

## DOCTOR OF PHILOSOPHY

### 3-Nitrotyrosine As An Indicator Of The Disease State Intermittent Claudication

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**3-Nitrotyrosine As An Indicator Of  
The Disease State Intermittent  
Claudication**

**Sadie Dean**

**A Thesis Submitted in Partial Fulfilment of the  
University's Requirements for the Degree of  
Doctor of Philosophy**

**2009**

**Coventry University**

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## Dedication

In memory of Leighton Parker and Gerald Mercer.

“Remembered with a smile”

To Andy and Jasmine who bring love and happiness to my life.

---

## **Abstract**

3-nitrotyrosine (3NT), a stable end product arising from the interaction of proteins and reactive nitrogen species such as peroxynitrite, is produced during periods of oxidative stress. 3NT is, therefore, of interest as a potential biomarker in a variety of disease states where oxidative stress is known to be involved in the pathology, for example intermittent claudication. The aim of this thesis was to develop sensitive and specific immunoassays to assess the levels of 3NT in plasma samples from claudicants and to investigate the protein nitration profile.

Clinical data and plasma samples were collected from claudicant (n=33) and control (n=6) subjects. Analysis of data confirmed the difficulty of using parameters such as ankle brachial index (ABI) in diagnosis, supporting the need for investigations into potential biomarkers.

Development of indirect and competitive ELISAs using electrochemically nitrated bovine serum albumin as the standard revealed that the detection of 3NT was dependent on the antibody being able to access the 3NT-residues within the protein. Various denaturing conditions and different types of microtitre plate were utilised during development. Initially the presence of 3NT in claudicant or control whole plasma samples could only be detected using dot blot immunodetection. Affinity purification techniques for the fractionation of the plasma proteins were therefore applied. Subsequently, 3NT-containing plasma proteins were found to be present in all of the claudicant and control samples using the developed competitive ELISA. Proteomic analysis of the 3NT-affinity purified samples, using MALDI-MS and LC-ESI-MS/MS, confirmed the presence of human serum albumin, serotransferrin and apolipoprotein A1 and A2 precursors within those protein bands staining immunopositive for 3NT on SDS-PAGE gels. The identification of apolipoprotein A1 within 3NT-immunopositive bands confirms previous reports suggesting the oxidative modification of HDL may contribute to the link between inflammation and the pathology of atherosclerosis.

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I would also like to thank my family for their support and patience and to Andy thank you so much without you I would not have been able to complete my PhD your support and encouragement made this happen.

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## **Abbreviations**

A	Absorbance
Ab	Antibody
ABI	Ankle brachial index
ACE	Angiotensin converting enzyme
AHA	American heart association
ANOVA	Analysis of variance
Anti-3NT Ab	Anti-3-nitrotyrosine antibody (polyclonal)
BMI	Body mass index
BSA	Bovine serum albumin
CAD	Coronary arterial disease
CVA	Cardiovascular accident
CVD	Coronary vascular disease
DBP	Diastolic blood pressure
EC	Electrochemical detection
EDTA	Ethylene diamine tetra acetic acid
ELISA	Enzyme linked immunosorbant assay
HDL	High density lipoprotein
HPLC	High performance liquid chromatography
HSA	Human serum albumin
IC	Intermittent claudication
IgG	Immunoglobulin G
KHz	Kilohertz
KLH	Keyhole limpet haemocyanin

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LC-ESI-MS/MS	Liquid chromatography - electrospray ionisation - tandem mass spectrometry
LDL	Low density lipoprotein
LV-PAD	Large vessel - peripheral arterial disease
MALDI-TOF	Matrix assisted laser desorption ionisation- Time of flight
MAP	Mean arterial pressure
MI	Myocardial infarction
MPO	Myeloperoxidase
MS	Mass spectroscopy
NBT/BCIP	Nitrobluetetrazolium/5-bromo-4-chloro- 3-indolyl phosphate
NO	Nitric oxide
3NT	3-nitrotyrosine
NT-BSA	Nitrated bovine serum albumin
NT-HSA	Nitrated human serum albumin
NT-PL	Nitrated human plasma (electrochemically)
OD	Optical density
ONOO <sup>-</sup>	Peroxynitrite
PAD	Peripheral arterial disease
PBS	Phosphate buffered saline
pNPP	p-Nitrophenol phosphate
rcf	Relative centrifugal force
RMM	Relative molecular mass
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SBP	Systolic blood pressure
SD	Standard deviation

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SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SMP	Skimmed milk powder
SV-PAD	Small vessel - peripheral arterial disease
TEMED	N,N,N',N'-tetramethylethylenediamine
TIA	Transient ischaemic attack
Tris	Tris[hydroxymethyl] aminomethane
Tryp	Trypsin
Tryp-NT-BSA	Trypsin digested nitrated BSA
Tyr	Tyrosine
U+M	Urea and mercaptoethanol
UV	Ultra violet
V	Volts

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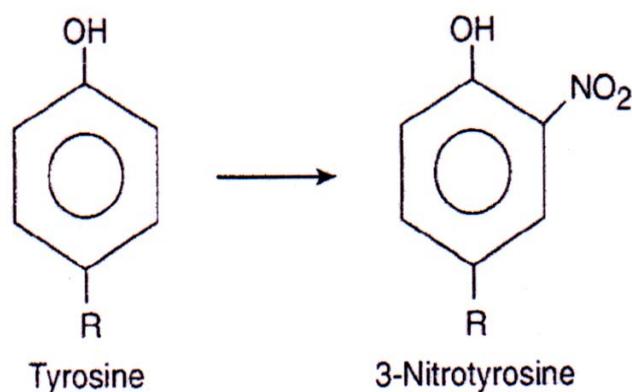
# **Chapter 1.**

## **Introduction**

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### 1.1. 3-Nitrotyrosine

3-nitrotyrosine (3NT) is formed by the addition of a nitro group ( $\text{NO}_2$ ) on the ortho position of the amino acid tyrosine (Figure 1.1.i.). Nitration can occur both in biological systems and can be produced chemically within the laboratory. Molecules with aromatic rings such as the amino acid tyrosine are more susceptible to nitration than most other molecules in biological systems. As a result of the fact that the nitro group is a stable chemical modification that dramatically changes the chemical properties of tyrosine it is thought that it may have a long lasting effect on protein function.



**Figure 1.1.i. Tyrosine nitration.**

Nitric oxide (NO) is an ubiquitous intercellular messenger that is synthesised enzymatically from the amino acid L-arginine in a number of tissues by NO synthase (NOS) of which there are three isoforms; inducible (iNOS), endothelial (eNOS) and neuronal (nNOS). NO has roles in both normal physiology and pathological conditions in humans. Under normal conditions NO is efficiently removed from the system by reacting with oxyhaemoglobin resulting in the formation of nitrite, even with the highest rates of NO

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synthesis. However data suggests that in specific subcellular compartments (when in close proximity to enzymes capable of production of reactive oxygen species (ROS) and iNOS) NO-dependent nitration of tyrosine is a physiologically relevant process (Heijnen, Van Donselaar, Slot *et al.* 2006) and 3NT was first proposed as an *in vivo* marker of reactive nitrogen species (RNS) by Ohshima *et al* (1990). Since this time protein nitration has been widely investigated as a stable marker of NO derived reactive species (extensively reviewed by Beckman and Koppenol 1996, Bruckdorfer 2005, Pacher, Beckman and Liaudet 2007).

Although the exact nitration pathways have yet to be completely elucidated it has been established that tyrosine nitration within biological systems is associated with NO and reactive oxygen species (ROS). Initial interest in biological nitration resulted from observations that chemicals such as peroxynitrite (ONOO<sup>-</sup>) nitrate tyrosine residues. In situations whereby an inflammatory response is occurring the simultaneous production of NO and superoxide (O<sub>2</sub><sup>-</sup>) results in the biological actions of NO being completely transformed and results in the formation of the potent oxidant ONOO<sup>-</sup>. At present there are many reported models/pathways which have been shown to result in nitration of tyrosine residues *in vitro* (Ischiropoulos, Zhu, Chen *et al.* 1992, Eiserich, Cross, Jones *et al.* 1996), however, very few have been replicated in conditions that mimic *in vivo*. It is likely to be the case that there are multiple pathways *in vivo* which lead to the nitration of tyrosine residues (Ischiropoulos 1998). Potential mechanisms of nitration which have been identified and extensively researched include ONOO<sup>-</sup> and myeloperoxidase (MPO).

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ONOO<sup>-</sup> is formed during periods of oxidative stress and is a powerful oxidant and cytotoxic agent, furthermore, its protonated form, peroxyntrous acid, is extremely unstable and cleaves rapidly to hydroxyl and NO<sub>2</sub> radicals potentially initiating further free radical reactions (Beckman, Chen, Ischiropoulos *et al.* 1994). At nanomolar concentrations ONOO<sup>-</sup> has been found to produce significant amounts of 3NT in both physiological and pathological events *in vivo* (Sawa, Akaike and Maeda 2000, Reiter, Teng and Beckman 2000). Initially it was thought that 3NT was a specific and stable marker of ONOO<sup>-</sup> activity, but this is now known not to be true (Eiserich, Cross, Jones *et al.* 1996). It has been shown that other species can and do nitrate tyrosine at physiological pH. For example the haem enzyme myeloperoxidase (MPO, a member of the animal peroxidases) in the presence of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and nitrite will nitrate tyrosine by radical coupling after one electron oxidations of both nitrite and tyrosinate anions. Since inflammatory disorders are typically associated with an infiltration of phagocytes, which contain high levels of haem peroxidases, this pathway has to be considered as a potential alternative pathway to ONOO<sup>-</sup> in mediating protein tyrosine nitration *in vivo*.

It has been known for some time that MPO acts to chlorinate tyrosine to chlorotyrosine (Hazen and Heinecke 1997) but it is now becoming apparent that MPO also acts to nitrate tyrosine in a nitrite-dependent peroxidase reaction in cytokine activated macrophages (Podrez, Schmitt, Hoff *et al.* 1999, Hazen, Zhang, Shen *et al.* 1999, Van Dalen, Winterbourn, Senthilmohan *et al.* 2000, Malle, Waeg, Schreiber *et al.* 2000, Pfeiffer, Lass, Schmidt *et al.* 2001, Gaut, Byun, Tran *et al.* 2002). It has also been highlighted that the reaction of

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nitrite, an auto-oxidation product of NO, with HOCl forms reactive intermediate species that are also capable of nitrating phenolic substrates such as tyrosine (Eiserich, Cross, Jones *et al.* 1996). As reviewed by Ischiropoulos (1998) many other potential protein nitration pathways are present in biological systems but the above are classed as the main probable routes, although it is likely that multiple nitrating agents function simultaneously.

Due to the complexity of biological systems it is necessary to evaluate the potential nitrating species responsible in every model of disease and it is more probable that there are multiple nitration pathways/agents operating at different times during the pathology of and progression of a disease state (Hazen, Zhang, Shen *et al.* 1999, Gaut, Byun, Tran *et al.* 2002). For example, in disease states that involve chronic inflammation such as atherosclerosis, MPO-dependent pathways must be considered (Podrez, Schmitt, Hoff *et al.* 1999). Thus the nitrating agent will be a function of the RNS and ROS present along with any secondary reactions with antioxidants and scavengers, and the presence of inflammatory cells (Ischiropoulos 1998). Hazen *et al.* (1999) generated a model of pathways available to monocytes for promoting LDL modification by RNS based on the findings of their research. This model highlighted the fact that MPO/H<sub>2</sub>O<sub>2</sub>, MPO-generated HOCl and ONOO<sup>-</sup> pathways were all potentially available to monocytes during conditions of oxidative stress.

As most proteins are thought to be composed of approximately 4% tyrosine residues it was initially thought that all proteins, along with free tyrosine, are potential targets for nitration in biological systems. However it has been reported that only a subset of proteins are actually nitrated suggesting that

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there are parameters such as a protein's innate properties that predispose it to nitration (Souza, Daikhin, Yudkoff *et al.* 1999). Neither the amount of a protein nor the number of tyrosine residues within a protein can be used to predict the likelihood of a protein being nitrated. For example human serum albumin is the most abundant protein in plasma and should, in theory, be a target for nitration. However, in actual fact it has been found that on a molar basis the extent of nitration for albumin is less than that for other plasma proteins (Ischiropoulos 1998). It is apparent that tyrosine nitration is a selective process and as such many factors are thought to contribute to the likelihood of tyrosine being nitrated, such as protein folding and the nitrating species involved (Ischiropoulos 1998). Although free tyrosine is abundant in plasma and does serve as a significant biological target for nitrating species, it is dynamic and subject to a relatively rapid turnover with a half life of about two weeks. Therefore, when quantifying the extent of nitration protein-bound 3NT should be more stable and consistent over time than free-3NT (Skinner, Crow, Skinner *et al.* 1997). It is probable that the specific disease pathology, nitrating agent involved and protein folding all contribute to the likelihood of a protein being nitrated and more specifically which tyrosine residues within a protein are nitrated.

NO-dependent nitration has been identified as being a physiologically relevant process localised in specific subcellular compartments in close proximity to enzymes capable of peroxidase activity, ROS and iNOS (Heijnen, Van Donselaar, Slot *et al.* 2006). Lanone *et al.* (2002) have shown that iNOS is susceptible to nitration both *in vitro* and *in vivo* leading to loss of activity and that this may serve as an endogenous mechanism of modulation of iNOS

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enzymic activity. iNOS expression in itself (through the production of large amounts of NO) has been implicated in extensive nitration of cellular proteins in transplant coronary artery disease, via ONOO<sup>-</sup> (Ravalli, Albala, Ming *et al.* 1998). Nitrated proteins, along with production of the iNOS inhibitor asymmetric dimethylarginine (ADMA), have been identified as being a feature of diffuse scleroderma, suggesting that this may be a reflection of abnormal NO regulation in systemic sclerosis (Dooley, Gao, Bradley *et al.* 2006). The susceptibility of a protein to be degraded has also been found to be enhanced by protein nitration (Souza, Choi, Chen *et al.* 2000) thus suggesting that nitration may have an important physiological role. Thus the identification of protein targets of nitration in human disease and animal models of disease is important in the understanding of the role of protein nitration in both normal physiology and mechanisms of disease progression. However, at present, data on the role of protein nitration in signal transduction events and in the pathogenic mechanism of diseases is extremely sparse when compared to the number of diseases 3NT has now been associated with.

Several *in vitro* methods are available to nitrate tyrosine residues; the majority of these are chemical methods of modification. Tetranitromethane is widely used to nitrate tyrosine residues in proteins, initiating nitration via a charge transfer/radical mechanism. Chemically formed ONOO<sup>-</sup> is also widely used to nitrate proteins in biological models. The major problem with chemical nitration is that the reactions are not easily controlled therefore making it difficult to nitrate a protein to a set degree of nitration. Electrochemical nitration will be used within this study and has been shown to specifically

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nitrate tyrosine residues within proteins (Kendall, Cooper, Heptinstall *et al.* 2001). The major advantage of this type of nitration is that using a potential to drive oxidation makes the reaction easier to control thus allowing for proteins to be nitrated to exact levels of nitration. Kendall *et al* (2001) suggest that electrochemical nitration of tyrosine residues may occur as a result of a radical/radical coupling reaction reliant upon the reaction of the protein to be nitrated and a nitrogen source (sodium nitrite) at the anode (see Figure 1.1.ii.).

**Figure 1.1.ii. Potential mechanism for electrochemical nitration of tyrosine as put forward by Kendall *et al* (2001)**

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## **1.2. Peripheral arterial disease and intermittent claudication**

Atherosclerosis is a disease affecting large and intermediate sized arteries and is defined as the build up of fatty deposits beneath the endothelium known as 'atheromatous' plaques. It is one of the most significant causes of health problems in the Western world and it is thought that almost half of all people in the USA and Europe die of atherosclerotic related complications (Rosamond, Flegal, Friday *et al.* 2007). When the arteries of the heart are affected by atherosclerosis it is known as coronary artery disease, CAD, and when affecting other arteries outside the heart and is then classified as peripheral arterial disease (PAD). PAD is a generalised term and affects four major areas; the arteries of the brain including the carotid arteries, kidneys, intestines and the legs. Although the main focus of PAD generally tends to be placed on the arteries of the legs. PAD is associated with a poor cardiovascular prognosis and is often considered an indicator of generalised systemic atherosclerosis. The main emphasis of this current study was to investigate the presence of 3NT in the plasma of patients suffering from intermittent claudication (IC) which is a clinical manifestation of PAD within the lower limbs.

Intermittent claudication (IC), from the Latin claudicare – to limp, is a direct result of atheromatous plaque build up within the arteries supplying the legs. IC tends to be the first and most common clinically significant manifestation reported by those suffering from lower extremity PAD; although it is known that many with PAD do not present with classic IC symptoms (Burns, Gough and Bradbury 2003). Most individuals tend not to present with IC symptoms until the artery has narrowed by at least 60%. The first indication of someone

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suffering from IC is that they experience a cramp like pain in their leg muscles upon mild exertion such as walking. This pain is a result of an inadequate supply of oxygenated blood to the muscle to meet its demands due to a blockage in the arteries supplying the leg. This cramp like pain is often reported within the calves but can also be present within the thighs and or buttocks depending on the position of the blockage. The affected artery can usually provide an adequate supply of oxygenated blood at rest in order to meet the muscles demands thus ceasing exercise results in the claudication pain stopping.

The development of atherosclerotic disease is thought to progress at a very slow rate beginning as early as childhood but, depending on the individual, after the fourth decade of life this progression can increase dramatically (Kiechl and Willeit 1999). Although the exact pathology of atherosclerosis and causative agents are not known it is thought to involve the proliferation of smooth muscle cells and the accumulation of fats, cholesterol, fibrin, platelets, cellular debris and calcium following endothelium damage. This accumulation of substances is then thought to act as a further stimulus resulting in the production of a variety of biological responses by the endothelium, including production of cytokines, leading to both local and systemic responses involved in the inflammatory response (Ross 1999). Atheromatous plaques consist of a mass of lipid-engorged monocytes covered by a fibrous cap protruding into the vessel lumen thus reducing the amount of blood being able to flow past this point and causing periods of ischaemia in the area affected. It is within these plaques that 3NT has been found to be present (Beckmann, Ye, Anderson *et al.* 1994).

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Atherosclerotic plaques have been found to have many different histological characteristics and as a result of this the American Heart Association (AHA) has devised a classification system (Table 1.2.i.). Although this system is a numerical classification it does not represent a severity scale or route of progression. Under normal circumstances it is generally true to say that types I-III lesions are small and clinically silent (Stary 2000) and can be found in the arteries of the young, potentially acting as precursors to more serious lesion types. With regards to lesions types IV-VI no correlation has been found regarding the size and composition of a lesion and the extent of lumen obstruction and associated clinical manifestations (Stary 2000). Thus any of the lesion types IV-VI have the potential of obstructing the lumen of an artery causing a clinical event such as a stroke (Wissler and Strong 1998).

LESION TYPE	HISTOLOGICAL CHARACTERISTICS
<b>I (initial lesion)</b>	Isolated macrophage foam cells
<b>II (fatty streak)</b>	Foam cells stratified in adjacent layers, few intimal SMCs containing lipid droplets, few T lymphocytes and isolated mast cells
<b>III (intermediate)</b>	Changes of type II and pools of extra-cellular lipid
<b>IV (atheroma)</b>	Lipid core, foam cells, fibrous cap rich in SMCs, A proportion of T lymphocytes and mast cells
<b>V (fibroatheroma)</b>	Lipid core and fibromuscular tissue layers produced
<b>VI (complicated lesion)</b>	As type IV with complications including surface disruption, haematoma, haemorrhage and thrombosis
<b>VII (calcific)</b>	As V but calcification predominates
<b>VIII (fibrotic)</b>	As V but fibrous tissue predominates

**Table 1.2.i. Classification of atherosclerotic lesions.**

**Adapted from that stated by the American Heart Association (AHA, [www.americanheart.org](http://www.americanheart.org)) and Stary (2000). SMCs – smooth muscle cells.**

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Current research has highlighted the fact that inflammation is a relevant process in the pathology of atherosclerosis. For example the importance of oxidants derived from NO, such as ONOO<sup>-</sup>, in the pathogenesis of atherosclerosis was highlighted by the identification in the early 1990's of nitrated proteins localised within human atherosclerotic lesions and vascular muscle (Beckmann, Ye, Anderson *et al.* 1994). Since this initial discovery much research has been undertaken regarding protein nitration and the pathology of atherosclerosis. Nitrated LDL has now been isolated from atherosclerotic lesions in human arteries (Leeuwenburgh, Hardy, Hazen *et al.* 1997). NO is produced by both endothelial cells and macrophages within the vasculature and appears to have both protective and atherogenic properties, depending on its source. Within the vasculature eNOS is the predominant NOS isoform and is thought to be responsible for producing most of the NO during normal NO signalling and has atheroprotective properties (Förstermann, Closs, Pollock *et al.* 1994). Evidence now suggests that under certain conditions, such as the absence of L-arginine or the cofactor tetrahydrobiopterin (BH4), the homodimer eNOS 'uncouples' and actually becomes an important source of ROS (O<sub>2</sub><sup>-</sup>) and thus proatherogenic (Vasquez-Vivar, Kalyanaraman, Martasek *et al.* 1998, Bevers, Braam, Post *et al.* 2006). iNOS is also thought to have a proatherogenic role and as the process of oxidative stress is associated with the disruption of NO signalling it is not surprising that the co-distribution of iNOS and Cox-2 with 3NT has been observed in macrophages and smooth muscle cells in both native and transplant atherosclerosis (Baker, Hall, Evans *et al.* 1999). Normal NO signalling is also thought to be affected during periods of oxidative stress due

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to the inhibition of eNOS (Cooke 2003). Thus all of the above can contribute to the disruption in NO signalling and supports the hypothesis that NO, via RNS such as ONOO<sup>-</sup>, may have a direct role in promoting atherogenesis. It has also been reported that MPO may serve as an important link between chronic inflammatory events and the development of atherosclerotic plaques (Malle, Waeg, Schreiber *et al.* 2000). For example nitrating intermediates formed by MPO, e.g. nitryl chloride formed from the reaction of nitrite with HOCl, have been found to mediate LDL protein nitration and peroxidation resulting in the conversion of LDL into an atherogenic form (Hazell, Arnold, Flowers *et al.* 1996, Podrez, Schmitt, Hoff *et al.* 1999). It has recently been highlighted that mitochondrial dysfunction may be of importance in the pathology of PAD thus providing further evidence of the significance of oxidative stress in PAD (Pipinos, Judge, Zhu *et al.* 2006). MPO-mediated oxidation of HDL (both chlorination and nitration) has recently been identified as having a potential role in the pathology of atherosclerosis and diabetes type II (Shao, Bergt, Fu *et al.* 2005, Hermo, Mier, Mazzotta *et al.* 2005).

Research regarding risk factors associated with the development of clinically significant atherosclerosis stems around the coronary and carotid arteries. Several risk factors have been associated with the formation of atherosclerotic plaques and can be split into two categories: major risk factors such as; hyperlipidaemia (McGill, McMahan, Malcom *et al.* 1997, Ridker, Stampfer and Rifai 2001) hypertension (McGill, Strong, Tracy *et al.* 1995, Murabito, D'agostino, Silbershatz *et al.* 1997), smoking (Morrow, Frei, Longmire *et al.* 1995, Waters, Lesperance, Gladstone *et al.* 1996, McGill, McMahan, Malcom *et al.* 1997, Murabito, D'agostino, Silbershatz *et al.* 1997, Petruzzelli, Puntoni,

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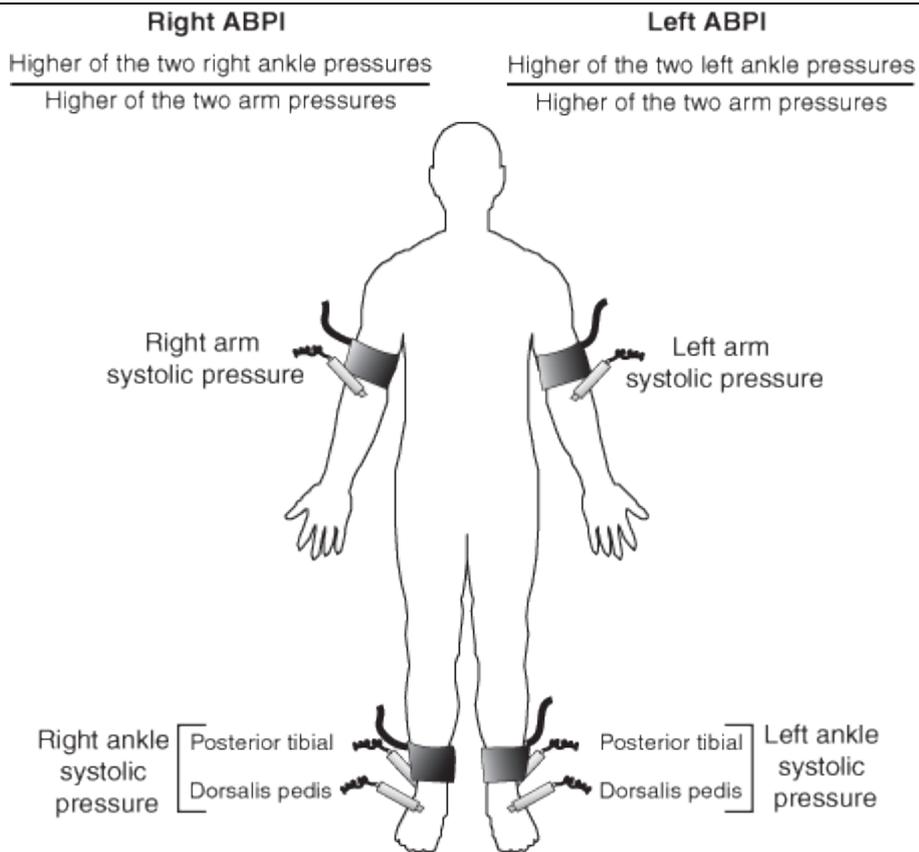
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Mimotti *et al.* 1997) and diabetes (McGill, McMahan, Malcom *et al.* 1995, MacGregor, Price, Hau *et al.* 1999, Pennathur, Wagner, Leeuwenburgh *et al.* 2001, Dolan, Liu, Criqui *et al.* 2002) and minor risk factors such as; age (McGill, McMahan, Tracy *et al.* 1998), sex (McGill, Strong, Tracy *et al.* 1995, Adams, Williams and Kaplan 1995, Murabito, D'agostino, Silbershatz *et al.* 1997, Kiechl and Willeit 1999), and obesity (McGill, McMahan, Malcom *et al.* 1995). Evidence has been found to suggest that precursor lesions (types I-III) may occur as early as adolescence but the frequency of definite lesions present remains low until the age of forty in men and the onset of the menopause in females, dependent on the individual (Kiechl and Willeit 1999). It is evident from the literature that more emphasis is now being made on researching risk factors directly associated with the progression of PAD, which can result in the development of clinically significant IC. Incidence of IC has been found to be more prevalent in those patients suffering from all or one of the following risk factors; high cholesterol levels, stage two or greater hypertension, diabetes, coronary heart disease and/or smoking (Murabito, D'agostino, Silbershatz *et al.* 1997). It has been highlighted that for the progression of large vessel PAD (LV-PAD) and small vessel PAD (SV-PAD) risk factors contribute differentially suggesting different pathology for the progression of PAD in large and small vessels. For example, smoking, lipid status and inflammation were found to contribute significantly to LV-PAD whereas for SV-PAD progression diabetes was the only significant predictor observed (Aboyans, Criqui, Denenberg *et al.* 2006). The duration of type II diabetes has also been found to be strongly associated with an increased risk of development of PAD in men (Al-Delaimy, Merchant, Rimm *et al.* 2004).

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A patient is usually assessed for the presence of IC upon reporting a history of cramp like pain upon mild exertion which can be relieved by a period of rest. Upon reporting such symptoms IC is predominantly diagnosed using non-invasive techniques, such as a thorough physical examination and recording the patient's medical history, i.e. history of leg and or heart symptoms, family history, to determine if they have any risk factors present. The patient's ankle-brachial index (ABI, a comparison of the arm and upper thigh, upper knee, calf and then ankle systolic blood pressure measurements) is then assessed (see Figure 1.2.i). This is a simple and relatively reliable tool in the diagnosis of PAD especially IC, and provides the doctor with a more accurate idea of where the atheromatous plaques are within the leg. A normal ABI range is classed as 0.96-1.2, however there is no consensus on a cut-off level (Meijer, Hoes, Rutgers *et al.* 1998). In a population study it has been found that 68.9% of over 55 year olds with IC had an ABI below 0.9, and also that they were more likely to be smokers, have hypertension, and have symptomatic/asymptomatic CVD (Meijer, Hoes, Rutgers *et al.* 1998). Although ABI is classed as a reliable test for diagnosis of IC, it may not be accurate in all patients and it is also important to note that a normal ABI cannot rule out the presence of IC.



**Figure 1.2.i. A schematic representation of the measurements taken in order to establish a patient's ankle brachial index.**

**Adapted from Meru *et al* (2006), ABPI: ankle brachial pressure index which is an alternative abbreviation for ankle brachial index (ABI).**

Another test for IC is a treadmill exercise test allowing the doctor to assess the severity of the symptoms being presented by the patient by comparing the ABI measurements before and after exercise. When combined these methods of diagnosis in most cases provide the doctor with the data to diagnose or rule out IC. A major problem in the diagnosis of IC is that even in advanced cases patients may present with atypical symptoms or may assume their symptoms are as a result of old age. Also, in the elderly, walking problems are often present due to other underlying medical conditions therefore causing potential problems in correct diagnosis (Murabito, D'agostino, Silbershatz *et al.* 1997).

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If left untreated IC can lead to significant functional impairment and an increased risk of cardiovascular morbidity and mortality (2.5 fold increase compared to an age matched population (Stewart and Lamont 2001)). That is IC is a symptomatic expression of lower extremity PAD and is therefore a marker of potentially more serious cardiovascular problems and generalised atherosclerosis. IC can lead to gangrenous lower limbs leading to amputation and in serious cases death. Furthermore, due to pain upon exercise the individual suffering from IC has a tendency to reduce their exercise levels that can result in further clinical problems. Atherosclerosis rarely produces symptoms until the extent of disease is severe and arteries are dangerously narrowed or in extreme cases completely occluded, therefore the earlier the diagnosis the better the prognosis. It is estimated that in the UK 1 in 5 middle aged individuals have PAD upon examination but very few of these (25%) are symptomatic e.g. presence of IC (Burns, Gough and Bradbury 2003). This clearly highlights that there is a discrepancy between the presence of clinically significant PAD and the reporting of symptomatic PAD such as IC.

Early diagnosis of PAD and IC is crucial with regards to reducing the mortality associated with IC and improving a patient's quality of life. With regards to claudication it has also been reported that there is no correlation between a patient's quality of life and standard parameters used to assess IC such as ABI (Spronk, White, Bosch *et al.* 2007). The recently published guidelines for the management of patients with PAD state that those over the age of 50 years who have atherosclerotic risk factors present should be assessed for the presence of PAD and IC (Hirsch, Haskal, Hertzler *et al.* 2006) however in reality this is not found to be the case. It is known that there is under diagnosis

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of PAD within primary care practices and that this may result in the lack of provision of effective risk factor management and treatment (Hirsch, Criqui, Treat-Jacobson *et al.* 2001). Thus it would be beneficial for the development of additional tests for the diagnosis of PAD and IC in order to improve the long term prognosis and quality of life of those afflicted with the disease. Another reason for the development of additional tests for the detection of IC (such as using 3NT as a biomarker) is that physician awareness of PAD diagnosis is low (Hirsch, Criqui, Treat-Jacobson *et al.* 2001).

### **1.3. Aims and objectives**

As it has been established that 3NT is present within the lesions of those suffering from atherosclerosis and since IC is a clinical manifestation of systemic atherosclerosis (PAD) it is hypothesised that those patients suffering from IC will have high circulating 3NT levels. Moreover, that there will be a correlation between circulating 3NT levels in patients and the degree of claudication.

As such the overall aim of this study was to investigate 3NT as a potential marker of oxidative stress in people suffering from IC. In order to establish the presence of 3NT within the plasma of claudicants, immunoassays, based on enzyme linked immunosorbant assay (ELISA), were developed along with techniques to visualise the presence of 3NT in plasma samples such as sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), Coomassie staining and Western blotting. Additional chromatography methods employing affinity purification were also be developed and used along with mass spectrometry to further confirm the presence/absence of 3NT and provide information regarding the nitroproteome within the disease IC.

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## **Chapter 2.**

# **Subject Recruitment and Clinical Diagnosis**

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## **2.1. Introduction**

Subjects who, following consultation with a vascular consultant, had been diagnosed as suffering from IC were enrolled onto this study in order to assess the validity of using 3NT as a marker of oxidative stress.

In general the claudicants enrolled onto this study had been referred to the vascular clinic after presenting to their general practitioner with symptoms of leg pain upon exertion. Upon referral to the vascular clinic the patient would initially meet with the consultant where a full patient history was taken. These patients then underwent a lengthy medical examination including the completion of a questionnaire by a nurse practitioner and taking a full medical history along with the examination for the presence/absence of pulses in the arms, neck and legs and if there were any bruits present. Haemodynamic variables were also measured and recorded at this point, which included brachial and ankle blood pressure measurements, to allow the patient's ABI to be ascertained. Once all of the necessary information had been gathered the nurse would then consult with the vascular consultant and a diagnosis would be made. If the diagnosis of IC was confirmed it was at this point that a patient was approached with regards to potential enrolment onto this study and a sample of blood taken. Medical information was recorded from that obtained by the nurse and consultant during the diagnostic process.

Within this chapter the medical information gathered from the claudicants enrolled, along with that gathered from the control subjects, was evaluated in relation to diagnosing IC. This was done in order to assess the appropriateness of the current diagnostic tools used in the diagnosis of IC and

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to highlight the potential need for additional diagnostic tools such as using 3NT as a biomarker of disease.

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## **2.2. Materials & methods**

### **2.2.1. Enrolment of claudicants**

Materials: 9ml EDTA blood tubes obtained from Starstedt. Flexi Dopplex Doppler, Huntleigh.

Methods: Ethical approval was obtained from the Wolverhampton District Local Research Ethics Committee, (see appendix A-C for proposal, information sheet, consent form and questionnaire). Patients attending the weekly vascular disease clinic at Wolverhampton New Cross Hospital were assessed for IC by the consultant vascular surgeon and support team after reporting leg pains upon exertion to their GP. This assessment included measurement of the patients' ABI (see 2.2.4. below) and taking a full medical history. Following consultation those diagnosed with IC were approached for enrolment onto the study. Volunteers signed an informed consent form, after reading an information sheet (see appendix B), and a blood sample was taken into 9ml EDTA tubes. Patients previously diagnosed with IC at the clinic who were making a return visit were excluded from enrolment. Samples were stored at 4°C during transit (maximum of 2 hours) until the plasma was separated from the whole blood using centrifugation for 5 minutes (700 x g). The plasma was then divided into two aliquots, one stored at -20°C and one at -80°C. The sample stored at -20°C was used as the working aliquot.

### **2.2.2. Enrolment of control subjects**

Ethical approval was obtained from Coventry University School of Science and the Environment Research Ethics Committee (see appendix D-F for

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proposal, information sheet, consent form and questionnaire). Healthy individuals, approximately age matched to the claudicants, were enrolled onto the study. Volunteers signed an informed consent form, and a blood sample was taken as before. Past medical history and the patients' ABI were also recorded. The plasma samples were then processed along with the patient plasma samples as described in chapter 4.

### **2.2.3. Body mass index (BMI)**

The BMI was calculated for all subjects, where height (cm) and weight (Kg) data were available, as follows:

$$\text{BMI} = \frac{\text{Weight (Kg)}}{\text{Height}^2 \text{ (m)}}$$

Each patient was then categorised using their BMI into the relevant classification system illustrated below in Table 2.2.i.

### **2.2.4. Blood pressure and ankle brachial index (ABI)**

Brachial blood pressure measurements were taken from both arms by use of a sphygmomanometer and stethoscope. The mean arterial pressure (MAP) was calculated as follows:

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$$\text{MAP} = \text{Diastolic blood pressure} + \frac{1}{3} \text{ pulse pressure}$$

(Where pulse pressure = systolic BP - diastolic BP)

Pressures were also measured for the posterior tibial and dorsalis pedis arteries using a sphygmomanometer and a hand held continuous Doppler probe. All pressures were recorded with the subjects in a supine posture. The ABI for each leg was calculated as follows:

$$\text{ABI} = \frac{\text{Highest ankle systolic pressure (mmHg)}}{\text{Highest brachial systolic pressure (mmHg)}}$$

The value obtained was then categorised using the classification system illustrated below in Table 2.2.ii.

**Table 2.2.ii. ABI classifications. (Daigle 2002)**

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## 2.3. Results

### 2.3.1. Claudicant and Control subjects

Patients diagnosed with IC (claudicants) were enrolled onto the study over a 12 month period between September 2002 and September 2003. In total, 33 patients (26 male and 7 female) were enrolled along with 6 generally fit and healthy control subjects from Coventry University (4 male and 2 female). A blood sample was collected from all participants along with a record of their medical history.

		Claudicants	Control
Age (years)	Male	64 ± 8	54 ± 8
	Female	74 ± 10	53 ± 2
	Total	66 ± 9	54 ± 6

**Table 2.3.i. Summary age data for claudicant and control subjects enrolled.**  
The above data was collated for both the claudicants (n=33) and the control subjects (n=6). Mean ± standard deviation.

With regards to gender and race the majority of the patients enrolled were male (79%) and 81% were Caucasian. For the control subjects 67% were male and all were Caucasian.

### 2.3.2. Body mass index

For those patients where height (metres) and weight (kilograms) were available their body mass index (BMI) was calculated and categorised (see Table 2.2.i.). The mean BMI for the claudicants was 27 (±5) and was thus situated within the overweight category (Table 2.3.ii). More than 50% of the male claudicants had BMI values which were situated within the overweight

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BMI range whereas the female claudicants had a greater percentage situated within the obese range (50%).

BMI	Male % (control %)	Female % (control %)	Total % (control %)
<18.5	0	25	4
<b>18.5-24.9</b>	32 (50)	0 (50)	26 (50)
<b>25-29.9</b>	53 (50)	25	48 (33)
<b>30-39.9</b>	16	50 (50)	22 (17)
>40	0	0	0
<b>Mean ± SD</b>	26 ±4 (26 ±3)	28 ±7 (32 ±11)	27 ±5 (28 ±6)

**Table 2.3.ii. BMI classifications for claudicants and control subjects.**  
For the claudicants n=23 (n=19 male & n=4 female). The data in brackets is that for the control subjects, (n=6, 4 male and 2 female).

### **2.3.3. Haemodynamic variables**

Bilateral brachial blood pressure measurements were obtained for each participant. Minimal differences in bilateral systolic and diastolic pressures were observed for the control subjects and the claudicants (Table 2.3.iii.). It was clear that there were noticeable differences in the values obtained both within and between gender (more so for systolic blood pressure). Systolic blood pressure (SBP) measurements >140mmHg were recorded in 85% of the claudicants, and 42% had measurements >160mmHg. With regards to SBP it was also observed that the female claudicants had somewhat higher recorded readings compared to their male counterparts (e.g. +19mmHg with regards to right hand side SBP measurements). There was a less noticeable difference when measuring the diastolic pressures (DBP), again the females had higher values. A degree of variation for SBP and DBP was observed

between subjects with regards to the control subjects enrolled along with an observed difference between the genders, although this was not so apparent (Table 2.3.iii). The mean arterial pressures (MAP) were calculated for all of the participants involved and these values further confirmed that the female claudicants and control subjects had higher and more variable blood pressure readings compared to their male counterparts, Table 2.3.iii.

		Claudicants		
		Male	Female	Total
Left (mmHg)	Systolic	145 ±20 (143 ±25)	161 ±36 (151 ±9)	148 ±25 (145 ±21)
	Diastolic	83 ±10 (83 ±15)	88 ±10 (105 ±5)	84 ±10 (90 ±17)
	MAP	104 ±11 (103 ±2)	112 ±17 (105 ±7)	106 ±13 (108 ±10)
Right (mmHg)	Systolic	148 ±21 (140 ±27)	167 ±19 (140 ±20)	152 ±22 (140±24)
	Diastolic	84 ±10 (83 ±15)	86 ±8 (95 ±15)	84 ±9 (87 ±16)
	MAP	105 ±12 (102 ±2)	113 ±10 (110 ±24)	107 ±12 (104 ±11)

**Table 2.3.iii. Summary brachial blood pressure measurements for claudicant and control subjects.**

The above data was collated for both the claudicants (n=33) and the control subjects (n=6). Data in brackets represents control subject data. MAP – mean arterial pressure; determines the rate of blood flow through the circuit. All claudicant blood pressure data was measured by a nurse practitioner, the control blood pressure readings were recorded by an experienced physiologist at Coventry University. All measurements were recorded using a mercury sphygmomanometer, Mean ± standard deviation.

#### **2.3.4. Claudication risk factors**

When diagnosing claudication it is important to identify common risk factors acknowledged to be connected to the development of such vascular disease.

Of the claudicants enrolled, 85% reported that they were or had been smokers (55% current smokers, 30% ex smokers). Hypertension (61%), hyperlipidaemia (52%) and family history of cardiovascular events (52%) were the other major risk factors identified in the majority of the claudicants enrolled (Table 2.3.iv). Within the control population all of the males reported that they were current or ex smokers and one male subject presented with pre-diagnosed hypertension. Furthermore 67% of the control subjects reported a family history of cardiovascular events.

Risk factors		Claudicants			Control		
		Male	Female	Total	Male	Female	Total
Smoking (%)	Current	50	71	55	50	--	33
	Past	38	--	30	50	50	50
	Never	12	29	15	--	50	17
Pre-diagnosed (%)	Diabetes	19	71	24	--	--	--
	Hypertension	62	57	61	25	--	17
	MI	12	--	9	--	--	--
	Angina	27	14	24	--	--	--
	CVA	12	14	12	--	--	--
	TIA	12	14	12	--	--	--
	Hyperlipidaemia	54	43	52	--	--	--
Family history	50	57	52	50	100	67	

**Table 2.3.iv. Risk factors associated with vascular disease reported to be present in claudicant and control subjects.**

Claudicants n=33 (26 male, 7 female). Controls n=6 (4 male, 2 female). This data was recorded from patient medical notes (where available) and self reported using a questionnaire (which was the same for both the claudicants and control subjects, see appendices C and F). Family history was determined by asking the individuals if there was a close family member (mother, father, siblings and/or grandparents) who had a history of any of the disorders listed in the table above.

MI - myocardial infarction; CVA - cardiovascular accident; TIA - transient ischaemic attack.

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For all of the claudicants a list of prescribed medications was obtained. It was clear from the medical notes of the claudicants enrolled that the majority were on a 'cocktail' of vasodilator and diuretic medication. These drugs can be used for the treatment of hypertension, arrhythmias and angina, although it was not always clear what they had been prescribed for. Other drugs being taken included anti-clotting drugs such as aspirin and drugs used for the direct treatment of angina, diabetes, gastric ulcers and urinary tract problems. In the case of the control subjects one male was on prescribed drugs for hypertension (angiotensin converting enzyme (ACE) inhibitor, calcium channel blocker and diuretic) and one male was on an anti-arrhythmia drug. No in depth analysis of the drugs being taken was carried out.

### **2.3.5. Claudication symptoms**

All of the claudicants enrolled (with the exception of one patient) reported that they had been suffering from symptomatic IC for a minimum of 1 year. Bilateral calf pain was the most predominant IC symptom reported by both the male and female claudicants enrolled (Table 2.3.v). Although the majority of the patients enrolled reported that the symptoms present were bilateral within the calf area (68%), bilateral IC pain was also experienced within the thigh/buttock region (33%) and foot (12%). The reported distance walked by the claudicants prior to the onset of IC pain was found to be somewhat variable with the female claudicants reporting a reduced distance compared to their male counterparts (Table 2.3.v). The period of rest needed for recovery from symptoms was again found to differ considerably between claudicants and was reported to be between 1-10 minutes with a mean value of 4 minutes (Table 2.3.v). No correlation was observed between the reported distance

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walked prior to the onset of pain and the recorded ankle brachial index (ABI, correlation coefficient 0.08 and 0.28 when compared to left and right hand side ABI measurements respectively). Again no correlation could be established when comparing the reported recovery time and the ABI measurements (left and right independently) nor the distance walked before the onset of pain and the recovery period required by an individual.

		Male	Female	Total
<b>Thigh/Buttock pain (%)</b>	<b>Left</b>	39	14	39
	<b>Right</b>	50	29	46
<b>Calf pain (%)</b>	<b>Left</b>	77	86	79
	<b>Right</b>	85	71	82
<b>Foot pain (%)</b>	<b>Left</b>	19	14	18
	<b>Right</b>	23	14	21
<b>Distance before onset of pain (m)</b>	<b>Mean <math>\pm</math>SD</b>	117 $\pm$ 99	83 $\pm$ 73	110 $\pm$ 94
<b>Recovery time (min)</b>	<b>Mean <math>\pm</math>SD</b>	4 $\pm$ 3	4 $\pm$ 3	4 $\pm$ 3

**Table 2.3.v. Symptoms of claudication reported by enrolled patients.**

**Claudicants n=33 (26 male, 7 female). The symptoms were self reported to a nurse practitioner during the initial consultation for the presence of IC; all of the subjects enrolled were consequently diagnosed with intermittent claudication. None of the control subjects enrolled reported any symptoms associated with the presence of claudication.  $\pm$  SD – Standard deviation.**

### **2.3.6. Ankle brachial index**

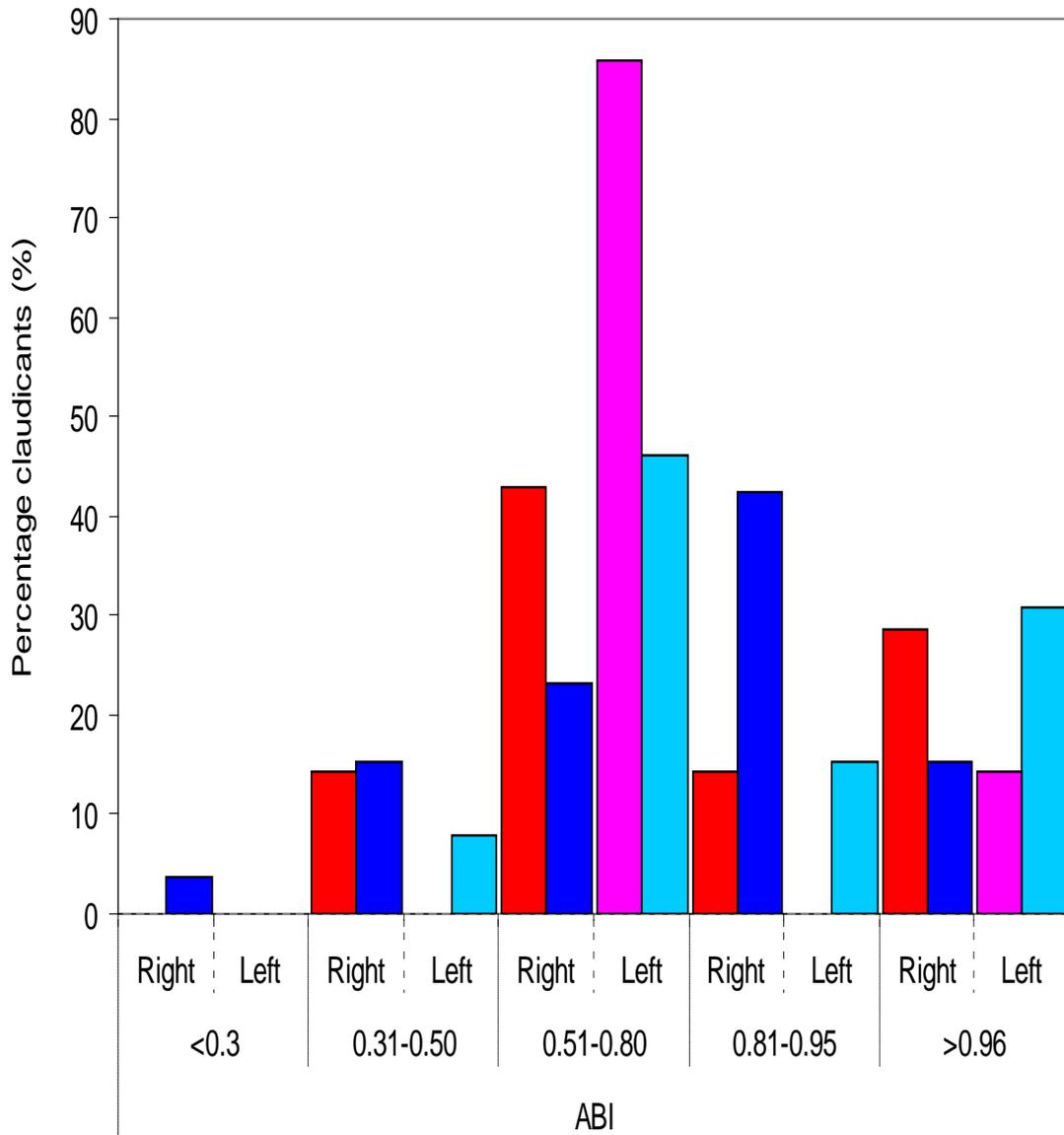
The ABI was ascertained for the claudicant and control subjects using the brachial SBP recordings and by measuring the ankle systolic pressure (the higher value of the dorsalis pedis or posterior tibial systolic pressure) using the calculation shown in section 2.2.4. For all patients the ABI value calculated was categorised as per Table 2.2.ii. (section 2.2.4.). In brief, an ABI below 0.96 was generally classified as being indicative of IC being present. It was found that for both the male and female claudicants the mean ABI

reflected moderate disease being present with an overall ABI for all claudicants of 0.77 (Table 2.3.vi. and Figure 2.3.i). No correlation was observed with regards to SBP and recorded ABI. Bilateral differences in the recorded ABIs were observed in both the male and female claudicants. For the male claudicants these values were situated within the same disease classification bracket however for the females the bilateral measurements fell within two different classification brackets (mild and moderate disease). As highlighted in Figure 2.3.i. a small number of the patients (18%) subsequently diagnosed with IC had ABI values that fell within the generally normal ABI classification as set out in Table 2.2.ii., section 2.2.4., although IC symptoms were being experienced. In a small percentage of claudicants the recorded ABI values also signified the presence of moderate-severe (3%) and severe disease (3%) (Figure 2.3.i).

All of the control patients enrolled, with the exception of the one male, lay within the >0.96 bracket (bilaterally), suggesting no disease was present. The male control subject who lay out of the normal range (bilaterally) was placed within the mild disease classification and had been pre-diagnosed with hypertension.

		Claudicants			Control		
		Male	Female	Total	Male	Female	Total
ABI Mean ±SD	Left	0.78 ±0.23	0.73 ±0.20	0.77 ±0.22	1.17 ±0.03	1.15 ±0.05	1.11 ±0.05
	Right	0.76 ±0.24	0.81 ±0.24	0.77 ±0.24	1.05 ±0.08	1.17 ±0.03	1.09 ±0.05

**Table 2.3.vi. Recorded ABI measurements for claudicant and control subjects.**  
**Claudicants n=33 (26 male, 7 female). ±SD – Standard deviation.**  
**Controls n=6 (4 male, 2 female). The above data represents the highest ABI recording for each leg; for a more detailed breakdown of ABI measurements recorded see Figure 2.3.i. below.**



**Figure 2.3.i. Percentage distribution of claudicants in each ABI classification.**  
 The above data represents the % ABI measurements for each leg and their severity classification. Claudicants n=33, 26 male (left leg (■), right leg (■)), 7 female (left leg (■), right leg (■)).  
 All of the control patients enrolled, with the exception of the one male, lay within the >0.96 bracket (bilaterally). The male control subject who lay out of the normal range (bilaterally) was placed within the mild disease classification (ABI range 0.81-0.95).

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## **2.4. Discussion**

### **2.4.1. Enrolment of claudicant and control subjects**

The claudicants enrolled onto this study are not necessarily representative of the whole claudicant population. It was originally intended to enrol approximately 200 subjects; however after 12 months of weekly clinic attendance only 33 claudicants were enrolled. The reasons for this were thought to be due more to the type of clinic being run by the consultant vascular surgeon involved rather than a reflection of the numbers of claudicants within the population as a whole. That is, the clinic from which the subjects were enrolled onto the study was a general vascular clinic where many of the patients attending had vascular problems other than IC e.g. severe leg ulceration. This resulted in only 4-5 new patients being enrolled a week, also those patients who were attending follow up consultations having been previously diagnosed with IC were excluded from this study due to treatment already having begun. Thus it is acknowledged that any assumptions and conclusions made from this data set may be limited.

It has been suggested that referral to a vascular surgeon may only be necessary when the primary care team are not confident in making such a diagnosis, or lack the resources necessary to monitor and treat the disorder, or where there are suspected weak/absent femoral pulses (Burns, Gough and Bradbury 2003). The low number of claudicants attending this clinic may therefore have been a reflection of low GP referral.

The time needed to diagnose a patient with claudication was somewhat high, approximately 30-45 minutes per patient which could also explain why only 4-

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5 new patients were seen at each clinic. The consultation consisted of a full patient history being taken by the consultant followed by a thorough medical examination and completion of a claudication questionnaire by a nurse practitioner. This included the examination for the presence/absence of pulses within the arms, neck and legs and to establish if there were any bruits present, especially within the aorta and the carotid arteries. Haemodynamic variables were also measured at this point which involved recording the brachial and ankle blood pressures, which then allowed the patient's ABI to be ascertained. A diagnosis would then be made following a review of the findings of the medical examination by consultant.

The time and resources needed for such a diagnosis to be made clearly highlights the need for complementary and perhaps more objective diagnostic tools e.g. a biomarker such as 3NT in order to aid the prompt diagnosis of IC. The implementation of such a diagnostic tool would allow for the blood test to be taken prior to the date of the consultation and the results used in conjunction with the current process which may speed up the diagnostic process, allowing more patients to be seen within each vascular clinic.

It is important to note that, although this study is based upon patients suffering from IC (a clinical manifestation of PAD), it is probable that those enrolled also had systemic atherosclerosis in which multiple arteries are affected. Although the severity of atherosclerosis in one area does not allow for the prediction of severity in arteries elsewhere it suggests multiple areas being affected. For example, one study suggests that further disease extension is almost obligatory once more than three atherosclerotic plaques pre-exist in a subject (Kiechl and Willeit 1999).

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#### **2.4.2. Claudicant and control subject data**

PAD has been found by others to disproportionately affect non-hispanic blacks (Newman, Shemanski, Manolio *et al.* 1999, Selvin and Erlinger 2004) but the sample size within this study was not big enough to determine this and information regarding ethnic origin was not always provided, as such no analysis with respect to the local population was made.

Regarding the pathobiological determinants of atherosclerosis in youth (PDAY) studies it has been well documented that early atherosclerotic lesions can be found in the arteries from a young age (McGill, McMahan, Herderick *et al.* 2000) and that the progression of these early plaques into clinically manifest atherosclerotic disease is significantly increased by the presence of many different risk factors including age and gender. Within this patient population the majority of those claudicants enrolled were male (79%), which concurs with previous reports showing that males are at a higher risk of atherosclerotic disease compared to their female counterparts (pre-menopause) (Adams, Williams and Kaplan 1995, Murabito, D'agostino, Silbershatz *et al.* 1997, Kiechl and Willeit 1999, Ridker, Stampfer and Rifai 2001). It has also been reported that women with PAD are less likely to report symptoms of IC than men (Meijer, Hoes, Rutgers *et al.* 1998, Meru, Mitra, Thyagarajan *et al.* 2006). This observed difference between genders is thought primarily to be due to hormonal differences and that these differences disappear within 5 years of the menopause after which the progression rate of any disease present is similar to that observed in males (Adams, Williams and Kaplan 1995, Kiechl and Willeit 1999). The age range of the claudicants enrolled onto this study was broad (49-87 yrs) for females; the mean age was

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found to be 10 years higher than for their male counterparts (74±10 yrs and 64±8 yrs respectively). Rosamond *et al* (2007) report cardiovascular events increase dramatically with age in females but 10 years later than their male counterparts, although this gap decreases with advancing age.

The mean age of the control subjects was lower than that observed in the claudicants (male 54±8 yrs, females 53±2 yrs) and as such was not entirely representative of the age range observed for the claudicant population enrolled. Originally the intention was to enrol healthy age matched hernia patients who were attending the same vascular clinic as the claudicants, as mentioned above. However, upon assessment, it was found that those approached, who were of comparable age to the claudicants, were symptomatic of PAD being present and as such were not suitable control subjects. The difficulties found enrolling appropriately age matched control subjects were not entirely surprising as it is well documented that the presence of PAD dramatically increases with advancing age in both sexes (Selvin and Erlinger 2004) and with respect to males the relative risk factor for the development of IC increases 2-3 fold with each individual decade of life (Dormandy, Heeck and Vig 1999).

It is clear that age and being male are unavoidable risk factors associated with the development of clinically significant arterial disease, such as IC, (Dormandy, Heeck and Vig 1999, Khan, Flather, Mister *et al*. 2007). It is however important that an assessment of the relative risk of the development of IC should not be solely made on the basis of age and gender but should be made in conjunction with the presence of other major risk factors such as those discussed below.

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### **2.4.3. Body mass index**

It has long been noted that a high BMI is associated an increased risk of atherosclerosis and associated vascular problems along with the development of other major risk factors such as diabetes type II and hypertension (Medalie, Papier, Goldbourt *et al.* 1975, McGill, McMahan, Malcom *et al.* 1995, Murabito, Evans, D'agostino Sr *et al.* 2005). Adiposity as measured by BMI or panniculus adiposus thickness has been found to be associated with more advanced and extensive atherosclerosis of the right coronary artery in the young which cannot be explained by either serum lipoprotein cholesterol levels or smoking (McGill, McMahan, Malcom *et al.* 1995). When assessing the body mass index (BMI) for those enrolled onto this study it was clear that there was little difference in the mean BMI values for the male and female claudicants, both being situated within the overweight category ( $26\pm 4$ ,  $28\pm 7$  respectively). These values are similar to those previously reported in a study of patients with PAD referred to UK vascular clinics (Khan, Flather, Mister *et al.* 2007), although some studies suggest a BMI  $>30$  as a risk factor for IC (Murabito, Evans, D'agostino Sr *et al.* 2005). It is important to note that there are differing arguments with regards to the direct effect of obesity on atherosclerosis. Although obesity (as determined by BMI) may be of importance, some studies have suggested that there is no evidence that a high BMI is directly associated with prevalent PAD when no other risk factors are present (Solberg and Strong 1983, Selvin and Erlinger 2004). A review of autopsy studies also highlighted the fact that there is no consistent direct association with regards to obesity and the degree of atherosclerosis present (Solberg and Strong 1983). Despite these contradictory reports, all of those

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claudicants whose BMI was outside of the normal range were advised to address this issue and start a weight loss program in order to reduce their BMI and thus risk of further complications.

#### **2.4.4. Claudication risk factors**

The findings of this study were not dissimilar to those previously reported regarding risk factors associated with the development of clinically significant atherosclerosis (McGill, Strong, Tracy *et al.* 1995, Morrow, Frei, Longmire *et al.* 1995, Waters, Lesperance, Gladstone *et al.* 1996, McGill, McMahan, Malcom *et al.* 1997, Murabito, D'agostino, Silbershatz *et al.* 1997, Petruzzelli, Puntoni, Mimotti *et al.* 1997, Selvin and Erlinger 2004, Leibson, Ransom, Olson *et al.* 2004). 95% of persons with prevalent PAD, presenting as IC for example, have at least one of the major risk factors attributed with traditional cardiovascular disease; namely current smoking status, diabetes, hypertension and hypercholesterolaemia (Selvin and Erlinger 2004, Leibson, Ransom, Olson *et al.* 2004). The findings of this study support this with current smoking and hypertension being the most prevalent.

It has been well documented that hypertension is a risk factor for the development of clinically significant atherosclerosis and is thought to be attributable to the development of PAD. During the PDAY studies it was highlighted that hypertension was positively associated with accelerated atherosclerosis in youth, with particular reference to the development of fibrous plaques (McGill, Strong, Tracy *et al.* 1995). Further reports by this group go on to suggest that in both men and women, in the third decade of life, hypertension augments atherosclerosis principally by accelerating the conversion of fatty streaks to raised lesions and importantly that the effect of

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hypertension increases with age (McGill, McMahan, Tracy *et al.* 1998). It is the conclusion of the PDAY study that by the beginning of the fourth decade of life those subjects who are hypertensive have approximately twice the extent of raised lesions (in their coronary arteries) when compared to normotensive subjects (McGill, McMahan, Tracy *et al.* 1998). This clearly highlights the importance of monitoring such haemodynamic variables and more so the need of non-invasive quick tests for the early diagnosis of the presence of atherosclerotic disease such as PAD before the onset of clinically significant symptoms such as IC.

Within the published literature it is clear that there are differing values used with regards to the cut-off limits used for the definition of the presence of hypertension. In general hypertension is defined as any of the following being present; SBP  $\geq 140$ mmHg and/or DBP  $\geq 90$ mmHg or where anti-hypertensive drugs are prescribed (Meijer, Hoes, Rutgers *et al.* 1998, Hirsch, Criqui, Treat-Jacobson *et al.* 2001, Rosamond, Flegal, Friday *et al.* 2007). Within this definition there are also subcategories for hypertension, for example within the Framingham heart study (FHS) blood pressure measurements were classified as normal (SBP  $< 130$ mmHg  $\pm$  DBP  $< 85$ mmHg), high normal (130-139mmHg  $\pm$  85-89mmHg), stage 1 hypertension (140-159mmHg  $\pm$  90-99mmHg), or stage 2 hypertension ( $\geq 160$ mmHg  $\pm$   $\geq 100$ mmHg) (Murabito, D'agostino, Silbershatz *et al.* 1997). In the current study mean SBP's recorded for the claudicants and control subjects suggested hypertension being present, with 85% of the claudicants having a SBP  $> 140$ mmHg. Although most of the claudicants enrolled were being prescribed anti-hypertensive drugs, the current recommended therapeutic goal to achieve  $< 140$ mmHg systolic over

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90mmHg diastolic (non-diabetics) in order to reduce the risk of serious cardiovascular events and death (Hirsch, Haskal, Hertzler *et al.* 2006, Khan, Flather, Mister *et al.* 2007) was clearly not being met. In general the female claudicants enrolled had SBP readings that were somewhat higher than their male counterparts (+19mmHg). This agrees with previously published reports in which it has been found that after 54 years of age a higher percentage of women have hypertension (Rosamond, Flegal, Friday *et al.* 2007) and higher SBP measurements in females suffering from PAD have been observed (Khan, Flather, Mister *et al.* 2007). Ness *et al.* (2000) reported that when hypertension is present there is a significant increased risk of developing PAD for both elderly males and females (2.2 and 2.8 times respectively).

Of the reported risk factors, current smoking status has been identified as an important independent risk factor for the development of PAD (Al-Delaimy, Merchant, Rimm *et al.* 2004) and in particular is the most powerful predictor of large vessel PAD progression (Aboyans, Criqui, Denenberg *et al.* 2006). Waters *et al.* (1996) demonstrated that atherosclerosis progressed much more rapidly in the coronary arteries of smokers compared to non-smokers. In this study 85% of the claudicants reported being current or ex-smokers and all of the male control subjects reported being current/ex smokers. Jenson *et al.* (2005) and Khan *et al.* (2007) also report a strong positive association between the prevalence of IC and current and/or previous smoking habits. Within this study no analysis was made of the duration of smoking and level of smoking (determined by pack/years) due to the documented unreliability of patient-offered smoking history (Hobbs, Wilkink, Adam *et al.* 2005).

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The presence of diabetes has long been classed as a risk factor for the development of atherosclerosis (McGill, McMahan, Malcom *et al.* 1995, Dolan, Liu, Criqui *et al.* 2002) and a significantly increased risk of developing PAD has been established in elderly men and women suffering from diabetes, type I and II (Ness, Aronow and Ahn 2000, Leibson, Ransom, Olson *et al.* 2004). It increases the incidence of and accelerates the clinical course of atherosclerosis within the coronary and extracranial carotid arteries along with the vascular beds of the lower extremities (Beckman, Creager and Libby 2002). For example atherosclerotic plaques have been found in the arteries of young adults identified as being in a pre-diabetic or early diabetic state, as indicated by elevated post-mortem glycohaemoglobin levels (McGill, McMahan, Malcom *et al.* 1995).

Type II diabetes represents more than 90% of those patients with diabetes and atherosclerosis (Beckman, Creager and Libby 2002). A questionnaire based study of males indicated that there is a strong correlation between the duration of diabetes (type II) and the risk of developing PAD. The study suggested that increased risk was further strengthened in men with hypertension and who were current smokers (Al-Delaimy, Merchant, Rimm *et al.* 2004). Diabetes is also thought to play a unique role in the progression of PAD in that it is a significant predictor for the presence of small vessel PAD progression (Aboyans, Criqui, Denenberg *et al.* 2006). In the claudicant population enrolled on this study 24% had been pre-diagnosed with diabetes which agrees with the findings of Khan *et al.* (2007).

In a study comparing lower extremity function & dysfunction in diabetics and non-diabetics with PAD it was found that although there was no observed

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difference in the recorded ABI values it was apparent that those participants suffering from both diabetes and PAD were less likely to report classical symptoms of IC. Overall it was identified that those patients in which PAD and diabetes were found to coexist tended to be younger in age, had a higher BMI, an increased prevalence of hypertension and poorer lower extremity function compared to those who had PAD alone (Dolan, Liu, Criqui *et al.* 2002). It has been suggested that a potential reason behind the higher prevalence of PAD in diabetic subjects compared to non-diabetic subjects may be due to increased mean levels of triglycerides and SBP (MacGregor, Price, Hau *et al.* 1999). Thus this clearly highlights that those with diabetes are a subset of patients in which the development of a biomarker to screen for the presence of PAD/IC may be extremely beneficial.

The current lipid status of the claudicants enrolled was not available and so no interpretation of this risk factor was possible, although 52% of those claudicants enrolled had been pre-diagnosed with hyperlipidaemia. A high percentage of claudicants and control patients reported a family history of cardiovascular events (67% and 52% respectively) and to a lesser extent had already suffered a cardiovascular event themselves (e.g. MI, angina, CVA/TIA). The findings relating history of prior coronary/cerebral artery disease were also similar to those recently reported by Khan *et al* (2007).

#### **2.4.5. Claudication symptoms and ABI**

Within the claudicant population enrolled onto this study (with the exception of one male patient) all reported suffering symptoms suggesting of IC being present for >1 year. The mean estimated claudication walking distance reported by the patients was 110±94 m which is similar to that reported by

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Khan *et al* (2007). There was a reduced reported walking distance in the females enrolled compared to their male counterparts (83±73 m and 117±99 m respectively). The calf area was found to be the main area in which IC symptoms were reported to be present upon mild exertion although some patients also reported experiencing claudication pain within the thigh/buttock region and foot. For all of these regions it was found that there was a slightly higher incidence of reported pain in the right leg compared to the left.

It has been well reported that a reduced ABI is an indicator of the presence of LV-PAD being present (Feigelson, Criqui, Fronek *et al.* 1994, Newman, Shemanski, Manolio *et al.* 1999, Hirsch, Criqui, Treat-Jacobson *et al.* 2001, McDermott, Greenland, Liu *et al.* 2002, Aboyans, Criqui, Denenberg *et al.* 2006). Patients with a reduced ABI are also generally regarded as having an unfavourable cardiovascular risk profile (Meijer, Hoes, Rutgers *et al.* 1998, O'hare, Katz, Shlipak *et al.* 2006) and an ABI<0.9 is an independent risk factor for incident and recurrent CVD and mortality (Newman, Shemanski, Manolio *et al.* 1999). In general the majority of patients with IC are expected to have ABIs ranging between 0.3-0.9 (Sacks, Bakal, Beatty *et al.* 2003) within this study the recorded ABIs were found to range from 0.18-1.10. The overall mean ABI for the claudicants enrolled was found to be 0.77 with only small bilateral differences observed for the male claudicants and a slightly higher right-hand sided mean ABI value for the female claudicants. Again these findings are similar to those that have been previously reported (Hirsch, Criqui, Treat-Jacobson *et al.* 2001, Khan, Flather, Mister *et al.* 2007). In contrast, unlike the findings of Khan *et al* (2007), no correlation was observed regarding the distance walked prior to the onset of claudication pain and the

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recorded ABI, reported recovery time nor SBP and ABI. This may have been due to the small claudicant population recruited onto this study.

Although it has been clearly highlighted that ABI measurement is an effective tool with regards to the diagnosis of PAD there are issues surrounding it that need to be highlighted. It is well known that there are instances in which an ABI measurement is not necessarily a good indicator of lower limb perfusion, for example within this study 18% of those enrolled had a mean  $AB\geq 0.96$ . If this were used as the only screening tool by a GP practitioner these patients could have been falsely regarded as not having PAD/IC. Artificially elevated ABI measurements can be obtained in patients who have heavily calcified non-compressible arteries, like those found in diabetics (Orchard and Strandness 1993, Sacks, Bakal, Beatty *et al.* 2003, Leibson, Ransom, Olson *et al.* 2004, Cassar 2006). McDermott *et al.* (2002) reported that due to having calcified lower extremity arteries, which resulted in artificially increased pressure, approximately 5% of persons with PAD have a normal ABI. The effects of artificially elevated ABI readings due to such issues could potentially result in underestimation of disease severity or even worse patients being misdiagnosed as being clear of PAD. As such this is a worrying finding when considering the elevated CV risk that is associated with the presence of PAD (Sacks, Bakal, Beatty *et al.* 2003). Another issue surrounding the use of ABI as an indicator of the presence of PAD is the fact there that there is no consensus regarding the cut-off levels currently being used to diagnose PAD. Although it is generally accepted that an ABI  $<0.9$  suggests large vessel disease being present this value is an arbitrary value and may result in some patients being incorrectly classified as having only mild atherosclerosis

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(Meijer, Hoes, Rutgers *et al.* 1998, Newman, Shemanski, Manolio *et al.* 1999).

This lack of consensus may help to explain the different levels of PAD prevalence reported within different studies (Meijer, Hoes, Rutgers *et al.* 1998).

The incidence of IC has been found to have declined within the general population since 1950 but it has been observed that the level of mortality has stayed the same. Within their study on the trends in the incidence of IC between 1950-1999 Murabito *et al* (2005) found that approximately 40% patients die within 10 years following initial diagnosis of IC. Due to this increased risk of mortality associated with the presence of symptomatic vascular disease such as IC, it is clear that such patients need to be diagnosed a lot earlier in their disease progression. Furthermore, although the prevalence of PAD has been demonstrated to be high within the elderly in primary care settings, very few of these have been found to report symptomatic leg pain which has led to a diagnosis of IC (Meijer, Hoes, Rutgers *et al.* 1998, Hirsch, Criqui, Treat-Jacobson *et al.* 2001, McDermott, Greenland, Liu *et al.* 2002). For example Hirsch *et al* (2001) report that of those found to be suffering from PAD only 8.7% reported classical claudication symptoms. This is important if patients are only referred to vascular surgeons for diagnosis on the basis of reporting classical claudication symptoms as it indicates that a potentially large percentage of PAD sufferers (85-90%) are not being diagnosed. This low level of IC incidence in those found to be suffering from PAD can in part be explained by the fact that the elderly may not be walking far enough to experience symptoms of IC due to concurrent medical conditions such as osteoarthritis

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and as such are not consulting their GP for treatment. It is also a concern that although there are current recommendations that the ABI test should be administered to all patients being evaluated for PAD (Sacks, Bakal, Beatty *et al.* 2003, Hirsch, Haskal, Hertzler *et al.* 2006) this is not always the case in a primary care setting, Findings suggest that clinicians who utilise a classic history of claudication alone to detect PAD or do not use an ABI test as routine in patients in which common risk factors are present may be missing a large percentage of PAD diagnoses (Hirsch, Criqui, Treat-Jacobson *et al.* 2001, Khan, Flather, Mister *et al.* 2007).

All of the risk factors identified within patients suffering IC, a major clinical manifestation of PAD, are also strongly associated with an increased risk of more serious atherosclerotic disease. This in itself should then act as a warning sign regarding an increased risk of a serious cardiovascular event/death occurring in these patients and therefore highlights the need for effective diagnosis and management of IC. Although ABI measurements have been shown to be an effective tool in the diagnosis of clinically significant PAD, in which blood flow is being obstructed, as highlighted in this study and by others (McDermott, Greenland, Liu *et al.* 2002) there are issues regarding cases where IC is present but an  $AB\geq 0.96$  is recorded. The data presented here agrees with that previously published and illustrates issues surrounding the effectiveness of current protocols in place for the early diagnosis of PAD prior to the onset of clinically significant manifestations such as IC. An  $ABI < 0.9$  is indicative of the presence of already diffuse and advanced atherosclerosis; as such ABI is more specific to advanced atherosclerotic disease and thus may not a useful screening test for early vascular disease

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(Newman, Shemanski, Manolio *et al.* 1999). This clearly highlights the need for additional screening methods to be developed in order to aid in the early diagnosis and administration of risk factor intervention and treatment of PAD/IC, the investigation of which is a major objective of this thesis.

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# **Chapter 3.**

# **ELISA Development and Evaluation**

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### 3.1. Introduction

Since Ohshima *et al* (1990) first reported 3-nitrotyrosine (3NT) as an *in vivo* marker of reactive nitrogen species, several methods have been developed in order to detect both free and protein-bound 3NT within human biological fluids and tissues. The methods developed range from simple spectrophotometric techniques through to mass spectrometry. In general, the specificity and sensitivity of the different methods available for the detection of 3NT varies considerably.

The concentration of 3NT can be quantified spectrophotometrically ( $A_{430}$ ) at alkaline pH due to the  $pK_a$  of the phenolic group of 3NT (7.5) being considerably lower than that of tyrosine (10). As a result of this difference 3NT has a peak at 430nm under basic conditions, in addition to the peak observed at 280nm. Unfortunately this method it is suitable only for isolated relatively 'pure' proteins due to the potential for interference from prosthetic groups which absorb in the 350-450nm region e.g. haem (Crow and Ischiropoulos 1996). Therefore more specific assay methods are required to detect 3NT-residues in tissue homogenates and plasma.

Within biological samples a number of different methods have been reported for the separation, detection and quantification of free and protein-bound 3NT. These include methods involving the use of high performance liquid chromatography (HPLC) combined with various detection systems. Methods using HPLC combined with an ultra violet (UV) detection system for the detection of 3NT have been reported (Kaur and Halliwell 1994, Van Der Vliet, Eiserich, Kaur *et al.* 1996, Crow and Ischiropoulos 1996). HPLC-UV detection of 3NT has the advantage of being able to simultaneously detect other

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metabolites of tyrosine such as dityrosine. A disadvantage of this method is its relative lack of sensitivity, only allowing for the detection of 3NT in samples of relatively high concentration (0.1nM) (Crow and Ischiropoulos 1996). When HPLC is combined with an electrochemical (EC) detector 3NT has been detected in amino acids, peptides and proteins with limits of detection as low as a few picomoles per sample (Shigenaga, Lee, Blount *et al.* 1997, Skinner, Crow, Skinner *et al.* 1997, Shigenaga 1999, Crow 1999, Richards, Silva and Devall 2006). HPLC-EC has the advantage of being 1000 times more sensitive than HPLC-UV. Although chromatographic detection systems such as HPLC-UV and HPLC-EC are potentially highly sensitive techniques they have the disadvantage of requiring relatively large amounts of protein. The sample preparation required for such analysis also presents problems as it involves acid hydrolysis of the proteins to amino acids. This hydrolysis can give rise to the artifactual production of 3NT which may result in the overestimation of 3NT content of a sample (Shigenaga, Lee, Blount *et al.* 1997). However, methods for the hydrolysis of proteins and subsequent derivatisation of 3NT and tyrosine (under alkaline conditions, thus preventing artifactual production of 3NT) have been developed (Frost, Halliwell and Moore 2000).

In recent years several techniques employing mass spectrometry have been developed for the quantification and identification of 3NT-residues in biological samples. For example Kendall *et al.* (2001) used high-resolution electrospray ionisation - Fourier transform ion cyclotron resonance - mass spectrometry (ESI-FTICR-MS) to confirm the presence of nitration within electrochemically nitrated horse heart myoglobin. Using NT-BSA as a test case, matrix assisted

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laser desorption ionisation - mass spectrometry (MALDI-MS) has been shown to be capable of identifying 3NT modifications in complex peptide mixtures (Sarver, Scheffler, Shetlar *et al.* 2001). Kanski *et al.* (2005) have also reported the identification of specific modified proteins in ageing skeletal muscle using nanoelectrospray ionisation - tandem mass spectrometry.

The main issue with regards to methods involving HPLC, with various detection systems, and mass spectrometry for the detection of 3NT is that these techniques involve the use of specialised equipment and require extensive sample preparation. As such the screening of large numbers of biological samples such as plasma could be an issue and thus highlights the need for a more robust and convenient screening tool.

Commercial and custom made polyclonal and monoclonal antibodies raised against 3NT are now readily available enabling the detection of 3NT using immunohistochemistry (Ye, Strong, Huang *et al.* 1996, Viera, Ye, Estevez *et al.* 1999) and immunoassays including enzyme linked immunosorbant assay (ELISA) (Khan, Brennand, Bradley *et al.* 1998, Ter Steege, Koster-Kamphuis, Van Straaten *et al.* 1998, Inoue, Hisamatsu, Ando *et al.* 2002). Immunohistochemical methods have been developed for the detection of 3NT in tissues using both polyclonal and monoclonal anti-3NT Abs. Using such methods, 3NT has been identified in atherosclerotic lesions in coronary arteries; for example using both mono- and polyclonal antibodies (Beckmann, Ye, Anderson *et al.* 1994, Baker, Hall, Evans *et al.* 1999). Although immunohistochemistry is extremely useful for the identification of the presence of 3NT within specific tissues, actual quantification of the 3NT is difficult. Immunohistochemical analysis of 3NT as a stand-alone assay is generally

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regarded as a less appropriate method for the quantitative measurement of 3NT.

Within this study the aim was to develop novel semi-quantitative immunoassays (indirect and competitive ELISA) in order to detect the presence of 3NT-containing proteins in the plasma of claudicants. Although several ELISA methods have been developed for the detection of 3NT in human plasma for other disease pathologies (Ter Steege, Koster-Kamphuis, Van Straaten *et al.* 1998, Khan, Brennand, Bradley *et al.* 1998, Inoue, Hisamatsu, Ando *et al.* 2002) none have been used for the detection of 3NT in the plasma of claudicants. This chapter reports on the development of both the indirect and competitive ELISAs. It highlights important issues these developments have identified regarding the appropriateness of using immunoassays, specifically ELISAs, for the detection of 3NT in human plasma.

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## **3.2. Materials & Methods**

All chemicals used were analytical grade and purchased from Fisher chemicals unless otherwise stated. Unless otherwise stated water was obtained by reverse osmosis (RO) to a resistance of greater than  $18\text{M}\Omega\cdot\text{cm}^{-1}$

### **3.2.1. Production of custom polyclonal anti-3-nitrotyrosine antibody**

Materials: An EG&G model 273 potentiostat/galvanostat was used for electrochemical nitration. A platinum basket anode was made and supplied by The Birmingham Metal company, Birmingham UK. A saturated Calomel reference electrode (SCE) was purchased from ABB Kent Taylor. Keyhole limpet haemocyanin (KLH, H8283) was obtained from Sigma (Poole, Dorset, U.K.). Polyclonal antibody raised against electrochemically nitrated KLH was produced by AbCam (Cambridge UK).

Methods: Electrochemically nitrated keyhole limpet haemocyanin (KLH) was used as an antigen to raise a polyclonal antibody against 3NT in rabbits.

In brief KLH (1mg/ml) was dialysed into 50mM sodium tetraborate, 20mM sodium nitrite pH 9, and then nitrated electrochemically using the method of Kendall *et al* (2001), to an absorbance of 0.446 at 430nm (see figure 3.2.i. for schematic diagram of apparatus). The nitrated KLH was desalted via dialysis into RO water and freeze-dried. This freeze dried product was then sent to AbCam (Cambridge) for production of a polyclonal anti-3NT antibody. The rabbit anti-3NT antibody (anti-3NT Ab) was aliquoted into Eppendorf tubes and stored at  $-80^{\circ}\text{C}$ . The working aliquot was diluted using an equal volume of glycerol and stored at  $-20^{\circ}\text{C}$ .

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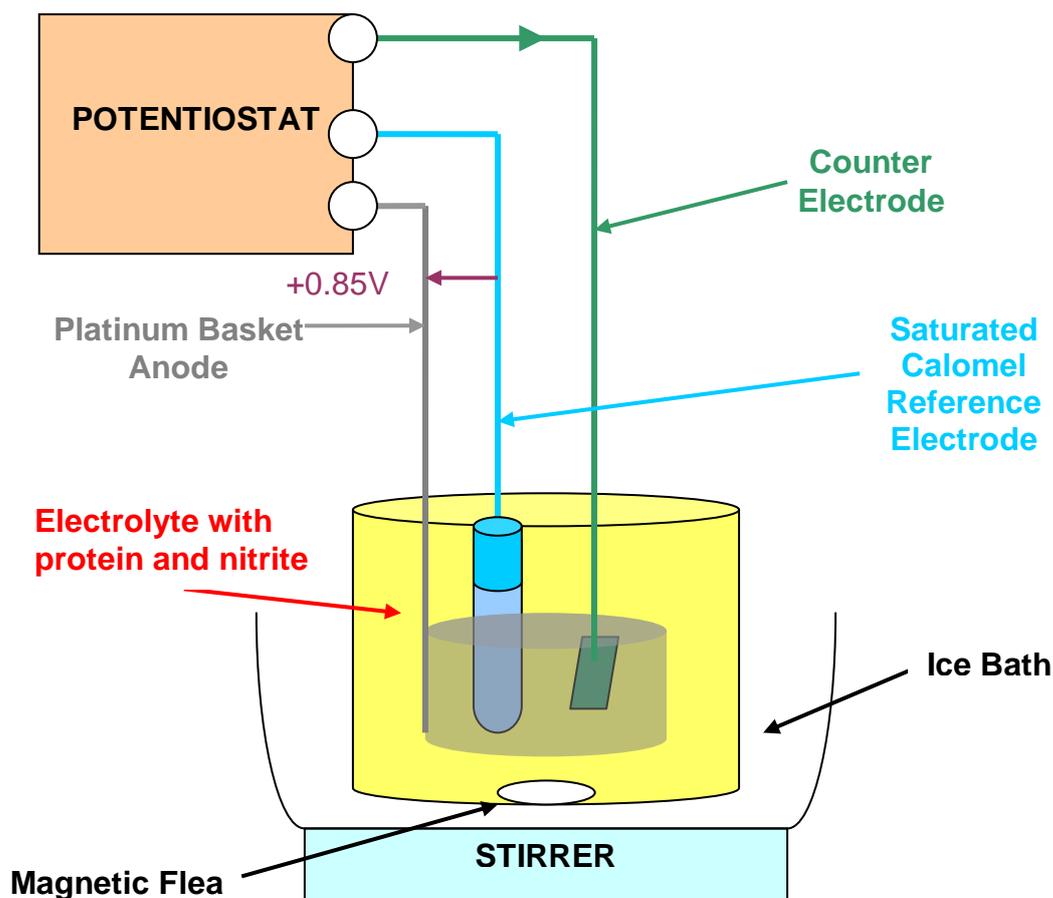


Figure 3.2.i. Electrochemical nitration apparatus

Electronitration (electrooxidation at an alkaline pH in the presence of nitrite) is an easily controlled method developed in house and using ESI-FTICR-MS it has been confirmed that the only reaction product is the nitration of tyrosine residues (Kendall, Cooper, Heptinstall *et al.* 2001). Kendall *et al* (2001) proposed that electronitration of tyrosine residues may occur as a result of the oxidation of both nitrite and tyrosinate anions at the anode followed by a radical/radical coupling reaction (See Figure 1.1.ii, Chapter 1). Although it was accepted that they could not rule out the fact that there may be no requirement for tyrosine oxidation by the anode in order for nitration to occur, due to the possibility of the oxidation of nitrite to  $\text{NO}_2^+$ , nor of the formation of  $\text{ONOO}^\cdot$  from the oxidation of aqueous nitrite.

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### 3.2.2. Spectrophotometric determination of 3-nitrotyrosine levels

The following calculation was used to determine the level of nitration in BSA and HSA samples nitrated either electrochemically or with peroxyxynitrite, as described in the following sections.

Using  $A_{430}$  to determine 3NT levels at alkaline pH.

The extinction coefficient ( $\epsilon$ ) for 3NT =  $4300\text{M}^{-1}\text{cm}^{-1}$  (Beckman, Chen, Ischiropoulos *et al.* 1994)

$$\text{Absorbance } 4.3 = 1\text{mM}$$

$$A_{430} 0.0043 = 1\mu\text{M } 3\text{NT}$$

Relative Molecular Mass of BSA = 66000

For example - An absorbance at 430nm of 0.262 for nitrated BSA

$$0.262/0.0043 = \mathbf{60.9\mu\text{M } 3\text{NT}}$$

$$\text{Concentration of BSA sample} = 1000\mu\text{g/ml} = 1000\text{mg/L}$$

$$= 1000/66000$$

$$= 0.015\text{mM}$$

$$= \mathbf{15\mu\text{M } BSA}$$

$$\text{Therefore level of nitration} = 60.9/15$$

$$= \mathbf{4.06 \text{ mol } 3\text{NT} / 1\text{mol } BSA}$$

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### **3.2.3. Electrochemical nitration protocol**

Materials: See section 3.2.1., Bovine serum albumin (BSA, A8022) and lyophilised human plasma (P9523) were obtained from Sigma (Poole, Dorset, U.K.).

Methods: Electrochemical nitration of bovine serum albumin (BSA), human serum albumin (HSA) and lyophilised human plasma was as described by Kendall *et al* (2001) with modifications.

In brief the protein was dissolved in nitration buffer to a final concentration of 1mg/ml. The nitration procedure was continued until the required absorbance had been achieved at 430nm (as described in section 3.2.2.). The nitrated product was not separated from any unreacted protein left in solution and was dialysed into phosphate buffered saline (PBS, pH 7.4). The samples were stored in small aliquots at  $-20^{\circ}\text{C}$ , diluted in an equal volume of glycerol.

NB: When nitrating plasma the level of nitration was difficult to ascertain due to the colour of plasma, moreover the sample is poly-disperse with respect to RMM. The required  $A_{430}$  was thus estimated on the assumption that HSA was the dominant protein, thus the equation as stated in 3.2.2. was used for human plasma also (i.e. assumed RMM of 66000).

### **3.2.4. Nitration with peroxyntirite**

Peroxyntirite was prepared as described by Beckman *et al* (1994). Using this peroxyntirite a BSA sample (1mg/ml) was nitrated as described by Khan *et al* (1998) to a level of 3mol 3NT / 1mol BSA (determined using  $A_{430}$ , section 3.2.2.).

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In brief, three additions of the prepared alkaline stock solution of peroxyxynitrite were added to a BSA solution (1mM final concentration of peroxyxynitrite, concentration determined optically at  $A_{302}$  where  $\varepsilon = 1670 \text{ M}^{-1}\text{cm}^{-1}$  (Ye, Strong, Huang *et al.* 1996)). The level of nitration was determined using the measured absorbance at  $A_{430}$  (as described in section 3.2.2. above). The nitrated BSA solution was dialysed against PBS and then stored as described in section 3.2.3. above.

### **3.2.5. Reduction of nitrotyrosine to aminotyrosine**

Aminotyrosine was produced in order to assess the polyclonal anti-3NT antibody specificity towards 3NT residues. The nitrated tyrosine residues within the electrochemically nitrated BSA (4mol 3NT / 1mol BSA) were reduced to aminotyrosine using sodium dithionite (Crow and Ischiropoulos 1996).

In brief, the  $A_{430}$  of the nitrated BSA sample (1ml, 500 $\mu\text{g}/\text{ml}$ ) was measured followed by the addition of approximately 100x molar excess of sodium dithionite (50 $\mu\text{l}$  of 100mM sodium dithionite, pH 9.5). The mixture was incubated for 30 minutes at room temperature. To ensure the nitrated tyrosine residues had been reduced to aminotyrosine the  $A_{430}$  was again measured to confirm the absorbance was zero at  $A_{430}$ . This reduction of nitrotyrosine to aminotyrosine was also confirmed via dot blot immunodetection with the polyclonal anti-3NT Ab (see section 3.2.14. dot blot immunodetection).

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### **3.2.6. Nitrated protein; ELISA pre-treatment protocols**

Nitrated protein samples were pre-treated with a variety of denaturants in order to assess the effects, if any, these had on availability of the nitrated epitopes to the anti-3NT Ab.

Materials: Mercaptoethanol (M7522) and trypsin (T1426, Chymotrypsin  $\leq 0.1$  BTEE units/mg protein) obtained from Sigma (Poole, Dorset, U.K.).

Methods:

Pre-treatment with urea and mercaptoethanol: To a sample of nitrated BSA, urea (final concentration 8M) and mercaptoethanol 0.5% (v/v), were added and incubated at 37°C for an optimal time (1 hour). These samples were prepared fresh when required.

Pre-treatment by boiling with Tween 20: Nitrated BSA was diluted in PBS + Tween 20 (0.2%, v/v) and placed in a water bath at approximately 100°C for 5 minutes. The sample was then diluted into an equal volume of incubation buffer used in the ELISA protocol (minus Tween 20) so that the final Tween 20 concentration was 0.1%. These samples were made fresh when required.

Pre-treatment by trypsin digestion: Nitrated BSA (0.5mg/ml) was dialysed into 0.1M ammonium bicarbonate. To this nitrated BSA solution (5.5ml), 10 $\mu$ g of trypsin (5 $\mu$ l of 2mg/ml trypsin in 20mM calcium chloride, 1mM HCl, pH 2.7) was added. The solution was then incubated at 37°C for 2 hours. Following this another 10 $\mu$ g of trypsin was added and the solution was again incubated at 37°C for 2 hours. The reaction was stopped by boiling the sample for

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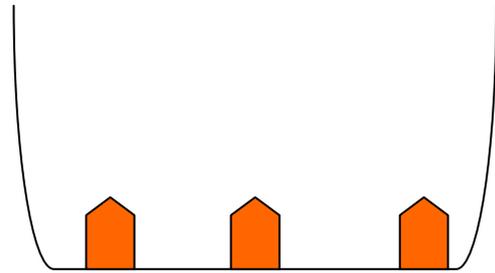
5minutes to denature the enzyme. The samples were stored in small aliquots at  $-20^{\circ}\text{C}$ , diluted in an equal volume of glycerol.

All of the above pre-treated samples were then used for indirect and competitive ELISA development.

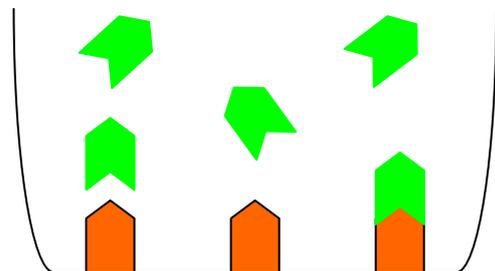
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### 3.2.7. Indirect ELISA

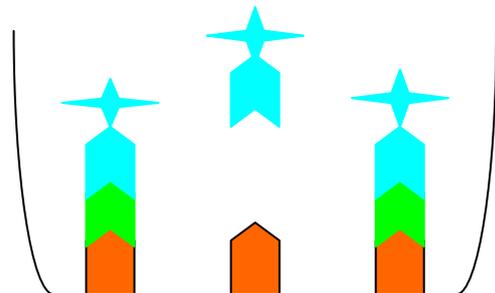
The microtitre plate is coated with the test antigen or standard nitrated BSA (🏠)



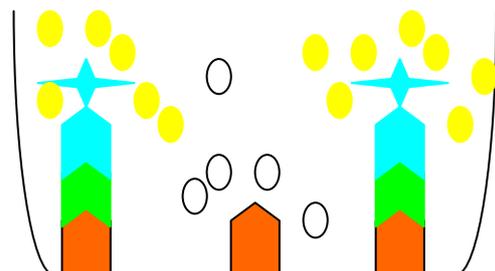
The unoccupied adsorption sites are then blocked with SMP. The anti-3NT Ab (🏠) is incubated with the antigen



Following a wash phase, incubation with the enzyme-labelled antibody (🌟, goat anti-rabbit IgG with alkaline phosphatase conjugate).



Following a final wash phase, incubation with p-nitrophenol phosphate substrate (○) and determination of the product (●)



**Figure 3.2.ii. A schematic representation of the indirect ELISA method.**

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Materials: Sero-Wel<sup>®</sup> 96 well flat bottom microtitre plates supplied by Bibby Sterilin, Stone, UK. Nunc Maxisorp<sup>™</sup> 96 well flat bottom microtitre plates, Nunc, part of Thermo Fisher Scientific, Denmark. Immulon 2HB<sup>®</sup> 96 well flat bottom microtitre plates, Thermo scientific. Carbonate buffer (C3041), L-tyrosine (T4446), 3-chloro-L-tyrosine (C5897), polyclonal goat anti-rabbit IgG (whole molecule alkaline phosphatase conjugate, A3812), p-nitrophenol phosphate substrate (N4645) and 30% hydrogen peroxide solution (H1009) were purchased from Sigma (Poole, Dorset, U.K.). Skimmed milk powder, (SMP, Oxoid). OPD tablets (BUF047B, AbD serotec). Rabbit anti human IgG with horse radish peroxidase conjugate (DAKO, Denmark). Anthos 2001 plate reader (Anthos labtec instruments).

All reagent concentrations were optimised using checkerboard titration. Indirect ELISAs were developed using different brands of commercially available microtitre plates (Sero-Wel<sup>®</sup>, Nunc Maxisorp<sup>™</sup> and Immulon 2HB<sup>®</sup>), differing nitration protocols as well as both native and pre-treated NT-BSA.

The general protocol used was as follows: 96 well microtitre plates were coated with 100µl of the test samples or nitrated BSA standards (calibration curves ranging from 40-0µg/ml) using carbonate buffer pH 9.6. The plates were then incubated overnight at 4°C. Wells were blocked with 130µl 5% skimmed milk powder (SMP) in phosphate buffered saline (PBS x10, pH 7.4; 80g sodium chloride, 2g potassium chloride, 28.9g sodium hydrogen phosphate (ortho) decahydrate, 2g potassium dihydrogen (ortho) phosphate made up to 1L) for 1 hour at 37°C. Following blocking the plates were washed

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with wash buffer (PBS and 0.05% Tween 20) three times. Polyclonal anti-3NT Ab (at optimal dilution as determined by checkerboard analysis) was then added and incubated for 2 hours at 37°C. The plates were then washed as above. Goat anti-rabbit IgG (whole molecule alkaline phosphatase conjugate), 1/5000 in incubation buffer, was added and incubated for 2 hours at 37°C. Following washing as above, substrate (p-nitrophenol phosphate substrate, 1mg/ml in 0.1M diethanolamine buffer, pH 10.3) was then added. The plates were placed in the dark at room temperature for 15 minutes to allow the colour to develop. The reaction was stopped using 100µl of 0.75M sodium hydroxide, and the absorbance was then read at 405nm using an Anthos reader 2001. All antibody dilutions were made in incubation buffer (PBS pH 7.4 with 0.05% Tween 20, 1% BSA (unless otherwise stated) and 5% SMP).

All analysis of data was carried out using Microsoft Excel<sup>®</sup> Office 2003 and SPSS v15. Prior to any analysis of the data all of the absorbance values at 405nm collected from each indirect ELISA were corrected by subtracting the mean blank value.

### **3.2.8. Testing secondary antibody for non-specific binding to BSA and NT-BSA**

The goat anti-rabbit IgG (whole molecule alkaline phosphatase conjugate) was assessed for non-specific binding to BSA and NT-BSA as follows:

A 96 well microtitre plate was coated with BSA and NT-BSA over a range of concentrations (80-0µg/ml) overnight at 4°C, using carbonate buffer pH 9.6. After this incubation period the wells were emptied and blocked with 130µl 5% SMP in PBS pH 7.4 for 1 hour at 37°C and washed with wash buffer three

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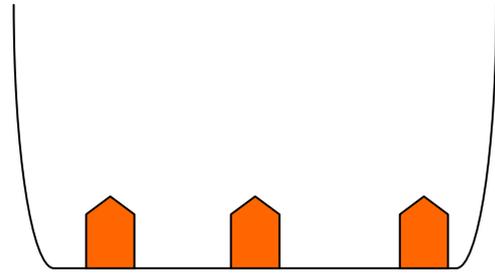
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times. The plate was then incubated with the goat anti-rabbit IgG (1/5000 in incubation buffer) for 2 hours at 37°C. Following a wash cycle as described above, substrate (p-nitrophenol phosphate substrate, 1mg/ml in 0.1M diethanolamine buffer, pH 10.3) was then added. The plates were placed in the dark at room temperature for 15 minutes to allow the colour to develop. The reaction was stopped using 100µl of 0.75M sodium hydroxide, and the absorbance was then read at 405nm using an Anthos reader 2001. All antibody dilutions were made in incubation buffer.

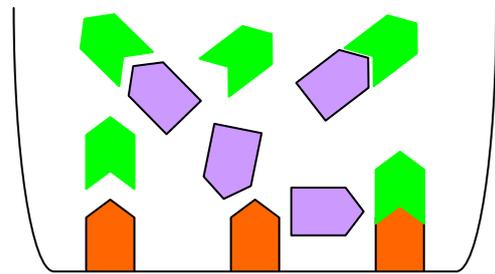
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### 3.2.9. Competitive ELISA

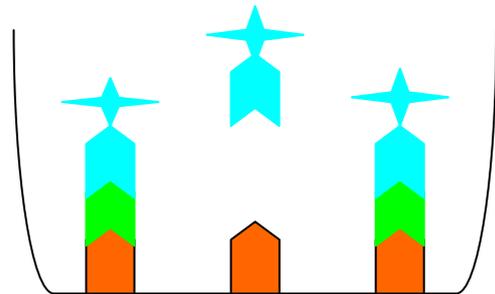
The microtitre plate is coated with the standard antigen (NT-BSA, )



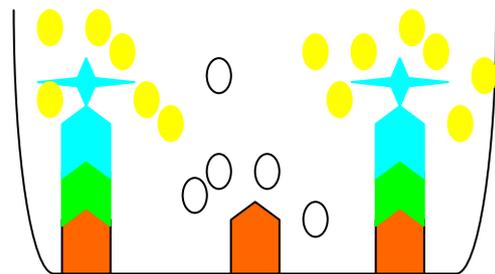
The unoccupied adsorption sites are then blocked. The anti-3NT Ab () is then incubated with the competitive antigen () and/or the standard ()



Following a wash phase, incubation with the enzyme labelled antibody (, goat anti-rabbit IgG with alkaline phosphatase conjugate).



Following a final wash phase, incubation with p-nitrophenol phosphate substrate () and determination of the product ()



**Figure 3.2.iii. A schematic diagram of the competitive ELISA method.**

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Materials: See section 3.2.7. for materials

All reagent concentrations and incubation times were investigated along with a variety of reagent formats. Three different commercially available microtitre plates were compared as before (Sero-Wel<sup>®</sup>, Nunc Maxisorp<sup>™</sup> and Immulon 2HB<sup>®</sup>). All volumes were 100µl unless otherwise stated.

The general protocol used was as follows: Optimally diluted nitrated protein in carbonate buffer, pH 9.6, was added to all wells of a microtitre plate and incubated at 4°C overnight. Plates were blocked with 5% SMP in PBS (130µl) for 1 hour at 37°C and then washed with wash buffer three times. The test sample or BSA standard (for the calibration curve), 50µl, were pipetted into the appropriate wells along with 50µl of the polyclonal anti-3NT Ab (at optimal dilution as determined by checkerboard analysis). The plates were incubated for 2 hours at 37°C and then washed with wash buffer as above. Goat anti-rabbit IgG (whole molecule alkaline phosphatase conjugate), 1/5000 in incubation buffer, was added and incubated for 2 hours at 37°C. Following washing, the substrate (p-nitrophenol phosphate substrate, 1mg/ml in 0.1M diethanolamine buffer, pH 10.3) was added to the plates which were then placed in the dark at room temperature for 15minutes to allow the colour to develop. The reaction was then stopped using 100µl of 0.75M sodium hydroxide, and the absorbance was then read at 405nm. All antibody dilutions were made in incubation buffer.

All analysis of data was carried out using Microsoft Excel<sup>®</sup> Office 2003. Prior to any in depth analysis of the data all of the absorbance values at 405nm

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collected from the competitive ELISA were standardised by subtracting the observed absorbance value from mean blank value. This resulted in a positive relationship between corrected absorbance and concentration of 3NT.

### **3.2.10. Competitive ELISA testing inhibition of anti-3NT antibody**

A microtitre plate was coated and run as described in the general protocol above (section 3.2.9.). During the competition phase of the assay a range of different substances were tested in order to assess the specificity of the polyclonal anti-3NT Ab. The substances tested in competition with the anti-3NT Ab over a range of concentrations were as follows: Tryp\_NT-BSA, NT-BSA, BSA, protein-bound aminotyrosine (see section 3.2.5.), free 3-nitro-L-tyrosine, free L-tyrosine and free 3-chloro-L-tyrosine.

### **3.2.11. Sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS-PAGE)**

Materials: 12% polyacrylamide gel pH 8 with a 4% stacking gel (SciePlas, Southam, Warwickshire). 4-20% gradient polyacrylamide Tris-HCl gel, 15 well, (BIORAD). Anachem size markers (Anamed Elektrophorese GmbH, Germany).

Methods: Protein samples were separated using 12% polyacrylamide running gel, pH 8, with a 4% stacking gel, or gradient polyacrylamide gels. The samples to be separated were diluted in an equal volume of sample buffer (12.5ml 1.5M Tris-HCl pH 8, 0.5g SDS, 5g sucrose, 0.25ml mercaptoethanol, 5mg bromophenol blue and made up to 50ml) and then loaded onto the gel. Once the samples were loaded, including Anachem size markers, the gels were run at 150V for 1 hour in Tris-glycine running buffer (6g Tris, 28.8g

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glycine (pH 8.48), 1g SDS (pH 8.42) made up to 1L RO water with no pH adjustment) or until the dye front had reached the bottom of the gel. The gels were then stained either with Coomassie blue R250 or processed by Western blotting.

### **3.2.12. Coomassie blue staining**

Materials: Coomassie brilliant blue R250 (B7920) Sigma (Poole, Dorset, U.K.). UVIttec image acquisition system including transilluminator and UVIdoc image acquisition software

Methods: Gels from SDS-PAGE, were fixed for 1hour using Coomassie fixer/de-stain solution (10% methanol, 10% acetic acid made up to volume with RO water). The gels were then placed in Coomassie brilliant blue R250 stain (0.15g Coomassie brilliant blue, 60ml methanol, 15ml glacial acetic acid, 75ml RO water and filter) and left over night. The gels were then left to de-stain (in fixer/de-stain solution) overnight, replacing with fresh de-stain periodically. Photographic representations of the gels were taken using an UVIttec image acquisition system including transilluminator and UVI doc image acquisition software.

### **3.2.13. Western Blot**

Materials: Hybond C nitrocellulose membrane (Amersham Pharmacia). BCIP/NBT substrate (B5655) Sigma (Poole, Dorset, U.K.).

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Methods: Separated proteins were transferred from SDS-PAGE gels onto Hybond C nitrocellulose membrane using a BioRad Mini Trans-Blot® Electrophoretic Transfer Cell as per manufacturer's instructions.

In brief the nitrocellulose membrane was equilibrated in Towbin buffer (3.03g Tris, 14.4g glycine, 200ml methanol and made up to 1L with RO water) and the cell was run at 100V for 1 hour. The membrane was blocked with 3% SMP in PBS overnight at 4°C. Once blocked the membrane was incubated for 1 hour at room temperature in a solution of polyclonal rabbit anti-3NT Ab, at optimised dilution, in PBS with 2% BSA. Following a thorough wash in PBS, the membrane was then placed in a solution of goat anti rabbit IgG alkaline phosphatase conjugated antibody (1:5000 in PBS with 0.5% BSA) and incubated for 45 minutes at room temperature. The membrane was again washed in PBS followed by rinsing with RO water to remove any residual phosphate. The presence of 3NT-containing proteins was detected colourimetrically by the addition of fast 5-bromo-4-chloro-3-indolyl phosphate / nitro blue tetrazolium (BCIP/NBT) substrate, as per manufacturer's instructions, and incubation of the membrane in the dark. The reaction was stopped by rinsing the membrane with RO water.

#### **3.2.14. Dot blot**

Materials: As for 3.2.13.

Methods: The patient plasma and electrochemically nitrated BSA tested were pipetted (4µl) onto Hybond C nitrocellulose membrane (which had been equilibrated in Towbin buffer). The samples were allowed to air dry and then treated as described above for western blotting (3.2.13.) starting with blocking the membrane with 3% SMP in PBS.

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### **3.3. Results**

#### **Indirect ELISA**

##### **3.3.1. The effect of protein integrity on 3NT detection**

Prior to developing a competitive ELISA for the detection and quantification of 3NT-containing proteins in human plasma samples, several parameters needed to be investigated; in particular the efficacy of the electrochemically nitrated protein (BSA) being used as the standard. These investigations were carried out during the development of an indirect ELISA.

Initial development of the indirect ELISA was performed using a stock electrochemically nitrated sample which had been stored at -20°C since 1999. Whilst using this sample the indirect ELISA being developed appeared to be relatively sensitive and accurate for the detection of 3NT containing proteins (based on the optical densities of the dilution curves recorded). However upon using a fresh electrochemically nitrated BSA sample it was apparent that the assay sensitivity and reliability was dramatically reduced.

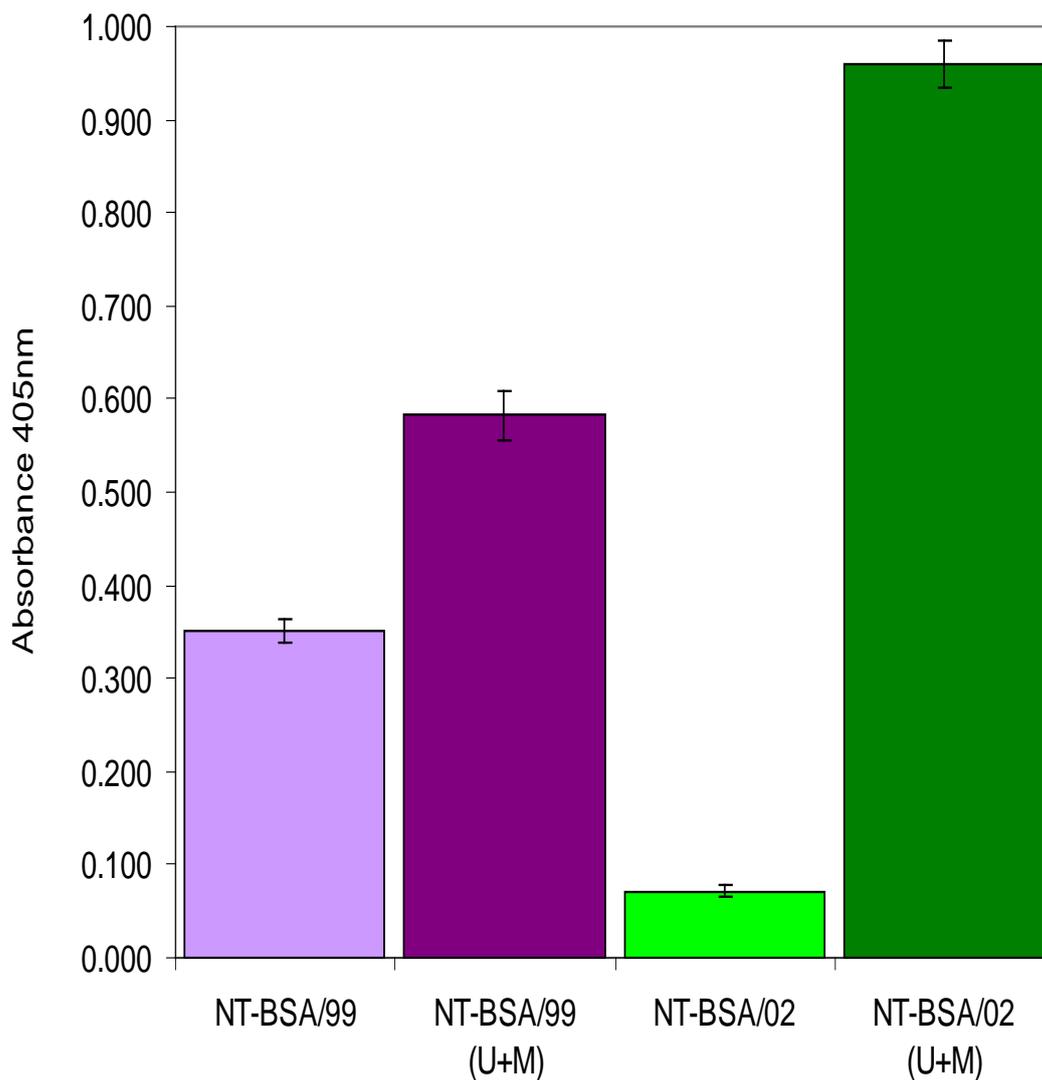
Due to the observations above the effects of storage on the protein integrity were investigated using two electrochemically nitrated samples. The first sample (NT-BSA/99), prepared in 1999, had been stored at -20°C and had gone through the freeze thaw cycle an unknown number of times. The second sample was a freshly nitrated sample stored at -20°C in glycerol (50:50) to prevent freezing (NT-BSA/02). Although the samples tested were of comparable nitration levels (as determined by  $A_{430}$ ) analysis using indirect ELISA revealed that there were differences in the detection of 3NT by our polyclonal anti-3NT Ab. Significantly higher optical densities were obtained

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when assaying the native NT-BSA/99 samples compared to native NT-BSA/02 at comparable nitrated-protein concentrations ( $P < 0.0001$  determined using single factor ANOVA).

Due to this obvious difference both samples were chemically denatured in order to allow maximal availability of the 3NT residues to the antibody. Several treatments were investigated as denaturants including; acetonitrile, urea, and iodoacetamide / mercaptoethanol. These investigations highlighted the fact that using 8M urea and mercaptoethanol (0.5%) in combination was the best treatment to use for denaturing NT-BSA samples. Time course experiments were carried out to establish the optimal incubation parameters with the urea and mercaptoethanol. In the case of NT-BSA an incubation time of 1 hour at 37°C was found to be sufficient to maximise the optical density obtained (results not shown). There was a significant improvement in sensitivity when using urea and mercaptoethanol (U+M) to denature the protein compared to their native counterparts (Figure 3.3.i.,  $P < 0.0001$ , ANOVA single factor). Tukey post hoc analysis highlighted that there was a significant difference between the native and denatured NT-BSA/99 and NT-BSA/02 samples ( $P < 0.05$ ) regardless of the fact that they were of comparable concentration and level of nitration.



**Figure 3.3.i. NT-BSA/99 and NT-BSA/02 with and without pre-treatment with urea and mercaptoethanol (U+M).**

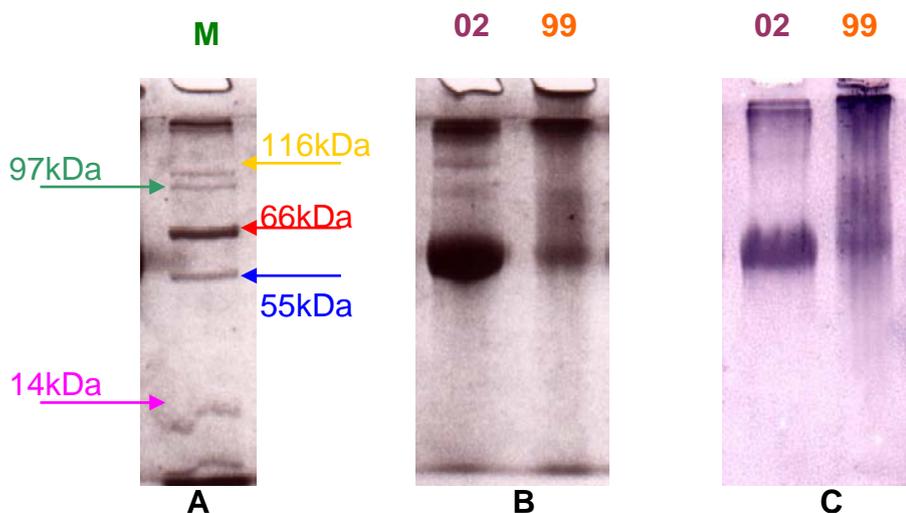
Four nitrated BSA samples were assayed to investigate the effects of storage and sample pre-treatment with U+M. NT-BSA/99 with no pre-treatment (■), NT-BSA/99 pre-treated with U+M (■), NT-BSA/02 (■) and NT-BSA/02 U+M (■). Mean ± SEM, 16 replicates for each protein sample.

The two nitrated samples (NT-BSA/99 and NT-BSA/02) were further analysed via separation on SDS-PAGE and visualised using Coomassie staining and Western blot immunodetection. From this separation it was established that there were differences in the integrity of the protein structure between the two

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nitrate samples (Figure 3.3.ii). The Coomassie stain highlighted that NT-BSA/99 gave no distinct bands, rather a smear from the bottom of the stacking gel to an overloaded band between 66-55kDa within the resolving gel. For NT-BSA/02 several bands were observed with a similar large distinct band at approximately 66-55kDa, (Figure 3.3.ii.B). Western blot analysis of the SDS-PAGE gel clearly showed the presence of 3NT (dark purple staining) in the whole of the smear for NT-BSA/99 whereas in the case of the NT-BSA/02 there were more distinctly stained bands. As with NT-BSA/99 the presence of 3NT was detected in more than one band (Figure 3.3.ii.C), mainly within the 66-55kDa band and additional smearing from the top of the resolving gel.



**Figure 3.3. ii. NT-BSA/99 and NT-BSA/02 visualised using Coomassie staining and Western blot following separation using SDS-PAGE.**

**M – Anamed ProteMix size marker; 02 – NT-BSA/02; 99 – NT-BSA/99. All samples were loaded onto the gel at approximately 1µg/µl. A – Size marker (Coomassie stain); B – Coomassie stain; C – Western blot.**

Due to the results above (see discussion) NT-BSA/02 was used in all further experimentation and will be referred to as NT-BSA from this point onwards.

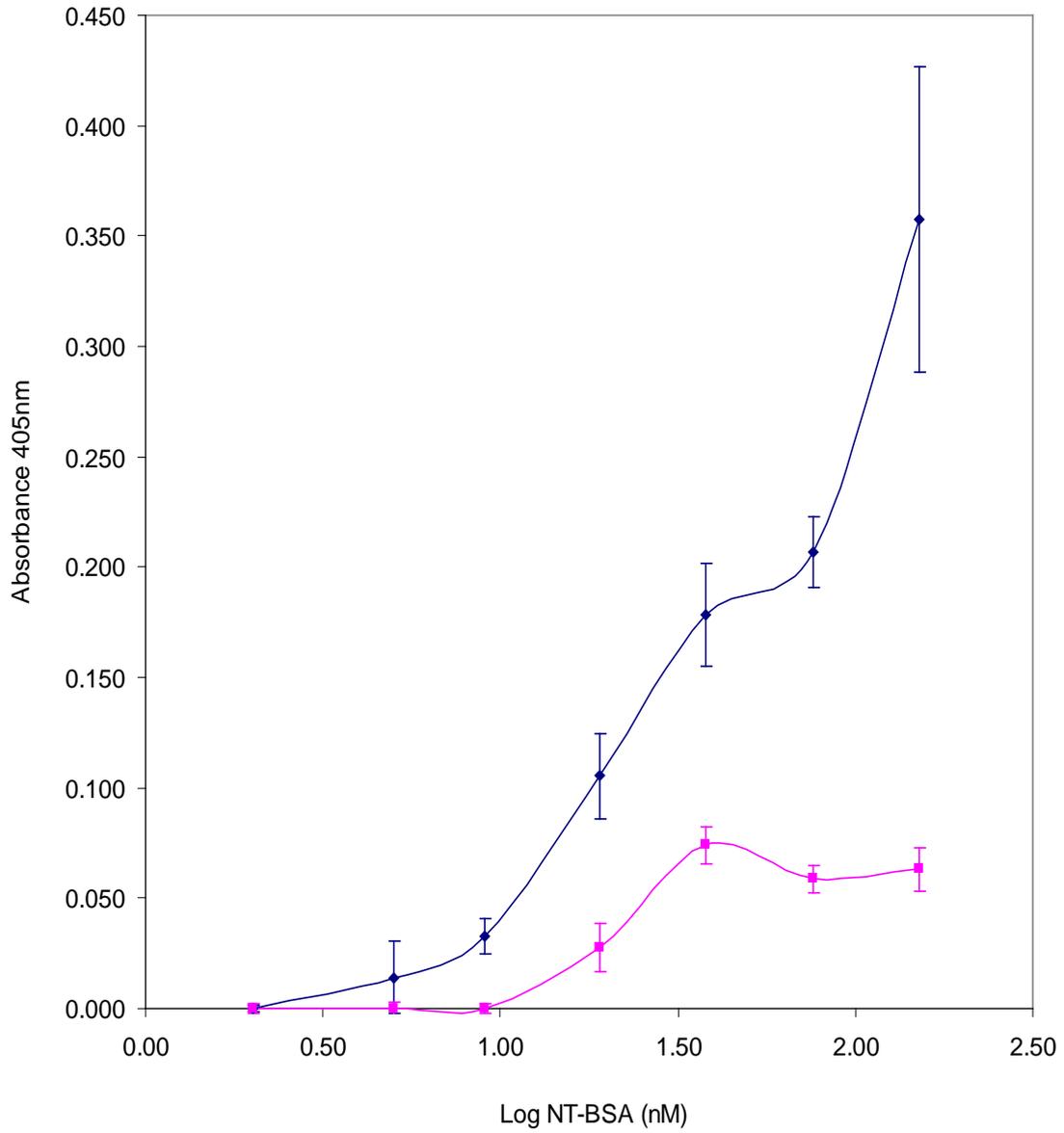
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### **3.3.2. A comparison of different nitration protocols**

Comparisons were made between electrochemically-nitrated BSA and peroxyxynitrite (ONOO<sup>-</sup>) -nitrated BSA using indirect ELISA. NT-BSA samples were nitrated either electrochemically or chemically to approximately the same level of nitration (4mol 3NT/mol BSA, as determined by A<sub>430</sub>) and were compared using Sero-Wel<sup>®</sup> microtitre plates by indirect ELISA and dot blot analysis (Figures 3.3.iii. and 3.3.v.). Upon initial testing, a higher response for 3NT was achieved with ONOO<sup>-</sup>-nitrated BSA compared to electrochemically-nitrated BSA at comparable concentrations and level of nitration (Figure 3.3.iii.). It was evident that for both electrochemically nitrated and ONOO<sup>-</sup>-nitrated BSA there was low assay sensitivity (approximately 10nM), although there was a significant difference between the two nitration protocols (P<0.0001, two way ANOVA with replication).

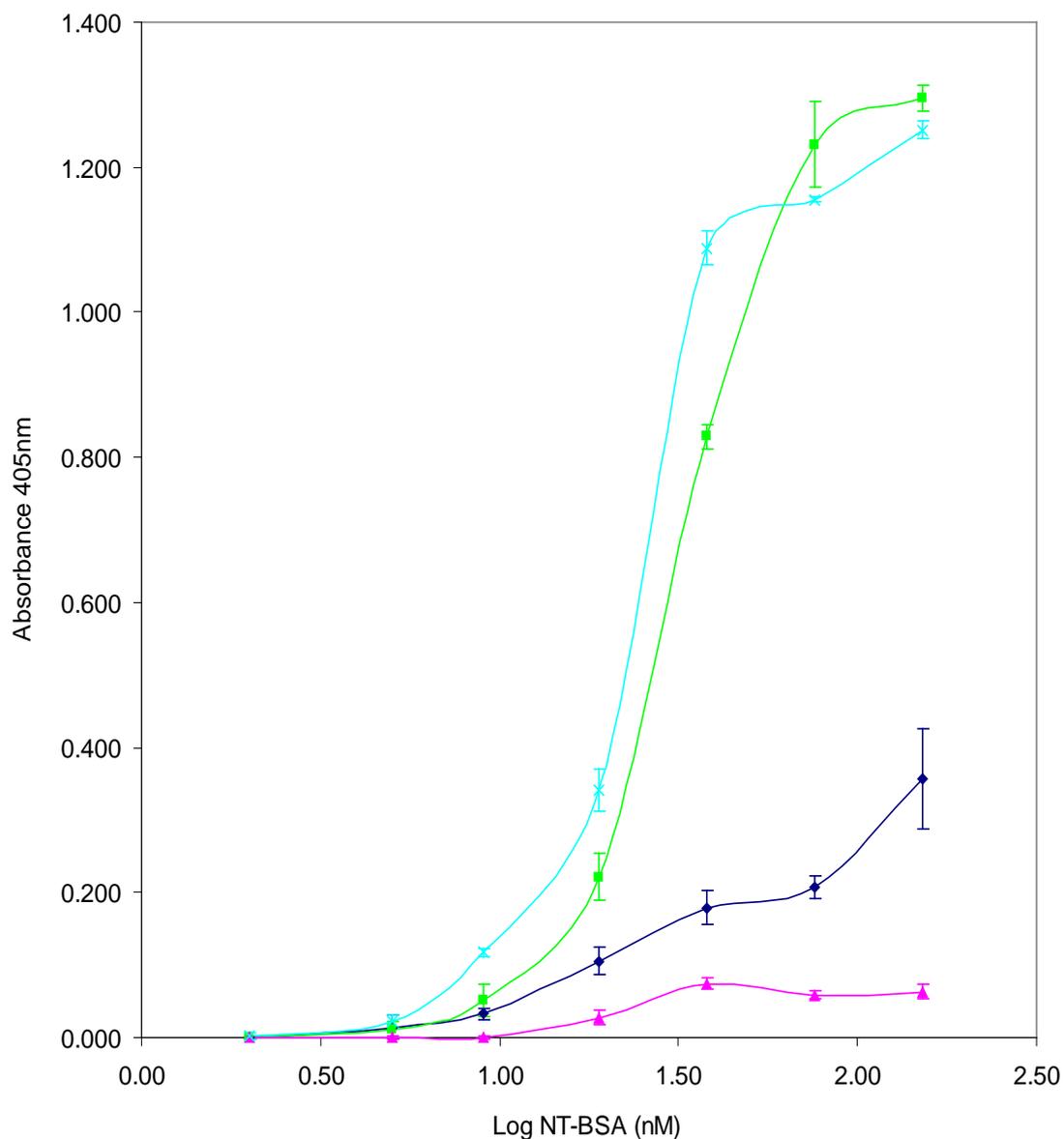
The nitrated proteins were then denatured using U+M in order to determine whether the differences observed were due to the availability of the 3NT residues to the anti-3NT Ab. After treatment with U+M it was clear that, as seen earlier, there was a significant increase in sensitivity of the assay for both of the nitrated samples (P<0.0001, for both). It was also evident that the initial differences in detection of 3NT-containing residues in ONOO<sup>-</sup>- and electrochemically-nitrated BSA were no longer observed. Indeed upon treatment with U+M the assay appeared to be marginally more sensitive to 3NT residues in electrochemically-nitrated BSA (Figure 3.3.iv.).



**Figure 3.3.iii. Nitrated BSA using different nitration protocols 3NT detection using indirect ELISA**

**Peroxynitrite-nitrated BSA (◆), electrochemically-nitrated BSA (■).**

**Mean ± SEM, samples ran in triplicate.**



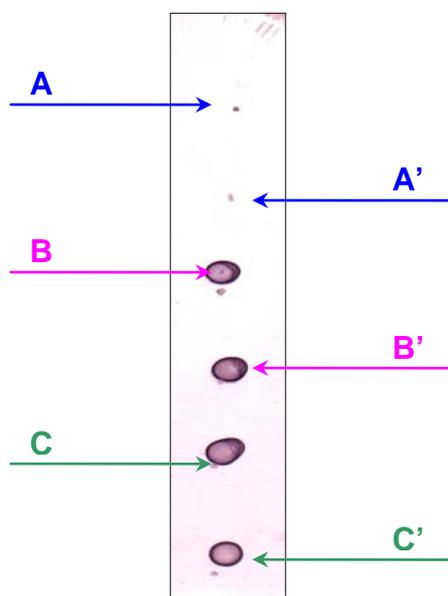
**Figure 3.3.iv. Indirect ELISA using electrochemically and peroxyntirite nitrated BSA treated or untreated with urea and mercaptoethanol.**

**Peroxyntirite nitrated BSA (◆), peroxyntirite nitrated BSA treated with U+M (■). Electrochemically nitrated BSA (▲), electrochemically nitrated BSA treated with U+M (✕). Mean ± SEM, samples ran in triplicate.**

When binding electrochemically- and  $\text{ONOO}^-$ -nitrated BSA on a nitrocellulose membrane it was observed, via dot blot immunodetection, that there was no need for sample pre-treatment with U+M. No obvious visual differences in the

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detection of 3NT between either nitration protocol both before and after treatment with U+M were observed (Figure 3.3.v).



**Figure 3.3.v. Comparison of different nitration protocols treated and untreated with urea and mercaptoethanol using dot blot.**

**A – Treated native BSA; A' – Untreated native BSA; B – Treated electrochemically nitrated BSA; B' – Untreated electrochemically nitrated BSA; C – Treated BSA nitrated using peroxynitrite; C' – Untreated BSA nitrated using peroxynitrite. All samples blotted 4 $\mu$ l of 1.25 $\mu$ g/ml.**

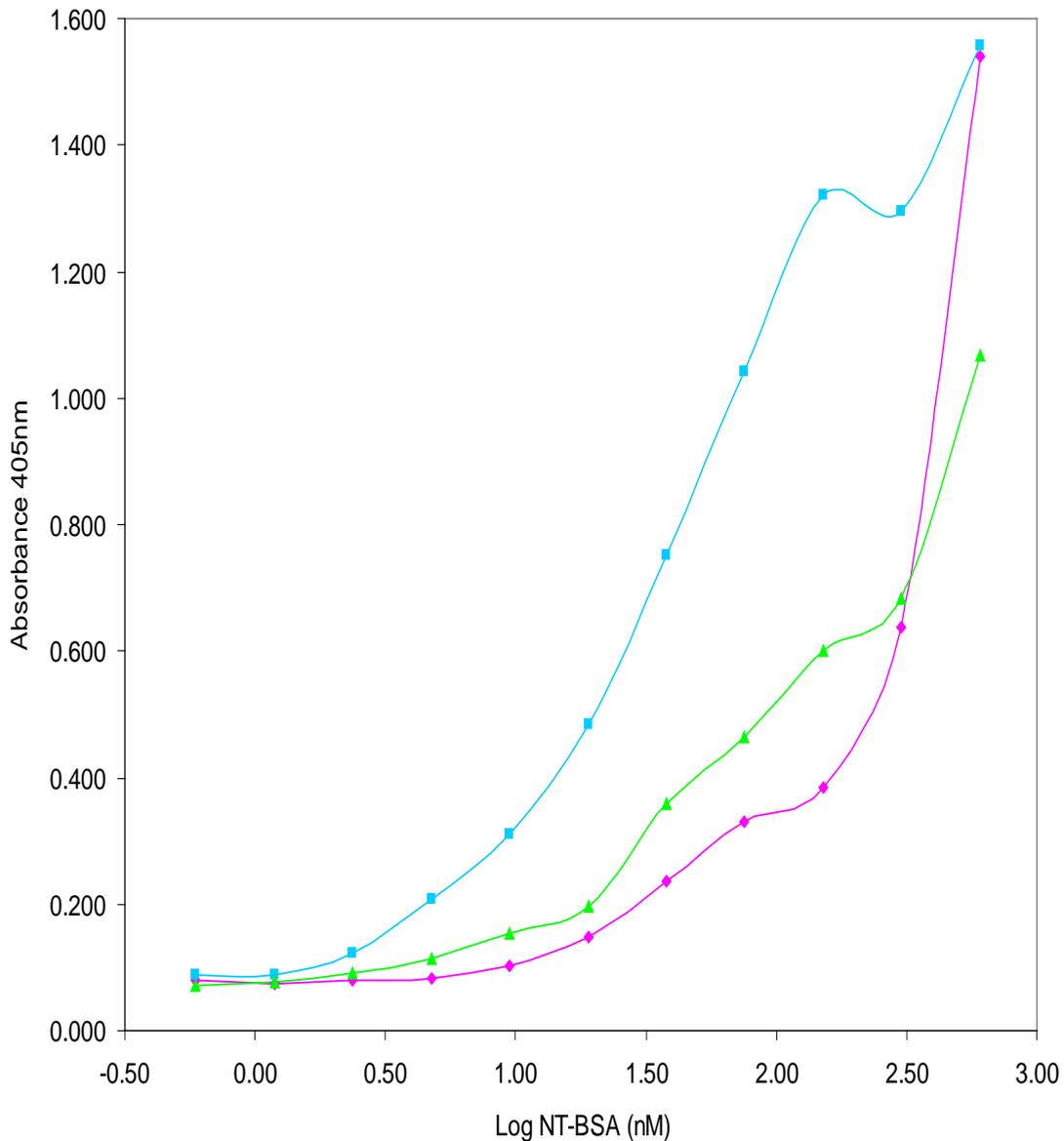
When nitrating BSA using the peroxynitrite protocol it was found that the reaction was extremely fast and the nitration level was therefore relatively difficult to control. Electrochemical nitration of BSA was a more lengthy process but it was easier to control the degree of nitration as the reaction could be paused periodically by removal of the applied potential in order to measure the absorbance of the sample at 430nm and calculate the degree of nitration. As a result of the findings above only electrochemically-nitrated BSA

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was used in all further experiments and will be referred to as NT-BSA. The level of nitration of the NT-BSA used was calculated to be 4mol 3NT / 1mol BSA.

### **3.3.3. Comparison of different denaturing protocols and microtitre plates**

Preliminary experiments produced differing results when using the indirect ELISA protocol with microtitre plates from different commercial sources. Three different brands of microtitre plates of varying cost were compared (Sero-Wel<sup>®</sup>, Immulon 2HB<sup>®</sup> and Nunc Maxisorp<sup>™</sup>). For initial comparisons between the different brands checkerboard indirect ELISAs were run using NT-BSA (with no prior denaturation) and anti-3NT Ab at varying concentrations. These preliminary comparisons suggested that the highest levels of assay sensitivity for the presence of 3NT were achieved when using the Immulon 2HB<sup>®</sup> plates (Figure 3.3.vi.). For the calibration curves run on the Immulon 2HB<sup>®</sup> plates optical densities obtained were over a broad range and were linear from 152-2.4nM with an anti-3NT Ab dilution of 1/1000. In the case of both the Sero-Wel<sup>®</sup> and Nunc Maxisorp<sup>™</sup> plates there was a narrower range of detection for 3NT residues (linear between approximately 152-9nM NT-BSA, anti-3NT Ab 1/1000). There was a significant difference in the levels of detection of 3NT-residues by the anti-3NT Ab for each plate ( $P < 0.001$ ).



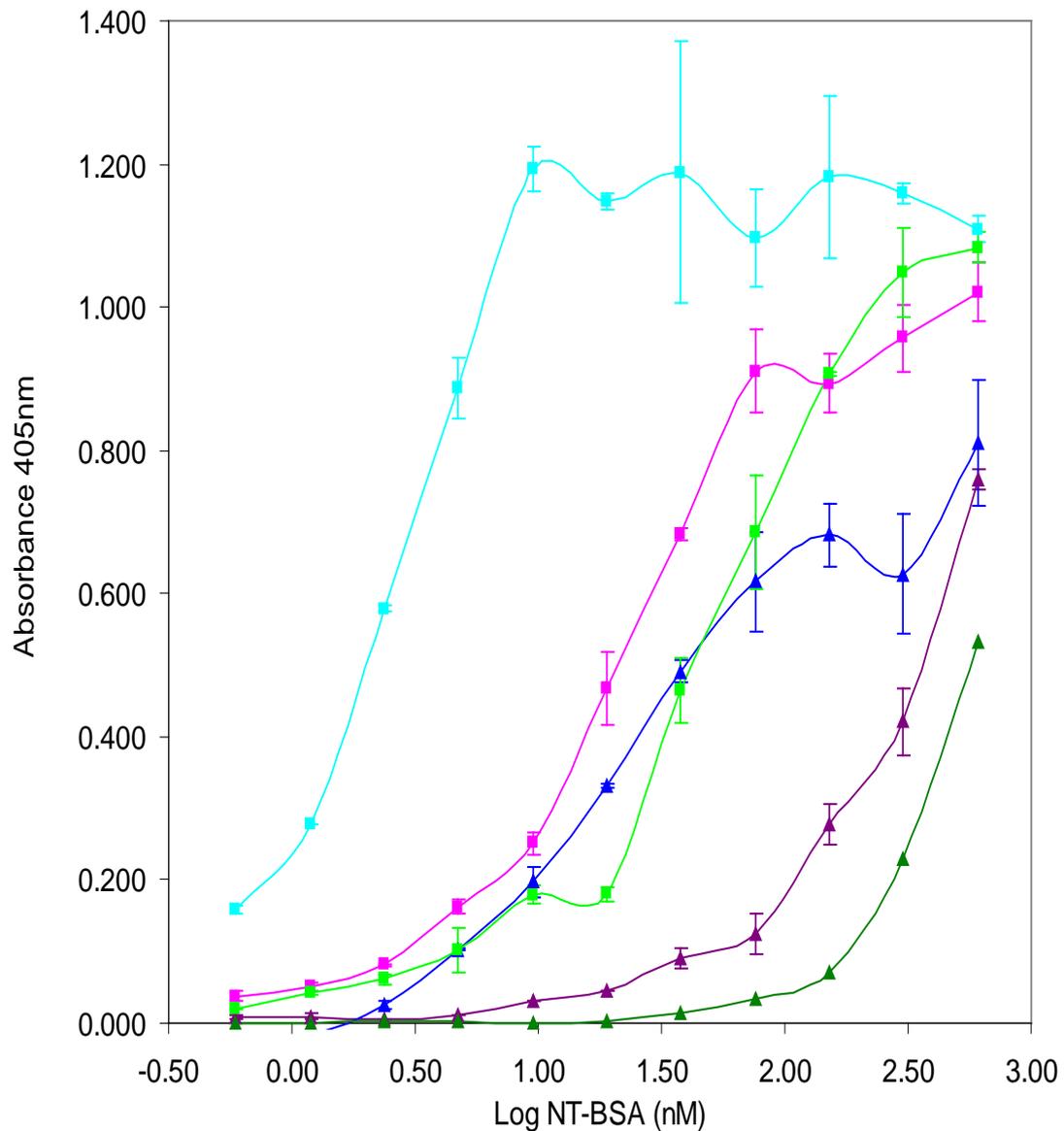
**Figure 3.3.vi. Summary of checkerboard indirect ELISA comparing different brand microtitre plates.**

**Nunc Maxisorp™ (◆), Immulon 2HB® (■) and Sero-Wel® (▲). The above data comes from checkerboard analysis using each brand microtitre plate. Single point data representing the values obtained when using the anti-3NT Ab at 1/1000 dilution, which had been determined as the optimal dilution. See appendices H for full checkerboard analysis.**

To examine the role of denaturation in the detection of 3NT residues in NT-BSA, U+M-treated NT-BSA was directly compared to NT-BSA on each of the plates. The results of this comparison are shown in Figure 3.3.vii. As before

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there was an increase in assay sensitivity for denatured NT-BSA, but there were also obvious differences between the different plates seen across the concentration range. With regards to the Sero-Wel<sup>®</sup> microtitre plates it was clear that the detection limits and assay sensitivity for both native and denatured NT-BSA was extremely low, as a result of which these plates were not used in further indirect ELISA development. When comparing the Immulon 2HB<sup>®</sup> and Nunc Maxisorp<sup>™</sup> microtitre plates smaller differences in sensitivity were observed. However, for both native and denatured NT-BSA higher optical densities were obtained when using the Immulon 2HB<sup>®</sup> plates (Figure 3.3.vii). As a result of these findings all further indirect ELISA analysis was carried out using the Immulon 2HB<sup>®</sup> microtitre plates.



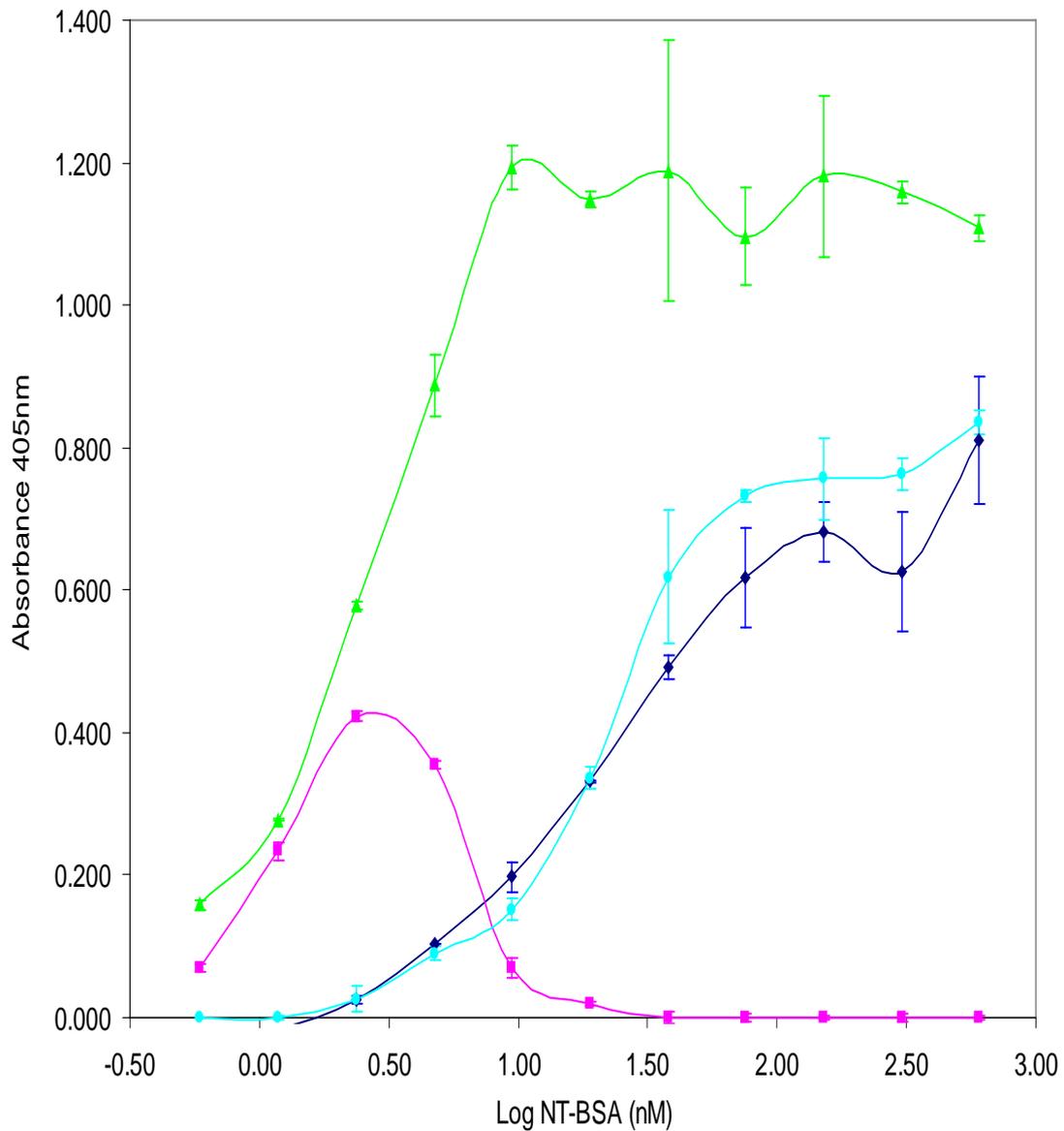
**Figure 3.3.vii. The effects of protein pre-treatment and different brand microtitre plates using indirect ELISA for the detection of NT-BSA.**

The plates tested were Immulon 2HB<sup>®</sup> (NT-BSA (▲) and U+M treated NT-BSA(■)); Nunc Maxisorp<sup>™</sup> (NT-BSA (▲) and U+M treated NT-BSA(■)); and Sero-Wel<sup>®</sup> (NT-BSA (▲) and U+M treated NT-BSA(■)). Mean  $\pm$  SEM, samples ran in duplicate.

The effects of protein denaturation were further investigated using alternative denaturants for use with NT-BSA. Proteins were denatured by boiling in the presence of a detergent (Tween 20) or treatment with trypsin to digest the

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protein. These were then investigated as potential procedures for a more sensitive ELISA method. A large number of trials of these different procedures were conducted and a summary is presented below. All denaturing treatments and native untreated NT-BSA were compared firstly in an indirect ELISA (Figure 3.3.viii.). None of these alternative denaturing treatments gave the same increase in assay sensitivity as seen when using U+M to denature the NT-BSA. It was evident that denaturing NT-BSA by boiling it in the presence of Tween 20 actually had a detrimental effect on the sensitivity of the assay. With regards to the trypsin digested NT-BSA there was no significant difference between it and the native NT-BSA; although, when using untreated NT-BSA it was a common finding that wells gave false negative results. This anomaly was found to be greatly reduced when using tryptically digested NT-BSA as the standard antigen. As a result of this tryptically digested NT-BSA was further investigated in the development of the competitive ELISAs.



**Figure 3.3.viii. Comparison of different protocols to denature NT-BSA for use in indirect ELISA**

The plates used to carry out these tests were Immulon 2HB<sup>®</sup> and the anti-3NT Ab was 1/1000. NT-BSA untreated (◆) and U+M treated NT-BSA (▲); NT-BSA boiled in the presence of Tween 20 (■) and trypsin digested NT-BSA (●), samples ran in duplicate. The above data is representative of multiple trials conducted when assessing the importance of different denaturing protocols.

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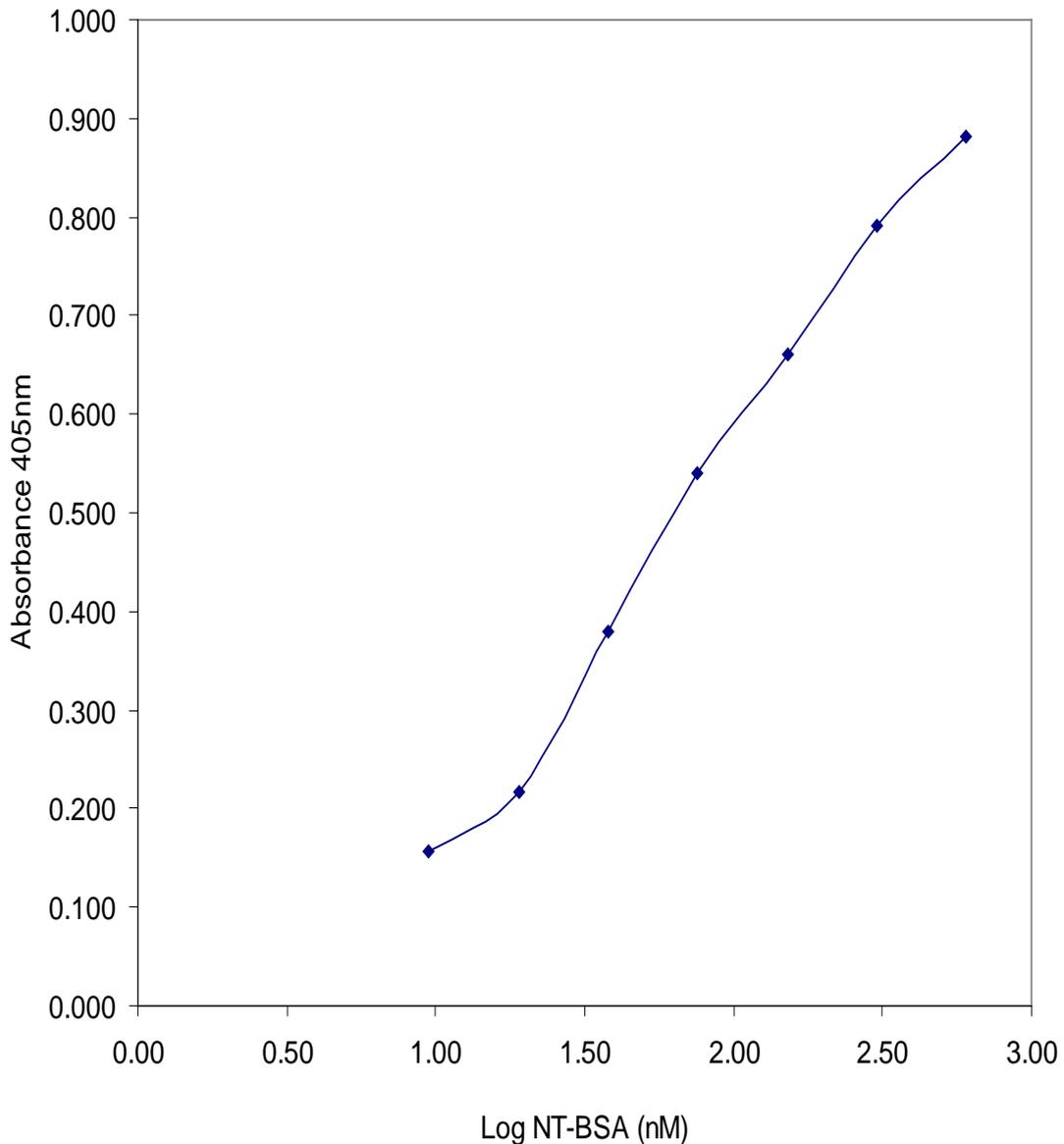
## **Competitive ELISA**

### **3.3.4. Competitive ELISA development**

All competitive ELISA development was carried out using electrochemically nitrated BSA (NT-BSA, 4mol 3NT/mol BSA) and Immulon 2HB<sup>®</sup> microtitre plates. The analysis of different brands of microtitre plates was repeated for the development of the competitive ELISA and the findings agreed with those reported in section 3.3.3., (results not shown).

Initially it was apparent that no competition could be achieved between the anti-3NT Ab and the NT-BSA in solution when it was freshly nitrated, (results not shown). However after the NT-BSA had been through a period of storage at -20°C and through the freeze thaw process a number of times, competitive checkerboard analysis suggested that the assay gave a linear response over a broad range of NT-BSA (606-9nM) when using 1/1000 anti-3NT Ab dilution (Figure 3.3.ix.). However there were problems regarding assay sensitivity, reproducibility and reliability when using untreated NT-BSA as both the coating antigen and as the standard in competition (results not shown).

Therefore the effects of different forms of sample pre-treatment were investigated in order to increase the exposure of the 3NT residues to the anti-3NT Ab. Either untreated NT-BSA or tryptically digested NT-BSA (tryp-NT-BSA) were coated onto the microtitre plates and used as the test antigen in competition. (Note: - Only tryp-NT-BSA was used as denatured sample since residual reagents from the other methods (e.g. U+M) could have potentially affected the antibody during the competitive stages of the assay.)



**Figure 3.3.ix. Checkerboard competitive ELISA on Immulon 2HB<sup>®</sup> microtitre plates**

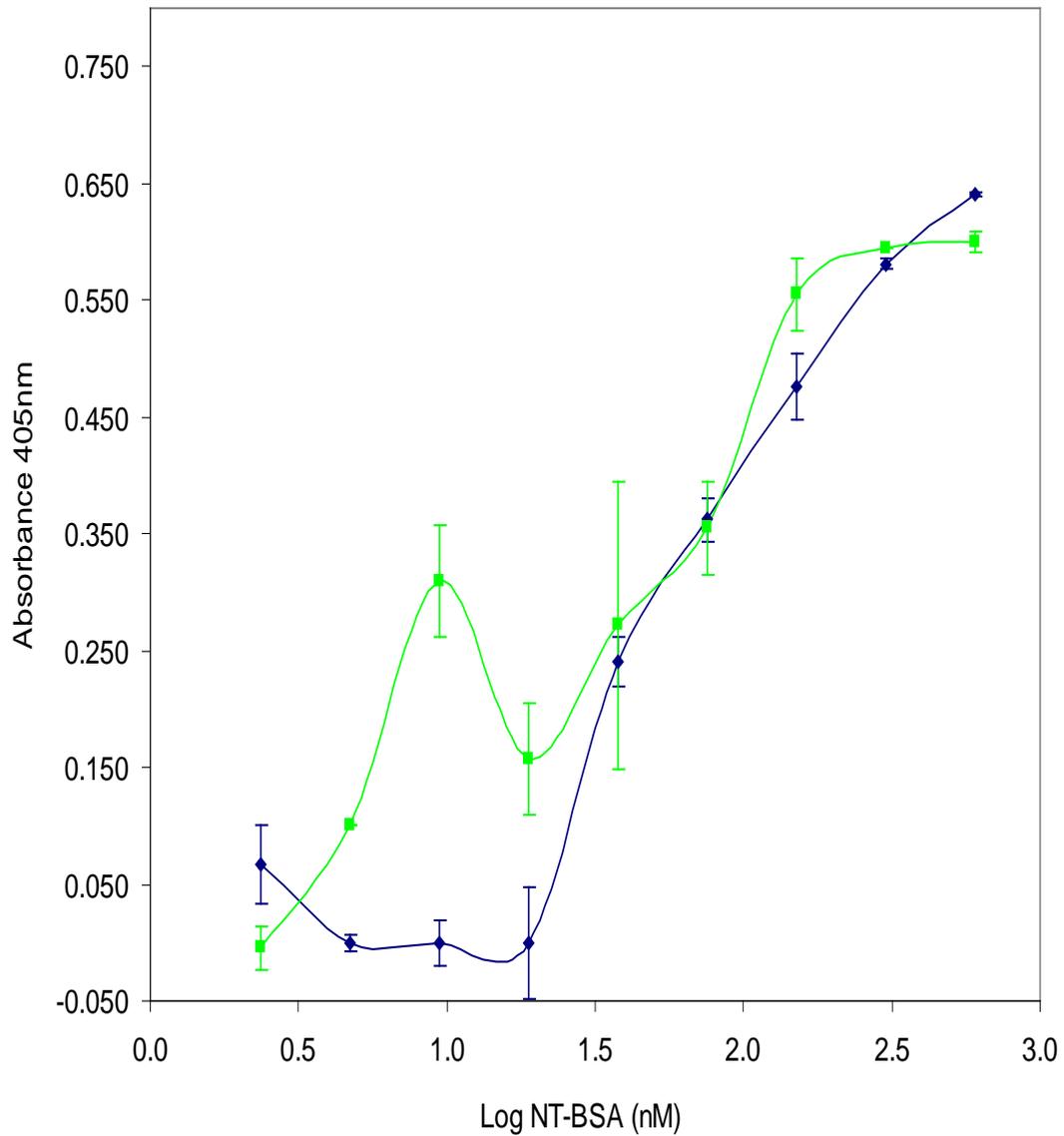
The above figure represents single point data taken from a checkerboard competitive ELISA over a range of NT-BSA (◆) concentrations in the competitive stage (anti-3NT Ab 1/1000). The microtitre plate was coated at 2µg/ml of NT-BSA. See appendices I for full checkerboard analysis data.

It was evident that when the plate was coated with native NT-BSA the levels of competition achieved for native and tryp-NT-BSA were low (see Figure 3.3.x.). When native NT-BSA was used as the coating antigen no there was

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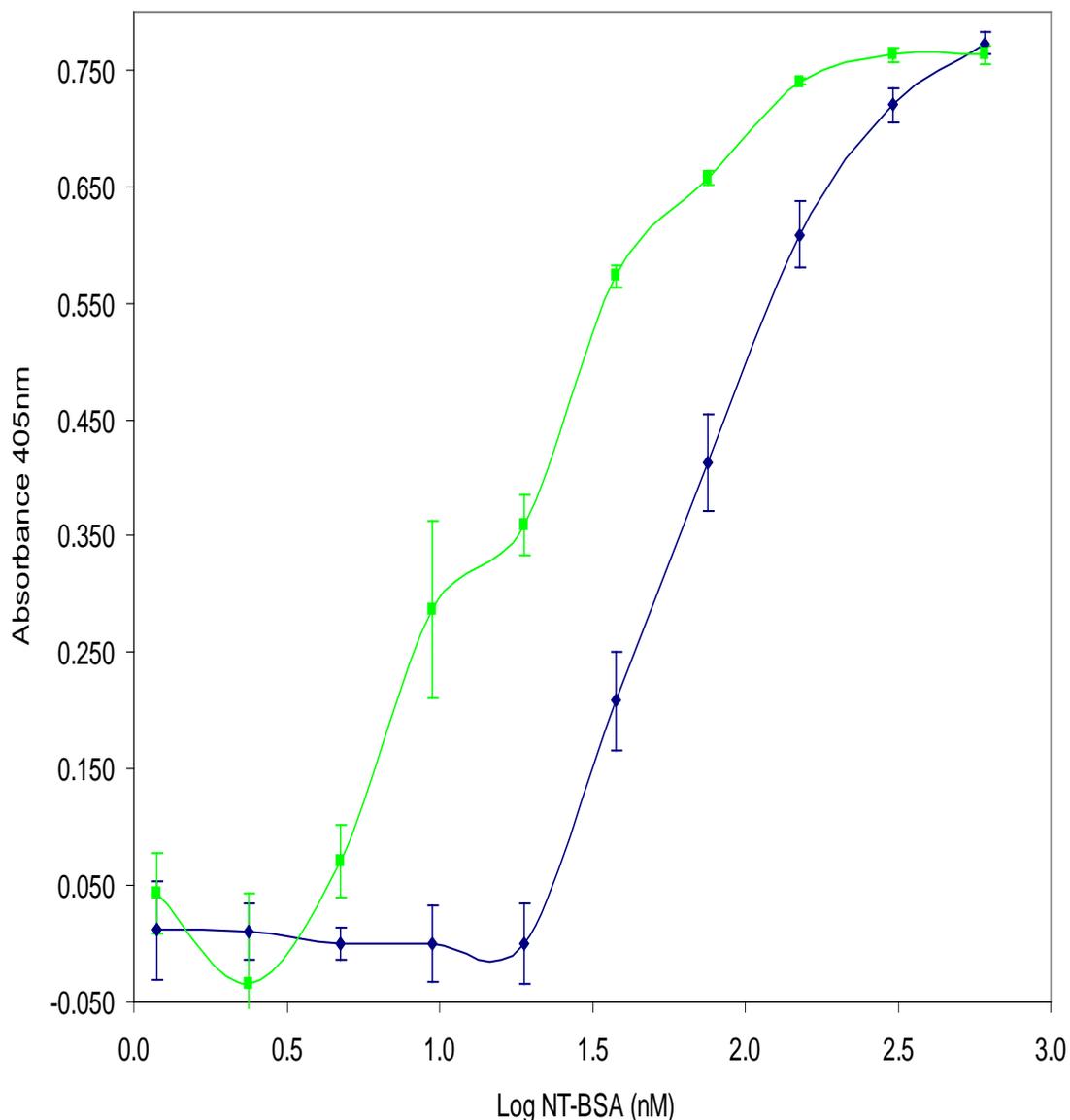
no statistical difference between the level of detection of 3NT residues in native NT-BSA compared to trypsin digested NT-BSA during competition with the anti-3NT Ab ( $P>0.05$ , two way ANOVA with replication). In contrast when the plate was coated with tryp-NT-BSA higher levels of competition were achieved (Figure 3.3.xi.). It was also evident that when tryp-NT-BSA was used as the coating antigen there was a reduced level of assay variation, as shown by the SEM. A significant increase in the level of detection of 3NT residues by the anti-3NT Ab was observed for tryp-NT-BSA compared to the native NT-BSA ( $P<0.0001$ , two way ANOVA with replication, Figure 3.3.xi.).

These findings were found to be consistently reproducible in subsequent assays. All further competitive ELISAs therefore used tryp-NT-BSA as the antigen for coating and as a standard.



**Figure 3.3.x. A competitive ELISA using NT-BSA as the coating antigen and competition with NT-BSA or trypsin digested NT-BSA**

The competing antigen was either NT-BSA (◆) or trypsin digested NT-BSA (■). Standard curves for both antigens were run from 606-0.3nM. (mean  $\pm$  SEM, samples ran in duplicate).



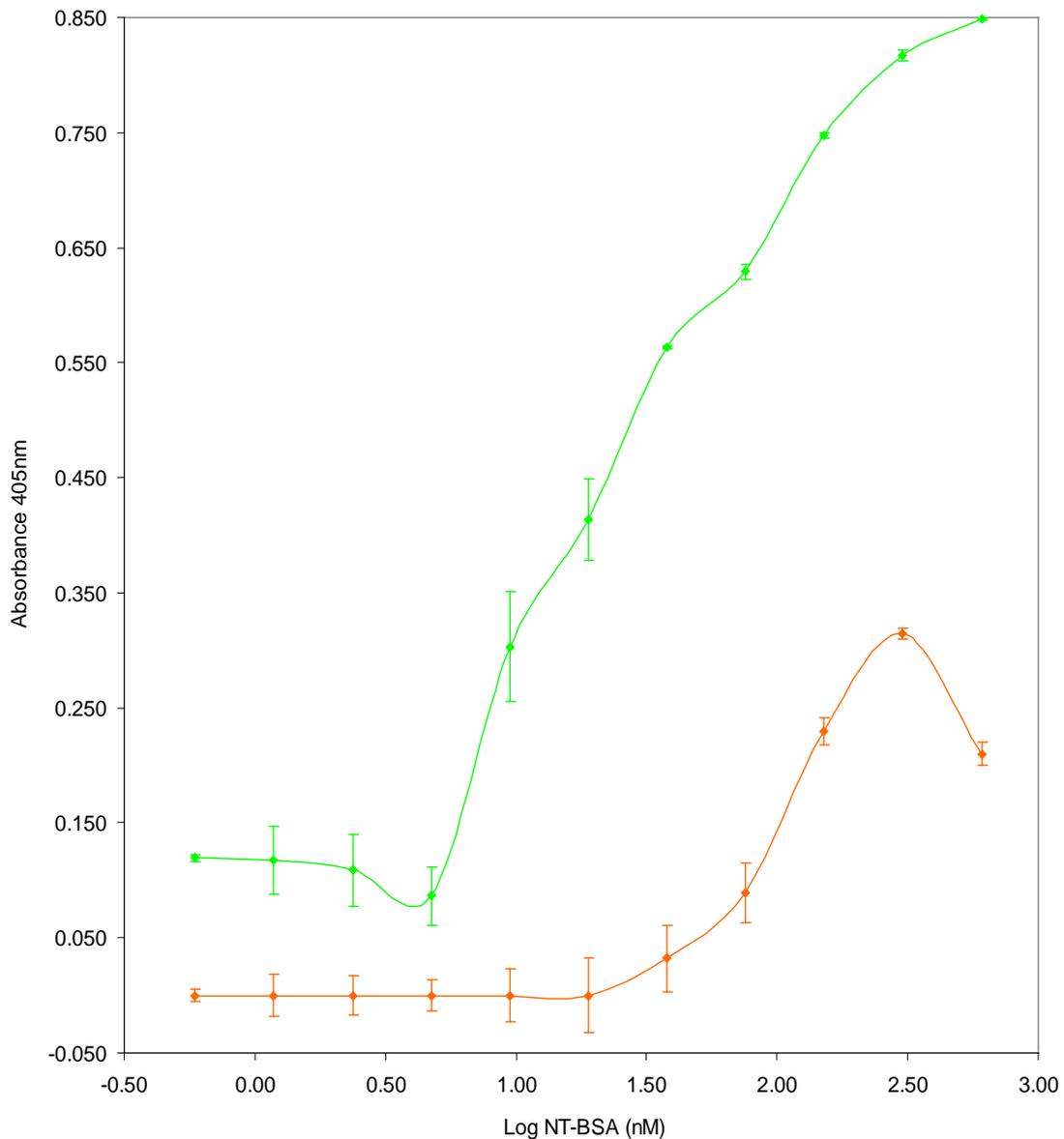
**Figure 3.3.xi. A competitive ELISA using trypsin digested NT-BSA as the coating antigen and competition with either NT-BSA or trypsin digested NT-BSA.**

**The competing antigen was either NT-BSA (◆) or trypsin digested NT-BSA (■). Standard curves for both antigens were run from 606-0.3nM. (mean ± SEM, samples ran in duplicate).**

To further improve the assay sensitivity, reliability and reproducibility several assay parameters were investigated. When the concentration of the coating antigen was increased from 2µg/ml to 4µg/ml of tryp-NT-BSA there was an

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obvious improvement in assay sensitivity (Figure 3.3.xii.). Furthermore the standard curve produced was linear over a larger concentration range (606-2nM), demonstrating improved assay sensitivity. Increased assay variation was observed, for both coating concentrations, as the competing tryp-NT-BSA concentration reduced. The continuing issue regarding assay sensitivity and reproducibility is further evident when comparing the observed corrected optical densities achieved when the coating antigen was 2 $\mu$ g/ml in Figure 3.3.xii. and those observed in Figure 3.3.xi.. These experiments were repeated on several occasions with more replicates in order to further assess the observed assay variation (results were as shown in Figure 3.3.xii). Although increasing the concentration of the coating antigen improved assay sensitivity, further investigations regarding the assay parameters were needed regarding assay variation.



**Figure 3.3.xii. Competitive ELISAs with different concentrations of trypsin digested NT-BSA to coat the plate.**

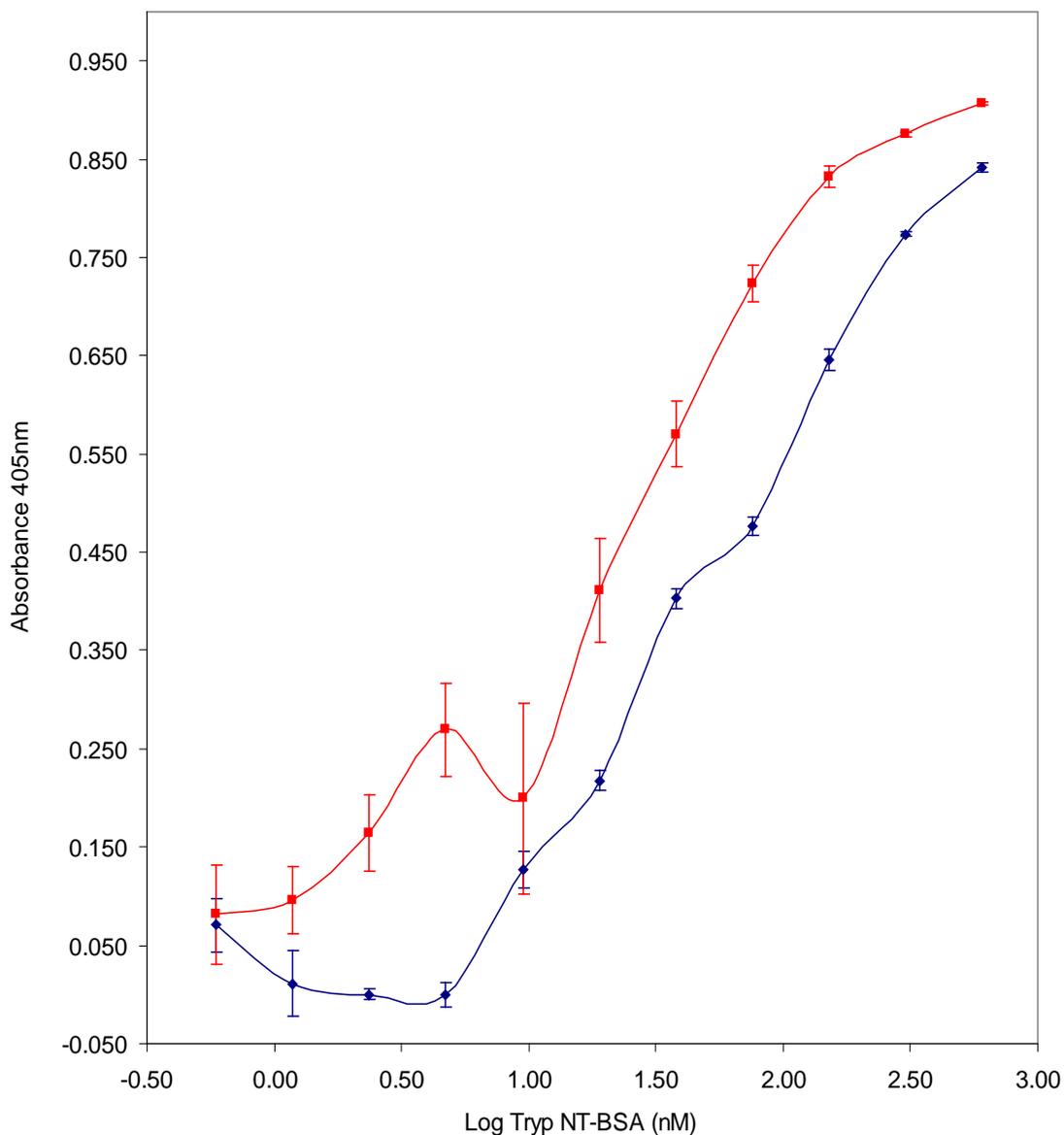
Tryp-NT-BSA coated at 4µg/ml (◆) and coated at 2µg/ml (◆). In competition the tryp-NT-BSA standard curve ran from 606-0.6nM. The above data is mean data ± SEM, samples ran in duplicate, anti-3NT Ab 1/1000 dilution.

The constituents of the incubation buffer used within the competitive assay were altered in order to assess the effects these had on sensitivity and reliability. Concentrations of BSA, ovalbumin and Tween 20 were varied. A

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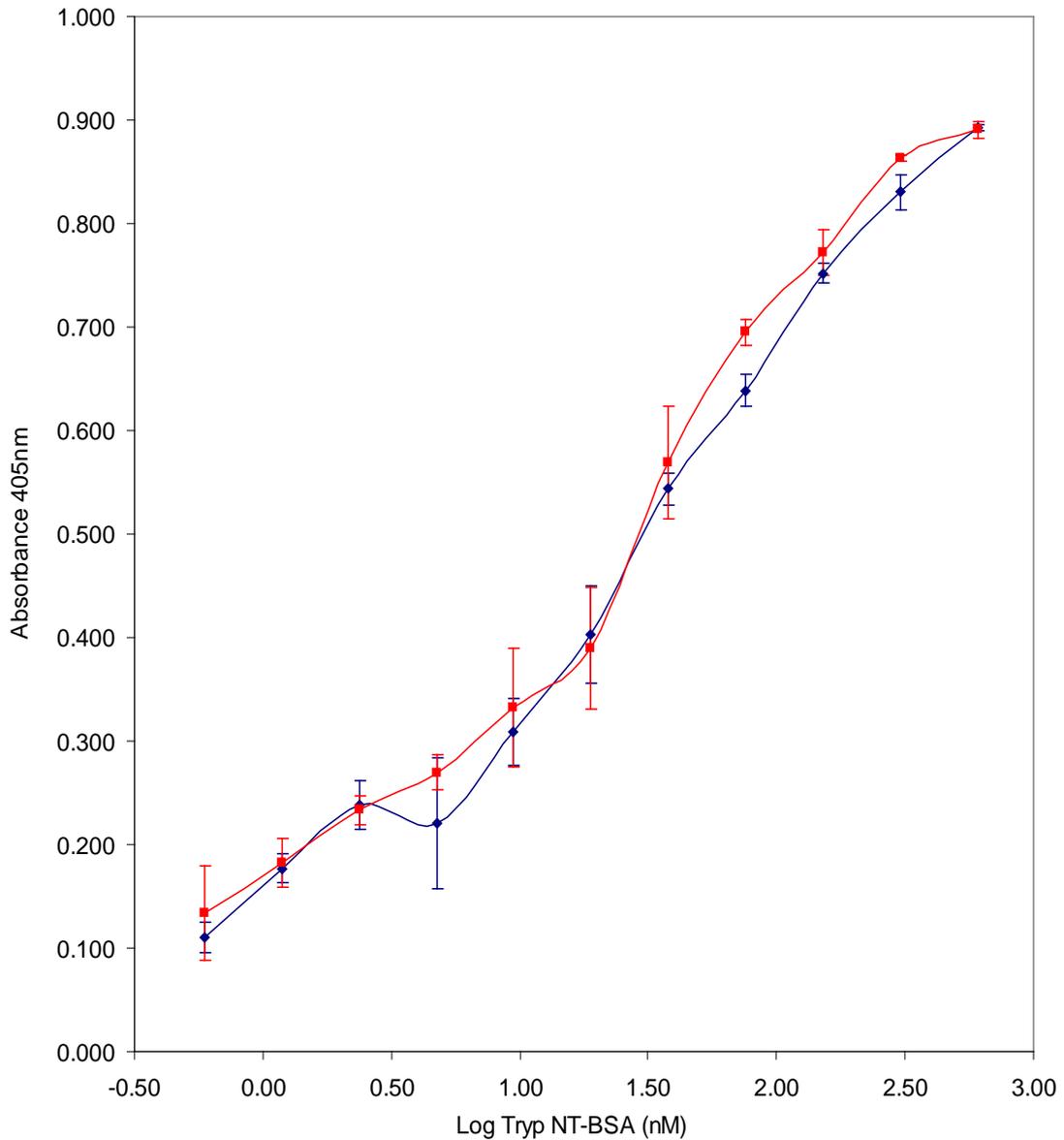
significant increase in the levels of competition was achieved when using incubation buffer containing 1% ovalbumin and 0.1% Tween 20 compared to incubation buffer containing 1% BSA and 0.1% Tween 20 ( $P < 0.0001$  two way ANOVA with replication). However a higher level of variation between replicates was observed with 1% ovalbumin and 0.1% Tween 20 (Figure 3.3.xiii.). There was no longer a statistical difference between the levels of competition achieved for tryp-NT-BSA diluted in incubation buffer containing 1% ovalbumin or BSA when there was a reduction of Tween 20 from 0.1% to 0.05% (Figure 3.3.xiv.,  $P > 0.05$  two way ANOVA with replication).

Therefore it was decided that for all further competitive ELISA assays the concentration of the coating antigen would be  $4\mu\text{g/ml}$  and the incubation buffer would contain 1% BSA and 0.05% Tween 20.



**Figure 3.3.xiii. The effects of changing the incubation buffer parameters on intra-plate variation (part 1).**

**A comparison of 1% BSA (◆) and ovalbumin (■) using 0.1% Tween 20 as incubation buffer components. The plates were coated with tryp-NT-BSA (4µg/ml) and the antigen in competition was tryp-NT-BSA run from 606-0.6nM (mean, ± SEM, 4 replicates per dilution).**



**Figure 3.3.xiv. The effects of changing the incubation buffer parameters on intra plate variation (part 2).**

**A comparison of 1% BSA (◆) and ovalbumin (■) using 0.05% Tween 20 as incubation buffer components. The plates were coated with tryp-NT-BSA (4µg/ml) and the antigen in competition was tryp-NT-BSA run from 606-0.6nM (mean, ± SEM, 4 replicates per dilution).**

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### 3.3.5. Competitive ELISA reliability studies

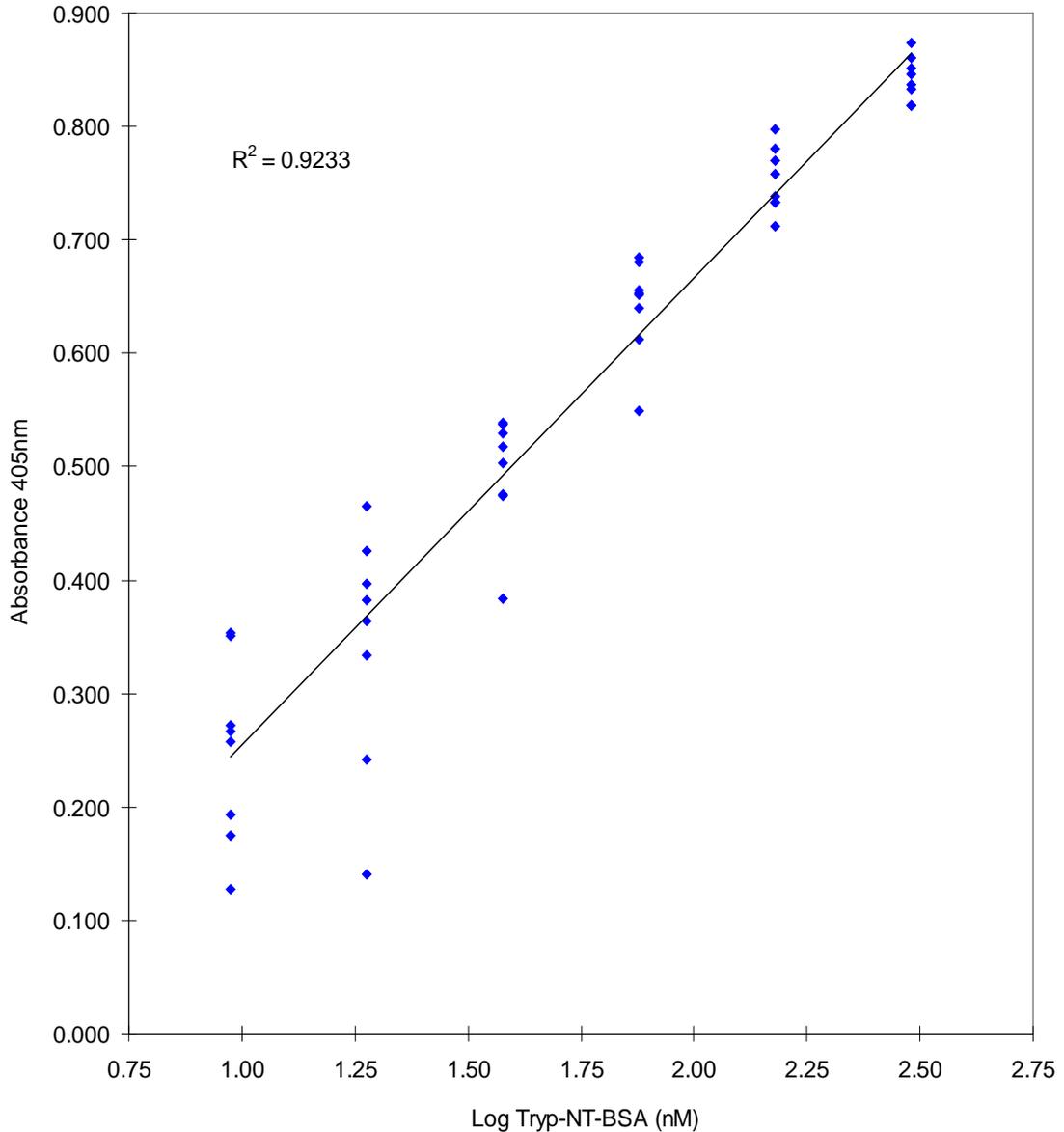
In order to investigate inter- and intra-plate variation in the competitive ELISA a number of repeat assays were performed. Standard curves were generated using tryp-NT-BSA as both the coating antigen and competitor. Control samples of known concentration of tryp-NT-BSA and of nitrated plasma (NT-PL, estimated concentration) were included in each plate. Regression analysis was used to calculate concentrations from the calibration curves.

Intra-assay variation was assessed with 8 replicates of each standard dilution on a plate and is presented in Figure 3.3.xv. and Table 3.3.i. The standard curve was linear over a range of 9.47-303nM ( $R^2 = 0.993$ ). However, with decreasing concentration of the standards the variation can be seen to increase (Figure 3.3.xv.) also seen as increasing coefficients of variation in Table 3.3.i. Percentage coefficients of variation values  $\leq 10\%$ , the accepted range, were only obtained for concentrations ranging between 75.8-303nM tryp-NT-BSA which further suggested that the assay lost precision at low levels of nitration.

<b>Tryp-NT-BSA (nM)</b>	303.0	151.5	75.8	37.9	18.9	9.5
<b>Log Tryp-NT-BSA (nM)</b>	2.5	2.2	1.9	1.6	1.3	1.0
<b>%CV</b>	2.3	3.8	6.8	10.5	30.6	32.3

**Table. 3.3.i. Percentage coefficient of variation within an assay for tryp-NT-BSA.**

The above data represents calculated percentage coefficient of variation (%CV) for serial dilutions of tryp-NT-BSA (n=8 replicates for each dilution).



**Figure 3.3.xv. Regression analysis for competitive ELISA using tryp-NT-BSA as coating and test antigen.**

**Above figure represents corrected optical densities measured in serial dilutions of the standard competing antigen (tryp-NT-BSA, ♦) and the resulting regression coefficient. 8 replicates per dilution.**

A similar approach was taken to assess inter-plate performance. Fourteen competitive ELISAs were run using tryp-NT-BSA as the competitor to produce the standard curves along with the controls (all samples were run in triplicate).

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Standard curves produced on each plate were generally found to be linear from 303-2.4nM trypan-NT-BSA. However, the linear range did vary slightly from plate to plate.  $R^2$  values were generally greater than 0.930 (range 0.805-0.976). Again variation increased as the concentration of trypan-NT-BSA decreased, which can be seen by the %CV values in table 3.3.ii.

<b>Trypan-NT-BSA (nM)</b>	303.0	151.5	37.9	9.5	4.7	2.4
<b>Log Trypan-NT-BSA (nM)</b>	2.5	2.2	1.9	1.6	1.3	1.0
<b>%CV</b>	4	8	22	52	80	170

**Table. 3.3.ii. Percentage coefficient of variations for inter-assay study using trypan-NT-BSA.**

The above data represents calculated percentage coefficient of variation (%CV) for serial dilutions of trypan-NT-BSA (n=3 replicates for each dilution, 14 plates).

For some assays the intra-assay %CV between replicates was well within 10% but for other plates %CVs of >20% were obtained. For inter-assay variation the %CV was found to be less than 10% for high concentrations of trypan-NT-BSA (303.0-151.5nM) but then increased significantly.

The lack of consistency observed within and between assays limits the reliability of concentration estimations of the controls. Triplicates of trypan-NT-BSA (76, 38 and 9nM of trypan-NT-BSA nitrated to a level of 4 mol 3NT / 1 mol BSA) and NT-PL (76nM estimated level of nitration equivalent to 4 mol 3NT / 1 mol BSA) were included on each plate. When using standard curves to estimate the levels of nitration in the test trypan-NT-BSA samples, only one of the fourteen competitive ELISAs provided acceptable estimated values (level of accuracy  $\pm 10\%$  of the known value) for the 76 and 9nM standards and two assays for the 38nM standard. The %CV for the samples within each plate varied between 0-44%, although the majority of assays had %CV values

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<20%. Estimates between each assay plate were less reliable with %CVs significantly above the accepted 10%.

Results were more reliable for the electrochemically nitrated plasma samples (NT-PL). Of the fourteen plates run, nine gave concentrations of comparable levels of nitration (exact levels cannot be quoted due to this being nitrated plasma). The average estimated concentration for the NT-PL test sample in these nine plates was  $92 \pm 24$  nM nitro-BSA equivalents. With the exception of one plate the intra-assay %CV for NT-PL was found to be  $\leq 20\%$  but the inter-assay %CV for NT-PL was higher. Although the assay appeared to be more precise and reliable regarding the NT-PL samples the intra- and inter-assay %CVs were still higher than the accepted  $\leq 10\%$ . These results suggest that the use of nitrated plasma in a competitive ELISA needed further investigation.

These assay plates were also those used to calculate the levels of nitration found within the claudicant and control plasma samples (affinity purified, see chapter 4, section 4.3.3. for these results).

### **3.3.6. Nitrated plasma as a potential coating and competitive antigen**

As a result of the findings above electrochemically nitrated plasma (estimated level of nitration equivalent to 4 mol 3NT / 1 mol BSA) was investigated as a potential alternative coating and competitive antigen for the developed competitive ELISA. During these investigations competitive ELISAs were coated with either NT-PL or tryp-NT-BSA at comparable concentrations and then serial dilutions of NT-PL or tryp-NT-BSA were used as competing antigens. A variety of assay parameters, e.g. incubation times, were also varied. It was evident that when NT-PL was used as the coating antigen the

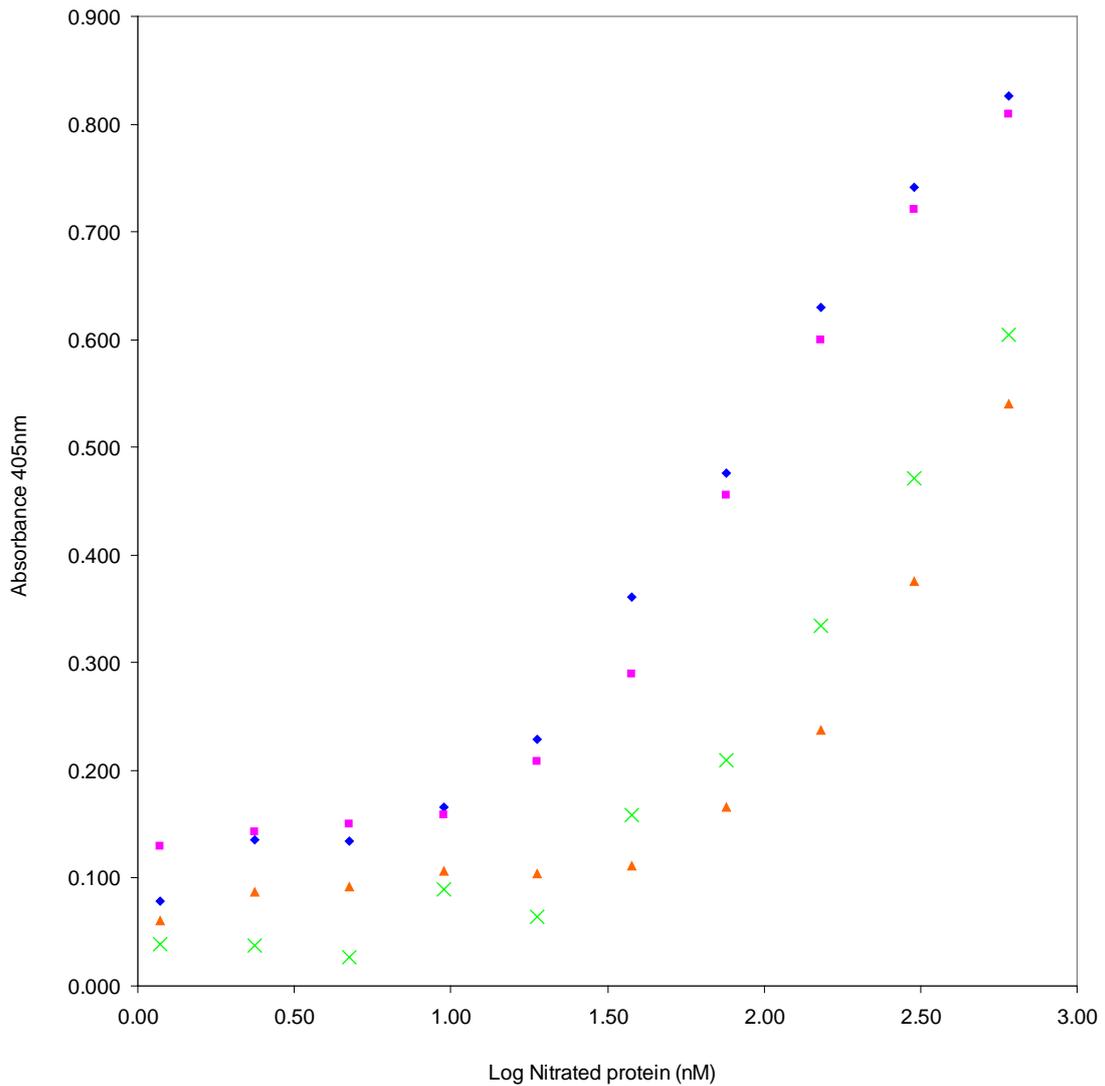
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assay sensitivity was reduced for both NT-PL and tryp-NT-BSA in competition (Figure 3.3.xvi.). NT-PL and tryp-NT-BSA were found to compete at comparative levels when the plates were coated with tryp-NT-BSA (Figure 3.3.xvi.). No improvements in intra- or inter-assay were observed when using NT-PL as the coating or the competing antigen, as determined by %CVs, compared to tryp-NT-BSA.

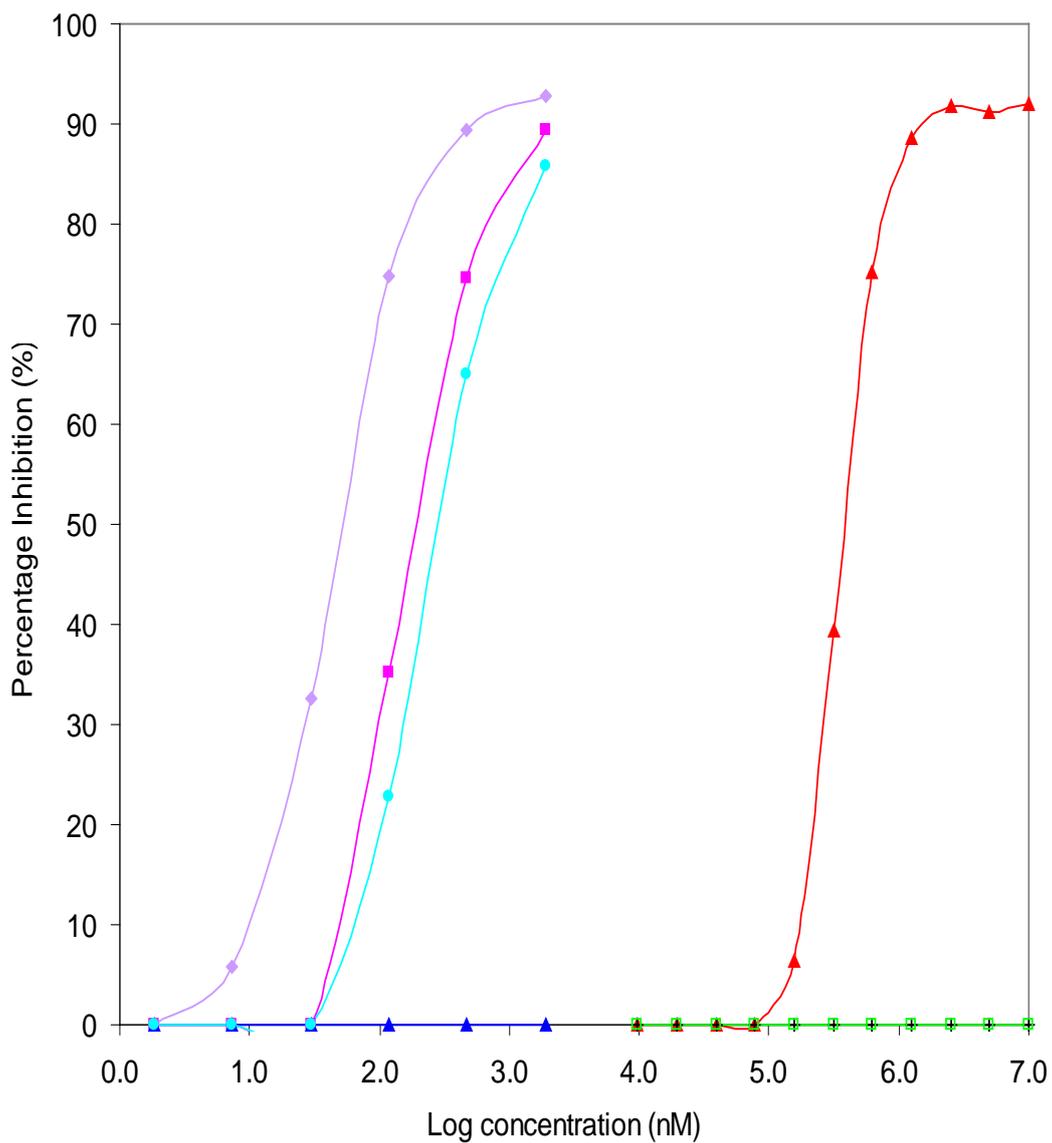
### **3.3.7. Specificity of the anti-3NT Ab within the competitive ELISA**

Percentage inhibition studies were carried out to assess the specificity of the anti-3NT Ab within the competitive ELISA assay. Several compounds of similar structure to tyrosine and 3NT were investigated and the percentage inhibition was noted. The anti-3NT Ab appeared to have the highest affinity for tryp-NT-BSA followed by NT-BSA, protein bound aminotyrosine and finally free 3-nitro-L-tyrosine. With regards to native BSA, chloro-L-tyrosine (free amino acid) and L-tyrosine (free amino acid) no inhibition of the antibody was observed (Figure 3.3.xvii.).



**Figure 3.3.xvi. Competitive ELISA using nitrated plasma and tryp-NT-BSA as coating and test antigens.**

Plates were coated with either nitrated plasma (NT-PL) or tryp-NT-BSA (4 $\mu$ g/ml at comparable levels of nitration). Serial dilutions of tryp-NT-BSA were run on the plates coated with tryp-NT-BSA (◆) and NT-PL (■) and serial dilutions of NT-PL were also run on the plates coated with tryp-NT-BSA (✕) and NT-PL (▲). N=3 plates per coating antigen with 4 replicates per dilution on each plate.



**Figure 3.3.xvii. Percentage inhibition study of anti-3NT antibody binding in the presence of different antigens in the competitive ELISA**

The plates were coated with trypt-NT-BSA (4 $\mu$ g/ml). The competing free antigens were: trypt-NT-BSA ( $\blacklozenge$ , n=3 experiments with 2 replicates on each plate); for the following antigens n=3 replicates on one plate NT-BSA ( $\blacklozenge$ ); BSA ( $\blacktriangle$ ); protein bound aminotyrosine ( $\blacklozenge$ ); free 3-nitro-L-tyrosine ( $\blacktriangle$ ); free L-tyrosine (+); free chloro-L-tyrosine ( $\square$ ).

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## **3.4. Discussion**

### **3.4.1. Indirect ELISA development**

A major objective of this thesis was to develop an immunoassay for the detection of 3NT in clinical samples, specifically the plasma of claudicants. The successful use of immunoassays for 3NT detection has been reported by a number of authors (Ye, Strong, Huang *et al.* 1996, Khan, Brennand, Bradley *et al.* 1998, Ter Steege, Koster-Kamphuis, Van Straaten *et al.* 1998, Inoue, Hisamatsu, Ando *et al.* 2002, Franze, Weller, Niessner *et al.* 2004). These studies used a variety of nitration protocols with either polyclonal or monoclonal antibodies. The results presented in this chapter have highlighted a range of issues concerning the development of a sensitive, reproducible and interpretable immunoassay for the detection of 3NT in biological samples and suggest that further work is required.

First of all there is the issue of availability of 3NT within the protein and its accessibility to an anti-3NT antibody. The results clearly demonstrated that an identical antigen stored at -20°C and freeze-thawed a number of times (NT-BSA/99) had a greater number of exposed 3NT-residues than a more recently produced antigen (NT-BSA/02) electrochemically nitrated to the same level.

Freeze thawing is known to denature proteins. Substantial protein denaturation can occur in proteins stored in sodium phosphate buffered solutions going through freeze-thaw cycles potentially due to the selective precipitation, and thus concentration, of the disodium phosphate as ice crystals form. This observed precipitation, when using sodium phosphate buffer solutions, causes a significant shift in pH which could potentially affect

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the stability of the dissolved proteins, although this has not been found to be the case within potassium phosphate buffered solutions (Pikal-Cleland, Rodriguez-Hornedo, Amidon *et al.* 2000). Strambini and Gabellieri (1996) have put forward an alternative hypothesis regarding the effects of storage on protein conformation involving protein-ice interactions. They demonstrated for the first time that the native fold of a protein is altered in frozen solutions due to the strain on the protein fold which can be attributed to the adsorption of the protein molecule onto the ice surface.

Further investigation of this was conducted by exploring different denaturation protocols, mainly using urea and mercaptoethanol (U+M). The use of urea as a denaturant allows the unfolding of protein molecules making cysteine residues available for reduction using mercaptoethanol (Zadow and Hardham 1980). Enhanced reliability and specificity of an ELISA via the use of mercaptoethanol to cleave/unfold an antigen in order to expose specific epitopes to the relevant antibody has been reported by Abass *et al* (2006). By chemically denaturing the nitrated samples (NT-BSA/99 and NT-BSA/02) both should be in similar degraded states and should, therefore, have allowed for maximal availability of the 3NT-epitopes to the anti-3NT Ab. However, although both showed an increase in OD after treatment a higher OD was obtained for the NT-BSA/02. This may have been due to protein aggregation in the older sample, something which is supported by the Coomassie and Western blot analysis. These clearly showed protein aggregates from the bottom of the stacking gel and within the resolving gel to approximately 55kDa whereas the new protein had distinct bands. During subsequent assay development all stock solutions were stored in small aliquots containing 50%

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glycerol to prevent ice crystals forming and used a maximum of five times before disposal.

To investigate availability of 3NT residues produced with different nitration protocols a comparison was made of electrochemically- and peroxyntirite (ONOO<sup>-</sup>) - nitrated BSA, at comparable levels of nitration. Several different protocols have been reported for the nitration of proteins *in vitro* that have subsequently been used as standards in ELISAs (indirect, competitive and sandwich). These methods include the use of proteins nitrated using tetranitromethane (Franze, Weller, Niessner *et al.* 2004), ONOO<sup>-</sup> (Ye, Strong, Huang *et al.* 1996, Khan, Brennand, Bradley *et al.* 1998), and incubation in the presence of sodium nitrite (Ter Steege, Koster-Kamphuis, Van Straaten *et al.* 1998), Inoue *et al.* (2002) used peroxidase conjugated free nitrotyrosine. These methods also report the use of different anti-3NT Abs including polyclonal (Ye, Strong, Huang *et al.* 1996, Khan, Brennand, Bradley *et al.* 1998, Inoue, Hisamatsu, Ando *et al.* 2002, Franze, Weller, Niessner *et al.* 2004) and monoclonal (Ye, Strong, Huang *et al.* 1996, Ter Steege, Koster-Kamphuis, Van Straaten *et al.* 1998, Franze, Weller, Niessner *et al.* 2004) antibodies all of which could also account for the differences observed in assay sensitivity.

The results demonstrated that the anti-3NT Ab initially gave a slightly higher response to the ONOO<sup>-</sup>-nitrated BSA but that after U+M treatment a significant increase in assay sensitivity occurred for both protocols. This suggests that it is not electrochemical nitration that affects 3NT detection.

Peroxyntirite activity results in products other than tyrosine residues which include nitrosylated (R-NO) derivatives as well as more traditional oxidation

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products (such as hydroxylated aromatics) (Beckman, Chen, Ischiropoulos *et al.* 1994). This should be considered when using ONOO<sup>-</sup>-nitrated BSA as a standard and anti-3NT antibodies that have been raised against ONOO<sup>-</sup>-nitrated KLH. This is because any antibody raised against a protein standard that has been nitrated using ONOO<sup>-</sup> could potentially also have antigenic specificity towards these products, thus resulting in an assay that is sensitive to products of ONOO<sup>-</sup> and not nitration exclusively. However, in the method developed by Khan *et al* (1998) they report that their assay was specific to the detection of 3NT-residues in proteins and peptides. Their assay showed no cross-reactivity with other ONOO<sup>-</sup> associated amino acid modifications such as oxidation of cysteine and methionine and oxidised/nitrated tryptophan. It has also been demonstrated by Kendal *et al* (2001) that, unlike the ONOO<sup>-</sup>-nitration protocol, 3NT is the only product of the electrochemical nitration protocol used within this study.

When testing proteins from both nitration protocols using dot-blot immunodetection on nitrocellulose membrane it was observed that the use of U+M denaturation made little difference to 3NT detection; therefore highlighting that the manner in which a nitrated protein binds to a solid phase directly affects the availability of 3NT-residues to the anti-3NT Ab. Following this, differences were also found in the binding capacities of different manufacturers' microtitre plates even after the NT-BSA had been denatured using U+M, with Immulon 2HB<sup>®</sup> producing the best results.

The reason behind such differences in the binding capacities of different manufacturer's microtitre plates is unclear although intra- and inter-assay variation in levels of protein adsorbing to microtitre plates is well known

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(Kricka, Carter, Burt *et al.* 1980). It was thought that such variation may be due to differences in the ability of the NT-BSA to adsorb onto the plate. The Immulon 2HB<sup>®</sup> and Sero-Wel<sup>®</sup> microtitre plates were sent away for analysis by infrared spectroscopy - mass spectrometry to establish if there was a chemical difference between the two plates. From this analysis it was found that both plates were made of polystyrene but that the Immulon 2HB<sup>®</sup> plates did appear to have some form of biological lipid layer present (results not shown). This lipid would aid in the binding of hydrophobic regions of proteins as well as act to denature the protein. Other factors that may vary between plates include desorption of the adsorbed NT-BSA from the solid phase due to poor protein-plate interactions (Kemeny and Challacombe 1988: 32-35). It has also been reported that the adsorption of BSA onto, and potential desorption from, polystyrene can cause irreversible changes in its secondary structure (Norde and Giacomelli 2000). They found that irreversible surface-induced conformation changes for BSA may have been as a direct result of aggregation of BSA molecules brought about by exposure to a hydrophobic surface. This could in some part explain the differences between the different brands of plate tested. Similar findings have also been reported with reference to conformational changes in C-reactive protein once adsorbed onto polystyrene microtitre plates resulting in altered antigen binding sites (Shields, Siegel, Clark *et al.* 1991).

Chemical denaturation with U+M was found to be an extremely effective mechanism of increasing 3NT-residue availability to the anti-3NT Ab in the indirect ELISA. However, it would not be suitable for a competitive assay due to the detrimental effect it would have on the anti-3NT Ab during the

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competitive stage. Other denaturation methods were investigated but none gave a similar increase in sensitivity. Denaturing the NT-BSA/02 via boiling in the presence of Tween 20 (0.1%) and digesting the protein using trypsin did not result in the same increase in assay sensitivity as U+M. However, tryptically digested NT-BSA did produce fewer false negative results. Denaturing NT-BSA by boiling it in the presence of Tween 20 was found to have a detrimental effect on the sensitivity of the assay possibly through a reduction in the ability of the protein to adsorb onto the microtitre well.

The indirect ELISA was a useful format to investigate assay reagents and parameters and revealed a number of issues. However, this format would always be limited for detecting 3NT in complex protein mixtures such as claudicant plasma. In order to quantify nitrated proteins where different proteins may contain different amounts of 3NT-residues a competitive assay was required. Franze *et al* (2004) also reported that indirect ELISAs have limited applicability, and sensitivity, with regards to the quantification of 3NT-containing proteins within complex samples such as those observed in plasma.

#### **3.4.2. Competitive ELISA development**

In the lengthy development of a competitive ELISA there were similar findings to the indirect ELISA. Immulon 2HB<sup>®</sup> microtitre plates were found to be the most suitable of those tested. Trypsin digested NT-BSA, as a potential coating and competing antigen, was found to increase assay sensitivity and reliability compared to native NT-BSA. This was probably due to the 3NT-residues in the resultant peptide fragments being more available to the anti-3NT Ab. Denaturation, or modification, protocols have not been reported in any of the

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other studies where similar immunoassays were developed (Khan, Brennand, Bradley *et al.* 1998, Ter Steege, Koster-Kamphuis, Van Straaten *et al.* 1998, Inoue, Hisamatsu, Ando *et al.* 2002) although some have reported differences in the limits of detection dependent upon the source of anti-3NT Ab being used (Ye, Strong, Huang *et al.* 1996, Franze, Weller, Niessner *et al.* 2004) and that their anti-3NT Abs have differing binding affinities for different nitrated proteins/peptides (Ye, Strong, Huang *et al.* 1996, Khan, Brennand, Bradley *et al.* 1998, Ter Steege, Koster-Kamphuis, Van Straaten *et al.* 1998). The sensitivity of the assay may not be solely due to the limited availability of the 3NT-residues to the Ab but may also be due to the effects of the local environment surrounding the 3NT-residue e.g. influences of amino acids adjacent to the 3NT residue (Khan, Brennand, Bradley *et al.* 1998). This brings into question the validity of the ELISA as a quantitative method for the estimation of 3NT-containing proteins and that the assay should be classed as a semi-quantitative screening tool (Khan, Brennand, Bradley *et al.* 1998).

Other assay parameters were evaluated to optimise the assay including antigen coating concentration and incubation buffer. Coating buffer and conditions were not explored as thought not to be significant, in fact binding proteins to plastic is thought to be independent of pH and ionic strength of the coating buffer (Kemeny and Challacombe 1988: 39). With respect to concentration of coating antigen, 4 $\mu$ g/ml of tryptically digested NT-BSA resulted in increased assay sensitivity, but was higher than that generally accepted for most proteins to form a monolayer of 1 $\mu$ g/ml (Cantarero, Butler and Osborne 1980).

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In order to prevent the non-specific binding of proteins to the microtitre plate, plates were initially blocked using skimmed milk powder (5%) with Tween 20. This differed to that used in other published assays in which most reported using BSA with Tween 20 as the blocking agent (Ter Steege, Koster-Kamphuis, Van Straaten *et al.* 1998, Inoue, Hisamatsu, Ando *et al.* 2002), although use of ovalbumin and goat serum have also been reported (Ye, Strong, Huang *et al.* 1996, Khan, Brennand, Bradley *et al.* 1998). Using blocking agents such as BSA and ovalbumin have the potential for non-specific binding occurring due to the presence of antibodies against these proteins in the plasma of humans (Siltanen, Kajosaari, Savilahti *et al.* 2002, Mogues, Li, Coburn *et al.* 2005). The incubation buffer used in all further dilutions for the assay was optimised and it was found that incubation buffer with BSA (1%) in the presence of Tween 20 (0.05%) was sufficient to reduce background interference brought about by non-specific binding of human anti-BSA antibodies and thus not impede assay sensitivity. However, the possible interaction between BSA and anti-BSA antibodies needs further investigation (as reported in Chapter 4).

With respect to other non-specific reactions involving protein bound aminotyrosine, a novel approach in this study was to reduce NT-BSA to amino-BSA for use in the competitive ELISA. Although other studies have screened amino-BSA on membranes, in an ELISA format it appears that only free aminotyrosine has been used and no cross-reaction reported (Khan, Brennand, Bradley *et al.* 1998). In our ELISA the antibody did bind to amino-BSA at similar levels to native NT-BSA. This could be explained by the epitope environment of protein bound aminotyrosine being similar to that for

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NT-BSA and it is that environment which is important for antibody binding. It may be that binding affinities to free aminotyrosine are similar to free 3NT which was seen in this and other studies (Khan, Brennand, Bradley *et al.* 1998, Ter Steege, Koster-Kamphuis, Van Straaten *et al.* 1998) to be significantly less compared to protein bound 3NT. As reported by ter Steege *et al* (1998) the increased sensitivity towards protein bound 3NT will provide an assay which gives a more accurate determination of the extent of nitration within a sample due to the relatively short half life of free 3NT. However, the detection of aminotyrosine in proteins should be noted. The reduction of the NT-BSA to amino-BSA was confirmed spectrophotometrically and using dot blot immunodetection. However it is possible that that the amino-BSA autooxidised back to NT-BSA prior to the assay being carried out. Ye *et al* (1996) have reported that protein solutions that have been reduced to aminotyrosine, using dithionite, will slowly autooxidise back to nitrotyrosine. Despite extensive evaluation and parameter modification the inter- and intra-plate variation was outside conventional limits of a %CV  $\leq$  10%. Variation was reasonable at higher concentrations of standard but poor below 76nM tryp-NT-BSA. Regression coefficients were consistently less than 0.99 and lower than those of Khan *et al* (1998) using ONOO<sup>-</sup>-nitrated BSA which were >0.99 but higher than those of ter Steege *et al* (1998). ter Steege *et al*'s (1998) assay differed in that it was a sandwich ELISA in which two monoclonal anti-3NT Abs were used. Monoclonals were not investigated in the study presented here but may be important for greater sensitivity and also reproducibility. Variation within and between plates of control samples (tryp-

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NT-BSA and nitrated plasma) were also greater than those reported for controls by Khan *et al* (1998) and ter Steege *et al* (1998).

To continue to improve assay performance other nitrated proteins, as potential coating and competing antigens, were investigated. The most notable of these being electrochemically-nitrated human plasma (NT-PL), nitrated to an estimated level of nitration similar to that of the NT-BSA standard. A benefit of using NT-PL was that it would provide a standard more comparable and representative of the claudicant plasma samples, i.e. with complex mixtures of protein species. That is the anti-3NT Ab would display similar binding kinetics to the NT-PL as it would to claudicant plasma.

Upon investigation it was clear that there was reduced assay sensitivity when using NT-PL compared to tryp-NT-BSA but intra- and inter-assay variation was comparable. Anti-3NT antibodies have differing binding affinities for 3NT-residues within different proteins (Khan, Brennand, Bradley *et al.* 1998, Ter Steege, Koster-Kamphuis, Van Straaten *et al.* 1998). This is another issue for these assays as the nitration profile may differ depending on the nature of the pathology of the disease involved.

In summary the results presented here have demonstrated that detection of 3NT in proteins is very sensitive to assay conditions, in particular to the accessibility of the residues to the antibody. The structural integrity of a protein is, therefore, important and for any sample that may contain nitrated proteins the level of 3NT detection would only be proportional to the accessible residues. This would further indicate that the conformation of a protein (thus availability of epitope) and binding parameters of the solid phase

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being used all influence the specificity of anti-3NT Abs. Thus an assay may underestimate the total nitrated protein population. These findings are important with respect to the validity of the use of ELISAs for detection of 3NT-containing proteins as described in previously published reports (Khan, Brennand, Bradley *et al.* 1998, Ter Steege, Koster-Kamphuis, Van Straaten *et al.* 1998, Inoue, Hisamatsu, Ando *et al.* 2002).

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# **Chapter 4.**

## **Detection of 3NT and Analysis of the Nitration Profile in Claudicants**

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## 4.1. Introduction

Atherosclerosis rarely produces symptoms until the extent of disease is severe and arteries are dangerously narrowed or in the worse case complete occlusion of the artery occurs, therefore the earlier the diagnosis the better the prognosis. IC is a symptomatic expression of lower extremity atherosclerotic disease (PAD) and as such is a marker of more serious cardiovascular problems and generalised atherosclerosis. If left untreated IC can lead to significant functional impairment and is indicative of an increased risk of cardiovascular morbidity and mortality (2.5 fold increase compared to age matched population (Stewart and Lamont 2001)). Also due to pain upon exercise the individuals suffering from IC have a tendency to reduce exercise levels which can result in further clinical problems. Therefore it is imperative that IC should be treated seriously and highlights the need for accurate tests to enable early diagnosis. Current diagnostic techniques used for the diagnosis of IC rely on the accurate reporting of symptoms by the patient to the GP in order to be referred to a consultant vascular surgeon (Chapter 2). As such it would be beneficial to diagnose the presence of PAD within an individual at an earlier stage in the disease process (e.g. when the patient is still asymptomatic) leading to more prompt intensive treatment and thus potentially a better prognosis.

Plasma is one of the most informative biological specimens from a medical viewpoint but is also one of the most difficult to analyse due to its complexity and enormous dynamic range (Anderson and Anderson 2002, Hortin, Jortani, Ritchie *et al.* 2006). Proteomics involves the identification and functional analysis of the protein complement (proteome) of a cell, tissue, or bodily fluid

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such as plasma at a given time. The analysis of biological fluids such as plasma and urine has been investigated using both qualitative and quantitative techniques for the identification of markers of disease for many years. For example, the first protein marker of disease reported was the Bence Jones protein in urine (Rosenfeld 1987). In the past 30 years the advent of more advanced analytical techniques has allowed for the simultaneous analysis of a large number of proteins within complex biological samples such as plasma. Although the terms proteome and proteomics were coined in the late 1990's by Marc Wilkins, techniques such as 2-dimensional polyacrylamide electrophoresis (2D-PAGE) were first applied to separate proteins in the 1970's. The high resolution separation of human plasma proteins using 2D-PAGE was first reported by Anderson, L. and Anderson N.G. (1977). Within proteomic studies proteins are resolved by 2D-PAGE and then further analysed and identified using mass spectrometry techniques including MALDI-TOF-MS and LC-ESI-MS/MS. Even with such advances there are still major challenges in the isolation and identification of proteins from complex samples such as plasma and it has been reported that the proteins identified are dependent upon the proteomic approach used (Omenn, States, Adamski *et al.* 2005).

Unlike the human genome which is static there are many different proteomes (e.g. plasma proteome) which are dependent upon time, location and importantly may be affected by disease pathology; therefore investigating the plasma proteome may provide useful biomarkers of disease. Protein targets of nitration have already been established *in vivo* within different disease states and disease models (Aulak, Miyagi, Yan *et al.* 2001, Lanone, Manivet,

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Callebert *et al.* 2002, Kanski, Hong and Schoneich 2005, Casoni, Basso, Massignan *et al.* 2005). It was the aim of the proteomics study herein to identify the presence/absence of a nitration profile within the plasma of claudicants. Within this study MALDI-TOF-MS and LC-ESI-MS/MS analysis were combined to identify proteins that had stained immunopositive for the presence of 3NT in corresponding Western blots, excised from 1D SDS-PAGE gradient gels.

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## **4.2. Materials and methods**

All chemicals used were analytical grade and purchased from Fisher chemicals unless otherwise stated.

Unless otherwise stated water was obtained by reverse osmosis to a resistance of greater than  $18\text{M}\Omega\text{ cm}^{-1}$

### **4.2.1. Determination of total protein concentration**

Materials: Bradford reagent (B6916), purchased from Sigma (Poole, Dorset, U.K.). Bicinchoninic acid (BCA<sup>TM</sup> Protein assay kit 23225, Pierce).

Methods:

The methods used for the determination of the protein concentration were dependent upon the sample being tested to due to presence of potential interfering chemicals.

Lowry (Folin-Ciocalteu) method (Lowry, Rosebrough, Farr *et al.* 1951): The Lowry method was used to determine the total protein concentration of the patient and control whole plasma.

BSA was used as the standard protein to prepare a calibration curve over a range of 0-200 $\mu\text{g/ml}$ . To 1ml of sample (diluted as necessary) or BSA (0-200 $\mu\text{g/ml}$ ), 5ml alkaline copper reagent was added (2%  $\text{Na}_2\text{CO}_3$  in 0.1M NaOH: 0.5%  $\text{CuSO}_4\cdot 5\text{H}_2\text{O}$  in 1% sodium potassium tartrate 50:1). This solution was mixed and allowed to stand for 10 minutes. To this solution 0.5ml Folin-Ciocalteu Reagent (diluted 1:1 with water, 1M in acid) was then added and mixed rapidly by inversion. Following a 30 minute incubation period to

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allow the colour to develop, the absorbance was read at 750nm. Total protein concentrations were then determined from the calibration curve.

Absorbance  $A_{280}/A_{260}$  method: The total protein concentration for each sample being tested was determined at  $A_{280}$  (Warburg and Christian 1942). The  $A_{280}$  and  $A_{260}$  were measured for each sample at room temperature. The  $A_{280}:A_{260}$  ratio was then calculated and used to determine the corresponding factor for the conversion of  $A_{280}$  to protein concentration. (See appendix G for factors)

Example calculation:

For sample 1  $A_{280} = 1.74$

$A_{280}:A_{260}$  ratio = 1.70

Factor for 1.70 = 1.118 (appendices G)

Therefore protein concentration (mg/ml) =  $A_{280} \times 1.118$   
= 1.945mg/ml

NB: Since nucleic acid contamination was very low in all samples, the factor used was invariably 1.118

Bradford method using microtitre plates: The total protein concentration for each of the three samples obtained from HSA fractionation, using dye-ligand affinity chromatography on immobilised Cibacron blue columns, was determined using the Bradford method adapted for use with microtitre plates following manufacturer's instructions.

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In brief, BSA was used as the standard protein and a calibration curve was prepared over a range of 0-1.4mg/ml. 5µl of sample (diluted as necessary) and BSA samples for the calibration curve (0-1.4mg/ml) were added to a 96 well microtitre plate. To this, 250µl of Bradford reagent was added, mixed for 30 seconds, and allowed to stand for 45 minutes at room temperature to allow the colour to develop, after which the absorbance at 600nm was read. From the calibration curve produced the total protein concentrations were calculated.

Bicinchoninic method using microtitre plates: The total protein concentration for each of the samples obtained from the affinity purification for 3NT-containing proteins was determined using the bicinchoninic acid (BCA™) method adapted for use with microtitre plates following the instructions provided by the manufacturer.

In brief, the working reagent was prepared by mixing 50 parts of BCA™ reagent A with 1 part BCA™ reagent B followed by mixing thoroughly to yield a clear green working reagent. Diluted BSA standards were prepared over a working range of 20-2000µg/ml. 25µl of each standard and unknown (in duplicate) was pipetted into 96 well microtitre plate to which 200µl of the working reagent was added. The plates were then mixed thoroughly on a plate shaker for 30 seconds, covered and incubated at 37°C for 30 minutes. The plates were then allowed to return to room temperature and the absorbance was measured at 620nm on a plate reader.

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#### **4.2.2. Dye-ligand affinity chromatography on immobilised Cibacron blue**

The claudicant and control plasma samples were fractionated using affinity purification in order to assess the plasma nitration profile, which potentially may have been masked by proteins present in high concentrations.

Human serum albumin (HSA) was separated from other plasma proteins in the patients' plasma using a method adapted from that described by Gianazza and Arnaud (1982).

Materials: Fast flow cross-linked agarose gel with covalently coupled Cibacron blue dye (C8321) purchased from Sigma (Poole, Dorset, U.K.).

Column preparation: Mini columns were set up using fast flow cross-linked agarose gel with covalently coupled Cibacron blue dye. The gel (0.5ml) was packed into a small column and equilibrated with five bed volumes of buffer 1 (0.03M phosphate buffer, pH 7; 10.74g disodium hydrogen orthophosphate dodecahydrate and 4.68g sodium hydrogen orthophosphate, made up to 1L).

Chromatography procedure: Patient plasma (500 $\mu$ l) was centrifuged (12100g) for 5 minutes after which the supernatant was loaded onto the column. Once loaded onto the column the sample was circulated continuously through the column for three equivalent column volumes, eluted and the column was washed using 3ml of buffer 1 (fraction F1). The column was then washed with 2ml of buffer 2 (0.03M phosphate buffer, pH 7 plus 0.5M NaCl), and the eluate was again collected following a wash of 3 bed volumes of buffer 1 (fraction

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F2). Finally the tightly bound HSA and lipoproteins were washed from the column using 2ml of 0.5M sodium thiocyanate followed by 3 bed volumes of buffer 1 (fraction F3). From this separation the three samples obtained were dialysed into 10mM Tris-HCl pH 8, diluted with an equal volume of glycerol and stored at -20°C. These samples were then further purified/analysed using a variety of techniques mentioned below and by ELISA (indirect and competitive).

#### **4.2.3. Affinity chromatography**

Samples from each of the separated fractions obtained from method 4.2.2. were further purified using affinity chromatography on immobilised rabbit anti-3NT antibody.

Materials: Cyanogen bromide activated agarose (C9210) and 3-nitro-L-tyrosine (N7389) Sigma (Poole, Dorset, U.K.).

#### Methods:

Column preparation: Affinity columns were set up using 0.25g cyanogen bromide-activated agarose reconstituted in 3ml 0.25M carbonate buffer, pH 9.5, to which anti-3NT Ab (100µl) was added. This solution was stirred gently overnight at 4°C to allow the antibody to bind. The gel slurry was placed into a small glass column and washed until the  $A_{280}$  returned to zero using 0.25M carbonate buffer, pH 9.5. The remaining active groups present were then blocked using 0.25M carbonate buffer plus 10mM ethanolamine, pH 9.5, run through the column for 2 hours at room temperature. The column was washed again using 0.25M carbonate buffer, pH 9.5. The column was prepared for

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storage by washing the column with phosphate buffer containing 0.02% (w/v) azide and was stored at 4°C.

Affinity purification procedure: The three fractions obtained from the Cibacron blue mini columns (F1, F2, and F3) were further purified using anti-3-NT antibody affinity purification. The column was equilibrated in carbonate buffer (0.25M, pH 9.5) after storage. 5ml of a fraction obtained from the Cibacron columns (e.g. F1) were then circulated through the column for 2 hours at room temperature after which the column was washed using carbonate buffer (0.25M pH 9.5), and the eluate was collected (unbound fraction U). The bound, nitrated plasma proteins were eluted from the column using 10mM 3NT (10ml), followed by washing the column using 0.25M carbonate buffer, pH 9.5 (bound fraction B). The two fractions were dialysed into 10mM ammonium acetate, pH 7, and freeze dried. The samples were then reconstituted in 1ml 10mM Tris-HCl pH 7, ready for further analysis. Each affinity column was used specifically for one plasma sample, after which it was discarded.

#### **4.2.4. Sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS-PAGE)**

For materials and methods see Chapter 3, section 3.2.11.

#### **4.2.5. Coomassie blue staining**

For materials and methods see Chapter 3, section 3.2.12.

#### **4.2.6. Western Blot**

For materials and methods see Chapter 3, section 3.2.13.

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#### **4.2.7. Silver stain**

Materials: Proteosilver™ Plus silver staining kit (PROT-SIL2) Sigma (Poole, Dorset, U.K.). Ultra pure water obtained by filtering RO water through an Elgastat spectrum water purifier/polisher (Elga Ltd, High Wycombe, Bucks. UK).

Methods: Following Coomassie staining the gradient gels were then additionally stained using Proteosilver™ Plus silver staining kit as described in the technical bulletin supplied.

In brief the gels were fixed overnight in fixing solution (50ml ethanol, 10ml acetic acid and 40ml ultra pure water). The gels were then washed in 30% ethanol solution followed by washing in ultra pure water. Following this the gels were placed in sensitizer solution for 10 minutes, again followed by a water wash. Silver equilibration then occurred followed by a brief water wash (no longer than 1.5 minutes). During gel development the gels were placed in developer solution for 3-7 minutes, after which the reaction was stopped using Proteosilver stop solution and left for 5 minutes. The gels were then washed and stored in ultra pure water.

#### **4.2.8. Mass spectrometric analysis of plasma proteins**

Following Coomassie and silver staining the gradient gels were sent to Warwick University for matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) and liquid chromatography electrospray ionisation tandem mass spectrometry (LC-ESI-MS/MS) analysis on excised bands. Protein bands which stained positive for 3NT (via western blot analysis) were identified and manually excised into a 96 well microtitre plate.

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(NB: not all bands were excised for further analysis due to limited time & resources, those bands excised are highlighted in the results section 4.3.3.).

**The following methodology was carried out by the Biological Mass Spectrometry and Proteomics Department, University of Warwick.**

Materials: MALDI-TOF-MS (MALDI micro MX).and MassPREP™ PROtarget™ plates purchased from Waters®. Liquid chromatography-electrospray ionisation-mass spectrometry with ionisation (LC-ESI-MS/MS, manufacturer unknown).

Methods:

General protocol for extracting the proteins from the excised bands was as follows: The gel plugs were de-stained, reduced, alkylated, and digested with trypsin. The resulting peptides were extracted using the Micromass MassPREP™ station running the standard digestion protocol supplied by manufacturer. The tryptic peptides were transferred to a cooled 96 well microtitre plate ready for spotting onto a MassPREP™ PROtarget™ plate™.

In brief the protocol for spotting the tryptic digests was as follows: the MassPREP™ PROtarget™ plate was conditioned by the addition of 1µl of acetonitrile to each active well followed by 1µl of trifluoroacetic acid (TFA) after 30 seconds. The plate was then left for 2 minutes, after which the conditioning solution was removed ready for sample addition. 10µl of the tryptic digest (in 0.1% TFA / 30% acetonitrile) was added to an active well, and left until it had completely dried. Once dry a further 10µl aliquot was

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spotted and allowed to air dry. The plate was then further made ready for MALDI-MS analysis by washing with approximately 5 $\mu$ l of aqueous 0.1% TFA, repeated x3. Once completely dry 1 $\mu$ l of matrix solution ( $\alpha$ -cyano-4-hydroxycinnamic acid (CHCA) 0.5mg/ml / 90% acetonitrile / 0.1% TFA) was added and allowed to dry. The plate was then analysed using MALDI analysis using a Micromass M@LDI<sup>®</sup> mass spectrometer fitted with a nitrogen UV laser (337nm) incorporating a time of flight (TOF) mass analyser. This data was further confirmed or rejected by means of LC-ESI-MS/MS. The MALDI-MS and LC-ESI-MS/MS data was then used to interrogate the IPI human protein database V.3.07 (or SwissProt where appropriate) using the Mascot search engine.

#### **4.2.9. Indirect and competitive ELISA protocols**

For materials and methods see Chapter 3, section 3.2.7. and 3.2.9.

#### **4.2.10. Testing for the presence of human antibodies against 3NT, BSA or HSA**

A microtitre plate was coated with BSA, NT-BSA, human serum albumin (HSA) or NT-HSA (4 $\mu$ g/ml), using carbonate buffer pH 9.6 and incubated overnight. The plate was then blocked with 5% SMP in PBS (130 $\mu$ l) for 1 hour at 37°C and washed with wash buffer three times. Each claudicant and control plasma sample (1/50, in duplicate) was pipetted into an appropriate well coated with BSA, NT-BSA, HSA or NT-HSA. The plate was then incubated overnight at 4°C. Following a wash cycle (as described in earlier ELISA protocols) the plate was probed using an anti-human IgG horse radish peroxidase conjugate (1/1000) and incubated for 1 hour at 37°C. Following

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another wash cycle OPD substrate was added and incubated for 15 minutes to allow the colour to develop. The reaction was stopped using 2M sulphuric acid and the absorbance was read at 492nm. All antibody dilutions were made in incubation buffer.

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### 4.3. Results

#### 4.3.1. Initial Screening of the patient and control plasma for 3NT-proteins

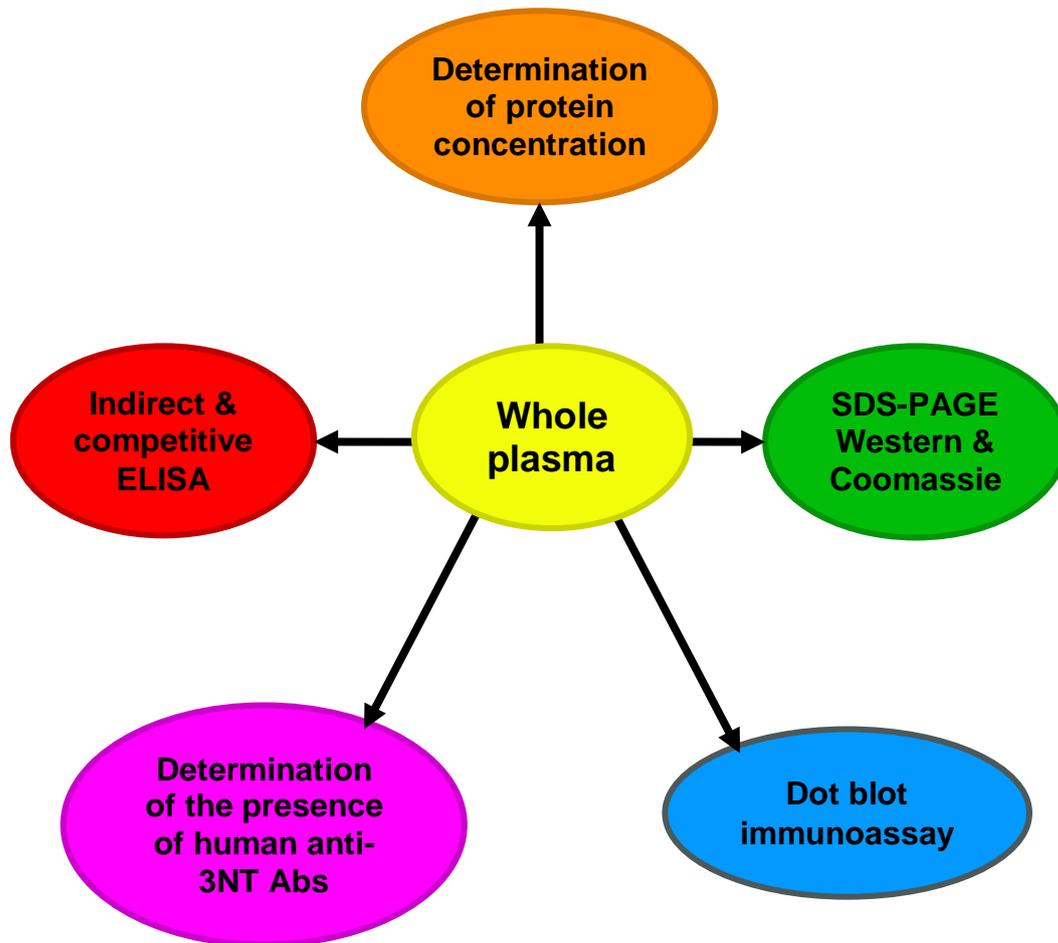


Figure 4.3.i. Flow diagram to illustrate how the whole plasma samples from the claudicants and control subjects were analysed.

The total protein concentrations for the claudicant and control plasma samples were determined using the Lowry method (Lowry, Rosebrough, Farr *et al.* 1951) and were found to range from 90-157mg/ml and 96-121mg/ml respectively (see Table 4.3.i. for mean plasma concentration  $\pm$ SD).

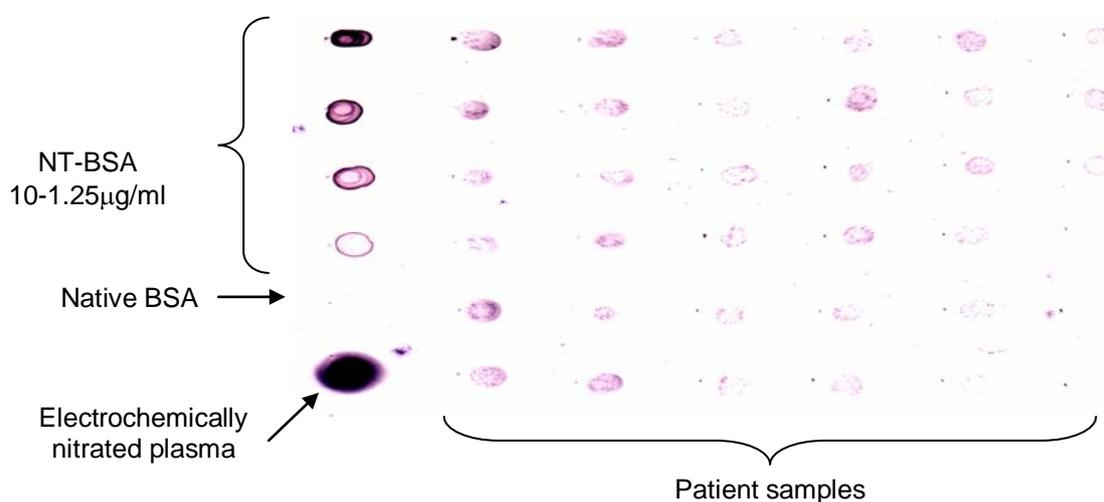
Total plasma protein concentration (mg/ml)	
Claudicants	Control subjects
102 ±13	104 ±9

**Table 4.3.i. Plasma protein concentrations for claudicant and control subjects.**

Total plasma protein concentrations were determined using the Lowry method (Lowry, Rosebrough, Farr *et al.* 1951). ± SD - standard deviation

The presence of nitrated proteins within these plasma samples was investigated using a variety of techniques; this will now be described below.

The claudicants' whole plasma samples were preliminarily screened for the presence of 3NT residues using dot blot immunodetection and 3NT was found to be present in all thirty three patient samples (Figure 4.3.ii.). When testing the whole plasma from the control subjects a similar colourimetric response was obtained, suggesting that there are 3NT-containing proteins present in the control plasma also (results not shown).



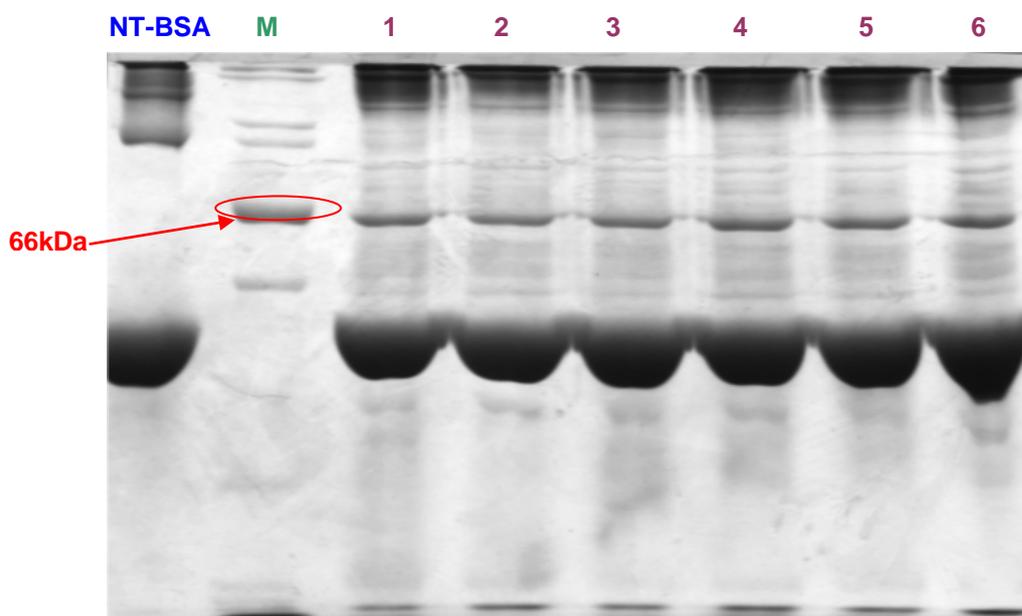
**Figure 4.3.ii. Dot blot immunodetection of claudicant plasma.**

Claudicants plasma (n=33) was loaded directly onto the nitrocellulose membrane to screen for the presence of 3NT residues. NT-BSA - 4 mol 3NT / mol BSA, all samples loaded at 4µl.

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In an attempt to identify the nitration profile within the claudicant whole plasma samples a selection (CS001-CS006) were separated using SDS-PAGE, visualised using Coomassie blue and probed for the presence of 3NT residues using Western blot immunodetection. For both the patient plasma samples and the NT-BSA standard a cluster of high RMM Coomassie-stained bands were observed at the base of the stacking gel and start of the resolving gel. An overloaded band was also observed for the NT-BSA standard and patient samples within the resolving gel, migrating faster than the 55kDa size marker. Although a Coomassie-stained band was observed within the patient samples at the same position to the 66kDa band within the size marker, no Coomassie-stained protein band was observed for NT-BSA (Figure 4.3.iii). Western blot immunodetection clearly highlighted the presence of 3NT within all of the bands present within the control NT-BSA sample but no 3NT-positive bands were detected in protein bands from the patient samples (results for Western blot not shown).

These results were contradictory to the initial findings using dot blot immunodetection, with regard to the presence of 3NT-proteins in the claudicant plasma as shown in Figure 4.3.ii. above. Further analysis was carried out on both the claudicant and control plasma samples in order to confirm the presence/absence of 3NT residues. Upon further analysis using both indirect and competitive ELISA, the presence of 3NT-containing proteins could not be established in either the claudicant or control plasma samples (as described in Chapter 3).



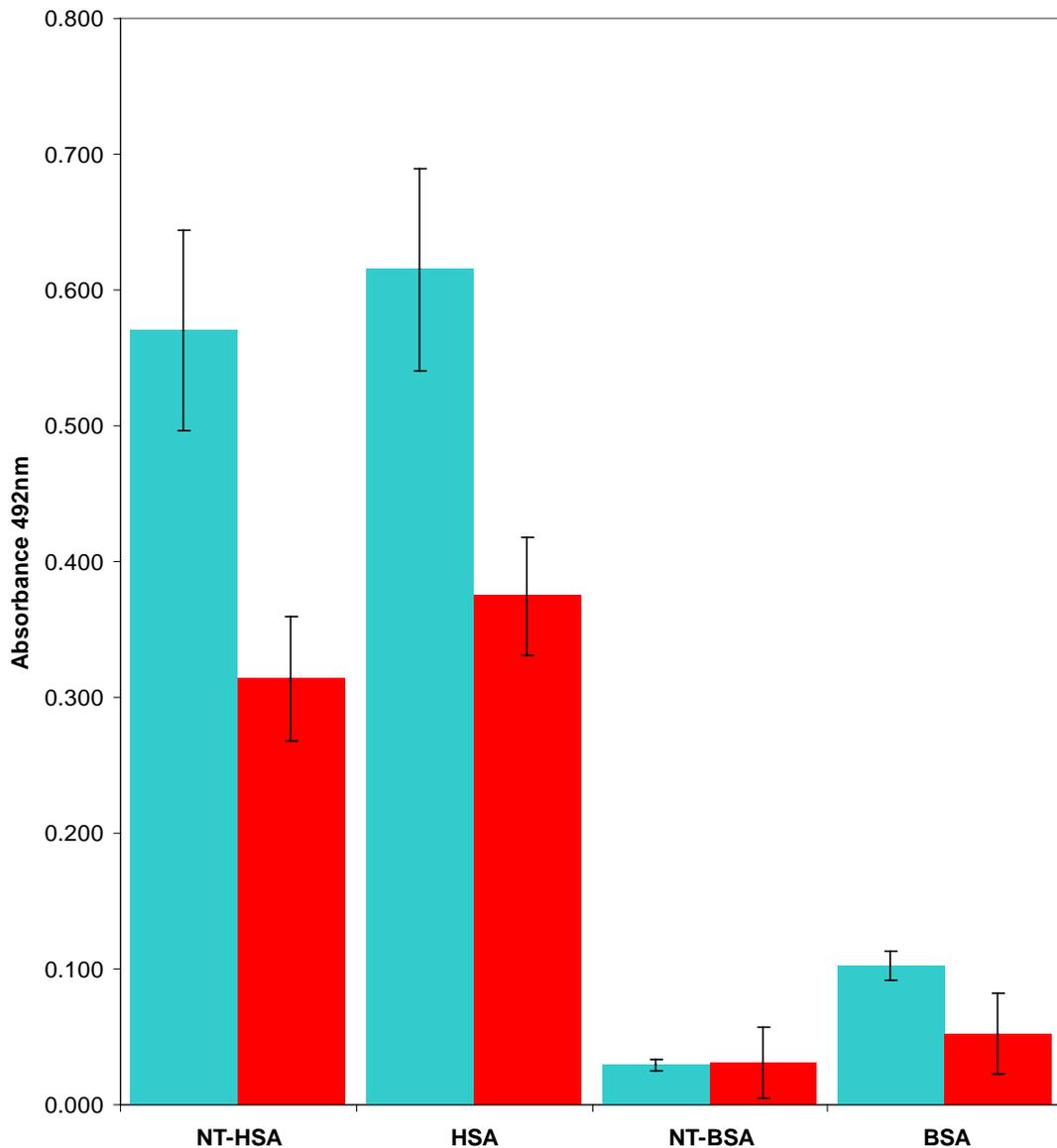
**Figure 4.3.iii. Claudicant whole plasma (CS001-CS006) separated on SDS-PAGE visualised using Coomassie blue staining**

Samples run on the gel were loaded at 4 $\mu$ l in sample buffer. Electrochemically nitrated BSA (NT-BSA) was used as a positive control and an Anamed size marker (M) was run for calculation of protein bands within samples. Patient sample CS001-CS006 (1-6) were separated on the gel. See Figure 3.2.ii. above for molecular masses of the other bands in the size marker.

The claudicant and control plasma samples were also screened for the presence of anti-3NT Abs using both native and nitrated BSA and native and nitrated HSA. With regards to the BSA a significantly higher mean antibody response was observed towards native BSA compared to NT-BSA in the 39 samples tested (2 tailed paired t-test, n=33 claudicants and n=6 control, p<0.001, Figure 4.3.iv.). This significant difference was also evident when testing the claudicant and control plasma samples independently, but only significant to a level p<0.05 in the case of the control subjects. A significantly

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higher mean antibody response towards native HSA was observed compared to NT-HSA in the 39 samples (2 tailed paired t-test, n=33 claudicants and n=6 control,  $p<0.001$ , Figure 4.3.iv.). Again when testing the claudicants and control subjects independently the mean Ab response to native HSA was found to be significantly higher than it was for NT-HSA (2 tailed paired t-test,  $p<0.001$ ). It was also clear that a higher antibody response was observed when using native and nitrated HSA compared to native and nitrated BSA for both the claudicant and control plasma samples. A higher antibody response against both the native and nitrated HSA/BSA was observed within the claudicant plasma samples compared to the control plasma (Figure 4.3.iv).



**Figure 4.3. iv. Presence of antibodies against nitrated BSA / HSA and native BSA / HSA in claudicant and control plasma.**

The above figure represents the mean absorbance values ( $\pm$  SEM) from an indirect ELISA screening for the presence of antibodies against nitrated BSA / HSA and native BSA / HSA in claudicant (n=33) and control (n=6) plasma samples. All absorbances have been corrected to the mean of the blank wells. Claudicant plasma samples (■, CS001-CS0033) and control plasma samples (■, CD001-CD006) were incubated in the presence of bound BSA and NT-BSA and with bound HSA and NT-HSA.

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4.3.2. Fractionation of human plasma using dye-ligand affinity chromatography on immobilised Cibacron blue

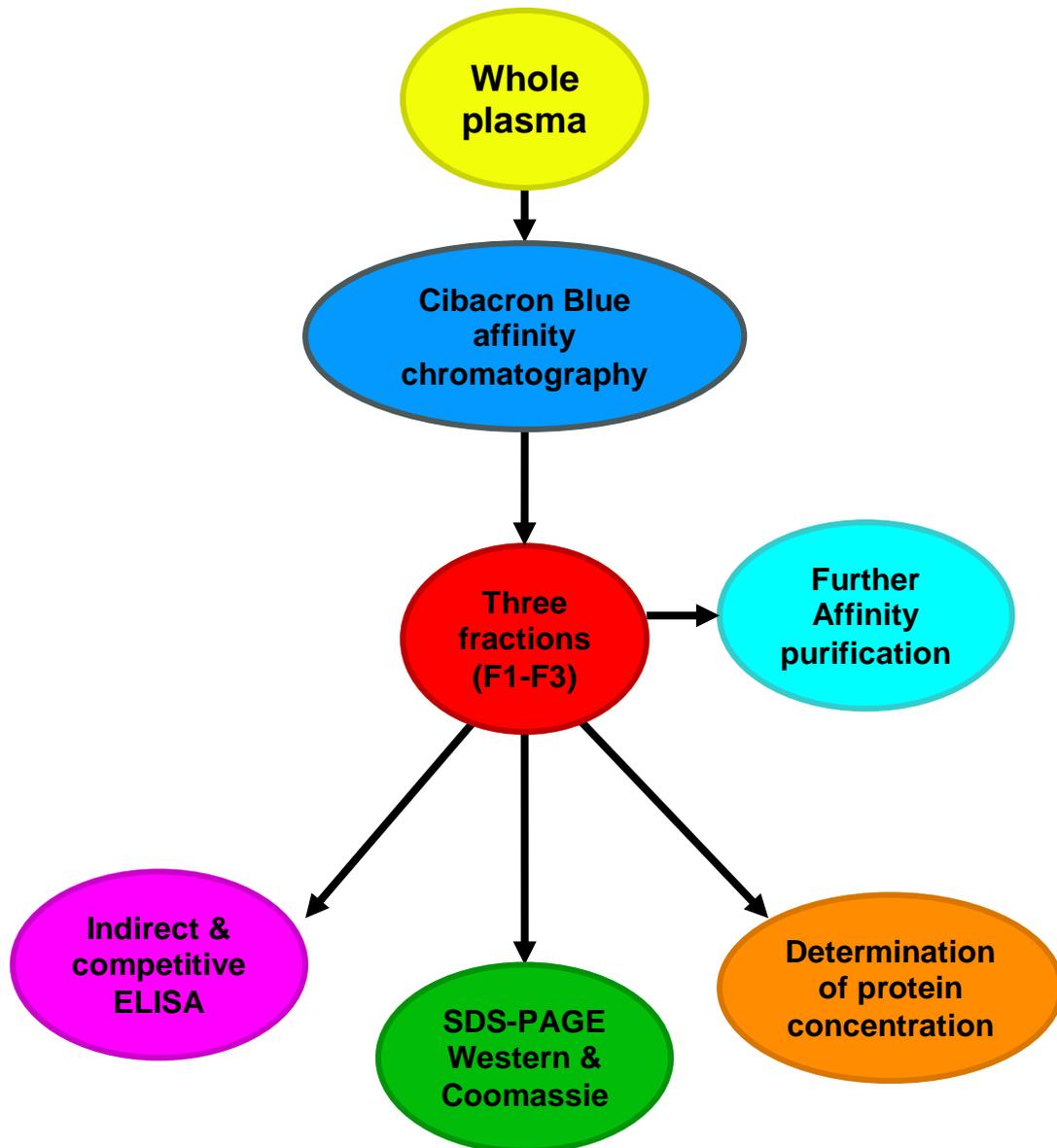


Figure 4.3.v. Illustration of the different analytical techniques applied to the claudicant and control plasma following Cibacron blue affinity purification.

To overcome the masking of other proteins by HSA the claudicant and control plasma samples were fractionated using dye-ligand affinity chromatography on immobilised Cibacron blue (Gianazza and Arnaud 1982). This fractionation method resulted in three protein fractions for each plasma sample (F1-F3, see table 4.3.ii. for protein concentrations in each fraction). Fraction 1 (F1) contained unbound plasma proteins which were eluted from the column using 0.03M phosphate buffer, fraction 2 (F2) contained loosely bound plasma proteins which were eluted from the column in the presence of salt (0.5M NaCl). Finally the third fraction (F3) consisted of tightly bound plasma proteins which were removed by 'stripping' the column using 0.5M NaSCN.

	Protein concentration (mg/ml)			
	F1	F2	F3	Total (F1-F3)
Claudicants	1.6 ±0.4	0.8 ±0.2	0.3 ±0.1	2.7 ±0.4
Control subjects	1.1 ±0.1	0.7 ±0.1	0.5 ±0.1	2.3 ±0.1

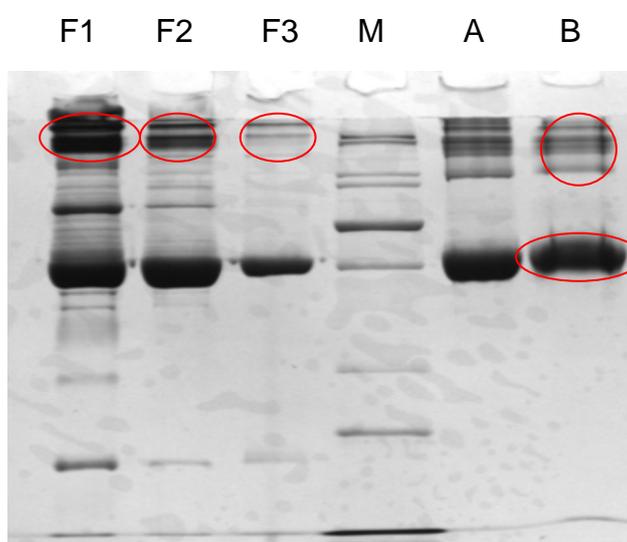
**Table 4.3.ii. Protein concentration for each fraction obtained following separation of the plasma samples using dye-ligand affinity chromatography on immobilised Cibacron blue.**

**Protein concentrations calculated using the Bradford method adapted for use on microtitre plates. F1 - unbound protein fraction, F2 - loosely bound protein fraction and F3 - represents the tightly bound protein fraction. Mean data ± standard deviation.**

These fractionated plasma protein samples were then further separated using SDS-PAGE and visualised via Coomassie staining. From this it was clear that the human serum albumin (HSA) had not been completely separated from the rest of the plasma proteins within the fractions (Figure 4.3.vi.). Following SDS-PAGE, the fractions were probed for the presence of nitrated-proteins using Western blot immunodetection. Very faint staining for the presence of nitrated

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proteins was identified in several bands in all three fractions for all claudicant and control subjects (Western blot not shown due to poor resolution). The corresponding areas in which positive bands for 3NT-containing proteins were identified by Western blot analysis have been circled (○) on the representative Coomassie stain shown in Figure 4.3.vi. Due to the nature of the assay being used and the low level response observed quantification of levels of 3NT present was not possible.



**Figure 4.3.vi. Visualisation of Cibacron blue affinity fractionated claudicant plasma, using Coomassie stain following SDS-PAGE separation.**

**Samples F1 – F3 represent the Cibacron blue fractions for claudicant CS001. M - Anamed size maker; A - native BSA; B - NT-BSA. A duplicate gel was probed for the presence of 3NT-containing proteins using Western blot immunodetection (positive bands identified above with ○). The above is representative of all of the fractions tested.**

The fractions obtained from the Cibacron affinity fractionation procedure were also screened for the presence of 3NT-containing proteins using both indirect and competitive ELISA (as described in Chapter 3). It was clear from this analysis that the presence of nitrated-proteins could not be detected in any of the Cibacron fractions tested (from either the claudicants or the control

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subjects) using indirect and competitive ELISA. These results did not concur with those findings obtained when using different immunodetection methods (Western blot immunodetection following SDS-PAGE). The three Cibacron fractions were therefore fractionated by affinity chromatography on an anti-3NT antibody column.

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4.3.3. Affinity chromatography – purification of nitrated and non-nitrated proteins

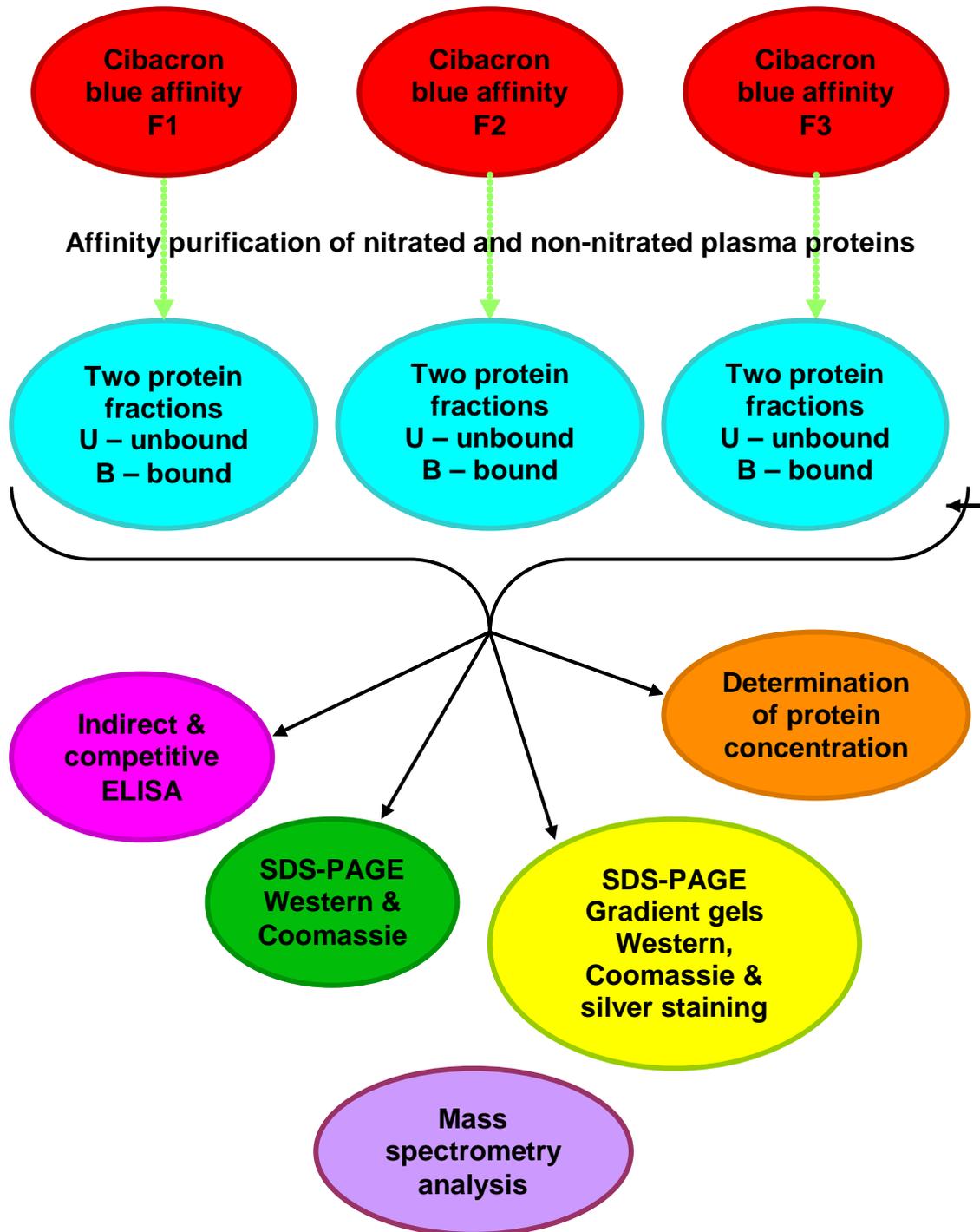


Figure 4.3.vii. A flow diagram to illustrate the further fractionation and analysis of the Cibacron blue affinity purification fractions for both the claudicant and control samples.

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The Cibacron fractions from plasma were further fractionated using anti-3NT Ab-affinity purification in order to separate nitrated plasma proteins from non-nitrated proteins. Each Cibacron fraction resulted in two affinity fractions, a non-nitrated unbound protein fraction (U) eluted from the column using carbonate buffer (0.25M, pH 9.5) and a bound nitrated protein fraction (B) eluted from the column in the presence of 10mM free 3-nitro-L-tyrosine. Table 4.3.iii. shows the protein concentrations of each fraction obtained.

	Protein concentration (mg/ml)						Total
	F1		F2		F3		
	U	B	U	B	U	B	
Claudicants	7.83 ±2.00	0.22 ±0.13	2.98 ±0.46	0.14 ±0.08	1.0 ±0.24	0.14 ±0.05	12.33 ±2.04
Control Subjects	6.57 ±1.00	0.10 ±0.15	2.56 ±0.18	0.06 ±0.14	1.50 ±0.17	0.09 ±0.13	10.88 ±1.58

**Table 4.3. iii. Protein concentration for each fraction obtained following affinity purification of 3NT-proteins from the Cibacron fractions from section 4.3.2.**

Protein concentrations were obtained using the BCA method adapted for use on microtitre plates. F1-F3 represents the fractions obtained from the dye-ligand affinity chromatography on immobilised Cibacron blue in section 4.3.2. above. Each of these fractions were further purified, producing an unbound (U, un-nitrated) fraction and bound (B, nitrated) protein fraction, using a column onto which the anti-3NT Ab had been bound. Mean data ± standard deviation.

The indirect and competitive ELISA methods developed were used to quantify the concentration of nitrated proteins within each affinity fraction for both the claudicants and the control subjects. When testing the affinity fractions using the indirect ELISA the presence of 3NT could not be established. However, when using the competitive ELISA developed, the quantification of the concentration of nitrated proteins within these affinity fractions was possible. From this analysis it was clear that there were varying levels of 3NT-containing proteins present within the different fractions and in general the

bound affinity fractions had significantly higher levels compared to the unbound fractions. With the exception of the unbound affinity fraction F2U the claudicant fractions were found to contain higher levels of 3NT-proteins (expressed as  $\mu\text{M}$  tryp-NT-BSA equivalents per mg protein) compared to the control subjects. It is however notable that nitrated proteins were found within the unbound proteins fractions moreover, high levels were also found in the equivalent control fractions (Table 4.3.iv).

	$\mu\text{M}$ Tryp-NT-BSA equivalents per mg protein						Mean
	F1U	F1B	F2U	F2B	F3U	F3B	
<b>Claudicants</b>	0.03 $\pm 0.04$	50.4 $\pm 48.6$	0.24 $\pm 0.13$	167.9 $\pm 308.1$	0.66 $\pm 0.97$	192.4 $\pm 191.7$	68.59 $\pm 91.59$
<b>Control subjects</b>	0.01 $\pm 0.02$	19.2 $\pm 28.8$	0.62 $\pm 1.22$	81.5 $\pm 38.3$	0.08 $\pm 0.04$	92.2 $\pm 89.4$	32.25 $\pm 26.30$

**Table 4.3.iv.** Estimated level of nitration in the affinity purified protein fractions for the claudicant and control subjects.

The above values are presented as tryp-NT-BSA equivalents ( $\mu\text{M}$ ) per mg protein as determined from standard curves constructed using tryp-NT-BSA standards on competitive ELISA. F1-F3 represent the fractions obtained from the dye-ligand affinity chromatography on immobilised Cibacron blue in section 4.3.2. above. Each of these fractions were further purified, producing an unbound (U, un-nitrated) fraction and bound (B, nitrated) protein fraction, using a column onto which the anti-3NT Ab had been bound. Mean data  $\pm$  standard deviation.

The proteins within each of these affinity fractions were again visualised using Coomassie stain and probed for the presence of nitrated-proteins via Western blot immunodetection following SDS-PAGE (Figure 4.3.viii.). The Coomassie stain highlighted that the majority of the protein was found to be located in the unbound non-nitrated fractions and that only very faint proteins staining was present within the bound fractions (Figure 4.3.viii. a). Upon probing for the presence of 3NT-containing proteins using Western blot immunodetection all of the affinity fractions, both unbound and bound, were found to contain

proteins which stained positive for the presence of 3NT. Although the Coomassie stain highlighted that the majority of the protein was present in the three unbound protein fractions a similar degree of staining was observed within all of the fractions during Western blot immunodetection. The majority of the protein bands that stained positive for the presence of 3NT-residues were of a molecular mass >100kDa (as determined from the Coomassie stain). Due to poor resolution of the bands, determination of the exact molecular mass was not possible, estimated to be in the region of 220-116kDa. Fainter staining was also observed at lower molecular mass within the bound affinity fractions (Figure 4.3.viii. b). No differences were observed between the claudicants and control affinity purified samples.

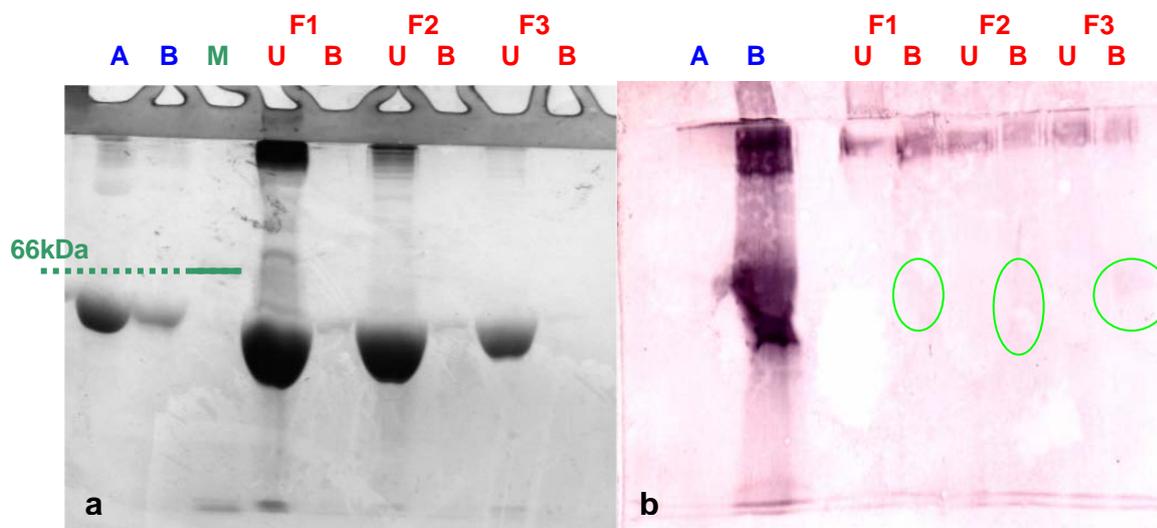
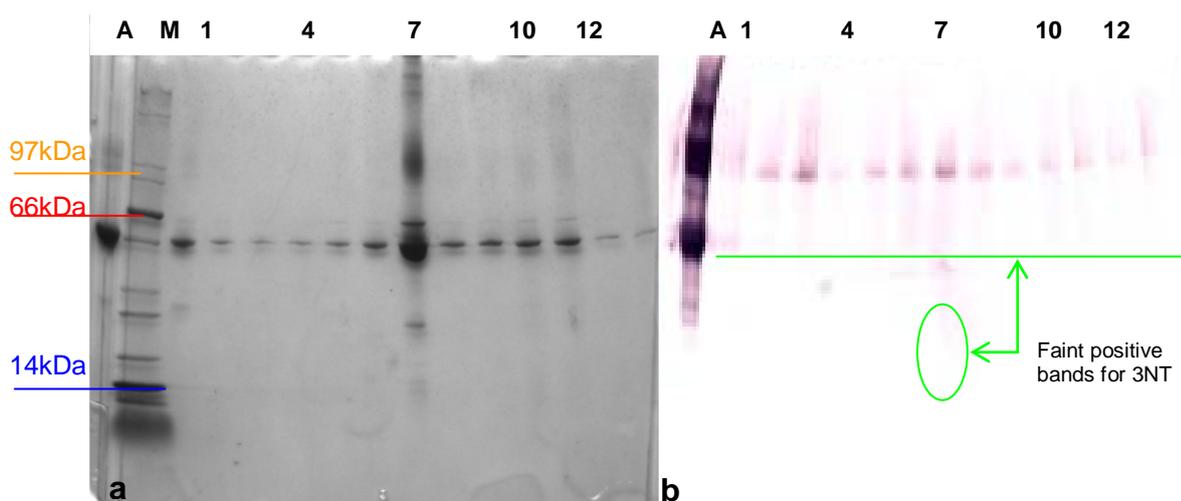


Figure 4.3.viii. Coomassie stain (a) and Western (b) of CS002 Cibacron fractions separated on an anti-3NT Ab affinity column

**F1U & F1B, F2U & F2B, F3U & F3B** = fraction 1-3 from Cibacron separation further separated by affinity purification where U represents the antibody unbound fraction and B represents the bound fraction.  
**A** – Native BSA; **B** – NT-BSA and **M** represents the size marker. Areas highlighted (○) indicate where faint staining was also found, not evident on figure above.

The bound fractions for each subject (F1B-F3B) were pooled, due to low protein concentration, and separated using SDS-PAGE on gradient polyacrylamide gels (4-20%) which resulted in a better resolution of the proteins present within each sample (Figure 4.3.ix. a and 4.3.ix. b). Several bands of varying molecular weight were found to stain positive for the presence of 3NT-containing proteins in all claudicant and control samples when using Western blot immunodetection (Figure 4.3.ix. b). It was evident that those bands which produced a strong signal for the presence of 3NT on the Western blot did not stain strongly on the Coomassie stained gel, Figure 4.3.ix. a and 4.3.ix. b.



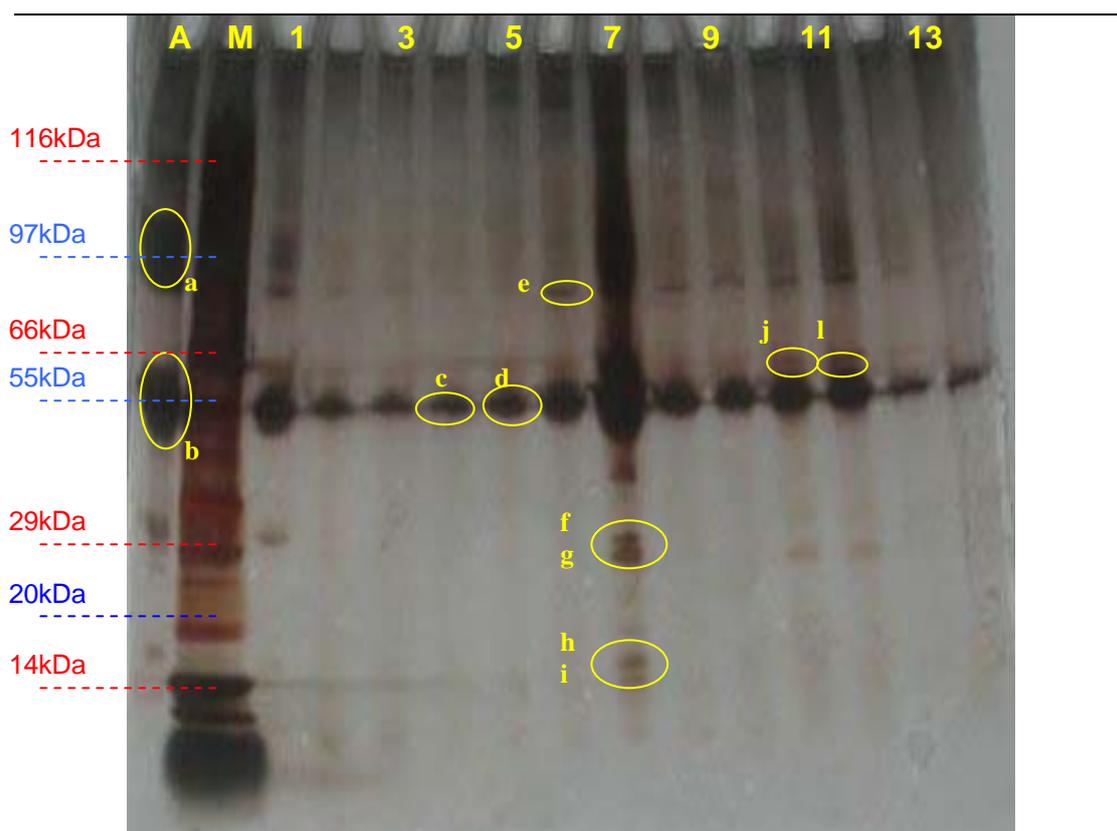
**Figure 4.3.ix. Affinity purified claudicant and control plasma fractions visualised using Coomassie stain and Western blot immunodetection using gradient SDS-PAGE**

The anti-3NT Ab antibody bound affinity purified fractions F1B-F3B for each subject were pooled together and loaded onto the gradient gel. a - Coomassie-stained gel; b- probed Western blot. Positive control - NT-BSA (A), protein size marker (M). Wells 1-11 - pooled affinity purified samples for 11 claudicants and wells 12-13 control subjects. Similar results were obtained for the other claudicant and control samples. Not all positive bands are clear on the figure above, areas highlighted (○ --) indicate where faint staining was also found.

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The gradient gels were further stained using the more sensitive silver stain in order to get a clearer representation of those bands which had been identified as containing nitrated proteins using Western blot immunodetection (Figures 3.4.x.-3.4.xii.). From the silver stain it was evident that the major stained proteins within the pooled samples were found to be situated in the resolving gel at  $RMM \approx 55kDa$ . Protein bands from these silver stained gels, that corresponded to bands identified as containing 3NT-proteins using Western blot immunodetection (Figure 4.3.ix.), were further analysed using MALDI-MS and LC-ESI-MS/MS (by the Biological Mass Spectrometry and Proteomics Department, University of Warwick). Those protein bands which were excised for MALDI-MS and LC-ESI-MS/MS analysis are highlighted in Figures 4.3.x.-4.3.xii; not all bands were excised from the gradient gels. All proteins were identified using the Mascot search engine which interrogated databases including the IPI human protein database (V.3.07) and SwissProt where appropriate. The proteins identified in within the bands excised from the gradient gels represented in Figures 4.3.x.-4.3.xii are shown in Tables 4.3.v.-4.3.vii. Several proteins were identified within each excised band with varying degrees of percentage sequence coverage by tryptic peptides for each protein. The presence of 3NT-residues (i.e. a mass increase of +45Da corresponding to +NO<sub>2</sub> -H) could not be confirmed within any of the proteins identified by either method. The main nitrated proteins identified in both the claudicant and control subjects were HSA, serotransferrin precursor, apolipoprotein A1 precursor and apolipoprotein A2 precursor (Tables 4.3.v.-4.3.vii).

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**Figure 4.3.x.** Silver stain of gradient SDS-PAGE gel for pooled affinity fractions for Claudicants CS001-CS011 and control subjects CD01-CD02.

A – NT-BSA; M – Anamed size marker; CS001-CS011 wells 1-11 and control subjects CD01-CD02 wells 12-13.

Band	Protein	Database No (IPI or SwissProt[P])	RMM	MS Coverage (%)
A	Serum albumin precursor (bovine)	P02769	71244	28
B	Serum albumin precursor (bovine)	P02769	71244	32
C	Albumin protein	00022434	73881	5
D	Albumin protein	00022434	73881	17
		00216773	46442	16
		00384697	48641	13
E	Albumin protein <sup>#</sup>	00022434	73881	8
	Serotransferrin precursor	00022463	79280	20
F	Apolipoprotein A1 precursor	00021841	30759	32
	Albumin <sup>#</sup>	00022434	73881	14
G	Apolipoprotein A1 precursor	00021841	30759	56
	Albumin protein <sup>#</sup>	00022434	73881	5
H	Apolipoprotein A1 <sub>I</sub> precursor <sup>#</sup>	00021854	11282	29
I	Haemoglobin β chain	00218816	16102	29
J & L	Serotransferrin precursor	N/A	N/A	N/A

**Table 4.3.v.** Summary data from figure 4.3.x. MALDI-MS & LC-ESI-MS/MS analysis.

The above table gives a summary of the proteins identified in the bands excised from the gradient gels shown in Figure 4.3.x. All of the proteins were identified using both MALDI-TOF-MS and LC-ESI-MS/MS unless otherwise stated (<sup>#</sup> - identified by LC-ESI-MS/MS only).

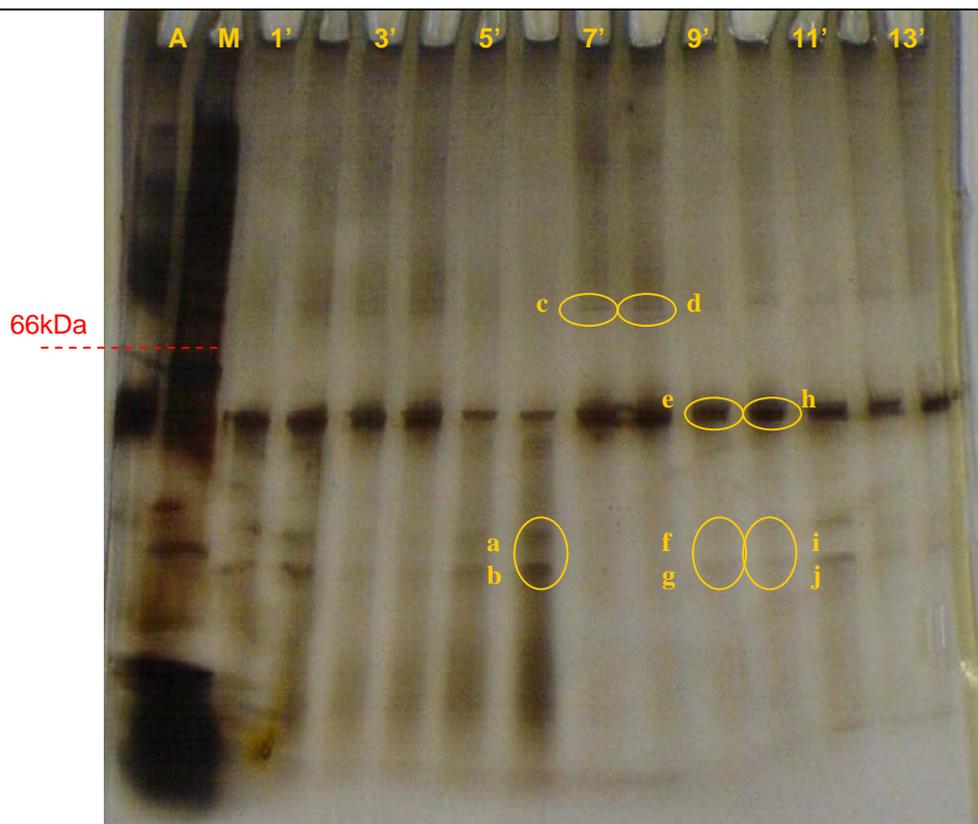


Figure 4.3.xi. Silver stain of gradient SDS-PAGE gel for pooled affinity fractions for Claudicants CS012-CS022 and control subjects CD03-CD04.

A – NT-BSA; M – Anamed size marker; CS012-CS022 wells 1'-11' and control subjects CD03-CD04 wells 12'-13'.

Band	Protein	Database No (IPI or SwissProt[P])	RMM	MS Coverage (%)
A	Albumin protein <sup>#</sup>	00216773	46442	21
B	Albumin protein <sup>#</sup>	00216773	46442	21
		00384697	48641	18
C, D, G & J	No significant hits	--	--	--
E, F, H & I	Albumin protein	00022434	73881	--

Table 4.3.vi. Summary data from figure 4.3.xi. MALDI-MS & LC-ESI-MS/MS analysis.

The above table gives a summary of the proteins identified in the bands excised from the gradient gels shown in Figure 4.3.xi. All of the proteins were identified using both MALDI-TOF-MS and LC-ESI-MS/MS unless otherwise stated (<sup>#</sup> - identified by LC-ESI-MS/MS only).

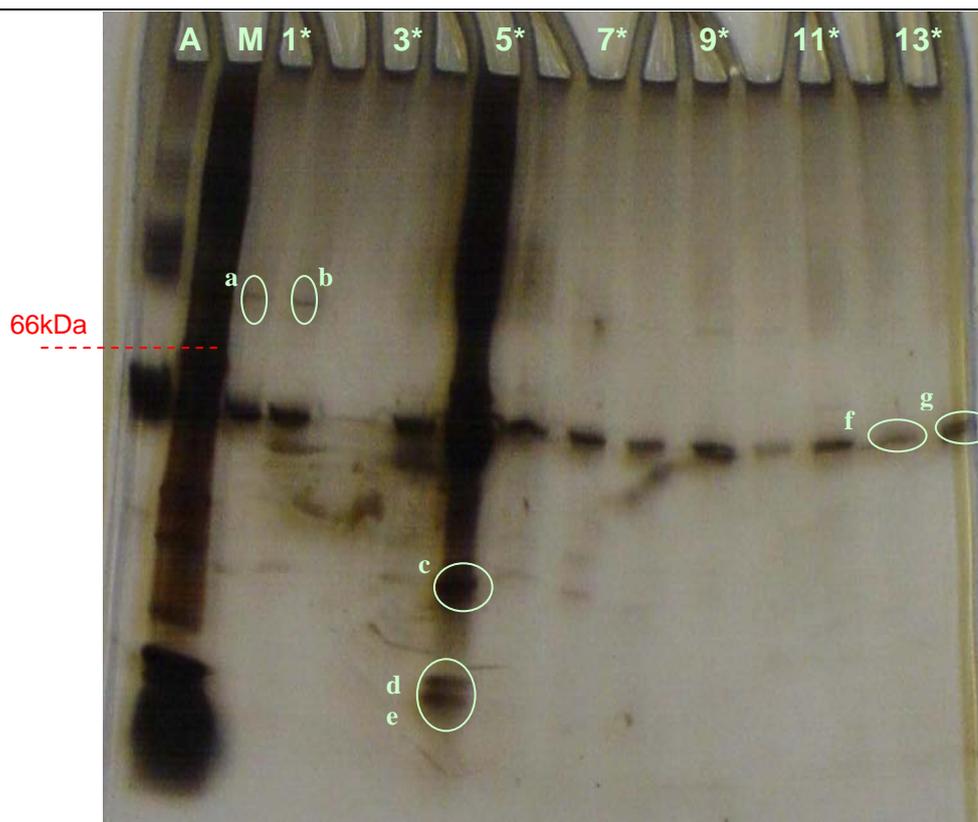


Figure 4.3.xii. Silver stain of gradient SDS-PAGE gel for pooled affinity fractions for Claudicants CS023-CS033 and control subjects CD05-CD06.

A – NT-BSA; M – Anamed size marker; CS023-CS033 wells 1\*-11\* and control subjects CD05-CD06 wells 12\*-13\*.

Band	Protein	Database No (IPI or SwissProt[P])	RMM	MS Coverage (%)
A	No significant hits	--	--	--
B	Apolipoprotein A1 precursor <sup>#</sup> Albumin protein <sup>#</sup>	00021841	--	--
C	Apolipoprotein A1 precursor	00021841	30759	70
D	Haemoglobin $\beta$ chain Apolipoprotein A1 <sub>I</sub> precursor <sup>#</sup>	00218816 00021854	16102 11282	23 46
E	Haemoglobin $\beta$ chain Haemoglobin $\delta$ chain <sup>#</sup> $\alpha$ 2 globin variant <sup>#</sup> Apolipoprotein A1 precursor <sup>#</sup>	00218816 00473001 00410714 00021841	16102 16028 15328 30759	51 30 16 12
F & G	Albumin protein	00022434	73881	--

Table 4.3.vii. Summary data from figure 4.3.xii. MALDI-MS & LC-ESI-MS/MS analysis.

The above table gives a summary of the proteins identified in the bands excised from the gradient gels shown in Figure 4.3.xii. All of the proteins were identified using both MALDI-TOF-MS and LC-ESI-MS/MS unless otherwise stated (<sup>#</sup> - identified by LC-ESI-MS/MS only).

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#### 4.3.4. Determination of the protein concentration of the whole plasma, dye-linked and 3NT-affinity purified fractions.

Several protein determination methods (e.g. Lowry, Bradford and BCA) had to be used to determine the total protein concentration of the different protein fractions as highlighted in Figure 4.3.xiii. below (for full data see Appendices J). Comparisons were made of the total protein values obtained when using different methods for the same samples from which it was clear that there were stark differences between the different methods (results not shown).

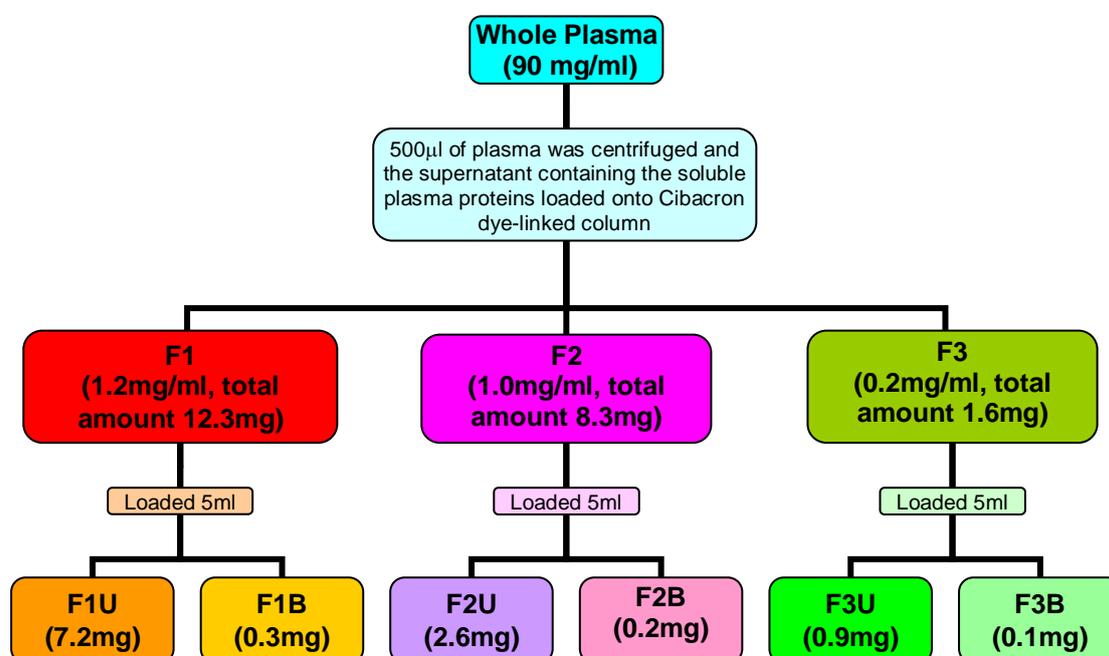


Figure 4.3.xiii. A schematic diagram to illustrate the protein concentrations determined for the different fractions obtained for a claudicant plasma sample.

The above values represent those calculated for claudicant CS002. The total protein concentrations for each set of samples were determined using different techniques: Lowry method (Lowry, Rosebrough, Farr *et al.* 1951) for the whole plasma; Bradford method adapted for use on microtitre plates was used for fractions F1-F3 (obtained from dye-linked affinity chromatography on immobilised Cibacron blue); BCA method adapted for use on microtitre plates was used for fractions F1U-F3B (obtained from 3NT-affinity purified fractions).

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#### **4.4. Discussion**

The presence of 3NT-containing proteins have been identified in many different diseases such as Alzheimer's (Smith, Richey Harris, Sayre *et al.* 1997), atherosclerosis (Beckmann, Ye, Anderson *et al.* 1994), celiac disease (Ter Steege, Koster-Kamphuis, Van Straaten *et al.* 1998), chronically rejected human renal allografts (Macmillan-Crow, Crow, Kerby *et al.* 1996), pituitary cancer (Zhan and Desiderio 2006), rheumatoid arthritis (Kaur and Halliwell 1994), and the inflammatory disorder systemic sclerosis (Khan, Brennand, Bradley *et al.* 1998). Within this study we can report that 3NT-containing proteins have been identified within the plasma of claudicants. The commentary below will now discuss the techniques employed in order to establish the presence of 3NT-residues.

##### **4.4.1. Initial screening for the presence of 3NT using immunodetection**

Initial screening using immunodetection (dot blot analysis) confirmed the presence of 3NT-containing proteins within all of the claudicant plasma samples tested. This finding was not unexpected as IC is a peripheral manifestation of systemic atherosclerotic disease and 3NT-residues have been identified within atherosclerotic lesions in coronary arteries using immunohistochemical staining techniques (Beckmann, Ye, Anderson *et al.* 1994) and lesions in transplant coronary artery disease (Ravalli, Albala, Ming *et al.* 1998). Interestingly dot blot immunodetection also signified the presence of 3NT-containing proteins within the plasma of the healthy control subjects enrolled onto this study. Currently there is a degree of controversy surrounding the presence of 3NT-containing proteins within the plasma of

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healthy individuals. While nitrated proteins have been confirmed in healthy individuals in some studies (Khan, Brennand, Bradley *et al.* 1998, Frost, Halliwell and Moore 2000, Inoue, Hisamatsu, Ando *et al.* 2002, Capeillere-Blandin, Gausson, Descamps-Latscha *et al.* 2004, Dooley, Gao, Bradley *et al.* 2006) the range of concentrations reported is highly divergent. Others have reported that nitration could not be identified in plasma from healthy individuals (Kaur and Halliwell 1994, Ter Steege, Koster-Kamphuis, Van Straaten *et al.* 1998). Although some of these differences can be explained by the analytical technique being used, differences in reported values have been observed for example in mass spectrometry-based techniques which are generally accepted as being inherently specific and accurate (Tsikas and Caidahl 2005). These differences could be explained by reports of 'denitrase' activity, thought to be an enzyme resulting in the reduction of 3NT immunoreactivity in proteins (Gow, Duran, Malcolm *et al.* 1996, Kamisaki, Wada, Bian *et al.* 1998, Irie, Saeki, Kamisaki *et al.* 2003) which provides evidence that the presence of 3NT-residues within proteins may be a dynamic process that can be regulated by the reversal of nitration by 'denitrase'. This supports the hypothesis that protein nitration, and the associated actions of 'denitrase', may not only be involved in disease pathology but may also be implicated in biological mechanisms such as cellular signal transduction (Gow, Duran, Malcolm *et al.* 1996, Kamisaki, Wada, Bian *et al.* 1998, Irie, Saeki, Kamisaki *et al.* 2003).

The level at which 3NT has been reported to be present in the plasma of healthy individuals may also be dependent upon the technique being used to quantify it and if free or protein-bound 3NT is being detected. For example

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Khan *et al* (1998) and Inoue *et al* (2002) reported similar levels of 3NT-containing proteins in healthy individuals (0.12 $\mu$ M nitro-BSA equivalents and 0.14 $\mu$ M respectively) by ELISA. Whereas Frost *et al* (2000), using a GC-MS method developed to avoid artifactual 3NT production, reported substantially lower levels of free 3NT in the plasma of healthy individuals (0.064 $\mu$ M).

As dot blot immunodetection is a purely qualitative technique it was not apparent from the results the extent to which the plasma proteins were nitrated. Further clarification was also required regarding the nitration profile of the plasma proteins. Therefore the plasma samples were analysed using the indirect and competitive ELISAs developed along with Western blot immunodetection following separation using SDS-PAGE. From this analysis it was clear that confirmation of the presence of 3NT-containing residues within the claudicant and control plasma samples was not possible. These findings are contradictory to those reported within this study when using dot blot immunodetection and with published reports in which 3NT-containing proteins have been shown to be present in human plasma using immunoassays (several ELISA formats) and Western blot immunodetection (Macmillan-Crow, Crow, Kerby *et al.* 1996, Khan, Brennand, Bradley *et al.* 1998, Ter Steege, Koster-Kamphuis, Van Straaten *et al.* 1998, Inoue, Hisamatsu, Ando *et al.* 2002, Capeillere-Blandin, Gausson, Descamps-Latscha *et al.* 2004, Dooley, Gao, Bradley *et al.* 2006, Khan and Siddiqui 2006).

It is well documented both within this study (Chapter 3) and by others (Ter Steege, Koster-Kamphuis, Van Straaten *et al.* 1998, Khan, Brennand, Bradley *et al.* 1998, Franze, Weller, Niessner *et al.* 2004) that there are quantitative differences regarding the antigen-antibody reaction for the detection of 3NT-

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epitopes within different proteins. When using immunodetection techniques such as Western blot analysis there is also the potential of the underestimation of 3NT-containing proteins due to reduction of the 3NT-residues during sample preparation (Pignatelli, Li, Boffetta *et al.* 2001). They reported that boiling protein fractions in sample buffer prior to separation using SDS-PAGE can result in the reduction of 3NT to aminotyrosine. This temperature-dependent reduction is thought to be a direct result of interference from thiols (e.g. mercaptoethanol) within the sample buffer which have been identified as agents that are capable of reducing 3NT to aminotyrosine (Balabanli, Kamisaki, Martin *et al.* 1999, Soderling, Hultman, Delbro *et al.* 2007). This sample preparation-induced reduction of 3NT-residues is not thought to be relevant to this study as the plasma fractions were not boiled in sample buffer prior to separation using SDS-PAGE. It is postulated that the observed differences between the ELISA and immunodetection techniques are more probably due to sample dilution and inaccessibility of the 3NT-residues rather than reduction of the 3NT-residues.

#### **4.4.2. Human anti-3NT Abs**

Several studies have reported the presence of anti-3NT Abs in different diseases including rheumatoid arthritis, osteoarthritis, systemic lupus erythematosus (Khan and Siddiqui 2006) and acute lung injury (Thomson, Christie, Vadseth *et al.* 2007). Although the presence of these antibodies could not be identified within this study their production in diseases such as IC cannot be ruled out. Khan and Siddiqui (2006) reported that anti-3NT Abs levels were higher within the synovial fluid compared to the serum in patients suffering from both forms of arthritis. Therefore it is credible that, if anti-3NT

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Abs are produced by claudicants, they may only be detectable during certain stages of the pathology of the disease, e.g. 3NT may only be produced/present in plasma during periods of oxidative stress, induced by ischaemia-reperfusion. At other times any 3NT-residues present may be located predominantly within the atherosclerotic lesions of the peripheral blood vessels as observed with atherosclerotic plaques within coronary arteries (Beckmann, Ye, Anderson *et al.* 1994). In studies such as that described by Thomson *et al* (2007), affinity purification steps were employed prior to confirmation of the presence of anti-3NT Abs however in this study only the whole plasma was assessed for the presence of anti-3NT Abs.

It has been acknowledged for some time that the presence of antibodies directed towards dietary proteins such as BSA exist especially, for example, in the young with insulin dependent diabetes mellitus (Hilger, Grigioni, De Beaufort *et al.* 2001, Mogues, Li, Coburn *et al.* 2005). Within this study both the claudicant and the control plasma samples were found to respond towards native BSA to a greater extent than that observed for NT-BSA. This diminished response observed when using NT-BSA would suggest that nitration of BSA actually serves to reduce the antigenic epitope on the BSA protein against which the anti-BSA Abs are selective. A similar pattern was observed when assessing HSA (both native and nitrated) although the response was somewhat higher in the claudicants' plasma compared to the control subjects. These findings regarding the presence of anti-BSA Abs within the plasma samples would give rise to non-specific binding when using BSA as an antigen within an assay such as an ELISA (e.g. NT-BSA). However, in the assay reported here the incubation buffer contained a

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sufficiently high concentration of BSA and as such should have prevented non-specific binding.

#### **4.4.3. Fractionation of human plasma using dye-ligand affinity chromatography on immobilised Cibacron blue**

Human plasma is a complex sample in which currently 289+ plasma proteins have been identified although many more are thought to be present, including proteins serving as messengers and those that leak into the blood due to tissue damage (as reviewed in Anderson and Anderson 2002). The complex nature of plasma can cause major problems when devising diagnostic techniques involving the characterisation and identification of modified proteins (e.g. nitrated proteins) such as the ELISA assay reported here. This is further confounded by the high concentration of large plasma proteins such as HSA potentially masking changes to proteins present in lower concentrations. Therefore when detecting protein markers within human plasma (e.g. nitrated-proteins) issues can arise as a direct result of HSA constituting approximately 55%, (35-50mg/ml), of the total protein (Anderson and Anderson 2002). This high concentration of HSA could result in the masking of 3NT-containing proteins present at lower concentrations. Therefore HSA was separated from the other plasma proteins using dye-ligand affinity chromatography on immobilised Cibacron blue.

Leatherbarrow and Dean (1980) and Gianazza *et al.* (1982) state that use of immobilised Cibacron blue is a technique which is specific for removal of HSA from plasma as it binds HSA with high specificity and the use of a chaotropic agent, such as sodium thiocyanate, is required to desorb it off the column. Unlike the reports above, within this study HSA was found to be present in all

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of the resulting protein fractions, albeit most was in the expected fraction. This may be due to the presence of high levels of fatty acids, within the plasma samples, which have been shown to interfere with the binding of HSA to the Cibacron blue dye (Leatherbarrow and Dean 1980).

Using a similar separation technique, Khan *et al.* (1998) reported the identification of 3NT-containing proteins within the plasma of healthy individuals and those suffering from the inflammatory disorder systemic sclerosis using competitive ELISA and Western blot immunodetection. Within the current study the presence of 3NT-proteins could not be confirmed in any of the plasma protein fractions obtained from this separation using the developed indirect and competitive ELISA methods. However, when using Western blot immunodetection, 3NT-containing proteins were observed in the protein fractions in all of the claudicant and control plasma samples (albeit a low level response). These observed differences in assay sensitivity may be due to alterations in the availability and accessibility of the 3NT-residues to the antibody brought about by changes in protein conformation and the binding capacities of the solid phase being used (similar to that discussed in Chapter 3).

#### **4.4.4. Affinity chromatography on anti-3NT antibody column – purification of nitrated and non-nitrated proteins**

The presence of 3NT-containing proteins was confirmed in both the purified nitrated and non-nitrated plasma fractions using competitive ELISA and Western blot immunodetection following separation using SDS-PAGE on linear and gradient gels.

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Due to the anti-3NT having differing binding affinities for various nitrated proteins, as reported in Chapter 3, the competitive ELISA could only be classed as a semi-quantitative technique with regards to detecting the presence of 3NT-containing proteins. Similar to that reported by Khan *et al* (1998), the results presented here can only be reported as tryp-NT-BSA equivalents ( $\mu\text{M}$ ) per mg of protein and not actual 3NT concentrations. This is because the standard used within the assay was a single electrochemically-nitrated protein (tryptically digested NT-BSA) and as such its reactivity with antibody could not be extrapolated to all potentially nitrated proteins in claudicant and control plasma.

Although nitration was found to be present within all of the affinity fractions (both bound and unbound fractions), in general elevated levels of nitration were observed within the plasma fractions of the claudicants compared to the healthy control subjects. This is similar to that reported by Khan *et al* (1998) who found elevated concentrations of nitrated proteins (again reported as NT-BSA equivalents) within the plasma of patients suffering from the autoimmune inflammatory disease systemic sclerosis compared to healthy individuals when using their competitive ELISA. This is the first time such separation and affinity purification techniques have been reported as being necessary to establish the presence of 3NT-residues.

Within this study the levels of nitration found to be present within the claudicant and control plasma fractions are somewhat higher than those reported previously in various disease pathologies using analytical techniques including ELISA (Khan, Brennan, Bradley *et al.* 1998, Ter Steege, Koster-Kamphuis, Van Straaten *et al.* 1998, Inoue, Hisamatsu, Ando *et al.* 2002,

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Dooley, Gao, Bradley *et al.* 2006) and HPLC-UV (Kaur and Halliwell 1994). For example in this study the level of 3NT within the HSA fractions of the control plasma samples was estimated to be 92.2 nmol tryp-NT-BSA equivalents per mg protein, and even 0.08 nmol tryp-NT-BSA equivalents per mg protein in the fraction which did not bind to the antibody column, compared to 0.085nmol nitro-BSA equivalents per mg protein (in Cibacron dye-linked affinity purified fractions) reported by Khan *et al* (1998). This observed difference could be as a result of the increased availability of the 3NT-residues to the anti-3NT Ab brought about by the separation and purification techniques used. However it is also possible that assay precision (highlighted in Chapter 3) and the potential contamination of the bound fractions with free-3NT (remaining from the affinity purification techniques) may have led to over estimation. The concentration of 3NT in the antibody-bound, Cibacron HSA fraction (3) was 92.2 nmol tryp NT-BSA equivalents per mg protein, a very high level, but this represented only 6% of the HSA in the total fraction 3 from the Cibacron column (Table 4.3.iii).

The estimated levels of nitration within the 3NT-affinity purified samples are not only dependent upon an accurate estimation of tryp-NT-BSA equivalents from the calibration curves constructed from the ELISA but also the accurate determination of the total protein concentration within each sample. Due to the nature of the plasma samples, the presence of incompatible substances (e.g. glycerol) and the number of samples needing to be processed following the affinity purification procedures, several different protein determination assays had to be used (Okutucu, Dincer, Habib *et al.* 2007). When comparing different protein assays using the same protein samples it was clear that there

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were differences in the determined protein concentrations obtained. The exact reason behind these differences is unclear. The accurate determination of the total protein concentration within a sample is reliant upon the accuracy of the protein assay being used. There are differences in the reliability of the different protein assays available with regards to the nature of the protein sample being tested (Nishi, Kestner and Elin 1985, Berges, Fisher and Harrison 1993, Okutucu, Dincer, Habib *et al.* 2007).

Western blot immunodetection, following separation using SDS-PAGE, confirmed the presence of 3NT-proteins within all of the affinity purified fractions (both bound and unbound) for all of the claudicant and control subjects. No apparent difference in the level and pattern of protein staining for the presence of 3NT was observed between the claudicant and control samples, similar to that findings reported by Capeillere-Blandin *et al* (2004) with reference to plasma from haemodialysed and control subjects. The major stained protein band observed on the Coomassie/silver stained gradient gels, which ran at a comparable distance to the NT-BSA sample (approximately 55kDa), was confirmed as being albumin (HSA) protein via MALDI-TOF-MS and LC-ESI-MS/MS analysis. This albumin protein band did stain positive for the presence of 3NT proteins when using Western blot immunodetection (albeit faintly) which differs to that reported by ter Steege *et al* (1998) who reported that no 3NT staining was identified at 66kDa representing HSA. This could be explained by the consistent observation in this study that albumin (both BSA and HSA) ran further than the 66kDa size marker on the gel for which no definitive reason can be suggested. Western blot immunodetection showed that those protein bands which staining strongly for the presence of

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3NT-residues were located within the resolving gel with molecular weights greater than 66kDa as also observed by ter Steege *et al* (1998) but in our case faintly stained bands of lower molecular weight were also observed. ter Steege *et al* (1998) report that their findings suggest only high molecular weight proteins in serum are subject to nitration. This does not agree with the current findings or with those of Khan *et al* (1998) where proteins staining positive for the presence of 3NT were observed at molecular weights both higher and lower than 66kDa. This difference in detection of nitrated proteins may in part be due to ter Steege *et al* (1998) only screening whole plasma whereas Khan *et al* (1998) screened dye-ligand purified plasma fractions and within this study samples were further purified into nitrated and non-nitrated fractions.

As determined by the competitive ELISA and Western blot immunodetection, nitrated proteins were found in both the nitrated and non-nitrated affinity fractions. The presence of 3NT-containing proteins in the unbound fraction (non-nitrated) could be due the 3NT-residues not being available to the antibody at the column fractionation stage. Also if our antibody has low affinity for certain nitrated-plasma proteins the quantitative removal of these proteins from the solution would not have been possible.

In recent years researchers have moved towards proteomic techniques in order to establish the protein targets of nitration *in vivo* within different disease states and disease models (Aulak, Miyagi, Yan *et al.* 2001, Lanone, Manivet, Callebert *et al.* 2002, Kanski, Hong and Schoneich 2005, Casoni, Basso, Massignan *et al.* 2005). For example using MALDI-TOF-MS analysis it has been found that inducible nitric oxide synthase (iNOS) in the skeletal muscles

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of septic patients is selectively nitrated (Lanone, Manivet, Callebert *et al.* 2002). iNOS has also been found to be expressed in both macrophages and smooth muscle cells in the lesions of transplant coronary artery disease where it is associated with extensive nitration of cellular proteins (Ravalli, Albala, Ming *et al.* 1998). Many proteins have now been identified as potential targets for nitration; there are however conflicting reports regarding the main sites of nitrated proteins with some studies reporting high levels within the cytosol of the cell (Kanski, Hong and Schoneich 2005) and others within the mitochondria (Aulak, Miyagi, Yan *et al.* 2001). Regardless of the location of the nitrated proteins it is probable that nitration of proteins has important implications in both physiological and pathological conditions.

In order to establish the importance of 3NT in the disease state IC it is imperative to establish the presence/absence of a nitration profile. MALDI-TOF-MS and LC-ESI-MS/MS analysis were combined within this study to identify proteins that were within PAGE bands that had stained immunopositive for the presence of 3NT-proteins. The main putative nitrated proteins identified in both the claudicant and control subjects were HSA, serotransferrin precursor, apolipoprotein A1 precursor and apolipoprotein A2 precursor (physiological relevance unknown) although the presence of 3NT-residues could not be established by mass spectrometry. As mentioned earlier it has been previously reported that HSA is a target for nitration (Khan, Brennand, Bradley *et al.* 1998) which has been further confirmed within this study. Apolipoprotein A1, the primary protein constituent of high-density lipoprotein (HDL) in plasma, is involved in the process of cholesterol esterification in plasma via activation of lecithin-cholesterol acyltransferase

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(LCAT). As such identifying that this protein may be a target for nitration within claudicants is of importance. Apolipoprotein A1 has also been identified as a target for nitration in diabetes type II (Hermo, Mier, Mazzotta *et al.* 2005). Shao *et al* (2005) identified tyrosine 192 as the predominant site of both nitration and chlorination with regards to oxidative damage resulting from both MPO and ONOO<sup>-</sup>. However, they could only establish reduced cholesterol efflux activity of apolipoprotein A1 following chlorination and not nitration.

As HSA appeared to be the predominant protein identified within the excised protein bands CLUSTAL W (1.83) multiple sequence alignment was carried out (See Figure 4.4.i.). In total three HSA accessions to the IPI database were identified, all having a signal sequence (pre-peptide 1-18) and a pro-peptide (inactivating sequence 19-24) by comparison to BSA. From the mass spectrometry analysis, HSA was identified as being present within bands of differing molecular mass (from >70kDa to 29kDa). The RMMs quoted for HSA are for the whole proteins, as coded in the DNA, but the proteins appearing in the plasma should be predominantly the mature versions without the N terminal sequences at 1-24 which are therefore >2kDa smaller. Proteins larger than these RMMs could be due to protein modification such as glycosylation thus giving variable larger sizes. However, smaller proteins must be truncated, presumably by proteolysis. For example, band G, in Figure 4.3.x. where albumin protein (IPI00022434) is apparently 29kDa, is identified in MS by only three peptides, covering 5% of the sequence, so this must be a fragment from the N-terminal end. This is due to the fact that the three peptides identified are at positions 66-75, 76-88 and 287-298 (numbers corresponding to the total protein).

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CLUSTAL W (1.83) multiple sequence alignment

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IPI22434 MKWVTFISLLFLFSSAYSF GVFRRDAHKSEVAHRFKDLGEENFKALVLI AFAQYLQQCPF 60
IPI384697 MKWVTFISLLFLFSSAYSF GVFRRDAHKSEVAHRFKDLGEEN----- 42
IPI216773 MKWVTFISLLFLFSSAYSF GVFRRDAHKSEVAHRFKDLGEEN----- 42
*****

IPI22434 EDHVKLVNEVTEFAKTCVADESAENCDKSLHTLFGDKLCTVATLRETYGEMADCCAKQEP 120
IPI384697 -----
IPI216773 -----

IPI22434 ERNECFLQHKDDNPNLRLVLRPEVDMCTAFHDNEETFLLKYLVEIARRHPYFYAPELLF 180
IPI384697 -----
IPI216773 -----

IPI22434 FAKRYKAAFTTECCQAADKAACLLPKLDLRLDEGKASSAKQRLKCASLQKFGGERAFKAWAV 240
IPI384697 -----FKAWAV 48
IPI216773 -----FKALVL 48
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IPI22434 ARLSQRFPKAEFAEVS KLVTDLTQVHTECCHGDLLECADDRAD--LAKYICENQDS--IS 296
IPI384697 ARLSQRFPKAEFAEVS KLVTDLTQVHTECCHGDLLECADDRAD--LAKYICENQDS--IS 104
IPI216773 IAFAQYLLQQCPFEDHVKLVNEVTEFAKTCVADESAENCDKSLHTLFGDKLCTVATLRETY 108
* * * * *

IPI22434 SKLKECCEKPLLEKSHCIAEVENDEMPADLPSLAADFVESKDVCKNYAEAKDVFLGMFLY 356
IPI384697 SKLKECCEKPLLEKSHCIAEVENDEMPADLPSLAADFVESKDVCKNYAEAKDVFLGMFLY 164
IPI216773 GEMADCCAKQEPERNECFLQHKDD--NPNLRLVLRPEVD--VMCTAFHDNEETFLLKYL- 163
* * * * *

IPI22434 EYARRHPDYSVVLRLRLAKTYETTLEKCCAADPHECYAKVFDEFKPLVEEPQNLIKQNC 416
IPI384697 EYARRHPDYSVVLRLRLAKTYETTLEKCCAADPHECYAKVFDEFKPLVEEPQNLIKQNC 224
IPI216773 -----YETTLEKCCAADPHECYAKVFDEFKPLVEEPQNLIKQNC 203
*****

IPI22434 ELFEQLGEYKFNALLVRYTKKVPQVSTPTLVEVSRNLGKVGSKCKHPEAKRMPCAEDY 476
IPI384697 ELFEQLGEYKFNALLVRYTKKVPQVSTPTLVEVSRNLGKVGSKCKHPEAKRMPCAEDY 284
IPI216773 ELFEQLGEYKFNALLVRYTKKVPQVSTPTLVEVSRNLGKVGSKCKHPEAKRMPCAEDY 263
*****

IPI22434 LSVVLNQLCVLHEKTPVSDRVTKCCTESLVNRRPCFSALEVDETYVPKEFNAETFTFHAD 536
IPI384697 LSVVLNQLCVLHEKTPVSDRVTKCCTESLVNRRPCFSALEVDETYVPKEFNAETFTFHAD 344
IPI216773 LSVVLNQLCVLHEKTPVSDRVTKCCTESLVNRRPCFSALEVDETYVPKEFNAETFTFHAD 323
*****

IPI22434 ICTLSEKERQIKKQTALVELVKHKPKATKEQLKAVMDDFAAFVEKCKKADDKETCFAEFG 596
IPI384697 ICTLSEKERQIKKQTALVELVKHKPKATKEQLKAVMDDFAAFVEKCKKADDKETCFAEFG 404
IPI216773 ICTLSEKERQIKKQTALVELVKHKPKATKEQLKAVMDDFAAFVEKCKKADDKETCFAEFG 383
*****

IPI22434 QKTCCCKSSCLRLITSHLKASQPTMRIRERK 627
IPI384697 KKLVAASQAALGL----- 417
IPI216773 KKLVAASQAALGL----- 396
* * *

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	PREPEPTIDE	PROPEPTIDE
IPI00022434	RMM 71704	Mature RMM 68810
IPI00216773	RMM 45160	Mature RMM 42265
IPI00384697	RMM 47360	Mature RMM 44466
BSA (P02769)	RMM 69293	Mature RMM 66432

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**Figure 4.4.i. CLUSTAL W (1.83) multiple sequence alignment of the HSA proteins identified using MALDI-MS & LC-ESI-MS/MS analysis by Warwick University.**

**The three RMMs for the total (unprocessed) protein sequences were calculated by software from ExPASy and are different from those given by Warwick University (Tables 4.3.v. - 4.3.vii.) in that the ExPASy values are smaller. The difference cannot be accounted for by isotope use, since the ones quoted from Warwick University are monoisotopic whereas those from ExPASy are average, the difference being only about 500Da in IPI00022434, moreover the average RMM (ExPASy) is larger than that calculated for the monoisotopic (Warwick University).**

Within this study the proteomic analysis was carried out from tryptically digested protein bands, which in corresponding Western blots had stained immunopositive for 3NT, excised from 1D SDS-PAGE gradient gels. Within each of these bands there was the possibility that there was more than one protein present and as such we cannot confidently report which of the proteins present were nitrated. It could be that proteins present in amounts sufficient to be detected by mass spectrometry of their tryptic peptides were not nitrated, whereas those below the level of MS detection may be nitrated and detected by antibody. Other studies which have identified the presence of many nitrated proteins (40+) have done so using MALDI-TOF-MS analysis of proteins separated on 2D gels (Aulak, Miyagi, Yan *et al.* 2001). Such analysis, separation by both RMM and pI, allows for more reliable assignment of single protein species to single spots (Molloy 2000), although the logic applied above to 1D gels still holds and there is a potential for there to be multiple occupants of a spot in which the nitrated species is not the one detected by MS. Confirmation can only be made by the isolation and sequencing of a nitrated tryptic peptide (+45Da).

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In summary it is reported here that the presence of nitration has been identified within the purified plasma of both the claudicant and control subjects. The need for plasma protein purification was apparent due to conflicting results obtained during this study regarding the presence of nitrated proteins when using differing analytical techniques. The levels of nitration reported within the affinity purified samples using our competitive ELISA are somewhat higher than that previously reported in whole plasma: although this analysis did show that higher levels were present within the purified affinity fractions from the claudicant samples compared to the control samples. Proteomic studies of the 3NT-affinity purified plasma fractions for both the claudicant and control subjects illustrated that the bands staining immunopositive for the presence of 3NT residues contained HSA, serotransferrin and apolipoprotein A1 and A2 precursors; although the presence of 3NT residues within a specific protein could not be confirmed.

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# **Chapter 5.**

# **Overall Discussion and**

# **Conclusions**

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The objective of this study was to develop immunoassays for the detection of 3NT, as a marker of oxidative stress and thus disease, in claudicants and to investigate the nitroproteome associated with this disease. Claudicants (n=33) and controls (n=6) were enrolled on to the study, their medical histories recorded and plasma collected.

It was clear that all of the risk factors associated with an increased risk of more serious atherosclerotic disease (Newman, Shemanski, Manolio *et al.* 1999) were present in this claudicant population. Measuring a patient's ABI has been shown to be an effective tool in the diagnosis of clinically significant PAD e.g. IC (Newman, Shemanski, Manolio *et al.* 1999, Hirsch, Criqui, Treat-Jacobson *et al.* 2001, Sacks, Bakal, Beatty *et al.* 2003). However, as also reported by McDermott *et al.* (2002), within this study the problem of the efficacy of the current protocols in place for the early diagnosis of IC and PAD patients were highlighted by the fact that patients with ABI recordings  $\leq 0.96$  were diagnosed with clinically manifest IC. ABI is also now thought to be more indicative of advanced atherosclerotic disease and as such is not a useful screening test for early vascular disease (Newman, Shemanski, Manolio *et al.* 1999). Therefore analysis of patient data has highlighted the need to develop additional screening methods to aid in the early diagnosis, administration of risk factor intervention and treatment of PAD.

In the indirect and competitive ELISA availability and accessibility of the 3NT-residues to the anti-3NT Ab was clearly an issue, demonstrated by changing the conformation or structural integrity of the protein either through denaturation or by use of substrates with different binding properties. Although the reasons are not given, ELISA methods have been published in which the

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standard 3NT-containing antigen was produced by nitrotyrosinylation (i.e. covalent attachment of 3NT) to a protein. Standards used in this way include BSA (Torreilles and Romestand 2001) and horseradish peroxidase (Inoue, Hisamatsu, Ando *et al.* 2002, Thomson, Christie, Vadseth *et al.* 2007). It is probable that nitrotyrosinylation of a protein would increase the availability of the 3NT-residue to an antibody (compared to nitration) within an ELISA, resulting in improved assay sensitivity and precision. Nevertheless, nitrotyrosinylation does not reflect the *in vivo* nitration process and, as such, introduces another variable into the assay since the conformation of the 3NT epitopes will be even less representative of those in the test sample (nitrated plasma) than those in nitrated BSA. Thus the reliance placed on absolute quantitative data from biological samples derived from an assay using a standard curve constructed using a nitrotyrosinylated standard is less secure, although comparative data are presumably as reliable as those from nitrated standards. The use of nitrotyrosinylation to enhance antibody-antigen recognition within an ELISA does suggest that the problems identified within this study regarding the availability of the 3NT-residue to the antibody and hence of assay reliability have been encountered by others but have not been published.

In a recent review Duncan (2003) states that an immunoassay for the detection of 3NT in biological samples will be dependent upon a variety of parameters including those highlighted within this current study e.g. the nature of the selectivity of the antibody and sample composition. As such the use of immunoassays for the detection of 3NT in biological samples such as plasma can only ever be classified as being semi-quantitative (Khan, Brennand,

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Bradley *et al.* 1998, Duncan 2003). Thus the findings reported in Chapter 3 have important ramifications regarding the implementation of an immunoassay as a diagnostic tool for the detection of 3NT-residues within biological specimens such as plasma. It also brings doubt on the accuracy of the results regarding previously reported levels of 3NT detection in other diseases using immunoassays such as ELISA (Khan, Brennand, Bradley *et al.* 1998, Ter Steege, Koster-Kamphuis, Van Straaten *et al.* 1998, Inoue, Hisamatsu, Ando *et al.* 2002).

The need for plasma fractionation and affinity purification in order to establish the presence of a nitroproteome in the plasma of the claudicants and control subjects is reported here for the first time. The difficulties in the detection of 3NT in human biological samples have not been reported by others using ELISAs (Ter Steege, Koster-Kamphuis, Van Straaten *et al.* 1998, Inoue, Hisamatsu, Ando *et al.* 2002). Khan *et al.* (1998) were able to determine the concentration of 3NT in whole plasma as 0.12 $\mu$ M. As presented, this represents a concentration of 3NT of 120pmol/ml plasma. The concentration of 3NT in HSA is reported as 0.085nmol/mg = 85pmol/mg, and, assuming a HSA concentration of 40mg/ml (mean values for HSA and prealbumin in plasma are 40 and 0.25 mg/ml respectively (Marshall and Bangert 2004), this would give a 3NT concentration in plasma of 3.4nmol/ml from HSA alone, as opposed to 0.12nmol/ml measured for the whole plasma. The relatively low value from whole plasma, compared to that from purified HSA is further evidence of the problems inherent in estimating 3NT concentration in complex protein mixtures in solution.

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The levels of nitration found to be present within all of the affinity purified fractions of the claudicant and control subjects were somewhat higher than those previously reported in the plasma of healthy and diseased individuals using similar techniques (Khan, Brennand, Bradley *et al.* 1998, Ter Steege, Koster-Kamphuis, Van Straaten *et al.* 1998, Inoue, Hisamatsu, Ando *et al.* 2002, Dooley, Gao, Bradley *et al.* 2006). There are many potential reasons behind such differences including assay precision discussed in Chapter 3, background contamination resulting from the presence of free 3NT from the affinity purification procedure. As stated in Chapter 4 it is also important to note that the values reported are for the affinity purified samples and not whole plasma and as such cannot be directly compared to most values reported by others. An exception is Khan *et al.* (1998) who did purify HSA from control plasma on Cibacron and measured nitration at 0.085nmol/mg protein. In the current project, the concentration of 3NT within the bound and unbound 3NT affinity-purified HSA fractions was 92.2 and 0.08nmol/mg protein respectively. Thus the mean 3NT concentration in the control plasma HSA would be  $(92.2 \times 0.06) + (0.08 \times 0.94) = 5.6\text{nmol/mg}$ , considerably higher than that found by Khan *et al.* (1998). Since both studies used a rabbit polyclonal antibody raised against nitrated KLH, the difference is unlikely to be substantially due to the antibody used. The results in the current study can only meaningfully be used comparatively, within the study. Tsikas and Caidahl (2005) recognised the potential limitations of using either free- or protein bound 3NT as biomarkers in diseases involving oxidative stress due to serious issues surrounding the published analytical techniques.

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The affinity purification of nitrated proteins from tissue homogenate isolated from human pituitary adenoma has been reported for analysis using tandem mass spectrometry (Zhan and Desiderio 2006). Within this study the bound fractions from the anti-3NT Ab affinity purification were analysed using mass spectrometry in order to establish the importance of nitration within the disease state IC. Using both MALDI-TOF-MS and LC-ESI-MS/MS, it was clear that although the proteins present within those bands that stained 3NT-immunopositive using Western blot immunodetection could be identified, confirmation of the presence of 3NT-residues (i.e. a mass increase of +45Da) could not. Most published reports regarding the identification of nitrated proteins in human samples appear to use mass spectrometry to identify the peptides/proteins in the corresponding gel spots or bands which have been found to stain 3NT-immunopositive using Western blot immunodetection (Aulak, Miyagi, Yan *et al.* 2001). The failure of such methods to characterise the presence of nitrated peptides is thought in part to be due to the low abundance of specific nitrated proteins along with the actual recovery of these proteins from the gels (Kanski, Hong and Schoneich 2005). Furthermore, the various mass spectrometry techniques themselves, particularly the detector, each define a different, albeit overlapping, series of proteins/peptides within a proteome. Even when a pure, small, mononitrated protein was used (lysozyme), and no gels were involved, Matters *et al* (2006) could not confirm the site of nitration using either ESI- or MALDI-FTICR or MALDI-TOF techniques. Success in finding the nitrated tryptic peptide was only achieved with ESI-TOF after “cleaning” (i.e. concentrating certain peptides) the peptides with a reversed-phase ZipTip. Nevertheless, some recent methods do report

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the identification of a mass shift of +45Da indicating the presence of 3NT modification using MALDI-TOF (Casoni, Basso, Massignan *et al.* 2005) and nanoelectrospray ionisation ion trap-MS/MS (Kanski, Hong and Schoneich 2005). An empirical observation is that the most likely mass spectrometry method to detect nitrated peptides is using ESI-ion trap (H. Ischiropoulos, personal communication, 2006).

There are numerous polymorphisms in the apoA-1 gene leading, usually, to pro-atherogenic variants. Thus mutations yield either impaired synthesis or the production of an abnormal apoprotein and result in premature atherosclerosis, xanthomatosis, and corneal opacities. In cases where no plasma apoA-1 is detected, the mutation is associated with increased coronary risk (Assmann, Von Eckardstein and Funke 1992). One proband who developed early coronary artery disease, had a deletion of the complete apoA-I/C-III/A-IV gene (Rosseneu and Labeur 1995). A Turkish girl had no detectable apoA-I and was affected by HDL deficiency with xanthomas. Patients with a nonsense mutation in codon 84 of apoA-I, which lead to apoA-I deficiency and low apoC-III levels, suffered from premature coronary heart disease and had planar xanthomas. A 5.5Kb inversion in the apoA-I/C-III gene leads to coronary artery disease, xanthomas, and corneal opacity. The truncation of apoA-I at residue 230 leads to corneal opacity and LCAT deficiency. The deletion of residues 145-160 results in HDL deficiency and xanthomas. A deletion in intron 2 causes coronary heart disease (Rosseneu and Labeur 1995). There are many other examples of missense mutations leading to atherosclerotic outcomes. However, in two other cases no

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increased risk for myocardial infarction could be demonstrated (Assmann, Von Eckardstein and Funke 1992). Even more unusually, there is an anti-atherogenic variant, apo A1 Milano, with an R173C mutation, found in some inhabitants of Limone on Lake Garda, which offers protection from coronary heart disease (Weisgraber, Rall, Bersot *et al.* 1983, Gualandri, Franceschini, Sirtori *et al.* 1985). It is tempting to speculate that some claudicants may have a pro-atherogenic polymorphism in the apo A1 gene region. Such a study is yet to be done.

The results in Tables 4.3.x and 4.3.xii show an apparent truncated apo A1 precursor at about 16kDa. The unprocessed apoA-1 of 267 amino acids has a RMM of 30,778 whereas the mature protein consists of 243 amino acids with a RMM of 28,079 (Swiss-Prot, P02647), but the truncated protein in the plasma is most likely to have arisen from proteolysis, since the same variants are found with HSA.

Apolipoprotein A-1 has previously been identified as a target for nitration within atherosclerotic lesions and the blood of those suffering from cardiovascular disease (Shao, Bergt, Fu *et al.* 2005) and those suffering from diabetes type II (Hermo, Mier, Mazzotta *et al.* 2005). MPO-mediated modification of apolipoprotein A-1 (nitration and chlorination) is directly linked to a reduction/loss in its ability to bind lipids and its reverse lipid-transport functions (Zheng, Settle, Brubaker *et al.* 2005, Shao, Bergt, Fu *et al.* 2005). Thus if the nitrated apoA-1 were confirmed by MS, the results from the current study would provide further evidence towards the hypothesis that impaired HDL function may have a direct role in the pathology of

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atherosclerosis. This is an area in which further research is required in order to identify the targets for nitration within apolipoprotein A-1 in claudicants.

Plasma is perhaps one of the most difficult specimens on which to carry out proteomic studies due to the wide dynamic range and complexity of proteins contained within it (Anderson and Anderson 2002). It has been suggested that there may be more than one plasma proteome present within an individual, e.g. the plasma proteome of arterial, venous and capillary plasma may differ significantly (Anderson and Anderson 2002). Therefore, a potential limitation of nitroproteome analysis may be that the blood samples used for analysis in this study were taken from the subjects' arm. This is due to the fact that there may be differences in the plasma proteome of claudicants near the site of the blockage (peripheral arteries of the legs), where the formation of nitrated proteins may take place, and the blood sampling site. Such sampling relies on the 'immediacy' of the translation of a protein marker from its site of origin to the rest of the systemic circulation (along with the active production of RNS and ROS). During this transition there is also the possibility that the 3NT-residues may be removed by anti-3NT Abs (Khan and Siddiqui 2006, Thomson, Christie, Vadseth *et al.* 2007) and/or modified by 'denitrase' (Kamisaki, Wada, Bian *et al.* 1998, Irie, Saeki, Kamisaki *et al.* 2003). Thus it is possible that the status of the disease present and the sampling method used may directly influence the ability for detection of 3NT in plasma. Also, different stages of a disease may directly affect the levels of circulating 3NT-proteins in the plasma. For example there are conflicting reports regarding the presence of 3NT-proteins in patients suffering from osteoarthritis (Kaur and Halliwell 1994, Khan and Siddiqui 2006) and at different stages of celiac disease (Ter

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Steege, Koster-Kamphuis, Van Straaten *et al.* 1998). In patients with acute disorders such as sepsis the duration of exposure of proteins to nitrating species may not be long enough for nitrated protein formation to appear in the circulation (Ter Steege, Koster-Kamphuis, Van Straaten *et al.* 1998). This may explain some of the variation in reported concentrations of 3NT-proteins within the plasma of different diseases.

Within the current study only the plasma of the claudicant and control subjects was investigated for the presence of 3NT-containing proteins. Although there is limited knowledge regarding the presence of nitrated proteins within the circulating blood cells *in vivo*, research has shown that nitration can occur as a result of ONOO<sup>-</sup> activity *in vitro* e.g. in platelets (Low, Sabetkar, Bruckdorfer *et al.* 2002, Sabetkar, Low, Naseem *et al.* 2002) and erythrocytes (Denicola, Souza and Radi 1998). It may be, therefore, that the production of NO and related RNS and ROS in the vasculature results in the nitration of circulating blood cells. Therefore it may be informative to investigate the presence of nitrated proteins within the whole blood as well as plasma in the future. Furthermore, the combined analysis of blood and urine for 3NT (and associated metabolites) maybe an effective diagnostic tool for pathological conditions where oxidative stress is involved such as IC, although the potential for selective diagnosis would appear to be limited. The presence of 3-nitro-4-hydroxyphenylacetic acid and 3-nitro-4-hydroxyphenyllactic acid (metabolites of 3NT) in the urine of humans was first reported by Ohshima *et al.* (1990) and the measurement of urinary free-3NT has also been suggested as a potential biomarker of oxidative stress in various pathological conditions (Schwemmer, Fink, Kockerbauer *et al.* 2000).

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Within this study only those diagnosed with clinically manifest PAD (IC present) were investigated for the presence of nitrated plasma proteins. It has been clearly established that the prevalence of IC has been found to be relatively low compared to the presence of PAD (Meijer, Hoes, Rutgers *et al.* 1998, Khan, Flather, Mister *et al.* 2007). Thus it may be of benefit in the future to assess the levels of nitration present within the plasma of those who have risk factors associated with development of atherosclerosis but are currently asymptomatic (no IC present). By doing this a wider cohort of patients would be eligible for enrolment onto the study increasing the chances of establishing any correlation there may be between the levels of circulating 3NT and the development of clinically significant atherosclerotic disease. It may be more appropriate to analyse a panel of biomarkers rather than relying on one protein marker of atherosclerotic disease. For example it has been reported that C-reactive protein, and the total cholesterol-HDL ratio are strong independent indicators for the development of PAD (Ridker, Stampfer and Rifai 2001). Furthermore it has been demonstrated that there is enhanced coagulation and endothelium activation in patients with PAD and that this activation increases with the severity of the PAD (Cassar, Bachoo, Ford *et al.* 2005). Thus by combining the measurement of 3NT with other markers of inflammation associated with atherosclerotic disease a more reliable and effective diagnostic tool may be developed.

In conclusion further research is required in order to establish the clinical relevance of nitrated proteins and PAD, specifically IC. The identification of the targets of nitration in a disease such as IC using proteomic analysis may

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be more clinically significant compared to the more traditional quantitative techniques such as immunoassays.

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# **Chapter 6.**

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# **Chapter 7.**

# **Appendices**

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## **APPENDICES A - Ethics proposal for Wolverhampton LREC**

### **WOLVERHAMPTON HEALTH EXECUTIVE**

#### **DESCRIPTION OF RESEARCH PROJECT FOR SUBMISSION TO THE WOLVERHAMPTON DISTRICT LOCAL RESEARCH ETHICS COMMITTEE**

##### **1 NAME(S) OF RESPONSIBLE INVESTIGATORS**

Sadie Mercer (postgraduate student), Dr M Cox (supervisor), Dr J Heptinstall (2<sup>nd</sup> supervisor), School of Science and the Environment, Coventry University, Priory Street, Coventry, CV1 5FB

Mr Andrew Garnham (consultant vascular surgeon). The Royal Wolverhampton NHS Trust. New Cross Hospital, Wolverhampton, WV10 0QP

##### **2 TITLE OF PROJECT:-**

3-nitrotyrosine as an indicator of the disease state in claudication

##### **3a Company or Co-ordinating Body with whom the Research is in Collaboration:-**

Coventry University with funding from the Engineering and Physical Sciences Research Council (EPSRC)

##### **3b Clinical Trial Certificate Reference or Exemption Certificate Reference:-**

Not Applicable

##### **4 SUMMARY (Objective, Hypothesis, Background, Study Design, Randomisation, Anticipated Benefits)**

###### **Objective –**

To investigate 3-nitrotyrosine as a potential marker of oxidative stress in people suffering from claudication. This will involve the development of novel immunoassays, based on enzyme linked immunosorbant assay (ELISA), for the detection of nitrated proteins and antibodies against these proteins. Using these assays 3-nitrotyrosine will be investigated to establish a possible correlation between 3-nitrotyrosine levels and the extent of the disease.

###### **Hypothesis –**

Atherosclerosis is associated with increased 3-nitrotyrosine levels in plasma and in low-density lipoproteins (LDL) recovered from atherosclerotic plaques. Since atherosclerosis is a concomitant condition of claudication it is

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hypothesised that patients suffering from claudication will have high circulating 3-nitrotyrosine levels. Moreover, that there will be a correlation between circulating 3-nitrotyrosine levels in patients and the degree of claudication.

### **Background –**

Peripheral arterial disease (PAD) –

PAD is an extremely common clinical manifestation of atherosclerosis in the lower limbs. In this disorder artery wall linings slowly become narrow leading to severely narrowed or, in extreme cases, blocked arteries. Atherosclerosis progresses through time at variable rates in individual patients depending on factors such as diabetes, hypertension, hyperlipidaemia, and smoking. For most individuals' symptoms do not appear until the artery has narrowed by at least 60%. In the majority of patients suffering from PAD the first physical indication that there is something wrong is a cramp like pain experienced in the muscles of the leg upon light exercise such as walking, known as Intermittent Claudication (IC). Relatively few population-based studies on the prevalence of PAD and IC have been performed.

Interference with blood flow leads to ischaemia/reperfusion episodes, as suffered by claudicants and yields oxidative stress damage including nitration of tyrosine both free and protein-bound. In turn 3-nitrotyrosine (3-NT) has been found in many disease conditions<sup>[1]</sup>, and the protein apoB from LDL, has been shown to be nitrated in atherosclerotic plaques<sup>[2-4]</sup>. The plasma concentration of 3-NT is a well-known marker of oxidative damage in vivo but this has not yet been determined in claudicants.

Protein oxidation and 3-NT formation –

Many proteins contain the amino acid tyrosine and therefore can potentially be oxidised to form 3-NT. 3-NT arrives from nitric oxide, a potent vasodilator with a diverse array of physiological functions within the body, but which has recently been identified as a key mediator in the pathogenesis of many inflammatory and degenerative diseases such as acute lung disease and atherosclerosis. It has also been implicated in and thought to contribute to tissue injury in inflammatory conditions via reactive nitrogen species derived from NO, which are powerful oxidants. LDL is of extreme interest in the study of atherosclerotic plaque formation and therefore IC. LDL can be oxidised in both its lipid (cholesterol, cholesterol ester, phosphoglycerides) and protein (apoB) components by complex reactions involving reactive nitrogen species generated during periods of oxidative stress. It has been demonstrated, by mass spectrometry, that there is a nearly 100-fold higher level of 3-NT in LDL recovered from atherosclerotic plaques compared to LDL isolated from plasma of healthy donors<sup>[2]</sup>. The precise nitrating intermediates formed and pathways for their generation in vivo, and the complete biological consequences of LDL nitration are still unknown. Nevertheless the multicellular components of atherosclerotic plaques are known to produce both superoxide ( $O_2^-$ ) and NO which react to produce peroxynitrite ( $ONOO^-$ ), which is known to promote the nitration of protein tyrosine residues in vitro; suggesting a possible pathway for LDL oxidation and for the presence of 3-NT in vivo<sup>[2 5-8]</sup>. Alternatively LDL nitration may involve the enzyme myeloperoxidase<sup>[3 9-12]</sup> (release from activated macrophages). Irrespective of

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the true nature of the nitrating agent responsible for the oxidation of LDL, it is an important marker of oxidative stress.

#### Intermittent Claudication (IC) -

IC is a common clinical manifestation of PAD that leads to significant functional impairment and an increased risk of cardiovascular morbidity and mortality (2.5 fold increase compared to age matched population)<sup>[13]</sup>. IC refers to cramping, fatigue or aching that develops in the legs of a sufferer upon exercise, this pain is caused due to a blockage within the arteries supplying the leg with oxygenated blood. Therefore, IC should be treated seriously and highlights the need for accurate tests to diagnose it. Studies have related smoking, diabetes mellitus, old age, elevated blood pressure and high cholesterol levels as risk factors in the development of IC<sup>[14 15]</sup>. Moreover at all ages men experience nearly double the rate of claudication of women. The risks attached to smoking were related to oxidative damage by free radicals<sup>[16]</sup>, particularly oxidative modification of low-density lipoprotein (LDL). It has also been found that the plasma of 55% of healthy smokers had 3-NT, a marker of oxidative damage, present compared to only 11% of healthy non-smokers<sup>[17]</sup>.

The tests for diagnosing IC are usually non-invasive. Firstly a thorough physical exam is performed and the patient's medical history is taken, noting exposure to and presence of risk factors. The ankle-brachial index (ABI), i.e. the ratio of ankle and arm systolic blood pressures, is a simple and reliable tool in the diagnosis of PAD, especially IC. A normal ABI range is classed as 0.95-1.2, however there is no consensus on a cut-off level<sup>[18]</sup>. A more advanced version of this test is where blood pressure measurements are taken from the upper thigh; upper knee, calf and then ankle and can indicate where the atherosclerotic lesions are within the leg. However ABI may not be accurate in all patients, for example patients with long standing diabetes mellitus or kidney disease may have rigid vessels resulting in potentially inaccurate ABI readings. A more searching test for IC is the treadmill exercise test; in which the patient has to walk slowly on a treadmill until it becomes too painful to continue. If there is a drop in the ankle blood pressures and ABI values after the treadmill exercise test then the patient is suffering from IC that is atherosclerosis is present. A major problem in IC diagnosis is that even advanced IC patients may present atypical symptoms, moreover in the elderly walking problems are often present due to other underlying medical conditions therefore causing potential problems in correct diagnosis<sup>[15]</sup>. Therefore a biochemical tool such as 3-NT detection, along with ABI and other indicators of IC, could prove valuable in diagnosis.

#### 3-nitrotyrosine (3-NT) detection –

There are a number of ways in which 3-NT can be detected and quantified. In general chemical methods (protein hydrolysis, high performance liquid chromatography (HPLC)) can suffer from artefacts. Enzyme linked immunosorbent assay (ELISA) is an enzyme immunoassay that combines the specificity of antibodies with the sensitivity of simple spectrophotometric assays and is relatively cheap and easy to undertake once established. It can be used to establish and quantify the presence/absence of the antigen in question (3-NT) within the serum of a patient. ELISA's to detect 3-NT based

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on a number of assay formats, e.g. competitive and direct, have been published<sup>[19 20]</sup>.

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### **Study Design –**

All patients who attend IC clinic for first time during the course of the study will be asked to give written consent to become enrolled. Blood samples are already routinely taken from each patient, on first visit and after a 3-month exercise regime, and at the same point a small sample of blood will be taken for use within this study (2ml). This will cause no additional discomfort to the patient due to the fact this sample will be taken at the same time as routine blood samples. The number of participants within the duration of this study (one year) will be approximately 250, all having two samples taken, as described above (based on the fact that around five new patients attend the clinic each week). Information, which is already gathered as part of the consultation, will be obtained as to the patients age, sex, height, weight and other contributing factors such as other diseases e.g. diabetes mellitus and their smoking status. At all times the patients confidentiality will be respected. Plasma will then be tested and quantified for the presence of 3-nitrotyrosine (3-NT) and comparisons will be made between the patients initial 3-NT levels and levels after the 3-month exercise regime. This will be carried out using a novel ELISA method that will be developed using polyclonal antibodies raised against 3-NT (using nitrated keyhole limpet haemocyanin) from rabbits. This technique will quantify the levels of 3-NT present in IC patients. Using this data and the information collected about the patients, e.g. age, sex, the patients will be sub-grouped and comparisons will also be made within these sub-groups. These findings will be extended using Western Blotting in which

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separated plasma proteins are probed with 3-NT antibody to produce a fingerprint pattern of nitrated proteins. Differences in the pattern of nitration may emerge that correlate with the disease origins and contributing factors.

### **Randomisation –**

No randomisation is required. All new patients that attend the IC clinic will be asked to partake in the study (n=250), two blood samples will be taken, one on the first consultation and the second on the 3-month check up, at the same time as routine samples are taken. The patients 3-NT levels being determined and compared before and after the 3-month training program, they will also be allocated into sub-sets depending on other contributing factors such as age, sex, weight, disease history, to make further comparisons to assess any trends in 3-NT plasma levels present. Control samples from healthy adult volunteers will be collected from healthy non-smoking staff at Coventry University and from an age matched population (hernia patients who have to have routine pre-operative blood samples taken, therefore causing no extra pain or discomfort) to assess base-line 3-NT levels.

### **Anticipated Benefits –**

The anticipated benefits of this study will be the development of a novel ELISA method to detect and quantify 3-NT in the plasma of patients with claudication and therefore aid in its diagnosis and to determine the extent of the disease. Oxidised protein profiling (via Western blotting and HPLC) may point to specific damage associated with different routes to the disease e.g. diabetes mellitus, hyperlipidaemia. This test may also prove valuable in monitoring the effects of the training program if it can be established that 3-NT levels decrease after the 3- month exercise regime.

## **5 STUDY SUBJECT**

### **5a Has the investigation been done previously with Human Subjects YES/NO**

No

### **5b Subjects:- How many will be recruited and how selected?**

N=250 all having two routine samples taken (1 on first consultation and 1 after 3-month exercise regime) based on the assumption that 5 new patients attend the clinic every week for one year.

**(a) By the Local Investigator - ALL**

**(b) In all study centres – N/A**

### **5c Controls:- How many are needed and how selected?**

Control samples will be taken from healthy non-smoking individuals based at Coventry University. Samples are routinely taken as part of other studies therefore a large panel of control samples will be available for use (n=30). Control samples will also be taken from an

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age-matched population, these will be hernia patients who have to have blood taken routinely as part of their pre-operative assessment therefore causing no extra pain or discomfort.

**6a**      **SUBSTANCES AND OTHER THERAPEUTIC INTERVENTIONS to be given to the subjects (physical or physiological interventions, special diets, drugs, isotopes, questionnaires etc.**

There will be no substances or other therapeutic interventions given to the patients.

**6b**      **If a randomised study, how will randomisation be performed and who will hold the randomisation records?**

N/A

**7**      **SAMPLES**

**7a**      **What samples will be taken from the subjects, what amount and what frequency?**

Routine blood samples are already collected from the patients, once at the first consultation and the second after the 3-month exercise regime, an additional blood sample will be taken at the same time as these routine blood samples. As a result of the samples being taken at the same time as routine samples those patients involved will experience no extra pain or discomfort.

**7b**      **Would the sample(s) be taken especially for this investigation rather than part of normal patient care?**

Samples will be taken at the same time as routine samples, therefore causing no extra pain or discomfort to the patient.

**7c**      **Identify any possible discomfort or inconvenience to be suffered by subjects and assess likely incidence.**

The patient should experience no extra discomfort or inconvenience due to the fact that the blood samples will be taken at the same time as routine samples.

**8**      **INFORMATION AND CONSENT**

**8a**      **How will the subjects be informed, who will provide the information and in what form will consent be obtained (Written, or in special circumstances witnessed verbal)**

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Patients will be informed both verbally and by letter with respect to the aims and objectives of this study and the potential beneficial outcomes of this study with respect to the future. Written witnessed consent will be obtained by all subjects.

- 8b The District Local Research Ethics Committee consider it to be mandatory to inform the patients' GPs of their inclusion in the study, how do you intend to do this? Please explain your reasons if you do not intend to inform the patients' GPs**

The GP's of the patients participating in this study will be informed via a letter sent by the clinic. A letter is routinely sent to the GP to inform them of the clinic's findings and this will be amended to include the relevant information about this study and the fact that the patients treatment will not be affected by participation in this study.

**9 FUNDING**

- 9a How will the cost of the drugs, substances and materials be met?**

EPSRC research grant

- 9b (i) Have the investigations to be performed been discussed with the appropriate laboratories or departments, and are they agreeable to performing these investigations?**

**NO – not applicable**

- (ii) If so, how will the cost of the investigations be met?**

**Not applicable**

- 9c Are any payments to be made for entering patients in this study and to whom:**

- (i) For the patient - No**

- (ii) To the researcher - No**

**10 ANALYSIS**

**How will the study be analysed and from whom is statistical advice being taken?**

All samples taken from the patients will have the 3-NT plasma levels determined and quantified using the ELISA technique developed. The samples will then be analysed further using Western blotting. All of the data collected will then be analysed

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using appropriate statistical packages to highlight any significant associations with attributable risk factors. Statistical expertise exists at Coventry University for all aspects of data analysis.

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11      **DATA PROTECTION ACT**

Are the researchers(s) registered under the Data Protection Act?

No

This information supplied above is to the best of my knowledge and belief accurate/ I understand my obligations and the rights of the patient, particularly the need to obtain freely given written informed consent, and to supply an annual progress and final report.

**Signature of Investigator**

**Date of submission**

.....

.....

**To be completed by the Consultant in Charge or Head of Department**  
(this section should be completed by the researcher's supervisor if the project forms part of a degree course).

I hereby endorse this application with my approval:-

Name ..... Department

.....

Signature ..... Date

.....

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## **APPENDICES B - Information leaflet and consent form for**

### **claudicants**

#### **STUDY INFORMATION LEAFLET FOR CLAUDICATION PATIENTS '3-NITROTYROSINE AS AN INDICATOR OF THE DISEASE STATE IN CLAUDICATION'**

We'd like to explain some of the research we're doing and then ask you if you would like to take part in it.

#### **PLEASE READ THIS LEAFLET CAREFULLY**

This study is being undertaken by PhD student Sadie Mercer, B.Sc, from Coventry University working in conjunction with Mr A Garnham consultant vascular surgeon at the royal Wolverhampton hospitals NHS trust

#### **1. Condition and treatment**

You have recently been informed that you suffer from claudication, which is a result of hardening of the arteries (atherosclerosis). Currently claudication is diagnosed by taking your medical history, measuring your ankle and arm blood pressure (known as ankle-arm index), and using a treadmill.

The aim of this study is to develop a simple blood test to improve the diagnosis of artery disease such as claudication in the future.

If you agree to take part in this study two small blood samples will be taken and tested for the presence of a modified protein and we will compare the levels of it with people without claudication. The particular modified protein, called 3-nitrotyrosine, is known to exist in similar diseases to that of claudication.

This study will not effect or improve your treatment for claudication.

#### **2. Your study medication**

If you take part in this study you will receive NO extra or different treatment.

#### **3. What the study involves**

This study will last up to 2 years, however you will only be involved for 3months. During this time you will be asked to give two blood samples (approximately 2 teaspoons of blood each time), which will be taken at the same time as your routine samples therefore causing you NO extra pain or inconvenience. One blood sample at your first appointment at the claudication clinic and one after the 3-month exercise program. You will not be required to visit the clinic extra time as a result of your participation in this study. All information obtained from you and some of your medical records will be treated confidentially and by using a coding system your identity will be protected at all times.

#### **Side effects**

Taking part in this study should not cause you any side effects because you will NOT receive any extra or different medication.

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## **Your rights**

The study will be explained to you in detail by your doctor and by myself. Please feel free to ask as many questions as you want. When you are satisfied with the information you have been given and wish to participate in the study, you will be required to sign the attached consent form. If after joining the study you wish to leave you can do so at any time without having to explain your decision. If you do not wish to be involved, you do not have to give a reason. Any decision you make will not affect your doctor/patient relationship.

Although it is not expected, your doctor may end your participation in this study if he/she decides that it is not in the best interest of your health to continue. You will be told of any new findings that may affect your decision to participate in this study. In the unlikely event you suffer any harm as a result of taking part in this study your doctor would be able to give you information about compensation and treatment.

It will be necessary for your medical records to be seen by authorised personnel associated with the study. All information collected during the study will be treated in confidence, and your personal details will not be revealed. Your hospital doctor will inform your family doctor/GP that you are involved in the study so he/she knows what is happening.

If you have any further questions regarding your participation in the study, or need any information or advice during the study, please do not hesitate to contact:

**Consultant:** Mr Andrew Garnham (For medical enquiries about study)

**Telephone Number (Day time):** 01902 642904

**Telephone Number (24 Hours):** 01902 642894

**Name of Investigator:** Sadie Mercer (For information about study)

**Telephone Number:** 024 7688 7609

**Email:** [apy088@coventry.ac.uk](mailto:apy088@coventry.ac.uk)

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**PATIENT CONSENT FORM FOR '3-NITROTYROSINE AS AN INDICATOR  
OF THE DISEASE STATE IN CLAUDICATION'**

I..... **(Print name)**  
**agree to participate in the '3-nitrotyrosine as an indicator of the disease  
state in claudication' study.**

I have read and understood the patient information sheet provided and any questions I had have been answered satisfactorily.

I understand that I am under no obligation to participate in this study, and have been informed that if I choose to participate in this study I can withdraw from the study at any time without prior notice and that this will not affect my normal treatment or relationship with my doctor.

I understand that participating in this study will not improve my treatment or disease state in any way.

I have been assured that any information I give to the investigator, Sadie Mercer, will be treated as strictly confidential and that my identity will be protected at all times.

I have been given adequate time to make my decision and am giving my consent to partake in this study freely

Printed:.....

Signed:..... Date:.....(Patient)

I declare that I have given a full explanation to the above patient of this study, answered all of their questions and that consent has been given freely.

Printed:.....

Signed:..... Date:.....(Sadie Mercer)

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## **APPENDICES C - Claudication questionnaire**

**STUDY NUMBER – CS \_\_\_\_\_**

### **CLAUDICATION QUESTIONNAIRE**

1. Date –
2. NHS Number -
3. Initials –
4. Surname –
5. DOB –
6. Ethnic origin –
7. Height –
8. Weight –
9. BMI –
  
10. History / Chief complaint –

11. History of present illness / duration –

Claudication -	Distance	Recovery	Rest pain
Thigh / buttocks			
Calf			
Foot			
Coldness of feet			
Ulcers			

12. Past medical history –

Diabetes	CVA	Others	Operative procedures
Hypertension TIA			
MI	Amaurosis Fugax		
Angina			

13. Medications –

14. Allergies –

15. Family history –

16. Psycho social History –

17. Risk factors identified –

Smoking	Diabetes	BMI
Hyperlipidaemia	Hypertension	Exercise levels

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18. BP → Rt  
          → Lt

Systolic BP with Doppler

19. Physical assessment of Pulses -

+present –absent ++bounding

20. Doppler assessment of pulses with ABI Readings –

	RT	LT
AT (leg)		
DP (foot)		
PT (ankle)		
Peroneal (side of calf)		
ABI		

21. Any additional comments -

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**APPENDICES D - Ethics proposal for School of Science and  
the Environment, Coventry University**

**SCHOOL OF SCIENCE AND THE ENVIRONMENT**

**FORM FOR THE ATTENTION OF  
DR VALERIE COX  
(SUBMIT VIA JAMES STARLEY BUILDING MAIN  
OFFICE)**

**COVENTRY UNIVERSITY – SCHOOL OF SCIENCE AND THE ENVIRONMENT**

**STUDENT SUBMISSION TO SCHOOL RESEARCH ETHICS COMMITTEE**

Students must submit the following forms if needed:

- Completed ethics form
- Completed subject informed consent form (or Subject Information leaflet if appropriate)
- Risk assessment form; note for taking blood samples you must use the STANDARD procedure(s) and append the standard risk assessment form
- Copies of any other questionnaires that will be issued to subjects/ questions that will be used in interviews

For more guidance please see School Ethics Handbook

Student's name:

SADIE MERCER

2. Course:

PhD Student

Title of project:

3-NITROTYROSINE AS AN INDICATOR OF THE DISEASE  
STATE IN CLAUDICATION

Name of supervisor

Dr Martin Cox

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**Aim of research**

To investigate 3-nitrotyrosine as a potential marker of oxidative stress in people suffering from claudication. This will involve the development of novel immunoassays, based on enzyme linked immunosorbant assay (ELISA), for the detection of nitrated proteins and antibodies against these proteins. Using these assays 3-nitrotyrosine will be investigated to establish a possible correlation between 3-nitrotyrosine levels and the extent of the disease.

**Subject population**

It is hoped that a minimum of six subjects, lecturers within school of science and environment, will be enrolled onto this study as control patients. These subjects will need to be older than 45 years of age and be healthy individuals. Information will be obtained as to the subject's age, sex, height, weight and other contributing factors such as other diseases e.g. diabetes mellitus and their smoking status. A blood sample will also be taken, approximately 9ml by someone qualified. At all times the subject's confidentiality will be respected and no identifiable data will be taken. The blood samples taken will then be tested and quantified for the presence of 3-nitrotyrosine (3-NT) and comparisons will be made between these control sample levels and levels found in the plasma of patients suffering from claudication. These samples have already been taken and ethical approval was obtained from Wolverhampton district local research ethics committee.

**Research site:**

This research will take place in School of Science and the Environment at Coventry University.

**Experimental procedure**

The subjects enrolled will be asked to give a blood sample (approximately 9ml, taken by a person trained in doing so from physiology department, adhering to the standard procedure), answer questions regarding their medical history, age, sex, race (see questionnaire attached) and will have

their blood pressure taken from their arm and ankle to establish their ABI (ankle brachial index).

**Foreseeable risks or discomforts and actions taken to reduce these**

The only foreseeable risk or discomfort for the subjects taking place on this study is potential bruising from blood sampling.

**Data protection and Consent**

No information gathered from this study will be available to people other than Sadie Mercer in such a way that the subject may be identified.

- Will the project involve patients(clients) and/or patient(client) data? Yes [ X ] No [ ]
- Will any invasive procedures be employed in the research? Yes [ X ] No [ ]
- Is there a risk of physical discomfort to those taking part? Yes [ X ] No [ ]
- Is there a risk of psychological distress to those taking part? Yes [ ] No [ X ]
- Will specific individuals or institutions (other than the University) be identifiable through data published or otherwise made available? Yes [ ] No [ X ]
- Is it intended to seek informed consent from each participant (or from his or her parent or guardian)? Yes [ X ] No [ ]

Student's signature:

Date:

Supervisor's signature:

Date:

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## **APPENDICES E - Information leaflet and informed consent for control subjects**

### **'3-NITROTYROSINE AS AN INDICATOR OF THE DISEASE STATE IN CLAUDICATION' STUDY INFORMATION LEAFLET & INFORMED CONSENT FORM FOR CONTROL PATIENTS**

We'd like to explain some of the research we're doing and then ask you if you would like to take part in it.

**PLEASE READ THIS LEAFLET CAREFULLY**

This study is being undertaken by Sadie Mercer, B.Sc, from Coventry University working in conjunction with Mr A Garnham consultant vascular surgeon at the royal Wolverhampton hospitals NHS trust

Thank you for agreeing to consider being a subject in this research.

#### **Purpose of research**

Claudication is a result of hardening of the arteries (atherosclerosis), and results in pain in the leg when exercising due to poor circulation. You have been selected because you do not have claudication. Currently claudication is diagnosed by a taking a patients medical history, measuring their ankle and arm blood pressure (known as ankle-arm index), and using a treadmill.

The aim of this study is to develop a simple blood test to improve the diagnosis of artery disease such as claudication.

If you agree to take part in this study a small blood sample will be taken to test for the presence of a modified protein and compare levels of it with people who suffer from claudication. This particular modified protein, called 3-nitrotyrosine, is known to exist in similar diseases to claudication.

#### **Participation in this research will involve**

You will be asked to give a single blood sample for this study (approximately 2 teaspoons of blood) to be used as a 'control' sample. This may result in slight discomfort where the blood sample is taken from. You will also have some medical history taken and your ankle arm index measured. This consists of your ankle and arm blood pressures being taken and compared, this is a pain free and fast procedure. This should take approximately half an hour.

All information gathered from you and parts of your medical records will be treated confidentially and your identity will be protected, as your name will not be recorded.

#### **Foreseeable risks or discomforts**

You will NOT receive any treatment other than you ankle-arm index being measured. You may experience some discomfort and mild bruising where the blood test will be taken from.

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**Benefits to the subject of participation**

You will not receive any benefits from participating in this study.

**What will happen to your data?**

Any data/ results from your participation in the study will be used by Sadie Mercer as part of their project work. The data will also be available to Dr M Cox...It may also be published in scientific works, but your name or identity will not be revealed. Confidentiality will be maintained as you will be allocated a code known only to yourself and Sadie Mercer and no record of your name will be made.

**If you have any questions or queries Sadie Mercer will be happy to answer them. If they cannot help you you can speak to Dr Martin Cox.**

If you have any questions about your rights as a participant or feel you have been placed at risk you can contact Dr J Henderson.

I confirm that I have read the above information. The nature, demands and risks of the project have been explained to me.

I have been informed that there will be no benefits/ payments to me for participation

I knowingly assume the risks involved and understand that I may withdraw my consent and discontinue participation at any time without penalty and without having to give any reason.

Subject's signature \_\_\_\_\_ Date \_\_\_\_\_

Investigator's signature \_\_\_\_\_ Date \_\_\_\_\_

**The signed copy of this form is retained by the student, and at the end of the project passed on to the supervisor.**

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## **APPENDICES F - Control questionnaire**

**STUDY NUMBER – CN\_\_\_\_\_**  
**CONTROL QUESTIONNAIRE**

Date –  
DOB –  
Ethnic origin –  
Height –  
Weight –  
BMI –

Do you ever have pain upon walking? YES/NO

If Yes please fill in data below –

Distance      Recovery      Rest pain

Thigh / buttocks  
    Calf  
    Foot  
    Coldness of feet  
    Ulcers

Past medical history –

Diabetes - YES / NO  
CVA (STROKE) - YES / NO  
Hypertension - YES / NO  
TIA (transient ischaemic attack, short lasting loss of/impaired vision) –  
YES/NO  
MI (Heart attack) – YES / NO  
Angina – YES / NO

Do you or have you ever smoked?  
    If yes please give details –

Others –

Operative procedures (in the last 12months)–

Are you on any medications if so please state below–

Do you have a family history of Heart attack / Stroke / vascular problems? –  
If yes please give details below -

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**APPENDICES G - Factors for  $A_{280}/A_{260}$  Warburg and Christian method**

Tabulated factors for  $A_{280}/A_{260}$  Warburg and Christian method

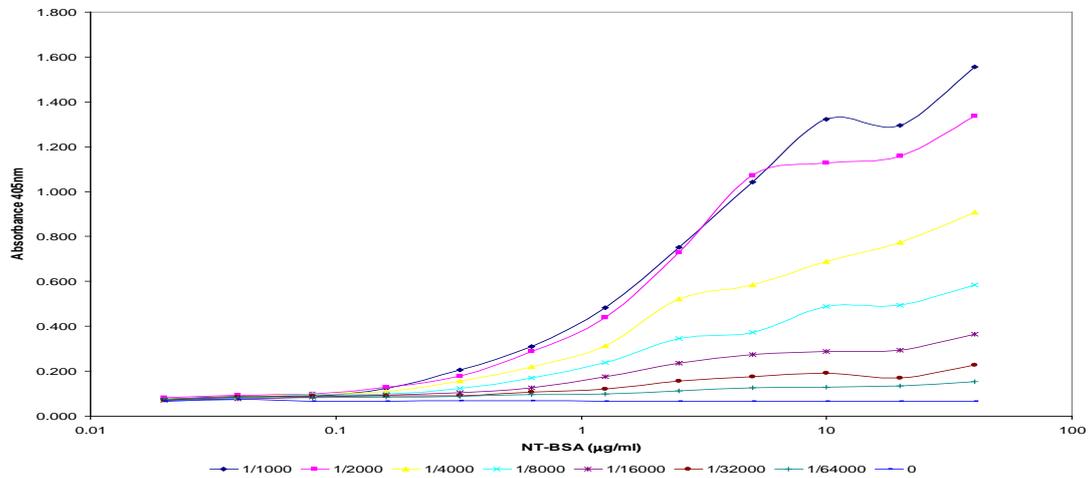
$A_{208} / A_{260}$	Nucleic acid (%)	Factor
1.75	0.00	1.118
1.60	0.30	1.078
1.50	0.56	1.047
1.40	0.87	1.011
1.30	1.26	0.969
1.25	1.49	0.946
1.20	1.75	0.921
1.15	2.05	0.893
1.10	2.40	0.863
1.05	2.80	0.831
1.00	3.30	0.794
0.96	3.70	0.763
0.92	4.30	0.728
0.90	4.60	0.710
0.88	4.90	0.691
0.86	5.20	0.671
0.84	5.60	0.650
0.82	6.10	0.628
0.80	6.60	0.605
0.78	7.10	0.581
0.76	7.80	0.555
0.74	8.50	0.528
0.72	9.30	0.500
0.70	10.30	0.470
0.68	11.40	0.438
0.66	12.80	0.404
0.64	14.50	0.368
0.62	16.60	0.330
0.60	19.20	0.289

## APPENDICES H - Checkerboard analysis - indirect ELISA

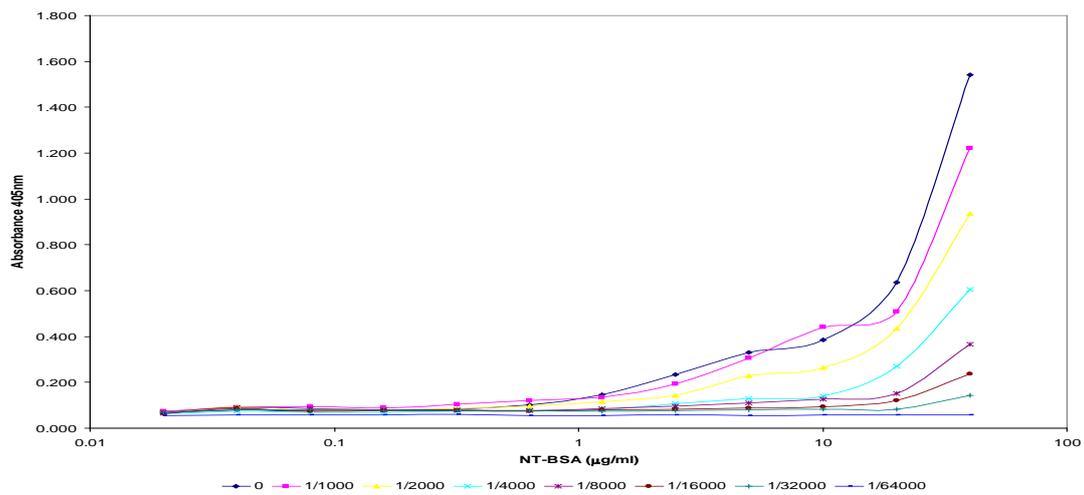
Full indirect ELISA checkerboard analysis for the different manufacturers microtitre plates using electrochemically nitrated BSA.

NB the legend represents the polyclonal anti-3NT Ab dilutions

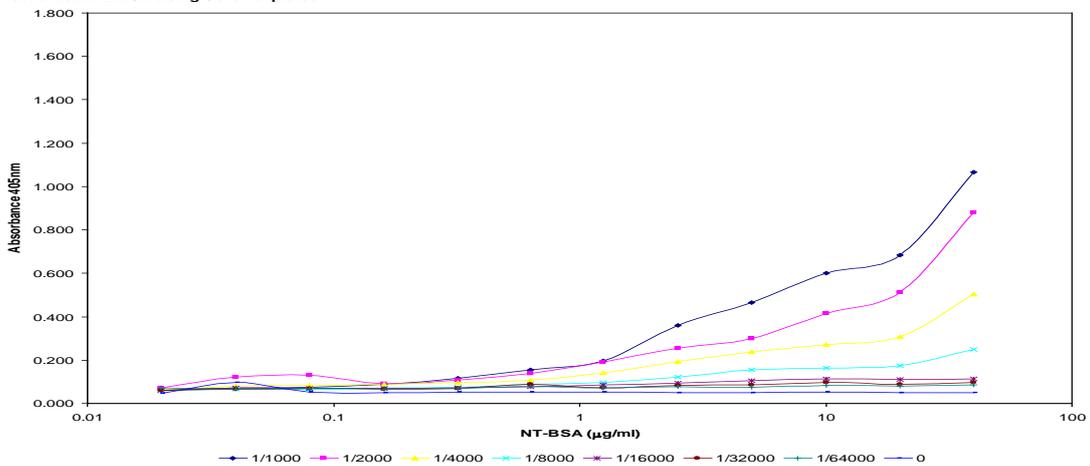
Checkerboard Indirect ELISA using Immulon plates



Checkerboard Indirect ELISA using Nunc plates



Checkerboard ELISA using Serowell plates

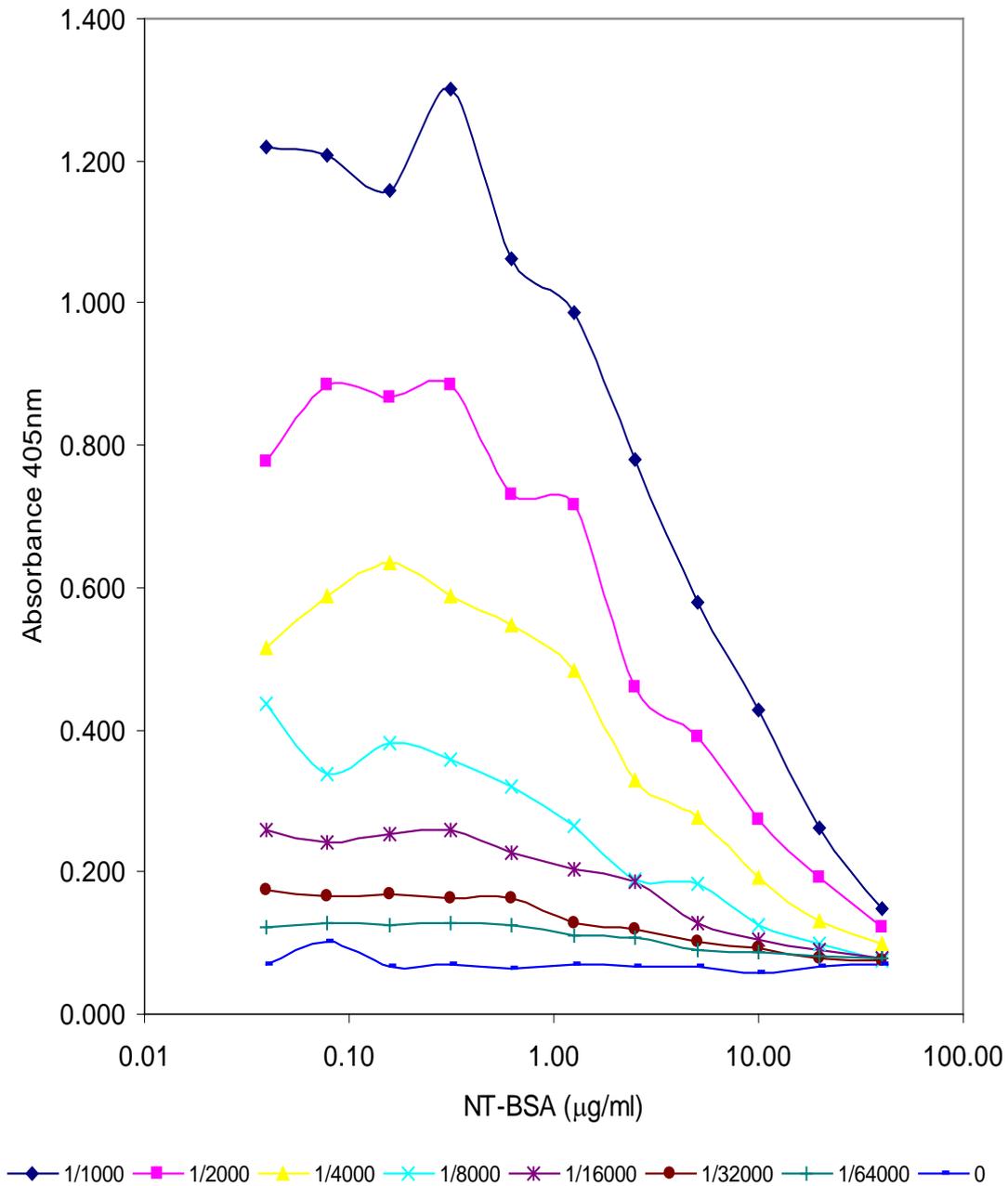


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## APPENDICES I - Checkerboard analysis - competitive ELISA

Full checkerboard analysis for competitive ELISA with Immulon 2HB microtitre plates using freshly nitrated BSA (electrochemically nitrated).

NB the legend represents the polyclonal anti-3NT antibody dilutions, this data has not been corrected for the mean blank values.



## APPENDICES J - Protein determination for the claudicant and control plasma samples (whole and affinity purified)

Sample	Plasma Conc' (mg/ml) Using Lowry		Cibacron (mg/ml) Using Bradford	Cibacron Total (mg)	Amount loaded on Affinity column (mg) Loaded 5ml		Affinity (mg/ml)	Affinity Total (mg) Using BCA Got 1ml	Calculated 3NT equivalents in AFFINITY fraction ( $\mu$ M per mg protein)
CS001	111	F1	1.8	12.8	9.2	F1U	5.2	5.1	0.018
						F1B	-0.1		
		F2	1.2	12.1	6.0	F2U	2.5	2.7	0.396
						F2B	0.2		188.678
		F3	0.3	2.2	1.3	F3U	0.7	0.9	0.534
				<b>27.1</b>	<b>16.6</b>	F3B	0.2	<b>8.7</b>	108.773
CS002	90	F1	1.2	12.3	6.2	F1U	7.2	7.4	0.007
						F1B	0.3		178.704
		F2	1.0	8.3	5.2	F2U	2.6	2.8	0.009
						F2B	0.2		172.080
		F3	0.2	1.6	0.8	F3U	0.9	1.0	0.354
				<b>22.2</b>	<b>12.1</b>	F3B	0.1	<b>11.3</b>	66.167
CS003	100	F1	1.0	10.3	5.1	F1U	5.5	5.8	0.002
						F1B	0.3		145.984
		F2	0.6	6.6	3.1	F2U	3.1	3.3	0.043
						F2B	0.2		235.901
		F3	0.3	2.8	1.3	F3U	1.5	1.6	0.025
				<b>19.7</b>	<b>9.5</b>	F3B	0.1	<b>10.7</b>	55.373
CS004	99	F1	1.2	12.3	6.0	F1U	5.2	5.3	0.190
						F1B	0.0		38.149
		F2	0.8	8.1	4.0	F2U	2.4	2.4	0.008
						F2B	0.1		35.155
		F3	0.4	4.0	1.8	F3U	1.0	1.1	0.012
				<b>24.3</b>	<b>11.7</b>	F3B	0.2	<b>8.8</b>	46.377
CS005	99	F1	1.5	15.5	7.6	F1U	8.7	8.9	0.016
						F1B	0.2		91.068
		F2	0.7	7.0	3.5	F2U	2.9	2.9	0.365
						F2B	0.1		250.699
		F3	0.2	2.0	0.9	F3U	0.8	0.9	0.185
				<b>24.5</b>	<b>12.0</b>	F3B	0.1	<b>12.7</b>	104.713
CS006	93	F1	1.1	12.0	5.6	F1U	7.6	7.9	0.100
						F1B	0.3		86.684
		F2	0.8	8.4	4.0	F2U	3.0	3.2	0.192
						F2B	0.2		15.427
		F3	0.2	2.4	1.1	F3U	0.9	1.1	0.636
				<b>22.8</b>	<b>10.7</b>	F3B	0.1	<b>12.2</b>	93.175
CS007	93	F1	1.3	13.3	6.4	F1U	7.6	7.9	0.118
						F1B	0.3		64.734
		F2	0.9	9.0	4.3	F2U	3.4	3.6	0.196
						F2B	0.2		13.458
		F3	0.2	2.4	1.1	F3U	0.9	1.0	0.588
				<b>24.7</b>	<b>11.8</b>	F3B	0.1	<b>12.5</b>	80.046
CS008	98	F1	1.6	16.0	8.0	F1U	8.6	8.9	0.014

						F1B	0.3		17.766
		F2	0.6	7.5	3.1	F2U	2.9	3.1	0.373
						F2B	0.2		20.166
		F3	0.2	3.7	1.2	F3U	0.9	1.0	4.864
				27.2	12.3	F3B	0.1	13.0	620.904
CS009	92	F1	1.4	16.6	6.9	F1U	7.8	8.0	0.012
						F1B	0.2		44.875
		F2	0.9	11.2	4.7	F2U	2.8	2.9	0.317
						F2B	0.1		150.778
		F3	0.3	3.5	1.3	F3U	1.6	1.8	2.240
				31.3	12.8	F3B	0.1	12.7	512.393
CS010	93	F1	2.1	12.5	10.4	F1U	13.1	13.4	0.029
						F1B	0.3		22.627
		F2	0.7	8.9	3.7	F2U	2.6	2.7	0.370
						F2B	0.1		455.375
		F3	0.3	3.4	1.3	F3U	1.0	1.1	0.128
				24.7	15.4	F3B	0.1	17.2	454.365
CS011	96	F1	2.1	12.5	10.4	F1U	11.8	12.2	0.017
						F1B	0.4		32.672
		F2	0.7	10.1	3.6	F2U	2.5	2.6	0.452
						F2B	0.1		322.329
		F3	0.3	4.9	1.7	F3U	1.0	1.0	0.638
				27.5	15.8	F3B	0.1	15.9	811.005
CS012	97	F1	1.5	14.7	7.3	F1U	8.4	8.5	0.055
						F1B	0.1		68.697
		F2	0.9	9.3	4.6	F2U	3.2	3.3	0.494
						F2B	0.1		307.078
		F3	0.3	3.2	1.6	F3U	1.2	1.4	1.424
				27.2	13.6	F3B	0.2	13.2	268.762
CS013	101	F1	1.9	21.3	9.7	F1U	10.8	11.2	0.012
						F1B	0.3		4.112
		F2	0.7	9.0	3.7	F2U	3.2	3.4	0.200
						F2B	0.2		57.281
		F3	0.2	2.4	1.0	F3U	0.5	0.8	2.504
				32.7	14.4	F3B	0.2	15.3	90.190
CS014	91	F1	1.5	18.9	7.3	F1U	7.5	7.7	0.010
						F1B	0.2		13.906
		F2	0.5	6.8	2.6	F2U	2.5	2.7	0.180
						F2B	0.2		6.925
		F3	0.2	2.2	1.0	F3U	1.2	1.4	0.115
				28.0	10.9	F3B	0.1	11.8	44.149
CS015	112	F1	1.2	15.4	6.1	F1U	6.7	6.9	0.007
						F1B	0.2		9.761
		F2	0.6	7.9	2.8	F2U	2.4	2.6	0.263
						F2B	0.2		11.204
		F3	0.2	1.8	0.8	F3U	1.3	1.4	0.084
				25.0	9.7	F3B	0.1	10.9	32.339
CS016	97	F1	2.1	20.2	10.5	F1U	9.2	9.4	0.045
						F1B	0.3		18.523
		F2	0.9	10.6	4.3	F2U	3.0	3.1	0.105
						F2B	0.1		16.809

		F3	0.3	3.0	1.3	F3U	0.7	0.8	0.389
				33.8	16.0	F3B	0.1	13.4	181.513
CS017	118	F1	2.5	24.0	12.5	F1U	10.5	10.7	0.034
						F1B	0.3		19.242
		F2	0.6	9.0	3.2	F2U	2.5	2.6	0.341
						F2B	0.1		59.920
		F3	0.3	3.2	1.3	F3U	0.8	0.9	0.262
				36.2	17.0	F3B	0.1	14.2	92.768
CS018	100	F1	2.1	24.0	10.3	F1U	9.5	9.8	0.006
						F1B	0.3		11.343
		F2	0.7	8.4	3.4	F2U	2.9	3.0	0.230
						F2B	0.1		47.354
		F3	0.2	2.9	1.2	F3U	0.9	1.0	0.610
				35.3	14.9	F3B	0.1	13.8	69.037
CS019	97	F1	2.2	25.4	11.1	F1U	8.9	9.4	0.013
						F1B	0.4		9.367
		F2	0.6	6.3	2.9	F2U	2.9	3.1	0.197
						F2B	0.3		19.110
		F3	0.3	3.2	1.4	F3U	1.2	1.5	0.613
				34.9	15.4	F3B	0.3	14.0	38.000
CS020	103	F1	1.8	20.7	8.9	F1U	8.1	8.4	0.013
						F1B	0.3		19.563
		F2	0.9	9.6	4.4	F2U	3.5	3.7	0.241
						F2B	0.2		82.043
		F3	0.2	2.5	1.1	F3U	1.2	1.4	0.292
				32.8	14.5	F3B	0.2	13.5	89.561
CS021	96	F1	2.0	24.4	10.0	F1U	8.7	9.0	0.013
						F1B	0.3		16.168
		F2	0.7	9.7	3.7	F2U	2.8	2.8	0.195
						F2B	0.0		
		F3	0.2	3.0	1.2	F3U	1.0	1.2	0.120
				37.1	15.0	F3B	0.2	13.0	85.008
CS022	117	F1	1.8	19.4	8.8	F1U	6.0	6.1	0.019
						F1B	0.2		12.577
		F2	0.7	9.8	3.5	F2U	2.6	2.7	0.180
						F2B	0.1		20.186
		F3	0.3	3.4	1.3	F3U	1.1	1.3	0.642
				32.6	13.6	F3B	0.2	10.1	63.073
CS023	109	F1	2.0	25.8	10.2	F1U	9.3	9.6	0.012
						F1B	0.2		9.924
		F2	0.7	9.4	3.7	F2U	2.7	2.8	0.230
						F2B	0.2		12.544
		F3	0.2	3.1	1.2	F3U	0.9	1.1	1.338
				38.4	15.2	F3B	0.2	13.5	81.636
CS024	100	F1	1.7	21.5	8.3	F1U	8.2	8.5	0.067
						F1B	0.3		34.514
		F2	0.7	7.6	3.3	F2U	3.1	3.3	0.451
						F2B	0.2		41.306
		F3	0.2	3.1	1.1	F3U	1.1	1.3	1.265
				32.1	12.7	F3B	0.2	13.0	29.072
CS025	106	F1	1.4	18.1	7.1	F1U	7.2	7.5	0.043

						F1B	0.2		28.203
		F2	1.3	15.9	6.3	F2U	3.5	3.7	0.425
						F2B	0.1		4.067
		F3	0.2	2.7	1.1	F3U	1.1	1.2	0.650
				36.7	14.4	F3B	0.1	12.4	145.241
CS026	93	F1	1.3	17.1	6.6	F1U	7.6	7.9	0.038
						F1B	0.2		33.040
		F2	0.8	9.7	3.8	F2U	3.0	3.2	0.367
						F2B	0.2		179.654
		F3	0.2	2.4	1.0	F3U	0.9	1.1	0.292
				29.2	11.3	F3B	0.1	12.1	288.943
CS027	91	F1	1.4	16.8	7.0	F1U	-0.2	4.2	
						F1B	4.4		0.116
		F2	0.8	9.1	4.1	F2U	3.4	3.6	0.197
						F2B	0.3		183.395
		F3	0.1	2.0	0.7	F3U	1.1	1.2	0.186
				28.0	11.9	F3B	0.1	9.0	357.999
CS028	96	F1	1.9	23.9	9.3	F1U	4.1	4.2	0.013
						F1B	0.1		34.738
		F2	1.0	11.0	5.1	F2U	3.2	3.2	0.220
						F2B	0.0		
		F3	0.3	4.7	1.7	F3U	0.8	0.9	0.089
				39.6	16.1	F3B	0.1	8.3	110.390
CS029	118	F1	1.8	21.2	9.1	F1U	7.5	7.6	0.012
						F1B	0.1		37.498
		F2	0.9	11.1	4.6	F2U	3.3	3.3	0.261
						F2B	0.0		243.170
		F3	0.3	4.7	1.7	F3U	0.8	0.9	0.150
				37.0	15.4	F3B	0.1	11.9	99.947
CS030	94	F1	1.4	16.7	6.8	F1U	5.4	5.5	0.006
						F1B	0.1		95.092
		F2	0.8	9.1	4.0	F2U	2.8	2.8	0.178
						F2B	0.0		1706.932
		F3	0.3	4.1	1.6	F3U	1.3	1.5	0.036
				29.8	12.4	F3B	0.1	9.8	253.858
CS031	103	F1	1.3	13.8	6.3	F1U	7.7	8.0	0.007
						F1B	0.3		63.422
		F2	1.1	12.2	5.3	F2U	4.7	4.9	0.076
						F2B	0.2		35.223
		F3	0.2	2.9	1.0	F3U	1.0	1.2	0.103
				28.9	12.7	F3B	0.2	14.1	154.145
CS032	157	F1	1.2	13.5	6.1	F1U	7.0	7.2	0.004
						F1B	0.2		130.740
		F2	1.1	15.4	5.7	F2U	3.4	3.5	0.127
						F2B	0.1		140.882
		F3	0.3	3.6	1.3	F3U	1.0	1.1	0.062
				32.4	13.2	F3B	0.1	11.9	393.343
CS033	109	F1	1.9	21.7	9.5	F1U	7.3	7.5	0.007
						F1B	0.2		167.991
		F2	1.1	14.5	5.6	F2U	3.2	3.3	0.153
						F2B	0.1		168.441

		F3	0.2	2.5	0.9	F3U	1.2	1.3	0.318
				38.7	16.0	F3B	0.2	12.1	426.373
CD001	121	F1	1.3	12.6	6.7	F1U	7.5	7.7	0.000
						F1B	0.2		0.377
		F2	0.7	9.7	3.5	F2U	2.6	2.8	0.004
						F2B	0.1		54.863
		F3	0.5	6.9	2.5	F3U	1.6	1.7	0.002
				29.2	12.6	F3B	0.2	12.2	15.795
CD002	96	F1	1.2	11.8	5.9	F1U	7.1	9.6	0.042
						F1B	2.5		0.724
		F2	0.7	9.9	3.5	F2U	0.3	0.5	3.119
						F2B	0.2		64.241
		F3	0.4	5.7	2.0	F3U	1.5	1.7	0.116
				27.3	11.4	F3B	0.2	11.8	70.102
CD003	98	F1	1.1	11.4	5.7	F1U	6.7	6.9	0.002
						F1B	0.2		14.238
		F2	0.6	9.1	3.2	F2U	2.9	3.1	0.115
						F2B	0.2		125.328
		F3	0.6	8.1	3.2	F3U	1.7	2.0	0.071
				28.6	12.2	F3B	0.3	12.0	190.553
CD004	108	F1	1.2	10.5	5.8	F1U	7.4	7.4	0.001
						F1B	0.0		61.295
		F2	0.8	11.5	4.1	F2U	2.5	2.5	0.121
						F2B	0.0		
		F3	0.4	5.5	2.0	F3U	1.6	1.5	0.094
				27.5	11.9	F3B	0.0	11.5	
CD005	105	F1	1.0	10.8	5.0	F1U	5.7	5.6	0.004
						F1B	-0.1		
		F2	0.7	9.6	3.4	F2U	2.4	2.4	0.201
						F2B	0.0		
		F3	0.5	6.5	2.5	F3U	1.3	1.3	0.127
				27.0	11.0	F3B	0.0	9.3	
CD006	98	F1	0.9	9.6	4.5	F1U	5.1	5.0	0.004
						F1B	-0.1		
		F2	0.9	11.9	4.6	F2U	2.4	2.3	0.185
						F2B	-0.1		
		F3	0.4	6.2	2.1	F3U	1.3	1.3	0.066
				27.7	11.3	F3B	0.0	8.5	