

Flow Mediated Dilatation Over the Natural History of Abdominal Aortic Aneurysms

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*A thesis submitted for the degree of
Master of Science by Research in the Surgical Sciences*

Trinity 2016

Abstract

Abdominal aortic aneurysm, an abnormal dilatation of the descending aorta, disproportionately affects males and is often asymptomatic until rupture, posing major risk of mortality. The natural history varies greatly in individuals; some patients remain at subclinical aortic diameters for decades, others rapidly progress to sizes indicating surgical intervention. Surgical mortality is associated with age, so it is advantageous to predict growth of AAA. The biochemical implications of wall degradation may have systemic inflammatory effects which adversely affect the endothelium, which regulates vasomotor tone. Vasodilation is modulated by the release of nitric oxide and the measurement of flow mediated dilatation (FMD) is a widely used proxy for NO bioavailability. The goal of this study is to examine changes of FMD over time and its predictive value in AAA growth. We hypothesise that FMD correlates with future growth rate of AAAs in humans.

In FMD, ultrasonography is used to measure the diameter of an artery before and after it is subjected to shear stress through temporary limb ischaemia. No past study of AAA patients has examined serial measurements of FMD. The OxAAA study collects serial measurements on AAA patients under surveillance and undergoing surgery. FMD predicts future aneurysm growth of AAA patients and FMD deteriorates over time. Due to its non-invasive and cost effective nature, FMD is useful in predicting the growth of AAA, and has also been shown to predict cardiovascular events, serving a two-fold purpose. In clinical contexts, it may be used to foresee imminent need for AAA repair, allowing for pre-surgical lifestyle changes. FMD may also indicate future growth, allowing for less frequent surveillance and reducing costs.

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Acknowledgements

My foremost gratitude goes to my supervisors, Professor Ashok Handa and Dr. Regent Lee, without whom this project would not have been possible. Throughout the course of this degree, they have always been at hand with motivation in the face of challenges and encouragement in the face of successes. They have set me on the path of a scientific career with a continual stream of insightful questions and areas of inquiry, and have taught me never to overlook even the smallest detail.

Any scientific endeavor is a team effort, and the Oxford Abdominal Aortic Aneurysm team, past and present, runs as a well oiled machine for which I'd like to thank my colleagues. Amy Jones, whose coordination of this study is truly unparalleled in organizational might, has been the human face of this project. Dr. Ismail Cassimjee has been invaluable in providing clinical insight into the application of this research, filling gaps in my budding medical knowledge. Nicholas Killough, who was with OxAAA at its inception, and Felicity Woodgate, who showed me my way through my first ultrasound scan, have both given this project many hours and much imaging experience. Their contribution to the corpus of data for this project has been immense.

Finally, I must thank my family and friends, who, despite being four thousand miles away, have never left my side. My parents, Nirmala and Vijay, and sister, Navya, are no farther than a phone call away, although the time difference means I have surely woken them at odd hours. Without their support, I cannot imagine embarking on this journey, and I will always look to them as my motivation to continue on.

Abstract

Abdominal aortic aneurysm, an abnormal dilatation of the descending aorta, disproportionately affects males and is often asymptomatic until rupture, posing major risk of mortality. The natural history varies greatly in individuals; some patients remain at subclinical aortic diameters for decades, others rapidly progress to sizes indicating surgical intervention. Surgical mortality is associated with age, so it is advantageous to predict growth of AAA. The biochemical implications of wall degradation may have systemic inflammatory effects which adversely affect the endothelium, which regulates vasomotor tone. Vasodilation is modulated by the release of nitric oxide and the measurement of flow mediated dilatation (FMD) is a widely used proxy for NO bioavailability. The goal of this study is to examine changes of FMD over time and its predictive value in AAA growth. We hypothesise that FMD correlates with future growth rate of AAAs in humans.

In FMD, ultrasonography is used to measure the diameter of an artery before and after it is subjected to shear stress through temporary limb ischaemia. No past study of AAA patients has examined serial measurements of FMD. The OxAAA study collects serial measurements on AAA patients under surveillance and undergoing surgery. FMD predicts future aneurysm growth of AAA patients and FMD deteriorates over time. Due to its non-invasive and cost effective nature, FMD is useful in predicting the growth of AAA, and has also been shown to predict cardiovascular events, serving a two-fold purpose. In clinical contexts, it may be used to foresee imminent need for AAA repair, allowing for pre-surgical lifestyle changes. FMD may also indicate future growth, allowing for less frequent surveillance and reducing costs.

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List of Abbreviations

AAA		Abdominal Aortic Aneurysm
FMD		Flow Mediated Dilatation
NMD		Nitroglycerin Mediated Dilatation
NO		Nitric Oxide
eNOS		Endothelial Nitric Oxide Synthase
GTN		Sub-lingual metered dose nitroglycerin spray
S1		Surveillance patients in Stream 1 of study
S2		Surveillance patients in Stream 2 of study
FMB		Image loop for baseline measure of arterial diameter
FMC		Image loop for cuff-inflation diameter of artery
TP-x		Time point indicator
SOP		Standard Operating Protocol
PAOD		Peripheral Arterial Occlusive Disease
EPC		Endothelial Progenitor Cell

1

Introduction

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1.1 Abdominal Aortic Aneurysms

The diagnosis, management, and treatment of Abdominal Aortic Aneurysms (AAA) is evolving with the development of imaging tools, population monitoring programs, and improvements in surgical outcomes. Methods to diagnose aneurysms without imaging equipment remain limited [1], and the discovery of biomarkers to evaluate

risk of rupture and growth in individual patients can be prognostically useful in preventing deaths from rupture. In the pre-screening era, incidental diagnosis was the most common method of detection for AAA [2]. By 1991, proliferation of imaging resulted in increased detection by ultrasound [3]. During the late 20th century, the sharp increase in diagnosis of AAA was chalked up to a number of factors, including rising prevalence of smokers [4]. The prevalence of AAA increased at the end of the 20th century and is now plateauing across the developed world, although the proliferation of screening programs and incidental diagnoses likely account for the increase[5]. A 2012 review of changing diagnosis patterns in England and Wales concluded that while overall prevalence remained relatively steady, new records of AAA indicated that diagnoses were trending toward older patients, offset by a drop in prevalence in younger patients [6].

1.2 Causes

Studies agree that smoking, hypertension, age, and gender are major correlates of aneurysm, although causative relationship is more tenuous. One study posits that, after age, "smoking is the only modifiable risk factor that is associated with the development, expansion and rupture of abdominal aortic aneurysm," implying that gender is significant only because the number of chronic male smokers far outweighs the number of chronic female smokers in the high risk age bracket [7]. As the gender ratio of smoker equilibrates, the epidemiology of AAA changes; a 2013 study of AAA prevalence in developed nations showed trends supporting this theory [8].

1.3 Treatment

Mortality from AAA typically occurs through rupture, which carries up to 80 percent mortality [9, 10]. Rupture risk reaches a critical point at 6.5 cm diameter, upon which yearly risk of death is over 10 percent. A symptomatic AAA is an typically indication for emergency surgery regardless of size [11]. When a patient presents with a 5.5 cm aneurysm, intervention is considered and if suitable, elective surgical repair options are presented to the patient [12]. Mortality risk associated with elective open repair is relatively low at 3.5 percent, and endovascular repair (EVAR) risk lower still at 1.4 percent; it is nonetheless a significant consideration in an elective and highly invasive proceeding [13]. Advanced patient age is a major contributing factor to peri-operative complications [14]. Prior to reaching surgical size, surveillance scans are the extent of medical intervention provided; no feasible and efficacious solution has been found to prevent the growth of AAA, much less reverse it [15]. Cessation of smoking and medication to lower blood pressure are recommended as general health measures, and in the case of the latter, to reduce rupture risk.

Although aneurysm size is the strongest correlate for rupture risk, it remains a relatively weak predictive method for future growth. Despite a small positive correlation between growth rate and aneurysm size [16], some patients with small aneurysms grow unexpectedly, and others with large aneurysms remain stable [17]. High blood pressure is another contributing factor - it is of significant value to demonstrate predictors of aneurysm growth rate [18]. One such potential predictor is overall vascular health. AAA is a pathology closely tied with atherosclerosis and

peripheral arterial occlusive vascular disease (PAOD) [19]. Whether atherosclerosis is a causative factor in AAA or is a frequently observed comorbidity is still debated [20]. However, systemic endothelial dysfunction may still yield valuable information about potential for growth and rupture risk [21]. One noninvasive test for endothelial dysfunction is flow mediated dilation (FMD). FMD uses ultrasound imaging of the brachial artery to gauge the vessel's response to shear stress, typically applied through compression of the forearm. It carries the advantage of using the same equipment as ultrasound screening, thereby requiring minimal investment in additional health infrastructure.

1.4 Aortic morphology

In comparison to peripheral arteries, the abdominal aorta bears a high proportion of cardiac output and suffers from relatively low support by surrounding musculature. Typical diameter increase can be up to 1 mm per year especially during adolescence and other periods of rapid growth, with a healthy physiological upper bound of 30 mm diameter in transverse section [22][23]. The aortic wall is composed of three layers, the intima, media, and tunica. During the normal ageing process a variety of seemingly pathological changes occur in these layers. However, studies posit that the neither the presence of structural degradation of the wall nor the extent of degradation have utility in prediction of aneurysm progress[24]

1.5 Function of the vascular endothelium

The vascular endothelium, a single celled layer of the tunica intima, lines the entire lumen of the circulatory system and precipitates many active functions of regulation. Endothelial cells promote inflammation when necessary, instigate angiogenesis, regulate vasomotor tone through vasoconstriction and vasodilation, and protect the underlying tissue layers from the shear force of blood flow [25]. Calcification and atherosclerotic plaques can attenuate the effectiveness of the functions of the vascular endothelium in a variety of ways, bringing about systemic dysfunction in regulatory behaviour, impacting the overall cardiovascular health of an individual. A number of studies covering a large range of metabolic, genetic, and cardiovascular diseases have investigated the properties of this cell layer in the pathogenesis and systemic effects of cardiovascular disease (CVD), and in many cases found a relationship between the health of this layer and the probability of adverse cardiac outcomes for the individual.

Vasomotor tone, brought about by the contraction and expansion of the smooth muscle of the tunica media, is one of the vital signalling functions of the endothelial layer. The level of dilatation in a vessel affects the perfusion of surrounding tissue, regulates metabolic processes through the supply of oxygen, and impacts long term organ health. Two methods of modulating vasomotor tone through the endothelium have been observed. The first pathway, NO-dependent vasodilation, is well known - the enzyme Endothelial Nitric Oxide Synthase (eNOS) facilitates the production of nitric oxide (NO) gas from an existing molecule, the amino acid

L-Arginine. The gas diffuses through the tissue layers to the smooth muscle to initiate the production of cyclic Guanosine Monophosphate through Guanylate Cyclase, resulting in vasodilation. When released into the blood, NO is also an inhibitor of clot formation, and retards the ability of cells to stick to the endothelial layer, protecting against occlusion[26]. In conditions where the bioavailability of NO precursors is poor or the levels of eNOS are insufficient to maintain such a response, the endothelium may revert to NO-independent signalling through the release of factors that cause hyper polarisation of muscle, also resulting in dilation. The pathways that restore baseline state of constriction following a dilatory event are separate from those causing dilation [27].

1.5.1 Endothelial Nitric Oxide Synthase

The enzyme eNOS is central to the study of endothelial function, as it can act in the beneficial capacity of regulation but can also facilitate the production of reactive oxygen species - the production of the latter naturally competes for production of NO, resulting not only in the presence of redox-causing superoxides, but also reduced dilation, tissue perfusion, and loss of anti-thrombotic protection.

In a study of eNOS knockout mice, five out of twelve male mice developed aortic aneurysm or dissection, as well as atherosclerosis in expected regions [21]. A follow up study by the same group confirmed that hypertension does not account for the increased prevalence of atherosclerosis and aneurysm in eNOS knockout mice [28]. These two studies are landmarks in implicating eNOS in vascular pathogenesis. A mouse model of knockout eNOS characteristics is, however, unlikely

to fully reflect pathogenesis, because NOS has three isoforms, each of which causes varying biological effects. In pathological states, the interactive effects caused by interconversion, rather than total removal of one isoform is likely to be significant. In some cardiovascular pathologies the level of NOS is actually increased in the body [29]. This, however, does not result in an increase of NO; rather, the superoxides and peroxides produced by excess eNOS bind to NO, rendering it useless and degrading the vasodilatory response. Diabetes, smoking, high blood pressure, and hypercholesteraemia all contribute to the subversion of eNOS for the production of radical species [30]. Both hypothesis ultimately reflect a shortage of NO - whether by binding to superoxides or by lack of production altogether - as the cause of failure in anti-thrombotic and anti-atherosclerotic pathways. Although atherosclerotic lesions are a localised process reliant on flow characteristics, their effects on endothelial function are systemic, so measurements of vasodilation and reactive hyperaemia in non-diseased vessel segments can be useful in characterising other pathologies [31].

A link between NO deficiency and AAA also appears when examining the eNOS G894T polymorphism [32]. Further studies on this allele showed a correlation between aneurysm size and the number of repeats of G894T; in a study group of 61 patients in Turkey, those with aneurysms of greater than 5 centimetres showed more repeats of the gene than patients with aneurysms fewer than five centimetres [33]. A strong correlation was found between the number of eNOS4 allele repeats and the need for surgical intervention [34].

Mechanical properties of the vessel wall can be compromised when NO production

is lowered [35] During uniform remodelling, such as during ageing, pressure outward on aortic walls remains consistent resulting in slow non-pathogenic expansion; during the pathogenic process of dynamic wall remodelling that results in aneurysm, stiffness of the vessel is variant, such that the outward force of blood causes a dilation in regions of compliant vessels while stiff segments remain at a normal diameter. In models, the junction between stiff and compliant areas of the aorta has the highest level of axial wall stress, resulting in AAA presentation in mouse models. A gradient in stress brought about by inconsistent regions of arterial stiffness has also been posited to be the cause of aneurysm formation [36]. The study of stiffness brought on by dysfunction of endothelial nitric oxide production leading to wall stiffness in non-aneurysmic vessel wall, adjacent within the central arterial system, or distant in the peripheral arterial system -such as the brachial artery- may therefore be of value in the understanding of AAA growth and rupture rates.

1.5.2 Measuring endothelial function

A variety of invasive and non-invasive methods exist to evaluate endothelial function and the bioavailability of NO in the body.

Among the non-invasive methods, flow mediated dilatation, pulse waveform analysis, exhaled NO production, and regional vascular studies are in common use. Flow mediated dilatation (FMD) uses ultrasound imaging to visualise the diameter of a peripheral artery before and after a stimulus, typically arterial occlusion. FMD is sensitive to inter-observer variances and technician skill. Pulse waveform analysis typically uses measurements of the radial pressure wave, as measured by applanation

tonometry. The interference of soft tissue between a transducer and vessel, as well as a steep learning curve have brought criticism to this method [37]. In regional vascular studies, target organs are examined with an ultrasound; blood vessels in the eye can be used to generate accurate blood flow velocity profiles that can indicate vascular function [38]. This method can allow for real time signal analysis, but is also somewhat more uncomfortable for patients and is still sensitive to operator skill [39]. Exhaled NO production during sub maximal exercise is another method of measuring nitric oxide; while far less sensitive to observer variability, this method does require subjects to be physically capable of exercise. In addition, there is evidence that despite frequent use in the detection of hepatic, pulmonary, and upper respiratory inflammation, it is not applicable to the measurement of endothelial function [40]. Invasive methods of detecting NO may involve coronary catheterisation, injection of NO modulators in FMD, quantitative analysis of NO in the blood and urine, and measurement of inflammatory markers of circulating endothelial progenitor cells in the blood. Coronary angiography, while accurate, is invasive and as a diagnostic test carries relatively high risk [41].

1.6 Flow Mediated Dilatation

The predominant non-invasive measure of the health of the vascular endothelium is flow-mediated dilatation, known as FMD. FMD, developed as a non-invasive test of endothelial dysfunction, is widely used in scientific practice. The methodology of FMD has been refined from requiring bulky equipment and cannulation to a

non-invasive, minimally painful ultrasound scan that can be carried out in any clinical centre with a sonography unit. In locations that scan for AAA, it is likely that the infrastructure to carry out FMD also exists or can be acquired at little cost.

1.6.1 History of FMD

FMD capitalises on the behaviour of the endothelium when subjected to shear stress; the typical response in a healthy individual is an observable dilation [42]. In 1933, the response of large arteries to shear stress was first observed, but this was not causally linked to a response from the endothelium until the 1980s. Original tests of endothelial function were highly invasive, requiring a catheterisation lab to acquire direct access to the heart for the injection of acetylcholine. Other studies used cannulation of the arm to administer eNOS antagonists, and eventually testing moved away from any invasive procedures, instead using sublingual nitroglycerine, which is commonly prescribed for angina. Shear stress quickly became an important field of study, as long term responses to shear stress resulted in arterial remodelling and development of more extensive vasculature in the affected area. In the case of atherosclerosis, an unevenly distributed stress load from inside the vessel resulted in areas of greater plaque impact. Many studies have found low shear stress to be correlated with low plaque impact, resulting in uneven stiffness across various regions of artery [43]. It was quickly discovered thereupon that external stress, such as compression of the artery from the skin surface, could result in the same provocation of the vasodilatory response that high shear stress from blood flow

induced, resulting in the development of FMD as a measure of responsiveness of the vascular endothelium [44].

The fundamental principle of FMD is to measure the availability of NO, the primary vasodilator in the human cardiovascular system, through the response produced [45]. NO is a modulator of atherogenesis, and is therefore considered the most salient in the class of human vasodilators, which include prostaglandins and endothelium derived hyper polarising factor. The most common method of measuring FMD through reactive hyperaemia is as follows: Ultrasonography is used to obtain images of the resting state artery; in most cases, the brachial artery is used for the sake of convenience and consistency, but the radial or femoral arteries can also be used for this purpose [46]. The brachial artery is superficial enough for convenient compression and measurement, large enough for clear imaging, and highly responsive in the healthy individual so it is the most desirable location. Nitroglycerine mediated dilatation, or NMD, is a test often paired with FMD. As discussed previously, FMD is meant to be an assay specifically for the bioavailability of nitric oxide [47].

However, the process from shear stress to vasodilation involved reception of the shear stress signal, production and release of NO, uptake of NO by smooth muscle cells, and induced cellular response to NO. FMD only measures induced response, so regardless of whether NO is produced or not, FMD may be absent if the receptor pathway for NMD in muscle cells is defective. Conversely, administration of L-NMMA, a nitric oxide synthase inhibitor, will inhibit the hyperaemia response [48].

1.6.2 Factors influencing FMD

FMD is influenced by a number of structural and behavioural factors. Limiting or controlling for the influence of these factors is a prerequisite of the collection of reliable clinical data regarding vascular function [48].

One of the primary variables in conducting FMD in patients is position of the occlusion site during induced limb ischaemia. A 2001 study assessed the varying response to both proximal and distal occlusion in healthy individuals in order to standardise a method. Forearm occlusion was more consistent in the evaluation of the availability of nitric oxide, and was therefore recommended as the clinical standard in the measurement of FMD. Local properties of the brachial artery may also confound results of FMD, which is meant to be an indicator of systemic function. Witte attempted to investigate the potential misapprehension of FMD values due to local stiffness in the brachial artery in a 2005 study [49]. Witte observed that if brachial FMD was disparate with predictions from age and smoking, then it was likely that local factors affect the measurement of endothelial function. 10.7% of the patients of this study exhibited abdominal aortic aneurysms. The findings of the study concluded that local stiffness in the brachial artery could indeed confound results, and in order to counteract this, strata based on smoking or age could be separated in clinical trials as rough approximations of true endothelial function. In recent studies, an important addition to the study of FMD was the examination of diurnal variation in measurements. A study of a Korean cohort measured FMD in the early morning and early evening, and yielded an average of 8.5%, with no

statistically significant difference observed between the morning and evening tests [50]. In a study by Hwang et al [51], the average FMD was 8.9%, and Imamura et al demonstrated an average FMD of 8.2%. Although the average value listed for healthy volunteers was between 6 and 12% in most studies, there was significant variation, as observed by Pyke. In all cases, however, FMD of the healthy population was significantly positive. In addition, further studies have proven that not only is diurnal variation an insignificant factor in FMD measurement, day to day variation in healthy volunteers also does not show significant statistical variation [52].

The sum of the variety of studies on individual factors affecting FMD have resulted in the convergence of the protocol for the test. A 2002 paper by Coretti et al [53] established methodology, later reviewed in 2011 by Thijssen [54]. In order to preserve consistency between trials, guidelines were set to minimize outside impact on study results. Patients are to be instructed to abstain from meals and exercise, as well as all stimulants (caffeine, etc) and depressants (alcohol, medications) for at least 6 hours prior to the study. In order to avoid signal noise from stimulation of the sympathetic nervous system, light, sound, and temperature fluctuations should be minimised, and care should be taken to maintain these conditions between study groups. As discussed earlier, cuff placement is optimal when distal to the artery being imaged, and it is agreed that an ischaemia time of 5 minutes is sufficient to induce hyperaemia while avoiding tissue damage. In the brachial artery, response to nitric oxide is relatively rapid in comparison to other peripheral conduit arteries, so it is recommended that FMD images be captured for 3 minutes following cuff deflation;

for other arteries, 5 minutes is the safest length to ensure peak diameter is captured.

1.6.3 Animal models of endothelial function

Independent of clinical studies, a number of animal models have linked factors involved in the pathogenesis of AAA to endothelial dysfunction, whether causatively through deprivation of nitric oxide bioavailability, or through correlation of FMD not with AAA itself, but with smoking or intracellular conditions.

In 2001, a mouse model of eNOS deficiency was found to induce spontaneous aortic aneurysm [21]. eNOS, or endothelial nitric oxide synthase, is an essential intermediate step in the release of nitric oxide gas, and by studying knockout mice without this protein, the immediate effects were studied. Mice were fed an atherogenic diet, and then dissected to examine for aortic lesions. While the absence of nitric oxide in itself was not the instigating factor in aneurysm development, growth rate and rupture risk were elevated compared to the baseline atherogenic ApoE deficient line, indicating that a measure of nitric oxide bioavailability could prove to have utility in predicting the natural course of an AAA in humans.

1.6.4 Human studies of FMD in AAA patients

Despite a proliferation of FMD as a research tool, the impact of FMD on AAA has largely remained in the shadows. Only a handful of clinical trials, spread out over several years, have collected data on the vascular response of patients with AAA, and fewer still have isolated the interaction of endothelial dysfunction and AAA.

The earliest trial to focus specifically on FMD as a tool to diagnose and distinguish AAA from other cardiovascular pathologies was conducted in 2002, by Knipp et al [55]. In a cohort of 30 patients, blinded FMD and NMD scans were conducted, and plasma nitric oxide was also evaluated using a biochemical assay. Of the 30 patients, one third had a diagnosed AAA, one third were controls, and one third had PAOD. Subjects with AAA exhibited the lowest FMD response when the ratio of maximum diameter to baseline diameter was observed, while simultaneously exhibiting elevated plasma nitric oxide, well above both the PAOD group and the healthy control group. The diminished vasodilatory response was observed even in the NMD portion of the study, and it remained distinct to the AAA group; the PAOD group, while showing somewhat diminished FMD response, showed no deviation from the healthy controls during NMD, suggesting that the source of the pathology lies in the bioavailability of nitric oxide and not an inherent failure of the vessel to respond. On the other hand, the AAA patients exhibited poor response even in high concentrations of plasma nitric oxide, whether naturally elevated or artificially. This would seem to indicate that a failure or degradation of the vascular smooth muscle is to be implicated in separating these two pathologies, though further study would be needed to demonstrate that it is truly unique to AAA. In addition, this study excluded current and recent smokers. Because AAA is so closely tied with smoking, it is possible that the small study cohort was not representative of the average case.

Shortly following the release of Knipp's work, another study related to FMD

and AAA was published, also in 2002 [56]. This study examined the risk of postoperative complications based on pre-operative FMD. Although this is unlikely to be of assistance in predicting the growth of AAA, it is nonetheless relevant when providing a recommendation towards or against elective interventional procedures. 24 patients in the study were undergoing such repair. The results found that patients who suffered postoperative consequences, including aneurysm rupture, had lower FMD percent and higher baseline arterial diameter. While no link between NMD and the risk of cardiovascular events was found, FMD was an independent predictor of operative risk. If the results of the Knipp study are to be applied, this risk assessment is not unique to AAA repair but is shared among the various pathologies included in this study, since Knipp indicated NMD to be a major differentiator of AAA and other arterial disease.

In 2005, another review by Abbularrage et al examined FMD in a number of disease states, AAA among them [57]. Only the 2002 study was identified as a source on this matter, further highlighting the need for work in the area. Abbularrage also posited that the elevated levels of nitric oxide in AAA patients may be attributable to a desensitisation of the endothelium to the vasodilatory signal, requiring ever increasing amounts to provoke a response.

Akamatsu et al provided two of three following works on this topic in 2007 and 2010. Their first study examined FMD in a cohort of 30 patients with AAA and 27 with PAOD [58]. Relevant concerns related to study design were brought up; peak NMD was found to occur at six minutes or more post administration, while in

previous studies recording was limited to only 3 or 4 minutes. This may have been a significant factor in the results of the Knipp study, as the NMD in AAA patients was found deficient. If, as suggested by Akamatsu, NMD was underestimated in that case, then nitric oxide sensitivity may be less of an issue than expected. In the second study by the Akamatsu group, FMD was compared between cohorts that had suffered a cardiovascular event such as a heart attack or AAA rupture, and those that had not [59]. This study also had a small cohort size, as there were only five subjects in the AAA group. However, across all pathologies it was evident that the incident group had lower NMD and FMD responses than the non-incident group. Both tests were independently predictive of adverse event risk, and additional information regarding time to maximal diameter was attained. This study found that maximum NMD was observed at 7.2 minutes following the first administration of nitric oxide or nitroglycerine. While these studies only tangentially address the effects of FMD and AAA together on outcomes and growth rates, they nonetheless indicate valuable areas of deficient study.

In 2009, the link between AAA size and FMD was investigated by Medina et al [60]. With a cohort of relatively small aneurysms averaging 43 mm, a negative linear correlation was discovered between aneurysm diameter and vasoreactivity. When large AAAs were compared with pre-surgical AAAs, a distinction between the FMD percent was observed, with the larger AAAs showing poorer response to shear stress. A comparison with CRP indicated an opposite correlation; the larger the aneurysm, the greater the concentration of pro-inflammatory factors, a conclusion supported

by literature. Medina posits that the two are related; that the increased levels of inflammation have an effect on the bioavailability of NO. However, this study neglects NMD measurements, which other studies have shown may be relevant to discerning the specific endothelial degradation occurring in AAA. The most recent study on this topic, in 2012, corroborates these results [61]. FMD was conducted on 78 subjects, who were then classified as healthy, small AAA, and large AAA. The large and small AAA group demonstrated significantly lower vasodilation than the control group, as well as a significant difference between the size groups. The study also used biochemical markers to peg FMD to a known risk factor, in this case the concentration of circulating endothelial progenitor cells (EPC). EPCs were evaluated by plating and counting colony clusters, and also through flow cytometry. EPCs, which positively correlate to cardiovascular health, were found deficient in patients with AAA compared to controls. Given that recent studies have not elaborated on the role of endothelium independent dilation, studied the effects of surgical intervention on endothelial function, or shown prognostic value in flow mediated dilatation, there is a wealth of territory to explore in this field.

1.7 Summary

Stratifying growth risk for abdominal aortic aneurysm through biomarker measurement would be an important step taking forward the treatment of aneurysms. AAA has a number of links to endothelial dysfunction, including its frequent concurrence with PAOD and atherosclerosis, size dependent correlation with genetic

markers related to shortages of nitric oxide synthase, and the potential link between mechanical factors and stiffness in pathogenesis brought about in part by NO shortages. Flow mediated dilatation is a convenient and non-invasive method to experimentally measure the response of arteries to shear stress, and when followed by nitroglycerine mediated dilatation, can distinguish NO bioavailability problems from smooth muscle degradation. FMD has the potential to improve upon baseline size as a method for predicting the future growth of diagnosed AAA.

Title and 1st Author	Year	Total cohort	AAA cohort	FMD% in HVs	FMD% in AAA	Conclusions
Impaired vasoreactivity despite an increase in plasma nitrite in patients with abdominal aortic aneurysms, Knipp	2002	30	11	2.96	-1.71	FMD and NMD are both lower in patients with AAA, and although FMD in patients with PAOD is not distinguishable from HVs, NMD is significantly decreased, possibly pointing to systemic processes involved in endothelial dysfunction.
Risk stratification for postoperative cardiovascular events via noninvasive assessment of endothelial function, Gokce	2002	187	24	-	-	Preoperative measurements of FMD were taken in a cohort of patients undergoing some type of vascular intervention. In patients that did not suffer perioperative complications, preoperative FMD was significantly higher than those that did, although NMD did not show any significant difference between the two groups.
Smooth Muscle Dysfunction in Patients Older than 54 Years of Age with Objective Evidence of Arteriosclerosis, Akamatsu	2007	75	30	3.3	2.10	In patients with AAA and PAOD, NMD and FMD were correlated. HVs had a significantly higher FMD than subjects, although the age of HVs was also significantly lower. There was no significant difference in NMD between subjects and HVs.
Relationship between Endothelium Dependent Vasodilation and Size of Abdominal Aortic Aneurysms, Medina	2010	30	30	-	3.92	Aneurysm diameter is significantly and negatively correlated with FMD. FMD was also negatively correlated with measurements of plasma CRP, while aneurysm diameter was positively correlated with CRP levels.
Nitroglycerin-mediated vasodilatation of the brachial artery may predict long-term cardiovascular events irrespective of the presence of atherosclerotic disease, Akamatsu	2010	93	37	3.1	2.10	FMD and NMD were both significantly lower in the group of patients that suffered a serious cardiovascular event between initial FMD and 47 month follow up.
Reduced number and impaired function of circulating endothelial progenitor cells in patients with abdominal aortic aneurysm, Sung	2013	78	63	8.8	5.71	Patients with large, small, and no AAA had significantly different FMDs, with FMD being inversely correlated with aneurysm diameter. Levels of endothelial progenitor cells (EPCs) in patients with AAA were significantly impaired.

Figure 1.1: Human studies of Flow Mediated Dilatation in AAA patients

2

Aims and Hypothesis

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2.1 Study aims

The aim of this study is to use flow mediated dilatation to evaluate the endothelial function of patients with abdominal aortic aneurysm over the natural history of the disease. Given the current clinical challenge of predicting the growth and unclear systemic effects of AAA, this study aims to find clinically relevant prognostic methods utilising FMD and characterise the progression and potential degradation of endothelial function in AAA patients.

2.2 Hypothesis

The primary hypothesis of this study is that impaired endothelial function as measured by flow mediated dilatation will be able to predict the future growth of an abdominal aortic aneurysm.

3

General Methods

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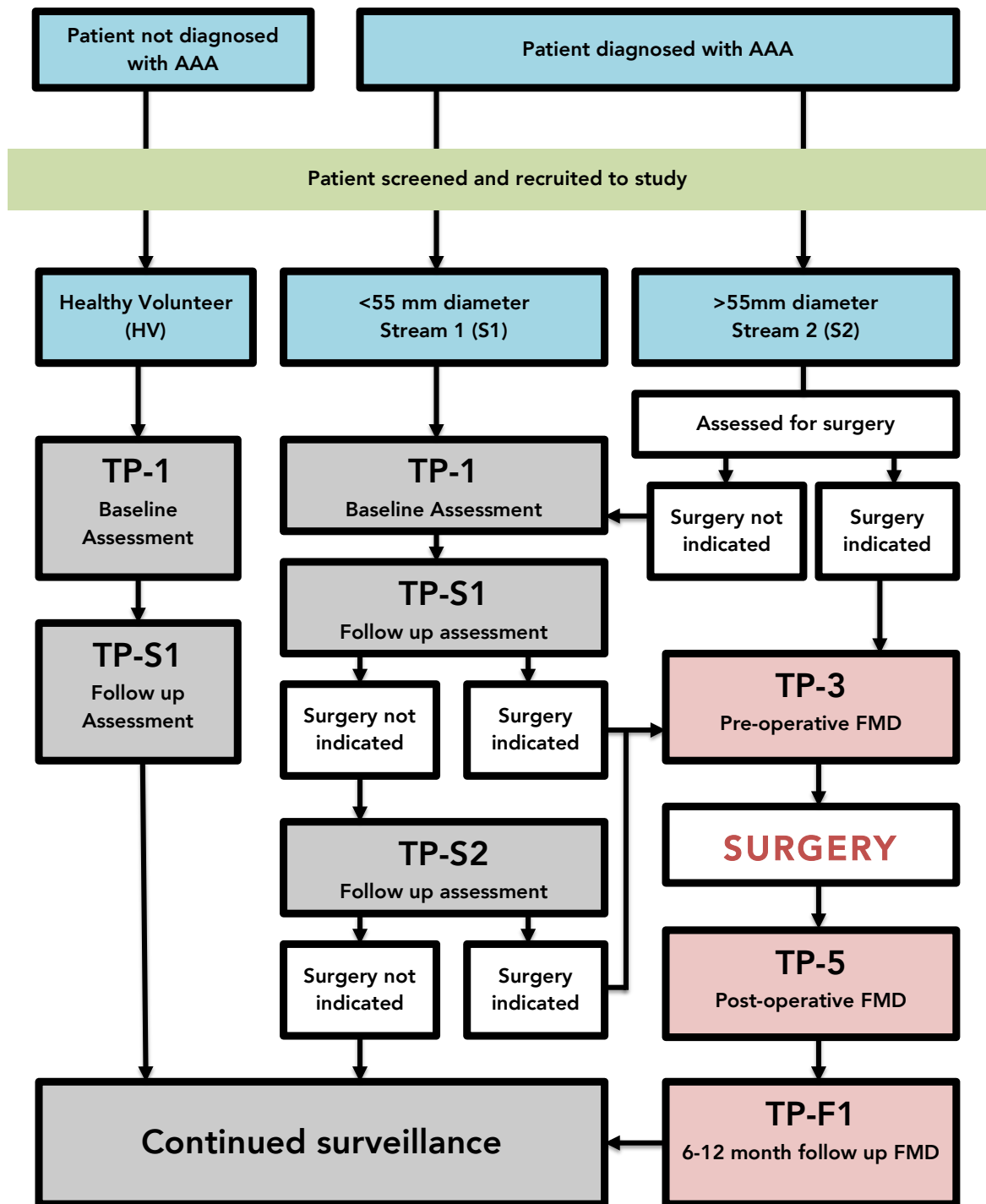


Figure 3.1: Study flowchart. Flowchart shows the longitudinal nature of the time point progression over two patient streams and one healthy volunteer stream

3.1 Study design

The Oxford Abdominal Aortic Aneurysm study (OxAAA) is constructed to longitudinally investigate the natural history of age-related AAA. Patients are recruited at the John Radcliffe Hospital, part of the Oxford University Hospitals NHS Foundation Trust (OUH). Exclusion criteria for the FMD sub-study include not being able to provide informed consent, and not being able to tolerate FMD, which can encompass inability to lie still, inability to lie flat for half an hour, and inability to observe verbal directions. Participants are approached after being identified through anonymized data in the OUH regional vascular unit database, or are identified by the clinical team during pre-operative preparations. If patients choose to participate, their study visits are synchronised with their regularly scheduled surveillance, surgical follow up, or other clinical visit to minimise inconvenience and transport costs.

3.1.1 Surveillance patients

Patients in the surveillance stream (S1) are those who enter the study with a diagnosed AAA but are not recommended for surgery. These patients are approached by letter for participation, are assigned a patient ID number and scheduled for their baseline assessment of FMD at time point on (TP-1). TP-1 is usually scheduled to coincide with their next scheduled surveillance with the Jackie Walton Vascular Studies Unit (JWVSU) of the John Radcliffe Hospital. The next surveillance time point, TP-S1, generally coincides with the patient's next aneurysm surveillance scan. As a result, the time span between TP-1 and TP-S1 is variable between

patients - those with the aneurysms closest to 55 mm are followed up at three month intervals, mid-sized aneurysms at 6 months, and low-sized aneurysms at 12 month intervals. At TP-S1, aneurysm diameter is also assessed. If the patient remains below surgical threshold, they will return for TP-S2 at the clinically indicated interval coinciding with their next scan, and afterwards will continue being seen at time points TP-Sx to coincide with future surveillance scans.

3.1.2 Healthy volunteers

Healthy Volunteers (HV) follow similar FMD scan intervals to surveillance patients, with a baseline TP-1 FMD at recruitment and a long term (>1 year) follow up TP-S1, and in future TP-S2 and TP-S3, etc. At all times, HVs also undergo abdominal ultrasound to confirm their eligibility for the HV category. As around eight percent of British men develop AAA, it is necessary to exclude diagnosis of AAA from potential HV participants.

3.1.3 Operative patients

Patients in the operative stream (S2) are typically recruited during their pre-operative assessment or on day of admission. The pre-operative assessment of FMD, typically the baseline for S2 patients, is time point three, or TP-3. Patients are then followed up eight to twelve weeks post-surgery for their time point five, or TP-5. Patients who are initially recruited to stream one and have baseline (TP-1) or further surveillance (TP-S1, etc) may also require surgery; in this case, stream one patients will have a TP-3 at their preoperative assessment, unless they recently

had a surveillance FMD which could also be used as a TP-3. Post-surgically, follow up intervals are labelled similarly in both S1 and S2 patients; a TP-5 at their follow up appointment typically 8-12 weeks following their surgery. At 6-12 months following surgery, patients return for a TP-F1 FMD scan. Longer term follow up intervals of TP-F2 and onwards are also planned for those patients willing to return to the hospital solely for study visits.

3.2 Study session

3.2.1 Materials

A Phillips CX50 ultrasound, which is used by the OUH for surveillance scans, is also utilised for the study with a curvilinear array transducer (L12-3) intended for peripheral vascular applications. For abdominal scans, a broadband curved array transducer (C5-1) is used. Images resulting from this ultrasound machine are in the DICOM file format.

For FMD, a blood pressure cuff, probe armature fixation device, and timing device are also required. The timing device used is an android touchscreen tablet using the Impetus timing app, which is programmed with the timing intervals for the FMD scan and will announce the commencement of each section of the scan automatically for improved accuracy. The specific timing and programming for the app may be found in the appendices. NMD also requires GTN spray at the full clinical strength of 400 micrograms. A full list of specific materials may be found in the standard operating protocols in Appendix 2.

3.2.2 Patient characteristics

After consent has been obtained, at the baseline scan (TP-1 or TP-3, depending on recruitment stream), a full clinical history is obtained. For the purposes of the study and abdominal surveillance scan, patients are asked to refrain from consumption of food or drink 6 hours prior to the study, especially coffee, which can affect vascular responses.

Baseline demographic data for each patient is recorded in a pre-defined clinical record form, which captures the history of smoking, cardiac disease, peripheral vascular disease, diabetes, surgery, alcohol consumption, familial risk, BP, height, weight, other medical conditions and medications were recorded. Blood and urine samples are obtained and prepared as part of the study protocol. The procedure for the scan is explained to the patient. NMD using GTN spray is performed as a part of the protocol unless there are concerns of its use due to clinical considerations. These may include low heart rate, low blood pressure, previous adverse reaction to GTN, known reactivity to nitro compounds, and susceptibility to migraines. Patients are also given an opportunity to refuse administration of nitroglycerine.

3.2.3 Aneurysm surveillance

The patient scans are anonymised through labelling with a patient ID. The OxAAA patient naming protocol is as follows: OxAAA-S'x'-'yyyyy' TP-S'z', to reflect the correct stream and number for each patient's substituted time point.

If the patient also requires an aneurysm surveillance scan, the typical JWVSU surveillance protocol is followed to obtain transverse and anteroposterior images

of the maximal diameter of the aneurysm.

3.2.4 Flow Mediated Dilatation

The protocol for FMD was first set out in 1992 by Celermajer and then refined in 2002 by Coretti [53]. The latter protocol has been in widespread use throughout flow mediated dilatation ever since. The protocol and timing for this study is adapted from established protocols by Leeson [62].

The patient is asked to lie supine and ECG tabs are affixed for the purpose of using R-wave gating during image collection. The patient is asked to stretch their right arm straight out, with the palm up, across a small cushioned table set to the same height as the bed.

The table is affixed to a stereotactic probe armature to assist in maintaining stable transducer position throughout the study. The brachial artery is visualized and maximal diameter transection is obtained. The ideal image included clear proximal and distal vessel boundaries, and the intima visible as a distinct layer. The closest zoom available on the CX50 is applied, and the gain and contrast is adjusted for clear and bright walls and dark, black lumen. Luminal artifacts might be indication that the vessel is not transected at the maximal diameter, and the probe should be adjusted to minimize them as shown in figure 3.3.

A full flowchart for the FMD and NMD studies may be found in the standard operating protocol in the appendices.

An ungated four second baseline image is captured (FMB-1 NG) as a backup in the event that gating fails during the gated baseline. An image without the zoom on,



Figure 3.2: Patient positioning during study. During FMD study, patient lies supine with arm outstretched, resting on two small cushions. The cushions elevate the arms to allow for better probe contact, and the space between the cushions allows for expansion of the cuff without displacing the arm or shifting the imaging plane

but with the position of the zoom box indicated, is captured to give a reference frame in the event that the vessel drifts during the zoomed in state. The gating module is turned on, and a sixty second baseline loop is captured (FMB-1). The radial pulse is located, such that occlusion can be confirmed following cuff inflation. The initial blood pressure measurement is reviewed, and the cuff is inflated at 20 millimeters Hg higher than the systolic pressure. The radial pulse is checked, and if not occluded, the cuff pressure is slightly increased until occlusion is obtained. The timer should be started at the initiation of cuff inflation; the period of cuff inflation should run five minutes and no longer, to prevent tissue damage from ischaemia. At five minutes, the cuff is deflated and immediately blood flow velocity is recorded (PHFM

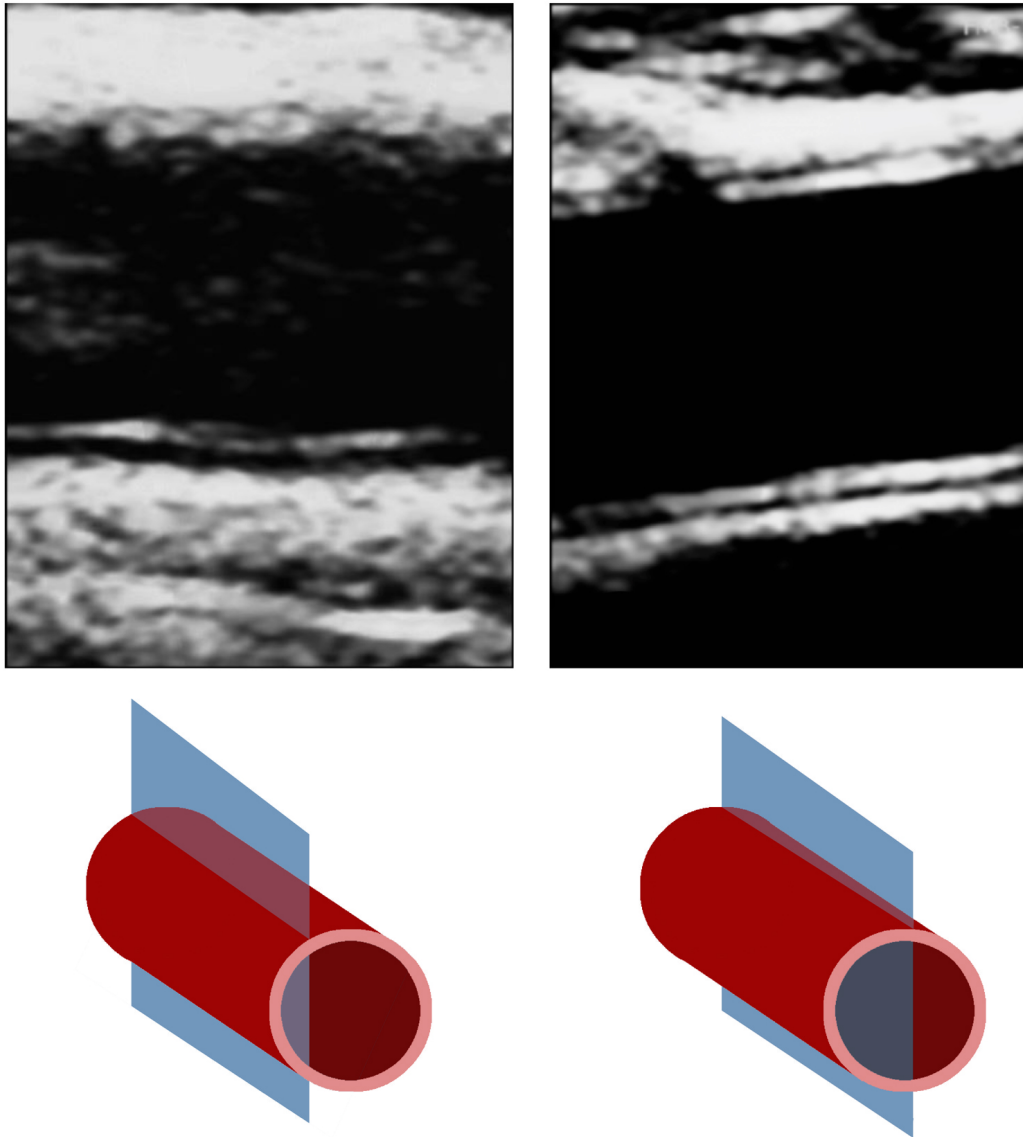


Figure 3.3: Imaging planes of the brachial artery Left: example of luminal artefacts and poor intimal visualisation, indicating failure of imaging plane to transect vessel at maximal diameter. Right: Accurate imaging plane showing clear intimal walls and absence of luminal artifacts.

VEL). Reactive hyperemia is easily evident during this recording. Immediately following, three minutes of gated image loop are obtained (FMD). Coretti observed that the maximal diameter frequently occurs at 45-60 second following cuff deflation, so three minutes should be a sufficient time frame for the capture of FMD.

If the subject has consented to the administration of GTN spray, there is a pause in the study to allow for return to baseline state. If NMD is contra-indicated or the subject refuses, the study session is complete.

3.2.5 Nitroglycerine Mediated Dilatation

Following a pause after FMD, a new gated 60 baseline loop is obtained (FMB-2). During the pause, the patient is encouraged to remain still, but if the vessel shifted during FMD, the probe can be adjusted to find the maximal and clear cross section. Once the baseline loop has been obtained, the first GTN spray of 25 micrograms is administered. If the patient does not suffer headache, dizziness, overheating, drop in heart rate, or other contraindicative signs, the next dose (400 micrograms) of GTN spray is administered sublingually after five minutes. After a waiting period, two and a half minutes of video loop are again captured. After NMD the patient is encouraged to sit up slowly, and is monitored for any adverse reactions.

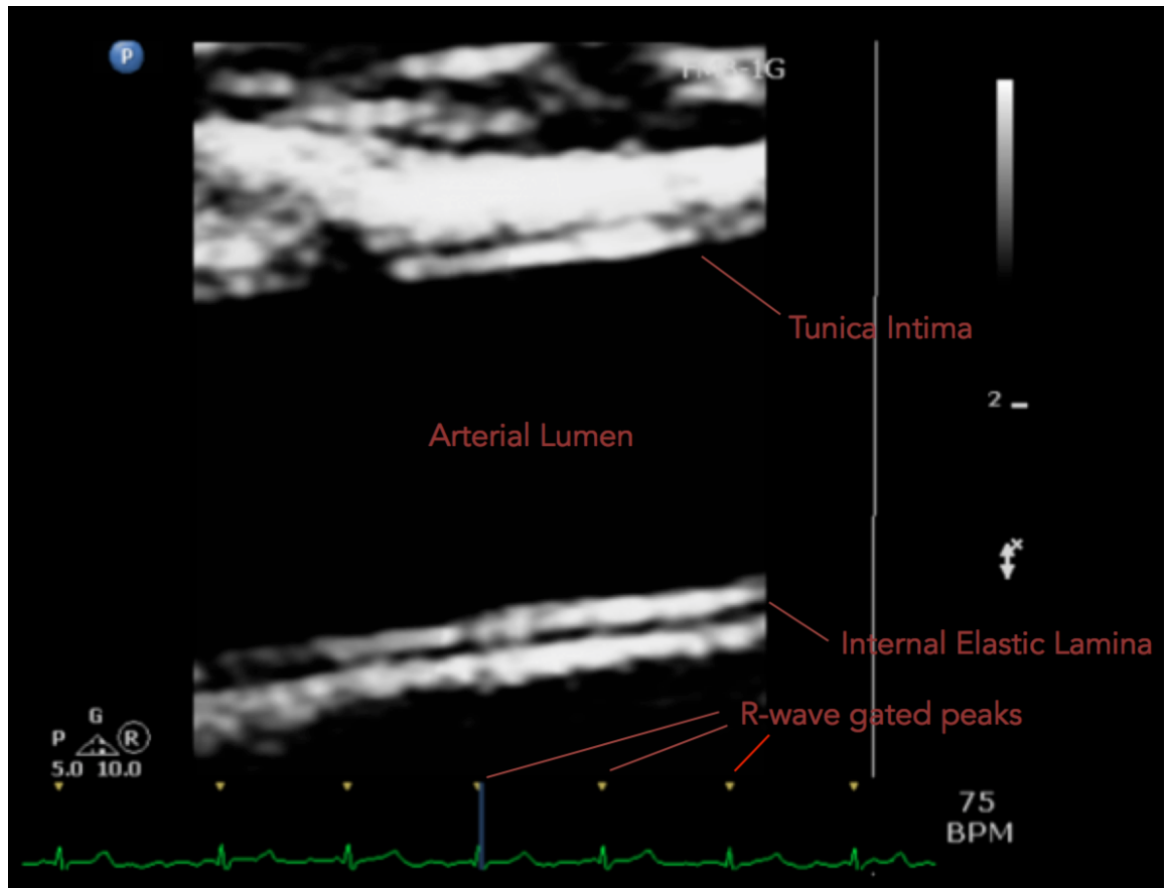


Figure 3.4: Example scan image. Image shows an arterial lumen free of artefacts. The intimal walls may be clearly visualized as separate from the media and outer layers of the vessel, with a dark internal elastic lamina showing as "tram lines" on the image. The bottom of the image shows the ECG trace used to gate the loops to R-wave peaks

3.3 Follow up

After the baseline assessment of FMD (TP-1), patients are followed up at the next clinically indicated interval depending on the size of their aneurysm. At the next FMD appointment, the clinical history is updated with any changes in medication, and a new height, weight, and BMI are observed. The FMD and NMD measurements are obtained as above, although if NMD was initially refused or counter indicated, it is not performed at subsequent study sessions. Patients also undergo their abdominal scan at each follow up surveillance appointment.

Surgical patients are followed up at their post-operative follow up appointment (TP-5) with their consultant; patients who underwent an EVAR procedure may have an abdominal scan, patients who underwent an open repair typically will not. Patients are asked to voluntarily return in 6-12 months for their long term follow up FMD measurement (TP-F1).

3.4 Image Analysis

The full protocol for image analysis specific to the images captured in this study may be found in Appendix 3.

3.4.1 Materials

Image analysis is done digitally through the use of the vessel tracking software Brachial Analyzer for Research Version 6.7.4, by Medical Imaging Applications LLC. This requires a computer with appropriate memory and access to the collection of scans from FMD and NMD studies. A full list of materials used for analysis may be found in the standard operating protocol in the appendix.

3.4.2 Image Retrieval

Images are retrieved from the Phillips CX50 ultrasound machine on a regular basis. A secure USB storage device is used to download all studies created since the last export are exported to the device. The images are moved to the OxAAA hard drive sorting folder. Detailed file storage and sorting process is included in the appendices.

3.4.3 Image Analysis

Image loops (FMB, NMB, FMD, etc) are opened in the Brachial Analyzer software. The region of interest (ROI) is selected based on the clearest available section of intima, and is set at the minimum width allowed by the software. A wider ROI risks capturing intermittent and non-parallel boundaries. The upper and lower boundaries of the ROI are edited to ensure that the yellow guidelines marking the vessel wall accurately reflect the inner edges of the intima. The analysis is first performed using

the internal automated process. Each frame is then manually viewed to look for aberrations in wall recognition. Frames that meet the rejection criteria are removed and only frames marked accurately by the computer are used in analysis.

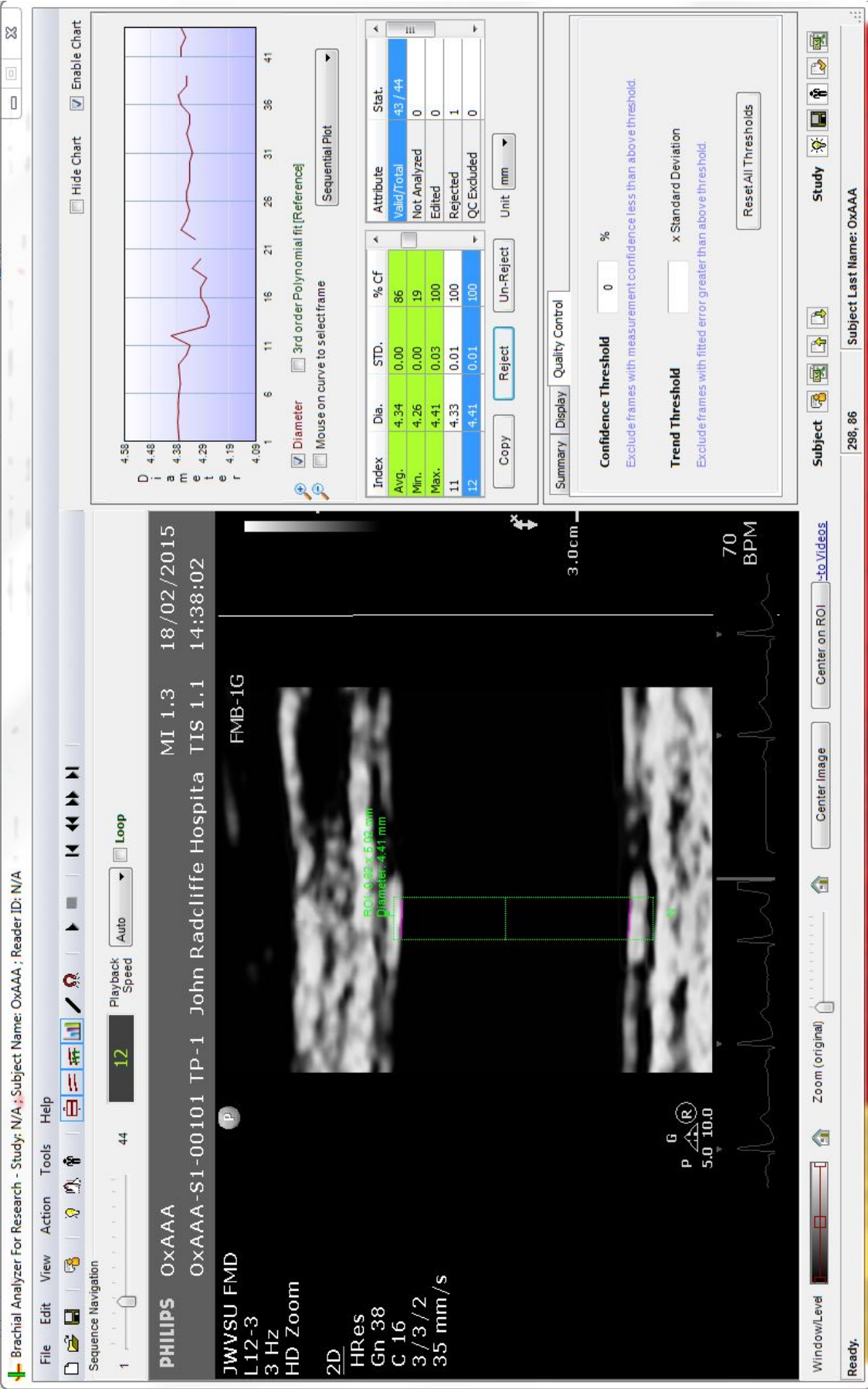


Figure 3.5: Image analysis example. Brachial Analyzer software showing change in diameter over time (upper right) in the region of interest selected

3.4.4 Calculation of percentage FMD

FMD is calculated as a percentage using the following equation reported in literature [53]:

$$\frac{(MaxDiameterinFMD - AverageDiameterinFMB)}{AverageDiameterinFMB} * 100$$

Max diameter in FMD, or FMD_{max} , is obtained by finding the single frame with the largest brachial artery diameter, while Average Diameter in FMB, or FMB_{avg} , is obtained by averaging the diameter of the brachial artery over each accepted frame in the baseline image loop. Figure 3.6 indicates on the left the maximum frame both numerically and at the maximum on the diameter v time plot produced during analysis of FMD, and on the right shows the frames used for obtaining average diameter in FMB.

For the calculation of average diameter in FMB, a minimum of ten frames is the threshold for an acceptable scan [54]. Because FMD uses the diameter from only one frame in the scan, it is especially important that this frame is clear and accurate. Usually, the maximum frame is very close to the next largest frame, and is NOT exceedingly larger than the next closest frames.

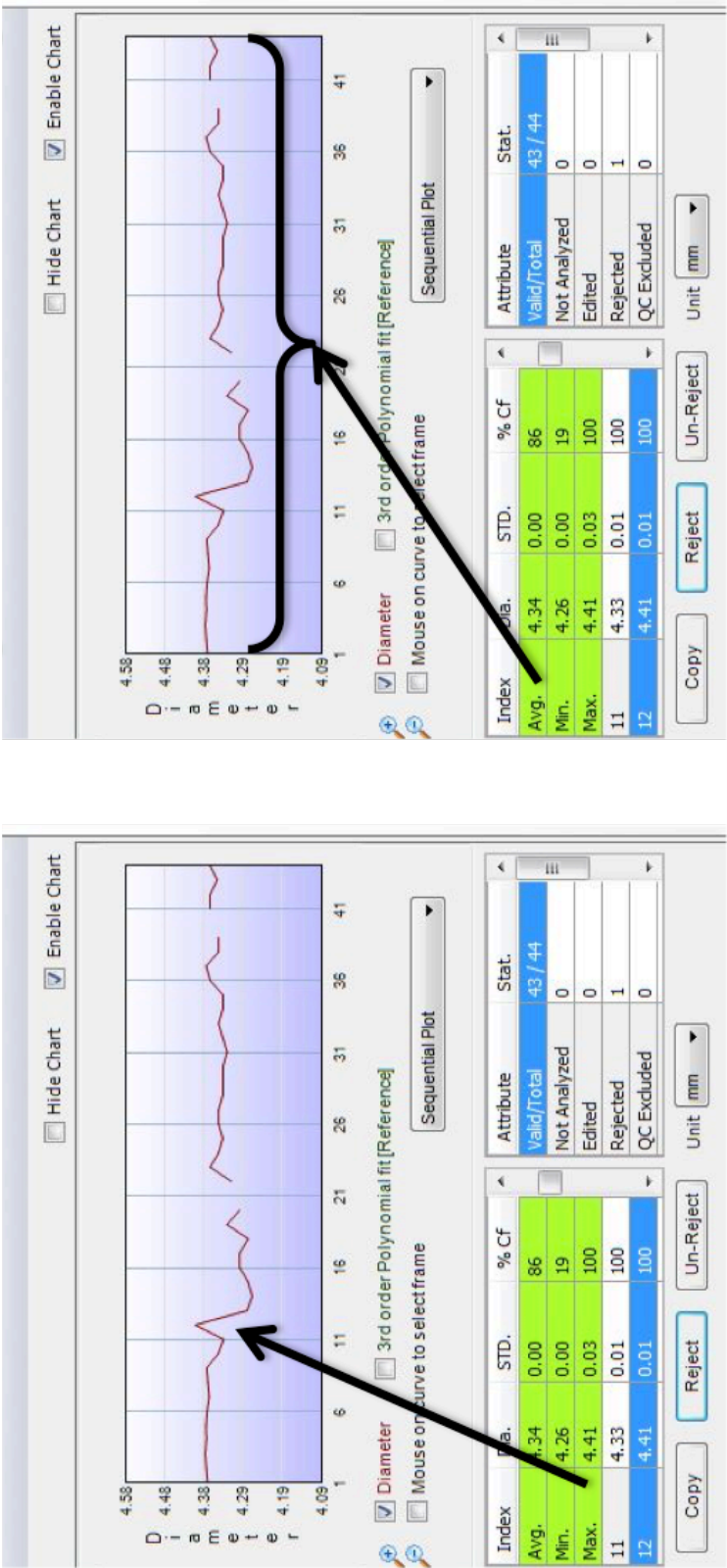


Figure 3.6: Example of obtaining FMD_{max} and FMB_{avg} during analysis. Image also indicates a gap in the graph where a frame has been rejected because it fit one of the rejection criteria outlined, and shows in the lower left hand corner the automatically calculated output for min, max, and average of frame sizes in green, as well as individual frame diameters in white. The buttons below the table can be used to select and reject individual frames based on either low confidence, visual mismatch, or other factors.

4

Methods Refinement

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4.1 Introduction

Although the initial methods were outlined in Chapter 3, over the course of initial analysis of 400 scans, as well as continual accrual of new scans, a number of refinements were made to both the scan and analysis methodology. Based on

quantitative and qualitative observations, analysis was repeated on all scans so that the final set would reflect a uniform methodology. In addition, a new method was tested and proposed for the ease of use of untrained observers, as a recommendation for future use. These observations are all reflected in the final SOPs found in the appendix, and have been incorporated into use for all current and future patients.

It has been demonstrated that consistent FMD measurements can be reproduced by different assessors [63]. We further sought to assess the reproducibility among different assessors within the research team. The FMD protocol and analysis protocol, both found in appendices, are constructed for minimal subjective interpretation. Reproducibility for FMD study was confirmed between observers. Intraobserver reproducibility for analysis was confirmed and used to refine the protocol for future analysis purposes, although all scans for the results in chapter 5 were analysed by a single observer.

4.2 Reproducibility methods

The reproducibility of FMD analysis was assessed to ascertain interobserver and intra-observer variability.

4.2.1 Scan interobserver reproducibility

All study sonographers were trained using the same protocol, as outlined in section 3.2.4 and the appendices. Inter observer FMD repeatability was tested by having two observers each run an FMD study on the same healthy volunteer, with a 10 minute rest period in between to allow recovery to baseline state.

4.2.2 Analysis

4.2.2.1 Interobserver variability

Interobserver variability was tested by repeating image analysis on a set of randomly chosen scans from both streams of the study by two separate observers.

4.2.2.2 Intraobserver variability

Intraobserver analysis was tested by repeating image analysis on a set of randomly chosen scans from both streams of the study by the same observer.

4.3 Consistency of FMD acquisition technique

The primary means of addressing error is through eliminating ambiguities and subjective interpretation in the SOP. The FMD SOP underwent a number of refinements to add cross-check measures. In the identification of the brachial artery, tracing back from the bifurcation, checking the pulse wave, and applying pressure to examine compressibility all ensure that the study is being performed on the correct peripheral vessel. In addition, the SOP was amended to include a check of the radial pulse preceding and following cuff inflation, since the same cuff pressure increase may fail to produce occlusion in some patients with advanced atherosclerosis. Ultrasound settings are programmed as a preset into the machine, and a picture of the location of the zoombox is acquired both before and after the FMD loop acquisition.

4.4 Results from optimization of FMD analysis protocol

While testing and training on the protocol described in Chapter 3, a number of observations were made and incorporated into final analysis method, which can be found in the SOP in the appendices. The most important points are highlighted as follows:

4.4.1 Region of interest selection

The selected region of interest should be on an unfragmented and unbroken section of the intima, and should remain consistent between analysis for FMB and FMD. The ROI should also be on a section of the vessel of moderate and consistent brightness, as brighter regions might be thickenings of imaging artifacts that do not accurately reflect vessel dilation.

4.4.2 Development of frame rejection criteria

The diameter of the vessel is dynamically tracked during analysis, resulting in a graph on the right hand side of the screen, shown in figure 3.5. Many of the aberrations and errors can be found in the spikes on this graph, so navigating to those frames first is the most effective way to remove rejected frames. A frame that is removed from analysis meets one of the following criteria: the computer recognized the media rather than the intima, the vessel shifting resulting in a blurry frame, the frame was not captured at the R-wave peak in the cardiac cycle, or intermittent intima, which is the most common reason for frame rejection.

The R-wave gating mechanism captures vessel diameter at end diastole, and usually results in a smaller diameter than when measured at end systole. Therefore, any scans in which the maximal diameter at FMD is captured in an ectopic heartbeat or otherwise not at an R-wave peak will usually have an inflated FMD percentage. On the other hand, scans in which the gating module in FMB was not accurate usually have deflated FMD percentages. This underscores the necessity to manually examine each frame for both accurate capture of vessel walls and accurate gating by the ultrasound machine. Even in atrial fibrillation patients, typically enough frames can be found to calculate FMD after rejecting unacceptable frames.

4.5 Reproducibility results

4.5.1 Scan Reproducibility

Interobserver reproducibility for sonography during the FMD study itself was confirmed by having each observer scan a set of ten healthy volunteers twice, with a break in between for recovery. Subjects were asked to abide by the same restrictions as study patients, including abstention from caffeinated beverages and food for six hours prior to the study.

It is important to note the patients RP-1 through RP-8 were recruited specifically for use only in reproducibility, their average age was much younger than the patient cohort and they were not registered as healthy volunteers. This explains the much higher FMDs that the RP group exhibits compared to both HV and S1 patients.

Analysis of scans were performed by a single, blinded, trained analyser using one ROI, to avoid multi-observer effects confounding effects. The results are shown

in table 4.4. There was found to be no significant difference between the two observers, and the two scans for each patient were found to be highly correlated in a spearman's correlation with a $p < .0004$. In addition, a Bland-Altman plot of reproducibility between two measures indicates that the mean difference of measures does not significantly differ from zero in a one-tailed t-test with $p > .05$.

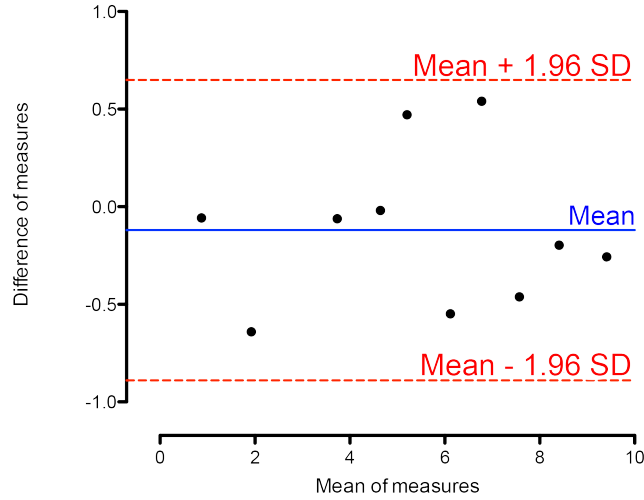


Figure 4.1: Bland-Altman plot for FMD reproducibility. This graph compares first and second FMD scans by a trained observers, showing that mean difference between measures does not significantly differ from zero in a one-tailed t-test ($p > 0.05$), $n=10$

Patient ID	Observer 1 FMD%	Observer 2 FMD%
RP-1	5.44	4.97
RP-2	3.70	3.77
RP-3	7.05	6.51
RP-4	5.84	6.39
RP-5	4.63	4.65
RP-6	7.34	7.80
RP-7	8.31	8.51
RP-8	9.28	9.54
HV-03	1.6	2.24
HV-24	0.85	0.90

Table 4.2: Reproducibility of FMD scan between two observers, analysis performed by single trained analyser

4.5.2 Analysis variability

4.5.2.1 Interobserver Variability

Results from two analysers were compared to examine variability between individuals. The analysers each examined 111 scans, blind to the other's results, and produced FMD values. A correlation is significant (Spearman's $\rho=0.71$, $p<0.001$). Longitudinal reproducibility of analysis across individuals is essential for a study with multiple time points, and increased correlation is expected as the new analyser becomes more proficient with the protocol.

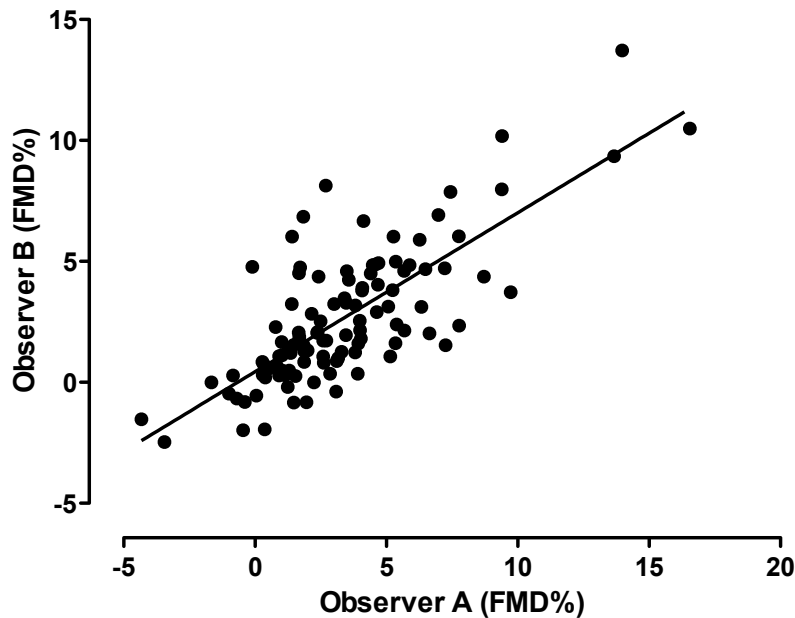


Figure 4.3: Comparison of FMD analysis results between two observers. Indicates a significant correlation ($p<0.001$, Spearman's $\rho=0.71$, $n=111$)

4.5.2.2 Intraobserver Variability

As described in the methods section, intraobserver analysis reproducibility was quantified through the repeat analysis of 37 randomly selected scans. The scans came from both stream one and stream two, and were from various time points. During the second analysis, the observer was blinded to the results of the first analysis, and images were analysed in a different order. The observer was well practiced, with hundreds of scans analysed. Analysis was performed by a trained analyser, using one region of interest, and the analyser was blinded between the first and second analysis. The results are shown below in a table and correlation graph between the first and second analysis. The correlation has a Spearman's Rho of .96 and in a two-tailed t-test, indicates that the two groups show no significant difference, with a $p < .0001$.

In addition, the repeated measurements may be compared in a Bland-Altman plot. The mean of the difference of measures was .008, indicating no significant skew toward higher or lower results upon second analysis. The general trend in data shows that larger values are distributed more sparsely around the mean difference of measures while at the lower end of FMD values, the difference of measures is smaller. This may indicate that systemic error is dependent on the magnitude of the values being measured.

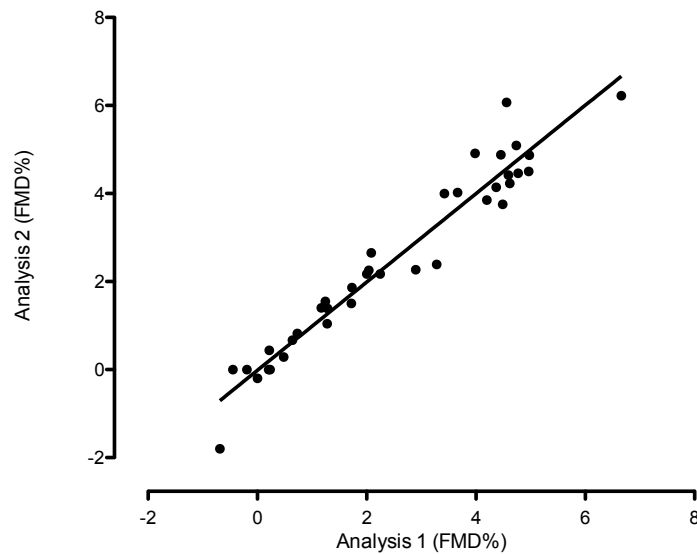


Figure 4.4: Correlation between first and second analysis. This graph shows the significant correlation between first and second analysis by a single, blinded observer. $p < 0.0001$, Spearman's $\rho = 0.96$, $n = 37$

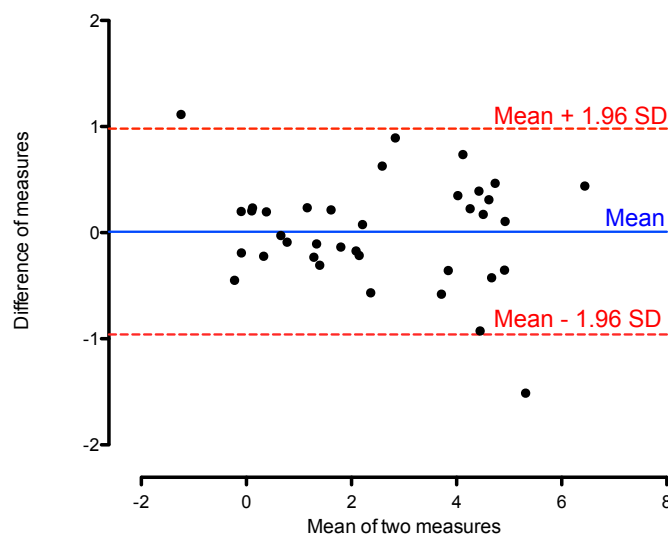


Figure 4.5: Bland-Altman plot for analysis variability. This graph comparing first and second analysis of scans by a single, blinded observer, showing no significant difference between the difference of measures and zero ($p < .001$), and 95 percent limits of agreement at .98 and -.96, $n = 37$

Patient ID	Time Point	Analysis 1 %FMD	Analysis 2 %FMD
S1-3	TP-1	2.90	2.27
S1-8	TP-1	2.25	2.17
S1-10	TP-1	4.20	3.85
S1-13	TP-1	-0.45	0.00
S1-16	TP-1	4.97	4.50
S1-17	TP-1	3.42	4.00
S1-19	TP-1	1.28	1.39
S1-22	TP-1	0.64	0.67
S1-26	TP-1	4.59	4.42
S1-32	TP-1	-0.69	-1.80
S1-37	TP-1	0.23	0.00
S1-39	TP-1	2.08	2.65
S1-43	TP-1	0.73	0.82
S1-48	TP-1	0.22	0.44
S1-51	TP-1	0.20	0.00
S1-56	TP-1	1.24	1.55
S1-63	TP-1	4.98	4.87
S1-69	TP-1	4.74	5.09
S1-71	TP-1	3.66	4.02
S1-77	TP-1	4.46	4.88
S1-80	TP-1	3.98	4.91
S1-87	TP-1	1.28	1.04
S1-90	TP-1	4.56	6.07
S1-98	TP-1	1.17	1.40
S1-103	TP-1	0.00	-0.20
S1-58	TP-3	2.26	2.04
S1-46	TP-3	0.00	-0.19
S2-6	TP-3	4.14	4.37
S2-41	TP-3	1.86	1.73
S2-14	TP-3	4.23	4.62
S2-18	TP-3	2.17	2.00
S1-58	TP-5	1.51	1.72
S1-46	TP-5	3.75	4.49
S2-6	TP-5	0.28	0.48
S2-41	TP-5	4.46	4.77
S2-14	TP-5	2.39	3.28
S2-18	TP-5	6.22	6.66

Table 4.6: Repeated analysis by single observer

4.6 Recommendation of FMD analysis method for future use

A significant challenge to reproducibility is the training of analysers. Selection of ROI is subjective and achieved mainly through practice over hundreds of scans, to find the optimal location balancing clarity and quantity of frames, as well as observing for minute shifts in the vessel that must be compensated for. Because vessels are inherently variable in diameter, it is absolutely vital to maintain the same ROI position between FMB and FMD, which, if the vessel shifts slightly, may not be the same relative position within the image. The surrounding fascia and vasculature may be used to compensate for shifts, but this is a subjective and difficult process. In addition, if the vessel is tortuous and transection is achieved in only one portion of the frame during baseline imaging, but is straightened or shifted in FMD observation, the FMD percent may be artificially deflated or inflated.

In order to compensate for training level between analysers, a set of ten clear scans was selected, and a skilled analyser obtained FMD percentage measurements using best judgement on ROI. Then, three arbitrary ROIs were selected, in the left, right, and centre of baseline and FMD loops, their position maintained between image loops. The FMD percent was calculated for each region and averaged for comparison between the skilled selection of ROI and arbitrary triplicate selection of ROI. The results are shown in the table below.

Table 4.7: Table of arbitrary triplicate ROI versus single skilled ROI selection

	Left ROI	Centre ROI	Right ROI	L/C/R avg	Skilled ROI
	%FMD	%FMD	%FMD	%FMD	%FMD
S1-98 TP-S1	2.67	2.64	1.53	2.28	2.8
S1-27 TP-S1	-2.68	-3.26	-2.98	-2.97	-2.47
S1-43 TP-S1	1.25	0.96	1.17	1.12	1.01
S2-6 TP-2	3.94	4.97	4.44	4.45	4.4
S2-31 TP-5	1.35	0.93	1.87	1.38	1.53
S1-28 TP-S2	2.95	3.04	3.30	3.10	2.97
HV-17 TP-1	2.90	2.74	2.36	2.66	2.5
S1-94 TP-S2	3.57	2.04	3.45	3.02	2.93
S1-112 TP-1	0.66	0.22	0.00	.29	0.43
S1-83 TP-S2	1.81	1.64	1.86	1.77	2.04

4.7 Discussion

Common criticisms of FMD address the influence of the observer in both the acquisition and analysis of images obtained in the study. It is a unique challenge of longitudinal studies to address the changing individuals involved. While it is possible to retain an observer for 2 or 3 years, following a pathology like AAA, which has been developing in some patients for decades, requires that substitution of observers does not influence study results. Of the studies that mentioned observer numbers at all in the human trials pertaining to AAA and FMD, the large majority specified that only one trained sonographer was obtaining the study images. In this study, reproducibility was assessed in a number of ways, and it is evident that the results of analysis and image acquisition are broadly reproducible between multiple observers. To date, the study has had three sonographers and four analysers, with overlap between the two groups.

Within one trained analyser (>400 scans experience), analysis results were

reproducible, with a confidence interval of about 1 percent in either direction, as evident in figure 4.4 and figure 4.5. However, given the high learning curve needed to acquire that much experience, it is also important that an untrained observer be able to replicate results in a reasonable timeframe. Otherwise, any observers added to the study would have to produce an entirely new set of analyses on the entire corpus of scans available, which is not ideal. The more stringent protocol makes strides toward making that a possibility. Figure 4.3 indicates a strong correlation between the first 100 analyses that a completely novice observer was able to produce and the matching analyses from the trained observer. While this is promising, the figure indicates that practice and subjectivity still play some role - while the two are strongly correlated in a linear fashion, the mean between the two observers are significantly different. The untrained observer had, in general, higher FMD percentages than observations from the experienced observer. However, this skew was evident across the entire spectrum and patterns and correlations in the data remained unchanged. The specific areas of discrepancy in following the protocol were discussed, and with more practice, the means will also likely converge. In future, using a triplicate method of ROI selection may also help to train new analysers and reduce variability.

Reproducibility of scans was more sound than reproducibility of analysis; when ten patients were scanned twice, by two different sonographers, and all scans analysed by a trained analyser, the confidence interval range was no wider than identical scans being analysed twice, as is evident when comparing figure 4.5 and

figure 4.1. Both sonographers were experienced, as would be expected of those administering a clinical examination. If there is observer bias introduced in FMD, it is not likely to be on the part of the ultrasound examination, but following it.

In general, reproducibility is confirmed for this study, although the training of new observers requires care and diligence, as well as stringent statistical confirmation that both the observed means and distributions are similar and correlated with prior results.

5

FMD as a Biomarker of AAA Progression

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	Aneurysm	Control
Number (male)	124(109)	23 (20)
Age, years (SD)	75(8)	68 (8)
Height, m (SD)	1.74(0.1)	2 (0.10)
Weight, kg (SD)	83(14)	85 (18.2)
Waist/Hip ratio (SD)	0.98(0.1)	1 (0.08)
Systolic BP, mm Hg (SD)	139(18)	141 (17.9)
Diastolic BP, mm Hg (SD)	78(13)	82 (10.6)
Smoking status, n (%)		
Current smoker	22(18)	1 (4)
Ex-smoker > 1 month	83(67)	12 (52)
Never smoked	19(15)	10 (43)
Diabetes mellitus, n (%)	21(17)	2 (9)
Hypertension, n (%)	80(65)	13 (57)
Hypercholesterolaemia, n (%)	73(59)	9 (39)
Past history of PVD, n (%)	29(23)	1 (4)
PVD intervention, n (%)	17(14)	0 (0)
Past history of IHD, n (%)	50(40)	3 (13)
IHD intervention, n (%)	23(19)	2 (9)
Past history of CVD, n (%)	20(16)	0 (0)
Valvular heart disease, n (%)	8(6)	0 (0)
Cardiac arrhythmia, n (%)	23(19)	2 (9)
Family history of IHD, n (%)	49(40)	5 (22)
Family history of aneurysms, n (%)	27(22)	13 (57)
Alcohol consumption, units/week (SD)	10.68(21)	13.00 (14)
Regular medication, n (%)		
Beta-blockers	49(40)	4 (17)
ACEI/A2RA	77(62)	8 (35)
Aspirin	75(60)	4 (17)
Anticoagulants	14(11)	2 (9)
CCBs	31(25)	6 (26)
Diuretics	28(23)	5 (22)
Gastro-restraint	44(35)	3 (13)
Steroids	14(11)	5 (22)
Statins	92(74)	12 (52)
Oral hypoglycaemic	15(12)	2 (9)
Baseline laboratory results, mean (SD)		
Total cholesterol	4.3(1.1)	4.8 (1.0)
HDL	1.2(0.4)	1.4 (0.4)
Calculated LDL	2.2(1.1)	2.5 (1.3)
Triglycerides	1.5(0.8)	1.3 (0.7)
HbA1c	40.8(9.7)	40.3 (8.3)
Creatinine	99.7(73)	81.5 (14.6)
Haemoglobin	14.1(1.5)	14.6 (1.2)
WCC	7.0(1.8)	6.9 (2.1)
Platelets	223.7(78)	235.9 (71.5)
CRP	4.3(1.1)	3.4 (4.8)

Figure 5.1: Cohort characteristics. Table illustrates demographic characteristics of S1 and control patients

5.1 Growth Rate Characteristics

5.1.1 AAA Size

At baseline, the average AAA size of all patients is 48 mm, with 14 patients at 55 mm or greater in S1 while still under surveillance, and 61 patients in the 40-55 mm range. The Society for Vascular Surgery and National Institute for Health and Care Excellence stratify AAAs into those lower than 40mm, 40-55mm, and greater than 55mm. It is agreed that those smaller than 40mm are at low risk, and those greater than 55mm are at high risk and should be referred for surgery. However, clinical treatment for the middle group is debated - they are kept on short surveillance intervals but are likely to require surgery eventually [64]. This group will henceforth be referred to as the clinically relevant group, as it is in their treatment that the most ambiguity arises.

5.1.2 Average Growth Rates

5.1.2.1 Is AAA growth dependent on size?

The data set was examined to determine if average retrospective growth rate was significantly different when stratified by AAA size. Retrospective growth is calculated from the first available AAA surveillance scan at which the patient was diagnosed, to the first FMD scan performed in the study. In the cohort of all patients in S1, the average growth rate is 2.19 mm/yr, and this is not significantly different in the 40-55 mm AAA size group, at 2.37 mm/yr. However, in the surveillance patients whose size at TP-1 is greater than 55, and likely progressed to surgery afterwards, the average growth rate was significantly higher, at 3.42 mm/yr.

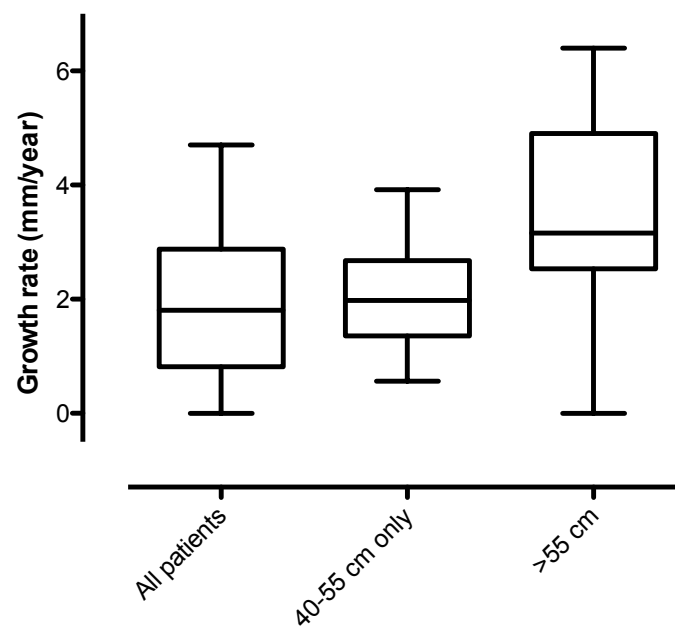


Figure 5.2: Retrospective growth of AAA size groups Growth rates are shown with interquartile range box and 10-90th percentile whiskers, and the group rate of the total cohort is not significantly different from the growth rate of the 40-55mm group, but growth is significantly higher in the >55mm group (Mann-Whitney unpaired t-test, $p < 0.05$)

Retrospective growth rates - all dates				Retrospective growth rates - all dates			
OxAAA Study ID	Dsize	Ddays	Growth rate (mm/year)	OxAAA Study ID	Dsize	Ddays	Growth rate (mm/year)
S100001	1.00	1057	0.35	S100060	1.00	377	0.97
S100002	3.00	716	1.53	S100061	23.00	2390	3.51
S100003	15.00	1166	4.70	S100062	0.00	92	0.00
S100004	1.00	1079	0.34	S100063	5.00	5801	0.31
S100005	9.00	956	3.44	S100064	1.00	1133	0.32
S100006	18.00	2891	2.27	S100065	7.00	1646	1.55
S100007	3.00	730	1.50	S100066	9.00	1632	2.01
S100008	6.00	535	4.09	S100067	1.00	686	0.53
S100009	11.00	2084	1.93	S100068	0.00	1077	0.00
S100010	7.00	721	3.54	S100069	8.00	2962	0.99
S100011	3.00	370	2.96	S100070	1.00	185	1.97
S100012	6.00	1171	1.87	S100071	6.00	1459	1.50
S100013	11.00	2186	1.84	S100072	0.00	167	0.00
S100014	21.00	3929	1.95	S100073	3.00	724	1.51
S100015	1.00	386	0.95	S100074	3.00	2178	0.50
S100016	2.00	504	1.45	S100075	17.00	2246	2.76
S100017	6.00	757	2.89	S100076	21.00	1399	5.48
S100018	4.00	2115	0.69	S100077	7.00	729	3.50
S100019	4.00	701	2.08	S100078	0.00	722	0.00
S100020	3.00	720	1.52	S100079	8.00	1448	2.02
S100021	4.00	1529	0.95	S100080	7.00	689	3.71
S100022	11.00	2599	1.54	S100081	7.00	2185	1.17
S100023	19.00	2449	2.83	S100082	0.00	1422	0.00
S100024	15.00	1660	3.30	S100083	3.00	370	2.96
S100025	7.00	1557	1.64	S100084	3.00	169	6.48
S100026	4.00	1211	1.21	S100085	1.00	178	2.05
S100027	8.00	1477	1.98	S100086	4.00	909	1.61
S100028	8.00	3012	0.97	S100087	3.00	791	1.38
S100029	7.00	1317	1.94	S100088	3.00	1074	1.02
S100030	10.00	1452	2.51	S100090	6.00	714	3.07
S100031	20.00	4262	1.71	S100091	0.00	356	0.00
S100032	2.00	2234	0.33	S100092	8.00	1220	2.39
S100033	12.00	2285	1.92	S100093	19.00	4685	1.48
S100034	1.00	738	0.49	S100094	7.00	757	3.38
S100035	15.00	1074	5.10	S100095	0.00	127	0.00
S100036	0.00	729	0.00	S100096	1.00	224	1.63
S100037	8.00	1665	1.75	S100097	5.00	359	5.08
S100038	2.00	847	0.86	S100098	3.00	1107	0.99
S100039	0.00	730	0.00	S100099	0.00	339	0.00
S100040	12.00	2185	2.00	S100100	4.00	414	3.53
S100041	0.00	1574	0.00	S100101	2.00	702	1.04
S100042	6.00	5123	0.43	S100102	2.00	351	2.08
S100043	7.00	1114	2.29	S100103	2.00	359	2.03
S100044	22.00	2714	2.96	S100104	2.00	378	1.93
S100045	2.00	708	1.03	S100105	2.00	400	1.83
S100046	17.00	503	12.34	S100106	0.00	206	0.00
S100047	2.00	102	7.16	S100107	1.00	374	0.98
S100048	4.00	547	2.67	S100108	3.00	337	3.25
S100049	7.00	543	4.71	S100109	2.00	1104	0.66
S100050	7.00	4795	0.53	S100110	4.00	538	2.71
S100051	10.00	2054	1.78	S100111	5.00	233	7.83
S100052	0.00	730	0.00	S100112	2.00	371	1.97
S100053	2.00	1287	0.57	S100113	1.00	253	1.44
S100054	3.00	4040	0.27	S100114	9.00	236	13.92
S100055	0.00	740	0.00	S100115	2.00	147	4.97
S100056	17.00	1639	3.79	S100116	12.50	777	5.87
S100057	13.00	2024	2.34	S100117	3.00	381	2.87
S100058	0.00	365	0.00	S100118	2.00	346	2.11
S100059	14.00	2547	2.01	S100119	8.00	1010	2.89

Figure 5.3: Total retrospective growth rates of S1 patients. Table illustrates variable interval length of surveillance, as well as typical growth rates across the surveillance cohort

5.1.2.2 Does total retrospective growth differ from recent growth in each cohort?

Data was also examined to see if between time points, growth patterns differed from total retrospective growth in each respective cohort. For aneurysms greater than 55 mm, there was a lower growth rate (1.87 mm/yr) between time points than retrospectively, though this can be accounted for by a shorter interval between first and last measurements as well as a low sample size since most AAAs larger than 55 mm are operated upon (Wilcoxon paired t-test, $p < 0.05$). The average total retrospective interval length was 1300 days (SD=1130) while the average recent interval length was 278 days. If growth occurred in spurts over the history of the pathology, it is likely that between time points, growth was stagnant. The prospective growth rate between time points for the 40-55 mm group was not significantly different from retrospective growth at 2.03 mm/yr (Wilcoxon paired t-test, $p > 0.05$)

5.2 FMD Results

5.2.1 Does FMD correlate to AAA size?

Comparisons were made between FMD percent and AAA size to establish their associations. Patients from both stream 1 and stream 2 were included, and the sizes of patients who were in the surgical stream were obtained from their diagnostic ultrasound or pre-operative CT scan from the hospital at which they were scanned. Patients in stream 1 had surveillance records available in the vascular studies surveillance records. No post-operative scans were included. FMD was inversely correlated to size in the total cohort in figure 5.4 (Spearman's $\rho = -0.3$, $p < 0.001$).

However, in the clinically significant cohort of AAAs sized between 40 and 55 mm, this correlation is absent. Figure 5.19 shows the distribution of FMD percent versus AAA, in which no clear pattern occurs.

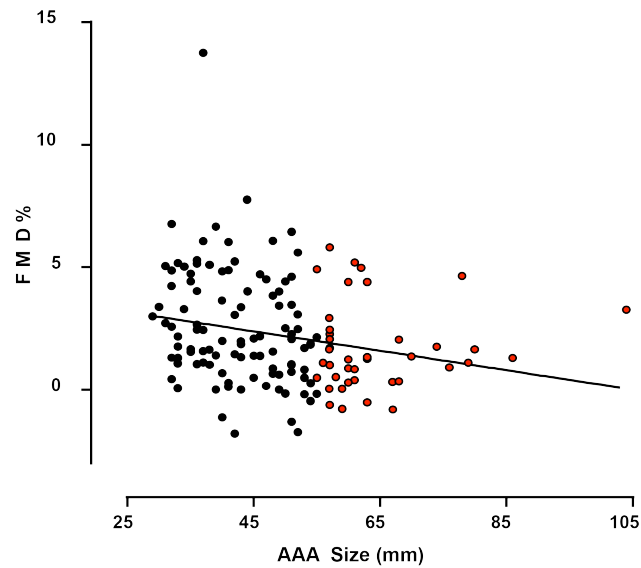


Figure 5.4: Flow Mediated Dilatation v. Size. Correlation of flow mediated dilation and size at first available time point. Red points indicate those patients above 55mm AAA diameter. Spearman's $\rho = -.03$, $p < 0.001$, $n = 143$

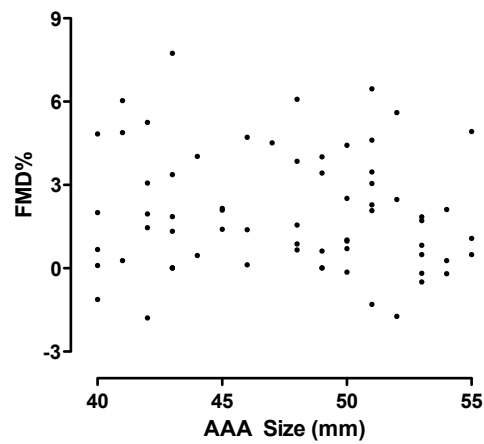


Figure 5.5: Flow Mediated Dilatation v. Size in 40-55mm AAA patients. Correlation of flow mediated dilation and size at first available time point in patients with AAA diameter 40-55 mm, Spearman's correlation $p > 0.05$, $n = 60$

5.2.2 Does FMD correlate to past growth?

In the group of all patients, FMD is compared to the total retrospective growth available leading up the point at which FMD was obtained. There is a significant and negative correlation between the FMD and retrospective growth rate; patients with high growth rates have poorer FMD in general. The Spearman's Rho for this correlation is -0.22, and $p < 0.05$, in figure 5.6. Growth in only the past year was also examined, as often only recent growth primarily informs clinical decisions. The past year's growth rate, however, was not correlated to FMD.

Neither the total retrospective growth nor the recent growth was correlated to FMD in the clinically relevant patients whose aneurysms were between 40 and 55 mm (figure 5.7).

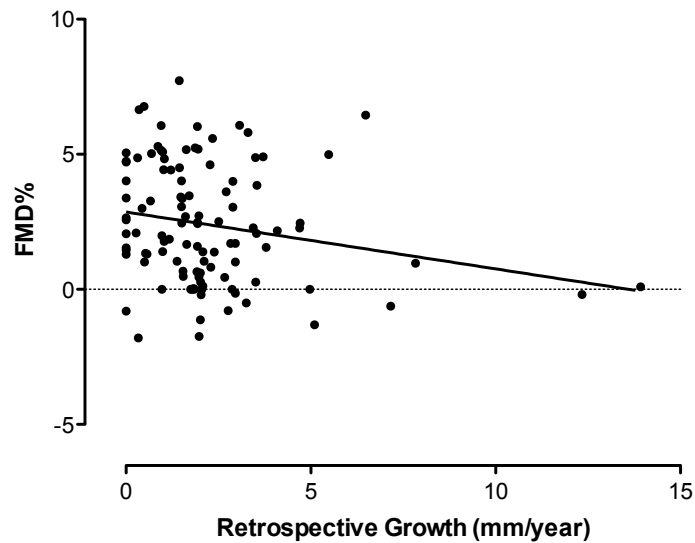


Figure 5.6: Flow Mediated Dilatation v. Total Retrospective Growth Rate Correlation between total retrospective growth rate and FMD at first available time point, Spearman's $\rho = -.22$, $p < 0.05$, $n = 107$

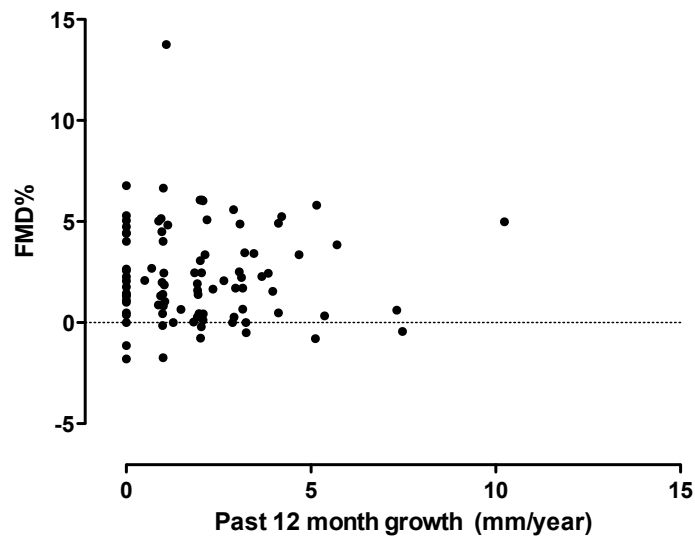


Figure 5.7: Flow Mediated Dilatation v. Recent Growth Rate Correlation between past 12 month growth rate and FMD at first available time point, Spearman's correlation $p > 0.05$, $n = 93$

5.2.3 Does FMD correlate to future growth?

Time point intervals of 12 months were found for as many patients as possible, and the growth rate between those intervals calculated using the anteroposterior measurements from surveillance data accompanying the time points. The FMD percent at the start of the interval was compared to the growth rate of the interval, and the results are shown in 5.8. In the set of all patients, FMD percent is strongly negatively correlated to the aneurysm's future growth behavior. The spearman's rho is -.41, with a $p < 0.001$.

The same correlation was examined for the set of clinically relevant patients, and the correlation persists with a spearman's rho identical to the full set and $p < 0.01$.

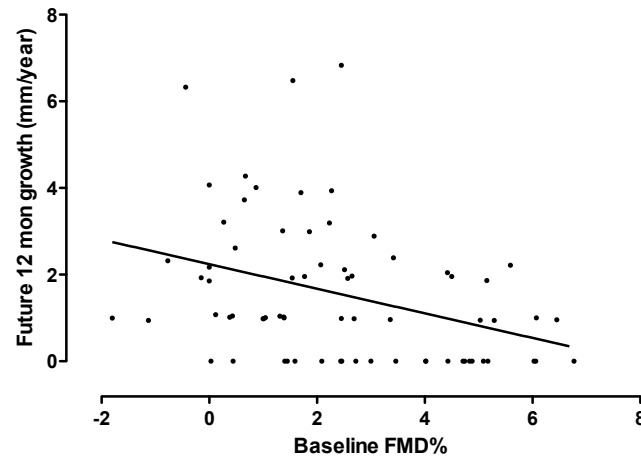


Figure 5.8: Flow Mediated Dilatation v. Prospective Growth Correlation between future 12 month growth rate and FMD percent, Spearman's $\rho=-0.41$, $p<0.001$, $n=71$

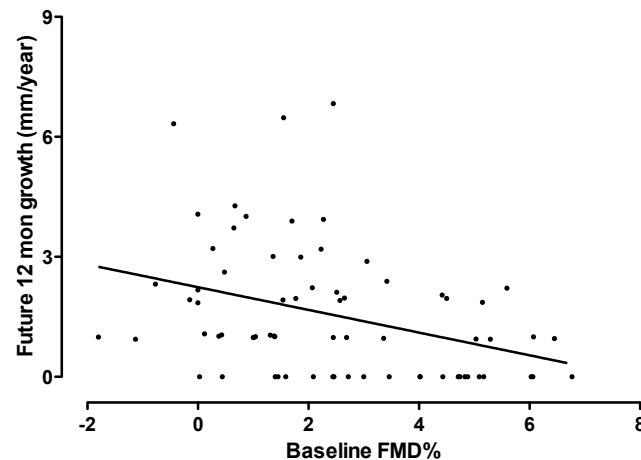


Figure 5.9: Flow Mediated Dilatation v. Prospective Growth in 40-55mm AAA patients. Correlation between future 12 month growth rate and FMD percent in patients with aneurysm 40-55 mm diameter, Spearman's $\rho=-0.41$, $p<0.01$, $n=39$

5.2.4 Does FMD change over time?

The progression of FMD over time in patients with multiple observation was examined for patterns of progression. Most patients in this group had an interval of between 6 and 12 months between their TP-1 and TP-S1 scans, and a significant drop in FMD was noted in a wilcoxon t-test ($p < .001$). However, because the time difference between patients is variable and dependent on their aneurysm size, which sets their surveillance interval, size could introduce a potential confounding factor in the observed difference.

In patients with three available FMDs, the progression was also compared, and appears in Figure 5.11. In this group of 32 patients, there is no significant degradation in FMD when examined using Friedman ANOVA. However, because interval lengths are variable, patients with larger aneurysms and thereby shorter surveillance interval lengths are likely to have more study time points. Therefore, the short interval length may be obscuring any changes in FMD.

To standardize this across all surveillance patients, an interval of approximately 12 months (average=362 days, SD=34 days) was found for each patient with multiple scans. For many patients, this was either the interval between TP-1 and TP-S1 or the interval between TP-1 and TP-S2. Rarely, it was the interval between TP-S1 and TP-S2. For some patients, such an interval did not exist, either because they were seen only every two years, or because the only available interval between serial scans was shorter than 9 months. Using a Wilcoxon t-test, there was found to be a significant drop in FMD over the course of the interval.

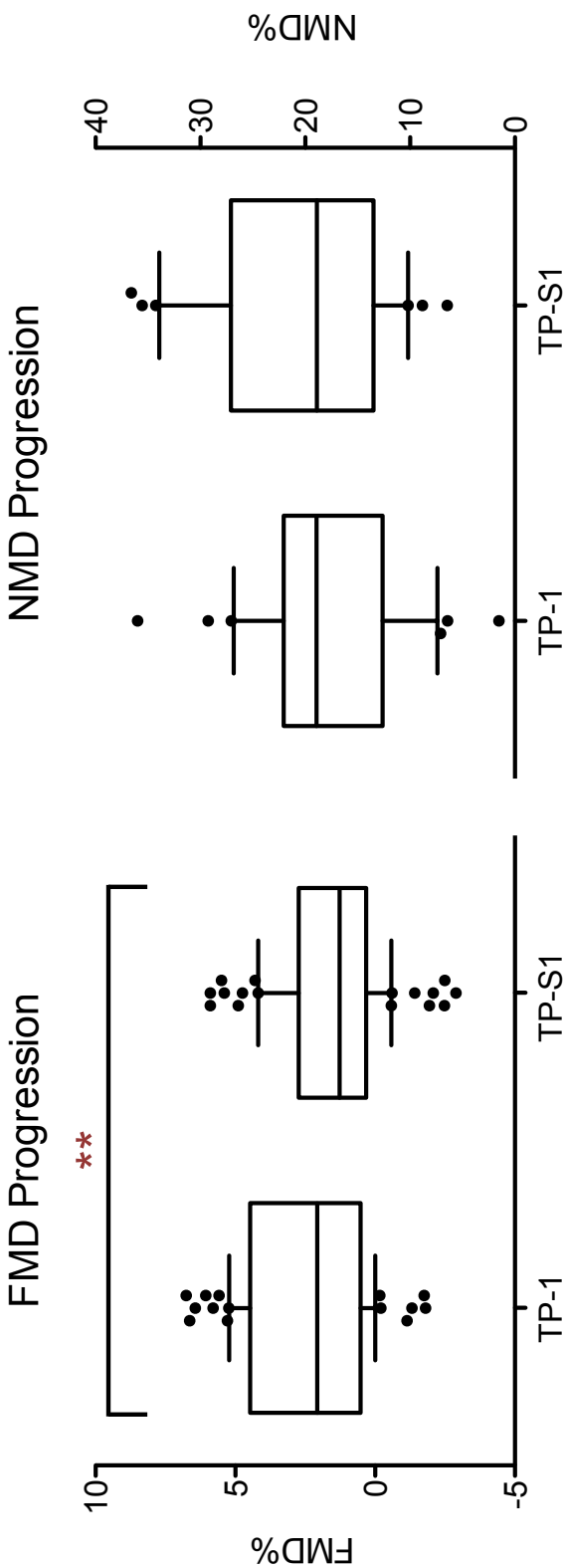


Figure 5.10: Progression of FMD and NMD between time point TP-1 and TP-S1 Significant drop in FMD, Wilcoxon t-test $p < 0.001$, $n = 80$. No significant drop in NMD observed in Wilcoxon t-test, $n = 32$

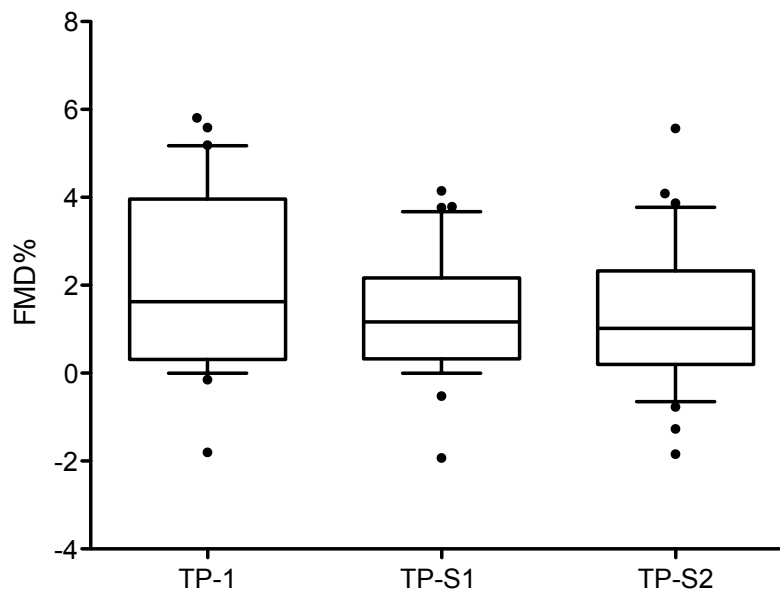


Figure 5.11: Progression of FMD and NMD between time point TP-1, TP-S1, and TP-S2 Groups show significant difference of means in decreasing fashion in a Freidman ANOVA, $p < 0.05$, $n = 32$

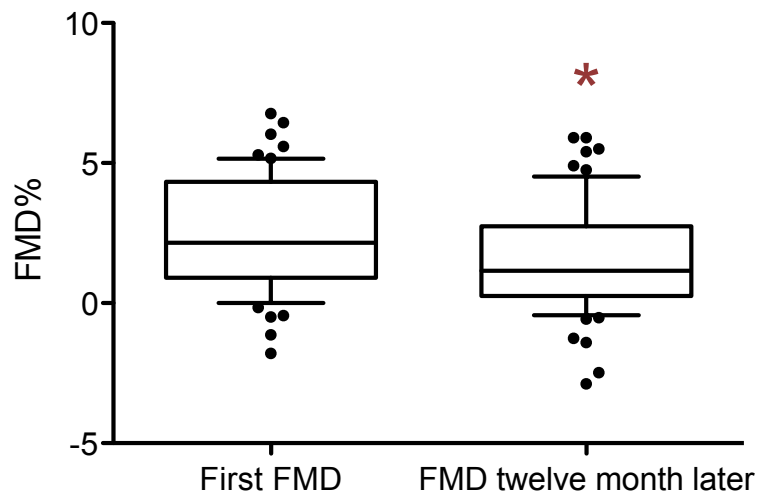


Figure 5.12: FMD change over 12 month interval No progression of FMD over a 12 month interval in surveillance patients, Wilcoxon t-test $p < 0.05$

5.2.5 Does the FMD of AAA patients differ from FMD of healthy volunteers?

The healthy volunteers enrolled in the study all have baseline FMD measurements, and those who consented to undergoing NMD also have NMD measurements. In a comparison of these measurements against the available FMD and NMD measurements for the S1 patients, there was a significant difference observed in a Mann-Whitney t-test between the FMD measurements of the healthy volunteers and S1 patients ($p < .0001$). The NMD measurements, however, were not significantly different between the two groups, and were markedly much higher than FMD averages, as is consistent with literature.

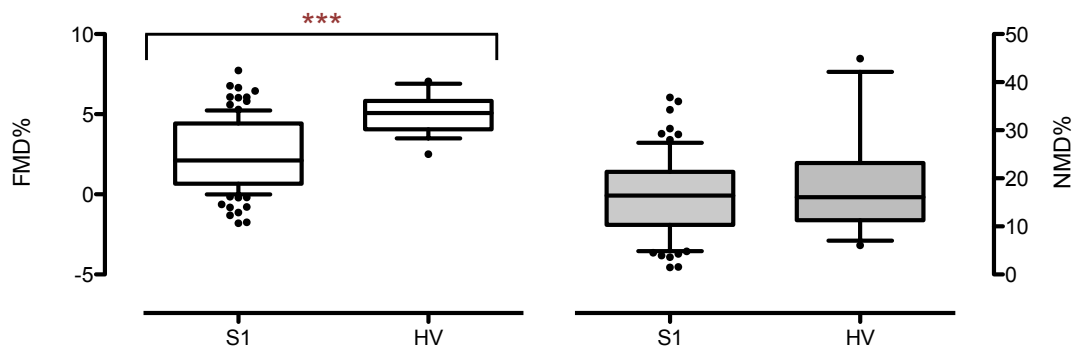


Figure 5.13: Healthy Volunteers v. S1 Patients Comparison of Healthy Volunteers and Stream 1 subjects indicates a significant difference, with AAA patients exhibiting significantly lower FMD percentages, Mann-Whitney t-test, $p < 0.0001$

5.3 Growth rate projection

Literature indicates that growth varies based on the size of AAA, with larger aneurysm diameters exhibiting faster growth [65]. Based on this, we examined the hypothesis that size will correlate to future growth, in order to compare past growth as a biomarker against FMD as a biomarker.

5.3.1 Does size correlate to future growth?

In the total group of patients (figure 5.14), size at the first available timepoint is strongly correlated to future growth ($p < .0001$). The Spearman's rho for this correlation is .533.

In the group of clinically relevant patients - between 40 and 55 mm in aneurysm diameter - however, this correlation is absent in Figure 5.15. There is no significant link between aneurysm size and future growth rate in this patient group.

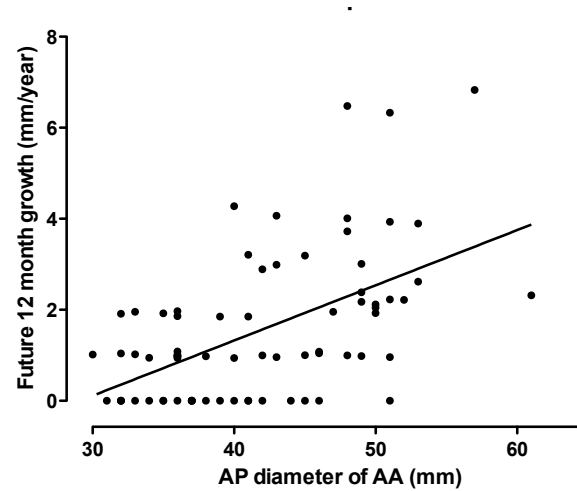


Figure 5.14: AAA Size v. Prospective Growth positive correlation present between anteroposterior diameter of AAA and future twelve months growth rate in mm/year, spearman's $\rho=0.533$, $p<0.0001$, $n=74$

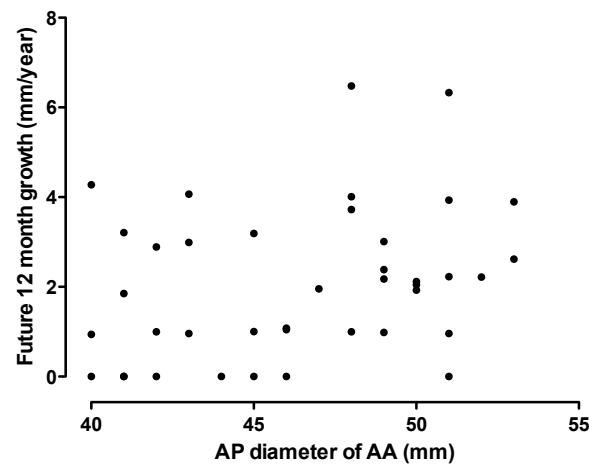


Figure 5.15: AAA Size v. Prospective Growth in Clinically Relevant Patients Lack of correlation of anteroposterior diameter of AAA and future twelve months growth rate in mm/year in patients with AAA between 40-55 mm diameter, Spearman correlation, $n=40$

5.3.2 Does past growth correlate to future growth?

One available resource for predicting future growth is past growth. Patients on surveillance may have records going back months to years, and if their growth rate has been consistently fast, it is possible this may affect assumptions about future growth. These records are readily available to clinicians from the regular surveillance ultrasounds, and can be used to calculate the retrospective growth rate of patients at the time they enter the study by dividing the total growth of the aneurysm by the amount of time a patient has been on surveillance.

However, literature indicates this may not be a sound method of prediction. Aneurysm growth has been shown to be volatile and unpredictable in mixed cohorts and clinically significant cohorts, although generally shows some relation to size. [66] [67]. Based on this, we hypothesize that total retrospective growth may correlate to future growth, but recent retrospective growth will not correlate to future growth due to temporal volatility in growth patterns, which would even out over lengthy observation.

Since most surveillance patients have multiple time points, the future growth rate from the first study point was calculated using a growth interval of approximately 12 months to control for non-representative growth rates from very short surveillance intervals. The retrospective growth rate was reasonably effective at predicting growth in the next year (figure 5.16), with a significant correlation ($p < 0.008$) and a Spearman's rho of 0.303.

The same graph was repeated in figure 5.17, including only the patients with an aneurysm between 40 and 55 mm at the first time point. In this group of patients, retrospective growth was not significantly correlated to growth in the future year (Spearman's correlation, $p > 0.05$)

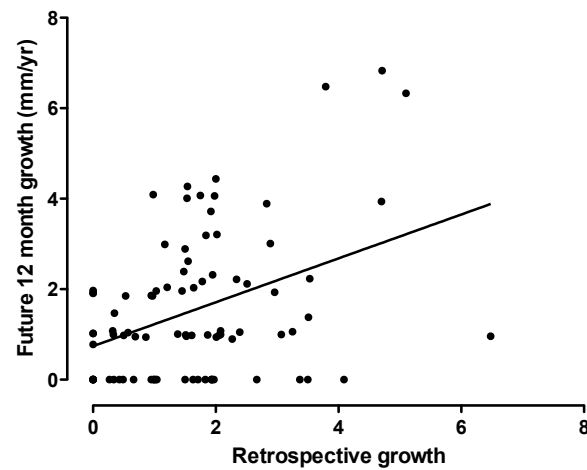


Figure 5.16: Retrospective v. Prospective Growth Rates in total cohort
Correlation between total recorded retrospective growth and future 12 month prospective growth, Spearman's $\rho=0.299$, $p<0.004$, $n=87$

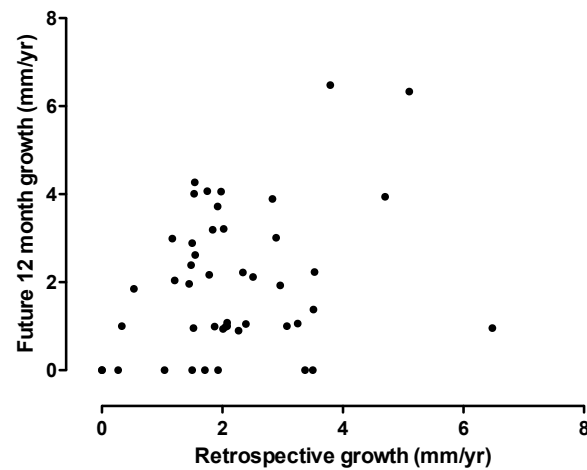


Figure 5.17: Retrospective v. Prospective Growth Rates in 40-55mm AAA Patients. Lack of correlation between total recorded retrospective growth and future 12 month prospective growth in patients with AAA between 40-55 mm diameter, Spearman's correlation, $p>0.05$, $n=46$

5.3.2.1 Does recent growth correlate to future growth?

Recent growth in (past twelve months) is also compared to growth in the future twelve months (Figure 5.18), centered around the first available study timepoint. The patients that fall in the shaded region of the graph are those whose future growth rate is within 75 percent greater or 75 percent lower than the past growth rate. All of the patients who lie outside the shaded region are those for which future growth cannot be predicted accurately using past growth, representing a majority of patients in this figure.

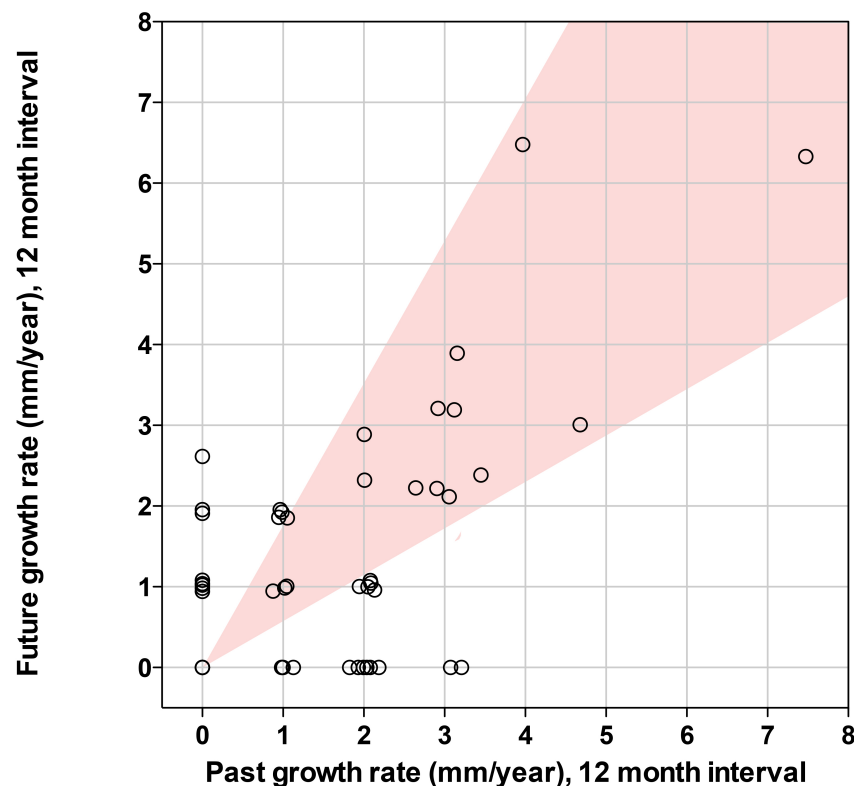


Figure 5.18: Retrospective v. Prospective Growth Rates in Clinically Relevant Patients. Lack of correlation between total recorded retrospective growth and future 12 month prospective growth in patients with AAA between 40-55 mm diameter, Spearman's correlation, $p > 0.05$, $n = 42$

5.4 Does FMD correlate with demographic data?

A large amount of demographic data is collected at the baseline study visit, including physical measurements of the patient, smoking patterns, hypercholesteremia, hypertension, history of CVD, history of stroke, diabetes status, surgical history, and a history of medical conditions in each bodily system. All, including blood test results, were examined for significant correlations with FMD measurement.



Figure 5.19: Demographic Correlations with FMD Correlations with FMD and demographic information provided in medical history. No significant correlation to history of Cardiovascular problems (Mann-Whitney, $p > 0.05$), significant correlation (Mann-Whitney, $p < 0.001$) with history of gastrointestinal problems, significant correlation (Mann-Whitney, $p < 0.05$) with history of musculoskeletal problems

The only correlations that appeared were in the history of each bodily system. Surprisingly, no correlation was observed with patients positively reporting history of cardiovascular conditions, however, FMD was significantly worse in a Mann-Whitney t-test ($p < 0.01$) in the group of patients who reported gastrointestinal conditions, when compared to those who did not report any gastrointestinal history. The same pattern was found in patients who reported a history of musculoskeletal disorders ($p < 0.05$).

6

Discussion

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6.1 Review of hypothesis and aims

Abdominal Aortic Aneurysms are a common pathology, affecting a significant portion of the population and posing significant risk of mortality. In the UK, 4000 deaths a year can be attributed to a ruptured AAA, and 8 percent of males have AAA. Yet, their pathogenesis remains poorly understood and the systemic characteristics of the illness relatively obscured.

The purpose of the Oxford Abdominal Aortic Aneurysm study is to gain greater

insight into the natural history of AAA by studying a variety of patients over a period of time. The OxAAA study aims to find patterns in biomarkers that will aid in clinical prognosis of this disease and better inform treatment.

The primary hypothesis of this study is that impaired endothelial function, as measured by flow mediated dilatation, will be able to predict the future change in diameter of an abdominal aortic aneurysm, especially in clinically relevant cases.

6.2 Summary of results

	Significant for total cohort	Significant for 40-55 mm cohort
Does FMD correlate to AAA size?	Yes	No
Does FMD correlate to total past growth?	Yes	No
Does FMD correlate to recent past growth?	No	No
Does FMD correlate to future growth?	Yes	Yes
Does size correlate to future growth?	Yes	No
Does past growth correlate to future growth?	Yes	No

Figure 6.1: Summary of correlations. Table illustrates correlations examined in results section, and their significance compared to $p=0.05$

6.3 FMD: predictive of growth?

6.3.1 FMD and size

Consistent with the literature [61] [61], FMD demonstrates a significant negative correlation with size in figure 5.4. This is especially evident when considering the pre-operative patients, for whom FMD is significantly lower compared to the surveillance patient population. A number of explanations have been proffered for the relationship between endothelial function and AAA. Pro-inflammatory molecules present in pathological aortic tissue, including matrix metalloprotease 9 [68], interleukins 1 and 6 and C-reactive protein [60], have also been linked to the dysfunction of endothelial cells [69] [70]. Furthermore, blood from healthy and unhealthy patients was used to seed plates for examination of the proliferation of endothelial progenitor cells (EPCs). EPCs were also found lacking in AAA patients [61].

As for the linear relationship with size, two explanations appear: A severe or progressing aneurysm could have occurred due to more unfavorable inflammatory molecules building up in the aortic wall, or alternatively, the larger surface area of a larger aneurysm could result in a proportional increase in the production of endothelium damaging biomolecules, causing the dropping FMD with rising AAA sizes. Both explanations would account for the trend, though the former explanation is likely if FMD is predictive of future progression of the pathology and the latter if FMD is indicative of past progression of the AAA.

In the clinically relevant group [64] (40-55mm AAA diameter) this correlation with size does not exist. Although as a group mid-size aneurysms have mid-range

FMDs. Within this group the severity of systemic effects is irrespective of size. Large aneurysms are those with severe wall degradations such that they are deemed at risk of rupture based on prior studies. Small aneurysms are relatively stable and in the early stages of disease, but mid size ones may have reached that stage through steady growth or sudden growth, hence the lack of correlation between FMD and size. It is also possible that the disappearing correlation is a function of shrinking group size - while the mid-size group makes up the largest portion of the total cohort, there is still a potential to examine this correlation at a larger sample size. The correlation, although consistent with literature, is particularly weak in the total cohort, and therefore more sensitive to sample size.

Growth rates also appear to fit these explanations - although some aneurysms of all sizes stagnate in growth, the general trend evident in figure 5.2 is that larger aneurysms progress more quickly. At the small end of the size scale, the average growth is a full millimetre per year slower than aneurysms at the larger end of the scale. However, there is also a greater spread of values in both the large and the small aneurysms, with mid size aneurysms having slightly more consistent growth rates. Figure 5.18 drives home the need for better biomarkers in the prediction of AAA growth - all of the points that lie outside the shaded region are those whose aneurysms grew much more slowly or quickly than the patient history would have led a physician to believe. As is evident, the majority of subjects lie outside the predictability region.

6.3.2 Utility of FMD compared to past growth data

Past growth and size data indicate two important conclusions: Past data is correlated but inaccurate in a large number of patients at predicting growth in the set of all patients, and in the 40-55 mm patient group, who are most clinically significant, past data completely fails to predict the growth of AAA. This is true in both cases for the prediction of future growth with aneurysm size, and in the prediction of future growth with past growth.

In figure 5.14 the correlation between aneurysm size and future growth rate is evident. This echoes the observation found in figure 5.2, where box and whisker plots replace the scatterplot. However, if just the middle bar is examined in a scatterplot - it has the least statistical variance in the box plot - the correlation is absent. Despite having a range of 15 mm in aneurysm diameter, there is no correlation between aneurysm size and future growth rate in figure 5.15. It appears that in aneurysms that may need surgery in the near future, there is startling unpredictability in future growth. There are AAA at 40 mm that exhibit a half-centimetre of growth in a year, and those which are 52 mm which exhibit no growth at all, and vice versa. These are the groups for which other biomarkers may be most useful.

Utilising past growth is a convenient and free method of attempting to anticipate future growth. In figure 5.16 it is clear that this would be a statistically sound method of prediction in the set of all aneurysm patients, but once again when narrowed down to only the clinically significant group, the correlation drops out to insignificance. The examination of both of these methods indicate that while

overall it may appear that AAA growth is predictable, it is volatile in clinically relevant patients. The test of efficacy for FMD will be its performance in predicting the developmental pattern of this clinically relevant group.

The same pattern exists in FMD's relation to retrospective growth - while there exists a strong correlation between past growth and FMD in the full cohort, there is no correlation in the clinically relevant cohort.

However, when examining the relation of FMD to future growth, the pattern is disrupted. In this case, both the full group and the clinically relevant group of patients have FMD correlated to growth in a prospective 12 month interval, indicating that a poor FMD even in the 40-55 mm group may be able to predict accelerated growth in the months to come. While size and past growth failed to correlate to future growth, FMD succeeded in doing so.

A potential explanation lies in the sensitivity of the endothelium - while inflammatory processes may take some time to cause degradation of structural proteins in the aortic wall, the endothelium is a single celled layer and significantly more susceptible to damage. Therefore, if wall-damaging inflammatory activity is occurring in the AAA, the endothelium may simply respond more quickly than the resilient aneurysm wall and therefore predict future growth more effectively than past growth.

FMD's predictive utility is less relevant in the non-clinically significant patients due to the large number of other factors that correlate with growth, however for the patients who are of most clinical interest at sub-surgical sizes, it may be an invaluable

and unparalleled prognostic tool. This finding supports the hypothesis that FMD can be used as a proxy for endothelial function to predict the future growth of AAA, and that it is of particular interest that the correlation persists in clinically relevant patients, in whom no other correlation using pre-existing measures exists.

6.4 Other conclusions

The degradation of FMD over time is visible in two separate ways, and called into question by a third. A comparison of FMD at TP-1 and TP-S1 in patients that have both time points indicates a large and significant drop from the first measurement to the second. If actual degradation of the endothelium is occurring, rather than simple deactivation of eNOS pathways, it may be an explanation for the decline in FMD. NMD remained similar between both time points.

A comparison of FMD at TP-1, TP-S1, and TP-S2 shows a different story - non difference at all between time points, with nearly identical means. However, time was considered as a possible confounding factor. Patients who had three time points were in general those who came in for surveillance more frequently, and FMD may not have had time to degrade. Alternatively as this would be in individuals with more severe disease, where the full extent of degradation may have already occurred.

Because of this inconsistency in comparing two versus three time points, another measure was used. Time points have inconsistent intervals between them, confounding results, so for the most accurate comparison, a 12 month interval was found for each patient and the FMD compared at the start and end of each

patient's interval. An interval was not available for every patient, so this comparison has a smaller sample size. However, in this case a marked drop in FMD was observed. Because this is the least confounded examination of FMD progression, it is likely that the drop in FMD over time is a phenomenon reflective of degrading vascular health in AAA patients.

A number of healthy volunteer are also registered with the study, providing two controls - a negative control against which to compare FMD values and a positive control against which to compare NMD values. As discussed previously the NMD test is used to check for medial dysfunction - if there is bioavailability of NO but the smooth muscle of the vasculature cannot respond to it, there will be a diminished NMD. However, this would not be reflective of endothelial dysfunction, which only affects the production of NO. A patient with endothelium only dysfunction should have a healthy NMD. In a comparison to the healthy volunteers, the AAA patients have significantly lower FMDs, but similar NMDs, confirming that it endothelial and not medial dysfunction observed in the study patients.

Finally, an examination of correlations with demographic data revealed only two significant correlations - patients that reported either musculoskeletal or gastrointestinal problems were more likely to have AAA. The latter could potentially be explained by the symptoms of AAA. While it is a traditionally asymptomatic pathology, it is possible that abdominal discomfort is caused by but not attributed to AAA, especially in those AAAs which more adversely affect endothelial function, manifesting in the correlation with FMD. The most typical musculoskeletal problems

are those that have a inflammatory root cause, such as arthritis. It follows then, that patients with more severe endothelial dysfunction, a symptom possibly caused by inflammatory compounds, would also have inflammatory disorders of the joint. Literature also hypothesizes this link, as the breakdown of the extracellular matrix in the pathological aorta by MMP-9 and other inflammatory markers is similar to the extracellular breakdown in rheumatoid arthritis patients [71].

6.5 Clinical applications

A larger multi centre study would be required to extrapolate the results of this pilot study to develop guidelines for interpreting the results of FMD. To expand from an observed correlation in a single-centre study to the development of system-wide standards for healthy and unhealthy FMDs would require longer term follow up of a much larger group of patients.

However, if the results are scalable, the clinical applications of these findings is important, especially in a health economy with scarce resources. This study confirms that patients with small aneurysms are low risk and can continue to be screened infrequently, and those with large aneurysms have poor FMDs. Poor FMDs have been significantly correlated to risk of peri-operative mortality, severe or fatal cardiovascular events, and other poor outcomes, underscoring the need for patients over the 55 mm size to undergo surgery. However, these findings are most relevant to the 40-55 mm patients, who may or may not reach threshold diameter for intervention.

Patients at the larger end of this range are aggressively screened every three months [64], partially due to the anxiety caused in patients by feeling as if they have "a ticking bomb" in them, and partially because of clinician uncertainty about the behaviour of the AAA [72]. Both of these could be significantly alleviated if the patient was found to have high FMD. It would save time, resources, and stress to reduce the number of surveillance visits the patient would have to make, and ease their mind of the possibility of surgery.

Alternately, if a patient had a very poor FMD, this could be an indication that three-monthly monitoring would be recommended for the patient. If at the next monitoring time point, FMD was measured and found to be poor again, this may be a good time to discuss surgical options with the patient. Age and infirmity are both significant threats to the success of surgery[73], especially one as invasive as an open aneurysm repair, so if it appears increasingly likely that surgery will be necessary, the patient would have more time to prepare by exercising, maintaining a healthy diet, and making their home conducive to recovery. In older patients, the surgery itself could be brought forward by up to a year, reducing age related risks and recovery hazards. This may even be used to inform the type of procedure - if a patient is more prepared and fit, they may be eligible for an open repair rather than an EVAR. An open repair reduces the chance of revision and re-operation, and is not at risk for endoleaks, causing it to be the more permanent, but more invasive solution.

6.6 Limitations

Flow Mediated Dilatation as a method of evaluating endothelial function comes under frequent scholarly criticisms for its sensitivity to a number of environmental factors [74] [75] [76]. Despite instructions to avoid the consumption of food and caffeine, non-compliance is always a risk in clinical studies. In addition, exercise - as little as the patient having climbed a flight of stairs, or briskly walked from the parking structure in the hospital - has the potential to affect endothelial function, especially depending on patient health [77]. In addition, light exposure, unexpected sounds, stress, and time of day are further factors that researchers have considered as potentially influencing FMD. While, ideally, all study scans would be performed after the patient has rested for an hour in complete silence and darkness without movement, this is not feasible due to time and location constraints. The patient may also suffer anxiety in hospitals, fear of needles, or apprehension of the results of their clinical surveillance scan. This is especially possible in pre-operative scans, as many patients receive their FMD on the morning of or evening prior to their surgery, undoubtedly a time of great stress for them.

A number of studies attribute this error in analysis to a variety of sources. Even in a perfectly static artificial blood vessel in a gelatine solution with the ultrasound probe in fixed position, variances in vessel diameter were observed upon analysis based on the settings of gain and contrast on the ultrasound machine itself [78]. Higher brightness levels can strengthen the appearance of the intimal walls, causing them to appear brighter and therefore thicker, causing the perception of a shrinking

lumen. Conversely, lower brightness levels can cause the edges of the intima to be very dim, causing the lumen to be over-measured. While this can be controlled to some extent by minimising the manipulation of these setting during the study, resulting in accurate percentage changes, the raw brachial artery diameter numbers cannot be accurately compared between scans. Due to the intricacies of sonography, it is impossible to have a fixed contrast and brightness setting for the entire study. Patients whose brachial artery is very superficial require lower settings and patients whose artery is more obscured under tissue layers require higher contrast settings.

There are technologies that circumvent the entire translation of ultrasound data to actual visible images and instead use a transducer with two crystals whose beams intersect in the lumen of the vessel [79]. Both crystals are positioned such that their doppler shifts are equivalent, and a short pulse is advanced from the distal to the proximal edge of the vessel to find the diameter. However, this method is only accurate to within 3 percent of the vessel diameter, so despite removing the extra graphic visualisation step, it probably only marginally improves accuracy if at all. Since the goal of the study is to find clinically available biomarkers, it is ideal that no specialised equipment be used to obtain FMD. The ultrasound machine used in the study is readily available in most imaging equipped healthcare centres, as is the occlusion cuff. The only equipment that a hospital would have to specifically acquire for FMD would be a stereotactic probe holder and perhaps a small table. Ideally, this would be as simple as getting an ultrasound surveillance scan for a AAA, and add minimal additional cost to the process.

The variations in individual's size can also pose a confounding factor that is difficult to control for. Some studies have found correlations between FMD percent and baseline diameter of the brachial artery; that is, smaller brachial artery baseline diameters tended towards larger FMDs. However, in this cohort, no correlation was found between brachial diameter and FMD, height and FMD, or weight and FMD. This does not rule out the possibility that any of these could be confounding - even the definition of AAA is dependent of a static value that does not account for variations in size. It is possible that in more petite women, a lower threshold for aneurysm or clinically significant aneurysm might be required. Further investigation in a larger cohort size could control for the influence of height or armspan, both one-dimensional values, to help better define presence and severity of aneurysm.

7

Conclusion and Future Directions

Flow Mediated Dilatation as a diagnostic and screening tool is an effective and non-invasive proxy for endothelial dysfunction, but has been limited in its application to study the natural history of AAA. FMD, chosen from among a number of methods for testing vascular responses, has been used in clinical studies and continually refined for 25 years, with most studies now using a standard method outlined in 2002. Prior studies indicate that while the most obvious effects of AAA are localized to the aorta, the inflammatory profile that exists during pathogenesis and development of the disease is similar to that which may be found in patients with atherosclerosis and other vascular dysfunctions.

Examining the presence and severity of endothelial function in patients with AAA may have clinical value in guiding a treatment plan. This study demonstrates that FMD is correlated to the future growth of AAA and supersedes current methods of predicting growth in clinically relevant patients. Further results localize the poor vasodilation in AAA patients compared to healthy volunteers to be most

likely linked to a problem in the production of NO, as patients showed no impaired response to administered nitroglycerin. Prior research links the severity of impaired endothelial response to aneurysm diameter in a group of patients, which is confirmed by this study, but also evident in the progression of the pathology over time in a single patient; as the diameter of the AAA grows, FMD worsens.

Because FMD requires no additional equipment, is non-invasive, consumes only ten to fifteen minutes of sonography time, and does not require a clinician presence, it could be a routine addition to the surveillance program of patients with moderate to large sized aneurysms. FMD has the potential to reduce the frequency of surveillance in patients with larger aneurysms but healthy vascular function, thereby outweighing any additional time and cost required to acquire it. Since FMD had also been shown by previous studies to be predictive of peri-operative cardiac complications, it fulfills a multi-purpose role to the physician, both to predict the likelihood of intervention in the future year, and to predict the success and potential risks of said intervention.

The future directions of study include further follow up of patients on surveillance to examine long term trends in AAA progression, and to examine possible methods of improving vascular function in AAA patients through exercise or medication. FMD prior to and just after the development of AAA may be particularly useful in preventative methods - if at-risk groups were monitored prior to the age of 65, statistically around 8 percent should develop an aneurysm, generating a significant amount of data on the changes in vascular function that precede or accompany

AAA development. In addition, further studies using other methods of evaluating vascular function could help to fortify conclusions about endothelial dysfunction in this patient group and aid in localizing the biochemical pathways through which AAA is linked to the failure of the endothelium. Operative patients also represent a significant group in the OxAAA study, and indicate the reversibility of damage to the endothelium. They may offer insights into reversing poor FMDs in pre-operative patients, perhaps lowering the risk of cardiovascular events and future growth of AAA.

Appendices

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.1 Patient Consent Form

The following is the document approved for recruitment and consent acquisition by institutional ethics approval.

Study Full Title	<i>Oxford Abdominal Aortic Aneurysm Study – OxAAA</i>
Patient ID	
Researchers	Prof. Ashok Handa, Dr. Regent Lee, Dr. Andrew Lewis, Ms Hayley Harvey (on behalf of the OxAAA investigators)

Please initial in the Box

1. I confirm that I have read and understand the information sheet (Version 2.0, 30.06.2014) 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 authorized individuals from the University of Oxford, from regulatory authorities or from the NHS Trust, where it is relevant to my taking part in this research. I permit these individuals access to my records. ☐
4. I understand why blood and other samples are being taken, how the samples will be collected, that giving samples for this research is voluntary and that I am free to withdraw my approval for use of the sample at any time without my medical treatment or legal rights being affected. I understand that data collected from the samples will be used in the results of the research study. I understand that results from relevant scans performed during my clinical care may be used in the research study. ☐
5. I understand that my samples may be used in genetic research aimed at understanding the genetic influence on blood vessel function, but that the results of these investigations are unlikely to have any implications for me personally. ☐
6. I agree to gift images and samples taken for the purpose of the research study to the University of Oxford. If a commercial product were developed as a result of this study, I will not profit financially from such a product. ☐
7. I understand that anonymised images from scans and data during my study participation may be used in research publications and can be passed on to other organisations which may include commercial organisation. I give consent for future use of images for any publications that may arise from the research. ☐
8. I understand that my GP will (with my permission) be informed of the results of medical tests performed as part of the research which are important for my health care. ☐
9. I agree to take part in the above study. ☐
10. (Optional) I agree to take part in the above study, but do not wish to undertake the following research assessment. Please specify: _____ ☐
11. (Optional) I agree to being contacted in the future to ask if I am interested in future related studies. ☐
12. (Optional) I agree for samples collected to be used in future studies which have been ethically approved. ☐

Name of participant

Signature

Date

Name of person taking consent

Signature

Date

For researchers (✓) to document: original kept & copy given to participant (_); copy inserted into medical notes (_)

.2 Standard Operating Protocol for FMD Image Acquisition

The SOP for FMD Image Acquisition is based on the commonly used protocol for FMD developed in 2002. Specific timing protocols and acquisition of all other study information has been integrated, as well as specific observations useful to the sonographer.

**NUFFIELD DEPARTMENT OF SURGICAL SCIENCES
UNIVERSITY OF OXFORD**



SOP Number: JWVSU_01_V2.2

SOP Title: OxAAA JWVSU FMD, NMD and AAA video loop measurements.

	NAME	TITLE	SIGNATURE	DATE
Original Author	Jonathan Diesch			
Review Author	Felicity Woodgate			
Review Author	Kirthi Bellamkonda			
Reviewer & Authoriser	Regent Lee			

Effective	22/02/2016
Review Date	22/02/2017

READ BY			
NAME	TITLE	SIGNATURE	DATE

1. PURPOSE

The purpose of this Standard Operating Procedure (SOP) is to describe the standard procedures to be followed when assessing flow mediated dilation (FMD), Nitroglycerin (NTG) mediated dilatation (NMD), and the process of capturing longitudinal and transverse AAA video loops in clinical studies sponsored by the Nuffield Department of Surgical Sciences, University of Oxford in conjunction with NIHR Oxford Biomedical Research Centre.

2. INTRODUCTION

This document outlines the correct procedures for recording flow mediated dilation (FMD) in the brachial artery, Nitroglycerin mediated dilatation of the brachial artery (NMD) and the recording of longitudinal and transverse AAA video loops within the Jackie Walton, Vascular Studies Unit (JWVSU) for the Oxford Abdominal Aortic Aneurysm Study (OxAAA).

3. SCOPE

This SOP applies to clinical research where the Nuffield Department of Surgical Sciences, University of Oxford has accepted the role of 'Sponsor', in the Jackie Walton Vascular Studies Unit. This SOP may or may not apply to commercially sponsored research or research sponsored by an external non-commercial organisation.

4. RESPONSIBILITIES

a. Investigator or delegate

- Ensure the subject understands the procedure as described below.
- Ensure that the subject will be continuously observed during the study.

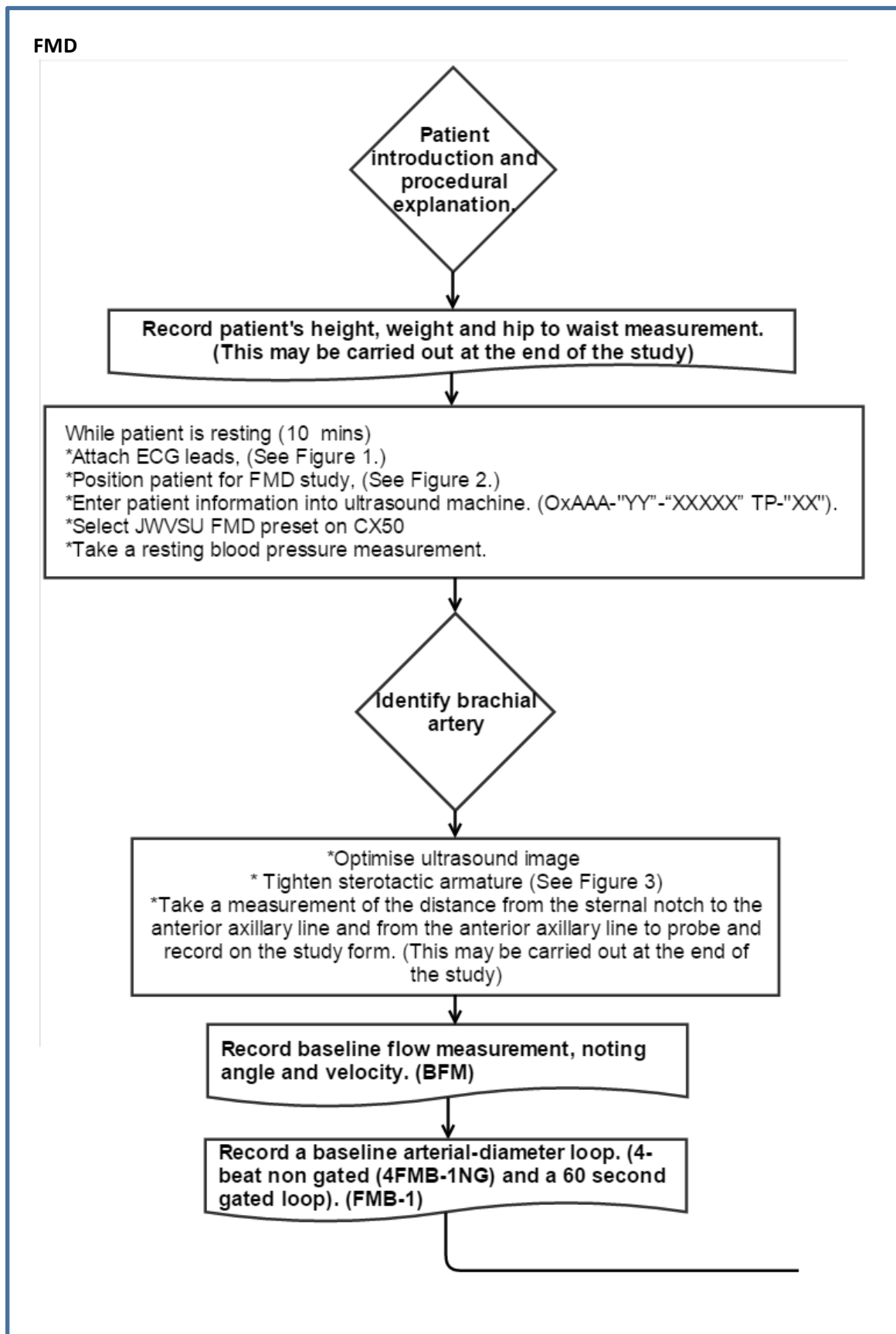
5. SPECIFIC PROCEDURE

a. Equipment

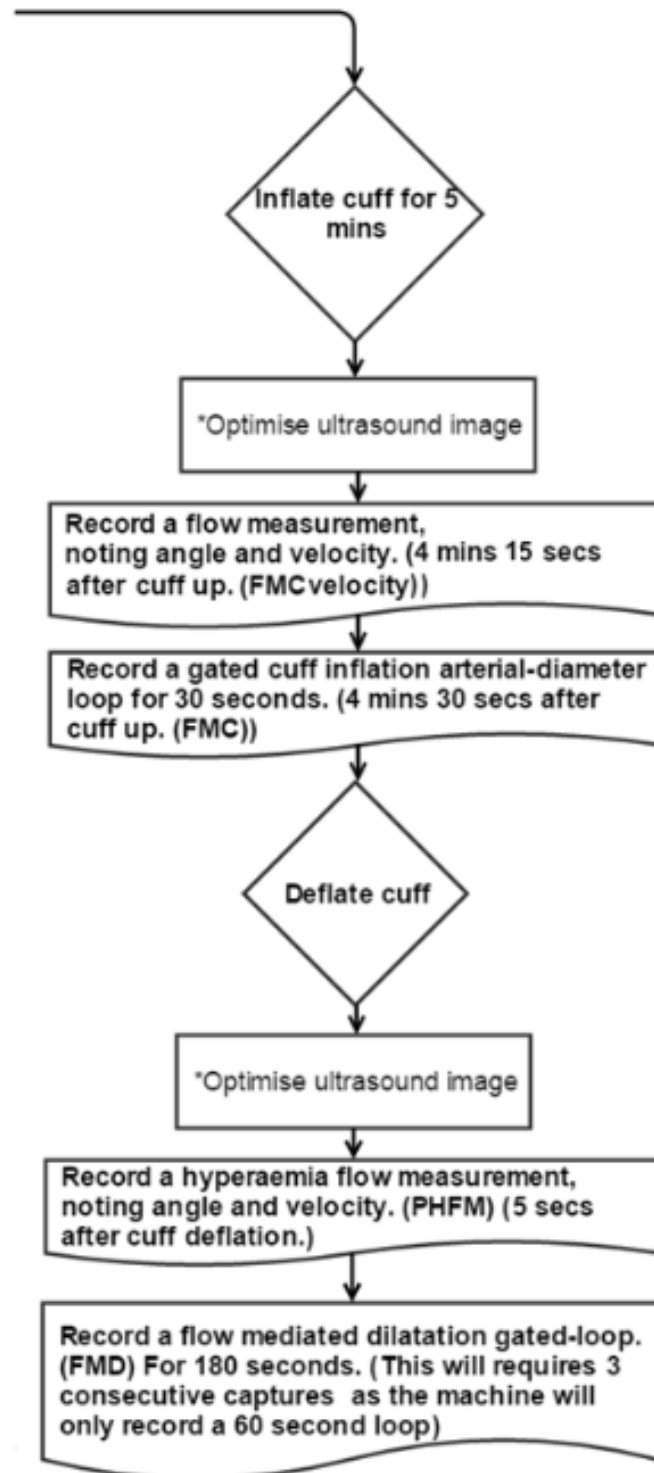
- Arm rest with stereotactic ultrasound probe-holder
- Adjustable rolled towel in a pillow case.X2
- Tourniquet or fabric bandage.
- ECG Lead (e.g. M1669A) and electrodes
- Ultrasonic gel
- Forearm-sized blood pressure cuff
- 400 microgram and 25 microgram metered dose sub-lingual NTG spray
- Pipettes
- Vascular ultrasound machine (e.g. Philips CX50)
- Vascular linear and curvilinear array ultrasound probe (Philips L12-3 and C5-1)
- Examination couch with variable height
- Android device with FMD/NMD timing program
- Study form v2.3
- Examination table – approx. 80 cm in height and 30 cm x 40 cm top
- Scales

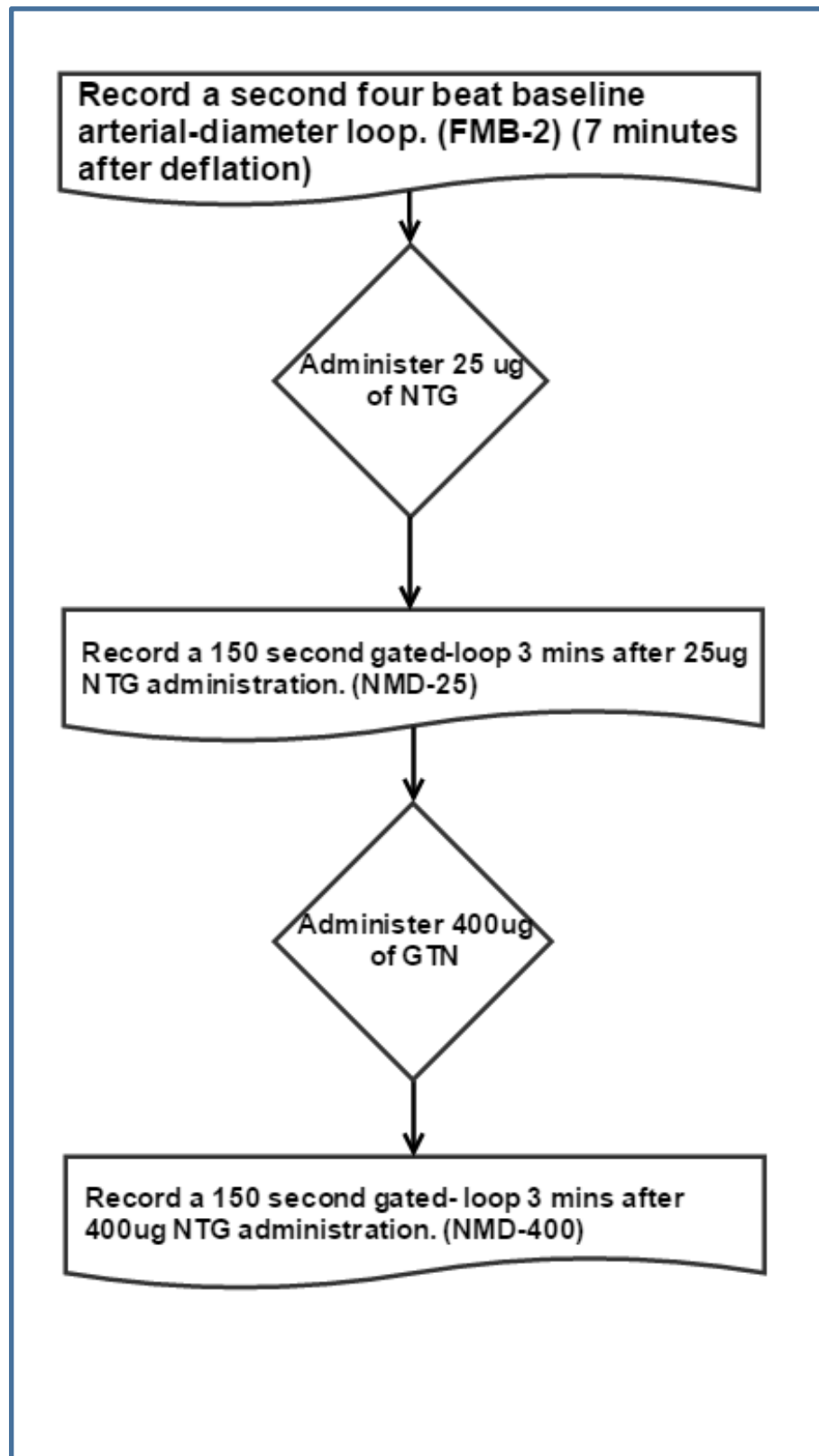
- Electronic blood pressure machine
- Tape Measure

b. FMD and NMD flow charts



FMD cont.





c. **Procedure for the routine AAA surveillance scan. Before the commencement of the procedure patients should confirm they have fasted for 6 hours and have not had any stimulant drinks for at least 24hours e.g. Coffee, cigarettes**

1. Measure and record the subject height, weight, hip (at top level of hip bone) and waist (at level of naval) measurement in the patient study form. (This may be carried out at the end of the study to ensure the patient has recovered from the NMD study).
2. Ask the subject to undress from the waist up unless they have a short sleeved shirt or top before placing them in the supine position on the bed in a quiet room. (~22°C)
3. Attach ECG electrodes in conventional positions at least 20cm from the heart and connect to machine. The red electrode should be placed on the right-hand side above the level of the heart, the yellow electrode should be placed on the left-hand side above the level of the heart, and the green electrode should be placed on the left-hand side below the level of the heart. (See figure 1.)

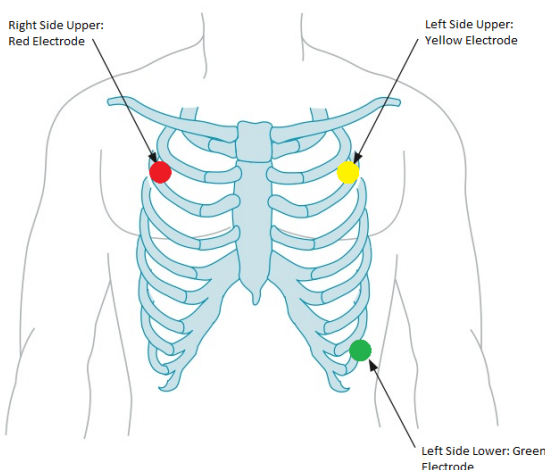


Figure 1.

4. Connect the Philips Curvilinear array ultrasound probe (C5-1) to the CX50 and lock in place on the right hand side of the machine.
5. Select **AbVasc pre-set imaging mode** on the CX50 console.
6. A routine AAA surveillance scan should be carried out in accordance with the local unit protocol and recorded on the JWVSU AAA surveillance form.
7. Under the **physio menu**, using the '**next arrow**' in the centre of the top row of the console switch between the menus and turn the ECG channel from '**off**' to '**internal**'. Then, using the next arrow again to switch menus, turn the trigger from '**off**' to '**on**'. Turn the physio menu off and then set the loop type from **time to beats (4 beats)**.
8. Ensure clear R wave tracing on ultrasound machine and record the patient's heart rate on the study form making note of irregularities.
9. A video loop recording, four beats long, should be obtained in transverse and longitudinal.

10. Wipe aqueous gel off subject and ultrasound probe with paper towel. Use specialised probe disinfectant as required.

d. Procedure for a standard FMD Study:

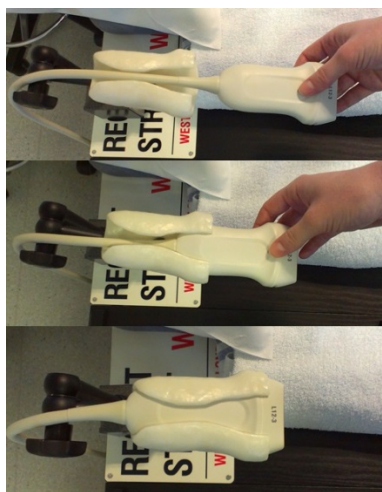
1. Take a resting blood pressure measurement and record on the study form.
2. Ask the subject to place their right arm outstretched upon the arm-rest. Ensure that the subject lies approximately horizontally, in a relaxed position with elbow and wrist completely supported by the rolled towels and a clear space underneath the forearm to ensure the blood pressure cuff does not move the arm when inflated. (See figure 2).

Figure 2.



3. Apply the correctly-sized blood pressure cuff to the forearm muscle mass, such that the top edge sits just below the cubital fossa (~3-5cm). Ensure that the cuff is wrapped snugly and evenly and is not touching either the armrest-bed or adjustable rolled towels.
4. Instruct the subject to keep as still as possible throughout the study.
5. Allow the subject to rest in supine position for **10 minutes** before commencing the rest of the procedure. In the meantime, do the following:
6. Enter subject details on ultrasound machine. Use the anonymised ID format for FMD study. This should be in the format of OxAAA-“YY”-“XXXXX” TP-“ZZ”. If you are unsure, check that an ID has been assigned.(YY is the study group, XXXXX the study number, TP ZZZ is the time point)
7. Select **JWVSU FMD pre-set imaging mode** on the CX50 console. (See appendices for the JWVSU FMD pre-set parameters)
8. Fit the ultrasound probe into the probe holder ensuring the leading edge is directed towards the subject's head (note: the probe should slide into the clip from underneath, do not press the probe in from the front or prise open the jaws of the clip). (See figure 3.)

Figure 3.



9. Apply the magnetic base of probe-holder to the armrest in line with the elbow so that the probe can comfortably reach the target artery. Ensure that the probe-holder's base does not touch the cuff, the subject's arm or the supporting towels. (See figure 4.)

Figure 4.



10. Apply sufficient ultrasound gel for the examination. Identify the brachial artery bifurcation through transverse views in both B mode and colour flow. Trace the brachial artery proximally to **approximately 5 cm** from the bifurcation.
11. Using the brachial artery as the axis, turn the probe $\sim 90^\circ$ so that it is in line with the longitudinal axis of the brachial artery.
12. Push down on probe slightly to ensure that vessel does not collapse, in order to confirm that the image depicts the brachial artery and not the adjacent vein.
13. Acquire an image of the brachial artery in longitudinal section using colour flow and pulse-wave Doppler to further ensure vessel is arterial.
14. Adjust the probe such that the artery appears straight, horizontal, and in the optimum focal zone of the machine.
15. Adjust the depth and optimise the image using the focus, B-mode gain (set to 96 on pre-set) and compression (set to 46 on pre-set). Ensure that only light pressure is applied to the arm by the probe, to avoid distorting the artery.
16. Tighten stereotactic armature, without moving the probe, such that the image position and quality are unaltered.
17. Take a measurement from the sternal notch to the anterior axillary line and from the anterior axillary line to the middle of the probe and record the results on the study form. (This may be carried out at the end of the study)

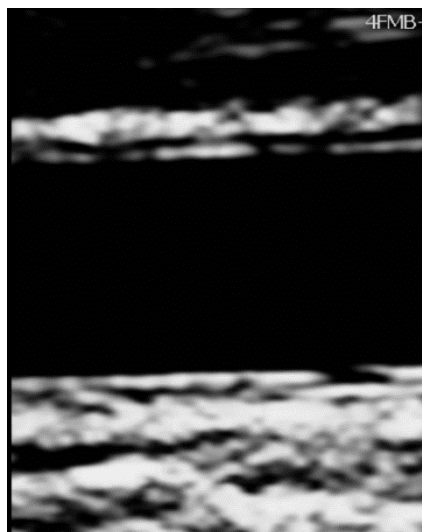
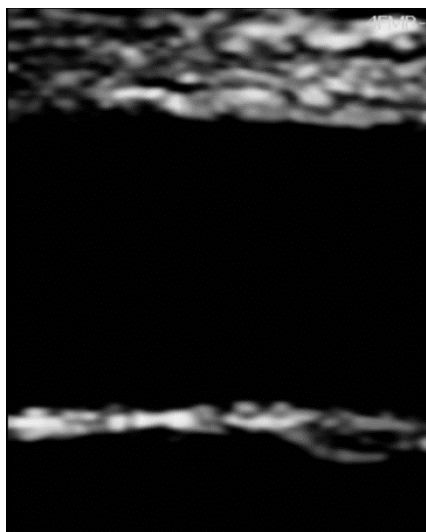
18. Using zoom function, enlarge the most clearly visualised and stable section of the vessel using the minimum size box of the zoom function (1.0 x 0.74 cm on CX50). (See Figure 5.)

Figure 5.

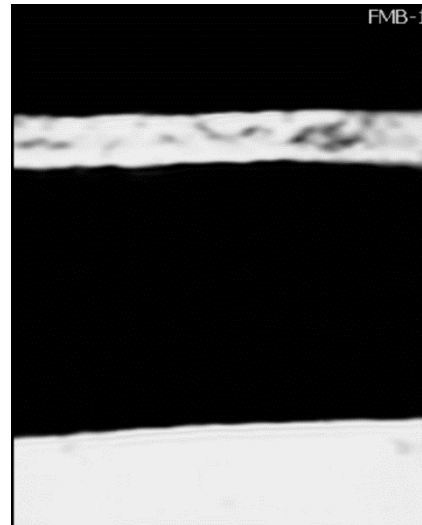
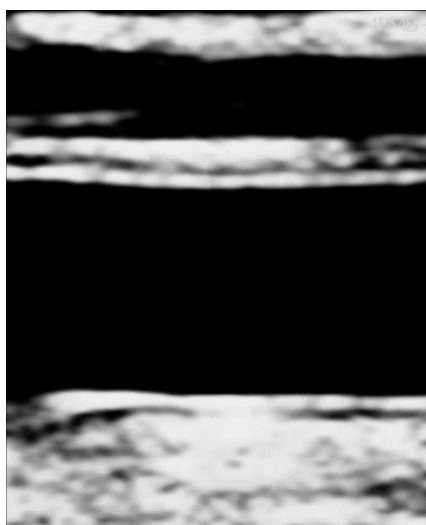


19. Optimise the image to the lowest compression and gain settings which maintain edge coherence whilst removing noise. Adjust compression before gain. See imaging guidelines below to ensure image is suitable for analysis.

IMAGING GUIDELINES FOR OPTIMAL ANALYSIS



The image on the left is not easily analysable and will not yield good results. The image on the right, while analysable, will have to be done mostly by hand as the software will pick up outer and inner walls intermittently.



The image on the left is a good example of visible vessel walls, and is sufficient for study. However, the analysis software may still intermittently pick up the outer wall. The image of the right will analyse very well, and is ideal for scans. In this situation, locate vessel walls, acquire an image that looks like the left, and then turn the gain up and conduct the study in image conditions on the right.

20. Make very fine adjustments to the probe position as required, to ensure the image is of the exact vessel diameter before fully tightening the armature.
21. Record a baseline flow measurement using pulsed wave Doppler positioned in the middle of the artery at the appropriate angle to minimise inaccuracy. (Try to keep the angle between 45-60°). (**BFM Velocity**)
22. Record a **four beat** baseline non-gated arterial-diameter loop (**4FMB-1NG**).
23. Press the zoom button twice to return to the zoomed out image with the zoom box region indicated, press freeze, acquire a still image (**FMB Location**), unfreeze the image, and press the zoom button once more to return to the previously selected zoom region.
24. **Under the physio menu turn the trigger from 'off' to 'on' and the loop type to time (60seconds)**. Record a 60 second gated arterial-diameter loop (**FMB-1**).
25. **Locate the radial pulse.**
26. Inflate BP cuff to the higher of the systolic pressures i.e. BP+50mmHg or 200mmHg, for **5 minutes**. The timer should be started on the android tablet FMD program once the cuff is inflated.
27. During cuff inflation, check that the previously located radial pulse has stopped, indicating occlusion of the artery. If radial pulse persists, slightly increase the cuff pressure.
28. Do not adjust the probe position during cuff-up.
29. If the image quality is not impaired by movement associated with cuff inflation record a flow mediated constriction velocity recording 4minutes 25 seconds post inflation using pulse wave Doppler. (**FMC Velocity**)
30. Record a further 30 second gated arterial-diameter loop at 30 seconds before cuff deflation, for measuring Flow-Mediated Constriction (**FMC**).
31. Deflate cuff
32. Adjust vascular probe (if required) to maintain image quality.
33. **It is highly important that the visualised segment of the brachial artery remains exactly the same throughout the examination** to ensure the vessel section in view has not changed from the baseline. Identification of the brachial bifurcation (step 12) is therefore highly important as it will allow for tracing of the same segment if the probe position is lost accidentally.
34. Record a peak hyperaemia flow measurement, approximately 5 seconds after the cuff is completely deflated, using pulsed wave Doppler positioned in the middle of the artery at an appropriate angle to minimise inaccuracy. (Try to keep the angle between 45-60° and aim to use the same angle as in step 22). (**PHFM Velocity**)
35. Record a 180 second gated arterial-diameter loop after cuff deflation starting 15 seconds after deflation. (**FMD**) (This will require 3 pushes of the capture button as the maximum loop time is restricted to 60 seconds. The ultrasound machine 'beeps' after each loop capture.)
36. Press the zoom button twice to return to the zoomed out image with the zoom box region indicated, press freeze, acquire a still image of the vessel following FMD (**FMD location**), unfreeze the image, and press the zoom button once more to return to the previously selected zoom region.
37. Prepare the patient for the next stage of the investigation.

38. Adjust the vascular probe if required to maintain image quality.

e. Procedure for sub-lingual NTG mediated dilation study. To be performed after the FMD study.

Exclusion Criteria:

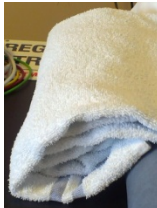
- Systolic BP below 100 mm Hg
 - Heart Rate below 60 bpm
 - No physicians present
 - Pharmacological contraindication (similar medications, previous reaction, etc)
1. Seven minutes after cuff deflation record a second 60 second gated baseline arterial-diameter loop. **(FMB-2)**
 2. Administer 25µg of sub-lingual NTG to the patient.
 3. Wait 3 minutes for the 25µg of sub-lingual NTG to take effect.
 4. Then record a 150 second gated arterial-diameter loop. **(NMD-25)** (This will require 3 pushes of the capture button as the maximum loop time is restricted to 60 seconds. The ultrasound machine 'beeps' after each loop capture.)
 5. Administer 400µg (1 full spray) of sub-lingual NTG to the patient. Provide warning that this may cause slight headache or light-headedness in some individuals for 10 to 15 minutes, but it will be gone before the procedure finishes.
 6. Wait 3 minutes for the 400µg of sub-lingual NTG to take effect.
 7. Then record a 150 second gated arterial-diameter loop. **(NMD-400)** (This will require 3 pushes of the capture button as the maximum loop time is restricted to 60 seconds. The ultrasound machine 'beeps' after each loop capture.)
 8. Measure distance from distal edge of probe to axillary crease and from axillary crease to sternal notch. Loosen probe holder and remove transducer from patient.
 9. Remove ECG electrodes and wipe aqueous gel off subject and ultrasound probe with paper towel. Use specialised probe disinfectant as required.
 10. The FMD study equipment should be moved to the side for the next stages of the investigation.
 11. Prepare the patient for the next stage of the investigation.

Appendix**Study equipment list**

Arm rest with stereotactic ultrasound probe-holder



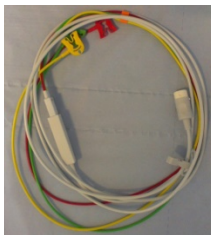
Rolled towel in pillow case



Tourniquet



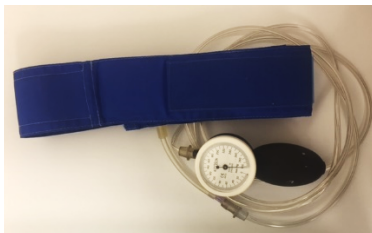
ECG Lead (e.g. M1669A) and electrodes



Ultrasonic gel



Forearm-sized blood pressure cuff



Sub-lingual NTG spray



Vascular ultrasound machine (e.g. Philips CX50)



Vascular linear array ultrasound probe (e.g. Philips L12-3)



Couch



Timing data sheet

(See FMD Study form v2.4)

JWVSU FMD Pre-set parameters Philips CX50

2D settings:

- 2D Opt, HRes
- Gain, Gn 96
- Compression, C 46
- Loop type, Beats
- Loop beat, 4
- Focal Zone, 1
- Harmonics, ON
- XRES, ON
- Sono CT, ON
- Res/Speed, S1
- Grey Scale mapping, 3
- Persist, 2
- Smooth, 3
- Power, -0.03dB
- iSCAN Gain, 0.0dB
- Chroma, OFF
- Biopsy, OFF
- Trapezoid, OFF
- TGC, ON
- LGC, OFF
- Zoom, smallest box, 1cm x 0.74cm
- ECG, internal

.3 Timing Protocol for FMD Image Acquisition

The timing for image acquisition during the FMD study is standardized by using the Impetus app for Android. The programming code for the app as well as the individual segment lengths for manual timing may be found in the following appendix.

Impetus Timing Protocol

Programming Impetus app:

```
1 <?xml version='1.0' encoding='UTF-8' standalone='yes' ?>
2 <presets>
3   <preset desc="JWVSU FMD & NMD" simple="0" summary="FMD & NMD">
4     <btimer desc="Inflate Cuff" pause="0" time="260000" work="1" />
5     <btimer desc="FMC velocity" pause="0" time="10000" work="1" />
6     <btimer desc="FMC gated loop" pause="0" time="25000" work="1" />
7     <btimer desc="Deflate cuff" pause="0" time="5000" work="1" />
8     <btimer desc="PHFM velocity" pause="0" time="10000" work="1" />
9     <btimer desc="FMD " pause="0" time="180000" work="1" />
10    <btimer desc="Wait" pause="0" time="230000" work="1" />
11    <btimer desc="4FMD2 gated loop" pause="0" time="60000" work="1" />
12    <btimer desc="Give GTN 25" pause="0" time="5000" work="1" />
13    <btimer desc="Wait" pause="0" time="175000" work="1" />
14    <btimer desc="NTG 25 NMD gated loop" pause="0" time="150000" work="1" />
15    <btimer desc="Give GTN 400" pause="0" time="5000" work="1" />
16    <btimer desc="Wait" pause="0" time="175000" work="1" />
17    <btimer desc="NTG 400 NMD gated loop" pause="0" time="150000" work="1" />
18  </preset>
19 </presets>
```

Timing protocol segments:

Timing Segment	Time (m:ss)
Inflate Cuff	4:20
FMC Velocity	0:10
FMC Gated Loop	0:25
Deflate Cuff	0:05
PHFM Velocity	0:10
FMD	3:00
Wait	3:50
4FMD2 Gated Loop	1:00
Give GTN 25	0:05
Wait	2:55
NTG 25 NMD Gated Loop	2:30
Give GTN 400	0:05
Wait	2:55
NTG 400 NMD Gated Loop	2:30

.4 Standard Operating Protocol for FMD Analysis

The SOP for image analysis reflects the guide for future analysis of FMD scans obtained in the OxAAA study and is geared toward untrained analyzers.

**NUFFIELD DEPARTMENT OF SURGICAL SCIENCES
 UNIVERSITY OF OXFORD**



SOP Number: JWVSU_03_V2.1
SOP Title: OxAAA JWVSU FMD and NMD analysis protocol.

	NAME	TITLE	SIGNATURE	DATE
Original Author	Kirthi Bellamkonda			
Reviewer & Authoriser	Regent Lee			

Effective Review Date	
------------------------------	--

READ BY			
NAME	TITLE	SIGNATURE	DATE
Matthew A Williams			16/05/2016

1. PURPOSE

The purpose of this Standard Operating procedure is to outline a consistent and reproducible method of analysis for Flow Mediated Dilatation and Nitro-glycerine Mediated Dilatation studies.

2. INTRODUCTION

This document outlines the correct procedures for organising study files, and the steps that should be taken to ensure accurate analysis of the flow mediated dilation (FMD) and Nitroglycerin mediated dilatation (NMD) images captured within the Jackie Walton, Vascular Studies Unit (JWVSU) for the Oxford Abdominal Aortic Aneurysm Study (OxAAA).

3. SCOPE

This SOP applies to the clinical research for which the Nuffield Department of Surgical Sciences, University of Oxford has accepted the role of 'Sponsor', in the Jackie Walton Vascular Studies Unit. This SOP may or may not apply to commercially sponsored research or research sponsored by an external non-commercial organisation.

4. RESPONSIBILITIES

a. Investigator or delegate

- Ensure the subject understands the procedure as described below.
- Ensure that the subject will be continuously observed during the study.

5. SPECIFIC PROCEDURE

a. Equipment

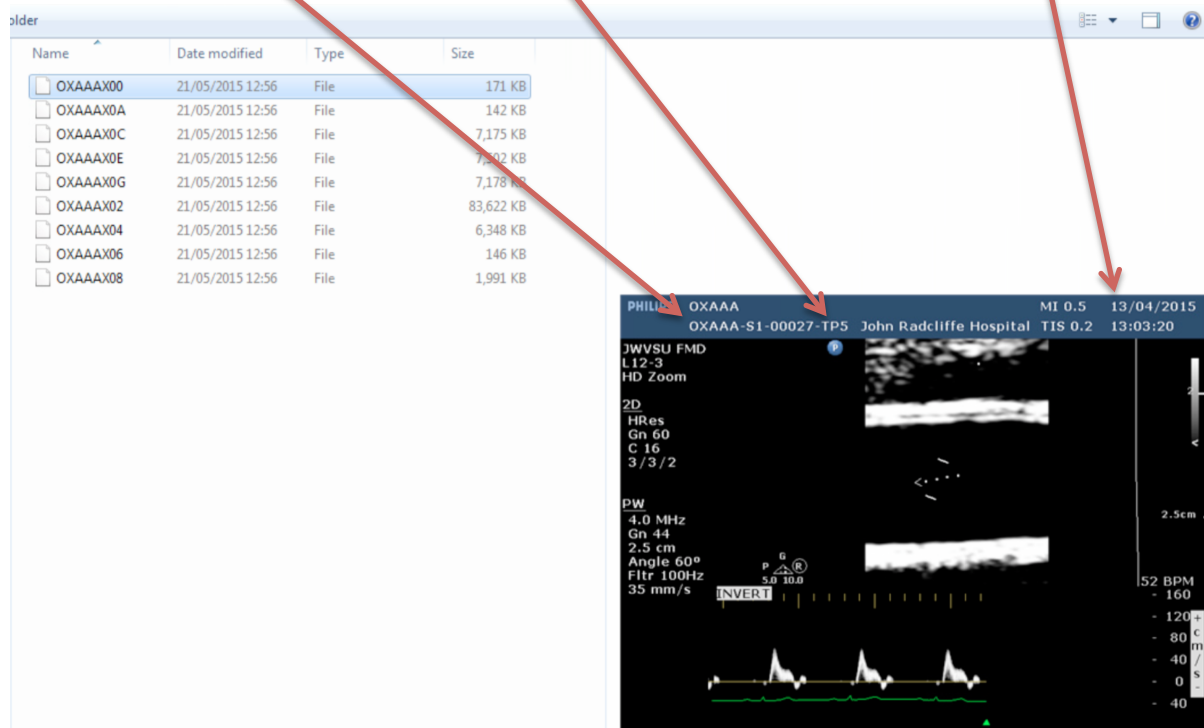
1. The research computer with the Brachial Analyzer for Research software installed
2. A designated, secure USB drive for transport of files
3. The OxAAA hard drive and backup hard drives
4. An extra Windows based PC
5. DICOM preview application (<http://www.microdicom.com/dicom-shell-extension.html>)
6. The blank folder structure available in the OXAAANDS1TB hard drive within the study image folder.
7. Access to an up-to-date version of the OxAAA FMD data mastersheet, or other excel document in which data is to be entered.
8. Access to OxAAA study appointment calendar and paper files
9. OxAAA backup hard drives

b. Retrieval of images for analysis

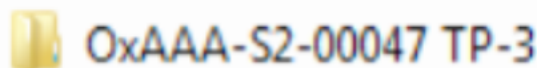
1. Initiate CX50 and allow 5 minutes for machine to fully start up. Although probe may capture images sooner, additional time is required for file retrieval functions to initiate.
2. Insert USB stick in port on the right hand side of machine.
3. In the top row of buttons on the keyboard, locate and press the “review” button.
4. Once in the review screen, hover the cursor over the top row of icons to locate the “export files” button, and press the left mouse button.
5. Select the “files created/modified since last export” option, and check the size of the files for export does not exceed the available space on your USB storage device. DO NOT directly download files to the main hard drive.
6. If not already selected, in the drop down menu, select “USB device”.
7. Click “export” and allow for media to be downloaded. This process typically takes an hour for several scans.
8. Once export has completed, remove USB stick from CX50, power down the machine, and close the cover.

c. Sorting of images

1. On a Windows PC with the DICOM preview software, insert the USB stick and the study hard drive.
2. Unlock the study hard drive by opening the unlocking application and entering the password.
3. Open an explorer window with the USB stick. You will see folders beginning with the extension “OXAA” followed by a letter and number string. Each folder contains all of the images from a particular scan.
4. Open the first folder and click once on the first image. Note the date of the scan, the patient ID, and the time point.



5. Refer to the appointment calendar for that day and verify that the correct patient ID and time point are present on the scan.
6. Relabel the folder in the form "OxAAA-S1-00123 TP-X," using the correct patient stream, patient number and time point, especially if the information on the DICOM file is not fully accurate. See image below for example.



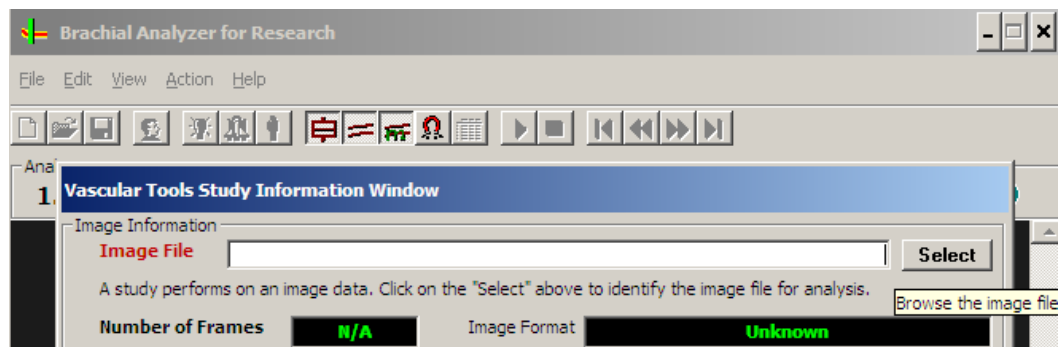
7. Repeat with all folders until each has a unique identifier in the correct form.
8. Open the OxAAA Hard drive and locate the FMD images file. Inside will be the Blank Folder Structure file. Open that, and copy the two folders – "FMD" and "AAA loops" into the newly labelled folders in the USB stick.
9. Click on each image, check the label on the preview window, and drop it into the appropriate folder in either the FMD or AAA loop folder.
 - i. Files will be labelled with hexadecimal identification and will not be in order when sorted by name in the filing system.
 - ii. However, examining the size of the file can be invaluable in speeding the sorting process. Typically, files will have up to 10 static images resultant from the AAA scan and 2 loops, one AP and one transverse.
 - iii. In order of size the files will be as follows:
 1. ~80,000-100,000 kB : Ungated baseline loop
 2. ~20,000-30,000 kB: AAA loops
 3. ~6,000-11,000 kB: FMB, FMD and NMD 60s loops
 4. ~2,000-3,000 kB: FMC and NMD 30s loops
 5. ~100-300 kB: Static images
 - iv. Below is an FMD scan without NMD once the AAA images have been sorted away. The first image of the scan is OXAAAX00, which, according to its size, is a static image. This will be BFM-Velocity. The scan then jumps to OXAAAX02, selected, which is the largest file and is the ungated baseline loop. From there, the images continue in order; next is the FMB gated loop, the static image for FMC-Velocity, the FMC loop. Having reached the end, the images start again at the top, after the BFM-velocity file, at the PHFM-Velocity image, and then the three FMD 60s loops in order.

Name	Date modified	Type	Size
OXAAAX00	21/05/2015 12:56	File	171 KB
OXAAAX0A	21/05/2015 12:56	File	142 KB
OXAAAX0C	21/05/2015 12:56	File	7,175 KB
OXAAAX0E	21/05/2015 12:56	File	7,502 KB
OXAAAX0G	21/05/2015 12:56	File	7,178 KB
OXAAAX02	21/05/2015 12:56	File	83,622 KB
OXAAAX04	21/05/2015 12:56	File	6,348 KB
OXAAAX06	21/05/2015 12:56	File	146 KB
OXAAAX08	21/05/2015 12:56	File	1,991 KB

- v. Examining several unsorted scans will quickly provide an intuitive understanding of this system and greatly speed sorting. In addition, if loops were mislabelled on the system, this pattern can help identify loops that are not labelled correctly based on their position in the order.
10. Copy a fresh version of the empty folder structure into the next folder and continue repeating this process until all images in all folders are sorted on the USB stick.
 11. Open the USB stick and the Hard drive in two separate windows side by side.
 12. Drag and drop each scan folder on the USB stick into the matching patient folder in the hard drive.
 13. Once it is certain that all scans have been copied, clear the USB stick of all scans.
 14. Maintain regular backups of the images folder on the OxAAA hard drive using the two designated backup drives.
 15. In addition to sorting scans into the file system and generating backups; it is also advisable to maintain a database of the full scan inventory.

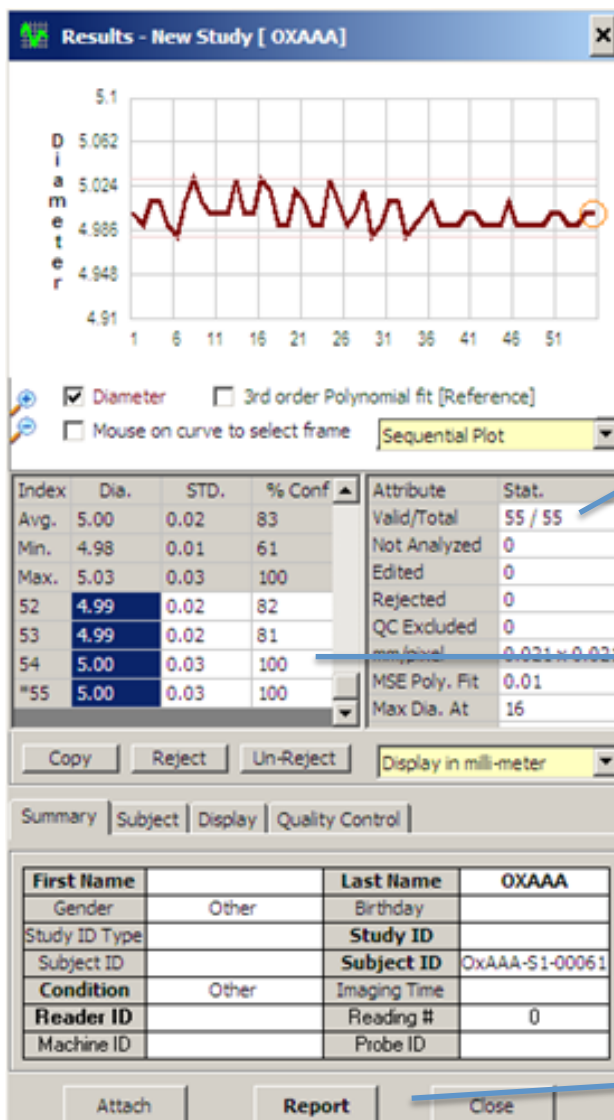
d. Running a basic analysis on an image loop

1. Plug hard drive in analysis laptop and navigate to "My Computer."
2. Open the external drive labelled "Application" and select the drive unlocking program.
3. Enter password to access hard drive.
4. Open the Brachial Analyzer Program.
5. Select the leftmost button representing a blank sheet of paper to create a new study file.
6. In the dialog box that opens, press the select button to open the file explorer.

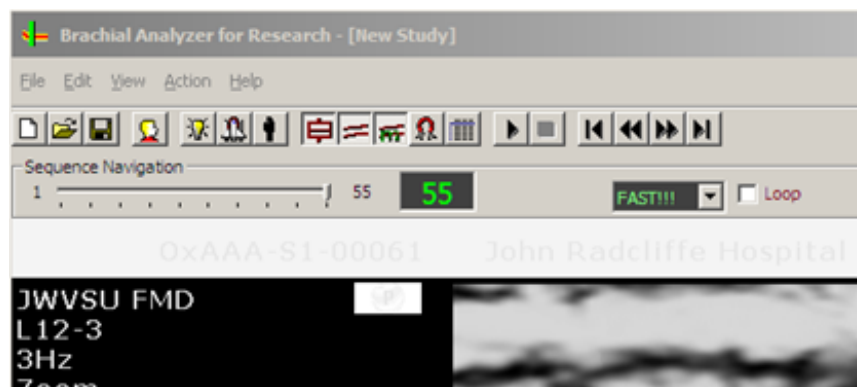


7. Navigate to the file you wish to choose, and double click to select the file.
8. Press "okay" in the open dialog box to proceed with analysis of image.

9. Play file from start to end to examine for any shifts in the vessel. Note the region of the image where the vessel is for the most frames.
10. Click Action in the upper menu, and then choose "Initialize." Alternatively, select the fifth button from the left, depicting the light bulb.
11. The red dotted box that appears is the region of interest, or the ROI. It is within these boundaries that the width of the vessel is measured. The circles can be used to extend the borders of the box, and the central line should always remain within the lumen of the vessel, as one border will be defined on either side of the center.
12. During Step 1, the ROI will jump around as adjustment is attempted, as the software will attempt to offer its own suggestions. Move as necessary, and if required press proceed. During Step 2, the software will interfere less with the placement of the ROI, and will also show yellow lines at the borders of the vessel. If these lines do not correspond to the edges of the vessel, adjust the ROI as required.
13. Once satisfied with the placement of the ROI in Step 2, press process. The analysis process will start automatically. Ensure that the speed is set on 20 fps for speedy analysis, as "FAST" will not proceed as quickly. On the right hand side of the screen, a new window will open will open with analysis statistics, showing a graph at the top of the window and the numerical data in the middle of the screen.



14. Once the graph has been acquired and all frame have analysed, frames that should be rejected can be rejected in two ways. Refer to the guidelines in the next section to decide which frames to reject.
- Select the frame on the graph or in the raw data table and press the reject button below the raw data table.
 - Navigate to the frame by clicking in the sequence navigation bar (shown below) and using the arrow keys or mouse to find the right image find the frame you wish to delete. Press “ctrl-X” on the keyboard and the frame will be rejected. This is best for loops in which many frames need to be deleted, as all frames can be examined by starting at the first frame and navigating through with the arrow key, deleting frames with the keyboard shortcut as required.



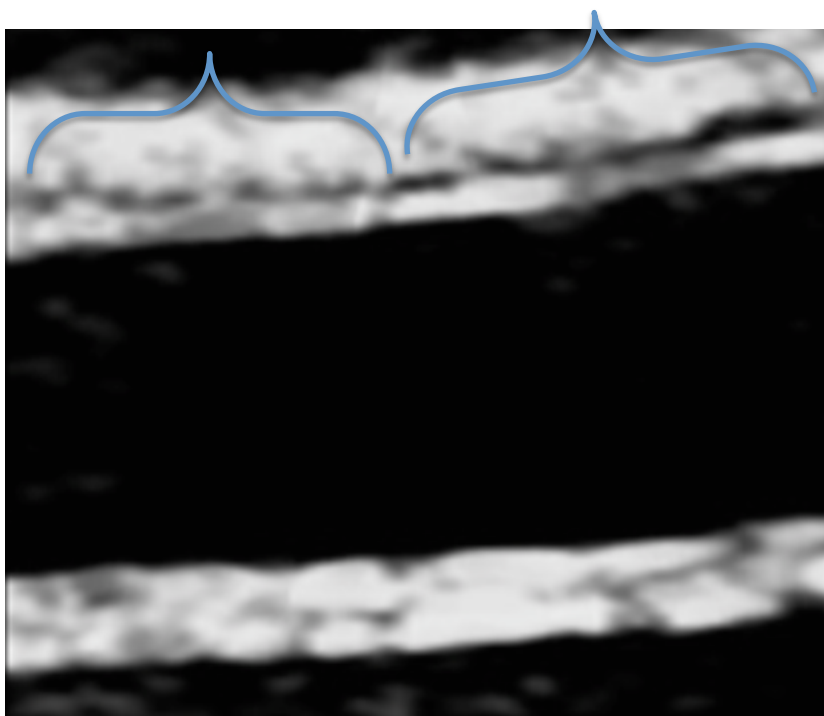
15. Once satisfied with the image, take note of data in appropriate location. Procedures for noting official data during analysis of an FMD scan will be detailed in the data management section below.
16. Save the study file by pressing the save button or choosing the option from the File menu.

e. Analysing an FMD scan

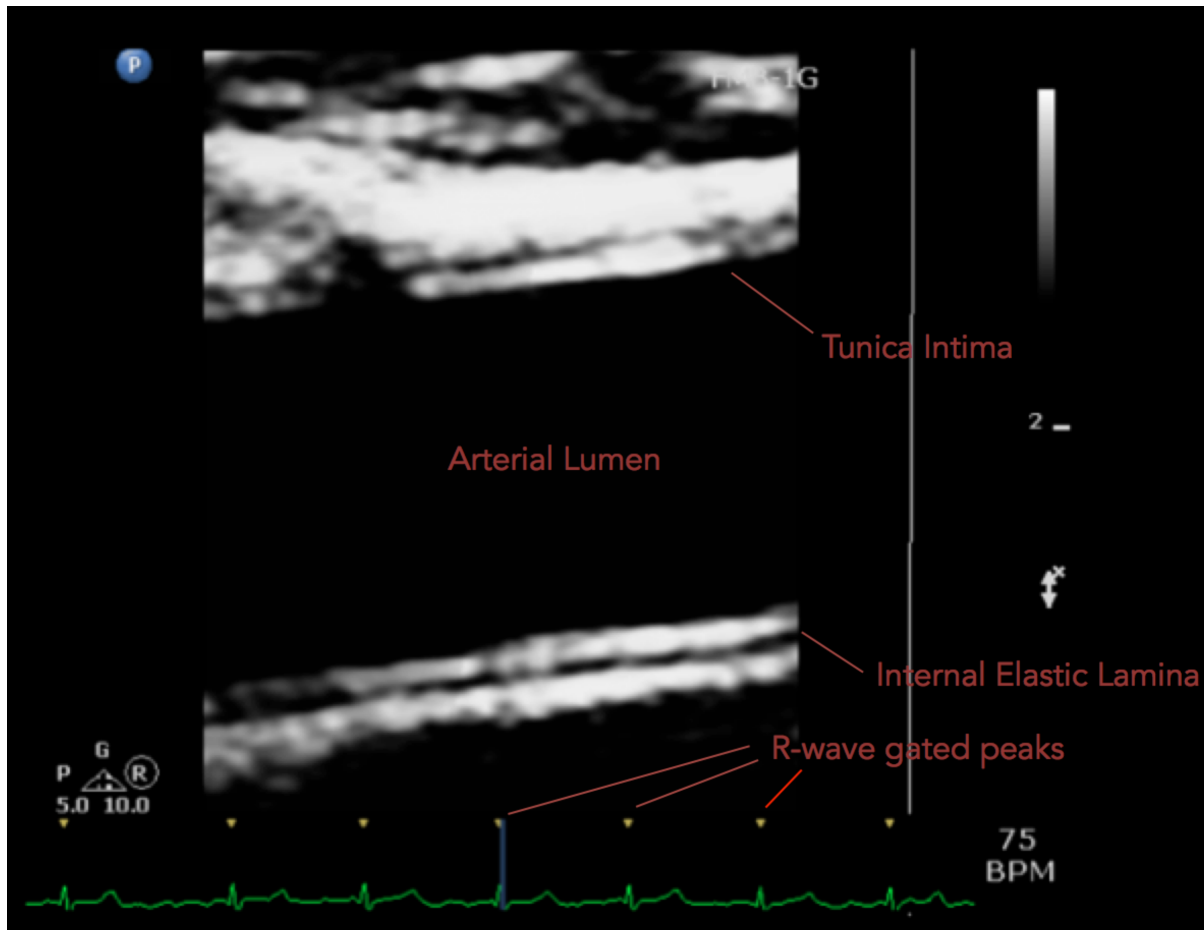
- With the study hard drive plugged in and unlocked, navigate to the scan to be analysed.
- Select the baseline, gated scan, in the FMB-1 folder. There should only be one baseline scan, but if there is more than one, select the later one. Ensure that if there are multiple, the chosen scan is of full length, or approximately 50-70

frames. The number of frames will be displayed when first selecting an image for a new scan, but before scan is opened.

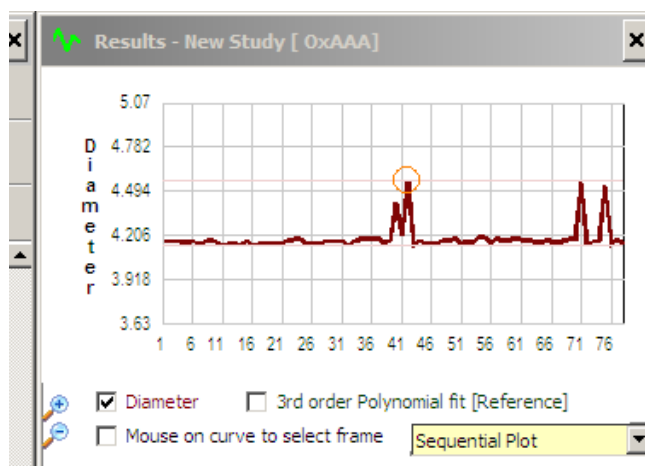
3. Examine the baseline scan to examine for vessel drifting.
4. If the vessel does drift slightly vertically, expand the length but not width of the ROI to encompass all the regions the vessel occupies over the course of the scan.
5. If the vessel shifts dramatically, find the section of consecutive frames during which the vessel is most stable. Click on the first frame of this section, and select the lightbulb or initialize option on this frame. This will allow for definition of ROI to occur in this frame, as opposed to on the first frame. All frames will still be analysed, so those not in the consecutive set of stable frames should be deleted according to the frame rejection protocol detailed.
6. Avoid excessively lumpy or unusually bright areas of the wall, as this could be caused by calcifications of the vessel or artifacting during imaging, causing aberrantly small readings. During the course of the loop, a section of previously stable wall may become excessively bright or lumpy; these frames should be deleted to avoid skewing the average. See below for an example of a vessel with a region of consistent brightness (left) and region of inconsistent brightness (right).



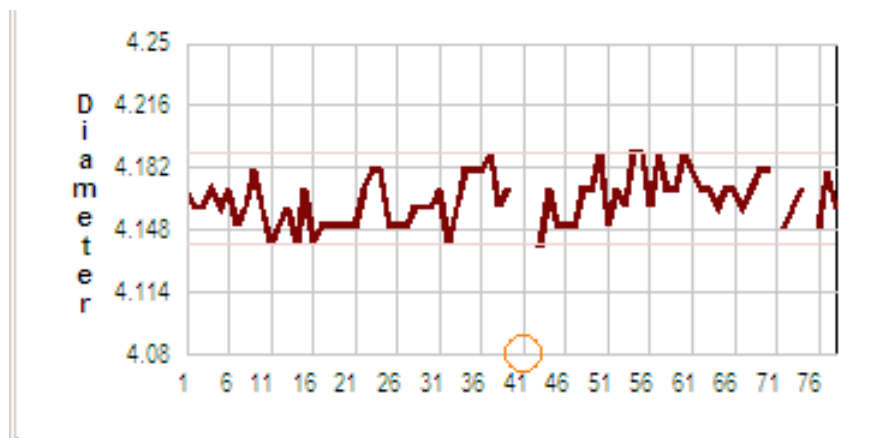
7. Select an ROI of minimal width in a clean and consistent region of the image, and proceed with analysis as detailed in section d. Ensure that the yellow lines of the ROI are capturing the intima of the vessel wall, as labelled in the image below.



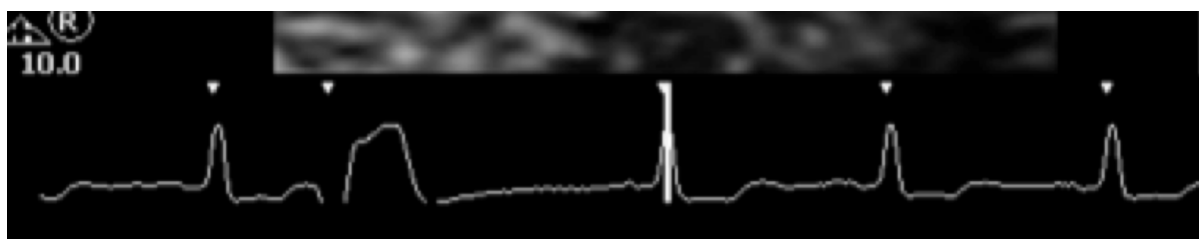
8. Once the analysis is complete, examine the graph.
 - i. The orange lines on the graph denote the maximum and minimum brachial diameter values. If they are within $1/10^{\text{th}}$ of a millimetre, there are likely no major aberrations in analysis.
 - ii. If the graph looks mostly like a straight line with one or a few peaks (below), those are the frames in which the analyser has failed to properly capture the walls of the intima. The orange lines will also have a much larger distance between them. Select those peaks on the graph and use the reject button to remove them.



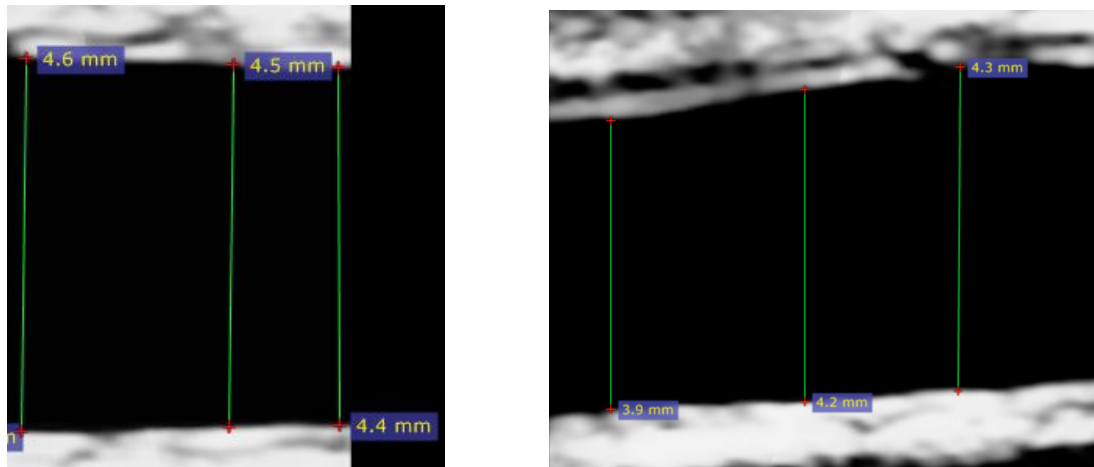
- iii. The graph dynamically adjusts the scale of the axis, so as the outlying aberrant points are all removed, the graph will readjust and the regions that previously looked straight will look far more jagged. A jagged graph is not a concern so long as the range is not very large; the physiological and imaging noise results in each frame having a slightly different diameter, which will average out during analysis of FMB-1. Below is an example, with gaps where frames have been rejected.



9. Check the ECG trace at the bottom of the screen to ensure that gating is functional and there are no ectopic beats. If there are, start at the beginning of the sequence navigation bar, and using the right arrow, scroll through each frame and delete those that are not gated at the peak of an R-Wave. Below is an example of an ECG in which the frame corresponding to the second beat much be removed.

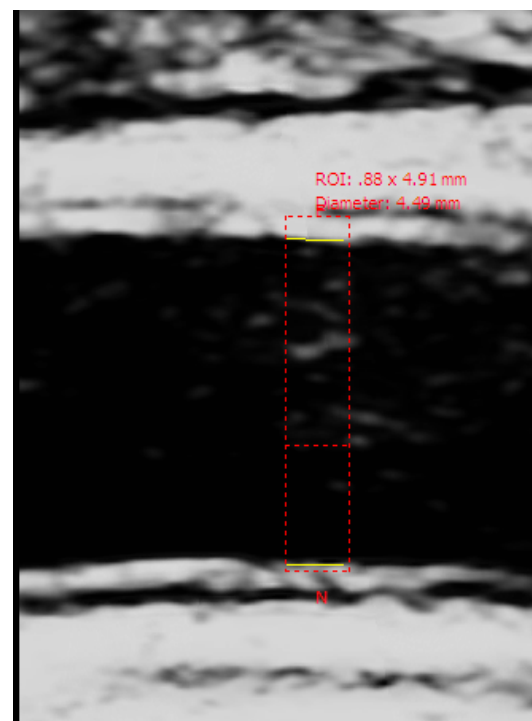
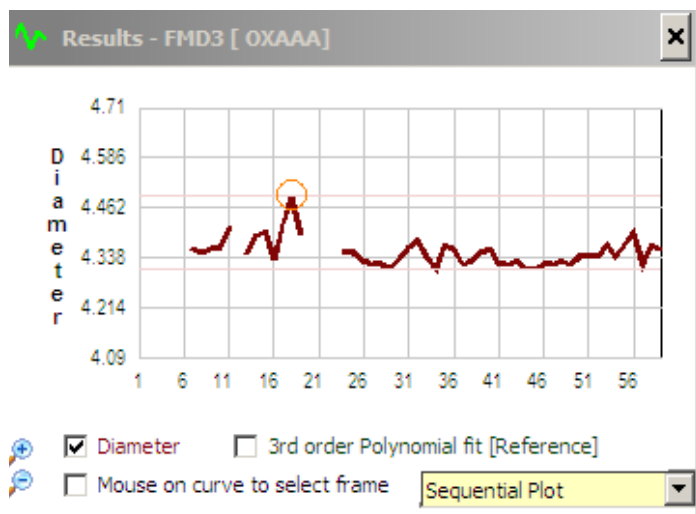


10. If the entire scan is ungated, as ones taken on EPIQ, allow analysis to run, and reject every single frame by selecting frame one in the raw data table, scrolling to the bottom, and pressing shift-click on the last frame. This will select all frames, and then the reject button or ctrl-X can be used to reject them all. There will be ~300 frames, so find the frames in which R-wave peaks occur by clicking along the navigation bar and pressing ctrl-Z or the un-reject button to restore accurate frames.
11. Once frames from regions in which vessel is not in the ROI, which are obviously aberrant on the graph, are on an ectopic heartbeat or not at the peak of an r-wave have been removed, flip through each frame starting from the beginning to ensure that that all remaining frames are acceptable; that is, they have caught the walls of the intima accurately on a frame that is clear and free of artefacts around the vessel walls in the ROI.
12. However it is generally accepted that for a sample to be useable it requires a minimum of 10 accepted frames.
13. Save the file to a new folder in the appropriate analysis folder, labelled in the form "OxAAA S100081 TP-S1," with the stream, time point, and patient ID as appropriate the scan that has just been analysed. Label the scan "FMB1."
14. Do not yet report the scan in the official spreadsheet or export it to excel, until FMD has been analysed and confirmed.
15. Open the first FMD image of the **SAME SCAN** in the brachial analyser tool.
16. Confirm that the vessel has not shifted between FMB and FMD. This can be done by opening the two scans side by side, examining the FMC scan for shifts, and looking for landmarks in the scan such as a characteristic bend in the vessel.
17. Once satisfied that the vessel has not shifted, select the lightbulb icon or "initialize" from the drop down menu.
18. The ROI will appear in the exact same place it was in the previously analysed FMB-1 scan. Adjust it as slightly as possible while still capturing the vessel walls.
19. **It is VERY important that the ROI is as close as possible between the FMB and FMD scan. If the same region of intima from the baseline scan is not visible in the FMD, then return to the baseline scan, select a region which is also visible on the FMD, and re-analyse the baseline. It may be helpful to examine the FMD scan prior to analysing FMB-1, in order to find regions which are clean in both scans. As shown below, the same vessel can have very different diameters depending on region.**



20. As with FMB-1, avoid regions of the intima which are lumpy. Once you are confident that the same region is being analysed in both scans and that the region has stable intima and is of consistent brightness and smooth, proceed with analysis of scan.
21. As before, examine the graph resulting from the analysis. While FMB-1 is based on an average and requires as many frames to be reasonably accurate as possible, FMD measurement is dependant ONLY on the maximum frame, and is therefore highly sensitive.
22. Start from the maximum frame – the point on the graph touching the orange line, and follow the checklist below:
 - i. Are the yellow lines touching the intima?
 - ii. Is the vessel wall free of imaging artefacts?
 - iii. Is this frame gated on a non-ectopic beat at the top of an R-wave?
 - iv. Is the measurement in this frame consistent with the frames preceding and following? i.e. no drastic or sudden jumps.
23. If all of these conditions have not been satisfied in the maximum frame, reject the frame and wait for the graph to readjust the scale and orange lines. This will happen quickly.
24. Select the new maximum frame, and apply the same checklist as above.
25. Repeat this process until you are satisfied that the maximum frame is acceptable.

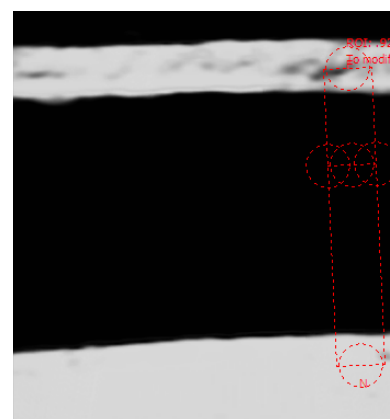
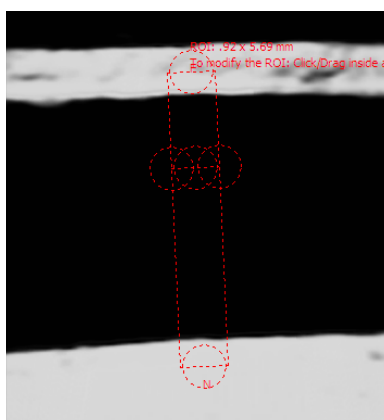
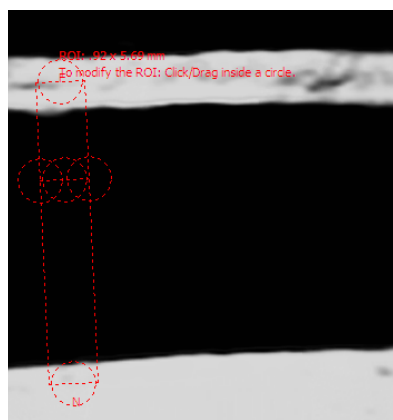
26. Examine the resulting graph. Does the value of the maximum frame occur multiple times? If yes, value is likely to be accurate. If the next highest frame/s are very close to the orange line, the maximum is also likely to be accurate.
27. If the clean maximum frame is far higher than the next highest clean frame (>3/100 mm) consult with another analyser to discuss whether frame should be discarded or kept. Shown below, this scan shows a peak 12/100ths of a millimetre above the next highest, which would cause a 2-3% difference in FMD. The decision will depend on the quality of the scan and the smoothness of the analysis graph.



28. Save the study in the previously created folder, named “FMD” and note the maximum diameter of the brachial artery, but do not yet report it in the excel spreadsheet.
29. Open the next FMD image file and repeat steps 13-23. If the resulting maximum diameter is higher than the first image of the FMD analysis, then save this analysis over the previous one. If the maximum diameter found in this scan is lower, do not save it.
30. Repeat step 25 with the final FMD scan of the study. Again, is the maximum diameter in this scan exceeds the previously record one, save this study file as

FMD, deleting the previous file. Note the maximum of the final file saved, which should be the maximum FMD brachial artery diameter between all FMD loops.

31. Compare the maximum FMD diameter found to the average baseline diameter noted. If FMD max diameter is lower, open the FMD study files saved and recalibrate the file as described below. This frequently fixes the problem. If, upon opening FMB1, calibration was requested, you **MUST** also calibrate each FMD file. If calibration is not explicitly requested, do not calibrate unless the FMD max is lower than FMB min.
 - i. To Calibrate, press the action menu and select calibrate.
 - ii. Ensure that vertical calibration is selected.
 - iii. Position the crosshair callipers on the top and bottom left-hand corners of the zoom box.
 - iv. At the top of the screen, enter 10 mm in the entry box, and press calibrate.
 - v. Repeat analysis process.
32. The two study files saved –FMB1 and FMD- should now both have the same ROI, and the FMD should be the file in which the largest diameter was found.
33. It is advisable to obtain measurements of FMB1 and FMD in several areas of the sample to provide an average due to irregularities in vessel lumen diameters. We recommend repeating steps 1-31 for regions within the sample to the left (L), centre (C) and right (R) of an analysable region within the frame. Save each file carefully. Ensure that the ROI analysed each time is consistent between the FMB and FMD scans. If there is not enough visible intima overlapping between both scans, only one or two analyses may be possible.



34. There should now be three scans labelled FMB1, and three scans labelled FMD. When saving, add an indicator of “L,” “C,” or “R,” to denote which of the three analyses is enclosed.

35. Open the first FMB1 file, record the minimum, maximum, number of valid frames, and average in a blank spreadsheet. Press the report button, choose Excel, and save the Excel report in the EXCEL REPORTS folder, under the appropriate filing. The filename should be the patient ID number, and L, C, or R, depending on which of the analyses is enclosed.
36. Repeat for each FMB1 scan with a new row, and use a formula to obtain the average minimum, maximum, frame number, and average.
37. Repeat this process for FMD.
38. In the OxAAA master spreadsheet under the tab for the correct study time-point, create a new row for the scan and place the short form of the study ID in the third column. Copy the DICOM tag from the bottom right hand side of the analysis window into the first column, labelled PID. If there were any notes during analysis, such as a gating issue or ambiguous frames, note them in column 2.
39. In the appropriate column, note the calculated values for minimum, maximum, frame number, and average.
40. FMD should first be calculated for each of the different vessel regions (L, C and R) as a percentage increase using the average FMB-1 value and the corresponding FMD max value. Then the 3 FMD percentages can be averaged to provide a measure of overall vascular function independent of irregular lumen diameter across the sample.
41. Step 39 can be completed concisely using the formula:

$$=IFERROR(100*((F4-C4)/C4 + (G4-D4)/D4 + (H4-E4)/E4)/3,"")$$
As used in the first row of data in the screenshot below.

	A	B	C	D	E	F	G	H	I
1			PRE-OP						
2			FMB1			FMD			
3	PID	NOTES	L	C	R	L	C	R	RESULT
4	OXAAAS100024		3.59	3.56	3.65	3.61	3.63	3.67	1.02
5	OXAAAS100027		3.34	3.44	3.34	3.28	3.29	3.2	-3.45
6	OXAAAS100065		4.18	4.16	4.22	4.45	4.2	4.19	2.24
7									-0.06
8									
9									

42. If NMD was completed on this patient, the same procedure should be repeated, treating FMB2 as the FMB1 and both NMD25 and NMD400 as the FMD scan. Unlike FMD, only one ROI/analysis needs to be completed for these scans.
43. Report these results in the same row as the FMD results were reported.

f. Tips on analysis

1. Position the ROI as close to the true intima as possible to avoid drifting to the internal elastic lamina or other artefacts – this will skew averages obtained for FMB1 measurements.
2. Use a standard ROI width – the OXAAA study defined this as 0.84mm (the point at which the ROI adjustment circles intersect).
3. Upon obtaining an anomalous result first check for ROI drifting. If this looks normal then check calibration as described in step 31 of section e. Note that the figure of 10mm listed in this SOP may be specific to the OXAAA CX50 ultrasound machine. It is advisable to obtain your own calibrating value by using the digital callipers available on most ultrasound machines.
4. If at any point you are unable to open a saved analysis through the Brachial Analyser, first check that the original sample file still exists. Either has it been deleted or was it stored on a removal storage device? The brachial analyser adopts a fixed file path utilisation mechanism and hence requires access to the initial loop in its original location. Therefore this issue can be resolved either through simply running a new scan or copying the sample file to its original location at the time of first analysing.
5. If the sample falls out of view when using the zoom feature press one of the 'centre image' or 'centre on ROI' buttons.
6. If the intima is faint or the contrast of the image is unfavourable for other reasons, this can be adjusted by using the 'window/level' slider bar in the bottom left of the screen.
7. Consider that the 'Brachial Analyser' occupies a large proportion of the CPU usage and it is therefore wise to run as few programs as possible in the background when conducting analysis.

.5 FMD and NMD Data Tables

The following data tables represent the raw FMD and NMD values from all available scans at the time of analysis, with the surveillance and operative patients included for all available time-points.

FMD and NMD data for S1 patients in from TP-1 to TP-S3

Patient ID	TP-1		TP-S1		TP-S2		TP-S3	
	FMD	NMD	FMD	NMD	FMD	NMD	FMD	NMD
S1-1	6.65		4.20					
S1-2			0.87	8.01	0.00		0.62	17.68
S1-3	2.27	9.02	0.96					
S1-4			1.11	0.41				
S1-5	2.28							
S1-6	4.61	5.88						
S1-7	3.06	12.67	1.36	11.36	4.09			
S1-8	2.17		2.46		0.19		2.14	
S1-9	2.47	8.04	-0.21	10.23				
S1-10	3.85	19.63	3.77	14.66	5.57			
S1-11	1.70	4.91	0.84					
S1-12	5.24	12.65	1.47	21.98				
S1-13	0.00	15.39	2.24	19.16	-0.30	33.73	-0.94	26.16
S1-14	5.19	22.27	0.00	6.47	-0.77	28.26	-1.94	30.08
S1-15	5.15	4.79	0.60		0.43			
S1-16	4.50	16.41	3.79	24.97	2.05		4.75	
S1-17	4.00	19.13	3.36	27.18	0.51		3.56	
S1-18	5.03	13.81	0.00		3.17			
S1-19	1.39	19.82	-0.52		-0.36	17.32	-0.23	23.58
S1-20	3.36	3.61	3.20					
S1-21	6.06	24.00			1.20			
S1-22	0.67	18.47	0.00	12.69	3.87		2.71	
S1-23	1.70	36.01	1.32	26.12	3.35			
S1-24	5.81	15.31	0.81		1.12			
S1-25	1.66	5.62						
S1-26	4.42	11.51			4.24			
S1-27	-1.74	19.18	-2.47	36.62				
S1-28	0.00	10.65	4.15		2.97			
S1-29	2.44	27.09	3.46	14.66	2.32	33.96		
S1-30	2.51	18.35			0.00			
S1-31	3.46	34.28			2.43	18.44	3.17	12.14
S1-32	-1.80	24.59	0.77	26.73	0.69	26.53		
S1-33	0.65	16.20			2.88	25.98		
S1-34	6.77	13.74	0.97	14.40				
S1-35	-1.31	29.28	-0.44	26.99				
S1-36	2.57	7.13	-1.40	18.03				
S1-37	0.00	1.56	-0.34	14.78				
S1-38	5.29	10.31	3.49	22.55				

S1-39	2.65	9.05	5.91	10.99				
S1-40								
S1-41								
S1-42	3.00	21.52	0.57	15.14				
S1-43	0.82	17.23	1.01		0.73			
S1-44	1.01	20.94						
S1-45	1.77	20.73	0.92	17.23	3.54	17.74		
S1-46	-0.19	8.43						
S1-47	-0.62							
S1-48	0.44	15.46	0.00	19.43	0.00		1.08	
S1-49	2.45	10.31						
S1-50	1.33	30.36						
S1-51	0.00		0.99		-1.84			
S1-52	5.05	20.75	0.95	32.67				
S1-53	1.31	26.25	0.00	34.28	2.33	23.72		
S1-54	2.09	4.53	0.84					
S1-55	1.45	8.70	2.20	8.84				
S1-56	1.55		2.12		-1.27		0.09	
S1-57	5.59	21.30	-1.93	33.20	1.97	18.79	3.00	6.04
S1-58	2.06	1.48						
S1-59	0.61	4.26						
S1-60	1.99	10.88						
S1-61	0.27	13.69	0.29	10.24	0.36	20.36		
S1-62	-0.81							
S1-63	4.87	8.04						
S1-64			13.76	25.24				
S1-65	0.48	14.94	-0.59					
S1-66	-1.13	12.89	-2.88					
S1-67								
S1-68								
S1-69	5.09	28.03	0.00					
S1-70	2.72	25.11						
S1-71	4.02	18.92	4.30					
S1-72	1.54	13.29	1.92		1.93			
S1-73	2.45	20.19	1.29					
S1-74	1.01		2.56					
S1-75	-0.79	10.31						
S1-76	4.99	21.35						
S1-77	4.88		5.91	4.25				
S1-78	4.02		2.29					
S1-79	0.27	23.57	0.83	28.57				
S1-80	4.91		3.55					

S1-81	1.86	6.45	1.84	13.21	1.00		0.69	
S1-82	3.38							
S1-83	-0.15	29.13	0.97		2.05			
S1-84	6.45	16.45	-0.57					
S1-85	0.00		2.12		1.33	27.22		
S1-86	2.69		1.45					
S1-87	1.04		0.34	16.73				
S1-88	4.43		5.41					
S1-89								
S1-90	6.07		3.69					
S1-91	1.30		1.05					
S1-92	1.38		0.43		0.22			
S1-93	3.42		4.75					
S1-94	6.03	24.41			2.93			
S1-95	4.71		2.18		1.04			
S1-96	5.17	19.54	1.64					
S1-97			0.48					
S1-98	1.40		2.80					
S1-99	4.74		-2.48					
S1-100	2.07		3.42					
S1-101	4.83		3.21					
S1-102	0.12	3.94	1.96		0.63	9.65		
S1-103	-0.20	17.38	-2.07					
S1-104	1.59	16.99	1.27					
S1-105	0.03	26.14	0.56	35.59				
S1-106			1.01					
S1-107			4.20					
S1-108	4.20	18.74	5.51	18.70				
S1-109	3.27	36.80	4.91					
S1-110	3.61	21.57						
S1-111	0.97	19.69	0.33	30.86				
S1-112	0.45							
S1-113	7.73							
S1-114	0.09		0.39	8.00				
S1-115	0.00		2.52	10.38				
S1-116								
S1-117	0.00		1.42					
S1-118	1.04	14.39						
S1-119	3.04							
S1-121	2.11							

FMD and NMD from time points TP-3 to TP-F1

Patient ID	TP-3		TP-5		TP-F1	
	FMD	NMD	FMD	NMD	FMD	NMD
S1-1						
S1-2						
S1-3			5.45			
S1-4						
S1-5			1.26	13.99	2.39	8.41
S1-6						
S1-7						
S1-8						
S1-9						
S1-10						
S1-11			4.84	24.35	3.17	
S1-12						
S1-13						
S1-14						
S1-15						
S1-16						
S1-17						
S1-18						
S1-19						
S1-20						
S1-21						
S1-22						
S1-23						
S1-24			-0.47		4.59	
S1-25						
S1-26						
S1-27			3.90	25.53	-0.83	
S1-28						
S1-29						
S1-30						
S1-31						
S1-32						
S1-33						
S1-34						
S1-35	2.08		3.80		13.72	
S1-36						
S1-37						
S1-38						
S1-39						

S1-40						
S1-41						
S1-42						
S1-43						
S1-44			-1.99			
S1-45						
S1-46			4.50		4.72	
S1-47			4.03			
S1-48						
S1-49	4.76		1.67		-0.84	
S1-50						
S1-51						
S1-52						
S1-53						
S1-54						
S1-55						
S1-56						
S1-57						
S1-58			1.73	7.13	4.51	
S1-59						
S1-60						
S1-61					2.54	20.19
S1-62			3.80		0.63	
S1-63						
S1-64						
S1-65	0.00	17.81				
S1-66						
S1-67						
S1-68						
S1-69						
S1-70						
S1-71						
S1-72						
S1-73						
S1-74						
S1-75			0.29			
S1-76			1.08		7.98	
S1-77						
S1-78						
S1-79						
S1-80	-0.39		1.55		4.84	
S1-81						
S1-82						

S1-83						
S1-84						
S1-85						
S1-86						
S1-87						
S1-88						
S1-89						
S1-90						
S1-91						
S1-92						
S1-93						
S1-94						
S1-95						
S1-96						
S1-97			0.56		2.05	
S1-98						
S1-99						
S1-100						
S1-101						
S1-102						
S1-103						
S1-104						
S1-105						
S1-106						
S1-107						
S1-108						
S1-109						
S1-110						
S1-111						
S1-112						
S1-113						
S1-114						
S1-115						
S1-116			4.24			
S1-117						
S1-118						
S1-119						
S1-121						
S2-6	4.37		0.48	19.02		
S2-7	-1.53		5.90	21.93	2.16	
S2-8						
S2-9						
S2-10						

S2-11	0.84		6.03		10.18	
S2-12						
S2-13			0.20		8.13	
S2-14	4.61		3.28			
S2-15	1.32		-0.67	28.22	2.35	25.64
S2-16	1.23					
S2-17						
S2-18	2.02		6.67			
S2-19	2.91		4.93		10.49	
S2-20	1.26	22.43	3.12		0.73	
S2-21	0.81		4.86		3.47	
S2-22	1.61		3.23			
S2-23						
S2-24			2.83			
S2-25	0.00		6.03			
S2-26						
S2-27	0.36		6.85			
S2-28						
S2-29			1.00		9.34	
S2-30			0.28			
S2-31	0.26		1.54			
S2-32	1.07		3.73			
S2-33	0.70	31.92	3.80			
S2-34						
S2-35	0.29		6.03			
S2-36	4.37					
S2-37	1.95	19.15	1.72	11.63		
S2-38						
S2-39						
S2-40	1.29		2.52			
S2-41	1.73		4.77			
S2-42	-0.56		4.68			
S2-43	1.61		3.13			
S2-44	1.54	6.99				
S2-45	0.48					
S2-46						
S2-47	0.30					
S2-48	1.07					
S2-49	3.23		6.92			
S2-50	2.14					
S2-51	1.88		2.30			
S2-52	1.81		7.87			

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