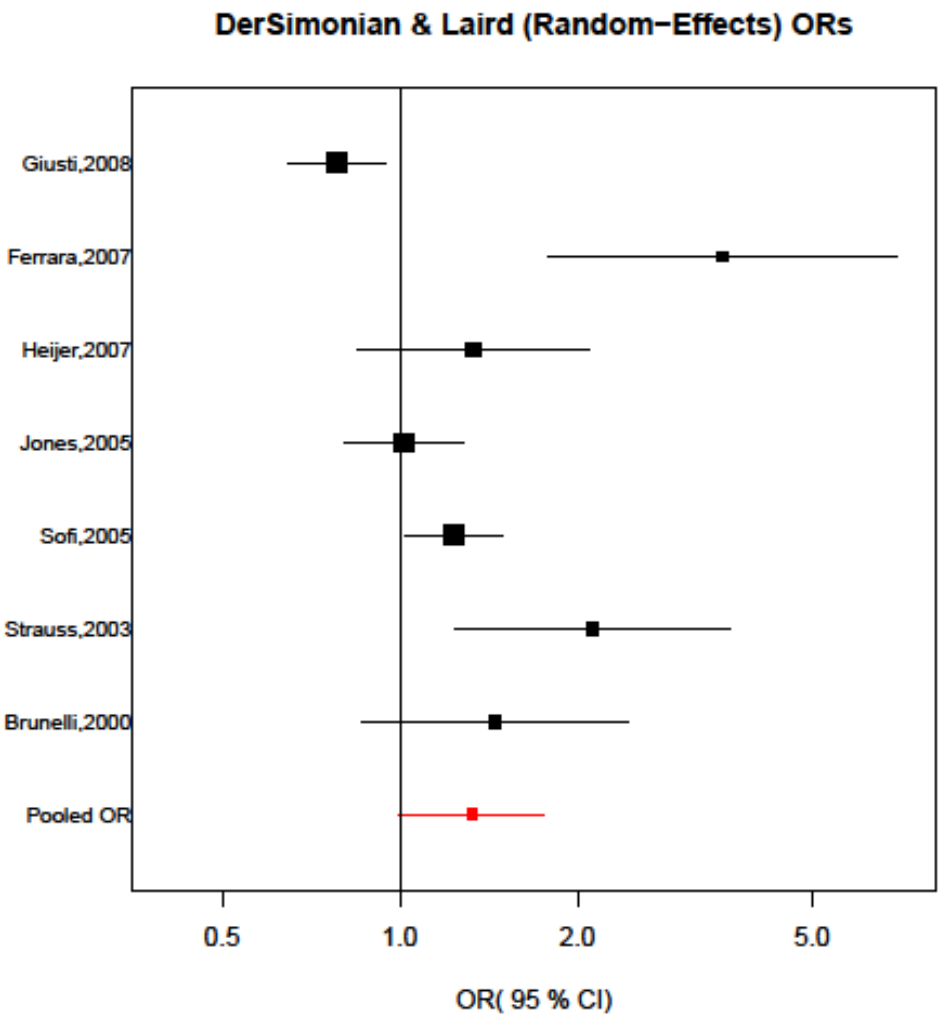

Figure 6: Random effects (RE) meta-analysis (M-A) of MTHFR C677T-AAA association studies. This is a forest plot of allele difference M-A. Each study is represented by a black line (95%CI) and a central square black box (point estimate).The red horizontal line at the bottom is the main effect of the pooled studies. (The square box represents the point effect whilst the long horizontal lines represent 95% CI.) If it touches the central vertical line (of equivalence) then the effect size is equivocal. However, if it crosses to the left side it implies no association. For there to be an association, the red line has to remain exclusively on the right side of the central vertical line marked with "1.0" at the bottom. The RE for this M-A is 1.325 (95%CI, 0.99-1.76) implying a lack of association.

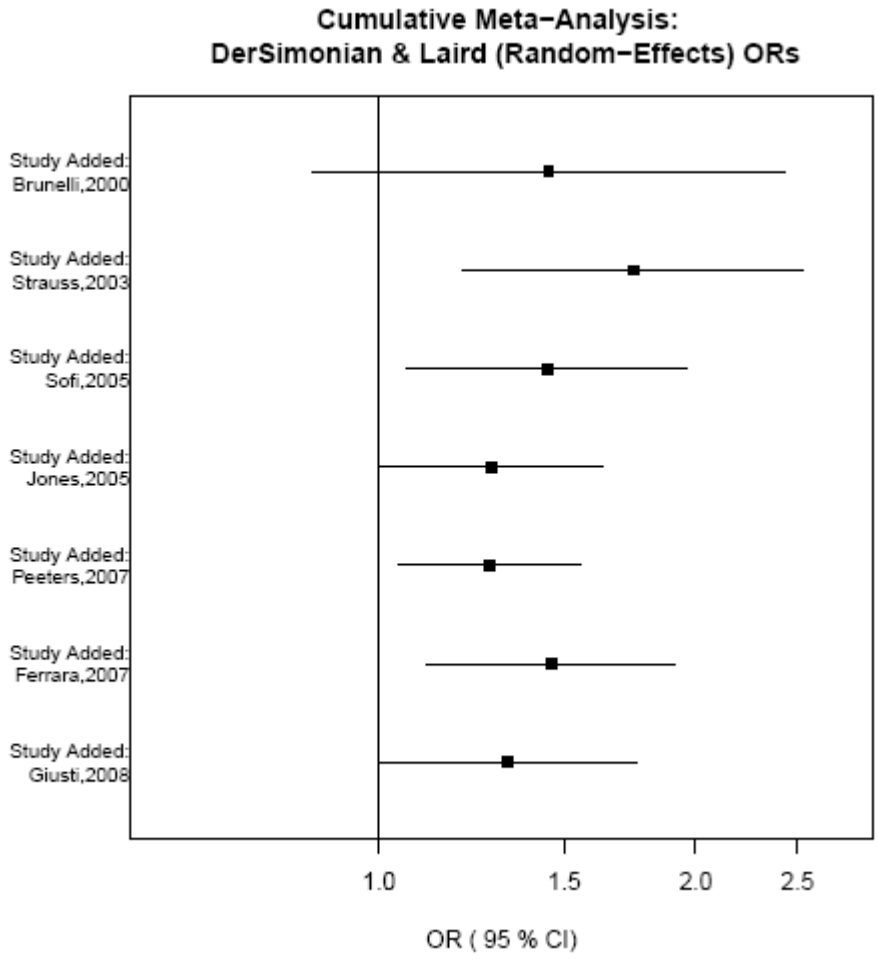

Figure 7: Cumulative RE Meta-analysis for published studies for MTHFR C677T-AAA association. A Cumulative M-A is a visual representation of progression of the evidence for association as the relevant studies are combined in temporal progression starting with earliest to the latest study. The first study does not contribute to total effect size until the subsequent studies are sequentially added. Note that there is no red line of main effect since the main effect is calculated at the point where a current study is added. Please note that at least three (3) studies are needed for M-A as a rule of thumb and from the forest plot the "evidence" would have changed with the study by Sofi et al in 2005 showing a positive association between MTHFR C677T and AAA. However, as further studies are added, an inconsistent pattern emerges until the final study by Giusti in 2008 in which the main effect line 95%CI abuts on the vertical line of equivalence showing that evidence for association is borderline at best.

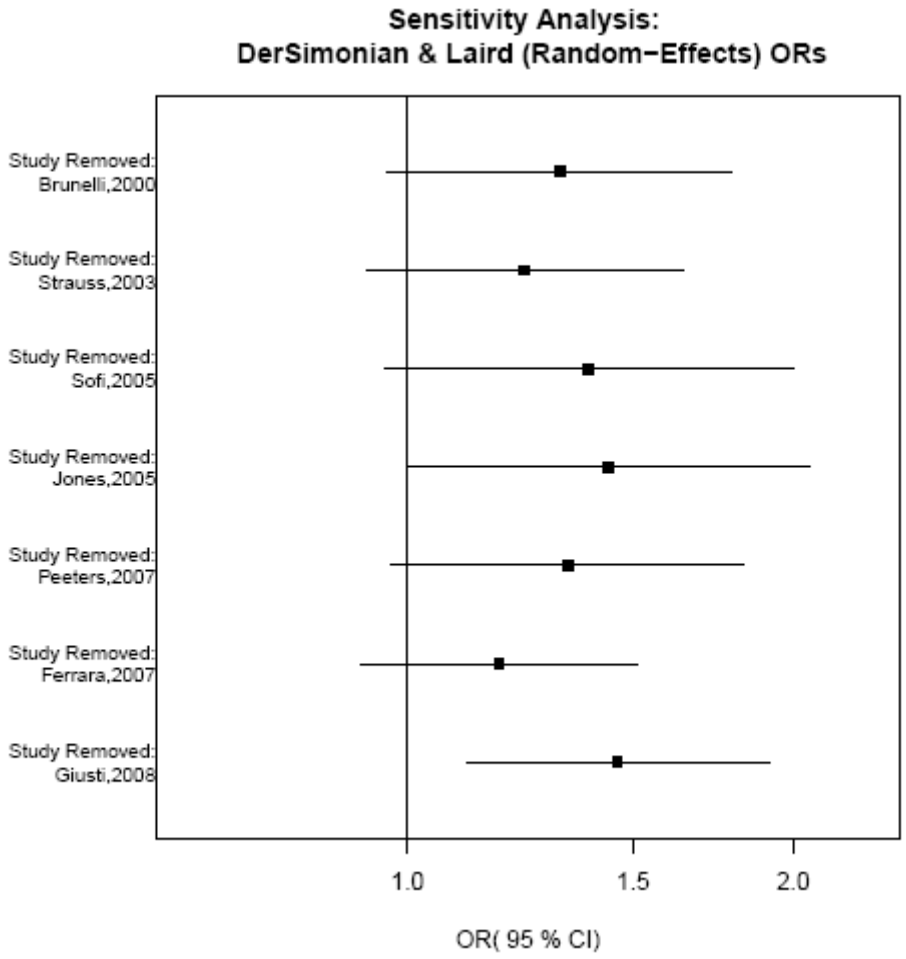

Figure 8: Sensitivity analysis of MTHFR C677T-AAA meta-analysis.With a sensitivity analysis, all the studies are pooled together and individual studies are thereafter sequentially removed starting with the earliest. If the 95%CI line crosses to the left when a study is removed this implies a strong positive contribution to the overall effect size by that study. For example, all the studies contributed strongly to overall effect size except the studies by Jones (2005) and Giusti (2008). The removal of the latter two studies showed an equivocal and positive (exclusively right-sided) main effect, respectively suggesting a strong negative contribution to the overall effect size.

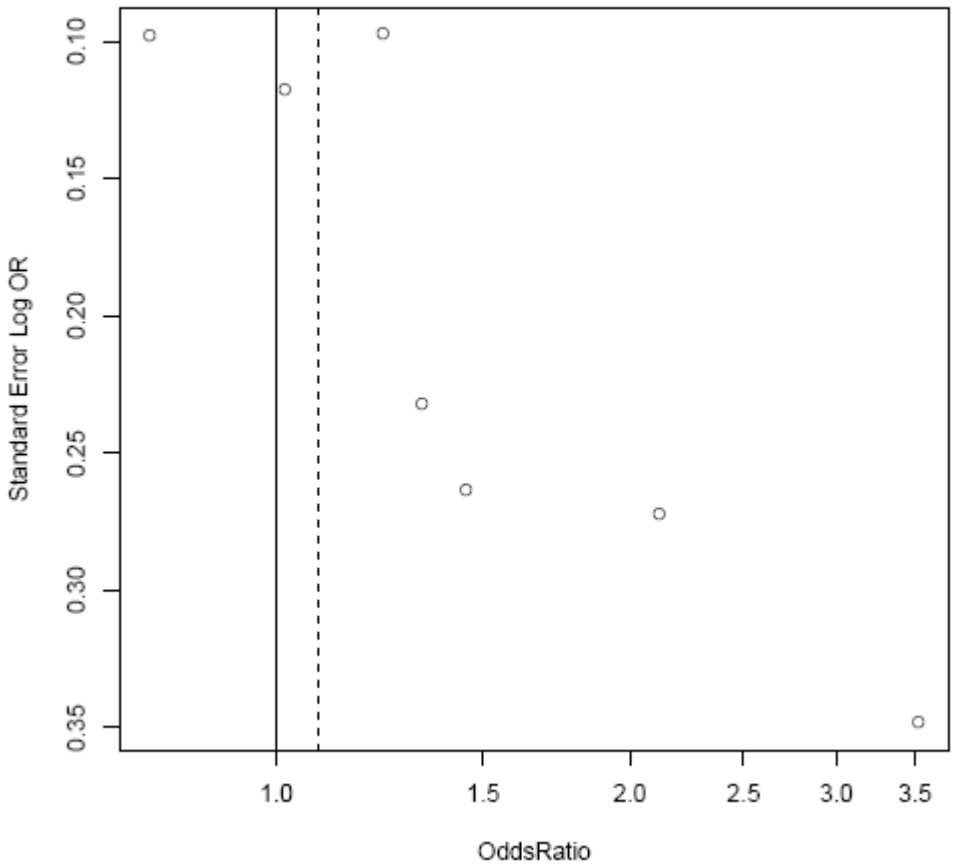

Figure 9: Funnel plot showing publication bias of MTHFR C677T-AAA association studies

[This is a funnel plot to show evidence of publication bias. The individual studies are represented by small circles on this graph. This is a standardized plot showing effect sizes of the studies are plotted on the x-axis at the bottom and standard errors of the log of their ORs are on the y-axis on the left. Mainly positive studies will cluster on the right side of the vertical line marked with "1.0" at the bottom of the graph. For publication bias to be excluded, the small circle representing the studies should be evenly spread about the vertical line marked "1.0" on either side. The dashed vertical line represent a fixed –effect point estimate (1.08) of the pooled studies. (The software used for this study does not plot RE funnel plots.)]

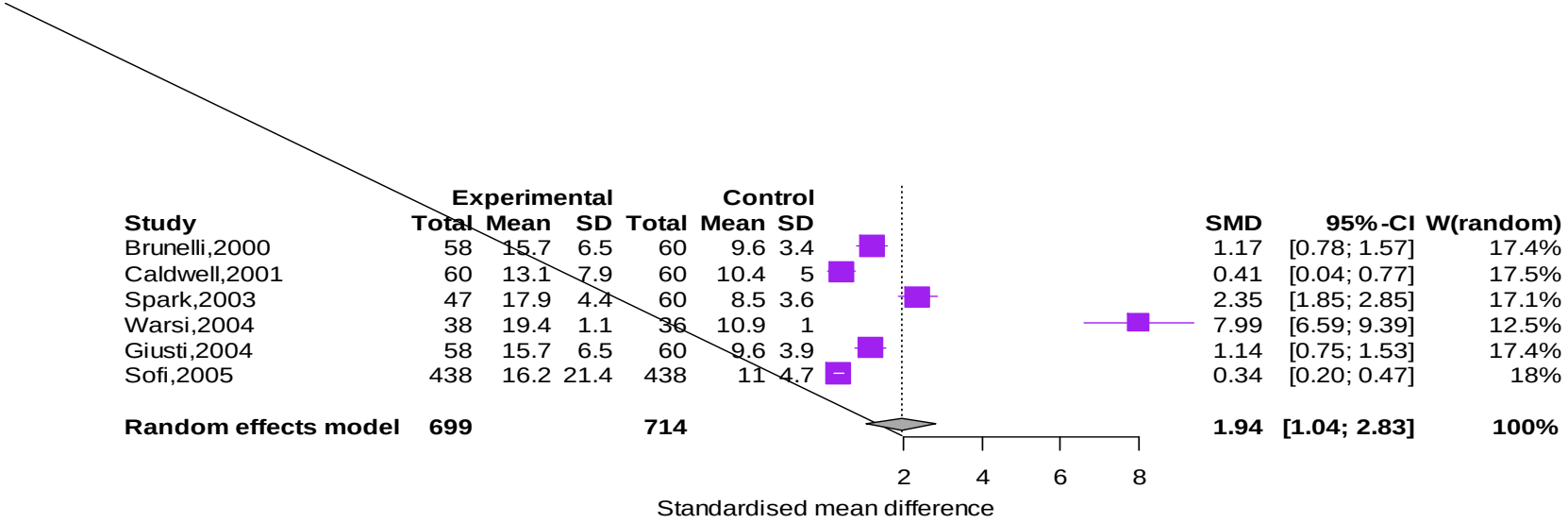

Figure 10: Random Effects Meta-analysis of Homocysteine and AAA. The combined studies are based on standardized plasma Hcy levels of the individual studies. The reported means are divided with their standard deviations (SD). Where no SD is reported, range or standard errors (SE) can be converted to SD according to standard guidelines. The figure is a forest plot which is a graphical representation of the combined studies (individual purple squares) and their main effect (grey diamond at the bottom). The horizontal breadth of the diamond reflects the 95%CI and this is shown on the right side of the figure. The point-estimate is 1.94 showing an ~ 2.0 fold risk or 94% risk of AAA with 1 standard deviation increase in plasma Hcy.

2.2.1 MTHFR and Homocysteine Metabolism

In the metabolic pathway of Hcy (**Figure 11**), methionine is converted to S-Adenosyl Methionine (SAM) which is then converted to S-Adenosyl Homocysteine (SAH).[287] Note worthily, SAM is an allosteric inhibitor of MTHFR and SAH dis-inhibits this inhibition (activates the MTHFR reaction). In contrast, both SAM and SAH inhibit the MFMT (methyltetrahydrofolate homocysteine methyltransferase) and BHMT (Betaine homocysteine methyltransferase) reactions but activate the transulfuration reactions. This latter event should theoretically reduce Hcy concentrations but SAH inhibits all SAM-dependent methyltransferases (including DNA methyltransferases, DNMT1 and DNMT3) and increase the hydrolysis reaction of SAHH to generate Hcy in conditions of high methionine. However, because SAHH catalyzes a reversible reaction, elevated Hcy will inhibit this enzyme and lead to increased SAH with the aforementioned sequelae. Current evidence suggests[288] that HHcy could also stimulate the transulfuration pathway leading to the production of Cystathionine which is further converted to hydrogen sulphide (H_2S) a recently suggested protective gaso-mediator in the cardiovascular system. However, HHcy is further implicated in the suppression of H_2S through the inhibition of Cystationine-γ-lyase (CSE) a H_2S generating enzyme.[289]

2.2.1.1 MTHFR C677T, Homocysteine and Global DNA Hypomethylation

There is evidence to show that total Hcy is higher in patients with vascular disease (atherosclerosis) compared to controls [10.4 μmol/L (95%CI, 8.8-12.7) vs. 7.5 μmol/L (95%CI, 6.5-8.9); $p<0.01$] in a study which compared 17 atherosclerotic patients to 15 controls matched for age and sex.[290] Further, total Hcy positively correlated with Adenosyl Hcy levels ($r = 0.81$;

p<0.0001) and global DNA hypomethylation (r=0.54; p<0.01) but inversely with Adenosyl Methionine/ Adenosyl Hcy ratio (r=-0.68; p<0.0001). It has been suggested that a raised SAH or decreased SAM/SAH ratio is associated with an aberrant genomic methylation characterized by global DNA hypomethylation and paradoxical CpG methylation in promoter regions[291, 292]
The CpG "islands" are not normally methylated as methylation is generally associated with promoter silencing. Interestingly, it is also suggested that this pattern of global hypomethylation also affects repetitive elements and imprinted loci resulting in their transcription. This pattern of altered genetic expression is referred to as epigenetics or alterations in genetic expression not dependent on genomic sequences (inheritable characteristic independent of genetic sequence). It is noteworthy that Hcy-dependent hypomethylation- induced VSMC proliferation applies mainly to neural crest-derived VSMC[293] and not to mesoderm –derived VSMC present in the infrarenal aorta. Furthermore, Hcy in clinically relevant doses induces oxidative stress in VSMC and apoptosis in a pathway dependent on endoplasmic reticulum stress. In a study in which high methionine was fed to Male Sprague-Dawley rats,[294] HHcy was associated with reduced SAM but elevated SAH in aortic tissue after 4 weeks compared to controls fed a normal diet. There was also an associated 3-to-4-fold reduction of the SAM/SAH ratio, increased expression of DNA methyl transferases (DNMT3a and DNMT3b) and reduced methyl-CpG-binding domain 2 (MBD2) consistent with foregoing observations compared to the control group.

2.2.1.1.1 Epigenetic dysregulation

Epigenetics refers to the non-genomic contribution to the gene expression. The formal definition means the "heritable changes in gene expression that occur without a change in DNA sequence".[295] These epigenetic changes are transmitted through each cycle of cell division and include changes like chromatin modification and remodelling, histone protein covalent modifications, chromatin arrangements and DNA methylation.[296]

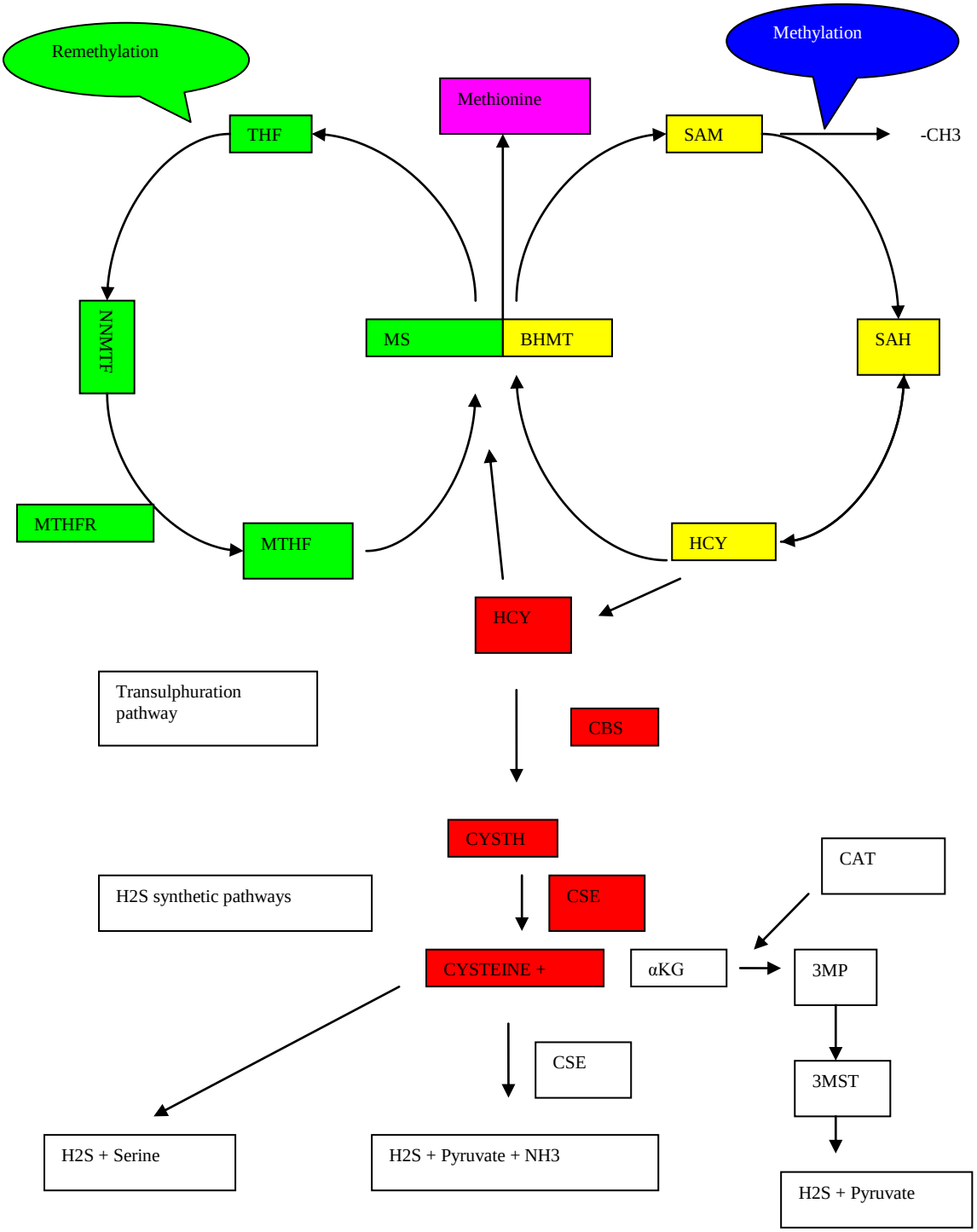

Figure 11: The homocysteine-methionine pathway showing the site of action of MTHFR. The pathway consists of three interlocking loops of remethylation, methylation and transulphuration mechanisms. The methylation pathway is strict a branch point from the other

two loops. Homocysteine is methylated to form methionine by the addition of a methyl group from methyl tetrahydrofolate which is generated from the action of MTHFR. The C677T polymorphism of the latter is associated with reduced enzyme activity at body temperature (37° C) resulting in the remethylation reaction and consequent build up of homocysteine with reduced methionine. Some of that homocysteine is diverted into the transulphuration pathway where it is converted to cysteine. Interestingly Cysteine is further converted in series of reactions to form hydrogen sulphide which is a gaso-mediator that acts as an antioxidant and antihypertensive in the cardiovascular system. Cysteine can also combine with αKG to form H_2S through the effect of CAT and MST.H_2S is further suspected to antagonize the effects of homocysteine. Conversely, elevated homocysteine suppresses the effects of the enzymes CBS and CSE thus reducing the production of this gas. See text for further discussion.

Abbreviations:3MP(3-Mercaptopyruvate); αKG(alpha Keto-glutarate); BHMT(Betaine: Homocysteine methyl transferase);CAT (Cysteine Aminotransferase); CYSTH(Cystathionine); Hcy (Homocysteine); CBS(Cystathionine β Synthase); γ-cyst(gamma-cystathioninase); HCY(Homocysteine);MTHFR (Methylene tetrahydrofolate reductase);NNMTHF(N5,N10-Methylene tetrahydrofolate) ; MTHF(N5-Methyl tetrahydrofolate); MS(Methionine Synthase);SAH(S-adenosyl homocysteine); SAM (S-adenosyl methionine); MST (3-Mercaptopyruvate sulfurtransferase);THF (Tetrahydrofolate);

Epigenetic marks in the genome are based on histone modifications and remodelling through acetylation[297], phosphorylation[298], methylation[299] and sumoylation.[300] Acetylation and phosphorylation of histone promote access for transcriptional machinery to activate gene expression whilst histone and DNA methylation has been associated with transcriptional silencing. Methylated histone (H3K9me) is tightly wound in nucleosomes as heterochromatin whilst acetylated chromatin (H3K9ac) is looser and lighter thus facilitating the transcription process. There is evidence of cross-talk between these epigenetic mechanisms.[301] For example Methylation of lys9 of H3 inhibits the acetylation of the same residue. Histone acetylating opens up chromatin from its tightly condensed formation (heterochromatin) to enable access for the transcription machinery. Within cancer cells, hypermethylation occurs in tumour suppressor genes whilst oncogenes and other genes are hypomethylated thus increasing their rates of transcription. Another layer of epigenetic control is via DNA hypermethylation.[302] Methylated DNA is transcriptionally silent. There is also evidence that there is a bridge between DNA methylation and lysine methylation in histones. This is because Methylated DNA recruits Methyl DNA binding proteins (MBD) like methyl CpG binding protein, MeCP2 which has also been implicated in the methylation of H3K9[303] perhaps using an adaptor protein, HP-1(Heterochromatin Protein -1) which binds to both DNA and methylated Histone H3K9. Methylated histone (H3K9me) is tightly wound in nucleosomes as heterochromatin whilst acetylated chromatin (H3K9ac) is looser and lighter thus facilitating the transcription process. There is evidence of cross-talk between these epigenetic mechanisms[304]. For example Methylation of lys9 of H3 inhibits the acetylation of the same residue. Histone acetylating opens up chromatin from its tightly condensed formation (heterochromatin) to enable access for the transcription machinery. There is cross regulation of DNA and histone modification. Methylated DNA are found in nucleosome complexes with methylated histones proteins (H2A, H2B, H3

and H4) in tightly wound complexes called nucleosomes which are densely packed in regions called heterochromatin

It has been reported that HHcy results in increased methylation of histone and non-histone proteins.[305] For example high concentrations of SAM a metabolite in the homocysteine metabolic cycle activates the enzyme protein arginine N-methyl transferase 1(PRNMT1) which methylates histone proteins at lysine and arginine residues. The methylation of arginine residues by this enzyme in histones and non-histone proteins can be either of two forms : symmetric dimethyl arginine or asymmetric dimethyl arginine (ADMA). Protein can also be methylated on either their lysine (K) or arginine residues (R). Methylation of Histones alters gene transcription depending on the histone subunit methylated and configuration adopted by the DNA-chromatin structure.[306] Furthermore, methylation of the "R" residues in proteins can affect protein function and this is catalyzed by a class of proteins known as Co-activator arginine methyltransferase-1 (CARM-1) and Protein arginine methyl transferrase-1 (PMRT-1).[307] It is suggested that histone subunit 3 (H3) K methylation at position 17 (H3K17me) is done by CARM1 as is the methylation of co-activator acetyl transferase, p300 and transcription factor Stat1a. Further, H4K3me is catalyzed by PRMT1 and these patterns of methylation are associated with activation of gene expression. On the other hand asymmetric dimethylarginine (ADMA) is a functionally active enzyme in the inhibition of eNOS in vascular endothelial cells.

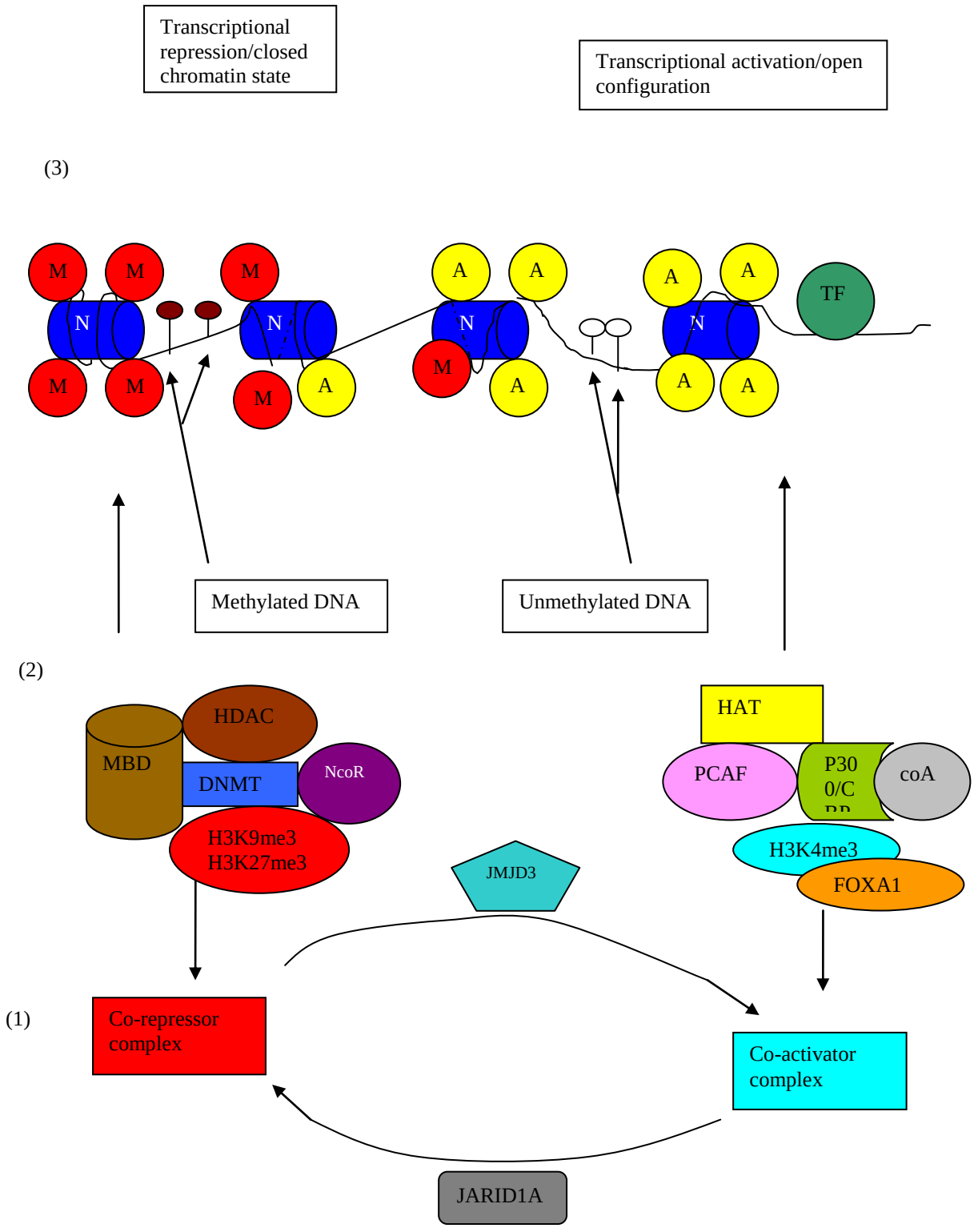

Figure 12: DNA methylation and co-regulator cycles. The red circles are methyl molecules with which DNMT3A silences DNA whilst the yellow circles are acetyl groups on histones

creating a transcriptionally permissive state. Histone 3 methylation at lysine 9 (H3K9me) and 27 (H3K27me) is associated with gene promoter silencing whilst the converse occurs with H3 lyisne 4 methylation (H3K4me). At the bottom on the right is the association of co-activator complexes with a permissive state whilst at the bottom left is the repressor complex associated with a silenced DNA. N=nucleosomes, MBD=methyl DNA binding protein, NcoR=nuclear co-repressor, HDAC=histone deacetylase, HAT=histone acetyl transferase, FOXA1=forkhead box A1, coA=Coactivator, JMJD3, JARID1A=Jumomji C family demethylases. The histone demethylases are the subject of current interest because they potentially link chronic inflammatory states like ageing and epigentics. For example, signalling via p38 MAPK or p53 pathway activates JMJD3 which demethylates H3K27me3 at the 9p21.3 leading to increased expression of p16 to induce senescence by G1-S cell cycle arrest.

Significantly, YY1 a transcription factor important in MMP-2 gene expression has been reported to target PRMT1 to chromatin.[308] Asymmetric dimethylation of STAT1 transcription factor by HHcy also modulates cytokine induced gene expression.[309] It should be noted that MMP-2 and MMP-9 gene promoters have STAT1 response elements in their promoter regions at approximately -900bp of transcription start site.

Methylation of H4 at the third arginine residue (R3) has been associated with increased transcriptional activity and cross activation of other transcription machinery.[310] For example, the H4R3me cross-activates the acetylation of H4K8 (8^{th} lysine residue) which has been reported to stimulate the activity of a transcription factor, TFIID a major component of the transcription machinery.[311, 312] However, H3K9 methylation also stimulates HDAC recruitment which results in deacetylation of histone with subsequent heterochromatin formation (via the recruitment of HP1 a protein that aids in heterochromatin formation) and vice versa. There is also evidence implicating widespread histone modification as a consequence of H4R3 methylation.

In a study involving chromatin immunoprecipitation (ChIP) experiments of the chick β-globin gene locus in 6C2 cell lines,[313] it was shown that deletion of the PRMT1 gene is associated with decreased global acetylation of H3K9, H3K14 and , particularly in insulator regions, increased methylation of H3K9 and H3K27. These features were reversed with the restoration of H4R3 methylation in vitro. Interestingly, methylation of another lysine residue at position 27 of H3 histone protein (H3K27) is also associated with transcriptional silencing. This histone methylation is effected by a group of evolutionarily conserved proteins called polycomb group (PcG) and these can form two multimeric polycomb repressor complexes – PRC1 and PRC2.

2.3 Angiotensin converting enzyme insertion-deletion (ACE I/D) polymorphism

The ACE gene consists of approximately 26 exons spaced over ~ 24kb (~16% coding sequences). It is duplicated with two homologous domains (exons1-12, 14-16). A tissue specific expression for a testicular is found in exon 13 with a promoter in intron 12.[314] The 287 base pairs (bp) Alu segment was inserted ~10^6 years ago resulting in an I/D polymorphism These are the INDEL (I/D) for Insertion ("I") and Deletion ("D") alleles. The "D" allele is the common wild type but risk allele whilst the "I" allele is the minor but protective allele corresponding to homogenous II and DD and heterozygote genotypes.[315]

Accumulating evidence suggests that the ACE locus and I/D polymorphism account for 26% - 64% and 9-28%, respectively of total plasma ACE variance. [316, 317, 318, 319] The Alu insertion is in intron 16 adjacent to, and in strong linkage disequilibrium with a functional locus in exon 17 which accounts for ~20% of total ACE plasma variance.[320]

Differential allele-specific gene expression of mRNA is a recognized phenomenon affecting 20-50% of all genes (3-30% of heterozygotes) resulting in between 1.3 and 1.4-fold difference between alleles.[321] This underscores evidence which suggests that the presence of the "D" allele is associated with increased ACE mRNA expression relative to the "I" allele with an expression ratio (D/I) of 1.79 which, however, does not correlate with plasma ACE activity.[322] Furthermore, the plasma ACE account for ≤ 10% of total ACE with the remaining ~ 90% in virtually all tissues of the body.[323]

One of the earlier studies,[324] conducted on the association between ACE INDEL polymorphism

and AAA, compared normal control (n=153) with cases with confirmed AAA (n=125) drawn from an Asian (Japanese) population matched for age, sex and cardiovascular co-morbid conditions, using PCR methodology. In this study the "I" and "D" allele frequencies for controls and cases were 124 (58%) vs. 99(60%) and 99 (42%) vs. 74 (40%) and were not statistically different between groups, p=0.774. Similarly, the II, ID and DD genotypes frequencies for controls and cases were 54 (35%), 70 (46%), 29 (19%) and 51(41%), 48 (38%), 26 (21%), respectively with no statistical difference in genotype distribution between groups (p=0.418). Notable in this study is the high degree of heterozygosity which might contribute to lack of Hardy-Weinberg equilibrium in the study population.

Interestingly another study with subjects drawn from Southern European (Italian) population compared elderly normotensive (n=56; mean age =73 ±10) and hypertensive (n=68; mean age=72 ± 9 years) AAA patients to elderly controls (n=112; mean age=71 ± 6 years).[325] The II, ID and DD genotypes distributions in controls, normotensive AAA and hypertensive AAA patients were 36 (32%) vs. 3(5%) vs.14 (21%); 48 (43%) vs. 14(25%) vs.32 (47%) and 28 (25%) vs. 39 (70%) vs. 22 (32%), respectively. A 3-way chi-squared analysis showed that there was a difference between normotensive AAA vs. controls (p=0.001) and between normotensive and hypertensive AAA (p=0.001) but not between controls and hypertensive AAA (p=0.22). The authors concluded that ACE DD and ID genotypes were independent risk factors for AAA in normotensive patients.

In another study from the Italian population, the authors compared 250 AAA with mean age of 72 years (range 50-83) with healthy age matched controls (n=250).[326] The frequencies of the "D" allele in controls and cases were 0.49 and 0.63 respectively. However, the II, ID and DD genotype frequencies (numbers) for controls and cases were 28% (70), 45.2% (113), 26.8% (67)

and 15.2 % (38), 43.2% (108), 41.6% (104) respectively. The adjusted OR for a recessive model DD vs. ID +II was 2.4 (95%CI, 1.3-4.2; p=0.003).

A more recent study examined the genotype by risk factor interaction in a case-control study of ACE I/D polymorphism and AAA. In this study there were 112 AAA cases and 50 controls.[327] The latter were all normotensives compared to only 47 normotensive AAA patients. The "D" allele frequency amongst NT, HT and controls were 0.77, 0.55 and 0.45 respectively. With an allele difference OR for NT vs. controls of 4 (95%CI, 2.2-7.4; p=0.0001). The II, ID and DD frequencies (%) for the NT, HT and controls groups were 6 (12.8%), 10 (21.3%), 31 (65.9%), and 14 (21.4%), 31 (47.7%), 20 (30.8%) and 17 (34%), 21 (42%), 12 (24%) respectively. For all cases and controls, the DD vs. ID +II (recessive model) was significant for HT (OR=2.6; 95%CI, 1.3-5.6; p<0.0002) and NT (OR=6.14; 95%CI, 2.5-14.9; p<0.0001), respectively. However, the corresponding OR for HT male smokers (n=74) and NT male smokers (n=32) vs. control were 3.6 (95%CI, 1.5-9; p<0.006) and 8.3 (05%CI, 2.7-25.1; p<0.0001) respectively. The combined association for HT females (n=38) and male non-smokers was not significant. Amongst the AAA patients there was a significant difference in "D" allele frequencies between NT and HT (p<0.0001).

The association of the ACE "D" polymorphism with HT was also corroborated by another study conducted on Polish subjects.[328] AAA (n=133) were compared with aorto-iliac occlusive disease (AIOD) subjects (n=152), controls (n=152) and a random population controls (n=392). The "D" allele frequency amongst AAA, AIOD, controls and random group were 0.519, 0.497, 0.510 and 0.504 respectively. Similarly the corresponding II, ID and DD genotype distributions for the same categories were 27 (20.3%), 74 (55.6%), 32 (24.1%); 34 (22.4%), 85 (55.9%), 33 (21.7%); 30 (19.7%), 89 (58.6%); 33 (21.7%); and 104 (26.5%), 181 (46.2%) and 107 (27.3%),

respectively with no differences in distributions amongst groups. However, there was a difference between hypertensive AAA (HT) vs. normotensive AAA patients using a recessive model (DD+ID vs. II; OR=3.08, 95%CI, 1.22-7.79; p=0.0147) and between HT vs. the random population using the same dominant model (OR, 2.56; 95%, 1.27-5.16; p=0.0066).

Interestingly, a study of 58 AAA patients did not show any differential expansion rates amongst different ACE I/D genotypes.[329] In this study, the mean baseline AAA diameter was 4.3 cm with an expansion rate of 0.35cm/year obtained by linear regression after a 28-mont follow-up. The genotype –specific expansion rates for the DD (n=14, 24%), ID (n=29, 50%) and II (n=15, 26%) genotypes were 0.22cm/year, 0.32cm/year and 0.30cm/year with no difference between groups (analysis of variance, p=0.6).

A recent case-control study analyzed the ACE I/D genotype distributions in three different populations using an additive (co-dominant) model.[330] In the UK study population, there was no difference in cases (n=298) and controls (n=912) in terms of genotype distributions (II (64), ID (157) and DD (76) vs. II (228), ID (423) and DD (236), respectively). Similarly, cases (n=576) and controls (n=472) in the New Zealand cohort were not significantly different in terms of ACE I/D distributions (II (112), ID (257) and DD (156) vs. II (85), ID (177) and DD (116), respectively). Similarly, a Western Australian cohort of 352 cases and 339 controls did not show any difference in terms of genotype distributions (II (81), ID (171) and DD (99) vs. II (92), ID (164), and DD (84), respectively). However, a pooled analysis of all three population data showed a significant heterozygote co-dominant association [(ID vs. II); OR, 1.33; 95%CI, 1.06-1.67; p<0.02]

In another study consisting of 201 cases and 252 controls,[331] the ACE I/D genotype distributions were significantly different between both groups [II (34), ID (82) and DD (85) vs. II (59), ID (122) and DD (71), respectively; P=0.006]. In a recessive model comparison, the "DD" genotype was found to be significantly associated with AAA (DD vs. ID +II; OR, 2.13; 95%CI, 1.06-4.28; P=0.03). Interestingly, the presence of transforming growth factor β receptor (TGFBR1) polymorphism 6A risk allele (compared to the 9A allele) increased the ACE "DD" predisposition to AAA (OR,5.09; 95%CI, 1.44- 18.02; P=0.01).

2.3.1 Cumulative Meta-analysis of ACE I/D association studies with AAA

An ACE I/D-AAA meta-analysis (cases= 2328; controls=2985) showed that this polymorphism was associated with AAA (OR, 1.268; 95%CI, 1.046-1.537; P=0.015) (Table 4;
Figure 13) and the cumulative meta-analysis indicated that the supportive evidence changed in 2009 implying that this present study is a replication study (

Table 5; Figure 14). Interestingly, the sensitivity analysis suggests that all the studies contributed equally to the meta-analysis (Table 6; Figure 15). The evidence suggests that there is marginal publication bias (Figure 16)

2.3.2 ACE I/D Genomics and the molecular basis of AAA

Accumulating evidence suggests that the ACE mRNA expression is subject to epigenetic regulation. In one study brains of foetuses of maternal protein deprived mice demonstrated 9-fold increased expression of ACE mRNA but with ~50 % reduction in ACE protein relative to controls but no change in mRNA and proteins of AT1R, ACE-2 and renin.[332] It was further demonstrated that the affected foetuses had ~ 50% decreased CpG island methylation at the ACE promoter. Furthermore, there was 3.3-fold and 8.8-fold increase in the respective concentrations miR-27a and miR-27b which target the ACE mRNA. In addition, there is also evidence suggesting the increase of H3 acetylation at the ACE promoter by trichostan A (TSA) a generic

histone deacetylase inhibitor (HDACi) thus suggesting combined histone modification and DNA methylation mechanisms regulate ACE tissue expression.[333]

The Alu repeats constitute about 10% of mammalian genome and about 50-60% of them are methylated in CpG islands. Alu elements can affect gene expression by acting as distal regulatory elements (enhancers or silencers) or through post-transcription modification of the mRNA.[334] Presence of alternative splicing sites within the intronic Alu element may be utilized by splicing machinery of the cell to recruit intronic Alu into mRNA to produce protein diversity.[335] In addition, embedded Alu also promote Adenosine to Inosine (A-I) editing in reactions characterized by ADAR (Adenosine Deaminase Acting on RNA) family of enzymes. A-I editing could affects RNA stability, splicing and protein translation. It is possible to speculate that the presence/absence of the Alu sequence could also be associated with rearrangement of genetic interaction networks with implication for other genetic expression because of evidence suggesting allele-specific genetic interactions in which a different (secondary) locus exerts an influence conditional on the presence of a particular allele.[336]

Alu insertions were previously regarded as "Junk DNA" serving no useful purpose but this thinking is being reversed with the identification of response elements (transcription sites) for the oestrogen ERα receptor, p53 (generated by deamination of methylated CpG to either Thymidine –Guanosine pair (TpG) or Cytosine-Adenosine pair (CpA)) and more recently, NF-κB. [337] [338] The foregoing suggests a functional role for this allele which is further underscored by the transcription of novel miRNAs.[339] Evidence also suggest that Alu miRNAs also mediate cis-suppression of nearby genes by binding to long intervening ncRNAs (lincRNA) like Evf2 to prevent the formation of pre-initiation complex.[340] Alu insertions induce suppression of nearby genes by a process of spreading genetic silencing through CpG methylation.[341] Conversely, these

methylation marks can erode with aging in a process referred to as epigenetic drift with consequence for gene expression and disease pathology.[342]

In one of the earlier association studies, the ACE I/D polymorphism in a cohort of Caucasian French subjects (n=80),[343] the mean (±SD) serum ACE levels was not significantly different between men (n=38; 408.5 ± 98.2 μg/L) and women (n=42; 417.1 ± 103.5 μg/L) but correlated with the genotypes [II (n=14; 299.3 ± 49.0 μg/L), ID (n=37; 392.6 ± 66.8 μg/L) and DD (n=29; 494.1 ± 88.3 μg/L), respectively]. The plasma levels were consistent with an additive genetic model. The ACE I/D genetic polymorphism were shown to account for about 47% of the variance of phenotypic ACE levels in this French cohort. However, in a Japanese association study between ACE I/D polymorphism and coronary artery disease (CAD),[344] plasma ACE levels in controls were correlated with the DD (16.6 ± 4.6 IU/mL; n=41), ID (14.5 ± 3.5 IU/mL; n=44) and II (11.4 ± 2.7 IU/mL; n=26) genotypes, respectively. There was a significant difference between DD and II ($p<0.01$) and between ID and II ($p<0.01$) but not between DD and ID genotypes. There was a significantly increased distribution of the "D" allele in the cases (n=178) in this study.

Table 4: RE M-A ACE I/D-AAA association studies

Study	Fixed-effects Ors	95% Conf. Intervals		Study weights
Hamano, 1999	0.7511	0.5391	1.0463	34.9482
Pola, 2001	1.6153	1.1361	2.2966	31.0227
Fatini, 2005	2.1539	1.4312	3.2416	22.9885
Walcszewski, 2007	2.1577	1.3364	3.4838	16.7384
Jones;NZ, 2008	1.0036	0.8319	1.2107	109.1309
Jones;UK, 2008	1.0891	0.9045	1.3112	111.4245
Jones; Aus, 2008	1.0954	0.8856	1.3548	85.0182
Lucarini, 2009	1.6801	1.2825	2.2006	52.7196
Korcz, 2009	1.0546	0.8064	1.3791	53.3737

Fixed effects OR=1.1745 (95%CI, 1.0775 -1.2802; P=0.0002)
Q statistics (Chi-sq)=35.9056; P=2.00E-05

Random effects or=1.2682 (95%CI, 1.0464-1.5371; P=0.0154)

[The large studies by Jones et al (2008) contributed strongly to the overall effect size of the RE M-A but a relatively large study by Fatini et al (2005) has a smaller weight compared to the relatively small studies of Lucarini et al (2009) whose sample size (n=285) is smaller than that of Fatini (n=500) . Notably, the studies by Jones et al (2008), Walcszewski et al (2007) and Lucarini et al (2009) are not in HWE.]

Table 5: Cumulative RE M-A ACE I/D-AAA association studies

Study Added	Random Effects	95% Conf. Intervals		RE Chi-square	P value
Hamano, 1999	NA	NA	NA	NA	NA
Pola, 2001	1.0988	0.5188	2.32749	0.0606	0.8055
Fatini, 2005	1.3667	0.7311	2.5543	0.9581	0.3276
Walcszewski,2007	1.5197	0.9042	2.5542	2.4962	0.1141
Jones;NZ, 2008	1.3767	0.9352	2.0266	2.6261	0.1051
Jones;UK, 2008	1.2999	0.9824	1.7201	3.3702	0.0663
Jones;Aus, 2008	1.2522	1.0001	1.5679	3.8434	0.0499
Lucarini, 2009	1.3046	1.0507	1.6198	5.7983	0.0161
Korcz, 2009	1.2682	1.0464	1.5371	5.8688	0.0154

[The evidence for a positive association between ACE I/D and AAA effectively changed in 2008 with addition of the Jones et al study (2008) where the RE M-A became 1.25 (95%CI, 1.001-1.567; P=0.049).]

Table 6: Sensitivity analysis of RE M-A ACE-AAA association studies

Study removed	Random Effects	95% Conf. Intervals		RE Chi-square	P value
Hamano, 1999	1.3406	1.1082	1.6217	9.1113	0.0025
Pola, 2001	1.2341	1.0083	1.5106	4.1621	0.0414
Fatini, 2005	1.1978	0.9983	1.4371	3.7708	0.0521
Walcszewski,2007	1.2093	1.0024	1.4588	3.9418	0.0471
Jones;NZ, 2008	1.3192	1.0588	1.6437	6.0985	0.0135
Jones;UK, 2008	1.3063	1.0392	1.6421	5.2444	0.0221
Jones;Aus, 2008	1.3029	1.0409	1.6309	5.3387	0.0208
Lucarini, 2009	1.2174	1.0018	1.4793	3.9156	0.0478
Korcz, 2009	1.3046	1.0507	1.6198	5.79834	0.0161

[The sensitivity analysis showed that all the studies except that for Fatini et al (2005) contributed equally to the overall effect size. Removal of the Fatini study shifted the overall effect size into negative territory showing that this study strongly contributed to the overall positive association which is not surprising since the study demonstrate an OR of 2.4 (95%CI, 1.3-4.2) with a recessive model of association (DD vs. ID +II). Removal of any of the other studies did not perturb the RE implying a relatively small contribution to the overall study.]

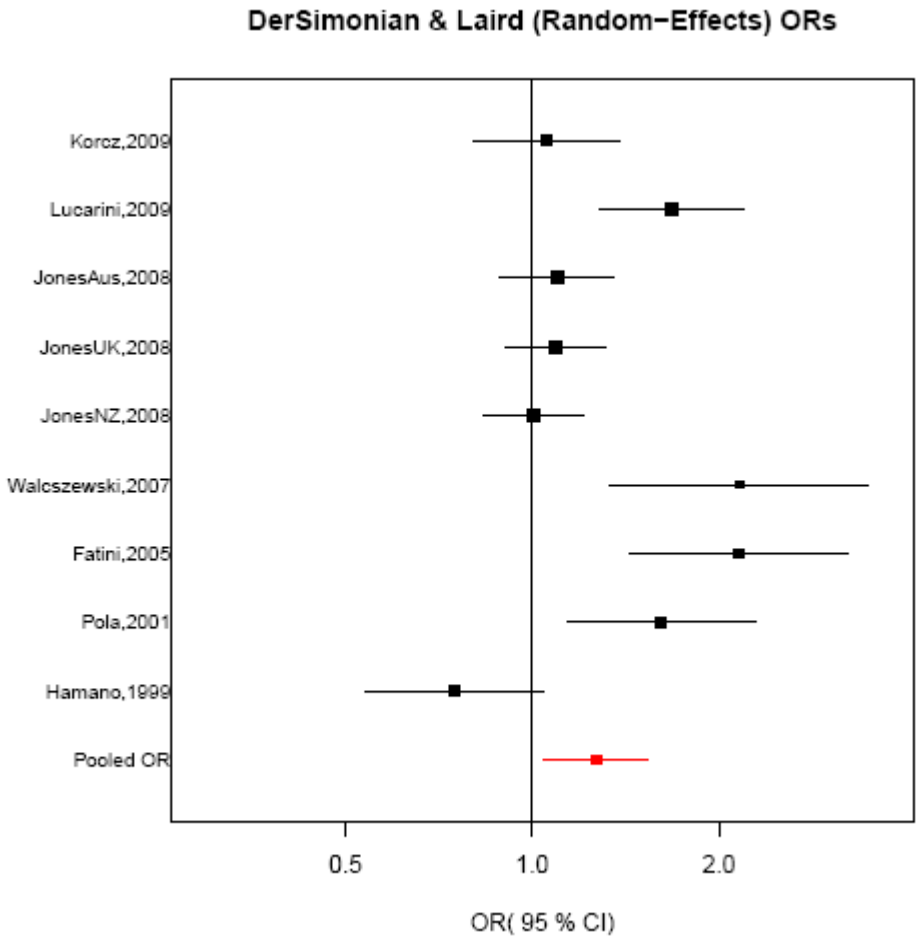

Figure 13: Forest plot of ACE I/D-AAA RE Meta-analysis.

The pooled estimate (red) line clearly shows an association between ACE and AAA from this RE M-A as indicated by the direction of the 95%CI lines which are exclusively on the right of the central vertical line of indifference (1.0). Note the strong contribution by the studies by Fatini (2005), Walcszewski (2007) and Lucarini (2009). It is noteworthy however, that most of these studies' control populations were not in Hardy-Weinberg Equilibrium (HWE)

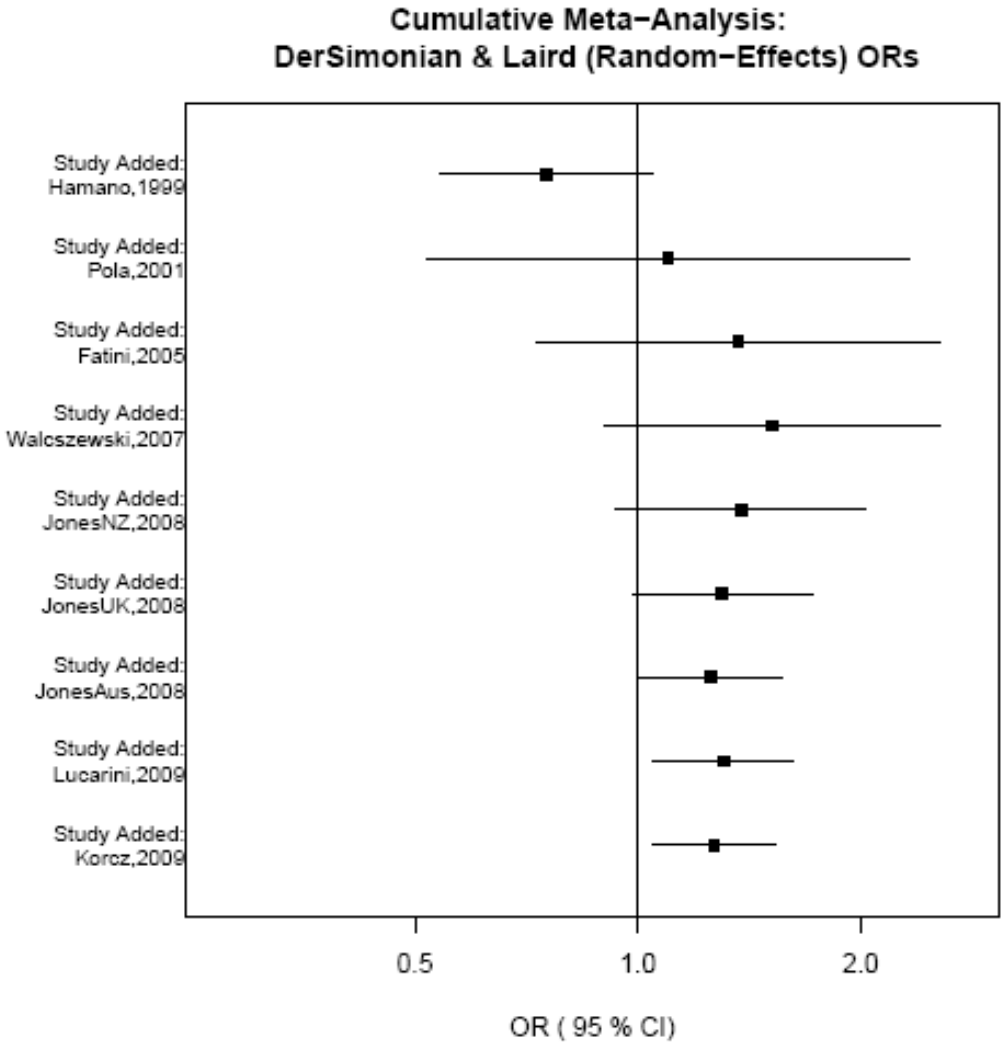

Figure 14: Cumulative RE Meta-analysis of ACE I/D-AAA association studies. A clear change in the direction of evidence for association is seen with the addition of the Lucarini (2009) study reflecting a positive association with combined studies up to that point because the 95%CI arms are exclusively to the right of the line of indifference (1.0).

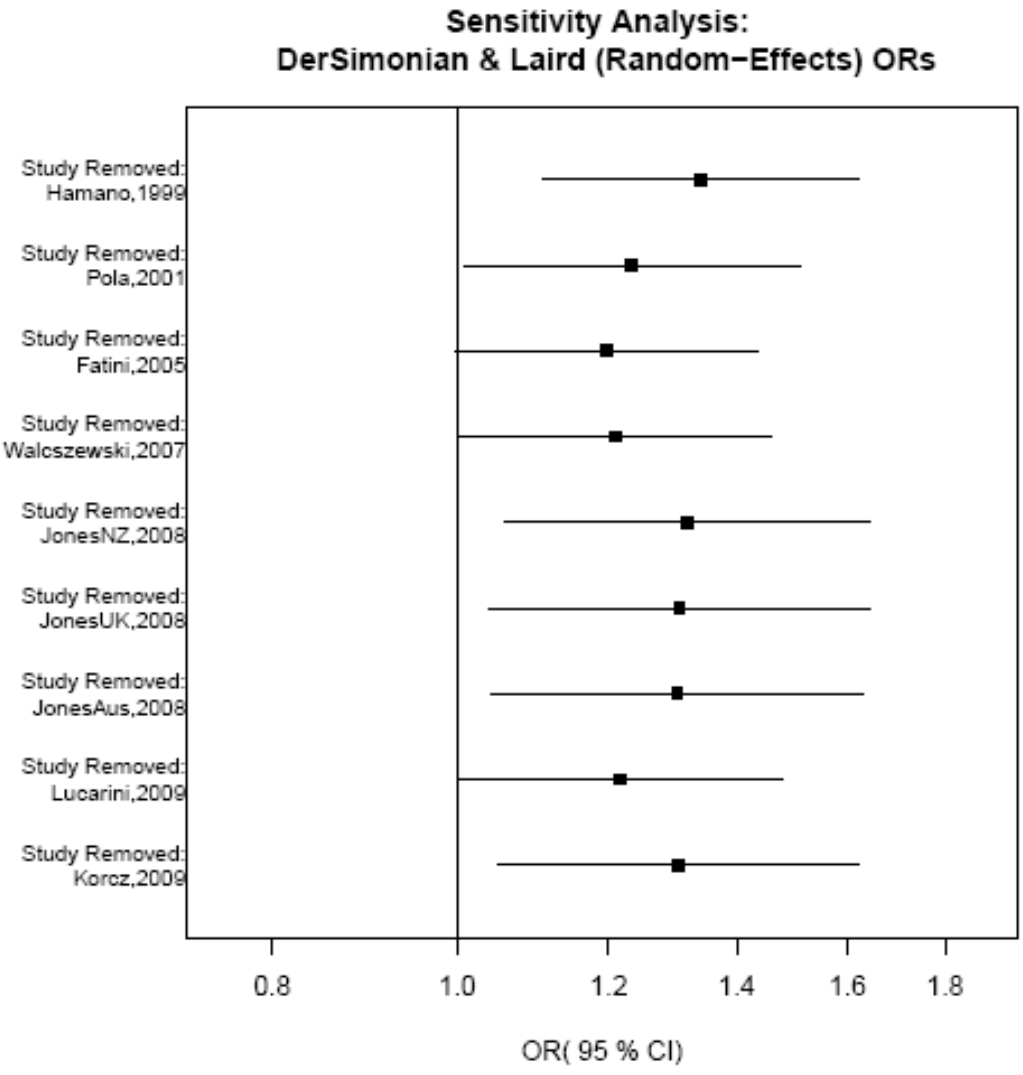

Figure 15: Sensitivity analysis of ACE I/D-AAA RE Meta-analysis. Removal of the studies by Fatini (2005), Walcszewski (2007) and Lucarini (2009) resulted in reduction of the effect size indicated by the 95%CI line touching or slightly crossing the central line of indifference (1.0). The foregoing suggests a strong contribution to the overall effect size by these 3 studies. However, Table 6 shows that only the study by Fatini et al strongly affected the overall effect size.

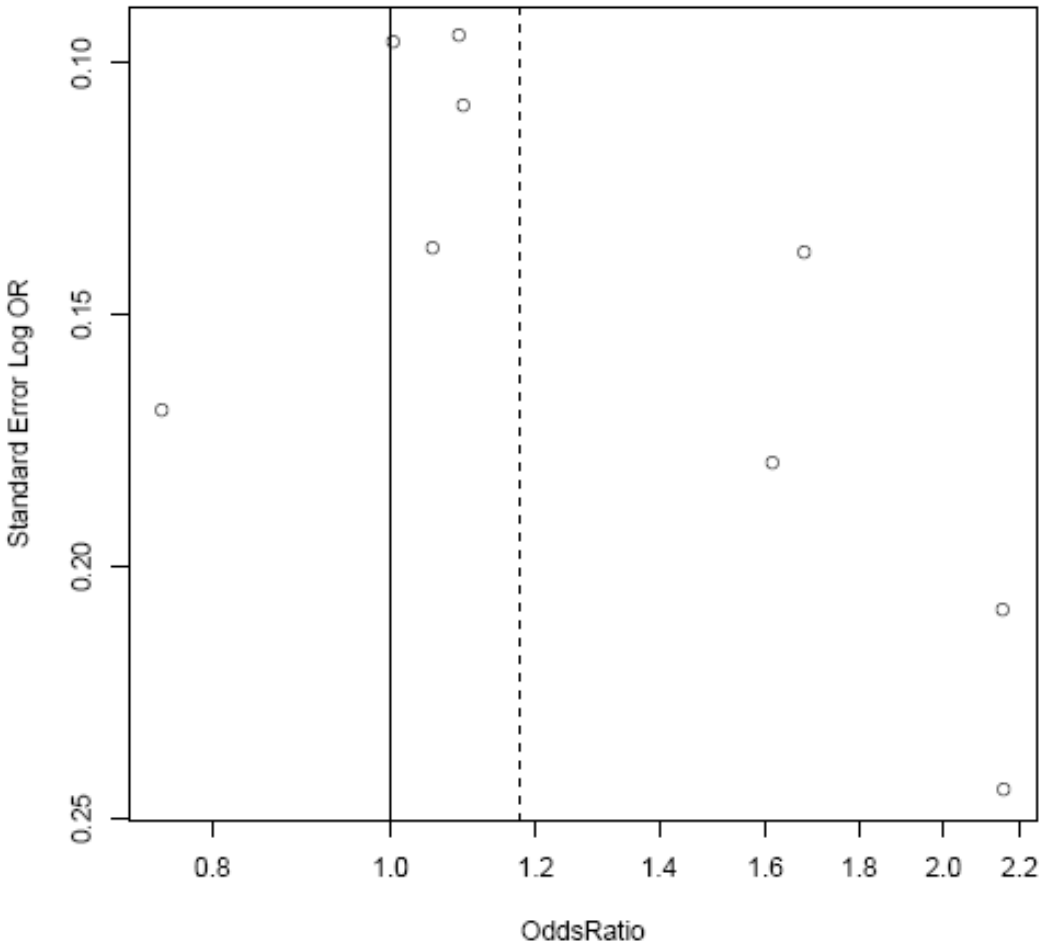

Figure 16: Marginal publication bias is evident from this funnel of ACE-AAA association M-A. The publication bias in this M-A is milder than observed one in the MTHFR RE M-A with a relatively more spread around the central line of equivalence ("1.0"). The dashed line as before is the line of Fixed Effect point estimate at 1.17

2.3.3 ACE molecular pathways and AAA

ACE, is the rate limiting enzyme for the production of Angiotensin II within the renin-angiotensin –aldosterone system (RAS) (Figure 17). ACE is an endothelial based mechano-sensor, responding to and activated by high, low or oscillatory shear stress.[345] It is worth mentioning that ACE has two recently discovered shear stress response elements at -251 to -195 of the transcription start site referred to as Barbie and GAGA which are suppressed by ~ 35% under laminar SS conditions.[346] However, ACE signalling is inhibited by normal (15 dynes/s) laminar shear stress (40 %) in association with concomitant reduction in its cytoplasmic tail phosphorylation (~50%) and ~40% JNK (c-Jun MAPK) phosphorylation activation respectively.[347] Accumulating evidence implicates H_2S as the inhibitor of endothelial ACE under laminar SS conditions. H_2S suppresses not only ACE activity (~50%) but also the gene expression of ACE.[348]

There is also evidence suggesting that ACE might function as a transcription factor able to activate the expression of src-homology protein (SHP) SHP-2, PDGFRβ, focal adhesion kinase (FAK) and Akt.[349] SHP-2 is a tyrosine phosphatase which unlike other phosphatases activates its targets by dephosphorylation and it is also capable of mediating cell-cell communication by activating the transmembrane SHPS-1 thereby coupling intracellular and intercellular communication.[350] SHP-2 has also been shown to be a downstream mediator of Angiotensin II activated AT_1R signalling to JAK2-STAT pathway.[351]

Angiotensin II activates the Ca2+ signalling pathway by binding to plasma membrane-bound ACE.[352] This pathway leads to the activation of NF-kB, c-Jun terminal (JNK) MAPK, NFAT

and calcium calmodulin kinase (Ca-CAMK) pathways which are implicated in AAA pathogenesis. Increased [Ca^{2+}] results in the activation of calcium sensors and activators amongst which are calcineurin, calmodulin, calpain, cyclophilin and S1000A12 proteins which have all been implicated in the pathogenesis of AAA.

ACE is predominantly tissue located but about 10% of ACE circulates in plasma while the remaining 90% is in tissues. ACE is found in virtually every tissue of the body because of the ubiquity of the RAS but it is predominantly found on endothelial surfaces. Accumulating evidence has implicated ACE in intracellular signalling pathways involving JNK MAPK,[353] NF-κB,[354] COX-2,[355] PPARγ and focal adhesion kinase (FAK).

ACE is also upregulated in pre-adipocytes and mature adipocytes[356] in humans where it is activated by cellular kinases like PKC and PKA unlike Casein Kinase II in endothelial cells to up regulate pro-apoptotic mediators like Caspase-3 and -4, E-Cadherin and non receptor tyrosine kinases like Syk which functions as a link in the TNFα signalling cascade to activate MAPK and NF-κB. There is also upregulation of CDK1 which can activate the pro-apoptotic BAD an important mediator in the intrinsic pathway of apoptosis. Similarly, ACE signalling also upregulates FLASH [FLICE (Fas-associated death domain-containing protein-like interleukin-converting enzyme)-associated huge protein] a member of the extrinsic apoptotic pathway.

It is the paradoxical activation of ACE by ACE inhibitors that led to the discovery of the intracellular ACE signalling pathway. It is also worth noting that ACE inhibition (ACEI) paradoxically increases ACE levels, ACE activation and upregulation of Angiotensin II from a de-repression of the feedback inhibition of renin by Angiotensin II.[357] Binding of ACEI to ACE is associated with activation phosphorylation of the cytoplasmic tail at a particular lysine residue (K1270) by Casein Kinase II (CK-2) a ubiquitous signalling kinase. Thereafter, JNK and NF-KB pathways become activated. Considering that proximal MAPKKK like TAK1 activates both

pathways, it is possible to speculate at this stage that this enzyme may be recruited to the phosphorylated ACE cytoplasmic tail to mediate the activation of both pathways.

Accumulating evidence also suggest that ACE upregulates and stimulate the activities of bradykinin receptors (BKB1R and BKB2R).[358] The BKB1R pathway activation leads to the increased angiogenesis via upregulated eNOS whilst BKB2R pathway activity leads to inflammation via PKC activation and Cytochrome P450 epoxygenase which stimulates ERK MPAK and NF-κB activation. The latter subsequently increases P-selectin, ICAM-1 and COX-2. It is also suggested that VEGFR2 is upregulated via increased BKB2R activity. There is also evidence for heterodimerization with ACE (BKB2R: ACE) and AT1R (AT1R:BKB2R) induced by Angiotensin (1-7) and increased Angiotensin II at these heterodimers.

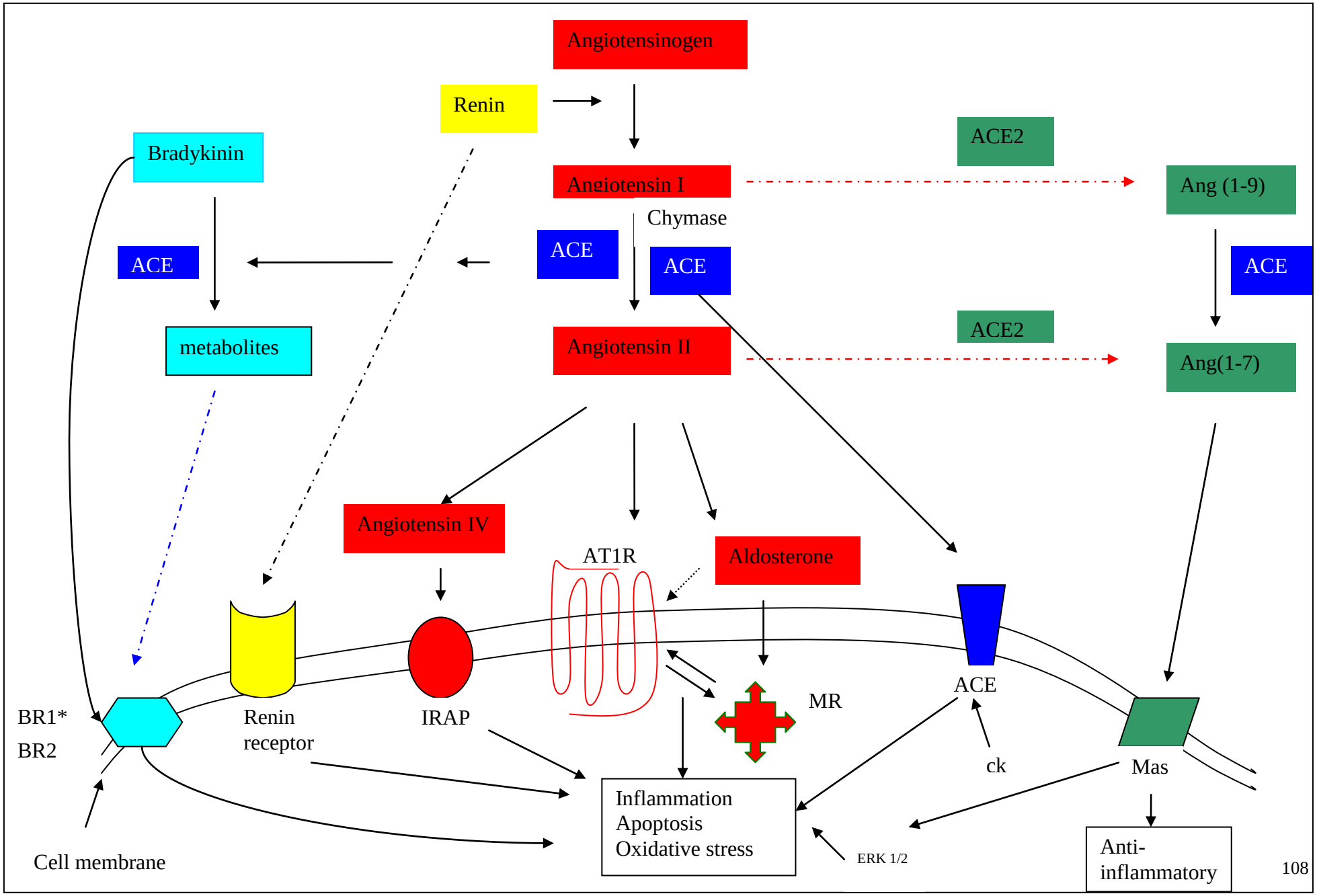
Angiotensinogen
Renin
Bradykinin
ACE2
Angiotensin I
Ang (1-9)
Chymase
ACE
ACE
ACE
ACE
metabolites
ACE2
Angiotensin II
Ang(1-7)
Angiotensin IV
AT1R
Aldosterone
BR1*
BR2
Renin
receptor
IRAP
MR
ACE
ck
Mas
Inflammation
Apoptosis
Oxidative stress
ERK 1/2
Anti-
inflammatory
Cell membrane

Figure 17: ACE signalling pathway. The red shaded structures are important for ACE1 which is the enzyme of interest in this study is an exopeptidase functional in the Renin-Angiotensin-Aldosterone System (RAS). ACE 1 also generically known as ACE converts angiotensin1 to angiotensin II and also cleaves bradykinin to its metabolites. It also converts a variant of Angiotensin 1 (Angiotensin 1-9) to Angiotensin 1-7 which binds to Mas, a receptor on inflammatory cells to mediate anti-inflammatory action. Angiotensin II, the main peptide hormone of this system can also be converted to Angiotensin IV and they both mediate pathological processes like inflammation, apoptosis and oxidative stress which are of pathognomonic significance in AAA pathology. Angiotensin binds to predominantly surface G-protein coupled receptor, Angiotensin II Type 1 receptor (AT_1R). ACE can also function as a plasma membrane receptor and is capable of being activated by fluid flow, Angiotensin II and paradoxically ACE inhibitors. When ACEI bind to ACEI they induce a conformational change which allow Casein Kinase II (CK) to phosphorylate ACE intracytoplasmic tail to induce a pro-inflammatory signalling and oxidative stress. (See text for more details)

Chapter 3. Methods

3.1 Case-Control study

This was a retrospective case-control study with the main aim of replicating previous studies with inconsistent association with AAA. These comprise the MTHFR C677T SNP and ACE I/D polymorphisms.

Hypotheses for this segment of the study were:

Ho: The ACE gene polymorphism is not associated with abdominal aortic aneurysms (null hypothesis)

HA: The ACE gene polymorphism is associated with AAA (alternative hypothesis)

Ho: The MTHFR C677T polymorphism is not associated with AAA (hull hypothesis)

HA: The MTHFR C677T polymorphism is associated with AAA (alternative hypothesis)

3.1.1 Sample Size requirements

Previous studies using ACE gene and MTHFR gene polymorphisms have used populations of varying sample sizes. Small sample sizes will encourage type II error whilst population heterogeneity, genotyping errors, bias and confounding can result in type I error. In calculating the sample size, adequate reference was made to the previously documented studies of the

genetic association between ACE and MTHFR genetic polymorphisms and AAA respectively. The largest sample sizes in those studies and the effect sizes were taken into consideration. The documented genotyping error rate for the ACE gene polymorphism is about 5% which was also included in the sample size calculation. One of the major assumptions I made is that the population that I will be sampling from is in Hardy-Weinberg equilibrium.

The calculated sample size for MTHFR C677T genotyping was 617 cases and 617 controls for a power of 90% and alpha of 5% ($p<0.05$).[359,360,361] For the ACE I/D genotyping, the calculated sample size incorporating a 5% genotyping error was 511 cases and 511 controls for a power of 80% and alpha of 5%.[362] [It is instructive that sample size for the ACE I/D polymorphism was further increased when this sample size during interim analysis was found not to be in Hardy-Weinberg Equilibrium in the study population. A further reduction of alpha to 1% increased sample size to 760 cases and 760 controls. Final sample size of 1107 cases approximated power of 80% and alpha of 0.1%

3.1.2 Participant recruitment:

The cases in this study are those participants who have had an imaging (duplex or computerized tomography scan) confirmed abdominal aortic aneurysm. Abdominal aortic aneurysm for the purpose of this study is defined as an infra-renal abdominal aorta with its maximum antero-posterior or lateral diameter equal to or greater than 2.5cm. Referrals from other hospitals or GP practices with AAA were also recruited into the study.

In contrast, the controls are those who participants who belong to the same demographic risk group and who have had a single scan and confirmed to be free from AAA. Every effort was made to match for potential confounding demographic variables like age, sex and ethnicity. My

controls were at least 65years of age and predominantly males (because of the recruitment from screening programme in GP practices). Only persons of white Caucasian origin were recruited for the study. Other sources of recruitment were from in-hospital admissions, vascular outpatient clinics, urology clinics and radiology department. Subjects who were unable to consent were not recruited.

Recruitment of study participants was through a cooperative recruitment scheme in conjunction with colleagues from the same Vascular Surgery Group who were engaged in AAA research. A recruitment rota was made to facilitate this process since some of us also had clinical engagement. In this manner we recruited into a common pool and MD candidates (Research Fellows) used samples from this common to satisfy their sample size requirements. This was a very helpful scheme. At any particular time, there were at least 2 Research Fellows available to go on recruitment drive. Ethics approval was obtained from the University Hospitals of Leicester Ethics committee.

3.1.2.1 Data Management, Quality Control and Safety

All samples were labelled with a unique code separate for both controls and cases
A separate demographic database was set up, different from phenotypic database. This is to ensure ethics compliance with access to confidential data

3.1.2.2 DNA Extraction:

Blood was taken in 7ml EDTA bottles from the study participants after a specific consent for the procedure was obtained. A buffy coat sample was obtained from the blood sample in the

following steps:

The blood in the EDTA bottles was spurn in the centrifuge at 2000 RPM at 4ºC for 10minutes. This allowed the blood to separate into a plasma layer supernatant and a dense cellular layer of red blood cells with an overlying buffy coat layer. The supernatant was then aspirated with a plastic pipette into small tubes and the buffy layer was then gently aspirated with plastic pipette and put in a small Eppendorf® tubes (in small aliquots of 0.5 to 1 ml) with a tight cover. This tube with its buffy coat content was appropriately labelled and then snap-frozen in liquid nitrogen for subsequent storage at -80ºC in the freezer.

3.1.2.3 Cell Lysis:

The frozen buffy coat was rapidly thawed in a water bath at 37ºC and added to a 15ml centrifuge tube containing 3 parts of Red Blood Cell (RBC) lysis solution obtained from Puregene®. The original protocol recommended using 250µl of buffy coat and 750 µl of the RBC lysis solution. This did not yield adequate DNA from such a small quantity of buffy coat. So the buffy coat from two blood sample bottles were added together in a tube containing 4.5ml of RBC lysis solution. The tube was inverted several times to adequately mix the contents and incubated overnight at room temperature. The original protocol recommended 10 minutes but I found that leaving it overnight yielded a higher quantity of DNA. Subsequently, the lysed sample was centrifuged at 2000g for 10 minutes at 4^{o}C and the supernatant discarded leaving behind a white cell pellet and about 100-200µl of residual fluid. This fluid was left intact as attempts to remove it risk loss of the white cell pellet. The tube was then vortexed vigorously to re-suspend the white cell pellet in the residual liquid. A further 3 ml of cell lysis solution was then added to the

solubilised pellet and the mixture incubated at 37 ^{o}C for 1-2hours until the solution was completely homogenous.

3.1.2.4 Protein Precipitation

During this stage structural protein materials like membrane proteins and other cellular proteins including histone proteins were removed to obtain pure DNA. The samples from the cell lysis solution were cooled to room temperature. 1 ml of protein precipitation solution was then added to the cell lysate and vortexed at high speed for 20 seconds. The mixture was then centrifuged at 2000g centrifugal force for 10minutes at 4 ^{o}C to form a tight whitish-brown pellet containing the precipitated proteins. However, if no brown pellet were formed at this stage as happened occasionally, a further 1ml of protein precipitate solution (ammonium acetate) was added followed by incubation on ice for 5minutes and further centrifugation. The DNA in the supernatant was then precipitated from solution.

3.1.2.5 DNA precipitation

The supernatant containing the DNA was then poured into a clean 15ml centrifuge tube containing 3ml of 100% isopropanol which precipitates the DNA as a cloudy band on top of the isopropanol solution. The sample was inverted about 50 times to obtain stringy DNA precipitates. This whole solution and precipitate was then centrifuged again at 2000g for 3 minutes at 21 ^{o}C to form a white DNA pellet at the bottom of the tube. The supernatant was then discarded and the tube dried by inversion on a clean absorbent paper. 3 ml of 70% ethanol was added to the dried pellet and the tube inverted several times to wash the DNA pellet to remove

residues of the previous salt solution. The tube was centrifuged at 2000g for 1 minute at 21 ^{o}C and the ethanol supernatant was discarded leaving behind the pure DNA pellet. The tube was inverted and drained on clean absorbent paper and allowed to dry for 10-15 minutes.

3.1.2.6 DNA hydration

To the DNA pellet obtained from the previous step, 250µl of DNA hydration solution was added. This should yield about 400µg/ml of DNA if the total yield was 100µg. The hydrated DNA was incubated at 65 ^{o}C for 1 hour then overnight at room temperature, although the protocol recommended 1 hr incubation only. My protocol increased DNA yield.

3.1.2.6.1 DNA storage

The freshly extracted DNA was quantified and stored at -20 ^{o}C in 1.5ml eppendorf tubes.

3.1.2.7 Quantification of the extracted DNA

An optical density method using a ultra-violet visible spectrum spectrophotometer machine is used in the DNA quantification process. A spectrophotometer is a convenient and sensitive way of measuring the concentration of a photo-absorbent solute such as DNA in solution. Within the spectrophotometer the DNA is exposed to UV light and a photo-detector measures the wavelength of the light rays passing through the solution. The more light that is absorbed by the sample, the less light is sensed by the photo-detector and therefore the higher the DNA concentration in the sample. To ensure that the extracted DNA was of the highest quality, an

optical density (OD) based quantification was carried out using the spectrophotometer. The materials required for this step in addition to the spectrophotometer include: stock DNA, sterile water and quartz cuvettes. Careful quality assurance steps were undertaken in this step to avoid possible contamination which may affect the optical density readings from the spectrophotometer machine. The procedure was carried out far from the DNA extraction bench and hand gloves were worn at all times. The pipettes were also changed after use at each step and the cuvettes were washed with distilled water and blotted dry between use. The following steps were carried out sequentially.

[1] 495 µL of distilled water was added to a clean quartz cuvette and this was used to obtain a base reading with the spectrophotometer.5µL of genomic DNA was then added to the cuvette content to make a total volume of 500 µL (1:99 dilution)
After which the cuvette was inverted a few times.

[2] Optical density scanner and readings were taken at 3 different wavelengths: 230nm, 260nm and 280nm. The OD reading at 280nm is used in the calculation of genomic DNA in the sample:

(OD260 – OD230) X 50 X100= DNA µg/ml

The DNA was further diluted to obtain aliquots ranging from 50-100 µg / µl
An example of the foregoing steps is instructive:
Suppose the following readings were obtained at the various wavelengths:

A230 = 0.048

A260 = 0.063

A280 = 0.110

Then the DNA purity can be calculated as:

ODA260/ODA280 =1.74

And the DNA content will be (0.063-0.048) x 50 x 100=75μg/ml

This is because an OD of 1.0 corresponds to a DNA concentration of 50 μg/mL

3.2 Protocol for Polymerase Chain Reaction (PCR) Methodology

3.2.1 PCR reaction

PCR was carried out in a series of cycles. Each cycle begins with a **denaturation** step to render the target DNA to be single stranded. This step is then followed by the **annealing** step during which the primers anneal to their complementary sequences so that their 3' hydroxyl ends face the target. Finally, each primer is **extended** through the target region by the action of Taq DNA polymerase. The optimal Ta was further used as a reference point in an experimental design matrix with varying concentrations of $MgCl_2$ to get the optimal combination of these two vital components for the PCR conditions (table 7). In the design experiment, each column represents increasing concentrations of $MgCl_2$ in a top-down manner whilst each row represents increasing temperature from left to right to bracket the optimal Ta (55ºC). Further modification of this step in a touchdown (TD) was done. The TD regime was incorporated a range of annealing temperatures on both sides of Ta. The final TD annealing temperature was 50-61ºC

3.2.1.1 Extension

The time for extension is dependent on the DNA polymerase and also on the length of the DNA to be amplified. As a rule of thumb, 1 minute per kilo base pair is the recommended elongation

rate. Usually the extending step is performed 70-75oC. Recommended extending time is 1 minute for the synthesis of PCR fragments up to 2 kb. When larger DNA fragments are amplified, the extending time is usually increased by 1 minute for each 1000 bp.

3.2.1.2 Optimization

The first optimization step was for the Ta as explained previously in conjunction with Mg^{2+}

3.2.1.2.1 PCR Enhancers:

It may be necessary sometimes to add certain enhancing agents to a PCR to facilitate the optimization steps. GC –rich templates can be problematic due to inefficient separation of the DNA strands or because of the tendency of the GC- rich primers to form intermolecular and intra-molecular secondary structures that compete with template annealing. Templates that form strong secondary structures can cause the polymerases to stall.

Dimethyl Sulfoxide (DMSO) was added to the reaction mix to further optimize the specificity of the reaction (i.e., primer-template pairing) and reduce the T of the primer-template pairing The common concentrations of these agents given to enhance poor reactions are DMSO (1-10%).

In order to determine the optimal or near optimal annealing temperature (Ta) conditional on the reaction mix, a gradient step in the PCR machine (STORM® thermocycler) was used with varying concentrations of $MgCl_{2\ in}$ a matrix format. The PCR products obtained from this step with the brightest intensity on gel electrophoresis was selected and this corresponded to $MgCl_2$ concentration of 1.5mM (**Error! Reference source not found.**) for MTHFR and 2.0mM for

ACE. The Ta associated with this concentration was chosen for the next step which is the touch-down step in the annealing step of the PCR process. The gradient steps are given below:

(50.1 ^{o}C) (50.4 ^{o}C) (50.9 ^{o}C) (51.9 ^{o}C) (53 ^{o}C) (54.3 ^{o}C) (55.6 ^{o}C) (56.9 ^{o}C)
(58.4 ^{o}C) (59.3 ^{o}C) (59.6 ^{o}C) (60.2 ^{o}C)

The matrix format for the joint evaluation of optimal Ta and $MgCl_2$ concentrations is as given below (Table 7). As seen in the table using the Ta gradient function of the thermocycling instrument (Thermocycler) and the manufacturer supplied melting temperature (Tm) of the primers (1 and 2), a range of temperatures is created in incremental steps around the Tm from below to above. These constitute the gradient(s) or step of temperature change during the annealing step of the PCR. Furthermore, 50 μL aliquots of PCR mix are pipetted into tubes with different $MgCl_2$ concentrations. These tubes are then placed in the 96-well plate of the thermocycler to correspond to the different temperature blocks . As noted from the matrix, the Ta increases from left to right in incremental steps whilst the $MgCl_2$ increase from top downwards in steps as well.

After the run of the PCR, a 2% gel electrophoresis was carried out for each of the samples corresponding to individual Ta/MgCl2 concentration combination. The sample with the highest intensity under ultraviolet light and electronic viewer was selected and that combination adopted for subsequent PCR. However, although the $MgCl_2$ remains the same for subsequent PCR runs, the selected Ta is further used as an index for a Touch-Down step in the annealing step of the definitive PCR. Touch –Down works in a similar manner to the gradient step but its purse was to exponentially increase the amplification yield .

Table 7: Design Matrix for $MgCl_2$ and Ta

	Ta (^oC) ⇨							
$MgCl_2$ Conc.(µL) ⇩	50.1 ⇩ ⇩	50.4 ⇩	51.9 ⇩	53.0 ⇩	55	….	….	60.2 ⇩
1.0	⇩ ⇨	⇩ ⇨	⇩ ⇨	⇩ ⇨	…	…	…	…
1.5	⇨	⇨	⇨	⇨	…	…	…	…
2.0	⇨	⇨	⇨	⇨	…	…	…	…
2.5	⇨	⇨	⇨	⇨				
3.0	⇨	⇨	⇨	⇨				
3.5	⇨	⇨	⇨	⇨				
4.0	⇨	⇨	⇨	⇨				

3.2.1.3 Ace insertion-deletion polymorphism genotyping

ACE I/D genotyping was done with an adaptation of the method of Rigat et al.[363]

Sense primer 5'-CTGGAGACCACTCCCATCCTTTCT-3'

Antisense primer 5'-GATGTGGCCATCACATTCGTCAGAT-3'

The reagents are as specified in Table 9. The reaction profile is:

$94^{o}C\ ^{5:00}\ [94^{o}C\ ^{1:00;}\ (60->50^{o}C)\ ^{1:00;}\ 72^{o}C\ ^{2:00}]_{35}\ ;72^{o}C^{5:00}$

A brief explanation of the above shorthand notation is needed. There is initial denaturation at $94^{o}C$ followed by 35 cycles of touch-down reaction , comprising sequential denaturation, anneal temperature touchdown step. During this step the thermocycler cycles through a descending range of temperatures from a maximum of $60^{o}C$ to $50^{o}C$ for I minute. This step is then followed by an elongation step at $72^{o}C$ lasting for 2 minutes. After 35 cycles of this process, there is a final elongation at 5 minutes before the PCR cycle automatically stops. This protocol was also found to be optimal for the confirmatory step (except for gradient step).

The obtained PCR products were processed with 2% gel electrophoresis.

3.2.1.3.1 Genotyping error

Mistyping of ID genotype as DD occurs preferentially in about 4-5 % of the cases. Genotyping error can be reduced by the addition of 5% DMSO or the use of Allele- Specific Oligonucleotide. The ASO method, though preferred in most cases, it is time consuming and

requires a second nested PCR to be performed on the DD samples obtained with the original methods. Some authors have adopted a second stage PCR in order to obviate the preferential amplification of the DD allele which may lead to a genotyping error of ~ 5% when the ID is amplified as DD genotype.[364,365] This second stage required an insertion (I) specific primer pair: (HACE 5A, HACE 5B) Those PCR products migrating as D bands on gel electrophoresis are subjected to a second round of PCR using the HACE primers:

HACE5A 5'-TGG GAC CAC AGC GCC CGC CAC TAC-3'

HACE5C 5'-TCG CCA GCC CTC CCA TGC CCA TAA-3'

Using identical PCR conditions except for an annealing temperature of 64°C, the reaction yielded a 335 bp product in the presence of the "I" allele and no product in the presence of DD homozygosity (Figure 20).

Using identical PCR conditions except for an annealing temperature of 64°C, the reaction yields a 335 bp product in the presence of an "I" allele and no product in the presence of DD homozygosity. The same genotyping conditions of $94^{o}C\ ^{0:40}\ [94^{o}C\ ^{0:30;}\ 64^{o}C\ ^{0:45;}\ 72^{o}C\ ^{1:30}]_{32;}\ 72oC^{3:00}$ were applied.

Table 8: ACE I/D PCR Mix

Polymerase Chain Reaction Mix

Total Volume 50uL	Volume	Final Concentration
Sterile H_2O	34.25uL	
Buffer (x10)	5uL	1x
$MgCl_2$ (50mM)	2.0uL	2.0mM
dNTPs (10mM)	1uL	200uM
Genomic DNA Template (10ng)	1uL	10ng
Primer 1(Sense)	1uL	10pM
Primer 2(Anti-sense)	1uL	10pM
DMSO	2.5uL	5%
KCL	1uL	50mM
TritonX-100 (5% stock)	1uL	0.10%
Enzyme (TAQ Polymerase)	0.2uL	1.5U

[The buffer solution was supplied by the manufacturer (Puregene) with the primers. The sense primer binds to the forward DNA strand whilst the Anti-sense primer binds to the opposite (reverse) allele of the DNA. The two primers were combined in a solution with 1 µL contributing both primers in equal proportion. The dNTPs are deoxyribonucleotides for incorporation into the amplicon.The volume for H_2O is variable to make up the 50µL volume whenever it was necessary to increase template DNA volume for 10ng of genomic DNA]

Table 9: Confirmatory PCR for ACE ID genotype (HACE)

Confirmatory Mix for ACE DD

Total Volume 50uL	Volume	Final Concentration
Sterile H_2O	34.5uL	
Buffer (x10)	5uL	1x
$MgCl_2$ (50mM)	2.5uL	2.5mM
dNTPs (10mM)	1uL	200uM
Genomic DNA Template (10ng)	1uL	10ng
HACE 5A	1uL	10pM
HACE 5B	1uL	10pM
DMSO	2.5uL	5%
KCl (2.5M)	1uL	50mM
TritonX-100 (5% stock)	1uL	0.10%
TAQ	0.Ul	0.5U

[The specific primers (HACE 5A and HACE 5B) used here specifically identify sequences within the Insertion or "I" sequence for amplification. Other materials are as previously described and in text]

3.2.1.4 METHYLENE TETRAHYDROFOLATE REDUCTASE C677T POLYMORPHISM

The primer pair combination for the MTHFR C677T PCR reaction is given below:

Sense primer: 5'-TGAAGGAGAAGGTGTCTGCGG GA-3'
Antisense primer: 5'-AGGACGGTGCGGTGAGAGTG-3'

PCR was similarly conducted in 50µL tubes using the 96-well plate thermocycler. Optimization for the MTHFR was done with/without DMSO using different values of $MgCl_2$ from 1.5-4.5mM with a temperature gradient of 50-60 in the following steps [50; 51.4; 52.8; 54.2; 55.6; 57.0]30secs. Interestingly, it was observed that there were products with bright signal intensity with lower concentrations of $MgCl_2$ (1.5-2.5mM) without DMSO. Note worthily, the reaction mix contained both TritonX-100 and Gelatine which have been shown to act like DMSO in improving specificity of primer-template pairing and lowering Ta. Subsequent PCRs were done without DMSO but at a lower $MgCl_2$ concentration.

3.2.1.4.1 MTHFR Digestion mix

Total Volume	25 µl
Restriction Enzyme (*Hinf l*)	1.0 µl (containing 10U of enzyme)
NE buffer II	2.5 µl
Sterile water were	11.5 µl
PCR products	10 µl

The above protocol was followed in order to yield a mixture 1:10 of enzyme volume to total volume. The digestion mix was left in the water bath for overnight digestion at 37^{o}C.
The Hinf1 restriction site is a "**G|A"** site [5'$(n)_{22}$…**G-**|-**A-**N-T-C…$(n)_{176}$ 3'; 5'$(n)_{22}$…C-T-N-**A-**|-**G**…$(n)_{176}$ 3'] in the amplicon resulting in two digestion products of 22bp and 176bp respectively. Whereas the manufacturer's instructions wee to digest in the water bath for 2 hours, higher product yields (evidence from UV viewing) were obtained from an overnight digestion Electrophoresis was carried out on 2.0 % agarose gel (+ 0.5mg/ml ethidium bromide). The 198 base pair product (CC) genotype and 176 base pair product (TT) genotype migrate close to the 200bp marker of the DNA ladder on electrophoresis (Figure 21). It was however difficult to identify the 22 bp product on the gel but its presence could be inferred by the presence of the 176bp product.

3.2.2 Preparation of the Gel 2% Agarose Gel

Add 400ml 10X TBE stock solution to 8gm of agarose to make 2% agarose gel. The solution is boiled preferably in a microwave oven and allowed to cool to ~ 60o C at room temperature. The solution is gently stirred whilst cooling and 10ml of ethidium bromide is added to each 400ml of gel solution (nitrile glove are worn because ethidium bromide is a mutagen). In addition this process was carried out in the fume chamber (cupboard).The solution is stirred to disperse the ethidium bromide. This mixture was then poured into the gel rack (96 well gel racks) and the comb inserted at one end of the gel about 5-10mm from the border and about 2mm above the gel plate. Another comb (26-well) was inserted in the gel at the middle giving a total of 52 wells. The comb was then removed as the gel cooled and solidified. It takes about 20-25 minutes to solidify in the cold room but ~ 45minutes at room temperature.

Table 10: MTHFR C677T PCR Mix

Polymerase Chain Reaction Mix for MTHFR

Total Volume 50uL	Volume	Final Concentration
Sterile H_2O	36.5uL	
Primer1	1.0 uL	100ng
$MgCl_2$ (50mM)	2.0uL	1.5mM
dNTPs (10mM)	1uL	200uM
Genomic DNA Template (40ng)	0.5uL	50ng
Primer2	1.0 uL	100ng
Gelatine	0.5uL	100ug/ml
KCl	1uL	50mM
TritonX-100 (5% stock)	1uL	1mg/mL
Enzyme (TAQ Polymerase)	0.5uL	1U
Buffer (x10)	5uL	1x

[The reaction components are as before except for the specific primers and the gelatine. Primer 1 is the template DNA for the sense strand of the DNA whilst primer 2 is the template for the anti-sense strand. Gelatine is an additive like DMSO to optimize the reaction. H_2O is added to make the 50µL volume or reduced if the volume of genomic DNA] had to be increased to make up the required quantity]

The solid gel is placed in an electrophoresis tank containing 1x TBE and the tank is filled up to cover the wells. One of the wells is loaded with 10 µl of the DNA ladder and another well is loaded with a control sample (amplified PCR sample without genomic DNA).About 10µl of PCR products is mixed with 2 µl of the blue juice loading buffer, pipetted and placed in the wells of the gel. The wells are situated in such a way to be closer to the anode (negative electrode) of the electrophoresis machine. This is because; DNA being negatively charged will migrate away from the anode towards the cathode (positive electrode) thus ensuring proper separation of the PCR products. For the ACE and MTHFR polymorphisms, gel electrophoresis was done at a voltage of 150 minutes for 1 hour. Wearing gloves and UV safety glasses, the gel is then removed from the buffer and viewed on the short wave UV trans-illuminator. Ethidium bromide fluoresce pink in the presence of DNA. The rate of migration in the electric field is a function of the molecular weight of the amplicons. The smaller products will migrate faster than the heavier products and the size of the product is then read relative to the DNA ladder.

3.2.2.1.1 Contamination Quality assurance

DNA sample preparation, reaction mixture assemblage and the PCR process are performed in separate areas and fresh gloves were worn between steps. Furthermore, reagents for PCR were prepared separately and solely for this purpose as when necessary

As part of quality assurance, a control PCR was done every time with each reaction. One of the wells did not contain DNA. In addition, reagents were prepared in one room but the thermocycling reaction was carried out in a different room whilst gel electrophoresis was done in a separate area.

Other precautions were:

- Long sleeve lab coats and clean set of gloves when handling PCR reagents or equipment
- Aliquot all reagents to avoid contamination of stocks
- Aerosol guarded (filter) tips for pipetting
- Non reuse of tips and tubes.
- Decontamination as per lab policy and protocols

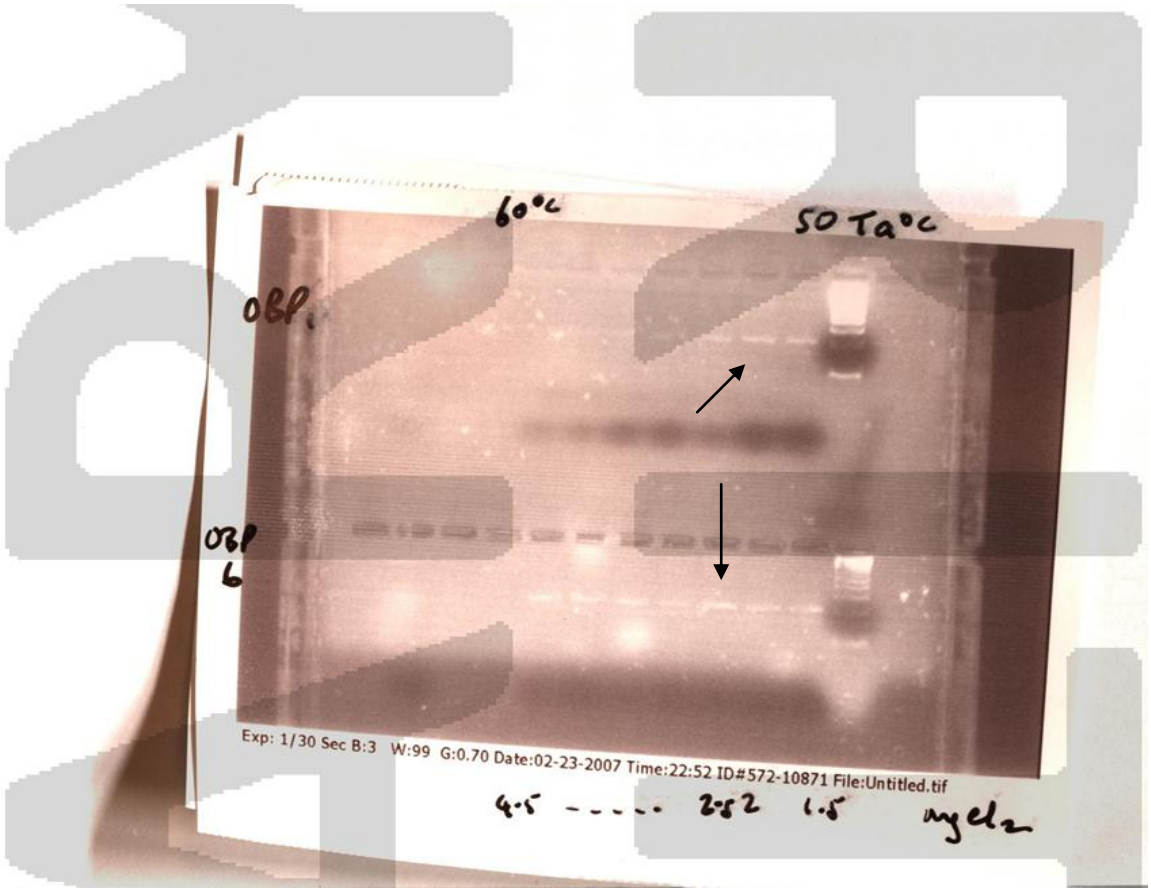

Figure 18: Design matrix for optimal annealing temperature (Ta) and $MgCl_2$. Arrows point to high signal intensity for optimal $MgCl_2$ and Ta combinations. OBP1 and OBP6 are identifiers for participants.

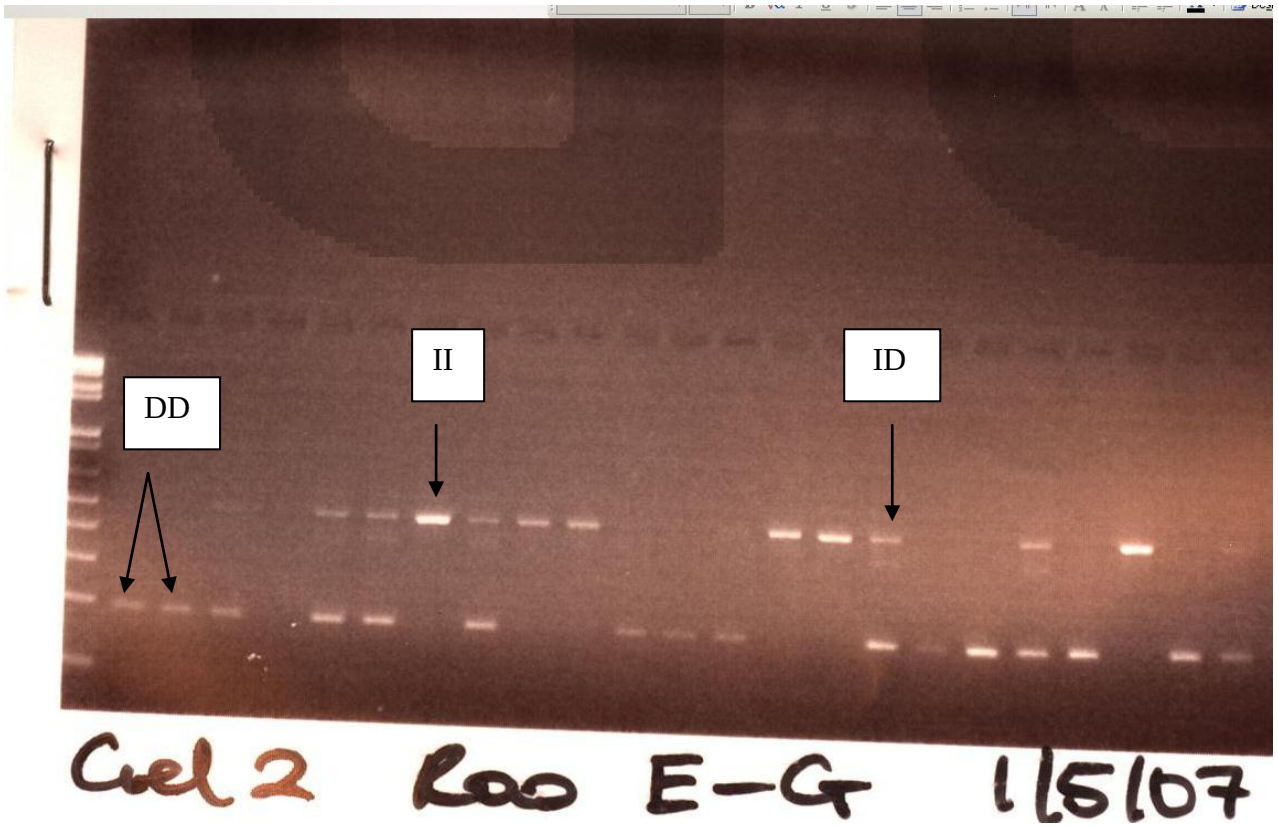

Figure 19: 2% Gel electrophoresis for ACE I/D. (II migrated at 480 bp and the D migrated at ~200bp)

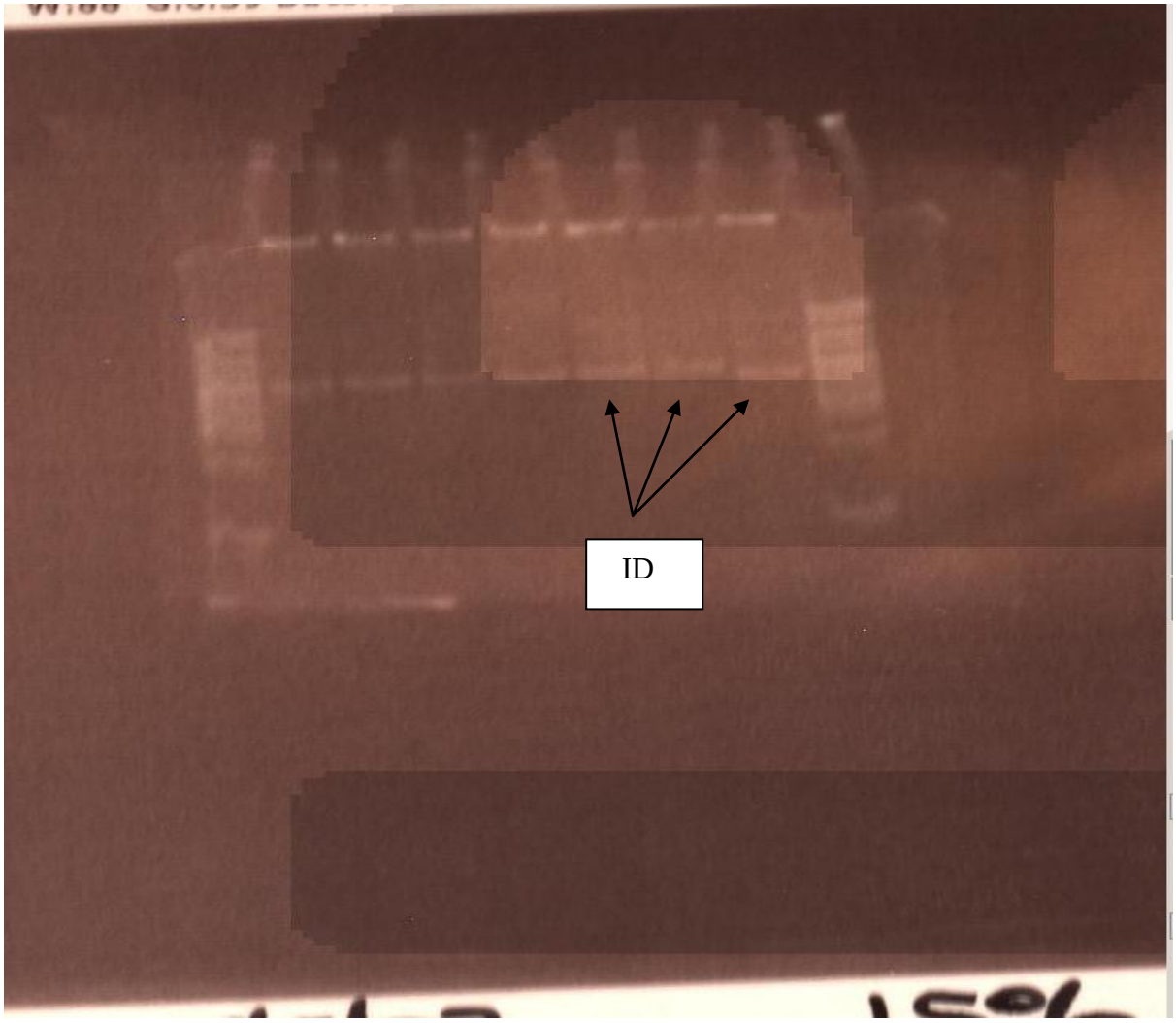

Figure 20: 2% gel electrophoresis for HACE. Only confirmed ID genotype showed bands (signals) when DD genotypes were repeated with confirmatory HACE step. True ID genotypes (falsely genotyped as DD in previous stage) will show signals as this step was specific for ID. True DD did not show any signal (not on this figure).

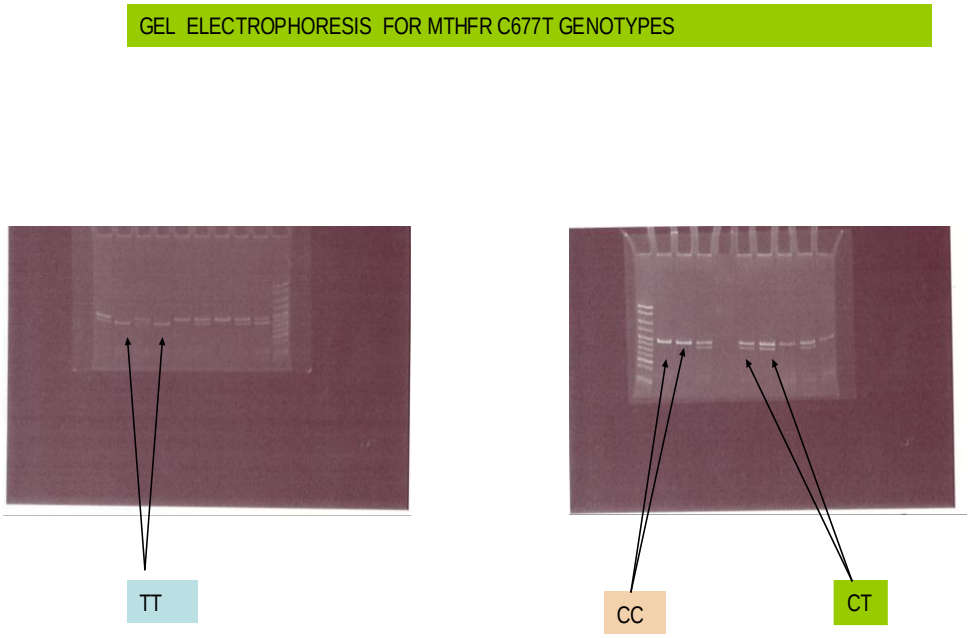

Figure 21: 2% gel electrophoresis for MTHFR C677T genotypes. CC migrates at 198 bp and TT at 176 bp.

3.2.3 Statistical analysis

All analyses were done in R software with user-written codes. Continuous variables were expressed as means (95% confidence intervals) and compared with student t-test. Allele frequencies were calculated from genotype frequencies and were compared using chi-squared (χ^2) statistics. *P* value < 0.05 was considered statistically significant.

3.2.4 Level of personal Involvement:

This was my project from conception to conclusion. The research topics were chosen after discussion with my supervisors. I did my literature review, calculated the different sample sizes and designed the study protocol with periodic discussion with my thesis committee from whom I received useful feedback. I recruited study subjects jointly with other researcher fellows who were conducting similar projects to mine in the unit. I introduced Mendelian Randomization as an underlying theory in genetic case-control studies in the AAA literature for the first time. To further contribute to the body of knowledge, I designed a matrix for a joint $MgCl_2$ and Ta as an optimization step for the two most important PCR variables. This has never been reported before. I also applied a second-stage confirmatory ACE I/D PCR step which is also a first in the ACE I/D-AAA association study. I designed the PCR steps (including component quantification) with the active involvement of the laboratory staff (Ms Julie Chamberlain and Jonathan Barber) both of whom instructed me in laboratory calculations and were also invaluable in checking my overnight PCR runs when I am otherwise engaged with clinical work or out recruiting participants. I also prepared my own gels for electrophoresis and recorded my data. I personally conducted the statistical analyses using programming codes which I wrote in R statistical programming software. The writing and editing were also my personal work.

Chapter 4. Results

There were in total 2095 subjects recruited of which there were 852 controls (41.85%) and 1184 cases (58.15%) (Table 11). (Only data for genotyped participants were included in analysis.) The mean age of the subjects was 70.21 years [95%CI, 64- 84years]. The mean age of controls and cases were 66.97 years [95%CI, 65 – 79 years] and 72.72 years [95%CI, 62 – 85 years], respectively suggesting that on the average, the cases were 6 years older. Males and females were coded as "0" and "1", respectively. In addition, covariates were coded as "1" if present or "0" if absent except for non-smoking, ex-smoking and current smoking which were coded as "0", "1" and "2", respectively. In addition ex-smoking and current smoking categories were further combined as ever_smoked with a category of "1" with non-smoking remaining "0" in this new variable.

Overall, 7% (137) of the subjects were females and 93% (1822) males implying that females constituted 2% (20) and 11% (117) of the controls and cases, respectively. Conversely, males constituted 98% (832) and 89% (990) of the controls and cases, respectively. Non-smokers, ex-smokers and current smokers constituted 19% (376), 20% (401) and 60% (1180), respectively of the study population. In addition, amongst controls, the same categories constituted 31% (267), 15% (127) and 54% (458), respectively whilst for the cases these were respectively, 10% (109), 25% (274) and 65% (722). There were disproportionately more ex-smokers and current smokers amongst cases compared to controls whilst there were more non-smokers amongst controls ($P<0.0001$).

When these figures are collapsed into a 2-level categorization, there were 19% (375) never smoked and 81% ever smoked (1582) amongst all subjects which translated into 90% (996) and 69% (586) of cases and controls had ever smoked compared to 10% (109) and 31% (266),

respectively who had never smoked. With this classification, subjects who had ever smoked were disproportionately represented in the sample ($P < 0.0001$). Of the total subjects, 19% (372) had ischaemic heart disease (MI) whilst 81% (1585) were free of ischaemic heart disease. Among controls, 10% (84) had MI and 90% (768) free of MI. The corresponding figures for cases were 26 % (288) and 74% (814), respectively implying that there were disproportionately more subjects with MI amongst the cases ($P< 0.0001$). There were more hypertensives amongst subjects representing 53% (1028) of all subjects with normotensives representing the remaining 47% (929). Amongst the controls, hypertensives accounted 39% (336) and normotensives for 61% (515). Conversely, hypertensives constituted 63% (692) of cases and normotensives 37% (414). The former were disproportionately more in cases compared to controls. There were 57% (984) cases and 43% (850) controls analyzed for Diabetes Mellitus out of whom there were 120 and 92 diabetics, respectively. The corresponding figures for cases were 57% (120) and 45% (92), respectively. The proportions of diabetics in cases were not significantly different from those in controls ($P=0.967$). Ninety-three percent (93%) of subjects (1684) did not have a family history (FH) of AAA compared to 7% (121) who had. Positive FH was present in 5% (38) and 7% (83) of controls and cases, respectively. Conversely, negative FH was obtained for 95% (745) and 93% (939) of controls and cases, respectively with a disproportionate representation of positive FH amongst cases ($P=0.007$). Of the total cohort, there were 60% (970) without hypercholesterolaemia and the remaining 40% (640) had no hypercholestrolaemia. The proportion of hypercholesterolaemia in controls and cases were 33% (221) and 45% (419), respectively. The corresponding respective proportions without hypercholesterolaemia were 67% (454) and 55% (516). Hypercholesterolaemia was disproportionately represented amongst cases ($P<0.0001$).

A visual representation of the risk factor distributions showed that there were more subjects amongst cases with HT who were smokers than could be expected under a model of independence of these two variables (Figure 22). Further quantitative analysis (Figure 23) showed that irrespective of smoking history, HT was significantly associated with AAA across strata of smoking suggesting that HT and Smoking could potentially be included in a logistic regression model with AAA as a dependent variable without biasing the results by confounding.

All available genotype data were analyzed. There were a total of 1829 subjects genotyped for the ACE I/D polymorphism (94%) of which there were 1017 cases and 812 controls (Table 12). The true (expected) genotype frequencies for II, ID and DD in controls [177 (179.71), 410 (404.58) and 225 (227.71)] and for cases [218 (228.91), 529 (507.17) and 270 (280.91)] were in Hardy-Weinberg Equilibrium (HWE), P=0.21. The total frequencies of the "D" and "I" alleles were 53% (1981) and 47% (1789), respectively. The "D" and "I" allele distributions in controls [53% (912) vs. 53% (1069)] and cases [47% (824) vs. 47% (965)] were not significantly different [OR, 1.001; 95%CI, 0.88 – 1.14; P=0.98] (Table 13).

The total II, ID and DD genotype distributions were 27% (507), 51% (969) and 22% (411), respectively (Figure 24; Tables 14 & 15). Furthermore, the ACE II, ID and DD genotype distributions in controls were 21.8% (177), 50.5% (410) and 27.7% (225). Similar distributions in cases were 21.4% (218), 52% (529) and 26.5% (270). There was no significant association between ACE I/D and AAA under a heterozygous (OR, 1.05; 95%CI, 0.83 – 1.33; P=0.70) or homozygous co-dominant model (OR, 0.97; 95%CI, 0.75 – 1.27; P=0.84). Similarly, under the recessive model (DD vs. ID +II), ACE I/D was not associated with AAA (OR, 0.94; 95%CI, 0.77 – 1.16; P=0.57). Furthermore, a dominant model did not show any significant association

between ACE I/D and AAA (OR, 1.02; 95%CI, 0.82 – 1.28; P=0.85). Adjustment for potential confounding variables did not alter these results.

A total of 1352 subjects were genotyped for MTHFR C677T polymorphism of which there were 674 controls and 678 cases (Figure 25;Table 16). The true (expected) frequencies of the CC, CT and TT genotypes for controls [358 (351.16), 257 (270.68) and 59 (52.16)] and cases [321 (321.67), 292 (290.67) and 65 (65.67)] were in HWE (P=0.4). The total "C" and "T" allele frequencies were 71% (1907) and 29% (797), respectively. The corresponding distributions in controls vs. cases were 72% (973) vs. 69% (934) and 28% (375) vs. 31% (422), respectively and were marginally different (OR, 1.172; 0.99 – 1.38; P=0.057].

The CC, CT and TT genotype distributions for MTHFR C677T were 50.3% (679), 40.5% (549) and 9.2% (124). The CC, CT and TT genotype distributions in controls were 53% (358), 38% (257) and 9% (59) and in cases were 47% (321), 43% (292) and 10% (65), respectively (Tables 17 & 18). Consequently, there is a significant association between MTHFR C677T with AAA under the heterozygote (CT vs. CC) co-dominant model (OR, 1.27; 95%CI, 1.01 – 1.59; P= 0.039) but not under the homozygote (TT vs. CC) co-dominant model (OR, 1.23; 95%CI, 0.84 – 1.80; P=0.29). With correction for multiple testing, P =0.104. There was no significant association under a recessive (TT vs.CT+CC) model (OR, 1.11; 95%CI, 0.76 – 1.60; P=0.59). Conversely, under the dominant model (TT+CT vs.CC), MTHFR C677T was significantly associated with AAA (OR, 1.26; 95%CI, 1.02 – 1.56; P=0.034). The Armitage trend test for the whole analyses was marginally significant (P=0.062). Interestingly, adjusting for potential confounders eroded these significant associations for the heterozygote co-dominant (OR, 1.29; 95%CI, 0.97 – 1.71) and dominant model (OR, 1.26; 95%CI, 0.96 – 1.65; P=0.089).

There is no evidence for an epistatic effect (P=0.41) of the recessive ACE I/D within MTHFR CC (OR, 0.80; 95%CI, 0.51-1.26), CT (OR, 0.97; 95%CI, 0.60 – 1.57) and TT (OR, 1.32; 95%CI, 0.50 – 3.5) (Table 19). Similarly, there was no evidence (P=0.81) for a dominant epistatic effect within MTHFR genotypes, CC (OR, 1.0; 95%CI, 0.62 – 1.61), CT (OR, 0.77; 95%CI, 0.45 – 1.24) and TT (OR, 1.25; 95%CI, 0.40 – 3.94) (Table 20). Furthermore, there was no significant (P=0.40) epistatic interaction between recessive MTHFR C677T within ACE I/D genotypes II (OR, 0.72; 95%CI, 0.25 – 2.1), ID (OR, 0.91; 95%CI, 0.44 – 1.91) and DD (OR, 1.26; 95%CI, 0.55 – 2.9) (Table 22). In addition, there was no evidence (P = 0.90) for epistatic interaction between dominant MTHFR within ACE I/D genotypes II (OR, 1.57; 95%CI, 0.87 – 2.84), ID (OR, 1.13; 95%CI, 0.77 – 1.67) and DD (OR, 1.59; 95%CI, 0.93 – 2.71) (Table 21).

A secondary random effect (RE) meta-analysis (M-A) under the allele difference model, showed a significant association between ACE I/D and AAA (OR, 1.22; 95% CI, 1.03 – 1.44; P= 0.017) for a total of 3853 controls and 3345 cases (Table 23). In addition, a secondary RE M-A for MTHFR C677T showed a significant association (OR, 1.26; 95%CI, 1.02 – 1.59; P=0.03) for 2082 controls and 2264 cases (Table 24). A cumulative RE M-A for MTHFR C677T showed that the evidence for significant association was positively altered with this study.

Table 11: Demographic and co-morbidity data

Variables	Categories	Controls (N=852)	Cases (N=1184)	P values
Gender/Sex	M(0)	832	990	
	F(1)	20	117	<0.0001
Age (Years)	Mean 95%CI)	66.97 (65-79)	72.72 (62-85)	<0.05
Hypertension (BP)	0	515	414	
	1	336	696	<0.0001
Myocardial Infarction	0	768	817	
	1	84	288	<0.0001
Family History (FH)	0	745	939	
	1	38	83	0.007
Hypercholesterolaemia	0	454	516	
	1	221	419	<0.0001
Smoking	0	266	109	
	1	127	274	
	2	458	722	<0.0001
Ever_smoked	0	266	109	
	1	586	996	<0.0001
Diabetes Mellitus	0	758	864	
	1	92	120	0.967

[From the above table, the total figures for controls and cases were not all genotyped and some variables also had missing data which overall were less than 5% and did not affect the overall effect sizes. Categorical variables were dichotomized into"0" for absence and "1" for present except for a 3-level "0" (non-smoking), "1" (ex-smoking) and "2" (current smoking) for smoking. All categorical variables were compared with Chi-square test with a $p<0.05$ accepted as indicative of significant difference. The continuous variable age was compared with t-test and $p<0.05$ was similarly accepted for significant difference between groups.]

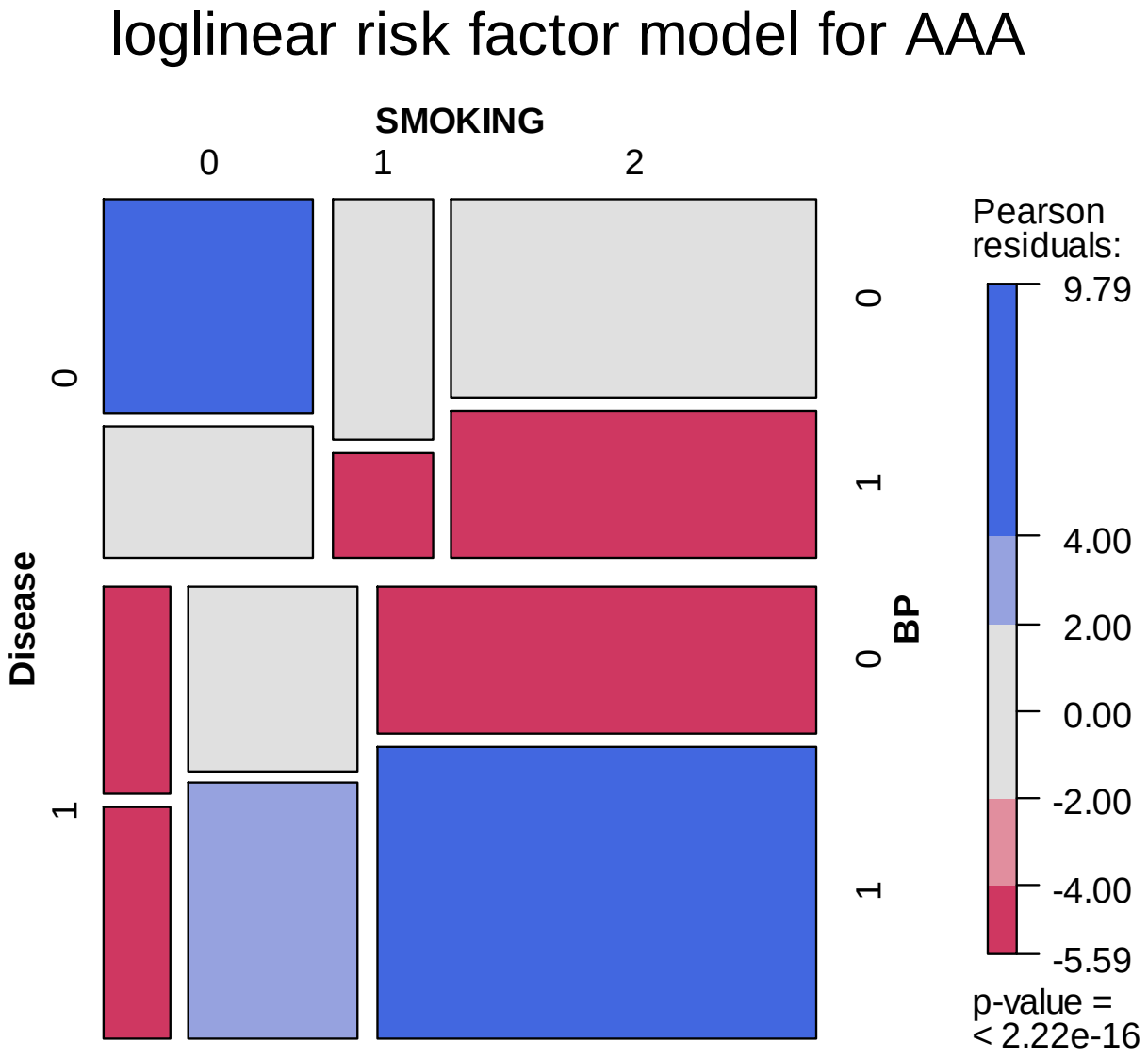

Figure 22: Mosaic plot of AAA (Disease) and HT (BP) and Smoking history. This plot is a visual display of interaction amongst variables stratified by a dependent variable under the null hypothesis of independence (no interaction) within the population. Significant deviation from independence is indicated by colour coding the direction of which is measured by the colour bar on the right. This means that persons who are current smokers are most likely to have HT amongst cases with Pearsons' residuals (PR) > 4.0 (95%CI significance vary ≤-2.0 and ≥+2.0). Similarly, ex-smokers were also likely to have HT among cases (PR>+2.0). Similarly, non-smokers were most likely to by normotensive amongst controls (PR>+4.0). Conversely, non-smokers with HT and normotensives who are current smokers were also less likely to have AAA (PR< -2.0).

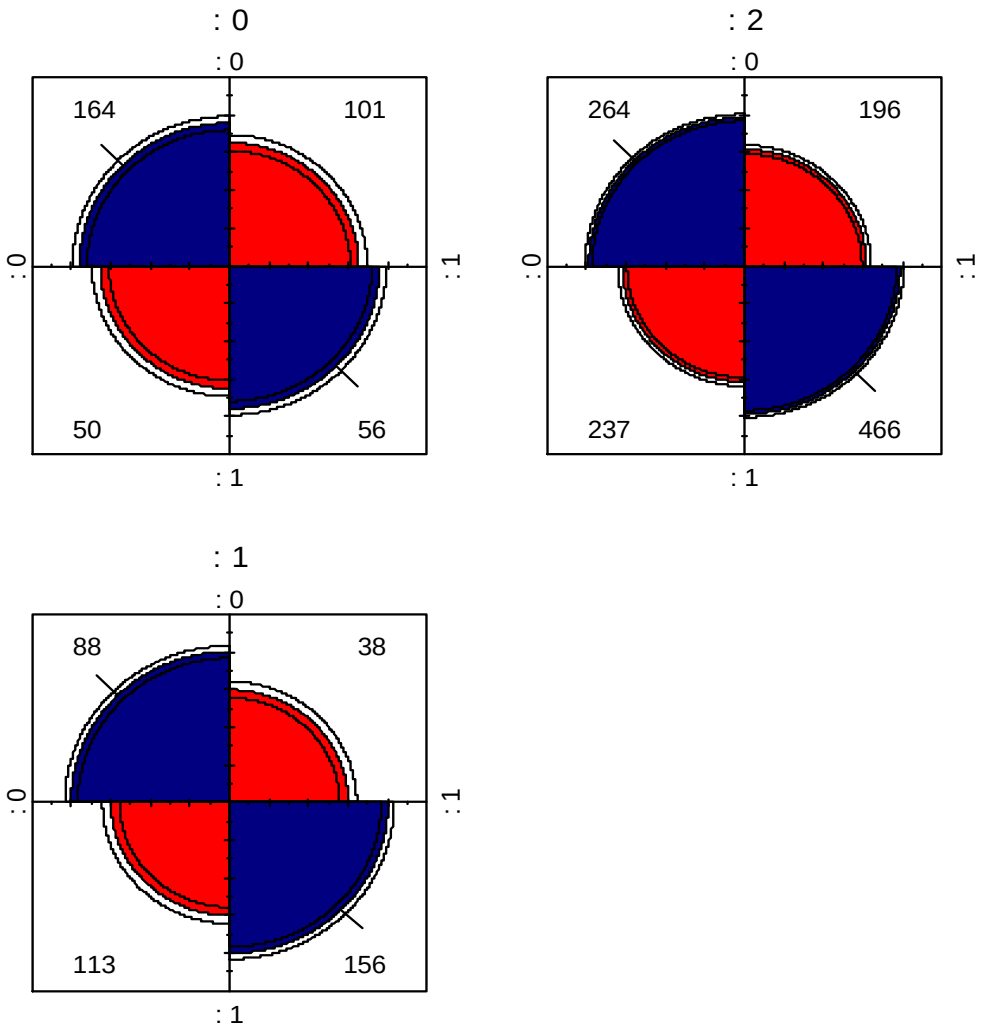

Figure 23: Four-fold plot of AAA vs. HT nested within Smoking. **The four-fold plot is a visual representation of the odds ratio (OR) of a 2 x 2 x k contingency table where "k" represents strata or populations and is of interest to see if the association between the first 2 variables are homogenous across strata (not confounded). The frequencies, f (i, j) of each cell is standardized whilst preserving the OR. The area of each quarter circle is proportional to this standardized frequency. If the quarter circles align, it means the OR is 1. Furthermore, the arcs of the quarter circles represent 99% CI rings. If these rings do not overlap then the ORs are significantly different from 1. Each of the large squares above represent different strata of smoking (non-smokers = 0, ex-smokers = 1 and current smokers = 2). AAA (rows) is further stratified by HT (columns) within smoking strata. Within non-smoking category (first square), AAA are represented by 50 +56 cells whilst controls are represented by 164 + 101 cells. HT (columns) is represented by 56 + 101 cells and non-HT is represented by 50 + 164. Therefore odds of AAA with HT is 50/101 and odds of without HT is 56/164 equating to OR of 1.77. The same principle applies to other strata of smoking.**

Table 12: Allele and Genotype distributions for ACE I/D and MTHFR C677T Polymorphisms

Variables	Categories	Controls (N=852)	Cases (N=1184)	HWE,P
ACE I/D	DD	225 (0.28)	270 (0.27)	
	ID	410 (0.50)	529 (0.52)	
	II	177 (0.22)	218 (0.21)	
	D	0.53	0.53	
	I	0.47	0.47	
HWE,P		0.73	0.19	0.21
MTHFR	CC	358 (0.53)	321 (0.47)	
	CT	257 (0.38)	292 (0.43)	
	TT	59 (0.09)	65 (0.10)	
	T	0.28	0.31	
	C	0.72	0.69	
HWE,P		0.21	1	0.4

Table 13: ACE I/D distributions

Alleles	Total (1829)	Controls (N=812)	Cases (N=1017)
D	1981(53%)	912(53%)	1069(53%)
I	1789(47%)	824(47%)	965(47%)
D vs. I	OR=1.001 [0.88-1.14]		
	P=0.98		

Table 14: ACE I/D tests of association

ACE I/D Polymorphism in Association with AAA

Model	Controls	%	Cases	%	OR	95% Conf. Interval	P-value	AIC
Codominant								
II	177	21.8	218	21.4	1			
ID	410	50.5	529	52	1.05	0.83 - 1.33		
DD	225	27.7	270	26.5	0.97	0.75 - 1.27	0.796	2518
Recessive								
II + ID	587	72.3	747	73.5	1			
DD	225	27.7	270	26.5	0.94	0.77 - 1.16	0.579	2516
Dominant								
II	177	21.8	218	21.4	1			
ID + DD	635	78.2	799	78.6	1.02	0.82 - 1.28	0.852	2516
Overdominant								
II + DD	402	49.5	488	48	1			
ID	410	50.5	529	52	1.06	0.88 - 1.28	0.517	2516
Log-Additive								
0,1,2	812	44.4	1017	55.6	0.98	0.86 - 1.12	0.807	2516

Table 15: Adjusted ACE I/D tests of association

ACE I/D Polymorphism in Association with AAA adjusted by smoking, Age, MI, HT, Sex

Model	Controls	%	Cases	%	OR	95% Conf. Interval	P-value	AIC
Codominant								
II	159	2.9	184	22.7	1			
ID	364	50.1	415	51.3	0.95	0.71 - 1.27		
DD	204	28.1	210	26	0.87	0.62 - 1.21	0.702	1704
Recessive								
II + ID	523	71.9	599	74	1			
DD	204	28.1	210	26	0.9	0.70 - 1.18	0.45	1702
Dominant								
II	159	21.9	184	22.7	1			
ID + DD	568	78.1	625	77.3	0.92	0.70 - 1.21	0.549	1702
Overdominant								
II + DD	363	49.9	394	48.7	1			
ID	364	50.1	415	51.3	1.02	0.81 - 1.29	0.868	1702
Log-Additive								
0,1,2	727	47.3	809	52.7	0.93	0.79 - 1.10	0.405	1702

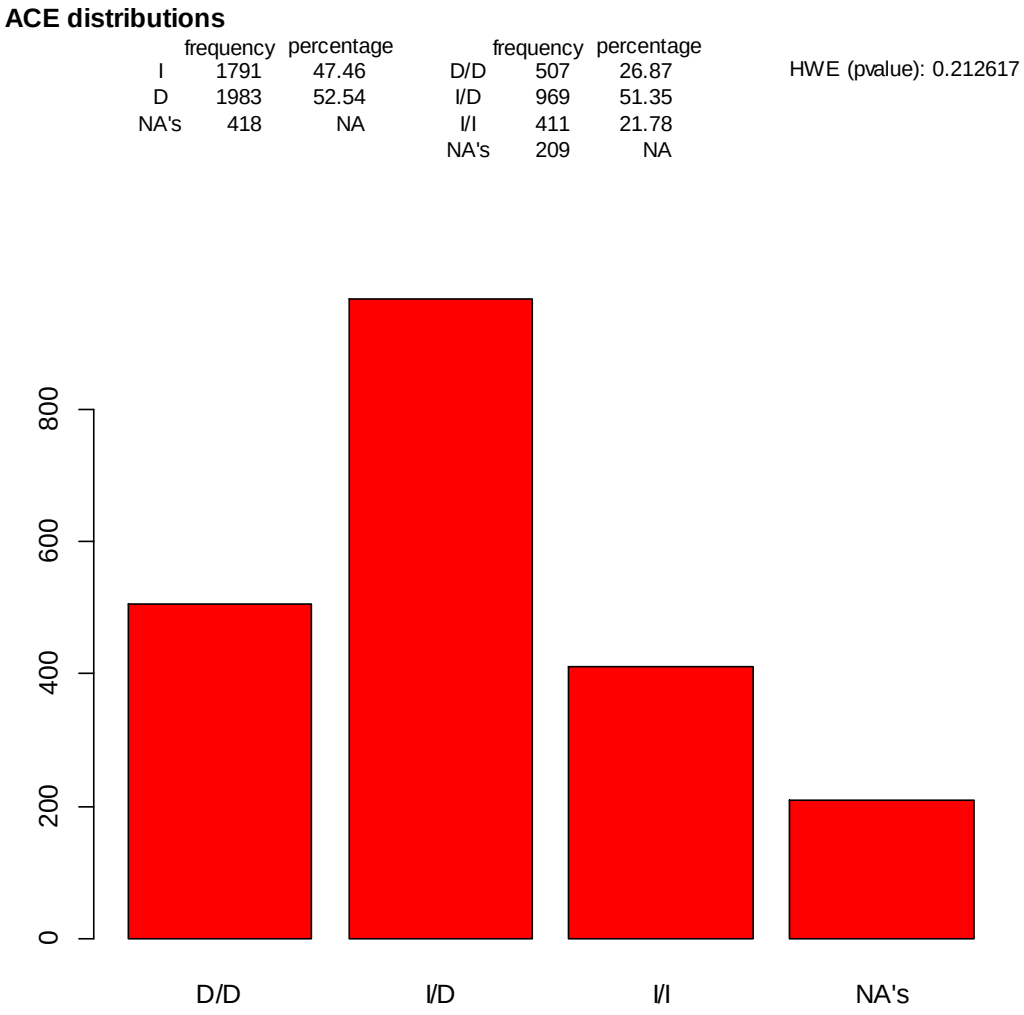

Figure 24: ACE I/D polymorphism distributions. The NA represent subjects that were not genotyped. The latter did not affect the results as the study was significantly powered.

MTHFR distributions

	frequency	percentage
C	1907	70.53
T	797	29.47
NA's	1488	NA

	frequency	percentage
C/C	679	50.22
C/T	549	40.61
T/T	124	9.17
NA's	744	NA

HWE (pvalue): 0.395276

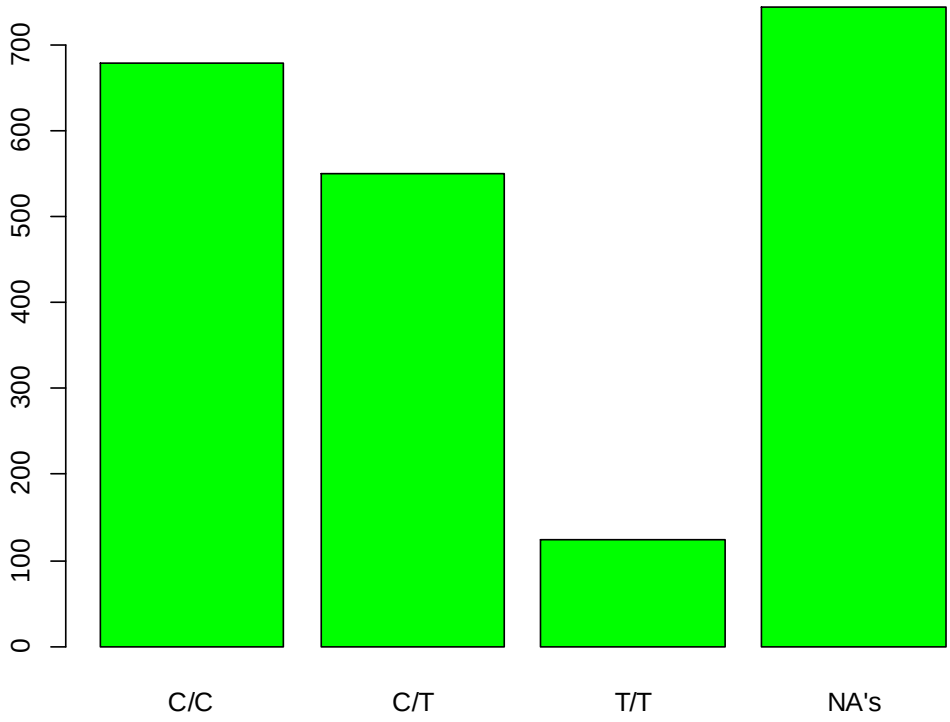

Figure 25: Distributions of MTHFR C677T genotypes. NAs as before represent those subjects that were not genotyped and do not form part of the analysis.

Table 16: MTHFR Allele distributions

Alleles	Total (N=1352)	Controls (N=674)	Cases (N=678)
C	1907 (71%)	973 (72%)	934 (69%)
T	797 (29%)	375 (28%)	422 (31%)
C vs. T	OR=1.172 (95%CI,0.99-1.38) P=0.057		

Table 17: MTHFR C677T tests of association

MTHFR C677T Polymorphism in Association with AAA

Model	Controls	%	Cases	%	OR	95% Conf. Interval	P-value	AIC
Codominant								
CC	358	53.1	321	47.3	1			
CT	257	38.1	292	43.1	1.27	1.01 - 1.59		
TT	59	8.8	65	9.6	1.23	0.84 - 1.80	0.104	1876
Recessive								
CC + CT	615	91.2	613	90.4	1			
TT	59	8.8	65	9.6	1.11	0.76 - 1.60	0.596	1878
Dominant								
CC	358	53.1	321	47.3	1			
CT + TT	316	46.9	357	52.7	1.26	1.02 - 1.56	0.034	1874
Overdominant								
CC + TT	417	61.9	386	56.9	1			
CT	257	38.1	292	43.1	1.23	0.99 - 1.53	0.064	1875
Log-Additive								
0,1,2	674	49.9	678	50.1	1.17	0.99 - 1.38	0.063	1875

Table 18: Adjusted MTHFR C677T tests of association

MTHFR C677T Polymorphism in Association with AAA adjusted by smoking, Age, MI, HT, Sex

Model	Controls	%	Cases	%	OR	95% Conf. Interval	P-value	AIC
Codominant								
CC	318	54	284	47.3	1			
CT	221	37.5	261	43.5	1.29	0.97 - 1.71		
TT	50	8.5	55	9.2	1.15	0.70 - 1.87	0.214	1294
Recessive								
CC + CT	539	91.5	545	90.8	1			
TT	50	8.5	55	9.2	1.02	0.64 - 1.65	0.922	1295
Dominant								
CC	318	54	284	47.3	1			
CT + TT	271	46	316	52.7	1.26	0.96 - 1.65	0.089	1292
Overdominant								
CC + TT	368	62.5	339	56.5	1			
CT	221	37.5	261	43.5	1.26	0.96 - 1.66	0.095	1293
Log-Additive								
0,1,2	589	49.5	600	50.5	1.15	0.94 - 1.42	0.176	1294

Table 19: Interaction between recessive ACE I/D and MTHFR

Multivariate adjusted Recessive ACE I/D within MTHFR

	Controls	Cases	OR	95% Conf. Interval
MTHFR_CC				
II + ID	229	189	1	
DD	85	62	0.8	0.51 - 1.26
MTHFR_CT				
II +ID	154	175	1	
DD	64	64	0.97	0.6 - 1.57
MTHFR_TT				
II + ID	34	30	1	
DD	16	19	1.32	0.5 -3.5

Interaction p-value: 0.41909

Table 20: Interaction between dominant ACE I/D within MTHFR

Multivariate adjusted Dominant ACE I/D within MTHFR

	Controls	Cases	OR	95% Conf. Interval
MTHFR_CC				
II	63	53	1	
II + ID	251	198	1	0.62 - 1.61
MTHFR_CT				
II	45	54	1	
ID +DD	173	185	0.774	0.45 - 1.24
MTHFR_TT				
II	12	11	1	
ID + DD	38	38	1.25	0.4 - 3.94

p trend: 0.818

Table 21: Adjusted Dominant MTHFR within ACE I/D

Multivariate adjusted Dominant MTHFR within ACE I/D

	Controls	Cases	OR	95% Conf. Interval
ACE_II				
CC	63	53	1	
CT + TT	57	65	1.57	0.87 - 2.84
ACE_ID				
CC	166	136	1	
CT + TT	131	140	1.13	0.77 - 1.67
ACE_DD				
CC	85	62	1	
CT + TT	80	83	1.59	0.93 - 2.71

p trend: 0.901

Table 22: Adj. Recessive MTHFR within ACE

Multivariate adjusted Recessive MTHFR C677T within ACE I/D

	Controls	Cases	OR	95% Conf. Interval
ACE_II				
CC+ CT	108	107	1	
TT	12	11	0.72	0.25 - 2.1
ACE_ID				
CC + CT	275	257	1	
TT	22	19	0.91	0.44 - 1.91
ACE_DD				
CC + CT	149	126	1	
TT	16	19	1.26	0.55 - 2.9

p trend: 0.404

Table 23: Secondary meta-analysis for ACE I/D-AAA studies

Study	Fixed-effects Ors	95% Conf. Intervals		Study weights
Hamano, 1999	0.7511	0.5391	1.0463	34.9482
Pola, 2001	1.6153	1.1361	2.2966	31.0227
Fatini, 2005	2.1539	1.4312	3.2416	22.9885
Walcszewski, 2007	2.1577	1.3364	3.4838	16.7384
Jones;NZ, 2008	1.0036	0.8319	1.2107	109.1309
Jones;UK, 2008	1.0891	0.9045	1.3112	111.4245
Jones; Aus, 2008	1.0954	0.8856	1.3548	85.0182
Lucarini, 2009	1.6801	1.2825	2.2006	52.7196
Korcz, 2009	1.0546	0.8064	1.3791	53.3737
Obukofe, 2009	0.9885	0.8658	1.1285	218.8962

Fixed effects OR=1.1158(95%CI, 1.0381-1.1994; P=0.0029)
Q statistics (Chi-sq)=40.4764; P=1.00E-05

Random effects OR=1.2225 (95%CI,1.0358 -1.4428; P=0.0174)

Table 24: Secondary meta-analysis for MTHFR C677T-AAA association studies

Study	Fixed-effects Ors	95% Conf. Intervals		Study weights
Brunelli, 2000	1.4496	0.8646	2.4303	14.3887
Strauss, 2003	2.1203	1.2442	3.6132	13.5201
Sofi, 2005	1.2319	1.0177	1.4913	105.2504
Jones, 2005	1.0161	0.8073	1.2786	72.7043
Peeters, 2007	1.3309	0.8441	2.0982	18.5342
Ferrara, 2007	3.5238	1.7801	6.9757	8.2372
Giusti, 2008	0.7804	0.6443	0.9452	104.6151
Obukofe, 2009	1.1699	0.9913	1.3805	140.1175
Fixed effects	1.112	1.0158	1.2155	P = 0.021
Q statistics (Chi-sq)	33.37			P = 2E-05
Random effects	1.2696	1.0163	1.5861	P = 0.035

[Secondary Random Effects (RE) Meta-Analysis (M-A) for the two polymorphisms were conducted by combining the sample sizes of these two polymorphisms with previous studies. For both the ACE I/D-AAA and MTHFR C677T-AAA association studies, the pooled estimates showed positive associations as indicated by the respective REs. Furthermore, since all previously combined studies for the latter polymorphisms showed a negative pooled association, a Cumulative RE M-A will necessarily show that the evidence for this polymorphism was significantly altered by this study.]

Chapter 5. Discussion

AAA is not formed from a single process but from a combination of different self-organizing complex (mal-) adaptive pathophysiological processes including haemodynamic and biomechanical interactions, aortic wall material property dysfunction, genetic and molecular network interacting simultaneously within a milieu of individual specific risk factors or predisposing factors.[366] Accumulating evidence also suggests that matrix metalloproteinases, inflammatory genes and their polymorphisms and autoimmune processes may be causal in the pathogenesis of AAA.[367] Furthermore, a recent review also suggested a potential role for epigenetic phenomena (non-genetic inherited predisposition to disease).[368] The latter becomes more instructive when a consistent pattern of genetic inheritance is elusive. The clustering of the disease within families suggested a genetic predisposition. Interestingly, recent studies have indeed showed that genetic factors account for 70-80% of the phenotypic variance.

Nevertheless, recent genome-wide association studies have identified very few predisposing loci some of which also suffer the fate of inconsistent replication emblematic of some previous genetic association studies. The MTHFR C677T and ACE I/D genetic polymorphisms which were the subject of this study were recently shown to be associated with AAA in two recent meta-analyses.[369, 370]

The ACE I/D polymorphism was not independently associated with AAA in this study. Furthermore, there was no evidence of epistatic interaction between ACE I/D and MTHFR C677T in this sample population with AAA. The results of this study are consistent with those of another large population case control study which was also a negative study for the individual populations [New Zealand (n=903), UK (n=1194) and Australia (n=691)] considered using the

co-dominant model.[371] However, combining all three populations (N=2788) showed a heterozygote co-dominant ACE I/D association with AAA (OR, 1.33; 95%CI. 1.06 - 1.67; P<0.02). Interestingly, a study of 58 AAA patients did not show any differential expansion rates amongst different ACE I/D genotypes.[372] In this study, the mean baseline AAA diameter was 4.3 cm with an expansion rate of 0.35cm/year obtained by linear regression after a 28-month follow-up. The genotype –specific expansion rates for the DD (n=14, 24%), ID (n=29, 50%) and II (n=15, 26%) genotypes were 0.22cm/year, 0.32cm/year and 0.30cm/year with no difference between groups (analysis of variance, p=0.6).

In contrast, MTHFR C677T was significantly associated with AAA on univariate test of association with allele difference, heterozygote co-dominant and dominant genetic models of associations. These effects disappeared when adjusted for potential confounders and multiple comparison corrections. This is arguably the largest completed study of its kind and similar lack of association was found with the moderately large studies of Giusti et al[285] and Sofi et al.[286]

The lack of significant associations between these polymorphisms and AAA in this study notwithstanding, there is compelling evidence implicating these genetic polymorphisms in the pathogenesis of AAA. From a biological perspective, these polymorphisms participate in molecular pathophysiological process which have been shown to be elemental to the pathogenesis of AAA. These processes include aortic wall inflammation and inflammatory cell recruitment, aortic wall oxidative stress, VSMC abnormalities (apoptosis, migration, senescence, and matrilysis) and adventitial remodelling.

The association between MTHFR C677T polymorphism and AAA could be a consequence of the previously little known global hypomethylation consequent upon on the reduced SAM/SAH ratio and second from the thermolability of the MTHFR enzyme resulting in HHcy. Mild HHcy (≥ 15μmol/L) is independently associated with AAA expansion (β, 0.03; 95%CI, 0.013-0.065; P=0.004).[373] It participates in the pathogenesis and expansion of the AAA (Figure 26) through pathological remodelling of all the layers of the aortic wall by inducing oxidative stress, increasing expression of proteolytic enzymes, cellular senescence, VSMC migration and apoptosis, pro-inflammatory signalling and inflammatory cell recruitment and adventitial remodelling.[374]

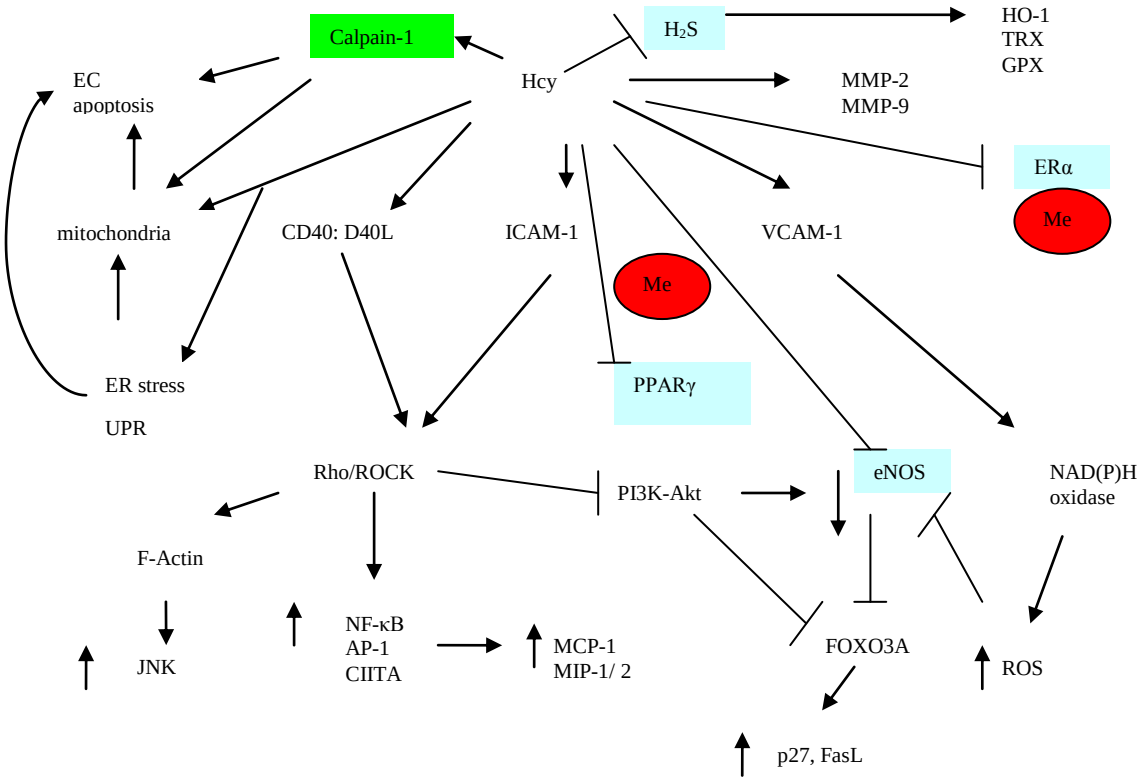

Figure 26: Signalling pathways of Hcy in AAA pathogenesis. The red ellipses show how Hcy can suppress PPARγ, ERα and eNOS to cause vascular injury since these three gene products predominantly mediate vascular protection. The mechanism of suppression is through gene promoter hypermethylation. Hcy can also suppress gaso-mediators like H_2S and carbon monoxide (CO) which like nitric oxide (NO) are vasculo-protective. Other mechanisms of vascular injury include senescence and apoptosis induced by suppression of the phosphoinositide -3-kinase (PI3K)-Akt survival pathway. Another increasingly recognized mechanism of Hcy-induced apoptosis in the vascular system is via the endoplasmic reticulum (ER)-associated unfolded protein response (UPR). Hcy can also activate pathways leading ultimately to the generation of ROS and other pro-inflammatory mediators like JNK MAPK and NF-κB

Homocysteine dependent VSMC migration is induced via several processes. Hcy induces the formation of MMP-2 and -9 from VSMC via NMDA receptors –dependent signalling.[375, 376] This may be associated with an NAD(P)H Oxidase - dependent generation of ROS.[377] The MMP-2 is vital for VSMC migration to the intima from the media via enlarged fenestrae in IEL. Similarly, Hcy induced resistin from Periadventitial Adipose Tissue (PAAT) also stimulates VSMC migration in a process dependent on upregulation of resistin.[378] It is noteworthy that resistin is significantly associated with AAA in obese males.[379]

Similarly, there was an association between serum adiponectin with AAA ≥ 3cm (OR, 1.26; 95%CI, 1.07-1.50; P) but not > 4cm (OR, 1.03; 95%CI, 0.77-1.39) but did not show any independent association with aortic diameter (β=0.06, P=0.07). This might have been an induced counter-regulatory response because adiponectin antagonizes the molecular pathophysiological processes operative in pathogenesis of AAA. Adiponectin stimulates endothelial proliferation whilst concomitantly suppressing EC apoptosis, mobilizes and activates EPC and modulate EC NO through an Akt-eNOS pathway;[380] suppress VSMC phenotypic switch;[381] suppress ceramide-dependent apoptosis,[382] and suppress ROS generation and NF-κB activation.[383] Interestingly, adiponectin expression from adipocytes is suppressed by Hcy[384] and inversely correlated with the 'T' allele of MTHFR C677T polymorphism in women.[385] Another possible mechanism of HHcy dependent VSMC migration is through the activation of calpain which promotes the VSMC invasive phenotype by increasing MMP-2 and cleaving intracytoplasmic structural proteins like vimentin, spectrin and α-SMA to dislodge VSMC from ECM anchorage.[386]

Another homocysteine –mediated link between oxidative stress and the pro-inflammatory is through the activation of the CD40-CD40Ligand pathway.[387] And the latter is implicated in the

pathogenesis of AAA possibly through the upregulation ROS-dependent NF-κB, MCP-1, MIF-1, VEGF, p38 MAPK, VCAM-1, MMP-2 and MMP-9.[388,389, 390] Similarly, Hcy also stimulate the production of IL-6 from VSMC via a NF-κB dependent pathway.[391] Similarly, Hcy-induced calcium sensing protein, S100A12, is associated with pro-inflammatory signalling and upregulation of MMP-9.[392] Furthermore, S100A12 is implicated in the pathogenesis of aortic aneurysms in transgenic murine studies.[393] Interestingly, Hcy also simulate the expression of another calcium sensing enzyme, calpain,[394] which is involved in pro-inflammatory signalling and oxidative stress,[395] aortic wall remodelling,[396] and VSMC migration and apoptosis in AAA pathogenesis in murine models.[397, 398] Mechanistic studies suggested that their role may involve the recruitment of CD68+ MØ and increased expression of MMP-12 in the aortic aortic wall.

Accumulating knowledge is consistent with the fact that MMP-9 genetic expression is epigenetically regulated through promoter methylation. Promoter methylation is associated with gene silencing. In contrast, reduced promoter methylation will result in increased gene expression. In addition, the chromosomal locus at 11q22 contains a cluster of MMPs like MMP-1, MMP-7, MMP-9, MMP-10, MMP-12, and MMP-13 which are collectively regulated by DNA methylation.[399] It is possible to speculate that MTHFR C677T global hypomethylation could lead to increased expression from this locus. Furthermore, DNA methylation is present at the MMP-9,[400] and TIMP-2[401] promoters.

Current evidence suggests that homocysteine affects the expression of the peroxisome proliferator activator receptors (PPAR) (PPARα and PPARγ) by promoter methylation induced silencing.[402] These two nuclear receptors belong to a group of endogenous transcription factors which are implicated in glucose and lipid metabolism. They function endogenously as anti-inflammatory,[403, 404] antioxidants and antihypertensives.[405] PPARγ has been associated with

attenuation of AAA in experimental murine AAA model possibly due to inhibition of osteopontin-mediated oxidative stress-dependent MMP-9 and suppression of Cathepsin S in MØ and VSMC, respectively.[406, 407] MTHFR C677T can also contribute to AAA formation by DNA methylation of the oestrogen ERα receptor.[408] The latter has been mechanistically linked to the attenuation of AAA in experimental murine AAA models. [409,410] ER mediates these functions by suppressing MMP-2, MMP-9 and oxidative stress , inflammatory and senescence in the aortic wall.[411] Similarly, MTHFR C677T suppresses Heme Oxygenase-1 (HO-1) by DNA promoter methylation.[412] This enzyme induces the formation of carbon monoxide (CO), a protective anti-inflammatory, antioxidant and anti-apoptotic gaso-mediator in the vasculature (Figure 27).[413]

The PAAT is a rich source of H_2S which is suspected to be the adipose tissue derived hyperpolarizing relaxing factor (ADHRF) due to its ability to activate membrane K_{ATP} channels in VSMC to cause relaxation.[414] Interestingly, the H_2S was also shown to reduce the aortic concentration of Angiotensin II in vitro. In the HHcy rat model (high methionine diet), it was demonstrated that HHcy was associated with a 43% decrease in H_2S content respectively in rat cardiac tissue.[415] The HHcy-mediated suppression of H_2S is associated with MCP-1 and MIP expression.[416] Current evidence suggests that H_2S attenuates atherosclerosis,[417] through its antioxidant and anti-inflammatory properties. It is equally likely that H_2S will attenuate AAA formation. Without an effective antioxidant defense system, the EC ROS activate the NF-κB pathway to induce the expression of IL-6, $TNF_{\alpha/\beta}$, IL-1, IL-17 and iNOS.[418] It is possible to speculate that the beneficial effects of statins in attenuating AAA expansion could be due in part to upregulation of these gaso-mediators. Statins have been shown to increase H_2S (~70%),[419] HO-1(~30%) and NO (100%) in the aorta of murine models.[420]

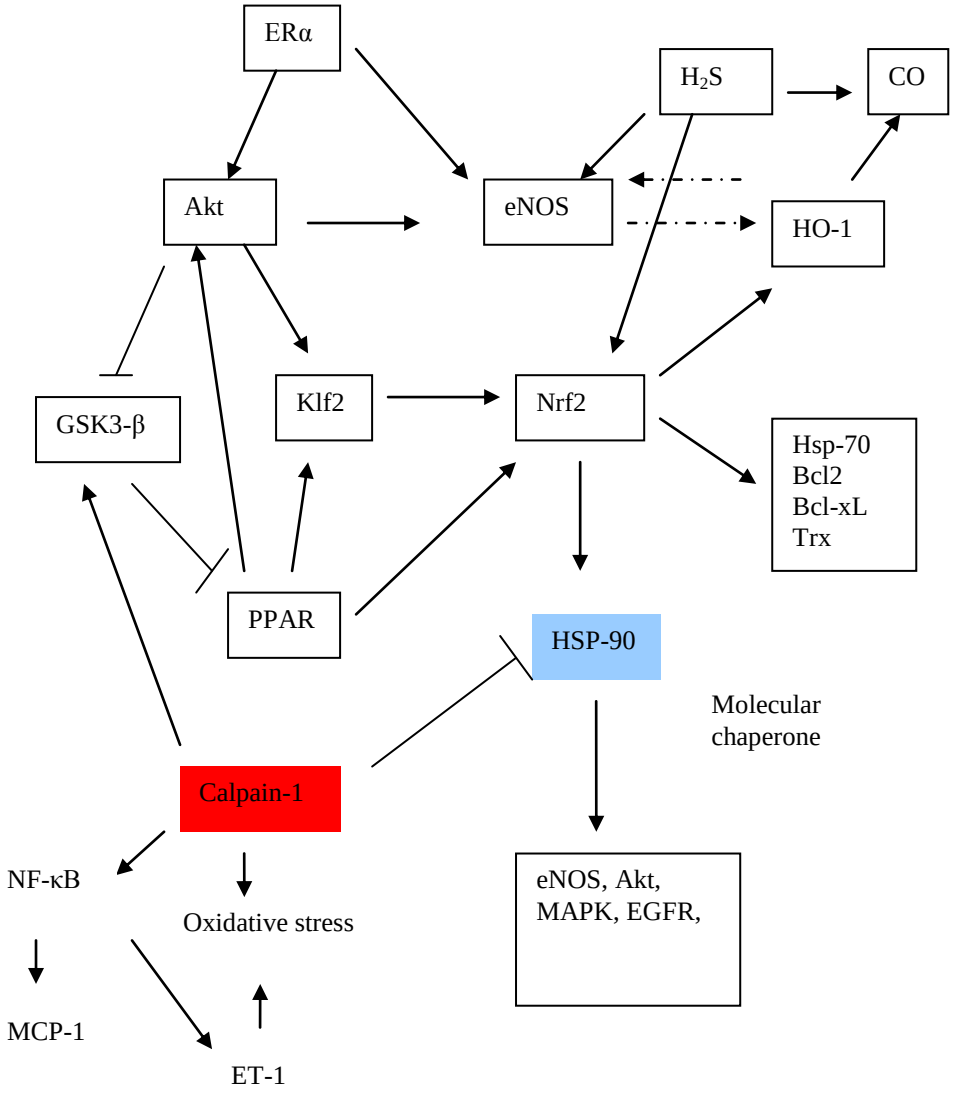

Figure 27: Homocysteine-dependent gaso-mediator pathophysiology in putative AAA pathobiology. The gaso-mediator loop involving the three vascular protective endogenous gases (NO, CO and H_2S) is shown with links to other protective mechanism. Nrf2 is a an anti-oxidant transcription factor which when induced by H_2S and kruppel-like factor 2 (KLF2) induces the expression of other protective enzymes like the heat shock proteins HSP-70 and -90 and anti-apoptotic proteins of the Bcl2 family. HSP-90 is the only known mammalian molecular buffer with thousands of known client proteins ranging from

surface membrane receptors to signal transduction proteins and nuclear transcription factors and receptors. Hence a compromised function has far reaching consequences as seen in the unfolded Protein Response attributable to Hcy-induced apoptotic process. Calpain-1 a calcium –dependent effector protein, is induced and activated byHcy and has been associated with AAA in some recent studies. Calpain cleaves HSP-90 resulting in up to 30% loss of function and through upregulation of GSK3β suppress the vasculo-protective PPARγ. Calpain-1 is also associated with inhibition of th prosurvival Akt signalling pathway.

Hcy recruits and activates neutrophils by binding to surface adenosine (A1, A2)[421] and N-Methyl-D-Aspartate (NMDA)[422] receptors leading to a ROS-dependent pro-inflammatory cascade.[423] It is noteworthy that activated neutrophils release glutamate which subsequently activates the surface endothelium NMDAr and metabotropic glutamate receptors (mGLUR1, 4, 5).[424] The activated endothelium thereafter recruit other pro-inflammatory cells like monocytes and Th17 lymphocytes which participate in AAA pathogenesis. [425,426] It is noteworthy that neutrophils are implicated in the pathogenesis and progression of AAA in experimental murine models.[427, 428, 429] Mechanistic studies in these models have suggested a fundamental importance of the neutrophil adhesion molecules like S-, L- and P-selectins in aneurysm pathology. Furthermore, systemic depletion of neutrophils is associated with a significant reduction in AAA formation in these elastase infusion mice AAA models.[430]

There is evidence to suggest that depletion of CD25+FOXP3+ T-regulatory lymphocytes (Tregs) is associated with AAA.[431, 432] A 50% reduction in peripheral blood Tregs was demonstrated in human AAA. It has also been established that HO-1 is fundamental to the proper functioning of Tregs and the former is upregulated by ERα- dependent FOXP3. [433, 434] However, there is in vivo evidence that FOXP3 is down-regulated by homocysteine thereby promoting a pro-inflammatory state.[435] It is possible that the beneficial effects of statins in suppressing AAA formation could be due in part to upregulation of $CD4^{+}CD25^{+}Foxp3^{+}$ Treg cells by a TGFβ dependent mechanism.[436] Interestingly, there is also evidence which suggests that Hcy polarizes naïve Th0 lymphocytes to the Th1 phenotype characterized by increased production of IL-2, IFNγ, TNFα, IL-18 and IL-12.[437] These T lymphocyte subsets have been implicated in AAA disease. [438, 439] $CD3^{+}$ T-lymphocytes expressed pro-inflammatory markers consistent with Th1 phenotype like IFNγ, TNFα and IL-6. Mechanistic studies further suggested that t $IFN\gamma^{-/-}$ and /or $CD4^{-/-}$ mice are resistant to AAA formation.[440] Lymphocytes are activated by Hcy on binding

to surface NMDAr and mGLUR III receptors resulting in the production of ROS and inflammatory cytokines like INFγ and TNFα thereby sustaining the inflammatory response.[441,442]

Other possible mechanisms of Hcy-induced oxidative stress in EC include the translocation of the NAD(P)H Oxidase[443] It has been shown that co-culture of rat heart microvascular EC (MVEC) with 0.5mM of Hcy for 12 hours is associated with cellular apoptosis with accompanying mitochondrial ROS generation.[444] This process was also accompanied by the increased expression of pro-apoptotic Bcl_2 protein, BAX, and a decrease in anti-apoptotic Bcl2 family proteins Bcl_2 and Bcl_{10} by 80% and 90% respectively.

It is noteworthy that endothelial concentration of asymmetric dimethyl arginine (ADMA) an enzyme which inhibits eNOS is stimulated by Hcy by the inhibition of its degrading enzyme, dimethylarginine dimethylaminohydrolase (DDAH) which is conversely stimulated by ligand (Telmisartan)-induced PPARγ-activated increased promoter activity.[445]
Hcy has been shown to activate endothelial oxidative stress through inhibition of GABA-A receptors resulting in increased production of ROS.[446] Furthermore, these ROS were shown in the same study to increase the expression of MMP-9 via an ERK 1/2 MAPK signalling cascade.[447] It is also noteworthy that Hcy can also activate other members of the classical MAPK pathway like JNK MAPK and p38 MAPK in an NAD (P) H Oxidase-dependent process to induce EC oxidative stress[448, 449]

Telomere length attrition is a documented feature of vascular ageing and a risk factor for cardiovascular pathologies like atherosclerosis with areas subject to elevated haemodynamic stresses more severely affected.[450] There is also evidence implicating homocysteine in telomere attrition in atherosclerosis.[451] Furthermore, telomere length shortening is implicated in AAA

pathogenesis in human AAA studies.[452] Telomere attrition is associated with senescence and aging. HHcy mediated oxidative stress has been implicated in apoptosis and DDR-dependent vascular senescence pathway. This is because, nuclear poly-(ADP)-ribose polymerase (PARP-1) is activated by Hcy induced oxidative stress.[453] There is evidence implicating HHcy in the pathogenesis of EC senescence by dose-dependently suppressing the EC levels of fibroblast growth factor -2 (FGF2) in bovine aortic endothelial cells (BAEC) in vitro via promoter methylation of CpG islands resulting in G1/S arrest.[454] Furthermore, HHcy can also lead to senescence via upregulation of tribbles-related protein 3 (TRB3) a Drosophilia homologue an endogenous inhibitor of Akt in vitro.[455] This mechanism may also be additive to the HHcy-induced oxidative stress leading to EPC apoptosis.[456]

Hcy can induce VSMC apoptosis through many plausible mechanisms. The first is through the upregulation of asymmetric dimethylarginine (ADMA) which is implicated in VSMC apoptosis by an oxidative stress-dependent p38MAPK pathway.[457] Hcy inhibits the metabolizing enzyme of ADMA, dimethylarginine dimethylaminohydrolase (DDAH), leading to increased ADMA. The latter uncouples NOS to produce ROS which through the ASK-1- dependent pathway activates p38 and JNK MAPK to induce apoptosis in VSMC.

Furthermore, Hcy is also capable of inducing the endoplasmic reticulum dependent unfolded protein response a direct cause of apoptosis. Interestingly, the recently discovered AIP1 participates in linking this pathway with ASK-1 and downstream activation of JNK and p38 MAPK in inducing apoptosis. Another mechanism of Hcy-mediated VSMC apoptosis is through the inhibition of the Akt-kinase signally pathway. This important pro-survival pathway is inhibited by Hcy through Trb3, an inhibitor of PI3-K-AKT resulting in the de-repression of FOXO3A and GSK-3β.

The progression of human atherosclerotic occlusive disease (AOD) to AAA is characterized by increased expression and activation of members of the RAS. ACE has been implicated in the pathogenesis of AAA possibly due to its ability to induce Angiotensin II formation. [458, 459] In addition, administration of ACEI attenuated AAA formation in murine AAA models by suppressing MMP-2 and -9 by suppressing the expression of MMPs by inflammatory cells independent of the recruitment of such cells. Interestingly, ACE increases the expression of JNK and COX-2 both of which have been implicated in AAA pathogenesis. [460, 461, 462] Accumulating evidence suggests that Angiotensin II plays a causal role in the pathogenesis of AAA as evidenced from numerous animal studies in which prolonged subcutaneous infusion of Angiotensin II is associated with the development of AAA in $ApoE^{-/-}$ hypercholesterolaemic mice models.[463, 464] Furthermore, administration of the Angiotensin II receptor type 1 blocker (ARB) Valsartan, in a murine elastase AAA model (1mg/kg/d x 4 weeks) resulted in attenuation of AAA temporal progression in association with significantly suppressed expression of MMP-2, -3,-9 and -12.[465] Similarly, there was reduced infiltration of MØ into the aortic wall accompanied by significantly inhibited elastin degradation.

Angiotensin II murine AAA models are characterized by recruitment and infiltration of MØ into the aortic wall.[466] These cells drive the intense inflammatory response accompanied by elaborate secretion of MMPs like MMP-9 and MMP-12. Angiotensin II induces the expression of TLR4 in VSMC resulting in the activation of proinflammatory cascades and MMP-9 production[467] Furthermore, members of the TLR2/4 signalling pathway like the adaptor protein, MyD88 has been mechanistically implicated in the pathogenesis of AAA are induced by Angiotensin II further underscoring the significance of the innate immune system in AAA pathogenesis.[468] The receptor for advanced glycation end products (RAGE) is a pro-inflammatory mediator induced

by Angiotensin II[469, 470] and together with high mobility group B1 (HMGB1)[471] (which is also upregulated by Angiotensin II via OPN)[472] induce NF-κB, Egr-1, MAPK, Akt and the JAK-STAT pathway in virtually all of the cells of the vascular wall.[473,474]

There is evidence from mechanistic studies implicating HMGB1 in AAA pathogenesis.[475] (Figure 29) There is evidence that ACE is very active in the bone marrow where it mobilizes primitive haemopoietic stem cells (HSC) like Colony Forming Units- Erythroid (CFU-E) and Lymphoid progenitors which express AT_1R on their surfaces in addition to Cathepsin G- and AT_1R -expressing CFU-GM.[476] It is noteworthy that the aortic intramural haematopoietic stem cells (HSC) are responsive to Angiotensin II stimulation with a propensity to form monocytes.[477.] This may well be the source of early MØ infiltration in early AAA formation. There is also evidence for Angiotensin II to mobilize bone marrow derived mononuclear cells from stem cells and early progenitor forms.[478,479, 480] It has been shown that Angiotensin II facilitates MØ in response to a chemotactic gradient established by VSMC, [481, 482, 483] adventitial fibroblasts proliferation[484, 485, 486] and periadventitial adipose tissue cells.[487] The role of the latter in the pathogenesis of AAA has been established in Angiotensin II infusion murine AAA model.[488]

Furthermore, differentiation of these CFU-M into MC (monocytes) occurs under the influence of stromal derived factor -1α (SDF-1α; CXCL12) which is produced by VSMC and other mesenchymal cells like adventitial fibroblasts following Angiotensin II stimulation.[489] Furthermore, Angiotensin II plays a very prominent role in this process since the macrophages on differentiation from monocytes acquire a functional RAS with surface expression of AT_1R and AT_2R.[490] Antagonism of the RAS with either ACEI or ARB results in polarization of M1 to M2 ($CD163^+$) with a downregulation of IFNγ, TNFα, IL-6, 1L-12 and stimulation of IL-4, IL-10, CCL17 and IL-1ra[491] There is evidence for the infiltration of AAA wall tissue by CD4+ T cells

and B cells with distinct sets of cell surface markers which may support an autoimmune phenotype[492] like T cell activation (CD69), memory cells($CD45RO^+CD45RA^-$) and T-cell receptor chains(TCRα/β). It is also worth noting that the cells express AT_1R and binding of Angiotensin II to these receptors results in the activation of Th_1 phenotype. Inhibition of these receptors by ACEI or ARB results in the suppression of the Th_1 and Th_{17} cells and activation of the $CD4^+FoxP3^+$ T regulatory cells with a decrease in the T1/Th2 ratio and NF-κB.[493]

Angiotensin II induced oxidative stress is a very important pathophysiological process in the pathogenesis of AAA (Figure 28). There is evidence suggesting that even Angiotensin II induced HT is due to the promotion of oxidative stress.[494,495] The Angiotensin II –mediated oxidative stress-dependent upregulation of PKC_δ plays a central role in this and is required for VSMC apoptosis,[496, 497] VSMC genotoxic stress,[498, 499] and oxidative stress-dependent pro-inflammatory response of the NF-κB pathway.[500] Furthermore, PKC_δ has been implicated in the pathogenesis of AAA in human studies. There is evidence that Angiotensin II stimulates the generation of the ROS through many interacting pathways.[501] Angiotensin II also stimulates the production of the p47phox production through an Ets-1 dependent pathway.[502] It has been shown that AT_1R signalling stimulates ROS generation in VSMC and fibroblasts through the stimulation of the phox47 and activation of Rac1 with consequent membrane translocation to form the complete NAD(P)H Oxidase oligomeric complex to generate superoxide O^{2-} and H_2O_2 the main ROS which also include $ONOO^-$, OH^- and lipid peroxides. [503] Reactive oxygen species generated by Rac1 is a downstream stimulator of ROCK (RhoA kinase) a molecule that has been implicated in aortic stiffness and cardiovascular disease.[504] There is evidence implicating ROCK in the pathogenesis of AAA in association with increased expression of MMP-2 and -9. In addition, Angiotensin II-dependent expression of calpain[505] (which promotes VSMC invasiveness) is implicated in AAA pathogenesis from mechanistic studies.[506 ,507] Angiotensin

II signalling is also associated with senescence which can also participate in AAA pathology.[508, 509] This senescence may be a consequence of oxidative stress-induced genotoxic stress, a pathophysiological mediator in AAA pathogenesis.[510]

There is emerging evidence for Angiotensin II-induced expression of miR-29b which is implicated in AAA pathogenesis. Angiotensin II significantly increases the expression of miR-29b in cardiac fibroblasts by an AT1R-dependent mechanism via Gαq/11-ERK1/2 pathway.[511] This miRNA is implicated in the pathogenesis of AAA by its ability to target interstitial (type 1) and basement membrane (type IV) collagens. Another study demonstrated increased expression of miR-29b with aging accentuated by Angiotensin II infusion in 18 months old mice (and 6 months ApoE$^{-/-}$ mice) with increased abdominal aortic dilatation.[512] Conversely, TGFβ a cytokine product of Angiotensin II signalling which attenuates the formation of AAA in experimental murine models suppress the expression of the miRNA-29b[513] and upregulate miR-155

Angiotensin II dependent elevated intracellular calcium is a contributes to VSMC phenotypic switch[514] and apoptosis possibly via mitochondrial-ER coupling.[515, 516] There is also evidence that Angiotensin II-activated NAD(P)H Oxidase system contributes to mitochondria-dependent intrinsic apoptotic mechanism.[517, 518] Angiotensin also upregulates Fas ligand in VSMC to activate the extrinsic apoptotic pathway.[519] Another mechanism of Angiotensin II induced apoptosis is through the AT2R dependent generation of ceramide in plasma and organellar membranes.[520] Ceramide have been implicated in golgi body dependent apoptosis or via transactivation of clustered death receptors like Fas.[521, 522] Another mechanism through which Angiotensin II depletes the tunica media is by stimulating net VSMC migration towards the intima along a chemotactic (cytokine, growth factor and chemokine) gradient towards the intima

in processes orchestrated by OPN, an Angiotensin II induced protein; VSMC autocrine loop[523] involving MCP-1/CCR-2 and TGFβ/MMP-2 and VSMC-Adventitial fibroblasts choreography.[524] In the latter case, Adventitial fibroblast expressed endothelin-1 upregulates VSMC CD40 which when stimulated by CD40 ligand (CD40L) produce GM-CSF, MCP-1, MCP-2, MIP-1α, MIP-2α, MIP-2β, MIP-3α.

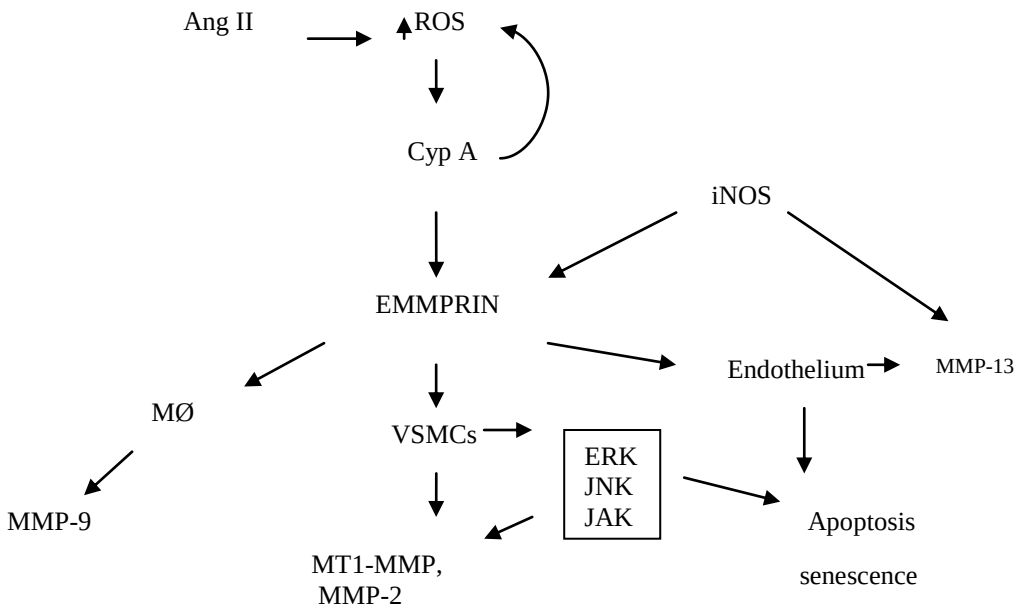

Figure 28: Angiotensin II pathways in AAA pathogenesis cluster around oxidative stress. CypA is cyclophilin A, a calcium sensor and mediator which is associated with AAA is commonly induced by Angiotensin II under inflammatory conditions. The induced MMPs contribute to AAA pathogenesis. EMMPRIN refers to extracellular Matrix Metalloproteinase inducer.

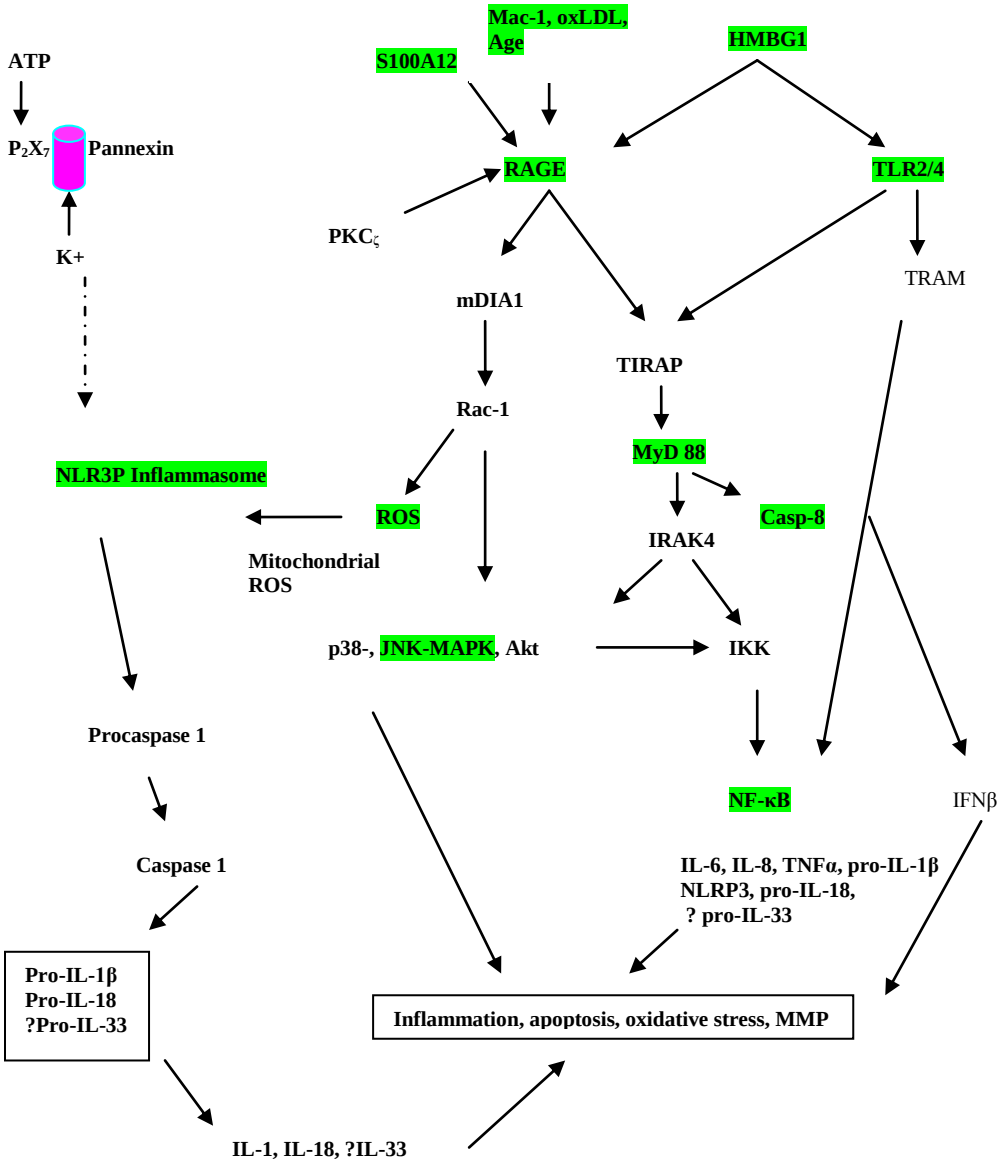

Figure 29: ACE and Angiotensin II dependent signalling cascades in macrophages implicated in AAA pathogenesis. Extracellular ATP is an endogenous alarm signal ("alarmin") which is transported via P_2X_7 channels into inflammatory cells like macrophages with concomitant exit of K^{+}. The ATP is recognized by intracytoplasmic pattern recognition molecules (NLR3P) leading to generation of proinflammtory mediators via a caspase-1 dependent pathway. This pathway links with the extracellular pattern recognition system via toll-like receptors (TLR2/4) and receptor for advance glycation end-products (RAGE) which activates the pro-inflammatory NF-κB MPAK (p38 and JNK) systems.

This is arguably the largest association study for MTHFR C677T and ACE I/D polymorphisms with AAA. They were adequately powered at 90% with alpha of 0.05 and 80% with alpha 0.01, respectively, to detect a relative risk of 2.0 in order to avoid types I (spurious association) and II errors (underpowered).

It is further suggested that the lack of replication of genetic association studies may in fact be due to the presence of epistasis and the recent availability of computational data mining techniques are making the identification of such gene-gene interactions easier and more frequent.[525] The commonest form of gene-gene interaction is referred to as epistasis and it implies a deviation from Mendelian segregation ratios[526] or deviations from additivity in a linear statistical model.[527]

However, a secondary (RE) meta-analysis (Figure 31) for MTHFR C677T inclusive of this study (cases=2264; controls=2082) showed a significant association (OR, 1.27; 95%CI, 1.016 – 1.586; P=0.035). This is a unique feature of genetic association studies, because of their relatively small effect sizes, larger sample sizes are required to show unequivocal effect sizes. Similarly, a secondary (RE) meta-analysis showed an association between the ACE I/D and AAA (OR, 1.22; 95%CI, 1.035 – 1.442; P=0.017) with combined 3345 cases and 3853 controls (Figure 30).

An interesting finding in this study from the MR paradigm is the potential for biased results when confounders are adjusted for as shown by the loss of association with MTHFR C667T with AAA under the dominant genetic model of inheritance from 1.26 (95%CI, 1.02-1.56; P=0.034) to 1.26 (95%CI, 0.96-1.65; P=0.089) when adjusted for confounders. An argument can be made for not controlling for these kinds of association studies since genes are not subject to confounding. Further studies are required to develop the argument further.

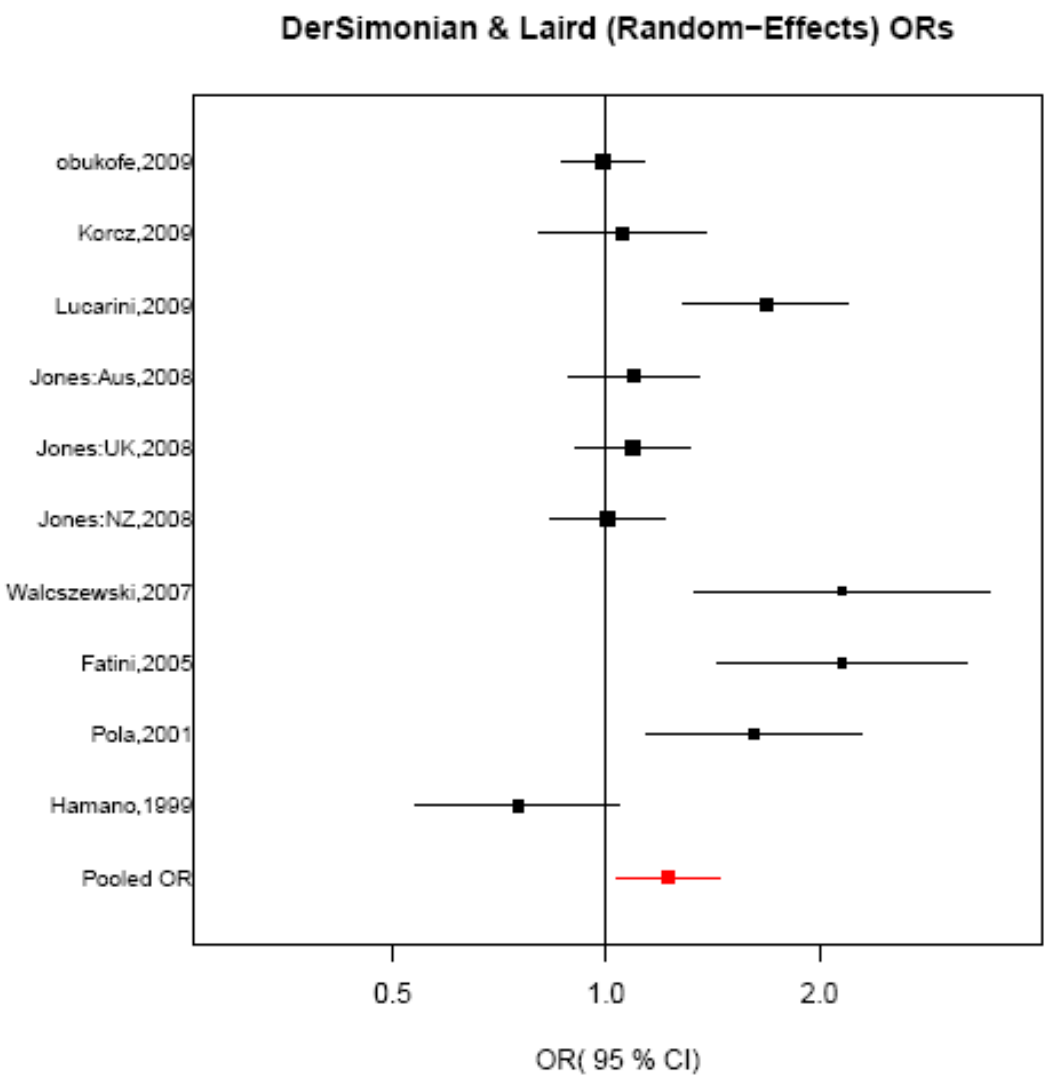

Figure 30: Secondary RE M-A of ACE I/D-Association. The effect size of 1.22 (95%CI, 1.035-1.44) is exclusively right-sided indicating a positive association.

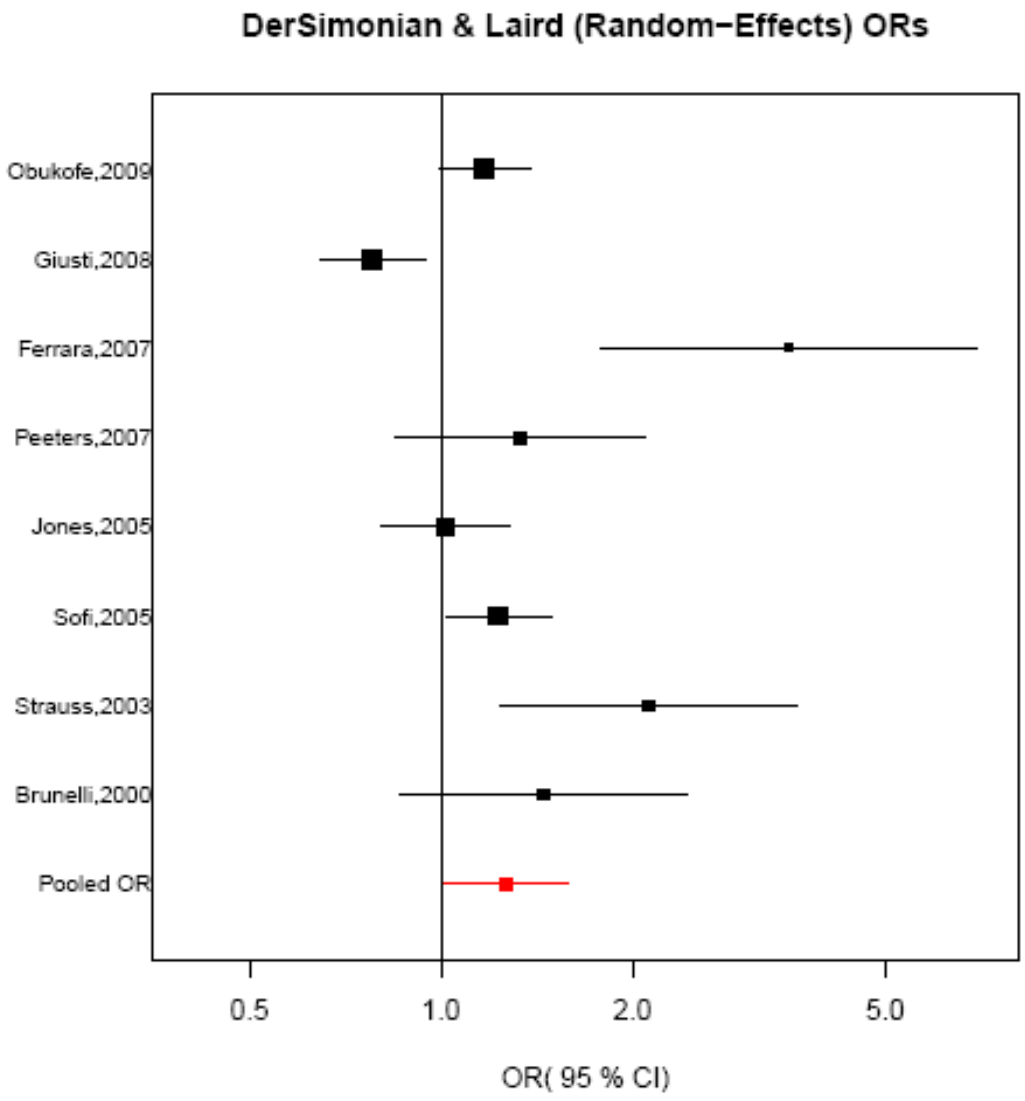

Figure 31: Secondary RE M-A for MTHFR C677T-AAA association. A RE of 1.26 (95%CI, 1.016-1.58) which is just on the right side of the line of equivalence ("1.0") shows a positive association.

There is evidence that ultrasonography is an accurate way to measure the AP diameter of AAA[528] although subject to a variability of 0.2-0.5cm.[529, 530] Thus the difference between a single duplex scan measurement of IAD of 2.7 cm and 2.3cm could be a measurement error creating bias in the analysis. The threshold aortic diameter taken for this study was 2.5cm.

With the instrumental variable (IV) approach, the formula for the naive OR for the presence of disease(Y) assumes that the causal odds ratio, ORYG is:[531]

$$OR_{YG} = \frac{P(Y=1|G=1)P(Y=0|G=0)}{P(Y=0|G=1)P(Y=1|G=0)} \qquad \text{Equation 7}$$

Thus, when G=1(causal gene present) is in the "controls" (Y=0|G=1) or in the denominator, the OR will be reduced thus biasing the results of the study. The second denominator term could also bias OR downwards if there are more cases without the risk genotype (Y=1|G=0) or if there more false positive cases as would a normal AA diagnosed as an AAA with a true protective allele (G=0). The foregoing gives rise to two potential causes of errors in a genotype-phenotype association studies and therefore requires a conclusive exclusion of AAA in controls preferably by repeat scans or a one-off scan after age 65 with a population defined threshold diameter. This becomes more instructive when it is appreciated that the measured infrarenal aortic diameter (IAD) varies with local practices. In a large epidemiological study (~70,000 subjects), 28% of the variability in measured IAD was due to local factors as compared to 5.5% due to age, sex, race and body size.[532]

Furthermore, no effort was spared to minimize genotyping error employing various techniques.

A factorial design almost similar to the matrix design used in this study has been found to quickly optimize the PCR components compared to a manual sequential optimization of individual PCR components.[533] In addition, varying Ta (annealing temperature) on either side of the optimal Ta conventionally referred to Touch-down PCR had been suggested to increase specificity and yield of PCR.[534] There is evidence for this approach as TD-PCR further increases yield in a previously optimized Ta.[535,536,537] The gradient step used in this study serves a similar purpose. DMSO was added to the reaction mix to further optimize the specificity of the reaction (i.e., primer-template pairing) and reduce the T of the primer-template pairing.[538,539] DMSO and formamide are thought to aid in the amplification of GC-rich templates in a similar manner by interfering with the formation of hydrogen bonds between the two DNA strands540 or by causing dehydration.[541] This method is shown to reduce the error rate to 2.6% comparable to the ASO-PCR and the addition of DMSO further reduces the error rate to 1%.[542]

It is noteworthy that 28% ACE ID genotype was wrongly amplified at the first stage PCR. Some authors have adopted a second stage PCR in order to obviate the preferential amplification of the DD allele which may lead to a genotyping error of ~ 5% when the ID is amplified as DD genotype.[543,544] It is noteworthy however, that none of the published ACE I/D association studies showed evidence for a confirmatory ASO-PCR.

One of the drawbacks of this study was the failure to fully match the cases and controls. This is a common drawback of retrospective studies of this sort. The necessity for an adequate matching was overcome by an overarching need for an adequately powered study. The cases in this study were older and more likely to have AAA associated cardiovascular risk factors like smoking, HT, COPD and IHD. Matching for an AAA genotype case-control will not be a trivial matter because of the reason given above: the infrarenal aorta grows throughout life and a control

subject might well be a case when measurement errors are considered as well. Consequently, the lack of standardized AAA measurement is a potential draw back for the reasons adduced.

Conclusion

The ACE I/D polymorphism does not adequately discriminate between case and controls in this study and was not independently associated with AAA. Conversely, there is insufficient evidence to refute an association between MTHFR C677T and AAA. There is a need for further studies with a larger sample size to confirm the association between these genetic polymorphisms and AAA. Furthermore, it may also be necessary to specifically test for epistatic interactions between these genetic polymorphisms in future studies.

Appendix [1] Violations of the MR

Under the instrumental variable approach of the MR paradigm, the genotype-phenotype associations need not be adjusted with confounders because of the potential for biasing results as shown in this study where moderate significant effects for MTHFR C677T association with AAA were eroded by covariate adjustment. Further violations of the MR could also result from situations of linkage disequilibrium (LD), gene-gene interaction (epistasis) and population stratification.[1]

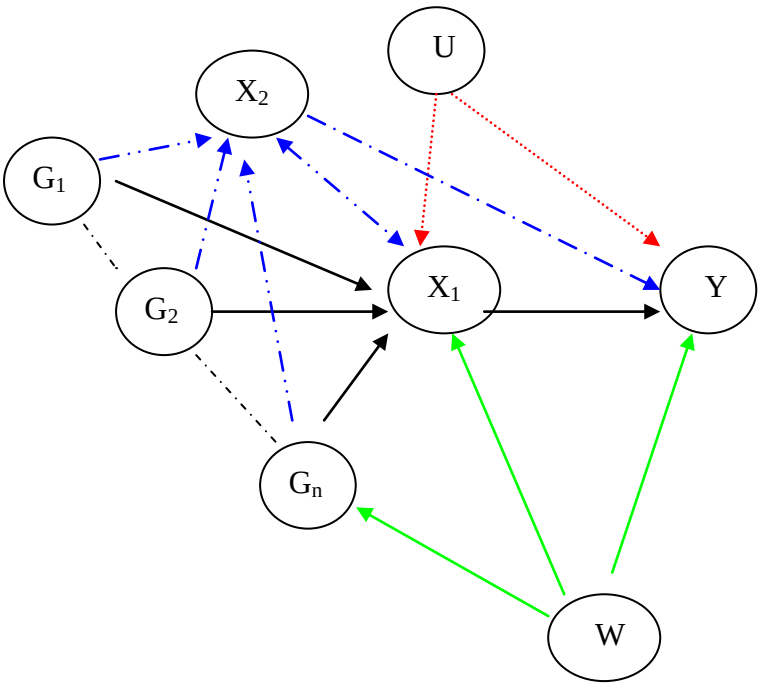

Figure 32: (Appendix) Violations of MR with the DAG showing complex inter-relationships. G_1-G_n are multiple genetic polymorphisms acting through multiple intermediate phenotypes, X_1 and X_2 which themselves may be confounded by U. W is the confounding due to population stratification, affecting the distribution of genotypes, intermediate phenotypes and the outcome of interest Y.

[1] Berzuini, C. 2006," Causal Effects in Integrative Genomics". Recent Advances in Statistical Genetics and Bioinformatics Workshop, Isaac Newton Institute of Mathematical Sciences, Cambridge.

Linkage Disequilibrium (LD): The various genes ($G_{1,...,}$ G_n) depicted in Fig , may be in LD. What this means is that the gene of interest may not be causal for the phenotype of interest but may be tightly linked on the same chromosome (or jointly inherited during meiosis) with the causal gene or variant. For example, G_1 and G_2 may be associated because they are on the same chromosome. But if G_2 is associated with Y and / or U, then Y is no longer independent of G_1 given (X,U) and /or G_1 and U are not independent anymore and all core conditions are therefore violated. But in a situation where any or all of the genes act through X_1, it doesn't matter which of the genes we use as instrumental variable all core conditions will remain the same and the backdoor criterion will hold.[2]

One study identified 78 polymorphisms within the ACE gene resulting in 13 distinct haplotypes.[3] The study showed that the I/D polymorphism is in absolute LD with 17 other polymorphisms within the ACE gene consisting of both coding (n=2) and noncoding (n=15) sequence variants. A constructed cladogram (haplotype tree) showed a major subdivision within the deletion (D) branch European-Americans branch, one of which might be associated with the cardiovascular traits. Expectedly, the "I" allele is associated with a lower ACE level compared to "D" allele and so the core conditions of the MR may still hold but this is not always the case.

The MTHFR C677T polymorphism is in LD with ~ 25% of the over 65 identified polymorphisms in the MTHFR gene, particularly the A1298C polymorphism with which it is in strong LD. The latter has a wild type allele "A" and the minor allele "C" which in most studies has a frequency equal to the "T" allele at 0.30. A unique feature of the A1298 polymorphism is that the CC genotype has been found to increase the MTHFR enzyme activity by ~110% but

[2] Sheehan NA, Didelez V, Burton PR and Tobin MD. Mendelian Randomization and Causal Inference in Observational Epidemiology. PLoS Med 2008; 5(8):e177

[3] Rieder MJ, Taylor SL, Clark AG et al. Sequence variation in the human angiotensin converting enzyme. Nature 1999; 22: 59-62

decreases enzyme activity by ~ 60% in combination with other polymorphisms.[4] It is noteworthy that relative to the wild type "CC" genotype, the "TT" genotype is associated with a 32% difference in total Hcy whilst the comparable reduction for the 1298CC genotype relative to the wild "AA" genotype was 5% in a population study of healthy 10,601 subjects.[5] Interestingly, it was shown that 677TT and 677CT genotypes accounted for 32% and 64% respectively of the enzyme activity of CC677 when the activity at the A1298 polymorphism is not taken into consideration.[6] Interestingly, there was no difference between the CC and AC genotypes of the A1298C MTHFR and the AA genotype (dominant model) when the C677T status is excluded from consideration. However, CC/AC, CT/AC, CC/CC and TT/AA compound genotypes of C677T/A1298C MTHFR exhibited 60%, 36%, 52% and 7.3% of the enzyme activity of the reference CC/AA compound genotype. Furthermore, A1298C polymorphisms *in cis* with the MTHFR "C677" allele can result in enzyme activity as low as 10% of controls in-vitro whilst the presence of the "667T" allele can further reduce the enzyme activity by another 50%.[7] Interestingly, the CC:CC double homozygote (CC677:1298CC) increased plasma Hcy by 17% when compared to 1298CC alone (16.87 vs. 14.45 µmol/L) and the increase due to the former is inversely correlated with plasma folate levels.[8] In another study, CC:AA, CC:AC and CT:AC combinations of the C677T and A1298C MTHFR polymorphisms were all significantly associated with raised tHcy levels in patients with DVT compared with controls and the CC:AC, CT:AC and CT:AA combinations showing differences between occlusive arterial disease as

[4] Martin YN, Salavaggione OE, Eckloff BW et al. Human methylenetetrahydrofolate reductase pharmacogenomics: gene resequencing and functional genomics. Pharmacogenet Genomics 2006; 15(4): 265-277

[5] Fredriksen Å, Meyer K, Ueland PM et al. Large-Scale Population-Based Metabolic Phenotyping of Thirteen Genetic Polymorphisms Related to One-Carbon Metabolism. Hum Mutat 2007; 28(9):856-865

[6] Chango A, Boisson F, Barbé F et al. The effect of 677C →T and 1298 A→C mutations on plasma homocysteine and 5,10-methylenetetrahydrofolate reductase activity in healthy subjects. Br J Nutr 2000; 83: 593-596

[7] Sibani S, Leclerc D, Weisberg IS et al. Characterization of Mutations in Severe Methylenetetrahydrofolate Reductase Deficiency Reveals an FAD-Responsive Mutation. Hum Mutat 2003; 21: 509-520

[8] Friso S, Girelli D, Trabetti E et al. The MTHFR 1298A>C polymorphism and genomic DNA methylation in human lymphocytes. Cancer Epidemiol Biomarkers Prev 2005; 14(4): 938-943

compared to controls.[9] In this study the presence of the homozygote TT genotype of the C677T MTHFR polymorphism was not associated with increased tHcy

Pleiotropy and heterogeneity: A genetic polymorphism may have more than one effect. For example the gene may affect more than one intermediate phenotype in the causal pathway of the phenotype of interest, a condition referred to as pleiotropy. This is commonly seen with ACE gene stimulates the expression of TGFβ (which protects against AAA formation) and Angiotensin II which promotes AAA formation. Furthermore, Angiotensin II stimulates the expression of miR-21 and miR-29b which individually protect and predispose to AAA pathogenesis, respectively.

The converse is that more than one gene may directly affect the intermediate phenotype. This latter situation is referred to as genetic heterogeneity or gene-gene interaction. From Fig, each of the genes may act through multiple intermediate phenotypes (X_1 and X_2) or the various genes may act through either X_1 or X_2 to affect Y. In this situation G_1 is associated with another intermediate phenotype X_2 which is also in association with Y and /or U. Then Y is no longer independent of G_1 given (X,U) and /or G_1 and U are not independent anymore and the core conditions are violated.

More than one genotype may affect the endophenotype of interest (X_1) and none of which is associated with the confounder (U) and Y. The genotype - phenotype association may be weak but all core conditions are still satisfied for any choice of genotype. For example, genetic loci other than the ACE or MTHFR genes (in this study) may affect the endophenotype. A recent

[9] Spiroski I, Kedev S, Antov S et al. Methylenetetrahydrofolat reductase (MTHFR-677 and MTHFR-1298) genotypes and haplotypes and plasma homocysteine levels in patients with occlusive artery disease and deep venous thrombosis. Acta Biochimica Polonica 2008; 55(3): 587-594

Genome Wide Analysis Study (GWAS)[10] involving 289 Nigerian families (N=2,079) showed that the ACE gene locus accounted for 26% of the phenotypic variance of plasma ACE whilst other chromosomal loci (These chromosomal loci corresponded to 4q28-31, 11p13 and 21q22.2-3) demonstrated suggestive linkage (Lod score, Z_{max} >1.0) with plasma ACE after adjusting for age, sex and ACE gene locus. Similarly, A recent genome-wide association study has also demonstrated the existence of unlinked genetic loci associated with plasma Hcy including 1p37, 2p21, 3p25, 3p27.3, 4q15, 9q34.3, 17q21.3 and 22q13.3 at a LOD score >1 in 100 Dominican families (n=1246) accounting for 22% of phenotypic variance ($h^2 = 0.44$) and covariates accounting for 50%.[11] The highest peak LOD score was at 17q21 (3.92; P=0.0022).

The commonest form of gene-gene interaction is referred to as epistasis and it implies a deviation from Mendelian segregation ratios[12] or deviations from additivity in a linear statistical model.[13] It is also noteworthy that some genes may have no independent main effects but associate with the phenotype under conditions of epistatic interactions.

Population stratification: For example, there could possibly be significant genetic variation in apparently homogenous populations[14] - it has been shown that across 3384 localities in Europe, 49 out of 59 alleles had substantial variation in allele frequencies. Baseline risk of the disease could also be correlated with allele frequency. Ignoring differences in baseline risks and allele

[10] McKenzie CA, Zhu X and Forrester TE. A genome-wide search replicates evidence of a quantitative trait locus for circulating angiotensin-1-converting enzyme gene. BMC Genomics 2008; 1(1): 23

[11] Della-Morte D, Beechan A, Rundek T et al. Genetic Linkage of Serum Homocysteine in Dominican Families: The Family Study of Stroke Risk and Carotid Atherosclerosis. Stroke 2010; 41: 1356-1362

[12] Bateson W. Mendel's Principles of Heredity. (1909) Cambridge University Press, Cambridge

[13] Fisher RA. The correlations between relatives on the supposition of Mendelian inheritance. Trans R Soc Edinburgh 1918; 52: 399-433

[14] Sokal RR, Harding RM and Oden NL. Spatial Patterns of human gene frequencies in Europe. Am J Phys Anthropol 1989; 80:267-294

frequencies could lead to confounding with increases in the chance of false positive findings as a result of distortion of the significant level.

With reference to the DAG, population stratification is represented by W. Allele frequencies and disease prevalence rates may be different within subgroups of population. Under these scenarios Y is no longer independent of G given only (X_1,U) and the core conditions are therefore violated. However, if only the endo-phenotype (X_1) depends on the subpopulation (of W) then Y is still independent of G given (X_1,U) and all core conditions will be satisfied. In this case we can use G for MR.

Furthermore, some authors have suggested that the conventional logistic regression model for binary phenotypes corrects for population stratification but other techniques like genomic control and nonparametric Bayesian methods have been advocated by others. There is evidence that that the ACE gene despite its many tightly linked polymorphisms has 2 ancestral break-points at the 5'-end and 3'-end resulting in different population substructures on the basis of different haplotypes. These haplotype blocks (cladotypes) have also been shown to predict plasma ACE levels.

In one of such studies in British population, three major clades (A, B and C) were constructed and the A clade contained "I" allele while both B and C clades contained the "D" allele.[15] It was inferred from the study that the C clade represents a recombination event between the 5'-end of A and 3'- end of B clades at a point between intron 5 and exon 8 (3' of position 6435). Clades B and C had similar average plasma ACE concentration with clade A being significantly lower suggesting that the major ACE-linked locus was downstream of the ancestral break point.

[15] Keavney B, McKenzie CA, Connell JMC et al. Measured haplotype analysis of the angiotensin –I converting enzyme gene. Hum Mol Genet 1998; 7(11): 1745-1751

Significantly, the ACE-linked clades accounted for 36% of the phenotypic variance of ACE trait. Another ancestral breakpoint was identified in the 3'-untranscribed region of the gene,[16] 16kb downstream of the intron 5 – exon 8 breakpoint involving a 655 bp interval region. This breakpoint identifies another clade (D) which differs from clade B by being similar to clade A in regions 3' of this latter breakpoint. It is noteworthy that haplotypes incorporating the D allele varied in frequency from 1% to 23% in this study. Interestingly, plasma ACE levels of clades B and D were similar and different from those in clades A and C with clade A demonstrating the lowest ACE levels. Furthermore, mean plasma ACE levels in clade C were only marginally lower than those in clade B suggesting that the18kb region between these two ancestral recombination points (which includes I/D) account for most of the plasma ACE variance. Furthermore, 10% and 22% of total and genetic variances are due to unlinked genetic loci outside of the ACE locus. The foregoing underscores the point the significance of population stratification as it affects the ACE gene and its polymorphisms. In addition, the ACE I/D polymorphism was not found to be significantly associated with ACE trait in 234 Nigerian families (n=765).[17] In this study, promoter and distal polymorphisms and 6 ACE coding region polymorphisms (A23495G, A31958G, 31839insC, A6138C, 29349delT and G28952C) were significantly associated with total plasma ACE accounting for 35% of the plasma variance (20% familial and 45% individual specific residual correlations).

[16] Soubrier F, Martin S, Alonso A et al. High-resolution genetic mapping of the ACE-linked QTL influencing circulating ACE activity. Eur J Human Genet 2002; 10: 553-561

[17] Cox R, Bouzekri N, Martin S, Southam L et al. Angiotensin -1-converting enzyme (ACE) plasma concentration is influenced by multiple ACE-linked quantitative trait nucleotides. Hum Mol Genet 2002; 11(23): 2969-2977

Appendix [2]: Presentations and Publications

Publications

[1] Eur J Vasc Endovasc Surg 2010 Oct; 40(4):457-60. Epub 2010 Jul 23.

Is there an association between angiotensin converting enzyme (ACE) genotypes and abdominal aortic aneurysm?

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Abstract

OBJECTIVES: There is strong evidence of a genetic predisposition to abdominal aortic aneurysm (AAA), however the genes involved remain largely elusive. Recently, two large studies have suggested an association between the angiotensin converting enzyme gene and AAA. This study aimed to investigate the possible association between the ACE insertion/deletion polymorphism and abdominal aortic aneurysm (AAA) in order to replicate the findings of other authors.

DESIGN AND METHODS: A case-control study was performed including 1155 patients with aneurysms and 996 screened control subjects. DNA was extracted from whole blood and genotypes determined in 1155 AAAs and 996 controls using a two stage polymerase chain reaction (PCR) technique.

RESULTS: The groups were reasonably matched in terms of risk factors for AAA. No association was found between the ACE gene insertion/deletion polymorphism and AAA in this study.

CONCLUSIONS: This study cannot support the findings of previous authors and provides evidence against a link between the ACE gene insertion/deletion polymorphism and AAA.

Oral Presentations:

[A] The 57th International Congress of the European Society for Cardiovascular Surgery. Barcelona, Spain. 24-26 April 2008

[1] Interact CardioVasc Thorac Surg (2008) 7 (Supplement 1): S39

V5bis-5 MODELLING BIOLOGICAL VARIABILITY AND NON-LINEAR GROWTH IN A COMPLEX PHENOTYPE: APPLICATION TO ABDOMINAL AORTIC ANEURYSM EXPANSION

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Objectives: Abdominal aortic aneurysm (AAA) expansion has both prognostic and surgical decision implications. Aneurysm diameter is an independent predictor of all cause mortality in people with AAA. People with higher AAA diameters are at significant risk from rupture. Hence the current practice of surgical intervention at a diameter of 5.5 cm. Previous attempts at modelling AAA expansion have assumed a linear process and ignored the highly correlated nature of repeated measurements. Our objective is therefore, to model AAA expansion process using a hierarchical non-linear mixed effects regression methodology.

Methods: We recruited 354 white male Caucasians with confirmed abdominal aortic aneurysms (AAA) into our study. We followed them up over a period of time and repeatedly measured their AAA maximum diameters with ultrasound and CT-scans. The maximum infra-renal aneurysm diameter was recorded at the time of measurement. People whose aneurysms exceeded 5.5 cm or became symptomatic were offered surgery. The minimum and maximum periods of follow-up were nine months and seven years, respectively. The minimum and maximum time-points of observations per person were 3 and 10. The data was organized in a longitudinal data format and missing data were assumed to be missing completely at random and therefore, were ignored. We used maximum likelihood methods accounting for the correlation between repeated measurements in individual patients. The choice of a non-linear multivariate regression technique was informed by the fact that a quadratic linear regression model out-performed a simple linear regression model in terms of information criteria. We adjusted for covariates such as sex, hypertension, smoking, ischaemic heart disease and chronic obstructive pulmonary disease (COPD).

Results: The AAA diameter at baseline was 3.036 cm (0.05) and the rate of expansion was 0.14 cm/year (0.008) $P \ll 0.0001$. At baseline, people with hypertension have AAA that are 9% higher in diameter as compared with normotensives. However, smoking accounts for a 5% increase in baseline AAA diameter and COPD, 3% ($P<0.001$). Using empirical bayes estimates we were also able to predict individual AAA diameters from their data. The individual specific variance was 11%.

Conclusions: Abdominal aortic aneurysm expansion shows inherent between-individual and within-individual variability and it is inappropriate to model this ‘stochastic’ behaviour with linear approaches. Using non-linear mixed effects regression methods we have been able to model AAA expansion and extract individual specific diameters. Our findings are consistent with the commonly quoted ‘small’ AAA expansion rates.

[2] Interact CardioVasc Thorac Surg (2008) 7 (Supplement 1): S89

V11-9 MENDELIAN RANDOMIZATION AND GENOTYPE-PHENOTYPE ASSOCIATION: THE CAUSAL RELATIONSHIP BETWEEN METHYLENE TETRAHYDROFOLATE REDUCTASE (MTHFR) C677T POLYMORPHISM AND ABDOMINAL AORTIC ANEURYSM (AAA)

B.A. **Obukofe**, M. Bown, R. Sayers, N. London (*UK*)
University of Leicester, Leicester, UK

Objectives: The Methylene Tetrahydrofolate reductase (MTHFR) C677T polymorphism results in a substitution of thymidine nucleotide for cytidine at position 677 of the MTHFR gene resulting in a change from the wild type 'C' allele to the risk (minor) 'T' allele. The latter results in homocysteinaemia which has been implicated in the pathogenesis of abdominal aortic aneurysm (AAA). Studies have also implicated the MTHFR genetic polymorphism in AAA formation although this is not consistent. Our aim is therefore, to determine if there is a causal association between MTHFR genetic polymorphism and AAA within the framework of Mendelian randomization. Mendelian randomization is the random assignment of an individual's genotype from his or her parental genotypes that occur before conception. A causal relationship between MTHFR and AAA is therefore, free from bias and confounding just like a randomized controlled study.

Methods: This is a retrospective case-control study involving white Caucasian participants. Our cases are those with imaging confirmed AAA whilst the controls are free from AAA. We recruited 816 study participants comprising 362 controls and 454 cases with a mean age of 70.21 years (CI: 69.587–70.55) We obtained some blood specimen from the participants from which we extracted genomic DNA. We subsequently genotyped the DNA sample for the MTHFR polymorphisms using a polymerase chain reaction (pcr) methodology. The pcr products were thereafter subjected to overnight restriction fragment length digestion with the restriction enzyme Hinf1. The resulting products were then run on a 2% agarose gel electrophoresis to yield the homozygote 'CC' and 'TT' genotypes and heterozygote 'CT' genotype.

Results: The distributions of the obtained genotypes (cases vs. controls) were as follows: CC (222 vs. 204), CT (189 vs. 132) and TT (43 vs. 26) accounting for allele frequencies of 0.70 (±0.015) and 0.75 (±0.016) respectively. The genotypes are in Harding-Weinberg equilibrium

(P=0.48 for controls and P=0.78 for cases). The Allele difference odds ratio between cases and controls is 1.275 (CI: 1.024–1.587, P=0.0296) and the genotype odds ratio (CT+TT vs. CC) is 1.349 (CI: 1.022–1.781, P=0.034) and 1.254 (P=0.032) after correction for multiple testing.

Conclusions: We have shown that the MTHFR C677T genetic polymorphism is causally associated with AAA pathogenesis. Hyperhomocysteinaemia resulting from this polymorphism is amenable to folate therapy. Therefore, offering MTHFR C677T polymorphism genotyping test to people with other risk factors for AAA formation could prove a useful risk reduction strategy.

[B] 44th Annual Scientific Meeting of the Vascular Society of Great Britain and Ireland, Liverpool, UK, 18-20 November 2009

[1] Br J Surg 2010; 97(S1):1

The association between methylene tetrahydrofolate reductase (MTHFR) genetic polymorphism and abdominal aortic aneurysm (AAA)

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Objective: The methylene tetrahydrofolate reductase (MTHFR) C677T polymorphism results in a substitution of thymidine nucleotide for cytidine at position 677 of the MTHFR gene resulting in a change from the wild type ‘C’ allele to the risk (minor) ‘T’ allele and this is referred to as single nucleotide polymorphism (SNP). Homozygosity for the ‘T’ allele has been associated with AAA, although this is not consistent. Our aim is to confirm the association between MTHFR SNP and AAA.

Method: We recruited 1352 predominantly male (99 • 94%) white Caucasians of whom there were 678 cases and 674 controls in a case-control study design with a mean age of 77.22 years. Both groups were evenly matched for demographic characteristics. Blood samples were obtained from both groups and genotyped for the MTHFR genotypes. The amplicons were subjected to an overnight restriction enzyme (Hinf1) digestion before gel electrophoresis. A Chi-square test of allele difference was done and $p < 0 • 05$ was accepted as significant.

Results: The MTHFR genotypes in the control group were in Hardy-Weinberg Equilibrium ($p = 0 • 211$) and distributed as follows: TT (59), CT (257) and CC (358). Conversely, the genotype distributions in cases were: TT (65), CT (292) and CC (321). The allele specific odds ratio (OR) was 1 • 260 [95% CI, 1 • 018–1 • 560; $p = 0 • 033$] and $\chi 2 = 4 • 50$. The Armitage Trend test OR was 1 • 144, $p = 0 • 062$.

Conclusion: We do not have enough evidence to refute the association between MTHFR genetic polymorphism and AAA.

[2] Br J Surg 2010; 97 (S1):8

The evidence for candidate gene polymorphisms in the pathogenesis of Abdominal Aortic Aneurysms (AAA)

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Objective: The Angiotensin Converting Enzyme (ACE) insertion-deletion (INDEL) and Methylene Tetrahydrofolate Reductase (MTHFR) single nucleotide polymorphism (SNP) may be implicated in the pathogenesis of AAA. The aim of this study was to determine any association between these genetic polymorphisms and AAA.

Method: We recruited 1185 patients with AAA and 910 screened controls

Nine hundred and eighty-five cases and 812 controls were genotyped for the ACE INDEL and 678 cases and 674 controls for the MTHFR SNP using polymerase chain reaction-based techniques. Primary analysis of the locally generated data and a secondary meta-analysis were both performed (combining our data with published genetic association studies of ACE and MTHFR), resulting in a pooled sample size of 7110 (cases = 3970, controls = 3140) for ACE I/D and 2621 for MTHFR (cases = 1400, controls = 1221).

Results: The distribution of the MTHFR genotypes for cases *versus* controls was as follows: CC (321 *versus* 358); CT (292 *versus* 257) and TT (65 *versus* 59). The controls were in Hardy-Weinberg Equilibrium (p = 0·211) and the allele specific odds ratio (OR) was 1·260 [95% CI, 1·018–1·560; p = 0·033] and $\chi 2 = 4{\cdot}50$. The pooled (meta-analysis) per-allele odds ratio (OR) was 1·435 [95% CI, 1·097–1·876; p = 0·0083]. For the ACE INDEL, genotype frequencies were as follows: DD (340 versus 283); ID (428 versus 352) and II (217 versus 177). The allele specific OR was 0·989 [95% CI, 0·866–1·129; p = 0·864] and $\chi 2 = 0{\cdot}03$. The pooled per-allele OR was 1·222 [95% CI, 1·035–1·443; p = 0·017].

Conclusion: We have demonstrated that ACE and MTHFR genetic polymorphisms are associated with AAA.

[3] Br J Surg 2010; 97 (S1): 10

Angiotensin converting enzyme inhibitors (ACEI) do not decrease abdominal aortic aneurysm (AAA) size or growth rate

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Objective: There has been a recent interest in the trials of pharmacological agents in the regression of AAA growth because this has both prognostic and surgical decision significance. Large AAA have significant comorbidities in addition to increased propensity to rupturing. Our

objective is to determine whether patients taking ACEI have smaller AAA with a decreased rate of growth.

Method: We recruited 509 predominantly male, white Caucasians with confirmed AAA in a longitudinal study with repeated measurements of the maximal infrarenal AAA diameter using ultrasound scanning. Demographic and clinical data were recorded at times of scanning. The mean age was 77 • 22 years. A maximum likelihood method was employed and p < 0 • 05 was accepted as significant.

Results: The baseline AAA diameter for patients who were not on ACEI was 2 • 950 cm (95% CI, 2 • 802–3 • 097 cm; p = 0 • 000) with a growth rate of 0 • 179 cm/year (95% CI, 0 • 155–0 • 203 cm/year; p = 0 • 000). For those taking ACEI, the baseline AAA diameter was 3 • 113 cm (95% CI, 2 • 729–3 • 298 cm; p = 0 • 363) whilst the growth rate of AAA in this category of patients was 0 • 203 cm/year (95% CI, 0 • 14–0 • 267 cm/year; p = 0 • 226). Adjustment for other risk factors and drugs did not alter these findings.

Conclusion: There is no evidence to conclude that ACEI reduce the size or growth rate of AAA.

Poster Presentations:

[A] The 57th International Congress of the European Society for Cardiovascular Surgery. Barcelona, Spain. 24-26 April 2008

[1] **Interact CardioVasc Thorac Surg (2008) 7 (Supplement 1): S123**

VP19 MENDELIAN RANDOMIZATION AND GENOTYPE-PHENOTYPE ASSOCIATION: THE CAUSAL RELATIONSHIP BETWEEN METHYLENE TETRAHYDROFOLATE REDUCTASE(MTHFR) C677T POLYMORPHISM AND ABDOMINAL AORTIC ANEURYSM (AAA) B.A. **Obukofe**, M. Bown, R. Sayers, N. London (*UK*) University of Leicester, Leicester, UK

Objectives: The Methylene Tetrahydrofolate reductase (MTHFR) C677T polymorphism results in a substitution of thymidine nucleotide for cytidine at position 677 of the MTHFR gene resulting in a change from the wild type ‘C’ allele to the risk (minor) ‘T’ allele. The latter results in homocysteinaemia which has been implicated in the pathogenesis of abdominal aortic aneurysm (AAA). Studies have also implicated the MTHFR genetic polymorphism in AAA formation although this is not consistent. Our aim is therefore, to determine if there is a causal association between MTHFR genetic polymorphism and AAA within the framework of mendelian randomization. Mendelian randomization is the random assignment of an individual's genotype from his or her parental genotypes that occur before conception. A causal relationship between MTHFR and AAA is therefore, free from bias and confounding just like a randomized controlled study.

Methods: This is a retrospective case-control study involving white caucasian participants. Our cases are those with imaging confirmed AAA whilst the controls are free from AAA. We recruited 816 study participants comprising 362 controls and 454 cases with a mean age of 70.21 years (CI: 69.587–70.55) We obtained some blood specimen from the participants from which we extracted genomic DNA. We subsequently genotyped the DNA sample for the MTHFR polymorphisms using a polymerase chain reaction (pcr) methodology. The pcr products were thereafter subjected to overnight restriction fragment length digestion with the restriction enzyme Hinf1. The resulting products were then run on a 2% agarose gel electrophoresis to yield the homozygote ‘CC’ and ‘TT’ genotypes and heterozygote ‘CT’ genotype.

Results: The distributions of the obtained genotypes (cases vs. controls) were as follows: CC (222 vs. 204), CT (189 vs. 132) and TT (43 vs. 26) accounting for allele frequencies of 0.70 (± 0.015) and 0.75 (±0.016) respectively. The genotypes are in Harding-Weinberg equilibrium (P=0.48 for controls and P=0.78 for cases). The Allele difference odds ratio between cases and controls is 1.275 (CI: 1.024–1.587, P=0.0296) and the genotype odds ratio (CT+TT vs. CC) is 1.349 (CI: 1.022–1.781, P=0.034) and 1.254 (P=0.032) after correction for multiple testing.

Conclusions: We have shown that the MTHFR C677T genetic polymorphism is causally associated with AAA pathogenesis. Hyperhomocysteinaemia resulting from this polymorphism

is amenable to folate therapy. Therefore, offering MTHFR C677T polymorphism genotyping test to people with other risk factors for AAA formation could prove a useful risk reduction strategy.

[B] International Surgical Congress of the Association of Surgeons of Great Britain and Ireland, Bournemouth, UK. May 14-16, 2008

[1] Br J Surg 2008; 95(Suppl 3): 189

Mendelian randomization and genotype-phenotype association: the causal relationship between methylene tetrahydrofolate reductase (MTHFR) C677T polymorphism and abdominal aortic aneurysm (AAA)

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Background: The MTHFR C677T polymorphism results in a substitution of thymidine nucleotide for cytidine at position 677 of the MTHFR gene resulting in a 'C' allele and a minor 'T' allele. The latter results in homocysteinaemia which has been implicated in the pathogenesis of AAA. Studies have also implicated the MTHFR genetic polymorphism in AAA formation although this is not consistent. Our aim is therefore to determine if there is a causal association between MTHFR genetic polymorphism and AAA within the framework of mendelian randomization. Mendelian randomization is the random assignment of an individual's genotype from his or her parental genotypes that occur before conception.

Methods: This is a retrospective case-control study involving white Caucasian participants in which our cases are those with imaging confirmed AAA. We recruited 816 study participants comprising of 362 controls and 454 cases with a mean age of 70.21 years (CI: 69.587 – 70.55).

We obtained some blood specimen from which we extracted genomic DNA from and genotyped the DNA sample for the MTHFR polymorphisms using a polymerase chain reaction (pcr) methodology. The pcr products were thereafter subjected to overnight restriction fragment length digestion with the enzyme Hinf1. The resulting products were then run on a 2% agarose gel electrophoresis to yield the homozygote 'CC' and 'TT' genotype and heterozygote 'CT' genotype.

Results:

Genotypes	CC	CT	TT	Allele frequencies
Controls	204	132	26	0.75 (± 0.016)
Cases	222	189	43	0.70 (± 0.015)

The genotypes are in Hardy-Weinberg equilibrium (P = 0.48 for controls and P = 0.78 for cases). The allele difference odds ratio between cases and controls is 1.275 (CI: 1.024 –1.587, P = 0.0296) and the genotype odds ratio (CT + TT versus CC) is 1.349 (CI: 1.022 – 1.781, P = 0.034) and 1.254 (P = 0.032) after correction for multiple testing.

Conclusion: We have shown that the MTHFR C677T genetic polymorphism is causally associated with AAA pathogenesis.

[2] Br J Surg 2008; 95(Suppl 3): 189

Is it time to make individual-specific predictions of abdominal aortic aneurysm (AAA) expansion?

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University of Leicester, Leicester

Background: Abdominal aortic aneurysm expansion has both prognostic and surgical decision implications. Previous attempts at predicting the population mean AAA expansion rate have assumed a linear expansion process and ignored the highly correlated nature of repeated

measurements. Our objective is therefore to model AAA expansion process using a hierarchical nonlinear mixed effects regression methodology.

Methods: We recruited 354 white male Caucasians with confirmed AAA into our longitudinal (prospective) study and followed them up over a period of time and repeatedly measured their AAA diameters with ultrasound and CT scans. The maximum infra-renal AAA diameter was recorded at the time of measurement. Participants whose AAA exceeded 5.5cm or became symptomatic were offered surgery. We adjusted for covariates such as hypertension, smoking, ischaemic heart disease and chronic obstructive pulmonary disease (COPD).

Results: The mean AAA diameters are given in the table.

Years of follow-up	0	1	2	3	4	4.5	5	5.5	6	6.25
AAA diameter (cm)	3.4	3.5	3.8	4.5	4.6	4.8	4.9	5.0	5.1	5.2

The adjusted mean population diameter at baseline was 3・036 cm (± 0.05) and the rate of expansion was 0・14 cm/year (± 0.008), $P < 0.0001$. At baseline people with hypertension have AAA 9% greater in diameter than normotensives. However, smoking and COPD account for 5% and 3% increase in baseline AAA diameter respectively when compared with non-smokers and people without COPD ($P < 0.0001$).We also used empirical Bayes estimates to extract individual predicted AAA diameters given baseline risk factors. The individual specific variance was 11%.

Conclusion: AAA expansion shows inherent between-individual and within individual variability. It is possible to model this nonlinear behaviour AAA expansion and also to make individual predictions of AAA expansion using current techniques.

Appendix [3]: Table of Abbreviations

Abbreviations used in this thesis

3'-UTR	3'-Untranslated region
5'-UTR	5'-Untranslated region
8-oxo-dG	8-oxo-deoxyguanosine
A7r5 VSMC	Vascular Smooth Muscle Cell line
AAA	Abdominal Aortic Aneurysm
ACE	Angiotensin Converting Enzyme
AD	Aortic Diameter
AGE	Advanced Glycation End Products
AIP1	Apoptosis Signal Kinase (ASK)-Interacting Protein -1
Akt	Ak thymoma (Protein Kinase B)
AMF	Adventitial Myofibroblasts
ANRIL	Antisense non-coding RNA in the INK4 locus
AP-1	Activator Protein-1
APAF1	Apoptosis Protein Activating Factor -1
ApoE-/-	Apolipoprotein E double knockout
ASK-1	Apoptosis Signal Kinase-1
ASO-PCR	Allele-Specific Oligonucleotide- Polymerase Chain Reaction
AT1R	Angiotensin II Type 1 Receptor
BAX	B-cell Lymphoma 2 (Bcl2)-Associated X protein

Bcl2	B-cell Lymphoma 2
Bcl-Xl	B-cell Lymphoma 2-extra Large
BMP	Bone Morphogenetic Protein
C57BL/6J	C57 Black 6 (inbred strain of laboratory mice)
C677T	Substitution of Thymidine for Cytosine at position 677 of MTHFR gene
CaCl2	Calcium Chloride
CAD	Coronary Artery Disease
Casp-8	Caspase-8
CCL19	C-C (type α) Chemokine Ligand -19
CCL2	C-C (type α) Chemokine Ligand -2
CCL21	C-C (type α) Chemokine Ligand -21
CCL3	C-C (type α) Chemokine Ligand -3
CCL5	C-C (type α) Chemokine Ligand - 5
CCL7	C-C (type α) Chemokine Ligand- 7
CCL8	C-C (type α) Chemokine Ligand - 6
CCR2	C-C (type α) Chemokine Receptor-2
CCR5	C-C (type α) Chemokine Receptor-5
CD25	Cluster of Differentiation 25
CD40/CD40L	Cluster of Differentiation 40/Ligand
Cdc42	Cell division cycle 42 GTP binding protein
CDKN1A	Cyclin Dependent Kinase Inhibitor 1A (p21)
CDKI	Cyclin Dependent Kinase Inhibitor
cIAP	cellular Inhibitor of Apoptosis
CIITA	Class II Major Histocompatibility Transactivator
CO	Carbon Monoxide

COL3A1	Collagen Type 3 A subtype 1 gene
COL4A1	Collagen Type 4 A subtype 1 gene
COPD	Chronic Obstructive Pulmonary Disease
COX-2	Cyclo-oxygenase-2
CXCL10	C-X-C (type β) Chemokine Ligand -10
CXCL12	C-X-C (type β) Chemokine Ligand -12 (stromal-derived factor-1α)
CXCL5	C-X-C (type β) Chemokine Ligand -5
CXCL9	C-X-C (type β) Chemokine Ligand -9
CXCR3	C-X-C (type β) Chemokine Ligand -3
DAB2IP	Disabled homolog (DAB)-2-Interacting Protein
DDR	DNA Damage Response
DMSO	Dimethyl Sulphoxide
DNMT1	DNA Methyltransferase Type 1
DNMT3	DNA Methyltransferase Type 3
dNTP	deoxy Nucleotide Triphosphate
DZ	Dizygotic
EC	Endothelial Cell
Egr-1	Early growth response -1 (transcription factor)
ELN	Elastin gene
EMMPRIN	Extracellular Matrix Metalloproteinase Inducer
eNOS	endothelial Nitric Oxide Synthase
ERK1/2	Extracellular signal-regulated Kinase -1, -2
ERα	Oestrogen Receptor alpha
ET-1	Endothelin-1

EZH2	Enhancer of Zeste homolog 2
Fas/FasL	Tumour Necrosis Factor Receptor Super family
FOXO3	Forkhead box O type 3 protein
FOXP3	Forkhead box P type 3 protein
GADD45A	Growth Arrest and DNA-damage -inducible 45 alpha
GM-CSF	Granulocyte Monocyte-Colony Stimulating Factor
GPX	Glutathione Peroxidase
GSK3-β	Glycogen Synthase Kinase-3-beta
H2O2	Hydrogen Peroxide
H2S	Hydrogen Sulphide
H3K27me3, me2	Histone 3 lysine 27 trimethylation, dimethylation
H3K4	Histone 3 lysine 4
H3K4me3,me2	Histone 3 lysine 4 trimethylation, dimethylation
H4K36me3	Histone 4 lysine 36 trimethylation
H4K39me2	Histone 4 lysine 39 dimethylation
Hcy	Homocysteine
HHcy	Hyperhomocysteinaemia
HLA-DR	Human Leucocyte Antigen-
HO-1	Heme Oxygenase -1
HOCL	Hypochloric Acid
HR	Hazard Rate/ Ratio
HSP-70	Heat Shock Protein 70
HSP-90	Heat Shock Protein 90
HT	Hypertension
I/D	Insertion/ Deletion Polymorphism (of ACE)

ICAM-1	Intercellular Adhesion Molecule-1
IFNβ	Interferon beta
IFNγ	Interferon gamma
IGFBP3	Insulin-like Growth Factor Binding Protein 3
IKK	Inhibitor of Kinase Kinase
IL-1β	Interleukin-1beta
IL-6	Interleukin 6
IL-8	Interleukin 8
INDEL	Insertion/ Deletion Polymorphism (of ACE)
iNOS	inducible Nitric Oxide Synthase
IQR	Interquartile Range
IRAK4	Interleukin-1 Receptor- Associated Kinase 4
IRE-1	Inositol Requiring Protein-1
JAK	Janus Kinase
JNK	c-Jun Kinase Mitogen Activated Protein Kinase
KLF2	Kruppel Like Factor -2
LOD	Logarithm of the Odds
MAPK	Mitogen Activated Protein Kinase
MAPKKK	Mitogen Activated Protein Kinase Kinase Kinase
MCP-1	Monocyte Chemoattractant Protein -1
MEKK	MAPK/ Extracellular Signal-Regulated Kinase Kinase
MgCl2	Magnesium Chloride
mGLUR	metabotropic glutamate receptor
MIP-1/2	Macrophage Inflammatory Protein-1/2
MLU	Medial Lamellar Unit

MMP	Matrix Metalloproteinase
MØ	Macrophage Inflammatory Protein-1/2
MR	Mendelian Randomization
MT1-MMP	Membrane Type-1- Matrix Metalloproteinase (MMP-14)
MTHFR	Methylene Tetrahydrofolate Reductase
MyD88	Myeloid Differentiation primary response gene 88
MZ	Monozygotic (Twin)
NAD(P)H	Nicotineamide Adenine Dinucleotide Phosphate (reduced)
NF-κB	Nuclear Factor kappa Beta
NLR3P	NOD-like receptor family, pyrin domain containing 3
NOX1	NADPH Oxidase 1
NOX2	NADPH Oxidase 2
NOXA	Latin for damage. A p53 inducible proapoptotic protein
Nrf2	Nuclear factor (erythroid-derived 2)-like 2 (antioxidant transcription factor)
O2-	Superoxide radical
OH.-	Hydroxyl radical
ONOO-	Peroxynitrite
OPN	Osteopontin
p15INK4B	Cyclin Dependent Kinase 4B Inhibitor
p16INK4A	Cyclin Dependent Kinase 4 A inhibitor
p19ARF	Alternative Reading Frame to p16
p21	protein 21
p22phox	protein 22 subunit of the neutrophil phagocyte oxidase
p38MAPK	protein 38 Mitogen Activated Protein Kinase
p47phox	protein 47 subunit of neutrophil phagocyte oxidase

p53	protein 53 (tumour suppressor protein)
p63	protein 63 (p53 family member)
p73	protein 73 (p53 family member)
PCR	Polymerase Chain Reaction
PDGFRβ	Platelet Derived Growth Factor Receptor beta
PI3K	Phosphoinositide Kinase
PKA	Protein Kinase A
PKCδ	Protein Kinase C subtype delta
PPARγ	Peroxisome Proliferator Receptor subtype gamma
PVD	Peripheral Vessel Disease
RAGE	Receptor of Advanced Glycation End-products
RECK	Reversion-inducing-cysteine-rich protein with kazal motifs
RhoA	Ras homolog gene family, member A
ROCK	Rho -associated Protein Kinase
ROS	Reactive Oxygen Species
S100A12	S100 calcium-binding protein A12 (calgranulin C, EN-RAGE, p6)
SAHF	Senescence Associated Heterochromatic Foci
SHP-2	Src-Homology Protein -2
SHPS-1	Src-Homology Protein Substrate -1
SIRT	Silent mating type Information Regulation
SNP	Single Nucleotide Polymorphism
SWSSG	Spatial Wall Shear Stress Gradient
TA	Thoracic Aorta
TAA	Thoracic Aortic Aneurysm
TAK1	Transforming Growth Factor beta (TGFβ)-Associated Kinase-1

TBE	Tris Base, Boric Acide and EDTA (Ethylamine Diacetic Acid)
TCRα/β	T- Cell Receptor type alpha/beta
TGFβ	Transforming Growth Factor beta
Th	Helper T cell
tHcy	total Homocysteine
TIF	Telomere Dysfunction-Induced Focus
TIMP-1	Tissue Inhibitor of Matrix Metalloproteinase
TIRAP	Tumour Necrosis Factor (TNF)-Receptor Associated Protein
TLR-2,-4	Toll-like Receptor -2, -4
TNFα	Tumour Necrosis Factor alpha
TRAF2	Tumour Necrosis Factor Receptor Associated Factor-2
TRAM	Toll/IL-1R domain containing adaptor-inducing IFNβ-related adaptor molecul
Treg	Regulatory T-Cell (Lymphocyte)
TRX	Thioredoxin
UPR	Unfolded Protein Response
UV	Ultraviolet
VCAM-1	Vascular Cell Adhesion Molecule-1
VEGF	Vascular Endothelial Growth Factor
VEGFR2	Vascular Endothelial Growth Factor Receptor 2
VIV	Vortex Induced Vibration
WSSG	Wall Shear Stress Gradient
xIAP	x-linked Inhibitor of Apoptosis
γ-H2AX	gamma variant histone

Appendix [4]: Ethics Documentation

Ethics Approval Forms:

NHS
Health Research Authority

NRES Committee East Midlands - Leicester
The Old Chapel
Royal Standard Place
Nottingham
NG1 6FS

Tel: 0115 8839425
Fax: 0115 8839294

01 October 2012

Mr Nikesh Dattani
Academic Fellow in Vascular Surgery
Department of Cardiovascular Sciences
University of Leicester
Robert Kilpatrick Clinical Sciences Building
Leicester Royal Infirmary
Leicester
LE2 7LX

Dear Mr Dattani,

Study title: **The Role of Cytokines and Cytokine Gene Polymorphisms in the Development of Abdominal Aortic Aneurysms**
REC reference: **6819**
Amendment number: **13**
Amendment date: **08 August 2012**

The above amendment was reviewed at the meeting of the Sub-Committee held on 07 September 2012.

Ethical opinion

The members of the Committee taking part in the review gave a favourable ethical opinion of the amendment on the basis described in the notice of amendment form and supporting documentation.

Approved documents

The documents reviewed and approved at the meeting were:

Document	Version	Date
Notice of Substantial Amendment (non-CTIMPs)	13	08 August 2012
Participant Consent Form: 1	1	01 August 2012
Participant Consent Form: 3	1	01 August 2012
Participant Consent Form: 2	1	01 August 2012
Participant Information Sheet: 3 Controls	1	01 August 2012
Participant Information Sheet: 2 Cases Non Operative	1	01 August 2012
Participant Information Sheet: 1 Cases Operative	1	01 August 2012

NRES Committee East Midlands - Leicester

Attendance at Sub-Committee of the REC meeting on 07 September 2012

Name	*Profession*	*Capacity*
Mr John Baker (Vice Chair)	Radiation Protection Advisor and Senior Lecturer (retired)	Lay
Dr Carl Edwards (Chair)	Implementation Fellow	Lay

Also in attendance:

Name	*Position (or reason for attending)*
Mrs Carol Marten	Co - Ordinator

NHS
Central Office for Research Ethics Committees
(COREC)

NOTICE OF SUBSTANTIAL AMENDMENT

For use in the case of all research other than clinical trials of investigational medicinal products (CTIMPs). For substantial amendments to CTIMPs, please use the EU-approved notice of amendment form (Annex 2 to ENTR/CT1) at http://eudract.emea.eu.int/document.html#guidance.

To be completed in typescript by the Chief Investigator in language comprehensible to a lay person and submitted to the Research Ethics Committee that gave a favourable opinion of the research ("the main REC"). In the case of multi-site studies, there is no need to send copies to other RECs unless specifically required by the main REC.

Further guidance is available at http://www.corec.org.uk/applicants/apply/amendments.htm.

Details of Chief Investigator:

Name:	RD Sayers
Address:	Robert Kilpatrick Clinical Sciences Building Leicester Royal Infirmary Leicester LE2 7LX
Telephone:	01162523252
E-mail:	Rs152@le.ac.uk
Fax:	01162523179

Full title of study:	**An investigation into candidate genes and protein profiling for abdominal aortic aneurysms**
Name of main REC:	Leicestershire, Northamptonshire and Rutland 1
REC reference number:	6819
Date study commenced:	February 2005
Protocol reference *(if applicable)*, current version and date:	Version 5.2, May 2012
Amendment number and date:	Amendment number 13, August 2012

of the REC is sought.

List of enclosed documents

Document	*Version*	*Date*
Patient information sheet 1	1	August 2012
Patient information sheet 2	1	August 2012
Patient information sheet 3	1	August 2012
Consent form 1	1	August 2012
Consent form 2	1	August 2012
Consent form 3	1	August 2012

Declaration

- I confirm that the information in this form is accurate to the best of my knowledge and I take full responsibility for it.
- I consider that it would be reasonable for the proposed amendment to be implemented.

Signature of Chief Investigator: .....

Print name: .....

Date of submission: .....

PARTICIPANTS INFORMATION SHEETS

An investigation into candidate genes and protein profiling for abdominal aortic aneurysms

PATIENT INFORMATION SHEET 1 (Cases Operative) (Version 1 August 2012)

For patients with aneurysms undergoing repair.

Principal Investigator

Mr Robert D Sayers MD FRCS

Professor of Vascular Surgery

University Hospitals of Leicester NHS Trust

Contact telephone number: 0116 2523135

Introduction

You are invited to participate in the above study, which will improve our understanding of aneurysms and hopefully lead to improvements in their future treatment. We are comparing a group of patients with aneurysms to a group who do not have aneurysms. Your participation as someone who has an aneurysm would be greatly appreciated.

Study Purpose

Arteries are blood vessels that take blood away from the heart. The aorta is the main artery in the body. An aneurysm occurs when the wall of the artery becomes weak and it stretches. Although aneurysms usually affect the aorta, they can also occur in other blood vessels such as the leg arteries.

We do not know why some people develop aneurysms and others do not. We do know however, that aneurysms are more common in some families. This may suggest a genetic

cause. We also know that different people produce different amounts of certain types of protein in the body that can result in an aneurysm.

Protein levels in the blood are controlled by genes (chemicals that contain genetic information). We know that some people have genes that cause high or low levels of circulating proteins. A recent study has suggested that patients who produce a low level of one protein may be more likely to develop aneurysms. We aim to do a similar study in more detail to clearly see whether there is a link between aneurysms, proteins and genes. We will compare a group of patients with aneurysms to a group without aneurysms.

You have been invited to participate in this study because you have been diagnosed by your doctor as having an aneurysm.

The operation that you are to undergo is designed to prevent your aneurysm leaking. It involves opening the wall of the aortic aneurysm and placing a synthetic tube (graft) inside the aorta to carry the blood. There is a smaller artery that comes off the aorta called the inferior mesenteric artery, and it supplies blood to part of the colon (intestine). During aneurysm repair this artery is disconnected because the colon has a good blood supply from other arteries.

Taking part

It is up to you to decide to join the study. We will describe the study and go through this information sheet. If you agree to take part we will then ask you to sign a consent form. You are free to withdraw at any time without giving a reason. This would not affect the standard of care you receive.

What will be involved if I take part in the study?

The study looks at the levels of naturally occurring proteins and the genes that control the amount of proteins produced. In order to do this we need to take blood and urine samples, both before our operation, and 6 months after. You will probably have had blood tests before as part of medical care. We will only need 15-20ml of blood. From this we can analyse protein levels and the genes. Your genes will be examined by extracting and analysing DNA (which contains genetic information) from your blood sample. At the same time we would take a 20-30 ml sample of urine in order to study proteins in urine as well. **We will also ask some questions regarding your general medical background.**

We would also like to take a small specimen (1x1cm) from the wall of the aneurysm and a sample of the inferior mesenteric artery (1cm), if it is disconnected by the surgeon performing your operation. This will have no effect on your operation or recovery. We will use the specimen to investigate the protein levels in the wall of the artery.

As new potential genes that cause aneurysms are discovered all the time the sample taken from you may be used in future studies of these newer genes.

What will happen to any samples I donate?

Any samples donated will be made anonymous prior to storage and use in this study. Storage will be in locked refrigerators/freezers in a locked room of a secure building accessible only by the research team. Samples will not be transferred outside of the UK. Any additional material not used in the original study may be

used in further research into a related field, which may require further research and ethics committee approval if deemed necessary.

Will the information obtained in the study be confidential?

All details recorded in the study will be in the strictest confidence. No details that could be used to identify you will be used in any records relating to the study.

What if I am harmed by the study?

Medical research is covered for mishaps in the same way as for patients undergoing treatment on the NHS i.e. compensation is only available if negligence occurs.

What happens if I do not wish to participate in this study or wish to withdraw from the study?

If you do not wish to participate in this study or if you wish to withdraw from the study you may do so without justifying your decision and your future treatment will not be affected.

What if I want to make a complaint?

If you have a concern about any aspect of the study you should ask to speak to the principle investigator, or your consultant who will do their best to answer your questions. If you remain unhappy and wish to complain formally then you can do this through the Patient Advice and Liaison Service (PALS).

The results of the research

Upon completion of the study the results will be published in peer reviewed journals. You, your consultant, and your GP will not receive any personal information regarding this study.

Who is organising and funding the research?

This research is supported by the University of Leicester.

Who has reviewed the study?

All research in the NHS is looked at by an independent group of people, called a Research Ethics Committee, to protect you interests. This study has been approved by the Leicestershire, Northamptonshire & Rutland 1 Research Ethics Committee.

An investigation into candidate genes and protein profiling for abdominal aortic aneurysms

PATIENT INFORMATION SHEET 2 (Cases Non-operative) (Version 1 August 2012)

For patients with aneurysms not undergoing repair.

Principal Investigator

Mr Robert D Sayers MD FRCS

Professor of Vascular Surgery

University Hospitals of Leicester NHS Trust

Contact telephone number: 0116 2523135

Introduction

You are invited to participate in the above study, which will improve our understanding of aneurysms and hopefully lead to improvements in their future treatment. We are comparing a group of patients with aneurysms to a group who do not have aneurysms. Your participation as someone who has an aneurysm would be greatly appreciated.

Study Purpose

Arteries are blood vessels that take blood away from the heart. The aorta is the main artery in the body. An aneurysm occurs when the wall of the artery becomes weak and it stretches. Although aneurysms usually affect the aorta, they can also occur in other blood vessels such as the leg arteries.

We do not know why some people develop aneurysms and others do not. We do know however, that aneurysms are more common in some families. This may suggest a genetic cause. We also know that different people produce different amounts of certain types of protein in the body that can result in an aneurysm

Protein levels in the blood are controlled by genes (chemicals that contain genetic information). We know that some people have genes that cause high or low levels of circulating proteins. A recent study has suggested that patients who produce a low level of one protein may be more likely to develop aneurysms. We aim to do a similar study in more detail to clearly see whether there is a link between aneurysms, proteins and genes. We will compare a group of patients with aneurysms to a group without aneurysms.

You have been invited to participate in this study because you have been diagnosed by your doctor as having an aneurysm.

Taking part

It is up to you to decide to join the study. We will describe the study and go through this information sheet. If you agree to take part we will then ask you to sign a consent form. You are free to withdraw at any time without giving a reason. This would not affect the standard of care you receive.

What will be involved if I take part in the study?

The study looks at the levels of naturally occurring proteins and the genes that control the amount of proteins produced. In order to do this we need to take blood and urine samples, you will probably have had blood tests before as part of medical care. We will

only need 15-20ml of blood. From this we can analyse protein levels and the genes. Your genes will be examined by extracting and analysing DNA (which contains genetic information) from your blood sample. At the same time we would take a 20-30 ml sample of urine in order to study proteins in urine as well. **We will also ask some questions regarding your general medical background.**

As new potential genes that cause aneurysms are discovered all the time the sample taken from you may be used in future studies of these newer genes.

What will happen to any samples I donate?

Any samples donated will be made anonymous prior to storage and use in this study. Storage will be in locked refrigerators/freezers in a locked room of a secure building accessible only by the research team. Samples will not be transferred outside of the UK. Any additional material not used in the original study may be used in further research into a related field, which may require further research and ethics committee approval if deemed necessary.

Will the information obtained in the study be confidential?

All details recorded in the study will be in the strictest confidence. No details that could be used to identify you will be used in any records relating to the study.

What if I am harmed by the study?

Medical research is covered for mishaps in the same way as for patients undergoing treatment on the NHS i.e. compensation is only available if negligence occurs.

What happens if I do not wish to participate in this study or wish to withdraw from the study?

If you do not wish to participate in this study or if you wish to withdraw from the study you may do so without justifying your decision and your future treatment will not be affected.

What if I want to make a complaint?

If you have a concern about any aspect of the study you should ask to speak to the principle investigator, or your consultant who will do their best to answer your questions. If you remain unhappy and wish to complain formally then you can do this through the Patient Advice and Liaison Service (PALS).

The results of the research

Upon completion of the study the results will be published in peer reviewed journals. You, your consultant, and your GP will not receive any personal information regarding this study.

Who is organising and funding the research?

This research is supported by the University of Leicester.

Who has reviewed the study?

All research in the NHS is looked at by an independent group of people, called a Research Ethics Committee, to protect you interests. This study has been approved by the Leicestershire, Northamptonshire & Rutland 1 Research Ethics Committee.

An investigation into candidate genes and protein profiling for abdominal aortic aneurysms

PATIENT INFORMATION SHEET 3 (Controls) (Version 1 August 2012)

For patients without aneurysms.

Principal Investigator

Mr Robert D Sayers MD FRCS

Professor of Vascular Surgery

University Hospitals of Leicester NHS Trust

Contact telephone number: 0116 2523135

Introduction

You are invited to participate in the above study, which will improve our understanding of aneurysms and hopefully lead to improvements in their future treatment. We are comparing a group of patients with aneurysms to a group who do not have aneurysms. Your participation as someone who does not have an aneurysm would be greatly appreciated.

Study Purpose

Arteries are blood vessels that take blood away from the heart. The aorta is the main artery in the body. An aneurysm occurs when the wall of the artery becomes weak and it stretches. Although aneurysms usually affect the aorta, they can also occur in other blood vessels such as the leg arteries.

We do not know why some people develop aneurysms and others do not. We do know however, that aneurysms are more common in some families. This may suggest a genetic cause. We also know that different people produce different amounts of certain types of protein in the body that can result in an aneurysm

Protein levels in the blood are controlled by genes (chemicals that contain genetic information). We know that some people have genes that cause high or low levels of circulating proteins. A recent study has suggested that patients who produce a low level of one protein may be more likely to develop aneurysms. We aim to do a similar study in more detail to clearly see whether there is a link between aneurysms, proteins and genes. We will compare a group of patients with aneurysms to a group without aneurysms.

You have been invited to participate in this study because you have been diagnosed by your doctor as not having an aneurysm.

Taking part

It is up to you to decide to join the study. We will describe the study and go through this information sheet. If you agree to take part we will then ask you to sign a consent form. You are free to withdraw at any time without giving a reason. This would not affect the standard of care you receive.

What will be involved if I take part in the study?

The study looks at the levels of naturally occurring proteins and the genes that control the amount of proteins produced. In order to do this we need to take blood and urine samples, you will probably have had blood tests before as part of medical care. We will

only need 15-20ml of blood. From this we can analyse protein levels and the genes. Your genes will be examined by extracting and analysing DNA (which contains genetic information) from your blood sample. At the same time we would take a 20-30 ml sample of urine in order to study proteins in urine as well. **We will also ask some questions regarding your general medical background.**

If you undergo any aortic surgery, we would also like to take a small specimen (1x1cm) from the wall of the aorta. This will have no effect on your operation or recovery. We will use the specimen to investigate the protein levels in the wall of the artery.

As new potential genes that cause aneurysms are discovered all the time the sample taken from you may be used in future studies of these newer genes.

What will happen to any samples I donate?

Any samples donated will be made anonymous prior to storage and use in this study. Storage will be in locked refrigerators/freezers in a locked room of a secure building accessible only by the research team. Samples will not be transferred outside of the UK. Any additional material not used in the original study may be used in further research into a related field, which may require further research and ethics committee approval if deemed necessary.

Will the information obtained in the study be confidential?

All details recorded in the study will be in the strictest confidence. No details that could be used to identify you will be used in any records relating to the study.

What if I am harmed by the study?

Medical research is covered for mishaps in the same way as for patients undergoing treatment on the NHS i.e. compensation is only available if negligence occurs.

What happens if I do not wish to participate in this study or wish to withdraw from the study?

If you do not wish to participate in this study or if you wish to withdraw from the study you may do so without justifying your decision and your future treatment will not be affected.

What if I want to make a complaint?

If you have a concern about any aspect of the study you should ask to speak to the principle investigator, or your consultant who will do their best to answer your questions. If you remain unhappy and wish to complain formally then you can do this through the Patient Advice and Liaison Service (PALS).

The results of the research

Upon completion of the study the results will be published in peer reviewed journals. You, your consultant, and your GP will not receive any personal information regarding this study.

Who is organising and funding the research?

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Who has reviewed the study?

All research in the NHS is looked at by an independent group of people, called a Research Ethics Committee, to protect you interests. This study has been approved by the Leicestershire, Northamptonshire & Rutland 1 Research Ethics Committee.

Participant Consent Forms:

PATIENT CONSENT FORM 1 (Version 1 August 2012)

An investigation into candidate genes and protein profiling for abdominal aortic aneurysms

Principal Investigator:
Mr Robert D Sayers MD FRCS
Professor of Vascular Surgery
University Hospitals of Leicester NHS Trust

This form should be read in conjunction with the PATIENT INFORMATION SHEET 1 (Cases Operative) dated AUGUST 2012.

Please initial box

1. I confirm that I have read and understand patient information sheet 1 dated August 2012 for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected. ☐

3. I understand that relevant sections of my medical notes and data collected during the study, may be looked at by the researchers, from regulatory authorities or from the NHS Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records. ☐

4. I understand that this research involves donating blood, tissue, and urine samples, and my genetic information being analysed. I understand that the tissue is a gift and that I will not benefit from any intellectual property that results from the use of the tissue. ☐

5. I give my consent for the anonymous samples that I donate for this research project to be used in other research projects in the future, including use by other research teams. ☐

6. I agree to take part in the above study. ☐

Name of Patient _____ Date_____Signature _____

Name of Person _____ Date_____Signature _____
taking consent

PATIENT CONSENT FORM 2 (Version 1 August 2012)

An investigation into candidate genes and protein profiling for abdominal aortic aneurysms

Principal Investigator:
Mr Robert D Sayers MD FRCS
Professor of Vascular Surgery
University Hospitals of Leicester NHS Trust

This form should be read in conjunction with the PATIENT INFORMATION SHEET 2 (Cases non-operative) dated AUGUST 2012.

Please initial box

1. I confirm that I have read and understand patient information sheet 2 dated August 2012 for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected. ☐

3. I understand that relevant sections of my medical notes and data collected during the study, may be looked at by the researchers, from regulatory authorities or from the NHS Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records. ☐

4. I understand that this research involves donating blood, tissue, and urine samples, and my genetic information being analysed. I understand that the tissue is a gift and that I will not benefit from any intellectual property that results from the use of the tissue. ☐

5. I give my consent for the anonymous samples that I donate for this research project to be used in other research projects in the future, including use by other research teams. ☐

6. I agree to take part in the above study. ☐

Name of Patient _____ Date _____ Signature _____

Name of Person _____ Date _____ Signature _____
taking consent

PATIENT CONSENT FORM 3 (Version 1 August 2012)

An investigation into candidate genes and protein profiling for abdominal aortic aneurysms

Principal Investigator:
Mr Robert D Sayers MD FRCS
Professor of Vascular Surgery
University Hospitals of Leicester NHS Trust

This form should be read in conjunction with the PATIENT INFORMATION SHEET 3 dated AUGUST 2012.

Please initial box

1. I confirm that I have read and understand patient information sheet 3 dated August 2012 for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected. ☐

3. I understand that relevant sections of my medical notes and data collected during the study, may be looked at by the researchers, from regulatory authorities or from the NHS Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records. ☐

4. I understand that this research involves donating blood, tissue, and urine samples, and my genetic information being analysed. I understand that the tissue is a gift and that I will not benefit from any intellectual property that results from the use of the tissue. ☐

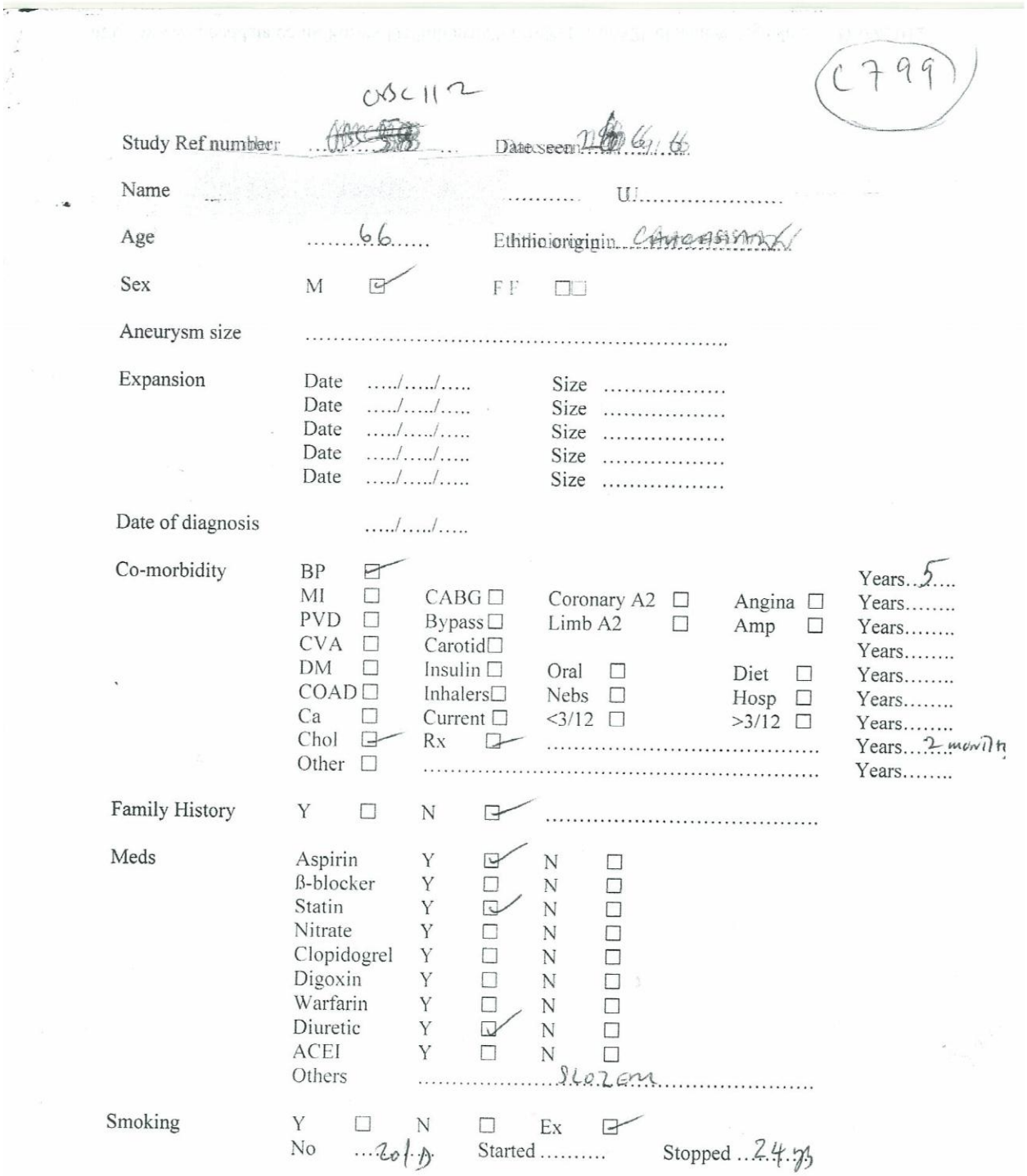
5. I give my consent for the anonymous samples that I donate for this research project to be used in other research projects in the future, including use by other research teams. ☐

6. I agree to take part in the above study. ☐

Name of Patient _____ Date_____Signature _____

Name of Person _____ Date_____Signature _____
taking consent

Data Abstraction Form:



C799

OSC112

Study Ref number ...OSC558... Date seen 21/6/06

Name ..... U.....

Age .....66..... Ethnic origin Caucasian

Sex M ☑ F ☐

Aneurysm size .....

Expansion
Date ...../...../..... Size .....
Date ...../...../..... Size .....
Date ...../...../..... Size .....
Date ...../...../..... Size .....
Date ...../...../..... Size .....

Date of diagnosis ...../...../.....

Co-morbidity

BP	☑				Years..5....
MI	☐	CABG ☐	Coronary A2 ☐	Angina ☐	Years.....
PVD	☐	Bypass ☐	Limb A2 ☐	Amp ☐	Years.....
CVA	☐	Carotid ☐			Years.....
DM	☐	Insulin ☐	Oral ☐	Diet ☐	Years.....
COAD	☐	Inhalers ☐	Nebs ☐	Hosp ☐	Years.....
Ca	☐	Current ☐	<3/12 ☐	>3/12 ☐	Years.....
Chol	☑	Rx ☑	.....		Years...2 months
Other	☐	.....			Years.....

Family History Y ☐ N ☑ .....

Meds

Aspirin	Y	☑	N	☐
ß-blocker	Y	☐	N	☐
Statin	Y	☑	N	☐
Nitrate	Y	☐	N	☐
Clopidogrel	Y	☐	N	☐
Digoxin	Y	☐	N	☐
Warfarin	Y	☐	N	☐
Diuretic	Y	☑	N	☐
ACEI	Y	☐	N	☐
Others	..... Slo2cm			

Smoking Y ☐ N ☐ Ex ☑
No ...20/d Started ..... Stopped ..24.7.3

Laboratory Biohazard Form:

Ben

University of Leicester

CARD/31

APPLICATION TO HANDLE HAZARDOUS BIOLOGICAL AGENTS

1, Department

Cardiovascular Sciences –
Vascular Surgery Research Group

Head of Department

Professor N. Samani
Professor N. London (Head of Res. Group)

2. Proposer

Mr M. Bown

Other Users

Ben Obukofe
Julie Chamberlain
Jonathan Barber

3. Nature of Agent (source, strains, concentrations to be handled)

Human blood

4. Known Risk to Humans (routes of infection, known incidence of laboratory infection)

Potential infection risk.
No known high risk samples to be used.
Possibility of needlestick injury.

5. Procedures: Describe handling procedures and control measures to be used to minimise exposure during planned use. Consider aerosol generating procedures such as ultrasonication and centrifugation and the hazards posed by the use of sharps. Indicate whether a microbiological safety cabinet will be used. If necessary, attach a protocol to this form.

Hep B vaccination recommended.
Laboratory coat and gloves to be worn at all times. Eye protection to be worn as appropriate.
All procedures to be carried out in Class II Microbiological Safety Cabinet.
Centrifugation carried out in sealed tubes &/or buckets.
Glass & plasticware either soaked overnight in 2,500 ppm free chlorine prior to washing, or autoclaved, or disposed of via University Clinical Waste contractor.
Blood/tissue waste disposed of via University Clinical Waste contractor.
Sharps used only when essential & disposed of immediately after use.

6. Is animal work proposed?

~~YES~~/NO (If YES, give details)

7. Areas where work is to be carried out (room nos., type of room - e.g. research laboratory, hot room)

Laboratories 238 & 239 RKCSB.

8. Storage. (How will the agent be stored? At what location?)

-20°C & -80°C freezers in the Department.

9. Action to be taken in the event of an emergency (Disinfectants to be used, protective clothing, respiratory equipment if appropriate).

Wear lab. coat & gloves at all times. Wear eye protection as appropriate.
Spills cleaned immediately with 10,000ppm free chlorine or equivalent granules.
All paper used for mopping to be autoclaved or disposed of via University Clinical Waste Contractor.
In case of needlestick injury encourage bleeding, wash with soap & water and cover with a waterproof dressing.

10. *

Proposed hazard group classification	Proposed containment level for this work
2	2

* Information on hazard groups and requirements for work at different containment levels is given in the University of Leicester publication Hazardous Biological Agents.

11. Declaration

The information provided in this form is to the best of my knowledge accurate and I undertake to notify Safety Services of any future changes to procedures or materials described.

Signature of Principal Investigator

27.4.6
Date

Office Only

Received 28.04.06 Safety Services CAR0/31
Reference

Actions

Assigned Hazard Group 2 Containment Level 2

Principal Investigator notified 28.04.06 Additional Comments Attached?

Reference:

1 Isselbacher EM. Diseases of the aorta . In: Braunwald E, Zipes DP, Libby P and Bonour RO (eds). Braunwald's Heart Disease (Saunder Philadelphia) 2004; 1403-1404.

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