THE SIGNIFICANCE OF GLUCOSE TRANSPORTERS IN THE PATHOGENESIS OF ABDOMINAL AORTIC ANEURYSMS

Thesis submitted for the degree of

Doctor of Medicine

At the University of Leicester

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January 2018

"Whatever we do in life will seem insignificant, but it's very important that we do it"

Mahatma Gandhi

Abstract

The significance of glucose transporters in the pathogenesis of abdominal aortic aneurysms

By

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Abdominal aortic aneurysm (AAA) is associated with significant mortality worldwide. At present, the only treatment involves an operation. Understanding the pathogenesis is important to help develop new drug therapies aimed at slowing aneurysm growth. Diabetes mellitus (DM) has been shown to be negatively associated with AAA however the mechanisms underlying this relationship are poorly understood.

This thesis first confirmed the robustness of this epidemiological relationship through a meta-analysis of >70 studies and then, using whole aortic tissue samples (WATS) and aortic smooth muscle cells (AoSMCs) from patients with and without AAA, examined the importance of glucose transporters (GLUTs), a group of proteins responsible for sugar transport across cell membranes, in the pathogenesis of AAA and in explaining the negative relationship between DM and AAA.

Comparing WATS from patients with and without AAA, gene expression of GLUT3 ($p=0.004$) and GLUT6 ($p=0.04$) and protein expression of GLUT1 ($p=0.002$), GLUT3 ($p=0.002$) and GLUT6 ($p=0.004$) were significantly higher in WATS from AAA patients. Comparing AoSMCs from patients with and without AAA, gene expression of GLUTs was similar between groups however GLUT activity was significantly higher in AoSMCs from patients with AAA ($p=0.02$).

To study the effect of DM on GLUTs, AoSMCs from patients with and without AAA were exposed to increasing levels of hyperglycaemia (4.6mmol/L - 50mmol/L). Hyperglycaemia was not associated with a significant change in the gene expression of GLUTs, cathepsins or TIMPs, however hyperglycaemia within the physiological range (up to 25mmol/L) was associated with a significant decrease in GLUT activity ($p=0.01$) selectively in the AoSMCs from AAA patients, independent of any hyperosmolar effect.

In conclusion, these results suggest that glucose transporters are important in the pathogenesis of AAA and may be involved in regulating the protective effect of DM on AAA. Targeting glucose transporters to slow aneurysm growth merits further investigation.

Acknowledgements

Thank you to my supervisors, Professors Matt Bown and Rob Sayers, for their constant support and supervision before, during and after my time in the lab.

Thank you to the colleagues that helped me collect the numerous patient tissue samples that were needed to conduct this research, especially Sarah Hosgood for remembering to call me whenever there was a cadaveric kidney arriving at the Leicester General and the Consultant Vascular Surgeons at the Leicester Royal Infirmary for allowing me to consent their patients.

Thank you to all those that helped me with the various experiments I performed but in particular Julie Chamberlain, Jon Barber & Ana Verissimo for keeping the aortic smooth muscle cells alive when I couldn't be around, Alan Bevington and Jez Brown for helping me with the glucose transport assays and Nic Sylvius for helping with the gene expression work. I'd also like to thank my fellow clinical research fellows (David, Phil, Ramesh, Vimal, Badri & Muhammed) for making my time in the lab enjoyable.

A special thank you to the Circulation Foundation for funding my research via the Surgeon Scientist Award, this helped me immensely.

I'd like to thank my parents for their support with all my studies, my son Arushan for being the enjoyment in my life and my grandfather who sadly passed away whilst writing up this thesis but whose wisdom I will never forget.

Finally, my *greatest* thanks must go to my wife Manisha whose love, support and understanding during this thesis has been immeasurable.

Statement of Originality

The work on which this thesis is based is my own independent work except where acknowledged.

Nikesh Dattani

January 2018

Funding, prizes, publications and presentations related to this thesis

Funding and prizes

1. **Surgeon Scientist Award 2012**, The Circulation Foundation, £55,000
2. **British Journal of Surgery prize 2014**, Vascular Society of Great Britain and Ireland 2014, £600

Publications

1. **Dattani N**, Sayers R, Bown M. Explaining the relationship between diabetes mellitus and abdominal aortic aneurysms: a review of the mechanisms. Accepted for publication to *Diabetes & Vascular Disease Research.*

2. **Dattani N**, Saratzis A, Sayers R, Bown M. Defining the relationship between diabetes mellitus and aortic aneurysm: a meta-analysis. *British Journal of Surgery* 2017; 107 (S5): 14.

3. Butt HZ, Sylvius N, Salem MK, Wild JB, **Dattani N**, Sayers RD, Bown MJ. Microarray-based gene expression profiling of abdominal aortic aneurysm. *European Journal of Vascular and Endovascular Surgery* 2016; 52 (1): 47 – 55.

4. **Dattani N**, Verissimo A, Bevington A, Sylvius N, Choke E, Sayers R, Bown M. Diabetes mellitus protects against abdominal aortic aneurysm via regulation of glucose transporters. *British Journal of Surgery* 2015; 102 (S2): 5.

5. Stather PW, Sylvius N, Sidloff DA, **Dattani N**, Verissimo A, Wild JB, Butt HZ, Choke EC, Sayers RD, Bown MJ. Identification of microRNAs associated with abdominal aortic aneurysms and peripheral arterial disease. *British Journal of Surgery* 2015; 102 (7): 755 – 66.

6. Sidloff D, Stather P, **Dattani N**, Bown M, Thompson J, Sayers R, Choke E. Aneurysm Global Epidemiology Study: Public Health measures can

further reduce Abdominal Aortic Aneurysm Mortality. *Circulation* 2014; 129 (7): 747 -53.

7. Stather PW, Sidloff DA, **Dattani N**, Gokani VJ, Choke E, Sayers RD, Bown MJ. Meta-analysis and meta-regression analysis of biomarkers for abdominal aortic aneurysm. *British Journal of Surgery* 2014; 101 (11): 1358 – 72.

Oral presentations

1. **Dattani N**, Saratzis A, Sayers RD, Bown MJ. Defining the relationship between aortic aneurysm and diabetes mellitus: a meta-analysis. The Vascular Societies ASM 2016. 30th Nov– 2nd Dec 2016
2. **Dattani N**, Bevington A, Sylvius N, Choke E, Sayers RD, Bown MJ. The significance of glucose transporters in the pathogenesis of abdominal aortic aneurysms. Midlands Vascular Society Research Meeting 2015. January 2015.
3. **Dattani N**, Verissimo A, Bevington A, Sylvius N, Choke E, Sayers RD, Bown MJ. Diabetes protects against abdominal aortic aneurysms via modulation of glucose transporters. The Vascular Societies ASM 2014. 26th – 28th Nov– 2nd Dec 2014.
4. **Dattani N**, Ali M, Aber A, Choke E, Bown MJ, Sayers RD. Popliteal artery aneurysm growth rates in patients with multianeurysmal disease. Society of Academic & Research Surgery AGM 2014. 8th – 9th January 2014.

Abbreviations

AA	aortic aneurysm
AAA	abdominal aortic aneurysm
ABF	aorto-bifemoral bypass
ABPI	ankle brachial pressure index
ACE	angiotensin converting enzyme
ACS	acute coronary syndrome
ADAM	Aneurysm Detection and Management
Adj	adjusted
ADP	adenosine diphosphate
AGE	advanced glycation end-product
AMPK	adenosine monophosphate-activated protein kinase
Ang II	angiotensin II
AOD	aorto-occlusive disease
AoSMCs	aortic smooth muscle cells
AP	anterior to posterior
aPKC	atypical protein kinase C
ApoE-def	apolipoprotein E-deficient
ARB	angiotensin receptor blocker
ARRDC4	Arrestin domain containing 4
AS160	Akt substrate of 160kDA
AT1	angiotensin type 1
ATBC	alpha-tocopherol, beta-carotene
ATP	adenosine triphosphate
BG	blood glucose
BMI	body mass index
BMT	best medical therapy
BSA	bovine serum albumin
CABG	coronary artery bypass grafting
CAD	cadaveric
CALIBER	cardiovascular disease research using linked bespoke studies and electronic health records
CC	case control
cDNA	complementary DNA
CI	confidence interval
cm	centimetres
CML	carboxymethyllysine
CNN2	calponin 2
CPRD	clinical practice research datalink
CT	computed tomography

Ct	cycle threshold
CTLA-4	cytotoxic T lymphocyte–associated protein 4
CV	cardiovascular
DDP	dipeptidyl peptidase
dL	decilitre
DM	diabetes mellitus
DMEM	dulbecco modified eagle medium
DNA	deoxyribonucleic acid
dpm	disintegrations per minute
DTT	dithiothreitol
dx	diagnosis
ECM	extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
Endart	Endarterectomy
ERK	extracellular signal-regulated kinases
EVAR	endovascular aneurysm repair
FCS	fetal calf serum
FDG	fluorodeoxyglucose
FPG	fasting plasma glucose
GAPDH	glyceraldehyde-3 phosphate dehydrogenase
GCA	giant cell arteritis
GDM	gestational diabetes mellitus
GIP-1	gastric inhibitory polypeptide 1
GLP-1	glucagon-like peptide 1
GLUT	glucose transporter
GO	gene ontology
GPRD	general practice research database
GWAS	genome wide association study
HbA1c	glycated haemoglobin
HBS	Hepes buffered saline
HDL	high-density lipoprotein
HEK	human embryonic kidney
HES	Hospital episode statistics
HLA	human leucoyte antigen
HMG-CoA	3-hydroxy-3-methyl-glutaryl-coenzyme A
HR	hazard ratio
HRP	horseradish peroxidase
hx	history
ICD	International classification of diseases
Ig	immunoglobulin
IHC	immunohistochemistry
IHD	ischemic heart disease
IL	interleukin

ILT	intraluminal thrombus
IMS	industrial methylated spirit
IPD	individual patient data
IR	insulin receptor
IRR	incidence rate ratio
IRS	insulin receptor substrate
ITI	inner to inner
IV	intravenous
JNK	c-jun N-terminal kinases
kDA	kilo dalton
kg	kilogram
LDL	low-density lipoprotein
LDLR	low-density lipoprotein receptor
LOMAS	Longitunidal Multilevel Analysis Scania
LRP1	low density lipoprotein receptor-related protein 1
M199	medium 199
M231	medium 231
MAPK	mitogen activated protein kinase
MASS	Multicentre Aneurysm Screening Study
MCP	monocyte chemoattractant protein
mg	milligram
MHC	major histocompatability complex
MINAP	myocardial ischemia national audit project
mins	minutes
mm	millimetre
mmol	millimole
MMP	matrix metalloproteinase
mRNA	messenger RNA
MSR	macrophage scavenger receptor
MZ	monozygotic
NAAASP	NHS AAA screening program
NaCl	sodium chloride
NAFLD	non-alcoholic fatty liver disease
NaOH	sodium hydroxide
NEFA	non-esterified fatty acid
NF-kB	nuclear factor kappa-light-chain-enhancer of activated B cells
NHS	National Health Service
NO	nitric oxide
NS	not specified
OC	observational cohort
OGTT	oral glucose tolerance test
OR	odds ratio
OSR	open surgical repair

OTO	outer to outer
P/S	penicillin / streptomycin
PAD	peripheral arterial disease
PAGE	polyacrylamide gel electrophoresis
PAI	plasminogen activator inhibitor
PBS	population based screening
PBS	phosphate buffered saline
PEI	polyethylenimine
PET	positron emission tomography
PGK1	Phosphoglycerate kinase 1
PI3	phospoinositide 3-kinase
PPAR	peroxisome proliferator-activated receptor
PRIMSA	Preferred Reporting Items for Systematic Reviews and Meta-analyses
Prosp	prospective
QALY	quality adjusted life year
QoL	quality of life
qPCR	quantitative real-time polymerase chain reaction
RAAA	ruptured AAA
RAGE	receptor for advanced glycation end-product
RAS	renin-angiotensin system
RCC	retrospective case-control
RCT	randomised controlled trial
REACH	Reduction of atherothrombosis for continued health
Retro	retrospective
RIN	RNA integrity number
RNA	ribonucleic acid
ROS	reactive oxygen species
rpm	revolutions per minute
RR	relative risk
sAGE	soluble advanced glycation end-product
SAVE	stroke and aneurysm vascular evaluation
SDS	sodium dodecyl sulphate
SE	standard error
SERPINB9	serpin peptidase inhibitor clade B member 9
shRNA	small hairpin RNA
siRNA	small intefering RNA
SLC2A	solute carrier family 2
SMART	secondary manifestations of arterial disease
SMC	smooth muscle cell
SMGS	smooth muscle cell growth supplement
SORT1	sortilin 1
sRANKL	soluble receptor activator of NF-κB ligand
SS	selective screening

STZ	streptozotocin
SUVmax	maximum standardised uptake value
T1DM	type 1 diabetes mellitus
T2DM	type 2 diabetes mellitus
TAA	thoracic aortic aneurysm
TAAA	thoracoabdominal aneurysm
TAAD	thoracic aortic aneurysm and dissection
TBP	TATA-box binding protein
TBS	Tris-buffered saline
TEMED	tetramethylethylenediamine
TGF	transforming growth factor
TIMP	tissue inhibitor of metalloproteinase
TMD	transmembrane domain
TNF	tumour necrosis factor
tPA	tissue plasminogen activator
Txnip	thioredoxin-interacting protein
TZD	thiazolidinedione
UHL	University Hospitals of Leicester
UKSAT	United Kingdom Small Aneurysm Trial
Unadj	Unadjusted
US	United States
USS	ultrasound scan
VA	veterens affairs
VDCC	voltage-dependent calcium channel
VEGF	vascular endothelial growth factor
vs.	versus
WATS	whole aortic tissue specimens
WHO	World Health Organisation
yrs	years
µL	microlitre

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Introduction

Chapter 1: Abdominal Aortic Aneurysms

1.1 Definition

The word 'aneurysm' derives from the Greek '*aneurysmos*' which translates as 'dilation'[1]. An aneurysm can be defined as an 'excessive localised swelling of any blood vessel in the body' since both arterial and venous aneurysms are documented in the literature[2]. More accurately, this refers to an increase in vessel diameter by >50% compared to the adjacent normal blood vessel with dilation <50% being referred to as ectasia[3].

An abdominal aortic aneurysm (AAA) refers to an aneurysm of the abdominal aorta which represents the portion of aorta below the diaphragm and is a continuation of the descending thoracic aorta. Anatomically the abdominal aorta lies between vertebral levels T12 and L4. At L4 it divides into left and right common iliac arteries. An arbitrary definition of $\geq$3 centimetres (cm) is used to define AAA[3], with abdominal aortas measuring between 2.5 to 2.9cm in diameter being referred to as subaneurysmal[4]. With respect to pathophysiology, AAA can develop as a result of atherosclerosis, infection or inflammation, with atherosclerotic AAA being the most common. With respect to extent, AAA confined to below the renal arteries are defined as 'infra-renal' which are most common, AAA extending to the level of the renal arteries are defined as 'juxta-renal' and AAA extending above the level of the renal arteries are defined as 'supra-renal'. Supra-renal AAA extending up to the level of diaphragm are termed type 4 thoraco-abdominal aneurysms (TAAAs) according to the Crawford Classification[5]. AAA can also extend distally to involve the common iliac arteries.

Distinguishing extent is important from a treatment point of view since more proximal extension is often associated with more complex treatment. Figure 1.1 depicts various aortic aneurysm configurations.

Figure 1.1. Various aortic aneurysm configurations. Reproduced from www.aorticdissection.com[6].

1.2 Epidemiology

1.2.1 Prevalence

AAA is more common in the elderly, male sex and in persons of white or European background. In the UK, the prevalence of AAA was reported as 7.7% amongst males aged 65 to 80 years old (4.9% for males 65 to 74 years old) in the Multicentre Aneurysm Screening Study (MASS) and 1.3% amongst women of the same age[7]. Examining males in their 65th year of life, the National Health Service (NHS) AAA screening program (NAAASP) reported a prevalence of 1.2%[8]. In the United States (US), Lederle *et al* screened male veterans aged 50 to 79 years old and found a prevalence of 4.6% for AAA $\geq$ 3cm, although prevalence rates were less for larger AAA[9]. However, the prevalence amongst the general population and women is far less. Kent *et al* reviewed questionnaire data from more than 3 million US individuals, and found a prevalence of only 0.77% in the general population[10]. Lederle *et al* found a prevalence of only 0.3% amongst women aged 50 to 79 years old[11]. In European populations, the prevalence rates seem to be similar to those in the UK. Lindholt *et al* performed a trial of screening Danish males aged 64 to 73 years and found a prevalence of 4%[12]. Svensjo *et al* screened only Swedish males in their 65th year of life and found a prevalence of 1.7%[13].

1.2.2 Incidence

The annual incidence rate for AAA diagnoses lies between 0.4% to 0.67% for Western populations and similar to prevalence rates, the incidence rate is higher in males and in those of white background[14]. Although previously a disease on

rise, western populations are now seeing a decrease in the incidence of AAA but also a shift to the older population. Norman *et al* reported a decline in the rate of hospitalization from ruptured and non-ruptured AAA in Australia between 1996 and 2006[15] and Sandiford *et al* reported seeing a similar trend in New Zealand between 1991 and 2007[16]. More recently, Laine *et al* has reported the incidence of AAA to be decreasing in Finland between 2003 and 2013[17]. In England and Wales, Choke *et al* and Anjum *et al* have shown a decline in the incidence of AAA since 1997 with the most rapid decline occurring in males <75 years old[18,19]. The reasons for this shift are still being investigated however changes in the prevalence of smoking seem to be one possibility[18].

1.2.3 Mortality

Epidemiological investigations reveal that mortality from AAA is decreasing. In England and Wales, the number of deaths due to aortic aneurysms has fallen from 6195 deaths in 2011 to 5886 deaths in 2013 and more recently to 5557 deaths in 2015[20]. Choke *et al* have studied these trends further and reported an annual decrease of 5.08% in age-standardised mortality from AAA in England and Wales. Furthermore they have suggested that significant reductions in the number of deaths from ruptured AAA (54.6% decline) are the principal component of decline[18]. Laine *et al* has reported a decline in mortality from AAA in Finland between 2003 and 2013[17] and studies from both Australia and New Zealand have reported similar decreases in AAA mortality of 38% and 53% respectively (in males) [16,21]. The authors from all studies have cited reductions in tobacco consumption in their respective countries as the most likely

explanation for these trends. Our own group has correlated trends in cardiovascular risk factors with trends in AAA mortality for various countries globally and found a significant positive correlation between trends in smoking prevalence and trends in AAA mortality for both males and females[22].

1.3 Risk factors

Risk factors for AAA are based on large-scale cross-sectional observational studies and can be stratified into risk factors for 1) AAA presence (prevalence and/or incidence), 2) AAA growth and 3) AAA rupture.

1.3.1 Age

The risk of developing AAA is positively associated with advancing age. One English study found that the risk of developing AAA increases by 40% for every 5-year increment beyond the age of 65[23]. Similarly, a large Norwegian study found that compared to individuals aged 65 to 69 years, individuals aged 70 to 74 years were 1.28-times more likely and individuals aged ≥75 years were 7.73-times more likely to develop AAA[24]. In contrast, increasing age is not a significant risk factor for AAA growth but maybe important for risk of rupture as was shown by the RESCAN collaborators (hazard ratio (HR) 1.04 per year increase in age) [25].

1.3.2 Sex

The risk of developing AAA is significantly higher amongst men. Vardulaki *et al* found the risk to be nearly 6-fold higher in men whereas the Tromso study report a more conservative odds ratio (OR) of 2.66[23,26]. Women naturally have smaller aortic diameters therefore using an absolute (aortic diameter of ≥3cm) rather than relative (>50% larger than diameter of adjacent normal vessel) definition would underestimate the true prevalence of aneurysms in women[27]. The reasons for this sexual dimorphism involve a combination of hormonal,

genetic and environmental factors however further research is required in this area[28,29]. Conversely, AAA growth rates might be higher amongst women with the Tromso study reporting rates nearly 1.5-fold those seen in men[30]. Rupture rates are also higher amongst women with the RESCAN collaborators reporting rupture rates for small AAA (<5.5cm) nearly 4-fold those seen in men.

1.3.3 Family history

AAA is known to have a strong hereditary component with some studies suggesting heritability accounts for as much as 70% of the risk of developing AAA[31]. In Sweden for example, the incidence of AAA amongst siblings of patients with AAA was found to be 13-fold that of siblings of patients without AAA[32]. Interestingly the risks were even higher when the first sibling developed AAA before the age of 50 suggesting that AAA in younger patients is likely to have a stronger hereditary component. The risks may also be gender-dependent with a recent study reporting relatives of female AAA patients having a 5.5-fold higher risk of developing AAA compared to relatives of male AAA patients having only a 2-fold increased risk of AAA[33]. Only one study from Japan has investigated the relationship between a family history for AAA and AAA growth rates[34]. The study found growth rates to be 2.2mm/year higher in those with a positive family history for AAA which would presumably lead to increase rates of AAA rupture although further research in this area is required.

1.3.4 Race & ethnicity

AAA seems to be a disease that predominantly affects persons of white or Caucasian background. In England, Bradford, Leicester and London are all cities with significantly ethnically diverse populations[35]. Spark *et al* studied a Bradford cohort of patients with AAA and found that none had occurred in patients of Asian origin[36]. Similarly, Salem *et al* studied a Leicester cohort of patients undergoing AAA screening and found a nearly 10-fold lower incidence of AAA in Asian men despite Asians constituting nearly 30% of the local population. Benson *et al* recently studied the cohort of patients in southwest London undergoing AAA screening and found that patients of white-British background were more than twice as likely to have an aneurysm than patients of black or Black-British background and nearly six times more likely than patients of Asian or Asian-British background[37]. Furthermore no AAA were identified in patients of Chinese background. In the US, LaMorte *et al* also noticed that, despite black patients requiring significantly more lower limb bypass operations than white patients, open AAA repair was rarely performed in black patients. The group calculated an OR of 0.29 for black patients undergoing open AAA repair versus white patients[38]. The effect of race on AAA growth or rupture rates is currently unknown but new insights may be gained by the results of the UK Aneurysm Growth Study which is ongoing.

1.3.5 Smoking

The association between smoking and AAA development is well-established[39-42] however only recently have the underlying mechanisms been elucidated[43].

Jahangir *et al* found that smoking was the strongest risk factor for the development of AAA in a cohort study containing nearly 19,000 patients (HR 5.55) and amongst females the risks were even more pronounced (HR 9.1) [44]. The group also showed that current versus former smoking was more risky for AAA development. In fact the results from the Atherosclerosis Risk in Communities (ARIC) study show that current smokers aged 45 have nearly a 1 in 9 chance of developing AAA in their lifetime. ARIC also shows that smoking cessation whilst middle-aged reduces the future risk of developing AAA by nearly 30%[45]. These findings are supported by the Tromso study which suggests smoking cessation for >20 years can lower the risk to near levels of non-smokers (HR 1.26) [46]. The benefit of early smoking cessation probably comes from the fact that duration of smoking rather than amount of smoking is the most important risk factor for AAA development[47] as confirmed in a recent cohort study which data on more than 3 million persons[10]. In addition to its effects on AAA development, smoking is one the most important risk factors for AAA growth and rupture; smoking increasing the growth rate of small AAA by 0.35mm/year and rupture rate by 2-fold[25].

1.3.6 Lipids

A number of studies have demonstrated that the risk of developing AAA increases in relation to rising total cholesterol levels. Iribarren *et al* found that patients with serum cholesterol levels between >240 mg/dL had a HR for AAA of 2.82 compared to those with normal serum cholesterol levels, whereas those with levels between 200 mg/dL to 239 mg/dL had a HR of 1.83[48]. Similarly, the

Tromso study found that patients with total cholesterol levels >7.55 mmol/L had more than a 2-fold risk of AAA whereas those with levels between 6.7 mmol/L – 7.5 mmol/L had only a 1.3-fold risk, compared to patients with normal levels[46]. A meta-analysis of 8 studies performed by Takagi *et al* found that elevated low-density lipoprotein (LDL) concentrations but decreased high-density lipoprotein (HDL) concentrations were associated with an increased risk of AAA presence[49] although HDL seems to be the more important of the two[50]. Total cholesterol levels have also recently been shown to predict AAA growth rates (r=0.38, p=0.01) [51]. No studies have assessed the relationship between cholesterol levels and AAA rupture rates however studies have shown that HMG-CoA reductase inhibitors (statins) are capable of slowing AAA growth and that their use is associated with lower rates of AAA rupture[52,53]. Given their numerous pleiotropic effects, it is possible that these benefits may be related to the non-cholesterol lowering actions of statins[54,55].

1.3.7 Blood pressure

Hypertension has been shown to increase the risk of AAA[48,56]. Vardulaki *et al* have reported a 30% - 40% increase in risk of AAA with hypertension[23] and Forsdahl *et al* reported an OR of 1.54 compared to patients with normal blood pressure[46]. Increased blood pressure, specifically increased mean arterial pressure, however does not seem to increase AAA growth rates but may increase AAA rupture rates (HR 1.32 for each 10mmHg increase in mean arterial pressure) [25]. The reasons for this disparity in risk are not yet fully understood and require further investigation.

1.3.8 Diet & Alcohol

The majority of evidence in this area comes from observations made on Swedish patients by researchers at the Karolinska Institute. Individuals consuming >2 servings of fruit per day were associated with 31% lower risk of non-ruptured AAA and 39% lower risk of ruptured AAA compared with individuals consuming no fruit each day[57]. Similar associations were not found between vegetable consumption and AAA risk. The same authors found that moderate consumption of alcohol (60g/week for women or 120g/week for men), in the form of beer or wine but not spirits, was associated with a reduced risk of developing AAA[58]. Research from the group in Western Australia sought to determine whether the 'Mediterranean paradox', which is seen in coronary artery disease patients, is also present for AAA. The study found that Australian migrants of Mediterranean origin had only a marginally lower prevalence of AAA than migrants not of Mediterranean origin and concluded that any Mediterranean paradox that exists for AAA is likely to be modest[59]. No studies examining the impact of dietary factors or alcohol consumption on AAA growth or ruptures rate have been performed.

1.3.9 Exercise

A recent study has shown that walking or cycling for >40minutes/day has been associated with a lower risk of AAA compared with almost never doing these activities (HR 0.59) [60]. The Tromso study also found lower levels of physical activity in both males and females with AAA compared to those without AAA (OR

0.79 to 0.80) [26]. Little is known about the effect of exercise on AAA growth and rupture rates.

1.3.10 Obesity

There have been numerous studies reporting a positive association between obesity and AAA presence. Wang *et al* followed up >25,000 men without AAA for over 10 years and found that incident AAA were more likely to develop in those with a baseline body mass index (BMI) $\geq$ 30kg/m^2 (HR 1.69) or 25 kg/m^2 to <30 kg/m^2 (HR 1.30) when compared to those with a BMI of <25 kg/m^2. They reported a 6% increase in the risk of AAA for every unit increase in BMI[61]. Kent *et al* reported similar findings in 'Life Line' screened patients with a BMI >25 kg/m^2 compared to those with a BMI of <25 kg/m^2 (OR 1.20). In contrast, Stackelberg *et al* were unable to find any association between BMI and AAA presence however using waist circumference as a marker of central adiposity, they reported a 15% increase in the risk of AAA for every 5cm increment in waist circumference (up to 100cm or 88cm in men or women respectively) [62]. Researchers from Western Australia have also confirmed a positive association between waist circumference and AAA presence (OR 1.14) in a cohort of >12,000 males[63]. Despite these studies, the most recent meta-analysis performed in 2015 by Takagi *et al* found no significant association between obesity and AAA prevalence (although there was a slight trend) [64]. The same group recently performed a systematic review on the relationship between BMI and AAA growth rates. They concluded that the current literature suggests that BMI is not associated with AAA growth although the review did not examine other

anthropometric measures such as waist circumference or waist-hip-ratio which are more closely related to levels of visceral adiposity[65]. In contrast, an individual patient data meta-analysis performed by the RESCAN collaborators found a negative association between BMI and AAA growth rates in unadjusted analyses (0.017 mm/year reduction AAA growth per unit increase in BMI) but this was lost after adjustment for co-variates (p=0.348) [25]. The same study also found a negative association between BMI and AAA rupture rates which was not lost after adjustment for co-variates (HR 0.93). The RESCAN authors postulated that increased BMI might be associated with lower AAA growth and rupture rates through an increase in the number of type 2 diabetics (diabetes was shown to be the strongest predictor of slower AAA growth) [25].

1.3.11 Diabetes Mellitus

Diabetes Mellitus (DM) is an established risk factor for atherosclerotic disease however various studies suggest DM may be negatively associated with the development, growth and rupture of AAA. A detailed analysis of the relationship between these two disease entities is provided in Chapter 3.

1.4 Natural history

The natural history of AAA is gradual expansion with eventual rupture unless prophylactic surgical repair is performed or the patient dies from an alterative cause of death. Both growth and rupture rates are higher amongst larger AAA however growth rates do not appear to increase in a linear fashion[66]. A recent meta-analysis examining small AAA growth and rupture rates concluded that on average AAA grow by 2.21 mm/year independent of age and sex but found that rupture rates were highly variable, ranging from 0.71 to 11.03 per 1000 person-years[25].

1.4.1 Clinical features

Non-ruptured AAA is usually asymptomatic but can cause abdominal or back pain if sufficiently large. Rarely patients can present with symptoms of lower limb ischemia secondary to distal embolisation or aneurysm thrombosis. In contrast, patients with ruptured AAA are nearly always symptomatic. The classical triad of abdominal or back pain, hypotension and a pulsatile abdominal mass are most common signs and symptoms. Other clinical features include flank or peri-umbilical bruising secondary to retroperitoneal haemorrhage, loss of consciousness, dizziness, sweatiness or shortness of breath.

1.5 Pathophysiology of AAA

The aetiology of AAA is complex with both genetic and environmental components. The Swedish Twin Study estimates the genetic component accounts for about 70% of the phenotypic variance in AAA whereas environmental factors such as smoking or infections only account for 30%[31]. Originally thought to be a manifestation of atherosclerosis, AAA seems to be a distinct disease entity with some authors suggesting that it forms part of a more widespread systemic dilating diathesis[67,68]. Evidence supporting such a hypothesis include observations of increased inguinal hernia rates amongst AAA patients suggesting an abnormality in collagen fibres[69], a 14% incidence of concomitant femoral or popliteal artery aneurysms in men with AAA[70] and the negative association between AAA and diabetes mellitus, a known atherosclerotic risk factor [71].

1.5.1 Histology

Histologically, AAA development is characterized by extracellular matrix (ECM) destruction, inflammatory cell infiltrate, smooth muscle cell apoptosis, oxidative stress and medial neovascularisation[72,73]. There is also often a degree of intimal atherosclerosis and laminated intra-luminal thrombus (ILT) [74]. These processes occur to varying degrees across 3 phases of AAA development namely the initiation, progression and rupture phases. During the initiation phase there is disruption to the normal helical organisation of elastin fibres within the tunica media as well as inflammatory cell infiltration[75,76]. As the aorta begins to expand, the progression phases ensues during which there is further loss of

elastin fibres as well as compensatory collagen fibre deposition[77,78]. The result is a loss in aortic wall elasticity and an increase in the expansion forces exerted on the aortic wall ultimately leading to further aortic dilatation during this phase. In addition, there is further inflammatory cell infiltration propagated by medial neovascularisation driven by the release of pro-angiogenic factors[79]. The rupture phase was originally thought to be purely a mechanical event occurring when the expansive forces of luminal blood pressure acting on a weakened aortic wall exceed its tensile strength[80]. However, AAA rupture is not confined to large aneurysms[41] and a number of studies also implicate biological factors in the process. Systemic[81] and localized[82] elevations of proteolytic enzymes as well as increased medial neovascularisation[83] and overexpression of proangiogenic cytokines[84] have all been reported as contributory. Ultimately there seems to be a shift in collagen balance from net production to net degradation which leads to aortic wall weakening and focal rupture so that the pathological hallmark of advanced AAA is ECM destruction.

1.5.2 Proteolytic enzymes

ECM destruction can largely be attributed to increased concentrations and activity of proteolytic enzymes in patients with AAA. Matrix metalloproteinases (MMPs), such as MMP-1, -2, -3, -8, -9, -12 and -13, cysteine proteases, such as cathepsin B, D, K, H, L and S, and serine proteases, such as tissue-type plasminogen activator (tPA) have all been implicated[85-87], although a recent meta-analysis suggests tPA may be less important[88]. MMPs are essentially endopeptidases which possess potent activity to ECM components within the

aortic wall including elastin and collagen[89]. Of those implicated in AAA, MMPs which act primarily on elastin (elastases) include MMP-2, -9 and -12 whereas MMPs which act primarily on interstitial collagen (collagenases) include MMP-1, -8 and -13[89]. Most cathepsins exhibit elastolytic activity and are endopeptidases although cathepsin H also possesses exopeptidase activity[90]. MMPs are inhibited by specific endogenous tissue inhibitors of metalloproteinases (TIMPs) of which there are 4 subtypes (TIMP1–4) whereas cathepsins are inhibited by cystatins[91,92].

MMP-2 and MMP-9 have received the most amount of attention in experimental studies. Tamarina *et al* studied MMP concentrations in aortic tissue samples from AAA patients and normal controls and found that MMP-9 was the predominant MMP expressed in AAA tissue, its mRNA level being more than 20-fold that of any other MMP measured including MMP-2[93]. Patel *et al* studied MMP-2, -3 and -9 levels in aortic smooth muscle cells (AoSMCs) from patients with AAA and compared them to levels in SMCs from control arterial tissues[94]. They reported significantly higher levels of MMP-2 and MMP-9 from the cells isolated from AAA tissue. Longo *et al* used murine knockout models to determine the role of MMP-2 and MMP-9. Mice genetically deficient of either MMP, but not wild type mice, failed to develop experimental AAA using the calcium chloride model[95]. It has been suggested that MMP-2 is responsible for the early development of AAA whereas MMP-9 is responsible for the more rapid growth found in larger AAA[96]. In addition, Takagi *et al* performed a meta-analysis of the association between MMP-9 and AAA presence in case-control studies and found significantly higher MMP-9 levels in patients with AAA versus

controls (standardised mean difference 0.70; p=0.004) suggesting that MMP-9 may also represent a useful biomarker for AAA[97].

More recently, a number of papers have looked at the role of cathepsins in AAA pathogenesis. Abisi *et al* examined cathepsin B, H, K, L and S, as well as cystatin A, B and C, expression and activity in aortic tissue from patients with AAA and atherosclerotic controls[90]. They found increased activity levels for cathepsin B, H, L and S but also of MMP-9 in the aortic wall of AAA patients. Increased expression levels were only present for cathepsin H whereas cystatin C expression was reduced in AAA samples. The authors postulated that cathepsins may influence AAA development through an ability to augment MMP-9 activity[90]. The group from Harvard (Boston) have studied cathepsins in AAA extensively[92,98,99]. They have shown cathepsin S, K and L expression in human AAA tissue and localized this expression to AoSMCs, macrophages and endothelial cells within the aortic wall[100,101]. Using the angiotensin II (AngII) apolipoprotein E-deficient (ApoE-def) mouse model of AAA, the group have shown increased expression of cathepsin S in AAA lesions and proven that cathepsin S gene knockout is associated with a reduction in incidence of AAA, aortic diameters, elastolysis and AoSMC apoptosis[102]. Using various murine models of AAA (porcine pancreatic elastase, calcium chloride peri-aortic injury) the group have reported similar findings for cathepsin K[103], cathepsin L[100] and, to some extent, cathepsin C[104], emphasizing the importance of cathepsins in AAA pathogenesis.

1.5.3 Genetic factors

Genetic factors are key determinants of AAA risk. Candidate gene studies have identified numerous risk loci for AAA however many of these studies are underpowered or have not adjusted for confounders, such as the presence of atherosclerosis[105]. Genome-wide association studies (GWAS) provide a more unbiased approach to identifying genetic risk factors for AAA. To date, GWAS and their meta-analysis[106,107] have identified 10 AAA risk loci: DAB2IP (DAB2 interacting protein), LRP1 (low-density lipoprotein receptor related protein 1), LDLR (low-density lipoprotein receptor), SORT1 (Sortilin 1) [108], IL6R (interleukin-6 receptor) [109], CDKN2B-AS1 (cyclin-dependent kinase inhibitor 2B antisense RNA 1) [110], SMYD2 (SET and MYND domain containing 2), LINC00540 (long intergenic nonprotein coding RNA 540), ERG (v-ets avian erythroblastosis virus E26 oncogene homolog) and another risk locus in chromosome 20 near the genes for PCIF1 (c-terminal inhibiting factor 1 of a protein called pancreatic and duodenal homeobox 1), MMP9 and ZNF335 (zinc finger protein 335) [107]. Epigenetic factors, such as DNA methylation, may also be important[111], particularly since smoking, the most important environmental risk factor for AAA, is a potent DNA methylation modifier[112]. Only 1 study with small patient numbers has examined the role of DNA methylation in AAA[113]. The study identified 2 genes (CNN2 (calponin 2) and SERPINB9 (serpin peptidase inhibitor clade B member 9)) which were differentially methylated in AAA patients however Kuivaniemi *et al* suggest that both genes represent plausible candidates for influencing AAA pathobiology since CNN2 may be an important regular of vascular SMCs and SERPINB9 has been shown to inhibit vascular SMC apoptosis[114].

1.5.4 Cigarette smoking

The mechanisms by which smoking affects AAA development are not completely understood however nicotine, a major component of cigarette smoke, may instigate AAA through an AMPK-α2-mediated activator protein-2α-dependent effect on MMP-2 expression in cultured vascular SMCs[43]. Jin *et al* subsequently examined the effect of tobacco smoke as a whole and found that alterations in the leucocyte response to aortic injury were the most important factors and that these alterations persisted even after cessation of smoking[115]. The authors postulated that these effects were more important than any effect via MMP modulation as has been reported by earlier studies.

1.5.5 Aortic smooth muscle cells

AoSMCs represent the subset of vascular SMCs residing in the aortic tree. These cells play a central role in the pathobiology of AAA since they are capable of orchestrating many of the processes described above[116]. Cultured AoSMCs have been shown to produce both elastin and collagen, the 2 key ECM components of the aortic wall[117,118]. Cultured vascular SMCs are also capable of producing MMP-1, -2, -3, -7, -9, -12 and -13 as well as cathepsins K, L and S, and various studies have reported differential expression and/or activity in AAA[100-102,116,119]. Furthermore AoSMCs have been shown to produce vascular endothelial growth factor (VEGF) and monocyte chemoattract protein 1(MCP-1), key factors responsible for medial neovascularisation and inflammatory cell recruitment,

respectively[120,121]. As such, cultured AoSMCs represent a well-established model in which to perform AAA pathophysiology studies[94,122,123].

1.6 Clinical management of AAA

1.6.1 Size thresholds

Current AAA management is size- and symptom-dependent. Asymptomatic AAA less than 5.5cm in diameter are nearly always managed conservatively. This involves AAA surveillance and provision of best medical therapy (BMT). Surveillance is usually ultrasound scan based[124]. BMT involves smoking cessation advice and where tolerated, prescription of an antiplatelet agent and HMG-CoA reductase inhibitor (statin) in order to reduce overall cardiovascular risk, although national guidance in this area is lacking[125]and therefore cardiovascular risk prevention is often lacking[126]. Size thresholds for asymptomatic AAA are based on the results of the UK Small Aneurysm Trial (UKSAT) which showed no mortality benefit for offering open surgical repair (OSR) for AAA <5.5cm compared to continued USS surveillance[127]. Practically however, there are a number of situations in which patients may still be offered surgical treatment for AAA <5.5cm. Rapidly expanding (>1cm/year) and symptomatic or tender AAAs are generally repaired expeditiously for fear of impending rupture[128,129], although most AAA presenting symptomatically are >5.5cm in size[130]. Women may also be offered repair at diameters <5.5cm and there is debate about whether the size thresholds for women should be different to men[27,131]. Proponents for this change argue that women naturally have smaller aortic diameters and that the UKSAT was based on data largely from male patients. The most recent European AAA guidelines recommend females be referred for vascular surgical consultation when AAA reaches 5.0cm on duplex ultrasound (DUS) and repair at 5.2cm in diameter although no level 1 evidence

exist to support these recommendations[132]. Other researchers have suggested using aortic diameters indexed to body size in females more accurately predicts risk of rupture [133].

1.6.2 Aneurysm repair

AAA repair can largely take one of two forms, traditional OSR or contemporary endovascular aneurysm repair (EVAR). OSR, in its present form (replacement of the abdominal aorta with a synthetic graft), was first successfully performed by Oscar Creech in 1966 in Houston [134]. It was not until September 1990 that Juan Carlos Parodi performed the first successful endovascular stent graft insertion[135] although Volodos and Balko were also exploring similar non-invasive methods of AAA repair experimentally 5 years earlier[136,137]. The technique, which has revolutionised the treatment of AAA, involves fixing a covered stent within the aneurysm sac thereby excluding it from the circulation and is now the mainstay of treatment for large AAA in modern vascular centres.

With the advent of less invasive technologies for AAA repair, researchers sought to determine whether the size thresholds for repair should be revised. The CAESAR trial (n=360) compared EVAR vs. continued surveillance for AAA <5.5cm but found that mortality and rupture rates were low and that early EVAR offered no clear survival advantage (except for treating a minority of AAA which might become anatomically unsuitable for EVAR with surveillance) [138]. Similarly, the PIVOTAL trial (n=728) found no increased risk of rupture (HR 0.99, p=0.99)

or mortality (HR 1.01, p=0.98) with continued USS surveillance vs. early EVAR for AAA 4cm – 5cm in size[139].

A number of large randomized controlled trials (RCTs) have compared long-term outcomes following OSR and EVAR: EVAR1 from the UK (n=1252) [140], DREAM from the Netherlands and Belgium (n=351) [141], ACE from France (n=306) [142] and OVER from the USA (n=881) [143]. Powell *et al* performed an individual patient data meta-analysis of these 4 trials looking at outcomes out to 5 years[144]. They found that, compared with OSR, EVAR was associated with an early survival advantage out to 6 months which was attributed to lower 30-day operative mortality rates in this group. Beyond 6 months, the survival advantage of EVAR began to become eroded and by 5 years the two treatment modalities had equal overall survival rates of 73.6%. Only looking at aneurysm-related mortality, OSR offers patients a significant survival advantage from as early as 3 years (HR 5.16, p=0.01) which probably explains at least some of the 'erosion' in overall survival benefit with time for EVAR. Patients with a low ankle-brachial pressure index (ABPI) also fared worse in the EVAR group beyond 6 months of randomization which may represent another factor responsible for this erosion in survival benefit[144]. Health-related quality of life, similarly, was better in the short-term but worse in the long-term in patients undergoing EVAR[145]. The EVAR1 trial, the largest of the 4 RCTs, recently published its 15-year outcomes [146]. This showed, similar to the IPD meta-analysis, that overall survival was superior amongst EVAR patients up to 6 months, however beyond 8 years of follow-up, OSR was associated with significantly better overall survival (HR 1.25, p=0.048) [146]. This was mainly due to an increased number of late AAA-related complications such

as sac rupture but also an increased rate of late cancer-related mortality in the EVAR group, the latter finding possibly a sequelae of higher levels of ionizing radiation exposure with EVAR.

Despite these findings, there is evidence from comparative studies between the UK and other countries that an increased use of EVAR is associated with better outcomes at a population level. Karthikesalingham *et al* compared in-hospital mortality for elective AAA repair between England and USA and correlated these with trends in EVAR uptake[147]. They found higher in-hospital mortality rates (4.09% vs. 1.96%, $p<0.01$) but also lower EVAR uptake rates (37.33% vs. 64.36%, $p<0.01$) in England vs. USA. They concluded that performing EVAR in high caseload centres that are able to offer EVAR would lead to an improvement in outcomes following elective repair[147]. The authors performed similar analyses for ruptured AAA (RAAA) repairs[148,149]. Comparing outcomes between England and the USA, they found higher in-hospital mortality rates (65.9% vs. 53.1%, $p<0.0001$) in England that were associated with lower overall intervention rates (58.5% vs. 80.4%, $p<0.0001$) but also lower EVAR uptake rates (8.5% vs. 20.9%, $p<0.0001$) [148]. Comparing outcomes between England and Sweden, they found higher 90-day (44.0% vs. 33.4%, $p<0.001$) and 5-year ($p<0.001$) mortality rates in England that were associated with lower EVAR uptake rates (9.5% vs. 16.4%, $p<0.001$) and a lower proportion of patients being managed in teaching hospitals ($p<0.001$) [149]. The suggestion that EVAR for RAAA is associated with better survival outcomes however is not supported by recent RCTs. Three RCTs comparing EVAR and OSR for RAAA have been performed to date: AJAX from the Netherlands (n=116), ECAR from France (n=107) and

IMPROVE from the UK (n=613) [150-152]. None of the trials reported any survival benefit for EVAR at 1-year and a meta-analysis of all 3 trials showed comparable mortality between EVAR and OSR (38.6% vs. 42.8%, p=0.209) [153]. The only long-term data in this area comes from interrogation of large registry databases. Robinson *et al* interrogated the Vascular Quality Initiative dataset and found that although EVAR seemed to be associated with reduced 1-year and 5-year mortality rates vs. OSR, these survival benefits were not maintained after adjustment for relevant confounders[154].

1.6.3 AAA screening

Screening for AAA is appropriate since the disease has a long lag phase during which repair is not required and during which the patient is asymptomatic. Furthermore AAA rupture (advanced disease) is associated with significant mortality therefore diagnosing AAA prior to rupture allows patients to be offered elective rather than emergency repair which is associated with significantly better patient outcomes. The MASS trial was a RCT performed in England to determine whether DUS screening of males aged 65 – 74 years for AAA was clinically beneficial[7]. The trial enrolled 67,800 participants and found a 53% reduction in mortality for patients who attended screening. This mortality benefit was maintained in the long-term (relative risk reduction 48% at 10-years) but also AAA screening was shown to be very cost-effective[155]. A meta-analysis of the four RCTs on AAA screening was recently performed which shows that screening reduces AAA-related mortality (OR 0.66, p=0.02) but does not reduce non-AAA related mortality (OR 1.00) [156].

The UK Department of Heath commenced a national publicly funded programme for AAA screening in 2009 which achieved full coverage in England in 2013. NAAASP invites all men in England to attend for ultrasound of their aorta in the year they turn 65 years old. Men >65 years can also electively present for screening[157]. Patients with an antero-posterior aortic diameter <3.0cm are discharged from screening, those with diameters between 3.0cm to 4.4cm are screened annually and those with diameters 4.5cm to 5.4cm are screening 3-monthly. Once the diameter reaches 5.5cm or larger, a referral is made to the vascular surgeon who reviews the patient to decide on treatment. Despite the

declining prevalence of AAA in the UK, economic analysis using contemporary costing models suggest that NAAASP will remain cost-effective (<£20,000 per quality-adjusted life-year (QALY) gained) down to a prevalence as low as 0.35%[158]. Analysis of the first 5 years of NAAASP (n=700,000 screened patients) reveal that average uptake was 78.1% but this varied significantly according to geography[159]. Further investigation into the reasons for these geographical differences in uptake revealed that men from more socially deprived areas were significantly more likely not to attend screening (OR 2.84 for uptake in least deprived vs. most deprived areas, p<0.0001) [160]. Over the coming years, NAAASP will be targeting such areas at a local level in an attempt to improve uptake of screening[161].

There are number of issues that NAAASP will need to consider. Women are currently not screened under the NAAASP which is keeping with computational analysis studies performed by the SWAN (Screening Women for Aortic Aneurysms) collaborative showing that female screening would not be cost-effective (£45,000 per quality-adjusted life-year gained) [162]. Furthermore screen-detected large AAA in women are less likely to be morphologically suitable for EVAR and operative mortality is significantly higher in women vs. men[163]. However further research is needed as to whether selective screening of women would be beneficial, for example women smokers >70 years old who have a 2% prevalence of AAA[164]. Another issue is screening intervals. Currently, a 4.0cm aorta would be screened annually however a recent meta-analysis by the RESCAN collaborators suggests that the intervals used for AAA surveillance could be extended for the majority of small AAA such that a 4.0cm aorta could

safely be scanned 2-yearly and 3.0cm aorta 3-yearly[66]. Thirdly the method used to measure aortic diameter is important. Two techniques for measuring the AP diameter on US have evolved: inner-to-inner (ITI) and outer-to-outer (OTO) measurement. The discrepancy between the 2 techniques has been reported to be in the region of 4mm[165] but can be as high as 6mm[166]. Since NAAASP uses the ITI diameter, some authors have suggested that patients with aortas screened to be just below the 3cm should still be entered into screening as their OTO diameter may in fact be >3cm[165]. Furthermore using the ITI measurement may delay surgical treatment for aortas >5.5cm in diameter as the UKSAT used the OTO method. The debate over which technique should be used as the gold standard continues although Hartshorne *et al*[167] reported that the ITI method was more reproducible which may explain why NAAASP has chosen to adopt this method. Recently, a third method of measurement has also been proposed, the leading edge to leading edge, which is used routinely in echocardiography and has been suggested as being even more reproducible than ITI or OTO[168]. Finally, a protocol for managing so-called 'subaneurysmal aortas' (25mm to 29mm) needs to be established since over two-thirds of these progress to true aneurysmal dilatation within 5 years and 26% progress to the size threshold for treatment within 10 years[4].

1.7 Medical therapies for abdominal aortic aneurysm

The advent of the NAAASP provides clinicians with a window of opportunity in which to slow or even halt AAA growth using pharmacological therapy. A detailed review of the literature surrounding pharmacological therapies for AAA is not possible however much of the work has focused on antibiotics, statins, β-blockers and angiotensin converting enzyme (ACE) inhibitors. To date, no such medications have made their way into mainstream vascular surgical practice.

1.7.1 Antibiotics

Pretreatment with doxycycline, a tetracycline antibiotic, has been shown to inhibit MMP expression and activity in patients undergoing AAA repair[169]. Recent work using experimental mouse models has shown that this effect is associated with a decrease in AAA expansion and appears to be mediated via an MAPK- and AKT-dependent mechanism[170]. Earlier evidence from a randomized, double-blinded, placebo-controlled study showed a negative effect of doxycycline on AAA expansion however this study was small (n=32) [171] and a larger RCT (n=286) showed no difference in AAA growth rates or requirement for AAA repair (between treatment and placebo groups) [172]. Other antibiotics which have been studied include the macrolides roxithromycin[173] and azithromycin[174].

1.7.2 Statins

Statins inhibit the enzyme 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase and although their principal action is to reduce serum cholesterol

levels, they are also known to possess pleiotropic effects. Their use has been shown to reduce aortic wall MMP expression in patients undergoing AAA repair[175] and small ultrasound surveillance studies suggest reduced AAA expansion in patients taking statins[176]. The only RCT attempting to investigate the beneficial effects of statins use in AAA patients was curtailed early due to high rate of statin use in the placebo arm of the trial and therefore there is no level 1 evidence to support the statement that statins reduce AAA growth rates[177]. The same group went on to show that more aggressive cholesterol lowering, through the addition of ezetimibe to patients already on simvastatin, was associated with a reduction in aortic wall MMP-9 and IL-6 concentrations but the study did not directly examine AAA growth rates and therefore the results cannot be translated into clinical practice[178].

1.7.3 β-blockers

Experimentally, β-blockers have been shown to decrease the growth rate of AAA by 0.27 – 0.32cm/year[179,180] however a large RCT comparing propranolol against placebo over a 2.5-year period showed no significant difference (p=0.11) in small AAA growth rates[181]. Furthermore β-blockade was associated with a reduction in quality of life making its clinical use difficult to justify[181]. These findings were confirmed by a Cochrane review published in 2012 which combined data from 3 RCTs comparing propranolol versus control [182].

1.7.4 Angiotensin converting enzyme inhibitors

The renin-angiotensin system seems to play a role in the development of AAA and ACE inhibitors have been shown to reduce aneurysm expansion in experimental models of AAA. Both captopril and losartan (an angiotensin receptor blocker (ARB)) have been shown to reduce aneurysm formation in rat and mouse models of AAA respectively[183]. Furthermore a large case-control study from Canada comparing medication use in patients with ruptured (n=3379) and non-ruptured (n=11,947) AAA found that ACE inhibitor use, but not β-blocker, statin or ARB use, was associated with a nearly 20% reduction in risk of rupture[184]. Despite this evidence, *post hoc* analysis of the UKSAT cohort did not suggest reduced AAA growth in those on ACE inhibitors[185] and examination of the Danish healthcare registry found no difference in AAA rupture rates amongst users and non-users of ACE inhibitors[186]. The only level 1 data on this subject comes from the AARDVARK trial, a randomised placebo-controlled trial comparing once daily 10mg perindopril against 5mg amlodipine (BP control) or placebo which was published in 2016[187]. At 2 years, AARDVARK found no impact for perindopril on small AAA growth rates (p=0.78 perindopril vs. placebo; p=0.89 perindopril vs. amlodipine) although the trial may have been underpowered for the actual observed growth rates in the study[187].

Chapter 2: An introduction to diabetes mellitus

2.1 Definition and classification

Diabetes Mellitus (DM) is defined as a condition characterised by chronically elevated blood glucose levels due to an absolute or relative lack of insulin production. Several types of DM exist. Type 1 DM (T1DM) is an autoimmune form due to insulin-producing β-cell destruction (absolute lack of insulin) whereas Type 2 DM (T2DM) is associated with insulin resistance, obesity and a subsequent impairment in β-cell function (relative lack of insulin) [188]. T2DM accounts for 90% of the cases in the UK[189]. The other types of DM are far less common and beyond the scope of this summary.

2.2 Clinical features and diagnosis

The onset of DM is often insidious however symptoms include polyuria, polydipsia, weight loss, hunger, deterioration in vision, sensorineural changes and increased rates of infection[190].

The World Health Organisation (WHO) published specific criteria for the diagnosis of DM in 2006 [191] based on plasma glucose concentrations. DM can be diagnosed by the presence of diabetic symptoms (see above) together with either a random venous plasma glucose concentration $\geq$ 11.1 mmol/L, a fasting plasma glucose concentration $\geq$ 7.0 mmol/L or a plasma glucose concentration $\geq$ 11.1 mmol/L two hours after 75g anhydrous glucose in an oral glucose tolerance test (OGTT) [192]. In the absence of diabetic symptoms, a diagnosis of DM can also

be made by two positive plasma glucose concentrations on two separate days [192]. As of 2011, the WHO have also recommended that a HbA1c value ≥ 6.5% (48mmol/mol) is diagnostic for DM although a value < 6.5% does not exclude a diagnosis of DM[193]. Finger prick HbA1c tests must be confirmed by laboratory venous HbA1c in all patients[193].

2.3 Epidemiology

The prevalence of DM is increasing globally as well as in the UK, driven by a surge in the number of T2 diabetics[194]. In 2010, an estimated 285 million people suffered from DM however by 2030 this figure is expected to reach 439 million (>4 million in the UK alone) [189,195]. The exponential growth in the number of T2 diabetics is thought to be due to an increase in the number of obese individuals (defined as a BMI >30) [196].

2.4 Complications

The sequelae of chronic hyperglycaemia can be divided into microvascular and macrovascular complications. Microvascular complications include diabetic retinopathy, neuropathy and nephropathy whereas macrovascular complications include coronary heart disease, cerebrovascular disease and peripheral arterial disease[197,198].

2.5 Glucose homeostasis and pathophysiology in diabetes

The control of blood glucose levels occurs at various levels. Firstly, absorption of carbohydrate-containing foods in the stomach stimulate the release of incretins which effectively prime the body to release insulin and reduce glucagon production[199]. As blood glucose levels increase, there is an influx of glucose into β-cells via the high capacity glucose transporter GLUT2 and glucose becomes phosphorylated by glucokinase which essentially acts as a glucose sensor by coupling insulin secretion with the prevailing glucose levels (see Figure 2.1) [200,201]. Insulin acts to control blood glucose levels by suppressing gluconeogenesis and glycogenolysis in the liver and stimulating glucose uptake (via GLUT4) in skeletal muscle and adipose tissues[202]. The mechanism by which insulin results in increased membrane expression of GLUT4 and hence uptake of glucose is summarised by Figure 2.2[203].

The pathophysiology of T1DM is likely a T-cell-mediated autoimmune phenomenon however the exact aetiology is still not fully understood[204]. In contrast, the pathophysiology of T2DM centres on the concept of insulin resistance which initially does not result in hyperglycaemia due to an increase in insulin secretion however as the demands for more and more insulin production surpass the secretory capacity of the pancreatic β-cell, insulin secretion begins to fall and a situation of overt hyperglycaemia ensues[205]. Obesity, specifically central obesity, is the main driver of insulin resistance via the production of non-esterified fatty acids (NEFAs) [206]. Specifically, NEFAs stimulate gluconeogenesis in the liver, inhibit glucose uptake and utilization in skeletal muscle, and can even directly affect the pancreatic β-cell[206].

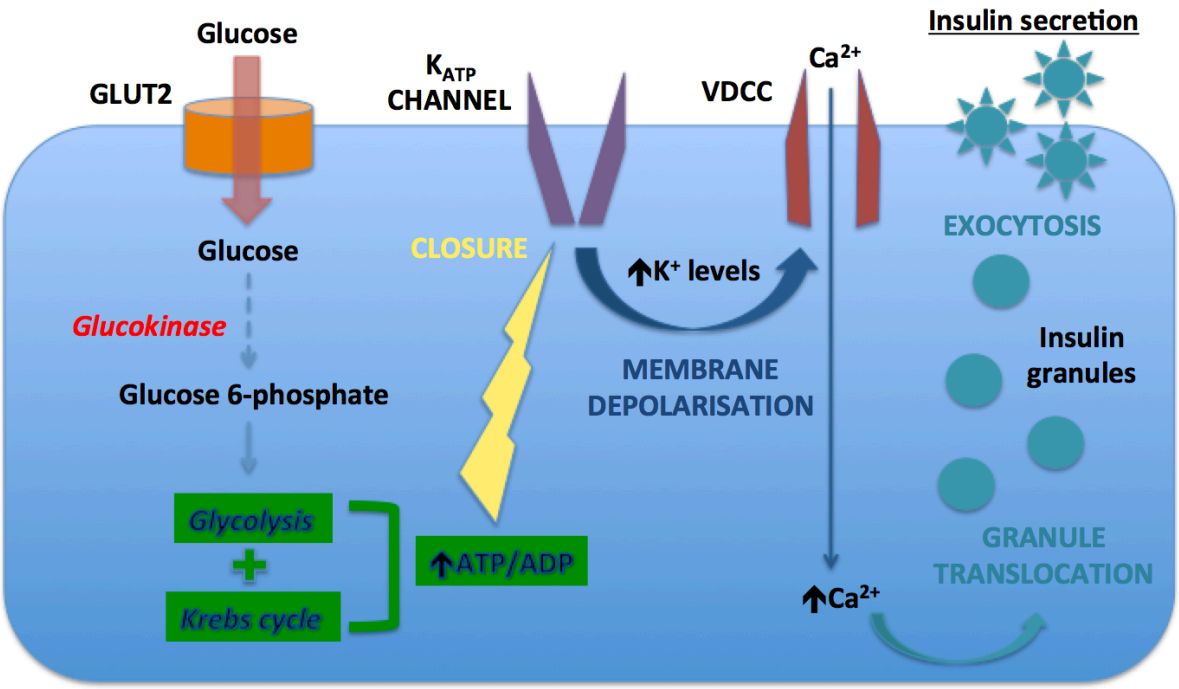


Figure 2.1. Intracellular mechanisms regulating insulin secretion in the β-cell. Glucose enters the cell via GLUT2, is phosphorylated by the enzyme glucokinase to produce glucose-6-phosphate which in turn is metabolised to generate adenosine triphosphate (ATP). The change in ATP/ADP ratio causes closure of ATP-sensitive K^+ channels, an increase in intracellular K^+ levels and subsequent membrane depolarisation. This in turn opens voltage-dependent calcium channels (VDCC) allowing calcium to enter the β-cell. The increase in cytosolic calcium initiates granule translocation and exocytosis.

Adapted from Karges B *et al* [201].

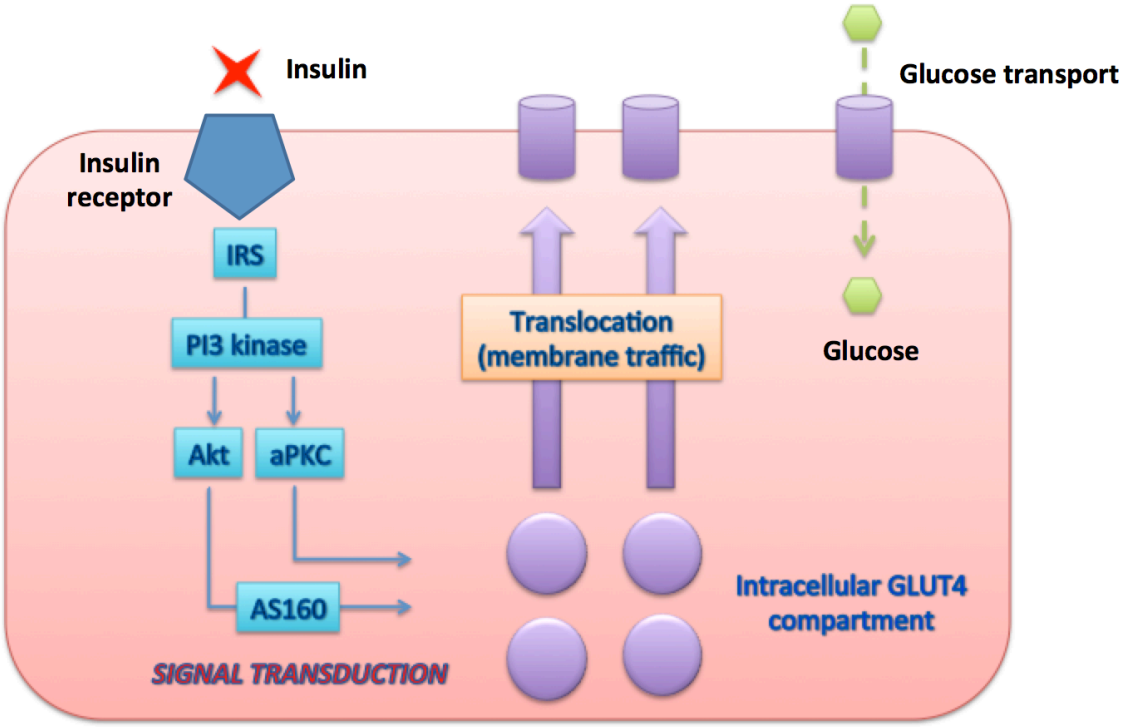


Figure 2.2. Insulin-stimulated GLUT4 translocation. IRS = insulin receptor substrate, PI3 = phospoinositide 3-kinase, aPKC = activated protein kinase C, AS160 = Akt substrate of 160kDA. Adapted from the University of Tokushima Institute for Enzyme Research webpage [207].

2.6 Treatment

The treatment of DM is multifactorial. T1 diabetics have an obligatory need for artificial insulin whereas T2 diabetics may often only require dietary control. A summary of the various forms of diabetic treatment is provided in Table 2.

Treatment type	Method of delivery	Mechanism of action	T1DM/T2DM	Examples
Insulin	Subcutaneous injection Fixed rate insulin pump Variable rate insulin pump (artificial pancreas)	Act on liver tissue to suppress gluconeogenesis and glycogenolysis, and on adipose and skeletal muscle to increase glucose uptake	T1DM T2DM	Mixtard
Insulin analogues	Subcutaneous injection Fixed rate insulin pump Variable rate insulin pump (artificial pancreas)	Similar to action of human insulin on liver, muscle and adipose tissue. Analogues can be shorter or longer-acting	T1DM T2DM	Novorapid Lantus
Amylin analogue	Subcutaenous injection	Inhibits action of glucagon Slows rate of digestion Decreases appetite	T1DM T2DM	Pramlintide
Anti-obesity drugs	Oral	Inhibits gastric and pancreatic lipases	Obese T2DM	Orlistat
Biguanides	Oral	Increase insulin sensitivity Decreases hepatic glucose production	Obese T2DM	Metformin
Meglitinides	Oral	Promotes insulin release from β-cell	T2DM	Repaglinide Nateglinide
Sulphonylurea	Oral	Promotes insulin release from β-cell	T2DM	Gliclazide Glimepiride
α-glucosidase inhibitors	Oral	Delay digestion and absorption of carbohydrate from gut	T2DM	Acarbose Miglitol
Thiazolidinediones	Oral	Increases adipocyte lipogenesis and decreases circulating fatty acids via activation of nuclear receptor PPAR-Y (cells become reliant on glucose for respiration leading to increase glucose uptake) Overall increase in insulin sensitivity	T2DM	Roziglitazone Pioglitazone
Dipeptidyl peptidase 4 inhibitors	Oral	Increase incretin levels and therefore inhibit glucagon release Decrease gastric emptying	T2DM	Sitagliptin
Incretin mimetics / GLP-1 analogues	Subcutaneous injection	Glucagon inhibition Decrease gastric emptying Increases insulin secretion	T2DM	Exenatide

Table 2.1. A summary of the medications used to treat diabetes mellitus.

Chapter 3: A meta-analysis on the association between diabetes mellitus and aortic aneurysm presence

3.1 Introduction

The relationship between DM and AAA is interesting[208]. Although a strong cardiovascular risk factor, a significant number of studies now suggest that DM may protect against AAA [71]. Recent research also suggests that this paradoxical relationship is not confined to the abdominal aorta but that a similar relationship exists between DM and aneurysms in the thoracic aorta[209-215].

LaMorte *et al* were the first to report an unusual relationship between DM and AAA. They compared risk factors for aneurysmal and atherosclerotic occlusive disease using Massachusetts hospital discharge data and found DM to be a strong risk factor for femoral artery bypass surgery but not for open AAA surgery (OR 2.76 vs. 0.78; p=0.03) [38]. Earlier studies had largely only found a lack of association between AAA and DM [216-218] and a few had even found DM to increase the risk of AAA [219]. Between 1997 and 2000, 4 further studies were published suggesting a negative relationship between DM and AAA[56,220-222] however it was not until the 2nd Aneurysm Detection and Management (ADAM) study was published[223], reporting an odds ratio (OR) of 0.5 between DM and AAA, that the results of previous studies were considered to be valid.

To date, there have been 5 meta-analyses examining the relationship between DM and AAA presence (encompassing prevalence and incidence) [71,224-227] and all

but the first[224], which did not include either of the ADAM studies, have shown DM to be negatively associated with AAA. For prevalence data, ORs range between 0.64 and 0.80 for meta-analyses of population-based screening studies and between 0.27 and 0.69 for meta-analyses of case-control studies, where as for incidence data, ORs range between 0.52 and 0.54 for meta-analyses of prospective cohort studies. The latest of these meta-analyses included studies published up to the 30th September 2015[227] however since this date there have been a number of large studies published which are not included in any of the published meta-analyses[61,228-230]. In addition, none of the published meta-analyses have included studies examining the relationship between DM and thoracic aortic aneurysm (TAA) [210,231] to allow quantification of the association between DM and aortic aneurysms in general. In response, a contemporary systematic review and meta-analysis of the literature to determine the association between DM and aortic aneurysm was performed.

3.2 Methods

3.2.1 Search strategy and results

A literature search of published studies was performed in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA) guidelines [232]. Both the Pubmed and MEDLINE online databases were searched for articles reporting on diabetes and aortic aneurysm. The following search strategy was used: (diabetes OR hyperglycaemia) AND (aortic aneurysm OR AAA OR TAA). No filters were used to restrict studies by date or study design and searches were up to date to the 30th June 2017. Titles and abstracts were personally reviewed and potential studies for inclusion were downloaded for full text review. Studies were included if they reported prevalence or incidence rates of aortic aneurysm or DM in patients with and without aortic aneurysm or DM. The reference lists of included articles and previous meta-analyses were also searched for additional articles. Studies were included twice under different sub-headings if they described 2 distinct patient populations suitable for meta-analysis [38,209,210,222,223,233]. Studies were excluded if they examined the impact of DM on outcomes following aneurysm repair, did not include a comparative group (either DM / AAA / TAA / thoracoabdominal aortic aneurysm) or were not published in the English language. Review articles, editorials, letters and case reports were only reviewed to check for missing studies but not included in the quantitative analysis.

Using the search strategy detailed above, 973 original articles were identified via database searching and an additional 25 articles were identified after manually

reviewing reference lists and previous meta-analyses. 881 were excluded after reviewing the title and abstract. Of the 117 articles undergoing full-text review, 81 were selected for final analysis (see Figure 3.1 for PRISMA diagram).

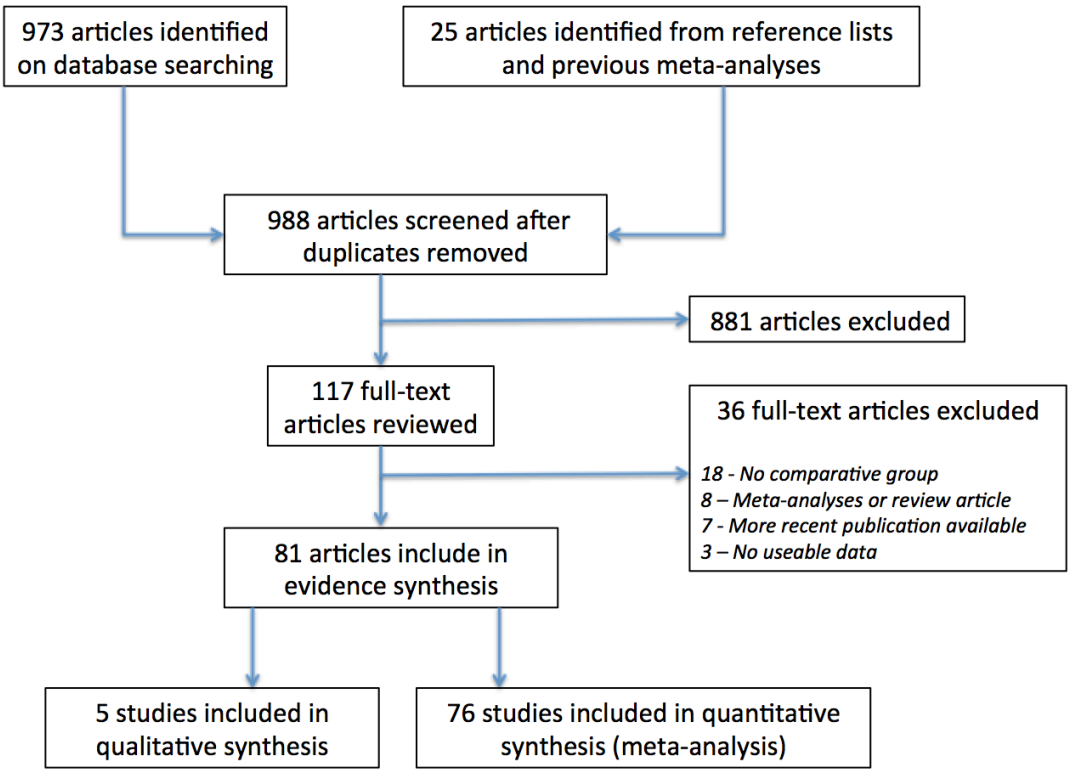


Figure 3.1. PRISMA diagram of how articles were selected for evidence synthesis.

3.2.2 Data extraction

The following data were extracted from each study using a pre-defined data extraction spreadsheet: Pubmed ID; first author surname; year of publication; type of aortic aneurysm; whether the study examined prevalence or incidence; whether the study included patients from unselected (e.g. general practice data,

large screening populations) or selected (e.g. coronary artery disease patients or vascular surgery clinic patients) patient populations; study design; study inclusion criteria; definitions used to define DM and aortic aneurysm; total number of patients studied; individual event rate data (where available); reported ORs and 95% CI for prevalence studies; reported hazard ratios (HRs) / incidence rate ratios (IRRs) and 95% CIs for incidence studies; whether the reported study estimates were unadjusted or adjusted for covariates (such as age, sex or other comorbidities). Studies were classified as including patients from selected populations even if the comparator group included patients from a selected population.

Of the 76 articles selected for meta-analysis, 46 studied unselected and 30 studied selected patient populations; 62 studied prevalence and 14 studied incidence; 68 studied AAA, 5 studied AAA + TAA combined and 3 studies examined TAA (2 studies in combination with thoracic aortic dissection). There were no studies examining thoracoabdominal aortic aneurysms. Of the prevalence studies, 34 were screening studies of varying size (national, regional, selected populations), 25 were case-control (CC) studies (21 retrospective, 4 prospective) and 3 used baseline data from other study types (2 prospective observational cohort studies and 1 international registry data). Of the incidence studies, 11 were prospective observational cohorts and 3 were retrospective observational cohorts. There were no randomised studies suitable for inclusion.

3.2.3 Data manipulation

Studies varied in design and how they reported data. Some studies reported the prevalence / incidence of DM in patient cohorts with and without aortic aneurysms (i.e. aneurysms as the 'denominator'). Others reported the prevalence / incidence of aortic aneurysms in patient cohorts with and without DM (i.e. DM as the denominator).

For prevalence studies comparing patient cohorts with and without aortic aneurysms (i.e. aneurysm-denominator studies), the following event rate data were collected where available:

The number patients with aortic aneurysm and with DM = (A)

The total number of patients with aortic aneurysm = (B)

The number of patients without aortic aneurysm but with DM = (C)

The total number of patients without aortic aneurysm = (D).

For prevalence studies comparing patient cohorts with and without DM (i.e. DM-denominator studies), the following event rate data were collected where available:

The number of patients with DM and with aortic aneurysm = (E)

The total number of patients with DM = (F)

The number of patients without DM but with aortic aneurysm = (G)

The total number of patients without DM = (H).

For ‘aneurysm-denominator’ studies where all 4 event rates were reported, i.e. (A) to (D), it was possible to calculate the corresponding ‘DM-denominator’ event rates, i.e. (E) to (H), using the following formulas:

(E) = (A)

(F) = (A) + (C)

(G) = (B) – (A)

(H) = (B + D) – (F)

For example, the population based AAA screening study by Chun *et al* reported that 18.6% of the 469 AAA patients (i.e. 87 patients) had diabetes compared with 27.4% of the 5673 patients without AAA (i.e. 1554 patients).

Therefore the aneurysm-denominator event rates for this study are:

(A) = 87 (number with aneurysm and DM)

(B) = 469 (total number of aneurysm patients)

(C) = 1554 (number without aneurysm but with DM)

(D) = 5673 (total number without aneurysm)

Using the formulas stated earlier, the corresponding DM-denominator event rates can be calculated to be:

(E) = 87 (number with DM and aortic aneurysm)

(F) = 87 + 1554 = 1641 (total number of diabetic patients)

(G) = 469 – 87 = 382 (number without DM but with aneurysm)

(H) = (469 + 5673) – 1641 = 4501 (total number without DM)

Similar event rate data was recorded for incidence studies except the number of new aneurysms detected during follow-up rather the number of aneurysms at baseline were recorded.

3.2.4 Data pooling

As recommended by the Cochrane Handbook for Systematic Reviews of Interventions[234], estimates adjusted for covariates such as age, sex and comorbidities are preferable to unadjusted estimates when considering non-randomised studies due to the higher risks of confounding. However, as reported by Voils *et al*, inclusion of only adjusted data during evidence synthesis can reduce the number of included studies and combining adjusted and unadjusted data into a single estimate increases heterogeneity[235].

Therefore adjusted estimates were combined for studies where available and for the remainder of studies unadjusted estimates were combined. For studies where adjusted estimates were not available, unadjusted estimates were preferentially calculated using event rate data. Only in cases where neither adjusted estimates nor event rate data were available were the reported unadjusted estimates used.

Studies examining unselected patient populations were pooled independently of studies examining selected patient populations. Studies examining AAA were first pooled independently, as these were the most common type of aortic

aneurysm, and then in combination with studies examining TAA to calculate overall pooled estimates for aortic aneurysms. Studies examining prevalence and incidence were also first pooled independently but then in combination to create a general estimate of 'aortic aneurysm presence'.

3.2.5 Sensitivity analyses

A sensitivity analysis was performed on the basis of study design and study size. For prevalence studies this was done by separately analysing 1) population screening studies and 2) studies with >10,000 patients. For incidence studies this was done by separately analysing 3) prospective observational cohort studies and 4) studies with >100,000 patients.

3.2.6 Statistical analysis

All statistical analyses were performed using Review Manager version 5.3 (Nordic Cochrane Centre).

For studies using adjusted data, the overall estimate (OR/HR) and corresponding 95% CI were combined in a conventional meta-analysis using the generic inverse variance option in Review Manager which is a method of aggregating random variables whilst trying to minimize the variance of the weighted average. This involved calculating the log odds ratios for case-control studies or log risk ratios for cohort studies and corresponding standard errors (SEs). Log ORs and SEs were calculated using 95% CIs where available. For studies not reporting adjusted outcomes, event rate data was first entered into Review Manager and

the ORs and corresponding standard errors were calculated which were then combined using the generic inverse variance option described above. For studies reporting both adjusted and unadjusted outcomes, the adjusted outcomes were preferentially used to reduce confounding.

Forest plots were calculated for each pooled estimate and data was presented using a diabetes-denominator format where possible to aid comprehension. A random-effects model was used in all cases owing to the heterogeneity between included studies on the overall analysis. Heterogeneity between studies was assessed using the Tau^2, Chi^2 and I^2 statistics. Tau^2 represents the between study variance in a random effects meta-analysis with a score >1 suggesting the presence of substantial statistical heterogeneity. Chi^2 assesses whether the observed differences in the results are compatible with chance alone. It has a lower power to detect heterogeneity hence the level of significance is set at $p<0.1$. I^2 represents the percentage of the total variation across the studies due to heterogeneity. An I^2 value >50% was taken to represent substantial heterogeneity [236]. The summary effect for a meta-analysis is determined by the Z score, with its associated *p* value considered significant if <0.05.

3.3 Study outcomes

3.3.1 Primary outcomes

The primary outcomes of the study were to pool data from studies examining the prevalence and/or incidence of diabetes mellitus and/or aortic aneurysms in patients from the general population (unselected population studies) in order to answer 3 key questions:

1) What is the impact of DM on the prevalence of aortic aneurysms in the general population?
2) What is the impact of DM on the incidence of aortic aneurysms in the general population?
3) What is the impact of DM on the presence of aortic aneurysms in the general population?

3.3.2 Secondary outcomes

The secondary outcomes of the study were to pool data from studies examining the prevalence and/or incidence of diabetes mellitus and/or aortic aneurysms in patients from selected populations with the aim of answering 3 questions:

1) What is the impact of DM on the prevalence of aortic aneurysms outside the general population?
2) What is the impact of DM on the incidence of aortic aneurysms outside the general population?
3) What is the impact of DM on the presence of aortic aneurysms outside the general population?

3.4 Results

3.4.1. Study demographics

The demographics of included studies are summarised in Tables 3.1 and 3.2.

3.4.2 Study data

The individual study event rates (reported and calculated) together with the individual study estimates from the included studies are summarised in Tables 3.3 and 3.4.

Table 3.1. Demographics of studies describing the association between diabetes and aortic aneurysms in the general population (this table spans 3 pages).

Author (extra detail)	Pub Year	Prevalence / Incidence	AAA/TAA	Study design	Patient population	DM definition	Aortic aneurysm definition
Krohn[219]	1992	Prevalence	AAA	PBS	Males >60yrs in private Norwegian health organisation	Casenote review	AAA ≥ 30mm on USS
Smith[218]	1993	Prevalence	AAA	PBS	Males 65 - 75yrs in Birmingham, UK	Self reported dx	AAA ≥ 30mm on USS
Pleumeekers[217]	1995	Prevalence	AAA	PBS	>55yrs in Rotterdam Study	BG ≥ 11mmol/L / antidiabetic med use	AAA ≥ 35mm or >50% larger than proximal aorta on USS
Simoni[237]	1995	Prevalence	AAA	PBS	65-75yrs in Genoa, Italy	NS	AAA ≥ 30mm on USS
Alcorn[238]	1996	Prevalence	AAA	PBS	>65yrs in Pittsburgh cohort of cardiovascular health study	BG > 7.8mmol/L	AAA ≥ 30mm on USS
Kanagasaby[216]	1996	Prevalence	AAA	PBS	65-80 years from UK population	Self reported at baseline	AAA > 30mm on USS
LaMorte[239]	1996	Prevalence	AAA	Retro. CC	Discharge data from Massachusetts Health Data Consortium	ICD-9-CM code for DM	ICD-9-CM code for AAA
Ishikawa[240]	1999	Prevalence	AAA	PBS	>60yrs old in Gumma, Japan	Not specified	AAA ≥ 30mm on USS
Blanchard[241]	2000	Prevalence	AAA	Prosp. CC	US referrals for AAA in Winnipeg, Canada	Self reported at baseline	AAA > 30mm on USS
Lederle (3-3.9cm)[223]	2000	Prevalence	AAA	PBS	50-79yrs in ADAM study	Self reported at baseline	AAA > 30mm - 39mm on USS
Lederle (4cm+)[223]	2000	Prevalence	AAA	PBS	50-79yrs in ADAM study	Self reported at baseline	AAA > 40mm on USS
Lederle[242]	2001	Prevalence	AAA	PBS	Females 50-79yrs in ADAM study	Self reported at baseline	AAA > 30mm on USS
Newman[243]	2001	Prevalence	AAA	PBS	>65yrs, Cardiovascular Health Study	BG > 7.8mmol/L	AAA ≥ 30mm on USS
Tornwall[244]	2001	Incidence	AAA	Prosp. OC	Male smokers 50 - 69yrs in ATBC cancer prevention study	Self reported at baseline	ICD-8/-9 codes for AAA
Bonamigo[245]	2003	Prevalence	AAA	PBS	Males >54yrs in Rio Grande do Sul, Brazil	Casenote review	AAA ≥ 30mm on USS or infra-renal 5mm > supra-renal aorta
Rodin[246]	2003	Incidence	AAA	Prosp. OC	Chicago Heart Association cohort	Self reported at baseline	ICD-8/-9/-10 codes for AAA
Jorgensen (males)[247]	2004	Prevalence	AAA	PBS	Males >55yrs in Tromso, Norway	Self reported at baseline	AAA ≥ 35mm on USS
Jorgensen (females)[247]	2004	Prevalence	AAA	PBS	Females >55yrs in Tromso, Norway	Self reported at baseline	AAA ≥ 35mm on USS
Wanhainen[248]	2005	Prevalence	AAA	Retro. CC	>65 years AAA patients vs. age/sex matched controls	Self-reported physician dx / antidiabetic med use	AAA ≥ 30mm on USS
Waterhouse[249]	2006	Prevalence	AAA	PBS	Males 50-75yrs in Dublin, Ireland	FPG > 6.9mmol/L	AAA ≥ 30mm on USS
DeRubertis[250]	2007	Prevalence	AAA	PBS	Women > 65yrs in SAVE program	Self-reported physician dx	AAA ≥ 30mm on USS
Iribarren[48]	2007	Incidence	AAA	Prosp. OC	Subscribers of Kaiser Permanente of North California attending multiphasic health checkups	Self-reported physician dx / antidiabetic med use	ICD-9 code for AAA
Le[251]	2007	Prevalence	AAA	PBS	Males>65years in Health in Men Study	FPG > 7mmol/L	AAA ≥ 30mm on USS

Author (extra detail)	Pub Year	Prevalence / Incidence	AAA/TAA	Study design	Patient population	DM definition	Aortic aneurysm definition
Morissey[252]	2007	Prevalence	AAA	Retro. CC	Hospital discharge data from New York and Florida states	ICD-9 code for DM	ICD-9-CM code for AAA
Wong[253]	2007	Incidence	AAA	Prosp. OC	Males 40-75yrs in Health professionals follow-up study	Self reported at baseline	Self-reported on 2-yearly questionnaires, confirmed on medical records
Lederle[11]	2008	Incidence	AAA	Prosp. OC	Post-menopausal females >50yrs in Womens Health Initiative	Self reported at baseline	Self-reported on standardised 6-monthly questionnaire
Parry[254]	2010	Prevalence	AAA	Retro. CC	Men >65yrs with small AAA and age/sex/race matched controls	Plasma glucose assay / 2-hr OGTT	AAA 30 - 54mm on USS
Ohrlander (females)[209]	2011	Incidence	Both	Prosp. OC	Women in LOMAS cohort	ICD-10 / ICD-9 code for DM	ICD-10 code for AAA / TAA
Ohrlander (males)[209]	2011	Incidence	Both	Prosp. OC	Men in LOMAS cohort	ICD-10 / ICD-9 code for DM	ICD-10 code for AAA / TAA
Svensjo[13]	2011	Prevalence	AAA	PBS	Males > 65yrs in Swedish national population registry	Self reported at baseline	AAA $\geq$ 30mm on USS (leading edge to leading edge)
Prakash[213]	2012	Prevalence	TAA	Retro. CC	>30yrs from 2006/2007 Nationwide Inpatient Sample (NIS)	ICD-9 code for DM	ICD-9-CM code for TAAD
Svensjo[255]	2013	Prevalence	AAA	PBS	Females > 70yrs in Swedish national population registry	Self reported at baseline	AAA $\geq$ 30mm on USS (leading edge to leading edge)
Barba[256]	2013	Prevalence	AAA	PBS	65yr old males in Northern Spain	BG > 6.7mmol/L, HbA1c $\geq$ 6.5% or antidiabetic med use	AAA $\geq$ 30mm on USS
Chun [257]	2013	Prevalence	AAA	PBS	AAA screening patients in VA North California health system	Casenote review	AAA $\geq$ 30mm on USS
Robson[212]	2015	Incidence	Both	Prosp. OC	GPRD database patients without history of AA	antidiabetic med / insulin syringe prescription	ICD-10 code for AAA / TAA
Shah[230]	2014	Prevalence	AAA	PBS	Lifeline study participants	Self-reported physician dx / antidiabetic med use	AAA $\geq$ 30mm / previous repair
Smelser[258]	2014	Prevalence	AAA	Retro. CC	Geisinger Health System patients (Central and Northeastern Pennsylvania)	ICD-9 code for T2DM	ICD-9 code for AAA
Lopez-de-Andres[229]	2015	Incidence	AAA	Retro. OC	Discharge data of all AAA patients >50yrs in Spain	ICD-9 code for T2DM	ICD-9-CM code for AAA
Shah[228]	2015	Incidence	AAA	Prosp. OC	>30yrs from CALIBER database	CPRD / HES code for DM	CPRD / HES / MINAP / Death registration code for AAA
Tsai[211]	2015	Incidence	Both	Retro. OC	Cohort >55yrs without previous AAA/TAA from National Health Insurance program in Taiwan	ICD-9 code for DM	ICD-9 code for AAA and TAA
Jimenez-Trujilo[231]	2016	Incidence	TAA	Retro. OC	Discharge data of all TAAD patients >50yrs in Spain	ICD-9 code for T2DM	ICD-9-CM code for TAAD
Takeuchi[233]	2016	Prevalence	AAA	Retro. CC	Patients with CT proven AAA and age- and sex-matched controls	Casenote review	CT $\geq$ 30mm
Wang[61]	2017	Incidence	AAA	Prosp. OC	US males physicians 40 -84yrs old	Self-reported physician dx	ICD-9 code / self-reported

Author (extra detail)	Pub Year	Prevalence / Incidence	AAA/TAA	Study design	Patient population	DM definition	Aortic aneurysm definition
Derezinski[259]	2017	Prevalence	AAA	PBS	>60yr old males, >65yr old females living in central Poland	Self-reported at baseline	AAA ≥ 30mm on USS
Stackelberg[60]	2017	Prevalence	AAA	PBS	65-75yr old males in central Sweden	Linkage to Swedish National Diabetes Register / self-reported	AAA ≥ 30mm on USS
Taimour[260]	2017	Prevalence	AAA	PBS	65yr old males in Malmo, Sweden	C-peptide >20IU/ml / HbA1c	AAA ≥ 30mm on USS

AAA = abdominal aortic aneurysm, ADAM = aneurysm detection and management, ATBC = alpha-tocopherol, beta-carotene, BG = blood glucose, CALIBER = cardiovascular disease research using linked bespoke studies and electronic health records, CC = case control, CPRD = clinical practice research datalink, CT = computed tomography, DM = diabetes mellitus, dx = diagnosis, FPG = fasting plasma glucose, GPRD = General Practice Research Database, HbA1c = glycated haemoglobin, HES = hospital episode statistics, hx = history, ICD = international classification of diseases, IHD = ischemic heart disease, LOMAS = Longitudinal Multilevel Analysis Scania, MINAP = myocardial ischemia national audit project, NS = not specified, OC = observational cohort, OGTT = oral glucose tolerance test, PBS = population based screening, Prosp. = Prospective, Retro. = Retrospective, SAVE = stroke and aneurysm vascular evaluation, TAA = thoracic aortic aneurysm, TAAD = thoracic aortic aneurysms and dissections, USS = ultrasound scan, VA = veterans affairs, vs. = versus, yrs = years

Table 3.2. Demographics of studies describing the association between diabetes and aortic aneurysms in selected populations (this table spans 2 pages).

Author (extra detail)	Pub Year	Prevalence / Incidence	AAA/TAA	Study design	Patient population	DM definition	Aortic aneurysm definition
Sumpio[261]	1985	Prevalence	AAA	Retro. CC	Open AAA repair vs. ABF repair	Casenote review	Open AAA repair
LaMorte (local)[38]	1995	Prevalence	AAA	Retro .CC	Open AAA repair vs. Fem Endart.	Casenote review	Open AAA repair
Wolf[262]	1995	Prevalence	AAA	SS	Lower extremity arterial evaluations	Self-reported physician dx / antidiabetic med use	AAA > 30mm on USS
Cahan[221]	1999	Prevalence	AAA	Retro. CC	Open AAA repair vs. AOD patients	Casenote review	Open AAA repair
Kang (0-49%)[222]	1999	Prevalence	AAA	Retro. CC	Carotid stenosis < 50% (ADAM study patients excluded)	Self reported at baseline	AAA ≥ 30mm on USS
Kang (50%+)[222]	1999	Prevalence	AAA	Retro. CC	Carotid stenosis > 50% (ADAM study patients excluded)	Self reported at baseline	AAA ≥ 30mm on USS
Shteinberg[263]	2000	Prevalence	AAA	Prosp. CC	Open AAA repair vs. AOD patients	Self reported at baseline	Open AAA repair
Bonamigo[245]	2003	Prevalence	AAA	SS	Males >54yrs, Cardiology clinic / severe IHD / previous coronary surgery	Hx of DM	AAA ≥ 30mm on US / infra-renal 5mm >supra-renal aorta
Raffetto[264]	2003	Prevalence	AAA	Retro. CC	Open AAA repair vs. AOD patients	Self-reported physician dx	Open AAA repair
Monney[265]	2004	Prevalence	AAA	SS	Males >60yrs awaiting CABG	Hx of DM	AAA ≥ 30mm on USS
Barba[266]	2005	Prevalence	AAA	SS	PAD clinic referrals	Random BG>7mmol/L / antidiabetic med use	AAA ≥ 30mm on USS
Madaric[267]	2005	Prevalence	AAA	Prosp. CC	Males > 60 with significant CAD	Casenote review	AAA ≥ 30mm on USS
Hanly[268]	2006	Prevalence	AAA	SS	Cardiology clinic referrals	Casenote review	AAA ≥ 30mm on USS
Westvik[269]	2006	Prevalence	AAA	Retro. CC	Patients undergoing open index vascular procedures	Casenote review	Open AAA repair
Koksal[270]	2007	Prevalence	AAA	Prosp. CC	AAA and AOD patients	Casenote review	Open AAA repair
Baumgartner[271]	2008	Prevalence	AAA	Registry data	REACH registry, outpatients >45yrs with thrombotic risk factors	Self-reported physician dx	Self-reported physician dx
Ito[272]	2008	Prevalence	AAA	Retro. CC	Elective aortic aneurysm repairs	FPG>7mmol/L, HbA1c≥6.5% / antidiabetic med use	AAA ≥ 30mm, TAA ≥ 40mm on CT
Vlek[273]	2008	Prevalence	AAA	Prosp. OC	SMART study group, clinically manifest CV disease / atherosclerotic risk factors	FPG≥7mmol/L / antidiabetic med use / hx of DM	AAA ≥ 30mm on USS / previous repair
Shirani[274]	2009	Prevalence	AAA	SS	CABG referrals in men	BG>6.6mmol/L twice / antidiabetic med use	NS
De'Ath[275]	2010	Prevalence	AAA	Retro. CC	Patients awaiting AAA repair vs. surgical patients without AAA	NS	Patients awaiting AAA repair / aorta >55mm but unfit for surgery

Author (extra detail)	Pub Year	Prevalence / Incidence	AAA/TAA	Study design	Patient population	DM definition	Aortic aneurysm definition
Dupont[276]	2010	Prevalence	AAA	SS	Elective CABG patients	FPG≥7mmol/L / antidiabetic med use	AAA ≥ 30mm on USS / infrarenal/suprarenal ratio > 1.5
Long[277]	2010	Prevalence	AAA	SS	ACS and coronary stenosis >50% patients	FPG≥7mmol/L / antidiabetic med use	AAA ≥ 30mm on USS
Cueff[278]	2012	Prevalence	AAA	SS	Acute MI patients	FPG≥7mmol/L / antidiabetic med use	AAA ≥ 30mm on USS
Robson[212]	2015	Incidence	Both	Prosp. OC	GPRD, GCA patients > 40yrs, no previous aortic aneurysm	Antidiabetic med / insulin syringe prescription	ICD-10 code for AAA and TAA
Stam-Slob[279]	2015	Prevalence	AAA	Prosp. OC	SMART study group, clinically manifest CV disease / atherosclerotic risk factors	FPG≥7mmol/L / antidiabetic med use / hx of DM	AAA > 30mm / previous repair
Tanaka (AAA)[210]	2015	Prevalence	AAA	Retro. CC	CABG patients	HbA1c≥6.5% / FPG>7 mmol/L / antidiabetic med / hx of DM	AAA ≥ 30mm on CT
Tanaka (TAA)[210]	2015	Prevalence	TAA	Retro. CC	CABG patients	HbA1c≥6.5% / FPG>7 mmol/L / antidiabetic med / hx of DM	TAA ≥ 40mm on CT
Tsialtas[280]	2016	Prevalence	AAA	Retro. CC	Surgical revascularization patients	NS	>5.5cm in diameter awaiting repair
Ultee[281]	2016	Prevalence	AAA	Retro. CC	Patients undergoing elective AAA repair / surgery for PAD	Self-reported physician dx / antidiabetic med use	AAA repair
Takeuchi[233]	2016	Prevalence	AAA	Retro. CC	Patients undergoing CT scanning for DM / CKD	Casenote review	AAA ≥ 30mm on CT

AAA = abdominal aortic aneurysm, ABF = aorto-bifemoral bypass, ACS = acute coronary syndrome, ADAM = aneurysm detection and management, AOD = aorto-occlusive disease, BG = blood glucose, CABG = coronary artery bypass grafting, CAD = coronary artery disease, CC = case control, CKD = chronic kidney disease, CT = computed tomography, CV = cardiovascular, DM = diabetes mellitus, dx = diagnosis, Endart = endarterectomy, FPG = fasting plasma glucose, GPRD = General Practice Research Database, GCA = giant cell arteritis, HbA1c = glycated haemoglobin, hx = history, IHD = ischemic heart disease, MI = myocardial infarction, , vs. = versus, NS = not specified, OC = observational cohort, PAD = peripheral arterial disease, Prosp. = prospective, REACH = reduction of atherothrombosis for continued health, Retro. = retrospective, SMART = secondary manifestations of arterial disease, SS = selective screening, yrs = years, TAA = thoracic aortic aneurysm, USS = ultrasound scan

Table 3.3. Prevalence and incidence data from studies describing the association between diabetes and aortic aneurysms in the general population (this table spans 2 pages).

Study	Year	Prevalence / Incidence	Study Total	Aneurysm-denominator data				DM-denominator data				OR / HR	95% CI	Unadj / Adj
				DM in AA	Total AA	DM in non-AA	Total non-AA	AA in DM	Total DM	AA in non-DM	Total non-DM			
Krohn	1992	Prevalence	500	3	41	10	459	3	13	38	487	-	-	-
Smith	1993	Prevalence	2597	11	219	382	2378	11	393	208	2204	-	-	-
Pleumeekers	1995	Prevalence	5304	8	112	509	5192	8	517	104	4787	-	-	-
Simoni	1995	Prevalence	1574	10	70	212	1504	10	222	60	1352	-	-	-
Alcorn	1996	Prevalence	4741	43	451	392	4290	43	435	388	4140	-	-	-
Kanagasaby	1996	Prevalence	5392	11	218	234	5122	11	245	207	5147	0.8	0.41-1.58	Adj
LaMorte	1996	Prevalence	10870	248	4682	184	3188	248	432	4434	10438	0.78	0.62-0.98	Adj
Ishikawa	1999	Prevalence	2514	8	43	173	2471	8	181	35	2333	-	-	-
Blanchard	2000	Prevalence	200	12	98	17	102	12	29	86	171	0.7 / 0.32	0.31-1.55 / 0.12-0.88	Unadj / Adj
Lederle	2000	Prevalence	117874	-	3455	-	114419	-	-	-	-	0.65	0.59-0.72	Adj
Lederle	2000	Prevalence	1568	-	1568	-	114419	-	-	-	-	0.52	0.45-0.61	Adj
Lederle	2001	Prevalence	3450	-	34	-	3416	-	455	-	2995	0.63	0.19-2.08	Unadj
Newman	2001	Prevalence	4734	43	416	505	4318	43	548	373	4186	-	-	-
Tornwall	2001	Incidence	29133	4	181	1161	28952	4	1165	177	27968	0.43	0.16-1.15	Adj
Bonamigo	2003	Prevalence	1012	1	17	72	995	1	73	16	939	-	-	-
Rodin	2003	Incidence	19274	72	418	2515	18856	72	655	346	18619	0.81	-	Unadj
Jorgensen (males)	2004	Prevalence	2586	8	251	93	2335	8	101	243	2485	-	-	-
Jorgensen (females)	2004	Prevalence	2806	3	59	110	2747	3	113	56	2693	-	-	-
Wanhainen	2005	Prevalence	175	3	35	17	140	3	20	32	155	-	-	-
Waterhouse	2006	Prevalence	1032	2	30	12	1002	2	14	28	1018	-	-	-
DeRubertis	2007	Prevalence	10012	10	74	1312	9938	10	1322	64	8690	1.2	0.6-2.36	Unadj
Iribarren	2007	Incidence	104813	-	605	-	104208	-	3321	-	101492	0.62	0.36-1.05	Adj
Le	2007	Prevalence	12203	103	933	1375	11270	103	1478	830	10725	0.79	0.63-0.98	Adj
Morissey	2007	Prevalence	174460	2735	22789	46691	151671	2735	49426	20054	125034	-	-	-
Wong	2007	Incidence	39352	7	376	-	38976	7	774	369	38578	0.58 / 0.55	0.27-1.22 / 0.26-1.17	Unadj / Adj
Lederle	2008	Incidence	161808	8	184	9536	161624	8	9544	176	152264	0.29	0.13-0.68	Adj
Parry	2010	Prevalence	165	13	75	2	90	13	15	62	150	-	-	-
Ohrlander (females)	2011	Incidence	139782	-	984	-	138798	-	3187	-	136595	0.41	0.19-1.88	Adj
Ohrlander (males)	2011	Incidence	106776	-	2351	-	104425	-	2712	-	104064	0.38	0.24-0.61	Adj
Svensjo	2011	Prevalence	14611	25	233	1754	14378	25	1779	208	12832	0.43	0.16-1.15	Adj
Prakash	2012	Prevalence	31255	924	7107	5216	24148	924	6140	6183	25115	0.56 / 0.47	0.52-0.60 / 0.43-0.51	Unadj / Adj

Study	Year	Prevalence / Incidence	Study Total	Aneurysm-denominator data				DM-denominator data				OR / HR	95% CI	Unadj / Adj
				DM in AA	Total AA	DM in non-AA	Total non-AA	AA in DM	Total DM	AA in non-DM	Total non-DM			
Svensjo	2012	Prevalence	5139	2	19	498	5120	2	500	17	4639	1.09	0.25-4.74	Unadj
Barba	2013	Prevalence	776	-	37	-	739	-	201	-	575	0.28 / 0.38	0.08-0.9 / 0.11-1.06	Unadj / Adj
Chun	2013	Prevalence	6142	87	469	1554	5673	87	1641	382	4501	0.6	0.46 - 0.77	Adj
Robson	2013	Incidence	41994	-	284	-	41710	-	3657	-	38337	0.54 / 0.32	0.3-0.97 / 0.18-0.58	Unadj / Adj
Shah	2014	Prevalence	3696778	-	73443	-	3623335	-	399884	-	3296894	1.40 / 0.86	1.37-1.43 / 0.84-0.88	Unadj / Adj
Smelser	2014	Prevalence	11411	-	888	-	10523	-	-	-	-	0.43	0.29-0.64	Unadj
Lopez-de-Andres	2015	Incidence	115020	19232	115020	-	-	19232	-	95788	-	0.2	0.19-0.21	Adj
Shah	2015	Incidence	1921260	62	3113	160329	1918147	62	34198	3051	1887062	0.46	0.35-0.49	Adj
Tsai	2015	Incidence	807101	282	2209	158182	804892	282	160391	1927	646710	0.85 / 0.65	0.83-0.87 / 0.56-0.74	Unadj / Adj
Jimenez-Trujilo	2016	Incidence	11594	1103	11594	-	-	1103	-	10491	-	0.57	0.53 – 0.60	Adj
Takeuchi	2016	Prevalence	522	44	261	91	261	44	135	217	387	0.31	0.19 – 0.49	Adj
Wang	2017	Incidence	25554	40	471	1906	25083	40	1946	431	23608	0.79	0.57 – 1.11	Adj
Derezinski	2017	Prevalence	922	0	2	187	920	0	187	2	735	-	-	-
Stackelberg	2017	Prevalence	14249	11	168	648	14081	11	659	157	13590	0.52	0.25 – 1.09	Adj
Taimor	2017	Prevalence	18953	17	299	674	18654	17	691	282	18262	0.38	0.08 – 1.73	Adj

AA = aortic aneurysm, Adj = adjusted data, CI = confidence interval, DM = diabetes mellitus, HR = hazard ratio, OR = odds ratio, Unadj = unadjusted data

Table 3.4. Prevalence and incidence data from studies describing the association between diabetes and aortic aneurysms in selected populations.

Study	Year	Prevalence / Incidence	Study Total	'AA denominator' data				'DM denominator' data				OR / HR	95% CI	Unadj / Adj
				DM in AA	Total AA	DM in non-AA	Total non-AA	AA in DM	Total DM	AA in non-DM	Total non-DM			
Sumpio	1985	Prevalence	103	4	79	3	24	4	7	75	96	-	-	-
LaMorte (local)	1995	Prevalence	380	17	102	130	278	17	147	85	233	-	-	-
Wolf	1995	Prevalence	531	5	32	78	499	5	83	27	448	-	-	-
Cahan	1999	Prevalence	240	28	139	38	101	28	66	111	174	-	-	-
Kang (0-49%)	1999	Prevalence	139	3	25	34	114	3	37	22	102	-	-	-
Kang (50%+)	1999	Prevalence	374	10	68	99	306	10	109	58	265	-	-	-
Shteinberg	2000	Prevalence	155	5	82	26	73	5	31	77	124	-	-	-
Bonamigo	2003	Prevalence	1269	8	67	65	1202	8	194	59	1075	-	-	-
Raffetto	2003	Prevalence	259	26	177	15	82	26	41	151	218	-	-	-
Monney	2004	Prevalence	395	4	40	77	355	4	81	36	314	-	-	-
Barba	2005	Prevalence	1166	29	151	279	1015	29	308	122	858	0.63	0.41-0.96	Unadj
Madaric	2005	Prevalence	109	1	16	36	93	1	37	15	72	0.11 / 0.12	0.01-0.83 / 0.01-1.03	Unadj / Adj
Hanly	2006	Prevalence	415	-	41	-	374	-	-	-	-	1.22	NS	Adj
Westwik	2006	Prevalence	68	1	16	24	52	1	25	15	43	-	-	-
Koksal	2007	Prevalence	80	5	40	7	40	5	12	35	68	-	-	-
Baumgartner	2008	Prevalence	68236	507	1722	29539	66514	507	30046	1215	38190	0.59	0.53-0.66	Adj
Ito	2008	Prevalence	343	83	211	30	132	83	113	128	230	2.2 / 1.85	1.36-3.64 / 1.06-3.3	Unadj / Adj
Vlek	2008	Prevalence	2196	30	170	351	2026	30	381	140	1040	-	-	-
Shirani	2009	Prevalence	2843	-	53	-	1907	-	91	-	2752	2.32	-	Adj
De'Ath	2010	Prevalence	50	0	25	5	25	0	5	25	45	-	-	-
Dupont	2010	Prevalence	217	6	15	57	202	6	63	9	154	-	-	-
Long	2010	Prevalence	306	4	20	87	284	4	91	16	215	-	-	-
Cueff	2012	Prevalence	193	3	9	43	184	3	46	6	147	-	-	-
Robson	2013	Incidence	6999		100	-	6899	-	689	-	6310	0.22 / 0.19	0.05-0.88 / 0.05-0.77	Unadj / Adj
Stam-Slob	2015	Prevalence	6841	28	247	1087	6600	28	1115	219	5726	-	-	-
Tanaka (AAA)	2015	Prevalence	279	5	23	139	256	5	144	18	135	-	-	-
Tanaka (TAA)	2015	Prevalence	320	23	61	139	259	23	162	38	158	-	-	-
Tsialtas	2016	Prevalence	1041	45	469	125	572	45	170	424	871	-	-	-
Ultee	2016	Prevalence	767	67	429	102	338	67	169	362	598	-	-	-
Takeuchi	2016	Prevalence	1526	2	30	398	1496	2	400	28	1126	-	-	-

AA = aortic aneurysm, Adj = adjusted data, CI = confidence interval, DM = diabetes mellitus, HR = hazard rati, OR = odds ratio, Unadj = unadjusted data

Primary outcome 1: prevalence of aortic aneurysms in the general population

Pooling studies reporting adjusted prevalence data

Pooling only studies that included patients from the general population and that reported adjusted outcomes regarding the prevalence of diabetes and aortic aneurysms resulted in a meta-analysis of 13 studies and 3,905,804 patients (Figure 3.2). Of these, 3,874,549 patients were from studies looking at AAA and 31,255 patients from studies looking at TAA. All 13 studies reported a lower prevalence of aortic aneurysms in patients with DM (OR range 0.32 – 0.86).

Nine of the studies were population-screening studies and 4 were case-control studies (3 retrospective, 1 prospective). Only 2 of the studies measured blood glucose (BG) or HbA1c levels to define DM with the remaining studies using International Classification of Diseases (ICD) codes, patient-reported diagnoses or casenote review. 10 studies used USS measurements to define an aortic aneurysm, 1 study used CT scans and 2 studies used ICD codes.

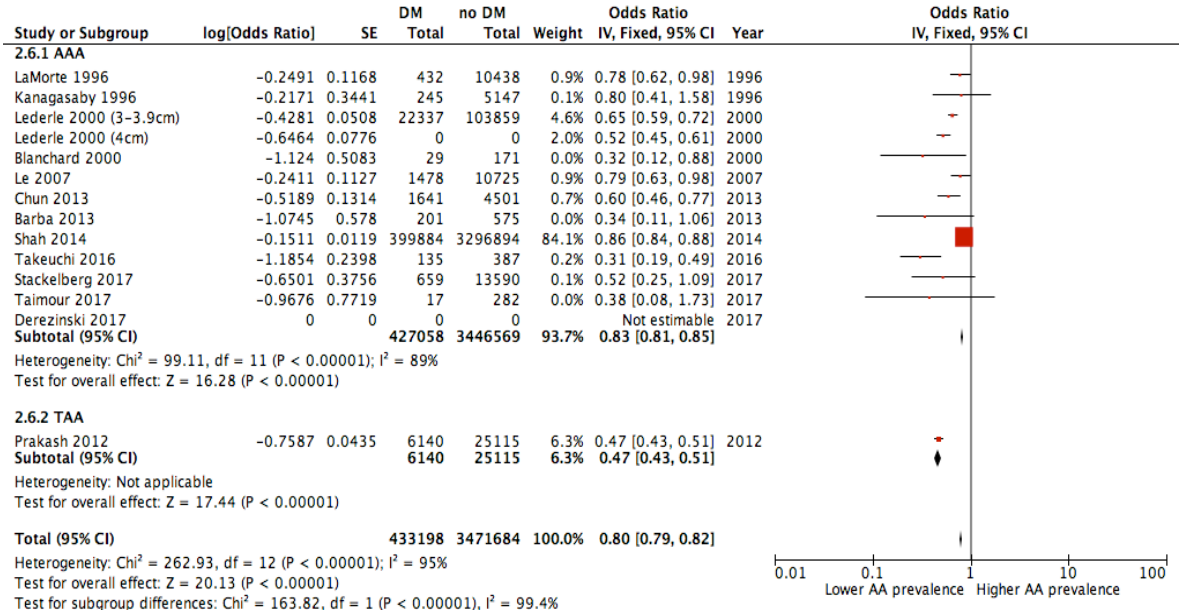


Figure 3.2. Forest plot comparing the prevalence of aortic aneurysms in patients with and without diabetes mellitus from the general population, only including studies reporting adjusted data. AA = aortic aneurysm, AAA = abdominal aortic aneurysm, CI = confidence interval, DM = diabetes mellitus, SE = standard error, TAA= thoracic aortic aneurysm.

The largest study included was by Shah *et al* [230] containing 3.69 million patients and has not been previously included in any published meta-analyses (OR 0.86; 95% CIs 0.84 – 0.88). Only the final report from the ADAM studies was included as this provided combined data from both studies[223]. Lederle *et al* reported separate ORs for AAA of different sizes (3cm to 3.9cm and ≥4cm) however it was not possible to determine the number of patients with DM in each diameter group therefore all patients (irrespective of AAA size) were stratified as DM or non-DM under the 3cm - 3.9cm entry. Prakash *et al*[213] included both TAA and thoracic aortic dissections and it was not possible to separate the data into aneurysms and dissections. There was significant heterogeneity amongst the included studies (I^2 statistic 89% for AAA studies, 95% for all studies).

The pooled estimate for the prevalence of aneurysms in DM patients was 0.83 for AAA studies (95% CI 0.81 – 0.85; p<0.0001). Combining the single study by Prakash *et al* reporting data on TAAs (OR 0.47; 95% CI 0.43 – 0.51; p<0.00001) resulted in the pooled estimate decreasing slightly to 0.80 (95% CI 0.79 - 0.82; p<0.0001) (see Figure 3.2).

Sensitivity analyses

Pooling only screening studies resulted in an OR of 0.84 (95% CI 0.82 – 0.85; I^2 statistic 90%) and pooling only studies with >10,000 patients resulted in an OR of 0.81 (95% CI 0.79 – 0.82; I^2 statistic 99%), which were not significantly different to the original result.

Pooling studies reporting unadjusted prevalence data

Pooling only studies that included patients from the general population and that reported unadjusted outcomes regarding the prevalence of diabetes and aortic aneurysms resulted in a meta-analysis of 19 studies and 248,823 patients (Figure 3.3). All studies examined patients with AAA. Ten studies reported a lower prevalence of aortic aneurysms in patients with DM (OR range 0.28 – 0.87) and 9 studies reported a higher prevalence in patients with DM (OR range 1.02 – 9.22).

Fifteen studies were screening studies and 4 were retrospective case-control studies. DM was defined using BG measurements in 5 studies, using a self-

reported diagnosis in 8 studies, using ICD codes in 2 studies and by case note review in 2 studies (2 studies did not specify how they defined DM). 17 studies used USS measurements to define AAA; 16 defined AAA as ≥ 30mm and 3 defined AAA as ≥ 35mm. Two studies used ICD codes to define AAA. There was significant variability in the individual ORs; 3 studies reported a significantly lower prevalence of AAA in DM, 3 studies reported a significantly higher prevalence of AAA in DM, 7 studies reported a non-significantly lower prevalence of AAA in DM and 6 studies reported a non-significantly higher prevalence of AAA in DM. There was substantial heterogeneity between studies (I^2 statistic 92%).

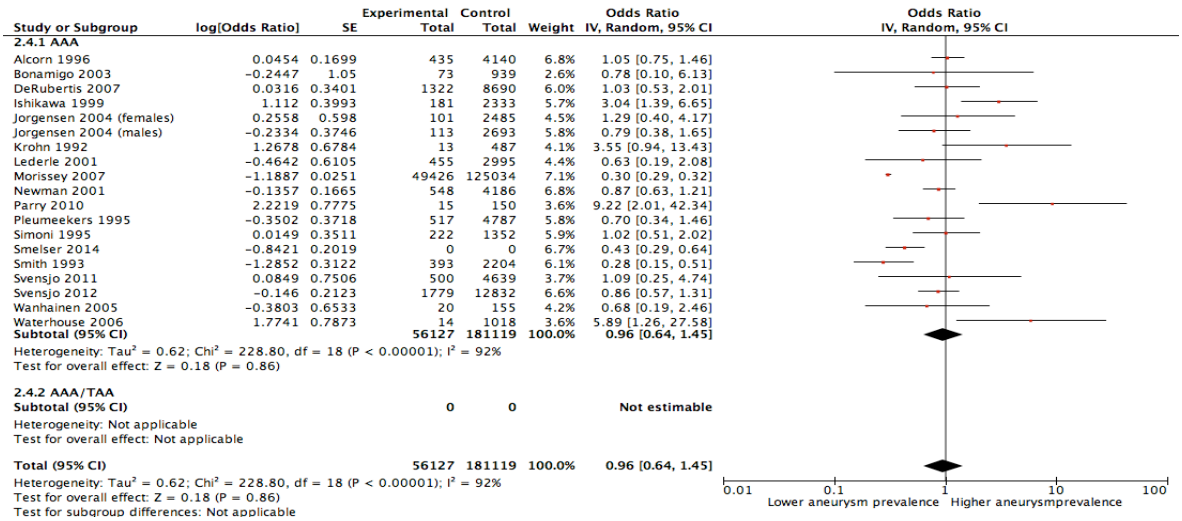


Figure 3.3. Forest plot comparing the prevalence of aortic aneurysms in patients with and without diabetes mellitus from the general population, only including studies reporting unadjusted data. AAA = abdominal aortic aneurysm, CI = confidence interval, DM = diabetes mellitus, SE = standard error, TAA= thoracic aortic aneurysm.

The largest study was by Morissey *et al* containing 174,460 patients and has not been included in previous meta-analyses (OR 0.30; 95% CIs of 0.29 – 0.32) [252].

The pooled estimate for the prevalence of AAA in patients with DM was 0.96 (95% CI 0.64 – 1.45; p=0.86) suggesting that DM is not associated with the prevalence of AAA when pooling unadjusted data (see Figure 3.3).

Sensitivity analyses

Pooling only screening studies resulted in an OR of 0.99 (95% CI 0.74 -1.33; I^2 statistic 61%) which did not change the overall result but reduced study heterogeneity. In contrast, pooling only studies with >10,000 patients led to a significant change in results (OR 0.47; 95% CI 0.27 – 0.83; I^2 statistic 87%).

Primary outcome 2: incidence of aortic aneurysms in the general population

Pooling studies reporting adjusted incidence data

Pooling only studies that included patients from the general population and that reported adjusted outcomes regarding the incidence of diabetes and aortic aneurysms resulted in a meta-analysis of 12 studies and 3,505,662 patients (Figure 3.4). Of these, 2,398,415 patients were from studies looking at AAA and 1,107,247 patients were from studies combining TAA and AAA. All 12 studies reported a lower incidence of aortic aneurysms in patients with DM (HR range 0.20 – 0.80).

Nine of the studies were prospective observational cohort studies and 3 were retrospective cohort studies. None of the studies measured BG or HbA1c levels to define DM. New AA were defined using ICD codes / HES data in 10 studies and using serial self-reported questionnaires in 2 studies. Study follow-up ranged between 5.5 years and 20 years. There was significant heterogeneity amongst studies (I^2 statistic 96% for AAA studies, 58% for TAA/AAA studies and 99% for all studies).

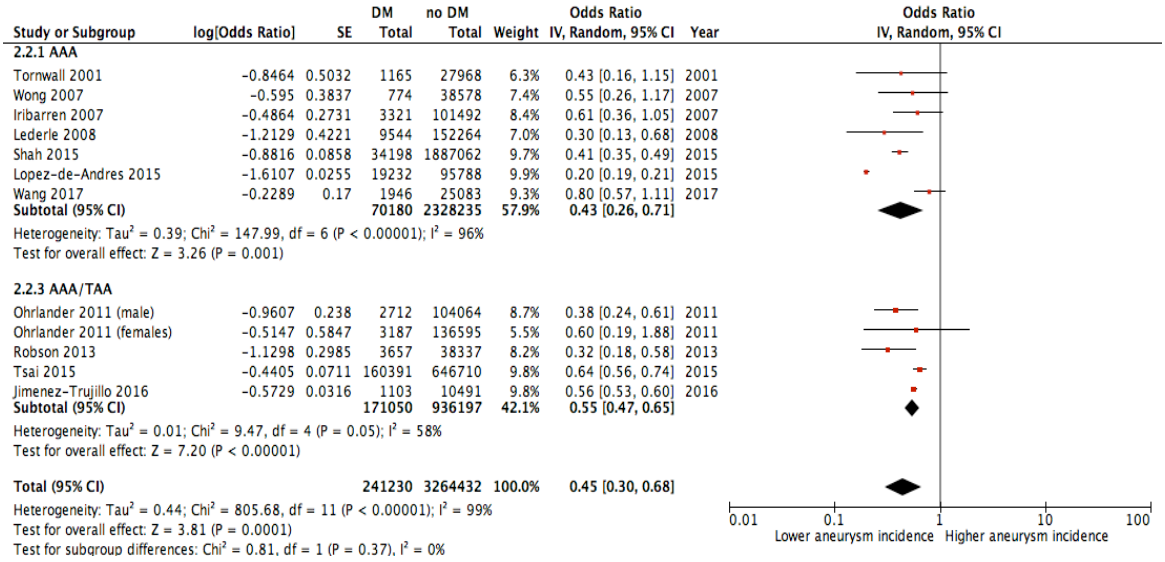


Figure 3.4. Forest plot comparing the incidence of aortic aneurysms in patients with and without diabetes mellitus from the general population, only including studies reporting adjusted data. AAA = abdominal aortic aneurysm, CI = confidence interval, DM = diabetes mellitus, SE = standard error, TAA= thoracic aortic aneurysm.

The largest study was by Shah *et al* containing 1.92 million patients and has not been included in previous meta-analyses (HR 0.46; 95% CIs of 0.35 – 0.49) [228]. Of note, the study by Ohrlander *et al* reported separate data for males and females which could not be combined and therefore had to be analysed stratified by gender[209].

The pooled estimate for AAA studies was 0.43 (95% CI 0.26 – 0.71; p<0.0001) whereas for combined TAA and AAA studies it was 0.55 (95% CI 0.47 – 0.65; p=0.05). The overall pooled estimate for all aortic aneurysm studies was 0.45 (95% CI 0.30 – 0.68; p<0.00001) (see Figure 3.4).

Sensitivity analyses

Pooling only prospective observational cohort studies resulted in an OR of 0.48 (95% CI 0.37 – 0.61; I^2 statistic 50%), narrowed the CIs further and reduced study heterogeneity. In contrast, only pooling studies with >100,000 patients (pooled OR 0.41; 95% CI 0.24 – 0.71; I^2 statistic 98%) widened the 95% CIs and increased the level of heterogeneity.

Pooling studies reporting unadjusted incidence data

There was only 1 article reporting unadjusted data on the incidence of aortic aneurysm in patients with DM[246]. Rodin *et al* assessed the relationship between various risk factors and the development of AAA in 19,274 participants (655 with DM, 18619 without DM) of the Chicago Heart Association Project and included an average of 30 years follow-up. The study reported a HR for AAA in DM of 0.81 (0.49 for men, 2.02 for women) suggesting a lower incidence of AAA in those with DM although this finding did not reach statistical significance. The authors did not report 95% CIs and therefore it was not possible to produce a forest plot.

Primary outcome 3: presence of aortic aneurysms in the general population

Pooling studies reporting adjusted prevalence or incidence data

Pooling studies that included patients from the general population and that reported adjusted outcomes regarding the prevalence or incidence of diabetes and aortic aneurysms resulted in a meta-analysis of 25 studies and 7,427,723 patients (Figure 3.5). All 25 studies reported a lower prevalence or incidence of AAA in patients with DM.

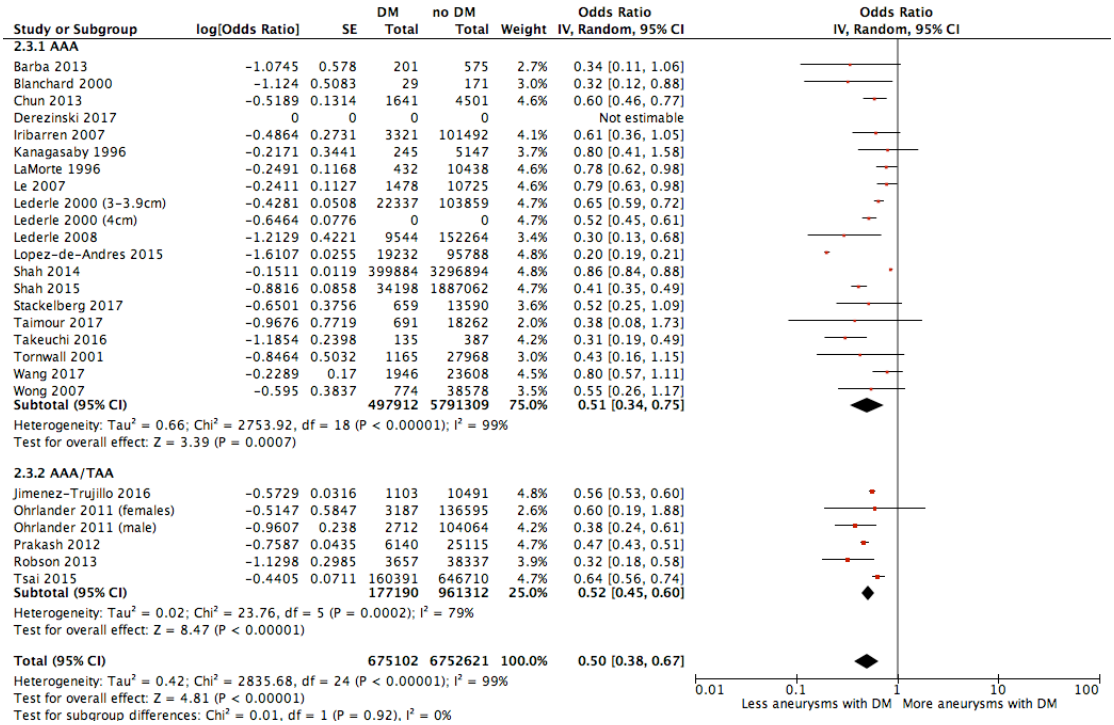


Figure 3.5. Forest plot comparing the presence of aortic aneurysms in patients with and without diabetes mellitus from the general population, only including studies reporting adjusted data. AAA = abdominal aortic aneurysm, CI = confidence interval, DM = diabetes mellitus, SE = standard error, TAA= thoracic aortic aneurysm.

The pooled estimate for AAA studies only was 0.51 (95% CI 0.34 – 0.75; p<0.00001) where for studies including TAA and AAA it was 0.52 (95% CI 0.45 – 0.60; p=0.0002). The pooled estimate including all 25 studies was 0.50 (95% CI 0.38 – 0.67; p<0.00001) (see Figure 3.5).

Pooling studies reporting unadjusted prevalence or incidence data

The pooled estimate result for this analysis was identical to the result shown in Figure 3.3 because the only study reporting incidence data did not provide 95% CIs and therefore could not be pooled together with the studies reporting prevalence data.

Secondary outcome 1: prevalence of aortic aneurysms in selected populations

Pooling studies reporting adjusted prevalence data

Pooling studies that included patients from selected populations and that reported adjusted outcomes resulted in a meta-analysis of five studies and 71,946 patients (Figure 3.6). All studies examined patients with AAA. Two studies reported a lower prevalence of aortic aneurysms in DM (OR range 0.10 – 0.59) and 3 studies reported a higher prevalence (OR range 1.22 – 2.32).

Three studies selected patients from those with a known cardiology disease background, 1 study from patients with known thrombotic risk factors and 1 study compared AAA patients against TAA patients. There were 2 screening studies, 2 case-control studies (1 prospective, 1 retrospective) and 1 study used registry data. Only 2 studies defined DM using BG / HbA1c measurements with DM being defined by case note review (n=2) or by self-reporting (n=1) in the remaining studies. AAA was defined using USS measurement in 3 studies, by recording self-reported physician diagnoses in 1 study and was not defined in 1 study.

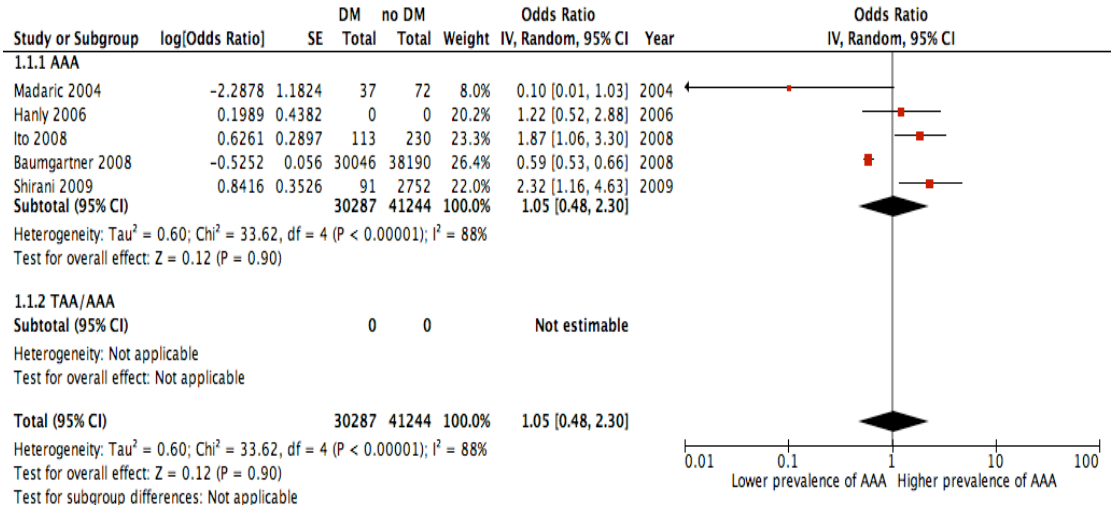


Figure 3.6. Forest plot comparing the prevalence of aortic aneurysms in patients with and without diabetes mellitus from selected populations, only including studies reporting adjusted data. AAA = abdominal aortic aneurysm, CI = confidence interval, DM = diabetes mellitus, SE = standard error, TAA= thoracic aortic aneurysm.

The largest study was by Baumgartner *et al* containing 68,326 patients which has been included in previous meta-analyses[271]. Hanly *et al* only reported adjusted OR data[268] and it was not possible to determine the number of patients with and without DM therefore no subgroup totals have not been displayed for this study.

The pooled estimate for AAA studies was 1.05 (95% CI 0.48 – 2.30; p=0.90; I^2 statistic 88%) suggesting no association between DM and the prevalence of aortic aneurysms in selected populations (Figure 3.6).

Sensitivity analyses

Pooling only screening studies (OR 1.77; 95% CI 0.48; I^2 statistic 23%) did not change the overall result but did reduce study heterogeneity. Only 1 study

included >10,000 patients which accounted for more patients than all the other 4 studies combined. This study suggested a reduced prevalence of AAA in patients with DM in selected populations (OR 0.59; 95% CI 0.53 – 0.66) [271].

Pooling studies reporting unadjusted prevalence data

Pooling studies that included patients from selected populations and that reported unadjusted outcomes resulted in a meta-analysis of 24 studies and 18,120 patients (Figure 3.7). Of these, 17,800 patients were from studies looking at AAA and 320 patients were from studies looking at both TAA and AAA.

Studies identified aortic aneurysm patients from a variety of patient populations; vascular surgery patients in 13 studies (5 aorto-occlusive disease, 4 peripheral arterial disease, 2 carotid artery disease, 2 mixed vascular), coronary artery bypass grafting patients in 4 studies, unspecified cardiological disease patients in 3 studies, mixed cardiovascular disease patients in 2 studies, chronic kidney disease patients in 1 study and colorectal cancer patients in 1 study. There were 7 screening studies, 15 case-control studies (13 retrospective and 2 prospective) and 2 prospective observational cohort studies (baseline data only). DM was defined using BG / HbA1c measurements in 8 studies, case note review in 8 studies, patient reported diagnoses in 6 studies and was not specified in 2 studies. Aortic aneurysms were identified using USS measurements in 11 studies, as a history of previous open AAA repair in 7 studies, as patients awaiting/undergoing AAA repair in 3 studies (2 studies AAA > 55mm, 1 study

AAA > 50mm) and by using CT measurement in 3 studies (AAA ≥ 30mm, TAA ≥ 40mm).

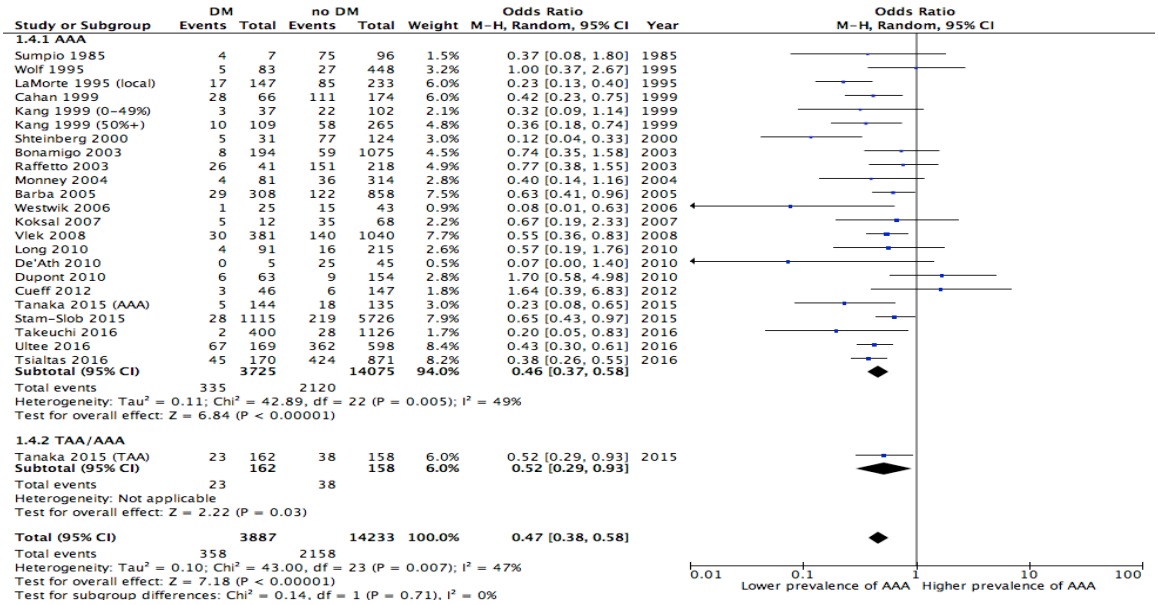


Figure 3.7. Forest plot comparing the prevalence of aortic aneurysms in patients with and without diabetes mellitus from selected populations, only including studies reporting unadjusted data. AAA = abdominal aortic aneurysm, CI = confidence interval, DM = diabetes mellitus, SE = standard error, TAA= thoracic aortic aneurysm.

The largest study was by Stam-Slob *et al* (6,841 patients) which has not been included in previous meta-analyses[279].

The pooled estimate for AAA studies was 0.46 (95% CI 0.37 – 0.58; p<0.005; I^2 statistic 49%) and only 1 study examined the prevalence of TAA (OR 0.52; 95% CI 0.29 – 0.93; p=0.03). The overall pooled estimate was 0.47 (95% CI 0.38 – 0.58; p<0.007; I^2 statistic 47%) suggesting that DM is associated with a lower prevalence of aortic aneurysms in selected populations (see Figure 3.7).

Sensitivity analyses

By only pooling screening and prospective observational cohort studies independently (OR 0.66; 95% CI 0.53 – 0.81; I^2 statistic 0%), the overall result remained the same (DM associated with a lower prevalence of aortic aneurysms) however the level study heterogeneity was significantly reduced.

Secondary outcome 2: incidence of aortic aneurysms in selected populations

Pooling studies reporting adjusted incidence data

There was only 1 study reporting adjusted data on the incidence of AA in patients with DM in selected populations[212]. Robson *et al* examined the incidence of aortic aneurysms in nearly 7000 patients >40 years old with giant cell arteritis obtained from the UK general practice research database (689 with DM vs. 6310 without DM) and examined up to 30 years of follow-up. The study reported an adjusted HR of 0.19 (95% CI 0.05 – 0.77; p=0.02) suggesting a lower incidence of aortic aneurysms in diabetic patients with giant cell arteritis (see Figure 3.8).

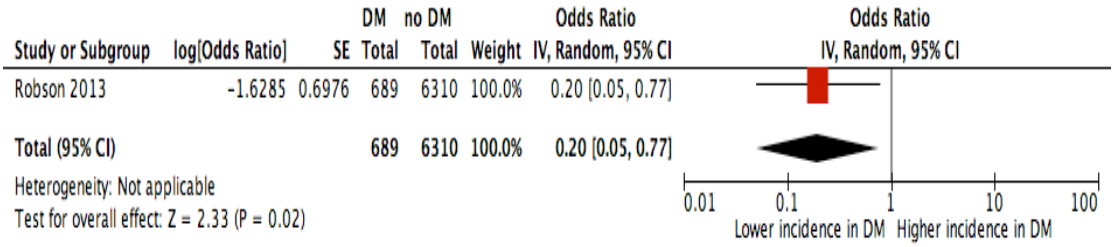


Figure 3.8. A Forest plot comparing the incidence of aortic aneurysms in with and without DM from selected populations only including studies reporting adjusted data. AAA = abdominal aortic aneurysm, CI = confidence interval, DM = diabetes mellitus, SE = standard error.

Pooling studies reporting unadjusted incidence data

There were no studies reporting unadjusted data on the incidence of aortic aneurysms in patients with DM in selected populations.

Secondary outcome 3: presence of aortic aneurysms in selected populations

Pooling studies reporting adjusted prevalence and incidence data

Pooling studies that included patients from selected populations and reported adjusted data on either the prevalence or incidence of aortic aneurysms resulted in a meta-analysis of 6 studies and 78,945 patients (Figure 3.9). In 3 studies, DM was associated with a decreased presence of aortic aneurysms and in 3 studies with an increased presence of aortic aneurysms.

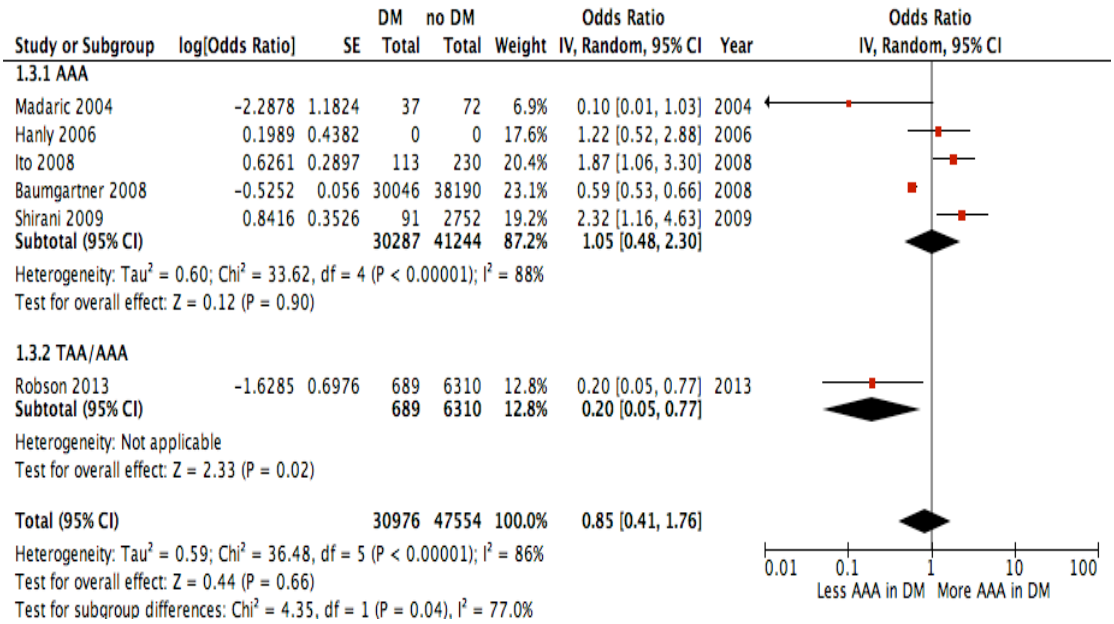


Figure 3.9. Forest plot comparing the presence of aortic aneurysms in patients with and without diabetes mellitus from selected populations, only including studies reporting adjusted data. AAA = abdominal aortic aneurysm, CI = confidence interval, DM = diabetes mellitus, SE = standard error, TAA= thoracic aortic aneurysm.

The overall pooled estimate was 0.85 (95% CI 0.41 – 1.76; p=0.66) suggesting no association between DM and aortic aneurysm presence in selected populations (see Figure 3.9).

Pooling studies reporting unadjusted prevalence and incidence data

There were no studies reporting unadjusted incidence data therefore the pooled estimate for this analysis did not change from that shown in Figure 3.7.

3.4.3 Other studies not suitable for meta-analysis

There were a number of other studies describing the relationship between aortic aneurysms and DM which were not suitable for meta-analysis. Bengtsson *et al* performed USS screening of males > 74yrs old for AAA in Sweden and found no relationship between hyperglycaemia and the presence of AAA however no event rate data, unadjusted or adjusted estimates were reported[282]. Hupp *et al* reported the results of vascular screening in individuals > 60 years old and found that DM was associated with larger aortic diameters and doubled the detection rate of AAA however also did not report any event rate, unadjusted or adjusted data [283]. Two other studies reported absolute aortic diameters or glucose levels without any reference to aortic aneurysm, AAA, TAA or DM rates and therefore could not be included in the meta-analysis as they would of required separate meta-regression. Cho *et al* examined the correlation between coronary artery calcium levels and aortic diameter and reported no significant association between DM and aortic diameter ($r = -0.026$, $p=0.614$) [214]. Persson *et al* examined the association between glucose levels at age 33 and abdominal aortic diameter at age 65 in a large population of Scandinavian men and found no association between glucose levels and aortic diameter ($r=0.035$, $p=0.146$) [284].

3.5 Discussion

This study examined the association between DM and aortic aneurysm prevalence and incidence. The principal findings from the study were that in the general population, the presence of diabetes mellitus reduces the odds of having an aortic aneurysm by 20% and of developing an aortic aneurysm by >50% when pooling studies reporting the most reliable type (adjusted) of data. The strength and validity of these findings is robust since each meta-analysis pooled over 3.5 million patients. Similar findings were not found when pooling studies reporting unadjusted data however unadjusted data is less reliable when analysing non-randomised studies and therefore this disparity may be due to the presence of confounding. Interestingly, pooling only large studies (>10,000 patients each) reporting unadjusted data did produce similar findings.

In selected populations, such as those where patients have concomitant cardiovascular comorbidities, the association between DM and aortic aneurysm prevalence and incidence is less clear. When pooling studies reporting adjusted outcomes, no association between DM and aortic aneurysm prevalence was found (OR 0.96 with CIs crossing 1.0) however when pooling (less reliable and generally smaller) studies reporting unadjusted outcomes, the presence of DM was associated a 50% lower prevalence of aortic aneurysm. As only 1 study had examined the relationship between DM and aortic aneurysm incidence in selected patient population (giant cell arteritis patients), more research is needed in this area to draw any clear conclusions.

The present meta-analysis is the first to examine the relationship between all aortic aneurysms and DM. Previous meta-analyses have focused on AAA [71,224-226] or TAA[285] in isolation. However individual studies have found similar negative associations between both disease entities[229,231] and there is evidence to suggest AAA are merely a localised manifestation of a more systemic dilating diathesis[68]. The present meta-analysis has also included significantly larger numbers of patients than previous meta-analyses despite general and selected population studies being analysed separately and also studies reporting adjusted and unadjusted data being analysed separately. This cannot be explained by the inclusion of TAAs as this group of patients only accounted for the minority proportion. The large numbers of included patients in the present meta-analysis are due to the inclusion of a number of recent publications examining national data and large patient cohorts [211,228-230] but also due to a meticulous searching of the literature which identified missed earlier publications [252].

There has only been one meta-analysis examining the association between DM and AAA specifically in selected populations[286] although De Rango *et al* did also comment on the association of DM and AAA in selected populations as part of a more comprehensive review [225]. Elkalioubie *et al* pooled 6 studies examining patients with coronary artery disease and reported no association between DM and the prevalence of AAA (OR of 0.85; 95% CI 0.53 – 1.36) [286]. In contrast, De Rango *et al* pooled 3 studies examining mixed patient populations (patients with carotid stenosis, ACS and CABG in 1 study each) and reported a negative association between DM and AAA prevalence (OR 0.59; 95% CI 0.35 – 0.99) [225].

The strength of the present meta-analysis lies in the fact that studies reporting adjusted and unadjusted data were pooled separately. In the absence of individual patient data, using adjusted outcome data is preferential over unadjusted outcome data when pooling non-randomised studies. In addition, where unadjusted outcome data was not available, the present meta-analysis preferentially used event rate data which is more reliable than using reported unadjusted estimates. Furthermore the present meta-analysis calculated 'DM-denominator' event rates where available and presented data in this format where possible. This is preferential to presenting data in an 'aneurysm-denominator' format because it is the impact of DM on aortic aneurysm prevalence or incidence that is of interest rather than the impact of aortic aneurysms on the prevalence or incidence of DM. Future studies in this area should aim to present data using a DM-denominator format.

The limitations of the present meta-analysis are due to the quality and variability of the included studies. There were no randomised studies that could be included and all analyses suffered from significant study heterogeneity although heterogeneity was mitigated to some extent in a few analyses by performing sensitivity analyses according to study type and size. Other limitations include the large number of AAA relative to TAA in the present study as well as the inability to differentiate between TAA and TAAD in some studies. This study has identified a need for further studies examining the epidemiology of TAA / aortic aneurysms in general.

3.6 Conclusions

Diabetes is strongly associated with a reduced prevalence and incidence of aortic aneurysms in the general population and may be associated with a reduced prevalence of aortic aneurysms in selected populations at high cardiovascular risk. Determining the relationship between diabetes and aortic aneurysm incidence in select populations requires further study. This may be through interrogation of existing large datasets that have collected data on specific patient cohorts.

Chapter 4: The effects of diabetes mellitus on abdominal aortic aneurysm growth and rupture

4.1 The relationship between diabetes and abdominal aortic aneurysm growth

In addition to being negatively associated with the presence of AAA, DM has been shown to be negatively associated with AAA progression/growth. The first study to report on this relationship was by Chang *et al* in 1997 who examined small AAA growth rates in 514 patients, 88 of whom were diabetic, however they found no significant reduction in growth rates amongst those with DM[287]. It was Brady *et al*, who analysed risk factors for AAA growth using the UKSAT dataset, that first reported a negative effect of DM on AAA growth (75 diabetics vs. 1668 non-diabetics; -0.79 mm/year) [288]. Thereafter at least a further 5 studies reported reduced growth of small AAA in patients with DM[139,289-292].

The first meta-analysis on the subject was performed by Sweeting *et al*, on behalf of the RESCAN collaborators[25]. They combined individual patient data on small AAA growth rates from 10 datasets[12,15,30,139,174,181,288,291,293], of which 1 was unpublished, and found that diabetics, on average, had a reduction in AAA growth rates of 0.51mm/year after adjustment for covariates which was consistent across all studies. This correlated with a nearly 25% reduction in AAA growth rates and was the largest determinant of AAA growth rates in the study, even more than smoking habits [25]. Since then, a number of other groups have also investigated this relationship. The CAESAR investigators, led by De Rango, studied patients enrolled

in the surveillance arm of the CAESAR trial and found that DM significantly reduced the likelihood of >5mm of small AAA (4.1cm – 5.5cm) growth by >60% (20 diabetes vs. 158 non-diabetics; HR 0.37, p=0.003) despite baseline AAA diameter being equal amongst the 2 groups[294]. Behr-Rasmussen *et al*. and Bhak *et al*. have also published data supporting a negative effect for DM on AAA growth with reported reductions in AAA growth rates ranging between 1.1mm/year – 1.4mm/year which translates into a 40% – 50% reduction in baseline growth rates[295,296]. Takagi *et al* has subsequently performed another meta-analysis on the subject, inclusive of the studies published since the first meta-analysis, which confirms the negative association between DM and AAA growth. Combining 18 studies reporting adjusted outcomes they found diabetics possessed a reduction in AAA growth rates of 0.29mm/year (p<0.0001) which persisted even after rigorous sensitivity testing however the authors did suggest the included studies suffered from publication bias [297].

Since the latest meta-analysis there have been 3 further publications on the subject. Lederle *et al* investigated the effect of various medications on AAA growth rates and found no effect for any of the medicines studied but reported that patients with a diagnosis of DM, on average, had lower AAA growth rates by 1.2mm/year (p=0.008) [298]. Hendy *et al* studied the effect of DM on AAA progression using aortic volume rather than diameter as the subject of measurement. They found that diabetics experienced less of an increase in aortic volume compared to non-diabetics (4.2%/year vs. 8.9%/year; p=0.003) [299]. In contrast to other studies which have only looked at DM as a dichotomous variable, Kristensen *et al* sought to determine whether the degree glycaemia might be important[300]. Using data from

the VIVA (Viborg Vascular) RCT, they confirmed reduced growth rates in those with DM (1.7mm/year vs. 2.7mm/year; p<0.001) but also found a significant negative correlation between AAA growth rate and HbA1c (p=0.002). Interestingly the latter finding was independent of patients' aortic stiffness indices or diabetic status[300].

Despite the numerous studies confirming a protective effect of DM on AAA growth, there have been no studies comparing the effect of type 1 versus type 2 diabetes mellitus. Furthermore, whether it is the disease process itself or the treatment for the disease that is mediating this protective effect is not fully understood. The relationship between antidiabetic medication use and AAA is further explored in Chapter 5.

4.2 The effect of diabetes on outcomes after endovascular aneurysm repair

Only two studies have examined the effect of DM on outcomes following EVAR. Diehm *et al* studied aortic neck dilatation in 6383 patients (810 diabetics vs. 5573 non-diabetics) from the EUROSTAR registry and found a trend towards reduced post-EVAR aortic neck dilatation amongst diabetics (incidence of aortic neck dilatation ≥8mm; diabetics 8.5% vs. non-diabetics 12.8%) but this did not reach statistical significance. Furthermore there was no difference in the incidence of type 1 endoleaks[301]. More recently, Png *et al* compared sac expansion and re-intervention rates in 183 diabetic and 810 non-diabetic patients post-EVAR. They found a significant decrease in aneurysm sac expansion (13.1% vs. 19.4%; p=0.02)

as well as a trend towards fewer re-interventions (23.5% vs. 28.4%; p=0.08) amongst diabetics compared with non-diabetics[302]. Similar to the study by Diehm *et al*, this was not associated with any difference in endoleak rates.

4.3 The relationship between diabetes and ruptured abdominal aortic aneurysm

The largest study examining the effect of DM on risk of AAA rupture comes from Taiwan which also examines TAA rupture rates. The study, which used medical claims data from the National Health Research Institute, compared rates of AAA/TAA with and without rupture in 160,391 patients with T2DM (282 combined AAA/TAA) and 646,710 patients without DM (1927 combined AAA/TAA) [211]. They found a reduction in the incidence of ruptured TAA (incidence rate ratio (IRR) 0.50; 95% CI 0.53 - 0.71) and ruptured AAA (IRR 0.53; 95% CI 0.40 – 0.69) in patients with advanced T2DM even after adjustment for confounders. The authors defined advanced DM as DM in the presence of DM-related diseases. Uncomplicated DM was also found to exert a beneficial effect on AAA rupture rates but not on TAA rupture rates.

The remaining studies examining this relationship are modest in size by comparison. The first meta-analysis on this subject was attempted by the RESCAN collaborators however they were only able to compare data from 62 ruptured AAA patients without DM against 4 ruptured AAA patients with DM (2 studies) [25]. Takagi *et al* recently performed a more useful meta-analysis of 9 studies (>800,000 patients) examining this relationship and found DM to be associated with a significantly lower prevalence/incidence of AAA rupture (HR 0.71; 95% CI 0.56 – 0.89; p=0.003) [303]. This finding persisted despite their rigorous sensitivity analyses (HR 0.67 – 0.78) which involved sequentially excluding each study from the meta-analysis.

One study by Chisci *et al*, which appears to have been missed by Takagi's meta-analysis, examined predictors of AAA rupture using CT and demographic data from 143 patients (73 non-ruptured AAA, 18 ruptured AAA, 52 non-aneurysmal aortas) [304]. The authors found DM to be the only protective factor against rupture in their study (OR 0.78). Most recently, Wierzba *et al* have published a study looking at the incidence rates of ruptured and non-ruptured AAA in patients with and without DM in Poland[305]. The study examined a large cohort of patients from the Polish National Health Fund (2388 non-ruptured AAA patients with DM, 5844 non-ruptured AAA patients without DM, 282 ruptured AAA patients with DM, 779 ruptured AAA patients with DM) and in contrast to other studies found that DM was associated with a significantly increased rate of ruptured AAA in both men (21.1 vs. 3.6 per 100,000 inhabitants; $p<0.0001$) and women (5.1 vs. 0.6 per 100,000 inhabitants; $p<0.0001$) [305]. The study also found a higher incidence of non-ruptured AAA in men (184.9 vs. 25.0 per 100,000 inhabitants) and women (35.4 vs. 5.6 per 100,000 inhabitants) with DM.

Chapter 5: Explaining the relationship between diabetes and abdominal aortic aneurysms

Clinicians will be well aware that arteries from patients with DM are often harder and more calcified than those from patients without DM. However increased vessel wall calcification does not appear to explain the reduced rate of aortic expansion seen in diabetics. The mechanisms underlying the protective effect of DM on AAA presence, growth and rupture are still the focus of considerable research however elucidation of these mechanisms is important since there are currently no medical therapies that can be used to slow or halt the progression of AAA. Numerous studies have been published in this area and the results can broadly be grouped into 4 main biological areas affecting: 1) extracellular matrix (ECM) volume, 2) ECM glycation and advanced glycation end-product (AGE) formation, 3) inflammation and oxidative stress and 4) intra-luminal thrombus biology. Significant research also suggests that the medications used to treat DM may play more of a role than the disease itself in explaining this relationship, therefore the literature in this area has also been reviewed.

5.1 Extracellular matrix volume

Many of the studies assessing the impact of hyperglycaemia on ECM volume have focused on patients with diabetic kidney disease. These studies show that chronic hyperglycaemia is associated with a progressive increase in ECM volume due to a decrease in ECM degradation and an increase in ECM synthesis[306]. In

the kidney this results in the development of diabetic nephropathy however in the aorta this probably results in arterial wall stiffening[307]. Astrand *et al* have shown that diabetic patients undergoing ultrasound of their aorta have a significantly thicker intima-media layer when compared to non-diabetic age- and sex-matched controls (0.89mm vs. 0.73mm; $p<0.001$) [308]. The same group found that this results in diabetic aortas being subjected to 20% less aortic wall stress (7.8×10^5 dynes/cm^2 vs. 9.7×10^5 dynes/cm^2; $p<0.001$) which may be one explanation for the reduced rate of AAA expansion amongst diabetics[297].

The metabolic milieu of DM can exert direct effects on the production of ECM proteins. Jones *et al* found that 25mmol/L of intermittent hyperglycaemia was capable of increasing collagen synthesis by 29% in cultured human tubulointerstitial cells[309]. In contrast, Shi *et al* found that hyperinsulinaemia, which is often present with hyperglycaemia in patients with insulin resistance, was associated with increased elastin gene expression via a PI3K-dependent pathway in cultured human aortic smooth muscle cells (AoSMCs) [310].

Matrix metalloproteinases (MMPs) are important in the pathogenesis of AAA since they are key regulators of ECM volume and structure. Although results are not uniform in this area, numerous studies have found hyperglycaemia to be associated with a reduced level of MMP expression. Death *et al* found that 25mmol/L of hyperglycaemia was able to down-regulate expression of MMP-3 in endothelial cells however expression of MMP-2, one the key MMPs in AAA, was up-regulated by hyperglycaemia in the same study[311]. Portik-Dobos *et al* used internal mammary artery specimens from patients with and without DM and

found significantly decreased levels of pro-MMP-1, -2 and total MMP activity ($p<0.05$) in specimens from diabetics[312]. In a different study, the same group reported decreased levels of MMP-1 and -9 in internal mammary and crural vessel specimens from diabetic patients undergoing lower limb amputation[313]. In the kidney, DM is associated with reduced MMP-2 expression[314], mediated through a pathway involving hyperglycaemia-induced angiotensin II production[315]. Angiotensin II in turn stimulates the expression of TGF-β1 which has been shown to down-regulate MMP-2 expression[316]. Plasmin is another regulator of MMP expression since plasmin catalyses the conversion of pro-MMP-2 and pro-MMP-9 to their active forms MMP-2 and -9. Dua *et al* has shown using the elastase infusion mouse model of AAA that hyperglycaemia can increase levels of plasminogen activator inhibitor-1 (PAI-1) which ultimately decreases plasmin expression and hence MMP levels[317].

5.2 Extracellular matrix glycation and advanced glycation end-product formation

Chronic hyperglycaemia is associated with non-enzymatic cross-linking of ECM proteins which results in the formation of so-called advanced glycation end-products (AGEs) which, in vascular tissues can increase vessel stiffness[318]. Chirinos *et al* performed pulse wave velocity measurements in patients with and without T2DM and found increased large artery stiffness, decreased reflection magnitude and increased pulsatile energy transmission to distal arterial beds amongst those with T2DM[319].

The importance of AGEs in protecting against AAA is complex. Norman *et al* reported lower circulating levels of carboxymethyllysine (CML) amongst diabetic males with AAA compared to those without AAA and hypothesised that increased AGE formation amongst diabetics may explain the protective effect of DM on AAA however the study was criticised since CML does not induce cross-linking[320,321]. In addition, Zhang *et al* found higher tissue concentrations of AGEs in aortic specimens from patients with AAA and suggested that AGEs together with their receptor (RAGE) are important for the development of AAA[322].

More recently, Koole *et al* performed a study comparing concentration of 3 AGEs (pentosidine, carboxyethyllysine and CML) in aortic wall biopsies from AAA patients with and without DM as well as non-AAA patients with and without DM to investigate the role AGEs play in protecting diabetic patients against AAA development further[323]. They found increased pentosidine concentrations were associated with DM in aortic tissues from AAA ($p=0.02$) and non-AAA ($p=0.02$) patients. Amongst diabetic AAA patients, pentosidine concentrations negatively correlated with aortic diameter ($r=-0.43$, $p=0.02$) and experimentally glycation of AAA tissue was associated with a significant reduction in MMP-induced, but not cathepsin-induced, proteolysis. No significant differences were observed for carboxyethyllysine or CML leading the authors to conclude that cross-linking AGEs like pentosidine, but not non-cross-linking AGEs, play a role in protecting against AAA progression in diabetic patients[323]. To study the diverging effects of DM on AAA and peripheral arterial disease (PAD) pathogenesis further, de Vos *et al* plans to perform a multicentre study that will measure the amount, type and

location of AGEs in the arterial wall of AAA and PAD patients with and without DM, and then correlate these findings with pulse wave velocity measurements[324].

AGEs also maintain vessel wall architecture by rendering ECM proteins less susceptible to protein degradation and by influencing the production of cathepsins and MMPs[325]. Grzebyk *et al* found that circulating levels of AGEs were higher amongst patients with T2DM ($p<0.001$) and that high AGE levels were associated with reduced plasma ($p<0.05$) and neutrophil-derived ($p<0.01$) levels of cathepsin B activity[326]. Zhang *et al* have also reported that AGEs can increase the production of MMP-9 in cultured macrophages but not in cultured AoSMCs[322,327]. This pathway is regulated by extracellular signal-regulated kinases (ERK) and nuclear factor kappa B (NF-κB) and can be attenuated by transforming growth factor (TGF)-β1[327]. Golledge *et al* have shown that human monocytes exposed to glycated collagen lattices decrease their production of MMP-2 and MMP-9 which are important in AAA development[290]. A similar phenomenon could be achieved by exposing monocytes to non-glycated but cross-linked collagen lattices which led the authors to suggest that it is the ability of hyperglycaemia to induce matrix protein cross-linking rather than hyperglycaemia itself that is responsible for the decrease in monocyte-derived MMP production[290].

5.3 Inflammation

The role of inflammation in explaining the negative relationship between DM and AAA is complicated. Up-regulation of inflammatory cell pathways is an

essential component of AAA formation[328]. DM is known to induce vascular inflammation in atherosclerotic tissues[329,330] however some studies also suggest a similar phenomenon occurs amongst aneurysmal tissues which would seem inappropriate given the relationship between DM and AAA[331]. Indeed, Arapoglou *et al* examined macrophage levels in patients undergoing open AAA repair and found significantly higher levels in aortic tissues from patients with DM compared to those without (p=0.02) and a positive correlation with serum glucose levels (r=0.21, p=0.04) [331].

Others however have reported reduced vascular inflammation in DM. Miyama *et al* have shown, using the ApoE-deficient AngII and porcine pancreatic elastase mouse models of AAA, that hyperglycaemia (produced using streptozotocin (STZ) administration) was associated with smaller AAA and a diminished inflammatory cell response characterised by a reduction in aortic macrophage infiltration (p=0.03) and peritoneal macrophage production[332]. In this study insulin administration was associated with abrogation of AAA expansion. Hurks *et al* investigated the relationship between AAA inflammation and atherosclerotic risk factors and found that lymphocyte-poor (i.e. low levels of inflammation) rather than lymphocyte-rich aortic specimens were more commonly associated with many traditional atherosclerotic risk factors including DM (22% vs. 9%; p=0.008) [333].

The exact mechanisms by which DM alters inflammation in AAA may involve the monocyte-macrophage system[332], activation of T-cell insulin receptors[334] or through production of C-peptide[335]. Tanaka *et al* have recently shown that

hyperglycaemia was able to suppress MMP-9 mRNA (17.9 +/- 2.5 vs. 50.5 +/- 9.7; p<0.01) and protein (3.6 +/- 0.5 vs. 6.4 +/- 0.3; p<0.01) expression in macrophage cell lines stimulated by tumour necrosis factor (TNF)-α and calcium-phosphate[336]. The authors went onto demonstrate that this suppression was mediated by hyperglycaemia-induced activation of the glucose-sensing nuclear receptor Nr1h2 (0.8 +/- 0.4 vs. 0.2 +/- 0.04; p<0.05) [336]. Patients with type 2 diabetes mellitus (T2DM) often have raised circulating c-peptide levels[337]. Cifarelli *et al* has shown that physiological levels of c-peptide are capable of reducing hyperglycaemia-induced vascular smooth muscle cell (SMC) proliferation, one of the primary lesions in diabetic atherosclerotic disease[338]. Haidet *et al* has also shown that addition of c-peptide to monocytic cell lines exposed to high glucose was able to reduce expression of various pro-inflammatory cytokines via a NF-κB-mediated pathway[339].

5.4 Intraluminal thrombus biology

The intraluminal thrombus (ILT) has been implicated in the pathogenesis of AAA and indeed patients with a greater arc of thrombus covering the aneurysm wall have been shown to exhibit more rapid AAA expansion (p<0.005) [340]. It is believed that MMPs within the thrombus are released during thrombus renewal, a process that involves fibrinolysis, therefore thrombi that are more resistant fibrinolysis may slow the rate of AAA expansion[71]. Dunn *et al* have shown that fibrin clots in patients with T2DM were denser, less porous and therefore less susceptible to fibrinolysis[341]. Hyperinsulinaemia, a feature of T2DM, may also exert a protective effect on AAA by preventing renewal of the ILT[342]. By

increasing PAI-1 levels, hyperinsulinaemia inhibits the conversion of plasminogen to plasmin which suppresses fibrinolysis[342] and also reduces MMP expression since plasmin is needed to convert pro-MMP to its active form[317,343].

5.5 Oral hypoglycaemic agents

The 3 classes of oral hypoglycaemic agents that have received the most attention with respect to explaining the protective effect of DM on AAA are the biguanides (in particular metformin), the thiazolidinediones (TZDs) and dipeptidyl peptidase-4 (DPP4) inhibitors (see Table 2.1).

Thompson *et al* analysed data on 1269 patients under AAA surveillance and was the first to report an association between medications used to treat DM and a reduction in AAA growth rates (56% reduction; p=0.003 even after adjusting for confounders). The two classes of anti-diabetic drugs that were particularly associated with a reduction in AAA growth rates in this study were the biguanides (p=0.05) and sulphonylureas (p=0.03) [289]. Fujimura *et al* performed a similar analysis using a much smaller cohort of patients but found that of the 11 classes of medications tested, only metformin demonstrated a significant negative association with AAA growth ($p<0.05$) [344]. The authors went on to perform various mechanistic studies. They found that metformin administration to mice infused with porcine pancreatic elastase resulted in less AAA formation which was associated with medial elastin and SMC preservation, reduced B cell, macrophage, CD4 and CD8 T cell accumulation and reduced mural neovessel concentrations on histological studies[344]. Similar findings were demonstrated by

Vasamsetti *et al* using the ApoE-deficient AngII mouse model[345]. They found that metformin administration was associated with a significant reduction in maximal aortic diameter, incidence of aneurysm formation and aortic wall and MMP-1 and MMP-9 levels. Using a monocytic cell line they also showed metformin reduced differentiation of monocytes into macrophages[345].

Hsu *et al* performed a contemporary analysis of Taiwan's National Heath Insurance database (>1.2 million patients) to identify 4468 patients with aortic aneurysms and 4468 matched controls[346]. They found metformin, sulphonylurea and TZD use were associated with a significantly lower risk of developing aortic aneurysms (OR 0.72, 0.82 and 0.82 respectively) and that the effects of metformin and sulphonylurea were dose-responsive ($p<0.001$ for trend with both). No protective effect was found for α-glucosidase inhibitors or DPP4 inhibitors[346]. Most recently, Golledge *et al* studied the effect of metformin, amongst other therapies, on AAA growth rates using 3 distinct patient cohorts. Prescription of metformin, but no other therapy for DM, was associated with a reduced likelihood of median or greater AAA growth in all three patient cohorts (OR range 0.13 – 0.59; $p<0.02$ in all cohorts) [347].

Kristensen *et al* were concerned that metformin prescription might reverse the protective effect of DM on AAA formation and therefore examined the risk of RAAA amongst individuals with and without DM using a nested case-control analysis of the national Danish registry data[348]. They found that metformin prescriptions were not associated with an increased risk of RAAA and may even

protect against RAAA although this did not reach statistical significance after correcting for confounders (OR 0.84; 95% CI 0.61 – 1.17) [348].

TZDs increase insulin sensitivity via activation of PPAR-Ɣ. Jones *et al* have shown pre- or post-treatment with rosiglitazone in ApoE-deficient mice prevented Ang II-mediated aneurysm rupture and reduced maximal aortic diameter. Pre-treatment was also associated with a reduction in expression of the inflammatory mediators TNF-α and IL-6[349]. The same group went onto demonstrate that the actions of rosiglitazone on aneurysm formation were mediated via a reduction in c-jun N-terminal kinase expression throughout the aorto-iliac tree and toll-like receptor 4 expression selectively in the suprarenal aorta which represents the site of aneurysm formation in the Apo-E deficient AngII model[350]. Using the same experimental mouse model, Golledge *et al* have produced similar reductions in aneurysm formation using another TZD pioglitazone (p=0.01) and the related compound fenofibrate (p=0.001) compared to using a vehicle control. Both compounds were also associated with reduced macrophage infiltration[351]. Cockerill's group suggested that pioglitazone may reduce experimental aneurysm formation via an action on the polycystic kidney disease 1 gene which itself is negatively controlled by early growth response protein-1[352].

The only study to test the effect of TZDs on AAA parameters in humans was performed by Motoki *et al*. This study found that pre-treatment of patients awaiting open AAA repair with 2 weeks of pioglitazone resulted in a significant

reduction in macrophage infiltration, MMP-9 and TNF-α levels in both aortic wall and periaortic fat samples[353].

DPP4 inhibitors work by augmenting the incretin effect. Lu *et al* have shown a positive correlation between the relative intensity of plasma DPP4 and AAA diameter ($p<0.05$) which may be mediated through an action of DPP4 on monocyte-macrophage differentiation[354]. Using the DPP4 inhibitor alogliptin, Bao *et al* were able to attenuate aneurysm formation in a dose-dependent fashion ($p<0.02$) using rodent models of AAA (intraluminal elastase and extraluminal calcium chloride). Furthermore they found that alogliptin treatment was associated with lower tissues levels of MMP-2 ($p<0.001$), MMP-9 ($p<0.001$) and reactive oxygen species ($p<0.0001$) and preservation of mural elastin[354]. Kohashi *et al* performed a similar study using the DPP inhibitor MK0626 in an ApoE-deficient AngII mouse model and found that treatment with MK0626, but not treatment with native incretins GIP-1 or GLP-1, was associated with a 30% reduction in aneurysm formation, significantly less IL-1β expression and an increased ratio of TIMP-2 to MMP-9. MK0626 did not affect aortic IL-6 or TNF-α expression in this study[355].

5.6 Additional mechanisms

Studies that do not fall into any of the biological pathways detailed above but do provide evidence that may aid our understanding of the relationship between DM and AAA are listed here for completeness. A genetic basis for the relationship between DM and AAA seems unlikely following the results of a

recent study using Mendelian Randomisation analyses [356] although post-genomic factors may be implicated. MicroRNAs are small non-coding ribonucleic acid (RNA) molecules that function in RNA silencing and regulating post-transcriptional gene expression. Maegdefessel *et al* reported increased expression of miR-29b in experimental models of AAA. Suppression of miR-29b, which may occur with medications for DM, has been shown to promote a fibrotic response which may therefore limit aneurysm expansion[357,358]. This is plausible since miR-29 is a key microRNA upregulated by hyperglycaemia and has important roles in insulin-stimulated glucose metabolism[359]. However there are no studies that have yet tested the effect of oral hypoglycaemic agents on miR-29 expression.

In addition to the increased arterial wall stiffness caused by cross-linking and calcification in diabetics, Madi *et al* have shown that DM alters the SMC phenotype to one which is more migratory and less proliferative which may also increase vessel stiffness[360]. In their study, saphenous vein SMCs from T2DM patients exhibited a phenotype that promoted vinculin-positive focal adhesions with disrupted actin cytoskeletons and disorganised α-actin networks[360], vinculin being an important focal adhesion protein coupling the ECM to the actin cytoskeleton[71].

Neovascularisation is one of the pathological hallmarks of AAA[361]. However, chronic hyperglycaemia results in microangiopathy and microvessel occlusion, which may explain why Miyama *et al* found reduced levels of adventitial neovascularisation in aortic aneurysm samples from hyperglycemic compared

with euglycaemic mice (12 +/- 8 vs. 20 +/- 6 vessels per high-powered field; p=0.02) [332].

Chapter 6: Glucose Transporters

6.1 Structure, function and classification

Glucose is the primary energy source for all mammalian cells and an important substrate for various biological processes. However due to the size and polar nature of glucose, it is unable to cross the plasma membrane by simple diffusion[362]. Glucose transporters (GLUTs), also known at solute carrier family 2 (SLC2A) proteins, represent a family of transmembrane proteins that facilitate the transport of glucose (and other hexoses) bi-directionally across plasma membranes. They were first described in 1977 in human erythrocytes (GLUT1) [363] however it was not until 1985 when Mueckler and colleagues cloned the 492-amino acid protein that their presence in other mammalian cells was discovered[364].

Fourteen members of the GLUT family have been identified to date, 9 of which were discovered following the sequencing of the human genome[365]. Each GLUT contains 12 transmembrane α-helices with intracellularly sited amino and carboxy terminals. There also exists a long loop connecting transmembrane domains (TMDs) 6 and 7 which resides on the cytosolic side (see Figure 6.1).

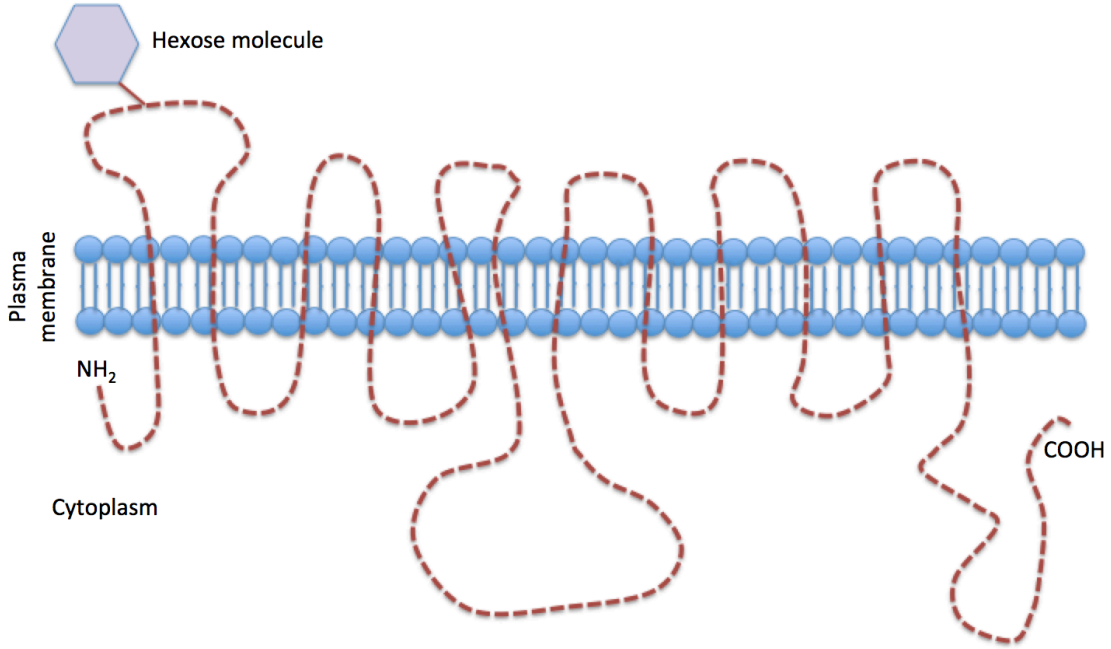


Figure 6.1. Two-dimensional molecular structure of a glucose transporter showing the arrangement of the 12 transmembrane helices within the plasma membrane as well as the binding site for glucose. Adapted from Bryant NJ *et al*[366].

Three-dimensionally, the TMDs combine to form a central aqueous channel through which hexose molecules can cross the plasma membrane. Zeng *et al* suggest that for GLUT1, either TMDs 3, 4, 7, 8 and 11 or TMDs 2, 5, 11, 8 and 7 line the aqueous pore, with the remaining TMDs surrounding these channel-lining TMDs [367].

GLUTs can be divided into 3 subclasses based on protein sequence, structural similarity and substrate specificity (see Table 6.1). Class I includes the originally identified GLUT1, GLUT2, GLUT3 and GLUT4 as well as the newly identified

GLUT14 which appears to have resulted from gene duplication of GLUT3 with which it shares significant sequence homology [368]. Class II includes the originally identified GLUT5 together with GLUT7, GLUT9 and GLUT11, all of which transport fructose in addition to glucose and none of which are capable of transporting galactose [369,370]. In addition, GLUT9 also seems to be capable of transporting urate [371]. Class III includes GLUT6, GLUT8, GLUT10, GLUT12 and GLUT13 although GLUT13 transports myoinositol rather than glucose [369,372]. Manolescu *et al* suggest the glycosylation site resides between TMD1 and TMD2 for Class I and II GLUTs but between TMD9 and TMD 10 for class III GLUTs [370].

Table 6.1. Characteristics of the different glucose transporters and diseases states related to each transporter. Adapted from Purcell *et al*[370].

GLUT	Tissue distribution	Substrate specificity	Pathophysiology
Class I			
GLUT1	Erythrocytes but ubiquitous	Glucose, galactose, DEHA	Upregulated in cancer. Autosomal dominant haplo-insufficiency mutations lead to seizures, delayed development, microcephaly. Upregulated GLUT1 may be related to HIV infection in T cells [369].
GLUT2	Intestine, kidney, liver, pancreatic β-cell	Glucose, fructose, galactose, glucosamine	Mutations in SLC2A2 gene cause Fanconi-Bikel syndrome (glycogen storage disease) characterised by poor blood glucose control [369].
GLUT3 (GLUT14)	Neuronal tissue, leucocytes, (testis)	Glucose, galactose, DEHA	No known specific disease states.
GLUT4	Adipose tissue, skeletal and cardiac muscle	Glucose, DEHA	Dysregulated in insulin resistance, obesity and T2DM. GLUT4 expression increased in liver cirrhosis [369,373].
Class II			
GLUT5	Intestine, kidney, muscle, sperm, brain	Fructose	GLUT5 expression levels altered in DM, hypertension, obesity and breast cancer [374].
GLUT7	Intestine (colon)	Glucose, fructose	Maybe involved in development type 1d glycogen storage disease [375].
GLUT9	Kidney, liver, placenta, colon	Glucose, fructose, urate	Polymorphisms in SLC2A9 gene responsible for development hyperuricaemia which is associated with gout and hypertension [365].
GLUT11	Muscle, heart, adipose, placenta, kidney, pancreas	Glucose, fructose	GLUT11 could be important for cell proliferation in multiple myeloma [376].
Class III			
GLUT6	Brain, spleen, leucocytes	Glucose	No known specific disease states.
GLUT8	Testis, brain, liver, adipose, spleen	Glucose, (fructose)	GLUT8 could be important for fructose-induced non-alcoholic fatty liver disease and cell proliferation in multiple myeloma [376,377].
GLUT10	Heart, Lung	Glucose	Mutations in SLC2A10 gene responsible for development of arterial tortuosity syndrome [378-381].
GLUT12	Insulin-sensitive tissues	Glucose, fructose, galactose	Expression levels increased in Alzheimer's disease. May also be involved in development of T2DM [382,383].

6.2 Mechanism of action

The mechanism by which glucose is transported across the plasma membrane is based on the Michaelis-Menten model of enzyme kinetics which states that the rate of glucose transport is defined by the initial glucose concentration, the binding affinity of the transporter for glucose and that there exists a maximum rate of transport [384].

LeFevre was the first to postulate that a specific component within the lipid bilayer was required for glucose transport[385]. However, it was not until 1952 when Widdas proposed the 'simple carrier' model based on his experiments using sheep placenta that a model to explain glucose flux across the plasma membrane existed[386]. This model stated that there were 4 transport stages: 1) glucose binds to an empty carrier site of one side of the membrane (*cis* side), 2) the substrate binding carrier site translocates to the other side of the membrane (*trans* side), 3) the carrier site releases the glucose molecule on the *trans* side and 4) the empty carrier site switches back to the *cis* side [386]. Various other models have also been proposed and therefore the exact mechanism by which glucose travels across the cell membrane is still under debate [369]. Importantly, the transport is facilitative (i.e. energy-independent) and bi-directional however a phenomenon known as trans-acceleration has also been observed with GLUT-dependent glucose transport which describes the ability of glucose to travel against its concentration gradient from the *cis* side to the *trans* side of the membrane and vice versa [387].

6.3 Glucose transporters: the link between diabetes mellitus and abdominal aortic aneurysms?

A review of the literature suggests that GLUTs are implicated in the pathophysiology of diabetes mellitus, its complications and aneurysmal disease making them suitably placed to regulate the protective effect of DM on AAA. There is evidence for a role for GLUT1, GLUT4 and GLUT12 in the pathogenesis of DM, GLUT1, GLUT2, GLUT3 and GLUT5 in the pathogenesis of diabetic microvascular complications, and GLUT1, GLUT3, GLUT4 and GLUT10 in the pathogenesis of aortic aneurysms.

6.3.1 Glucose transporters and diabetes mellitus

Polymorphisms in the GLUT1 gene have been shown to be associated with the presence of T2DM in patients of Malaysian origin[388]. In contrast, GLUT4 mutations are relatively rare in patients with T2DM[389]. Partial GLUT4 inhibition, via single allele knockdown of the SLC2A4 gene, in mice is associated with the onset of insulin resistance and diabetes mellitus[390]. Complete GLUT4 knockout, via a Cre/Lox-dependent mechanism, results in severe insulin resistance, glucose intolerance and fasting hyperglycaemia[391]. Conversely, overexpression of GLUT4 in adipose tissue or skeletal muscle decreases insulin resistance and hyperglycaemia in both normal and diabetic mice[392-396]. Clinically, a decrease in adipose-derived GLUT4 levels is seen in individuals with obesity, insulin resistance and T2DM[373] whereas exercise training, which is known to improve insulin sensitivity, is associated with an increase in skeletal muscle-derived GLUT4 levels in both non-diabetic and diabetic cohorts[397]. Clinical evidence to support a role for

GLUT12 is lacking however overexpression of GLUT12 in murine models is associated with a 70% improvement in insulin sensitivity in adipose tissue and skeletal muscle when tested using a hyperinsulinaemic-euglycaemic clamp[398]. Importantly, these actions of GLUT12 were found to be independent of any effect on GLUT4.

6.3.2 Glucose transporters and diabetic complications

Adeshara *et al* has shown GLUT1 expression to be 2.0-fold and 2.6-fold higher in erythrocytes from T2 diabetic patients (n=160) with and without vascular complications (20% retinopathy, 13% neuropathy, 40% nephropathy, 27% cardiovascular disease) compared to non-diabetic controls (n=60) [399]. Studies focusing on diabetic nephropathy also suggest a role for GLUT1. Diabetic nephropathy is characterised by basement membrane thickening and excessive ECM deposition which is mediated via TGF-β1[400,401]. Inoki *et al* have shown that TGF-β1-mediated ECM accumulation occurs by up-regulating expression of GLUT1 mRNA with an associated increase in 2-deoxy-D-glucose uptake in cultured mesangial cells. Furthermore addition of a TGF-β-neutralising antibody inhibits this increase in glucose uptake[402]. These observations support earlier work from Heilig *et al* which showed that overexpression of GLUT1 in rat mesangial cells cultured at normoglycaemia led to an increase in basal glucose transport and augmented fibronectin and collagen expression mimicking the effect of hyperglycaemia[403]. The same group also showed that GLUT1 antisense mRNA was able to protect mesangial cells from glucose-induced fibronectin deposition [404].

Chichger *et al* have also found up-regulation of GLUT2 expression and activity in the proximal convoluted tubule of type 2 diabetic rats as well as non-diabetic insulin-resistant rats[405]. The effect of GLUTs in the pathophysiology of diabetic microvascular complications seems to be tissue-specific. In a study of type 1 diabetics, Hodgkinson *et al* found that polymorphisms in the regions surrounding the SLC2A1 gene, encoding for GLUT1, were associated with an increased odds of concomitant diabetic nephropathy but not retinopathy (54.5% vs. 2.7%; $p<0.0001$) [406].

Looking at studies examining the role of GLUTs in diabetic retinopathy, Knott *et al* found a reduction in GLUT1 and GLUT3 mRNA expression in cultured human retinal endothelial cells at 25mmol/L of hyperglycaemia[407,408]. Interestingly a similar effect was not seen at 15mmol/L of hyperglycaemia suggesting that the degree of hyperglycaemia is also important[408]. Kim *et al* performed similar studies using retinal pigment epithelial cells and found hyperglycaemia to decrease glucose uptake in a dose-dependent manner which was associated with a decrease in GLUT1 protein expression. This effect was found to be dependent on PKC-mediated activation of Akt[409]. More recently, Lu *et al* has found that pharmacological inhibition of GLUT1 in diabetic mice was associated with reduced glucose uptake, retinal glucose levels and early biomarkers of diabetic retinopathy. The authors concluded that GLUT1 be may be a promising therapeutic target for the prevention of diabetic retinopathy[410].

Only 1 study has examined the role of GLUTs in the pathophysiology of diabetic neuropathy. Asada *et al* compared the sciatic nerve expression of GLUT5 in normal

and STZ-induced diabetic rats and found increased expression of GLUT5 in Schwann cells and axons from diabetic rats at 5 weeks after STZ injection which normalised following 2 weeks of insulin therapy. The authors concluded that overexpression of GLUT5 might be a trigger for the development of diabetic neuropathy[411].

Although not technically a microvascular complication of DM, hypoglycaemia is a well-recognised metabolic complication of DM, especially amongst type 1 diabetics[412]. Sato *et al* recently studied the mechanisms underlying the development of exercise-induced hypoglycaemia using STZ-induced diabetic rats[413]. They observed a significant decrease in blood glucose levels in diabetic rats made to exercise, but not in control rats made to exercise or sedentary diabetic rats. These changes were associated with a delay in counter-regulatory hormone responses (glucagon, adrenalin and noradrenaline secretion) but also prolonged enhancement of GLUT4 translocation in the exercised diabetic rat cohort[413].

6.3.3 Glucose transporters and abdominal aortic aneurysms

A number of studies suggest that GLUTs are involved in aneurysmogenesis however only one study has examined their role in explaining the negative relationship between DM and AAA[414].

Low *et al* have shown that exposure of cultured rat aortic SMCs (AoSMCs) to AngII, which experimentally is capable of inducing AAA formation and is significantly associated with AAA in candidate gene studies, increases GLUT1 mRNA expression

4-fold and glucose uptake 3-fold[415]. Bown *et al* have discovered that AAA is associated with polymorphisms in the gene coding for LRP1 and indeed SMC-specific LRP1 inactivation is associated with elastic layer disruption and aneurysm formation experimentally[416]. Jedrychowski *et al* have identified LRP1 as an essential component of GLUT4 storage vesicles, LRP1 interacting with the insulin-signaling pathway via AS160 (Akt substrate of 160kDA). Depletion of LRP1 reduces GLUT4 expression and insulin-stimulated glucose uptake in 3T3-L1 adipocytes and adipose-specific LRP1 knockout decreases GLUT4 expression in mice[417]. Animal and human studies have also confirmed that mutations in the SLC2A10 gene, coding for the protein GLUT10, are responsible for the development of arterial tortuosity syndrome, an autosomal recessive condition characterised by abnormal elastogenesis which results in tortuosity, elongation and aneurysm formation in medium-to-large sized arteries[378,379]. To date, 21 mutations in the SLC2A10 gene have been found[418].

18F-fluorodeoxyglucose (18F-FDG) positron emission tomography (PET) imaging assesses glucose uptake using a fluorescence-labelled glucose tracer (18F) and is thus a surrogate marker of GLUT activity. Increased FDG-PET uptake has been found in a number of cardiovascular diseases, such as transient ischemic attack and stroke[419], however for AAA the literature is divided. Sakalihasan *et al* were the first to utilize 18F-FGD-PET imaging in AAA patients and they found increase uptake to be associated with increased risk of AAA expansion and a requirement for more urgent repair[420]. Three subsequent case-control studies have shown increased 18F-FDG-PET uptake in AAA patients compared with controls[421-423] however four case-control studies have shown no difference or even decreased 18F-FDG-PET

uptake in AAA patients[424-427]. Two recent systematic reviews in this area have concluded that further work is still needed to define the role of 18F-FDG-PET imaging in predicting AAA growth and rupture risk[428,429].

Only 2 studies have measured GLUT expression and/or activity in patients with AAA. Tsuruda *et al* compared 18F-FDG-PET uptake in 17 patients awaiting AAA repair with 23 age-matched controls and also correlated aortic tissue expression of GLUTs 1 to 4 from AAA patients with their corresponding 18F-FDG-PET scores (SUVmax) [430]. They reported significantly higher glucose 18F-FDG-PET uptake in the abdominal and descending thoracic aortas of patients with AAA compared with controls ($p<0.01$ at maximal infrarenal aortic diameter, $p<0.05$ just below renal arteries and $p<0.05$ in descending thoracic aorta). The authors found significant positive correlations between SUVmax and GLUT1 ($n=35$; $r=0.71$; $p<0.0001$), SUVmax and GLUT3 ($n=35$; $r=0.42$; $p=0.013$) and aortic tissue MMP-9 expression and GLUT3 expression ($r=0.38$; $p=0.024$). No significant correlations were found for GLUT2 or GLUT4. The use of glycolysis inhibitor 2-deoxyglucose was also shown to attenuate aneurysm formation in 2 mouse models of AAA which led the authors to conclude that enhanced glycolytic activity within the aortic wall contributes to the pathogenesis of aneurysm development. More recently, Kurihara *et al* have investigated the effect of hyperglycaemia on GLUT1 expression in murine macrophages cell lines[414]. They found that pre-treatment of macrophages with 15.5mmol/L of glucose for 7 days abrogated the soluble receptor activator of NF-κB ligand (sRANKL)-induced increase in glucose uptake as well as the sRANKL-induced GLUT1 membrane translocation when compared to macrophages pre-treated with normoglycaemia (5.5mmol/L). Furthermore,

hyperglycaemia pre-treatment was associated with a decrease in MMP-9 and insulin receptor expression in sRANKL-activated macrophages. Using the GLUT inhibitors apigenin (a flavonoid) and cytochalasin B, the authors demonstrated dose-dependent (0.1 μmol/L - 10μmol/L) suppression of sRANKL-induced macrophage activation and glucose uptake[414]. These data support the hypothesis that GLUTs may play a role in regulating the protective action of DM on AAA formation.

Aims, Hypotheses & Design

Chapter 7: Thesis Aims

Abdominal aortic aneurysm (AAA) represents a significant health problem. It accounted for more than 5200 deaths in 2016 in England and Wales alone, mainly due to aneurysm rupture[20]. The reason why some individuals develop AAA whilst others do not is due to a complex interplay between genetic and environmental factors. Risk factors for the development and growth of AAA are similar to those for atherosclerosis however individuals with diabetes mellitus (DM), a condition defined by elevated blood glucose levels, seem to be relatively protected from AAA. The results of our own meta-analysis examining the epidemiological relationship between DM and AAA suggest that individuals with DM are nearly 20% less likely to have an abdominal aortic aneurysm and more than 50% less likely to develop one in the future, compared to individuals without DM.

The scientific basis for this relationship is incompletely understood. Improving our understanding of the mechanisms involved is important since this could lead to the development of novel drug therapies aimed at reducing AAA growth. The need for such drug therapies is paramount since the only effective treatment for AAA available at the moment involves an operation. Furthermore the introduction of the National Health Service AAA screening programme, which achieved national coverage in 2013, has led to the detection of a greater number of small AAA (<5.5cm) that are not yet suitable for operation[157]. This provides clinicians with a window of opportunity in which to slow or even halt aneurysm growth.

There is evidence from the literature that glucose transporters (GLUTs), a family of proteins responsible for the movement of glucose molecules across plasma membranes, are important in the pathophysiology of diabetes mellitus and its associated complications such as diabetic retinopathy, nephropathy and neuropathy. I postulated that the protective effect of DM on AAA development might also be a 'complication' of having DM, albeit a favourable one, and that the same group of proteins (GLUTs) might also be involved in regulating this protective effect. In addition I discovered a small but growing body of evidence linking dysfunction in glucose transporters with the development of arterial aneurysms.

The aims of this thesis were two-fold. Firstly to characterise the gene and protein expression profiles of GLUTs in aortic tissue samples and aortic smooth muscle cells (AoSMCs) obtained from patients with and without AAA. This will seek to confirm the importance of GLUTs in the pathophysiology of AAA. AoSMCs were selected as a model in which to study GLUT involvement because these cells are known to express GLUTs[431] and have previously been shown to be key orchestrators of many of the processes involved in AAA development[116]. Secondly to investigate the metabolic effects of DM on the expression and activity of GLUTs as well as on the expression of enzymes involved in extracellular matrix degradation (cathepsins and tissue inhibitors of matrix metalloproteinases (TIMPs)) using aortic smooth muscle cells cultured from patients with and without AAA. This will seek to determine whether GLUTs

could function as mediators of the protective effect of DM on AAA.

Chapter 8: Hypotheses

The study hypotheses were two-fold:

1. The expression and/or activity of glucose transporters will be up-regulated by the AAA phenotype.
2. The expression and/or activity of glucose transporters will be down-regulated by the diabetic phenotype.

The null hypotheses are therefore:

1. The expression and/or activity of glucose transporters will NOT be up-regulated by the AAA phenotype.
2. The expression and/or activity of glucose transporters will NOT be down-regulated by the diabetic phenotype.

Chapter 9: Study Design

The aims of this thesis will be delivered using a two-phase study design (see Figure 9.1), each phase addressing each of the main aims. Phase 1 of the study will use whole aortic tissue specimens (WATS) as well as cultured aortic smooth muscle cells (AoSMCs) from patients with and without AAA (cadaveric controls) to compare GLUT expression and activity. Expression will be quantified at both the mRNA and protein level. Phase 2 of the study will use cultured AoSMCs only and is designed to assess the metabolic effects of diabetes mellitus, namely hyperglycaemia, on GLUT expression and activity as well as on the expression of various factors known to regulate extracellular matrix degradation/synthesis.

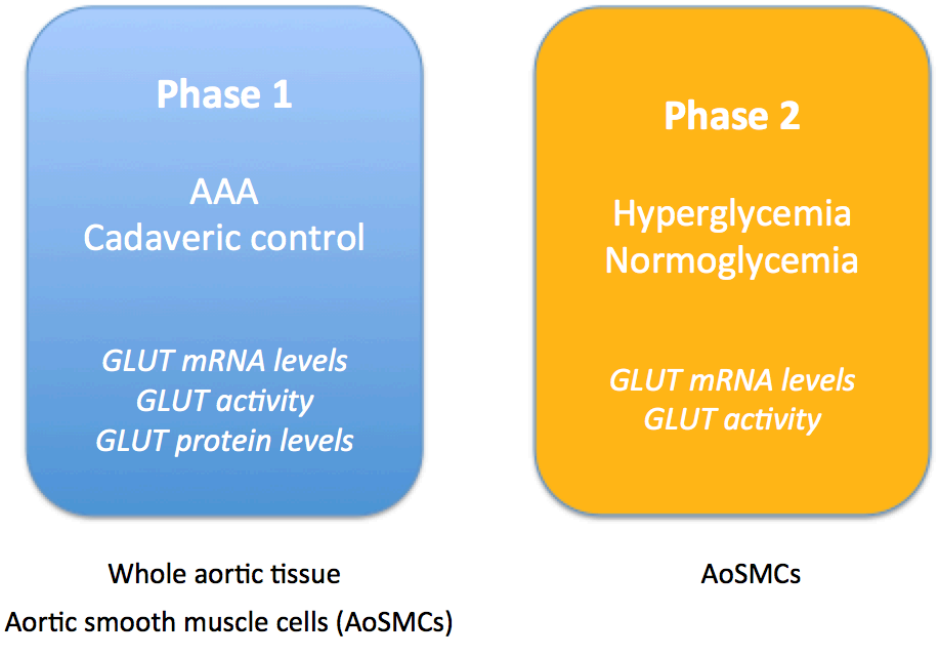


Figure 9.1. Diagrammatic summary of the two-phase study design outlining the experimental models to be used and comparisons to be made in each phase.

Sample sizes

Formal power calculations for each experiment could not be performed since there were no previous similar studies in the literature. Therefore all experiments were treated as pilot studies and used as many samples as available.

Experimental Work

Chapter 10: Governance and Tissue preparation

10.1 Approvals

Ethical approval to allow the collection of aortic tissues from patients undergoing open AAA repair at University Hospitals of Leicester (UHL) NHS Trust was already in place (Leicestershire Northamptonshire and Rutland Research Ethics Committee reference 6819). An ethics application to allow the collection of control aortic tissues from cadaveric organ donors was made in May 2012 and approval was obtained on the 1st November 2012 (Research Ethics Committee reference 12/EM/0355). Approval for the use of cadaveric tissues to study AAA pathophysiology was also obtained from NHS Blood and Transplant. Approval from the Research and Development department at UHL NHS Trust to allow the study to begin locally was obtained on the 17th December 2012 (UHL 11202) and the first control aortic specimen was successfully collected on the 2nd February 2013.

10.2 Consent process

Patients undergoing elective open AAA repair were consented the evening before surgery by a member of the research team. This was mainly by myself however occasionally this was by one of my research fellow colleagues (Mr Philip Stather, Mr David Sidloff or Mr Badri Vijaynagar). In addition to consenting for their tissue to be collected, patients were asked to complete a questionnaire regarding their general medical health (see Appendix 1) and donate a blood sample (used by other members of the research team).

Cadaveric organ donors were not directly consented by members of our research team as the organs were usually obtained from hospitals outside of Leicester. Therefore as per our ethics approval, aortic tissue was only obtained from cadaveric organ donors where the organ arriving at the University Hospitals of Leicester NHS Trust was accompanied by a NHS Blood and Transplant consent form agreeing for the tissue to also be used for "NHS approved research". This required a specific box to be ticked on the consent form which was checked by myself. NHS Blood and Transplant consent forms are completed by local transplant coordinators at the hospital where the patient's organs are harvested and a copy of this consent form accompanies the donated organ to the recipient hospital.

10.3 Tissue collection

Whole aortic tissue specimens (WATS) were collected from AAA patients during open surgical repair. WATS were always obtained from the infra-renal aortic sac after the graft had been secured proximally and distally and were always full thickness specimens. WATS were placed in sterile PBS at room temperature and agitated gently by hand to remove blood contaminants. The samples were then transported in phosphate buffered saline (PBS) to the tissue culture lab for immediate sample preparation (<30 minutes from collection to preparation).

Aortic tissue samples from cadaveric organ donors were either obtained from theatre during renal transplantation or from the Leicester University transplant laboratory in cases where the donor kidney was deemed not suitable for in vivo use but still had consent for NHS approved research. Kidneys and their accompanying vascular tissues were always transported in ice-cold organ preservation solution (University of Wisconsin solution or Hyperosmolar Citrate) from recipient to donor hospitals and therefore aortic tissue specimens obtained from these organs were also placed in organ preservation solution rather than PBS. The median cold ischemia time for these tissues was 34 hours (range 5 hours – 100 hours). These tissues were rarely blood stained due to the long times spent in cold solution prior to collection for research. The collection to processing time was longer for cadaveric tissues compared to AAA tissues due to the difficulties in predicting when organ donors might become available as well as the need to transport tissues across different hospital sites. The median collection to processing time was therefore 60 mins (range 15 mins – 24 hours).

10.4 Tissue preparation

WATS from both AAA patients and cadaveric organ donors (control specimens) were processed in an identical manner once collected. Under sterile conditions (using a class II tissue culture hood), specimens were divided into 3 sections and placed into containers pre-filled with RNALater® solution (ThermoFisher Scientific), 10% formalin or Medium 199 (Gibco) with added penicillin and streptomycin (+ P/S) respectively. A fourth section was snap frozen (-80°C) to allow further work to take place if needed in the future.

10.4.1 Preparing tissue for RNA work

Placement of the WATS in RNALater® stabilises and protects the tissue against RNA degradation by inactivating ribonucleases (RNases). The solution also minimises the need to immediately process the tissues samples or freeze samples in liquid nitrogen. WATS were left in RNALater® overnight at 4°C which was then drained off the following day as per the manufacturer instructions prior to storage of tissues at -80°C until needed (see Chapter 11).

10.4.2 Preparing tissue for histological work

WATS in 10% formalin were given 24 hours to fix and then underwent preparation by the pathology laboratory in the University of Leicester Core Biotechnology Services department to create sections of aortic tissue that could be used for immunohistochemical studies. Briefly, samples are prepared using the Leica ASP3000 automated vacuum tissue processor (see Appendix 1 for processing schedules), embedded in paraffin wax and sectioned (see Appendix

1). Calcified samples (determined by myself at the time of dividing the WATS into 3 pieces) also underwent a process of decalcification prior to sectioning (see Appendix 1). The WATS sections were then stored at room temperature until used for immunohistochemical studies examining the protein expression of GLUTs in WATS (see Chapter 12).

10.4.3 Preparing tissue for cell culture work

Medium 199 contains components such as adenine, adenosine, hypoxanthine, thymine and additional vitamins but does not contain any growth factors, proteins or lipids. WATS were placed in medium 199 + P/S as a generic medium in which to store the tissue prior to performing tissue explant culture in more specific culture mediums with the aim of growing aortic smooth muscle cells. (see Chapter 13)

Chapter 11: Gene expression of glucose transporters in whole aortic tissue

11.1 Introduction

Gene expression studies aim to quantify the expression of messenger RNA (mRNA). mRNA is the product of gene transcription and represents the first step towards synthesis of the protein encoded by a specific gene which is known as the central dogma (see Figure 11.1).

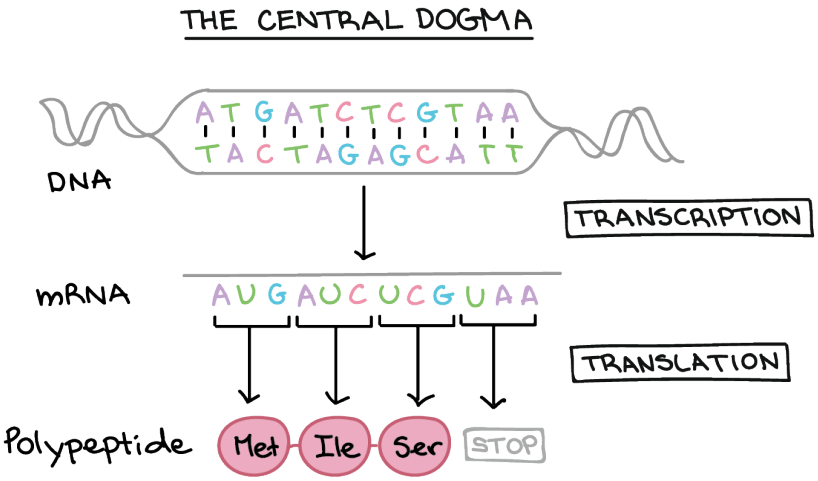


Figure 11.1. The transition from DNA code to protein synthesis known as the central dogma. Adapted from The Khan Academy [432].

The purpose of this experiment was to determine whether differential expression of glucose transporters (GLUTs) mRNA exists in whole aortic tissue samples (WATS) obtained from AAA patients compared with tissues obtained from non-AAA controls. The data obtained from this experiment will allow me to support or reject the first null hypothesis of this thesis which states that "the

expression and/or activity of glucose transporters will not be up-regulated by the AAA phenotype".

The methods used to quantify mRNA expression of GLUTs in WATS included homogenisation of aortic tissue, extraction of total RNA, reverse transcription, primer design and quantitative real-time polymerase chain reaction (qPCR).

11.2 Quality control

RNA samples are susceptible to degradation so precautions to prevent degradation of RNA were used throughout experiments. These included the wearing of clean lab coats, making regular glove changes, using RNase-free water and decontaminating gloves, surfaces and pipettes with RNaseZap® (ThermoFisher Scientific) prior to use.

Gene expression studies rely on sufficient quantities of high-quality RNA. RNA quantity was assessed using a Nanodrop™ spectrophotometer (ThermoFisher Scientific) which measures ultraviolet light absorbance at 260nm and 280nm wavelengths to determine the quantity of RNA and protein respectively. In addition, the 260/280 ratio allows determination of the purity of extracted RNA with ratios between 1.9 to 2.1 generally accepted as sufficiently pure RNA for downstream processing [433]. Samples with extracted RNA 260/280 ratios outside this range were excluded from the study. An example Nanodrop spectrum is shown in Figure 11.2.

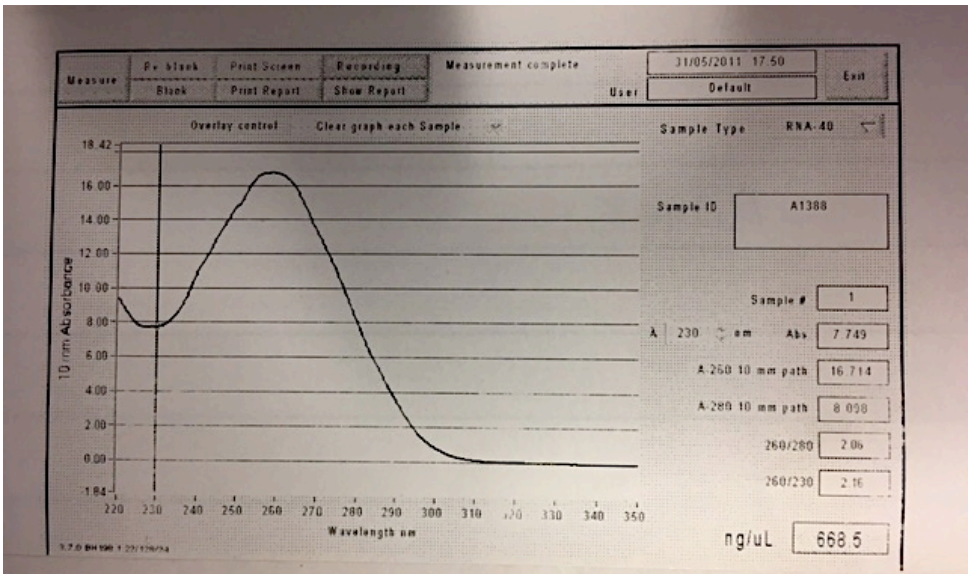


Figure 11.2. Example of a Nanodrop spectrum for an aortic tissue sample with an RNA concentration of 668.5 ng/µL and a 260/280 ratio of 2.06.

For a selection of samples, the quality of RNA was also assessed using an Agilent 2100 bioanalyser (Agilent Technologies), mainly to prove the validity of the techniques. This machine uses 2 different assay systems, electrophoresis and flow cytometry, to provide information about the size, quantity and quality of RNA (and DNA). Together with the Agilent RNA analysis kit (Agilent Technologies), it was possible to calculate the RNA integrity number (RIN), which provides a reliable assessment of RNA quality. RNA integrity is graded on a scale of 1 to 10, with 10 representing the best RNA quality.

11.3 Homogenisation

Tissue homogenisation is the first step for all RNA extraction protocols. This step required multiple attempts at optimisation.

11.3.1 Pestle and Mortar

Homogenisation was initially performed using a pestle and mortar to grind the aortic tissue into a fine powder however this method required the use of large amounts of frozen tissue (often the whole piece) since a significant amount of tissue was lost as it 'jumped' out of the mortar during grinding. Furthermore this method required constant cooling of the tissue in the mortar with liquid nitrogen to prevent RNA degradation at room temperature. This method produced reasonable quantities of RNA (see Table 11.1 for results) but was impractical and inefficient and was therefore abandoned.

Table 11.1. Nanodrop results from homogenising whole aortic tissue using a pestle and mortar (6 samples).

Sample	Concentration (ng/µl)	260/280 ratio
1	10.8	1.95
2	45.7	2.08
3	46.4	1.98
4	23.7	2.00
5	19.0	1.96
6	5.1	2.14

11.3.2 Ultra-Turrax

Homogenisation was next performed using an electric tissue homogeniser called the Ultra-Turrax® (Ika-Werke GmbH). With this method, coarsely crushed WATS were placed into a test tube filled with TRIzol® reagent (Life

Technologies) and the handheld Ultra-Turrax®, with its rotating head, was used to homogenise the tissue further in solution. Initially the tissue was coarsely crushed without a pestle, mortar and liquid nitrogen but later this was used together with the Ultra-Turrax®.

This technique did not result in as much tissue wastage as the pestle and mortar method and could be performed at room temperature once the coarsely crushed tissue was obtained since TRIzol® maintains the integrity of RNA whilst disrupting cells and dissolving cell components. However, with this method it was impossible to ensure no cross-contamination between samples when serially homogenising multiple samples because of difficulties in cleaning the Ultra-Turrax® head (visible particulate matter remaining on the head despite cleaning). This method of homogenisation produced overall better quantities of total RNA than using a pestle and mortar alone, although the quantity was more variable (see Table 11.2 for results), and ultimately this method was also abandoned due to the issues of cross-contamination.

Table 11.2. Nanodrop results from homogenising whole aortic tissue using an Ultra-Turrax®, with or without concomitant use of a pestle, mortar and liquid nitrogen (20 samples).

Sample	Method	Concentration (ng/µl)	260/280 ratio
1	Without pestle, mortar and liquid nitrogen	48.3	1.55
2	Without pestle, mortar and liquid nitrogen	83.1	1.59
3	Without pestle, mortar and liquid nitrogen	82.4	2.02
4	Without pestle, mortar and liquid nitrogen	151.2	1.99
5	With pestle, mortar and liquid nitrogen	59.0	1.96
6	With pestle, mortar and liquid nitrogen	68.7	1.99
7	With pestle, mortar and liquid nitrogen	668.5	2.06
8	With pestle, mortar and liquid nitrogen	148.3	2.08
9	With pestle, mortar and liquid nitrogen	725.4	2.02
10	With pestle, mortar and liquid nitrogen	54.8	2.03
11	With pestle, mortar and liquid nitrogen	189.5	2.04
12	With pestle, mortar and liquid nitrogen	171.6	2.06
13	With pestle, mortar and liquid nitrogen	577.5	2.11
14	With pestle, mortar and liquid nitrogen	78.9	2.02
15	With pestle, mortar and liquid nitrogen	10.8	1.95
16	With pestle, mortar and liquid nitrogen	45.7	2.08
17	With pestle, mortar and liquid nitrogen	46.4	1.98
18	With pestle, mortar and liquid nitrogen	23.7	2.00
19	With pestle, mortar and liquid nitrogen	19.0	1.96
20	With pestle, mortar and liquid nitrogen	5.1	2.15

11.3.3 Precellys bead homogeniser

In an attempt to overcome the issues above, a new bead homogeniser (Precellys system) at the University of Leicester Cardiovascular Research Centre was trialled (see Figure 11.3). This system uses bead technology to homogenise up to 24 samples at a time. This method of homogenisation overcame the issues with cross-contamination seen with the Ultra-Turrax® homogeniser since each sample underwent homogenisation in a single-use container. Furthermore the system allowed homogenisation of tissue in tri-reagent solution therefore reduced degradation and importantly required significantly less frozen tissue

than for either of the two methods described above since homogenisation took place in 'closed system'.

Figure 11.3. The Precellys 24 bead homogeniser.

A number of bead types (glass, ceramic and stainless steel), bead sizes (0.1mm, 0.5mm, 1.4mm, 2.8mm and 6.8mm) and tube sizes (0.5ml, 2ml, 7ml and 15ml) were available for use with this machine therefore advice from the Precellys representative was sought in order to determine the optimum bead type and tube size. The representative recommended ceramic beads in a 2ml system based on previous experience with 'arterial tissues' however was unsure as to whether 1.4mm or 2.8mm beads would be best for whole aortic tissue samples. Therefore an experiment was conducted to determine the optimum bead size (1.4mm or a mixture of 1.4mm and 2.8mm ceramic beads) for homogenisation of WATS (see Table 11.3 for results).

Table 11.3. Nanodrop results from homogenising whole aortic tissue using the Precellys bead homogeniser with different bead sizes (4 samples).

Sample	Bead type	Concentration (ng/µl)	260/280 ratio
1	Ceramic 1.4mm	174.1	2.15
2	Ceramic 1.4mm	155.1	2.16
3	Ceramic 1.4mm	82.2	2.25
4	Ceramic 1.4mm	34.2	2.62
1	Ceramic 1.4mm/2.8mm	0.0	n/a
2	Ceramic 1.4mm/2.8mm	0.0	n/a
3	Ceramic 1.4mm/2.8mm	0.0	n/a
4	Ceramic 1.4mm/2.8mm	0.1	n/a

Based on the results, the 1.4mm ceramic beads were chosen for homogenising WATS since the addition of 2.8mm beads were likely too aggressive and resulted in little or no RNA being extracted.

11.4 RNA Extraction

11.4.1 Optimisation

Initial experiments used a manual method involving TRIzol reagent for extraction of RNA (see Tables 11.1 and 11.2). However with the decision to use the Precellys bead homogeniser, it was decided that the use of a kit to extract RNA would be easier. Four different extraction kits were tested as part of the optimisation experiment. These included the GeneJET® RNA purification kit (ThermoScientific), the RNeasy® mini kit (Qiagen), the miRNeasy® mini kit (Qiagen) and mirVana™ Paris kit (Ambion). The same 3 aortic tissue samples (2 aneurysmal, 1 cadaveric) were used for each kit to enable direct comparison. The results of this optimisation experiment are shown in Table 11.4 and Figure 11.4.

Table 11.4. A comparison of total RNA extraction from 3 whole aortic tissue samples using 4 proprietary kits (ThermoScientific GeneJET® RNA purification kit, the Qiagen RNeasy® micro Kit, the Qiagen miRNeasy® mini kit, and the Ambion miRVana™ Paris kit).

Sample	Kit	Concentration (ng/µl)	260/280 ratio	RIN
1	Qiagen® RNeasy micro kit	17.59	1.53	N/A
2	Qiagen® RNeasy micro kit	7.095	1.79	N/A
3	Qiagen® RNeasy micro kit	30.83	2.17	7.6
1	ThermoScientific® GeneJET RNA purification kit	3.978	6.71	N/A
2	ThermoScientific® GeneJET RNA purification kit	15.95	2.26	3.2
3	ThermoScientific® GeneJET RNA purification kit	25.79	2.21	N/A
1	Qiagen® miRNeasy mini kit	113.8	2.07	6.2
2	Qiagen® miRNeasy mini kit	194.0	2.08	5.0
3	Qiagen® miRNeasy mini kit	367.7	1.96	5.0
1	Ambion™ miRVana Paris kit	34.21	2.62	N/A
2	Ambion™ miRVana Paris kit	82.21	2.25	1.8
3	Ambion™ miRVana Paris kit	155.1	2.16	N/A

RIN = RNA integrity number

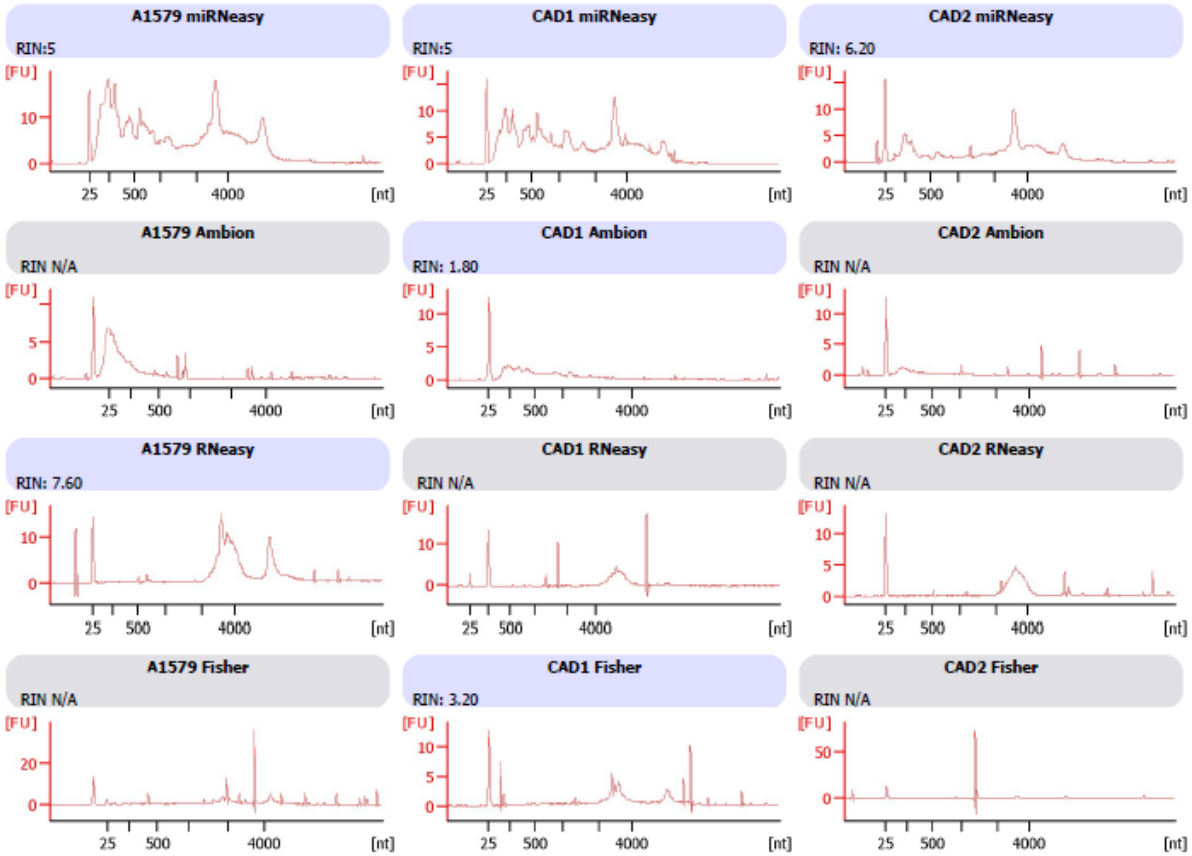


Figure 11.4. Electropherogram of RNA integrity using Agilent RNA 6000 Nano Kit on the 2100 Bioanalyser. Comparison of 4 different RNA extraction kits in 3 patients (ThermoScientific GeneJET® RNA purification kit, the Qiagen RNeasy® micro Kit, the Qiagen miRNeasy® mini kit, and the Ambion miRVana™ Paris kit). Nt = fragment size in nucleotides. FU = fluorescence units.

The results show that the mean RNA concentration was highest when using the Qiagen miRNeasy® mini kit. The RIN was also consistently highest using the Qiagen miRNeasy® mini kit. In addition the 260/280 ratio ranged from 1.96 to 2.08 when using the Qiagen miRNeasy® mini kit therefore this kit was chosen and taken forward for further optimisation.

Although the Qiagen miRNeasy® mini kit yielded the best results, in view of the lower RIN values compared to those achieved following RNA extraction from blood (results from colleague; 17 blood samples, average RIN value 8.07, range 6.9 to 8.8), it was decided that DNA digestion should be attempted to try and improve the RNA quality.

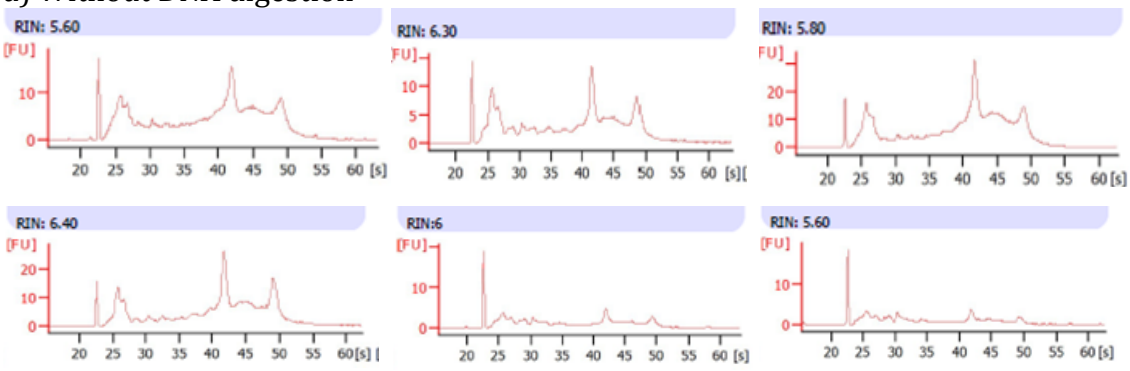
DNase (Qiagen) was initially added to the eluted RNA, however this did not improve the RIN value and only diluted the samples (sample 1 - No RIN, sample 2 RIN - 1.7, sample 3 - RIN 2.2). Therefore a further experiment comparing 6 aortic tissue samples (3 AAA, 3 cadaveric) with and without DNase digestion performed within the RNA extraction according to the Qiagen miRNeasy® mini kit protocol was performed. The results of this experiment are shown in Table 11.5 and Figure 11.5.

Table 11.5. Results from total RNA extraction using the Qiagen miRNeasy® mini kit, with and without DNA digestion.

Sample	With or without DNA digestion	Concentration (ng/ul)	260/280 ratio	RIN
1	Without	285	2.08	5.6
2	Without	84.7	1.68	6.3
3	Without	288.3	1.99	5.8
4	Without	246.7	2.00	6.4
5	Without	95.4	1.92	6.0
6	Without	86.7	1.91	5.6
1	With	166.6	2.01	6.4
2	With	93.9	2.04	6.6
3	With	455.4	1.97	6.0
4	With	173.5	2.04	6.7
5	With	98.5	2.04	8.0
6	With	158.3	1.88	6.8

RIN = RNA integrity number.

a) Without DNA digestion



b) With DNA digestion

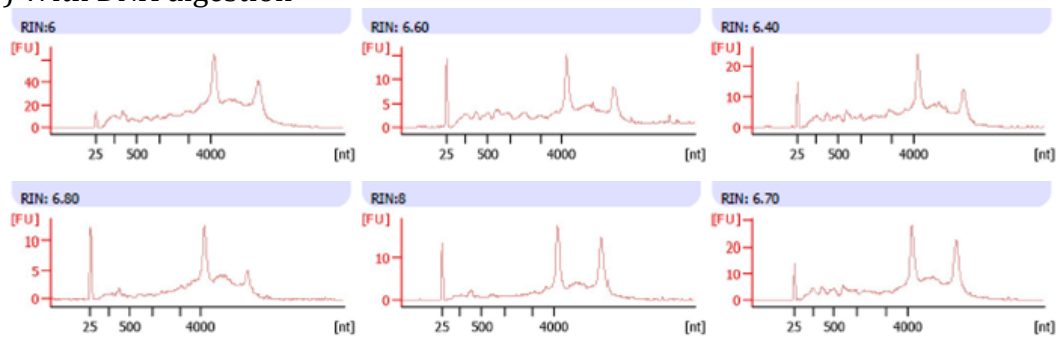


Figure 11.5. Electropherogram of RNA integrity using Agilent RNA 6000 Nano Kit on the 2100 Bioanalyser for total RNA extracted from samples using Qiagen miRNeasy® mini kit without DNA digestion (a) and with DNA digestion (b). Nt = fragment size in nucleotides. FU = fluorescence units.

The results show a marginal but significant improvement in RIN value with DNase digestion (mean RIN without DNA digestion 5.95, mean RIN with DNA digestion 6.75; p=0.02 Mann-Whitney U test).

Based on these optimisation experiments, the Qiagen miRNeasy® mini kit with a DNase digestion step incorporated into the extraction protocol were chosen a the final methods for extracting RNA from WATS and are detailed in the next section. The methods for the other kits used in the optimisation experiments are detailed in Appendix 1.

11.4.2 Final extraction methods used

50mg of full thickness aortic tissue was homogenised using the Precellys tissue homogeniser with 1.4mm ceramic beads in 700µL of QIAzol lysis buffer for 10 seconds twice with a 10 second pause in between, followed by 15 seconds at 6500rpm. The sample was left at room temperature for 5 minutes to promote dissociation of nucleoprotein complexes. 140µL chloroform was then added to the sample, vortexed for 15 seconds and left at room temperature for 3 minutes. The sample was then centrifuged for 15 minutes at 12,000g at 4°C. The upper aqueous phase of the sample was then transferred to a new 1.5ml processing tube and 1.5x the volume of the sample of 100% ethanol was added and mixed using a pipette. The sample was then placed into the miRNeasy® mini spin column in 700µL aliquots and centrifuged at 8,000g for 15 seconds. 350µL of buffer RWT was added to the spin column and centrifuged at 8,000g for 15 seconds. 80µl of DNase was then added to the spin column and allowed to work for 30 minutes. A further 350µl of buffer RWT was then added to the spin column and centrifuged at 8,000g for 15 seconds. 500µL of buffer RPE was then added to the spin column and centrifuged at 8,000g for 15 seconds. 500µL of buffer RPE was then added to the spin column and centrifuged at 8,000g for 2 minutes. The spin column was then transferred to a new 2ml processing tube and centrifuged at 8,000g for 1 minute to dry the membrane. The spin column was then transferred to a new 1.5ml collection tube and 30µL of RNase free water was added directly to the spin column membrane. The sample was then centrifuged at 8,000g for 1 minute and the elute immediately cooled on ice prior

to quantifying using a NanoDrop™ 8000 Spectrophotometer (ThermoScientific) and then storing at -80°C.

11.5 Quantifying RNA expression

RNA expression was quantified by way of polymerase chain reaction (PCR). This was performed using the two-step method where the RNA was first reverse transcribed into complementary DNA (cDNA) in one tube before being amplified in another tube and quantified using a real-time PCR. The two-step method was chosen so that primers did not have to be sequence specific.

11.5.1 Reverse transcription

Methods

cDNA synthesis was performed using the Applied Biosystems High-Capacity cDNA Reverse Transcription kit. A 2x mastermix consisting of 2µl 10x RT buffer, 0.8µl 25x dNTP mix, 2ul 10x RT random primers, 1µl Multiscribe reverese transcriptase and 4.2µl nuclease-free water per reaction was made up. To each well of a 96-well plate, 10ul of 2x mastermix and 10µl of RNA (500ng of extracted RNA in 10µl of nuclease-free water) per reaction was then added and mixed together by pipetting up and down. The 96-well plate was then sealed with an optically clear adhesive film and centrifuged at 1,500 rpm for 1min to remove any air bubbles. The plate was then transferred to a G-Storm GS1 thermal cycler programmed to run at 25°C for 10mins followed by 37°C for 120mins.

Calculating volumes

A Microsoft Excel spreadsheet was created to calculate the volumes of eluted RNA needed to reverse transcribe 1500ng of eluted RNA from each patient sample, 1500ng representing 3 x 500ng reactions as per the Applied Biosystems High-Capacity cDNA reverse transcription kit. Where there was not a sufficient volume of eluted RNA to reverse transcribe 1500ng, either 1000ng or 500ng was reverse transcribed. In addition the Excel spreadsheet was used to determine the volumes of other transcription kit reagents needed which was dependent on the number of samples being reversed transcribed in one go (see Figure 11.6).

	A	B	D	O	P	Q	R	S	T	U	V	W	X	Y	Z	AA	AB	AC	AD
1	Well	Sample ID	Conc.																
2	Whole tissue			Reaction volume	ng of RNA	RNA volume that contains 1500ng	H2O volume	# of reactions	Double check 1/2 volume	Total # of reactions	Total # of reactions + 2	Total volume of 2x mastermix (µl)	2 µl of 10x RT buffer	0.8 µl of 25x dNTP mix	2 µl of 10x random primer	1 µl of multiscribe RT	4.2 µl of RNase free water	Double check 1/2 volume	
3	A1	CAD12	164.2	60	1500	9.14	20.86479903	3	30.00	24	26	520	52	20.8	52	26	109.2	260	
4	B1	CAD13	77.67	60	1500	19.31	10.68752414	3	30.00									520	
5	C1	CAD14	181.8	60	1500	8.25	21.74917492	3	30.00										
6	D1	CAD19	181.4	60	1500	8.27	21.73098126	3	30.00										
7	E1	CAD21	127.1	60	1500	11.80	18.19826908	3	30.00										
8	F1	CAD23	46.01	60	1500	32.60	-2.601608346	3	30.00	it's ok, use 30 ul									
9	G1	CAD25	179.9	60	1500	8.34	21.66203446	3	30.00										
10	H1	CAD27	124	60	1500	12.10	17.90322581	3	30.00										
11	A2	C1340	151.2	60	1500	9.92	20.07936508	3	30.00										
12	B2	C1341	392.6	60	1500	3.82	26.17931737	3	30.00										
13	C2	C1298	156.7	60	1500	9.57	20.4275686	3	30.00										
14	D2	A1580	7.198	60	1500	208.39	-178.3912198	3	30.00										
15	E2	A1505	283	60	1500	5.30	24.69964664	3	30.00										
16	F2	A1608	386.7	60	1500	3.88	26.12102405	3	30.00										
17	G2	A1665	297.3	60	1500	5.05	24.95459132	3	30.00										
18	H2	A1557	125	60	1500	12.00	18	3	30.00										
19	A3	A1670	289.5	60	1500	5.18	24.81865285	3	30.00										
20	B3	A1636	155.7	60	1500	9.63	20.36608863	3	30.00										
21	C3	A1449	54.24	60	1500	27.65	2.345132743	3	30.00										
22	D3	A1527	218.7	60	1500	6.86	23.14128944	3	30.00										
23	E3	A1579	367.8	60	1500	4.08	25.92169657	3	30.00										
24	F3	A1570	689.6	60	1500	2.18	27.82482599	3	30.00										
25	G3	NGH1	308.5	60	1500	4.86	25.13776337	3	30.00										
26	H3	NGH2	194.8	60	1500	7.70	22.29979466	3	30.00										
27																			
28																			
29																			
30																			
31																			
32																			
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41																			
42																			
43																			
44																			

Figure 11.6. An example of the Excel spreadsheet used to calculate the volumes needed to perform a reverse transcription experiment according to the concentration of RNA in each sample and the number of reverse transcription reactions being performed.

11.5.2 Primer design

All primers were custom designed and human specific (see Table 11.6). The UCSC Genome Browser website (http://www.genome.ucsc.edu) was used to obtain the genomic sequence of those genes of interest. An intron-spanning section of each genomic sequence was then entered into the Primer 3 website (http://www.primer3.ut.ee) to design specific complimentary primers. These primer sequences were then 'blated' against the human genome to check correct identification of the gene of interest with 100% specificity. Primers were ordered through the Eurofins MWG Operon website (http://www.eurofinsgenomics.eu). Primers were delivered as lyophilized powders which were then dissolved in nuclease-free water to produce a stock primer concentration of 100pmol/L as per the manufacturer's instructions.

Table 11.6. Sequences of forward and reverse primers designed for gene expression studies.

Gene (Protein) of interest	Forward primer	Reverse primer
ACTB (β-actin)	GCACCCAGCACAATGAAGA	CGATCCACACGGAGTACTTG
B2M (β2-microglobulin)	TGACTTTGTCACAGCCCAAG	GAGCTACCTGTGGAGCAACC
SLC2A1 (GLUT1)	AGGCTTCTCCAACTGGACCT	CCTCGGGTGTCTTGTCACTT
SLC2A2 (GLUT2)	CATGTCAGTGGGACTTGTGC	CTAAAGCAGCAGGACGTGGT
SLC2A3 (GLUT3)	GCCCTGAAAGTCCCAGATTT	TTCATCTCCTGGATGTCTTGG
SLC2A4 (GLUT4)	CTGGACGAGCAACTTCATCA	GAAGGCAGCTGAGATCTGGT
SLC2A5 (GLUT5)	CATCACTGTTGGCATCCTTG	CCGCTTCGTCTTTCTTCTGA
SLC2A6 (GLUT6)	TGCTCATGTCTGAGGTCCTG	GAAGGACTCGATCTGCTCCA
SLC2A7 (GLUT7)	CTGGCTCACCAACTTCATCA	CCCAGCATCAATGGTTTCTT
SLC2A8 (GLUT8)	GTCCTCACCAACTGGCTCAT	CCTCAAAATGGGCTGTGATT
SLC2A9 (GLUT9)	ATCCCGTTCATCTTGACTGG	AAATGCCTGGCTGATTTCTG
SLC2A10 (GLUT10)	CTGGACCTTCCTGCTCTACG	TCCAGAATTTCCAGGCAGAC
SLC2A11 (GLUT11)	GCCTGGGATTTCCCTTTATC	GGATAACCTCCAGGCTCCTC
SLC2A12 (GLUT12)	CAAAGGGATGCTCTTTGGAA	CAGAAGGTGTTGAGGCCATT
SLC2A13 (GLUT13)	CCCTTTGGGCAAGAAGTACA	GAAAAGGAGTCCCACAGCAG
INSR (Insulin Receptor)	GCTTGGCAGAACAGCCTTAC	GGAAGAACGACACCTCTGGA
TIMP1 (TIMP1)	GGATGGACTCTTGCACATCA	TTTTCAGAGCCTTGGAGGAG
TIMP2 (TIMP2)	GATGCACATCACCCTCTGTG	GTGCCCGTTGATGTTCTTCT
CTSB (Cathepsin B)	AGAATGGCACACCCTACTGG	TGCATTTCTACCCCGATCTC
CTSH (Cathepsin H	GGATCCCTTACTGGATCGTG	CAGTGGATCTCCCCACAACT
CTSL (Cathepsin L)	ACATGGATCATGGTGTGCTG	CACAATGGTTTCTCCGGTCT
CTSS (Cathepsin S)	CCCCAAGACCATAGGGAAAT	ACACAAACCAGCCGTTTCA

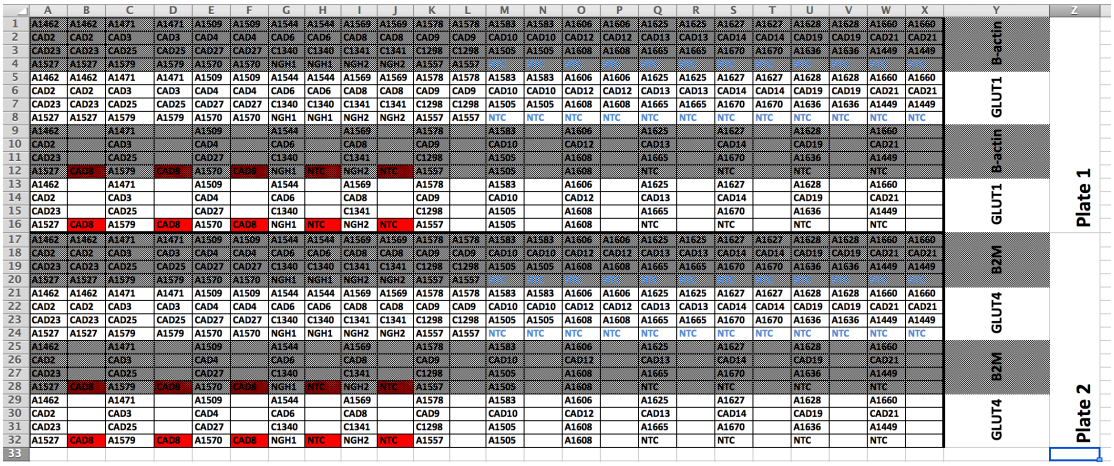
11.5.3 Quantitative real-time polymerase chain reaction

Gene expression was quantified in real-time using SYBR green probe technology (Maxima SYBR Green/ROX qPCR MasterMix) on the LightCycler 480 (Roche) platform (qPCR). SYBR green technology was chosen due to it being easy to use and relatively economical compared to other fluorescence probes such as Taqman. The principles of this technology are that when SYBR green binds to the double-stranded DNA of PCR products, it will emit light upon excitation. The intensity of this fluorescence will increase as the PCR products accumulate.

Methods

Initially, working cDNA solutions were prepared by diluting 20µl of stock cDNA samples in 80µl of nuclease-free water and working primer solutions were prepared by diluting 7.5µl of forward primer and 7.5µl of reverse primer in 985µl of nuclease-free water to yield a final primer concentration of 0.15µmol/L. A mastermix consisting of 2.5µl SYBR green, 1µl primer and 0.5µl nuclease-free water per reaction was made up and reaction numbers were calculated based on samples always being run in triplicate. Each well of a LightCycler 480 384-multiwell plate then received 4µl of mastermix followed by 1µl of cDNA as per a pre-determined layout (see Figure 11.7). Plates were sealed using an optically clear adhesive film specifically designed for the LightCycler 480 system and centrifuged at 1,500 rpm for 1min to remove any air bubbles. Plates were then run on the LightCycler 480 using a two-step thermal cycling program with data acquisition taking place during the annealing/extension step (45 amplification cycles). Melt curve analysis was performed using the proprietary software to check the specificity of any PCR products formed. Where melt curves showed

multiple peaks, these samples were rejected for further analysis due to the likelihood of multiple PCR products having been created.



	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X	Y	Z
1	A1462	A1462	A1471	A1471	A1509	A1509	A1544	A1544	A1569	A1569	A1578	A1578	A1583	A1583	A1606	A1606	A1625	A1625	A1627	A1627	A1628	A1628	A1660	A1660	B-actin	Plate 1
2	CAD2	CAD2	CAD3	CAD3	CAD4	CAD4	CAD6	CAD6	CAD8	CAD8	CAD9	CAD9	CAD10	CAD10	CAD12	CAD12	CAD13	CAD13	CAD14	CAD14	CAD19	CAD19	CAD21	CAD21		
3	CAD23	CAD23	CAD25	CAD25	CAD27	CAD27	C1340	C1340	C1341	C1341	C1298	C1298	A1505	A1505	A1608	A1608	A1665	A1665	A1670	A1670	A1636	A1636	A1449	A1449		
4	A1527	A1527	A1579	A1579	A1570	A1570	NGH1	NGH1	NGH2	NGH2	A1557	A1557	NTC	NTC	NTC	NTC	NTC	NTC	NTC	NTC	NTC	NTC	NTC	NTC		
5	A1462	A1462	A1471	A1471	A1509	A1509	A1544	A1544	A1569	A1569	A1578	A1578	A1583	A1583	A1606	A1606	A1625	A1625	A1627	A1627	A1628	A1628	A1660	A1660	GLUT1	
6	CAD2	CAD2	CAD3	CAD3	CAD4	CAD4	CAD6	CAD6	CAD8	CAD8	CAD9	CAD9	CAD10	CAD10	CAD12	CAD12	CAD13	CAD13	CAD14	CAD14	CAD19	CAD19	CAD21	CAD21		
7	CAD23	CAD23	CAD25	CAD25	CAD27	CAD27	C1340	C1340	C1341	C1341	C1298	C1298	A1505	A1505	A1608	A1608	A1665	A1665	A1670	A1670	A1636	A1636	A1449	A1449		
8	A1527	A1527	A1579	A1579	A1570	A1570	NGH1	NGH1	NGH2	NGH2	A1557	A1557	NTC	NTC	NTC	NTC	NTC	NTC	NTC	NTC	NTC	NTC	NTC	NTC		
9	A1462		A1471		A1509		A1544		A1569		A1578		A1583		A1606		A1625		A1627		A1628		A1660		B-actin	
10	CAD2		CAD3		CAD4		CAD6		CAD8		CAD9		CAD10		CAD12		CAD13		CAD14		CAD19		CAD21			
11	CAD23		CAD25		CAD27		C1340		C1341		C1298		A1505		A1608		A1665		A1670		A1636		A1449			
12	A1527	CAD8	A1579	CAD8	A1570	CAD8	NGH1	NTC	NGH2	NTC	A1557		A1505		A1608		NTC		NTC		NTC		NTC			
13	A1462		A1471		A1509		A1544		A1569		A1578		A1583		A1606		A1625		A1627		A1628		A1660		GLUT1	
14	CAD2		CAD3		CAD4		CAD6		CAD8		CAD9		CAD10		CAD12		CAD13		CAD14		CAD19		CAD21			
15	CAD23		CAD25		CAD27		C1340		C1341		C1298		A1505		A1608		A1665		A1670		A1636		A1449			
16	A1527	CAD8	A1579	CAD8	A1570	CAD8	NGH1	NTC	NGH2	NTC	A1557		A1505		A1608		NTC		NTC		NTC		NTC			
17	A1462	A1462	A1471	A1471	A1509	A1509	A1544	A1544	A1569	A1569	A1578	A1578	A1583	A1583	A1606	A1606	A1625	A1625	A1627	A1627	A1628	A1628	A1660	A1660	B2M	Plate 2
18	CAD2	CAD2	CAD3	CAD3	CAD4	CAD4	CAD6	CAD6	CAD8	CAD8	CAD9	CAD9	CAD10	CAD10	CAD12	CAD12	CAD13	CAD13	CAD14	CAD14	CAD19	CAD19	CAD21	CAD21		
19	CAD23	CAD23	CAD25	CAD25	CAD27	CAD27	C1340	C1340	C1341	C1341	C1298	C1298	A1505	A1505	A1608	A1608	A1665	A1665	A1670	A1670	A1636	A1636	A1449	A1449		
20	A1527	A1527	A1579	A1579	A1570	A1570	NGH1	NGH1	NGH2	NGH2	A1557	A1557	NTC	NTC	NTC	NTC	NTC	NTC	NTC	NTC	NTC	NTC	NTC	NTC		
21	A1462	A1462	A1471	A1471	A1509	A1509	A1544	A1544	A1569	A1569	A1578	A1578	A1583	A1583	A1606	A1606	A1625	A1625	A1627	A1627	A1628	A1628	A1660	A1660	GLUT4	
22	CAD2	CAD2	CAD3	CAD3	CAD4	CAD4	CAD6	CAD6	CAD8	CAD8	CAD9	CAD9	CAD10	CAD10	CAD12	CAD12	CAD13	CAD13	CAD14	CAD14	CAD19	CAD19	CAD21	CAD21		
23	CAD23	CAD23	CAD25	CAD25	CAD27	CAD27	C1340	C1340	C1341	C1341	C1298	C1298	A1505	A1505	A1608	A1608	A1665	A1665	A1670	A1670	A1636	A1636	A1449	A1449		
24	A1527	A1527	A1579	A1579	A1570	A1570	NGH1	NGH1	NGH2	NGH2	A1557	A1557	NTC	NTC	NTC	NTC	NTC	NTC	NTC	NTC	NTC	NTC	NTC	NTC		
25	A1462		A1471		A1509		A1544		A1569		A1578		A1583		A1606		A1625		A1627		A1628		A1660		B2M	
26	CAD2		CAD3		CAD4		CAD6		CAD8		CAD9		CAD10		CAD12		CAD13		CAD14		CAD19		CAD21			
27	CAD23		CAD25		CAD27		C1340		C1341		C1298		A1505		A1608		A1665		A1670		A1636		A1449			
28	A1527	CAD8	A1579	CAD8	A1570	CAD8	NGH1	NTC	NGH2	NTC	A1557		A1505		A1608		NTC		NTC		NTC		NTC			
29	A1462		A1471		A1509		A1544		A1569		A1578		A1583		A1606		A1625		A1627		A1628		A1660		GLUT4	
30	CAD2		CAD3		CAD4		CAD6		CAD8		CAD9		CAD10		CAD12		CAD13		CAD14		CAD19		CAD21			
31	CAD23		CAD25		CAD27		C1340		C1341		C1298		A1505		A1608		A1665		A1670		A1636		A1449			
32	A1527	CAD8	A1579	CAD8	A1570	CAD8	NGH1	NTC	NGH2	NTC	A1557		A1505		A1608		NTC		NTC		NTC		NTC			
33																										

Figure 11.7. An example layout of two 384-plates for qPCR showing how samples were performed in triplicate.

11.6 Data analysis

The proprietary LightCycler 480 software was used to determine the cycle threshold (Ct) values for each reaction. The Ct is the cycle at which the fluorescence achieves a defined threshold and corresponds to the cycle at which a statistically significant increase in fluorescence (known as the ΔRn) is first detected. Ct values were averaged in Microsoft Excel and the logarithmic scale used for Ct values was removed using the formula $2^{-(\text{average Ct value})}$. Expression values for each sample were then determined relative to the geometric mean expression of 2 reference genes, β-actin and β-2-microglobulin (B2M). The geometric mean is a measure of the central tendency of a set of values, calculated as the *n*th root of a product of *n* numbers. β-actin and B2M were chosen as reference genes since previous studies examining GLUT gene expression in vascular smooth muscle cells have shown these to represent valid reference genes [431].

The relative expression values of all samples within a cohort were then averaged and compared with another cohort by way of fold change (AAA/Control) and an independent samples *t*-test (SPSS version 22). GraphPad Prism version 7 (GraphPad software) was used to graphically compare the relative expressions between AAA and control samples.

11.7 Results

Gene expression of GLUTs was compared between WATS obtained from 24 patients with and 15 patients without AAA. The demographics of these patients are detailed in Table 11.7.

Table 11.7. Demographics of patients used to compare glucose transporter gene expression in whole aortic tissue samples.

	AAA (n=24)	**Control (n=15)**
Median age, years (range)	66 (50 – 77)	58 (33 – 77)
Male sex, n (%)	19 (90.5)	10 (66.7)
Current smoker, n (%)	4 (19.0)	8 (53.3)
Ex smoker, n (%)	16 (76.2)	4 (26.7)
Diabetes, n (%)	2 (9.5%)	0
Median AAA diameter, cm (range)	6.0 (3.1 – 10.0)	-

Expression of GLUTs was highly variable in WATS overall with Ct values ranging between 24 - 27 for more abundant GLUTs such as GLUT1 and GLUT3 to 28 - 34 for less abundant GLUTs such as GLUT4, GLUT10 and GLUT13. The expression of GLUT2, GLUT7, GLUT8, GLUT9 and GLUT11 was either not detected or not reliably detectable using the current primers (multiple peaks on melt curve analysis). Table 11.8 shows the mean relative expression values of the various GLUTs studied and Figure 11.8 provides visual comparisons by way of scatterplots.

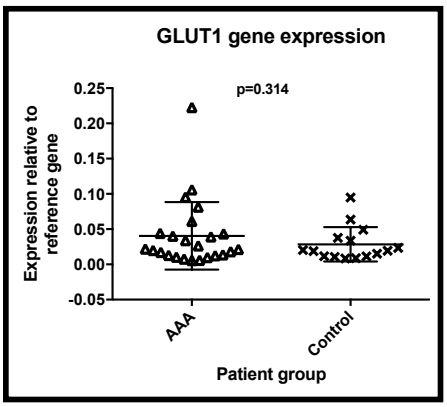
GLUTs that were significantly differentially expressed between AAA and cadaveric tissues include GLUT3 (9-fold difference, p=0.004, Figure 23) and GLUT6 (3-fold difference, p=0.04, Figure 25). There was a trend towards lower

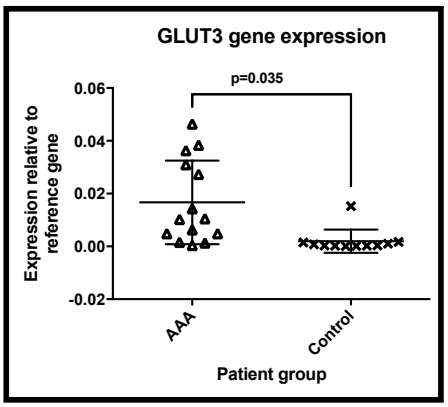
gene expression levels of insulin receptor in the tissues from patients with AAA (40% lower, p=0.08, see Figure 28) however this did not reach statistical significance.

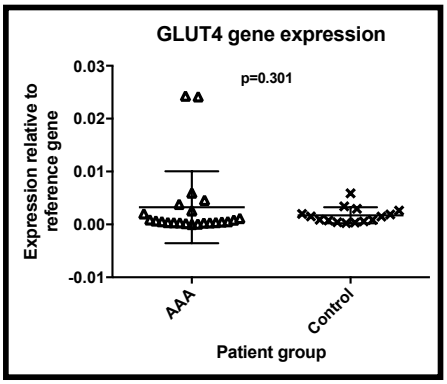
Table 11.8. A comparison of gene expression for various glucose transporters in whole aortic tissue samples from AAA and control patients by way of qPCR expression values relative to the geomean of β-actin and β2M reference genes, fold changes and an independent samples *t* test between groups.

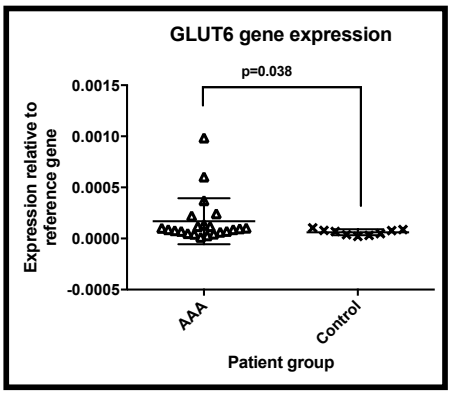
Gene of interest	Mean relative expression * 100 (AAA)	Mean relative expression * 100 (Controls)	Fold change (AAA/Control)	P value
GLUT1	4.256	2.843	1.50	0.314
GLUT3	1.895	0.197	9.64	0.004
GLUT4	0.341	0.173	1.98	0.301
GLUT6	0.017	0.006	3.01	0.043
GLUT10	0.221	0.188	1.17	0.688
GLUT12	0.002	0.015	0.12	0.017*
GLUT13	0.107	0.123	0.87	0.503
Insulin Receptor	0.770	1.311	0.59	0.085

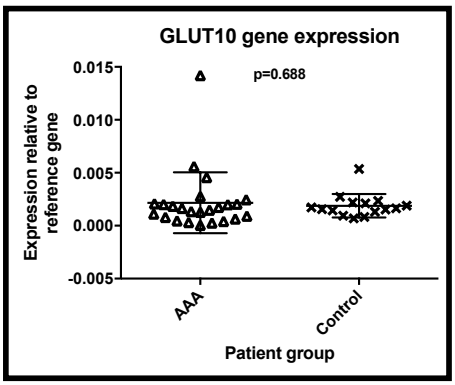
* Result should be interpreted with caution since only 50% of samples were available for analysis.

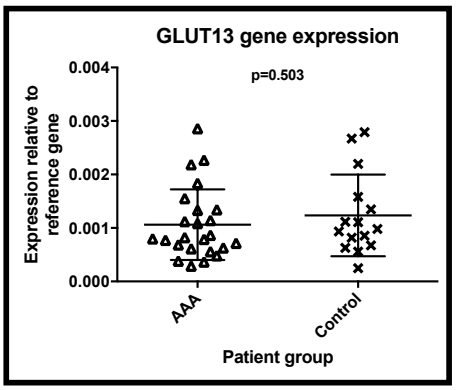












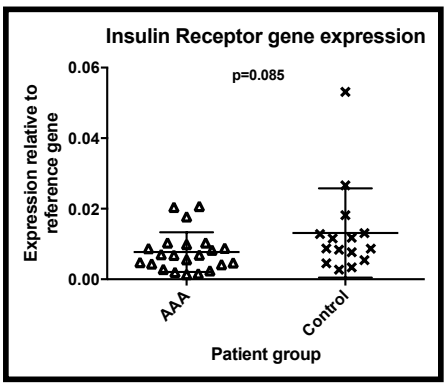


Figure 11.8. Scatterplots comparing relative gene expressions of various glucose transporters and insulin receptor in whole aortic tissue samples from patients with and without AAA.

11.8 Discussion

The aim of this experiment was to determine whether differential gene expression for glucose transporters exists in WATS obtained from AAA patients compared with tissues obtained from non-AAA controls. The results have shown that gene expression of GLUT3 and GLUT6 are significantly elevated in WATS from AAA patients. These findings are novel since previous research in this area is limited and has only studied GLUTs in whole aortic tissue specimens from AAA patients without a comparator group[430]. Tsuruda *et al* found a positive correlation between levels of GLUT1 and GLUT3 and fluorodeoxyglucose (FDG)-positron emission tomography (PET) markers of glucose transporter activity in WATS from AAA patients.

Our results partially support Tsuruda's hypothesis that the aneurysmal abdominal aorta is a metabolically active organ with increased glucose demands however we did not observe a significant increase in gene expression for GLUT1 which is known to be the constitutively expressed glucose transporter. Therefore the increased rates of GLUT3 and GLUT6 gene expression found in WATS from AAA patients are likely to represent either up-regulated expression within specific aortic wall cells capable of expressing GLUT3 and GLUT6, or tissue infiltration with cells known to express GLUT3 and GLUT6. One of the key orchestrating cells in AAA pathogenesis are aortic smooth muscle cells (AoSMCs). AoSMCs are capable of expressing GLUT3 and GLUT6 and therefore increased activity in this cell type could account for the increased levels of GLUT3 and GLUT6 however the predominant cells to express these particular GLUTs are leucocytes and inflammatory cell infiltration is a characteristic feature

of AAA pathobiology. It will therefore be interesting to determine whether AoSMCs from AAA patients also demonstrate similar increases in GLUT gene expression.

11.8.1 Conclusion

In conclusion, the results of this experiment suggest that the expression of GLUTs is up-regulated by the AAA phenotype and therefore allow me to reject the first null hypothesis of this thesis.

Chapter 12: Protein expression of glucose transporters in whole aortic tissue

12.1 Introduction

Protein expression studies aim to quantify the expression of a specific protein which represents the end-product in the central dogma (see Figure 11.1). The reason for studying glucose transporters both at the mRNA and protein level is because not all genes that are expressed at the mRNA level are necessarily translated and hence expressed as a protein. Importantly it is usually expression at the protein level that determines function of a gene.

The purpose of this experiment is to determine whether differential expression of glucose transporter proteins exists in whole aortic tissue samples (WATS) from patients with AAA compared to those from controls. The results of this experiment will again allow me to support or reject the first null hypothesis of this thesis which states that "the expression and/or activity of glucose transporters will not be up-regulated by the AAA phenotype".

The methods that will be used in this experiment include performing immunohistochemistry (IHC) for GLUTs on the prepared WATS sections, digitally photographing the stained sections of tissue and analysing the images systematically.

12.2 Methods

12.2.1 Selecting patients

As was noted in the previous experiment (see Chapter 11.7), control WATS compared to AAA WATS were more likely to come from female patients as well as from patients with a greater range of ages. The reasons for this are because cadaveric organ donors are often procured from patients suffering with significant non-salvageable brain injury, such as hypoxic brain injury or intracranial haemorrhage, which can occur at any age. Therefore whilst selecting the samples to include in this experiment, the decision was made to try and grossly match samples for age (>50 years old) and sex (males). In addition an attempt was made to match patients for smoking status however it was only possible to match patients with respect to the number of current smokers as nearly all patients in the AAA group were either ex-smokers or current smokers, whereas patients in the control group were either current smokers or non-smokers. The aim of this matching was to reduce the effect of confounding.

12.2.2 Immunohistochemistry

After obtaining WATS slides from the Core Biotechnology Services department, the slides were labelled with a pencil and the sections dewaxed and rehydrated using the following protocol. The slides were first immersed in xylene twice for 3 minutes each, then in 99% industrial methylated spirit (IMS) twice for 1 minute each, then in 95% IMS once for 1 minute and finally the slides were washed under running tap water for 5 minutes.

During formalin-fixation, methylene bridges form between proteins in the tissue which masks the epitopes needed to perform IHC. Prior to performing IHC, it is important to break these methylene bridges in order to unmask the epitopes within the tissue thereby allowing antibodies to bind them.

Heat-induced epitope retrieval was performed using the microwave retrieval method which involved placing the slides in a plastic slide rack in a plastic dish filled with 10mM sodium citrate (pH 6.0) and loosely covering the dish with a plastic lid before microwaving the dish for 23 minutes at 700 watt whilst ensuring to keep the sodium citrate solution topped up. The slides were then removed from the microwave and allowed to cool to room temperature before being placed in phosphate buffered saline (PBS).

Many cells and tissues contain endogenous peroxidase therefore blocking endogenous peroxidase is important to reduce non-specific background staining when using horseradish peroxidase (HRP)-conjugated antibodies. Therefore the slides were treated with freshly made 0.3% hydrogen peroxide (0.5ml 30% H2O2 + 49.5ml water) for 15 minutes to block endogenous peroxidase and then washed in PBS for 1 minute.

The slides were then treated with a blocking buffer made of 10% fetal calf serum + 3% bovine serum albumin in PBS tween (PBST) (0.1% Tween 20) for 1 hour to block non-specific binding. Primary antibodies were made up in this same blocking buffer and the slides were treated with their respective antibodies overnight at 4^{o}C in a humidified chamber.

Based on the results of the previous experiment which examined mRNA expression in WATS (see Chapter 11.7), six proteins were chosen for immunohistochemical analysis: GLUT1, GLUT3, GLUT4, GLUT6, GLUT12 and insulin receptor (IR). Optimisation of primary antibody concentration was performed when the suggested dilution on the product specification sheet produced either excessive non-specific background staining (see Figure 12.1) or no staining at all. Table 12.1 summarises the final primary antibody dilutions used for each protein and the host species for each antibody.

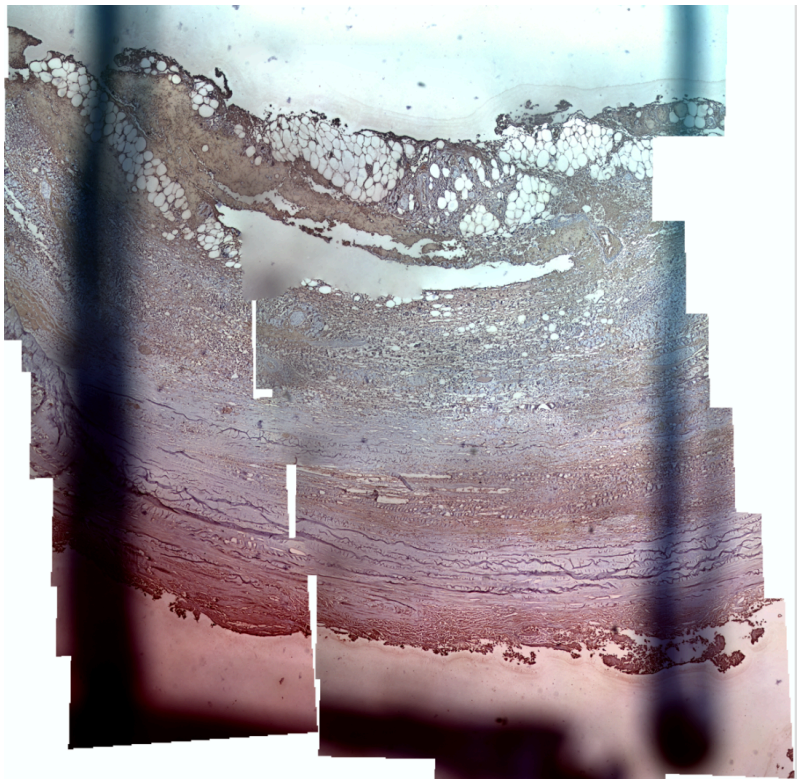


Figure 12.1. An example slide with excessive non-specific background staining (GLUT6 antibody used at 1:10 dilution).

Table 12.1. Final primary antibody dilutions and host species used in immunohistochemistry of whole aortic tissue samples.

Protein	Primary antibody dilution	Host species
GLUT1	1:200	Rabbit
GLUT3	1:50	Rabbit
GLUT4	1:500	Mouse
GLUT6	1:50	Mouse
GLUT12	1:10	Rabbit
Insulin Receptor	1:50	Rabbit

Next the slides were washed with PBST three times for 5 minutes each before being treated with the species-specific HRP-conjugated secondary antibody for 1 hour at room temperature. Secondary antibodies were also made up in the blocking buffer described above. The slides were then washed with PBST three times for 5 minutes each before treating the slides with NovaRed™ dye (Vector Labs) for 10 - 15 minutes. Care was taken to ensure that background tissues did not all stain brown by serially reviewing the slides every 5 minutes. The slides were then washed in water for 3 minutes and the slides dehydrated once again by washing them in hot water for 5 minutes, 99% IMS for 3 minutes and then xylene for 3 minutes. Finally the slides were air-dried and mounted using DPX (a mixture of distyrene, plasticizer and xylene).

12.2.3 Photographing stained slides

Stained WATS were digitally photographed using a microscope camera connected to a computer running the Leica Applications suite. Photographs

were taken using the microscope objective set to 20x and the camera exposure set to 1.43 milliseconds. Multiple photos were taken of the section from left to right ensuring the entire width of the sample is photographed. Sequential images were slightly overlapped which allowed them to be stitched together in Adobe Photoshop (Adobe Systems) using the 'photomerge' and 'blend' functionality.

12.2.4 Data analysis

Processed images were initially run through the Materials Image Processing and Automated Reconstruction (MIPAR) software package using the 'immunohistochemistry recipe' with the aim of automatically quantifying brown staining and hence protein expression. This was performed with the help of Dr Ana Verissimo at the University of Leicester. However the software did not specifically quantify staining in the stained parts of the image and often missed large areas of staining. This remained the case despite a few attempts at recipe manipulation. Therefore the more conventional approach of 2 independent observers was used to quantify staining in the images. A recent study has shown good correlation between computer and visual quantification of slide staining (spearman correlation of 0.88 – 0.90) [434].

Level of brown staining was graded using the standard 4-point scoring system where '-' indicated no staining, '+' indicated mild staining, '++' indicated moderate staining and '+++' indicated strong staining. Separate scores were given for the intima, inner media, outer media, boundary (between outer media

and adventitia) and adventitia.

To prevent bias, slides were first graded by Observer 1 (Jonathan Barber) without Observer 2 (myself) being present. Observer 1 then provided photographs to Observer 2 but without any accompanying patient or sample details. Observer 2 then graded the slides without observer 1 being present and without any knowledge of Observer 1's grades. Observer 1 then revealed the patient and sample details as well as their own grades to the Observer 2, still without knowledge of Observer 2's grades. The 4 possible grades (-, +, ++, +++) were then assigned numerical scores (0, 1, 2, 3). Both observer numerical scores were then averaged (mean) to create an average score for each section of the slide (e.g. intima, inner media, etc.). The section averages were also summated to create a 'total score' for each slide. Median section and total scores for AAA and control cohorts were then calculated and comparisons made between cohorts using a Mann Whitney U test (SPSS version 22).

12.3 Results

Protein expression of GLUTs was compared between WATS obtained from 6 patients with and 6 patients without AAA. The demographics of these patients are detailed in Table 12.2.

Table 12.2. Demographics of patients used to compare GLUT protein expression in WATS.

	AAA (n=6)	**Control (n=6)**
Median age, years (range)	65.5 (56 – 69)	57 (55 – 75)
Male sex, n (%)	6 (100)	6 (100)
Current smoker, n (%)	3 (50)	3 (50)
Ex smoker, n (%)	3 (50)	0 (0)
Diabetes, n (%)	0 (0)	1 (16.7)
Median AAA diameter, cm (range)	6 (5.5 – 10)	-

Comparing GLUT protein expression between AAA and controls revealed significantly higher overall levels of GLUT1, GLUT3 and GLUT6 in the WATS from patients with AAA (see Table 12.3). Table 12.3 outlines the median scores for each section of the slide as well as the median total scores for each protein. The results of Mann Whitney U testing between cohort also provided and significant differential expression is highlighted in bold.

Representative photographs of WATS stained for each GLUT for patients with and without AAA are shown in Figures 12.2 and Figures 12.3 respectively.

Table 12.3. A comparison of average GLUT protein expression scores in whole aortic tissue samples from patients with (n=6) and without (n=6) AAA stratified according to subsection of aortic wall as assessed by 2 independent observers.

		Median expression scores (ranges) and *p* values											
		Intima	***p***	***Inner media***	***p***	***Outer media***	***p***	***Boundary***	***p***	***Adventitia***	***p***	**TOTAL**	***p***
GLUT1	*AAA*	1.5 (0.5 – 3.0)	**0.002***	1.5 (1.0 – 2.0)	**0.015***	1.75 (1.0 – 2.5)	**0.041***	1.5 (1.0 – 2.5)	0.240	2.25 (1.5 – 2.5)	**0.009***	8.25 (7.5 – 10)	**0.002***
	Control	0 (0)		0.5 (0 – 1.5)		1 (0 – 1.5)		1 (0 – 2.5)		0.5 (0.5 – 2.0)		3.5 (1 – 6)	
GLUT3	*AAA*	1.5 (1.0 -3.0)	**0.002***	1 (0 – 1.5)	0.065	1.25 (1.0 -2.0)	**0.004***	0.5 (0 -1.5)	0.589	1.5(1.0 – 2.5)	0.093	6 (4.5 – 8.5)	**0.002***
	Control	0 (0 – 0.5)		0 (0 -1.0)		0 (0 -1.0)		0.5 (0 -1.0)		0 (0 -2.0)		1.25 (0 – 4.0)	
GLUT4	*AAA*	0.75 (0 – 2.5)	0.065	1 (0 -2.0)	0.818	1.25 (0 – 1.5)	0.180	1 (0 – 2.5)	0.485	1.25 (1.0 -2.0)	0.394	5.75 (2.0 – 8.0)	0.240
	Control	0 (0 – 0.5)		0.75 (0.5 – 1.5)		0.75 (0.5 – 1.0)		0.75 (0.5 – 1.0)		1 (0.5 – 1.5)		3 (2.5 – 5.5)	
GLUT6	*AAA*	2.75 (0 – 1.5)	**0.002***	1.5 (1 – 2.0)	**0.041***	1.75 (1.0 – 2.5)	**0.026***	1.5 (0.5 – 2.0)	0.240	2.75 (1.5 – 3.0)	**0.041***	10.25 (7.5 – 11.5)	**0.004***
	Control	0.25 (0 – 1.5)		0.75 (0 -1.5)		1 (0.5 – 1.5)		1.5 (1.5 – 2.5)		2 (1.0 – 2.5)		6 (3.5 – 8.0)	
GLUT12	*AAA*	0.5 (0 – 1.5)	0.310	0.5 (0 – 1.5)	0.180	0.5 (0 -1.0)	0.485	0.5 (0 – 1.5)	0.589	0.75 (0.5 – 2.0)	**0.041***	3 (1.0 – 5.5)	0.093
	Control	0 (0 – 0.5)		0.25 (0 – 0.5)		0.25 (0 – 1.0)		0.5 (0 -1.0)		0 (0 -1.0)		1.75 (0.5 – 3.0)	
IR	*AAA*	0.25 (0 -1.0)	0.310	1 (0.5 – 1.0)	**0.041***	0.75 (0 – 1.5)	0.937	0.5 (0 – 1.5)	0.240	0.5 (0.5 – 1.5)	0.240	3.5 (2.0 – 5.0)	0.589
	Control	0 (0 - 0.5)		0.5 (0 -1.0)		0.75 (0 – 2.0)		1 (0.5 – 2.5)		0.5 (0 – 0.5)		2.5 (0.5 – 5.0)	

IR = insulin receptor. Mann Whitney U test between AAA and control groups. Statistically significant *p* values asterisked in bold.

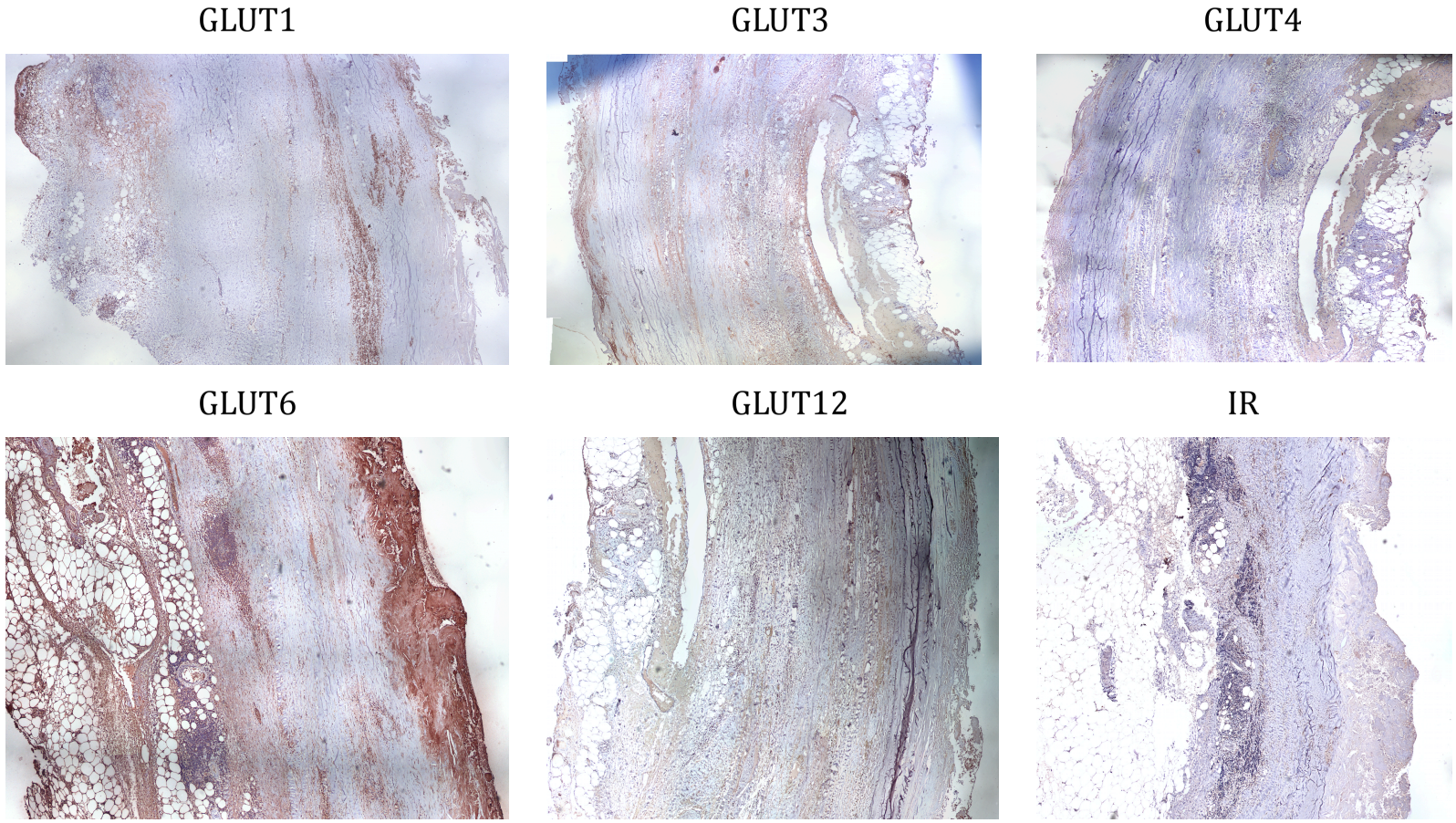


Figure 12.2. Representative photographs of whole aortic tissue samples from patients with AAA stained for GLUT1, GLUT3, GLUT4, GLUT6, GLUT12 and insulin receptor. Taken at 20x magnification

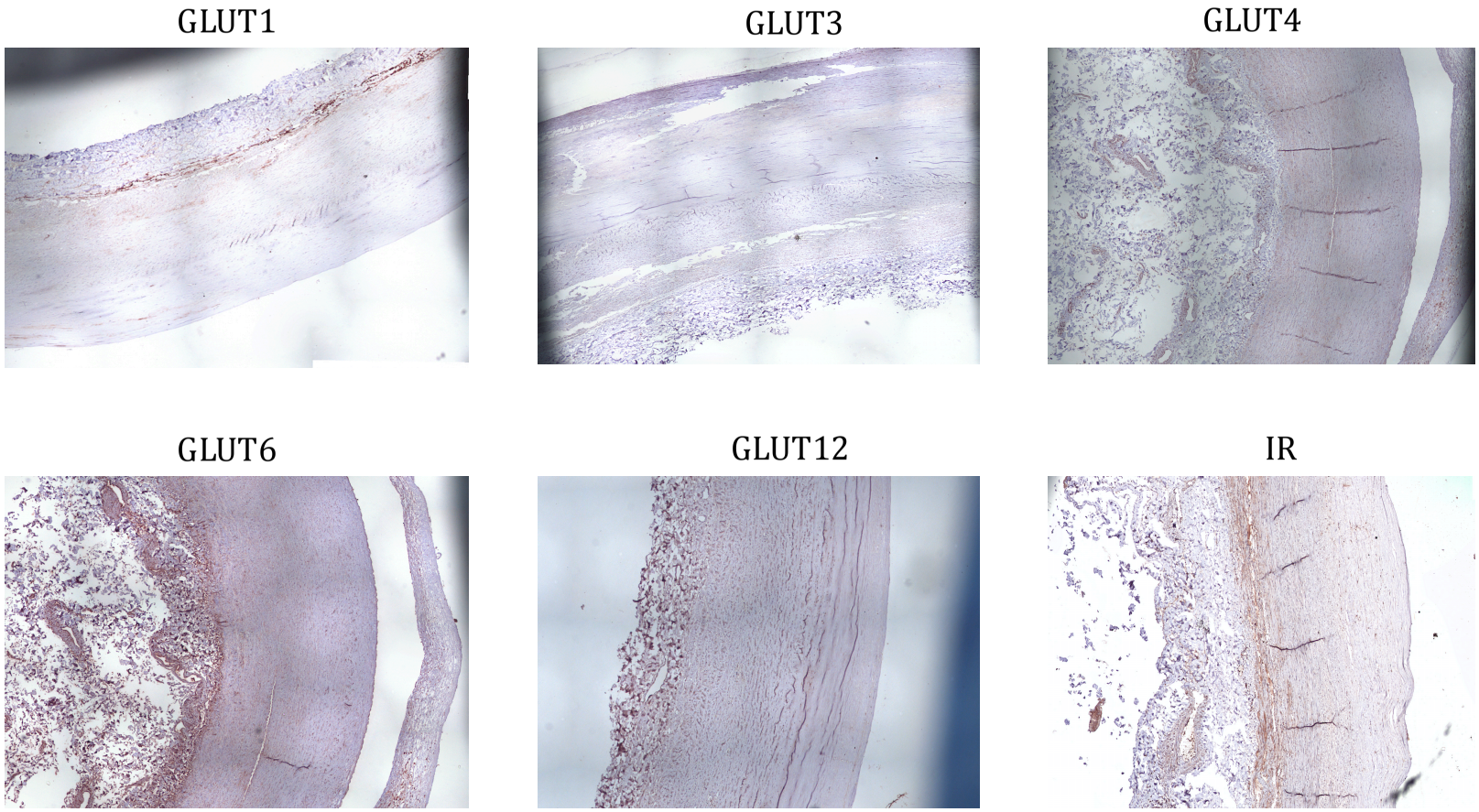


Figure 12.3. Representative photographs of whole aortic tissue samples from patients without AAA stained for GLUT1, GLUT3, GLUT4, GLUT6, GLUT12 and insulin receptor. Taken at 20x magnification.

12.4 Discussion

The aim of this experiment was to determine whether differential protein expression for glucose transporters exists in WATS obtained from AAA patients compared with tissues obtained from non-AAA controls. The results have shown that, overall, the protein expression of GLUT1, GLUT3 and GLUT6 is significantly elevated in WATS from AAA patients.

GLUT1 is constitutively expressed suggesting that aortic tissues from AAA patients have a higher basal rate of glucose uptake compared to their non-AAA counterparts. This supports Tsuruda's hypothesis that the aneurysmal abdominal aorta is a metabolically active organ with increased glucose demands[430]. The findings of increased GLUT3 and GLUT6 protein expression are in keeping with the results of earlier experiments examining gene expression in WATS from patients with and without AAA (see Chapter 11.8). As mentioned both GLUT3 and GLUT6 are heavily expressed by leucocytes and inflammatory cells, both of which are known to infiltrate aortic tissues during AAA development in vivo.

With the exception of GLUT3, the increased expression of GLUTs in AAA tissues seems to be localised to all layers of the aortic wall (intima, media and adventitia). GLUT3 was found to only be overexpressed within the intimal and medial layers, the latter corresponding to the main layer in which inflammatory cell infiltration is seen. The finding of increased GLUT protein expression in the intimal and medial layers also corresponds to the layers in which endothelial and

aortic smooth muscle cells are usually resident, both of which are have been implicated in the pathogenesis of AAA (particularly aortic smooth muscle cells).

12.4.1 Conclusion

In conclusion, the results of this experiment suggest that the expression of GLUTs is up-regulated by the AAA phenotype and therefore allow me to reject the first null hypothesis of this thesis.

Chapter 13: Gene expression of glucose transporters in aortic smooth muscle cells

13.1 Introduction

Aortic smooth muscle cells (AoSMCs) reside within the tunica media layer of the aortic wall and are well recognised as being one of the key cell types in AAA pathogenesis. They are capable of synthesising not only enzymes important for matrix degradation such as matrix metalloproteinases and cathepsins, but also vascular endothelial growth factor and monocyte chemoattractant protein 1, key mediators responsible for medial neovascularisation and inflammatory cell recruitment respectively.

Previous research has shown that AoSMCs express a number of glucose transporters (GLUTs) [431]. It is therefore likely that glucose is internalised and processed via the GLUT family of proteins in AoSMCs and that AoSMCs use glucose as an energy substrate in order to help synthesise many of the enzymes and mediators detailed above. Up-regulated expression of GLUTs would therefore represent one mechanism by which AoSMCs might augment AAA development.

The purpose of this experiment was to determine whether differential expression of GLUT mRNA exists in AoSMCs cultured from AAA patients compared with AoSMCs cultured from non-AAA controls. The results obtained from this experiment will allow me to support or reject the first null hypothesis

of this thesis which states that “the expression and/or activity of glucose transporters will not be up-regulated by the AAA phenotype”.

The methods used to determine mRNA expression of GLUTs in AoSMCs include whole aortic tissue explant culture, cell culture of AoSMCs, extraction of total RNA, reverse transcription, primer design and quantitative real-time polymerase chain reaction (qPCR).

13.2 Explant culture

Explant culture refers to the technique of culturing cells from pieces of tissue or an organ removed from a plant or animal. Whole aortic tissues specimens (WATS) were kept in medium 199 (Sigma Aldrich) with added penicillin and streptomycin (+ P/S) at 4^{o}C for up to 24 hours until a suitable time could be found to perform explant culture (median 1 hour, range 0.5 – 24 hours).

13.2.1 Optimisation steps

Protocol 1

My first attempts at isolating AoSMCs from WATS were performed using a draft protocol inherited from a previous research fellow (Miss Saima Ehsan). This protocol involved isolating the tunica media from the WATS, dividing the specimen into 'small pieces' and placing them in medium 199 supplemented with 20% fetal calf serum (FCS), P/S and amphotericin B (an antifungal agent) into 35mm wells of a 6-well plate. The explanted tissue was then covered with glass cover slips with the aim of increasing the surface area for cell growth. The plates were incubated at 37^{o}C and left undisturbed for 2 to 3 days after which the floor of the culture plate and the cover slips were checked for the presence of adherent cells. Using sterile forceps, the cover slips were then re-submerged in 1 to 2ml of fresh medium 199 in fresh 6-well plates with the cell coated surface facing upwards. The cells were then left to incubate for 7 to 10 days after which the medium was changed and then changed twice weekly until the cells had reached approximately 70% confluence at which point the cells were passaged and checked for AoSMC identity using α-actin immunofluorescence. During

medium changes, two-thirds of the conditioned medium was removed (to avoid disrupting the adherent cells) with this protocol and replaced with fresh medium.

Using this protocol however I found it particularly difficult to grow sufficient numbers of AoSMCs and more often failed to achieve 70% confluence. In the few cases where 70% confluence was achieved I found that the passaged cells failed to adhere to the flask during following the passage which meant that the lineage naturally died out.

Protocol 2

In order to improve my success with explant culture of AoSMCs, I visited Dr Karen Porter's laboratory (at the University of Leeds) who has a successful explant culture program for AoSMCs (although not from aneurysmal WATS). Dr Porter recommended using Dulbecco Modified Eagle Medium (DMEM) instead of medium 199, supplementing with 10% FCS rather than 20% FCS and avoiding the use of cover slips. She also advised that I use culture flasks (T25 or T75 depending on the amount of tissue available for explant culture) rather than using 6-well plates and only take out 1ml old medium and replace with 1ml of fresh medium during medium changes. Dr Porter also recommended staining for vimentin in addition to α-actin since myofibroblasts, a cell type distinct from AoSMCs, also stain positive for α-actin but are negative for vimentin.

For the next 5 cases, I incorporated Dr Porter's recommendations into my explant culture protocol however this did not improve the density of AoSMCs in

my experience. In addition I noticed the growth of other cells which were not AoSMCs based on immunofluorescence for α-actin and vimentin.

Protocol 3

Due to the difficulties encountered in performing explant culture for AoSMCs with generic culture mediums, a smooth muscle cell specific medium was searched for. Medium 231 (M231) (Life Technologies) is a commercially available complete culture medium containing essential and non-essential amino acids, vitamins, other organic compounds, trace minerals and inorganic salts needed for the growth of normal human vascular smooth muscle cells. According to the manufacturer, M231 is designed to be used with smooth muscle cell growth supplement (SMGS) (Life Technologies) which contains growth factors, hormones, and tissue extracts needed for the growth of normal human vascular smooth muscle cells. SMGS contains 5% v/v fetal bovine serum, recombinant human basic fibroblast growth factor, recombinant human epidermal growth factor and insulin. Protocol 3 involved using M231 and SMGS as per the manufacturers instructions.

Optimisation experiment

In order to determine the efficacy of protocol 3 to grow AoSMCs, an experiment was designed comparing M231, medium 199 and DMEM. Using aortic tissue from a single AAA patient, explant culture was attempted in 5 x T25 flasks each with 5ml of a different culture medium / serum combination.

1. M199 with 20% FCS
2. M199 with 5% FCS

3. DMEM with 10% FCS
4. DMEM with 5% FCS
5. M231 with SMGS (serum concentration 4.9% according to the manufacturer).

Flasks were left for 5 days before inspecting the flasks for cell growth. AoSMCs were confirmed visually by the presence of spindle shaped cells sprouting from the edge of the tissue. The presence of non-AoSMCs was confirmed using immunofluorescence staining for α-actin and vimentin and cell density was confirmed using a haemocytometer (see Chapter 13.3 for protocols). The results of this experiment are shown in Table 13.1.

Table 13.1. A comparison of different explant culture methods for isolating aortic smooth muscle cells from whole aortic tissue specimens.

Medium/serum combination	Spindle shaped sprouting cells	Presence of cells negative for α-actin OR vimentin	Cell density (cells/ml)
M199 + 20% FCS	Y	Y	13,000
M199 + 5% FCS	Y	N	4,000
DMEM + 10% FCS	Y	Y	8,000
DMEM + 5% FCS	Y	Y	6,000
M231 + SMGS	Y	N	14,000

Overall the flask containing complete M231 produced the highest density of cell growth on visual inspection which was confirmed to be AoSMC growth on immunofluorescence. This medium was therefore chosen as the optimised medium for growing AoSMCs.

13.2.2 Optimised protocol

The tunica media was isolated from the full thickness specimen by removing the tunica adventitia from the abluminal side and atherosclerotic plaque from the luminal side using a pair of fine sterile forceps and a dissecting microscope. The tunica media was then placed in M231 supplemented with SMGS and P/S and then divided into 4 to 6 equal sized portions. Each portion was then cut into as many fine squares (<1mm^2) as possible using a number 10 scalpel blade and gently aspirated together with 5ml of complete M231 (= M231 + SMGS) + P/S and placed in a T25 flask for incubation at 37^{o}C with 5% carbon dioxide (CO_2). Flasks were left untouched for at least 5 days before checking on them to ensure proper adherence of tissues to the flask.

13.3 Cell culture

The method described above produced visually identifiable AoSMC growth in most cases within 2 weeks of setting up the explant culture. Once the AoSMCs were about 70% confluent, they were harvested and the combined cell growth from all T25 flasks was moved into a single T75 flask ensuring that the explant tissue was left behind. The cells were set up in complete M231 without P/S at this point and this was designated as the first passage (P1). AoSMCs were thereafter grown exclusively in complete M231 without P/S.

13.3.1 Passaging

Passaging involved removing the old medium, washing once with phosphate buffered saline (PBS), adding 2ml of warmed 2x trypsin/EDTA (Invitrogen), giving the trypsin 3 to 5 minutes in the incubator to free the cells from the surface of the flask, adding 10ml of PBS with 5% FCS to stop the trypsin, spinning the cells down (5 mins at 1250 rpm), removing the supernatant, re-suspending the cells in M231 and then plating them in a new T75 flask. Cells were grown up this way up until P3.

13.3.2 Counting cells

If cells required plating at a specific density it was necessary to calculate the number of cells per ml of solution beforehand. This was performed by mixing 12µL of cells in solution with 12µL of trypan blue and then counting the number of cells in 2 diagonal sets of 16 squares of a Neubauer haemocytometer (see

Figure 13.1). Multiplying this number of cells by 10,000 allowed calculation of the number of cells in 1ml of the original solution.

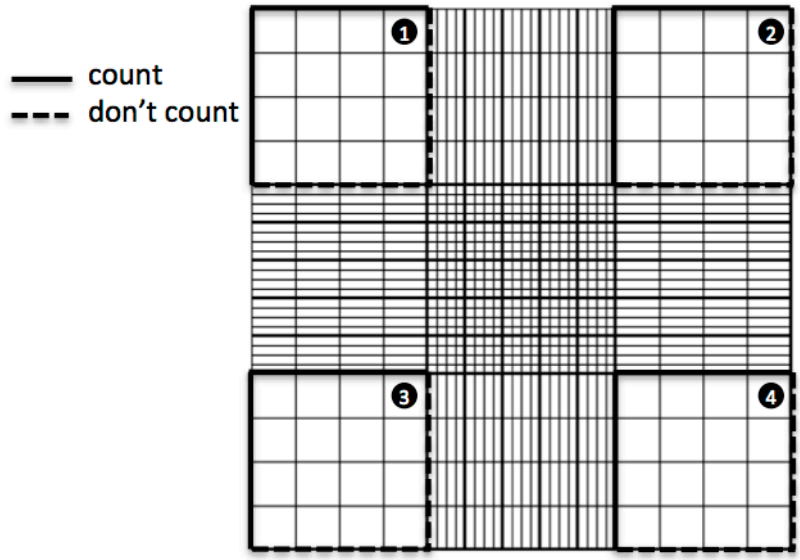


Figure 13.1. Diagram showing the counting grid of a Neubauer haemocytometer including the 2 diagonal sets of 16 squares as well as the areas where cells are counted and not counted. Taken from www.haemocytometer.org with permission.

13.3.3 Confirming aortic smooth muscle cell identity

In addition to confirming the identity of AoSMCs visually, cells from each patient also underwent identification using immunocytochemistry (ICC). This was performed at the P1 stage for the first 10 cases (6 AAA, 4 control) after which ICC was only performed where there was doubt about the identity of the passaged cells. Using the protocols outlined above produced cells which stained positive for α-actin and vimentin in all 10 cases confirming their identity as AoSMCs[435].

The methods are detailed below. All steps were performed at room temperature unless otherwise stated.

A subset of cells from the T25 flasks were seeded onto coverslips placed in 35mm wells of a 6-well plate and the cells allowed to adhere for at least 48 hours. The medium was then removed, the cells washed twice with PBS and the cells fixed with 4% paraformaldehyde for 20 mins at 4^{o}C. The cells were then washed three times with Tris-buffered saline (TBS), each wash lasting 2 mins. The cells were then permeabilised using TBS/0.5% Triton for 10mins and then washed three times with a wash buffer (TBS/0.1% Triton), each wash lasting 5mins. The cells were then incubated with a blocking buffer made of TBS/0.1% Triton/2% bovine serum albumin (BSA) for 10mins and then washed twice in PBS.

The cells were then sequentially incubated with both primary antibodies for 1 hour each in the dark; firstly against α-smooth muscle actin (Sigma Aldrich) at a dilution of 1:600 and then against vimentin (R&D systems) at a dilution of 1:400 after 3 PBS washes. After removing the second primary antibody and washing three times with PBS, the secondary antibodies against each primary antibody were added simultaneously and left for 1 hour in the dark. FITC-conjugated anti-mouse IgG (Sigma Aldrich) was used against α-smooth muscle actin and NorthernLights™ NL557-conjugated anti-rat IgG (R&D systems) was used against vimentin. The cells were then washed with PBS three times, each wash lasting 5 mins and then washed once with double-distilled water. The coverslips were then removed and mounted onto glass slides using VectaShield mountant

containing DAPI (Vector Labs) for simultaneous staining of the nuclei. Epifluorescence microscopy was then used to confirm AoSMCs identity which needed to stain positive for both α-smooth muscle actin and vimentin to be identified as AoSMCs (see Figure 13.2).

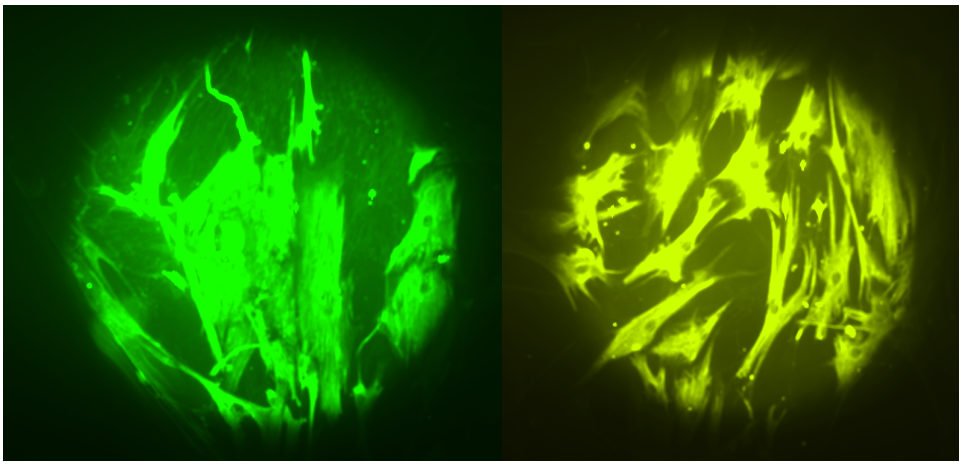


Figure 13.2. Example epifluorescence microscopy photographs of aortic smooth muscle cells staining positive for α-smooth muscle actin (left) and vimentin (right).

13.4 RNA extraction

At the P4/P5 stage, AoSMCs were transferred from T75 flasks to 12-well plates and plated at a density of 100,000 cells per well. AoSMCs were grown in complete M231 for 48 hours and then grown in low serum M231 (0.49% serum) for 48 hours before being harvested using the manual RNA extraction method (without the use a kit) using the method below.

After removal of culture medium in a sterile hood, 700µl of Trizol LS reagent (Life Technologies) was added to each well of a 12-well plate. After 60 seconds, the cells were lysed by pipetting the Trizol LS up and down at least 5 times and the lysate collected in 1.5ml eppendorf tubes. These were frozen at -80^{o}C until adequate samples had been collected to perform batch extractions.

To extract RNA, the samples were thawed, incubated at room temperature for 10 minutes and vortexed. The samples were then centrifuged at 12,000rpm for 5mins and 140µl of chloroform was added to each tube before shaking the tubes vigorously. Following 10mins of incubation at room temperature, the tubes were centrifuged at 12,000 rpm for 10mins and the upper aqueous portion was carefully transferred to a new tube. 0.7 volumes of ice-cold isopropanol were then added to each tube and the RNA allowed to precipitate overnight at -20^{o}C. The tubes were then centrifuged at 12,000 rpm for 30mins at 4^{o}C, the supernatant removed and the RNA pellet washed with 70% ethanol. The tubes were then centrifuged again at 12,000 rpm for 15mins at 4^{o}C after which the ethanol-containing supernatant was removed and the pellets allowed to air-dry for 20 to 30mins. Once all the ethanol had evaporated, the final RNA pellet was

re-suspended in 20µl of nuclease-free water prior to quantifying the RNA quantity on a NanoDrop spectrophotometer (ThermoScientific) and storing at -80^{o}C.

13.5 Quantifying RNA expression

When required, the samples reverse transcribed and RNA expression quantified using real-time PCR as detailed in Chapter 11.5. The same primers used to quantify gene expression in whole aortic tissue specimens were used for AoSMCs (see Table 11.6). β-actin and β2-microglbulin were used as reference genes and have been validated by others quantifying GLUT mRNA expression in AoSMCs[431]. Data analysis was performed as detailed in Chapter 11.6.

13.6 Results

Gene expression of GLUTs was compared between AoSMCs obtained from 8 patients with and 16 patients without AAA. The demographics of these patients are detailed in Table 13.2.

Table 13.2. Demographics of patients used to compare glucose transporter gene expression in aortic smooth muscle cells.

	AAA (n=8)	**Control (n=16)**
Median age, years (range)	65 (50 – 76)	59.5 (32 – 77)
Male sex, n (%)	6 (75)	9 (64.3)
Current smoker, n (%)	2 (25)	6 (42.9)
Ex smoker, n (%)	5 (62.5)	3 (21.4)
Diabetes, n (%)	0 (0)	0 (0)
Median AAA diameter, cm (range)	5.7 (5.5 – 10)	-
Median passage number (range)	4 (3 - 4)	4.5 (3 – 6)

Expression of GLUTs was highly variable with Ct values ranging between 23 to 28 for more abundant GLUTs such GLUT 1 to 30 to 36 for less abundant GLUTs such as GLUT6. Table 13.3 shows the relative expressions of the various GLUTs detected in AoSMCs. Figure 13.3 provides visual comparisons using scatterplots.

No difference was found in the relative expression of any GLUTs between the 2 groups including GLUT3 (1.7-fold difference, p=0.096) or GLUT6 (0.9-fold difference, p=0.864), which were differentially expressed in whole aortic tissue.

Table 13.3. A comparison of gene expression for various glucose transporters in aortic smooth muscle cells from AAA and control patients by way of qPCR expression values relative to the geomean of β-actin and β2M reference genes, fold changes and an independent samples *t* test between groups.

Gene of interest	Mean relative expression * 100 (AAA)	Mean relative expression * 100 (Controls)	Fold change (AAA/CAD)	P value
GLUT1	13.06	15.91	0.821	0.586
GLUT3	0.334	0.199	1.679	0.096
GLUT4	0.078	0.078	1.00	0.991
GLUT5	0.847	0.283	2.99	0.116
GLUT6	0.023	0.025	0.944	0.864
Insulin Receptor	0.380	0.487	0.780	0.363

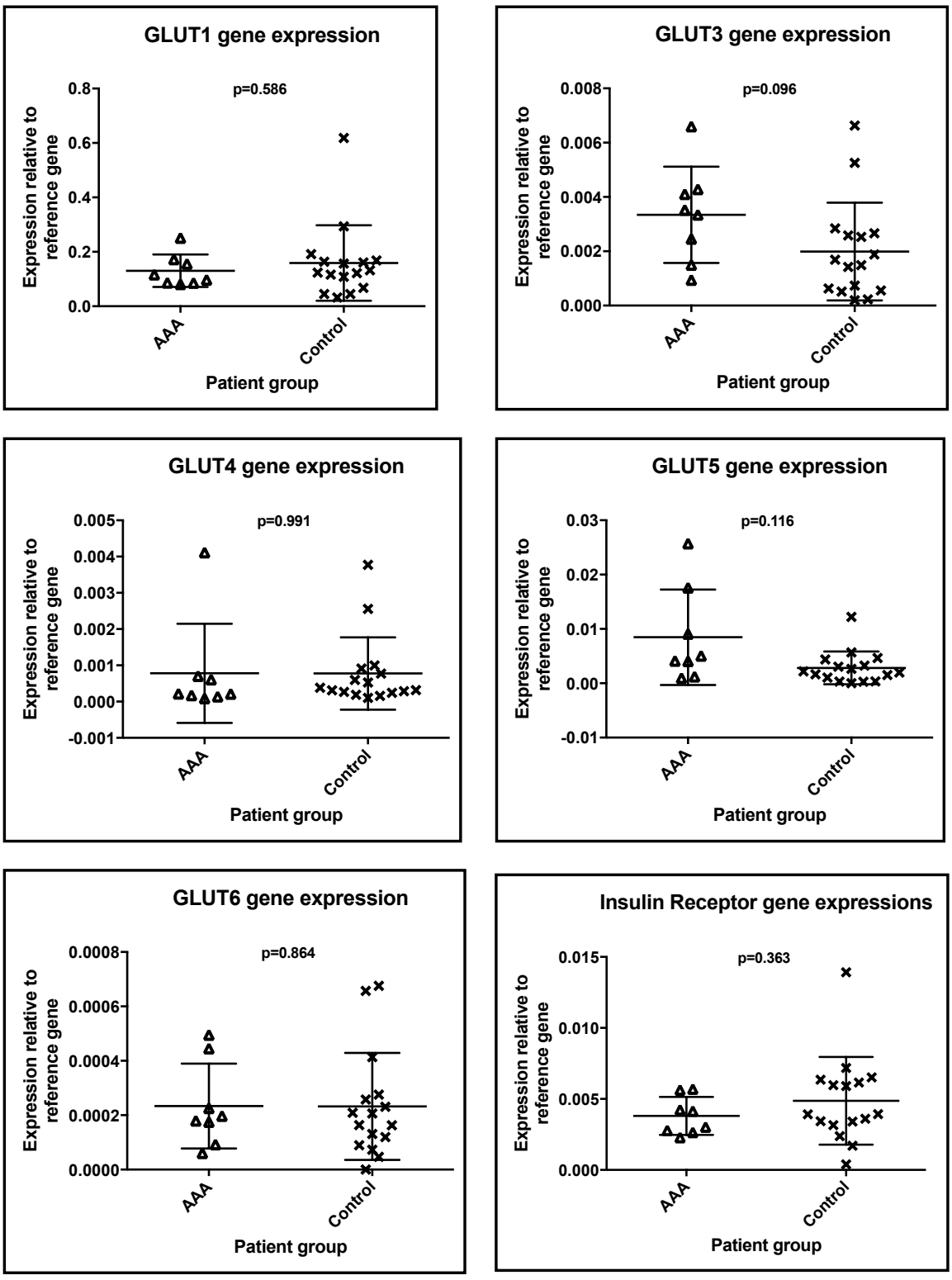


Figure 13.3. Scatterplots comparing relative gene expressions of various glucose transporters and insulin receptor in aortic smooth muscle cells from patients with and without AAA.

13.7 Discussion

The aim of this experiment was to determine whether differential mRNA expression for glucose transporters exists in AoSMCs obtained from AAA patients compared with cells obtained from non-AAA controls. The results have shown that there was no difference in the mRNA expression of 5 different glucose transporters or insulin receptor in AoSMCs from AAA patients versus controls. Taken together with the results of earlier experiments from Chapter 11, this experiment suggests that elevated GLUT gene expression in aortic wall smooth muscle cells are not the reason for the increased overall expression of GLUTs in whole aortic tissue specimens from patients with AAA. Therefore it is likely that increased expression within another cell type found within the aortic wall of AAA patients, such as leucocytes, is responsible for the previous finding. Since there are no previous studies comparing expression of GLUTs in WATS, AoSMCs or leucocytes from patients with and without AAA, the results of the current experiment are unable to support or refute previous observations.

The current experiment was limited by the number of available AoSMCs for analysis and therefore it was not possible to test for differential expression of all GLUTs previously analysed in Chapter 11. Therefore this experiment did not determine if differential expression exists for GLUT10, GLUT12 or GLUT13; to answer this, further experiments with increased cell numbers are needed.

13.7.1 Conclusions

In conclusion, the results of this experiment do not suggest that the expression of GLUTs is up-regulated by the AAA phenotype and therefore do not allow me to reject my first null hypothesis.

Chapter 14: Activity of glucose transporters in aortic smooth muscle cells

14.1 Introduction

To determine the importance of glucose transporters (GLUTs) in the pathogenesis of AAA, my earlier experiments have sought to determine whether differential expression of GLUTs exists, either at the mRNA or protein level, in cells or whole tissue from patients with and without AAA. However, given that the function of GLUTs is transport glucose across plasma cell membranes, an important comparison to make is whether function or activity of glucose transporters is different between AAA and control patients.

Studying activity of a transporter protein is difficult at a whole tissue level due to the difficulties in keeping the whole tissue 'functioning' whilst activity is being assayed. As a result, I decided to measure GLUT activity in cultured aortic smooth muscle cells (AoSMCs) from patients with and without AAA. The importance of AoSMCs in the pathogenesis of AAA has already been emphasised (see Chapter 1.5) and previous research has shown surrogate markers of GLUT activity to be increased in patients with large versus small AAA. However, no studies have directly compared cellular glucose transporter activity in AAA and control patients.

The aim of this experiment was to determine whether GLUT activity was different between AoSMCs from AAA and control patients. The results of this experiment will allow me to support or reject the first null hypothesis of this

thesis which states that ""the expression and/or activity of glucose transporters will not be up-regulated by the AAA phenotype".

The methods used to quantify GLUT activity in AoSMCs include explant culture, cell culture, a glucose transport activity assay, a protein concentration assay and data analysis.

14.2 Explant and cell culture

For this experiment, the explant culture and cell culture techniques were identical to optimised protocols described in Chapters 13.2 and 13.3 except that the once the AoSMCs were transferred from T75 flasks to 12-well plates at the P4/P5 stage, plated at a density of 100,000 cells per well and grown in complete medium 231 for 48 hours, the cells were then transferred into low serum medium 231 for 42 hours rather than 48 hours as described above.

14.3 Glucose transport activity assay

Glucose transport activity assays were performed in collaboration with Dr Alan Bevington's laboratory at the University of Leicester who have an existing optimised protocol to perform the technique. The assay utilises the principles of liquid scintillation counting which is a method to quantify the radioactivity of low-energy isotopes, mostly β-emitting and α-emitting isotopes. The University of Leicester protocol utilises the radioactive isotope of hydrogen Tritium (^{3}H).

14.3.1 Principles of assay

The principles of the glucose transport activity assay are as follows:

1) Cells are incubated in a medium containing a radiolabelled glucose tracer for a set period of time.
2) Cells uptake glucose including tracer as part of cellular respiration and to facilitate metabolic processes.
3) After a set period of time, the medium is removed and further metabolism is stopped by rapidly cooling the cells.
4) Cells are lysed which releases radiolabelled glucose from inside the cells
5) Lysed cells are added to a liquid scintillation cocktail composed of an aromatic organic solvent and a scintillator or fluor. The energy released from radiolabelled tracer excites the aromatic solvent which is then transferred to and absorbed by the scintillator within the liquid scintillation cocktail. This produces an excited states of electrons. When the electrons decay to their ground state they produce a pulse of light energy termed luminescence. The amount of luminescence, which is

proportional to the amount of ionising radiation and hence glucose transported, is then detected by a photomultiplier tube within a scintillation counter (see Figure 14.1).

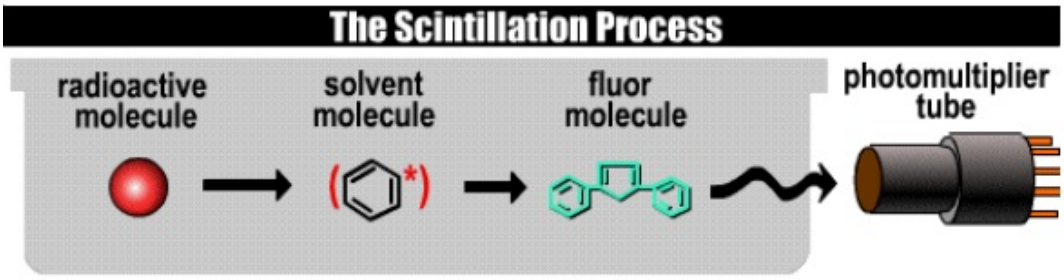


Figure 14.1. Diagram showing the stages in the scintillation process. Reproduced from https://wiki.uio.no/mn/safe/nukwik/index.php/LSC_Principles with permission.

14.3.2 Determining the best method of glucose transport inhibition

Prior to performing the main glucose transport activity assays, a brief experiment to determine the best method of inhibiting glucose transport was conducted. Inhibiting glucose transport is important to determine how much radiolabelled tracer enters the cell even when standard transport pathways (GLUT proteins) are blocked as this amount must be subtracted from final GLUT activity values.

Using AoSMCs from a single patient, the impact of inhibiting glucose transport using a non-radiolabelled glucose analogue (2-deoxyglucose) was compared against inhibiting glucose transport using an organic compound which targets carrier mediated transport (cytochalasin B). Both methods use competitive

pharmacodynamics to inhibit glucose transport but cytochalasin B also has non-competitive inhibitory properties.

The results showed that although glucose transport was inhibited some to extent using both low and high concentrations of non-radiolabelled 2-deoxyglucose, use of cytochalasin B produced a greater level of glucose transport inhibition (see Figure 14.2). Accordingly, cytochalasin B was selected as the method for glucose transport inhibition for all subsequent experiments.

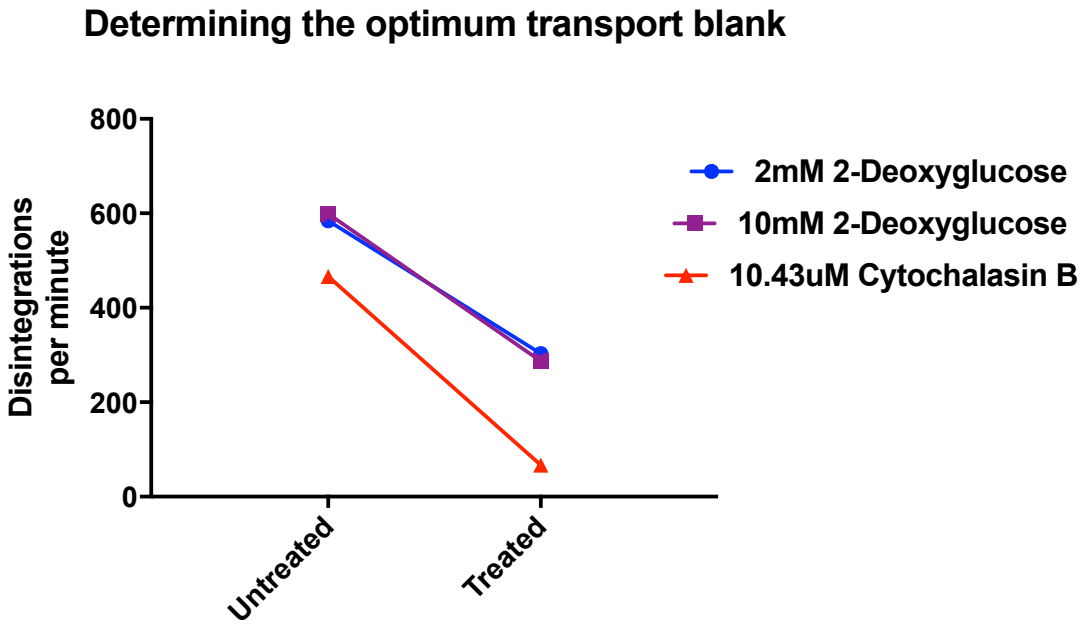


Figure 14.2. The effect of inhibiting glucose transport in aortic smooth muscle cells using the unlabelled 2-deoxyglucose at 2mM (blue line), unlabelled 2-deoxyglucose at 10mM (purple line) or the organic compound cytochalasin B at 10.43µM (red line). The graph shows the most potent inhibition of glucose transport with cytochalasin B.

14.3.3 Assay methods

As detailed earlier, AoSMCs were grown in 12-well plates at a density of 100,000 cells/well and mostly used at the P4/P5 stage. Whilst the AoSMCs were incubating in the test mediums, a bottle of Hepes Buffered Saline (HBS) was warmed up to room temperature (see Appendix 1 for methods on making up HBS), a bottle of 0.9% sodium chloride (NaCl) solution was placed on ice and a digital thermometer was set to monitor the room temperature.

A 'diluted glucose label' constituted from 5% radiolabelled 2-Deoxy-D-[^{3}H]-glucose (Perkin Elmer), 5% non-radiolabelled 2-deoxy-D-glucose (Sigma Aldrich) and 90% HBS was made up over an isotope tray in the culture hood to preserve the sterility of the radiolabelled glucose stock. Sufficient diluted label was made up each time to allow 10µl to be added to each well of the 12-well plate. 40ml of 'transport medium' and 40ml of 'blank medium' were made up by diluting 4µl of dimethyl sulfoxide (DMSO) stock in 40ml of HBS or 4µl of 50g/L cytochalasin B in 40ml of HBS respectively.

After 18 hours of incubation at 37^{o}C with 5% CO_2 in low serum M231, the cells were removed from the culture incubator and the test mediums aspirated. Working as quickly as possible, each well was rinsed twice with 1ml of HBS at room temperature and then 500µl of transport medium or blank medium was added to each well of the 12-well plate as per the layout shown in Figure 14.3. Immediately afterwards, 10µl of diluted glucose label was rapidly added to each well using a BCL 8000 pipette and the plates swirled to mix the ^{3}H label with the medium. A 5-minute timer was started at this point and the temperature in the

room recorded. At the end of the 5-minute incubation period, the plates were put on ice, the ^{3}H-labelled medium was rapidly aspirated into an isotope waste trap and the wells washed 3 times with ice-cold 0.9% NaCl solution to stop any further glucose transport.

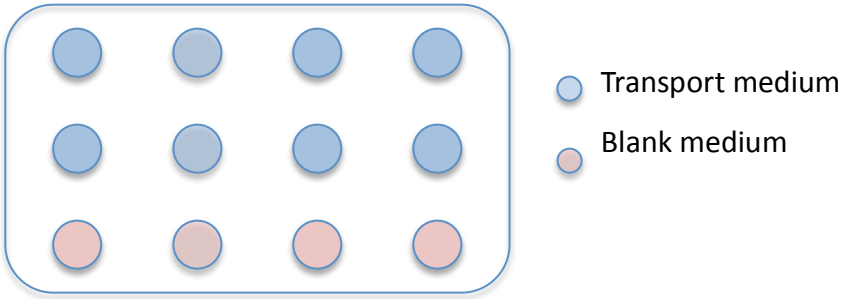


Figure 14.3. Layout of 12-well plate during glucose transport assay demonstrating the use of technical duplicates for wells containing transport medium.

After aspirating the last NaCl wash, 200µl of 0.05M sodium hydroxide was added to each well and the cells scraped using the plunger of a 1ml syringe. The scrapings were then transferred to newly labelled 1.5ml eppendorf tubes and the tubes incubated at 70^{o}C for 30mins in a water bath to digest the cells. 4ml of Ecoscint A scintillant (National Diagnostics) was then added to newly labelled scintillation vials followed by 150µl of cell digest added respectively. The scintillation vials were then incubated for 2 hours in the dark to allow the chemiluminescence to decay before the vials were counted using a Wallac 1219 Rackbeta liquid scintillation counter (LKB Instruments) set to parameter group A1.

14.4 Lowry protein concentration assay

A protein concentration assay was performed to standardise the counts from each well to the amount of total protein in the well, thereby overcoming the issues with marginally different cell counts in each well.

The Lowry assay is a chromogenic protein assay which relies on the reactivity of nitrogen ions in proteins pre-treated with copper ions under alkaline conditions causing a spectral shift and hence change in colour when exposed to the phosphomolybdatephosphotungstic acid present in Folin reagent (see Figure 14.4).

Figure 14.4. Diagram showing the steps involved in performing a Lowry protein concentration assay including the blue colour of the end-product. Reproduced from http://microamaze.blogspot.co.uk/2015/07/protein-estimation-by-folin-lowrys.html

14.4.1 Assay methods

A number of test tubes equal to the number of wells were set up in a rack (i.e. for 2 x 12-well plates, 24 test tubes were set up). To each tube was added, 10µl of nanopure water, 25µl of 0.95M NaOH and 25µl of cell digest (in 0.05M NaOH). Additionally 12 test tubes with known concentrations of BSA were set up (10µl nanopure water, 50µl BSA standard dissolved in 0.5M NaOH). Then to all test tubes was added 600µl of Folin reagent C (made up from Folin reagents A and B mixed in a 50:1 ratio) and 60µl of a Folin-Ciocalteau solution (made up from nanopure water and Folin-Ciocalteau's phenol reagent mixed in a 2:1 ratio) thereby giving each test tube a final volume of 720µl. The test tubes were vortexed to ensure thorough mixing and 300µl from each tube was then added to each well of a 96-well plate. Using a Thermo Scientific colorimetric plate reader set to read at the 650nm wavelength, the plates were read 40mins after the point of vortexing. The protein concentrations within the test samples were then calculated using the equation of the line generated by plotting the colorimetric values of the BSA standards against their known protein concentrations.

14.5 Data analysis

Data about the counts from each scintillation vial in disintegrations per minute (dpm) were first exported to Microsoft Excel 2010. The dpm values for the transport blank well were then subtracted from each transport medium well. Since only 150µL of the cell digest was added to scintillation vials, the dpm values were divided by 150 and multiplied by 200 to obtain the actual dpm value per well (termed "R").

A scatterplot of the optical density values against the protein concentrations of the BSA standards was then created and a linear regression line drawn in the form $y = mx + c$. The R^2 value of the line was also calculated to determine how good the fit of the line was to the plotted points.

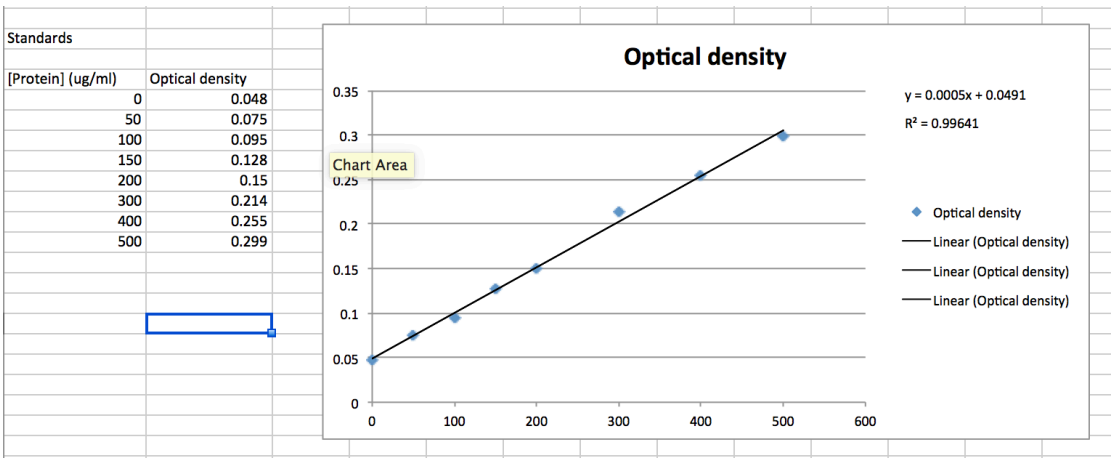


Figure 14.5. An example of the optical density versus protein standard curve in Microsoft Excel which was used to determine a linear regression equation of $y = 0.0005x + 0.491$ and an R^2 value of 0.996 suggesting a good fit.

Using the equation re-arranged to $x = \frac{y-c}{m}$ it was possible to determine the protein concentrations (in µg/mL) of each test samples from their optical density

values. To determine the amount of protein in each well (i.e. μg/well), the above protein concentrations were divided by 5 (as the cells were in 200μL of sodium hydroxide rather than 1mL) and then multiplied by 2 (to correct for the 2x dilution within the Lowry protein concentration assay). This value was termed "P".

Finally, "R" was divided by "P" to determine the GLUT activity in disintegrations per minute per microgram of protein for each test well. Since technical duplicates of each test well were performed (see Figure 14.3), these were then averaged (mean) to determine the average GLUT activity value for each patient.

14.6 Practice experiments

Before performing the experiments outlined above, I used 4 sets of non-essential AoSMCs to practice the techniques and optimise the culture conditions needed to successfully perform glucose transport assays in AoSMCs. This was important given my naivety with the above techniques, in particular to the speed and precision with which the techniques needed to be performed in order to obtain accurate results. During these practice experiments I was supervised by Jeremy Brown from the Bevington Laboratory.

These practice experiments utilised AoSMCs from AAA patients but had been plated at a density of 10,000 cells/well. In addition the AoSMCs were used at passage numbers 9 and 10. Although 3 out 4 sets of AoSMCs produced some results, none of these 'practice runs' were included in the final analysis since the AoSMCs were not felt to be representative of those used in the main experiment. In addition, 1 out of 4 sets of AoSMCs produced no results, probably due to loss of cells during scraping due to low plating density and in 2 out of 4 sets of AoSMCs the standard curves in the Lowry protein assays had a poor R^2 value of <0.90.

14.7 Results

GLUT activity was compared between AoSMCs isolated from 10 patients with and 10 patients without AAA. The demographics of these patients are outlined in Table 14.1.

Table 14.1. Demographics of patients used to compare GLUT activity in AoSMCs.

	AAA (n=10)	**Control (n=10)**
Median age, years (range)	68 (65 – 82)	59.5 (36 – 77)
Male sex, n (%)	7 (70)	7 (70)
Current smoker, n (%)	3 (30)	3 (30)
Ex smoker, n (%)	6 (60)	3 (30)
Diabetes, n (%)	0 (0)	0 (0)
Median AAA diameter, cm (range)	6.0 (5.5 – 7.5)	-
Median passage number (range)	5 (4 – 6)	4 (3 – 4)

Under standard culture conditions, GLUT activity was significantly higher in the cells cultured from patients with AAA (26.3 dpm/µg of protein vs. 17.8 dpm/µg of protein, 48.2% higher, p=0.023, Figure 14.6).

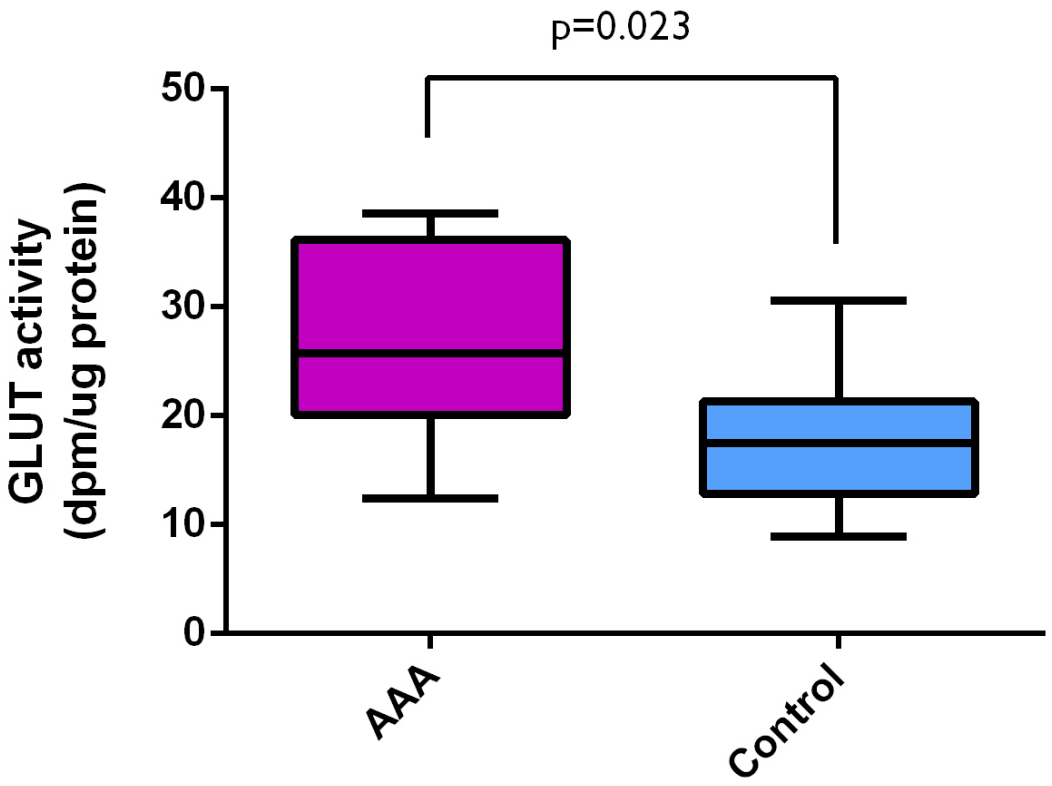


Figure 14.6. Glucose transporter activity in aortic smooth muscle cells cultured from patients with and without AAA (n=10 vs. 10). Mann Whitney U test used to compare groups. Data shown as median, interquartile range, minimum and maximum values. Dpm = disintegrations per minute.

14.8 Discussion

The aim of this experiment was to determine whether activity of glucose transporters differed between AoSMCs isolated from patients with and without AAA. The results have shown that GLUT activity is nearly 50% higher in the AoSMCs isolated from patients with AAA when measured using standard radio-isotope uptake studies. Since the results are standardised for protein content this cannot be explained by subtle differences in plating density or cell growth that invariably occur with cell culture experiments.

These findings support the hypothesis from Tsuruda *et al* which suggest that the aneurysmal abdominal aorta is a metabolically active organ with increased glucose demands[430]. The results of this experiment suggest that vascular smooth muscle cells residing within the aortic wall represent one of the cell types that become metabolically up-regulated. Taken together with the results of the previous experiment (Chapter 13), it would seem that although AoSMCs within the aortic wall do not increase their gene expression of GLUTs, the GLUTs which are present there already, seem to be up-regulated in terms of activity. It is unlikely that increased protein expression of GLUTs in AoSMCs can explain this finding since the current results are adjusted for overall protein content as previously mentioned. However, to confirm this, formal protein expression studies of GLUTs in AoSMCs are needed.

The limitations of this experiment are that it only focused on GLUT activity within AoSMCs. Other cell types known to infiltrate or reside within the aortic

wall such as leucocytes, in particular macrophages, have not been studied but may also demonstrate similar alterations in GLUT activity.

14.8.1 Conclusions

In conclusion, the results of this experiment suggest that the activity of GLUTs is up-regulated by the AAA phenotype and therefore allow me to reject my first null hypothesis.

Chapter 15: The effect of hyperglycaemia on gene expression of glucose transporters and proteolytic enzymes in aortic smooth muscle cells

15.1 Introduction

Circulating hyperglycaemia is the characteristic feature of diabetes mellitus (DM) which has been shown to be negatively associated with the presence, growth and rupture of AAA. The mechanisms underlying this protective effect are however incompletely understood.

Data from my earlier experiments have shown that the expression of certain glucose transporters (GLUTs) is increased in whole aortic tissue samples (WATS) from patients with AAA. Although subsequent experiments did not find increased gene expression of these proteins in aortic smooth muscle cells (AoSMCs) isolated from patients with AAA, there was up-regulation in the activity of these proteins. Regulation of these proteins in AoSMCs, either in terms of their expression or in terms of their activity, therefore could represent one mechanism by which hyperglycaemia protects against the development of AAA. The experiments described in this and the following chapter represent Phase 2 of the thesis and are designed to test this theory (see Chapter 9).

Previous researchers have shown that hyperglycaemia is capable of influencing the expression of matrix metalloproteinases (MMPs), which are known to be important in the development of AAA. Tanaka *et al* have shown that

hyperglycaemia reduces both mRNA and protein expression of MMP-9 in stimulated macrophages cell lines[336]. Miyama *et al* has shown that circulating hyperglycaemia during aneurysm induction using an experimental mouse model of AAA was associated with a reduction in AAA diameter but also in the activity of MMP-9 in the aortic walls of hyperglycaemic mice[332]. However, none of the studies investigating the protective effect of hyperglycaemic on AAA have examined the effect of hyperglycaemia on the expression of endogenous tissue inhibitors of MMPs (TIMPs), which have been shown to be important in the pathogenesis of AAA[91,93,436]. In addition none of the studies in this area has examined the influence of hyperglycaemia on the expression of cathepsins, another family of proteolytic enzymes implicated in AAA pathogenesis[90,100].

The aim of this experiment was to determine whether hyperglycaemia influences the mRNA expression of glucose transporters in AoSMCs isolated from patients with or without AAA. The results of this experiment will allow me to support or reject the second null hypothesis of this thesis which states that "the expression and/or activity of glucose transporters will not be down-regulated by the diabetic phenotype". In addition this experiment aims to determine whether hyperglycaemia influences the mRNA expression of cathepsins and/or TIMPs in AoSMCs from patients with or without AAA.

The methods used to perform this experiment include explant culture from WATS, cell culture of AoSMCs, extraction of total RNA, reverse transcription, primer design and quantitative real-time polymerase chain reaction (qPCR).

15.2 Explant culture

The explant culture of AoSMCs from WATS was performed as per the optimised protocol detailed in Chapter 13.2.

15.3 Phase 2 cell culture

AoSMCs were grown in complete medium 231 in T75 flasks between passages P1 to P3/4 after which they were transferred into 12-well plates at a density of 100,000 cells/well as described in Chapters 13.3 and 13.4. However, in comparison to Phase 1 where only 6 wells were needed per patient, Phase 2 required 24-wells per patient. One 12-well plate was used for glucose transport activity assays (see Chapter 16) and another used to perform gene and protein expression studies.

Phase 2 involved exposing AoSMCs to varying degrees of hyperglycaemia. This was done using AoSMCs at the P3/4 stage and followed a 96-hour protocol. On day 0, AoSMCs from each patient were plated into two 12-well plates at a density of 100,000 cells/well in complete M231. After 48 hours in complete M231, the culture medium was replaced for one containing a low serum concentration (= M231 with $1/10^{th}$ concentration SMGS = 0.49% FCS). After 24 hours of quiescence, the culture medium was replaced again with one of four test mediums each differing by their respective glucose concentration only.

The use of 12-well plates allowed exposure of each sample to four different test mediums in triplicate (see Figure 15.1). The first contained low serum M231 with no alterations made to the glucose concentration (= 4.6mmol/L) whereas the second, third and fourth consisted of low serum M231 with added analytical grade D-glucose (Sigma Aldrich) to produce test mediums with a glucose concentration of 12.5mmol/L, 25mmol/L and 50mmol/L respectively. Glucose

transport activity assays and RNA extractions were performed after 18 hours and 24 hours of incubation in the test mediums respectively.

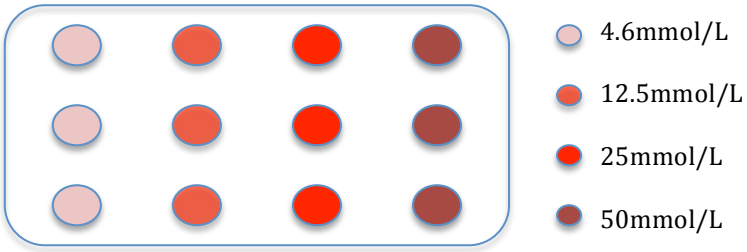


Figure 15.1. Distribution of test mediums in 12-well plates during Phase 2 studies. Medium 231 used with varying concentrations of glucose as shown in diagram.

15.3.1 Creating hyperglycaemic test mediums

The calculation and methods used to create the various hyperglycaemic test mediums were somewhat involved and therefore detailed in Appendix 1.

15.4 RNA extraction

Total RNA extraction from AoSMCs was performed using the manual method as described in Chapter 13.4.

15.5 Quantifying RNA expression

After extracting total RNA from the AoSMCs, the samples were reverse transcribed and mRNA expression quantified using real-time PCR as detailed in Chapter 11.5. The same primers sequences detailed in Table 11.6 were used to quantify gene expression in this experiment.

Phosphoglycerate kinase 1 (PGK1) and TATA-box binding protein (TBP) were tested as references genes for this experiment however using them produced highly variable relative expression values within each group (see Appendix 2). Therefore the references genes used in Chapter 13 were used again in this experiment (β-actin and β2-microglbulin) since these have also been validated previously by others examining GLUT gene expression in AoSMCs[431]. Data analysis was performed as detailed in Chapter 11.6 except that fold changes were not compared between AAA and controls but rather relative expressions were compared between the 4 test mediums by way of Kruskal-Wallis testing.

15.6 Results

AoSMCs from 5 patients with and 5 patients without AAA were exposed to varying degrees of hyperglycaemia and gene expression of GLUTs, cathepsins and TIMPs measured. The demographics of patients are outlined in Tables 15.1.

Table 15.1. Demographics of patients used to examine the effect of hyperglycaemia on mRNA expression of glucose transporters, cathepsins and tissue inhibitors of matrix metalloproteinases in AoSMCs.

	AAA (n=5)	**Control (n=5)**
Median age, years (range)	65 (56 – 71)	56.5 (50 – 67)
Male sex, n (%)	5 (100)	3 (60)
Current smoker, n (%)	1 (20)	1 (20)
Ex smoker, n (%)	4 (80)	4 (80)
Diabetes, n (%)	0 (0)	0 (0)
Median AAA diameter, cm (range)	7.2 (5.5 – 10.0)	-
Median passage number (range)	5 (4 – 5)	5 (4 – 5)

Exposure of AoSMCs from patients with AAA to increasing levels of hyperglycaemia was not associated with a significant change in the mRNA expression of GLUT1, GLUT3, GLUT4, GLUT5 or insulin receptor (IR) (see Table 15.2). Visually, there was a trend towards decreasing mRNA expression levels of GLUT1 and GLUT3 with increasing hyperglycaemia within the physiological range (4.6mmol/L to 25mmol/L) (see red bars in Figure 15.2 GLUT1 and GLUT3 graphs) however these trends were not statistically significant on Kruskal-Wallis testing ($p=0.962$ for GLUT1 and $p=0.506$ for GLUT3).

Table 15.2. Mean gene expression values of various glucose transporters and insulin receptor in aortic smooth muscle cells from AAA patients (n=5) exposed to varying levels of hyperglycaemia. Kruskal-Wallis testing between different test mediums.

	Mean relative gene expression				
Test mediums	**GLUT1**	**GLUT3**	**GLUT4**	**GLUT5**	**IR**
4.6mmol/L	0.0990	0.00172	0.00405	0.00437	0.00913
12.5mmol/L	0.0826	0.00169	0.00509	0.00252	0.00870
25mmol/L	0.0613	0.00149	0.00340	0.00401	0.00620
50mmol/L	0.0936	0.00136	0.00592	0.00173	0.00889
Kruskal-Wallis test	P=0.962	P=0.506	P=0.328	P=0.818	P=0.336

Exposure of AoSMCs from patients without AAA revealed similar results. Hyperglycaemia was not associated with a significant change in the mRNA expression of GLUT1, GLUT3, GLUT4, GLUT5 or IR (see Table 15.3). Visually, there was a trend towards lower mRNA expression levels of GLUT1, GLUT3 and GLUT4 with increasing hyperglycaemia (see blue bars in Figures 15.2 GLUT1, GLUT3 and GLUT4 graphs) which was not confined to glucose concentrations within the physiological range only however these trends were not statistically significant on Kruskal-Wallis testing (p=0.853 for GLUT1, p=0.962 for GLUT3, p=0.473 for GLUT4).

Table 15.3. Mean gene expression values of various glucose transporters and insulin receptor in aortic smooth muscle cells from control patients (n=5) exposed to varying levels of hyperglycaemia. Kruskal-Wallis testing between different test mediums.

	Mean relative gene expression				
Test mediums	**GLUT1**	**GLUT3**	**GLUT4**	**GLUT5**	**IR**
4.6mmol/L	0.172	0.00151	0.0118	0.00142	0.0129
12.5mmol/L	0.120	0.00145	0.0115	0.00095	0.00540
25mmol/L	0.107	0.000972	0.0104	0.00131	0.00722
50mmol/L	0.089	0.000997	0.00534	0.00266	0.00550
Kruskal-Wallis test	P=0.853	P=0.962	P=0.473	P=0.536	P=0.744

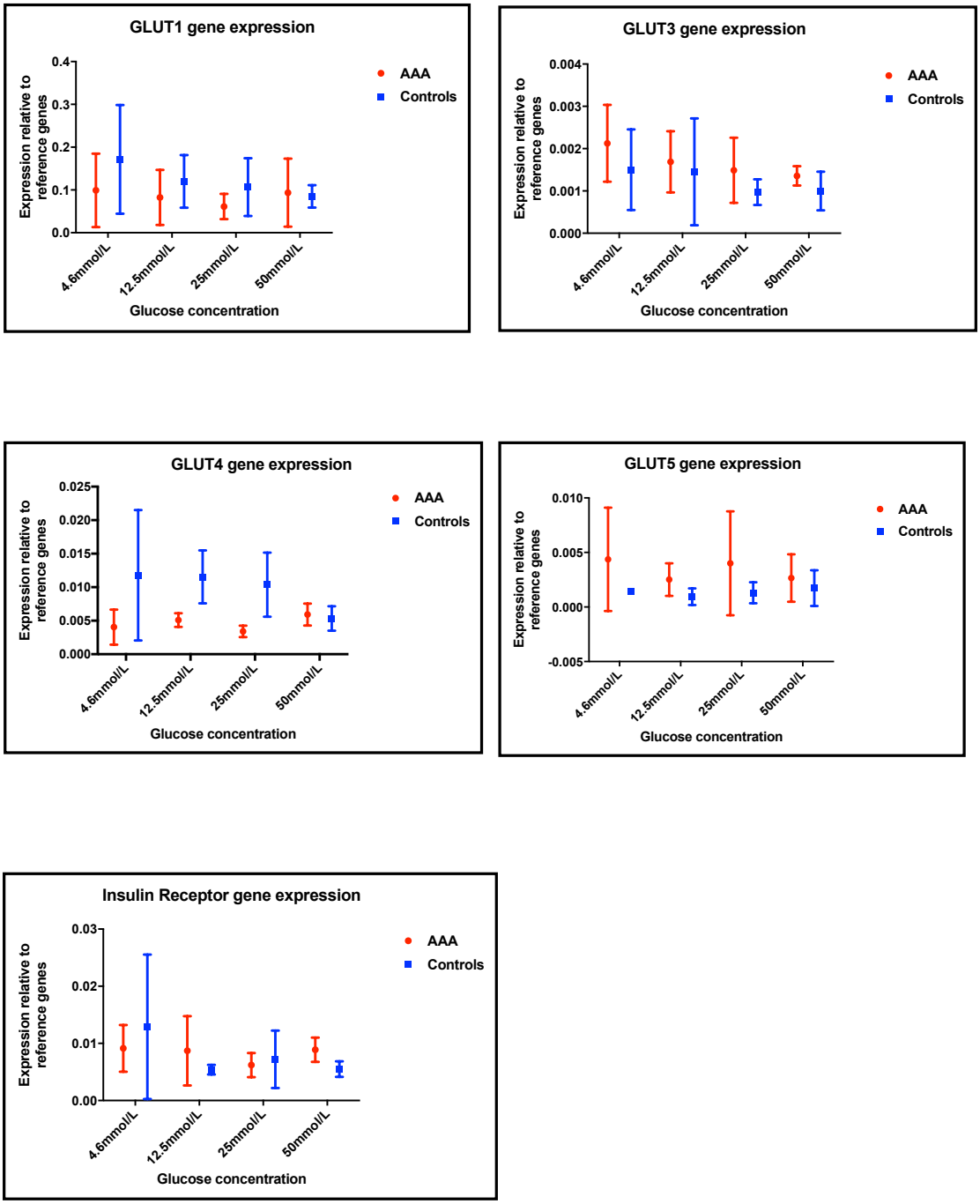


Figure 15.2. The effect of incremental hyperglycaemia on the mRNA expression of various glucose transporters and insulin receptor relative to β-actin and β2-microglbulin references genes in aortic smooth muscle cells from patients with (n=5) and without (n=5) AAA. Data are shown as mean and standard deviation.

No reliable gene expression data was obtained for GLUT6 and there was insufficient total RNA available to study gene expression of other GLUTs.

The effect of increasing hyperglycaemia on the mRNA expression of various cathepsins and tissue inhibitors of MMPs (TIMPs), which are implicated in the pathogenesis of AAA, was also examined. Exposure of AoSMCs from patients with and without AAA to incremental hyperglycaemia was not associated with any significant alterations in the mRNA expression of cathepsins B, H or L, nor was it associated with any significant alterations in the mRNA expression of TIMP-1 or TIMP-2 (see Tables 15.4 and 15.5 and Figure 15.3). No reliable gene expression data was obtained for cathepsin S.

Table 15.4. Mean gene expression values of various cathepsins and tissue inhibitors of matrix metalloproteinases in aortic smooth muscle cells from AAA patients (n=5) exposed to varying levels of hyperglycaemia. Kruskal-Wallis testing between different test mediums.

	Mean relative gene expression				
Test mediums	**Cathepsin B**	**Cathepsin H**	**Cathepsin L**	**TIMP-1**	**TIMP-2**
4.6mmol/L	0.150	0.00088	0.014	0.133	0.071
12.5mmol/L	0.198	0.00263	0.021	0.114	0.092
25mmol/L	0.171	0.00123	0.017	0.141	0.090
50mmol/L	0.163	0.00113	0.019	0.154	0.089
Kruskal-Wallis test	P=0.991	P=0.967	P=0.643	P=0.925	P=0.859

Table 15.5. Mean expression values of various cathepsins and tissue inhibitors of matrix metalloproteinases in aortic smooth muscle cells from control patients (n=5) exposed to varying levels of hyperglycaemia. Kruskal-Wallis testing between different test mediums.

	Mean relative gene expression				
Test mediums	**Cathepsin B**	**Cathepsin H**	**Cathepsin L**	**TIMP-1**	**TIMP-2**
4.6mmol/L	0.085	0.00053	0.0158	0.085	0.048
12.5mmol/L	0.140	0.00150	0.0165	0.143	0.078
25mmol/L	0.146	0.00078	0.0152	0.130	0.058
50mmol/L	0.079	0.00094	0.0116	0.095	0.058
Kruskal-Wallis test	P=0.840	P=0.891	P=0.780	P=0.773	P=0.596

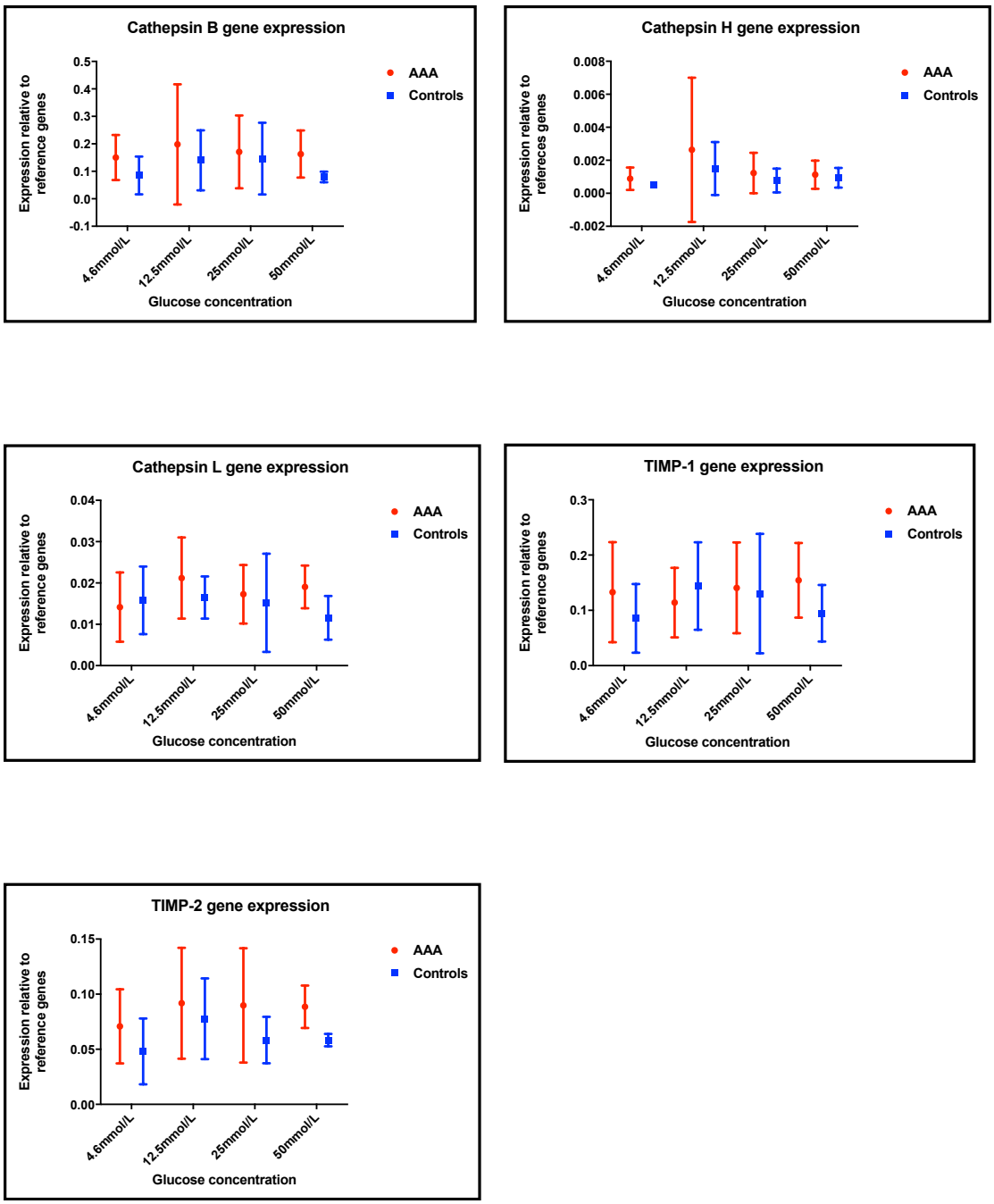


Figure 15.3. The effect of incremental hyperglycaemia on the mRNA expression of various cathepsins and tissue inhibitors of matrix metalloproteinases relative to β-actin and β2-microglbulin references genes in aortic smooth muscle cells from patients with (n=5) and without (n=5) AAA. Data are shown as mean and standard deviation.

15.7 Discussion

The main aim of this experiment was to determine whether hyperglycaemia, a characteristic feature of diabetes mellitus, is capable of significantly influencing the gene expression of glucose transporters and/or insulin receptor in aortic smooth muscle cells. In addition this experiment sought to determine whether hyperglycaemia might also alter the gene expression of other factors implicated in the pathogenesis of AAA. The effect of hyperglycaemia on the gene expression of matrix metalloproteinases has already been shown by previous researchers therefore this experiment chose to focus on endogenous tissue inhibitors of MMPs, known as TIMPs, as well as the family of proteolytic enzymes known as cathepsins.

The main results of this experiment were that incremental hyperglycaemia did not significantly influence the mRNA expression of GLUT1, GLUT3, GLUT4, GLUT4 or IR in AoSMCs from patients with or without AAA. Although on graphical analysis of the data there appeared to be a visual trend towards lower expression values for certain GLUTs with increasing hyperglycaemia, these trends were not statistically significant and therefore cannot be used to draw any further conclusions. In addition this experiment has shown no significant effect of incremental hyperglycaemia on the mRNA expression of cathepsins B, H or L, or TIMP-1 or TIMP-2, in AoSMCs from patients with or without AAA.

The findings from this experiment are in keeping with the findings from experimental work detailed in Chapter 13 which found no significant difference in the baseline mRNA expression of various GLUTs or insulin receptor between

AoSMCs from patients with AAA versus AoSMCs from patients without AAA. However, the findings from Chapter 13 were based on observations of larger patient numbers (n=8 versus n=16) compared to the number of patients in the current experiment (n=5 versus n=5). The reason for this was because a significantly greater amount of total RNA from each patient was needed to perform the experiments detailed in this chapter (8 times as much). This was due to both more genes being studied as well as gene expression being studied across 4 different glucose concentrations. Therefore, it is possible that the lack of a significant difference in gene expression between normoglycaemic and hyperglycaemic test mediums in the current experiment was as a result of a type 2 error. The results of this experiment should therefore be treated as pilot data and used to power future studies involving larger patient numbers.

15.7.1 Conclusions

In conclusion, the results of this experiment do not suggest that the expression of GLUTs and IR is down-regulated by the diabetic phenotype and therefore do not allow me to reject the second null hypothesis of this thesis. In addition, the expression of cathepsins and tissue inhibitors is not significantly altered by the diabetic phenotype.

Chapter 16: The effect of hyperglycaemia on activity of glucose transporters in aortic smooth muscle cells

16.1 Introduction

Circulating hyperglycaemia is the characteristic feature of diabetes mellitus, a condition negatively associated with the presence, growth and rupture of abdominal aortic aneurysms (AAA). The mechanisms underlying this relationship are poorly understood.

Previous experiments from this thesis have shown increased expression of glucose transporters (GLUTs) in aortic tissue from AAA patients as well as increased activity of GLUTs in aortic smooth muscle cells (AoSMCs) from AAA patients. Gene expression of GLUTs not was increased in AoSMCs from AAA patients at baseline and exposure of AoSMCs to culture conditions mimicking a diabetic milieu (see Chapter 15) did not significantly alter GLUT gene expression. However, a change in the activity of GLUTs in the absence of a change in expression is possible, as was shown in Chapters 13 and 14.

The aim of this experiment was to determine whether hyperglycaemia influences the activity of GLUTs in AoSMCs isolated from patients with and without AAA. The results of this experiment will allow me to support or reject the second null hypothesis of this thesis which states that "the expression and/or activity of glucose transporters will not be down-regulated by the diabetic phenotype".

Glucose transport activity assays will also be performed using an osmotic control to determine whether any findings from this experiment are related to hyperglycaemia or hyperosmolarity. In addition to using AoSMCs from patients with AAA, this experiment will also assess GLUT activity in normal AoSMCs which have been knocked down for the low-density-lipoprotein-related receptor protein 1 (LRP1) gene. LRP1 functions to maintain vascular wall integrity and therefore these modified AoSMCs should represent cells with an aneurysmal phenotype[416].

The majority of the methods used to perform this experiment have been described in earlier chapters but include explant culture from WATS, cell culture of AoSMCs, glucose transport activity assays and protein concentration assays. LRP1 knockdown in AoSMCs will be achieved through plasmid preparation, human embryonic kidney (HEK) 293T cell transfection and AoSMC infection.

16.2 Explant and cell culture

The explant culture of AoSMCs from WATS was performed as per the optimised protocol detailed in Chapter 13.2.

The culture of AoSMCs was performed as per the Phase 2 methods detailed in Chapter 15.3. Briefly, passage 4 (P4)/P5 AoSMCs were plated at a density of 100,000 cells/well in a 12-well plate in complete medium 231 (M231). After 48 hours the medium was changed to low serum M231 and the cells grown for 24 hours after which one of 4 test mediums was added to each well according to a specific plate layout. Each test medium was constituted of low serum M231 with a different concentration of glucose. AoSMCs were kept in test mediums for 18 hours before glucose transport activity assays were performed.

16.2.1 Time course experiment

The decision to incubate AoSMCs for 18 hours in hyperglycaemic test mediums was on the basis of a time course experiment.

Methods

Using P5 AoSMCs from a single patient (68 year old male), a time-course experiment was performed comparing the effect of incubating AoSMCs in hyperglycaemic medium over 3 different time points (18 hours, 10 hours and 1 hour) on GLUT activity. Briefly, AoSMCs were plated in 12-well plates at 100,000 cells/well in complete M231 and then serum starved with low serum M231 after 48 hours. After 24 hours test mediums were added to the 18-hour incubation

plate, after 32 hours test mediums were added to the 10-hour incubation plate and after 41 hours test mediums were added to the 1-hour incubation plate. Glucose transport activity assays were then performed for all 3 plates at the same time point as detailed in Chapter 14.3. All values were corrected for protein content as detailed in Chapters 14.4 and 14.5.

Results

There was a positive correlation between length of exposure and GLUT activity when AoSMCs were exposed to glucose levels within the physiological range (4.6mmol/L to 25mmol/L) (see Figure 16.1). At supraphysiological glucose levels (50mmol/L) this relationship between length of exposure and GLUT activity was not present suggesting that supraphysiological hyperglycaemia might be associated with disruption to normal cellular function.

Although this experiment was based on AoSMCs from only a single patient, the results also suggested that exposure to increasing levels of hyperglycaemia might be associated with a reduction in GLUT activity (see Figure 16.1). This phenomenon was most pronounced for AoSMCs exposed to 18 hours of test medium: comparing 25mmol/L vs. 4.6mmol/L test mediums; 32.3%, 19.8% and 18% decrease in GLUT activity with 18 hours, 10 hours and 1 hours exposure to hyperglycaemia respectively.

On the basis of these observations, 18 hours was chosen as the optimum time period in which to incubate AoSMCs in test mediums prior to performing GLUT activity assays.

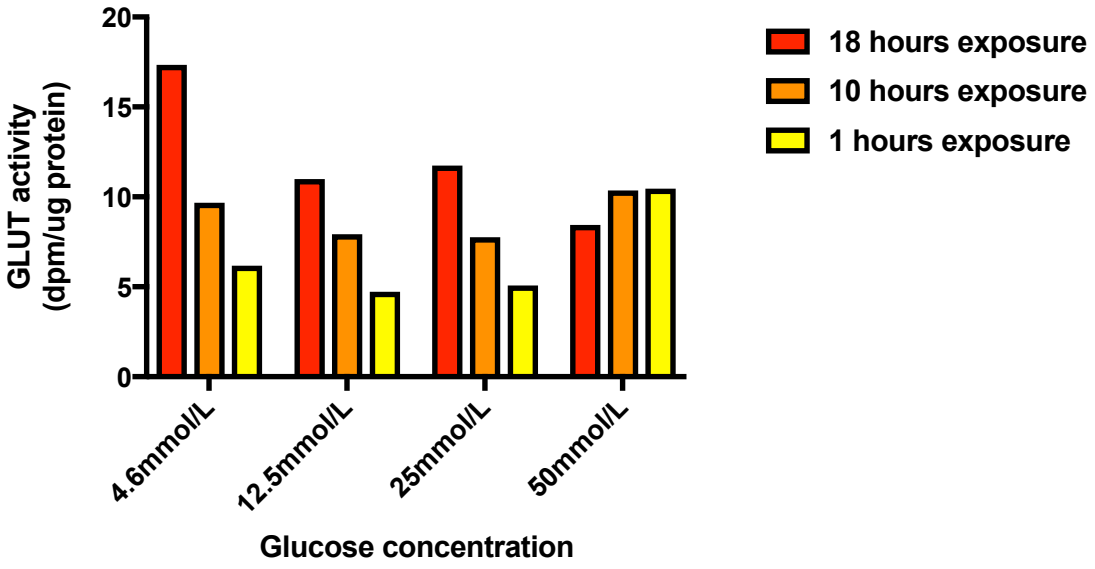


Figure 16.1. The effect of glycaemic test medium exposure over varying time periods (18 hours = red bars, 10 hours = orange bars and 1 hour = yellow bars) on glucose transporter activity in cultured aortic smooth muscle cells from a patient with AAA (n=1). Dpm = disintegrations per minute.

16.3 Main results

AoSMCs from 10 patients with and 10 patients without AAA were exposed to varying degrees of hyperglycaemia for 18 hours and GLUT activity measured. The demographics of patients are outlined in Table 16.1.

Table 16.1. Demographics of patients used to examine the effect of exposing aortic smooth muscle cells to hyperglycaemia on glucose transporter activity.

	AAA (n=10)	**Control (n=10)**
Median age, years (range)	68 (65 – 82)	59.5 (36 – 77)
Male sex, n (%)	7 (70)	7 (70)
Current smoker, n (%)	3 (30)	3 (30)
Ex smoker, n (%)	6 (60)	3 (30)
Diabetes, n (%)	0 (0)	0 (0)
Median AAA diameter, cm (range)	6.0 (5.5 – 7.5)	-
Median passage number (range)	5 (4 – 6)	4 (3 – 4)

Exposure of AoSMCs from patients with AAA to increasing levels of hyperglycaemia was not associated with a significant difference in the GLUT activity when comparing all 4 test mediums (see Table 16.2). However, visually there appeared to be a clear decrease in GLUT activity with increasing hyperglycaemia within the physiological range (i.e. up to 25mmol/L) which was lost at supraphysiological glucose levels (50mmol/L). Comparing GLUT activity between AoSMCs exposed to normoglycaemia and physiological hyperglycaemia revealed a significant decline in GLUT activity (4.6mmol/L vs. 25mmol/L test mediums; 25.42 vs. 18.11 dpm/µg protein; 28.8% decrease, p=0.013 on Mann Whitney U testing; see Figure 16.2).

Exposure of AoSMCs from control patients was not associated with a significant change in GLUT activity (see Table 16.2) and there did not appear to be a significant trend on visual analysis of the data (see Figure 16.2).

Table 16.2. Mean glucose transporter activity values in aortic smooth muscle cells from AAA (n=10) and control (n=10) patients exposed to varying levels of hyperglycaemia. Kruskal-Wallis testing between different test mediums.

	Mean GLUT activity values (dpm/µg protein)	
Test mediums	**AAA patients**	**Control patients**
4.6mmol/L	26.32	17.76
12.5mmol/L	23.06	17.56
25mmol/L	19.13	17.66
50mmol/L	21.34	24.40
Kruskal-Wallis test	P=0.211	P=0.565

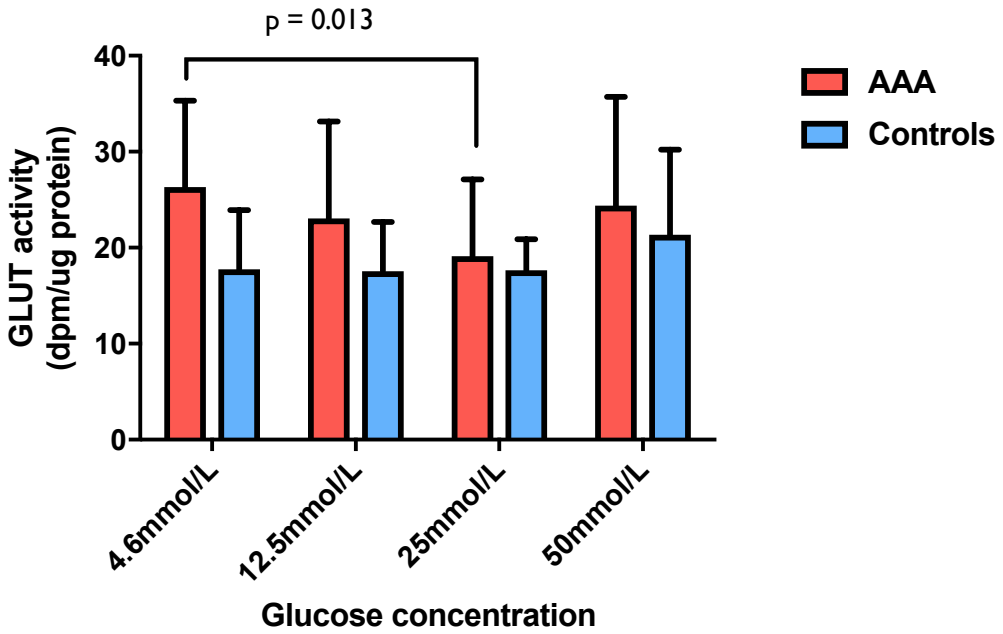


Figure 16.1. The effect of increasing hyperglycaemia on glucose transporter activity in aortic smooth muscle cells from patients with (n=10, red bars) and without (n=10, blue bars) AAA. Data are shown as mean and standard deviation. Mann Whitney U test between test medium glucose concentrations. Dpm = disintegrations per minute.

16.4 Osmotic control experiment

To determine whether the changes seen in GLUT activity described above were due to the effect of elevated glucose levels or due an increase in the osmotic pressure created by elevated glucose levels, an experiment substituting D-glucose for D-mannitol (molecular weight 182.17) (Sigma Aldrich) was performed. D-mannitol is an inert sugar alcohol and is frequently used to perform osmotic control experiments. Instead of incubating AoSMCs in hyperglycemic test mediums, hyperosmotic test mediums were created and used to incubate cells in under identical conditions to those described in Chapter 16.2.

16.4.1 Creation of hyperosmotic mediums

The hyperosmotic mediums were created in a similar fashion to the hyperglycaemic mediums (see Appendix 1) and therefore also contained a final FCS concentration of 0.49%.

In summary, to create a 10x stock of 12.5mM hyperosmotic M231, 143.91mg of D-mannitol were added to 10ml of complete M231, to create a 10x stock of 25mM hyperosmotic M231, 371.62mg of D-mannitol were added to 10ml of complete M231 and to create a 10x stock of 50mM hyperosmotic M231, 827.05mg of D-mannitol was added to 10ml of complete M231. Of note, no D-mannitol was added to the 10x stock solution of complete M231 used to create the 4.6mM test medium.

16.4.2 Results

AoSMCs from 2 patients with AAA were simultaneously exposed to 4 different levels of hyperglycaemia and 4 different levels of hyperosmolarity and GLUT activity compared.

There was a stepwise decrease in GLUT activity in the AoSMCs exposed to increasing hyperglycaemia within the physiological range (4.6mmol/L to 25mmol/L). However for the cells exposed to increasing hyperosmolarity, there was marginal decrease in GLUT activity between 4.6mmol/L and 12.5mmol/L (15.02 to 12.95 dpm/µg protein) which was not maintained out to 25mmol/L (13.96 dpm/µg protein) or 50mmol/L (14.81 dpm/µg protein) (see Figure 16.2). No statistical testing was performed due to the small number of patients within each treatment group (n=2).

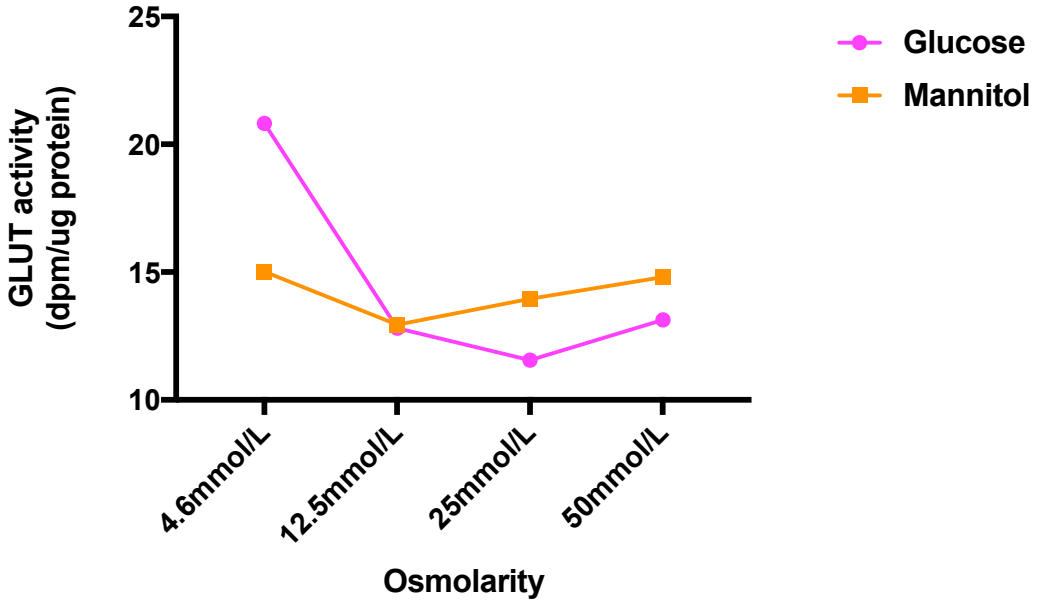


Figure 16.2. The effect of increasing osmolarity using D-glucose or D-mannitol on glucose transporter activity in AoSMCs (n=2 in each group). Data shown as mean value at each glucose concentration. Dpm = disintegrations per minute.

16.4.3 Conclusions

The results of this experiment suggest that the changes in GLUT activity seen with incremental hyperglycaemia in cultured AoSMCs from patients with AAA appear to be principally a hyperglycaemic effect rather than a hyperosmolar effect.

16.5 LRP1 knockdown in aortic smooth muscle cells

16.5.1 Introduction

Low density lipoprotein (LDL) receptor-related protein 1 (LRP1) is a 600 kDA receptor protein that is a member of the LDL receptor family and is found in the plasma membranes of cells involved in receptor-mediated endocytosis. It is encoded by the LRP1 gene which is situated on chromosome 12 in humans[437]. A recent genome-wide association study from the University of Leicester has found a significant association between a common genetic variant in the LRP1 gene and AAA even after adjustment for other cardiovascular risk factors[106]. Previous research suggests LRP1 is essential for vascular integrity and indeed LRP1 knockout is associated with aneurysm formation in mice[416].

Given the function of LRP1, it was hypothesised that knock down of LRP1 in normal AoSMCs would produce an aortic smooth muscle cell model with a similar phenotype to cultured AoSMCs from patients with AAA. The model was created with help and guidance from Dr Ana Verissimo using the methods detailed below.

The aim of this experiment was two-fold: firstly to confirm the aneurysmal phenotype of these modified AoSMCs and secondly to use these cells to validate the glucose transport activity findings seen in cultured AoSMCs from patients with AAA.

16.5.2 Methods

AoSMCs knocked down for LRP1 were created using small hairpin RNAs (shRNAs) targeting LRP1 and delivered by lentivirus. shRNAs are artificial RNA molecules with a tight hairpin turn that can be used to silence target gene expression by RNA interference (see Figure 16.3).

Figure 16.3. The molecular structure of siRNAs and shRNAs. A) siRNAs are short RNA duplexes with characteristic 2 nucleotide 3' overhangs. B) shRNAs consist of sense and antisense sequences separated by a loop sequence. Reproduced from online methods by O'Keefe[438].

The benefits of using shRNA over small interfering RNAs (siRNAs) to produce RNA interference are that shRNAs can produce interference in cells which are traditionally difficult to transfect and also that silencing via shRNA is more efficient since incorporation into the target cell genome takes place.

E.Coli growth and plasmid preparation

Large scale preparation of plasmids was performed from 200ml cultures of *E.Coli* bacteria grown in Luria-Bertani medium overnight using the GenElute™ Plasmid Maxiprep kit (Sigma Aldrich) according to the manufacturer's instructions.

Polyethylenimine (PEI) transfection of HEK 293T cells

Two lentiviral carrier plasmids containing the sequences for LRP1-targeting shRNA, as well as a non-targeting negative control were purchased from Open Biosystems.

Three million HEK 293T cells were seeded on 10cm plates, covered with complete DMEM medium and incubated at 37^{o}C for 24 hours. On the following day, the medium (without antibiotics) was replaced 2 hours before transfection. For the transfection, 9µg of DNA plasmid was mixed with 1ml of OptiMEM medium (ThermoFisher Scientific) and 36µL of PEI (1mg/ml). The DNA fraction was composed of 4.39µg of pGIPZ transfer vector (encoding green fluorescent protein (GFP), puromycin resistance and LRP1-targeting shRNA or non-targeting control shRNA), 3.29µg of packaging vector (encoding HIV Gag, Pol and Rev proteins) and 1.32µg of pMD2.G viral envelope vector. The tubes were then briefly vortexed and left to rest for 10 minutes to allow the PEI/DNA complexes to form. This solution was then added to the medium and gently mixed in. The cells were incubated at 37^{o}C for 48 hours and the virus-containing supernatants collected to further infect human normal aortic smooth muscle cells, after filtered (0.45µm) and supplemented with Polybrene (10µg/ml) (see Figure 16.4).

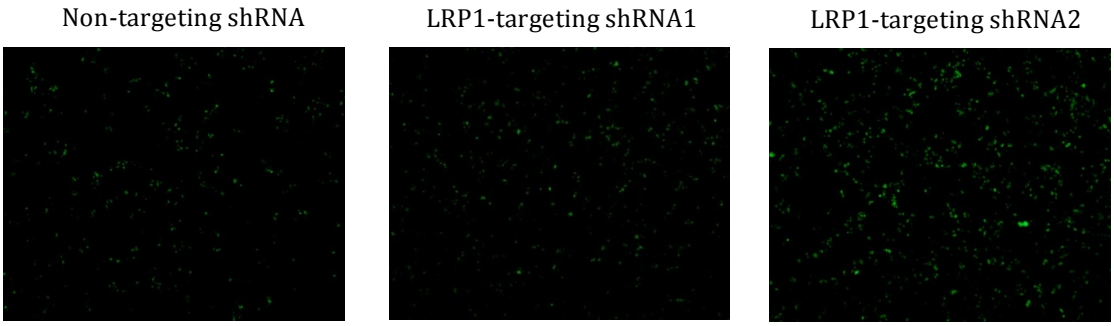


Figure 16.4. HEK 293T cells transfected with DNA plasmids encoding either non-targeting shRNA, LRP1-targeting shRNA1 or LRP1-targeting shRNA2. Green fluorescence indicates successful transfection. Images courtesy of Dr Ana Verissimo. Taken 48 hours after transfection at 40x magnification.

Normal AoSMC infection

One million normal commercially available P3 AoSMCs (ThermoFisher Scientific) were seeded onto 10cm plates, covered with complete medium 231 (M231) and incubated at 37°C for 24 hours. On the following day the medium was replaced with lentivirus-containing supernatants and incubated for 12 hours. Thereafter fresh M231 was added. The infected cells were allowed to grow for 48 hours and then 1µg/ml of puromycin was added to select a pure line of infected cells. The selection was performed over a period of 3 to 6 days with fresh puromycin-containing medium changes every 2 days (see Figure 16.5). Infected AoSMCs were then plated at a density of 50,000 cells/well in 12-well plates in M231 to test the effect of LRP1 knockdown on GLUT activity using the 90-hour protocol detailed in Chapter 16.2. A subset of infected cells was used to confirm LRP1 knockdown and aneurysmal phenotype.

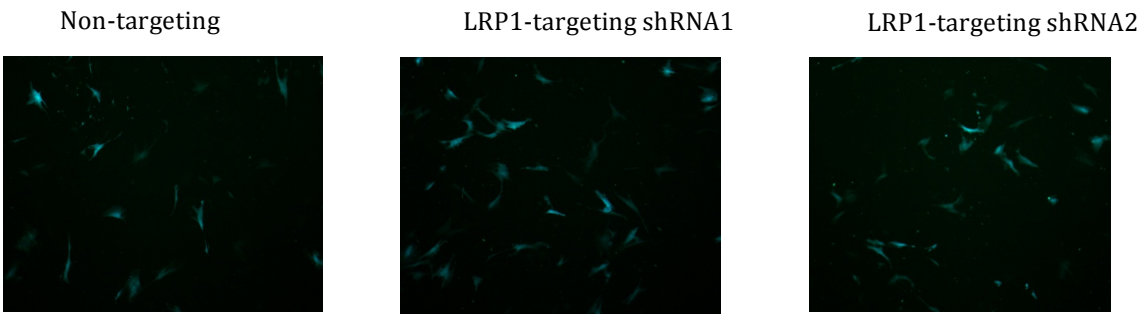


Figure 16.5. Normal aortic smooth muscle cells infected with lentivirus produced in HEK 293T cells, after puromycin selection. Green fluorescence indicates successful infection. Images courtesy of Dr Ana Verissimo. Images taken 96 hours after infection at 40x magnification.

Confirming LRP1 knockdown and aneurysmal phenotype

After confirming successful AoSMC infection, infected AoSMCs were lysed in Trizol LS and total RNA extracted as detailed in Chapter 13.4. Gene expression of LRP1 and MMP-2 was quantified using quantitative polymerase chain reaction (qPCR) and SYBR green technology as detailed in Chapter 13.5. Successful knockdown of the LRP1 gene was achieved using both LRP1-targeting shRNA sequences (35% to 50% knockdown relative to control; see Figure 16.6). In addition, mRNA expression of matrix metalloproteinase (MMP-2), one of the key enzymes regulating extracellular matrix degradation in AAA pathogenesis, was significantly increased by LRP1 knockdown (see Figure 16.5).

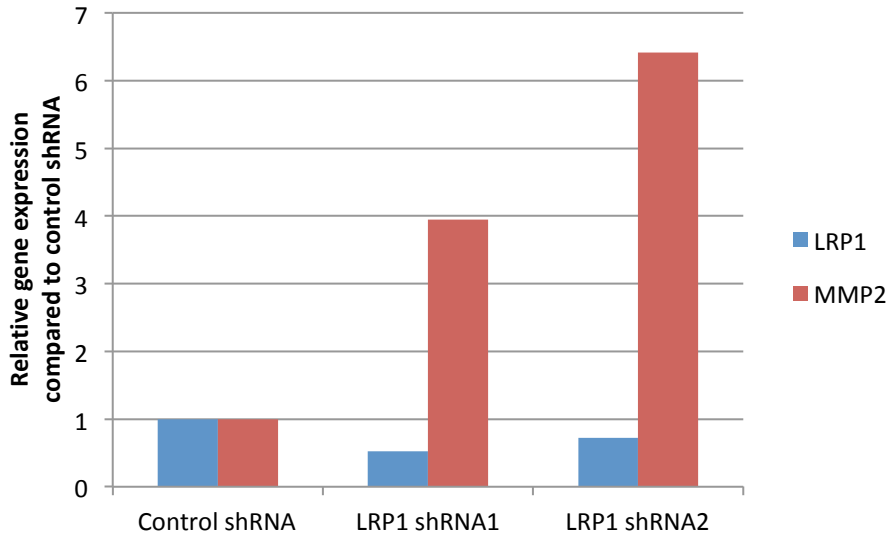


Figure 16.6. Relative mRNA expression of LRP1 (blue bars) and MMP-2 (red bars) genes in normal human aortic smooth muscle cells infected with lentivirus produced in HEK 293T cells, after puromycin selection. Figure courtesy of Dr Ana Verissimo. shRNA = small hairpin ribonucleic acids.

16.5.3 Results

GLUT activity assays were performed on infected AoSMCs grown in both normoglycaemic (4.6mmol/L) and hyperglycaemic (25mmol/L) M231 to test the effect of hyperglycaemia.

LRP1 knockdown using shRNA sequence 1 or 2 was associated with increased GLUT activity in normal AoSMCs compared to non-targeting control shRNA (see Figure 16.7). For AoSMCs cultured in normoglycaemic medium, LRP1 knockdown using shRNA-1 increased GLUT activity by 33.6% versus control shRNA whilst knockdown using shRNA-2 increased GLUT activity by 161.1% versus control shRNA (see Figure 16.7). For AoSMCs cultured in hyperglycemic medium, this effect was still present but much less pronounced; LRP1

knockdown using shRNA-1 increased GLUT activity by 19.2% whereas using shRNA-2 increased GLUT activity by 79.1% (see Figure 16.7).

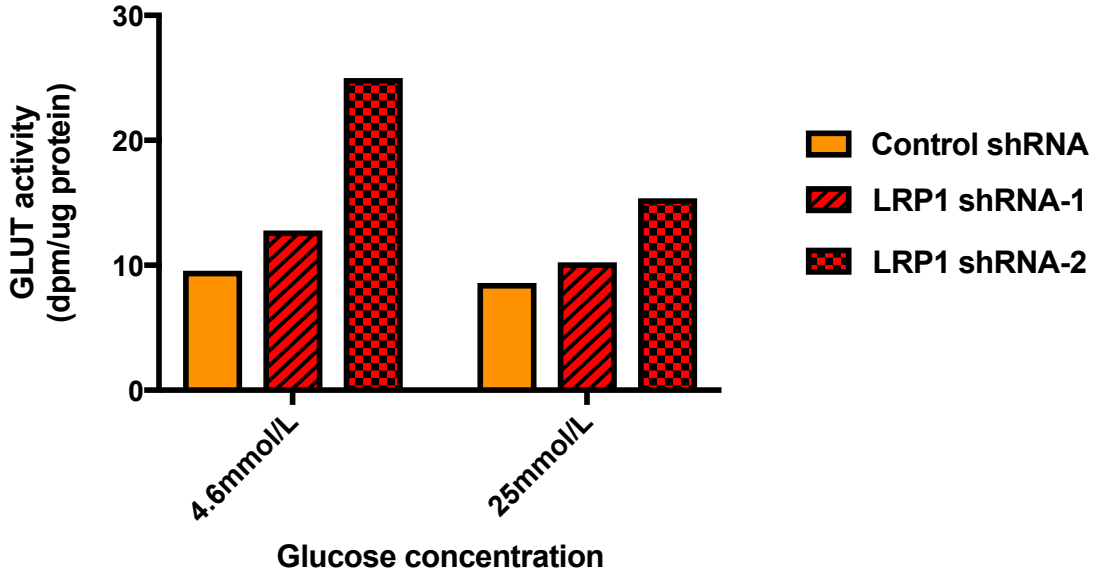


Figure 16.7. The effect of LRP1 knockdown using 2 different LRP1-targeting shRNAs on glucose transporter activity in normal aortic smooth muscle cells cultured in normoglycaemic (4.6mmol/L) and hyperglycemic (25mmol/L) medium 231. Data are shown as actual glucose transporter activity values. Dpm = disintegrations per minute.

16.5.4 Conclusions

In conclusion, LRP1 knockdown in normal AoSMCs was feasible and produced AoSMCs with an aneurysmal phenotype, characterised by elevated MMP-2 expression and increased GLUT activity. Exposure of these AoSMCs to 25mmol/L of hyperglycaemia was associated with a reduction in GLUT activity, which supports the findings from the main experiment detailed in Chapter 16.3 utilising AoSMCs from patients with AAA.

16.6 Discussion

Previous experiments have suggested that hyperglycaemia does not significantly influence mRNA expression of glucose transporters (GLUTs) in cultured AoSMCs from patients with or without AAA. The aim of this experiment was to determine whether hyperglycaemia was associated with a change in the activity of GLUTs in AoSMCs from patients with or without AAA.

This main results of this experiment were that hyperglycaemic pre-conditioning of AoSMCs was associated with a significant decline in the activity of GLUTs in cultured AoSMCs from patients with AAA but not in AoSMCs from patients without AAA. An osmotic control experiment suggested that this relationship was likely to mediated via hyperglycaemia per se rather than hyperosmolarity. Furthermore this relationship was only true for hyperglycaemia within the physiological range (up to 25mmol/L) but was lost at supraphysiological levels of hyperglycaemia (50mmol/L). The reasons for this are unclear but maybe related to such high glucose concentrations inducing a state of metabolic stress within the cells thereby causing them to transport more glucose.

The results of this experiment are novel since they show selective suppression of GLUT activity in AoSMCs from patients with AAA. Whether this is because AoSMCs from AAA patients have higher levels of GLUT activity under baseline conditions than cells from non-AAA patients (see Chapter 14) and therefore hyperglycaemic pre-conditioning is simply 'normalising' the GLUT activity in these cells in unclear. An alternative hypothesis would be that AoSMCs from AAA patients become phenotypically different to AoSMCs from non-AAA patients

and are therefore 'programmed' to respond to glucose differently. Indeed a recent study from Dr Karen Porter's group in Leeds has suggested that AAA development is associated with a progressive change in the phenotype of AoSMCs to one characterised by premature vascular ageing however this study did not specifically examine their response to glucose[439]. Further research is still needed in this area.

To validate the main findings of this experiment, I also performed GLUT activity assays in normal AoSMCs which had undergone lentiviral-mediated knockdown of the LRP1 gene using 2 different shRNAs. With both LRP1-targeting sequences there was a corresponding increase in the expression of MMP-2 and activity of GLUTs, compared to the negative control shRNA, suggesting that increased GLUT activity is characteristic of the AAA phenotype. In addition, exposure of the cells knocked down for LRP1 to physiological hyperglycaemia was associated with a marked decrease in GLUT activity thereby supporting the main results of this experiment.

16.6.1 Conclusions

In conclusion, the results of this experiment suggest that GLUT activity is down-regulated by the diabetic phenotype, at least in AoSMCs from patients with AAA, and therefore allow me to reject the second null hypothesis of this thesis.

Preliminary work

Chapter 17: Protein expression of glucose transporters in aortic smooth muscle cells

17.1 Immunocytochemistry

Quantification of GLUT protein expression in AoSMCs was initially attempted using immunocytochemistry however despite multiple attempts at optimisation over a 6 week period, successful immunocytochemistry for GLUTs or insulin receptor could not be achieved (no demonstrable immunofluorescence for GLUT1, GLUT3 or insulin receptor).

Optimisation experiments included:

1) Using different plating densities (50,000 cells/well, 100,00 cells/well, 200,000 cells/well)

2) A trial of manual (Gilson) versus electric pipettes to see if this affected cell adherence to cover slips

3) A change of wash buffer and antibody diluent from Tris-buffered saline with 0.1% Triton to phosphate buffered saline as Triton can acts as a detergent and can therefore affect proteins with the cell membrane such as glucose transporters

4) A trial of staining commercially available AoSMCs (Invitrogen).

None of these optimisation experiment yielded successful visualisation of GLUTs or insulin receptor and therefore I eventually abandoned this method of quantification and decided that western blotting for glucose transporters would be more appropriate.

17.2 Western blotting

17.2.1 Principles of western blotting

Western blotting is a technique used for the detection and analysis of proteins based on their ability to bind to specific antibodies. The first step involves separation of proteins based on their molecular weight by making them pass through a polyacrylamide gel using an electrical current, a process known as sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE). Smaller proteins move towards the anode faster than bigger proteins. Prior to SDS-PAGE, chemicals such as 2-mercaptoethanol or dithiothreitol (DTT) are added to the samples to reduce disulphide bonds between proteins as well as a sample buffer containing SDS such as Laemelli buffer which is negatively charged and binds to the proteins. After the gel has run, it is placed against a membrane and a current is run across the gel thereby transferring the proteins onto the membrane (Figure 17.1) [440].

The membrane is then washed in a non-specific protein which binds to the membrane where protein is not already present. Next the membrane is washed with specific antibody to the protein of interest (the primary antibody) which recognises a specific amino acid sequence. The unbound primary antibody is removed and the membrane then washed with another antibody that recognises the primary antibody (the secondary antibody). The secondary antibody is usually conjugated to an enzyme such as horseradish peroxidase (HRP) which is used to create a chemiluminescent reaction on addition of a chemiluminescent

substrate to the membrane. The emitted light from this reaction is detected using photographic film or a digital equivalent (Figure 17.2) [440].

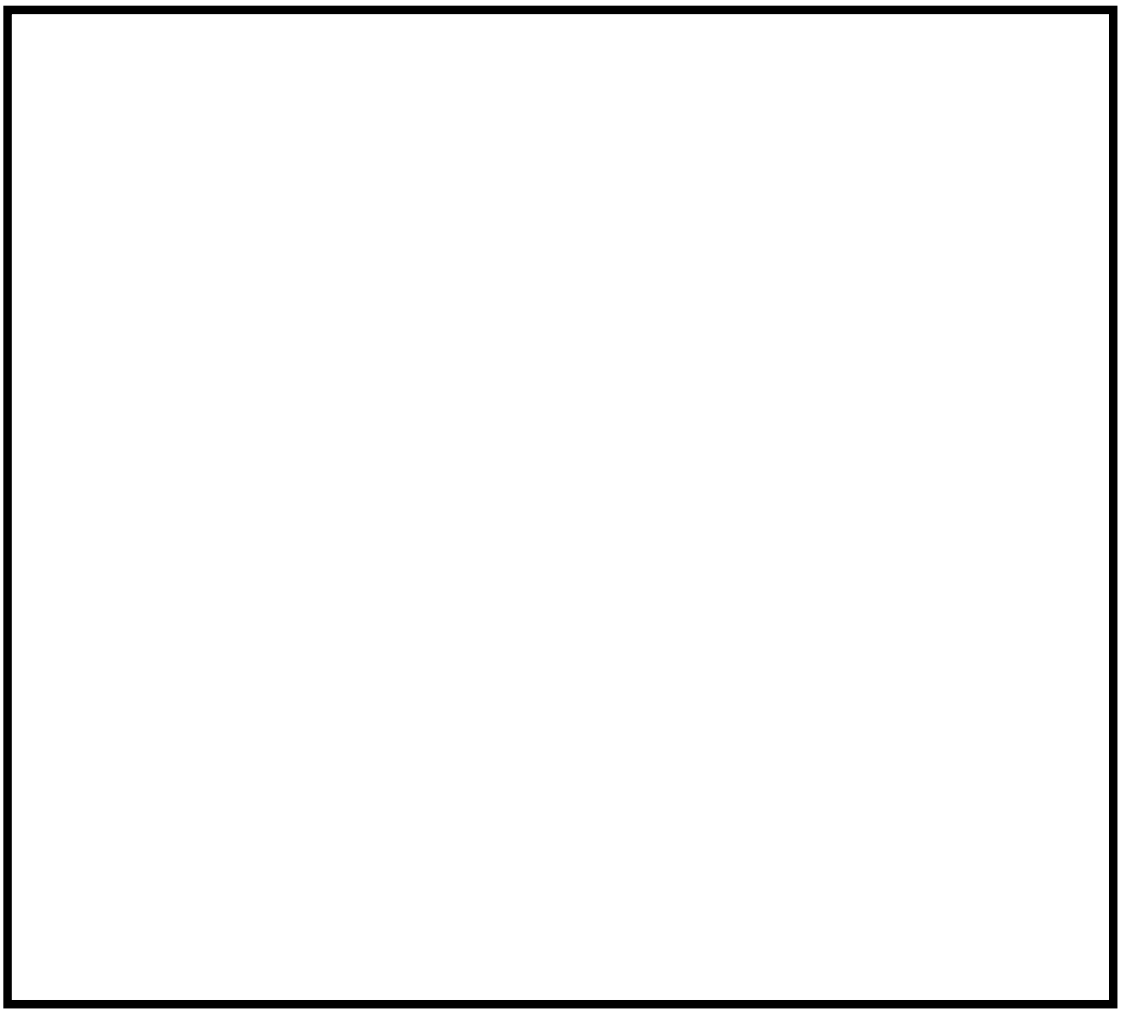


Figure 17.1. Top image, Representation of SDS-polyacrylamide gel electrophoresis showing movement of proteins from the negative to the positive electrode. Bottom image, Representation of the semi-dry method for electrophoretic transfer of proteins from the gel to the membrane. Taken from www.vlab.amrita.edu with permission[440].

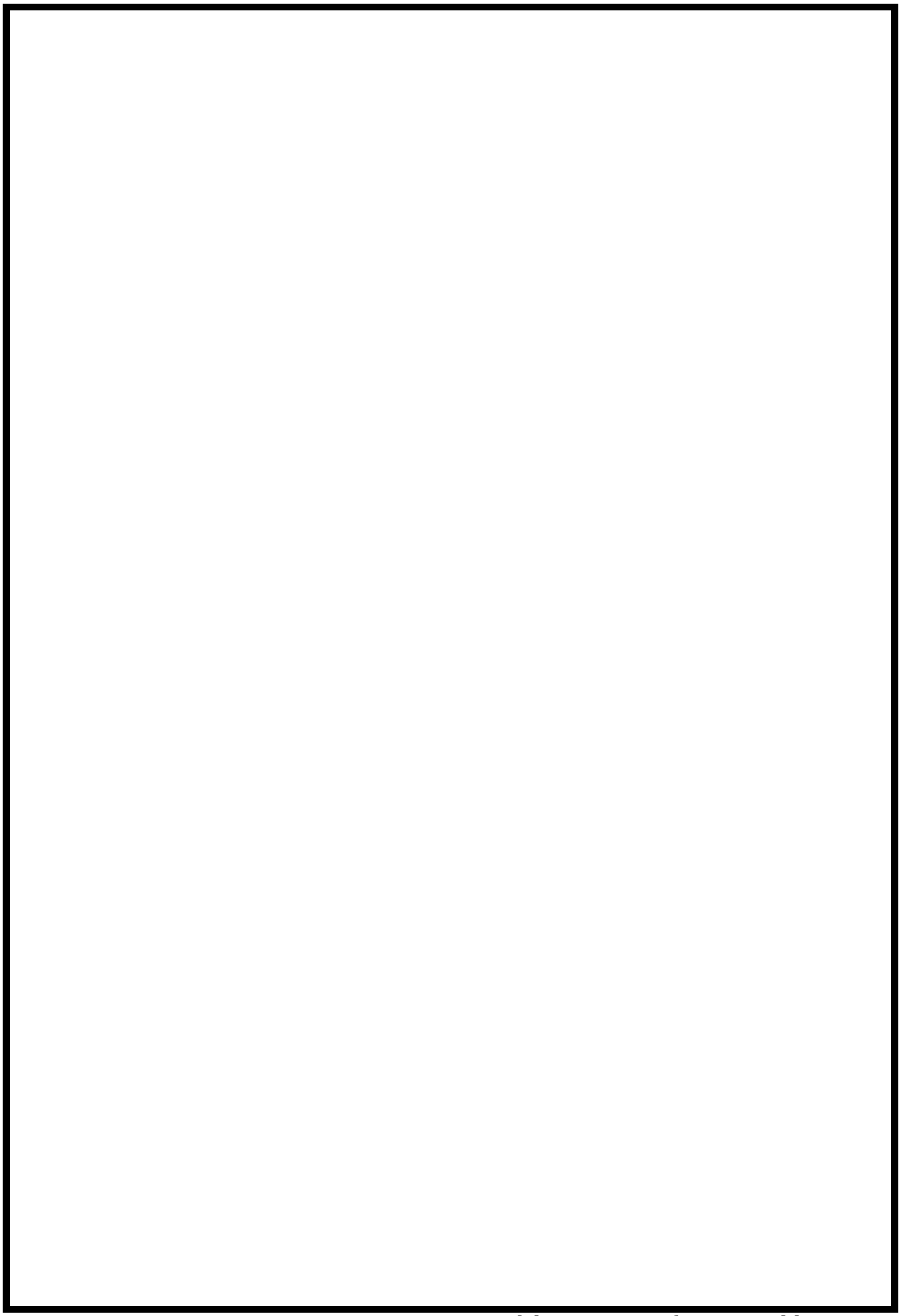


Figure 17.2. Diagrammatic representation of the process of Western blotting including non-specific protein blocking (top insert), primary and secondary antibody incubations (second and third inserts), and addition of chemiluminescent substrates (fourth insert) to allow quantification. Taken from www.vlab.amrita.edu with permission[440].

17.2.2 Collecting samples for western blotting

I discussed using western blotting to quantify protein content with one of the post-doctoral fellows in our laboratory, who advised that I should collect the AoSMCs in a lysis buffer prior to western blotting. I was recommended a homemade protein lysis buffer composed of:

- 1% NP40 (ThermoFisher),
- 1% Octyl-beta-d-glucopyranoside,
- 20mM Tris-HCl pH 7.5,
- 300mM NaCl
- 2mM EDTA
- 1% protease inhibitor cocktail

AoSMCs were harvested for western blotting in this protein lysis buffer using the following protocol. After culturing AoSMCs as per the 96-hour protocol outlined in Chapter 15.3, test medium was removed from each well of a 12-well plate and 200µL of the homemade protein lysis buffer was added to 2 of the 3 wells at each glucose concentration (1 of the 3 wells was used to harvest cells in Trizol for gene expression studies). Whilst tilting the 12-well plate to 45^{o}, the buffer was washed across of the face of each well at least 10 times before pipetting the cells up and down for 20 seconds and collecting the cell lysates in eppendorf tubes. Cell lysates were stored at -80^{o}C.

Given the lack of experience in performing western blotting for GLUTs in our laboratory as well as the difficulties I encountered in successfully performing immunocytochemistry for GLUTs, I decided that collaborating with another

laboratory more experienced at performing western blotting for GLUTs would be sensible. Therefore I arranged a meeting with Professor Geoff Holman at the University of Bath who agreed that I should come and learn how to perform western blotting for glucose transporters in his laboratory. However during the meeting, Professor Holman advised that octyl-beta-d-glucopyranoside was a non-ionic detergent and therefore not ideally suited for running SDS-PAGE. He suggested that I switch to using Novex Tris-glycine SDS-sample buffer (ThermoFisher) as a lysis buffer for future samples but advised that I bring a subset of my cell lysates in NP40/octyl-beta-d-glucopyranoside to Bath to see how they compared.

17.2.3 Methods used during optimisation experiments

Samples

In total, 16 samples were taken to Bath to learn how to perform western blotting for GLUTs. 8 of these samples were AoSMC lysates in Novex Tris-Glycine SDS-sample buffer (4 each from patients with and without AAA), 4 were cell lysates in homemade NP40/octyl-beta-d-glucopyranoside lysis buffer (2 each from patients with and without AAA) and 4 were cell lysates in homemade NP40/octyl-beta-d-glucopyranoside lysis buffer that had already been mixed 1:1 with SDS-sample buffer and boiled (2 each from patients with and without AAA).

SDS-PAGE

First I learnt how to create 10% SDS-PAGE gels for use with the BIO-RAD mini-gel electrophoresis system. The compounds outlined in Table 17.1 were

sufficient to create 2 10% resolving gels and 4 stacking gels. 10% resolving gels were chosen since they allow ~50kDA glucose transporter proteins to reach the middle of the gel. I learnt that the ammonium persulphate and tetramethylethylenediamine (TEMED) need to be added just prior to adding to the solution to the glass moulds as these are the catalysts for acrylamide polymerisation and also that adding a small volume of propan-2-ol on top of the resolving gel solution in the mould will to get rid of any air bubbles thereby creating a gel with a smooth line. A comb was used to create 10-lanes in the stacking gel.

Table 17.1. Compounds and quantities required to create two mini-gels for use with the Bio-Rad mini-gel electrophoresis system.

Stock solution	**Resolving Gel**	**Stacking Gel**
	10 %	*6%*
Acrylamide stock solution (ml)	5	3.4
Resolving gel buffer (ml)	5	-
Stacking gel Buffer (ml)	-	5.0
Double-distilled water (ml)	5	7.5
Ammonium persulphate (μl)	100	100
TEMED (ml)	8	20

The ingredients used to create the buffers listed above are shown in Appendix 1.

Eight gels were created in total - five gels used to run the 8 samples in Novex Tris-Glycine SDS-sample buffer and three gels used to run the 8 samples in NP40/octyl-beta-d-glucopyranoside. Prior to loading the samples into the gel, DTT was added to each sample in a 1:50 dilution. The 10 lanes of each gel were

loaded as shown below and gels were run at a constant current (25 mA) for approximately 15 hours until the bromophenyl blue tracking dye had just run off the gel.

Lane 1 – Novex Sharp Protein Standard used as a ladder (8µL)

Lane 2 – Cell lysate 1 (40µL)

Lane 3 – Cell lysate 2 (40µL)

Lane 4 – Cell lysate 3 (40µL)

Lane 5 – Cell lysate 4 (40µL)

Lane 6 – Cell lysate 5 (40µL)

Lane 7 – Cell lysate 6 (40µL)

Lane 8 – Cell lysate 7 (40µL)

Lane 9 – Cell lysate 8 (40µL)

Lane 10 – Adipocyte lysate used as a positive control (20µL)

Note, 40µL of cell lysate was added to each well as this was previously shown to approximately equate to 20µg of total protein in my samples.

Electrophoretic transfer to nitrocellulose

Next the gels were transferred to a nitrocellulose membrane using the semi-dry method detailed below. The area of gel was measured (usually 8.5 cm × 5.5 cm for minigels) and then 2 sheets of filter paper and 1 sheet of nitrocellulose membrane (GelmanSciences) cut to size (for BioRad mini-gels cut 9cm x 5.5cm). The gel was then soaked in SDS-Transfer Buffer. SDS-Transfer Buffer was made by dissolving 11.63g of Tris, 5.89g of glycine, and 0.75g of SDS in 1600 ml of distilled water and adding 400ml of methanol to create a solution with final pH 8.

Then 9 sheets of cut filtered paper were soaked in the transfer buffer and placed on the anode surface of the BioRad electrophoretic transfer unit, wetting the surface beforehand with distilled water. The air bubbles were smoothed out with a pasteur pipette. The process was repeated for the sheet of nitrocellulose, the gel, and another 9 sheets of filter paper, each component placed on top of one another, in that order. The cathode surface of the transfer unit was then wet before placing it on top of the anode.

The area of the gel (in centimeters) was multiplied by 0.8 to obtain the current (in mA) and the electrophoresis was run for an hour and three-quarters with low voltage. After transfer, the nitrocellulose membrane was rinsed in distilled water before staining with a small amount of 0.1% Ponceau S in 3% trichloroacetic acid (see Figure 17.3 for example). The membrane was rinsed again with distilled water again and air-dried before marking the molecular weight markers on the membrane with a pencil.

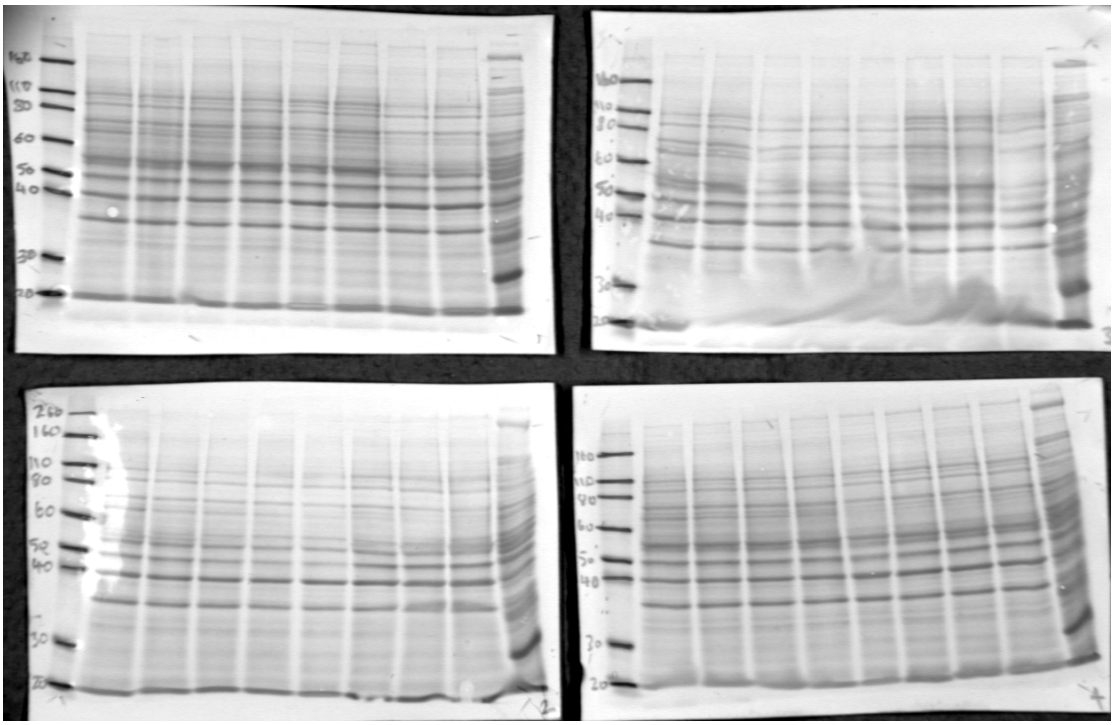


Figure 17.3. Photograph of the nitrocellulose membranes from gels 1 to 4 stained with 0.1% Ponceau S showing a good spread and transfer of proteins to the membranes.

Western blotting

Finally western blotting of the nitrocellulose membrane for the proteins of interest (GLUT1, GLUT3, GLUT4, GLUT6, GLUT10, insulin receptor (IR) and GAPDH) was performed using the protocol detailed below.

The nitrocellulose membrane was first washed in Tris-buffered saline buffer (0.09% NaCl, 1M Tris-HCl, pH 7.4) containing Tween-20 at a final concentration of 0.1% (TBS-T) to remove the Ponceau S stain. Next the membranes were placed in blocking solution consisting of 5% dried skimmed milk powder (Marvel) in TBS-T, and incubated with gentle rocking for 30-60 min at room temperature or overnight at 4°C. This procedure was to block non-specific binding.

The nitrocellulose membranes were next rinsed in TBS-T and the membranes cut at 40kDA so that a single membrane could be used to blot for both the respective GLUT (~50+ kDA) as well as GAPDH (37kDA), which was used as a loading control. In addition, for the membrane used to blot against GLUT4, the membrane was additionally cut at 70kDA so that the top portion could be blotted separately for the IR β subunit (MW 95kDA) without needing to run a second gel (see Figure 17.4).

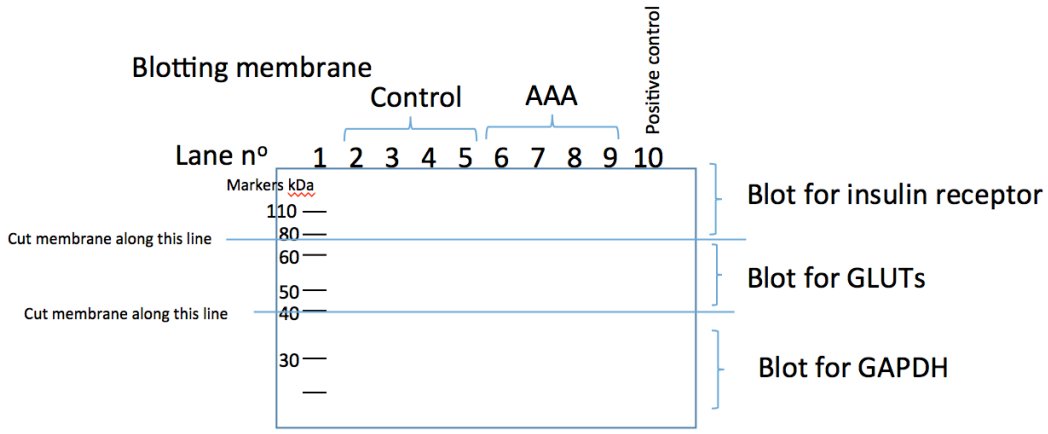


Figure 17.4. Schematic representation of the nitrocellulose membrane from gel electrophoresis of 8 aortic smooth muscle cell lysates (4 each from patients with and without AAA) used to blot against GLUTs, insulin receptor and GAPDH.

The respective part of the membrane was then incubated with the respective primary antibody at the dilutions shown in Table 17.2, in TBS-Tween containing 1% bovine serum albumin overnight at 4°C. The membranes were then washed extensively in TBS-T (at least 6 washes of 5 min each with big volumes of TBS-T) before incubating the membranes for 60 min at room temperature in the

appropriate secondary antibody, according to host species (see Table 17.2), at the required dilution (either anti-rabbit IgG-HRP conjugate at 1:4000 or anti-mouse IgG-HRP conjugate at 1:4000) in 5% milk TBS-T solution. Finally the membranes were washed again extensively before being exposed with a standard ECL agent (ThermoFisher) for 1 or 5 minutes depending on the signal strength. For blots with poor signal after 5 minutes exposure using the standard ECL, Supersignal West Dura Extended duration substrate (ThermoFisher) was used to improve the signal further.

Table 17.2. Dilutions, host species and source of primary antibodies used in western blotting for various proteins during optimisation experiments.

Protein of interest	Dilution	Host species	Source
GLUT1	1:1000	Rabbit	Holman group (in-house)
GLUT3	1:1000	Rabbit	Abcam
GLUT4	1:5000	Rabbit	Holman group (in-house)
GLUT6	1:500	Mouse	Abcam
GLUT10	1:1000	Rabbit	Novus Biologicals
Insulin Receptor	1:500	Rabbit	Santa Cruz
GAPDH	1:1000	Mouse	Abcam

17.2.4 Results

Gels 1 and 5

The nitrocellulose membranes from gel 1 and gel 5 were used to blot expression of GLUT4 and IR, however gel 1 was loaded with samples harvested in Novex Tris-Glycine SDS-sample buffer whereas gel 5 was loaded with samples harvested in NP40/octyl-beta-d-glucopyranoside. GAPDH was also blotted for as a loading control. Using standard ECL for 1 minute produced blots with poor signal for GLUT4 and IR and therefore these membranes were incubated for 5 minutes in ECL to produce good quality blots (see Figures 17.5) for the samples harvested in Novex Tris-Glycine SDS-sample buffer. For the samples harvested in NP40/octyl-beta-d-glucopyranoside, good quality blots could not be achieved using the same ECL conditions (see Figure 17.6) and using SuperSignal West Dura Extended duration substrate for 5 minutes did not significantly improve this.

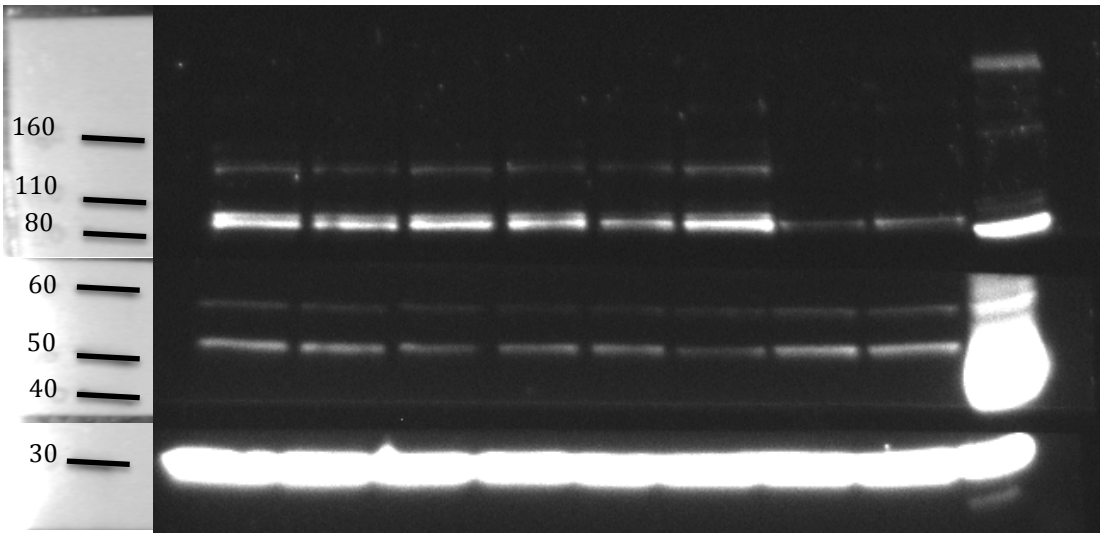


Figure 17.5. Western blot of gel 1 with superimposed ladder (lane 1) showing high intensity band for GAPDH at ~35kDA in lanes 2 to 10, weak intensity bands of GLUT4 at ~54kDA in lanes 2 to 9 and medium intensity bands for IR at ~95kDA in lanes 2 to 9. Lane 10 used as positive control (rat adipocyte lysate). Samples harvested in Novex Tris-Glycine SDS-sample buffer. Taken after 5 minutes exposure to standard ECL.

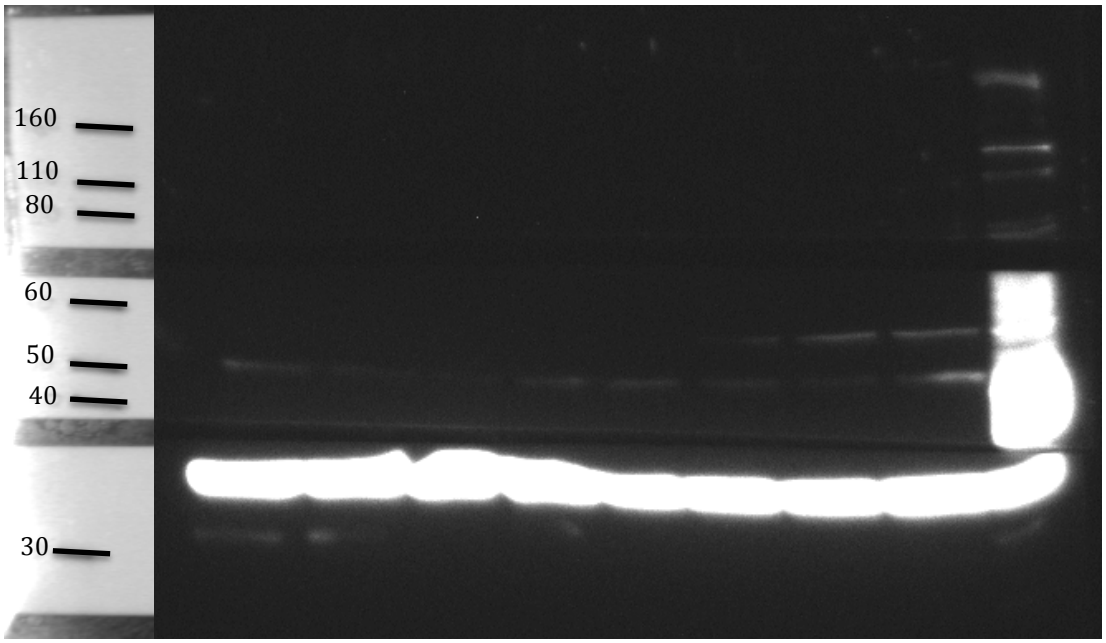


Figure 17.6. Western blot of gel 5 with superimposed ladder (lane 1) showing high intensity band for GAPDH at ~35kDA in lanes 2 to 10, no significant bands for GLUT4 at ~54kDA in lanes 2 to 9 and no bands for IR at ~95kDA in lanes 2 to 9. Lane 10 used as positive control (rat adipocyte lysate). Samples harvested in NP40/octyl-beta-d-glucopyranoside. Taken after 5 minutes exposure to standard ECL.

Gels 4 and 6

The nitrocellulose membranes from gel 4 and gel 6 were used to blot expression of GLUT1, however gel 4 was loaded with samples harvested in Novex Tris-Glycine SDS-sample buffer whereas gel 6 was loaded with samples harvested in NP40/octyl-beta-d-glucopyranoside. Using standard ECL for 1 minute produced blots with low signal for GLUT1 and therefore these membranes were incubated for 5 minutes in standard ECL to produce visible blots. Comparing Figure 17.7 and 17.8 it can be seen that the bands are markedly more discrete in Figure 17.7 which used samples collected in Novex Tris-Glycine SDS-sample buffer. This confirms the findings from Figures 17.5 and 17.6 which also show clearer blots using samples collected in Novex Tris-Glycine SDS-sample buffer.

Although lanes 2 to 9 do show a band at around ~54kDA, which corresponds to the molecular weight for GLUT1, these experiments used rat adipocyte lysates as the positive control rather than erythrocyte membranes which are more specific and hence a better positive control for western blotting GLUT1. This was due to the unavailability of prepared erythrocyte membranes whilst performing the optimisation experiments in the Holman laboratory. In addition both Figure 17.7 and 17.8 show bands are molecular weights that are not typical for GLUT1. The nature of these bands is unclear.

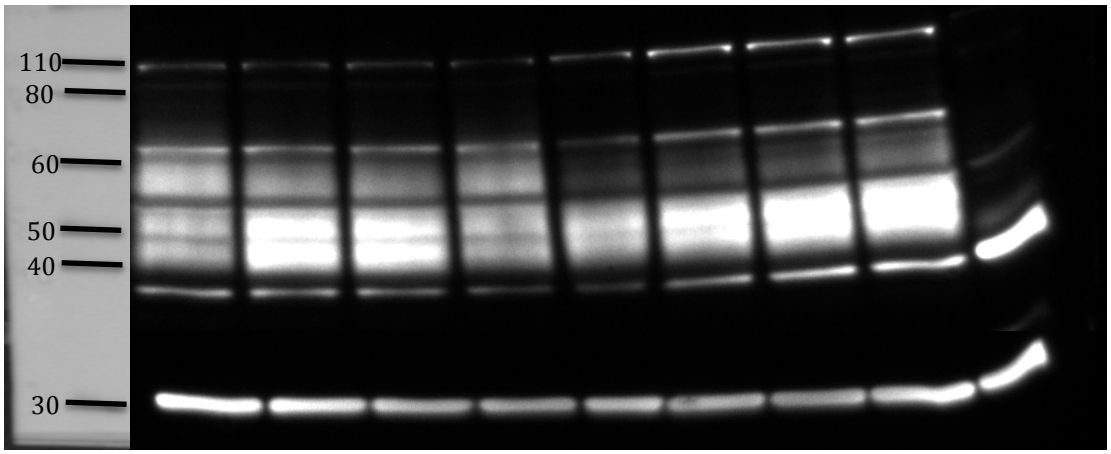


Figure 17.7. Western blot of gel 4 with superimposed ladder (lane 1) showing prominent band for GLUT1 at~54kDA and GAPDH at ~30kDA but also bands at ~70kDA, ~45kDA and ~38kDA in lanes 2 to 9. Lane 10 only shows band at ~38kDA however rat adipocyte lysate used as positive control rather than erythrocyte membranes which are more specific for GLUT1. Samples harvested in Novex Tris-Glycine SDS-sample buffer. Taken after 5 minutes exposure to standard ECL.

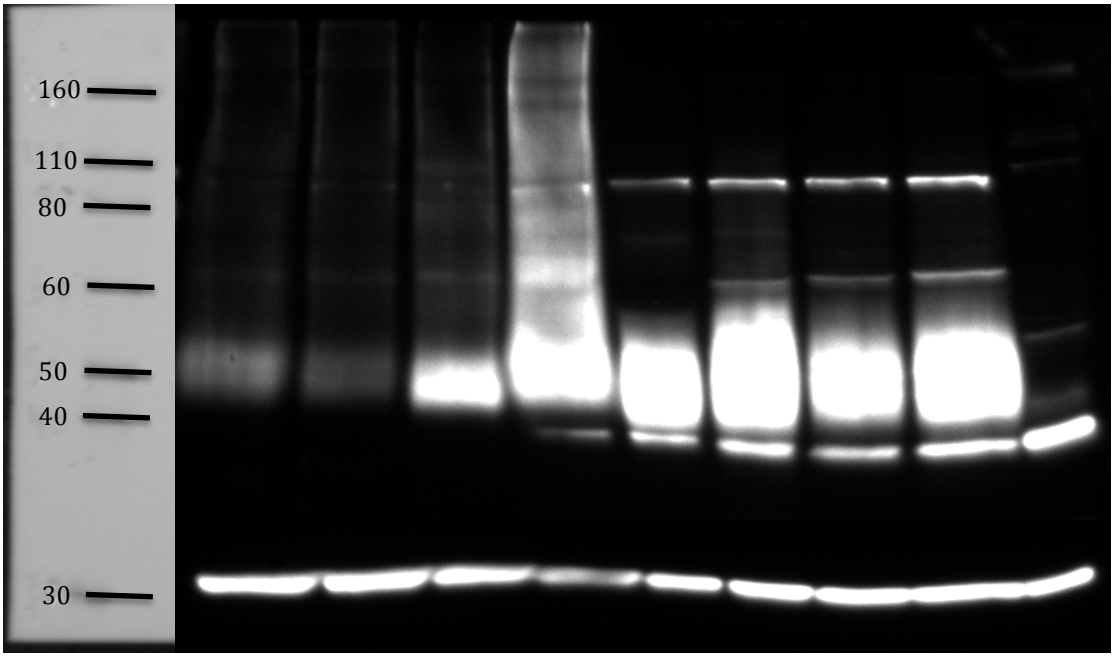


Figure 17.8. Western blot of gel 6 with superimposed ladder (lane 1) showing bands at~110kDA, ~54kDA and ~38kDA in lanes 2 to 9 however the bands are less discrete than those in gel 4. GAPDH shown at ~30kDA. Lane 10 only shows band at ~38kDA however rat adipocyte lysate used as positive control rather than erythrocyte membranes which are more specific for GLUT1. Samples harvested in NP40/octyl-beta-d-glucopyranoside. Taken after 5 minutes exposure to standard ECL.

Gels 7 and 8

The nitrocellulose membranes from gels 7 and 8 were used to blot expression of GLUT10, however gel 7 was loaded with samples harvested in Novex Tris-Glycine SDS-sample buffer whereas gel 8 was loaded with samples harvested in NP40/octyl-beta-d-glucopyranoside (only 4 samples loaded). GAPDH was blotted as a loading control only for gel 7. Using standard ECL for 1 and 5 minutes produced blots with low signal for GLUT10 and therefore these membranes were incubated for 5 minutes in SuperSignal West Dura Extended duration substrate for 5 minutes to produce visible blots (see Figures 17.9 and 17.10). GAPDH was only exposed for 1 minute using standard ECL.

Interestingly, both samples in Novex Tris-Glycine SDS-sample buffer and NP40/octyl-beta-d-glucopyranoside produced bands for GLUT10 of similar quality. As there was no specific positive control available for GLUT10 during the optimisation experiments, such a mitochondrial isolate, it can only be speculated rather than confirmed that the bands at ~50kDA represent GLUT10.

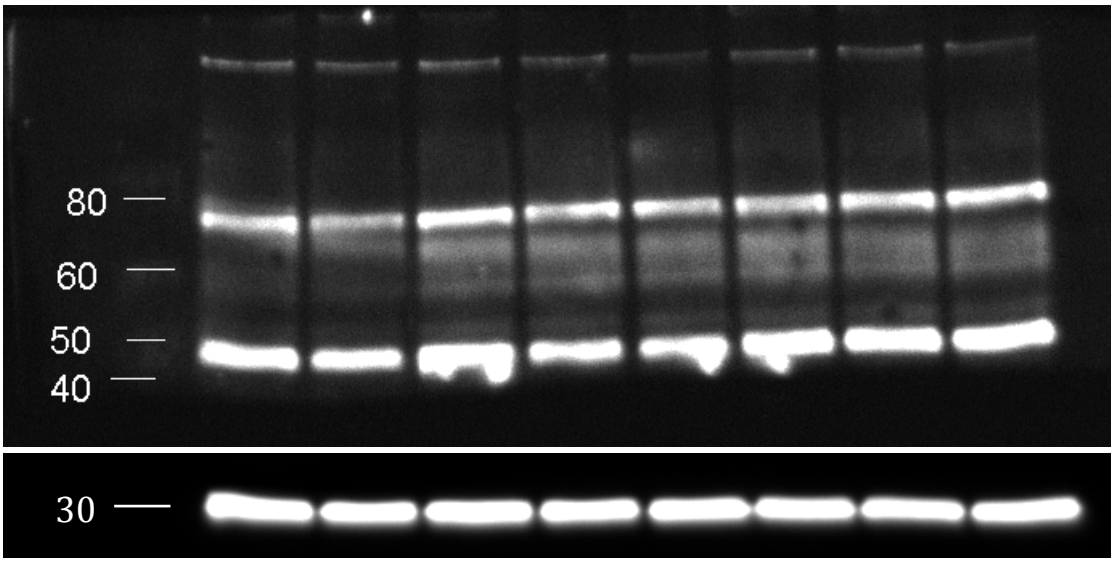


Figure 17.9. Western blot of gel 7 with superimposed ladder (lane 1) showing bands at ~75kDA, ~50kDA (for GLUT10) and ~30kDA (for GAPDH) in lanes 2 to 9. No positive control loaded therefore lane 10 empty. Samples harvested in Novex Tris-Glycine SDS-sample buffer. Upper blot taken after 5 minutes exposure to SuperSignal West Dura Extended duration substrate, lower blot after 1 minute exposure to standard ECL.

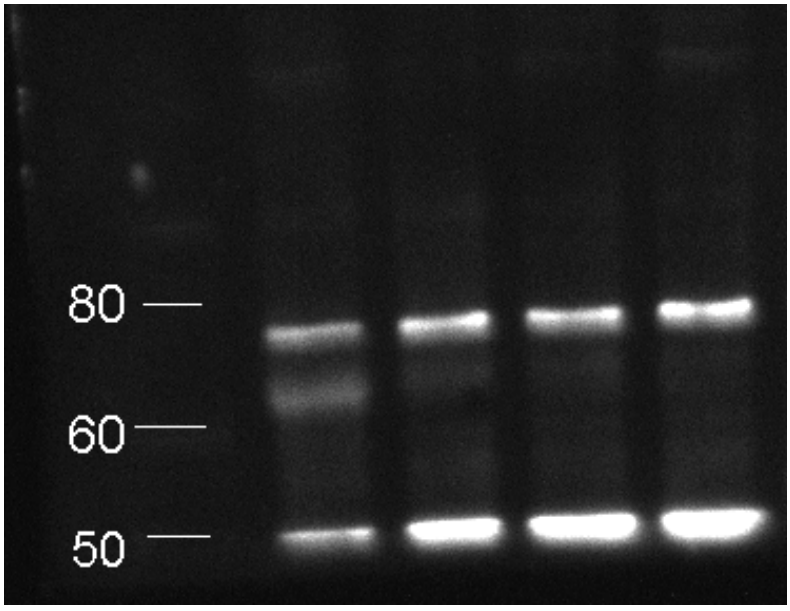


Figure 17.10. Western blot of gel 8 with superimposed ladder (lane 1) showing bands at ~75kDA and ~50kDA (for GLUT10) in lanes 2 to 5. No positive control loaded. Samples harvested in NP40/octyl-beta-d-glucopyranoside. Taken after 5 minutes exposure to SuperSignal West Dura Extended duration substrate.

Gels 2 and 3

The nitrocellulose membranes from gels 2 and 3 were used to blot expression of GLUT6 and GLUT3 respectively, however only samples harvested in Novex Tris-Glycine SDS-sample buffer were used in both gels. Using standard ECL for 1 and 5 minutes produced blots with low signal for GLUT6 and GLUT3 and therefore these membranes were incubated for 5 minutes in SuperSignal West Dura Extended duration substrate for 5 minutes to produce visible blots (see Figures 17.11 and 17.12).

Looking at Figure 17.11, there were at least 2 sets of discrete bands at ~60kDA and ~50kDA, either of which could of represented GLUT6, although the band at ~50kDA was more likely. Rat adipocyte lysate was used as a positive control which is known to express GLUT6[441] and did show bands at these molecular weights. Use of neuronal tissue may have been more specific for GLUT6 however unfortunately the Holman laboratory did not have access to this during my optimisation experiments. In Figure 17.12, there was only 1 set of non-discrete bands at ~50kDA which most likely represents expression of GLUT3 however the signal strength of these bands was weak, even after 5 minutes exposure to SuperSignal West Dura Extended duration substrate, suggesting low expression of GLUT3 is low in aortic smooth muscle cells.

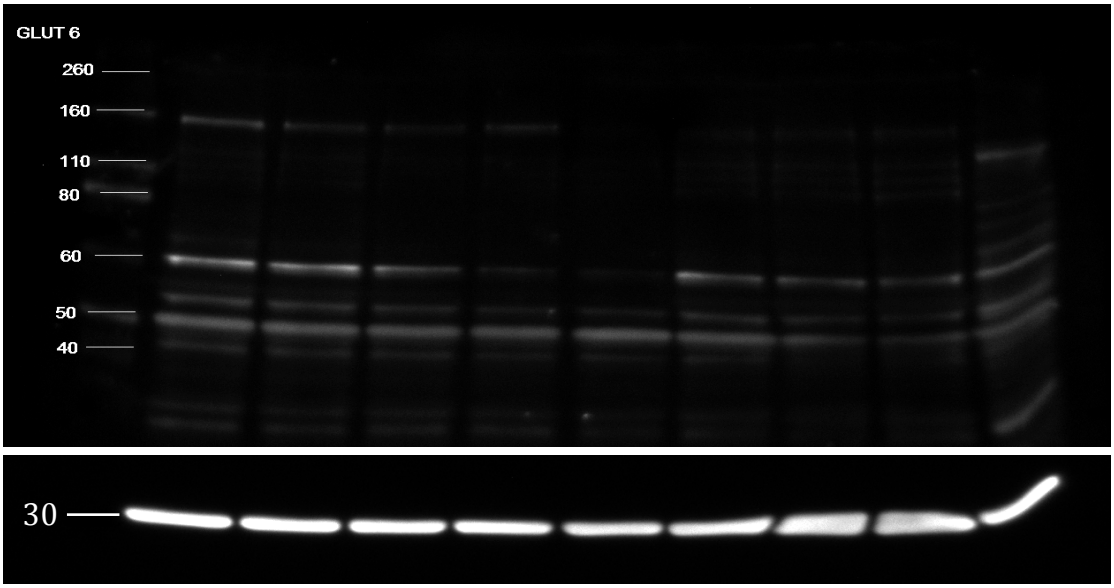


Figure 17.11. Western blot of gel 2 with superimposed ladder (lane 1) showing bands at ~60kDA, ~50kDA (GLUT6) and ~30kDA (GAPDH) in lanes 2 to 9. Lane 10 used as positive control however rat adipocyte lysate used rather than a more specific GLUT6 control. Samples harvested in Novex Tris-Glycine SDS-sample buffer. Taken after 5 minutes exposure to SuperSignal West Dura Extended duration substrate.

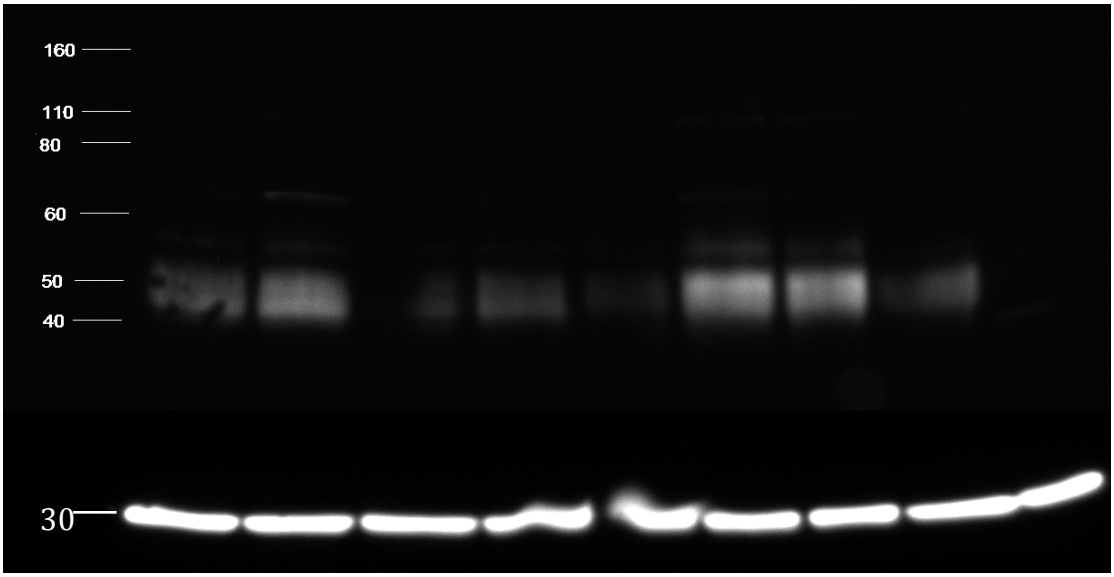


Figure 17.12. Western blot of gel 3 with superimposed ladder (lane 1) showing bands at ~50kDA for GLUT3 and ~30kDA for GAPDH in lanes 2 to 9. Rat adipocyte lysate loaded in lane 10 however no expression for GLUT3 found. Samples harvested in Novex Tris-Glycine SDS-sample buffer. Taken after 5 minutes exposure to SuperSignal West Dura Extended duration substrate.

17.2.5 Conclusions

The aim of this preliminary experiment was to determine the feasibility of performing western blotting for glucose transporters and insulin receptor in cultured aortic smooth muscle cells (AoSMCs) from patients with and without abdominal aortic aneurysm. This preliminary experiment did not specifically compare GLUT / IR protein expression in AoSMCs from a cohort of patients with AAA versus a cohort without AAA, nor did it test the effect of incremental hyperglycaemia on expression of these proteins. AoSMCs from 3 control patients and 4 AAA patients exposed to incremental hyperglycaemia had been collected in Novex Tris-Glycine SDS-sample buffer by the time I had finished my time in the laboratory. Although AoSMCs from at least 3 further patients in each group would have been needed, the next step within this chapter was to perform comparative western blots to determine differential protein expression for GLUTs / IR between AAA and control patients as well as in response to hyperglycaemia. However, due to time constraints, these experiments were not performed and therefore only the results of the feasibility studies could be presented in this chapter.

Given the lack of experience in performing western blotting for these proteins in our own laboratory, I sought the help and expertise of another laboratory which routinely measures protein expression of GLUTs using western blotting, albeit in a different cell model. With the benefit of this collaboration I was able to perform successful western blotting for glucose transporters (GLUT1, GLUT3, GLUT4, GLUT6 and GLUT10) and insulin receptor (β-subunit) in AoSMCs. This also showed me that collecting AoSMCs in our homemade protein lysis buffer

containing NP40 and octyl-beta-d-glucopyranoside was not appropriate for western blotting and that future AoSMCs needed to be collected in Novex Tris-Glycine SDS-sample buffer. However these experiments also showed the importance of having specific positive controls for each protein being quantified, some of which were not available whilst performing these optimisation experiments. Since performing these experiments, a protocol to isolate erythrocyte membranes has been obtained (specific positive control for GLUT1) and sources of GLUT3 and GLUT10 positive controls have been identified.

Chapter 18: Microarray of aortic smooth muscle cells exposed to hyperglycaemia

The second aim of this thesis was to determine whether the expression and/or activity of glucose transporters was significantly influenced by the diabetic phenotype, or more specifically by hyperglycaemia. Accordingly, focused experiments were conducted to fulfil these aims that followed a candidate gene approach.

However, research into the mechanisms underpinning the negative relationship between diabetes mellitus and abdominal aortic aneurysms is relatively sparse and therefore it was decided that performing a gene expression microarray might identify avenues for future research. Using the same aortic smooth muscle cell (AoSMC) model that has been used in previous experiments, AoSMCs were exposed to normoglycaemia and 25mmol/L of hyperglycaemia before being subjected to microarray analysis.

18.1 Principles of microarray

Nucleic acid microarrays are a technology that emerged in the late 1990s in which thousands of short DNA sequences are bound to a solid surface such a glass bead or silicone chip in a specific order which can then be used to determine parallel gene expression analysis within a solution in a high-throughput manner[442]. There are 3 main types of microarray: spotted arrays, self-assembled arrays and in-situ synthesised arrays[443] however a discussion of the differences between each is beyond the scope of this review and therefore only the generic principles are discussed below.

The principal application of microarray is gene expression profiling although it can also be used for transcription factor binding analysis or genotyping[443]. The steps involved in performing a microarray include sample preparation, hybridisation, washing and image acquisition and data analysis[442].

Sample preparation starts by isolating total RNA containing messenger RNA (mRNA) that ideally represents a quantitative copy of the genes expressed at the time of sample collection. The sample mRNA is then converted to complementary DNA (cDNA) using a reverse-transcriptase enzyme before each cDNA is labelled with a fluorescent tracking molecule such as Cy3 or Cy5.

Hybridisation involves the joining together of two complementary strands of DNA to form a double-stranded molecule. The fluorescently labelled cDNA is first purified to remove contaminants such as primers and unincorporated nucleotides using filter spin columns before being exposed to cDNA molecules

attached to a solid support such as beads in microwells on a chip [444]. Ideally each molecule in the labelled sample will only bind to its complementary target sequence on the immobilised array.

The slides or chips are washed to remove any labelled cDNA that did not hybridise and then imaged using a laser scanner to determine how much labelled cDNA is bound to each target spot, the laser exciting the hybridised targets to yield fluorescence with a specific emission spectra that can be measured using a confocal laser microscope.

18.2 Methods

AoSMCs from 6 patients were exposed to 24 hours of normoglycaemia or 24 hours of hyperglycaemia using the protocol detailed in Chapter 15.3 for RNA studies. AoSMCs were selected for microarray based on specific patient inclusion criteria (male sex, >50 years old and non-diabetic). Initially AoSMCs from 3 AAA patients and 3 control patients were selected based on this inclusion criteria however due to poor-quality RNA from one of the control patients, AoSMCs from an additional AAA patient had to be included.

RNA was extracted from AoSMCs using the manual method of extraction as described in Chapter 13.4. RNA quality was checked on an Agilent 2100 Bioanalyser as described in Chapter 11.2 and only RNA samples of high quality were used (RIN >7) (see Table 18.1). Total RNA samples were then processed with assistance from Dr Nic Sylvius at the University of Leicester Genomics Core Facility.

Table 18.1. RNA integrity numbers (RIN) of total RNA extracted from aortic smooth muscle cells (AoSMCs) used in microarray studying effect of normoglycaemia versus hyperglycaemia on whole genome gene expression.

	RIN values of total RNA from:	
Patient	***AoSMCs exposed to normoglycaemia (4.6mmol/L)***	***AoSMCs exposed to hyperglycaemia (25mmol/L)***
1	9.7	9.8
2	7.7	9.9
3	9.9	9.6
4	9.8	10.0
5	9.8	9.7
6	9.7	9.9

A total of 250ng of total RNA was prepared using the TargetAmp™ - Nano Labeling Kit for Illumina® Expression BeadChip (Epicentre) according to manufacturer instructions. This kit converts total RNA in the sample first to single-stranded cDNA, then, via the formation of antisense RNA, to double-stranded cDNA which is termed cRNA. The cRNA is then converted into biotin-aRNA which is suitable for microarray on the Illumina platform (see Figure 18.1).

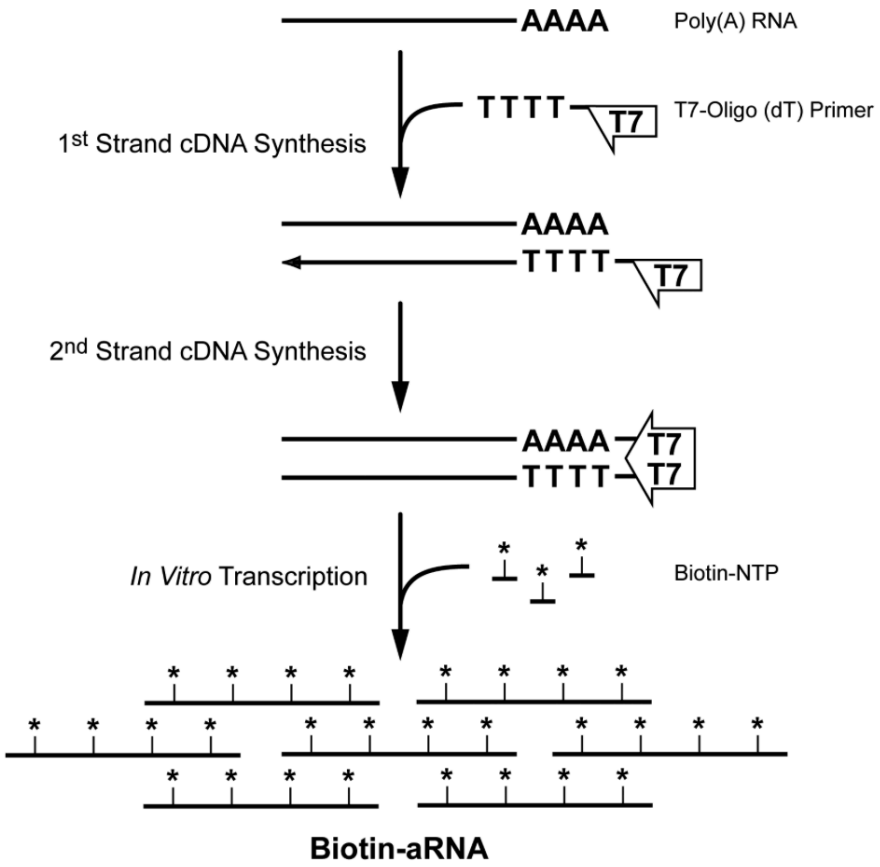


Figure 18.1. Overview of the TargetAmp™ - Nano Labeling Kit for Illumina® Expression BeadChip. Reproduced from manufacturers protocol on www.epibio.com.

Whole genome gene expression analysis was then performed using the HumanHT-12 v4 Expression BeadChip Array (Illumina) (see Figure 18.2) which

allowed quantification of about 47,000 transcripts based on the National Centre for Biotechnology Information Reference Sequence database Release 38 (November 2009). The beadchips were scanned on an Illumina Iscan. The raw data was normalised by quantile normalisation using the Illumina Genome Studio v2011.1.

Figure 18.2. An image of the Illumina HumanHT-12 v4 BeadChip showing the 12 sample sections arranged horizontally. Reproduced from www. illumina.com.

18.2.1 Data Analysis

After quantile normalisation, the probes confidently detected in all samples (signal intensity >120) were filtered in. The probes confidently detected in all samples of one group and not in the other comparison group were also selected. Significant probes were selected following a t-test using log2 transformed raw signal intensity values. As the correction for multiple testing (Holm-Bonferroni) was too stringent on the small dataset available, a cut-off p-value of 0.01 was used instead.

The fold-change of expression between groups was calculated using raw signal intensities on the normal scale. Gene Ontology (GO) terms enrichment analysis was performed using the WEB-based GEne SeT AnaLysis Toolkit [445]. The significant genes with an absolute minimum fold difference of expression of 1.25 were used as the input list. Significant GO terms were selected using the hypergeometric test. The 10 most significant terms within each GO category were selected for final reporting. The primary comparison of interest was differentially expressed genes between AoSMCs exposed to normoglycaemia versus AoSMCs exposed to hyperglycaemia.

18.3 Results

In total, 12 samples were microarrayed from 6 patients (4 AAA and 2 control) matched for age, sex and diabetic status (males > 50 years, non-diabetic) were included.

Comparing microarray data from AoSMCs exposed to normoglycaemia versus hyperglycaemia (n=6 vs. n=6) generated a list of 10 significantly differentially expressed genes (1 up-regulated, 9 down-regulated; see Table 18.2). Selectively comparing AoSMCs from patients with AAA (n=4 vs. n=4), generated a list of 11 significantly differentially expressed genes (see Table 18.3), the majority of which were discrete to those identified in Table 18.2. A selective comparison of AoSMCs from patients without AAA (n=2 vs. n=2) was not performed due to the low number of cases. No informative pathway enrichment analyses were possible for comparisons between normoglycaemia versus hyperglycemic as the lists of differentially expressed genes were too small.

Table 18.2. Differentially expressed genes discovered by microarray following aortic smooth muscle cell exposure to normoglycaemia versus hyperglycaemia (n=6 versus n=6).

Symbol	Definition	Fold change	P value
Up regulated genes by hyperglycaemia			
ARRDC4	Homo sapiens arrestin domain containing 4 (ARRDC4)	1.33	0.00223
Down regulated genes by hyperglycaemia			
ACAT2	Homo sapiens acetyl-Coenzyme A acetyltransferase 2 (ACAT2)	-1.38	0.00220
IDI1	Homo sapiens isopentenyl-diphosphate delta isomerase 1 (IDI1)	-1.41	0.00259
WDYHV1	Homo sapiens WDYHV motif containing 1 (WDYHV1)	-1.25	0.00410
FASN	Homo sapiens fatty acid synthase (FASN)	-1.53	0.00433
SC4MOL	Homo sapiens sterol-C4-methyl oxidase-like (SC4MOL), transcript variant 2	-1.28	0.00491
FDFT1	Homo sapiens farnesyl-diphosphate farnesyltransferase 1 (FDFT1)	-1.28	0.00685
ILMN_1909886	601452348F1 NIH_MGC_66 Homo sapiens cDNA clone	-1.30	0.00707
ALDH3B1	Homo sapiens aldehyde dehydrogenase 3 family, member B1 (ALDH3B1), transcript variant 1	-1.30	0.00837
INSIG1	Homo sapiens insulin induced gene 1 (INSIG1), transcript variant 2	-1.59	0.00895

Table 18.3. Differentially expressed genes discovered by microarray following AoSMC exposure to normoglycaemia versus hyperglycaemia selectively in aortic smooth muscle cells from patients with AAA (n=4 vs. n=4).

Symbol	Definition	Fold change	P value
Up regulated genes by hyperglycaemia			
NUCKS1	Homo sapiens nuclear casein kinase and cyclin-dependent kinase substrate 1 (NUCKS1)	1.39	0.0000931
LOC90120	PREDICTED: Homo sapiens hypothetical gene supported by AK023162, transcript variant 4 (LOC90120)	1.30	0.00498
DLEU1	Homo sapiens deleted in lymphocytic leukemia 1 (non-protein coding) (DLEU1), non-coding RNA	1.27	0.000440
TMEM184B	Homo sapiens transmembrane protein 184B (TMEM184B)	1.27	0.00235
YES1	Homo sapiens v-yes-1 Yamaguchi sarcoma viral oncogene homolog 1 (YES1)	1.25	0.00552
Down regulated genes by hyperglycaemia			
D2HGDH	Homo sapiens D-2-hydroxyglutarate dehydrogenase (D2HGDH), nuclear gene encoding mitochondrial protein	-1.26	0.00198
ZNF26	Homo sapiens zinc finger protein 26 (ZNF26)	-1.27	0.00976
TERF1	Homo sapiens telomeric repeat binding factor (NIMA-interacting) 1 (TERF1), transcript variant 1	-1.27	0.00599
LOC100129668	PREDICTED: Homo sapiens hypothetical protein LOC100129668 (LOC100129668)	-1.30	0.00934
ILMN_1909886	601452348F1 NIH_MGC_66 Homo sapiens cDNA clone IMAGE:3856355 5	-1.38	0.00563
FASN	Homo sapiens fatty acid synthase (FASN)	-1.44	0.00393

A secondary comparison of microarray data from AAA (n=8) versus CAD (n=4) patients was also performed for interest which generated a list of 160 significantly differentially expressed genes (see Appendix 2). The list of significant genes was exported to Ingenuity in order to perform a pathway enrichment analysis. The results of this analysis are shown in Table 18.4.

Table 18.4. Gene Ontology analysis of differentially expressed genes identified by microarray in aortic smooth muscle cells from patients with and without abdominal aortic aneurysm.

Biological process	**GO number**	**P value**
Protein O-linked glycosylation	GO:0006493	0.00060
Protein glycosylation	GO:0006486	0.00270
Macromolecule glycosylation	GO:0043413	0.00270
Glycosylation	GO:0070085	0.00290
O-glycan processing	GO:0016266	0.00330
Oestrogen metabolic process	GO:0008210	0.00550
Glycoprotein biosynthetic process	GO:0009101	0.00780
Positive regulation of pathway-restricted SMAD protein phosphorylation	GO:0010862	0.00840
M phase of meiotic cell cycle	GO:0051327	0.00980
Meiotic cell cycle	GO:0051321	0.01070

18.4 Discussion

The main aims of this experiment was to discover new targets for research regarding the mechanisms underpinning the negative relationship between diabetes and abdominal aortic aneurysms. These aims were delivered by way of a microarray of aortic smooth muscle cells (AoSMC) exposed to normoglycaemia versus hyperglycaemia. The main findings of the microarray were that at least 10 genes were differentially expressed in the aortic smooth muscle cells exposed to 25mmol/L hyperglycaemia.

Although a detailed analysis of each differentially expressed gene was not performed, and given the relatively small number of differentially expressed genes no pathway enrichment analysis was possible, it can be seen from Table 18.2 that many of the genes appear to be involved in cellular and energy metabolism. For example, acetyl-coA C-acetyltransferase and fatty acid synthase are enzymes involved in fatty acid metabolism and synthesis of ketone bodies[446,447] and insulin induced gene 1 which encodes an endoplasmic reticulum protein that regulates cholesterol metabolism, lipogenesis and glucose homeostasis[448]. These genes were all down-regulated in the presence of hyperglycaemia.

Only one gene was found to be significantly up-regulated in the presence of hyperglycaemia, Arrestin domain containing 4 (ARRDC4), with a fold change of 1.33 ($p=0.002$). This gene encodes an adapter protein which that has a role in endocytosis and/or ubiquitination of activated G-protein coupled receptors but interestingly has been shown to inhibit glucose uptake when overexpressed[449].

In humans, ARRDC4 belongs to a family of 6 proteins known as the α-arrestins which includes thioredoxin-interacting protein (Txnip) and ARRDC1 -5 however very little is known about the function of these proteins[450]. Patwari *et al* have shown, using lentiviral overexpression of ARRDC4 in skin fibroblasts, that ARRDC4 overexpression is associated with a significant decrease in glucose uptake (71% decline, $p<0.001$) which was seen to a lesser extent with Txnip overexpression (37% decline, $p<0.01$) but not with ARRDC3 overexpression[451]. In addition, both ARRDC4 and Txnip overexpression was associated with a significant reduction in lactate production in human embryonic kidney 293T cells[451].

The limitations of this study are mainly related to the small sample size (n=6 vs. n=6). As with all microarray experiments the findings need to be validated using formal quantitative PCR testing of the transcripts of interest using a larger cohort of patients. For this reason, the results from this microarray can only be regarded as 'preliminary data' however the results, particularly the finding of increased ARRDC4 gene expression with hyperglycaemia, open up interesting and exciting possibilities regarding the exact mechanisms by which diabetes protects against abdominal aortic aneurysm. If the function of ARRDC4 is the same in AoSMCs as it is skin fibroblasts, these results may help explain the earlier findings from this thesis that pre-incubation of AoSMCs in 25mmol/L of hyperglycaemia was associated with a significant decrease in glucose transporter (GLUT) activity without a corresponding decrease in GLUT gene expression. ARRDC4 may therefore represent the missing link between hyperglycaemia (which increases ARRDC4 gene and hence protein expression)

and a reduced GLUT activity in AoSMCs (ARRDC4 protein functions to decrease glucose transport). Future research should aim to determine the effect of incremental hyperglycaemia on ARRDC4 protein expression in AoSMCs but also investigate whether ARRDC4 overexpression can reduce aneurysm formation experimentally.

18.4.1 Conclusions

This microarray has identified new areas for future research including those related to energy and lipid metabolism as well as the α-arrestin family of proteins.

Conclusions and Future work

Chapter 19: Conclusions

19.1 Introduction

There is now a significant body of evidence from epidemiological studies supporting the protective role of diabetes mellitus (DM) on abdominal aortic aneurysm (AAA) prevalence, incidence, expansion and rupture. Mechanistic studies examining this relationship are however relatively few despite a number of recent editorials emphasizing the need for such studies to be undertaken[208,452]. Elucidating the mechanisms underpinning this relationship are important because currently the only treatment for AAA involves an operation and therefore such knowledge could aid the development of novel pharmacological therapies for AAA. Furthermore, with the introduction of the NHS AAA screening programme in England, there now exist a growing number of patients diagnosed with AAA but not yet suitable for operative repair. These patients represent ideal candidates for a drug therapy that slows or halts aneurysm progression since such a medication could prevent these patients ever requiring surgery which in its self has significant risks.

This thesis focused on the significance of glucose transporters in the pathophysiology of AAA. Knowledge of the fact that glucose transporter (GLUT) proteins have been implicated in the pathophysiology of DM and its microvascular complications formed the initial basis such a study. It was postulated that if the protective effect of DM on AAA was merely another 'complication' of having DM, then it was possible that similar mechanisms to

those regulating the (unfavourable) microvascular complications of DM might also be involved in regulating this more favourable 'complication'.

The main experiments in this thesis were set out using a 2-phase approach which firstly sought to assess the importance of glucose transporters in AAA and secondly to assess whether their involvement might be involved in regulating the negative relationship between DM and AAA.

19.2 Main results

The principal findings from this thesis were 3-fold:

1) GLUT mRNA and protein expression was higher in aortic tissue specimens from patients with AAA compared to cadaveric controls,
2) GLUT activity was higher in AoSMCs from patients with AAA compared to AoSMCs from patients without AAA,
3) Physiological levels of hyperglycaemia were associated with a reduction in GLUT activity selectively in AoSMCs from patients with AAA

The finding that GLUT3 and GLUT6 mRNA expression was 9-fold and 3-fold higher respectively in WATS from AAA patients has not been previously described. There was also a trend towards lower insulin receptor (IR) mRNA expression however this did not reach statistical significance. Similar differences in GLUT or IR mRNA expression were not seen when AoSMCs were studied in isolation suggesting that the increase in GLUT3 or GLUT6 expression within the aortic wall of AAA patients is probably due to increased expression from another cell type.

GLUT3 and to some extent GLUT6 are the predominant GLUTs expressed by leucocytes[365,453]. Inflammatory cell infiltrate, which is predominantly monocyte- and macrophage- driven, is one of the characteristic features of AAA[72,76] and may explain the increased expression of GLUT3 and GLUT6 mRNA in whole aortic tissue samples (WATS) from AAA patients in the present study.

The present study also found elevated GLUT1, GLUT3 and GLUT6 protein expression in WATS from patients with AAA. Specifically, protein expression was higher for these proteins in the intimal and medial subsections of the aortic wall. Tunica media infiltration with inflammatory cells is characteristic of AAA however this layer is also the predominant layer where AoSMCs reside. The only group to have previously studied GLUT protein expression in aortic tissue were those at the University of Miyazaki[430]. They found a positive correlation between GLUT1 and GLUT3 protein expression and FDG-PET markers of glucose transporter activity in patients with AAA. However this study did not contain a comparator group and therefore was unable to determine if GLUT protein expression was elevated specifically in patients with AAA. Furthermore this study did not attempt to define which layer of the aortic wall GLUTs were expressed in.

The present study found that AoSMCs from patients with AAA had nearly 50% higher levels of GLUT activity at baseline. This result supports the findings from various studies comparing 18F-FDG-PET uptake in patients with and without AAA. Reeps *et al* compared uptake in patients with symptomatic AAA, asymptomatic AAA and age-matched controls without AAA. They found increased 18F-FDG uptake in patients with symptomatic vs. asymptomatic AAA ($n=3$ vs. $n=12$; SUVmax 7.5 vs. 3.5; $p<0.001$) as well as in asymptomatic AAA vs. age-matched controls ($n=12$ vs. $n=24$; 3.5 vs. 3.0; $p<0.05$). Similarly Tegler *et al* found increased 18F-FDG uptake in the infrarenal aorta of patients with large vs. small AAA ($n=7$ vs. $n=5$; SUVmax 2.7 vs. 1.8; $p<0.001$). Such findings of increased glucose uptake in AAA tissue and cells support the theory that the aneurysmal

abdominal aorta is a metabolically active organ which has increased glucose demands (to support the processes that drive expansion).

Circulating hyperglycaemia is the characteristic feature of DM. Phase 2 of this thesis tested the effect of pre-treating AoSMCs with increasing levels of hyperglycaemia and found that hyperglycaemia within the physiological range (i.e. 12.5mmol/L or 25mmol/L) was associated with a sequential decrease in GLUT activity selectively in AoSMCs from patients with AAA. These findings were independent of any effect on osmolarity since similar changes were not observed when AoSMCs were pre-treated with euglycemic mannitol solutions. In addition, the decrease in GLUT activity with hyperglycemic pre-treatment was time-dependent with longer hyperglycemic pre-treatment times resulting in greater suppression of glucose transport activity.

The reason why suppression of GLUT activity occurred selectively in the cells from AAA patients is not fully understood. One possible explanation is that the development of AAA augments AoSMC GLUT activity to above-normal levels whilst the development of DM (hyperglycaemia) then leads to normalisation of this increase. Indeed in the current study, the degree of suppression with 25mmol/L of hyperglycaemia pre-treatment was sufficient to 'normalise' GLUT activity in AoSMCs from AAA patients to levels seen in AoSMCs from non-AAA patients ($p=0.673$ between groups). The reasons why this effect was lost at glucose concentrations outside the physiological range is not fully understood but maybe because such high levels of hyperglycaemia (50mmol/L = 900mg/dL)

induce a pro-inflammatory environment via a hyperosmotic effect [454] that in turn increases cellular glucose demand.

The concept of GLUT activity suppression selectively in AoSMCs from patients with AAA was supported by additional work using low-density lipoportein 1 (LRP1) knockdown cell models. LRP1 has been shown to have a pivotal role in maintaining vascular wall integrity and in the prevention atherosclerosis. Inactivation of LRP1 in murine vascular SMCs causes overexpression of platelet-derived growth factor (PDGF) and abnormal PDGF receptor signalling which then results in medial elastin disruption, abnormal SMC proliferation and aneurysm formation[416]. Lentiviral-induced LRP1 knockdown in normal AoSMCs was associated by development of AAA-phenotype characterised by increased matrix metalloproteinase 2 gene expression and increased GLUT activity, a phenomenon that did not occur when using non-targeting shRNA. Exposure of AoSMCs to physiological hyperglycaemia for 18 hours was associated with suppression of GLUT activity selectively in the AoSMCs knocked down for LRP1, thereby supporting the concept that hyperglycaemia only suppresses GLUT activity in AoSMCs with a AAA-phenotype (characterised by above-normal levels of GLUT activity).

Suppression of GLUT activity with hyperglycemic pre-treatment however is not unique to aneurysmal AoSMCs. Kurihara *et al* found that pre-treatment of RAW264.7 murine macrophage cells with hyperglycaemia (15.5mmol/L) for 7 days was able to suppress the increase in glucose uptake stimulated by sRANKL which they suggested was due to impaired translocation of GLUT1 to the plasma

membrane. However this study assayed residual glucose concentrations in the conditioned medium rather than intra-cellular glucose levels using a radiolabelled tracer which is a more precise method of determining cellular GLUT activity[414]. Quinn *et al* studied the effect of angiotensin II (AngII) and glucose on glucose uptake in rat thoracic vascular SMCs. They found that exposure to AngII, a bioactive peptide capable of inducing AAA experimentally, increased vascular SMC glucose uptake compared to controls (1192 vs. 592 pmoles/10^6 cells; $p<0.05$) but also that pre-incubation of vascular SMCs to physiological levels of hyperglycaemia (25mmol/L) for 18 hours was able to significantly supress the increase in glucose uptake mediated by AngII ($p<0.05$) [455]. Interestingly, a similar effect was also seen in vascular SMCs not exposed to AngII suggesting that selective suppression of GLUT activity in aneurysmal AoSMCs might be unique to those from the abdominal aorta.

19.3 Additional findings

The present study found that hyperglycaemic pre-treatment of AoSMCs was not associated with a significant decrease in GLUT mRNA expression. To explain the decrease in GLUT activity, this suggests the possibility of post-transcriptional down-regulation of GLUT expression and although AoSMC GLUT protein expression was not formally measured in the present study (only performed as preliminary work), all measures of GLUT activity were adjusted for absolute protein content making this explanation less plausible.

The alternative is an intrinsic decrease in the activity of GLUT proteins, independent of any change in mRNA or protein expression, which is well-described[456,457]. However, examining the data in more detail, there was a non-significant trend towards lower GLUT1 and GLUT3 mRNA expressions in the AoSMCs from AAA patients with increasing levels of physiological hyperglycaemia, which means the possibility of a type 2 error cannot be excluded, particularly since there were only AoSMCs from 5 patients in each group. Kaiser *et al* has shown reduced expression of GLUT1 in AoSMCs harvested from descending thoracic calf aorta when exposed to hyperglycaemia[458] and Kipmen-Korgen *et al* have shown that peripheral blood leucocytes from diabetic patients exhibited significantly lower GLUT3 mRNA and protein levels than leucocytes from non-diabetic counterparts[459].

A microarray study comparing whole genome expression in AoSMCs exposed to normoglycaemia or hyperglycaemia was also performed to identify potentially novel areas of research. This identified 10 differentially expressed genes (1 up-

regulated, 9 down-regulated). Although none of these belonged to the SLC2A family of genes, the only gene that was up-regulated (fold change 1.33) coded for the protein Arrestin domain-containing protein 4 (ARRDC4). The UniProt database suggests that ARRDC4 functions as an adapter protein that may have a role in endocytosis and/or ubiquitination of activated G protein-coupled receptors[460]. However Patwari *et al* has shown that overexpression of ARRDC4 in primary human skin fibroblasts is associated with a 71% decrease in glucose uptake and proposes the arrestins as a novel family of metabolic regulators[451].

The identification of hyperglycaemia-induced up-regulation of ARRDC4 in AoSMCs has not previously been reported. This finding may represent the mechanism by which hyperglycaemia decreases GLUT activity in AoSMCs from patients with AAA and may account for why no significant decreases in GLUT mRNA expression were observed. Analysis of the negatively differentially expressed genes found that nearly all were involved in lipid metabolism, cholesterol biosynthesis or alcohol metabolism.

A comparison of whole genome expression was also performed between AoSMCs from patients with and without AAA which revealed 160 differentially expressed genes (129 up-regulated, 31 down-regulated). Pathway enrichment analysis of these genes revealed a significant number of biological processes that were related to glycosylation, glycoprotein biosynthesis and glucose handling. Despite the same proportion of AoSMCs (50%) being exposed to hyperglycaemia in each comparison group, it is interesting that the differentially expressed genes were still related to glucose metabolism. This may suggest that the development of

AAA also alters the biological processes by which AoSMCs deal with glucose such as glycosylation of proteins.

19.4 Strengths and limitations

The present study focused on an important issue in aneurysm pathobiology, the mechanisms by which DM protects against AAA. The study made use of aortic tissue and cells from actual patients with and without AAA which improves generalisability of the results since they are not based on experiments using vascular tissues from non-aortic sources or on cell lines as were used by the only other study investigating the role of GLUTs in this context[414]. Furthermore all experimental protocols used in this study had undergone optimisation and most experiments had adequate internal controls (for example Lowry protein assays and osmotic control assays).

Despite these strengths, a number of limitations to the experiments exist. The results from this study suggest that GLUTs might be involved in mediating the protective effect of DM on AAA however it is important to note that these experiments were testing association and not causation. To identify causation would require further experiments utilising animal models of AAA as well as methods of GLUT inhibition.

During the study, a large number of WATS were obtained and therefore experiments using this tissue had access to sufficient numbers of samples. However, the demographics of WATS from patients with AAA versus those from cadaveric controls could not be perfectly matched; AAA patients were more likely to be male, of an older age and have a past history of smoking. In addition the ability to isolate and grow sufficient numbers of AoSMCs from WATS was sometimes an issue, particularly in the case of AoSMCs from patients with AAA.

Where AoSMCs were grown, these were preferentially used to perform glucose transporter activity assays which resulted in insufficient cell numbers to test and retest mRNA expression in AoSMCs, such as when initial attempts yielded poor results, which may ultimately have resulted in a type 2 error as discussed earlier.

Finally, protein expression studies in AoSMCs were planned and the techniques to do so had been optimised (see Chapter 17) however due to the issues with obtaining sufficient cell numbers as well as time constraints on the research, these experiments could not be completed. The completion of these experiments might of added further insight into the post-transcriptional expression of GLUTs in AoSMCs in response to hyperglycaemia.

19.5 Future avenues

There are a number of avenues that could be pursued to build on this research. It would be useful to determine whether the increase in GLUT3 and GLUT6 mRNA and protein expression in WATS is due to an increase inflammatory cell infiltrate. This could involve co-localisation studies such as staining for the macrophage-specific marker CD68 prior to staining for GLUTs. Completing the experiments examining the effect of hyperglycemic on GLUT protein expression would also provide useful insights into whether hyperglycaemia in AoSMCs also influences post-transcriptional GLUT expression.

Hyperinsulinaemia is often observed in addition to hyperglycaemia in patients with T2DM. Studying the effects of hyperinsulinaemia on GLUT expression and activity in AoSMCs may provide further insights into the mechanisms by which DM protects against AAA, particularly since insulin has recently been shown to induce the production of new elastin in cultures of AoSMCs[461].

To determine the importance of AoSMC GLUT activity on AAA formation, experimental models of AAA utilising methods of GLUT inhibition could be studied. GLUT inhibition can be performed pharmacologically using compounds such as Fasentin (GLUT1), Apigenin (GLUT1), Cytochalasin B (non-selective), Reservatrol (mainly GLUT1) or Genistein (combined GLUT1 and GLUT4). Kurihara *et al* have shown that Apigenin, a flavonoid capable of inhibiting GLUT1, suppressed sRANKL-induced macrophage activation in a dose-dependent manner as well as NFATc1 (nuclear factor of activated T cells cytoplasmic 1) expression, a master transcription factor responsible for

activation of cathepsin K and MMP-9 genes in macrophages[414]. Similar findings were also observed with Cytochalasin B. Similar experiments could be performed using AoSMCs from patients with AAA or those knocked down for LRP1.

However such studies do not test the effect of GLUT inhibition on AAA development or expansion. This would require the use of experimental animal models of AAA such as the ApoE-deficient AngII mouse model. Alternatively GLUT inhibition could be performed genetically although systemic inhibition of GLUT1 is unlikely to be compatible with life therefore generation of SMC-specific GLUT1 knockout (smGLUT1$^{-/-}$) mice would be required. Importantly, the GLUT1 gene (SLC2A1) has already been floxed in a mouse (SLC2A1$^{flox/flox}$)[462]and the group responsible for this has agreed to collaborate with our group and share their mouse model. Creation of this model would involve breeding with transgenic mice expressing Cre-recombinase under the control of mouse transgelin promoter (sm22Cre$^{-/-}$), the latter of which is available commercially (Jackson Laboratories, USA). However to incorporate this genotype then into a mouse model susceptible of forming AAA would require a large number of mice which would be costly, both financially and in terms of sacrificed mice (an estimated 160 mice would be required to generate a pair of smGLUT1$^{-/-}$ smLRP1$^{-/-}$ mice for example). Once created however, the benefit of this model would be that, unlike pharmacological GLUT inhibition, this model could test the effect of complete (rather than partial) inhibition of smGLUT1 on AAA development.

Finally, the effect of stretching AoSMCs on GLUT expression and activity would also be interesting to investigate since aortic wall stretching is a feature of AAA. Studies using mesangial cells, which are central to development of diabetic nephropathy, have shown that cell stretching using a Flexercell strain unit for only 33 hours is associated with a 40% increase in GLUT1 protein levels compared to unstretched mesangial cells[463]. It is possible that AoSMC stretching also contributes to the increase in GLUT activity seen in cells from patients with AAA.

19.6 Postulated mechanisms for glucose transporter involvement in abdominal aortic aneurysm pathophysiology

The mechanisms by which GLUTs are believed to regulate AAA pathogenesis are summarised in Figure 19.1. In the presence of risk factors for AAA, vascular SMCs and inflammatory cells within aortic wall are stimulated to produce proteolytic enzymes such as MMPs and cathepsins. These processes are energy dependent and therefore the cells increase their metabolic demand. To support the increase in metabolic demand, the cells increase their uptake of glucose via increased GLUT activity. The end result is extracellular matrix destruction which weakens the aortic wall and results in aortic expansion. Furthermore, the stretching of cells within the aortic wall may also precipitate increases in GLUT activity, as occurs in hypertensive nephropathy[463], which then positively reinforces the process itself.

In presence of diabetes mellitus however, these mechanisms are altered as is shown in Figure 19.2. Aortic SMCs and leucocytes within the aortic wall are exposed to circulating hyperglycaemia due to the presence of vasa vasorum. Hyperglycaemia, when chronic, down-regulates cellular GLUT activity which reduces the intracellular uptake of glucose and is likely to protect against AAA expansion via 2 main mechanisms. Firstly, the reduced cellular glucose uptake combined with the increased circulating levels of glucose will result in increased aortic tissue concentrations of glucose. This is likely to result in the formation of advanced glycation end-products (AGEs) between ECM proteins thereby stiffening the aortic wall directly. Secondly, the reduction in intracellular glucose

will reduce the ability of these cells to produce the various proteolytic enzymes that induce AAA expansion.

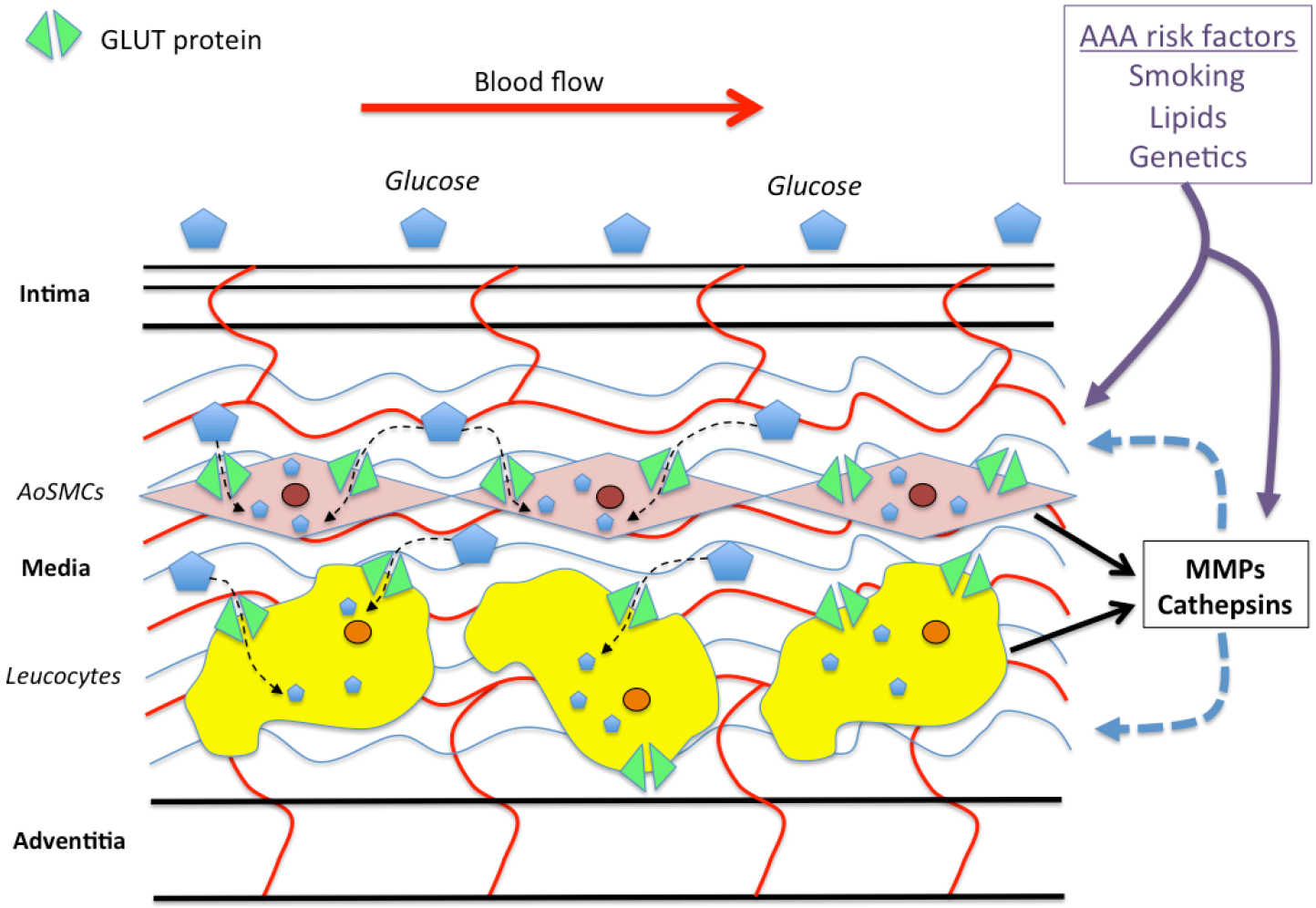


Figure 19.1. Postulated mechanisms by which glucose transporters are involved in the pathogenesis of abdominal aortic aneurysms.

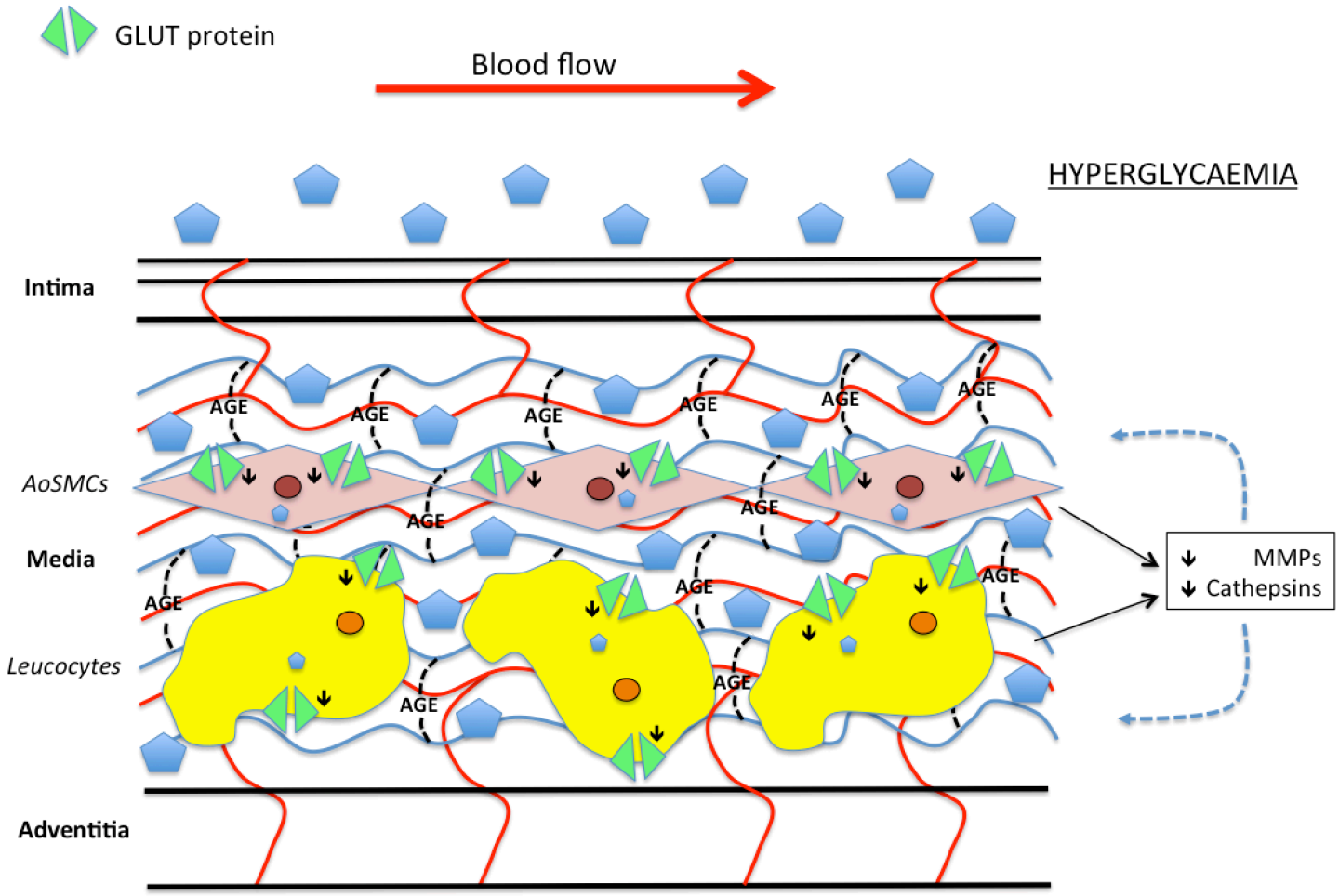


Figure 19.2. Postulated mechanisms by which glucose transporters regulate the protective effect of diabetes on abdominal aortic aneurysms.

19.7 Therapeutic and clinical implications

This thesis suggests that targeting GLUTs may represent a novel pathway by which to slow AAA growth. Further research using experimental animal models is needed before any trials of GLUT inhibition can be performed in humans. Importantly such studies should seek to determine whether pharmacological GLUT inhibition results in a situation of impaired glucose tolerance with or without overt DM because this may limit the clinical utility of such a drug therapy. In addition the current research provides a plausible biological explanation to support the suggestion that surveillance intervals for small AAA patients with concomitant DM could be increased.

Appendices and Bibliography

Appendix 1: Supplementary protocols

A1.1 Consent forms and data collection proforma

University Hospitals of Leicester NHS
NHS Trust

Caring at its best

PATIENT CONSENT FORM 1 (Version 2 December 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 DECEMBER 2012.

Please initial box

1. I confirm that I have read and understand patient information sheet 1 dated December 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

Figure A1.1. Consent form used to consent patients for aortic tissue collection prior to open abdominal aortic aneurysm repair.

An Investigation into Candidate Genes and Protein Profiling for Abdominal Aortic Aneurysms

PROFORMA (Revised September 2013)

Demographics

Date: PATIENT ID LABEL:

Reason for admission:

Blood sample taken: Y / N

Consented for study: Y / N UNIQUE STUDY ID:

Aneurysm size:

Date of operation:

Date of last scan:

Size at diagnosis:

Expansion Date:..... Size:..... Type of scan:.....
Date:..... Size:..... Type of scan:.....
Date:..... Size:..... Type of scan:.....
Date:..... Size:..... Type of scan:.....

Co-morbidities

Smoking: Y / Ex / N Start date: End date: No/day:
Cigarettes / Cigars / Pipe:

Cholesterol: Y / N Duration:

BP (HTN): Y / N Duration:

MI: Y / N Details:

Angina: Y / N Details:

Coronary Angio: Y / N Details:

CABG: Y / N Details:

Claudication: Y /N Details (leg, distance):

Lower limb Bypass: Y / N Angioplasty: Y / N Amputation: Y / N

CVA/TIA: Y / N Details:

Carotid Endartectomy: Y / N Details:

Diabetes: Y / N Details (diet, tablet, insulin, duration):

COPD: Y / N Details (inhalers, nebulisers, hospitalised):

Malignancy: Y / N Details (type, when):

Others:

Family history (AAA): Y/ N Details:

An Investigation into Candidate Genes and Protein Profiling for Abdominal Aortic Aneurysms

Medication

Aspirin:	Yes	No	Duration
B-blocker:	Yes	No	Duration
Statin:	Yes	No	Duration
Nitrates:	Yes	No	Duration
Clopidogrel:	Yes	No	Duration
Digoxin:	Yes	No	Duration
Warfarin:	Yes	No	Duration
Diuretic:	Yes	No	Duration
ACEI:	Yes	No	Duration
Insulin:	Yes	No	Duration

Others:

Sampling

Position: Supine Sitting Semi-recumbent

Site (specify anatomically):

Fasting: Y / N

Any deviation from protocol: Y / N

Recruited from: Vascular inpatients Vascular Outpatients Other

Figure A1.2. Proforma used to collect patient demographics and medical history from open AAA patients during consenting.

A1.2 Tissue processor schedules

Table A1.1. LEICA ASP3000 automated vacuum tissue processor processing schedule.

		OVERNIGHT	WEEKEND
Bottle 1	10% Formalin	1 Hour	Delay
Bottle 2	99% IMS	1 Hour	1 Hour
Bottle 3	99% IMS	1 Hour	1 Hour
Bottle 4	99% IMS	1 Hour	1 Hour
Bottle 5	99% IMS	1 Hour	1 Hour
Bottle 6	99% IMS	1 Hour	1 Hour
Bottle 7	99% IMS	1 Hour	1 Hour
Bottle 8	99% IMS	1 Hour	1 Hour
Bottle 9	Xylene	1½ Hours	1½ Hours
Bottle 10	Xylene	1½ Hours	1½ Hours
Wax bath 1	62°C	1 Hour	1 Hour
Wax bath 2	62°C	1½ Hours	1½ Hours
Wax bath 3	62°C	1½ Hours	1½ Hours
Cleaning reagents			
Bottle 11	Xylene		
Bottle 12	99% IMS		
Bottle 13	Tap water		

A1.3 Protocol for preparation and storage of paraffin wax samples

Table A1.2. Steps for preparation and storage of paraffin wax samples. Written by Angie Gillies and reviewed by Linda Potter at the University of Leicester Core Biotechnology services.

Step	Details
1	Prior to cutting, blocks should be thoroughly chilled on ice.
2	Carefully remove a disposable blade from its box and clean with a tissue moistened with xylene, being careful not to damage the knife edge.
3	Place blade in the knife holder on the microtome and clamp in place. Check that there is no movement of the blade.
4	Clamp the wax block in the chuck, tissue outermost and with the bevelled edge of the cassette pointing to the right of the microtome. Check that there is no movement of the block in the chuck.
5	Check that when the chuck is released the block will come down behind the blade. Release the safety lock on the rotary wheel.
6	Using the coarse advance, bring the block forward so that it is just behind the knife. Check that there is clearance all along the block.
7	Trim excess wax away from the surface of the tissue to expose a full face, by advancing the block up and down and using the coarse advance.
8	Polish the surface of the block by cutting a few sections at 4µm. Return the block to ice.
9	Clamp block in the chuck. Set section thickness at required setting depending on the tissue and the procedures to be carried out on the sections, usually 4µm.
10	Bring the block down just behind the knife. Turn the wheel all the way around so that the surface of the block is cut. Adjust speed of cutting as necessary to obtain flat, even sized sections. Friction of the block on the blade will cause subsequent sections to adhere to form a ribbon.
11	When a ribbon of sections is obtained, stop cutting and clamp the wheel. Using a small paint brush and forceps, grasp the free end of the ribbon with the forceps and gently lift off the blade with the paint brush using an upwards movement.
12	Float the ribbon on the water bath. Remove creases and bubbles from sections using forceps. Float sections until smooth and flat but not cracked or expanded.
13	Label a microscope slide with the reference number of the block. Separate sections using the forceps. Pick up sections on the microscope slide positioning the section centrally on the clear glass.
14	Allow slides to drain for a few minutes before putting in a slide rack and drying in the 37^{o}C incubator. Sections on coated slides should be dried overnight. Sections mounted on untreated slides for H & E staining may be usable after drying for 30mins.
15	As scores become apparent in the sections, move the blade along to a new section.
16	Return block to ice between each sectioning.

17	Clean water bath between each 'floating out' by sweeping a tissue across the surface.
18	Sweep excess wax trimmings away from the blade and microtome into a waste bucket using a brush.
19	After cutting, remove the blade and dispose of or store for further use in a closed box. Clean the microtome and bench using the brush and tissues.
20	When sections are dry proceed with required protocol or store at room temperature in a slide tray or box to keep dust free.

A1.4 Protocol for decalcification of fixed tissue

Table A1.3. Protocol for decalcification of whole aortic tissue samples. Written by Angie Gillies and reviewed by Linda Potter at the University of Leicester Core Biotechnology services.

Step	Detail
1	Ensure tissue is completely fixed in 10% Formalin (48 hours – 1 week)
2	Discard Formalin and add 10% Formic acid
3	Depending on the tissue and the level of calcification, leave in Formic acid for 1 or more days before endpoint testing
4	Change the Formic acid daily
5	When the endpoint test is required, take 5ml of the Formic acid (before the daily change) and place in a glass universal
6	Add a small piece of red litmus paper
7	Add concentrated Ammonia, dropwise, until the litmus paper turns blue
8	Allow to stand for 1 minute
9	Add 1ml saturated ammonium oxalate
10	Leave to stand for 30 minutes
End point testing	
Cloudy liquid	= decalcification incomplete
Clear liquid	= decalcification is complete and tissue can be processed

A1.5 Methods for different RNA extraction kits

Table A1.4. Protocols for three RNA extraction kits tested during optimisation experiment to determine best kit for extracting total RNA from whole aortic tissue samples. Details are a summary of the manufacturer's instructions.

ThermoScientific GeneJET RNA purification kit	30mg of frozen whole aortic tissue was placed in 300uL of propriety lysis buffer supplemented with β-mercaptoethanol. The sample, lysis buffer and β-mercaptoethanol were homogenised using the Precellys tissue homogeniser with CK14 beads for 10 seconds followed by a 10 second pause, a further 10 seconds followed by a 10 second pause, and a final 15 seconds at 6500rpm. Regular pauses are required due to the rapid generation of heat within the homogenisation process which could denature the homogenate. Thereafter 600uL of diluted proteinase K was added to the homogenate to digest contaminating proteins, and this was vortexed for 15 seconds then incubated for 10 minutes at room temperature. The homogenate was then centrifuged at 12,000g for 10 mins, and the supernatant was transferred to a new RNase free processing tube. 450uL of 100% ethanol was added to the supernatant and mixed using a pipette. The sample was then passed through the GeneJET RNA purification column in 700uL aliquots by centrifuging the column for 1 minute at 12,000g, with all flow-through discarded. The column was then washed with 700uL of wash buffer 1 for 1 minute at 12,000g, followed by washing with 600uL of wash buffer 2 for 1 minute at 12,000g, then a further 250uL of wash buffer 2 for 2 minutes at 12,000g. The total RNA was then eluted in 80uL of RNase free water by centrifuging for 1 minute at 12,000g and collected in a 1.5ml processing tube and the elute immediately cooled on ice.

Qiagen RNeasy micro kit	5mg of full thickness aortic tissue was homogenised using the Precellys tissue homogeniser with CK14 beads in 350uL of lysis buffer RLT (containing β-mercaptoethanol) for 10 seconds followed by a 10 second pause, a further 10 seconds followed by a 10 second pause, and a final 15 seconds at 6500rpm. The lysate was then centrifuged for 3 minutes at 15,000g, and the supernatant was removed and transferred to a fresh processing tube. An equal volume of 70% ethanol was added to the clear lysate and mixed by pipetting. The sample was then transferred to the RNeasy spin column in 700uL aliquots and centrifuged at 8,000g, with the flow-through discarded. 700uL of buffer RW1 was added to the spin column and centrifuged at 8,000g for 15 seconds. 500uL of buffer RPE was then added to the spin column and centrifuged at 8,000g for 15 seconds. 500uL of 80% ethanol was then added to the spin column and centrifuged at 8,000g for 2 minutes. The spin column was then transferred to a new 2ml processing tube and centrifuged at 8,000g for 5 minute to dry the membrane. The spin column was then transferred to a new 1.5ml collection tube and 14uL of RNase free water was added directly to the spin column membrane. The sample was then centrifuged at 8,000g for 1 minute and the elute immediately cooled on ice.
Ambion mirVana Paris Kit	50mg of full thickness aortic tissue was homogenised using the Precellys tissue homogeniser with CK14 beads in 300uL of ice cold cell disruption buffer for 10 seconds followed by a 10 second pause, a further 10 seconds followed by a 10 second pause, and a final 15 seconds at 6500rpm. The homogenate was mixed with an equal volume of 2X denaturing solution and stored on ice for 5 minutes. An equal volume of Acid-Phenol and chloroform was then added to the solution, and the sample vortexed for 1

	minute. The sample was then centrifuged at 10,000g for 5 minutes, to compact the interphase, and the aqueous upper phase was transferred to a fresh processing tube and measured. 100% ethanol was then added at a volume of 1.25 x the volume of the aqueous upper phase. The lysate ethanol mix was then transferred to a filter cartridge in 700uL aliquots and centrifuged at 10,000g for 30 seconds, with the flow-through discarded. 700uL of miRNA wash solution 1 was then applied to the filter and centrifuged at 10,000g for 15 seconds with the flow through discarded. 500uL of wash solution 2/3 was then applied to the filter and centrifuged at 10,000g for 15 seconds with the flow through discarded, and this step was repeated. Finally, a further centrifugation at 10,000g for 1 minute was carried out to remove any residual fluid. The filter cartridge was then transferred to a fresh collection tube and 100uL of 95°C RNase free water was applied directly to the filter membrane. The sample was centrifuged for 30 seconds, with the elute collected and immediately cooled on ice.

A1.6 Making Hepes-buffered saline

Component	Formula Weight	Weight (g)	Concentration (mM) when made up to 1L
Sodium Chloride	58.44	8.1816	140
Hepes Acid	238.3	4.7660	20
Hydrous Magnesium sulphate	246.5	0.6163	2.5
Potassium Chloride	74.55	0.3728	5.0
Hydrous Calcium Chloride	147.02	0.1470	1.0

The above reagents were weighed and dissolved in 750ml of freshly drawn nanopure water together with 0.010g of Phenol Red and the solution stirred using a magnetic stirrer bar. The pH of the solution was adjusted to 7.4 by titrating with NaOH. The pH-adjusted solution was made up to 1 litre in a volumetric flask with more nanopure water and stored at 4^{o}C until use.

A1.7 Creating hyperglycaemic test mediums

Table A1.5. Steps used to create hyperglycaemic test mediums during Phase 2 experiments.

STEP	DETAILS
1	The desired final glucose concentrations of test mediums were decided as 4.6mmol/L (normoglycaemia), 12.5mmol/L (mild hyperglycaemia), 25mmol/L (severe hyperglycaemia), 50mmol/L (supraphysiological hyperglycaemia). Test mediums were designed to contain only a low concentration of serum so that any effect on the AoSMCs could be attributed to the effect of glucose rather than the serum.
2	Initial attempts to create small volumes (10ml) of each test medium were not possible because a minute quantity of D-glucose needed to be measured out to achieve the desired glucose concentration. This was technically not possible using the equipment available (e.g. 0.01422g of D-glucose to 10ml of M231 to create a 12.5mmol/L hyperglycaemic solution).
3	Creating larger quantities of test mediums (100ml or 1000ml) was not deemed appropriate due to the risk of test mediums becoming non-sterile with repeated use.
4	Therefore it was decided that 10x stock concentrations of test mediums would be created (i.e. 125mmol/L, 250mmol/L and 500mmol/L) and that these would be diluted 1:10 with serum-free M231 (incomplete M231) to achieve the desired final concentrations of glucose but with 1/10th the amount of serum (which is contained in the SMGS).
Creating a 12.5mmol/L test medium	
5	To calculate the amount of glucose needed to create a stock solution which when diluted 1:10 would create a 12.5mmol/L solution the calculation below was performed: *1ml of X mmol/L stock + 9ml of 4.6mmol/L incomplete M231 = 10ml of 12.5mmol/L test medium* *Calculating the amount of glucose in each solution,*

	X mg in 1ml + 7.452mg in 9ml = 22.5mg in 10ml *X mg in 1ml = 22.5mg – 7.452mg* *X mg = 15.048mg in 1ml of stock* Of note, the molecular weight of glucose is 180 g/mol and the concentration of complete M231 is 4.6mmol/L. Therefore 1L of complete M231 contains 180 x 4.6 = 828mg of glucose and 10ml of complete M231 contains 8.28mg of glucose.
6	Therefore to create 10ml of a 10x stock solution would require 150.48mg of glucose. However since each stock solution was to be constituted from 10ml of complete M231, which itself contains 8.28mg of glucose, this amount of glucose needs to be subtracted i.e. 150.48 – 8.28 = 142.2mg
7	Therefore 142.2mg of glucose was added to 10ml of complete M231 to create 10ml of a 10x stock solution which when diluted with incomplete M231 would produce a final test medium with glucose concentration 12.5mmol/L and serum concentration of 0.49%.
8	Similar calculations were performed to calculate the amount of glucose needed to create 10x stock solutions of the 25mmol/L and 50mmol/L test mediums. The 4.6mmol/L test medium did not require creating of a stock solution the glucose concentration was fixed.
Creating a 25mmol/L test medium	
9	367.2mg of glucose was added to 10ml of complete M231 to create 10ml of a 10x stock solution which when diluted with incomplete M231 would produce a final test medium with glucose concentration 25mmol/L and serum concentration of 0.49%.
Creating a 50mmol/L test medium	
10	817.2mg of glucose was added to 10ml of complete M231 to create 10ml of a 10x stock solution which when diluted with incomplete M231 would produce a final test medium with glucose concentration 50mmol/L and serum concentration of 0.49%.

Creating a 4.6mmol/L test medium	
11	A 4.6mmol/L test medium was created by diluting 1ml of complete M231 in 9ml of incomplete M231 to produce a final test medium with glucose concentration 4.6mmol/L and serum concentration of 0.49%.

A1.8 Making SDS-PAGE buffers

Table A1.6. Creating stock buffers for SDS-PAGE.

BUFFER DETAILS	INGREDIENTS FOR VARIOUS FINAL VOLUMES			
Resolving gel buffer: 1.5 M Tris-HCl, 0.4 % (w/v) SDS, pH 8.8	Tris-HCL	45.43g	90.86g	181.7g
	0.4% SDS	1.0g	2.0g	4.0g
	Final volume	250ml	500ml	1000ml
Stacking gel buffer: 1.5 M Tris-HCl, 0.4 % (w/v) SDS, pH 8.8	Tris-HCL	15.14g	30.28g	
	0.4% SDS	1g	2g	
	Final Volume	250ml	500ml	
Electrophoresis running buffer: 0.025 M Tris-HCl, 0.1 % (w/v) SDS, 0.2 M glycine, pH 8.3	Tris-HCL	75.75g		
	0.1% SDS	25g		
	Glycine	360g		
	Final volume	5l (5x Stock)		

The above stock solutions were made and stored at 0 - 4 °C.

Appendix 2: Supplementary results

A2.1 GLUT gene expression experiment in AoSMCs

Table A2.1. Results from qPCR experiment using PGK1 and TBP as reference genes demonstrating the highly variable relative gene expressions of various glucose transporters within each group.

Sample	Case/Control	[Glucose] conc	GLUT1 RelExp	GLUT3 RelExp	GLUT4 RelExp	GLUT6 RelExp	GLUT10 RelExp
1	Case	4.6		0.650302903	4176572.067	0.116478468	0.119552298
2	Case	4.6	895.6458844	7.790545127		0.255312047	0.423435138
3	Case	4.6	0.017326879	6.339581927	173.3420745	0.081949213	0.306063624
4	Case	4.6	38.20434512	94.29358721	10156.82304	0.413757721	0.649960384
5	Control	4.6		11.64669073		2.579291502	0.180099009
6	Control	4.6	395110537.9	35.00173367		0.804209983	0.259204753
7	Control	4.6	4.149867575	18.50507859		0.360845766	0.864422918
1	Case	12.5	1.496198603	16.54023503	0.407810213	0.401766763	2.511809007
2	Case	12.5	0.325950493	17.38526241	0.699230947	0.056430957	1.171415112
3	Case	12.5	1.453350761	7.526134663		0.057514052	0.780352875
4	Case	12.5	1.314437385		38102.59869	0.031189203	0.205069624
5	Control	12.5	219076.2566	4.493302241	0.926966277		1.020283951
6	Control	12.5	22111.7507	6.002984771	3109.758316	0.108771441	0.252600398
7	Control	12.5	3.588943634	8.032238761		0.167519113	0.565942033
1	Case	25	21268.07592	20.38143938	1.335114596	0.586839972	1.622713299
2	Case	25	100302875.9	48.5271239	128492.1864	0.218969761	1.665489958
3	Case	25	1.0901243	6.87880471	0.00436183	0.118885348	0.456441433
4	Case	25	2.984960952	29.16287828	0.007874151	0.075389272	0.56046001

5	Control	25		18.97887579		3.227515369	0.049635323
6	Control	25	40703965.56	7.717693277		0.10898592	0.147140831
7	Control	25	1.310664004	10.51386569		0.406517217	1.117473893
1	Case	50	54.89600674	12.72123265	26678.96202	0.299154887	0.591126581
2	Case	50		0.582446209	0.007936536	0.014934577	0.023465518
3	Case	50	0.250477393	26.58534354	5230.857817	0.17316859	0.397135083
4	Case	50	1.111527792	22.56733762	3054.533717	0.176117691	1.029993308
5	Control	50	0.648893157	1.222954679	3675434.875	0.08051973	0.243261654
6	Control	50	0.627633121	8.984775003	10984.18741	0.061220376	1.359714785
7	Control	50	0.163567989	2.741927944		0.009121422	0.311908917

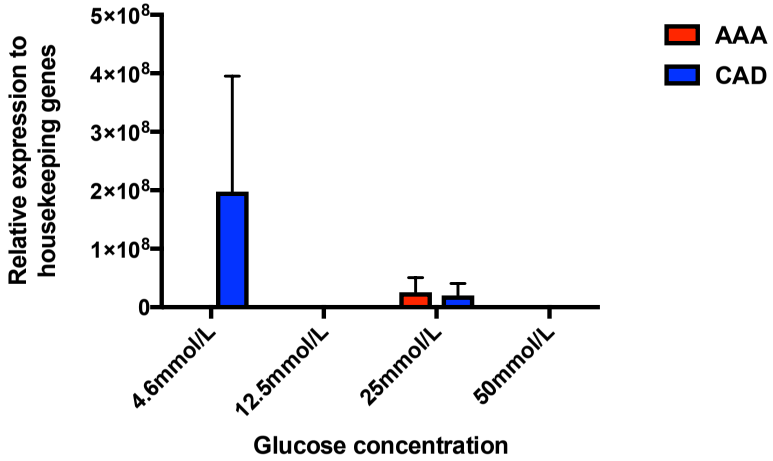


Figure A2.1. Graphical representation of highly variable relative expression of GLUT1 mRNA in aortic smooth muscle cells exposed to hyperglycaemia using PGK1 and TBP as housekeeping genes.

A2.2 Microarray data (secondary comparison)

Table A2.2. Differentially expressed genes discovered by microarray in aortic smooth muscle cells from patients with and without abdominal aortic aneurysm (n=8 vs. n=4).

Symbol	Definition	Fold Change	P value
Up-regulated in AAA			
LRIG1	Homo sapiens leucine-rich repeats and immunoglobulin-like domains 1 (LRIG1), mRNA.	1.80	0.00687
LRRFIP1	Homo sapiens leucine rich repeat (in FLII) interacting protein 1 (LRRFIP1), mRNA.	1.63	0.00745
CHRNA5	Homo sapiens cholinergic receptor, nicotinic, alpha 5 (CHRNA5), mRNA.	1.60	0.00257
LOC100128510	PREDICTED: Homo sapiens hypothetical protein LOC100128510 (LOC100128510), mRNA.	1.59	0.00206
LMOD3	Homo sapiens leiomodin 3 (fetal) (LMOD3), mRNA.	1.59	0.00519
LOC728903	PREDICTED: Homo sapiens hypothetical LOC728903, transcript variant 1 (LOC728903), mRNA.	1.59	0.00199
LOC401098	PREDICTED: Homo sapiens misc_RNA (LOC401098), miscRNA.	1.57	0.00140
SHROOM4	Homo sapiens shroom family member 4 (SHROOM4), mRNA.	1.57	0.00269
TMEM17	Homo sapiens transmembrane protein 17 (TMEM17), mRNA.	1.57	0.00178
LOC647389	PREDICTED: Homo sapiens hypothetical protein LOC647389 (LOC647389), mRNA.	1.56	0.00061
SEMA3E	Homo sapiens sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3E (SEMA3E), mRNA.	1.53	0.00722
DEM1	Homo sapiens defects in morphology 1 homolog (S. cerevisiae) (DEM1), mRNA.	1.53	0.00459
BLZF1	Homo sapiens basic leucine zipper nuclear factor 1 (BLZF1), mRNA.	1.53	0.00096
LOC729120	PREDICTED: Homo sapiens hypothetical LOC729120 (LOC729120), mRNA.	1.52	0.00173
CREB1	Homo sapiens cAMP responsive element binding protein 1 (CREB1), transcript variant A, mRNA.	1.52	0.00698
LOC100133516	PREDICTED: Homo sapiens hypothetical protein LOC100133516 (LOC100133516), mRNA.	1.51	0.00304
LOC100129502	PREDICTED: Homo sapiens hypothetical protein LOC100129502 (LOC100129502), mRNA.	1.51	0.00458

ZNF483	Homo sapiens zinc finger protein 483 (ZNF483), transcript variant 2, mRNA.	1.51	0.00690
PTGR2	Homo sapiens prostaglandin reductase 2 (PTGR2), mRNA.	1.51	0.00410
ILMN_1904980	xj89b12.x1 Soares_NFL_T_GBC_S1 Homo sapiens cDNA clone IMAGE:2664383 3, mRNA sequence	1.50	0.00867
LOC100128288	Homo sapiens hypothetical protein LOC100128288 (LOC100128288), non-coding RNA.	1.50	0.00984
ILMN_1904980	zr87e09.r1 NCI_CGAP_GCB1 Homo sapiens cDNA clone IMAGE:682696 5, mRNA sequence	1.50	0.00201
C3orf34	Homo sapiens chromosome 3 open reading frame 34 (C3orf34), mRNA.	1.49	0.00612
XRCC2	Homo sapiens X-ray repair complementing defective repair in Chinese hamster cells 2 (XRCC2), mRNA.	1.49	0.00496
TDRD1	Homo sapiens tudor domain containing 1 (TDRD1), mRNA.	1.49	0.00452
LOC100128098	PREDICTED: Homo sapiens hypothetical protein LOC100128098 (LOC100128098), mRNA.	1.48	0.00341
C14orf153	Homo sapiens chromosome 14 open reading frame 153 (C14orf153), mRNA.	1.48	0.00619
KIAA1751	Homo sapiens KIAA1751 (KIAA1751), mRNA.	1.48	0.00809
FAM63A	Homo sapiens family with sequence similarity 63, member A (FAM63A), transcript variant 1, mRNA.	1.48	0.00479
C15orf63	Homo sapiens chromosome 15 open reading frame 63 (C15orf63), mRNA.	1.48	0.00551
FKBP14	Homo sapiens FK506 binding protein 14, 22 kDa (FKBP14), mRNA.	1.47	0.00612
HNRNPU	Homo sapiens heterogeneous nuclear ribonucleoprotein U (scaffold attachment factor A) (HNRNPU), transcript variant 2, mRNA.	1.47	0.00388
ILMN_1900734	MR1-GN0172-061100-005-h03 GN0172 Homo sapiens cDNA, mRNA sequence	1.47	0.00351
LOC100130835	PREDICTED: Homo sapiens misc_RNA (LOC100130835), miscRNA.	1.46	0.00855
CCBE1	Homo sapiens collagen and calcium binding EGF domains 1 (CCBE1), mRNA.	1.46	0.00208
CATSPER2	Homo sapiens cation channel, sperm associated 2 (CATSPER2), transcript variant 4, mRNA.	1.46	0.00708
LOC730313	PREDICTED: Homo sapiens hypothetical LOC730313 (LOC730313), mRNA.	1.46	0.00596
ZMAT3	Homo sapiens zinc finger, matrin type 3 (ZMAT3), transcript variant 2, mRNA.	1.45	0.00944
LOC100129211	PREDICTED: Homo sapiens hypothetical protein LOC100129211 (LOC100129211), mRNA.	1.45	0.00598
NLRP8	Homo sapiens NLR family, pyrin domain containing 8 (NLRP8), mRNA.	1.45	0.00474
LOC100131989	PREDICTED: Homo sapiens hypothetical protein LOC100131989 (LOC100131989), mRNA.	1.45	0.00643
LOC100132391	PREDICTED: Homo sapiens hypothetical protein LOC100132391 (LOC100132391), mRNA.	1.45	0.00871
IL17RD	Homo sapiens interleukin 17 receptor D (IL17RD), transcript variant 1, mRNA.	1.45	0.00771
ARL16	Homo sapiens ADP-ribosylation factor-like 16 (ARL16), mRNA.	1.45	0.00223

ZNF69	Homo sapiens zinc finger protein 69 (ZNF69), mRNA.	1.45	0.00484
DUSP19	Homo sapiens dual specificity phosphatase 19 (DUSP19), mRNA.	1.44	0.00696
C14orf85	Homo sapiens chromosome 14 open reading frame 85 (C14orf85), non-coding RNA.	1.44	0.00363
DDX51	Homo sapiens DEAD (Asp-Glu-Ala-Asp) box polypeptide 51 (DDX51), mRNA.	1.44	0.00565
BMS1P5	Homo sapiens BMS1 pseudogene 5 (BMS1P5), non-coding RNA.	1.43	0.00297
USP49	Homo sapiens ubiquitin specific peptidase 49 (USP49), mRNA.	1.43	0.00072
FAM119A	Homo sapiens family with sequence similarity 119, member A (FAM119A), mRNA.	1.42	0.00981
GSTTP2	Homo sapiens glutathione S-transferase theta pseudogene 2 (GSTTP2), non-coding RNA. XM_941198 XM_945014 XM_945016	1.42	0.00388
GALNT10	Homo sapiens UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 10 (GalNAc-T10) (GALNT10), transcript variant 2, mRNA.	1.42	0.00737
SLC35E1	Homo sapiens solute carrier family 35, member E1 (SLC35E1), mRNA.	1.42	0.00585
LOC100132585	PREDICTED: Homo sapiens hypothetical protein LOC100132585 (LOC100132585), mRNA.	1.42	0.00937
MAGT1	Homo sapiens magnesium transporter 1 (MAGT1), mRNA.	1.42	0.00242
TRIM13	Homo sapiens tripartite motif-containing 13 (TRIM13), transcript variant 4, mRNA.	1.42	0.00511
LOC730990	PREDICTED: Homo sapiens hypothetical LOC730990 (LOC730990), mRNA.	1.41	0.00330
DUXAP3	Homo sapiens double homeobox A pseudogene 3 (DUXAP3) on chromosome 10.	1.41	0.00385
C11orf63	Homo sapiens chromosome 11 open reading frame 63 (C11orf63), transcript variant 2, mRNA.	1.41	0.00179
PPM1K	Homo sapiens protein phosphatase 1K (PP2C domain containing) (PPM1K), mRNA.	1.41	0.00396
SULT1A1	Homo sapiens sulfotransferase family, cytosolic, 1A, phenol-preferring, member 1 (SULT1A1), transcript variant 3, mRNA.	1.41	0.00159
LOC399900	Homo sapiens hypothetical gene supported by AK093779 (LOC399900), mRNA.	1.40	0.00157
LOC100129362	PREDICTED: Homo sapiens hypothetical protein LOC100129362 (LOC100129362), mRNA.	1.40	0.00726
SPTLC1	Homo sapiens serine palmitoyltransferase, long chain base subunit 1 (SPTLC1), transcript variant 2, mRNA.	1.40	0.00510
LOC100127975	PREDICTED: Homo sapiens misc_RNA (LOC100127975), partial miscRNA.	1.39	0.00574
MCART1	Homo sapiens mitochondrial carrier triple repeat 1 (MCART1), mRNA.	1.38	0.00784
LOC728620	PREDICTED: Homo sapiens misc_RNA (LOC728620), miscRNA.	1.38	0.00470
LOC728809	PREDICTED: Homo sapiens hypothetical LOC728809 (LOC728809), mRNA.	1.38	0.00271
LOC100128084	PREDICTED: Homo sapiens hypothetical protein LOC100128084 (LOC100128084), mRNA.	1.37	0.00348

TNFSF15	Homo sapiens tumor necrosis factor (ligand) superfamily, member 15 (TNFSF15), mRNA.	1.37	0.00381
LOC653566	PREDICTED: Homo sapiens similar to Signal peptidase complex subunit 2 (Microsomal signal peptidase 25 kDa subunit) (SPase 25 kDa subunit), transcript variant 3 (LOC653566), mRNA.	1.37	0.00147
HSD17B7	Homo sapiens hydroxysteroid (17-beta) dehydrogenase 7 (HSD17B7), mRNA.	1.37	0.00765
ZNF738	PREDICTED: Homo sapiens misc_RNA (ZNF738), partial miscRNA.	1.36	0.00983
DMC1	Homo sapiens DMC1 dosage suppressor of mck1 homolog, meiosis-specific homologous recombination (yeast) (DMC1), mRNA.	1.36	0.00809
LOC728554	PREDICTED: Homo sapiens similar to THO complex 3 (LOC728554), mRNA.	1.36	0.00901
LOC100128126	PREDICTED: Homo sapiens hypothetical protein LOC100128126 (LOC100128126), mRNA.	1.36	0.00856
PPA2	Homo sapiens pyrophosphatase (inorganic) 2 (PPA2), nuclear gene encoding mitochondrial protein, transcript variant 2, mRNA.	1.35	0.00500
C8orf45	Homo sapiens chromosome 8 open reading frame 45 (C8orf45), mRNA.	1.35	0.00608
FLJ46309	Homo sapiens hypothetical protein LOC649598 (FLJ46309), mRNA.	1.35	0.00745
LOC645452	PREDICTED: Homo sapiens similar to hCG1782414 (LOC645452), mRNA.	1.35	0.00739
TMEM106A	Homo sapiens transmembrane protein 106A (TMEM106A), mRNA.	1.35	0.00887
LOC644852	PREDICTED: Homo sapiens hypothetical protein LOC644852, transcript variant 2 (LOC644852), mRNA.	1.35	0.00925
MGC26356	PREDICTED: Homo sapiens misc_RNA (MGC26356), miscRNA.	1.34	0.00143
POFUT1	Homo sapiens protein O-fucosyltransferase 1 (POFUT1), transcript variant 1, mRNA.	1.34	0.00688
MYO3B	Homo sapiens myosin IIIB (MYO3B), mRNA.	1.34	0.00369
RBM3	Homo sapiens RNA binding motif (RNP1, RRM) protein 3 (RBM3), transcript variant 2, mRNA.	1.34	0.00638
C21orf58	Homo sapiens chromosome 21 open reading frame 58 (C21orf58), mRNA.	1.34	0.00739
LOC643509	PREDICTED: Homo sapiens similar to Dihydrofolate reductase, transcript variant 1 (LOC643509), mRNA.	1.33	0.00352
PHAX	Homo sapiens phosphorylated adaptor for RNA export (PHAX), mRNA.	1.33	0.00863
SLC5A8	Homo sapiens solute carrier family 5 (iodide transporter), member 8 (SLC5A8), mRNA.	1.32	0.00793
ILMN_1915783	FNPANH10 FNP Homo sapiens cDNA, mRNA sequence	1.32	0.00852
LOC653829	PREDICTED: Homo sapiens similar to Williams Beuren syndrome chromosome region 19, transcript variant 6 (LOC653829), mRNA.	1.32	0.00598
LOC100128505	PREDICTED: Homo sapiens similar to hCG2021201 (LOC100128505), mRNA.	1.32	0.00745

FLJ44124	Homo sapiens hypothetical protein LOC641737 (FLJ44124), mRNA.	1.32	0.00181
DEPDC7	Homo sapiens DEP domain containing 7 (DEPDC7), transcript variant 1, mRNA.	1.31	0.00674
ILMN_1857081	te46f04.x1 Soares_NhHMPu_S1 Homo sapiens cDNA clone IMAGE:2089759 3, mRNA sequence	1.31	0.00429
SYAP1	Homo sapiens synapse associated protein 1, SAP47 homolog (Drosophila) (SYAP1), mRNA.	1.31	0.00981
LOC646996	Homo sapiens hCG2040201 (LOC646996), non-coding RNA.	1.30	0.00763
NDUFB6	Homo sapiens NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 6, 17kDa (NDUFB6), nuclear gene encoding mitochondrial protein, transcript variant 1, mRNA.	1.30	0.00576
KIAA0408	Homo sapiens KIAA0408 (KIAA0408), mRNA.	1.30	0.00990
CREB1	Homo sapiens cAMP responsive element binding protein 1 (CREB1), transcript variant A, mRNA.	1.29	0.00713
XPNPEP3	Homo sapiens X-prolyl aminopeptidase (aminopeptidase P) 3, putative (XPNPEP3), mRNA.	1.29	0.00436
SLC16A12	Homo sapiens solute carrier family 16, member 12 (monocarboxylic acid transporter 12) (SLC16A12), mRNA.	1.29	0.00343
C2orf69	Homo sapiens chromosome 2 open reading frame 69 (C2orf69), mRNA.	1.29	0.00351
LOC202781	PREDICTED: Homo sapiens hypothetical protein LOC202781 (LOC202781), mRNA.	1.29	0.00039
GLIPR2	Homo sapiens GLI pathogenesis-related 2 (GLIPR2), mRNA.	1.29	0.00955
ZNF557	Homo sapiens zinc finger protein 557 (ZNF557), transcript variant 2, mRNA.	1.29	0.00839
LOC100134159	PREDICTED: Homo sapiens similar to Coiled-coil domain containing 144B (LOC100134159), mRNA.	1.28	0.00361
THOC3	Homo sapiens THO complex 3 (THOC3), mRNA.	1.28	0.00161
	xm52h06.x1 NCI_CGAP_GC6 Homo sapiens cDNA clone IMAGE:2687867 3, mRNA sequence	1.28	0.00348
FLJ25363	PREDICTED: Homo sapiens similar to hypothetical protein FLJ25976 (FLJ25363), mRNA.	1.28	0.00737
GDPD1	Homo sapiens glycerophosphodiester phosphodiesterase domain containing 1 (GDPD1), mRNA.	1.27	0.00196
SNAPC1	Homo sapiens small nuclear RNA activating complex, polypeptide 1, 43kDa (SNAPC1), mRNA.	1.27	0.00292
PCDHB9	Homo sapiens protocadherin beta 9 (PCDHB9), mRNA.	1.27	0.00310
LOC729090	PREDICTED: Homo sapiens similar to Eukaryotic translation elongation factor 1 alpha 1 (LOC729090), mRNA.	1.26	0.00849
LOC729603	Homo sapiens calcium binding protein P22 pseudogene (LOC729603), non-coding RNA.	1.26	0.00604
XAF1	Homo sapiens XIAP associated factor 1 (XAF1), transcript variant 2, mRNA.	1.26	0.00361
GALNT3	Homo sapiens UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 3 (GalNAc-T3) (GALNT3), mRNA.	1.26	0.00223
CSF2RA	Homo sapiens colony stimulating factor 2 receptor, alpha, low-affinity (granulocyte-macrophage) (CSF2RA), transcript	1.26	0.00807

	variant 6, mRNA.		
DTWD2	Homo sapiens DTW domain containing 2 (DTWD2), mRNA.	1.26	0.00443
LOC728105	PREDICTED: Homo sapiens misc_RNA (LOC728105), miscRNA.	1.25	0.00683
LOC100134868	Homo sapiens hypothetical LOC100134868 (LOC100134868), non-coding RNA.	1.25	0.00892
ZNF430	Homo sapiens zinc finger protein 430 (ZNF430), mRNA.	1.25	0.00453
Down-regulated in AAA			
STAMBPL1	Homo sapiens STAM binding protein-like 1 (STAMBPL1), mRNA.	-1.26	0.00016
DAB2	Homo sapiens disabled homolog 2, mitogen-responsive phosphoprotein (Drosophila) (DAB2), mRNA.	-1.26	0.00576
TRAK1	Homo sapiens trafficking protein, kinesin binding 1 (TRAK1), mRNA.	-1.27	0.00451
LOC392437	PREDICTED: Homo sapiens misc_RNA (LOC392437), miscRNA.	-1.29	0.00316
SLC1A5	Homo sapiens solute carrier family 1 (neutral amino acid transporter), member 5 (SLC1A5), mRNA.	-1.29	0.00025
CD164	Homo sapiens CD164 molecule, sialomucin (CD164), mRNA.	-1.29	0.00857
LRRC58	Homo sapiens leucine rich repeat containing 58 (LRRC58), mRNA.	-1.29	0.00803
GRN	Homo sapiens granulin (GRN), mRNA.	-1.29	0.00596
RHOQ	Homo sapiens ras homolog gene family, member Q (RHOQ), mRNA.	-1.30	0.00181
ANAPC13	Homo sapiens anaphase promoting complex subunit 13 (ANAPC13), mRNA.	-1.30	0.00511
KLF11	PREDICTED: Homo sapiens Kruppel-like factor 11 (KLF11), mRNA.	-1.30	0.00072
FMN2	Homo sapiens formin 2 (FMN2), mRNA.	-1.32	0.00343
LOC100132106	PREDICTED: Homo sapiens hypothetical protein LOC100132106 (LOC100132106), mRNA.	-1.32	0.00125
PRPS2	Homo sapiens phosphoribosyl pyrophosphate synthetase 2 (PRPS2), transcript variant 1, mRNA.	-1.33	0.00033
FVT1	Homo sapiens follicular lymphoma variant translocation 1 (FVT1), mRNA.	-1.34	0.00225
SH3D19	Homo sapiens SH3 domain containing 19 (SH3D19), mRNA.	-1.34	0.00965
IMPA2	Homo sapiens inositol(myo)-1(or 4)-monophosphatase 2 (IMPA2), mRNA.	-1.35	0.00454
TRIM4	Homo sapiens tripartite motif-containing 4 (TRIM4), transcript variant beta, mRNA.	-1.35	0.00855
PLAC9	Homo sapiens placenta-specific 9 (PLAC9), mRNA.	-1.36	0.00122
PRCP	Homo sapiens prolylcarboxypeptidase (angiotensinase C) (PRCP), transcript variant 1, mRNA.	-1.36	0.00403

AUH	Homo sapiens AU RNA binding protein/enoyl-Coenzyme A hydratase (AUH), nuclear gene encoding mitochondrial protein, mRNA.	-1.39	0.00252
RALGPS2	Homo sapiens Ral GEF with PH domain and SH3 binding motif 2 (RALGPS2), transcript variant 2, mRNA.	-1.41	0.00502
NT5DC1	Homo sapiens 5'-nucleotidase domain containing 1 (NT5DC1), mRNA.	-1.42	0.00441
VPS41	Homo sapiens vacuolar protein sorting 41 homolog (S. cerevisiae) (VPS41), transcript variant 1, mRNA.	-1.44	0.00324
ANKRD33	Homo sapiens ankyrin repeat domain 33 (ANKRD33), mRNA.	-1.50	0.00647
LARGE	Homo sapiens like-glycosyltransferase (LARGE), transcript variant 1, mRNA.	-1.51	0.00815
KCND2	Homo sapiens potassium voltage-gated channel, Shal-related subfamily, member 2 (KCND2), mRNA.	-1.52	0.00653
FAM110B	Homo sapiens family with sequence similarity 110, member B (FAM110B), mRNA.	-1.54	0.00532
USP53	Homo sapiens ubiquitin specific peptidase 53 (USP53), mRNA.	-1.54	0.00110
SASH1	Homo sapiens SAM and SH3 domain containing 1 (SASH1), mRNA.	-1.55	0.00969
ACVR2A	Homo sapiens activin A receptor, type IIA (ACVR2A), mRNA.	-1.68	0.00105
BEX2	Homo sapiens brain expressed X-linked 2 (BEX2), mRNA.	-1.77	0.00782
CTSF	Homo sapiens cathepsin F (CTSF), mRNA.	-1.91	0.00475
KIAA1324L	Homo sapiens KIAA1324-like (KIAA1324L), transcript variant 2, mRNA.	-2.31	0.00789
ADM	Homo sapiens adrenomedullin (ADM), mRNA.	-2.48	0.00647
MOXD1	Homo sapiens monooxygenase, DBH-like 1 (MOXD1), transcript variant 2, mRNA.	1.80	0.00687

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