School of Nursing

A Cross-Sectional Study of the Peripheral Circulation in Patients with Nephrosis

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ABSTRACT

Background: Lipid abnormalities are a common feature of the nephrotic syndrome that is also characterised by oedema, hypoalbuminaemia, proteinuria, and hypercoagulability. Concern has arisen over the increased incidence of cardiovascular disease that has been reported in individuals with nephrotic syndrome, particularly since the syndrome may occur early in life and become a chronic illness. The presence of proteinuria is a prognostic indicator for the progression of renal disease, but its possible contribution as a cardiovascular risk factor in patients with nephrotic syndrome is not known. In contrast, disordered lipoprotein metabolism, in isolation, is a conventional risk factor for the development of atherosclerosis. An early phase of atherosclerosis, vascular endothelial dysfunction, has been identified. Endothelial function can be measured non-invasively using ultrasonography and plethysmography allowing the impact of risk factors to be assessed *in vivo*.

Aim: To test the hypothesis that endothelial dysfunction occurs in the nephrotic syndrome primarily as a consequence of dyslipidaemia.

Methods: A cross-sectional design was used to study vascular function of the peripheral circulation in 45 individuals: fifteen patients with nephrosis (NP), 15 control subjects with primary hyperlipidaemia (HL) and 15 normolipidaemic controls (NC). The groups were matched for age, gender and body mass index. The NP group and the HL group had similar serum lipid and lipoprotein concentrations. High-resolution ultrasonography assessed endothelial function of the brachial artery. This non-invasive technique measured post-ischaemic flow-mediated dilatation (FMD) and endothelium-independent vasodilatation in response to glyceryl trinitrate (GTNMD). Post-ischaemic microcirculatory function was measured using venous occlusion strain gauge plethysmography.

Results: Post-ischaemic FMD of the brachial artery was significantly lower in the NP and HL groups, compared with the NC group, with no significant difference found between the former two groups. There were no significant differences in GTNMD of the brachial artery, or in microcirculatory responses. In the patients with nephrosis, non-esterified free fatty acids were inversely associated with FMD, and maximal blood flow with insulin resistance.

Conclusion: Dyslipidaemia is associated with endothelial dysfunction in patients with nephrosis and provides a plausible basis for the increased risk of cardiovascular disease observed in individuals with nephrotic syndrome.

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STATEMENT OF CONTRIBUTION BY THE CANDIDATE

For the purpose of this thesis, I managed the care and co-ordination of the patients and volunteers in this study. I carried out all of the forearm blood flow studies and analysed these data. Dr David Playford and Dr Gusharan Dogra performed the brachial artery ultrasound examinations and Dr David Playford described the ultrasound method and software analysis of the scans. Dr Dogra and myself carried out the analysis of these studies using the specially designed edge detection software. I was responsible for the statistical analysis of data derived from the blood flow studies and laboratory measurements.

ACKNOWLEDGEMENTS

CHAPTER 1 INTRODUCTION

1.1 Overview

In 1860 Virchow first suggested an association between lipid abnormalities and the pathogenesis of renal disease. In renal tissue acquired during the autopsy of patients with what was then called 'Bright's Disease', he observed 'fatty degeneration'. Fifty years later Munck used the term 'lipid nephrosis' to describe fat bodies in the urine and fatty changes in the tubules (as cited by Kamanna, Roh, & Kirschenbaum, 1993). It is the lipid abnormalities seen in the disorder, nephrotic syndrome, and their potential to affect blood vessel function in affected individuals that has stimulated the undertaking of the following thesis.

The nephrotic syndrome is characterised by the excessive excretion of plasma proteins across the glomerular capillary membrane into the urine. Albumin is the primary protein excreted leading to a common systemic feature of the disorder, hypoalbuminaemia. Other features of the syndrome include generalised oedema, dyslipidaemia and hypercoagulability. Although the primary cause of nephrotic syndrome, in non-diabetic individuals, is membranous glomerulonephritis, it can arise from any condition that impairs the permeability of the glomerular capillary membrane, for example, systemic lupus erythematosus, and more rarely the human immunodeficiency virus. Globally, the most common cause is malaria. (Orth & Ritz, 1998)

The most frequently reported lipid abnormality in patients with nephrosis is mixed hyperlipidaemia resulting from increased secretion and decreased clearance of apo-B containing lipoproteins (Cameron, 1998; De Sain-Van Der Velden et al., 1998; Kayson 1991; Warwick et al., 1991). Qualitative changes in the composition of low-density lipoprotein cholesterol particles (LDL-C) which deliver cholesterol to peripheral tissue may be altered in a manner that increases the atherogenicity of the particles (Deighan et al. 1998). Other metabolic changes related to maturation of high-density lipoprotein cholesterol (HDL-C) and the transfer of lipid moieties between particles may also play a part in the disorder (Braschi et al., 1997; Kayson & De Sain-van Der Velden, 1999). Both hypoalbuminaemia and proteinuria appear to contribute to the pathogenesis of

nephrotic hyperlipidaemia (Joven et al., 1996). The quantity of protein lost in the urine of patients with nephrotic syndrome and the degree of glomerular damage, appear also to be directly related to the extent of dyslipidaemia (Warwick & Packard, 1993).

Dyslipidaemia has the potential to injure the vascular endothelium, which is a single layer of endothelial cells lining the blood vessels and separating them from the other tissues. An active endocrine-like organ, the endothelium secretes substances necessary for, among other things, lipid and glucose metabolism, blood clotting and homeostasis of blood pressure. Surface receptors on the endothelium can respond to both mechanical stimuli such as blood flow (shear stress) and chemical stimuli from substances in the blood and surrounding tissue by initiating the formation of nitric oxide (NO) (Moncada, Palmer, & Higgs, 1991).

In order to understand the pathogenesis of atherosclerosis, it is necessary to appreciate the balance maintained by the healthy endothelium. By secreting factors such as NO and endothelin which promote, respectively, vasodilatation and vasoconstriction of small arteries, the endothelium can influence migration and proliferation of smooth muscle cells which can thicken arteries (Moncada, Palmer, & Higgs, 1991).

Low-density lipoproteins are purported to initiate atherogenesis, the process of creation of a lipid-laden plaque, by entering the intima of large arteries forming a 'fatty streak'. That this happens is a consequence of the normally anti-atherogenic lining of the blood vessels, the endothelium, becoming dysfunctional in response to injury (Ross, 1999).

Atherosclerosis has been linked with the development of progressive glomerular disease. In each of these phenomena the mechanisms appear similar with the development of lipid-laden foam cells and the proliferation of smooth muscle cells in both atherosclerosis and glomerulosclerosis (Diamond, 1991; Moorhead, Brunton, & Varghese, 1997). Furthermore, the concept of lipoprotein mediated exacerbation of renal disease is supported by growing experimental evidence (Attman, Alaupovic, & Samuelsson, 1999; Keane, O'Donnel, Kasiske, & Schmitz, 1990; Scoble, 1999; Suzuki et al., 1997).

The evidence that prolonged dyslipidaemia, particularly elevated LDL-C, in the general population results in endothelial dysfunction and atherosclerosis is considerable (Flavahan, 1992; Gordon, 1999; Vogel, Corretti, & Gellman, 1998), although it is uncertain how it contributes to the pathogenesis of coronary disease in the nephrotic patient. There is, however, agreement that atherosclerotic disease is accelerated in this group (Kasiske, O'Donnell, Kim, & Keane, 1993; Ordonez, Hiatt, Killebrew, & Fireman, 1993). Whether this is due solely to a pattern of dyslipidaemia or some other mechanism such as the accompanying heavy proteinuria, has not yet been clarified.

While the importance of proteinuria as a prognostic marker for the progression of renal disease has been established, its significance as a risk factor for cardiovascular disease is less widely appreciated (Kannel, Stamfer, Castelli, & Verter, 1984). Urinary secretion of albumin is considered an independent risk factor for cardiovascular disease (Danesh, Collins, Appleby, & Peto, 1998; Phillips, Shaper, & Whincup, 1989) and it has been suggested that microalbuminuria may be a renal manifestation of endothelial dysfunction (Ruilope, 1997). In individuals with diabetes it has been hypothesised that the presence of albuminuria indicates a widespread vascular process involving the retina, the intima of large vessels and the glomeruli in the kidney (Deckert et al., 1989). In addition to dyslipoproteinaemia and hypoalbuminaemia, hyperfibrinogenaemia is recognised as a cardiovascular risk factor in non-renal populations (Danesh et al., 1998; Phillips et al., 1989).

Endothelial function can be studied non-invasively using the techniques of strain gauge plethysmography which assesses vascular responses in the forearm microcirculation and brachial artery ultrasound, a non-invasive technique originally devised for use in paediatric research (Sorensen et al., 1994). The former technique can incorporate the use of vaso-active mediators such as acetylcholine. The latter technique is used as a measure of endothelium-dependent flow-mediated dilatation, an indicator of endothelial function.

Endothelial dysfunction of the brachial artery is associated with abnormal vasomotor responses of the coronary circulation (Anderson et al., 1995) and cardiovascular risk factors such as smoking, hypertension, dyslipoproteinaemia, hyperhomocysteinaemia,

diabetes and obesity (Celermajer et al., 1992; Ferro & Webb, 1997; Hashimoto et al., 1998; O'Brien et al., 1997; Tawakol et al., 1997). More recently endothelial dysfunction has been shown to predict cardiovascular events (Al Suwaidi et al., 2000). Impaired endothelial dysfunction can be improved with lipid-lowering medication, smoking cessation, exercise and dietary modification (Dupuis et al. 1999; LaRosa 1998; O'Driscoll, Green, & Taylor 1996; Vogel, Corretti, & Plotnik, 1995).

The potential influence of proteinuria on endothelial function has been studied in patients with nephrosis using venous occlusion strain gauge plethysmography and intra-arterial infusion of vaso-active mediators (Stroes, Joles, Chang, Koomans, & Rabelink, 1995a). Although this study concluded that nephrotic range proteinuria is accompanied by impaired endothelium-dependent vasodilatation in the microcirculation, a similar degree of endothelial dysfunction was also reported in patients with primary hyperlipidaemia (Stroes et al., 1995a; Stroes, Koomans, de Bruin, & Rabelink, 1995b). Accordingly, it is possible that the endothelial dysfunction seen in primary nephrotic syndrome may be as a consequence of dyslipidaemia or some other physiological alteration that occurs in the presence of nephrosis, such as proteinuria.

Forearm microcirculatory function has been studied previously in patients with proteinuria and end-stage renal disease (Demuth et al., 1998; Pannier et al., 2000; Stroes et al., 1995a). Brachial artery ultrasonography has been used to study endothelial dysfunction in children with chronic renal failure (Kari et al., 1997). The present study will investigate both the forearm microcirculation and a peripheral conduit vessel (the brachial artery) in patients without renal failure, in an ambulatory setting.

1.2 Hypothesis and Aims

Hypothesis: Patients with nephrotic syndrome have endothelial dysfunction and this occurs primarily as a consequence of dyslipidaemia.

Primary Aims:

- To test the observation that endothelial dysfunction as manifested by impaired post-ischaemic flow-mediated dilatation of the brachial artery and resistance vessels is

more common in patients with nephrotic syndrome than in healthy, normolipidaemic controls.

- To test the observation that the impairment in patients with nephrotic syndrome is comparable to that seen in patients with hypercholesterolaemia.

Secondary Aims

- To explore associations between endothelial dysfunction and a spectrum of variables in patients with nephrotic syndrome, as a secondary method of testing the study hypothesis and generating new hypotheses for future research.

1.3 Significance of The Study

Although pharmacological and lifestyle interventions may reduce the risk of atherosclerosis, researchers and clinicians assert that more clinical trials are needed to study the effect of these interventions on the regression of morphological and functional atherosclerosis (Gordon, 1999; Vogel et al., 1998). Keane (1994) observed that specific recommendations for treatment are difficult because of a paucity of studies assessing cardiovascular endpoints of dyslipidaemia, a common feature in patients with persistent proteinuria. Hoffart (1995) in the arena of nursing research, concurred. In her analysis of research avenues chosen by nephrology nurses, she identified the need for research into areas such as cardiovascular complications of renal disease, treatment modalities and patient education. The author also noted the trend towards descriptive rather than experimental designs. She recommended that if non-nephrology researchers can contribute to experimental studies that would lead to new interventions for chronic illness they should be welcomed. It is anticipated that this study will provide new insights into the relationship between cardiovascular complications of proteinuric renal disease.

The mechanisms that regulate the development of dyslipidaemia in severe proteinuria are complex. However, many of the lipid studies in this area have been performed in animal models and whether they can be extrapolated to humans has been questioned as the plasma lipoprotein profile of humans is considered to be unique (Warwick & Packard, 1993).

In summary, nephrotic syndrome is characterised by loss of protein in the urine and dyslipidaemia. Dyslipidaemia is known to induce endothelial dysfunction in the general population and there is evidence that this dysfunction precedes the development of atherosclerosis. Proteinuria may exacerbate the risk of endothelial dysfunction and as a consequence place patients with nephrosis at greater risk of coronary artery disease than individuals with hyperlipidaemia and no proteinuria. Researchers have identified a need for *in vivo* studies exploring the relationship between proteinuria, dyslipidaemia and endothelial dysfunction, stating that animal studies may be inadequate models for comparison with humans. Furthermore, researchers seek to improve the treatment of lipid disorders in patients with renal disease in order to reduce their risk of developing cardiovascular complications and slow the progression of renal disease. This study will explore the relationship between endothelial dysfunction, proteinuria and dyslipidaemia.

1.3.1 Significance to nursing

Hoffart (1995), in the February editorial of the American Nephrology Nurses Association Journal, described how research traditionally evolves from the descriptive to the exploratory and finally to the experimental. Commenting on data describing nephrology nurse researchers and their work, she made the observation that topics that may present challenges to nurses, such as cardiovascular complications of renal disease, were largely absent. Many studies carried out by nurses were descriptive and few were experimental. She further asserted that nurses need research based knowledge related to, among other things, physiological aspects of disease processes and that they should not be limited practically by the need to apply nursing theories and models. (Hoffart, 1995)

The application of physiology to nursing care is of importance to curriculum development in nursing. Jordan and Reid (1997) conducted research that evaluated the physiology component of a post registration nursing diploma in terms of its impact on patients, finding that nurses reported delivering a higher standard of care as a consequence of the course. It was observed that by increasing the nurses' knowledge of biological parameters, care delivery to patients could be augmented.

Clearly, dissemination of knowledge gained by research and experience remains critical. It is this writer's view that if nurses can conduct research rather than conduct data collection alone, then they are more likely to feel ownership of that knowledge and incorporate it into their practice and their experiential knowledge leading to enhanced patient care and increased professional satisfaction.

1.3.2 An opportunity for nurses

The U.S. Department of Health and Human Services has recognised the potential for the nursing profession to offer dyslipidaemic patients safe, cost effective and compliance-enhancing care by supporting the role of the lipid nurse specialist (Cofer, 1997). Within the profession the need for post graduate nursing expertise in the area of coronary heart disease screening and management of lipid disorders at a community level has been identified (Lindsay, Robb, & Gaw, 1995).

The role of the lipid nurse specialist is seen to combine knowledge related to the physiology and treatment of dyslipoproteinaemia with psychological and behavioural principles, for example, learning readiness, processes of change, change maintenance and facilitation. Nurses have a role in assessing new patients, stratifying risks, monitoring medication and assisting patients to set goals in relation to lifestyle changes. This can be achieved in a multidisciplinary setting (Cofer, 1997). Facilitating compliance to treatment regimens may also be seen as a nursing role but recent interest has been directed not so much at the notion of compliance which is seen by some to be a value laden word implying yielding or conformity, but to adherence. Adherence is interpreted as characterising patients as autonomous individuals engaged in a more active pursuit of health (Lutfey & Wishner, 1999). This may well be a semantic argument. Individualising patient care and engaging patients in setting realistic health seeking behaviours are nursing goals and it is the pursuit of these goals that may prove most effective in illness prevention and health promotion.

In conclusion, this study will provide nurses with knowledge of vascular endothelial function, lipid metabolism, atherosclerosis and the link with glomerulosclerosis and nephrotic syndrome. This knowledge may improve patient teaching and/or provide the

biological science knowledge needed to make clinical judgements. Indirectly this knowledge may augment experience and form the basis of protocols for patient care and nurse education (Jordan & Reid, 1997).

1.4 Philosophical Perspective

The paradigm (framework) of this study is scientific, with roots in logic in so far as a specific hypothesis will be tested. This framework would, however, be incomplete if it did not incorporate ethical reasoning and the nursing dimension. Building in an ethical dimension beyond the research question allows the researcher to ensure that the effects or outcomes of techniques or treatments under testing are morally acceptable (Wulff, Pederson, & Rosenberg, 1986).

Beyond science and ethics are the nursing dimensions of patient advocacy and patient teaching. Nurses need to act to enhance nursing roles that embrace multidisciplinary, patient-centred research and outcomes of care. Finally researchers must reflect on their findings with appropriate criticism and inform practitioners of their activities, findings and recommendations (Stevens, 1997).

1.4.1 Epistemology

This research seeks to build on the body of knowledge of the relationship between endothelial dysfunction, dyslipidaemia and proteinuria. Understanding these relationships may lead researchers to develop appropriate interventions for what is a significant health problem for the individual and the community.

1.4.2 Logic

"The science of reasoning" is a common definition for the word logic (Copi, 1986). The tools of logic are seen to be deduction and induction. The purpose of deductive argument is to make clear the relationship between the premise (proposition) of a valid argument and its conclusion. In this study a logical inferential process commenced with the proposition by earlier researchers (Stroes et al., 1995a), that endothelial dysfunction in nephrotic individuals resulted as a consequence of proteinuria, independent of the

effect of hyperlipidaemia, and concluded with a proposition that provided a premise or proposition for further research. In effect, the conclusion then became the basis for an inductive argument based on the concept of probability (Copi, 1986).

The case-control design of this study is based on a form of inductive inference recommended by the British philosopher, John Stuart Mill (1806-1873) (Copi, 1986). The Method of Agreement is based on the concept that if instances of a phenomenon have in common but one circumstance, and if in that circumstance all the instances agree, then that circumstance is the cause or effect of the phenomenon observed (Copi, 1986). Another of Mill's ideas was that of the Method of Difference in which the phenomenon under study may have circumstances in common save one and where those instances differ is part of the cause or effect of the phenomenon. Used together these methods, in theory, strengthen the probability of the conclusion. A third argument attributed to Mills is that of the Method of Concomitant Variation in which, when one phenomenon varies in a manner contemporaneously with another phenomenon it is seen to be either the cause, effect or is connected with it through some other causal influence. It is important to recognise that the conclusion of an inductive argument provides an inference of probability rather than certainty.

1.4.3 Ethics

An outcome of the Nuremberg trials after World War II was the Declaration of Helsinki, which established in international law the ethical guidelines for medical research and demanded that all research projects must have the approval of an independent ethics committee (Wallace, 1995). The International Council of Nurses has issued a statement " A Code for Nurses: Ethical Concepts Applied to Nurses" which provides a code for nurses in all aspects of professional conduct (Wallace, 1995). Changing societal values also influence expectations of what are acceptable ethical guidelines and researchers are beholden to keep pace with these expectations. The concepts of autonomy, informed and valid consent, and the legal requirements for a valid consent propounded by the Declaration, provide the ethical guidelines for researchers in the present day.

The consenting process is complex and should contain discussion involving the purpose, procedure, risks, and direct and indirect benefits. Alternatives to participation in the study can be discussed at this time. Confidentiality is another important element. A subject should understand that s/he can choose not to participate or withdraw at any time during the study without influencing any medical care that they may be receiving. It is not sufficient for the researcher to merely "inform" the potential participant about the research. An essential component of informed consent is to ascertain that the subject has understood the nature of his/her involvement in the proposed research. (Titus & Keane, 1996)

The individuals in this study were asked to participate in research from which they may not directly benefit. Although the tests were non-invasive, venepuncture can cause some minor discomfort and it was possible that headache could arise from administration of glyceryl trinitrate during the ultrasound procedure. These aspects were included in the information sheet and consent form. Those patients on lipid-lowering therapy were asked to discontinue it for six weeks prior to the study. It was these issues that were pertinent to address in this study within the framework of the standard ethical requirements that pertain to all human research.

CHAPTER 2 PHYSIOLOGY AND PATHOPHYSIOLOGY

2.1 Introduction

The significance of the endothelium in mediating diseases such as atherosclerosis and coronary heart disease lies in understanding the homeostatic mechanisms responsible for maintenance of vasomotion, vascular growth, inflammatory mediators and those factors that promote thrombosis and haemostasis (Rubanyi, 1993). An understanding of the putative relationship between the nephrotic syndrome and endothelial dysfunction can be achieved by reviewing the physiology of endothelial function, atherosclerosis, lipid metabolism and their associations with nephrotic syndrome. Some basic renal physiology will also be described in this chapter to provide background to the syndrome of nephrosis.

2.2 The Vascular Endothelium & Atherosclerosis

2.2.1 Physiological aspects of endothelial cells

Although Malphigi was the first known physician to describe the physical separation of blood from other tissue, and the existence of a network of blood vessels, it was von Reckingausen in the 1800's, who confirmed that cells lined these vessels. We now know that the endothelium is a single layer of cells that lines vessels in every organ system including the heart (Born, Rabelink, & Smith, 1998; Cines et al., 1998).

The emergence of electron microscopy in the 1950's allowed physiological studies to describe the interaction between the components of the circulation and the endothelium. Together with *in vitro* studies of cultured endothelial cells and more recently genetic recombination studies in mice, these studies have led to our current knowledge of the endothelium as being a sophisticated entity with secretory, metabolic and immunologic functions. (Cines et al., 1998; Moncada et al., 1991; Rubanyi, 1993)

Structurally, endothelial cells contain some of the contractile attributes of muscle cells. These components contribute to the cell's ability to maintain the plasma membrane, immobilise proteins and express selective permeability to various substances. The endothelial cell also expresses membrane-bound receptors found in vesicular structures

known as caveoli, which influence the cell's regulation of various molecules and blood cells. These receptors include those for proteins, lipoproteins, and metabolites such as nitric oxide and hormones (Born et al., 1998; Cines et al., 1998). Endothelial cells are heterogenous, for example, the endothelial cells lining vessels in the brain and retina have tight junctions that maintain the blood brain barrier. The liver, spleen and bone marrow sinusoids have discontinuous endothelial cells with intercellular gaps. The intestinal villi, endocrine glands and kidneys are lined with fenestrated endothelium to allow for secretion, absorption, and filtering (Cines et al., 1998).

As well as genetic influences on endothelial cell (EC) phenotype, there is also evidence to suggest that EC heterogeneity develops in response to numerous stimuli. These stimuli can be mechanical, or as a result of growth promoters and inhibitors, plasma lipids and proteins such as thrombin and antibodies. (Cines et al., 1998). It is also useful to understand that endothelial cells from different tissues vary in their expression of surface proteins and adhesion properties. A good example of this is that tumour cells preferentially adhere to specific endothelial cells that exhibit similar propensities to the tumour cells themselves (Cines et al., 1998).

2.2.2 Vasoactive mediators

An important function of the endothelium is that of modulating vascular tone. It does this by secreting and absorbing vasoactive substance such as nitric oxide and endothelin which result in dilation and constriction of vascular beds in response to specific stimuli (Rubanyi, 1993). See Table 2.1. Nitric oxide (NO) is the most potent of the vasodilator substances. It is expressed constitutively and in response to the shear stress exerted by the circulation or a reduction in oxygen tension. In addition, activation of surface receptors by substances such as acetylcholine, bradykinin and histamine will initiate a G-protein signal transduction pathway which stimulates endothelial NO synthase (e-NOS) to form NO and citrulline from L-arginine and molecular oxygen. Nitric oxide then crosses cell membranes to target the activity of enzymes such as soluble guanylate cyclase (GC). This causes an increase in cyclic guanosine monophosphate (cGMP) production in vascular smooth muscle cells resulting in reduced intracellular calcium levels leading to vasorelaxation. (Moncada et al., 1991; Rubanyi, 1993)

Nitric oxide, endothelium-derived hyperpolarizing factor (EDHF), prostacyclin and C-type natriuretic peptide are known as endothelium relaxing factors with the latter substances supporting the role of NO. Endothelin, angiotensin II, thromboxane and prostaglandin H2 are endothelium derived constrictors. In the kidney, nitric oxide acts as a tonic dilator on the afferent arteriole of superficial glomeruli opposing angiotensin II- induced vasoconstriction (Kone, 1997).

Homeostasis of vascular tone is achieved by a negative feedback system which in health, favours vasodilation (Born et al., 1998; Drexler, 1997). In physiological conditions, nitric oxide is released by the endothelial cells not only to the sub-endothelial space to be taken up by smooth muscle cells but also into the lumen of the blood vessel. If disrupted, this process may result in inhibition of monocyte and platelet adhesion, oxidation of lipoproteins and synthesis of inflammatory cytokines that are pro-atherogenic processes. (Flavahan, 1992; Rubanyi, 1993)

Table 2.1 Dilating and constricting factors released by the vascular endothelium

Vasodilators	Vasoconstrictors
Nitric oxide	Endothelin
Endothelin-derived hyperpolarising factor	Angiotensin II
Prostacyclins	Thromboxane A2
C-type natriuretic peptide	Prostaglandin H2

Born, et al. (1998)

2.2.3 Endothelial dysfunction: an early phase of atherosclerosis

Rubanyi (1993, p. S8) defines endothelial dysfunction as "an imbalance between relaxing and contracting factors, between anti- and pro-coagulant mediators or growth-inhibiting and growth-promoting factors." The morphologic and structural changes that typify endothelial dysfunction are considered to be an early manifestation of atherosclerosis (Rubanyi, 1993).

However, the pathogenesis of atherosclerosis involves multiple mechanisms. A reduction in the amount of NO available to the endothelium can be caused by abnormalities of endothelial signal transduction, lack of the necessary substrate or the expression of the enzyme nitric oxide synthase (Flavahan, 1992; Shimokawa, 1999). The endothelium is also particularly susceptible to injury by smoking, hypertension, hyperlipidaemia and diabetes mellitus, which are the conventional risk factors for atherosclerosis (Celermajer, Sorenson, Bull, Robinson, & Deanfield, 1994; Drexler, 1997; Vogel et al., 1998). Novel atherosclerotic risk factors include prothrombotic substances such as homocysteine and inflammatory mediators such as C-reactive protein (Farouque, O'Brien, & Meredith, 2000). More recently a theory for insulin resistance as a causal factor in the initiation of endothelial dysfunction in diabetes has been proposed (Pinkney, Stehouwer, Coppack, & Yudkin, 1997; Playford & Watts, 1999).

The endothelium itself produces superoxide anion, a vasoconstrictor and a metabolite of arachidonic acid (a polyunsaturated fatty acid). It breaks down nitric oxide, forming peroxynitrate which is a free radical that has the potential to oxidize lipids (Cines et al., 1998). Oxidant stress caused by oxidized low-density lipoproteins has been put forward as a common mechanism of endothelial injury in the atherogenic process (Flavahan, 1992; Liao, Shin, Lee, & Clark, 1995). Clearly the risk factors for the development of endothelial dysfunction agree with those established for atherosclerosis.

In summary, if the endothelium is damaged and NO production is impaired, then vasoconstriction, smooth muscle proliferation, white cell and platelet adhesion become the dominant partners in the feedback mechanism resulting in endothelial dysfunction an early stage of atherosclerosis (Drexler, 1997; Rubanyi, 1993). Furthermore in overt atherosclerosis, the altered vascular reactivity resulting from endothelial dysfunction may increase the haemodynamic stress on atherosclerotic plaques making them unstable (Sattar, Petrie, & Jaap, 1998).

2.2.4 Plaque formation

In developed countries, atherosclerosis is the most prevalent vascular disease and the underlying condition associated with peripheral vascular disease, stroke and coronary artery disease (Gordon, 1999). It is thought that spontaneous atherogenesis occurs at sites of low shear stress where LDL cholesterol enters dysfunctional endothelium triggering the aggregation of lipids, macrophages and T-lymphocytes forming, what is known commonly as a 'fatty streak'. These streaks, found in the intima of large arteries, are the first identifiable lesions of atherosclerosis developing into layers of macrophages and smooth muscles cells and ultimately into fibrous plaques. The mature plaques consist of a cap of connective tissue overlying a core of lipid and necrotic debris. These plaques may protrude into the lumen of the artery impeding flow and creating turbulence. They may rupture, releasing thrombi resulting in cerebrovascular or myocardial infarction (Ross, 1993). That atherosclerosis arises in response to endothelial injury was suggested over 20 years ago. In early atherosclerosis the endothelium has been found to be morphologically and functionally impaired. Later as the disease progresses, it can be denuded of endothelial cells. There are many predisposing factors to atherosclerosis including smoking, hyperlipidaemia, hypertension, diabetes, inflammation and increasing age. (Cines et al., 1998; Gordon, 1999)

2.2.5 Relationship of atherosclerosis to renal disease– 'glomerular atherosclerosis'

In large arteries, atherosclerotic plaques develop in areas of high shear stress where the endothelium has become dysfunctional in association with a disturbance of normal laminar blood flow, for example, in the coronary circulation at bends or branch points (Schussheim & Fuster, 1999). In a larger conduit vessel, the brachial artery, atherosclerosis has also been detected (Sorensen, Kristensen, & Celermajer, 1997). In individuals with renal disease atherosclerotic coronary disease is common and the hypercholesterolaemia seen in people with nephrosis is thought to contribute to this. However, agreement with this view is not universal, as nephrosis and its accompanying dyslipoproteinaemia, may be short-lived. Consequently, there are researchers who believe that there may be other risk factors contributing to the atherogenic profile of these patients (Moorhead, Brunton, & Varghese, 1998).

Whatever the mechanism responsible for the increased cardiovascular disease seen in patients with nephrosis and other non-diabetic renal disease, an analogy has been made between development of atherosclerosis and sclerotic disease in the glomerulus and tubular interstitial tissue (Diamond, 1991). This is perhaps not surprising as the microvasculature of the glomerulus possesses similar cell types to that of the macrovasculature, namely endothelial cells, monocytes and the mesangial cell which resembles the smooth muscle cell (Moorhead et al., 1997). It has been demonstrated in animals that the healthy glomerular membrane is protected from lipoproteins. This is supported by the observation that patients with hyperlipidaemia and normal renal function do not usually develop renal insufficiency (Attman, 1998). However, when the glomerular basement membrane is altered, exposure to even low levels of lipoproteins may contribute to the progression of renal disease (Moorhead et al., 1998). Whether this is due to compositional changes in the lipids caused by the underlying renal disease or alternatively the disease resulting in changes that allow accumulation of lipids in mesangial cells, or both, remains to be clarified (Attman, 1998).

2.3 Lipids and Lipoproteins

2.3.1 Chemistry and physiology

The major lipids in plasma are fatty acids, cholesterol esters, triglycerides (Tg), and phospholipids. Free fatty acids are bound to albumin and are present in plasma in both an esterified and a free form and are also stored in adipose tissue as triglyceride. An ester is the product of a reaction between an acid and an alcohol molecule, in this instance, a fatty acid bound to glycerol. The other lipids are bound to specific apolipoproteins and transported as dynamic lipoprotein complexes that are constantly remodelled (Ganong, 1993; Thompson, 1994).

Lipoproteins are spherical and contain varying proportions of lipid and protein. The hydrophobic core of the molecule contains predominantly Tg and cholesterol esters. The hydrophilic outer layer consists of phospholipids, free cholesterol and surface proteins. Conventionally, the proportion of lipid in the lipoprotein particle determines its buoyant density and therefore its classification. More recently, advances in laboratory techniques

have led to discrete sub-groups within existing classes of lipoproteins (Contois & Huang, 1997; Warwick & Packard, 1993).

Although centrifugation separates lipoproteins by their density, each lipoprotein class is a continuum of particles that are constantly remodeled. It is, however, useful to classify the various particles in order to study the various interactions and pathophysiology (Moorhead et al., 1998).

2.3.1.1 Cholesterol

Cholesterol is a steroid, a large lipid molecule contained by all animal cell membranes, a constituent of bile and a precursor of steroid hormones and vitamin D. Cells require cholesterol for maintenance of cell membranes, cell growth and division. It exists in both a free and an esterified form with free cholesterol principally present in most tissues. Cholesterol esters predominate in the plasma, adrenal cortex, liver and intestinal lymph. Atheromatous plaques are also predominantly free cholesterol. (Thompson, 1994)

The cell obtains cholesterol from exogenous, dietary sources such as meat, dairy products and eggs. Endogenous synthesis of cholesterol occurs in the liver as a result of mevalonic acid conversion. Primarily LDL and other lipoprotein complexes circulate endogenous cholesterol to extra hepatic tissue, whereas exogenous cholesterol is carried by the chylomicron. See Figure 2.1. (Thompson, 1994)

Control of cholesterol within the cell is by a feedback mechanism involving the two proteins 3-hydroxy-methylglutaryl-coenzyme A reductase (HMG-CoA reductase), acyl cholesterol acyltransferase (ACAT) and receptors on the surface of the cell membrane. In plasma, esterification of cholesterol is achieved by the enzyme lecithin: cholesterol acyltransferase (LCAT).
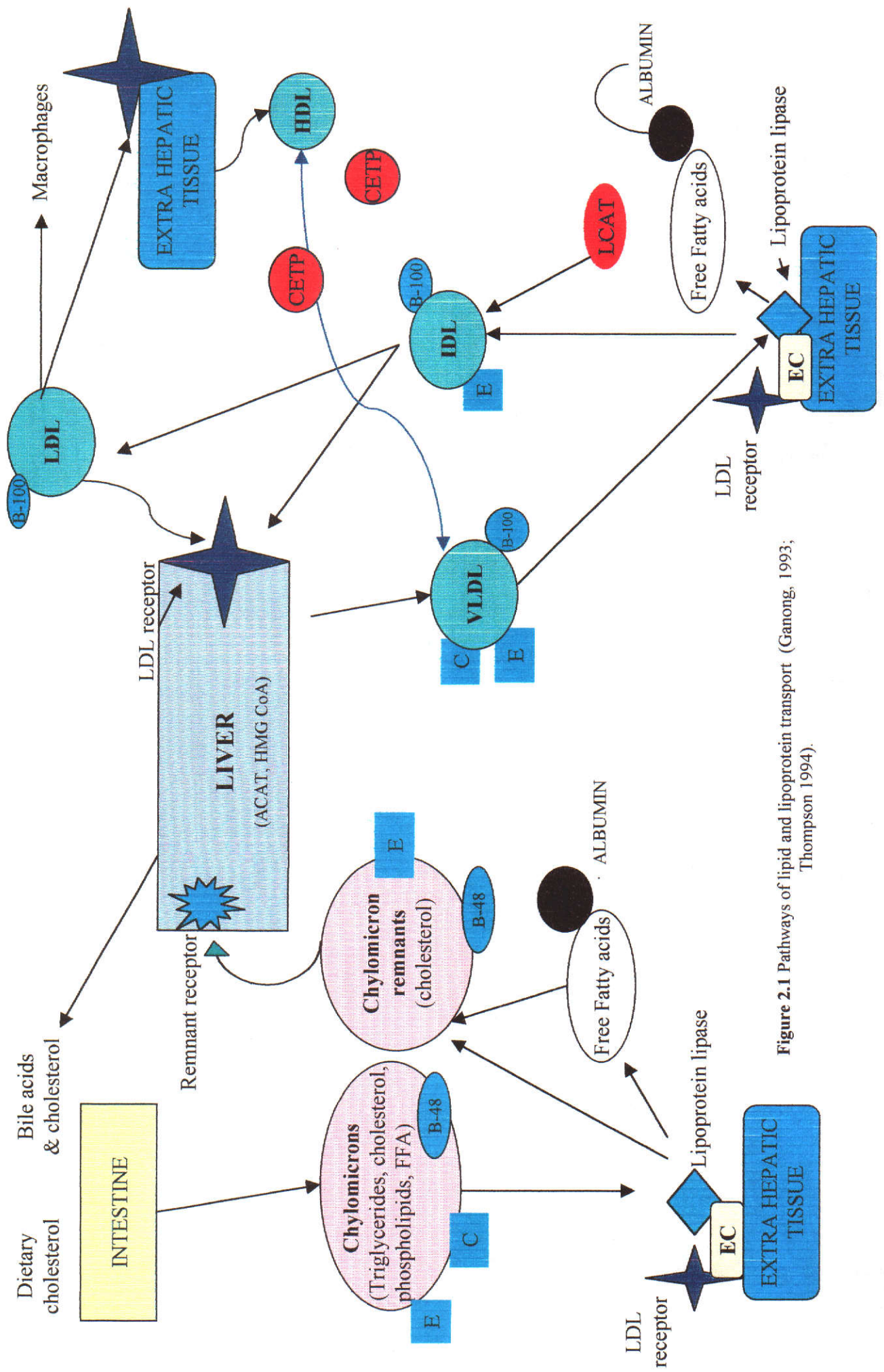
Figure 2.1 Pathways of lipid and lipoprotein transport (Ganong, 1993; Thompson 1994).

When the cell has a sufficient supply of cholesterol, the number of receptors on the cell membrane fall and as a result, less cholesterol is brought into the cell. 3-hydroxy-methylglutaryl-coenzyme A reductase (HMG-CoA reductase), which is active in the conversion of mevalonic acid to cholesterol, is inhibited and consequently less cholesterol is synthesized. Any cholesterol remaining in the cell is altered to cholesterol ester by ACAT for future use. The reverse effect can occur when cholesterol levels in the cell are low (Warwick & Packard, 1993).

Absorption and synthesis of cholesterol is balanced by faecal excretion of sterols and bile acids under the control of feedback mechanisms in the liver. Absorption of dietary cholesterol and reabsorption of biliary cholesterol help limit the rate of hepatic synthesis (Thompson, 1994; Wieland, Marz, & Nauck, 1994).

2.3.1.2 Triglycerides

Triglycerides consist of two to three different fatty acids, palmitic, linoleic and oleate. Exogenous or dietary triglyceride is synthesised in the small intestine and is carried by chylomicons to the liver. Endogenous triglyceride is secreted by the liver as very low-density lipoprotein (VLDL). It is absorbed by adipose tissue after hydrolysis involving the lipolytic enzymes. (Ganong, 1993)

2.3.1.3 Phospholipid

Phospholipid synthesis occurs in most tissues but the plasma phospholipids of lecithin and sphingomyelin are derived principally from the liver. Dietary phospholipid is hydrolysed in the small intestine by pancreatic lipase as is bile. Like cholesterol, phospholipids are part of all cell membranes and provide lipoproteins with the ability to remain soluble in the aqueous environment of plasma. This is because of the structure of the phospholipid which has a polar head and a non polar, fatty acyl chain giving the molecule both hydrophilic and hydrophobic properties. (Thompson, 1994)

2.3.2 Major lipoprotein groups

The major lipoprotein groups are chylomicrons (CM), very low-density lipoproteins (VLDL), low-density lipoprotein (LDL) high-density lipoprotein (HDL) and lipoprotein (a) (Lp(a)). Chylomicrons transport ingested dietary cholesterol and triglycerides and together with their remnant particles form the exogenous lipoprotein system. The other lipoproteins complexes are synthesised by the liver and as such form an endogenous system. See Figure 2.1.(Ganong, 1993)

2.3.2.1 Apolipoproteins

Surface proteins, termed apolipoproteins (apo), act as ligands or binding agents, between the lipoprotein complexes and the enzymes (lipases) that break down the molecule for use by the cells. Apolipoproteins can activate or inhibit enzymes and in that way regulate the constant remodelling of the lipoprotein particle. They are therefore critical in determining the serum concentrations of lipoproteins. For example, apo-C-II activates lipoprotein lipase catalysing chylomicrons and removing Tg for use by the cell. The major apolipoproteins are apo-E, apo-C, apo-B-100, and apo-B48 (Ganong, 1993; Wieland et al., 1994).

The most important ligands of atherogenic lipoproteins are apo-B and apo-E. Apolipoprotein E is a multifunctional glycoprotein that facilitates the hepatic uptake of particles that are too small to be manipulated by lipoprotein lipase and CM remnants. Apolipoprotein E facilitates the catabolism of VLDL remnants and the conversion of IDL to LDL. It has a role in the uptake of apo-E containing HDL in the liver. Apo-E mRNA is located in many cell and tissue types. It also interacts with the LDL receptor and the LDL receptor related protein (RLP). (Ganong, 1993; Wieland, Marz, and Nauck, 1994)

Six different apo-E phenotypes can occur and for each isoform there are homozygous and heterozygous individuals. Polymorphism of apo-E is a clinically useful marker. For example, E-2 homozygosity demonstrates Type III hyperlipidaemia and E-4 is over represented in persons with Alzheimer's disease. Apolipoprotein B is the major structural protein of LDL and is also found in IDL and VLDL and Lp (a). The mRNA

of apo-B is found in the liver and intestine. Hepatic apo-B is called apo-B-100 and intestinal apo-B is apo-B-48. (Contois and Huang 1997)

2.3.2.2 Receptors and lipases

The lipoprotein receptors are LDL receptor, the LDL receptor related protein and scavenger receptors responsible for the elimination of modified lipoproteins. The major role of the LDL receptor is to provide cholesterol to the cell for cell membrane synthesis and to organs for use as a substrate for the production of hormones, steroids and bile acids (Thompson, 1994).The LDL receptor related protein (LRP) is responsible for clearing CM remnants. The scavenger receptor is found in macrophages and hepatic endothelial cells and appears to degrade modified LDL. (Thompson, 1994)

Extra-hepatic lipase, known more commonly as lipoprotein lipase, is found on the luminal surface of capillary endothelium in adipose and skeletal muscle tissue. Lipoprotein lipase is responsible for the catabolism of the triglyceride rich lipoproteins, chylomicrons and VLDL. Within adipose tissue a hormone sensitive lipase breaks down stored Tg producing glycerol and fatty acids which are released into the circulation. The expression of both extra-hepatic and hepatic lipase is influenced by the presence of hormones such as noradrenaline and glucocorticoids. Hormone sensitive lipases are hormones activated by acute stress, lack of insulin and prolonged fasting. Free fatty acids are oxidised in the liver, the heart and skeletal muscle, they may be reesterified by the liver to form triglyceride or phospholipid and/or converted to other fatty acids. (Ganong, 1993; Thompson, 1994)

2.3.2.3 Chylomicrons

The chylomicron (CM) is the least dense of the lipoprotein classes and also the largest particle. It is formed in the intestinal epithelium from ingested dietary triglycerides and cholesterol and consists of 90% Tg, absorbed steroids and phospholipids with a coating of protein. The major protein of the CM is apo-B-48, a low molecular weight molecule synthesized by intestinal cells. Without apo-B-48 the CM particle cannot be formed (Contois & Huang, 1997). For each CM particle there is one apo-B-48 molecule which remains attached to the CM from its formation in the intestinal cells to its removal by the liver, making it a useful measure of the

number of circulating CM particles (Sethi, 1997). Other apolipoproteins contained by the CM are apo-A-1, A-IV and C, with apo-E and other apo-C's being acquired when the CM particle passes through the lymphatic system and into the subclavian vein via the thoracic duct (Ganong 1993).

Once the chylomicrons have picked up apo-C, the particles are capable of attaching to lipoprotein lipase that punches a hole through the phospholipid layer and hydrolyzes the triglyceride in the core of the complex. The altered chylomicrons, still cholesterol rich, are known as remnants and they move to the liver where receptors on the surface of hepatocytes attach them and absorb them into the cell via endocytosis. Three to six hours after ingesting a fatty meal, peak chylomicronaemia occurs with rapid removal of the CM particles from plasma and normally undetectable levels after a 12 hour fast. (Thompson, 1994)

2.3.2.4 Very low-density lipoproteins

Like chylomicrons, VLDL carries mostly triglyceride. The main difference is that chylomicrons consist of exogenous or dietary triglyceride and cholesterol whereas VLDL synthesis is primarily endogenous occurring mostly in hepatocytes which secrete the triglyceride rich lipids (formed from fatty acids and carbohydrates) with their protein moieties, apo-B-100, CII, CIII and E. Very low density lipoproteins transport triglycerides to extra-hepatic tissue where, by the action of lipoprotein lipase, triglyceride is removed and absorbed into the cell. As a result of this depletion, some particles remodel to form an intermediate density lipoprotein (IDL) and others are taken up by the liver (Thompson, 1994; Wieland et al., 1994).

2.3.2.5 Intermediate-density lipoproteins

Intermediate density lipoproteins (IDL), enabled by the circulating enzyme, lecithin cholesterol acyltransferase (LCAT), pick up cholesterol esters that are formed from cholesterol carried by HDL. The liver then takes up some of the IDL particles with the remainder becoming LDL which lose apo E but retain apo B-100. (Thompson, 1994; Wieland et al., 1994)

2.3.2.6 Low-density lipoproteins

Low-density lipoprotein particles contain 70% of circulating cholesterol and when in excess are associated with an increased risk of atheroclerosis. Although LDL particles also transport Tg, their primary function is to supply cholesterol to peripheral tissues. Low-density lipoprotein receptors on the surface of these tissues recognise and bind with apo B-100 (the primary protein constituent of LDL) and apo-E. The cell, via endocytosis, absorbs the particle and the cholesterol is metabolised (Contois & Huang, 1997).

2.3.2.7 Lipoprotein (a)

Lp (a) are LDL like particles with an extra glycoprotein molecule, apo (a), connected to the apo-B component of the particle. An increased incidence of cardiovascular disease is associated with high Lp (a) concentration. (Warwick & Packard, 1993)

2.3.2.8 High-density lipoprotein

The liver, the small intestine and the kidney, synthesise and secrete high-density lipoprotein (HDL), the most numerous of the lipoprotein species. High-density lipoprotein functions to modulate the de-lipidation of chylomicrons, VLDL and IDL and transport cholesterol from peripheral tissues back to the liver. This is the so-called reverse cholesterol transport system (Moorhead et al., 1998; Warwick & Packard, 1993). This is achieved by the transference of cholesterol ester between HDL and VLDL by a protein called cholesterol ester transfer protein or CETP. See Figure 2.1. (Thompson, 1994)

High serum concentrations of HDL-cholesterol (HDL-C) have been associated with decreased cardiovascular risk. Factors associated with an increase in HDL-C concentration are exercise, alcohol consumption, and oral ingestion of exogenous oestrogen. Lower levels of HDL-C have been associated with obesity and cigarette smoking. An inverse correlation exists between HDL-C and LDL-C. (Gordon, 1999)

2.4 Dyslipoproteinaemia: Aetiology

2.4.1 Genetic factors

Inherited genes account for approximately 50% of the variability of the major risk factors for coronary artery disease. Furthermore risk factors such as diet may interact with genetic factors to mediate cardiovascular risk. The cloning and characterisation of genes that encode proteins involved in lipoprotein and lipid metabolism has facilitated the diagnosis and treatment of lipoprotein abnormalities, which are commonly known as dyslipoproteinaemia. (Contois & Huang, 1997)

Cardiovascular disease is rarely related to a single genetic locus, it generally involves the interaction of multiple genes and a number of environmental factors. However, premature cardiovascular disease and a family history of cardiac death before the age of 65, imply a genetic influence. Monogenic lipoprotein disorders do occur and usually involve an enzymatic, receptor or transport related defect of metabolism. A monogenic defect affecting cholesterol metabolism is estimated to occur in 1 in 500 individuals and is termed familial hypercholesterolaemia or FH. In individuals with FH, binding of LDL to the LDL receptor is defective leading to high circulating levels of total cholesterol, low density lipoprotein and intermediate density lipoprotein. (Contois & Huang, 1997)

Dyslipidaemia may manifest as hypercholesterolaemia which is usually due to elevated LDL-C. Combined hypercholesterolaemia and hypertriglyceridaemia is a result of elevated LDL and VLDL, and hypertriglyceridaemia a consequence of elevated VLDL and/or remnant particles. (Contois & Huang, 1997)

Defects of enzymes, receptors and transfer proteins involved in lipoprotein metabolism will also have physiological consequences. For example, enzyme lecithin cholesterol acyltransferase (LCAT) deficiency results from a failure of LCAT to esterify cholesterol in plasma leading to unesterified plasma accumulating in tissue and premature atherosclerosis. In cholesterol ester transfer (CETP) deficiency,

individuals are unable to transfer triglyceride and cholesterol esters between lipoproteins. Low activity of lipoprotein lipase can lead to hypertriglyceridaemia. These disorders are known as primary hyperlipidaemias, however hyperlipidaemia may accompany other disorders and these are known as secondary hyperlipidaemias. Examples of secondary hyperlipidaemias are hypothyroidism, diabetes, obesity, proteinuria and renal failure. (Contois & Huang, 1997)

2.4.2 Lifestyle factors

More commonly, however, plasma lipids are influenced by lifestyle factors such as diet, cigarette smoking, alcohol consumption and exercise. High levels of dietary intake of saturated fats increase total LDL-C and VLDL cholesterol. High carbohydrate intake promotes VLDL synthesis by the liver as a consequence of an increase in free fatty acids providing the necessary substrate (Thompson, 1994). A diet deficient in fresh fruit and vegetables and therefore anti-oxidant vitamins, although not changing levels of circulating cholesterol, may lead to oxidation of LDL and a resultant atherogenicity of the LDL particle (LaRosa, 1998).

Cigarette smoking is known to induce dyslipoproteinaemia by reducing HDL levels (Gordon, 1999) as well as causing other damaging vascular changes (Celermajer et al., 1994). Alcohol ingestion has been associated with increases in both triglyceride and HDL. The former is seen to be an adverse effect, in contrast with increases in HDL which is considered beneficial, a 'negative risk factor' (1993). Exercise has been shown in epidemiological, observational and clinical studies to lower LDL-C and raise HDL-C levels. (Gordon, 1999; LaRosa, 1998)

2.5 Role of Lipids and Lipoproteins in Endothelial Dysfunction

The endothelial cell expresses LDL receptors and lipoprotein lipase. The latter releases free fatty acids into the circulation by hydrolysing triglyceride. Both elevated free fatty acids and triglycerides have been implicated in endothelial dysfunction (Lewis, Dart, and Chin-Dusting 1999; Sattar, Petrie, and Jaap 1998; Steinberg et al. 1997). Notably, LDL in its small dense form as seen in insulin resistance, truncal obesity, dyslipoproteinaemia and hypertension, is susceptible to oxidisation and

uptake of macrophages. Principally, oxidised LDL has been shown to diminish nitric oxide synthase expression (eNOS) as opposed to native LDL, and consequently NO release by the endothelium (Flavahan, 1992). Low-density lipoproteins may also enhance the synthesis of endothelin (Boulanger et al., 1992) and this may be of significance for patients with established CVD as endothelin may have a role in promoting plaque instability (Vogel et al., 1998). Furthermore, increased levels of Lp(a) may induce a pro-thrombotic situation and increase cardiovascular risk (Sorensen et al., 1994). The notion that hypercholesterolaemia or atherosclerosis-associated endothelial dysfunction results as a consequence of increased nitric oxide breakdown and not decreased production has also been proposed (Vogel et al., 1998).

2.6 Significance of Dyslipidaemia for Renal Disease

Renal disease is often accompanied by qualitative and quantitative alterations in lipoprotein metabolism. The character of the lipid abnormality is generally determined by the nature of the renal disorder and many glomerular diseases result in proteinuria which may provide an additional stimulus to the liver to synthesise lipoproteins (Moorhead et al., 1998). Dyslipoproteinaemia has been implicated as contributing to the progression of renal insufficiency contemporaneously with the development of atherosclerosis (Attman et al., 1999). Hyperlipidaemia alone is possibly not sufficient cause for renal injury however, it is the exposure of damaged glomerular cells that would normally be protected in the healthy kidney, to circulating lipids that induces injury (Moorhead et al., 1997).

Lipid deposits have been found in diseased kidneys. This is thought to happen after initial damage to the glomerular barrier that normally protects the mesangial cells from inflammation or infection. As a result of this large particles such as lipids infiltrate (Attman et al., 1999). Experimental evidence points to elevated apo-B containing lipoproteins, particularly triglyceride rich particles as opposed to cholesterol enriched, as being associated with the progression of renal disease (Attman, Samuelson, & Alaupovic, 1997; Keane et al., 1990). Suzuki et al. (1997) proposed that glomerular deposition of Lp(a) is involved in coagulation and fibrinolysis in the glomeruli of patients with a range of glomerular disorders.

Interestingly, the healthy kidney metabolises, catabolises and excretes HDL. Low-density lipoprotein oxidation in the kidney occurs via activity of the mesangial cell, which may produce oxygen radicals and glomerular macrophages, creating potential for inflammation. (Moorhead et al., 1998).

Glomerular responses to alterations in lipoprotein status are influenced by a number of factors, including glomerular disease, glomerular haemodynamics, and the nature of the lipoprotein particle as well as the duration of exposure (Moorhead et al., 1998). Recent research suggests a role for 3-hydroxy-methylglutaryl-coenzyme A reductase (HMG-CoA reductase) inhibitors or 'statins' in modulating intra-cellular signalling systems involved in the development of glomerulosclerosis (Oda & Keane, 1999).

2.7 Treatment of Dyslipidaemia

2.7.1 Diet and exercise

Coronary atherosclerosis is a disease begun in adolescence or early adulthood. Diet, exercise and weight control has a role to play, not only in CVD prevention, but also in the treatment of dyslipoproteinaemic individuals without clinical evidence of coronary heart disease. Indeed, debate exists as to whether recommendations for levels of LDL-cholesterol should be lowered below the current guidelines (LaRosa, 1998; Vogel, Corretti, & Plotnik, 1995).

The most important dietary factor contributing to hypercholesterolaemia, is the ingestion of large amounts of saturated or animal fats. Limiting dietary cholesterol and saturated fat in the diet will lower total cholesterol and HDL cholesterol and promote weight loss. Substituting monounsaturated fats such as olive oil for saturated fat lowers LDL-cholesterol with no reduction in HDL-cholesterol. However, as all fat contains 9 calories per gram of fat regardless of the source, weight loss will not be achieved (LaRosa, 1998). Consumption of fish and the reduction of coronary risk are strongly associated. Omega-3 fatty acids, a form of poly-unsaturated fats found in deep-water fish, are thought to effect lipoproteins quantitatively and qualitatively and decrease platelet adhesiveness (LaRosa, 1998). The weekly consumption of at least

three fish meals is recommended by the National Heart Foundation. Equally, a diet high in fibre, especially from oats or psyllium, has some cholesterol lowering effect (LaRosa, 1998). Plentiful consumption of fruit and vegetables and the moderate consumption of alcohol, known to be high in anti-oxidants, is associated with a lower risk of coronary disease. The value of anti-oxidant supplements such as Vitamins C and E, both in studies of endothelial function and coronary artery disease, have also been reported, however this issue remains contentious (Investigators, 2000; Jialal & Devaraj, 2000; Vogel et al., 1998).

Exercise has a number of favourable effects on coronary risk factors, including lowering blood pressure, enabling weight loss and promoting increased glucose tolerance. In relation to plasma lipids it has been shown to lower LDL-cholesterol and raise HDL-cholesterol levels. (LaRosa, 1998).

2.7.2 Pharmacotherapy

Diet and exercise are considered to be important preventative measures and may be the first line of treatment for individuals without evidence of CVD and with few risk factors. For patients with CVD and other accompanying risk factors, aggressive lipid lowering therapy is the current treatment (LaRosa, 1998). However, target serum lipid concentrations are dependent on individual cardiovascular risk profiles and recommendations vary between North America, Europe and Australia. Hyperlipidaemia, as well as increasing the risk of atherosclerosis, can result in tendon xanthomas and pancreatitis. Use of lipid modifying drugs in those people without CHD or hyperlipidaemia of genetic origin, is generally as an adjunct to dietary modification. Other measures such as weight control, exercise, restriction of alcohol intake and management of underlying disorders such as hypothyroidism and diabetes mellitus are considered. However, in those persons with established CHD even mild dyslipoproteinaemia is an indication for treatment (Thompson, 1994; Vogel et al., 1998).

Choice of drug is influenced by the nature of the lipoprotein abnormality, evidence of benefit from an individual drug, cost, and potential side effects and contraindications, for example, in women of childbearing age and children. The main classes of lipid lowering medication are statins, bile acid binding resins, fibrates, nicotinic acids and fish oils. (Thompson, 1994)

Statins are a class of drugs that are similar in structure to 3-hydroxy-methylglutaryl-coenzyme A reductase (HMG-CoA reductase), a cholesterol precursor. They inhibit the HMG-CoA reductase enzyme resulting in a reduction of serum LDL-C cholesterol and an 'up' regulation of the LDL receptor (Knopp, 1999). Statins have also been associated with improvements in endothelial function and regression of atheromatous plaque (O'Driscoll, Green, & Taylor, 1996; Simon et al. 1998; Vogel, Corretti, & Plotnik, 1995).

Bile acid binding resins are generally used as an adjunct to statin therapy when that therapy alone has not resulted in the desired cholesterol lowering effect. This class of drugs binds bile acids in the intestine facilitating faecal excretion. Bile acid synthesis is stimulated resulting in an increased demand for cholesterol by the liver. Consequently, hepatic LDL receptors are expressed by the hepatocytes, this is known as 'upregulation', resulting in increased removal of LDL-cholesterol from plasma (Thompson, 1994).

Fibrates are effective triglyceride lowering drugs that lower VLDL levels, may lower or increase LDL-cholesterol levels, but reduce total cholesterol. They may also increase HDL-cholesterol concentrations. These drugs work by stimulating lipolysis and increase biliary cholesterol secretion (Thompson, 1994).

Nicotinic acids have anti-lipolytic properties that result in a reduced supply of free fatty acids to the liver. Consequently, there is decreased synthesis of VLDL and a fall in LDL levels. High density lipoprotein concentrations are raised, possibly because of a decrease in HDL breakdown. This class of drugs has also been found to reduce Lp(a) when given in high doses. (Thompson, 1994).

Fish oils are oils derived from the bodies of fatty fish. These contain eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), also known as omega-3 fatty acids, which reduce VLDL levels, may decrease or increase LDL-cholesterol and may increase HDL-cholesterol levels. Fish oils act by decreasing the rate of hepatic synthesis of VLDL. Although some studies have shown that there is a concomitant rise in LDL apo-B. (Thompson, 1994)

Apart from these lipid lowering drugs, oral oestrogen therapy, when given to post-menopausal women, has been shown to lower serum LDL-C by approximately ten percent and raise serum HDL-C concentration. However, the attendant risk for such therapy is venous thrombosis and the risk of recurrent disease is not reduced (Knopp, 1999).

2.8 Renal Physiology

A primary function of the kidney is to maintain homeostasis by regulating the volume and composition of blood. It does this by controlling electrolyte metabolism, secreting hormones and by excreting and reabsorbing water. (Noble, 1998; Watts, 1996).

The functional unit of the kidney is the nephron of which there are approximately 2-3 million in both kidneys with the number declining with age (Watts, 1996). Each nephron consists of a partially convoluted tubule. The beginning of the tubule is an expanded chamber called Bowman's capsule, and a knot or tuft of entwined capillaries surrounds this with blood arriving at the tuft via an afferent arteriole and leaving via an efferent vessel. This is the glomerulus. The wall of the glomerulus is a selective filtration membrane. The glomerulus together with Bowman's capsule is known as the renal corpuscle. The first convolution after the glomerular tuft is the proximal, separated from the second convolution, the distal, by a loop known as the Loop of Henle. The distal tubule is connected to a collecting duct that receives filtrate from more than one nephron. Nephrons differ in structure according to their location in the kidney. Cortical nephrons, which account for 85% of all nephrons, are found in the superficial cortex of the kidney, the Loops of Henle of these nephrons do

not extend deeply into the medulla. Juxtamedullary nephrons comprise the remaining 15% with their loops extending into the renal pyramids; these are important for concentration and formation of urine. Within the nephron the tubular segments vary in structure and function. (Martini, 1995; Noble, 1998)

The kidneys receive about 1.25 litres of blood per minute via the renal arteries and the glomeruli produce an ultrafiltrate of plasma at a rate of approximately 80-125 ml/minute under resting conditions. However, statistics of renal function are conditional on gender, age and pathological changes within and without the kidney. (Noble, 1998; Watts, 1996)

In order to move from the plasma into the chamber of Bowman's capsule, the filtrate must pass firstly through the blood capillary endothelial layer. This layer is fenestrated allowing diffusion of solutes and plasma proteins but not blood cells. The second layer is the glomerular basement membrane (GBM) which is thicker than a typical basement membrane and surrounds the capillary layer. It restricts large plasma proteins but not diffusion of smaller proteins and solutes. A complex layer of epithelial cells called podocytes or foot processes form the last membrane and are attached to the GBM. The arrangement of the podocytes forms slits through which passes the glomerular filtrate. These specialised cells appear to have contractile properties allowing them to vary the slit width. In healthy individuals none of the larger plasma proteins would pass through this membrane apart from some albumin molecules usually smaller than 7 nm. Selectivity of the filter depends on the net negative charge of the glomerular structures as opposed to the charge carried by molecules such as albumin, which is negative at a physiological pH. The molecular size and shape of the molecules is also a factor influencing membrane selectivity. The structural integrity of the glomerulus depends on the intrinsic intercellular and inter-membrane adhesive properties involving, for example, proteins like immunoglobulins and proteoglycans such as heparin sulphate. All or any one of the filtration barriers can be altered by glomerular injury. (Martini, 1995; Noble, 1998; Wiseman, 1991)

An important component of the glomerulus is the mesangium whose cells and intracellular matrix can be found in the centrilobular region of the glomerular tuft (Noble, 1998). Mesangial cells have a role in regulating glomerular function by providing structural support for the capillaries, contracting and relaxing to alter capillary diameter and possibly mediating immunological mechanisms of renal disease (Martini, 1995; Noble, 1998). The juxtaglomerular apparatus is made up of the macula densa, which is a group of epithelial cells that forms part of the distal convoluted tubule and abut the juxtaglomerular smooth muscle fibres of the afferent arteriole. This group of cells forms an endocrine structure that secretes renin and erythropoietin. The importance of renin in the kidney is that it generates angiotensin II (Ang II), a powerful vasoconstrictor within the renal vasculature. Angiotensin-converting enzyme inhibitors or angiotensin receptor antagonists can block the effects of Angiotensin II. The consequence of administering these drugs is a reduction in renal vascular resistance and an increase in renal blood flow. (Martini, 1995; Noble, 1998)

Fluid movement between vascular and interstitial spaces is dependent on the opposition of hydrostatic pressure and colloid osmotic pressure. In the glomerulus, hydrostatic pressure is approximately 45-44 mmHg, this is the pressure of the blood pressing against the capillary walls which favours filtration, the pressure being slightly less at the efferent end of the vessel. Within Bowman's capsule the hydrostatic pressure is about 15-20 mmHg opposing filtration. Capillary colloid osmotic pressure is exerted by proteins in plasma and opposes filtration, however most of the total osmotic pressure of blood plasma is due to sodium and chloride ions. Because the concentrations of these are similar in the plasma and the filtrate, the osmotic gradient is not as a result of these substances. In effect and under normal circumstances, because water is filtered and protein stays in the capillary, becoming more concentrated, colloid osmotic pressure is greater at the efferent than in the afferent arteriole. The net filtration rate is 10 mmHg. It is important to appreciate the complexity of filtration in order to understand the implication of pathological processes when they occur. (Martini, 1995; Noble, 1998)

2.9 Nephrotic Syndrome

Proteinuria can occur as a consequence of various primary renal and non-renal conditions. Nephrotic syndrome (NS) is characterised by urinary protein loss exceeding 3.5g per 1.73m^2 of body surface area in 24 hours (Orth & Ritz, 1998). The significantly increased proteinuria characteristic of nephrotic syndrome results in hypoalbuminaemia, oedema, hypercoagulability, altered immunity, dyslipidaemia and lipiduria (Cameron 1998). Diabetes is the commonest cause of NS in Western societies but several primary glomerular diseases can cause nephrosis in patients without diabetes. The aetiology of NS varies according to age. (Orth & Ritz, 1998).

Minimal-change nephropathy (MCN) is responsible for 20% of nephrotic syndrome in adults and 90% in children and is more common in boys than girls (Mason & Pusey, 1998). In MCN light microscopic examination of the glomeruli appears normal but using electron microscopy the podocytes exhibit fusion (Orth & Ritz, 1998). Focal segmental glomerulosclerosis (FSGS) of idiopathic origin causes up to 15% of cases of nephrotic syndrome and is more likely to be associated with hypertension than MCN. Pathological features of FSGS include podocyte fusion and focal and segmental glomerular scarring. Juxtamedullary glomeruli are normally affected first (Mason & Pusey, 1998). In adults the most common cause of nephrotic syndrome is membranous glomerulonephritis. Membranoproliferative or mesangiocapillary glomerulonephritis is relatively uncommon (Orth & Ritz, 1998). Membranous glomerulonephritis may be associated with chronic infections, drugs, and in older patients, neoplasms. Most frequently it occurs without a specific disease association (Orth & Ritz, 1998).

Glomerular damage results in the pathological passage of plasma proteins normally retarded across the glomerular basement membrane. The defect in MCN is thought to be due to loss of charge selectivity, while the defect in membranous glomerulonephritis is due to a loss of size selectivity (Orth & Ritz, 1998). One of the sequelae of this large urinary protein loss is hypoalbuminaemia. Albumin is normally synthesised by the liver at a rate of 8-14 g/day and oncotic pressure is one control

stimulus for its production (Wiseman, 1991). Hypoalbuminaemia also appears to provide a major stimulus to the liver to synthesise excess lipoprotein leading to dyslipoproteinaemia, another usual manifestation of the nephrotic syndrome.

The hypercoagulability of nephrotic syndrome, due to increased quantity or activity of pro-coagulant function or loss of anti-thrombotic function can lead to hyperviscosity and thromboembolic complications. Immune dysfunction due to IgG deficiency and hypogammaglobulinaemia can lead to infections (Wiseman, 1991).

2.9.1 Clinical course and progress

The most common presenting feature of nephrotic syndrome is oedema. Oedema is thought to be caused by primary retention of sodium by the kidney rather than the accompanying decrease in serum albumin (Orth & Ritz, 1998). Initial diagnosis is confirmed by the finding of nephrotic range proteinuria and a low serum albumin less than 25 g/l. Renal biopsy is usually recommended for definitive diagnosis in adults because variation in the pathology of the disorder results in differing responses to treatment (Dr A. B Irish, personal communication, July, 2000).

Membranous nephropathy is associated with a spontaneous remission in 20-30% of patients. While 40% remain stable or achieve a partial remission, 30% develop progressive renal failure, predicted by male gender, age greater than 50 years, impaired renal function at diagnosis and heavy proteinuria. Treatment with both steroids and cytotoxic drugs has been demonstrated in controlled trials to achieve remission and preserve renal function in affected individuals. In adults and children with focal segmental glomerulosclerosis (FSGS), up to 40% will achieve remission in response to a prolonged course of steroids and in those patients 95% will achieve 5 year renal survival. Treatment of idiopathic membranoproliferative glomerulonephritis is more difficult and retrospective studies have not demonstrated a conclusive role for steroids or cytotoxic therapy in this clinical setting. (Mason & Pusey, 1998)

More recently, Probucol, a drug with potential to inhibit oxidative changes of LDL-C was found to reduce urinary protein excretion in 15 patients with membranous nephropathy and nephrotic syndrome (Haas, Kerjaschki, & Mayer, 1999). Treatment of nephrotic syndrome of any cause, with an angiotensin converting enzyme (ACE) inhibitor may reduce urinary protein excretion and lower accompanying plasma cholesterol levels (Keilani, Schlueter, Levin, & Batlle, 1993; Vega, Toto, & Grundy, 1995).

Practical management of nephrotic syndrome also involves symptom control. For example, the treatment of oedema, hypertension, and hyperlipidaemia. The accompanying lifestyle problems that may complicate nephrotic syndrome, include obesity and smoking (Wiseman, 1991).

2.9.2 Nursing management

Nursing management of the patient with nephrotic syndrome is multifaceted. Dietary education in tandem with a dietician is appropriate. In the dyslipoproteinaemic patient dietary cholesterol restrictions are necessary. However, other changes may be indicated. For example, alterations in protein, sodium and potassium intakes, and perhaps supplemental vitamins and iron may be needed (Wiseman, 1991). Monitoring of hydration and oedema status by regular weighing is warranted. Immunologic dysfunction caused by nephrotic syndrome also dictates nursing assessment of skin, wound and secretions for signs of infection. Corticosteroid therapy can also cause skin to become fragile and may mask symptoms of infection (Wiseman, 1991). Monitoring of other medications taken by these patients will allow early detection of side effects. For example, statins may cause myalgia, and ACE inhibitors may cause an irritating cough and hyperkalaemia. Patients who are newly diagnosed with nephrotic syndrome may be quite shocked and concerned about being diagnosed with kidney damage and find it difficult to process information given to them early in their illness. The role of the nurse involves providing emotional and informational support to the patient and family beyond the acute stage of the illness (Wiseman, 1991).

2.9.3 Dyslipidaemia in the nephrotic syndrome

The most frequently reported lipid abnormalities seen in the nephrotic syndrome are increased serum concentrations of cholesterol and triglyceride rich apo-B containing lipoproteins (Kayson, 1991; Warwick & Packard, 1993; Warwick et al., 1991). These abnormalities occur primarily as a result of increased synthesis of these lipoproteins by the liver in response to hypoalbuminaemia and albuminuria (Kayson & De Sain-van der Velden, 1999; Warwick et al., 1991). There is evidence to suggest, however, that the stimulus for LDL-C synthesis may not be related to albumin (De Sain-Van Der Velden et al., 1998; Kayson & De Sain-van der Velden, 1999) and that an alternative pathway exists. Decreased clearance of lipoproteins also contributes to the hyperlipidaemia in nephrotic syndrome and this is thought to be partly due to reduced expression of hepatic and lipoprotein lipases (Kayson & De Sain-van der Velden, 1999; Liang & Vaziri, 1997). The enzyme, LCAT, may be dysfunctional in the nephrotic syndrome leading to a failure of the HDL particle to mature affecting catabolism of VLDL cholesterol and chylomicrons (Kayson & De Sain-van der Velden, 1999). The level of HDL may be high, normal or low in these patients and the relative concentrations of sub-groups of HDL, that is HDL2 and HDL3 may also be disrupted (Keane, 1994; Cameron, 1998). Cholesterol ester transfer protein (CETP) activity is also increased in nephrotic syndrome causing increased exchange of cholesterol esters from HDL for triglyceride from remnant VLDL yielding LDL-C (Braschi et al., 1997; Kayson & De Sain-van der Velden, 1999). Furthermore, the relative proportion of lipoprotein-bound nonesterified fatty acids (NEFAs) may be increased in nephrotic syndrome (Braschi et al., 1997).

Increased levels of circulating lipoprotein a (Lp(a)), recognised as an independent risk factor for the development of atherosclerosis, have been detected in patients with nephrosis (Keane, 1994; Sorensen et al., 1994).The severity of the nephrotic syndrome in terms of the degree of glomerular damage and resultant albuminuria, may influence the lipid profile of these patients (Warwick et al., 1991). For example, the level of hypertriglyceridaemia is directly related to the severity of proteinuria (Keane, 1994). Another theory accounting for the dyslipoproteinaemia seen in

nephrosis is that a lipo-regulatory substance, such as apo-C-11, is excreted in the urine (Kayson, 1991; Vega et al., 1995).

It is perhaps useful to keep in mind that many of the lipid studies in this area have been performed in animal models. Whether they can be extrapolated to the unique human lipoprotein profile is uncertain (Warwick & Packard, 1993).

A study by Vega et al. (1995), described data indicating that metabolism of low-density lipoproteins is different between two forms of nephrotic dyslipoproteinaemia, hypercholesterolaemia and combined hyperlipidaemia, which is an elevation of both cholesterol and triglyceride levels. This was an important observation as it implies that the kinetic activity of the two types is different, and when studying elevated dyslipidaemia in the nephrotic syndrome these differences should be considered.

Importantly, the atherogenicity of the LDL particle in heavy proteinuria has also been researched. Small dense LDL is considered to be more atherogenic than larger less dense particles. In a study of 12 proteinuric patients (> 2.5 g/24 hr) and 23 matched controls, concentrations of small dense LDL particles were found to be greater in the proteinuric group versus controls (Deighan et al., 1998). This observation suggests that heavy urinary protein loss may contribute to the atherogenic profile seen in individuals with nephrotic syndrome.

2.10 Conclusion

It is evident that patients with nephrotic syndrome have many of the risk factors that have been associated with the development of cardiovascular disease. It is also apparent that endothelial dysfunction is an early stage of atherosclerosis. Less clear are the relative contributions of these factors to endothelial dysfunction in the nephrotic syndrome and the overall risk of accelerated atherosclerosis in this group of patients.

CHAPTER 3 REVIEW OF ENDOTHELIAL FUNCTION

3.1 Introduction

This chapter will present an overview of research conducted, *in vivo*, in the area of endothelial function with particular attention given to those studies related to proteinuric renal disease. To highlight differences between the techniques used to study conduit vessels and the forearm microcirculation, and the variability of responses between these vascular beds, the topics are discussed under separate headings.

3.2 Measurement of Endothelial Function

3.2.1 Brachial artery ultrasonography

Assessing endothelial dysfunction involves the measurement of arterial diameter in response to an increase in shear stress, which results in endothelium dependent vasodilatation, a phenomenon attributed primarily to the action of nitric oxide (Joannides et al., 1995; Playford & Watts, 1998; Shimokawa, 1999). In 1992, the first non-invasive, *in-vivo* tests of endothelial function were carried out on the brachial and femoral arteries, conduit vessels, using high-resolution ultrasound (Celermajer 1997; Celermajer et al. 1992; Sorensen et al. 1994). During this procedure a pneumatic tourniquet placed around the forearm is inflated to supra-systolic pressure for five minutes resulting in forearm ischaemia. Release of the cuff produces an increase in shear stress in the brachial artery inducing reactive hyperaemia or flow-mediated dilatation. The maximum dilatation of the artery is measurable. The technique will be discussed in more detail in Chapter Four.

Endothelium-independent vasodilatation of arterial diameter can be measured after the sub-lingual administration of glyceryl trinitrate. The lipophilic NO produced by the endothelium is able to diffuse through the cell membrane of smooth muscle cells to mediate vascular relaxation (Shimokawa, 1999). The technique has been used to study vascular function *in vivo* with the aim of assessing cardiovascular risk in various high-risk groups.

The findings of Celermajer et al. (1992) generated much interest. They studied 100 subjects of whom there were 50 controls (aged 8-57 years), without vascular risk factors, 20 cigarette smokers (aged 17-62 years), 10 children with familial hypercholesterolaemia (FH) (aged 8-16 years) and 20 patients with coronary artery disease (CVD). Their results, and later those of Sorensen et al. (Sorensen et al., 1994), indicated that flow-mediated dilatation was reduced or absent in comparison to control subjects in smokers, children with FH and adults with CAD. The technique appeared to have potential as a screening device for individuals at risk of CVD, and appeared to be a good predictor of coronary endothelial dysfunction (Anderson et al., 1995).

Another important finding of Celermajer et al. (1992) was in relation to the interpretation of ultrasound measurements of brachial artery responses to increases in flow. They observed that changes in vessel diameter were inversely related to vessel size. For example, the greater the increase in flow-mediated dilatation the smaller the resting diameter of the vessel. As the brachial artery response is expressed, conventionally, as percent increase in vessel diameter from resting diameter, statistical analysis must allow for this phenomenon, especially when comparing groups or evaluating responses to interventions. (Celermajer et al., 1992).

Anderson et al. (1995) confirmed this after they investigated the sensitivity and specificity of the technique. They studied patients (N = 50) with coronary artery disease. Two techniques were used in their study. Firstly they examined coronary vasomotor response to serial intra-coronary infusions of the endothelium dependent agonist acetycholine. Secondly, they studied changes in diameter of the brachial artery in response to reactive hyperaemia using ultrasonography. Using stepwise multivariate analysis, they reported that the strongest predictors of reduced brachial dilator responses were baseline brachial artery diameter, coronary endothelial dysfunction as assessed by the first test, presence of CAD and cigarette smoking (R^2 = .47, p = <.01). The positive predictive value of abnormal brachial dilatation, defined as less than 3 percent dilatation, was found to be 95 percent. Of further interest was their finding that the sensitivity of detecting coronary endothelial dysfunction using ultrasonography was 49 percent and the specificity was 92 percent.

They concluded that endothelial dysfunction is a generalised process that may occur in arteries not usually manifesting clinical atherosclerosis. The authors observed that brachial artery ultrasonography is perhaps better suited to the study of small groups matched for artery diameter size than individual patients because of the relationship of dilatation to baseline diameter (Anderson et al., 1995).

Lieberman et al. (1996) elucidated this further in a study of brachial artery dilatation using ultrasonography. They measured brachial artery diameter in the same subjects on two occasions. On the first occasion the measurements were made above the cubital fossa and the mean diameter was 3.9 mm. On the second visit the measurement was made below the cubital fossa and the mean diameter was 2.4 mm. Dilatation was found to be 6.2% vs 21.1% respectively (Lieberman et al., 1996). In the same study these researchers also examined responses of the brachial artery, using ultrasound, to acetylcholine and sodium nitroprusside before and after the administration of L-NMMA. They compared a group of healthy men ($\underline{n}$ = 11), mean age 34 yrs ± 1, to men with CAD ($\underline{n}$ = 14), mean age 36 yrs ± 1. They found that post-ischaemic dilatation was significantly reduced compared with controls; L-NMMA inhibited flow-mediated dilatation (FMD) and FMD in response to acetycholine, but did not affect response to nitroprusside, the responses to which were similar between groups. The authors concluded that flow induced dilatation is endothelium dependent and mediated by nitric oxide and that this mechanism is impaired in young men with CAD (Lieberman et al., 1996).

In concordance with these findings, Lundman, et al. (1997) in a study of FMD of the brachial artery, found that transient triglyceridaemia decreased vascular reactivity in seven young, normolipidaemic men with no apparent risk factors for coronary heart disease. In 1995, Vogel et al. (1995), in the wake of research showing abnormal FMD in patients with hypercholesterolaemia, studied flow-mediated brachial vasoactivity in seven healthy normocholesterolaemic men to determine whether lowering cholesterol levels below the then current guidelines would result in a change in vasoactivity. Subjects were studied on six occasions: at baseline; at two, four and twelve weeks on simvastatin; and on two follow-up visits off cholesterol lowering therapy. Flow-mediated dilatation increased from baseline when on

simvastatin and returned to baseline after 12 weeks off medication. Statistically, vasoactivity was found to correlate with cholesterol levels (r = -.47, p = 0.004) (Lieberman et al., 1996). These findings were reproduced in a slightly larger study (N =10) that included two women using intra-arterial cannulation and venous occlusion plethysmography (O'Driscoll, Green, & Taylor, 1996). Other research has also identified improvements in endothelial function after lipid lowering interventions (Simon, Sullivan, Simons, & Celermajer, 1998; Treasure et al., 1995; Vogel et al., 1995).

Later researchers wrote of the gender differences that account for variations in FMD between men and women, as well as the change in vasoactivity that accompanies menopause (Perregaux et al., 1999). These differences are thought to be as a consequence of the modulating effect of oestrogen on the expression of nitric oxide synthase (Drexler, 1997). Impairment of flow-mediated dilatation has also been associated with low birthweight (Leeson et al., 1997), hyperhomocysteinaema (Tawakol et al., 1997) and obesity, particularly the accumulation of intra-abdominal visceral fat (Hashimoto et al., 1998).

Kari et al. (1997) measured endothelial function in 23 children with chronic renal failure (CRF) and no known cardiac risk factors matched with 23 healthy controls using brachial artery ultrasonography. Endothelial physiology, specifically nitric oxide metabolites and endogenous NO synthetase (NOS) were also studied. Their findings that brachial artery response to an increase in blood flow was reduced in children with chronic renal failure compared with controls (no difference was found in responses to GTN) was interesting as the children had normal serum cholesterol, triglyceride, HDL and LDL levels. Twenty-four hour urinary protein excretion and serum albumin levels were also within the normal range. No correlation between percent FMD and any of the lipid sub-fractions was found. However, the CRF group expressed antibodies to oxidised LDL suggesting a contribution of oxidative stress not found in the controls. Furthermore, endogenous NOS inhibitors were higher in patients with chronic renal failure (Kari et al., 1997).

3.2.2 Venous occlusion plethysmography.

Volume plethysmography as a technique for studying vasomotor response, has been used for over 50 years. Early methods involved encasing a limb or part of, in a rigid jacket with fluid filling the space between the limb and the jacket. Volume changes in the limb would correspond with volume displacement of the fluid in the space between the jacket and the limb. This method proved cumbersome and invalid for measuring vasomotor changes over time (Whitney, 1953). This early research led to the development of strain gauge plethysmography. Although the technique has been refined with the advent of the computerisation, the principles underlying the method remain essentially unchanged.

The strain gauges currently in use are made of mercury-in-silastic and are placed around the forearms distal to occlusive cuffs placed over the upper arms. The cuffs occlude venous return whilst allowing arterial inflow resulting in swelling of the forearm and producing stretch of the strain gauges. It is this stretch that reflects changes in smooth muscle tone in small arteries and arterioles provided arterial blood pressure remains constant during the measurement period (Benjamin et al., 1994).

The advantage of forearm venous occlusion plethysmography is that it allows the *in vivo* study of vascular physiology and pathophysiology under the influence of physiological mediators which may be local or circulating (Benjamin et al., 1994). This method will be described in Chapter Four.

Nitric oxide, prostacyclin and bradykinin mediate dilatation of conduit vessels, such as the brachial artery. The contribution of these substances to vascular responses in the microcirculation, for example the forearm microvasculature, has been of research interest (Benjamin et al., 1994; Gilligan et al., 1994; Meredith et al., 1996). Drugs known to have vaso-active effects can be infused via an intra-arterial cannula into the brachial artery where they produce a local effect on blood flow in the forearm without affecting systemic blood pressure (Benjamin et al., 1994). Vaso-active drugs used in these studies include the nitric oxide synthase inhibitor NG monomethyl-L-arginine (L-NMMA), a specific antagonist to EDRF/NO production by the

endothelium and acetylcholine and bradykinin seen to be endothelium-dependent agonists, and sodium nitroprusside an endothelium-independent vasodilator (Benjamin et al., 1994; Meredith et al., 1996). The reason for using different agonists in the same study is that they use different signal transduction pathways to induce nitric oxide production by the endothelial cell (Gilligan et al., 1994) thus allowing study of endothelium-dependent and independent responses.

Reactive hyperaemia or flow debt repayment is the vasodilation and transient rise in blood flow that occurs as a consequence of ischaemia caused by arterial occlusion (Engelke, Halliwell, Proctor, Dietzt, & Joyner, 1996). The rise in blood flow is confined to that area and experimental evidence would suggest that the phenomenon does not depend on the area having intact innervation (Engelke et al., 1996). This supports the theory of a mechanism of endothelium-dependent vasodilation separate from that of endothelium independent vasodilation, primarily a response of smooth muscle cells. However, the mechanisms responsible for reactive hyperaemia appear to involve both myogenic and vaso-active substances (Meredith et al., 1996).

Of particular relevance to the current project is research carried out by Stroes et al. (1995a; 1995b) in two separate studies. Based on the knowledge that proteinuria is associated with increased cardiovascular morbidity and that nitric oxide is protective of vascular endothelial function, they tested the hypothesis that proteinuria is associated with a defect in nitric oxide dependent vasodilatation (Stroes et al., 1995a). Endothelial function was compared in three groups. Included were patients with nephrotic range proteinuria (> 3.5 g/24 hrs) and normal renal function ($\underline{n}$ = 9), patients with low range proteinuria (< 1.0 g/24 hrs), with normal renal function and active glomerulonephritis ($\underline{n}$ = 8), and healthy volunteers ($\underline{n}$ = 10). After cannulating the brachial artery, they compared forearm vascular responses between groups to an infusion of L-NMMA to inhibit, initially, basal nitric oxide inhibition, followed by infusions of serotonin and nitroprusside. Inhibition of endogenous NO by L-NMMA was similar between groups and was explained by the authors as arising from the fact that basal nitric oxide release is influenced by more than one regulatory mechanism, for example, shear stress and other receptor dependent pathways. Serotonin induced vasodilatation was impaired in nephrotic patients compared with the control groups.

Administration of nitroprusside to elicit an endothelium independent response was, however, similar between groups. The groups were matched for age, gender, smoking status, body mass index (BMI), and forearm volume. The two renal groups had higher blood pressure than the healthy group. The nephrotic patients had dyslipoproteinaemia and higher total phospholipids as well as hypoalbuminaemia compared with the two other groups. The authors observed that the vascular responses of the nephrotic patients were similar to those of hyperlipidaemic patients and in a follow up study they presented data that supported the view that impaired NO mediated vasodilatation was a consequence of hyperlipidaemia. A detrimental role was also suggested for lysophosphatidylcholine (lyso-PC), a molecule normally bound to albumin but in these patients attached to LDL. (Stroes et al., 1995a; Stroes et al., 1995b)

Stroes et al. (1995b) also noted that the impaired serotonin response observed in another study of hyperlipidaemic patients (Gilligan et al., 1994) was improved by the administration of L-arginine, a substrate for nitric oxide synthase. They found that this intervention had no effect in their study group of patients with nephrosis. They surmised that the mechanism for endothelial dysfunction in nephrosis may differ from that observed in hyperlipidaemia in the absence of proteinuria and hypoalbuminaemia. Alternatively the lipoprotein composition seen in nephrosis may differ in some respect. Of particular concern, were the cardiovascular implications of their findings in what was a relatively young group of individuals, and they recommended further work in this area (Joles, Stroes, & Rabelink, 1999; Stroes et al., 1995a; Stroes et al., 1995b).

More recently in a case-control study, associations were found between plasma levels of endothelin (ET) and forearm blood flow responses after reactive hyperaemia of the forearm microcirculation and common carotid intima media thickness (CCA-IMT) in 76 patients with end-stage renal disease (ESRD) (Demuth et al., 1998). Correlations presented were, ET and flow debt repayment, ($r = -.36$, $p = <.01$), ET and maximal blood flow ($r = -.49$, $p = <.001$), ET and CCA-IMT ($r = .41$, $p <.001$).

Using a similar method for measuring reactive hyperaemia, Pannier et al. (2000) presented concordant data. In another cross-sectional study of 60 patients with ESRD and 34 controls matched for age, gender and blood pressure they demonstrated statistically significant associations between flow debt repayment (FDR), and carotid intima media thickness (r = -.47, p = <.001) and FDR and serum albumin (r = .55, p = <.01) (Pannier et al., 2000). These studies were important because they demonstrated that endothelial dysfunction may have a role in influencing morphological and functional changes within the cardiovascular system of patients with end stage renal disease (Pannier et al., 2000).

3.3 Conclusion

In summary, endothelial dysfunction in the resistance arteries, as measured by venous occlusion plethysmography, has been associated with hypercholesterolaemia, hypertension and proteinuria and end stage renal disease (Chowienczyk et al., 1992; Demuth et al., 1998; Ferro & Webb 1997; Gilligan et al., 1994; Pannier et al., 2000; Stroes et al., 1995a; Stroes et al., 1995b). The method is, however, only a surrogate measure of coronary endothelial function (Celermajer, 1997). Intra-arterial studies with administration of vaso-active agents are invasive and not suitable for use in children and not optimal in some patient groups where vascular access is of issue, such as those with renal disease or the human immunodeficiency virus. Clinical assessment of coronary artery and microvasculature endothelial function is possible using quantitative angiography and the administration of small vessel vasodilator substances (Celermajer, 1997). These techniques, particularly for serial studies, are not considered suitable for assessing those individuals who are at high risk of developing atherosclerosis but have no clinical signs and symptoms (Celermajer, 1997).

Endothelial dysfunction has been described in the presence of classical cardiovascular risk factors such as dyslipidaemia and, less well researched, proteinuria (Joles et al., 1999). Nephrotic range proteinuria is associated with an increased risk of coronary artery disease and between a five to six fold risk of myocardial infarction (Ordonez et al., 1993). Research in this area also suggests

dyslipidaemia, by promoting glomerulosclerosis, may increase the rate of progression of renal injury (Haas et al., 1999). The non-invasive vascular techniques of brachial artery ultrasound and plethysmography, utilising reactive hyperaemia, appear suitable for assessing endothelial function in dyslipidaemic, renal patients. Furthermore, these tests may be potentially useful for serial studies of responses to therapy in these patients.

CHAPTER 4 METHODS

4.1 Introduction

This chapter will provide details of the subjects included in the study, the study design and clinical and laboratory methods. The procedures for the vascular function tests are also described. Further information related to procedures and the data collection forms is provided in the appendices.

4.2 Subjects

The study sample comprised 45 participants who were divided into three groups. The patient group was fifteen individuals with nephrotic syndrome (NP) referred to the study from the Nephrology Clinic at Royal Perth Hospital and the private rooms of interested nephrologists. The two control groups were fifteen individuals with primary hyperlipidaemia (HL) referred from the Lipid Clinic of the same hospital and fifteen healthy, normolipidaemic (NC) people recruited by advertisement or by hearing of the study by word of mouth. The three groups were matched for age, gender and body mass index. The nephrotic and hyperlipidaemic groups were selected to have similar serum total cholesterol (TC), triglycerides (Tg) and low-density lipoprotein cholesterol (LDL-C) levels. In this study, nephrosis referred to a primary glomerulopathy resulting in classical nephrotic syndrome or an associated residual nephrotic range proteinuria. Primary hyperlipidaemia was defined as hyperlipidaemia in the absence of a recognised precipitating cause with or without classical clinical stigmata and a family history of coronary disease. All participants attended a screening visit and had a full clinical examination, ECG, urinalysis and blood tests to screen for liver and renal abnormalities, hypothyroidism, diabetes mellitus and anaemia. Patients and hyperlipidaemic controls were studied off lipid lowering drugs (for at least 4 weeks) and aspirin. Patients were not excluded for taking angiotensin converting enzyme (ACE) inhibitors, diuretics, or a stable dose of other anti-hypertensive drugs, since these treatments constitute best clinical practice and patients were studied in an outpatient setting. Participants were also given advice related to consumption of dietary cholesterol and encouraged to restrict intake of

saturated fats. The blood flow studies were scheduled to take place 4-6 weeks after a screening visit attended by each participant.

4.3 Study Design

A cross-sectional design was chosen for this study in order to ascertain whether, in patients with nephrosis, endothelial function was impaired to a degree seen in patients with primary hyperlipidaemia. A healthy control group was necessary to establish a normal range for endothelial function as measured by brachial artery ultrasonography and venous occlusion plethysmography.

4.4 Clinical and Laboratory Data

On the study day, blood pressure and heart rate were measured using a Dinamap (Critikon Ltd, Tampa, Florida, USA). Urinary protein excretion (g/L) was assessed in the nephrotic patients by a 24-hour collection and in the two control groups by dipstick urinalysis at the screening visit and an early morning specimen for urinary protein:creatinine ratio on the study day. Height was measured without shoes and weight in light clothes. Body mass index (BMI) was calculated as kg/m^2. Venepuncture was carried out after a 12-hour fast, with the subject in the recumbent position and with minimal venous stasis to maximise precision of lipid assays. Serum cholesterol (TC) and triglyceride (Tg) were measured by enzymatic, colorimetric methods. High-density lipoprotein cholesterol (HDL-C) was estimated after precipitation of apolipoprotein B-100 (apo-B 100) containing lipoproteins with heparin/ manganese. Serum low-density lipoprotein cholesterol (LDL-C) was calculated by the Freidewald equation, except in subjects with Tg greater than 4.5mmol/L in which it was estimated directly by an enzymatic colorimetric assay using reagents from Boehringer Mannheim (LDL-C, Boehringer Mannheim GmBH, Mannheim, Germany) on a Hitachi 917 analyser. Homocysteine was measured by a fluorescence polarisation immunoassay (Axis Biochemicals ASA, Oslo, Norway).

Serum glucose was assayed by the hexokinase method, and serum and urinary creatinine by the modified Jaffé reaction. Glomerular filtration rate was calculated by the Cockcroft and Gault formula: {(140-age) * weight (in kg) / creatinine} * K, where K is 1.0 for men and 0.85 for women (Cockcroft & Gault, 1976). Serum

albumin and urinary protein were measured in standard auto-analysers (Hitachi 917 Biochemical analyser, Hitachi Limited, Tokyo, Japan). Serum insulin levels were measured by a solid phase two-site chemiluminescent enzyme-labelled immunometric assay (Immulite, DPC, Los Angeles, CA). Insulin resistance was calculated by the HOMA Model: insulin resistance = $\text{insulin}/(22.5e^{-\ln \text{glucose}})$ (Matthews et al., 1985).

Non-esterified free fatty acids (NEFAs) were determined by a colorimetric method using a commercial kit (Boehringer Mannheim). Lipoprotein (a) (Lp(a)) and apo-B 100 were assayed by immunonephelometric methods. LDL particle diameter was determined using non-denaturing gel electropheresis (O'Neal et al., 1998). Fibringen was assayed by the Clauss method (Clauss, 1957). Analysis of insulin, LDL particle size, apo B 100, Lp(a), plasma NEFAs, and fibrinogen were from stored plasma and serum sampled on the study day. See Appendices B and C for laboratory methods, precision of assays and protocol for sample storage.

Vascular function studies were carried out in the morning after a 12 hour fast from food and caffeine containing beverages, and after resting in the supine position for at least fifteen minutes. Satisfactory brachial artery ultrasound scans were obtained from all patients. Suitable plethysmographic recordings, without movement artefact, were obtained from 14 of the patients with nephrosis, 13 of the hyperlipidaemic and 14 of the normolipidaemic controls. Smoking was not allowed on the day of the tests.

Table 4.1 Screening visit

Ten – Twelve hour fast from food and caffeine containing fluids - tap water allowed.
Medical examination (if not already done in clinic).
ECG, if not previously done in last three months.
Assessment of urinary protein → 24-hr collection for patients with nephrosis.
→ Dipstick urinalysis for all potential control subjects.
Venous blood specimens to RPH Core Laboratory: Fasting cholesterol, triglyceride, LDL-C, HDL-C, urea and electrolytes, FBC, albumin, ALT, AST, TSH.
Dietary advice.

Table 4.2 Study day (six weeks after screening test)

Ten – Twelve hour fast from food and caffeine containing fluids, tap water allowed.
Assessment of urinary protein → 24-hr collection for patients with nephrosis.
→ First early morning urine sample for control subjects.
Venous blood specimens to RPH Core Laboratory: Fasting cholesterol, triglyceride, LDL-C, HDL-C, urea and electrolytes, albumin.
Venous blood samples for storage
Brachial artery ultrasound
Venous occlusion plethysmography

4.4.1 Brachial artery ultrasonography

During the ultrasound procedure, subjects rested supine in a quiet, temperature controlled (24 degrees celsius) room. The left arm was immobilised in a foam cast and supported comfortably in extension. A 12-mHz transducer connected to an Acuson Aspen™ System (Acuson Pty Ltd, Mountain View, CA 94039, USA) was employed for ultrasound, but a few early scans were carried out using a 7.5 mHz linear array, high resolution vascular transducer connected to a Toshiba SSA-270A ultrasound system (Yoshiba Corp, Tokyo, Japan). Continuous ECG monitoring was performed in all studies. The transducer was placed 5-10 cm proximal to the anti-cubital crease and fixed in position by a stereotactic clamp. After good images were obtained, the edge-to-lumen interface was further optimised using depth and gain controls, and an edge enhancement function. Images were recorded on s-VHS videotape (Sony MQSE 180) for retrospective analysis. A pneumatic tourniquet was placed around the left forearm, and after recording the baseline images for two minutes, the cuff was inflated to 200 mmHg for 5 minutes. Release of the cuff induced forearm reactive hyperaemia and images were recorded from 30 seconds before to 4 minutes after cuff release. A second resting scan was obtained at least 10 minutes after cuff deflation to ensure that the brachial artery diameter returned to the basal level. Four hundred µg of glyceryl trinitrate (GTN) was administered sublingually by spray and the artery was subsequently scanned for a further 5 minutes to assess glyceryl trinitrate mediated vaso-dilatation (GTNMD) (Dr D. A. Playford, personal communication, July 2000).

Analysis of post-ischaemic flow and glyceryl trinitrate mediated dilatation of the brachial artery was carried out using semi-automated edge detection software recently developed and validated within the University Department of Medicine at Royal Perth Hospital. During s-VHS playback into a digital frame-grabber, images were digitised into a personal computer. A rectangular region of interest (ROI) was drawn round the most representative section of the artery. A second ROI was selected around the ECG tracings, and a third ROI for calibration of the diameter measurements. The computerised edge detection and wall tracking software system then automatically determined brachial artery diameter at end diastole, each frame corresponding to up to 300 individual diameter measurements. A third order polynomial function was applied to the curve of serial, end diastolic diameter measurements, to drive maximal FMD and GTNMD of the brachial artery. Responses were calculated as percent change in brachial artery diameter from baseline. The reliability of the measurement was addressed by using two experienced observers to carry out all analyses. The analytical (intra-observer) variation of the computerised technique is of the order of 6% compared with at least 35% using more conventional visual estimations employing callipers. (Dr D. A. Playford, personal communication, July 2000). See Appendix F for examples of videotaped images obtained during brachial artery ultrasound examination and analysis of brachial artery responses.

4.4.2 Strain gauge plethysmography

Although the development of strain gauge plethysmography and the advent of computerisation has refined the technique of measuring volume changes in limbs, the principles underlying the method remain essentially unchanged. Whitney (1953, p. 13), stated that the reliability of the strain gauge method for measuring changes in limb volume "depends, first, on the accuracy with which girth changes can be recorded; and, secondly, on the validity of the assumed relationship between girth and volume". These statements remain consistent with currently held concepts of reliability and validity (Portney & Watkins, 1993a). Strain gauge plethysmography estimates the total flow in the forearm from the wrist cuff to the upper arm collecting

cuff and is expressed most commonly as ml/100ml/forearm /minute (Benjamin et al.,
1994).

As temperature and anxiety are known to influence vasomotor responses subjects
were studied after ten minutes rest in a supine position in a quiet room with the
temperature controlled at approximately 24 degrees Celsius (Petrie et al., 1998).
Measurement of forearm blood flow (FABF) was carried out using venous occlusion
plethysmography with mercury in silastic strain gauges calibrated electronically and
placed 5cm below the ante cubital crease of the left arm (Hokansson, EC4
Plethysmograph, Bellevue Washington, USA connected to Maclab/4e,
ADInstruments, Sydney, Australia). Strain gauges were chosen to be 2 cm smaller
than the circumference of the widest part of the forearm. To isolate the forearm blood
flow from the hand, blood flow in the hand being primarily through the skin
(Benjamin et al., 1994), a wrist cuff was inflated to 200 mmHg during measurement
periods. The upper arm cuff was inflated rapidly, using a commercially available air
source, to 40 mmHg to impede venous outflow but allow arterial inflow, causing
swelling of the forearm and stretch of the strain gauge. The cuff was set to inflate for
10 seconds and deflate for 7 seconds. Stretch of the strain gauge causes a change in
the voltage of the gauge and the Maclab software records this deflection.

Subjects were acclimatised to the cuff inflation and deflation, prior to the
measurement of forearm blood flow. This was obtained by recording a mean of three
representative curves and is expressed as ml/100 ml of forearm/min. Forearm blood
flow was calculated in Maclab by selection of the steepest gradient of each curve or
deflection, assuming change in flow to be directly proportional to change in voltage
with respect to time. Flow is considered to be an estimate of the contraction of the
vascular smooth muscle providing that blood pressure does not alter significantly
throughout the procedure (Benjamin et al., 1994). Blood pressure was measured
before and after the procedure using a semiautomatic sphygmomanometer (Dinamap
Critikon Ltd, Tampa, Florida, USA).

To initiate the ischaemic stimulus, a sphygmomanometer cuff was placed over the
left upper arm and inflated to 40 mmHg above systolic pressure to induce forearm

ischaemia for four minutes. After releasing the cuff, blood flow was measured continuously for four minutes. The initial peak after the ischaemic period was defined as the hyperaemic response or maximal blood flow expressed as ml/100 ml of forearm/minute. These absolute values were entered into a Prism (Prism, Graph Pad Software, Inc) spreadsheet and non-linear regression analysis was carried out. Flow debt repayment, defined as the area under the blood flow curve, expressed as ml/100 ml of forearm, was derived using this method. The formula used is span/(k * 60) where span is an estimate of the absolute peak flow, k is the rate of decay of the curve and 60 is time in seconds. Forearm vascular resistance (FVR) was measured using the formula: mean arterial pressure/FABF. The formula gives an approximation of the contractile state of the small arteries and arterioles (Benjamin et al., 1994). Maximum forearm vascular resistance (MAX FVR) was also measured using the formula; FABF/ mean arterial pressure. Forearm length (l) and forearm circumference (c) were measured in order to calculate forearm volume (c^2 * l)/4π). The intra-observer reliability of this method is represented by a coefficient of variation of less than 5% (Watts, Herrmann, & Riches, 2000).

4.5 Statistical Analysis

Data analysis will be performed using SPSS, version 9.0. The alpha level for significance for all statistical analyses will be .05. Frequencies (f), means (M), standard deviations (SD) or standard error of the mean (SE) will be computed on the demographic and clinical characteristics of the three groups. Normality of distribution will be tested using the Shapiro-Wilk statistic, a significance level for testing normality in sample sizes less than 50 (Coakes & Steed, 1999). If the significance level is less than .05 on any variable a natural logarithmic transformation will be used in an attempt to normalise distribution.

Comparison of means will be by analysis of variance for independent samples (ANOVA) of three or more groups. The assumptions of concern for ANOVA are population normality within each of the groups, which can be tested by the Shapiro-Wilk statistic and homogeneity of variance, which can be assessed using the Levene's test. With equal sample sizes the ANOVA is considered to be a "robust"

tool even when assumptions of normality and homogeneity may not be met (Portney & Watkins, 1993). Where the assumption of equality of variance is not met the Tamhane's T^2 post hoc pairwise comparison that is based on a t-test will be applied (Coakes & Steed, 1999). Applying the Bonferroni comparison will control the likelihood of a Type I error occurring with ANOVA. This test makes adjustments for the number of comparisons made within a set of data, for example, in this study, .05/3 (Portney & Watkins, 1993). The non-parametric Kruskal-Wallis test will be used where variables do not meet the stringent assumptions of ANOVA. The Mann-Whitney test will used to test for any potential differences within the NP group.

The Pearson product moment coefficient of correlation (r) (two-tailed) will be computed to identify relationships between variables and those variables that are statistically significant or that are considered *a priori* to influence vascular function. If significant relationships are identified in the nephrotic group between stimulated blood flow variables and any other variable, partial correlations will be used to control for the potential influence of, in the case of FMD% and GTNMD%, baseline artery diameter. Regression analyses will establish the predictive power of any variables shown to be significantly associated in correlational analysis with stimulated blood flow variables in the patients with nephrosis.

The major assumptions to be met before carrying out multiple regression analysis include the following: normality, linearity, homoscedasticity, and independence of residuals. Plotting the predicted values against the residual values and examining the scatterplots that are generated can test these assumptions. When using regression analysis, the ratio of cases to independent variables should not exceed five times the number of cases to independent variables.(Coakes & Steed, 1999)

A *post hoc* issue that might arise in this study is that of multicollinearity and singularity. Condition indices, tolerance values and the variance inflation factor (VIF) will assess multicollinearity effects (Hair et al., 1998). Another *post hoc* issue in this study could be that of "interaction". Interaction can occur in linear regression analysis when an independent variable has a differential effect on the dependent variable within each group. This effect can be examined by creating a new variable

that is the product of the two variables of interest. The effect is then tested with the F statistic for the improved fit in the regression model. Altman (1991) advises using this analysis with caution as it may increase the chance of a spurious finding.

In order to calculate flow debt repayment, non-linear regression analysis will be carried out on blood flow data derived from venous occlusion plethysmography. This analysis was described in section 4.4.2, 'Strain gauge plethysmography'.

4.6 Human Subject Protection

This study received the approval of the Royal Perth Hospital, and the Curtin University of Technology Human Research Ethics Committees, both of which are based upon NH& MRC guidelines. This researcher was available in person to answer all enquiries that eligible persons made regarding their participation in the study. Prior to obtaining voluntary informed consent, participants were provided with written information concerning the study. See Appendix A. They were informed that should they choose to participate in the study they had the right to withdraw from the study at any time and that any medical care would not be influenced by their decision to withdraw. Patients on lipid lowering medication were asked to discontinue this 4-6 weeks before the blood flow study date. Other medications remained unaltered. This was done with the prior knowledge and approval of physicians involved with the medical care of the renal and lipid patients participating in the study.

Participants' anonymity was protected in this thesis, by coding. As the study was undertaken in a clinical setting, relevant clinical details collected as part of the study and results of blood tests were entered, where appropriate, into the patients' hospital records. Publications arising from this study will not identify individuals. In accordance with University policy, original data will be held in a secure place for at least five years after completion date.

CHAPTER 5 RESULTS

5.1 Introduction

The following chapter provides a description of the histological features, renal function and treatment of the patients with nephrosis, and demographical, clinical and biochemical characteristics of the patients and control subjects ($\underline{N}$ = 45). Presented are results of blood flow studies carried out in the patients with nephrosis (NP $\underline{n}$ =15) and in hyperlipidaemic (HL $\underline{n}$ =15) and normolipidaemic (NC $\underline{n}$ =15) control groups. The hypothesis to be tested in this chapter is that patients with nephrotic syndrome have endothelial dysfunction and that the dysfunction occurs primarily as a consequence of dyslipidaemia.

5.2 Demographic and Clinical Characteristics of the Three Groups

The demographic and clinical characteristics of the subjects with nephrosis (NP) and the hyperlipidaemic (HL) and normolipidaemic (NC) control groups are shown in Table 5.1. None of the patients with nephrosis reported being hyperlipidaemic prior to developing renal disease. The HL subjects had either familial hypercholesterolaemia, familial combined hyperlipidaemia or common hypercholesterolaemia. Two of the nephrotic patients were smokers and all of the HL and NC subjects were non-smokers.

Chi square analysis revealed that the proportion of males to females was not statistically different among the groups. Analysis of variance (ANOVA) indicated that the mean ages, weights and body mass indices of the three groups were not statistically different from each other. There were also no significant differences in diastolic blood pressure, pulse pressure or heart rate. However, systolic blood pressure was significantly higher in the NP than the NC group but was not in the hypertensive range ($\underline{F}$ (2, 42) = 4.11, $\underline{p}$ = .030). Mean arterial pressure (mmHg) was also found to be significantly higher in the subjects with nephrosis compared with the NC group alone ($\underline{F}$ (2, 42) = 4.09, $\underline{p}$ = .028).

Table 5.1 Frequency (f), mean ($\underline{M}$) and standard error of the mean ($\underline{SE}$) for the demographic and clinical characteristics of the three groups ($\underline{n}$ = 15 for each).

Variable	NP f	HL f	NC f
Male/female	10/5	11/4	8/7
Smokers	2	0	0
ACE-inhibitor	5	0	0

Variable	NP $\underline{M}$	NP $\underline{SE}$	HL $\underline{M}$	HL $\underline{SE}$	NC $\underline{M}$	NC $\underline{SE}$
Age, yr	45.5	3.7	41.8	3.8	47.6	3.4
Weight, kg	76.8	4.2	78.8	3.8	73.7	3.1
BMI, kg/m^2	27.3	1.6	26.1	0.9	25.3	1.0
SBP, mmHg	123*[a]	3.4	114	2.3	112	3.1
DBP, mmHg	75	2.6	69	2.2	67	1.8
MAP, mmHg	90.8*[a]	2.7	83.9	1.9	81.8	2.2
Pulse pressure, mmHg	48.7	2.4	45.2	1.5	44.8	2.3
Heart rate bpm	65	2.8	64	2.1	58	2.3

* $\underline{p} < .05$,

a = NP vs NC.

Abbreviations: BMI = body mass index, SBP = systolic blood pressure, DBP = diastolic blood pressure, MAP = mean arterial pressure, ACE = angiotensin converting enzyme.

5.3 Clinical Characteristics, Renal function and Treatment of The Patients With Nephrosis

The clinical characteristics, renal function and treatment of the patients with nephrosis are shown in Table 5.2. Two of the patients had focal segmental glomerulonephritis, three membranous glomerulonephritis, one mesangiocapillary glomerulonephritis, five minimal change disease, one mesangioproliferative, one IgA nephropathy and two patients had not been biopsied at the time of the study. Disease duration ranged from 1-56 months (mean ($\underline{M}$) = of 27.1 months and standard deviation ($\underline{SD}$) = 43.4, median = 6 months). Twenty-four hour urinary protein excretion was in the nephrotic range at $\underline{M}$ = 6.3 g/24 hr, ($\underline{SD}$ = 4.6). Mean serum albumin was 26.1 g/L (7.2). Mean serum creatinine and glomerular filtration rates were 99.3 μmol/L (31.9) and 76.8ml/min (33.4) respectively. Five patients were being treated with an ACE-inhibitor, either enalapril, quinapril, perindopril, trandolapril or lisinopril. Two other patients were taking an angiotensin II receptor antagonist (irbesartan, losartan), and two had just commenced steroid treatment.

Table 5.2 The clinical characteristics, renal function and treatment of the patients with nephrosis.

Case	Glomerular histology	Disease duration, months	24 hr Urinary protein, g/24hr	Serum albumin, g/L	Serum creatinine, umol/L	Calculated GFR ml/min	ACE inhibitor
1	Focal and segmental glomerulonephitis	48	1.4	40	137	56.96	yes
2	Membranous glomerulonephritis	5	6.4	26	89	67.67	yes
3	Mesangiocapillary glomerulonephritis Type 1	156	1.0	32	67	69.23	no
4	Minimal change disease	2	2.2	26	56	136.62	no
5	No biopsy	2	15	26	154	36.42	yes
6	Minimal change disease	4	5.5	19	66	92.41	no
7	IgA nephropathy	84	4.8	33	136	46.20	no
8	Mesangioproliferative glomerulonephritis	6	6.9	26	70	76.50	no
9	Membranous glomerulonephritis	12	1.4	33	90	90.53	yes
10	Focal and segmental glomerulonephritis	24	3.3	28	135	28.52	yes
11	No biopsy	1	7.4	15	83	73.95	no
12	Minimal change disease	7	7.5	20	102	65.33	no
13	Minimal change disease	1	16.2	20	118	70.81	no
14	Membranous glomerulonephritis	54	5.5	32	66	153.77	no
15	Minimal change disease	1	9.3	15	120	87.45	no
	Mean	27.1	6.3	26.1	99.3	76.8	
	Standard Deviation	43.4	4.6	7.2	31.9	33.4	

Abbreviations: GFR = glomerular filtration rate; ACE = angiotensin converting enzyme.

5.4 Plasma Lipids, Lipoproteins and Biochemical Characteristics of the Three Groups

The means (M) and standard errors (SE) of the three groups serum lipid, lipoprotein, glucose, insulin, creatinine and albumin concentrations and urinary protein:creatinine ratio mg/mmol are shown in Table 5.3. Criteria for normality were met for homocysteine, NEFAs, calculated GFR, serum creatinine and albumin. Criteria for normality were not met for cholesterol, triglyceride, LDL-cholesterol, HDL-cholesterol, LDL particle size, apo-B, Lp(a), insulin, HOMA score, and serum glucose. After log transformation, the significance level of the Shapiro-Wilk statistic was more than .05 for triglyceride, LDL-C, HDL-C, Apo-B and Lp(a). However, cholesterol (NP group), LDL particle size (NP group), HOMA score (HL group), insulin (HL group), serum glucose (HL group) and urinary protein:creatinine ratio were not able to meet the criteria for normality.

Table 5.3 Means (M) and standard error of the mean (SE) for the plasma lipids, lipoproteins and other biochemical measurements in the three groups (n = 15 for each group).

Variable	NP		HL		NC	
	M	SE	M	SE	M	SE
Cholesterol, mmol/L	10.4***[a]	0.8	8.6***[a]	0.5	4.7	0.2
Triglycerides, mmol/L	3.6***[a]	0.9	2.0***[a]	0.3	0.8	0.1
LDL-Cholesterol, mmol/L	6.9***[a]	0.7	6.3***[a]	0.5	2.8	0.2
HDL-Cholesterol, mmol/L	1.6	0.1	1.3	0.1	1.5	0.07
Apo B 100, g/L	1.9***[a]	0.2	1.5***[a]	0.1	0.8	0.05
Lp(a), g/L	0.6*[c]	0.2	0.2	0.04	0.1†	0.03
NEFAs, mmol/L	0.27	0.02	0.37	0.02	0.36†	0.04
[kw]LDL-C particle size, nm	25.8†	0.2	25.9†	0.1	26.2	0.06
[kw]Glucose, mmol/L	5.2*[c]	0.2	5.2*[c]	0.09	4.7	0.09
Insulin, mU/L	12.5	1.8	8.9	2.4	6.8	1.0
HOMA score	2.94*[c]	0.43	2.19	0.24	1.44	0.2
Serum albumin, g/L	26***[ab]	1.9	42.0	0.6	43.0	0.9
Serum creatinine umol/L	99.3	8.2	87.0†	3.5	82.9	3.2
Fibrinogen, g/L	4.2**[bc]	0.5	1.9	0.1	2.5	0.2
Homocysteine mmol/L	10.8	1.0	8.7	0.6	9.2†	0.7
Urinary protein:creatinine ratio, mg/mmol	541.6***[ab]	88.6	3.8	1.0	3.3	0.5

*p < 0.05
** p < .01
a = < .01vs NC, b = < .01 vs HL, c = < .05 vs NC.
Comparison of means by one-way analysis of variance (ANOVA) or Kruskall Wallis test.
Abbreviations: LDL = low density lipoprotein, HDL= high density lipoprotein, NEFAs = non-esterified free fatty acids. [kw] = Kruskall Wallis
Missing values: = †, Lp(a) 2 from NC group, NEFAs 3 from NC group, LDL-C particle size 1 from NP and 3 from HL group, serum creatinine 1 from HL group, homocysteine 1 from NC group.

The Levene's test indicated that the means for triglyceride, LDL-C, HDL-C, apo-B, Lp(a), insulin and HOMA score could be said to be from the same population. These variables were analysed using ANOVA with the Bonferroni correction. The assumption of homogeneity of variance was not met for cholesterol (log transformed), serum albumin and serum creatinine. These variables were analysed using ANOVA with Tamhane's pairwise comparison. The non-parametric Kruskal Wallis test was used to compare the values between groups for serum glucose, and LDL particle size.

5.4.1 Analysis of lipids and lipoproteins

The variables found to be significantly higher in the NP and HL subjects compared with the healthy controls were, total cholesterol (F (2, 42) = 49.24, p < .01), triglyceride (F (2, 42) = 16.68, p < .01), LDL-C (F (2, 42) = 36.35, p < .01) and apo-B (F (2, 42) = 36.20, p < .01). Lipoprotein (a) was significantly higher in the NP group than in both the control groups, (F (2, 40) = 4.41, p = .019). Serum HDL-C concentration, LDL particle size and non-esterified fatty acid concentrations did not differ significantly between groups

5.4.2 Analysis of other biochemical characteristics

Serum glucose was significantly higher in both the NP and HL groups compared with the NC group (χ^2 (2, N = 45), p = 0.042). Serum insulin tended to be higher in the nephrotic than the control groups but the difference did not attain statistical significance (F (2, 42) = 3.13, p = .054). However, insulin resistance as assessed by the HOMA score was increased in the patients with nephrosis in comparison with the NC group (F (2, 42) = 3.58, p = .032).

Serum creatinine, calculated glomerular filtration rate and homocysteine levels did not differ significantly among the groups. As expected, the patients with nephrosis had a significantly lower serum albumin concentration compared with the controls (F (2, 42) = 63.39, p < .01). Plasma fibrinogen was notably elevated in the nephrotics compared with the two control groups and this was statistically significant (F (2, 42) = 12.19, p < .01). Urinary protein:creatinine ratio, was higher in the NP group

compared with the HL and NC subjects ($\underline{F}$ (2, 42) = 251.15, $\underline{p}$ < .01), there being no significant differences between the latter groups.

5.5 Post-ischaemic Responses of the Brachial Artery and Forearm Microcirculation.

5.5.1 Brachial artery

The analysis of the post-stimulatory changes in brachial artery diameter between groups were carried out using ANOVA, these are shown in Figure 5.1 and Figure 5.2. There were no significant differences in brachial artery diameter, mean ($\underline{M}$) and standard error ($\underline{SE}$), among the three groups (NP $\underline{M}$ = 3.56 mm, $\underline{SE}$ = 0.1; HL $\underline{M}$ = 3.80 mm, $\underline{SE}$ = 0.18; NC $\underline{M}$ = 3.29 mm, $\underline{SE}$ = 0.14). Post-ischaemic flow-mediated dilatation (FMD, percent increase) of the brachial artery was significantly lower in both the NP ($\underline{M}$ = 4.91%, $\underline{SE}$ = 0.8), and HL ($\underline{M}$ = 4.53%, $\underline{SE}$ = 0.6) groups compared with NC group, ($\underline{M}$ = 8.45%, $\underline{SE}$ = 0.5), ($\underline{F}$ (2, 42,) = 10.57, $\underline{p}$ < .01). There was no significant difference between the former two groups. To determine if the higher mean arterial pressure observed in the patients with nephrosis had influenced this outcome, one-way analysis of covariance (ANCOVA) was carried out. This test showed that when statistically controlling for mean arterial pressure, the difference in FMD observed between the NP and HL groups compared with the NC group remained significant. The responses to glyceryl trinitrate mediated dilatation (GTNMD, percent increase) of the brachial artery among the three groups were not significantly different. The means and standard errors are as follows: NP $\underline{M}$ = 21.78%, $\underline{SE}$ 1.4; HL $\underline{M}$ = 18.36%, $\underline{SE}$ 1.8; NC $\underline{M}$ = 23.34%, $\underline{SE}$ = 2.3. After removing the two patients with nephrosis who were cigarette smokers from the data set and repeating the statistical comparisons the results did not alter.

Subsequently a *post hoc* power analysis was carried out. This analysis demonstrated that with a sample size of $\underline{n}$ = 15 per group, equal variances and a common sample standard deviation of 2.53, the study had over 90% power to detect a difference of 2.35% in FMD between groups.

5.5.2 Forearm microcirculation

There were also no significant differences among the groups in forearm blood flow, maximal blood flow, flow debt repayment, forearm vascular resistance, or maximal forearm vascular resistance of the forearm microcirculation. Forearm volume was also similar among the groups. These data are shown in Table 5.4. A Mann-Whitney test was carried out in the NP group to determine if blood flow responses were affected by treatment with ACE inhibitors. No significant difference was found in FMD%, GTNMD%, maximal blood flow and flow debt between the patients treated with ACE inhibitors and those not.

Table 5.4 Means (M) and standard error of the mean (SE) for the vascular function in the forearm microcirculation in the three groups.

Variable	NP ($\underline{n}$ = 14) M	SE	HL ($\underline{n}$ = 13) M	SE	NC ($\underline{n}$ = 14) M	SE
FABF, ml/100 ml/min	1.98	0.2	2.13	0.1	2.31	0.2
Maximal blood flow ml/100 ml/min	18.07	2.3	21.25	2.6	19.26	2.2
Flow debt, ml/100 ml	4.84	1.3	5.23	0.7	4.89	0.6
Forearm vascular resistance, mmHg/ml/100 ml/min	50.9	4.4	41.5	2.9	39.6	4.3
Maximal forearm vascular resistance, mmHg/ml/100 ml/min	6.74	1.2	5.27	1.1	5.70	1.2
Forearm volume	1536	96.6	1627	101.0	1413	71.0

Abbreviations: FABF = forearm blood flow. Comparison of means by one-way analysis of variance (ANOVA)
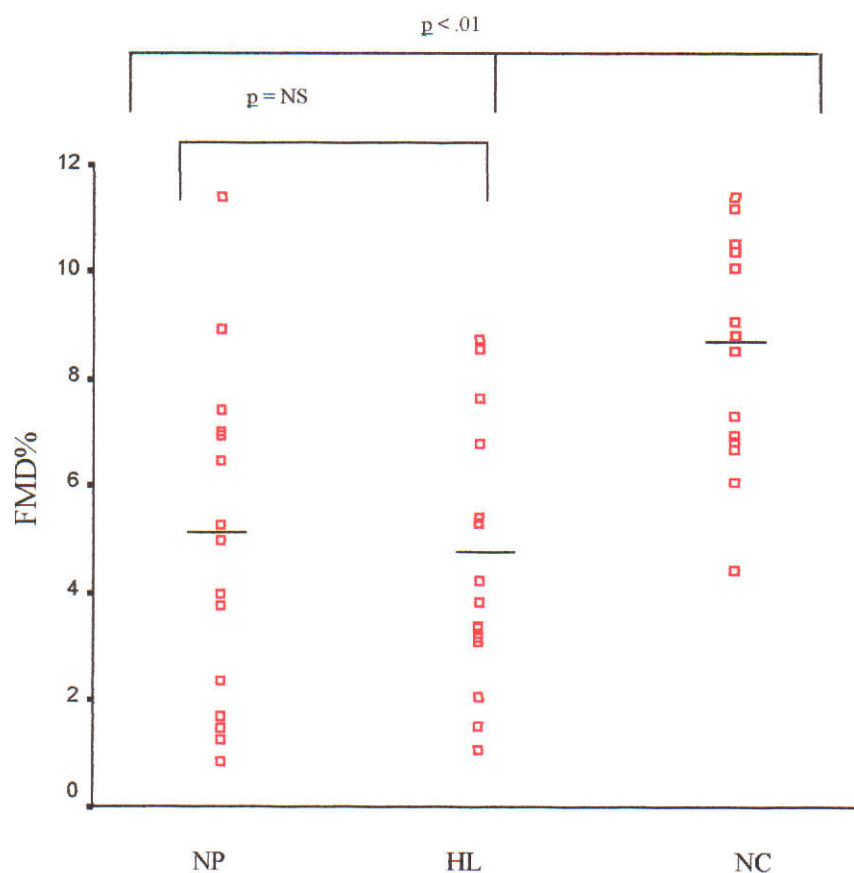
Figure 5.1 Post-ischaemic flow- mediated dilatation of the brachial artery, percent increase (FMD%) in the three groups. Horizontal bars represent mean values for each group.

Abbreviations: NP = patients with nephrosis, HL = hyperlipidaemic control group, NC = normolipidaemic control group. NS = not significant.
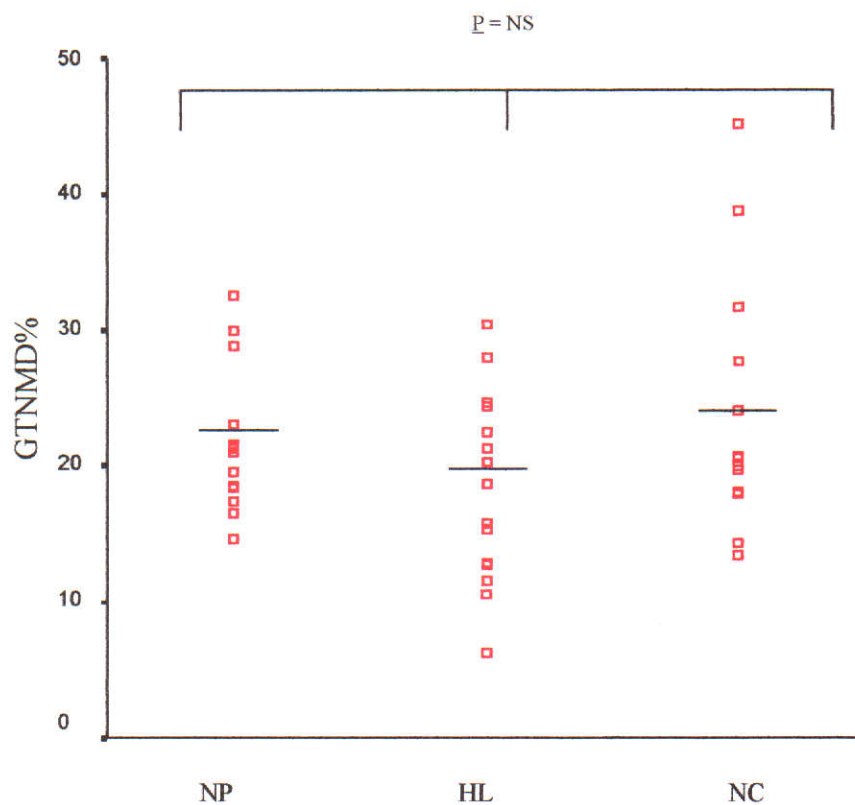
Figure 5.2 Glyceryl trinitrate mediated dilatation of the brachial artery, percent increase (GTNMD%) in the three groups. Horizontal bars represent mean values for each group.

Abbreviations: NP = patients with nephrosis, HL = hyperlipidaemic control group, NC = normolipidaemic control group. NS = not significant.

5.6. Correlational Analysis in the Patients with Nephrosis

Pearson's product-moment correlation coefficient (two-tailed) was used to determine relationships between stimulated blood flow variables and demographic and clinical parameters in the patients with nephrosis. The results presented for FMD% and GTNMD% are before adjustment for brachial artery diameter. These relationships are shown in Table 5.5.

In the patients with nephrosis, post-ischaemic FMD% was significantly and inversely associated with non-esterified fatty acid levels (r = -.61, p = .016), but not with other variables, including serum lipid, lipoprotein, albumin and creatinine concentrations, age, blood pressure, use of ACE-inhibitor and insulin resistance. After partial correlational analysis adjusting for brachial artery diameter, the inverse association between FMD and NEFAs remained statistically significant (r = -.577, p = .03). In further analysis of partial correlations between FMD and NEFAs, correcting for age, BMI, cholesterol, Tg, apo-B, LDL, HDL, SBP, MAP, serum insulin, HOMA score, serum albumin and 24 hr urinary protein the association remained significant. None of the aforementioned correlations were seen in the HL and NC groups.

Furthermore, in the patients with nephrosis, both maximal blood flow and flow debt repayment were significantly and inversely associated with age (r = -.574, p < .05, and r = -.543, p < .05, respectively). After correcting for the influence of age, maximal blood flow was seen to be inversely correlated with serum insulin and insulin resistance (r = -.596, p < .05; r = -.602, p < .05, respectively). Adjusting for potential influence of body mass index showed that the associations remained significant (r = -.672, p < .01; r = -.692, p < .01, respectively).

Table 5.5. Patients with nephrosis: Pearson's correlations between stimulated blood flow variables and demographic and clinical characteristics **before** adjustment for brachial artery diameter.

Variables	FMD%	GTNMD%	Maximal blood flow	^{ln}Flow debt
Age	-.116	-.131	-.574*	-.543*
^{ln}BMI	.243	-.308	-.150	.129
Systolic BP	.162	-.401	-.307	.064
Diastolic BP	.069	-.416	-.078	.122
MAP	.114	-.440	-.065	.104
Pulse pressure	.156	-.118	.036	-.047
^{ln}Cholesterol	-.119	-.157	-.012	.133
^{ln}Triglyceride	-.395	-.095	-.396	-.220
LDL-Cholesterol	-.093	-.107	.456	.269
HDL-Cholesterol	.232	-.053	-.219	-.345
^{ln}Apo-B	-.182	-.172	.314	.274
^{ln}Lp(a)	.319	-.375	.178	.121
NEFA	-.611*	.033	.006	-.233
LDL particle size	.106	.042	.326	-.033
Serum glucose	.294	-.310	-.240	-.147
Insulin	.004	-.484	-.607*	-.294
HOMA score	.058	-.530	-.616*	-.322
Calculated GFR	.419	-.034	.414	.439
Serum albumin	.093	-.301	-.323	-.157
Serum creatinine	-.166	-.215	-.356	-.194
Fibrinogen	-.015	.286	.182	.278
Homocysteine	-.120	-.438	-.036	-.109
24hr Urinary protein	.022	.069	-.067	-.077
Up:creat ratio	-.079	.030	-.215	-.358

Significance (two tailed)
* <.05

Abbreviations: ^{ln} = log transformed. BMI = body mass index, BP = blood pressure, MAP = mean arterial pressure, LDL = low density lipoprotein, HDL = high density lipoprotein, GFR = calculated glomerular filtration rate. Up:creat ratio = urinary protein to creatinine ratio.

5.6.1 Tests for potential interaction effects

Because NEFA levels were not statistically different between groups and an association was found between FMD% and NEFAs only in the NP group, it was hypothesised that the effect of NEFAs on FMD% in the NP group was different to that effect in the two control groups. To test this hypothesis a standard regression model was fitted. The following variables were entered simultaneously: i1 and i2, being the indicator variables constructed to test for group differences (i1 gives the comparison of the NP to the HL group and i2 the comparison of the NP to the NC group); baseline artery size; the main effect variable which were NEFAs; and the variables 1_11NEFAs and 1_12NEFAs which are the product of i1 x NEFAs and i2 x

NEFAs, respectively. These last two variables are the interaction terms. (See Table 5.6). To determine whether the addition of the interaction terms contributed significantly to the model, a subsequent analysis was carried out with these terms entered last. The results showed that the addition of the interaction terms contributed significantly to the model ($\underline{F}$ change (2, 38) = 4.829, $\underline{p}$ < .01). The adjusted R^2 indicated that the model accounted for 52% of the variability in FMD%. Both interaction terms were significant, indicating that the effect of NEFAs on FMD% in the NP group was significantly different from its effect on FMD% in both the HL and NC groups. Figure 5.3 shows a scatter plot that illustrates the differential effect of NEFAs on FMD% in the three groups.

Table 5.6 Simultaneous regression analysis of NEFAs on FMD% after adjusting for group assignment, baseline artery size and potential interaction effects.

Variables	B	SE B	Beta	t	Sig
Constant	16.228	2.505		6.479	.000
i1	-4.709	2.426	-.727	-.941	.060
i2	.156	1.424	.024	.110	.913
Baseline artery size	-2.023	.567	-.408	-3.566	.001
NEFAs	-15.170	4.823	-.622	-.3.145	.003
1_l1NEFAs	17.097	7.049	1.048	2.425	.020
1_l2NEFAs	13.388	4.599	.787	2.911	.006

$R^2 = .582$ $R^2_{adj} = .516$

Abbreviations: NEFAs = non esterified fatty acids. Adj = adjusted.

The other variables tested for differential effects on FMD were LDL-C, triglycerides, Apo-B, serum glucose, serum insulin, HOMA score, calculated GFR, serum albumin, fibrinogen, homocysteine and up:creat ratio. No significant interactions were observed in these analyses.

The finding that HOMA score and serum insulin levels were associated with maximal blood flow in the patients with nephrosis was also tested for potential interaction. A standard regression analysis was carried out in a similar fashion to that described for the NEFA interaction. The first analysis included all the cases. This found that the first interaction term was significant, indicating that the maximal blood flow response in the NP group differed from that of the HL group. To determine if there was an influence from the outlying case previously identified in the

HL group, this case was removed from the analysis. Consequently, no significant interaction was found between the three groups.
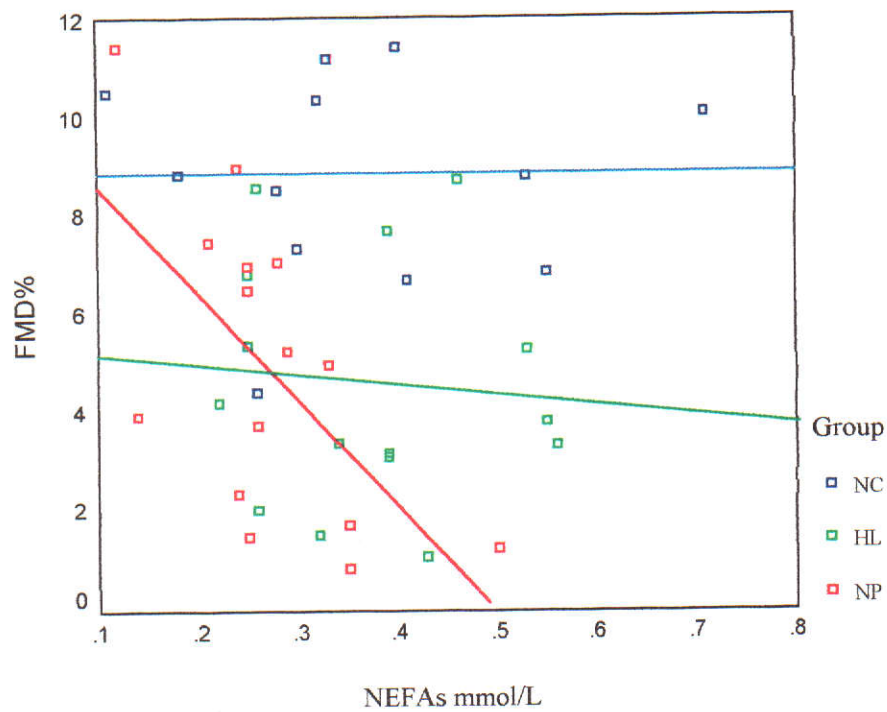
Figure 5.3 Scatter plot of non-esterified fatty acids (NEFAs) on FMD% in the three groups.
Abbreviations: NP = patients with nephrosis, HL = hyperlipidaemic control group, NC = normolipidaemic control group. FMD% = Post-ischaemic flow-mediated dilatation of the brachial artery, percent increase.

5.7 Conclusion

The results support the hypothesis that endothelium-dependent vasodilatation is impaired in patients with nephrosis compared with healthy control subjects. The impairment was comparable to that seen in hyperlipidaemic, non-proteinuric individuals. Within the NP group, increases in non-esterified free fatty acids were associated with reduced flow-mediated vasodilatation suggesting an injurious role for these lipids on vascular function in individuals with nephrotic syndrome.

Glyceryl trinitrate mediated vasodilatation was not different between groups suggesting that endothelium independent responses were unimpaired in both the nephrotic and hyperlipidaemic groups. Forearm microcirculatory function was also not different between groups. There was, however, in the patients with nephrosis, an inverse association between maximal blood flow and age, and flow debt and age. Maximal blood flow was also inversely associated with a degree of insulin resistance in these patients that remained significant after adjusting for age. As the patients with nephrosis were demonstrably insulin resistant in comparison with the normal control group, insulin may have a role in mediating forearm vascular responses in individuals with nephrosis.

CHAPTER 6 DISCUSSION AND CONCLUSIONS

6.1 Introduction

The previous chapter presented the results of the vascular function and laboratory tests obtained in the three groups studied. The principal finding supports the hypothesis that endothelial dysfunction occurs in patients with nephrosis and that the impairment is primarily due to dyslipidaemia. The relevance of these results will be discussed followed by an outline of the limitations of the study and recommendations for future research.

6.2 Findings

The results presented in this study add to the existing body of knowledge concerning vascular function in patients with nephrotic proteinuria. The study found that patients with nephrosis exhibited impaired endothelial function of the brachial artery similar to that seen in individuals with primary hyperlipidaemia, and that the impairment was associated with relative increases in plasma NEFAs. The abnormality appeared confined to an endothelium-dependent mechanism, as no difference was found between the groups in endothelium independent responses to the administration of glyceryl trinitrate. The endothelial dysfunction seen in the brachial artery was also not apparent in the forearm microcirculation between the three groups studied. However, although no difference between groups in the responses of the resistance vessels was observed, in the patients with nephrosis, maximal blood flow was inversely related to the degree of insulin resistance found in this group. There was also an apparent attenuation of maximal blood flow and flow debt by increasing age in this group that was not seen in the two control groups.

6.3 Previous Research

A number of studies have demonstrated endothelial dysfunction of the conduit and resistance arteries in patients with renal failure (Bradley, Evans, & Cowley, 1988;

Demuth et al., 1998; Joannides et al., 1997; Kari et al., 1997; Pannier et al., 2000). The present study, however, differs from earlier work in this area because it has focused on lipid abnormalities in the presence of proteinuria but in the absence of potential confounding factors, for example, influences such as raised serum creatinine, anaemia and the cardiovascular complications characteristic of end stage renal disease.

Stroes, et al. (1995a) studied vascular function in patients with nephrotic range proteinuria, dyslipidaemia and normal renal function; patients with active glomerulonephritis, low range proteinuria and normal renal function; and healthy volunteers. They demonstrated impaired microcirculatory responses in the patients with nephrotic range proteinuria compared with the control groups. Endothelium-independent function of the three groups in response to sodium nitroprusside was not, however, found to be different among the groups. Although their vascular measurements were carried out in the forearm microcirculation alone, their findings appear consistent with the findings of the current study which also found no difference between groups in endothelium-independent responses.

In the current study, the observations of Stroes, et al. (1995a) have been extended by studying a larger group of nephrotic patients and matching them, as closely as possible, for age, gender and body mass index with control subjects. In this study, two control groups were used for comparison with the nephrotic group, one with hyperlipidaemia and without evidence of proteinuria, and a second group of normolipidaemic subjects also without evidence of proteinuria. In a further attempt to add power to the study design, the hyperlipidaemic group was selected to have a similar lipid profile to that of the group with nephrosis. In addition, this study has examined the relative contributions of variables such as fibrinogen, LDL particle size, homocysteine and NEFAs to vascular endothelial dysfunction in the nephrotic patients. Furthermore, non-invasive tests of vascular function have been carried out in two different vascular beds.

6.4 Vascular Responses: Mechanisms

Joannides, et al. (1995) observed that large arteries, by increasing their internal diameter, oppose neurogenic and myogenic vasoconstriction and function to maintain shear stress by accommodating changes in blood flow. Post-ischaemic dilatation of the brachial and other conduit vessels results primarily from the endothelial release of nitric oxide in response to shear stress (Joannides et al., 1995; Playford & Watts, 1998; Vogel et al., 1998).

Although continuous basal release of nitric oxide is responsible for resting vascular tone in the microcirculation, the hyperaemic responses of forearm resistance arteries are mediated by agonists other than NO, including prostaglandins and adenosine as well as myogenic influences (Meredith et al., 1996; Tagawa et al., 1994). These authors observed that forearm blood flow was maintained above basal levels after a period of ischaemia in the presence of NO (Meredith et al., 1996; Tagawa et al., 1994). It is also possible, however, that the initial increase in blood flow (maximal blood flow) after release of the cuff may be due to an ischaemia induced reduction in oxygen tension that results in vaso-relaxation rather than the response to a vasoactive substance (Joannides et al., 1995). The brachial artery, in contrast with the resistance vessels, may develop atherosclerosis, which makes it a useful surrogate for coronary and carotid vessels in research programs (Sorensen et al., 1997).

The apparent differences in physiology between the two vascular beds may account for the divergent findings in the brachial and forearm arteries in the present study. It is also probable that forearm hyperaemic responses have a larger within patient variability (Playford & Watts, 1998). Had this study used a pharmacological stimulant of NO rather than an ischaemic stimulus to assess responses in the forearm microcirculation the findings may have been different.

6.5 Mechanisms of Endothelial Injury: Lipoproteins and Lipids

The mechanisms by which lipoproteins can impair endothelial function, an early phase of atherosclerosis, were discussed by Flavahan (1992). Signalling within the

cell involves G-proteins that attach membrane bound receptors to subcellular systems. It is the activation of G-proteins that causes the release of NO, initiating endothelium-dependent relaxation. Early endothelial dysfunction is manifested by an impairment of NO synthesis and release via a specific G-protein (Gi) signal transduction pathway which may be associated with increased release of endothelial constricting factors that are antagonistic of NO. It has been suggested that endothelial dysfunction caused by hypercholesterolaemia may involve loss of endothelial G-proteins or signal transduction processes. Of significance to the development of atherosclerotic plaques is the notion that lipids may accumulate in the endothelial cells as a consequence of disruption of normal signal transduction (Flavahan, 1992).

Triglycerides may impair endothelium dependent vasodilatation by a pathway common to that of elevated LDL-C (Lewis, Dart, & Chin-Dusting, 1999). A pathway suggested by these researchers was via small dense LDL particle size that is seen in both hypercholesterolaemia and hypertriglyceridaemia. Small dense LDL, is susceptible to oxidative modification and has also been associated with endothelial dysfunction, as has low HDL-C (O'Brien et al., 1997; Zeiher et al., 1994). It is interesting that in the present study no statistical difference was found in LDL particle size or HDL-C in the three groups. As it is primarily triglyceride concentrations that influence LDL particle size, it might have been expected that the dyslipoproteinaemic groups be different from the normal control group. Missing data from three cases in the hyperlipidaemic group and one case from the group with nephrosis, may have contributed to this negative finding. It is also possible, that in this sample, other factors such as genetic background, hormonal status, and diet may have effected LDL particle size. Alternatively, in patients with heavier proteinuria and higher triglycerides, the finding may have been different (Deighan et al., 1998).

Acutely elevated triglycerides and free fatty acids have also been associated with impairment of vascular reactivity independently of hypercholesterolaemia in insulin sensitive subjects (Lundman et al., 1997; Steinberg et al., 1997). The postulated mechanisms for these responses included: decreased diffusion of NO through the cell wall; oxidation of NO by elevated fatty acids; and the interference of free fatty acids in prostaglandin synthesis and the consequent imbalance between vasoconstrictor and

vasodilator factors (Lundman et al., 1997). However, Steinberg et al. (1997) commented that although the mechanism for FFA induced endothelial dysfunction has not been fully established because insulin mediated dilatation is dependent on nitric oxide, it seems likely that in insulin resistant individuals FFAs cause endothelial dysfunction by affecting the nitric oxide system. Although in the current study total fatty acids were not elevated in the patients with nephrosis compared with controls, they coexisted with a degree of insulin resistance not seen in the normal control group.

The relationship between plasma NEFAs and impaired FMD in the patients with nephrosis was an unexpected finding. However, the interaction analysis (Figure 5.3) indicated that the impact of NEFAs on variation in FMD% was not the same in the three groups. This suggests a potentially unique role for NEFAs to influence endothelium-dependent vasodilatation in this group of patients with nephrotic dyslipidaemia. Research by Braschi et al. (1997) in the course of a study of CETP activity in fifteen patients with nephrotic syndrome and twenty-two controls, found similar levels of plasma NEFAs between the groups. However, they observed that the patients with nephrosis showed a significant increase in the relative proportion of lipoprotein bound NEFAs compared with the controls.

In the present study the levels of NEFAs in the nephrotic group were partly a consequence of low serum albumin to which NEFAs are normally bound (Cameron, 1998). However, low serum albumin was not significantly correlated with impaired FMD within the nephrotic group. It may be postulated that increased substrate delivery of unbound NEFAs to the endothelium contributes to impaired NOS activity in the nephrotic patients. Alternatively an increase in the proportion of lipoprotein bound NEFAs may alter the nature of the lipoprotein particle, as observed by Braschi et al. (1997) and increase its potential for injuring the endothelium. It has been observed *in vitro* that LDL-C transfer across endothelial cells is facilitated by exposure to elevated NEFA levels (Hennig, Shasby & Spector, 1985). Furthermore, in an *in vitro* study by Laughton et al. (1988) serum NEFA concentrations and NEFA/albumin molar ratios but not cholesterol and triglyceride concentrations, were strongly associated with cellular lipid concentration in human arterial smooth muscle

derived cells exposed to sera containing elevated NEFAs. This suggests NEFAs may be a factor in atherosclerotic plaque formation and therefore endothelial dysfunction. Research with a larger number of patients would contribute more information to either support or refute the validity of the findings in the current study.

In the patients with nephrosis, NEFAs and FMD% were correlated but not NEFAs and maximal blood flow in the resistance vessels, which was associated with insulin resistance. This divergent finding may be reflective of the multiplicity of factors influencing the vasotonic responses in the resistance vessels that has been observed by other researchers (Meredith et al., 1996; Tagawa et al., 1994). It is highly probable that reactive hyperaemia is an incomplete measure of endothelium-dependent vasodilatation.

Insulin has been found to stimulate endothelial cells to produce both endothelin and nitric oxide in the forearm microcirculation (Cardillo et al., 1999) and LDL-C also enhances the synthesis of endothelin (Boulanger et al., 1992). Secretion of endothelin, a vasoconstrictor, is polarised towards vascular smooth muscle (Cardillo et al., 1999). This may explain in part, the inverse association of insulin resistance and maximal blood flow observed in the patients with nephrosis. In patients with insulin resistance it may be hypothesised that an imbalance of the release of endothelin and NO may contribute to endothelial dysfunction. Elevated plasma endothelin has also been observed in patients with end-stage renal disease and was associated with reduced flow debt repayment and increased common carotid artery intima media thickness in comparison with a control group (Demuth et al., 1998). In the current study levels of endothelin were not measured.

The tendency to insulin resistance seen in the nephrotic patients may be a consequence of abnormal fatty acid transport or of dyslipoproteinaemia (Boden, 1997) or some other mechanism. Insulin resistance has also been shown to be present in patients with renal disease and glomerular filtration rates within the normal range (Fliser et al., 1998). That insulin resistance was positively correlated with body mass index in the patients with nephrosis in the current study suggests that insulin

resistance may be due to variation in adiposity (Laakso et al., 1990). However, this observation should be accepted with caution because of the small size of the study sample.

6.6 Other Potential Causal Mechanisms for Endothelial Dysfunction

In the patients with nephrosis, it might be that impaired NO release was caused by low serum albumin. Serum albumin has been suggested as a reservoir of NO in plasma and this may account for its vaso-relaxant properties (Minamiyama, Takemura, & Masayasu, 1996). Acute brachial artery vasodilatation, however, results from release of NO from the endothelium that has been stimulated by shear stress. Therefore, it seems unlikely that this vaso-relaxant property attributed to serum albumin would contribute in a major sense to flow mediated dilatation in the current study. Furthermore, endothelium-dependent responses were similar to hyperlipidaemic subjects with normal serum albumin.

Another putative causal explanation for endothelial dysfunction given in the literature is oxidative stress. Oxidised LDL-C can inhibit endothelium-dependent relaxation and it is thought to do so in a similar fashion to that of hypercholesterolaemia by selectively interrupting the processes of endothelial receptor signal transduction. Furthermore, as concentrations of LDL-C increase the dysfunction may become less specific (Flavahan, 1992). Oxidative stress has been previously assessed in the nephrotic patients in the current study by measuring plasma and urinary F_2-isoprostanes. It was concluded that, using this measure, there was no evidence of increased oxidant stress (Dogra et al., 2000).

Stroes et al. (1995a) suggested a contributary role in the initiation of endothelial dysfunction by lysophosphatidylcholine (lyso-PC). This is produced from the lecithin component of the lipoprotein particle in the process of LDL-C oxidisation (Stroes et al., 1995a). This was not measured in the current study, therefore, oxidative stress assessed with another method may have had a causal role in impairing FMD in this group of nephrotic patients.

Another mechanism for the endothelial dysfunction seen in the nephrotic patients may be elevated plasma concentrations of Asymmetric Dimethylarginine (ADMA), an endogenous inhibitor of nitric oxide synthase which has been found in hypercholesterolaemic humans (Boger et al., 1998). This potential mechanism was not explored in the current study. It is also possible that increased plasma concentrations of remnant lipoproteins (Kugiyama et al., 1998) played a role but these were not measured in the current study. Others have found an association between increased levels of Lp(a) and impaired brachial artery responses (Sorensen et al., 1994) but this was not observed in the groups.

Also not assessed were the fitness levels and duration of dyslipidaemia in the patients with nephrosis. These factors might have partly explained the observation that not all of the patients with nephrosis exhibited impaired endothelial function. The variability of the ultrasound technique may also have contributed to differences between patients. This factor was addressed by the use of an edge detection algorithm to enhance precision of the brachial artery diameter changes. The use of the algorithm allows for detection of small differences in the responses of this conduit vessel between groups of relatively small sample size.

6.7 Potential Confounding Factors

Physiologically, the findings of the study may have been confounded by hypertension, although the research findings on the link between hypertension and endothelial function are not universally concordant (Cockcroft et al., 1994). Use of ACE inhibitors in the nephrotic patients may have confounded the findings, but statistical analysis did not find evidence for this. It is also necessary to acknowledge the variable effects of different ACE inhibitor types on endothelial function (Anderson, 1999; Creager & Roddy, 1994).

In regard to variations in blood pressure, a potential problem when comparing forearm microcirculation between the three groups might have been the tendency of the nephrotics to have a higher mean arterial pressure than the normal controls suggesting different starting conditions (Benjamin et al., 1994). Nevertheless, there

were no differences in forearm blood flow and basal forearm vascular resistance between the groups making the possibility of blood pressure confounding the results less likely.

The relative contribution of factors, such as homocysteine and fibrinogen, to the endothelial dysfunction seen in the patients with nephrosis cannot be excluded but neither were significantly correlated with any of the stimulated blood flow variables in the within group analysis. In contrast, the significant inverse association between maximal blood flow and insulin resistance in the patients with nephrosis suggests that this variable has some role in predicting post-ischaemic vascular responses in resistance vessels and is worthy of further research.

6.8 Limitations of The Study

The study had a two-fold purpose. Firstly, to determine whether patients with nephrosis had endothelial dysfunction and secondly, whether the abnormality was comparable to that seen in the patients with primary hyperlipidaemia. The study was a pilot for a second study of the patients with nephrosis only. Nevertheless, the cross-sectional design and the small sample size of this study could be considered limitations. However, employing two control groups in order to partition the relationships between endothelial dysfunction, dyslipidaemia and proteinuria gave a greater degree of experimental control than if only one comparison group had been used. Taken in this context the design is appropriate.

A potential weakness of the study pertains to the selection of patients and controls. The availability of nephrotic patients was limited and recruitment of patients took place over two years. An attempt was made, however, to recruit controls contemporaneously with the patients to limit the influences of improvement in technique of the operators carrying out the blood flow studies.

Furthermore, two types of nephrotic dyslipidaemia have been identified, predominant hypercholesterolaemia and mixed hyperlipidaemia (Vega et al., 1995). Studying these phenotypes separately may have given more information. Due to the small numbers

of patients this was not possible. In an effort to compensate for this, the subjects in the hyperlipidaemic control group were selected to match the lipid phenotype of the nephrotic group.

Matching the groups for lipid levels and ensuring that none of the control subjects was proteinuric were primary objectives. The group means were not statistically different in indices of age, gender, weight and body mass. However, the variability within groups as a consequence of the small sample size and the necessity to select subjects on the basis of the attribute variables of the patient group, was necessarily large. In addition, selection of the healthy normolipidaemic control group was not by random sampling. These subjects volunteered for the study and it might be argued that they were driven by health seeking behaviour patterns to join the study indicating that as a group, they may not be representative of the population.

Forearm microcirculatory function may have been more rigorously assessed by the infusion of NO agonists via intra-arterial cannulation. However this was not considered ethically justified by referring renal physicians. There are some inherent problems with measurement of forearm microcirculatory, blood flow responses at peak hyperaemia, which may impact on the reliability and validity of the technique. Regarding reliability, the curves that are obtained immediately on release of the occlusive cuff are steep. The steepest slope of the curve is chosen by the operator, highlighted and selected in MacLab, which then transfers the selection to a text pad. Although the same practiced operator performed all these measurements, even slight deviations can result in differences of calculated flow. When the curves are less steep measurement estimating the steepest part of the curve is easier. Thus measurement error may be less in studies where flow rates are lower. Employing computerised software that could measure the precise slope could increase the reliability of the technique. Petrie et al. (1998) found that even at rest forearm blood flow was responsive to changes in ambient temperature and sympathetic nervous system activity. An attempt to control for these variables was made in this study by maintaining an ambient room temperature of 24C, allowing the subjects to acclimatise to the technique, and by not conversing during the blood flow measurements.

6.9 Recommendations

Despite the limitations of the study, the results present a plausible explanation for the increased risk of cardiovascular disease in nephrosis reported by Ordonez et al. (1993) and suggest a mechanism for endothelial injury as a precursor to atherosclerosis. They also support the argument for treating dyslipidaemia in patients with nephrosis. The contributory roles of NEFAs and insulin resistance to vascular dysfunction require further investigation.

Reduced bio-availability of NO not only results in impaired flow-mediated dilatation and consequent vasotonicity but also platelet aggregation and smooth muscle proliferation in arterial walls (Keaney & Vita, 1995). In the patient with nephrosis, abnormalities of coagulation and dyslipidaemia co-exist and compound risk of atherothrombosis (Cameron, 1998) and potentially, coronary events (Rubanyi, 1993). Acute elevations of plasma lipids have been shown to alter endothelial function (Lundman, Eriksson, Schenk-Gustafsson, Karpe, & Tornvall, 1997; Steinberg et al., 1997). Endothelial dysfunction of the brachial artery is correlated with changes in epicardial coronary artery responses (Anderson et al., 1995) and more recently has been shown to predict coronary events (Al Suwaidi et al., 2000).

The results of this study contribute support for the evidence that patients with nephrosis are at risk of developing cardiovascular disease, possibly prematurely, and should be treated accordingly. These findings also recommend that research should continue into developing and evaluating interventions that will result in improvements in the management of cardiovascular complications of renal disease. Nurses may have a role to play in this research which, hopefully, will not only benefit patients, but enhance professional practice for nurses. Another important, facet of care is that of providing informational and emotional support for patients who require treatment interventions where lifestyle changes alone may be insufficient to treat dyslipidaemia.

6.10 Questions for Future Research

There are some important questions raised by the study. How do the qualitative changes in lipid metabolism that occur in individuals with nephrosis, influence their risk of developing cardiovascular disease? Are NEFAs a marker for some other factor that may be causing, directly or indirectly, vascular injury? Does the exposure of mesangial cells to lipids and in particular apolipoprotein B, containing lipoproteins, contribute to the progression of renal disease (Attman et al., 1997; Keane et al., 1990), and if so, is this reversible? Furthermore, is impaired NO release of the peripheral arteries reflective of abnormal NO release in the kidney where it may influence autoregulation of renal blood flow? (Romero & Strick, 1993). Another question that arises is whether individuals with a lesser degree of proteinuria with or without dyslipidaemia, hypoalbuminaemia or hypertension have endothelial dysfunction and are at increased risk of developing cardiovascular disease. Are public health initiatives adequate in addressing these questions?

6.11 Conclusion

This study permits the conclusion that patients with nephrosis have endothelial dysfunction that is comparable to patients with primary hyperlipidaemia, and that the abnormality may be causally related to dyslipidaemia. Forearm microcirculatory function, in this group of patients, appears to be mediated by insulin. Other potential factors that might have influenced endothelial function in these patients have been explored but many questions remain to be answered. It is acknowledged that in a larger study sample the findings may have been different. Lipid-lowering medication, and lifestyle changes such as cessation of smoking, diet and exercise modification have been shown to correct dyslipidaemia and improve endothelial function in dyslipidaemic, non-proteinuric individuals. Further research into appropriate interventions for nephrotic patients with dyslipidaemia to lower their apparent risk of premature cardiovascular disease is recommended.

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APPENDIX A

Information Sheet and Consent Form

Dyslipidaemia and arterial wall function in the nephrotic syndrome

You are invited to participate in a research study that will examine blood vessels in people with nephrotic syndrome. The nephrotic syndrome is a type of kidney disease in which large amounts of protein are lost in the urine, this does not happen in healthy kidneys. As a result of this protein loss some changes take place in the blood. One of these changes is called dyslipidaemia (dis-lippid-eemiah), which means high blood fats.

High blood fats are a risk factor for the development of heart disease. It is not certain why blood fat levels rise in people with nephrotic syndrome and whether the effect on the blood vessels is similar to the effect of high blood fats on people without nephrotic syndrome. The researchers in this study would like to compare the blood vessel function of people with protein in the urine with another group of people with high blood fats but no protein loss in their urine. Both of these groups will then be compared with a third group of people with no protein loss and normal blood fat levels.

It is hoped by doing this research that the results may guide doctors towards appropriate treatments for people with nephrotic syndrome and add to knowledge about the causes of heart disease and the relationship between renal and heart disease.

There will be two kinds of blood flow measurements performed for this particular study, plethysmography and ultrasound. Both tests are non-invasive and do not require any anaesthetic. In these techniques you will be asked to lie flat on a bed for up to 45 minutes and blood pressure cuffs will be inflated at frequent intervals to measure blood flow. On one occasion the blood pressure cuff will be inflated for up to eight minutes and this may induce some discomfort in the arm. In the ultrasound test you will also be asked to spray a substance called glyceryl trinitrate under the tongue and this may cause a mild headache lasting up to ten minutes. Before these tests you will be asked to fast for 12 hours.

If you are taking cholesterol lowering medication you will be asked to discontinue the medication for six weeks prior to the test. Other medications will not be changed. Up to 20 ml (4 teaspoons) of blood will be taken to measure fat levels, liver and kidney function. On the day of the blood flow studies you will be asked to have a blood test that will be refrigerated and stored for analysis at the end of the study. Up to 80 ml of blood (approximately 3 tablespoons) will be required. The tests performed will be for blood fat, vitamin and enzyme analysis. You will also be asked for your permission to store a small amount of blood, 15 ml (3 teaspoons) for DNA testing. This will be discussed with you and further information given at the time.

The blood flow studies and the blood tests will be performed on the same morning at the University Department of Medicine at Royal Perth Hospital. You are free to withdraw from the study at any time and should you choose to do so your routine care will not be influenced in any way. All information will be kept strictly confidential, and any publications arising from the study will not include your name or any identifying features. The Ethics Committee of Royal Perth Hospital has given approval for this study and any concerns regarding the study can be directed to Professor J. A. Millar, Chairperson, Ethics Committee, C/- Medical Administration Royal Perth Hospital, Wellington Street, Perth WA 6001. Any questions concerning the study can be directed to Dr Gerald Watts, Dr David Playford or Susan Herrmann RN, at the Department of Medicine, Royal Perth Hospital on 9224 0245, or Dr Ashley Irish in the Department of Nephrology RPH on 9224 2550.

I   (Name of participant)   have read the information above and questions I have asked have been answered to my satisfaction. I agree to participate in this activity realising that I may withdraw at any time. I agree that the research data gathered for the study may be published provided that my name is not used.

Signature of patient: Signature of investigator

Date:

APPENDIX B

Details of Laboratory Methods

(Tests carried out in the Core Laboratory at Royal Perth Hospital and details provided by Ken Robertson)

Analyte: Serum Cholesterol

Methodology: Enzymatic, colorimetric method

Cholesterol ester is acted upon by cholesterol esterase to produce cholesterol.

Cholesterol is then acted upon by cholesterol oxidase, producing hydrogen peroxide.

The production of 4-(p-benzoquinone-monoimino)-phenazone produced when hydrogen peroxide, 4-aminophenazone and phenol are then acted upon by peroxidase, is then measured. The colour measured is proportional to the cholesterol concentration of the serum.

Reagents: Boehringer Mannheim Cholesterol CHOD-PAP reagents, Boehringer Mannheim Pty Ltd, Mannheim, Germany.

Instrumentation: Hitachi 917 Biochemical Analyser, Hitachi Limited, Tokyo, Japan.

Precision: Interassay Coefficient of Variation: At level of 3.2 mmol/L, CV = 1.5%, at level of 6.8 mmol/L, CV = 1.2%,

Sample type: Serum. Separated within 2 hours of collection and analysed within 24 hours of collection.

Reference: Boehringer Mannheim Cholesterol reagent package insert. Hitachi 917 Operator's Manual.

Analyte: Serum Triglycerides

Methodology: enzymatic, colorimetric method.

Glycerol is split from triglyceride by the action of lipoprotein lipase. Glycerol, under the action of glycerol kinase, is converted to glycerol-3-phosphate which is in turn acted upon by glycerol phosphate oxidase, resulting in the production of hydrogen peroxide. The production of 4-(p-benzoquinone-monoimino)-phenazone produced when hydrogen peroxide, 4-aminophenazone and phenol are then acted upon by

peroxidase, is then measured. The colour measured is proportional to the cholesterol concentration of the serum.

Reagents: Boehringer Mannheim Triglycerides GPO-PAP reagents, Boehringer Mannheim Pty Ltd, Mannheim, Germany.

Instrumentation: Hitachi 917 Biochemical Analyser, Hitachi Limited, Tokyo, Japan.

Precision: Interassay Coefficient of Variation:- At level of 0.95 mmol/L, CV = 1.8%, at level of 2.1 mmol/L, CV = 1.7%,

Sample type: Serum. Separated within 2 hours of collection and analysed within 24 hours of collection.

Reference: Boehringer Mannheim Triglyceride GPO-PAP reagent package insert. Hitachi 917 Operator's Manual.

Analyte: Serum HDL-Cholesterol

Methodology: Homogenous, enzymatic, colorimetric method

Sulphated a-cyclodextrin and dextran sulphate form water soluble complexes with LDL-C, VLDL-C & chylomicrons. These are resistant to PEG-modified enzymes. HDL-Cholesterol esters are acted upon by PEG-cholesterol esterase, releasing cholesterol. Cholesterol is then acted upon by PEG-Cholesterol oxidase resulting in production of hydrogen peroxide. Peroxidase then acts upon hydrogen peroxide, 4-aminophenazone and N-(2-hydroxy-3-sulphopropyl)-3,5-dimethoxyaniline to form a purple-blue pigment which is measured. The colour intensity is proportional to the cholesterol concentration of the serum.

Reagents: Boehring Mannheim HDL-C plus reagents, Boehringer Mannheim Pty Ltd, Mannheim, Germany.

Instrumentation: Hitachi 917 Biochemical Analyser, Hitachi Limited, Tokyo, Japan.

Precision: Interassay Coefficient of Variation: At level of 1.2 mmol/L, CV = 3.0%, at level of 2.0 mmol/L, CV = 2.2%.

Sample type: Serum. Separated within 2 hours of collection and analysed within 24 hours of collection.

Reference: Boehringer Mannheim HDL-C plus reagent package insert. Hitachi 917 Operator's Manual.

Analyte: Serum LDL-Cholesterol

Methodology: Calculated value based upon the following modified Freidewald Formula: LDL-C = Total Cholesterol - (0.46 x TG) - HDL-C where all values are in mmol/L.

Reagents: Not applicable

Instrumentation: Not applicable

Precision: Dependent upon other assays.

Sample type: Serum. Separated within 2 hours of collection and stored at -20 deg C until assayed.

Reference: Friedewald et al., (1972). *Clin Chem 18*: 499-502. Friedlander et al., (1982). *Israel J Med Sci 18*: 1242-1252.

Analyte: Serum Creatinine

Methodology: Rate blanked Jaffe method without deproteinisation.

Reagents: Hitachi 917 Creatinine reagents (1 730 304)

Instrumentation: Instrumentation: Hitachi 917 Biochemical Analyser, Hitachi Limited, Tokyo, Japan.

Precision: Interassay Coefficient of Variation: At level of 115 mmol/L, CV = 3.0%, at level of 490 mmol/L, CV = 2.2%,

Sample type: Serum. Separated within 2 hours of collection and stored at -20 deg C until assayed.

References: Hitachi 917 Operator's Manual. Bartels, et al., (1972). *Clin Chem Acta, 37*:193.

Analyte: Serum Albumin

Methodology: Bromcresol Green technique measuring the absorbance of the green albumin/bromcresol green complex formed.

Reagents: Boehringer Mannheim Albumin (BCG) reagents (1 551 256)

Instrumentation: Instrumentation: Hitachi 917 Biochemical Analyser, Hitachi Limited, Tokyo, Japan.

Precision: Interassay Coefficient of Variation: At level of 26 g/L, CV = 0.9%, at level of 39 g/L, CV = 1.2%.

Sample type: Serum. Separated within 2 hours of collection and stored at -20 deg C until assayed.

Reference: Hitachi 917 *Operator's Manual*. Tietz, N.W. (1986). *Textbook of clinical chemistry.*

Analyte: Urinary Protein

Methodology: Alkaline Benzathonium Chloride method.

Reagents: Urine Protein Reagent, Roche Diagnostics, Australia.

Instrumentation: Hitachi 917 Biochemical Analyser, Hitachi Limited, Tokyo, Japan.

Precision: Interassay Coefficient of Variation:At level of 0.15 g/L, CV = 13.3%, at level of 0.53 g/L, CV = 7.5%.

Sample type: 24 hour urine collection, no preservative.

Reference: Hitachi 917 Operator's Manual.

Analyte: Urinary Albumin

Methodology: Immunoturbidimetric assay.

Reagents: Boehringer Mannheim Tina-quant™ Albumin 1875400

Instrumentation: Hitachi 917 Biochemical Analyser, Hitachi Limited, Tokyo, Japan.

Precision: Interassay Coefficient of Variation: At level of 13.9 mg/L, CV = 7.2%, at level of 88.0g/L, CV = 2.3%.

Sample type: 24 hour urine collection - no preservative

References: Hitachi 917 Operator's Manual. Boehringer Mannheim Tina-quant™ Albumin Reagent package insert.

Analyte: Insulin

Serum insulin was measured by a solid phase two site chemiluminescent enzyme-labeled immunometric assay (Immunolite, DPC, Los Angeles, CA). Assay sensitivity, defined as the concentration two standard deviations above the response at zero dose was 2 mIU/L. No significant cross reaction is reported with C-peptide, glucagon of proinsulin.

Reference Range: The reference interval obtained from healthy volunteers is 6-27 mIU/L.

Precision: The imprecision of the insulin assay is 5.2% at 6 mIU/L (n = 6) and 7.1% at 33 mIU/L (n = 9).

Analyte: Free Fatty Acids

Instrumentation: Cobas Mira analyser, Roche Diagnostics, Basle, Switzerland.

Reagents: Roche Diagnostics/Boehringer Mannheim Free Fatty Acid-Half-micro Test (Cat No 1383 175), Boehringer Mannheim, Mannheim, Germany.

Precision of Assay: At the level of 0.35 mmol/L. the between run coefficient of variation is < 2.0%.

Reference Range: The following ranges were obtained on a group of apparently healthy volunteers.

Males: 0.0-0.47 mmol/L

Females: 0.0-0.59 mmolL.

Reference: Boehringer Mannheim Free Fatty Acids, half-micro test package insert. *Cobas Mira Operators' Manual.* Shimizu, S., et al. (1980). *Anal. Biochem. 107*, 193-198. Harris, R.J. (1974) *J Pediatr. 84*, 578-5843. Eastham, R.D. (1978) *Biochemical values in clinical medicine.*

Analyte: Plasma Homocysteine

Methodology: Fluorescence Polarisation Immunoassay

Reagents: Abbott IMx Homocysteine kit B3D390 (Manufactured by Axis Biochemicals ASA, Ulvenveien 87, N-0581 Oslo, Norway.

Instrumentation: Abbott IMx Analyser (Abbott Australasia, North Ryde, NSW 2113, Australia)

Precision: Coefficient of Variation: At level of 5.9 mmol/L, between run CV = 5.2%, within run CV = 2.2% At level of 10.8 mmol/L, between run CV = 4.1%, within run CV = 1.9%. At level of 21.6 mmol/L, Between run CV = 3.7%, Within run CV = 1.4%.

Sample type: EDTA plasma. Sample collected on ice, separated within 2 hours of collection, and frozen at and storage: -20 deg C until assayed.

References: Abbott IMx Homocysteine kit package insert. Abbott IMx Operator's Manual.

APPENDIX C - Blood Storage Protocol

| TEST	TUBE	AMOUNT	CENTRIFUGE	PROCESS	ALIQUOT	STORE	ASSAY AT
Total serum oxidisability	yellow	2x6ml	Jouan	3500 rpm/10min/RT	2x0.5ml starstedt	-80	UDM
NO	yellow		Jouan	3500 rpm/10min/RT	2x0.5ml starstedt	-80	SCGH
Insulin	yellow		Jouan	3500 rpm/10min/RT	2x0.5ml starstedt	-80	BIOCHEM
Apo A-1, Apo B, Lp (a)	yellow		Jouan	3500 rpm/10min/RT	1x1ml starstedt	-80	ST VIN
Sialic acid	yellow	1x3.5ml	Jouan	3500 rpm/10min/RT	2x1ml starstedt	-80	BIOCHEM
LDL particle size	EDTA	2x9ml	Jouan	3000rpm/15min/4deg	1x4ml polypro	-80	ST VIN
Vit E, CoQ	EDTA		Jouan	3000rpm/15min/4deg	2x1ml starstedt	-80	BIOCHEM
Selectins P and E	EDTA		Jouan	3000rpm/15min/4deg	2x0.5ml starstedt	-80	HAEM
vWF	blue	1x3.5ml	Jouan	3000rpm/15min/4deg	1x1ml starstedt	-80	HAEM
DNA (once only)	EDTA	1x9ml & 1x3,5ml			Freezer direct	-40	UDM
Plasma B48	EDTA	1x5ml	Jouan	2500rpm/10min/1deg	1x1ml+ starstedt	4 overnight	
Fibrinogen	blue	1x3.5ml	Hettich	4500rpm/15min/14deg	2x1ml starstedt	-80	UDM
8 isoprostanes (plasma)	5ml syringe	10ml flat polypro and 50ul BHT glutathione		3000rpm/15min/4deg	2x1ml starstedt and 5ul/BHT/ethanol	-80	UDM
8 isoprostanes (urine)	10ml				and 50ul/BHT/ethanol	-80	UDM
Creatinine	5ml						
Total protein	5ml				10ml flat polypro	-80	UDM

NB. Not all of these samples have been analysed for the current study

APPENDIX D

Ultrasound and Computer Software Images

200899

10:00

NS2

| 1 | 6 | 1 | 0 |
| 0 | 59 | 34 | 0 |
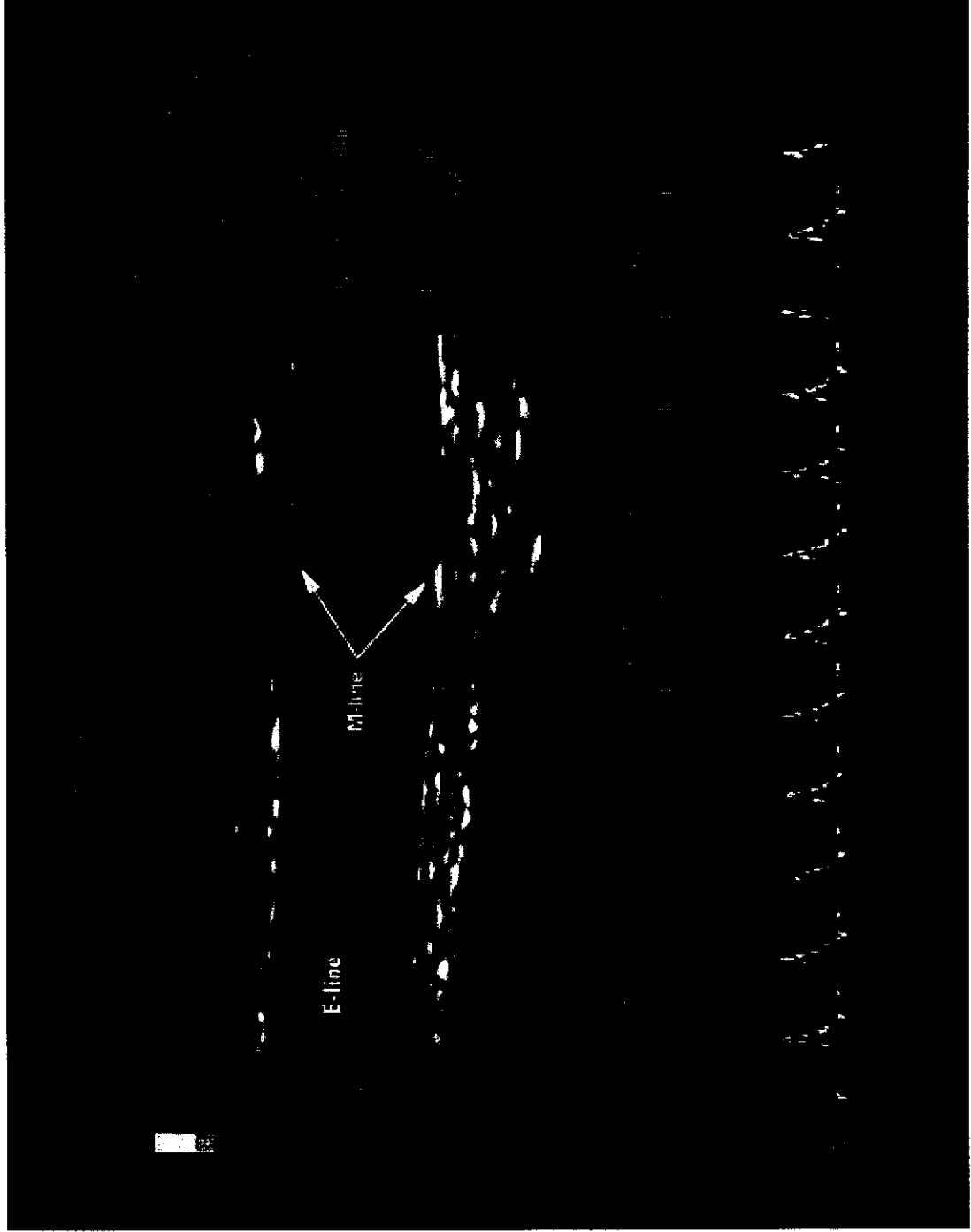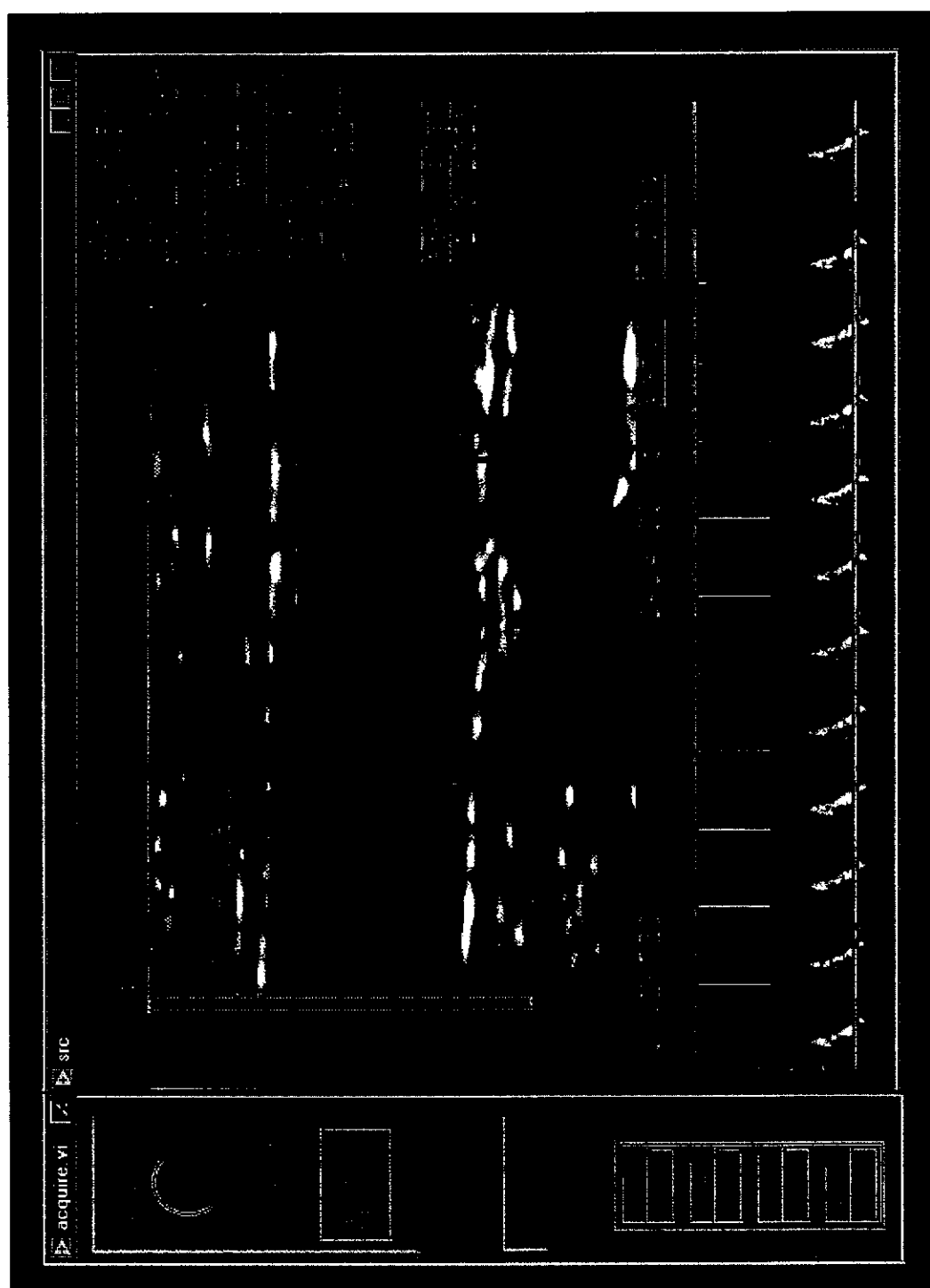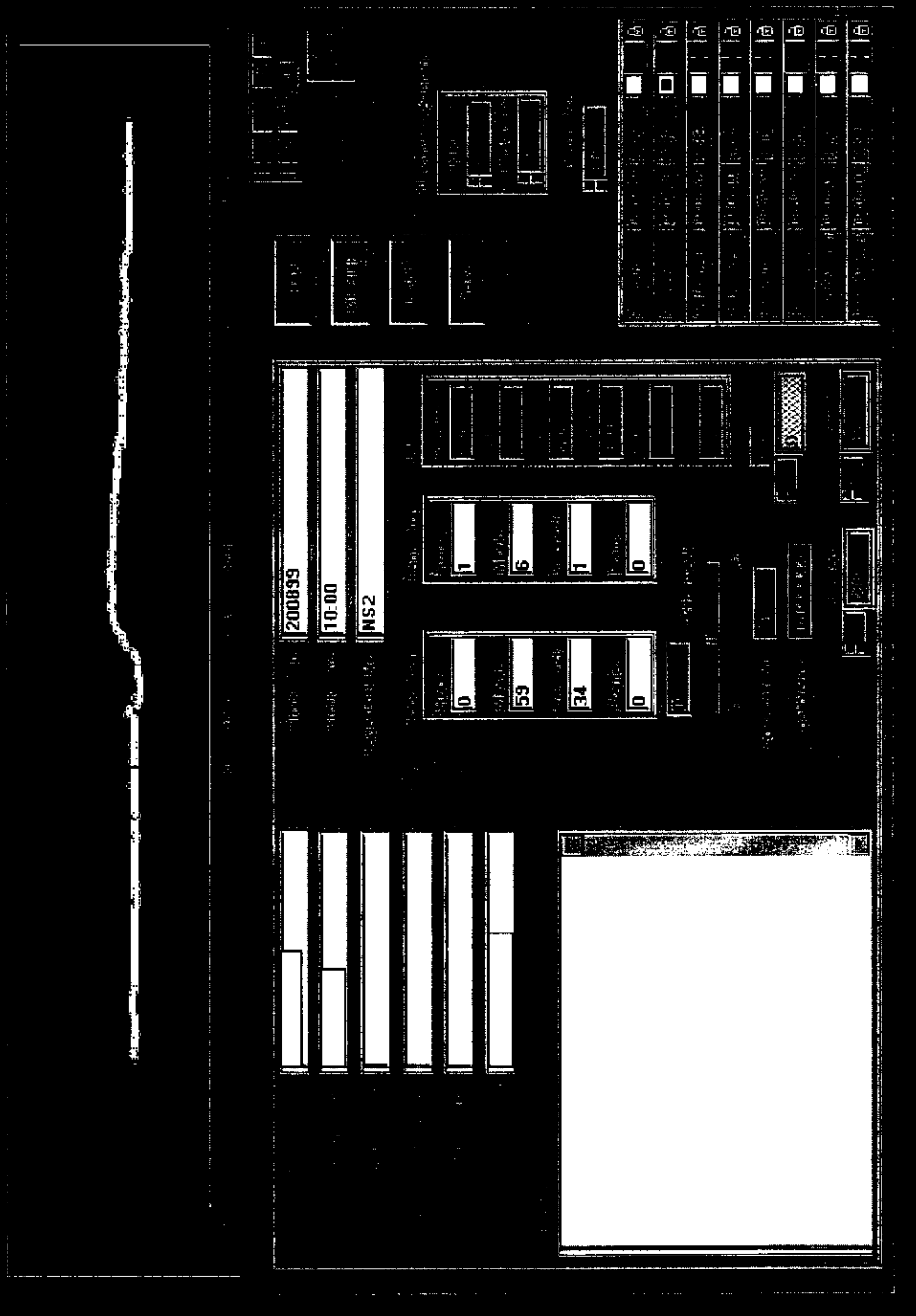
Procedure for Forearm Hyperaemia Using Strain Gauge Plethysmography

Before the patient arrives:

Check the equipment: that all tubes are connected to the machinery and where they enter the cuffs, check that they are not splitting.

Turn on:

- The air cylinder and ensure that there is sufficient gas in the bottle to complete the procedure.

- The computer and printer and find the appropriate file. Name and date it select 'Save as'

- The two voltmeters.

- The Hokanson, EC4 Plethysmograph connected Maclab/4e, ADInstrument.

- The Dinamap (Dinamap, Vital Signs Monitor 1846 SX).

- Record the room temperature and the time of day.

When the patient arrives:

When the patient arrives make sure it is the person for whom the file is named. Explain the procedure. Ask the patient to remove watch and /or bracelets as these may occlude flow. Making provision for privacy, ask the patient to remove his/her shirt. Provide a sheet or blanket to cover the patient asking him/her to lie supine. Position the patient's arms in the supports so that the forearms are just above the level of the heart. Make the patient comfortable.

Allow the patient time to settle, about 10 mins, ensure comfort and warmth.

Measure blood pressure (BP) every one minute for five cycles using the Dinamap. Take an average of the middle three systolic pressures. This will provide you with the baseline blood pressure. Name the print-out, noting that it is pre-ischaemic and attach it to the patient's details. Remove cuff.

Measure forearm length of both arms from the medial epicondyle to the crease of the wrist with a tape measure, record measurement. At a level approximately 5 cm below the medial epicondyle, measure the forearm circumferences and record. Choose a

pair of strain gauges 2 cm smaller than the forearm circumference and note the size and code of these with the other measurements. The same strain gauges will be used for subsequent visits.

Apply the two wrist and two upper arm impedence cuffs to each arm. These should be firm but not occlusive. The tubes for the wrist cuffs are pointing towards the feet. For the upper arm cuffs they are towards the head. Place the arm supports under each arm ensuring that they are comfortable and not impeding flow. The palms should be facing upwards.

Hold the strain gauges up so that they fall into alignment. Gently place them around the patient's arms at the spot marked, they should not be slack or stretched. Plug them into the voltmeters. Explain to the patient that it is important for the accuracy of the recording that he/she remains still. Ensure the patients comfort.

Balance the two voltmeters to zero.

Switch the two automatic wrist cuffs on first, then the upper cuffs inflator.

Press "Start".

Type "test" or "base 1" on the computer and press "Return".

This test period allows the volunteer time to acclimatise to the procedure and the operator to check equipment and adjust cuffs if necessary.

Check the quality of the image, there should not be a lot of tremor. Although it is possible to smooth the curve later it is better to have a good quality image to start with as this may reduce measurement error.

Press "Stop".

Allow at least one minute to pass before repeating procedure.

Switch the two automatic wrist cuffs on first, then the upper cuffs inflator.

Press "Start". Type "base 1 or 2". Obtain 3-4 satisfactory curves.

Press "Stop".

Allow at least one minute to pass before repeating procedure.

Switch the two automatic wrist cuffs on first, then the upper cuffs inflator.

Press "Start". Type "base 1 or 2". Obtain 3-4 satisfactory curves.

Press "Stop".

Reactive hyperaemia:

Explain to patient the sensation experienced during the ischaemic episode.

Apply tourniquet of sphygmomanometer over the upper cuff of the left arm and inflate to 40 mm Hg above the average BP recording taken earlier. Set timer for required time which may differ between protocols.

At the end of the required ischaemic period:

Press "Start".

Just before releasing the sphygmomanometer cuff:

1. Calibrate the voltmeters to zero.
2. Inflate the two wrist cuffs.
3. Release the sphygmomanometer cuff and simultaneously switch on the rapid cuff inflator. (check that the volt meters are on zero otherwise you will miss the initial inflection, the maximum curve).
4. Record for 4 minutes continuously
5. Press "Stop" and select "Save".

Remove impedence cuffs and apply Dinamap tourniquet. Take 2 blood pressure recordings, label the output and attach to patient's details. Remove cuff. Advise the patient to sit for a moment on the edge of the bed before standing. Thank them for their cooperation.

Turn off the computer and all the machinery.

Turn off air cylinder.

Trouble-shooting (the following are my own observations).

Tremor on the trace: too much movement of the upper impedence cuffs may cause this or the strain gauges not being firm enough. Flat trace: Strain gauges are too loose. Flattened curve: strain gauges are positioned too high. Note that the number of baseline measurements and the length of the hyperaemic period will vary according to the protocol. Avoid conversing with the patient during the procedure as this may increase blood pressure.

APPENDIX F

Medical Form

Screening Questionnaire   Date_____ Patient Code_____

Name_____

Unit No_____

Age_____ DOB_____   Gender_____

Address_____

_____

Post Code_____

Telephone Home_____Work_____

Attending clinic at RPH, SCGH or Fremantle (please circle).

Name of Doctor _____

Name and address of General Practitioner

_____

Are you on a special diet or fluid restriction? Yes/No.
If yes what kind?_____

Do you smoke? Yes/No. If yes how many per day? _____

Have you ever smoked? Yes/No. If Yes when did you stop?

_____

How   much   alcohol   would   you   consume   on   a   normal   day   or   week?

_____

Are you currently taking any medications prescribed by your doctor? Yes/No.
If so what are these?

_____

Are you currently taking medication you buy at a health food shop, grocery shop or chemist?
Yes/No. If so what are these?

_____

Are you taking or have you ever taken cholesterol lowering medication? Yes/No. If yes what was it called?

_____

When was the last time you had your blood fat levels measured?

_____

Do you know what they were?

_____

Are you taking or have you ever taken medication for high blood pressure? Yes/No. If yes what was it called?

_____

Are you taking or have you ever taken immunosuppressive medication, for example, cyclosporin or cyclophosphamide? Yes/No. If Yes what was it called?

_____

Have you ever been told by a doctor that you have any of the following conditions?

Diabetes   **Yes/No**
Thyroid disease Yes/no
Liver disease   Yes/No
Gall bladder disease or gallstones Yes/No
Muscular disease Yes/No
Rheumatic disease Yes/No
G.I. disease Yes/No
Heart problems ie angina, MI. or stroke Yes/No
High blood pressure Yes/No   Low blood pressure Yes/No
Asthma Yes/No
Blood diseases Yes/No
Allergies Yes/No
Nervous disorders Yes/No
Recent major surgery

Renal disease Yes/No
When diagnosed and treatment
Onset of proteinuria if known
Biopsy date

Height   Weight   Waist   Hip   BMI

Blood pressure   Pulse

Urinalysis_____

ON EXAMINATION

JVP

NAD   Comments_____

PRECORDIUM

NAD   Comments_____

LUNGS

NAD   Comments_____

ABDOMEN

NAD   Comments_____

NEUROLOGICAL

pupils   NAD
reflexes   NAD
gait   NAD
fundoscopy   NAD

ECG

Urine: 24 hr urine collection for nephrotic patients.

First early morning specimen for controls on study day.

Screening bloods: UEBG Cr, FBP, CRP, LFT (AST and GGT), folate, uric acid, CK, chol, Tg, HDL, LDL.

Baseline bloods: Chol, Tg, HDL, LDL, Lp (a), CRP serum albumin, UEGB Cr, uric acid, CK. homocysteine, free and bound.

Informed Consent?

Eligible/ Ineligible

Next appointment

Lipid Clinic   Renal Clinic   UDM

Signed