ENDOTHELIAL DYSFUNCTION AND CORONARY HEART DISEASE RISK FACTORS IN WOMEN WITH SYSTEMIC LUPUS ERYTHEMATOSUS (SLE)

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It appears indeed by no means improbable that the internal coat of arteries may be one of those parts over which some specific diseases may exert their peculiar influence.

Joseph Hodgson

1815

Contents

List of tables

List of Figures

Abstract

Introduction:

Systemic lupus erythematosus (SLE) is a chronic multi-system immuno-inflammatory disease. With improved survival over the recent decades, the problem of accelerated atherosclerosis has emerged as a major cause of morbidity and mortality in SLE. The prevalence of CHD in large clinic series is 6 – 10% and the mean age at the time of CHD events is around 48 years. Autopsy and studies of sub-clinical atherosclerosis showed a prevalence of 30 – 40%. Although the classical Framingham risk factors are more prevalent in SLE, there is evidence that even after adjustment for these factors, the risk of CHD is approximately 8 fold higher compared to the general population. Therefore, additional novel metabolic risk factors may also contribute to the risk of CHD in SLE. Factors associated with SLE it self such as disease activity, anti-phospholipid (APL) antibodies and steroid therapy have also been implicated. Recent evidence suggests that atherosclerosis represents low grade inflammation in the arterial wall and also that endothelial dysfunction is an important mechanism in the initiation and progression of atherosclerosis. In order to study endothelial function in SLE, we sought to establish and validate an ultrasound technique to determine whether impaired flow mediated dilation (FMD) responses in the brachial artery, a surrogate for endothelial dysfunction, is prevalent in SLE. We also aimed to determine whether endothelial dysfunction in SLE is explained by classical CHD risk factors, and whether endothelial dysfunction is associated with carotid intima media thickness (IMT); a marker for sub-clinical atherosclerosis. With regard to novel metabolic risk factors, we aimed to study insulin sensitivity in the context of SLE. We also examined the frequency of a relevant polymorphism in the E-selectin gene, which is associated premature atherosclerosis in the general population and is located within the linkage area for SLE.

Methods:

Consecutive women with SLE were recruited from the Lupus Clinic in the Rheumatism Research Centre at the Manchester Royal Infirmary. Healthy

controls were recruited from the secretarial and nursing staff at Manchester Royal Infirmary and from the staff at the ARC Epidemiology Research Unit. For the insulin sensitivity study, additional community controls were from a recently completed cardiovascular study undertaken at Manchester Royal Infirmary. Demographic and classic risk factor data was collected and in patients, disease and treatment-related parameters were also assessed including disease activity and damage using standard instruments. We used doppler ultrasound measurement of FMD to assess endothelial function and dilation following glyceryltrintrate (GTN) to assess endothelium independent dilation. SLE patients also had a study of the carotid arteries to measure intima-medial thickness (IMT) and examine for atherosclerotic plaques. We used polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP) to genotype the E-selectin gene A561C polymorphism in SLE patients from three distinct populations (British, Spanish and Turkish) and their respective and controls.

Results:

Women with SLE aged < 50 years have significantly impaired FMD compared to controls. There was no difference in GTN dilation. We included all our patients (n=62) and controls (n=38) as a one group of 100 subjects in a linear regression model. In univariate analysis, factors significantly associated with impaired FMD were systolic pressure, resting diameter, 10-year risk of CHD and SLE. In stepwise multiple regression model, factors independently associated with percentage FMD were systolic pressure, BMI and SLE. Within SLE patients, in univariate analysis factors associated with FMD were resting diameter, systolic blood pressure, SLEDAI and carotid IMT. In stepwise multiple regression analysis, carotid IMT alone was independently associated with FMD. We also found that euglycaemic SLE patients have evidence of decreased insulin sensitivity, increased fasting insulin and increased pancreatic β-cell function. Steroid therapy was not a major factor to explain reduced insulin sensitivity. The E-selectin gene A561C polymorphism was significantly associated with SLE in the British and Spanish but not in the Turkish population. There was no association between the C allele and vascular function.

Conclusion:

SLE patients display evidence of endothelial dysfunction, which cannot be fully attributed to the classic CHD risk factors i.e SLE is an independent predictor of impaired endothelial function. Endothelial dysfunction is associated with markers of early atherosclerosis in SLE and therefore is a valid surrogate for early atherogenesis in SLE. Further prospective studies to determine the mechanisms and factors associated with endothelial dysfunction and to design interventions to improve endothelial function in SLE are needed. The presence of insulin resistance may represent a link between inflammation and the atherogenic metabolic changes observed in SLE and may also contribute to the increased CHD risk in SLE. The E-selectin polymorphism A561C may be directly implicated in the disease susceptibility. The significance of two latter factors will need to be further studied in a larger prospective cohort to determine their association with CHD risk in SLE.

Declaration:

I declare that no portion of work referred to in this thesis has been submitted in support of an application for another degree or qualification of this or any other university or institute of learning.

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Preface:

I am medically qualified graduated from Medical School in Benghazi, Libya 1984. I completed my rotation in medical departments at Tripoli Central Hospital. I had a grant from the Ministry of Health in Libya for a PhD degree in clinical research field. I joined the ARC Epidemiology Research Unit at University of Manchester as PhD student. On completion of my studies in the UK, I plan to return to Libya to specialize in Rheumatology and continue to develop my academic interest in connective tissue disease.

Publications and presentations arising from the work in this thesis.

Publications:

El-Magadmi M, Alansari A, The LS, Ordi J, Gul A, Inanc M, Bruce IN, Hajeer A. Association of the A561C E-selectin Polymorphism with Systemic Lupus Erythematosus in two independent populations. (J Rheumatol: 2001;28:2650-2).

El-Magadmi M, Bodill H, Ahmad Y, Durrington PN, Mackness M, Walker MG, Bernstein R, Bruce IN. Systemic lupus erythematosus: an independent factor for endothelial dysfunction in Women. Circulation (Submitted).

El-Magadmi M, Ahmad Y, Juleuka Wajed, Ian Bruce. The Bimodal Mortality Pattern of Systemic Lupus Erythematosus. Current Medical Literature (2003); 22,1-6.

Published Abstracts:

Abstract (poster), ACR Annual Scientific Meeting in Philadelphia, Pennsylvania, Oct 28-Nov 2, 2000. Hajeer A, **El-Magadmi M**, Alansari A, Bruce IN. E-selectin is a susceptibility gene for Systemic Lupus Erythematosus (SLE). Arthritis Rheum 2000; 43, 9 (Suppl): S362.

Abstract (poster), EULAR, Stockholm, Sweden, 12-15 June 2002. **El-Magadmi M,** Bodill H, Ahmad Y, Bernstein R, Walker MG, Bruce IN. Impaired Endothelium Dependent-Flow Mediated Dilation of the Brachial Artery in Patients with Systemic Lupus Erythematosus (SLE). Ann Rheum Dis 2002; 61(Suppl 1):97.

Abstract (poster). American College of Rheumatology (ACR) Annual Scientific Meeting in New Orleans, October 24- 29, 2002. **El-Magadmi M**, Turkie W, Yates AP, Sheikh N, Laing ID, Bernstein RM, Mackness M, Durrington PN, Bruce IN. Hyperinsulinemia and Insulin Resistance in Women with Systemic Lupus Erythematosus (SLE). Arthritis Rheum 2002; 46 (9) (Suppl): S395.

Abstract, British Society of Rheumatology (BSR), Manchester, UK, 1-4 April 2003. **El-Magadmi M**, Yasmeen A, Yates AP, Sheikh N, Laing ID, Bernstein RM, Mackness M, Durrington PN, Bruce IN. The Metabolic Syndrome, A Link Between Inflammation and Atherosclerotic Potential in Women with Systemic Lupus Erythematosus (SLE). Rheumatol 2003; 42 (Suppl 1): 16.

Abstract (poster), ACR Annual Scientific Meeting in Orlando. October 24- 29, 2003. **El-Magadmi M**, Bodill H, Ahmad A, Bernstein RM, Walker MG, Bruce IN. Systemic lupus erythematosus: an independent factor for endothelial dysfunction in women. To be published in Arthritis Rheum 2003; 49, (9) (Suppl).

Presentations:

European Atherosclerosis Society, Geneva 8-11 March 2001, (Podium).
A preliminary study of the E-selectin A561C polymorphism, a candidate risk factor for atherosclerosis in SLE.

American College of Rheumatology (ACR) Annual Scientific Meeting, New Orleans, October 24- 29, 2002, (Podium). Hyperinsulinemia and Insulin Resistance in Women with Systemic Lupus Erythematosus (SLE).

British Society of Rheumatology (BSR), Manchester, UK, 1-4 April 2003, (Podium). The Metabolic Syndrome, A Link Between Inflammation and Atherosclerotic Potential in Women with Systemic Lupus Erythematosus (SLE).

North West Regional Rheumatology meeting, Liverpool, UK, April 2002.
Systemic Lupus erythematosus is associated with impaired endothelial function.

Plan of the thesis:

The broad aim of this thesis is to study factors that may contribute to the increased CHD risk in SLE, in particular, endothelial dysfunction as an early marker of increased CHD risk. We planned to study whether endothelial dysfunction occurs more frequently in SLE compared to healthy controls. Also, whether endothelial dysfunction in SLE is explained by classical CHD risk factors and whether it correlates with carotid IMT a marker of sub-clinical atherosclerosis. In addition, we aimed is to explore insulin sensitivity in SLE and some other novel risk factors for CHD including a study of E-selectin A561Cgene polymorphism.

The first four chapters cover the literature search, which includes discussion of the epidemiology of SLE including mortality and a consideration of premature CHD in SLE. They also consider some novel risk factors for CHD in the general population and the role of endothelial dysfunction as a mechanism involved in the process of atherogenesis. The fourth chapter also discusses assessment of endothelial function using flow-mediated dilation. Chapter five is a brief overview of the specific aims of the project and chapter six includes some of the methods used throughout the study.

Chapters 7 – 10 provide the main results chapters in this thesis. Chapter seven describes our SLE cohort in detail. Chapters 8 – 10 cover the three main studies in this thesis flow mediated dilation, insulin sensitivity and E-selectin A561C polymorphism. Each study chapter has a description of subjects, detailed methods, results and a summary of the main points. The final chapter includes a summary of the main followed by separate discussion of the technique of measuring flow mediated dilation as well as the implications of our results in SLE and how they inform our understanding of atherogenesis in this context. The results of our findings on insulin sensitivity and E-selectin genetics are also considered in details.

Glossary of abbreviations

ACL	Anti-cardiolipin.
ACR	American College of Rheumatology.
ATP III	Adult Treatment Panel III.
AM's	Antimalarials.
ANA	Anti-nuclear antibody.
APL	Anti-phospholipid.
Apo-A1	Apolipoptortein A1
ATII	Angiotensin II
BMI	Body mass index.
CAMs	Cellular adhesion molecules.
CHD	Coronary heart disease.
CHF	congestive heart failure.
CVA	Cerebrovascular accidents.
CVD	Cardiovascular disease.
DBP	Diastolic blood pressure.
ds-DNA	Double stranded deoxyribonucleic acid.
EC's	Endothelial cells.
EDD	Endothelium dependent dilation
EDRF	Endothelium derived relaxing factor.
ENDD	Endothelium non-dependent dilation
eNOS	endothelial nitric oxide synthase
ET	Endothelin.
FBG	Fasting blood glucose.
FFA's	Free fatty acids.
FMD	Flow mediated dilation.
GTN	Glycerine trinitrate
H_2O_2	Hydrogen peroxide
HDL-C	High density lipoprotein cholesterol.
HOMA	Homeostasis model assessment .
HOMA-B	Beta cell function.
HOMA-S	Insulin sensitivity.

HQC	Hydroxychloroquine.
HSP's	Heat shock proteins.
ICAM-1	Intercellular cell adhesion molecule-1.
IDL-C	Intermediate density lipoprotein cholesterol.
IGT	Impaired glucose tolerance.
IL	Interleukin.
IMT	Intima media thickness.
INF-α	Interferon alpha.
iNOS	Inducible nitric oxide synthase
IRS	Insulin resisteance syndrome
ISDN	Isosorbide dinitrate.
LAC	Lupus anticoagulants.
LDL-C	Low density lipoprotein cholesterol.
LNMMA	N-monomethyl-L-arginine.
Lp(a)	Lipoprotein (a)
LpL	Lipoprotein lipase.
Lyso-PC	Lyso-phosphatidylcholine.
MCP-1	Monocyte chemotactic protein-1.
MI	Myocardial infarction.
MMPs	Matrix metalloproteinases.
NF-κB	Nuclear factor-kappa B.
NIDDM	Non-insulin dependent diabetes mellitus.
NO	Nitric oxide
NOS	Nitric oxide synthase
O^-_2	Superoxide anion.
$OONO^-$	Peroxynitrite.
OR	Odds ratio.
Ox-LDL-C	Oxidized Low density lipoprotein cholesterol.
PCR	Polymerase chain reaction.
PDGF	Platelet derived growth factor PDGF.
PHLP	Post-heparin lipase activity.
RLFP	Restriction length fragment polymorphism.
RR	Relative risk.

SBP	systolic blood pressure.
SLE	Systemic lupus erythematosus.
SLEDAI	Systemic Lupus Erythematosus Disease Activity Index.
SLICC/ACR	Systemic Lupus International Collaborating Clinics damage index.
TC	Total cholesterol.
TGs	Triglycerides.
TNF-α	Tumour necrosis factor alpha.
tPA	Tissue plasminogen activator.
tPAI-1	Tissue plasminogen activator inhibitor-1.
TRL's	Triglycerides-rich lipoproteins.
UAE	Urinary albumin excretion rate.
VCAM-1	Vascular cell adhesion molecule-1.
VLDL-C	Very low density lipoprotein cholesterol.
VSMCs	Vascular smooth muscle cells.

Chapter 1

1 Epidemiology and Mortality in Systemic Lupus Erythematosus

1.1 Introduction:

SLE is predominantly a disease of young women with a male to female prevalence ratio of about 1:9. It is a multi-system autoimmune disease characterised by production of autoantibodies directed against intracellular antigens and immune-complex deposition in tissues, which induces immune-mediated inflammation. Both humoral and cellular immune mechanisms are involved in SLE. The clinical course is of relapses and remissions that vary in severity and frequency from mild infrequent episodes of disease activity to a severe progressive course that result in tissue damage.

This chapter reviews the epidemiology of SLE in Europe and discusses world wide trends in the prevalence of SLE. In addition, the major causes of mortality are reviewed including the increasingly recognized problem of atherosclerosis

1.2 UK and European studies of the prevalence and incidence of SLE:

The first study in the UK by Hochberg (1987) estimated the overall prevalence of SLE in England and Wales to be 6.5/100,000 and the prevalence in females 12.5/100,000. This study used only diagnosed cases and therefore was likely to have under-estimated the true prevalence.

1.2.1 Birmingham UK:

During 1991 in Birmingham, UK, Johnson *et al* (1995) identified 242 cases (227 females and 15 males) in the population over 18 years of age (900,000). Thirty-three cases were diagnosed during 1991. The incidence rate was 3.8/100,000/year. The point prevalence rate on the 1st Jan 1992 was 27.7/100,000 overall and in females 49.6/100,000. The female to male prevalence ratio was 13:1. The peak age-specific prevalence for females was in the 40-49 years age group (83.2/100,000). Caucasians represented 85.8% of the population with a prevalence of 20.7/100,000 (Johnson *et al* 1995).

1.2.2 Nottingham UK:

Hopkinson *et al* (1993) studied the incidence and prevalence of SLE in Nottinghamshire during the period 1989-1990. In this population 95.9% were Caucasians. Multiple sources of case ascertainment were employed including: central immunology registry, physician notification of suspected cases, renal unit registry of patients on dialysis, hospital records for SLE discharges including acute psychiatric admissions screened for anti-nuclear antibodies.

Among 37 cases diagnosed over a 2-year period, 23 satisfied the ARA criteria (19 females and four males). The overall incidence rate was 3.7/100,000/year, (6.1/100,000 for females and 1.3/100,000 for males). The peak age-specific incidence rate was in the 50-59 years age group for both sexes. The median age at diagnosis was 47 years for females and 55.5 years for males. The one-year period prevalence was 24.6/100,000. The age standardised one-year prevalence rate for females was 45.4/100,000 and 3.7/100,000 for males.

1.2.3 Other studies in the UK:

In Leicester, where Asians represent 22% of the total population of 285,000, Samanta *et al* (1992) identified hospital diagnosed cases from different sources. The overall prevalence rate was 26.1/100,000, 20.2 /100,000 in whites and 50.4/100,000 in Asians). In a large survey which involved the population of Northern Ireland (1.7 million), Gourley *et al* (1997) also used multiple sources of case ascertainment (Northern Ireland regional connective tissue clinics, medical consultants, Regional nephrology clinics, a database of clinical immunology consultation, the Northern Ireland Lupus UK branch and the regional immunology laboratory) and the capture-recapture method in their analysis, which gives an estimate of the number of missed cases. Since Northern Ireland's population is almost all Caucasian in origin, only one of 422 referrals was not Caucasian. The estimated point prevalence on 1st August 1993 was 25.4/100,000 (Table 1.1).

1.2.4 SLE in migrant populations in the UK:

The ethnic distribution of the population studied in Birmingham was 85.8% Caucasian, 8.8% Asian and 5.1% Afro-Caribbean. The prevalence in these

ethnic groups was 20.7, 46.7 and 111.8/100,000 respectively, giving a prevalence ratio of 1: 2: 5. The female predominance also differed between the three ethnic groups. It was 11:1 in Caucasians, 23:1 in Asians and 31:1 in Afro-Caribbeans. There was no significant difference in incidence and prevalence in the ethnic groups according to the place of birth (Johnson *et al* 1995).

In Nottingham, the prevalence rate was highest among the Afro-Caribbeans followed by Asians and whites, being 207, 48.8 and 20.3/100.000 respectively. The Afro-Caribbeans represented only 2.1% of the population, but contributed to 13% of the cases; Asians were 2% of the population and represented 4.8% of the cases (Hopkinson *et al* 1994). In a recent study in south of London, 205 SLE cases were identified from the records of the four hospitals serving that area. The prevalence in women aged 15 -64 years in Afro-Caribbeans, recent migrants from West Africa and whites were 177, 110 and 35/100,000 respectively (Molokhia *et al* 2001) (Table 1.2).

1.3 Scandinavian studies:

In Sweden, Jonsson *et al* (1990) studied the prevalence and incidence of SLE in Lund, which has a population of 160,000, during the period between 1980 and 1986. The overall incidence of SLE was 4/100,000/year and the point prevalence at the end of 1986 was 42/100,000. The peak incidence rates in females occurred in the 55-64 years age group. A recent study from the same population between 1987 and 1991 showed a similar incidence of 4.5/100,000/year with a median age at diagnosis of 47 years and the point prevalence in December 1991 was 68/100,000 (Stahl-Hallengren *et al* 2000).

In a nation-wide study in Iceland (239,498 population) Gudmundsson and steinsson (1990) reported a total incidence rate of 3.3/100,000/year and a prevalence of 35.9/100,000 in 1990. The peak incidence rates in females occurred in the 50-74 years age-group. In Central Denmark, Voss *et al* (1998) studied the prevalence and incidence of SLE in a population of 387,841 during the period between 1980-1994. The prevalence in 1995 was 21.7/100,000. The incidence of definite SLE had increased from 1.0 to 3.6/100,000 during the study period 1980-1994. The prevalence was 37.9/100,000 in females and 4.7/100,000 in males. The peak-age incidence was in the 30-40 years age

group. Whereas, in a nation-wide survey in Finland, the reported prevalence in 1978 was 28/100,000 (Helve 1985) (Tables 1.3, 1.4).

Table 1.1 Prevalence of SLE in white Europeans, UK studies:

Region	Year of study	Reference	Prevalence/ 100,000/year	95% CI ****
Leicester	1989*	Samanta (1992)	20.2	(13 – 27.2)
Nottingham	1989-90*	Hopkinson (1994)	20.3	(16.6 – 24)
Birmingham	1991*	Johnson (1995)	20.7 ***	(17.5 – 24)
N Ireland	1993*	Gourley (1997)	25.4	(22.1 – 28.7)
London	1999	Molokhia (2001)	35**	(26 – 43)

* Used multiple cases ascertainment.
** Women aged 15 – 64 years
*** Adults > 18 y
**** 95% confidence interval

Table 1.2 Prevalence of SLE in the non-white UK population:

Region	Reference	Year of study	Ethnicity	Prevalence/100, 000 (95%CI)**
Nottingham	Hopkinson (1994)	1989-90	A-Caribbeans South Asians	207 (111 – 302) 48.8 (10.5 – 87)
Birmingham	Johnson (1995)	1991	South Asians A-Caribbeans	46.7 (31.5 – 62) 111.8 (81 – 143)
Leicester	Samanta (1992)	1992	South Asians	50.4 (27.7 – 73)
London	Molokhia (2001)	1999	Africans* A-Caribbeans*	110 (58 – 163) 177 (135 – 220)

* Women between 15-64 years of age only.
** 95% confidence interval

Table 1.3 Prevalence of SLE in white Europeans, Scandinavian studies:

Region	Reference	Year of study	Prevalence /100,000
Finland	Helve (1985)	1978	28
Iceland	Gudmundsson (1990)	1984	35.9
Denmark	Voss (1998)	1995*	21.7
Sweden	Jonsson (1990) Stahl-Hellgren (2000)	1986* 1991*	42 68
Arctic Norway	Nossent (2001)	1996*	44.9

* Used multiple case ascertainment.

Table 1.4 Incidence of SLE in the UK and European studies:

Region	Reference	Year of study	Incidence/ 100,000/year
Iceland	Gudmundsson (1990)	1984	3.3
Denmark	Voss (1998)	1994*	3.6
Sweden	Jonsson (1990) Stahl-Hellgren (2000)	1986* 1991*	4.0 4.5
Arctic Norway	Nossent (2001)	1996*	2.6
Nottingham Birmingham	Hopkinson (1993) Johnson (1995)	1989-90* 1991*	3.7 3.8

* Used multiple case ascertainment.

1.4 Trends in the epidemiology of SLE:

1.4.1 Incidence and prevalence:

There appears to have been a dramatic increase in the incidence of SLE since the early studies in the 1950s. Uramoto *et al* (1999) compared the incidence in Rochester, Minnesota, over the two periods 1950-1979 and 1980-1992. In this 99% white population, the average incidence rate has increased almost three-fold, from 1.51/100,000 in the 1950-79 period to 5.56/100,000 in 1980-92. This could be mainly attributed to the much improved ability to diagnose SLE.

Several other studies have however suggested that the incidence of SLE has been stable over the last two decades. Stahl-Hallengren *et al* (2000) compared two cohorts using the same methods, during 1981-86 and 1987-91 and reported that the incidence was constant during the eleven years of the study and that the prevalence had increased from 42/100,000 in the first to 68/100,000 in the second cohort. McCarty *et al* (1995), in Pennsylvania, reported a stable incidence between 1985 and 1990. Similarly, Hochberg (1985) also reported a stable incidence rate over the study period 1970-1977.

In studies of Northern European populations the incidence among Caucasians was broadly comparable within the UK, whereas, the prevalence was higher in Sweden, Iceland, Finland and Arctic Norway (Table 1.3). This difference could be related to difference in the level of case detection or to differences in the size of the population surveyed (much smaller in Scandinavian studies compared to UK studies). Nevertheless, it may also be related to genetic or some environmental factors associated with exposure to high northern latitude, which has been observed in other autoimmune diseases such as multiple sclerosis and insulin dependent diabetes. In support of this suggestion is the high prevalence of SLE (92/100,000) in the Native North American population of Alaska (Boyer *et al* 1991), which is more than twice that in most white populations. In addition, the three Native North American tribes with the highest known prevalence are all residing in the North West region of the USA (Morton *et al* 1976). In addition it is worth pointing out that native North Americans are descended from Asian Mongolians, therefore part of the increased prevalence could be related to this genetic background. The gradient associated with latitude may, however, suggest the presence of an additional environmental

trigger. However, a study in the two most northern counties in Norway (1978 to 1996) showed that the incidence was constant and rather low at 2.7/100,000/year and the prevalence was 44.9/100.000 (Nossent 2001).

Table 1.5 Female peak-age incidence in the UK and Scandinavian studies:

Place of study/year	Peak-age of onset (years)	Peak-age incidence
Nottingham 1990	50 – 59	18/100.000
Birmingham 1992	20 – 72	7.0/100.000
Sweden 1986	55 – 64, 65 – 74	7.5/100.000
Iceland 1984	50 – 74	15.2/100.000
Denmark 1994	50 – 59	4/100.000

1.4.2 Age at onset:

There is a trend in Scandinavia and the UK towards a higher age at diagnosis in white females compared to North American studies. In Iceland 29% of cases had their first disease manifestation after the age of 50 years and there was a sharp rise in incidence from 7 to 16/100,000 between the age groups 40 – 49 and 50 – 59 years. The peak incidence in Sweden of 7/100,000 was reported at age groups of 55 – 64 and 65 – 74 years and in Nottingham of 18/100,000 in age group 50-59 years. The median age at diagnosis in the Birmingham study was lower compared to Nottingham 37 vs 47 years, which could be related to the earlier disease onset in Afro-Caribbeans, who contributed more to the incidence in Birmingham (21%) compared to Nottingham (13%) (Table 1.5).

In the USA, the peak-age incidence in Pittsburgh was highest at 30-39 years (McCarty *et al* 1995) and in Baltimore at 25-54 years for white females and at 25-34 years for black females (Hochberg 1985). In Rochester Minnesota,

Michet *et al* (1985) showed two peaks in age-specific incidence of 6.3 and 5.0/100,000 in 25-44 and in ≥ 65 years age groups respectively.

1.4.3 Ethnic gradients in SLE prevalence:

The prevalence of SLE in central and West Africa has been estimated to be very low, based on the number of hospital diagnosed cases in Nigeria, (Greenwood 1968), Adebajo (1992) Ghana and Cote d'Ivoire (Bae *et al* 1998). However, as the prevalence among the generations of West Africans who have migrated to the Caribbean and North America is high, a prevalence gradient hypothesis has been suggested. This could be related either to exposure to new environmental factors in the new land, or that Africans are genetically susceptible to SLE but certain environmental factors in Africa, such as parasitic infections prevent disease expression. Adebajo *et al* (1992) have suggested that malarial infection is associated with high levels of tumour necrosis factor-alpha (TNF-α), which may have a protective effect against autoimmune diseases, particularly SLE. Alternatively, genetic admixture between Africans and Caucasians could render Africans more susceptible to develop SLE. This is supported by the fact that genetic admixture is a risk factor for rheumatoid arthritis in African Americans (Fraser *et al* 1996). The estimate of genetic admixture based on frequency of red blood cell antigens, ranges from 10% to 50% (Adams and Ward 1973). The prevalence of SLE is high in native Asians compared to whites. In Auckland, New Zealand, Hart *et al* (1983) estimated the prevalence in the whites and Polynesians to be 14.6 and 50.6/100,000 respectively. In China, the prevalence has been estimated to be between 40/100,000 and 70/100,000 (Chang Nia-cheng 1983).

In contrast to the prevalence gradient noted for people of African origin across the Atlantic, the prevalence among Chinese who migrated to other parts of the world remained high. In Kuala-Lumpur, where Chinese represent 36% of the population, Frank (1980) estimated that the prevalence among Chinese would be 1.5 times higher than that in natives of Malay or Indian origins. In Hawaii, which consists of whites and mixed Oriental populations, Serdula and Rhoads (1979) and Catalano and Hoffmeier (1980) confirmed the higher prevalence of SLE both in Chinese and in Oriental races as a whole compared to the white

population. In 1980 the prevalence rates among whites, Chinese, Filipinos and Japanese were 10.3, 33.5, 44.0 and 27.5/100,000 respectively. In contrast, in San Francisco, Fessel (1974) found no difference in the prevalence of SLE between Chinese and white populations.

1.5 Mortality and morbidity in SLE:

1.5.1 Survival trends in SLE:

There has been significant improvement in survival in SLE over the last four decades (Table 1.6). An early report at John Hopkins University hospital by Merrell (1955) estimated the 5-year survival rate to be less than 50%. Whereas, in the Toronto cohort, the recently reported survival rates at 5, 10, 15 and 20 years were 93%, 85%, 79% and 68% respectively (AbuShakra *et al* 1995a). Urowitz *et al* (1997) studied 720 patients in Toronto who were followed-up over three intervals; 1970-77, 1978-85 and 1986-94. The mean age at diagnosis was 32 years in the three intervals and the total number of deaths was 130. The standardised mortality ratio (SMR-the ratio of number of deaths among patients compared to sex and age matched deaths in the general population) calculated after eight years of follow-up in each of the three groups was 10.1, 4.8 and 3.3 respectively. Compared to age-specific mortality in the general population, SLE patients showed a 9.2-fold increase in risk of death in the age-group 0-54 years, and a 2.8-fold increase in those aged ≥ 55 years. In a large multi-centre study in Denmark (1975 –1995), the SMR was 4.6 and the 5-year survival was 91%, which is comparable to that in the Toronto study. However, lower 10 and 15-year survival at 76% and 64% were reported, which could be related to the high proportion of older patients in this cohort (Jacobsen *et al* 1998). In other more recent studies, with cases diagnosed between 1980 and 1996, survival at 5,10 and 15 years was 97%, 92% and 87% respectively. Stahl-Hallengren *et al* (2000) from Sweden reported survival in a cohort of 81 patients diagnosed between 1981 and 1991. The 5 and 10-year survival was 93% and 83% respectively.

Several factors have been suggested to account for the observed improvement in survival in SLE, which include:

- Improvement in the treatment of disease complications as a result of wider use of steroid-sparing immune-suppressive regimes, effective antibiotic therapy, antihypertensive drugs and improvement in replacement therapy for advanced renal disease.
- General improvement in health that resulted in increased survival in the general population as a whole. This was supported by the lower survival rates of SLE in developing countries and among low socioeconomic classes in the developed countries.
- Improved ability for diagnosis, as a result of wide availability of immunological tests and increased awareness of the disease has helped in diagnosis of more mild cases.

1.5.2 Causes of death:

Causes of death in SLE can be divided into:

1. Disease activity related: these include nephritis, severe vasculitis leading to central nervous system (CNS) disease, intestinal perforation, carditis and progressive pulmonary fibrosis.
2. Infections: this is closely associated with disease activity and high dose immuno-suppressive therapy.
3. Atherosclerotic cardiovascular disease (CVD); coronary heart disease (CHD) and cerebrovascular accidents (CVA)
4. Thrombosis secondary to antiphospholipid antibodies.
5. Thrombocytopenia or haemolytic anaemia secondary to autoantibodies.
6. Unrelated causes.

In most studies in North America and Europe (Table 1.7) the main causes of death were active disease (16-52%), infection (17-33%), and atherosclerotic cardiovascular diseases (6-25% of cases).

Deaths due to disease activity were mainly due to severe renal and CNS involvement. Ward *et al* (1995a) showed that, out of disease activity-related causes of death, nephritis followed by CNS involvement were the most common and increased the relative risk of death by 2.34 and 1.77-fold respectively.

Fatal infections are commonly due to septicaemia, pneumonia or meningitis; opportunistic and viral infections were reported with much less frequency.

Infections mostly occur in patients on high doses of steroids during high disease activity. Causative organisms are mainly typical gram+ve or –ve bacteria, whereas herpes zoster is the most common viral infection.

Atherosclerosis in the context of SLE will be discussed further in the next chapter.

1.6 Factors associated with mortality and morbidity:

These can be divided into demographic and disease related factors:

1.6.1 Demographic factors:

1.6.1.1 Gender:

The effect of gender on prognosis has been controversial. In Toronto (AbuShakra *et al* 1995a), in a large multi-centre study in the USA (Ginzler *et al* 1982) and in North Carolina (Studenski *et al* 1987) there was no significant effect of gender. On the other hand in California, Ward *et al* (1995b) and (Wallace *et al* 1981) found a lower survival in males compared to females.

1.6.1.2 Ethnicity and socioeconomic status:

The effect of race has been studied extensively in the USA. Reveille *et al* (1990) and Studenski *et al* (1987) reported that black race was an independent risk factor for decreased survival. On the other hand, Ward *et al* (1995b), Wallace *et al* (1981) and Ginzler *et al* (1982) have found, in multivariate analysis, that socioeconomic status and type of medical insurance were independent risk factors for increased mortality and the racial difference observed in univariate analysis in these studies could be explained by the significant difference in SE status between blacks and whites. The latter study represents the average survival rates of nine centres with wide variation in the percentage of blacks and public funding. It is quite generalizable within the USA across different regions and types of health care utilisation (Table 1.8). Some studies showed higher frequency of renal disease among blacks (Studenski *et al* 1987, Reveille *et al* 1990). From the John Hopkins cohort, which consisted of 198 patients (58% blacks and 42% whites), Petri *et al* (1991) assessed the effect of race, SE status and patient's compliance (physician global assessment of compliance) on morbidity as measured by the presence of important renal or neurological

disease and number of hospitalisations. The prevalence of renal disease was higher among the blacks (31% vs 18%), but blacks were less compliant and had a higher prevalence of hypertension (47.8% vs 33.7%). In the multivariate model, independent factors for morbidity were hypertension, physicians' global assessment of compliance and high steroid doses.

1.6.1.3 Age of onset:

SLE of childhood onset has been considered more severe, with higher frequency of renal involvement and worse prognosis (Wallace *et al* 1981). The effect of age at onset differs between the studies. Studenski *et al* (1987) showed no difference in survival in the younger compared to older age groups, defined by age at diagnosis before and after 55 years. However, Reveille *et al* (1990) and Ward *et al* (1995b) showed that older age of onset was associated with decreased survival.

Table 1.6 Mortality studies in SLE in North America and Europe:

Author	Number in cohort	Years of study	Number of deaths	Survival %					
				1 y %	2 ys %	5 ys %	10 ys %	15 ys%	20 ys%
Kellum and Haserick 1964	299	1949 - 1960	86	89	80	69	54	-	-
Ginzler *et al* 1982	1103	1965 – 1978	222	90	86	77	71	-	-
Wallace *et al* 1981	609	1950 – 1980	128	98	96	88	79	74	61
Halberg *et al* 1987	184	1965 – 1983	51	98	97	89	80	-	-
Reveille *et al* 1990	389	1975 – 1984	89	96	-	88	83	80	-
Ward *et al* 1995b	480	1969 - 1991	144	-	-	82	71	63	-
AbuShakra *et al* 1995b	665	1970 – 1993	124	98	96	93	85	79	68
Jacobsen *et al* 1998	513	1975 – 1995	122	97	-	91	76	64	-

Table 1.7 Causes of mortality in SLE in selected studies:

Study	Ward *et al* 95a 1969-1991 California	Abushakra *et al* 95a 1970-1993 Toronto	Helve *et al* 85 1970 – 1983 Finland	Rosner *et al* 82 1965-1978 USA	Cervera *et al* 99 1990-1995 Europe
Mean study duration/yrs	11	10	6	3.6*	5
Number of deaths	144	124	155	222	45
Causes of death: Infection	32 (22%)	40 (32%)	24 (17%)	74 (33%)	13 (29%)
Active disease	49 (34%)	20 (16%)	74 (52%)	68 (31%)	13 (29%)
Cerbrovascular disease (CVA)	8 (6%)	5 (4%)	17 (12%)	6 (3%)	5 (11.1%)**
Cardiovascular disease (CVD)	23 (16%)***	26 (21%)	-	-	-
Myocardial infarction (MI)	13 (9%)	13 (10.5%)	9 (6%)	6 (3%)	3 (6.6%)
Unrelated	-	13 (10.5%)	14 (10%)	20 (9%)	-
Unknown	10 (15%)	13 (10.5%)	-	29 (13%)	7 (15%)

* Mean duration of follow-up from study entry.

** In addition to deaths due to CVA and MI, thrombosis was the cause of death in 12 (26.7%), pulmonary thrombosis was the cause in three deaths and one was related to other causes. *** CVD deaths include 13 MI, seven CHF, two valvular heart disease and one sudden death.

In contrast Ginzler *et al* (1982) reported that older age at diagnosis was associated with better survival, and AbuShakra *et al* (1995a) showed only a mild decrease in survival in those with an age of onset above 50 years. In a meta-analysis of studies of the clinical manifestations in subjects with older-onset SLE, Ward *et al* (1989) have found that serositis, interstitial lung disease, anti-La antibodies and symptoms of Sjogren's syndrome significantly more frequent in older-onset SLE, whereas Raynaud's, fever, lymphadenopathy, neuropsychiatric symptoms and low complement were less frequent.

1.6.2 Disease factors:

Several studies have assessed the effect of disease parameters at study entry on survival. Ginzler *et al* (1982) found that impairment of renal function, low haematocrit level and a higher number of ARA criteria were independent risk factors for increased mortality. In the Toronto cohort, renal damage, thrombocytopenia, lung involvement and high disease activity (measured by SLE disease activity index (SLEDAI) >20) at presentation were predictive factors for mortality in univariate as well as in multivariate analysis. Whereas, CHD and hypertension were associated with higher mortality only in univariate analysis (AbuShakra *et al* 1995b). Overall disease activity and creatinine level at the time of renal biopsy have also been shown to be significant predictors for increased mortality (McLaughlin *et al* 1994).

Wallace *et al* (1981) reported that during the period from 1970 to 1980, five and ten-year survival rates for those with nephritis were 86% and 76% compared to 93% and 93% for those without nephritis. Jacobsen *et al* (1998) has found that independent risk factors predicting increased mortality were ischaemic heart disease, seizures, azotaemia, haemolytic anaemia, non-fatal infections, arthritis and pericarditis. However, interestingly, photosensitivity correlates negatively with mortality.

Reveille *at al* (1990) showed, in univariate analysis, that renal and neuropsychiatric involvement adversely affected survival. In multivariate analysis, only moderate to severe thrombocytopenia, was an independent risk factor for increased mortality. The impact of moderate to severe thrombocytopenia (≤100.000) on survival was markedly high during the first two

years after diagnosis. In another Danish study, the presence of a high number of ARA criteria within the first year, as well as proteinurea and azotaemia within two years of diagnosis were associated with reduced survival (Halberg *et al* 1987).

1.6.3 Morbidity and Damage:

As survival in SLE has improved dramatically over recent decades, there have been changes in the patterns of mortality with increasing deaths due to cardiovascular disease and decreasing deaths due to active disease. In view of the chronic inflammatory nature of SLE, changes in morbidity and distribution of end-organ damage are also expected as a result of longer disease duration and longer exposure to therapy. In recent years a scoring system has been developed to assess the total cumulative permanent organ damage, which allows studies to focus on the outcome of damage rather than relaying completely on mortality to study trends in disease. The Systemic Lupus International Collaborating Clinics/ American College of Rheumatology Damage Index (SLICC/ACR DI), which defines damage as irreversible impairment of function that lasts for at least six months and is not related to potentially reversible impairment due to disease activity (Gladman *et al* 1992).

SLICC damage index scores nine systems renal, neuropsychiatric, pulmonary, cardiovascular, gastrointestinal, peripheral vascular, skin, musculoskeletal and occular system and, in addition, scores are also given to premature gonadal failure, diabetes and malignancy. This system has been used to study the clinical factors associated with early organ damage and poor outcome in SLE. Rivest *et al* (2000) studied 200 patients with mean (range) disease duration of 3.8 (2-7) years. In multivariate analysis, older age at diagnosis correlated with cardiovascular, pulmonary, musculoskeletal and ocular damage. Longer disease duration correlated with cardiovascular and renal damage and higher disease activity correlated with renal, pulmonary and musculoskeletal damage. Stoll *et al* (1996) retrospectively calculated the damage score in 80 patients at 1, 5 and 10 years after diagnosis. Fifty three were Caucasians, 15 Afro-Caribbeans and nine were Asians. The mean renal damage score one year after diagnosis significantly predicted end-stage renal failure and

Table 1.8 Demographic and disease related predictors of mortality:

Abushakra et al 1995 N = 665	Ward et al1995 &1996 N = 408	Ginzler et al 1982 N = 1103†
Sex (NS)	Sex (NS)	Sex (NS)
Whites/Blacks (NS)	Whites/Blacks (NS)	Whites/Blacks (NS)
Age at diagnosis ≥ 50 years**	Age**(10 year increment)	
	Socioeconomic status and type of medical insurance **	Type of medical insurance**
Thrombocytpenia**	Thrombocytopenia**	
Renal impairment**	Nephritis**	Urinary protein excretion**
SLEDAI at presentation ≥ 20**	Seizures**	Higher serum creatinine**
Lung involvement**	Haemolytic anaemia*	Lower haematocrit**
Hypertension*	Psychosis*	Number of ACR criteria at study Entry**
MI/ angina *	Serositis*	

† Predictors assessed only at study entry, (NS) = not significant, * predictors in univariate analysis.** Independent predictors in multivariate analysis.

mean pulmonary damage score significantly predicted death within ten years of diagnosis. Ten patients had died on average 4.9 (±3.4) years after diagnosis. One patient died of myocardial infarction (MI) 8.1 years after diagnosis and four patients died of MI with associated end-stage renal failure four years after diagnosis. At ten years, the cumulative prevalence of damage in the neuropsychiatric system was 22.1%, musculoskeletal 22.1%, renal 32.4%, cardiovascular 8.8% and pulmonary 7.4%.

Damage in SLE in association with steroid therapy has been addressed by Zonana-Nacach *et al* (2000), in a study of 539 patients form the Baltimore SLE cohort. High cumulative steroid dose (defined as ≥ 36.5 gm, which is equivalent to 10 mg/day for ten years) was significantly associated with osteoporotic fractures (RR =2.5, 95% CI 1.7 – 3.7), symptomatic CHD (RR =1.7, 95% CI 1.1 – 2.5) and cataract (RR= 1.9, 95% CI 1.4 – 2.5).

The use of this index has allowed us to understand further the range of morbidity associated with SLE. Furthermore, this index highlights the importance of atherosclerosis related outcomes in patients with SLE.

1.6.4 Summary:

In general, the prevalence of SLE in Europe is between 20-30/100,000 of the total population and the incidence rates are similar and appear to have been stable over the last two decades. The higher prevalence in some studies from Scandinavia could be related to the detection of more milder cases or to the small size of the populations studied. It may also be related to genetic factors or environmental changes associated with northern latitudes. The prevalence in the UK is significantly higher in Afro-Caribbeans (5 – 6 fold) and in South Asians (2 – 3 fold) compared to white Caucasians, but since they represent a small minority, about 5%, they do not significantly affect the population estimates as a whole. The prevalence of SLE in recently migrated Africans is intermediate between that found in white Caucasians and the Afro-Caribbeans.

The incidence of SLE appears to have been constant during the last two decades. In view of a significant improvement in survival and a trend towards higher age of onset of SLE, the prevalence is expected to increase especially among older age groups. As a result long-term morbidity will become an

increasingly important problem to focus on in these patients. In particular, atherosclerosis and its consequences will represent an important clinical problem for these patients. As the overall prevalence of SLE increases, it will also, in absolute terms, become a great problem for rheumatologists to focus on and address.

Chapter 2

2 Atherosclerosis and Coronary Heart Disease (CHD) in SLE

2.1 The burden of CHD in SLE:

2.1.1 CHD mortality:

As survival in SLE patients improves, several new problems have emerged that affect long-term morbidity and mortality. One such problem is premature atherosclerosis and its consequences, especially CHD.

Cardiovascular disease due to accelerated atherosclerosis has been increasingly recognised as an important cause of morbidity and mortality in SLE. Urowitz *et al* (1976) were the first to describe a bimodal mortality pattern in SLE. In their 5-year study of 81 patients, eleven patients died. The six patients who died in the first year after diagnosis had active disease and were on a high steroid dose. Those who died after one year were on a low steroid dose with an average disease duration of 7.2 years. All had had a recent MI and only one had active disease at the time of death.

Several further analyses of this cohort confirmed this bimodal pattern (Urowitz and Gladman 1980, Rubin *et al* 1985). More recently, in an analysis of 120 deaths in this cohort, it was reported that 37% of deaths occurred at < 5 years after diagnosis and 63% occurred ≥ 5 years after diagnosis. Cardiovascular deaths accounted for 17% and 30% of the early and late deaths respectively. In 25.8% of all deaths, CVD was the primary cause of death and the majority were related to atherosclerosis, which include MI, CVA, congestive heart failure (CHF) or sudden death (Abu-Shakra *et al* 1995 b). Ward (1999) analysed 134 deaths in SLE patients from Duke University Medical Center in California, USA. Mean disease duration at death was 6.3 ± 5.5 years. Twenty three (16%) deaths were due to CVD, these include 13 MI, seven congestive heart failure, two valvular heart disease and one sudden death. A further eight (6%) deaths were caused by cerebrovascular disease. These patients were significantly older at the time of death compared to those who died due to infection or active disease. Compared to the expected death rates due to CVD in the general population, there was a two-fold increase in cardiovascular deaths in this cohort. In a large multi-centre study in the USA, with a short mean duration of

follow-up from study entry to death of 3.6 years, among 222 deaths only 3% died of MI and 3% of CVA (Rosner et al 1982). Similarly in a multi-centre prospective study in Europe, which included 1000 patients, during five years of follow up, 45 patients had died. In twelve (26.7%) of these the cause of death was related to thrombosis (coronary 6.7% and cerebral 11.6%) (Cervera *et al* 1999). On the other hand, a study with a long duration (13 years) of follow-up from the Pittsburgh cohort, showed that among the 54 deaths recorded, MI was the cause of death in six (11%) (Manzi *et al* 1997) (Table 2.1).

Since most of the cardiovascular deaths occur late after diagnosis, studies with a longer duration of follow-up will include more cardiovascular deaths than studies of short duration. Similarly, deaths due to disease activity may be under-estimated if studies do not include patients early after their diagnosis.

2.1.2 CHD morbidity:

Long-term follow-up of patients with SLE, mainly in large North American cohorts, have demonstrated a high burden of CHD as a late complication of SLE. In addition, it is now clear that, compared to women in the general population, patients with SLE have increased risk of CHD. In a study from New York (SUNY Health Science Centre in Brooklyn USA) 15% of 200 SLE patients had symptomatic CHD. MI was clinically diagnosed in 13 (6.5%) and angina alone in 17(8.5%) patients. These patients had their events at a mean age and disease duration of 47.5 and 14 years respectively (Sultan *et al* 1994).

In the Toronto Lupus Cohort, the prevalence of symptomatic CHD was reported as 8.9% (45 out of 507 patients) (Gladman *et al* 1987). Angina, MI or both were diagnosed in 17/45 (37%), 10/45 (22%), and 18/45 (40%) patients respectively. The mean age at presentation of MI was 48 (25-73) years and disease activity was low at the time of the ischaemic event in 40 (88.8%) of patients. The incidence rate of MI in SLE patients was 5/1000/year compared to 1/1000/year in the female population of Ontario. Thus, SLE patients have an overall 5-fold risk of developing CHD (Bruce *et al* 2000a).

Table 2.1 Cardiovascular deaths in SLE in selected studies:

Study	Duration	Number of patients	Number of deaths	Cardiovascular deaths n (%)
Urowitz *et al* (1976)	1976-1974	81	11	5/11 (45%)
Wallase *et al* (1981)	1950-1980	609	128	26/128 (20%)
Rosner *et al* (1982)	1965-1972	1103	222	12/222 (5.4%)
Jonson *et al* (1989)	1979-1986	86	9	4/9 (44.5%
Reveille *et al* (1990)	1975-1984	389	89	8/89 (10.8%)
Petri *et al* (1992)	1987-1992	229	10	2/10 (20%)
Abu-Shakra *et al* (1995 b)	1969-1995	665	124	31/124 (25%)
Ward *et al* (1995a)	1969-1983	408	144	32/144 (22%)
Cervera *et al* (1999)	1990-1995	1000	45	8/45 (17.7%)
Stahl-Hallengren *et al* (2000)	1981-1986	121	17	13/17 (76%)

In the Pittsburgh Lupus Cohort, the prevalence of symptomatic CHD was 7.3%, with a mean age at presentation of 48 years; 50% were premenopausal at the time of the first event. This study compared the incidence rate of MI in different age groups in SLE patients with age-matched women in the Framingham offspring study. The rate ratio in the 35-44 years age group [(8.39/1000 person-year) / (0.16/1000 person-year)] was 52.4 (95%CI 21.6 – 98.5), which means that SLE patients in this age group were 52.4 times more likely to have MI than matched women from the general population (Manzi *et al* 1997).

Further confirmation of the increased risk of CHD in SLE comes from analysis of hospital admission registries in the State of California. In this population it was found that for subjects 18-44 years old, MI, CHF and CVA were 8.5, 13.1 and 10.7 times more common in SLE compared to women in the general

population. In the 45 – 60 years age group the ratios were 2.8, 2.5, and 3.3 (Ward 1999). This observation has also been noted in a Northern European population. Jonsson *et al* (1989) found that, during a follow-up of 86 patients between 1980 and 1986, MI was diagnosed in eight patients; two of the affected women were 36 years of age. Compared to the expected rate of MI in a control population over the same period, MI was nine times more common in SLE patients. SLE patients have at least a 5 – 9 fold increased risk of developing CHD compared to healthy women in the general population. The prevalence of symptomatic CHD in SLE patients is approximately 6 – 10%. CHD in SLE appears to be premature and accelerated in nature since the mean age at presentation is below 50 years, and frequently affects women in their premenopausal or perimenopausal years. This suggests that SLE is a strong risk factor for CHD and that SLE abolishes a woman's premenopausal protection from CHD.

2.1.3 Sub-clinical CHD:

2.1.3.1 Autopsy studies:

The first indication of the magnitude of sub-clinical atherosclerosis in SLE came from an early autopsy report of 35 unselected SLE cases who died of different causes at a mean age of 32 years. This report showed at least 50% narrowing of one or more coronary arteries in 42% of cases (Bulkley and Roberts 1975).

Similarly, Haider and Roberts (1981) studied 21 young female patients with SLE, aged 16 to 37 years, and found >75% narrowing of one or more coronary arteries in ten of 21 young women with SLE who died between the ages of 16-37 years.

Rubin *et al* (1985) reviewed 51 cases that died between 1970 and 1983 in the Toronto cohort. Autopsy was performed on 27 unselected cases with a mean age of 46.9 years. Significant atherosclerosis was detected in the coronary arteries and aorta in eleven (41.7%) cases. There was no difference in CHD risk factors profile between the autopsy cases and the rest of the study group. More recently, from the same cohort autopsy of 40 cases showed moderate to severe atherosclerosis in 21(54%) cases regardless of the cause of death (Abu-Shakra *et al* 1995 b).

2.1.3.2 Carotid artery plaques:

Using B-mode Doppler of carotid arteries, Manzi *et al* (1999) studied the prevalence of carotid plaques in 175 unselected consecutive patients from the Pittsburgh SLE Cohort (mean age and disease duration 44.9 and 8.6 years respectively). Focal carotid plaques were detected in 70 (40%) patients. The mean age of those with and without plaques were 50.5 and 40.4 years respectively. In addition, 15% of those with plaques had clinical CHD compared to 3.8% in those without. The prevalence of carotid plaques increased with age, from 21% in those aged ≤ 35 years to 100% in those aged > 65 years. Carotid plaques were detected in 27.6% of the 98 premenopausal patients.

From the same cohort, Manzi and Wasko (2000) have studied 158 SLE patients who were normotensive, non-diabetic and with no past history of cardiovascular disease and 99 healthy matched controls. Carotid plaques were detected in 29% of the patients and in 16% of the controls (P=0.02). When 106 premenopausal SLE patients were compared with 73 matched healthy controls, the prevalence of plaques was 20% vs 8% (P=0.02). After adjusting for age, blood pressure and menopause, SLE patients were still more likely to have plaques (OR=2.37, 95% CI (1.1-5.09)). Premenopausal SLE patients were especially more likely to have plaques OR=3.7 (95% CI 1.37-10.2). In a smaller controlled study, Roman *et al* (2001) have compared 18 SLE patients, 4 patients with primary anti-phospholipid syndrome and 48 controls matched for age and hypertension. Carotid atherosclerosis was 4-times more common in patients compared to controls (41% vs 9% p<0.005). A history of hypertension was an independent risk factor for carotid atherosclerosis.

Increased pulse wave velocity (PWV) as a measure of vascular stiffness is a potential early maker of vascular disease in large arteries. A study of 220 SLE patients from the Pittsburgh cohort (Selzer *et al* 2001) showed increased PWV in common carotid and femoral arteries.

2.1.3.3 Isotope myocardial perfusion Imaging:

Myocardial perfusion imaging has also been used to detect asymptomatic involvement of coronary arteries. Hosenpud *et al* (1984) used post-exercise thallium 201 perfusion imaging technique in a study of 26 unselected SLE

patients aged less than 50 years and with no past history of CHD. Segmental perfusion defects were detected in ten (38%) patients; five had reversible defects, four had irreversible defects suggesting previous infarction or fibrosis and one had both defects. Perfusion defects were significantly more frequent in patients with, compared to those without a past history of pericarditis (50% vs 19%, $p<0.05$). The reason for this was unclear, but the authors suggested that it might be due to extension of inflammation from the pericardium to the epicardial vessels or due to associated myocarditis. However, segmental and sub-segmental defects noted in this study corresponded with the distribution of the larger epicardial vessels and not with the diffuse involvement of small vessel disease seen in myocarditis.

Bruce *et al* (2000b) used single photon emission computed tomography (SPECT) and dual isotope myocardial perfusion imaging (DIMPI) during rest and following dipyridamole stress, on 130 consecutive SLE patients. The mean age and disease duration were 54.1 and 14.6 years respectively. 13 (10%) patients had a history of angina or MI. Perfusion defects were found in 52 (40%) of the overall patients, and in 41(35%) of those with no history of CHD. In 47(90%) of cases the perfusion defects were reversible. In 37(71%) cases the perfusion defects were seen in the region of single vessel territory. respectively. Eighteen (14%) patients had an ejection fraction <50%. Myocardial perfusion defects have also been detected in paediatric lupus patients who would be expected to have less CHD risk factors and shorter disease duration. Gazarian *et al* (1998) studied a group of asymptomatic patients with a median age of 15.9 (10.5-19.5) years using exercise thallium scan, radionuclide angiography with multiple gated acquisition (MUGA). Five of 31 patients (16%) had perfusion abnormalities.

Overall, these studies demonstrate a high burden of subclinical disease in SLE. Approximately 35-40% of unselected patients have early evidence of atherosclerosis. The frequency when using different imaging techniques is more or less similar to that noted in autopsy studies.

2.2 Risk factor studies in SLE

2.2.1 Risk factors identified in clinical and subclinical studies:

Since the prevalence of SLE in the general population is low, most clinical studies of CHD in SLE are of limited size. The number of patients with symptomatic CHD identified in SLE studies ranges between 19 and 45, compared to hundreds or more in studies of CHD in the general population. Therefore, it has not been possible to study a large number of potential risk factors in any of the SLE studies and only a few independent factors can be expected in any study. On the other hand, detection of subclinical disease provides a larger group for assessment of a wider range of risk factors.

In the previous three large clinical studies from SLE cohorts in North America (Toronto, Baltimore and Pittsburgh), hypercholesterolaemia and older age at diagnosis were significantly associated with CHD in all three studies. In the Baltimore and Pittsburgh studies, disease duration, steroid therapy and hypertension were also significant factors (Table 2.2). Despite differences in the cut off level for definition of hypercholesterolaemia that contributed to the wide variation in its prevalence (16% to 44%), and despite differences in ethnic distribution between the studies (Caucasians represent 54% in Baltimore and 87% in the Pittsburgh cohort) hypercholesterolaemia has been identified as a strong predictive factor for both clinical and subclinical disease. The association of hypertension with vascular events, MI, angina, CVA, and peripheral vascular disease (PVD) in SLE has been addressed in a prospective study from the Toronto cohort (Rahman *et al* 2000). Seventy-five hypertensive and 75 normotensive SLE patients, diagnosed between 1980 and 1988 and seen within one year of diagnosis, were followed up prospectively, seventeen hypertensive patients (22.7%) developed at least one vascular events (7 CHD, 5 CVA and 5 PVD) compared to six (8.0% $P<0.05$) in the normotensive group (3 CHD, 2 CVA and 1 PVD). The hypertensive group had significantly higher total cholesterol, serum creatinine and use of steroid therapy. In multivariate logistic regression, the best predictor of vascular events was hypercholesterolaemia. Therefore, the effect of hypertension was confounded by hypercholesterolaemia in the hypertensive patients. In a Swedish cohort (Jonsson *et al* 1989), patients who developed MI, compared to the rest of the patients, had a longer median

disease duration (19.5 vs 6.5 years) and longer exposure to steroid therapy, 88% vs 46% of the observation time.

In a univariate analysis, factors associated with carotid plaques in the Pittsburgh cohort (Manzi *et al* 1999) were age, body mass index, menopausal state, longer disease duration, high systolic and diastolic blood pressure, total and low density lipoprotein cholesterol, fibrinogen, C-reactive protein, damage score (SLICC), disease activity, longer duration, higher cumulative dose of steroids and previous coronary event. In a logistic regression model, independent risk factors for plaques were older age at study (OR 1.85), increased systolic blood pressure (SBP) (OR 1.03), increased LDL-cholesterol (OR 2.0) and longer duration of steroid therapy (OR 1.1). In a smaller study by Roman *et al* (2001), hypertension was also an independent risk factor for carotid plaques. In a study of risk factors associated with abnormal myocardial perfusion defects (Bruce *et al* 2000d). Perfusion defects were detected in 43% of 140 SLE patients. Factors significantly associated with perfusion defects included known clinical CHD (OR=8.5, $p<0.01$), postmenopausal state (OR=2.7, $p<0.05$), hypertension (OR-2.7, $p<0.01$), previous steroids side effects (OR=2.4, $p<0.05$), anti-malarial therapy ever (OR=2.8, $p<0.05$) and total cholesterol/high density lipoprotein ratio (TC/HDL-C) ratio (OR=1.7 for each increase of 1.0, $p<0.05$).

2.2.2 Dyslipidaemia and its implication in CHD:

2.2.2.1 Cholesterol and subsequent CHD:

Evidence for the influence of hypercholesterolaemia on subsequent development of CHD came from a study from the Toronto SLE cohort (Bruce *et al* 1999) in which 134 patients, diagnosed between 1974 and 1987, were followed up. The patients were divided into three groups according to total cholesterol levels in the first three years of diagnosis. Normal total cholesterol (TC) (<5.2 mmol/l) was found in 24%, variable hypercholesterolaemia in 35% and sustained hypercholesterolaemia in 40.3% of the patients. MI or angina occurred in one (3%), three (6.4%) and 15(27%) of the groups respectively and 79% of CHD events occurred in the sustained hypercholesterolaemia group.

Most of the studies of CHD risk factors in SLE included only total cholesterol as a variable and did not include the whole lipid profile or lipid sub-fractions. In

view of the systemic inflammation and autoimmune activity associated with SLE, this may have an influence on the normal interaction between the arterial wall and the lipid sub-fractions.

2.2.2.2 Dyslipidaemia and disease activity:

Lipid profile abnormalities are common in SLE and have been described during both active and inactive disease. Borba and Bonfa (1997) showed in a study of 36 SLE patients and 30 controls, that, during inactive disease, there was significantly higher triglycerides (TGs), very low density lipoprotein-cholesterol (VLDL-C) and low high density lipoprotein-cholesterol (HDL-C) levels in the patients compared to controls. In the presence of active disease these changes become further aggravated. With active disease 79% of patients had an at-risk level of HDL-C compared to 29% during inactive disease. Total cholesterol (TC) and low density lipoprotein-cholesterol (LDL-C) were significantly higher during active, compared to inactive disease, but there was no significant difference between levels in patients during inactive disease and controls (Borba and Bonfa 1997). In addition to this pattern of a high TGs and VLDL-C combined with low HDL-C, low level of apolipoprotein-A1 (ApoA-1) also have been reported in a group of paediatric lupus patients before initiation of steroid therapy. After initiation of steroids there was a further significant rise in TGs, VLDL-C and HDL-C compared to pre-treatment levels (Ilowite *et al* 1988). In a cohort study, Bruce *et al* (2000c) found that TGs and VLDL-C levels were significantly higher in SLE patients compared to community controls, however levels of HDL-C and LDL-C were not different between the two groups.

Impaired activity of lipoprotein lipase (LPL), the enzyme which hydrolyses TGs in TGs-rich lipoproteins, chylomicrons and VLDL-C, has a strong influence on lipid profile. In a large controlled study, Henderson *et al* (1999) reported a 22% decrease in post-heparin lipase activity (PHLA) in patients with CHD compared to healthy controls and that PHLA correlated negatively with TGs and positively with HDL-C. Inflammatory cytokines, such as interleukin-1 (IL-1), interleukin-6 (IL-6), tumour necrosis factor-α (TNF-α) and interferon-α (IFN-α) are involved in the pathogenesis of SLE (Linker-Israeli *et al* 1991, Kim *et al* 1987) and have been shown by Feingold *et al* (1994) and Ehnholm *et al* (1982) to down regulate

the enzyme LPL in animal models. Low activity of LPL may explain the disordered lipoprotein metabolism in SLE. Kawanishi *et al* (1977), have found a low levels of PHLA with a strong negative correlation with immunological parameters of active disease in SLE patients. A recent study of chylomicron metabolism in SLE also showed abnormalities both in lipolysis of TGs in the chylomicron core and the up-take of chylomicron remnants by the liver. In this study a lower PHLA activity in serum samples from SLE patients compared to controls, was also noted (Borba *et al* 2000).

2.2.2.3 Dyslipidaemia and disease therapy:

The effect of steroids and anti-malarial drugs on the lipid profile in SLE has also been a subject of interest in several studies. Ettinger *et al* (1987) studied 46 SLE patients and 30 controls. Lipid profiles in patients showed significantly higher TGs, TC, LDL-C and lower HDL-2 sub-fraction. Patients on steroid therapy, in particular, had significantly higher TGs and LDL-C compared to those not on steroids. Wallace *et al* (1990) studied the effect of the anti-malarial drug hydoxychloroquine (HCQ) in 155 women with SLE. HCQ therapy was associated with lower TC, TGs and LDL-C, but showed no significant effect on HDL-C.

The effect of steroids on the lipid profile appears to be dose dependent. Leong *et al* (1994) found that patients on steroid doses of ≥30 mg/day had significantly higher TGs, TC, LDL and TC/HDL ratio compared to those on <30mg/day. In another study of 64 patients, the lipid profile was not adversely affected in patients taking <10mg prednisolone/day. In contrast, patients on >10mg/day had significantly higher TGs and apolipoprotein B than controls (MacGregor *et al* 1992). In a study of 134 patients who had their TC level checked several times during the first three years of diagnosis. Bruce *et al* (1999) found that 75% of patients had at least one elevated TC level. This was elevated at most visits (sustained) in 40.3% of patients. The best predictors of sustained hypercholesterolaemia were cumulative steroid dose and no anti-malarial therapy. Thus, while steroids may adversely affect the lipid profile, anti-malarials (AM's) may help improve it.

In a large prospective study of 264 SLE patients, high TC was present in 66% of patients on a steroid dose of ≥10mg/day and in 49% of patients on <10mg/day. After adjusting for factors known to affect cholesterol level, steroid doses of <10mg, 10 – 20mg and >20mg/day were associated with 5%, 20% and 33.3% increases in TC levels respectively. In multivariate analysis, predictors of high TC, other than steroids, included female sex, older age and proteinuria. A 10 mg change in prednisolone dose was associated with a mean change of 0.19 mmol/l in serum TC, a 5.5lb change in weight and a 1.1mmHg change in blood pressure. In contrast HCQ therapy was associated with a lower mean (+/-SE) TC (0.23 mmol/l +/- 0.09) in both uni- and multivariate regression models and, in effect, counter-balanced the effects of 10 mg of prednisolone (Petri *et al* 1992, 1994).

Rahman *et al* (1999) also found that the initiation of AM's in patients on a stable dose of steroids reduced TC by 11.3% at three months and 9.4% at six months. Similarly, initiation of steroids to patients on AM's caused less of an increase in TC than when steroids were started without AM's. The lipid lowering effects of AM's were less marked when not used with steroids, which suggests that AM's have their major effect on steroid treated patients.

2.2.3 Do traditional risk factors explain all the excess risk in SLE?

Traditional risk factors for CHD are important in the development of atherosclerosis in SLE. Only a few studies have systematically studied the prevalence of such risk factors in SLE. More importantly, which factors are over-represented in SLE population has not been extensively studied.

In the Johns Hopkins lupus cohort in Baltimore, Petri *et al* (1992) studied the prevalence of the seven key traditional CHD risk factors in 225 patients (92% females, 54% blacks and 44.4% whites). The mean age ± SD was 38±12 years and the mean disease duration was 8.1 years. Only 3% of the cohort had no risk factors. In contrast, 53% had ≥3 risk factors and 30% had two risk factors. The most common combination in the two-risk factor group was sedentary life style and hypercholesterolaemia. In the three risk-factor group the most common combination was sedentary life, hypercholesterolaemia and family history of CHD. The most prevalent risk factor was sedentary life style (70%),

which was related to fatigue, anaemia, arthritis and treatment-related complications. The prevalence of hypercholesterolaemia (>5.2mmol) was 56.3%. Hypercholesterolaemia was associated with increased steroid dose and increased age. Significant obesity was present in 37% and it was more common in blacks. In general, this study has suggested a high prevalence of traditional risk factors in SLE, but it did not compare the prevalence in SLE with that of the general population in Baltimore. However, it showed that traditional CHD risk factors were significantly more prevalent in blacks than in whites (mean± SD: 3.8±1.3 vs 3.6± 1.4, P=0.02).

Bruce *et al* (2003) studied the prevalence of traditional risk factors in 250 SLE patients and 250 controls matched for age and ethnicity. Hypertension and diabetes were both significantly more frequent in the patients (33% vs 13% and 5.0% vs 1%) respectively. Sedentary life style was significantly more frequent in SLE patients (15% vs 9%). The overall number of risk factors/patient was significantly higher in the patients compared to controls (1.84 vs 1.54, P <0.01).

In patients with SLE, the disease or its therapy can have a significant influence on traditional risk factors for CHD. Disease activity status, steroid therapy and proteinuria all have unfavourable effect on the lipid profile. Steroid therapy may induce or aggravate existing hypertension and glucose intolerance. In addition, renal disease is also associated with hypertension.

Although, traditional CHD risk factors are more prevalent in SLE, they do not explain the whole risk observed. In two Canadian prospective SLE cohorts, Esdaile *et al* (1998) compared the incidence rate of MI and stroke in SLE patients with the expected outcomes according to their traditional risk factor profiles. Even after adjusting for the effect of traditional Framingham risk factors, the relative risk of MI and stroke remained high at 8.3, (95%CI 4.9-12.4) and 6.7 (95% CI 3.6-10.9) respectively. They concluded that a diagnosis of SLE is the strongest known risk factor for MI and stroke. In another study Rahman *et al* (1998) found that the mean number of traditional risk factors per each ischaemic event in females with SLE has been shown to be 2.0 ± 0.77 compared to 2.9±1.19 in females with premature CHD. Therefore, compared to female patients with premature CHD in the general population, SLE patients, on average develop CHD with one risk factor less than expected.

Table 2.2 Significant risk factors associated with clinical coronary artery disease in three large North American lupus cohorts:

Toronto cohort	Baltimore cohort	Pittsburgh cohort
Older age at diagnosis of SLE	Older age at diagnosis of SLE*	Older age at diagnosis of SLE*
Hypercholesterolaemia	Hypercholesterolaemia*	Hypercholesterolaemia*
Hypertension	Hypertension*	-
Pericarditis	Longer duration of steroid therapy*	Longer duration of steroid therapy
Myocarditis	Longer disease duration	Longer disease duration
Congestive heart failure	Antihypertensive therapy	Postmenopausal status
Diabetes	Obesity*	-
Hypertriglyceridaemia	-	-

* Significant in multivariate analyses.

Table 2.3 Prevalence of traditional risk factors in two large studies from Baltimore USA and Toronto Canada:

Risk factor	Petri *et al* 1992 n = 225	Bruce *et al* 2003 n = 250
Sedentary life	70%	15%*
Hypertension	41%	33%
Diabetes	7%	5.0%
Hypercholesterolaemia	56%†	34%†
Family history of CHD	41%	20%
Current smokers	35%	17%

* Used physical activity index questionnaire. † TC > 5.2 mmol/l.

2.3 The potential influence of disease related factors to atherosclerosis development in SLE:

2.3.1 Influence of disease activity

Endothelial cells (EC's) activation is associated with increased expression of cellular adhesion molecules (CAMs), secretion of inflammatory cytokines and increased recruitment of leukocytes into the vascular wall. Disease activity in SLE correlates with the degree of endothelial activation with increased expression of CAMs (Belmont *et al* 1994) and higher expression of inflammatory cytokines IL-6, IL-10 (Waszczykowska *et al* 1999, Stuart *et al* 1995). There is evidence from animal models that IL-1 and TNF-α play a role in atherosclerosis, since blockage of TNF-α and IL-6 in apolipoprotein-E deficient mice was found to be protective against atherosclerosis (Elhage *et al* 1998).

Atherosclerosis is currently considered an inflammatory process in the arterial wall with inflammatory cell infiltration present in different stages of atherosclerotic lesions. There is good evidence from large prospective studies that the basal rate of inflammation, reflected in measured inflammatory markers, predicts future risk of developing CHD in the general population (Ridker *et al* 2000). In view of the chronic systemic inflammatory nature of SLE, recurrent flares and/or chronic sub-clinical disease activity may be an important factor that contribute to the development of atherosclerosis in SLE. Higher CRP was found to be associated with the presence of carotid plaques in SLE patients (Manzi *et al* 1999), and also a history of pericarditis or myocarditis was associated with presence of clinical CHD (Gladman *et al* 1987). Similarly, a history of pericarditis was significantly associated with myocardial isotope perfusion defects (Hosenpud *et al* 1984). More recently, from the Toronto Lupus Cohort, lung involvement was found to predict CHD in SLE (Bruce *et al* 1999).

In vitro studies also suggest that other mechanisms associated with SLE may play a role in atherogenesis. There is evidence of specific endothelial cell damage in active SLE, since incubation of normal polymorphonuclear leukocytes in sera from patients with SLE can be shown to be cytotoxic to cultured human vascular EC's (Hashimoto *et al* 1992).

Carvalho *et al* (1999) found that antibodies to EC's induce EC's to secrete inflammatory cytokines, including IL-1, and express CAMs intercellular adhesion

molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and E-selectin that increase leukocyte adhesion and migration into the vessel wall. Lai *et al* (1996) demonstrated the ability of anti-DNA antibodies in vitro to increase the expression of E-selectin, ICAM-1 and VCAM-1. Furthermore, incubation of normal human aortic smooth muscle cells with sera from SLE patients accelerated cholesterol uptake 1.5-6.0 times compared to incubation with normal sera (Kabakov *et al* 1992). Incubation of SLE sera with cultured human aortic EC's have been shown to down-regulate the enzyme sterol 27-hydoxylase in the EC's and also in monocytes. This enzyme converts cholesterol to a non-atherogenic compound 27 hydroxycholesterol and promotes cholesterol efflux from monocytes thus decreasing foam cell formation (Reiss *et al* 1998).

2.3.2 Oxidised low density lipoprotein antibodies (anti-ox-LDL):

Oxidative modification of LDL-C generates different forms of oxidised short chain fatty acids that bind to lysine residues on apolipoprotein-B of LDL-C, and it is the oxidized LDL-C (ox-LDL-C) molecules which are taken up by macrophage scavenger receptors, leading to lipid accumulation and foam cell formation (Witztum 1994) and is recognised by anti-ox-LDL-C antibodies (Palinski *et al* 1990). Since LDL-C contains proteins and phospholipids, and anti-ox-LDL-C antibodies bind to lipid-protein complexes, therefore anti-ox-LDL-C antibodies can be considered within the group of antiphospholipid (APL) antibodies. Anti-ox-LDL-C antibodies are heterogeneous and some of them cross-react with anti-cardiolipin (ACL) antibodies, which suggests a possible link between the thrombosis and atherosclerosis in SLE (Vaarala *et al* 1993, 1996).
Anti-ox-LDL-C antibodies occur frequently in SLE patients. In a series of 118 SLE patients the prevalence of anti-ox-LDL-C and anti-beta-2-glycoprotein-I (anti-ß2GP-I) antibodies was 53% and 17% respectively (Romero *et al* 1998). In another series of 61 patients, raised levels of anti-ox-LDL-C and ACL antibodies were found in 80% and 46% of patients respectively (Vaarala *et al* 1993)
The pathogenic role of anti-ox-LDL-C antibodies in atherosclerosis has been suggested by *in vitro* studies, which showed that they enhance up-take of LDL-C by the macrophages via an Fc-receptor mediated mechanism (Lobes-Virella

et al 1997). Immune complexes of ox-LDL-C and anti-ox-LDL-C have been detected in atherosclerotic plaques (Yla-Herttuala *et al* 1994).

The increased production of anti-ox-LDL-C antibodies in SLE may represent an accelerated rate of ox-LDL-C generation due to increased oxidative stress in the arterial wall. In addition, general enhancement of autoantibody production in SLE may also contribute to higher levels of anti-ox-LDL-C. Clinical studies have shown that the serum levels of anti-ox-LDL-C antibodies correlate with the progression of carotid atherosclerosis (Salonen *et al* 1992) and predict MI in the general population (Puurunen *et al* 1994, Wu *et al* 1997). Autoantibodies to apo-A1, the major protein constituent of HDL-C, has been studied in a series of 186 SLE patients by Dinu *et al* (1998a), 18.3% of the patients tested positive for anti-apo-A1 antibodies and had significantly lower HDL-C, TC and apo-A1 levels. Thirty-two percent of patients with anti-apo-A1 antibodies had carotid plaques despite having lower LDL, LDL-C, diastolic blood pressure (DBP) and younger age. Therefore, anti-apoA1 antibodies may independently increase the risk of atherosclerosis in a subgroup of patients with SLE. Recently, Hyka *et al* (2001) have found that apo-A1 has an anti-inflammatory effect, decreasing production of IL-1β and TNF-α through inhibition of contact mediated activation of monocytes by activated T-lymphocytes.

In another study Dinu *et al* (1998 b) reported positive anti-apo A1 antibodies in 32.5% of SLE patients and 22.9% of patients with primary APL syndrome. These antibodies were associated with anti-ß2GP-I antibodies and showed high affinity for mature HDL-C in vitro.

2.3.3 Anti-phospholipid anti-bodies:

Antiphospholipid (APL) anti-bodies are a group of autoantibodies directed against negatively charged phospholipids and co-factors, especially ß2GP-I. Functionally APL antibodies are associated with prolonged phospholipid-dependent clotting tests, the so-called lupus anticoagulant (LAC). APL antibodies associated with autoimmune diseases or APL syndrome are mainly directed against ß2GP-I (Matsuura *et al* 1992). A role for anti-ß2GP-I antibodies in atherosclerosis has been suggested mainly by evidence from *in vitro* studies and experimental models of atherosclerosis. Anti-ß2GP-I antibodies can induce

expression of CAMs E-selectin, ICAM-1 and VCAM-1, enhance monocyte adhesion and stimulate secretion of proinflammatory cytokines IL-1 and IL-6 (Meroni *et al* 1996, Simantov *et al* 1996).

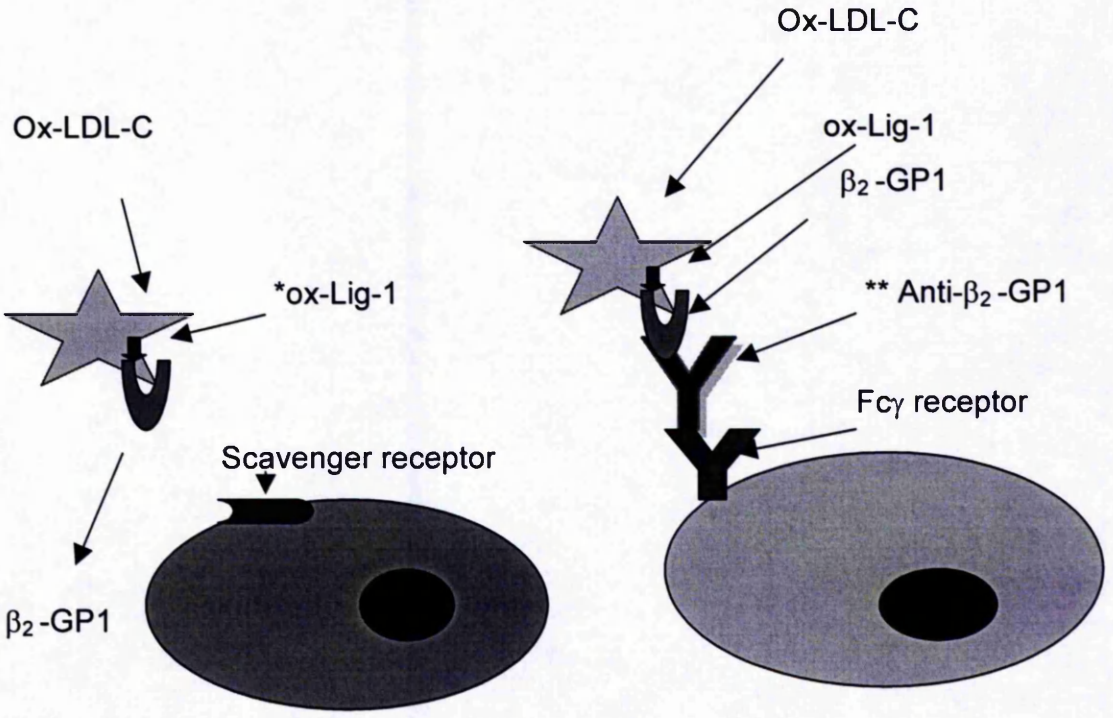
Hasunuma *et al* (1997) showed *in vitro* that the unbound ß2GP-I inhibited ox-LDL-C uptake by macrophages. But when ß2GP-I and monoclonal anti-ß2GP-I antibodies where added simultaneously, the uptake of ox-LDL-C increased significantly via Fc-γ receptor. This suggests that anti-ß2GP-I antibodies may have a pro-atherogenic effect while ß2GP-I may have an anti-atherogenic effect (Figure 2.1). Animal studies also suggest a role for the immune response against ß2GP-I in the development of atherosclerosis. Immunisation of LDL-C receptor deficient or apolipoprotein-E (apo-E) deficient mice with human ß2GP-I led to enhanced development of atherosclerosis (George *et al* 1998, Afek *at al* 1999).

Epidemiological studies have shown that anti-cardiolipin (ACL) antibody is an independent risk factor for MI (Vaarala *et al* 1995) and CVA (Tuhrim *et al* 1999) in the general population. A large multi-centre study of post-MI patients showed that elevated IgG anti-CL and IgM anti-CL antibodies are independent risk factors for recurrent cardiac events, but there was no significant association with anti-ß2GP-I antibodies (Bili *et al* 2000).

Romero *et al* (1998) showed in a study of 118 SLE patients including 40 with secondary anti-phospholipid syndrome that anti-ß2GP-I antibody was positive in 17% of patients. Presence and titers of anti-ß2GP-I antibody were strongly associated with history of arterial thrombosis. A higher prevalence of anti-ß2GP-I antibody in SLE (36%) was reported Viard *et al* (1992). In that study, eight out of nine patients with history of thrombosis had anti-ß2GP-I antibody and lupus anticoagulants. In 18 patients with anti-cardiolipin antibody and without anti-ß2GP-I antibody or lupus anticoagulant, only one patient had thrombosis.

Petri and Hamper (1997) found that APL antibodies were important predictors of atherosclerosis in both clinical and carotid plaques studies in SLE. An association of APL antibodies with atherogenesis in SLE has not been as clearly demonstrated. This may be due to small study sizes.

Figure 2.1 Oxidized LDL-C interactions with β_2-GP1:



* Binding of β_2-GP1 to ox-Lig-1 on ox-LDL inhibits binding to scavenger receptor.

** In the presence of β_2-GP1 and anti-β_2-GP1 binding to Fcγ receptor enhanced.

2.3.4 Other Immunological factors:

Recent studies have suggested that the initial events in the process of atherogenesis involve an autoimmune reaction to the endothelium. Several other inflammatory mechanisms of relevance in SLE may also be associated with the development of atherosclerosis in this context.

2.3.4.1 CD40:

SLE is associated with increased expression of CD40 on antigen-specific B cells and CD40-ligand on the activated T-cells. The binding of CD40 to the CD40-ligand enhances production of the autoantibodies (Desai-Mehta *et al* 1996). Furthermore, inflammatory cytokines induce up-regulation of CD40 on

the EC's of patients with lupus nephritis and enhance interaction between CD40 with CD40-Ligand positive T-cells, which induce expression of CAMs E-selectin, VCAM1 and ICAM1 (Karmann *et al* 1995). The role of CD40 in atherosclerosis has been suggested from a study on murine models of atherosclerosis with hypercholesterolaemia, where treatment with blocking antibodies of CD40 significantly reduced the atherosclerotic lesions (Mach *et al* 1998).

2.3.4.2 Heat shock proteins (HSP's):

These have also been implicated as possible autoantigens. Surface expression of HSP's has been shown to play a role in the generation of autontibodies. HSP's 60/65 are over-expressed on activated endothelium and can induce an immune response, which results in the development of anti-HSP60/65 antibodies and infiltration of the arterial wall with T-cells reacting with HSP 60/65 (Wick *et al* 1995). Shear stress has been shown *in vitro* and *in vivo* to induce HSP60 in endothelial cells (Hochleitnr *et al* 2000). There is a high degree of sequence homology between human HSP's and several species including bacteria, parasites and viruses. Human HSP60 has a high homology with chlamydia, H pylori and E coli (Jones *et al* 1993). An immune response to HSP60/65 has been proposed as a mechanism that links exposure to infective organisms to atherosclerosis. Antibodies to HSP60 have been shown to be cytotoxic to activated EC's (Schett *et al* 1995), and are detected in high titers in subjects with atherosclerosis (Metzler *et al* 1997). Furthermore, levels of soluble HSP60 are also elevated in subjects with atherosclerosis in the general population (Xu *et al* 2000), and immunisation of rabbits with recombinant mycobacterial HSP60 has resulted in the development of atherosclerosis (Wick *et al* 1995).

The frequency of anti-HSP's in SLE has been reported in several studies, but the frequency varied between studies and these antibodies were not significantly increased in SLE compared to healthy subjects (Tishler and Shoenfeld 1996). There is, however, evidence in animal models and human SLE that HSP90 is expressed on lymphocyte surfaces prior to the onset of clinical disease and that, antiHSP90 antibodies are significantly increased.

Their expression is further enhanced by IL-6 and IL-10 (Stephanou *et al* 1998, Ripley *et al* 1999)

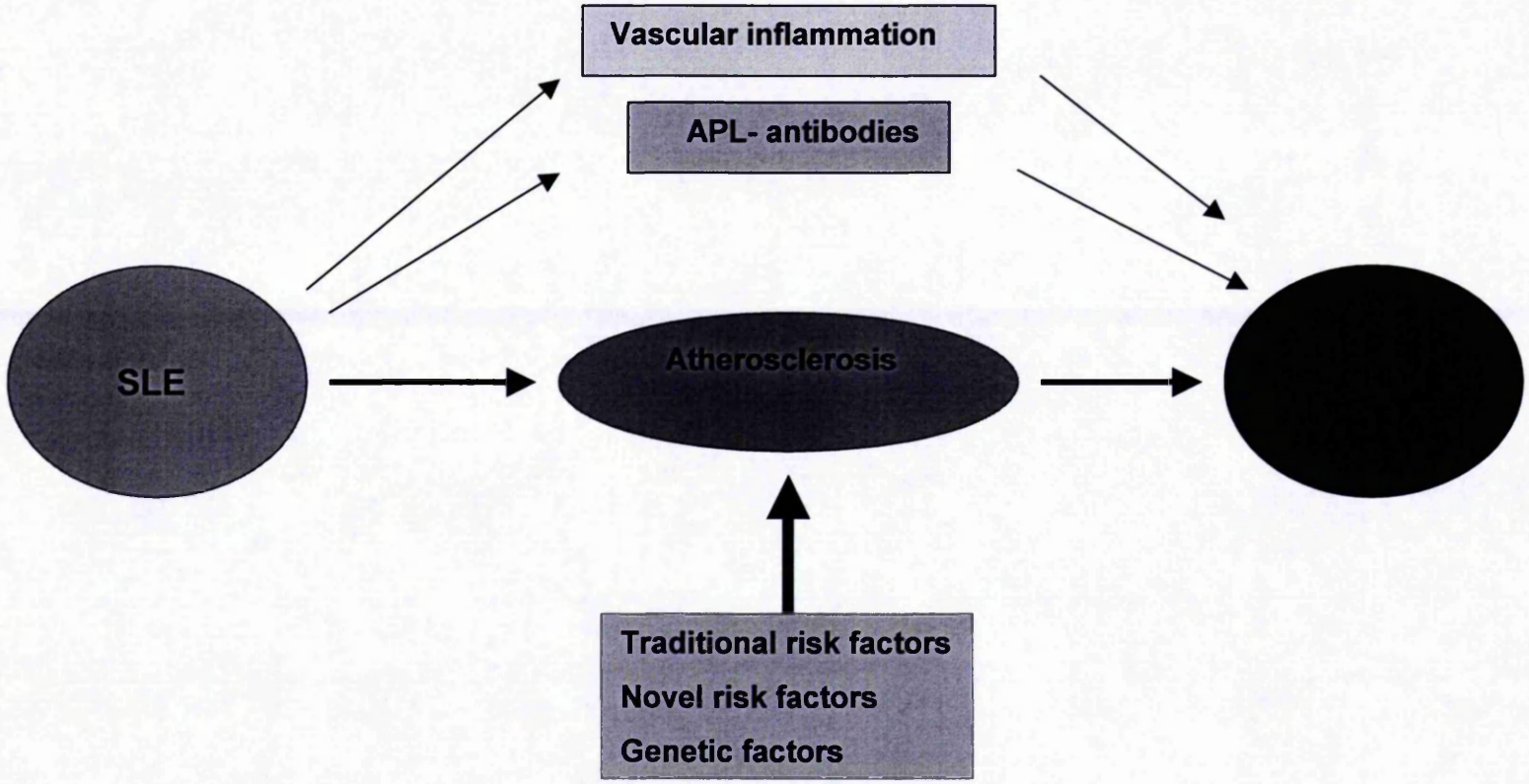
2.3.5 Haemostatic factors:

The thrombotic tendency in SLE is largely due to the presence of LAC and APL antibodies. Additional factors may also be of importance. Since in primary anti-phospholipid syndrome, arterial thrombosis can occur even in apparently normal arteries, in SLE the additional presence of systemic inflammation and atherosclerosis would increase the tendency to thrombosis.

Fibrinogen is a pro-coagulant factor and acute phase reactant and is significantly raised in several conditions associated with increased risk of CHD, such as diabetes, smoking and obesity (Kannel *et al* 1992). Fibrinogen, therefore, may explain part of the link between inflammation and atherosclerosis. Ames *et al* (2000) measured fibrinogen levels in 96 SLE patients and 39 healthy controls; levels correlated with age in both patients and controls but were significantly higher in the patients. Fibrinogen levels were independent of disease activity. Defects in the fibrinolytic system reflected in high levels of tissue plasminogen activator inhibitor-1 (tPAI-1) and low levels of tissue plasminogen activator (tPA), are associated with increased risk of thrombosis. In addition, they are also implicated in the progression of atherosclerosis through enhancing fibrin deposition (Hamsten *et al* 1995). Defects in the fibrinolytic system have been described in conditions with increased risk of CHD such as insulin resistance (Prins and Hirsh 1991). Significantly raised levels of TPA-1 have been found in several connective tissue disorders, including SLE and rheumatoid arthritis and have been associated with ACL antibodies or incidence of thrombosis (Jurado *et al* 1992).

The aetiology of Atherosclerosis in SLE is more likely to be multi-factorial (Figure 2.2). In addition to traditional and novel risk factors, SLE specific factors such as vascular inflammation and APL antibodies may also contribute to the increased risk of atherosclerosis.

Figure 2.2 Coronary heart disease in SLE:



Chapter 3

3 Novel risk factors for CHD in the general population

3.1 Introduction:

CHD is the leading cause of death in the developed western countries. The association between CHD and the classic Framingham risk factors is well established. However, CHD-related events not uncommonly occur in subjects with normal lipid profiles who otherwise appear to have a low risk. It has been reported that the traditional factors explain the risk in only about 50% of patients (Braunwald 1997).

This chapter reviews some of the more recently elucidated novel risk factors for CHD including hypertriglyceridaemia, insulin resistance syndrome, lipoprotein (a), basal systemic inflammation and paroxonase enzyme activity.

3.2 Hypertriglyceridaemia:

The main TGs-carrying lipoproteins are chylomicrons and VLDL, with lesser content in intermediate density (IDL-C) and LDL-C. In the fasting state, VLDL-C accounts for about 60% of total plasma TGs. Chylomicrons are not normally present after a12 hour fast.

The atherogenic effect of the TGs has been shown by *in vitro* formation of foam cells when human macrophages were incubated with IDL and VLDL remnants (Lechleitner *et al* 1994). Gianturco *et al* (1994) showed that the uptake of TGs-rich lipoproteins (TRL's) was via a specific receptor, other than the scavenger receptor for acylated LDL-C. Recently, a new receptor that binds apo-B48 has been detected in monocytes and macrophages, which can internalise TRL's such as VLDL. Immune reactivity for anti-bodies against apo-B48 receptors has been detected in human atherosclerotic lesions containing foam cells (Tanaka *et al* 2001). Earlier epidemiological studies have pointed to the high prevalence of elevated TGs among patients with CHD (Brunner *et al* 1977). However, evidence that hypertriglyceridaemia is an independent risk factor for CHD was not conclusive, as some studies have found that elevated TGs was not an independent factor after adjustment for other risk factors (Hulley *et al* 1980).

There is now stronger epidemiological evidence in favour of TGs being an independent risk factor for CHD. The Prospective Cardiovascular Munster study (PROCAM) involved an eight-years follow-up of 17,437 middle-aged men and 8,065 women. In a multivariate analysis with adjustment for age, significant and independent associations were found for serum TGs, HDL-C and LDL-C with CHD events (Assmann *et al* 1998).

In a meta-analysis of 17 population-based prospective studies published between 1965 and 1994, with a total number of 46,413 men and 10,864 women followed up for more than 8 years, elevated TGs was confirmed to be an independent risk factor for CHD. When the six studies, which adjusted for other risk factors, including HDL-C, were analysed, the risk decreased but remained significant. These latter studies included 22,000 men and 6000 women and estimated that the relative risk associated with a 1.0 mmol/l increase in TGs to be 1.14 and 1.37 for men and women respectively (Hokanson and Austin 1996). In the Framingham study, a high TGs level (>1.7 mmol/l) and low HDL-C level (< 1.3 mmol/l) frequently coexisted with the presence of normal total cholesterol and LDL-C. Subjects with this combination were at a significantly increased risk of CHD, with a relative risk of 2 in men and > 2 in women (Castelli 1992). In the Prospective Copenhagen Male Study, which involved 3,000 subjects, when TGs levels were stratified by HDL-C level there was a clear dose-response gradient of CHD risk within each level of HDL, supporting the independent effect of TGs. This study also showed that, after adjustment for other risk factors, the relative risk of CHD associated with TGs levels in the middle and upper thirds of TG level, compared with the lower third, was 1.5 and 2.2 respectively. Similar results have been reported from the Collaborative Heart Disease Study in the UK, which showed a relative risk of 2.2 when TGs levels in the upper 20% were compared with the lower 20% of the distribution (Bainton *et al* 1992).

The atherogenecity of TRL's appears to be related to their size. A smaller size may enhance their ability to penetrate into the sub-endothelial space. Larger chylomicrons, which are absorbed from the intestine, are rich in TGs but have not been associated with an increased risk for CHD. In contrast, chylomicron remnants, derived from chylomicrons by lipolysis of their TGs by the enzyme LpL in the serum, have been implicated in progression of CHD (Karpe *et al*

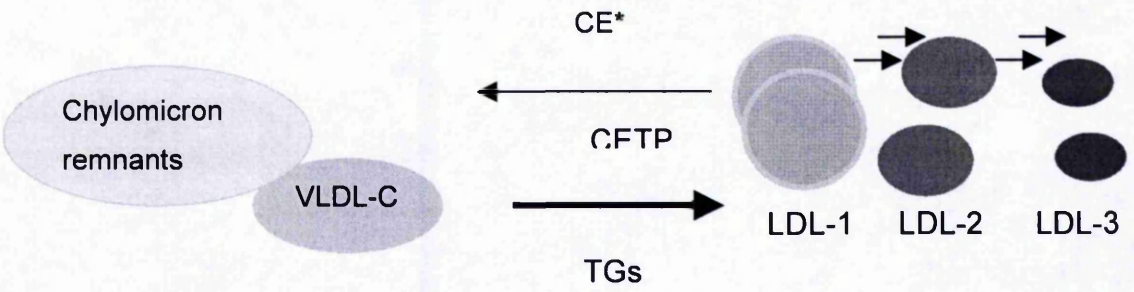
1994). Similarly, large size VLDL-C, which are secreted from the liver and contain endogenous TGs, may not be atherogenic, however the smaller size IDL-C produced by the action of LpL on VLDL-C are highly atherogenic (Gotto 1998, Davignon and Cohn 1996). Postprandial lipaemia, or the rate of clearance of postprandial lipoproteins from the serum, also has been implicated in the development of atherosclerosis and found to be impaired in a group of normolipidaemic patients with CHD compared to controls (Groot *et al* 1991). Impaired LpL activity has been suggested to explain derangement in postprandial lipoprotein metabolism. In a study of 730 patients with CHD and 75 healthy normolipidaemic controls, LpL activity was lower by 22% in CHD patients compared to controls (Henderson *et al* 1999). Impaired chylomicron clearance and lipoprotein lipase activity have been described in patients with SLE (Borba *et al* 2000).

Hypertriglyceridaemia is commonly associated with the abnormally dense small particle size type of LDL-C (type B) and low HDL-C. This lipid profile has been given the term "atherogenic dyslipidaemia" or atherogenic lipid profile and is strongly associated with insulin resistance syndrome (Austin *et al* 1990). TGs levels of >1.5 mmol/l have been described as the critical level for the development of small dense LDL-C particles (Griffin *et al* 1994). The mechanism of this TGs threshold is shown in (Figure 5.1). Usually there is an equimolar balanced transfer of TGs from TRL's chylomicrons and VLDL-C into LDL-C and HDL-C in exchange for cholesterol esters. At higher TGs levels (>1.5 mmol/l), there is an excess transfer of TGs to LDL-C, which become transiently rich in TGs and rapidly hydrolysed in the liver by hepatic lipase producing smaller and denser LDL-C particles (LDL-3) (Packard and Shepherd 1997). This mechanism forms part of the process by which cholesterol is transferred from the peripheral tissues to the liver for excretion.

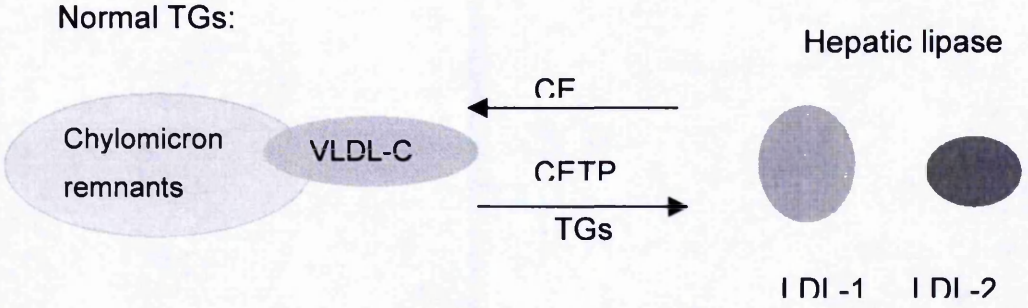
Figure 3.1 Mechanism of neutral lipids, TGs and cholesterol esters exchange between TRL's and cholesterol-rich lipoproteins.

In the presence of high TGs there is excess transfer of TGs in exchange for cholesterolester to LDL-C, which become rapidly hydrolysed by hepatic lipase into smaller and denser LDL-C particles, LDL-3.

High TGs:



Normal TGs:



**CE; cholesterol ester, **CETP; cholesteryl ester transfer protein.*

In vitro evidence for atherogenecity of small-particle size LDL-C has been attributed to its increased ability to cross into the sub-endothelial space (de Graaf *et al* 1991), its increased binding to proteoglycans in the arterial wall (Anber *et al* 1997) and its greater susceptibility to oxidation compared to the other, larger and lighter, LDL-C particles (Chait *et al* 1993).

In addition to its association with small dense LDL-C, moderately high TGs levels in atherogenic lipid profiles also contribute to the risk of CHD via its association with slower clearance of chylomicrons that lead to accumulation of the atherogenic chylomicrons remnants (Karpe *et al* 1994)

An inverse relationship between LDL-C particle size and CHD risk, independent of TGs and HDL-C, has been reported from the Stanford Five-City project (Gardner *et al* 1996). In a meta-analysis of three recent prospective studies (Quebec cardiovascular study, Physicians' Health study, and Stanford Five-City project) and using nested case-control designs, multivariate analysis showed after adjustment for TGs, HDL-C and other variables that, a ten angstroms decrease in LDL-C size was associated with a 30% increase in risk of CHD (Austin *et al* 1999).

3.2.1 Triglycerides in SLE:

Lipid profile abnormalities can be detected in the majority of SLE patients. A high TGs, VDL-C and low HDL-C pattern is the most frequently observed. This pattern is aggravated by disease activity and steroid treatment (Borba and Bonfa 1997, Ilowite *et al* 1988). It has been suggested that high TGs in SLE results from decreased activity of LpL enzyme. This has been reported in SLE patients (Kawanishi *et al* 1977) and in patients with CHD from the general population (Henderson *et al* 1999). Inflammatory cytokines IL-6 and TNF-α have been shown to down-regulate LpL. Chylomicron metabolism abnormalities have been described in SLE. Using infusion of chylomicrons like emulsions containing labelled cholesterol esters and labelled TGs, Borba *et al* 2000 showed that lipolysis of TGs in the chylomicron core was significantly impaired in SLE patients and the up-take of chylomicron remnants by the liver was 3-fold lower in SLE patients compared to controls. They also showed that *in vitro* lipolysis was 50% lower in SLE patients compared to controls.

Recently, Reichlin *et al* (2002) found in a study of 105 patients with SLE that 47% of had antibodies to LpL enzyme, and there was a strong positive correlation between the levels antibody to LpL and TGs levels.

3.3 Inflammation:

There is increasingly strong evidence that atherosclerosis represents a low grade inflammatory process in the arterial wall. Inflammatory mechanisms are involved in initiation, progression and clinical presentation of atherosclerosis. T-lymphocytes are the earliest mononuclear cells to infiltrate the intima at the sites of atherosclerotic lesions (Emeson and Robertson 1988). In the early stages of atherogenesis, fatty streaks consist mainly of monocyte-derived macrophages and T-lymphocytes (Stary *et al* 1994). An increase in the number of inflammatory cells in the shoulder region of a developed plaque increases the risk of rupture (Ross 1999). The inflammatory response in the arterial wall is regulated by several interacting factors such as levels of circulating and locally produced inflammatory cytokines, endothelial and smooth muscle cells, oxidized LDL-C and attracted inflammatory cells.

Acute phase reactants, such as C-reactive protein (CRP), fibrinogen and amyloid-A, are sensitive markers of systemic inflammation. IL-6 is the main regulator of acute phase response proteins production in the liver (Gauldie *et al* 1990). With the availability of a highly sensitive assay for CRP (hs-CRP), it is now possible to detect levels of inflammation within the low normal range. Several large prospective studies of healthy individuals have examined the relationship between baseline hs-CRP and the future risk of CHD in the general population. From the prospective Physicians Health Study, a nested case-control of 543 men who subsequently developed MI, stroke or thrombosis and 543 who did not, showed that basal hs-CRP was significantly higher in subjects with MI or stroke. Subjects with hs-CRP levels in the upper quartile had a 3-fold increase in risk of MI and 2-fold increase in risk of stroke compared to subjects with levels in the lower quartile after adjustment for other classical risk factors. (Ridker *et al* 1997).

This study also showed that aspirin use was associated with 55.7% reduction in risk of MI in subjects with hs-CRP in the upper quartile and insignificant reduction in subjects with hs-CRP levels in the lower quartile.

The predictive value of hs-CRP may be even stronger in women. In a nested case-control study from the Women Health Study involving 122 women who subsequently develop MI or CVA during the first three years of the study and in

a control group of 244 age and smoking-matched women who remained free of cardiovascular disease. Base line hs-CRP was significantly higher in women who developed cardiovascular disease (p=<0.001). When hs-CRP level was divided into quartiles defined by distribution in control subjects, and after adjusting for other risk factors such as diabetes, hypertension, total cholesterol and family history, the risk of future vascular events increased significantly with each quartile of hs-CRP (p for trend <0.001). Women with highest compared to lowest quartile had a 5-fold increase in risk of any vascular event (RR=4.8 95%CI 2.3-10, P<0.001) and 7-fold increase in risk of MI or stroke (RR=7.3, 95%CI 2.7-19.9, P<0.001). Similarly, The trend for increase in risk with each quartile was found also when analysis was limited to women with low risk [normal lipid profile (RR=3.9), non smokers (RR=4.5), no hypertension (RR=2.8), no diabetes (RR=4.9) or no family history (RR=6.6)] (Ridker *et al* 1998). In the same study, out of the 12 markers measured, which included other inflammatory markers such as serum amyloid A, IL-6, ICAM-1, homocysteine and a variety of lipid profile sub-fractions, in univariate analysis hs-CRP was the strongest predictor of cardiovascular events (nonfatal MI, stroke or the need for coronary re-vascularisation). In multivariate analysis, the only markers that independently predicted risk were hs-CRP and TC/HDL-C ratio (Ridker *et al* 2000).

The distribution of CRP levels in the general population is not normally distributed but skewed to the right. From a large study of > 5,000 Americans without known CHD, Ridker *et al* (2001) showed that the adjusted relative risk for a future CHD event for each quintile increase in hs-CRP was 26% (95%CI 1 – 44% $P < 0.005$) for men and 33% (95% CI 13 – 56% $P < 0.001$) for women. The median hs-CRP was 0.16 mg/dL and the five quintile ranges were 0.01 - 0.069, 0.07 – 0.11, 0.12 – 0.19, 0.20 – 0.38 and > 0.38. In another nested case-control study from a prospective cohort of 14, 916 healthy male physicians aged 40 to 84 years, 140 subsequently developed symptomatic peripheral vascular disease (PAD) and were compared to 140 age and smoking status-matched men who remained free of vascular disease during an average of 9-year follow-up period. Using baseline levels of eleven metabolic risk factors for atherosclerosis in a multivariate analysis, Ridker *et al* (2001) found that, the

best independent predictors of PVD were TC/HDL-C ratio and hs-CRP with a relative risk and 95% CI for those in the highest vs lowest quartile of 3.9 (1.7 – 8.6) and 2.8 (1.3 – 5.9) respectively.

Hs-CRP and amyloid A levels on admission to hospital significantly predict a poor outcome in patients with unstable angina (Liuzzo *et al* 1994). The pathogenic role of CRP in atherosclerosis is not known, whereas, the effect of fibrinogen may be mediated via an increase in plasma viscosity, platelets aggregation and stimulating smooth muscle proliferation (Ernst *and* Resch 1993).

3.4 Insulin resistance:

Insulin resistance implies a decreased ability of insulin to facilitate peripheral up-take of glucose in tissues, such as skeletal muscles and adipose tissue. Insulin is an important regulator of metabolic activity, gene transcription and cell growth by modulating the activity of several intracellular signalling pathways. The cellular response to insulin is mediated via two pathways. One is the activation of phosphatidylinositol-3-kinase pathway, which mediates the metabolic effects of insulin. The other is the activation of mitogen-activated protein (MAP) kinase, which is associated with the mitogenic affects of insulin that regulates cell growth and proliferation. In insulin resistance, the effect of insulin on phosphatidylinositol-3-kinase is impaired whereas the effect on mitogen-activated protein (MAP) kinase is maintained (Cusi *et al* 2000).

Glucose transport involves the glucose transport protein (GLUT-4). In the resting state 90% of GLUT-4 is accumulated in small vesicles in the cytoplasm. On binding of insulin to its receptor phosphatidylinositol-3-kinase is activated, this generates phosphatidylinositol triphosphate from insulin receptors, which promotes translocation of the GLUT-4 from the cytoplasmic vesicles to the plasma membrane (Holman and Kasuga 1997). About 70-80% of insulin-stimulated glucose uptake occurs in the skeletal muscles (DeFronzo 1981).

Insulin resistance syndrome (IRS) describes clustering of lipid and non-lipid risk factors for CHD which are closely related to the generalized metabolic disorder of insulin resistance and these include hyperinsulinaemia, glucose intolerance, non-insulin dependent diabetes mellitus (NIDDM), hypertension, dyslipidemia,

central obesity, haemostatic abnormalities, microalbuminuria, increased basal inflammation and endothelial dysfunction. These metabolic abnormalities are more likely to occur together rather than separately and tend to cluster in the presence of hyperinsulinemia or insulin resistance. In a large 16-year follow-up study, Wilson *et al* (1999a) studied the impact of clustering of six metabolic risk factors (HDL-C, BMI, SBP, TGs, fasting blood glucose (FBG) and TC) on the incidence of CHD. Clustering of three or more risk factors was associated with twice the rate expected by chance and was associated with a relative risk (95% CI) for CHD of 2.4 (1.6-3.7) and 5.9 (2.5-13.7) for men and women respectively

In 1998, the WHO proposed the "metabolic syndrome" as a new definition to replace insulin resistance syndrome. According to the WHO definition, the components of the syndrome are: hypertension (defined as BP of >160/90 and/or anti-hypertensive therapy), dyslipidaemia (TGs ≥ 1.7mmol/l) and/or HDL (<0.9mmol/l in men or<1.0 in women), obesity (defined as body mass index (≥ 30 kg/m^2) and/or waist/hip ratio (>0.9 in men and >0.85 in women), microalbuminuria (urinary albumin excretion rate AER ≥ 20 µg/min).

The metabolic syndrome is present if a subject has two of the previous criteria plus either:

- Type II diabetes or impaired FBG/impaired glucose tolerance.
- Insulin resistance with normal glucose tolerance, which is defined as HOMA-S in the upper quartile (Alberti and Zimmet 1998).

Recently, the Executive Summary (2001) of the Third Report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III) (ATPIII) has drawn attention to the importance of the metabolic syndrome and has defined the syndrome as the presence of ≥ 3 of the following criteria are present:

- Abdominal obesity (waist circumference >102 cm for men and >88 cm for women).
- TGs ≥1.69 mmol/l.
- HDL-C <1.04 mmol/l for men and <1.29 mmol/l for women
- Blood pressure ≥ 130/ ≥85 mmHg.
- FBG ≥ 6.1mmol/l

Subjects using anti-hypertensive or anti-diabetic medication are counted as having high blood pressure or diabetes.

Using ATPIII criteria for identification of the metabolic syndrome, in the USA, Ford *et al* (2002) estimated the prevalence of the metabolic syndrome in a cross-sectional study of 8,814 men and women aged >20 years in. Overall the unadjusted and age adjusted prevalence was 21.8% and 23.7% respectively. The prevalence increased from 6.7% in the age group 20-29 years to 43% in the 60-69 year age group.

Clustering of risk factors with insulin resistance suggests the presence of some common pathogenic pathways. In the next paragraphs we discuss the associations and the mechanisms of the components of the metabolic syndrome:

3.4.1 Impaired glucose tolerance and hypertension:

To maintain a normal glucose level, decreased insulin sensitivity is compensated for initially by an increase in pancreatic beta cell function. However, in the presence of beta cell dysfunction, impairment of glucose tolerance and progression to NIDDM will eventually occur. About 50% of subjects with essential hypertension are insulin resistant (Zavaroni *et al* 1992). The mechanisms underlying hypertension associated with insulin resistance are not clearly defined. Intravenous insulin causes vasodilation in normal subjects, but not in subjects with type II diabetes or with insulin resistance (Laakso *et al* 1992). Hyperinsulinaemia is associated with increased absorption of sodium in the proximal renal tubules (DeFronzo *et al* 1981) and increased sympathetic output (Landsberg 1999).

3.4.2 Dyslipidaemia:

The lipid profile in IRS is characterised by high TGs, low HDL-C and a predominance of small dense LDL-C particles. The main defect in lipid metabolism is an increase in free fatty acid (FFA's) secretion from adipose tissue because of the lack of the inhibitory effect of insulin on hydrolysis of TGs to FFA's and glycerol. Increased FFA's delivery to the liver stimulates secretion of VLDL-C, excess VLDL-C stimulates exchange of TGs in VLDL-C for

cholesterol esters in HDL-C, and LDL-C. Excess TGs in HDL-C facilitates dissociation and loss of Apo-A1 through renal excretion, thus reducing availability of HDL-C for reverse cholesterol transport. TGs-enriched LDL-C undergoes lipolysis by hepatic lipase and becomes more dense and smaller in size (Kahn and Flier 2000). Recent studies have shown that high FFA's impairs insulin mediated vasodilation and endothelial NO production (steinberg *et al* 2000). VLDL-C also stimulates PAI-1 production (Banfi *et al* 1999).

3.4.3 Obesity and inflammation:

Inflammatory cytokines IL-6 and TNF-α and CRP correlate with insulin sensitivity, TGs, low HDL-C, hypertension and measures of obesity (Yudkin *et al* 1999). Adipose tissue is a potent source of TNF-α and IL-6 in healthy subjects. About 30% of total circulating IL-6 originates from adipose tissue (Mohamed-Ali *et al* 1997). Adipocytes from obese subjects show a 2.5 fold increase in production of the TNF-α messenger RNA (m-RNA) compared to subjects with normal weight, and there was a strong positive correlation between levels of TNF-α m-RNA and the degree of hyperinsulinaemia. Weight reduction was associated with a 45% reduction in TNF-α m-RNA (Hotamisligil *et al* 1995). TNF-α impairs the function of the insulin-signalling pathway through interference with the phosphorylation of insulin receptor and its substrates (Hotamisligil *et al* 1996). TNF-α is a strong inducer of IL-6, which is the main mediator of acute phase response. Both TNF-α and IL-6 inhibit the action of lipoprotein lipase, stimulate lipolysis and increase hepatic VLDL-C secretion (Hardardottir *et al* 1994, Feingold *et al* 1994). TNF-α and IL-6 also induce endothelial dysfunction (Mohamed *et al* 1995, Romano *et al* 1997). This may explain the strong association between inflammation, obesity, dyslipidaemia, and insulin resistance syndrome.

In a study of CHD patients who were non-obese, normotensive and with normal glucose tolerance, decrease in insulin sensitivity correlated significantly with an increase in white blood cell count and microalbuminuria (Piedrola *et al* 2001). Data from the Insulin Resistance Atherosclerosis Study (IRAS) also suggested that a high basal rate of inflammation is part of insulin resistance syndrome as CRP protein, fibrinogen and white blood cell count correlated with other

components of the syndrome. A strong association was found between insulin resistance and BMI, weight/height ratio and fasting insulin in non-diabetic patients and there was a linear increase in CRP with increase in the number of metabolic components of insulin resistance syndrome (Festa *et al* 2000b).

3.4.4 Impaired fibrinolysis:

Insulin resistance is associated with low fibrinolytic activity. In a study of 756 men and women from the MONICA study, Lindahl *et al* (1996) showed a strong positive correlation between insulin resistance and the activity tPAI-1 as well as a negative correlation between insulin resistance and activity tPA. Subjects in the upper tertile of insulin resistance had a 3-fold increase in PAI-1 activity. Insulin resistance is also associated with increased levels of protein C and protein S, which could be a counteracting mechanism for the associated decreased fibrinolysis (Agewall *et al* 2000).

Hyperinsulinaemia may directly impair fibrinolysis. *In vitro* studies have shown increased synthesis of PAI-1 from hepatic and endothelial cells in response to increased levels of insulin, and from endothelial cells following incubation with VLDL-C (Eriksson *et al* 1998). Studies in humans also have shown increased PAI-1 secretion in response to insulin infusion (Calles *et al* 1998).

3.4.5 Microalbuminuria;

Microalbuminuria may be secondary to endothelial dysfunction in the glomerular capillaries and is defined as an increase in urinary albumin excretion rate (UAE) in the presence of normal renal function. It is a strong risk factor for progressive renal disease in type I diabetes (Viberti *et al* 1982). However, in type II diabetes it is associated with an increased risk of CHD, rather than renal disease (Macleod *et al* 1995). There is association between CRP and fibrinogen with the UAE rate in type II diabetes and also in non-diabetics (Festa *et al* 2000a). In a study of 368 healthy men, the UAE rate correlated with carotid intima media thickness (Agewall and Bjorn 2002).

3.4.6 Endothelial dysfunction:

Dysfunctional endothelium expresses atherogenic and pro-coagulant effects and recently has been implicated in the alteration of insulin action.

Insulin induces NO-dependent vasodilation of the skeletal muscle vessels and this effect is decreased or absent in insulin-resistance states, even in the absence of NIDDM (Steinberg et al 1994). The same group also reported a reduction of 40% and 55% in leg blood flow following metcholine infusion into the femoral arteries in obese insulin resistant subjects and in patients with NIDDM respectively, when compared to healthy controls. Euglyacemic induced hyperinsulinaemia enhanced leg blood flow by 50% in the controls, but not in insulin resistant subjects or patients with NIDDM (Steinberg *et al* 1996).

It has been reported that the haemodynamic action of insulin is responsible for as much as 40% of its effect on peripheral glucose uptake (Yudkin 1997). The role of arteriolar vasodilation in the regulation of insulin-mediated glucose delivery has been suggested to explain why treatment with an ACE inhibitor was associated with a reduced incidence of new onset diabetes in the participants of the Heart Outcome Prevention Evaluation Study (HOPE) (Yusuf *et al* 2000).

The degree of insulin resistance in a group of healthy normotensive non-diabetic subjects was found to correlate significantly with the levels of soluble forms of CAMs E-selectin, ICAM-1 and VCAM-1, as well as with the degree of endothelial dysfunction (Chen *et al* 1999).

Several studies have shown that "insulin resistance" is a predictor of CHD. In the Paris Prospective Study, a cohort of 7028 men was followed for eleven years and 26 deaths were reported as due to CHD. Impaired fasting and 2-hour post oral glucose load was an independent predictor of CHD in addition to hypertension, smoking and high cholesterol (Fontbonne and Eschwege 1991). The Helsinki Policemen Study involved a 22-year follow up of a cohort of 970 men aged between 34 and 64 years, who had no history of CHD or diabetes. During the study, 164 major CHD events (death or nonfatal MI) occurred. Area under the plasma insulin response curve during oral glucose tolerance test was used to reflect plasma insulin levels. Men in the highest area under curve insulin quintile compared to those in the combined lower four quintiles, had significantly

higher adjusted hazard ratio for major CHD events after adjustment for age and other risk factors including cholesterol, hypertension, smoking, TGs and measures of obesity. The hazard ratios diminished with increased length of follow-up from 2.33 (1.0 – 1.97) at 5-ears to 1.32 (0.09 – 1.97) at 22 years (Pyorala *et al* 1998).

In a prospective study of 2103 men followed between 1985 and 1990 in Quebec City, Canada (Depress *et al* 1996), one hundred fourteen developed CHD events. These were matched for age, BMI and smoking with subjects who remained free of CHD by 1989. Fasting insulin at baseline was 18% higher in those with CHD ($P<0.001$). In logistic regression after adjustment for hypertension and family history of CHD, fasting insulin showed association with CHD with an increase in odds ratio (95% CI) of 1.7(1.3-2.4) with each one SD increase in insulin concentration. Further adjustment for TGs, apolipoprotein B, LDL-C and HDL-C in multivariate analysis also showed significant association between high fasting insulin and CHD with OR (95%CI) of 1.6 (1.1-2.4).

In a recent meta-analysis of 17 studies Ruige *et al* (1998) found a weak positive association of fasting insulin with cardiovascular disease with a RR (95% CI) of 1.18 (1.08 – 1.29) for a difference in fasting insulin of 50 pmol/l or equivalent to the difference between 75^{th} and 25^{th} percentiles. The RR risk in studies of white populations was 1.42 (0.23 – 1.65).

There is a controversy whether hyperinsulinaemia by itself is atherogenic. A lot of experimental evidence from studies on cultured arterial EC's supports that insulin has a direct atherogenic effect. This effect is mediated via stimulation of vascular smooth muscle cells (VSMCs) proliferation and migration. Cholesterol synthesis and binding of LDL-C to its receptor on VSMCs is enhanced by insulin (Stout 1991, Pfefile and Ditschuneit 1981). Insulin also enhanced adhesion of neutrophils to EC's via increased expression of ICAM-1 (Okouchi *et al* 2002). Hyperinsulinaemia and hyperglycaemia after a glucose tolerance test has also been associated with greater neointimal proliferation following coronary stent implantation in non-diabetic patients (Takagi *et al* 2000). Conversely, exogenous administration of insulin for 24 weeks to cholesterol fed rabbits did not increase atherosclerosis (Nordestgaard *et al* 1997).

Insulin resistance appears to be important in the general population in the context of cardiovascular risk. Little is known about the prevalence of insulin resistance and its effects on disease outcomes in patients with SLE. Paolisso *et al* (1991) reported a study on a small group of patients with connective tissue diseases, including eight subjects with rheumatoid arthritis, five with SLE, three with systemic sclerosis and ten healthy controls. They found increased fasting insulin levels and diminished insulin sensitivity in the patients compared to the controls.

3.4.7 Effects of anti-malarial therapy on insulin metabolism:

Choroquine has several effects on insulin metabolism. It enhances the effect of insulin through inhibition of insulin dissociation from its receptor, inhibition of degradation of insulin in endosomes and inhibition of recycling of insulin receptors from endosomes to the plasma membrane. These effects result in an increase in the half-life of the active insulin-receptor complex (Bevan *et al* 1997). There has been a case report of hypoglycaemia developing in a patient with type II diabetes, who was on a stable dose of insulin, within two weeks of starting on 400mg of HCQ and 5mg of prednisolone. He had two attacks of hypoglycaemia requiring emergency treatment and his insulin requirement had to be reduced by 37% (Shojania *et al* 1999).

HCQ was given to 20 patients with NIDDM in a dose of 250mg/day for three days and resulted in a significant decrease of FBG, increase in fasting insulin, a 39% decrease in insulin clearance and 17% increase in C-peptide secretion (Powrie *et al* 1991). However, previous studies did not show increase in insulin secretion, since there was no change in C-peptide level (Phillips *et al* 1986, Smith *et al* 1987).

In a clinical trial, Quatraro *et al* (1990) studied the effect of HCQ on 22 patients with uncontrolled type II diabetes on insulin therapy and 16 patients taking the oral hypoglycaemic drug glibenclamide. The groups were randomised to take HCQ 600 mg/day or placebo. The addition of HCQ to insulin resulted in a significant reduction in insulin requirement by an average of 30%. This effect was noted at two weeks and persisted for six months. Interestingly, there was no change in fasting or glucagon-stimulated C-peptide, which further supports

the previous observation that the decrease in insulin requirement was not due to increased insulin secretion.

From the Baltimore Lupus cohort, Petri *et al* (1994) reported that 10% of SLE patients have glucose intolerance requiring treatment, and that significant predictors of glucose intolerance were age, steroid therapy, absent HCQ therapy and family history of diabetes. These data suggest a protective role for HCQ.

3.5 Lipoprotein (a) Lp(a):

Lp(a) is structurally similar to LDL-C in lipid content and the apolipoprotein apo B-100. In addition, it has a large glycoprotein, apo(a), attached to apo B-100 (Scanu 1995). Apo(a) consists of a number of ring structures known as kringles, which have high sequence homology with the kringle number IV of plasminogen. Plasma levels of Lp(a) are mainly controlled at the genetic level and vary widely up to 1000-fold between individuals. This variation is a result of genetic polymorphisms involving the number of kringle IV class 2 repeats, which is inversely related to the serum level (Rosby and Berg 2000). Lp(a) levels are not influenced by diet or exercise.

Several epidemiological studies have shown that high levels of Lp(a) are associated with CHD. In a prospective study involving 6,002 men aged 40-60 years followed for 5 years, Lp(a) was an independent risk factor for CHD and ranked fifth behind LDL-C, family history of CHD, fibrinogen and HDL-C (Cremer *et al* 1994). In another large prospective study of 9,936 men and women who were free of CHD, Lp(a) significantly predicted future CHD (Orth-Gomer *et al* 1997). Lp(a) levels increase by about 25% in postmenopausal women, which may contribute to the increased risk of CHD in this group of women (Kim *et al* 1996). Although African-Americans have higher mean level of Lp(a) compared to whites, this is not reflected in an increase in CHD risk (Moliterno *et al* 1995)

Plasma levels of >30mg/dl are associated with the presence and extent of CHD (Budde *et al* 1994) and levels >39mg/dl were found to be the most common dyslipidaemia in a study of patients who developed CHD below the age of 60 years (Genest *et al* 1992).

Although Lp(a) has been described as having atherogenic and thrombogenic properties, the underlying mechanisms have not been clearly defined. Lp(a) is susceptible to oxidation in the arterial wall and oxidation increases its atherogenecity (Naruszewicz *et al* 1992). The thrombogenic effects of Lp(a) may be related to interference with fibrinolysis via competition with plasminogen for binding to fibrin (Hajjar *et al* 1989). Inhibition of activation of plasminogen by tPA and increased expression tPAI-1 has been reported (Takami *et al* 1998).
In a study of 34 Brazilian SLE patients and 66 controls, Lp(a) was significantly higher in patients with levels of ≥ 30mg/dl found in 65% of patients and 30% of the controls (Borba *et al* 1994). In a prospective study of 166 SLE patients (91% females and 55% afro-American) from the Baltimore Lupus Clinic, Petri *et al* (1995) found that the mean (SD) of Lp(a) levels in SLE patients was 22,3 (22.9) mg/dl, with a range from 0 to 126 mg/dl. The Lp(a) level was significantly higher in patients compared to matched controls and was predictive of myocardial infarction, with a mean level of 33.5 (±5.9) vs 21.2 (±1.8) in those with and without myocardial infarction respectively. LP(a) was not predictive of thrombotic events and correlated negatively with APL antibodies. Another study of 77 SLE patients also showed significantly higher Lp(a) levels in patients compared to controls and in patients with active compared to inactive disease. In this study Lp(a) correlated positively with TC and proteinuria and negatively with serum albumin (Okawa *et al* 1996). The Lp(a) level was also found to be significantly higher in SLE patients with myocardial or cerebral infarctions than in patients without (Kawai *et al* 1995). In primary or SLE-associated APL syndrome, patients with higher levels of Lp(a) showed lower fibrinolytic activity compared to those with normal Lp(a), which suggests that Lp(a) may also contribute to the thrombotic tendency in SLE (Atsumi *et al* 1998). However, in a recent large controlled study from Canada which involved 229 unselected Caucasian SLE patients and 235 controls, Bruce *et al* (2000) found no difference in the LP(a) level between patients and controls.

3.6 Paroxonase activity:

The name of the paroxonase enzyme reflects its ability to hydrolyse paroxon, a toxic metabolite of the organophosphate insecticide parathion. Paroxon binds

irreversibly and destroys serum esterases, such as peudocholinesterase and acetylcholineesterase, at neuronal synapses and neuromuscular junctions. Paroxonase is not present in fish, birds and insects, which explains why they are killed by organophosphate insecticides. In serum, paroxonase is exclusively bound to the lipoprotein HDL-C (Durrington *et al* 2001). Animal studies have shown that paroxonase-1 (PON1) deficient mice are highly sensitive to the toxic effects of organophosphates. HDL-C, isolated from PON1-deficient mice, was unable to prevent *in vitro* oxidation of LDL-C and these mice were more susceptible to atherosclerosis. Both LDL-C and HDL-C from these mice were more susceptible to oxidation by cultured cells than lipoproteins from normal mice (Shih *et al* 1998).

Mackness *et al* (1991) were first to show that PON1, isolated from HDL-C enzymes was responsible, at least in part, for the ability of HDL-C to prevent accumulation of lipid peroxides on LDL-C and the authors postulated that this antioxidant effect of PON1 may, therefore, modulate the risk of CHD.

Paroxonase activity is partly controlled at the genetic level. Two single nucleotide polymorphisms have been described in the coding region that result in an amino acid substitution; glutamine to arginine at position 192 (Q192R) and methionine to leucin at position 55 (M55L). The Q192R polymorphism has a much greater effect on activity than M55L, and the two are not in linkage disequilbrium. The difference in PON1 activity resulting from these two polymorphisms is substrate dependent, with the R and Q alleles expressing different enzyme activity toward different organophosphate substrates. The R allele has a higher activity towards paroxon than the Q allele, whereas the Q allele hydrolyses nerve gases and diazoxin faster than the R allele (Davis *et al* 1996). The R allele has been shown by Mackness *et al* (1998b) to be less efficient in retarding the oxidation of LDL-C compared to the Q allele. The activity of R allele in hydrolysing lipid peroxides is opposite to that toward paroxon, which is the most commonly used substrate to assay PON1 activity. Hydrolytic activity of PON1 towards paroxon was highest with RR/LL and lowest with QQ/MM haplotypes, while heterozygotes showed intermediate activity. HDL-C or PON1 isolated from QQ/MM homozygote, was the most effective in protecting LDL-C, while HDL-C from RR/LL was the least effective (Mackness

et al 1997 and Mackness *et al* 1998b). These finding have suggested that PON1 polymorphism could be a risk factor for CHD.

PON1 activity and concentration varies widely between different healthy populations and within each genotype in the same population. This may be related to variation in the number and composition of HDL-C particles (Blatter *et al* 1994 and Mackness *et al* 1996). Nutritional factors can affect PON1 activity. In animal studies, PON1 activity is reduced by cholesterol-rich diet. Mackness *et al* (2000) have reported a 50% reduction of PON1 activity in rabbits fed on high cholesterol diet for 14 days. Significant postprandial reduction in PON1 activity was also found in a cross over study of 10 healthy subjects after a meal rich in oxidized lipid from fats used in repeated deep frying (Sutherland *et al* 1999). There is some experimental evidence to suggest that PON1 activity decreases as part of an acute inflammatory response (Van Lenten *et al* 1995 and Feingold *et al* 1998). Daily moderate alcohol consumption in middle-aged men has been shown to increase PON1 activity (van der Gaag *et al* 1999).

PON1 activity is reduced in patients with CHD or with classical risk factors for CHD. Mackness *et al* (2001) have found significantly lower PON1 activity and concentration in 417 patients with proven CHD compared to 282 healthy controls. Mackness *et al* (1998a) also reported low activity and concentration of PON1 in 252 patients with non-insulin diabetes mellitus compared to 282 controls. PON1 activity is reduced in subjects with familial hypercholesterolaemia and in patients with renal failure (Hasselwander *et al* 1998). Lower PON1 activity and concentration is independently associated with current smoking, which normalise after a short time of cessation (James *et al* 2000).

Several case control studies have found an association between the R allele of PON1 and CHD (Ruiz *et al* 1995, Serrate and Marian 1995), while other groups found no association (Herrmann *et al* 1990, Antikainen *et al* 1996). In a meta-analysis, Durrington *et al* (2001) showed a significant association with an overall odds ratio of 1.5.

In a study of 417 patients with proven CHD and 282 healthy controls Mackness *et al* (2001) have found that PON1 activity and concentration were significantly lower in patients with CHD compared to controls, irrespective of the genotype.

There was no difference in frequency of the two polymorphisms of PON1. In myocardial infarction patients PON1 activity reduced within two hours of the onset of pain and did not change up to 42 days after infarction. This is not in parallel with the decline in the acute phase response associated with infarction, which suggests that reduced activity may have preceded the acute event. The decrease in PON1 activity was more than can be explained by differences in genotype between patients with myocardial infarction and healthy controls. The authors suggest that in the context of myocardial infarction, PON1 activity and concentration would be more important than the genotype. In a small study of 36 patients with positive ACL antibodies, Lambert *et al* (2000) have found, that patients who developed thrombosis had markedly decreased PON1 activity and increased prevalence of RR genotype. More recently, Delgado *et al* (2002) found in SLE patients with APL syndrome that, paroxonase activity correlates inversely with IgG anti-ß2GP-I and IgG anti-HDLC antibodies

Chapter 4

4 The Endothelium in Health and Disease

4.1 Introduction:

The endothelium is the cellular monolayer lining the blood vessels and separates blood constituents from the sub-endothelial layers. EC's are metabolically very active and play an essential role in the regulation of vascular tone and the regulation of homeostasis by functioning in normal states as a surface with anti-thrombotic properties. During tissue damage it exhibits procoagulant properties to limit blood loss and helps in the repair process. The endothelium also plays an essential role in the regulation of leukocyte trafficking across the endothelium, normally to facilitate leukocyte migration to sites of infection or entry of foreign antigens, and pathologically in autoimmune driven inflammation.

4.2 Regulation of the vascular tone:

EC's produce several vasodilator and vasoconstrictor substances that control the vascular tone and, hence, regulate blood flow to the tissues according to the physiological demand. The balance between the two maintains the vascular tone in the normal state.

4.2.1 Vasodilator factors:

4.2.1.1 Nitric oxide (NO):

NO, or the endothelium derived relaxing factor (EDRF), was first discovered by Furchgott and Zawadzki (1980) who noted that an intact endothelium is essential for the relaxation of a rabbit's aorta in response to acetylcholine. Later on EDRF and the active metabolite of nitrate vasodilators was found to be NO, which is a highly reactive, rapidly diffusible free radical gas with a short half-life of 6-30 seconds. NO shares some properties with oxygen as it binds to the haem group in the enzyme guanylate cyclase in VSMCs and is inactivated by haemoglobin (Palmer *et al* 1987). NO is produced in the endothelial cells by the enzyme nitric oxide synthase (NOS) as a by-product in a reaction converting the amino acid L-arginine into L-citrulline. NOS can be inhibited by different

analogues of L-arginine. The most widely used is N-monomethyl-L-arginine (LNMMA), which is a competitive and specific inhibitor for NOS enzyme. LNMMA is therefore used to confirm that the endothelium dependent dilatation in humans is dependent on the generation of NO (Palmer *et al* 1988). NO acts either directly on calcium-dependent potassium channels or through activation of guanylate cyclase in the smooth muscle leading to increased concentration of cyclic guanine monophosphate (cGMP). This results in activation of guanine monophosphate-dependent kinases that results in the decrease of intracellular calcium and smooth muscle relaxation (Bolotina *et al* 1994). Several vasodilators are known to stimulate receptors on the endothelium to release NO, these include serotinin, bradykinin, substance P, adenine nucleotide, norepinephrine and thrombin (Harrison 1993). Shear stress, produced by blood flow, activates potassium channels that also lead to the release of NO (Cooke *et al* 1991). Normal shear stress also has been shown to up-regulate endothelial nitric oxide synthase (eNOS) gene expression (Sessa *et al* 1994) and to stabilise the eNOS mRNA (Davis *et al* 2001).

Three isoforms of the NOS enzyme have been described:

1. Type I or neuronal (nNOS), produced in the brain and skeletal muscles.
2. Type III or endothelial (eNOS), other than in endothelial cells, it also produced in the platelets (Sase and Michel 1995), placenta (Myatt *et al* 1993), myocytes (Feron *et al* 1996) and bronchial epithelium (Shaul *et al* 1994). eNOS and nNOS are produced constitutively to maintain basal levels of NO; they are calcium and calmodulin dependent and inhibited by the L-NMMA.
3. Type II or inducible (iNOS) is present in macrophages, endothelial cells, smooth muscles, myocytes, keratinocytes and hepatocytes. It is a calcium and calmodulin independent enzyme and it is not inhibited by L-NMMA. iNOS activity is induced by inflammatory cytokines, IL1, IL 2 and TNF$\propto$ during inflammation and tissue injury (Chester *et al* 1998). It is also inhibited by steroids (Di Ross *et al* 1990).

The mechanisms underlying basal production of NO are not well understood. Continuous shear stress generated by the blood flow may be an important stimulus for the release of NO in conduit arteries via activation of ion channels

(Lansman 1988), however more NO is produced in the resistance rather than the conduit blood vessels and NO production continues in cultured endothelial cells not expressed to shear stress.

4.2.1.2 Prostacycline (PGI2):

Prostacycline is one of the arachidonic acid derivatives in the cyclooxygenase pathway. It is a potent vasodilator and has both anti-thrombotic and anti-platelet effects (Moncada *et al* 1977).

4.2.1.3 Endothelium derived hyperpolarising factor (EDHF):

This factor is derived from arachidonic acid by the action of cytochrome p450 enzyme, released from the endothelium, and causes hyperpolarisation of the underlying smooth muscle membrane via activation of potassium channels. It is stimulated by bradykinin and acetylecholine and has been suggested to play an important role in the coronary microcirculation (Bauersachs *et al* 1994).

4.2.2 Vasoconstricting factors:

4.2.2.1 Endothelins:

Three types of endothelins have been described in humans. Endothelin-1 (ET-1), the most important one, is synthesised in the endothelium and acts on the adjacent vascular smooth muscles. It has a potent and prolonged vasoconstrictor effect ten times more potent than angiotensin II (Vane *et al* 1990). ET-1 is produced at a basal rate by the action of an endothelin-converting enzyme, which is stimulated by epinephrine, angiotensin II, IL-1, transforming growth factor beta (TGF-ß) and thrombin. ET-1 acts on two receptors. Binding to endothelin receptor-A stimulates phospholipase leading to release of intracellular calcium and smooth muscle contraction, while binding to endothelin receptor-B activates calcium channels through a G-protein. endothelin receptor-B is mainly expressed in resistant arteries controlling peripheral resistance and, therefore, is important in blood pressure control. Calcium channel blockers antagonise this effect (Luscher *et al* 1992).

4.2.2.2 Angiotensin II (AT-II):

Angiotensin-II is the active product of the renin-angiotensin system, produced from Angiotensin-I by the action of angiotensin converting enzyme (ACE). AT-II stimulates release of a vasoconstrictor substance, thromboxane A_2 from EC's. Manabe *et al* (1989) noted in an experiment on dogs, that AT-II caused endothelium dependent vasoconstriction, which could be inhibited by cyclooxygenase inhibitor aspirin. AT-II has been shown to induce apoptosis of human umbilical vein endothelial cells, an effect that could be inhibited by NO (Dimmeler *et al* 1997a).

4.2.2.3 Superoxide anion (O^-_2):

Superoxide anion (O^-_2) is a one-electron reduced form of oxygen. It is a highly reactive oxygen free radical produced by the endothelial, inflammatory and vascular smooth muscle cells via different oxidative enzyme systems. The most important sources are NADH/NADPH oxidoreductase, cyclooxygenase, xanthine oxidase and eNOS. O^-_2 has a direct vasoconstrictor effect on smooth muscles. In an experiment on canine cerebral arteries, Katusic and Vanhoutte (1989) found that O^-_2, produced by xanthine and xanthine oxidase in the presence of catalase, caused smooth muscle contraction that can be inhibited by superoxide dismutase enzyme (SOD). O^-_2 also acts indirectly by altering the balance between the vasodilating and vasoconstricting factors, which reduces the bioavailability of NO (Cosentino *et al* 1994).

O^-_2reacts rapidly with NO to form peroxynitrite ($OONO^-$), a highly reactive molecule and is also converted readily to hydrogen peroxide by the action of SOD.

4.2.2.4 Vascular tone as an active process:

Thus, in the normal state, vasomotor tone is maintained mainly by a balance between endothelium derived vasodilators NO, EDHF, prostacycline (PGI2), and the endothelium derived vasoconstrictors, ET1, AT-II and thromboxane A2, with the net effect on resting vessels towards mild vasodilatation.

4.3 Regulation of haemostasis:

Normally functioning endothelium exhibits anti-thrombotic features through several mechanisms:

1. Expression of heparan sulphate on the endothelial surface activates anti-thrombin III, an inhibitor of thrombin, which inhibits the conversion of fibrinogen to fibrin (Busch *and* Owen 1982).
2. Synthesis of a thrombomodulin receptor, which binds to thrombin thus reducing its procoagulant activity. In addition, thrombomodulin-thrombin complex activates protein-C, which inhibits the action of several activated coagulation factors (Grinnell and Berg 1996).
3. NO also inhibits platelets adhesion to the endothelium. Radomski *et al* (1987) showed *in vitro* that this effect is potentiated by SOD, which inactivates O^{-}_{2} anion and thus increases the availability of NO. They also noted that prostacycline and NO have a synergistic effect in inhibiting platelets aggregation.

4.4 Regulation of inflammation:

Inflammation is a complex process, naturally designed to control infection, limit tissue damage and promote regeneration. Inflammation is regulated by the release of inflammatory cytokines from endothelial and inflammatory cells and the expression of CAMs on endothelial cells.

Expression of CAMs plays a pivotal role in recruitment of leukocytes at the sites of inflammation. There are two main groups of CAMs, the selectins group E-, L- and P-selectin, which are expressed on endothelial cells, leukocytes and platelets and endothelial cells respectively. They bind to specific carbohydrate molecules on leukocytes. The other group of CAMs include, ICAM-1 and VCAM-1, which are members of the immunoglobulin super-family and bind to specific glycoproteins (integrins) on the leukocyte surface (Bevilacqua 1993).

CAMs are expressed in response to chemotactic stimuli released during tissue damage, such as leukotriene B4, complement component C5a, chemotactic peptide and platelet-activating factor (PAF) (Tonnesen 1989). E-selectin expression is tightly regulated by inflammatory cytokines, mainly IL-1 and TNF-α. On cytokine activation E-selectin appears within one hour, reaches a

maximum at six hours and then gradually returns to the basal level (Wellicome *et al* 1990).

Leukocyte recruitment is a multi-step process; initially E-selectin expression increases the number of rolling leukocytes and enhances their contact with endothelium-bound chemokines. This triggers binding of β2 integrin on the leukocyte surface to the ICAM-1 leading to firm adhesion, which is then followed by transmigration (Simon *et al* 2000). A recent study showed that endothelium-bound chemokines also trigger binding of another integrin (VLA-4) on lymphocytes to the VCAM-1, leading to massive lymphocyte migration (Cinamon *et al* 2001).

Several genetic polymorphisms have been described in the CAMs (E-, P- and L-selectin, ICAM-1 and VCAM-1) (Wenzel *et al* 1996). E-selectin gene is located on chromosome 1q 23 within the linkage area for SLE in murine and human studies (Moser *et al* 1998, Shai *et al* 1999). A single nucleotide polymorphism from adenine to cytosine at position 561 (A561C) in E-selectin gene result in substitution of serine for arginine at position 128 (S128R) in the EGF domain of the E-selectin molecule. This polymorphism is of particular importance, since the C allele has been found in higher frequency in patients with premature CHD compared to the general population (Wenzel *et al* 1997) and Ye *et al* 1999) (Table 3.1). The association of C allele with CHD was even stronger in 40 patients aged ≥ 40 years with a frequency of 21% compared to 8.7% in the controls, P= 0.003 (Wenzel *et al* 1994). Using electron beam computed tomography, Ellsworth *et al* (2001) found in a study of 294 a symptomatic males and 314 females that in women aged 50 years, coronary artery calcification was detected in 27% of those with the C allele compared to 6.7% in those without. Where as in women aged > 50 years, coronary calcification as detected in 38% of those with the C allele compared to 39% in those without.

Table 4.1 Frequency of C allele of the E-selectin gene in patients premature CHD and in the general population:

Study	C-allele frequency		P-value
	Patients	Controls	
Wenzel *et al* (1997)	15.5% (n=113)*	8.7% (n=103)	< 0.05
Ye *et al* (1999)	19.5% (n= 82)	10.6 (n=71)	< 0.05

* Patients aged ≤ 50 years.

EC's and fibroblasts secrete monocyte chemotactic protein (MCP) within three hours of activation by IL-1ß and TNF-α (Rollins *et al* 1990, Strieter *et al* 1989). In addition, IL-4 secreted by T-lymphocytes specifically serves to increase the binding of endothelial cells to T-lymphocytes. This selective recruitment of immune cells occurs at the sites of chronic inflammation (Thornhill *et al* 1990). Thrombin and bradykinin stimulate prostcyclin and NO release, which enhance blood flow to the site of inflammation. In normal conditions, the inflammatory reaction is controlled by a down regulating mechanism to prevent excessive tissue damage and to promote resolution. Interleukin-8, secreted by activated endothelial cells selectively, inhibits further adhesion and promotes rapid detachment of adherent neutrophils from human umbilical vein endothelial cells (Luscinskas *et al* 1992).

4.5 Endothelial dysfunction:

Endothelial dysfunction describes a state of reduced basal production or avialability of NO. Several studies have demonstrated endothelial dysfunction in patients with established atherosclerotic vascular diseases and in asymptomatic patients with risk factors for CHD. Features of endothelial dysfunction include:

1. Impaired regulation of the vasculature, which manifests as impaired dilation of the blood vessels in response to endogenous or exogenous agonists that act by stimulating NO release or to an increase in shear stress caused by increased blood flow.

2. Increased expression of cellular adhesion molecules, which increase adhesiveness and promote migration of leukocytes to sub-endothelial space.
3. Increased production of cytokines and growth factors.

Currently it is widely accepted that endothelial dysfunction represents the earliest stage in the development of atherosclerosis.

4.5.1 Role of oxidant stress in endothelial dysfunction:

In normally functioning endothelium there is a crucial balance between NO and O^-_2 production. There is strong experimental evidence to support the hypothesis that oxidative stress, generated by excess production of O^-_2 in the arterial wall, is involved in the pathogenesis of atherosclerosis. Excessive O^-_2 production impairs endothelial function and enhances lipoprotein oxidation. O^-_2 anion is produced in the cells by several enzyme systems, mainly NADH/NADPH oxidase, xanthine oxidase and uncoupled eNOS. NADH/NADPH oxidase is regulated by inflammatory cytokines, hormones and shear stress (Warnholtz *et al* 1999). Xanthine oxidase expression is enhanced by INF-γ (Dupont *et al* 1992) and its inhibition has been shown to improve endothelial function in hypercholesterolaemic rabbits (Ohara *et al* 1993) and in hypercholesterolaemic patients (Cardillo *et al* 1997).

Uncoupling of eNOS occurs in states of substrate (L-arginine) or co-factor tetrahydrobiopterin (BH_4) depletion where eNOS can produce O^-_2 and H_2O_2 (Vasques-Vivar *et al* 1998). Intra-arterial infusion of BH_4 has been shown to improve endothelial function in chronic smokers and in patients with Type-II diabetes (Heitzer *et al* 2000).

The reactions of O^-_2 can also lead to the generation of additional, often more highly reactive, free radical species. For example O^-_2 readily reacts with NO leading to a decrease in the bioavailability of NO and production of $OONO^-$ a highly reactive molecule which enhances oxidative damage to the cell membrane and oxidative modification of LDL-C. Hydrogen peroxide (H_2O_2) is produced by the action of SOD enzyme on O^-_2. Hydoxyl group (OH^-) is produced by the catalysis of H_2O_2 in the presence of transitional metals (Liao 1998). O^-_2 produced in cultured endothelial cells increases by four to five-fold the oxidation rate of human LDL-C compared to the rate in cell-free incubation.

This oxidation is significantly inhibited by transfection of endothelial cells with cDNA for SOD or by adding exogenous SOD (Fang *et al* 1998, Takatsu *et al* 2001). Miller *et al* (1998) also reported that O^{-}_{2} production was three times higher in atherosclerotic compared to normal rabbits and similarly transfection of the atherosclerotic group with the SOD gene significantly reduced endothelial O^{-}_{2} production. Interestingly, this did not improve endothelium-dependent dilatation, which suggests that in atherosclerosis, O^{-}_{2} is produced not only by the endothelium but also in the media.

Hypertension causes an increase in the stretch forces on the endothelium and increased production of O^{-}_{2}. Inoue *et al* (1998) used special dishes to study the effect of stretch on the ability of rat aortic smooth muscle cells to oxidize LDL-C. Stretch resulted in a 150% increase in the rate of LDL-C oxidation and a concomitant increase in O^{-}_{2} production. LDL-C oxidation was inhibited by adding superoxide dismutase or by chelation of metal ions in the culture medium. Furthermore, the enhanced LDL-C oxidation by stretch force was inhibited by diphenyliodonium, an inhibitor of NADH/NADPH oxidase system, indicating that excessive shear stimulates O^{-}_{2} production via activation of NADH/NADPH oxidase.

Increased oxidant stress enhances inflammation via activation of transcription factors such as nuclear factor-kappa B (NF-κB), activator protein-1 and early growth response factor (egr-1). This induces transcription of CAMs, inflammatory cytokines and growth factors such as monocyte colony stimulating factor (Collins 1993). Therefore, increased oxidant stress enhances inflammation and increases oxidation of LDL-C in the arterial wall.

I suggest that the interaction between oxidative stress, inflammation and oxidation-susceptible lipoproteins in the pathogenesis of atherosclerosis can be viewed as the three sides of "atherosclerosis triangle", which bear analogy to classic "fire triangle" in nature represented by heat, oxygen and fuel (Figure 4.2).

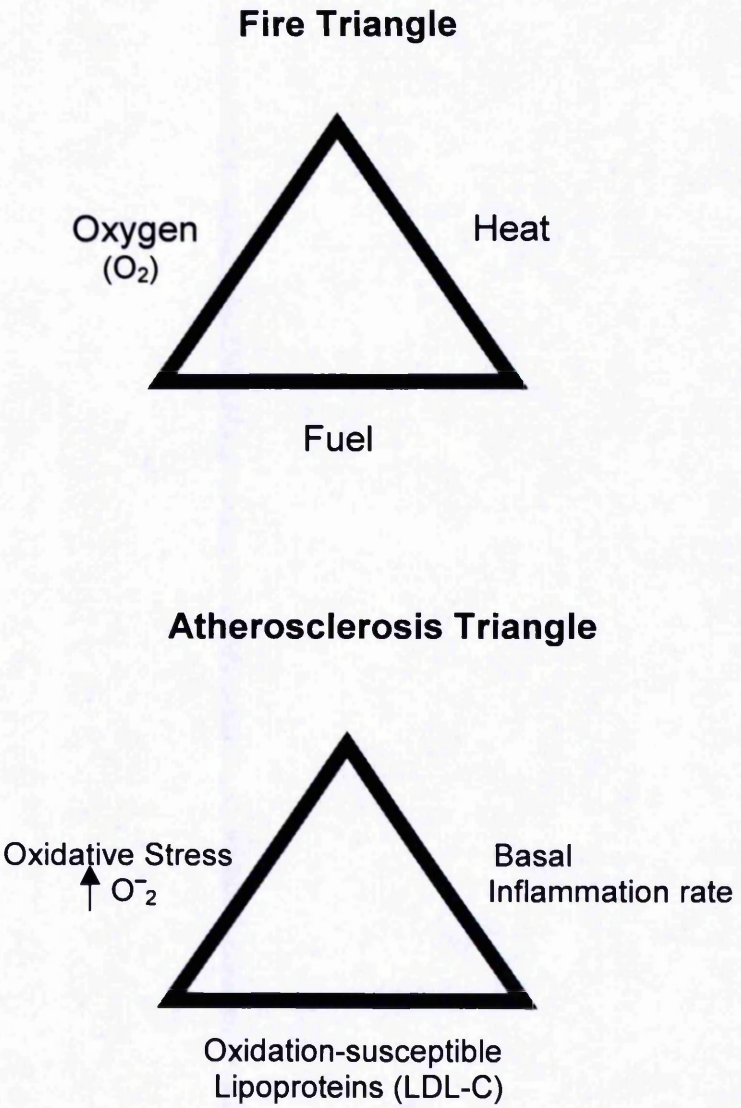
Recent studies have shown that the isoprostanes (isomers of prostaglandin) are produced by an enzyme-independent reaction of free radicals with the arachidonic acid compounds in the cell membrane. These are stable compounds and have been isolated from the plasma and urine of animals and

humans with conditions associated with high oxidant stress (Lawson *et al* 1999). Assays of F_2-isoprostanes offer a sensitive non-invasive method for the measurement of the oxidative stress and their levels *in vitro* have correlated with the degree of LDL-C oxidation (Lynch *et al* 199, Delanty *et al* 1996). SLE patients also showed enhanced urinary excretion of isoprostanes, which was more pronounced in patients positive for anti-phospholipid antibodies (Iuliano *et al* 1997). In addition, immunohistochemical staining of human atherosclerotic lesions showed strong immune reactivity for the isoprostanes 8-epi $PGF_{2\alpha}$ and $IPF_{2\alpha}$-1 in monocytes, macrophages and vascular smooth muscle cells (Pratico and Fitz Gerald *et al* 1996).

4.6 Role of endothelial dysfunction in the pathogenesis of atherosclerosis:

Atherosclerosis was believed to result from a response to damage or loss of endothelial integrity, the so-called response to injury theory. However, recent evidence suggests that endothelial dysfunction, rather than loss or damage to the endothelium, is the earliest stage in the development of atherosclerosis. On a rabbit model of atherosclerosis, Mano *et al* (1996) showed that endothelial dysfunction, manifested as impaired endothelium dependent vasodilation, can be demonstrated at a very early stage when only microscopic changes of atherosclerosis are present in the intima. This group also demonstrated a vasoconstrictive response to infusion of acetylcholine into angiographically normal coronaries of patients with established CHD. Endothelial dysfunction is associated with increased adhesiveness of monocytes and increased oxidative stress in the arterial wall, which enhances LDL-C oxidation within the endothelial cells and other components in the sub-endothelial space. Oxidative and chemical modification products of LDL-C lead to further impairment in endothelial function and recruitment of monocytes to the sub-endothelial space. Monocytes transform to macrophages which take-up the ox-LDL-C through specific scavenger receptors that mainly recognize the acetylated forms of ox-LDL-C. Macrophages then transform into foam cells, which are the predominant microscopic feature of early atherosclerotic lesions (Goldstein *et al* 1979).

Figure 4.1 Triangle analogy between fire in nature and atherosclerosis in life:



Goldstein and Brown (1977) showed that endothelial cells have a specific high affinity receptor for LDL-C. About 85% of LDL-C normally enters the arterial wall and diffuses back, whereas a higher proportion of the ox-LDL-C is trapped in the sub-endothelial space (Schwenke and Carew 1989). Henriksen *et al* (1981) reported that incubation of LDL-C with cultured endothelial cells for 12-16 hours resulted in different modified forms of LDL-C, which were taken up by cultured macrophages at a 3-10 times greater rate than native LDL-C. The atherogenic effect of ox-LDL-C is mediated through different oxidation products, such as lysophosphatidylcholine (Lyso-PC), lysolicithin and oxidized sterols. These products promote inflammation in the arterial wall by the following steps:

4.6.1 Recruitment of monocytes to the sub-endothelial space:

Quinne *at al* (1985) showed that Lyso-PC, generated by the activity of phospholipase A2 enzyme during LDL-C oxidation or from membrane phospholipids of damaged cells, is a strong chemotactic factor for circulating monocytes and also enhances localization of monocytes to the sub-endothelium space during the early stages of atherosclerosis. Lyso-PC has been shown to selectively induce expression of VCAM-1 and ICAM-1 in cultures of human and rabbit arterial endothelial cells, and also to induce growth factor transcription in endothelial cells (Kume *et al* 199, Kume and gimbrone 1994). Ox-LDL-C stimulates endothelial and smooth muscle cells to secrete monocyte chemotactic protein-1 (MCP-1) and macrophage colony stimulating factor (M-CSF), which promote chemotaxis and differentiation of monocytes into macrophage (Ross *et al* 1990).

MCP-1 plays an important role in the initiation and progression of atherosclerosis. In one study, LDL receptor deficient mice were also made MCP-1 deficient and then fed on a cholesterol-rich diet. Compared to control LDL receptor deficient mice fed on the same diet, MCP-1 deficient mice showed 83% less lipid deposition and fewer macrophages in the aorta (Gu *et al* 1998). *In vitro* studies show that shear stress and minimally oxidized LDL-C also increase production of MCP-1 (Shyy *et al* 1994). MCP-1 selectively attracts monocytes and memory T-lymphocytes (Fuentes *et al* 1995). Oxidative products of LDL-C also activate transcription factors, such as NF-κB, activator

protein-1 and early growth response factor, which control transcription of CAMs and several cytokines and growth factors (Collins 1993). Recently, Wilson *et al* (2002) reported further evidence for the role of inflammation in the progression of atherosclerosis, by detecting NF-κB immune reactivity in coronary atherosclerotic plaques removed by direct atherectomy from 32 CHD patients. Plaques from 16 patients with unstable angina showed greater immune reactivity to NF-κB, with more inflammatory cells and loose intimal proliferation, whereas those with stable angina showed less reactivity and more fibrosis.

4.6.2 Proliferation and migration of vascular smooth muscle dells (VSMCs):

Proliferation and migration of VSMCs into the intima play a critical role in progression of the atherosclerotic lesions and formation of the fibrous cap. This process is regulated by a number of cytokines and growth factors, such as platelet derived growth factor (PDGF) (Raines and Ross 1993). PDGF has a strong chemotactic and mitogenic effect on smooth muscle cells (Grotendorst *et al* 1982). Lyso-PC has been shown *in vitro* to stimulate the release of PDGF from smooth muscle, macrophages and endothelial cells (Stiko-Rahm *et al* 1992, Kume and Gimbrone 1994). VSMCs also express receptors for ox-LDL-C and can take the appearance of foam cells (Moncada *et al* 1977). VSMCs produce large amounts of extracellular matrix, accumulate lipids and are the predominant cells in the fibrous cap of atherosclerotic lesions. VSMCs proliferation controls the size of the fibrous cap and the propensity of the plaque for rupture (Burke and Ross 1979, Ross 1986).

4.6.3 Alteration of the intercellular matrix:

Alteration of the intercellular matrix involves matrix metalloproteinases (MMPs), a large family of enzymes, which degrade collagen and connective tissue matrix in the vessel wall and mediate remodelling in response to haemodynamic changes. Normally there is a balance between MMPs and tissue inhibitors of MMPs (Brew *et al* 2000). MMPs, produced constitutively by endothelial and smooth muscle cells, have been implicated pathologically in plaque disruption, formation of stenotic lesions and aneurysmal dilation (Hanemaaijer *et al* 1993,

Galis *et al* 1995a). Macrophages are an important source of MMPs (Galis *et al* 1995b). Expression of MMPs in macrophages has been stimulated *in vitro* by incubation with ox-LDL-C (Xu *et al* 1999) and in response to inflammatory cytokines (Libby and Galis 1995). Oxygen free radicals O^{-}_{2} and the highly reactive product $OONO^{-}$ both activate stored MMPs zymogens and inhibit tissue inhibitors of MMPs. Degradation of collagen in the fibrous cap by MMPs produced by macrophages, weakens the cap and predisposes to plaque rupture. This has been shown in human plaques and in animal model of atherosclerosis (Shah *et al* 1995, Rekhter *et al* 2000).

4.6.4 Cytotoxicity:

Ox-LDL-C has been shown *in vitro* to have cytotoxic activity for dividing VSMCs, macrophages, fibroblasts and endothelial cells (Chatterjee 1992). Bjorkerud and Bjorkerud (1996) demonstrated that minimally oxidized LDL-C stimulate growth whereas strongly modified LDL induce cell death by apoptosis, and that this differential effect may explain excessive alternating areas of intimal growth and necrosis in typical atherosclerotic lesions. Davies *et al* (1976) have demonstrated with the use of scanning electron microscopy loss of endothelial integrity over fatty streaks. Recently, ox-LDL has been shown to induce apoptosis of cultured endothelial and vascular smooth muscle cells through the Fas-Fas ligand interaction pathway (Sata and Walsh 1998, Lee and Chau 2001).

4.6.5 Generation of immune response:

Early in the modification process of LDL-C antigenic epitopes are formed when oxidized short chain fatty acids combine with lysine residues of apo-B forming lipid-protein adducts. Generation of antibodies to ox-LDL-C may lead to accelerated uptake of immune complexes through Fc-γ receptors of macrophages at an earlier stage in the process of oxidation before recognition by acetyl LDL-C or scavenger receptors (Steinberg *et al* 1989).

4.7 Role of Nitric oxide:

Normal basal production of NO by the eNOS enzyme from the endothelial cells is critical for maintaining normal endothelial function and has been shown to have anti-atherogenic effects, which include:

4.7.1 Anti-inflammatory effects:

NO inhibits expression of cellular adhesion molecules and production of proinflammatory cytokines via inhibition of NF-κB transcription (Peng *et al* 1995). De Catrina *et al* (1995) showed *in vitro* that NO significantly reduced expression of VCAM-1, E-selectin and, to a lesser extent, ICAM-1 on cytokine activated EC's. This was associated with reduction in adhesion of monocytes to the endothelium and also decreased secretion of the pro-inflammatory interleukins (IL-6, and IL-8).

4.7.2 Anti-platelet effect:

The anti-platelet effect of NO is mediated via two mechanisms. Direct activation of guanine cyclase enzyme to increase cGMP in the platelets and NO-mediated activation of cyclooxygenase to produce prostacycline, an arachidonic acid derivative, which has an anti-platelet and vasodilator effect (Radomski *et al* 1987, Salvemini *et al* 1996). Inhalation of nitric oxide has been shown to prolong bleeding time and reduce agonist-stimulated platelet aggregation (Gries *et al* 1998).

4.7.3 Antiproliferative effect:

In vitro studies have shown that NO inhibits DNA synthesis in VSMCs (Nakaki *et al* 1990). Janssens *et al* (1998) in balloon-injured carotids in rats showed that transfection of lesions with cloned eNOS restored NO production and inhibited VSMCs cells proliferation and neo-intima formation.

4.7.4 Regulatory effect on VSMCs and extracellular matrix:

NO has a regulatory effect on collagen synthesis by smooth muscles. in a culture of coronary endothelium and vascular smooth muscles, Myers *and* Tanner (1998) showed a spontaneous gradual increase in collagen type-I and

decrease in type-III, while inhibition of NO resulted in a marked increase in type-I and mild increase in type-III. Gurjar *et al* (1999, 2001) found that NO inhibits induction of MMP-9 by IL-1 in rat aortic VSMCs. Similarly, NO inhibition in cultured rat aortic VSMCs resulted in a dose dependent increase in expression and synthesis of MMP-9 (Upchurch *et al* 2001).

4.8 Nitric oxide in SLE:

NO is an important mediator of inflammation and is produced in excessive amounts by the inducible NOS (iNOS) enzyme in the endothelium and vascular smooth muscles in response to different inflammatory cytokines including IL-1, TNFα and INFγ (Chester *et al* 1998). Studies on the MRL-lpr/lpr mice model of SLE showed higher excretion rate of NO metabolites nitrites and nitrates in the urine compared to normal strains. In addition, peritoneal macrophages showed increased activity of iNOS and an immune reactivity of iNOS was detected in different tissues. Furthermore, treatment with NO inhibitors inhibited nitrite secretion and prevented development of significant renal disease (Weinberg *et al* 1994). SLE patients have high serum levels of nitrite reflecting higher production of NO and their levels correlate with disease activity and with increased expression of iNOS in endothelial cells and keratinocytes (Belmont *et al* 1997).

Therefore, Excessive production of NO by iNOS may contribute to the pathogenesis of SLE via increase in production of cytotoxic free radicals such as $OONO^-$ and OH^-, which promote inflammation, increase capillary permeability and induce cellular apoptosis (Clancy *et al* 1995).

4.9 Assessment of endothelial function

The study of endothelial function in clinical research depends largely on assessment of the vasomotor aspect of endothelial function. Usually both endothelium dependent dilation (EDD) and endothelium non-dependent dilation (ENDD) are measured in the conduit arteries. Measurement of EDD is based on the ability of the endothelium to produce NO in response to pharmacological stimulation by vasoactive agonists, such as acetylcholine and bradykinin, or to increase in shear stress induced by physiological increase in blood following

reactive hyperaemia. Endothelial dysfunction is demonstrated as reduced or absent dilation or even constriction. Endothelium non-dependent dilation is assessed by the use of glycerine trinitrate (GTN) or nitroprusside, which directly release NO to the smooth muscles, and reflects the functional state of the vascular smooth muscles.

Ludmer *et al* (1986) were the first to use serial angiography in patients with CHD to assess coronary artery response to intra-coronary infusion of acetylcholine. Further studies used another technique, which involved brachial and femoral artery cannulation and infusion of increasing doses of acetylcholine for assessment of EDD or infusion of GTN for assessment of ENDD. The change in forearm and leg blood flow was then measured using venous occlusion plethysmography technique. This technique has also been used to assess the basal rate of NO production using intra-arterial infusion of L-NMMA, a competitive inhibitor of the constitutive NO production by endothelial nitric oxide synthase. Less reduction in forearm blood flow is observed when there is impaired basal production of NO.

Obviously, these invasive techniques for assessment of endothelial function will limit the number of subjects entering studies and may be inappropriate in asymptomatic children or young adults. More importantly, patients with SLE are more prone to infections and thrombosis, especially if they are on immunosuppressive therapy or positive for APL antibodies respectively. Therefore, intra-arterial cannulation for research purposes in these patients may be unethical.

4.9.1 Flow mediated dilatation (FMD):

Flow mediated dilation is the dilation that occurs in conduit arteries in response to an increase in blood flow. Measurement of FMD is a non-invasive method to assess endothelial function and is currently widely used in clinical research of vascular diseases. Percentage of FMD is calculated from the formula;

$$\text{Percentage FMD} = \frac{(\text{Post-dilation diameter} - \text{resting diameter})}{\text{Resting diameter}} \times 100.$$

High resolution B-mode ultrasonography is used for diameter measurement of superficial arteries such, as the brachial or femoral arteries. Rubanyi *et al*

(1986) were first to suggest that FMD is mediated by the release of endothelium relaxing factor. FMD depends on the presence of a normally functioning endothelium and is abolished by physical or pharmacological injury to the endothelium (Pohl *et al* 1986, Smiesko *et al* 1985). Joannides *at al* (1995) showed that FMD is mediated through the release of NO from endothelial cells in response to an increase in shear stress and can be inhibited by L-NMMA, a competitive inhibitor of NOS. The mechanism by which the endothelial cells sense increase in shear stress is not well understood. Boo *et al* (2002) found that laminar shear stress stimulates phosphorylation of eNOS enzyme at position Ser (635) by a protein kinase dependent mechanism. Physiological levels of shear stress also increase expression of Cu/Zn SOD (Inoue et al 1996).

Increase in blood flow in the brachial artery is induced by inflation of a blood pressure cuff around the forearm to induce ischaemic vasoconstriction distal to the site of measurement. Immediately after cuff release the blood flow in the artery increases by several fold, resulting in increased shear stress, stimulation of NO release and vasodilation. Different protocols for the measurement of FMD have used different sites and durations of cuff occlusion, as well as different time points for taking measurements after cuff release. Cuff position on the upper arm produced higher FMD compared to a forearm position but is associated with greater discomfort (Mannion *et al* 1998, Berry *et al* 2000). Leeson *et al* (1997) tested different occlusion durations, ranging from 30 seconds to eight minutes, and the effect of different doses of sublingual isosorbide dinitrate (ISDN) 10-400µg on normal subjects. They recorded a maximum dilatation after eight minutes occlusion; after an occlusion time of 4.5 minutes the dilation produced was near maximum (96%) and lasted for one minute. The peak blood flow occurred at around 15 seconds after cuff release. With regard to ISDN doses, they found that no significant extra dilatation occurred with doses of > 200 µg. In a study of healthy subjects Sinoway *et al* (1989) found that after occlusion for one minute, there was maximum dilatation with 37% increase in diameter above the baseline (0.33 to 0.45 cm). There was little further increase with longer occlusion times.

The technique we used for the measurement of FMD in this study follows that of Celermajer *et al* (1992) and is presented in detail in the methods section.

4.9.2 Accuracy and reproducibility of FMD measurement:

Several studies have shown that the technique of FMD measurement is accurate and reproducible. Using phantom arteries Sorensen *et al* (1995) showed that a 7 MHz B-mode ultrasound could reliably detect diameter differences of 0.1-0.2 mm in vessels measuring 2-5 mm in diameter. Phantom arteries with a 0.1-0.2 mm difference in diameter, were correctly measured in 61% of a total of 264 measurements and no error was more than 0.1 mm. In 40 healthy subjects, aged 22-51 years, who were studied on four occasions, the overall coefficient of variation of percentage FMD for all subjects was 1.8% (1.9% for men and 1.6% for women, P=0.18). In 85% of subjects, repeated measurements were within 2.5% of overall mean FMD% for each subject. Only 5% of measurements on any subject were more than 4% from the mean percentage FMD. The overall coefficient of variation for percentage GTN dilation was 2.8%. The two subjects with most consistent percentage FMD on four occasion had 10%, 10%,10%,11% and 4%,4%,4%,4%, whereas the two subjects with most inconsistent results had percentage FMD of 9%, 10%, 11%, 0% and 1%,1%,7%, 8%. Within subject variability of percentage FMD was mainly from day to day (2.8% variance) with less variation observed over longer intervals of weeks or months (the variances of percentage FMD were 0.1% and 1.3% respectively). Celermajer *et al* (1992) reported a low coefficient of variation of 1-3% for diameter measurements between observers. The mean (SD) of interobserver difference for the measurement of percentage FMD was 1.7% (1.4%) with a range between 0-7%. With repeated measurements on more than one visit, the mean (SD) difference in percentage FMD was 2.8 (2.3) with a range between 1-10%. The estimated coefficient of variation between repeated measurements was 2.3%. However, other studies reported wide variation of percentage FMD between studies. In a study of 19 healthy subjects studied on 2 occasions Hardie *et al* (1997) reported high variability in percentage FMD between studies. The mean (SD) difference of percentage FMD was 0.57% (6.8%). The mean (SD) of difference in percentage FMD within

and between observers was low at 0.13 (2.1) and 0.06 (2.17) respectively. In a study of 75 healthy volunteers, Hijmering *et al* (2001) evaluated the reproducibility of a wall tracking system using a radio-frequency processing technique, which allows a resolution of 2-8 μm for moving targets.

The accuracy of this system is comparable to that of intravascular ultrasound. Reproducibility of baseline diameter was good with intrasession variability of 1.1% (0.06 – 2.0%). Intersession variability was 3.6% and the interobserver variability was 4.1%. Intra-individual variability in percentage FMD between sessions was high with coefficient of variation of 13.9% for FMD and 9.3% for GTN dilation.

To increase the accuracy and improve reproducibility of FMD measurements, stereotactic devices to tried to ensure more stability of the transducer over the artery. Various viewing techniques were used including longitudinal, cross sectional B-mode views and, more recently the edge tracking system software, which allows continuous monitoring of the diameter and measurement of peak response (Woodman *et al* 2001). In addition, automated and semi-automated image analysis soft wares have been used. Herrington *et al* (2001) reported high reproducibility and substantial reduction in analysis time with the use of automated image analysis in a large population-based study of 4,040 subjects. In this study the base line diameter was inversely related to %FMD. Age correlated directly with base line diameter and inversely with %FMD. These modifications, may in part, explain the differences in the normal range values for percentage FMD reported in different studies.

Endothelial function, reflected by percentage FMD, is sensitive and may be influenced by several factors and vary during the daytime. Adjustment for factors such as time of day, fasting state, avoidance of smoking and alcohol and drugs that may alter percentage FMD may all increase the accuracy of endothelial function assessment.

There are several ways to report percentage FMD:

1. Continuous numeric variable.
2. Categorical variable with endothelial dysfunction defined as present or not when percentage FMD is higher or lower than a given cuff-off value.

4.9.3 Endothelial dysfunction in established coronary heart disease:

Endothelial dysfunction is a systemic condition and peripheral endothelial function assessed by FMD in the brachial artery has been shown to correlate closely with coronary artery endothelial function. In a study of 50 patients referred for coronary catheterization, Anderson *et al* (1995) found that patients with coronary dysfunction, as indicated by a constrictor response to sequential intra-coronary infusion of acetylcholine, had significantly impaired percentage FMD in the brachial artery. In multivariate analysis, the independent predictors of impaired percentage FMD were baseline brachial artery diameter, coronary endothelial dysfunction, presence of CHD and smoking. The positive predictive value of percentage FMD of <3% in predicting coronary endothelial dysfunction is 95%.

In 122 patients undergoing coronary angiography for suspected CHD, Enderle *et al* (1998) found that the mean (SD) percentage FMD in those with and without CHD was 3.7 (4.1)% vs 7.0 (3.5)% respectively. Using receiver operating characteristic analysis, an percentage FMD of ≤4.5% predicted CHD with a sensitivity of 71% (95% CI 0.61-0.80) and a specificity of 0.81 (95% CI 0.58-0.95). From the same study of patients with suspected CHD, Schroeder *et al* (1999) compared the predictive value of percentage FMD of ≤4.5% to that of angina pectoris, exercise electrocardiogram and perfusion imaging in predicting any degree of atherosclerosis. The positive predictive value was 0.95 (72 out of 76) and the negative predictive value was 0.41(17 out of 46). Percentage FMD of ≤ 4.5% had a high sensitivity and the best specificity.

Neunteufl *et al* (1997) also reported a strong correlation between percentage FMD and the angiographic extent of CHD in a study of 74 patients with angina who under-went coronary angiography and brachial artery FMD study. In a multiple regression analysis, the extent of CHD and basal diameter were independent factors associated with percentage FMD. Kuvin *et al* (2001) studied 94 men and women who underwent diagnostic exercise myocardial perfusion imaging. Percentage FMD in the brachial artery was significantly impaired in 23 subjects found to have CHD (MI or ischaemia) compared to 71 without CHD (mean ± SEM 6.3 ± 0.7% vs 10.5 ± 0.6 P <0.001). Each 1% decrease in percentage FMD predicted CHD with an odds ratio (OR) of 1.32.

When percentage FMD of 10% was taken as a cut off point, 21 out of 23 subjects with CHD had percentage FMD <10% (91% sensitivity). Only two of 40 subjects with percentage FMD >10% had CHD, which gave a negative predictive value of 95%.

In a prospective study of 147 patients who had coronary endothelial function assessed invasively using infusion of acetylcholine and GTN to test for EDD and ENDD, Schanchinger *et al* (2000) found that coronary artery endothelial dysfunction has a prognostic value in predicting future progression of CHD and ischaemic events. All the abnormal tests of coronary endothelial vasoreactivity were independent predictors of poor prognosis, even after adjustment for traditional risk factors for CHD.

4.9.4 Endothelial dysfunction and FMD and CHD risk factors:

Impairment of FMD has also been associated with the presence of risk factors for CHD in adults. In subjects with a high risk of CHD, impairment of FMD is detected in early childhood. Celermajer *et al* (1994) demonstrated impairment of FMD in asymptomatic hypercholesterolaemic children as young as eight years old and in smokers as young as 17 years old, as well as in patients with established CHD. In this study of 500 asymptomatic non-hypertensive subjects aged between five and 73 years, impaired percentage FMD was associated in univariate analysis with cholesterol level, smoking, hypertension, male gender, larger resting diameter and family history of CHD.

4.9.4.1 Hypercholesterolaemia:

Steinberg *et al* (1997) reported a continuous and inverse relationship between impairment of EDD and cholesterol level in their study of the response to graded infusion of methacholine chloride into the femoral artery in a population with a wide range of cholesterol levels. There was a significant difference in lower limb blood flow and an approximately 50% reduction in EDD between the two groups of high normal and low normal cholesterol.

4.9.4.2 Family history of CHD:

A history of premature CHD in a first degree relative is one of the established risk factor for CHD. Clarkson *et al* (1997) studied 50 young (aged 28 ± 8 years) healthy first-degree relatives of patients who developed premature CHD, which they defined as males aged ≤ 45 years and females aged ≤ 55 years and 50 healthy age matched controls. They found that EDD was significantly impaired in those with a family history of CHD compared to controls. Absence of CHD risk factors in the subjects with premature CHD is a associated more impairment of percentage FMD in their first-degree relatives. This may suggest that some genetic factors play an important role in endothelial dysfunction.

4.9.4.3 Smoking:

Cigarette smoking is associated with endothelial dysfunction and there is an inverse relationship between lifetime amount of cigarettes smoked (pack years) and FMD. Celermajer *et al* (1993) studied FMD in 80 current smokers, 40 ex-smokers and 80 healthy controls. All were normotensive, non-diabetic, with no family history of CHD and had normal cholesterol. Percentage FMD in the healthy controls was 10 ± 3.3% compared to 6.6 ± 4.05, 4.0 ± 3.1%, 3.2 ± 3.2% and 2.6 ± 1.2% in the very light, light, moderate and heavy smokers respectively. The percentage FMD was also reduced in former smokers compared to healthy controls. Passive smoking was also found to be associated with impairment of FMD in a dose dependent relationship, whereas GTN dilation was unaffected (Celermajer *et al* 1996). In a study of 35 chronic smokers and 16 healthy non-smokers using forearm occlusion plethysmography, McVeigh *et al* (1996) compared the response to methcholine, sodium and nitroprusside infusion and cuff occlusion on forearm blood flow. There was no significant difference between the smokers and non-smokers. However, infusion of L-NMMA resulted in a significantly lower reduction in flow in the smokers, which suggests that in long term smokers there is impairment of basal, but not the stimulated, NO production.

4.9.4.4 Hypertension:

Normally FMD response serves to reduce excessive shear stress on the arterial wall. Li *et al* (1997) reported significant impairment of %FMD (4.6% ± 2.8% vs 12.4% ± 2.9) in 21 patients with essential hypertension compared to 21 healthy controls. Panza *et al* (1990) showed that infusion of L-NMMA, an inhibitor of endothelial NOS, into the brachial artery of hypertensive patients produced less reduction in blood flow compared to normal controls, suggesting a defect in the basal production of NO in hypertensive patients. McAllister *et al* (1999) also reported impaired basal production of NO in a group of normotensive offspring of parents with essential hypertension, compared to age matched normotensive offspring of normotensive subjects. This suggests that endothelial dysfunction may be a risk factor for hypertension and not a consequence of hypertension.

4.9.4.5 Diabetes and insulin resistance:

Diabetes mellitus and insulin resistance are associated with endothelial dysfunction. McVeigh *et al* (1992) studied 29 patients with non-insulin dependent diabetes and 21 healthy controls. This study employed venous occlusion plethysmography and brachial artery infusion of graded doses of acetylcholine and GTN. The forearm blood flow responses to acetylcholine and GTN were significantly lower in patients compared to control, which suggests that both endothelium dependent and non-dependent dilation were impaired in patients with NIDDM. Similarly, Steinberg *et al* (1996) reported 40% and 55% reduction in leg blood flow following infusion of methcholine into the femoral artery in obese patients with insulin resistance and in patients with NIDDM compared to the healthy controls. Meeking *et al* (1999) found impaired FMD in patients with insulin dependent diabetes, either with or without microalbuminuria, compared to healthy non-diabetic controls.

4.9.4.6 Systemic lupus erythematousus:

At the time of developing the protocol for this study, there was only one previous study of FMD in SLE, by Lima *et al* (1997), which included 20 premenopausal SLE patients with a mean age of 30 years and 14 healthy controls. This study was expanded and subsequently reported on 69 patients

and 35 healthy controls. The percentage FMD was significantly impaired in SLE patients compared to controls (5.0 ± 5% vs 12.0 ± 6.0% p =<0.001). There was no difference in baseline diameter between SLE patients and controls. Percentage FMD was not related to disease activity, disease duration, history of hypertension, anti-cardiolipin antibody steroid therapy or Raynuad's phenomenon The GTN dilation was significantly lower in patients positive for ACL antibody (11.9 ±4.0% vs 16.3 ±6.0%, p<0.05) (Lima *et al* 2002).

4.10 Reversibility of endothelial dysfunction:

The reduction in cardiovascular morbidity and mortality observed in the large clinical trails of lipid lowering therapy (statins), has been achieved more rapidly than can be explained by regression or stabilization of the atherosclerotic plaques. One suggestion is that this could be related to the effect of statins in improving endothelial function. Several randomized placebo-controlled clinical trials have demonstrated improvement in endothelial function following interventions to reduce CHD risk factors. In a study of 60 patients with acute MI or unstable angina randomized to take pravastatin or placebo for six weeks, Dupuis *et al* (1999) reported 42% improvement in percentage FMD following lowering of TC and LDL-C by 23% and 33% respectively. Tamai *et al* (1997) showed a significant improvement in blood flow response to acetylcholine even with single apheresis of LDL-C and ox-LDL-C. ACE inhibitors are used in the treatment of heart failure, hypertension and MI. In the TREND (Trail on Reversing Endothelial Dysfunction), patients with CHD who were normotensive and without heart failure or major lipid abnormalities were randomized to quinapril (n=51) or placebo (n=54). Using intra-coronary infusion of acetylcholine and quantitative coronary angiography at baseline, the constrictive response to acetylcholine was similar in the two group. After six months of treatment, patients in the quinapril group showed significant improvement in coronary artery endothelial function as demonstrated by progressive dilation in response to increasing concentration of acetylcholine (12 ± 3% vs -0.8 ± 2.9%, p=0.002) (Mancini *et al* 1996). Anti-oxidant therapy may increase the bioavailability of NO by reducing its degradation by O^{-}_{2}. Intra-arterial infusion of vitamin C has been shown to significantly improve forearm blood flow in

response to intra-arterial methacholine in a group of patients with insulin-dependent diabetes (Timimi *et al* 1998). In a study on 23 patients with type II diabetes, McVeigh *et al* (1993) showed a significant improvement of forearm blood flow in response to intra-arterial infusion of acetylcholine after 6 weeks treatment with fish oils.

4.11 Summary:

The endothelium is metabolically very active, secreting various vasodilator and vasoconstrictor substances. The best characterized is NO. In addition to regulation of the vascular tone, constitutive production of NO is essential for maintaining other aspects of normal endothelial function, most importantly is its anti-atherogenic and anti-thrombotic properties. Endothelial dysfunction is present at an early stage in the process of atherogenesis and precedes the appearance of pathological changes. Assessment of FMD using B mode ultrasound is a non-invasive tool for assessment of endothelial function. Several studies have shown that endothelial dysfunction, detected by impaired FMD, is present not only in patients with established CHD but also in asymptomatic young individuals with risk factors for CHD. In addition, impaired FMD has been found to correlate with invasive testing of coronary endothelial function and has a high predictive value for angiographic evidence of CHD. Indeed it has comparable sensitivity and even better specificity than exercise electrocardiogram in this regard. Thus, the ability to detect impaired endothelial function early in the process of atherosclerosis opens a wide area for research to determine metabolic or genetic factors associated with endothelial dysfunction, and to plan early interventions to improve or reverse endothelial dysfunction with the aim to prevent or retard CHD progression.

There are, however, problems with accuracy and reproducibility of FMD measurement. The accuracy and interobserver variability can be improved by conducting studies with experienced sonographers and by the use of computer software for automated continuous image analysis. The problem of reproducibility is more important in longitudinal studies and clinical trials that aim to detect improvement in percentage FMD after interventions. Although, most studies showed good reproducibility of percentage FMD on repeated

measurements over time, some studies showed poor reproducibility on repeated measurement. In the case of cross sectional studies that compare groups of subjects, the problem of reproducibility is less important. Since, variation in percentage FMD will be non differential or non-systematic, its effect will be in the direction of reducing the chance of detecting a difference. Any significant difference is therefore likely to represent a real difference. Larger arteries tend to dilate less than smaller arteries and the resting diameter correlates negatively with and influences percentage FMD. There is a progressive increase in resting diameter with age. This may be related to general structural and functional changes in the arterial walls that occur with age, which may be accelerated by the atherosclerotic process. This is more evident at sites of atherosclerotic narrowing.

In SLE there are several vascular pathologies that may influence FMD such as vasculitis, vasospasm and atherothrombotic risk associated with APL antibodies. It is not known whether impaired percentage FMD in SLE is related to the presence of clinical CHD and traditional risk factors for CHD. It is also important to determine which potentially novel risk factors have an influence. One study prior to developing this hypothesis suggested that FMD is impaired in SLE (Lima *et al* 1997). Such a finding requires confirmation. It is also important to determine whether endothelial dysfunction is related to other markers of sub-clinical atherosclerosis and CHD risk factors. In addition, the influence of the other vascular pathologies observed in SLE also requires further investigation as this may provide additional clues to the pathogenesis of atherosclerosis in this context.

Chapter 5

5 Specific aims of the project

5.1 Aims:

Given the background to vascular dysfunction and excess atherosclerosis in SLE discussed above, our main aims in this project are:

- Assessment of endothelial function by doppler ultrasound measurement of flow-mediated endothelium dependent dilation of the brachial artery in response to reactive hyperaemia, and also endothelium independent dilation in response sublingual spray of GTN in SLE patients and healthy controls.
- To determine predictors of endothelial dysfunction in SLE. Classical and novel risk factors of CHD as well as disease related factors such as disease activity, steroid therapy, APL antibodies and Raynaud's on endothelial dysfunction will be examined. Also to determine whether SLE is an independent predictor of endothelial dysfunction.
- Assessment of novel risk factors for CHD, which include:
    - Hyperinsulinaemia and insulin resistance using the homeostasis model assessment to derive insulin sensitivity and pancreatic β-cell function from fasting insulin and glucose.
    - To compare insulin sensitivity in SLE patients and healthy controls.
    - To study the effect of disease activity and therapy (steroid and anti-malarial therapy) on fasting insulin and insulin sensitivity.
    - To study the effect of fasting insulin on vascular function.
- Assessment of the some novel risk factors for CHD and their potential influence on endothelial dysfunction:
    - Hypertriglyceridaemia,
    - High sensitive CRP (hs-CRP).
    - Interleukin-6 (IL-6),
    - Lipoprotein(a),
    - Paroxonase activity
    - Ox-LDL-C.
- To determine the frequency of the A561C E-selectin gene polymorphism in SLE patients and in controls from 3 different ethnic populations (British,

Spanish and Turkish) and to examine associations of the mutant C allele with organ involvement and vascular function.

5.2 Hypothesis:

The above aims were to test the hypotheses that patients with SLE:

- Have impaired endothelial function that is related to the presence of atherosclerosis.
- Classic CHD risk factors may not fully explain endothelial dysfunction in SLE and therefore.
- Other factors including novel metabolic and genetic risk factors for CHD as well as disease specific factors will also contribute to endothelial dysfunction and increased risk of CHD in SLE.

Chapter 6

6 Methods

6.1 Introduction:

The experimental work in this thesis is divided into three parts. The first part was the assessment of endothelial function and measurement of the intima medial thickness. This part was done in the vascular laboratory of the Vascular Surgery Department at Manchester Royal Infirmary. We used high-resolution ultrasound to measure flow mediated dilation in the brachial artery. We also measured by B mode ultrasound, intima-media thickness of the common carotid artery. The techniques used in this study are described in details in the flow mediated dilation study (chapter 8). The second part was measurement of insulin and other metabolic factors. The third part of the experimental work was in genetics, which involved DNA extraction, use of restriction fragment length polymophism and polymerase chain reaction technique for studying the E-selectin gene A561C polymorphism. The techniques are described in detail in the genetic study (chapter 10).

In this chapter, I will describe several laboratory methods of relevance to other chapters, and in more details, the immunoassay techniques for measurement of oxidised LDL-C and lipoprotein (a), which I have performed in the Lipid Laboratory, Department of Medicine, University of Manchester.

6.2 Introduction to immunoassay:

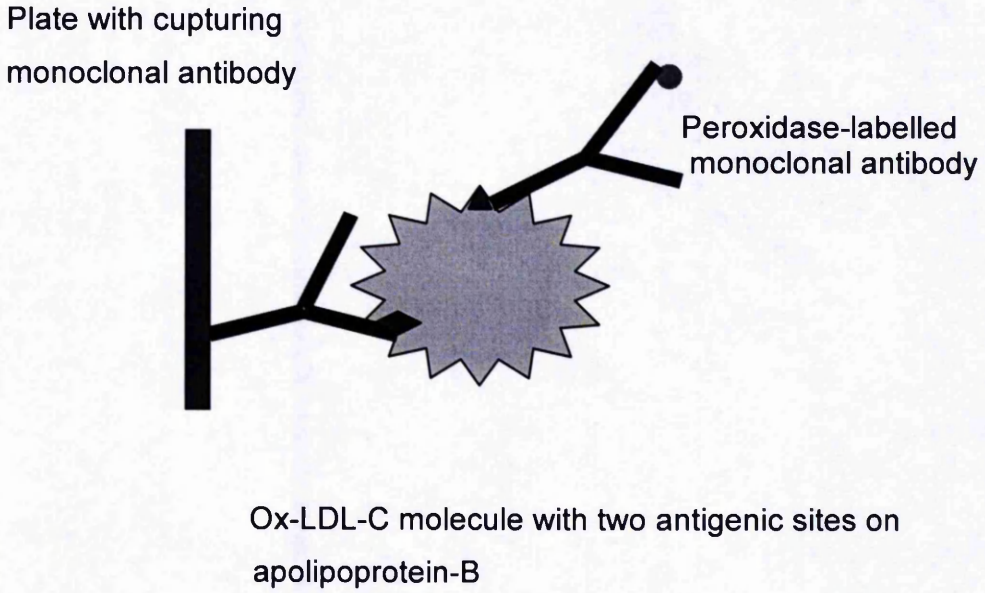
Radioimmunoassay describes several techniques that use radiolabelled reagents to detect antigens or antibodies. Antibodies to be tested in solution are first bound to plates sensitised with specific antigens. The antibodies are then detected by adding a radiolabelled ligand specific for that antibody. The amount of ligand bound to the plate is proportional to the amount of antibodies to be measured. The classic technique used to assay antigens is competitive radioimmunoassay in which plates with specific antibodies are used to bind both the unlabelled antigen in solution and the added labelled antigen. Both labelled and unlabelled antigens will then compete for the binding site on the antibody. The amount of washed out labelled antigen is proportional to the bound

unlabelled antigen. Enzyme linked immunosorbent assay (ELISA) is a similar technique for detection of antibodies or antigens, but the labelling agent is an enzyme instead of a radioisotope agent. Peroxidase and phosphatase enzymes are usually used. Another technique is the sandwich immunoassay in which plates with specific antibodies are used to capture the antigen to be measured. Another specific labelled antibody that binds to a different antigenic site on the antigen is added.

6.3 Assay of oxidized LDL-C:

Ox-LDL-C is an assay based on a two-site ELISA (sandwich technique), in which two monoclonal antibodies are directed against separate antigenic sites on apolipoprotein molecule on LDL-C particle (Figure 6.1). During incubation ox-LDL-C in the samples binds to monoclonal anti-ox-LDL-C fixed to plates in microtitration wells. After washing to remove unbound plasma, a peroxidase-conjugated monoclonal anti-apolipoprotein B antibody was then added to the wells, which binds to a separate site on apolipoprotein B fixed on the plates. A second wash will then remove unbound labelled antibody.

Figure 6.1 Sandwich technique of ELISA:



The amount of bound conjugate is detected by a reaction with 3,3',5,5'-tetramethylbenzidine (TMB) which react with peroxidase to generate a colorimetric reaction. This reaction is stopped after a defined period by adding an acid to give a colorimetric endpoint that can be read by spectrophotometer at 450 nm.

6.3.1 **Oxidized LDL-C ELISA assay reagents and buffers:**

Kits for ox-LDL ELISA were supplied by Mercodia AB Sweden (Ref 10-1143-31). Each kit is supplied with:

1. Mouse monoclonal antibody to ox-LDL-C microtitration strips in plates of 69 wells.
2. Assay buffer, one 12 ml vial ready for use.
3. Four standard concentrations of lyophilized ox-LDL-C, each supplied in a separate vial with labelled concentration. During the assay 1ml of redistilled water is added to each vial.
4. Two (low and high concentration) control samples of ox-LDL, which are also reconstituted by adding 1ml of redistilled water.
5. Ox-LDL-C standard 0 sample, ready for use.
6. Peroxidase conjugated mouse monoclonal anti-apolipoprotein-B, one vial of 1.2 ml.
7. Anti-apolipoprotein-B conjugate stock solution (12 ml vial). This is added to the 1.2 ml vial of peroxidase conjugated mouse monoclonal anti-apo-B to prepare the conjugate solution.
8. Samples buffer; two bottles of 100 ml for dilution of plasma samples
9. Washing solution concentrate; one bottle of 40 ml, which is diluted to 1:21 by adding 20 parts of redistilled water.
10. Peroxidase substrate (TMB), ready for use, supplied in a vial of 22 ml.
11. Stop solution 1M of sulphuric acid (H_2SO_4).

6.3.2 Dilution of plasma samples:

Samples are diluted on the same day as the assay with two tubes for each patient's sample. The dilution is performed in two steps:

1. To 25μl of plasma we added 2000μl of sample buffer and mixed to make 1/81 dilution.
2. To 25μl of 1/81 dilution we added 2000 ml of buffer sample and mixed to make a final dilution of 1/6561.

6.3.3 **Assay procedures:**

Each sample is tested in duplicate, and this includes unknown plasma, standards and controls. Forty plasma samples were tested using one 96-well plate. The procedures we used consisted of:

1. Addition to the 96-well plate of 25μl of plasma samples, standards and controls.
2. Addition of 100μl of assay buffer.
3. Incubation of the plate on a shaker for two hours at room temperature.
4. Washing six times, using an automatic washer.
5. Addition of 100μl of conjugate solution.
6. Incubation for one hour at room temperature.
7. Washing six times using an automatic washer.
8. Addition of 200 μl of peroxidase substrate.
9. Incubation for 15 minutes at room temperature without shaking.
10. Addition of 50μl of stop solution and then putting the plate on a shaker for 15 seconds to ensure mixing of substrate and stop solution.
11. Measuring the absorbance at 450 nm by spectrophotometer and calculation of the results.

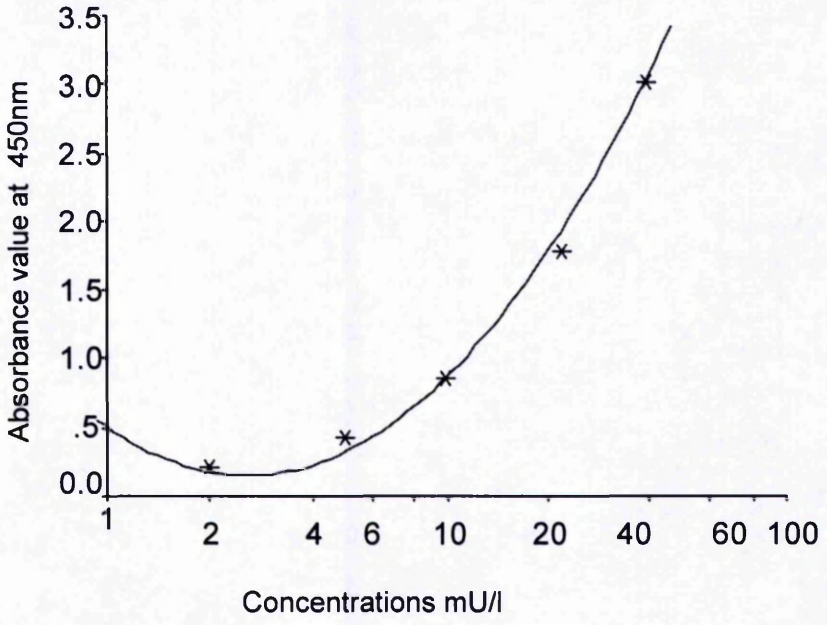
6.3.4 **Calculation of the results:**

This can be done manually by:

1. Plotting absorbance values of the standards against the concentrations of the standards on a linear/logged paper with 0.0 – 100 logarithmic scale on the X-axis (Figure 6.2).
2. Reading the concentration of the samples and controls from the curve.
3. Multiplying concentration of the samples and controls by the dilution factor of 6.6.

Figure 6.2 Standard curve of ox-LDL-C:

Obtained by plotting measured absorbance of the standards at 450 nm against their concentration:



Calculation is also done by a computer program using the linear regression equation y = b*x +a, where b is the coefficient of regression which equals 0.075 and represent the amount of change in absorbance by each unit change in concentration, and (a) is the constant and equals 0.096 [y = 0.075*X + 0.096]. The squared correlation coefficient of the model equals 0.992, which means that 99% of the change in absorbance is explained by change in concentration. In this work we used a Multiskan system with Ascent V1.24 software, which executes three functions; automatic washing of the plates, photometric reading and calculation of the results

There is no international reference for ox-LDL-C levels so it is calibrated relative to arbitrary units (U/l). Intra-assay and inter-assay coefficients of variation calculated from three to eight repeated assays of three samples in one setting and repeated on 20 different occasions, were <8% and <7% respectively. The lower detection limit is < 1mU/l.

6.4 Lipoprotein (a) Assay:

For the assay of lipoprotein(a) we used the same technique of sandwich ELISA provided by Mercodia. Two monoclonal antibodies that recognize different antigenic sites on apolipoprotein(a) were used. During the first incubation apolipoprotein(a) in the sample binds to anti-apolipoprotein(a) antibody fixed on the microtitration plates and to peroxidase-conjugated anti-apolipoprotein(a) antibody. This is then washed once to remove unbound labelled antibody. The apolipoprotein conjugate is detected by adding 3,3',5,5'-tetramethylbenzidine (TMB) which induce a calorimetric reaction with peroxidase. This reaction is stopped by adding an acid to give a colorimetric endpoint at 450 nm, which is read by spectrophotometer.

6.4.1 Reagents and buffers:

Each kit is used for 86 well plate and is supplied with:

1. Anti-apolipoprotein(a) microtitration strips fixed in a 96 well plates.
2. Apolipoprotein(a) standards; four vials of 0.5 ml which are reconstructed by adding 500µl of redistilled water.
3. Apolipoprotein(a) standard 0, one vial of 0.5ml.
4. Controls (high and low) apolipoprotein(a) concentration; twos vial to be reconstructed by adding 500µl of redistilled water.
5. Peroxidase conjugated mouse monoclonal anti-apolipoprotein(a) antibody (conjugate stock solution).
6. Conjugate buffer; one vial of 7 ml.
7. Pre-treatment solution; 5 ml vial
8. Sample dilution tablets; two tablets each to be dissolved in 250 ml of redistilled water.
9. Stabilization solution; 5 ml vial.
10. Washing solution concentrate, 40 ml bottle to be diluted by adding 800 ml of redistilled water (1:21).
11. Peroxidase substrate (TMB); one vial of 22ml.
12. Stop solution; 1M of sulphuric acid, 7ml vial.

6.4.2 Preparation of conjugate solution:

This is prepared by mixing 25µl of conjugate stock solution with 500 µl of conjugate buffer for each strip. The total amount is mixed in a urine container.

6.4.3 Preparation of sample diluent:

This is prepared by dissolving each of the sample diluent tablets in 250 ml of redistilled water, mixing with the use of a magnetic stirrer and adding 2.5 ml of stabilization solution.

6.4.4 Preparation of the samples;

This is done for all the samples; unknown, standards and the controls:

1. Adding 25µl of pre-treatment solution to 25µl of the sample.
2. Mixing and incubating for one hour at room temperature
3. Adding 1.5 ml of prepared sample to give a 1:61 dilution.
4. Adding 0.2 ml of 1:61 sample solution to 0.6 of diluent to give a 1:244 dilution.

6.4.5 Assay procedures:

Each test of the unknown samples, controls and standards are measured in duplicate.

1. Pipetting of 25µl of standard, control and unknown sample into their respective wells.
2. Adding 50µl of conjugate solution to each well.
3. Incubation on a shaker for one hour at room temperature.
4. Aspiration of the reaction solution and washing four times with 350µl of washing solution. Complete aspiration after the last wash.
5. Adding 200µl of peroxidase substrate.
6. Incubation for 15 minutes
7. Addition of 50µl of stop solution and putting the plate on a shaker for one minute.
8. Measurement of absorbance at 450 nm by spectrophotometer.
9. Plotting the standard curve and calculation of the results.

6.4.6 Calculation of the results:

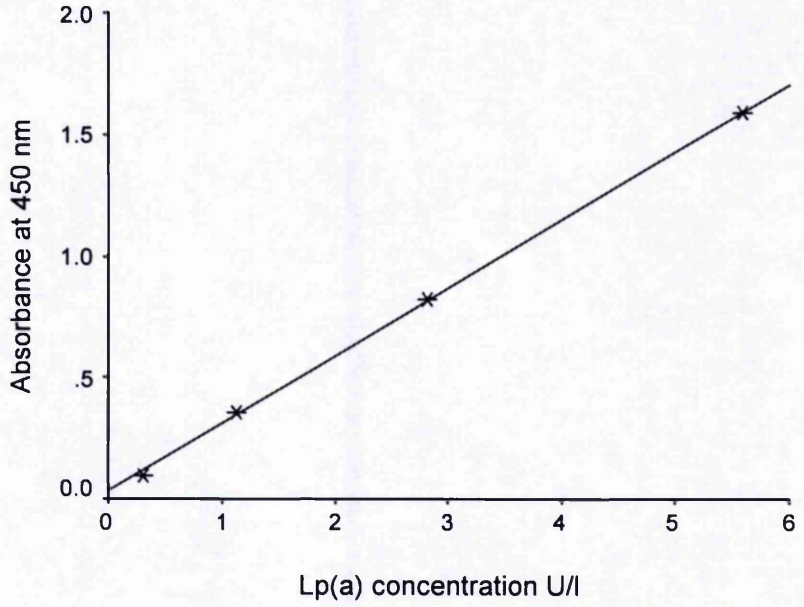
This can be done manually by:

1. Plotting absorbance values of the standards against their known concentration on a linear/linear paper (Figure 6.3).

2. Concentration of the unknown samples can be read from the curve.
3. Concentration of the samples and controls to be multiplied with the dilution factor of 244.

Figure 6.3 Standard curve of lipoprotein (a):

Obtained by plotting measured absorbance of the standards at 450 nm against their concentration in U/l:



Computerized calculation: This is done by a computer program using the linear regression equation y = b*x + a, where (b) is the coefficient of regression and equals 0.0278 and (a) is the constant and equals 0.0305 [y = 0.0278*X + 0.0305]. The squared correlation coefficient of the model equals 0.999, which means that 99.9% of the change in absorbance is explained by change in concentration.

To convert Lp(a) U/l to mg/dl: Lp(a) mg/dl = U/l x 0.07.

The lower detection limit of the assay is 0.05U/l.

6.5 Paroxonase activity assay:

The Paroxonase assay involves dealing with the highly toxic substance paroxon. Extra care and wearing of protective clothing and gloves is needed. I was advised by Dr Mackness not to do the assay by myself, so one of his experienced technicians in the lipid laboratory did the assay.

Before staring the assay, a decontamination solution of >2M sodium hydroxide is prepared. This solution is also harmful and is stored in a labelled sealable bottle. The steps of the assay were:

6.5.1 Preparation of stock solution:

The bottle of paroxon was removed from the protective tin in a fume cupboard. Then 18.8 µl of paroxon (O-O-Diethyl-*p*-nitrophenolphosphate) was pipetted into a glass container and mixed with 20ml of a tris/HCl buffer (100mmol/l pH 8.0) containing 2mM of $CaCl_2$.

6.5.2 Assay:

0.5 ml of paroxon solution is added to 25µl of serum sample. Paroxonase in sample cleaves paroxon in the colourless stock solution. The rate of generation of *p*-nitrophenolphosphate, which gives a yellow colour, was determined at 405nm 25°C using a continuously recording spectrophotometer.

6.5.3 Decontamination

Excess paroxon solution and all instruments used in the assay were placed into a>2M NaOH overnight in a fume cupboard.

1. Calculation of activity:

Paroxonase activity measured as nmol of p-nitrophenolphosphate produced /minute /ml of serum, was calculated using the following formula:

$$\frac{OD/min}{{}^{E}405^{(15.1)}} \times \frac{\text{volume of the assay (ml)}}{1000} \times \text{fractional volume (ml)}$$

- OD = change in absorbance/minute.
- ${}^{E}405^{(15.1)}$ = Extinction coefficient, which is the colour change caused by production of 1mM of the colour product.
- Volume of the assay (ml)= 0.5 ml of paroxon solution and 25µl of serum = 0.525 ml
- Fractional volume = 40 (1ml of sample = 25µl x 40)

6.6 Assay of Lipoproteins:

Total serum cholesterol, triglycerides and HDL-C were measured in the hospital laboratory with the use of a Hitachi Modular (P + E) autoanalyser. Calibrator (C.f.a.s) and reagents were supplied by Roche diagnostics, UK. Quality controls were supplied by RioRad laboratories in Hemel Hempstead, UK. Total cholesterol, triglycerides and HDL-C were measured in the hospital laboratory as a routine investigation. LDL-C and VLDL-C were measured in the Lipid Laboratory, Department of Medicine at University of Manchester.

6.6.1 Total serum cholesterol:

Total serum cholesterol was measured by the enzymatic colorimetric test CHOD-PAP method, which involved enzymatic reactions, that first cleaved cholesterol ester into cholesterol and fatty acid by cholesterol esterase, then generating hydrogen peroxide (H_2O_2) cholesterol and oxygen by cholesterol oxidase. H_2O_2 reacted with 4-aminophenazone and phenol to produce a red quinoneimine dye in a reaction catalysed by peroxidase. The colour intensity was determined by its absorption at 500nm using a spectrophotometer.

6.6.2 Triglycerides:

Triglycerides were also measured using the enzymatic colorimetric test GPO-PAP method, which involved first hydrolysis of triglycerides into glycerol by lipoprotein lipase, then hydrogen peroxide is generated from glycerol by glycerol phosphate oxidase enzyme. Hydrogen peroxide reacted with 4-aminophenazone and phenol to produce a reaction catalysed by peroxidase to red coloured quinoneimine dye and the colour intensity is determined by spectrophotometer.

6.6.3 High density lipoprotein cholesterol:

HDL-C was measured using the direct method, which uses polyethylene glycol modified cholesterol esterase and cholesterol oxidase enzymes. These modified enzymes show selective catalytic activity towards different lipoproteins, with the reactivity increasing in order as: cholesterol in LDL-C < VLDL-C = chylomicrons < HDL-C. In the presence of magnesium ions, dextran sulphate

forms water soluble complexes with chylomicrons, LDL-C and VLDL-C, which further reduces reactivity of cholesterol in these lipoproteins. The cholesterol in HDL-C is then determined enzymatically using modified cholesterol esterase and cholesterol oxidase to produce hydrogen peroxide. In the presence of peroxidase, hydrogen peroxide reacts with 4-amino-antipyrine and HSDA to form a purple-blue dye. The colour intensity is directly proportional to the HDL-C concentration.

6.6.4 Low density (LDL-C) and very low density lipoprotein cholesterol (VLDL-C)

VLDL-C is measured by the ultra-centrifugation method. This involves ultra-centrifugation (at 144,000 x **g** for 18 hours at 4.0 C°) of the plasma to D = 1.006g/ml (β-quant). In fasting plasma samples, the supernatant of a density of < 1.006g/ml is almost all VLDL-C. Cholesterol is assayed in the supernatant to determine VLDL-C. The amount of cholesterol in the infranatant, which contains HDL-C and LDL-C is assayed. HDL-C in the infranatant is then precipitated by heparin/manganese sulphate and assayed. LDL-C cholesterol is calculated by: cholesterol in the infranatant – HDL-C.

6.7 High sensitive-CRP:

This assay was performed by Dr I Laing in the Radioimmunoassay laboratory at Manchester Royal Infirmary. Briefly, high sensitive CRP was assayed with the two-site ELSA technique. Reagents were supplied by DAKO DK-2600 Glostrup, Denmark. Diluted unknown serum samples, test standards and the low and high quality controls were added to microtiter plates coated with rabbit anti-human CRP. After two hours of incubation and washing out the plates, peroxidase-conjugate rabbit anti-human CRP was added to the plates followed by incubation for a further two hours. After a second washing, a chromogenic substrate was added and, after 30 minutes, sulphuric acid was added to the reaction and the resulting colour is read at 492 nm by a spectrophotometer. The sensitivity of the assay was 0.1mg/l

6.8 Interleukin-6:

This assay was also performed by Dr I Laing in the Radioimmunoassay laboratory. IL-6 was measured using the two-site chemiluminescent ELISA technique (Quantiglo, R&D Systems Europe, 19 Barton Lane Abingdon OX 14 FA UK). A monoclonal antibody specific for IL-6 was fixed to microplates. The standards and the unknown samples were added to the plates, the IL-6 in the samples is bound to the immobilized antibody. After washing away unbound proteins, an enzyme-linked polyclonal antibody specific for IL-6 was then added to the wells. After three hours incubation at room temperature a second wash was carried out to remove the unbound antibody-enzyme reagent. An enhanced luminol/peroxide substrate (40) solution was added to the wells, followed by incubation for 20-40 minutes. The light is produced in proportion to the amount of IL-6 bound in the initial step. A microplate luminometer was used to measure the intensity of the light emitted as relative light units (RLUs) for each well. The sensitivity of the assay was determined by adding 2.5 standard deviations of the zero standard, was 1pg/ml and the coefficient of variation was 15% at an IL-6 of 4 pg/l.

The methods used in the study of E-selectin gene polymorphism, included DNA extraction and polymerase chain reaction and restriction fragment length polymorphism (PCR-RLEP). These are presented in details in chapter 10.

Chapter 7

7 Description of SLE patients of this study

7.1 Introduction:

The studies in this thesis are mainly based on analysis of a cohort of patients with SLE from Manchester Royal Infirmary. These patients are routinely followed up in the Department of Rheumatology. This chapter outlines the important clinical features of this group of patients.

7.2 Recruitment of SLE patients:

Inclusion criteria:

The following patients were eligible for recruitment:

1- SLE patients fulfilling ≥ 4 of the 1997 updated ACR criteria for SLE (Hochberg *et al* 1997) (Appendix 1).
2- Patients with three criteria with no alternative diagnosis, who have had a clinical diagnosis of SLE made by their treating physician.

Exclusion criteria:

1- Male patients.
2- Age less than 18 years.
3- Active arthritis of the right elbow or wrist joints.
4- Fixed flexion deformities of the elbow joints.
5- Pregnancy or lactation within the previous 6 months.
6- Infection in the previous month.

Medical records of all patients were reviewed and subjects fulfilling the study criteria were contacted directly in the clinic or by mail and requested to participate in the study. Those who agreed to participate were invited and given appointments. This study was approved by Central Manchester Local Research Ethics Committee. Written informed consent was obtained from each participant. The study consisted of two parts. Firstly patients underwent an interview, clinical assessment and collection of fasting blood samples in the Rheumatology Department. This was performed by me under the supervision of

Dr IN Bruce Consultant Rhreumatologist. They then had a vascular study performed to assess endothelial function in the brachial artery and measurement of intima media thickness (IMT) of carotid artery (see chapter 8).

7.3 Clinical Assessment:

The clinical assessment included a full history asking about demographic factors such as age, ethnicity, marital status, years in education and occupation. We collected data on SLE criteria, date of diagnosis as well as specific features such as Raynaud's phenomenon and APL syndrome. We recorded patients' menstrual status and whether or not they were on any hormonal agents. In addition a history of diabetes and hypothyroidism was ascertained. We also gathered information about any history of cardiovascular disease, including CHD (angina, myocardial infarction, etc) and cerebrovascular disease and inquired about known classic risk factors. Information was also gathered on tobacco and alcohol consumption.

A physical examination was then performed to determine relevant CHD risk factors as well as to assess SLE disease activity and irreversible damage.

The data collection forms we used to record this information is shown in Appendix 2.

7.3.1 Clinical assessment of disease activity:

Disease activity was assessed by two disease activity indices:

1. Systemic Lupus Erythematosus Disease Activity Index (SLEDAI): this activity index was originally designed in Toronto in 1985 and modified in 1992 (Bombardier *et al* 1992). The SLEDAI consists of 24 items covering nine organ- systems; points are scored for different items within different systems with a maximum total score of 105 points (Appendix 3). The items are scored as being present or absent in the ten days preceding the assessment. Some items, such as renal and neurological involvement, are weighted more heavily than others. This index is well validated and widely used in studies in North America.
2. British Isles Lupus Assessment Group (BILAG) index: this activity index was developed in the United Kingdom in 1984 (Symmons *et al* 1988) according

to the principle of the physician's intention to treat (Appendix 4). The BILAG index consists of 86 items in eight organ systems (general, mucocutaneous, musculoskeletal, cardiopulmonary, neurological, vasculitis, renal and haematology), each item is scored as present or not in the last month, and most items are scored as new, improved, same or worse. Alphabetic scores are allocated to each system and each system is scored either A,B,C,D or E where:

- A: Active disease that requires starting or increasing steroid dose, prednisolone > 20 mg/day or use of other immunosuppressive drugs.
- B: Less active disease requiring close follow-up and anti-malarial or anti-inflammatory drugs i.e steroid dose <20 mg/day.
- C: Stable and mild disease.
- D: Previous involvement of a system that is currently inactive.
- E: No previous involvement of that system.

The BILAG index scores disease activity in specific organs compared to the SLEDAI, which provides a global score of disease activity.

7.3.2 Assessment of organ damage:

Organ damage was assessed by the ACR/ Systemic Lupus International Collaborating Clinics (SLICC/ACR) damage index (Appendix 5). This index was developed to assess cumulative damage in SLE and defines damage as a non-reversible change, not related to ongoing disease activity that is present for at least six months and has occurred since the diagnosis of SLE. Assessment includes 12 organ systems (ocular, neuropsychiatric, renal, pulmonary, cardiovascular, peripheral vascular, gastrointestinal, musculoskeletal, skin, gonads, diabetes and malignancy) with a potential maximum score of 49. Damage is ascertained clinically, by simple investigations or by radiography. This scoring index does not require attribution of damage to SLE, as damage related to therapy, surgery and malignancy is also included.

7.4 Blood samples:

A blood sample was obtained on the morning of study after an overnight fast and avoidance of alcohol for 48 hours. As part of the routine assessment,

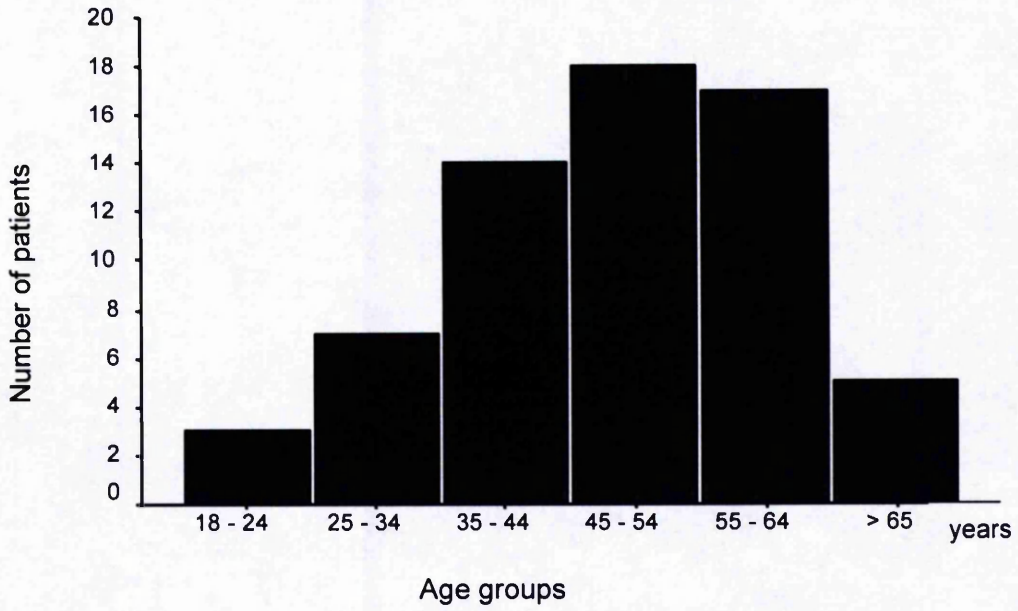
patients had a biochemical profile to include creatinine, full blood count, liver function tests, TC, HDL-C, TC's and fasting glucose. In addition an autoantibody profile was performed, which included levels of C_3, C_4 complement, ds-DNA and cardiolipin antibodies. As well as the routine bloods, we also collected serum and plasma and whole blood for DNA extraction. The separated plasma and serum were divided into 1 ml aliquots and stored at -20°C until later analysis. The experimental methods used for individual assays and experiments performed in this study will be outlined in the relevant chapters.

7.5 Demographic features of SLE patients in this study:

We studied 64 patients in total; the study population, were by definition, all females. Their median (range) age and disease duration from the time of diagnosis was 48.5 (21 – 73) and 11 (1.0 – 40) years, 32(50%) were in the 35 – 54 years age group, 22 (34%) were > 54 years old and 10 (16%) were < 35 years old (Figure 7.1). Table 7.1 shows the education attainment, marital status of patients along with their ethnic background and menstrual status. The median years of education was 12 (0 – 19) years, 16 (25%) had college or university education level (Table 7.1), 45 (66%) were married or living as married, eleven were single and eight were divorced, separated or widowed (Table 7.1).

Fifty-three (83%) were white Caucasians from the British Isles. Thirty-one (48%) patients were post-menopausal, eleven (36%) of whom were on hormonal replacement therapy and six patients had menopause at ≤ 50 years (Table 7.1).

Figure 7.1 Age distribution of the 64 SLE patients:



7.6 SLE features of patients in this study:

7.6.1 ACR criteria

Of the 64 cases studied, 58 (92%) had ≥4 and six (8%) had three of the ACR criteria. The details of these six patients with three criteria are included in table 7.3. Overall in the whole group the median number of criteria was 5.0 (range 3.0 – 10). The frequency of ACR criteria is shown in table 7.2. Arthritis and positive antinuclear antibody (ANA) were the most frequent single features, with both were reported in 93% of patients. Renal involvement was reported in ten (16%) patients in this group. Thirty-six (56%) patients had a history of Raynaud's phenomenon, defined as the occurrence of at least two out of three responses to cold stress namely pallor, cyanosis and reactive hyperaemia.

7.6.2 Autoantibody profiles:

In addition to the ANA, patients had other antibodies checked. Table 7.4 summarizes the antibodies positive in these patients.

Twenty-nine (45%) had IgG ds-DNA antibody ever, of which 22 (34%) had IgG ds-DNA on the day of study. Similarly, on the day of study 20 (31%) had increased IgG ACL antibodies, and 12 (19%) had positive LAC. Overall, 24

(38%) had either ACL antibodies or LAC on day of study. The commonest additional immunological features were antibodies to Ro (35%) and low complement (29%) (Table 7.4). Six patients (9%) satisfied the Sapporo criteria for the diagnosis of APL syndrome (Table 7.4) (Wilson *et al* 1999b) (Appendix 6).

Table 7.1 Demographic features in 64 SLE patients:

Education:	
Median (range) years of education	12 (0.0 – 19)
No formal education n (%)	1 (2)
School education n (%)	47 (73)
College/University n (%)	16 (25)
Marital status: n (%)	
Married or living as married	45 (70)
Single	11 (17)
Divorced or separated	7 (11)
Widowed	1 (2)
Race/Ethnicity: n (%)	
White Caucasian	53 (82.8)
South Asian	3 (4.7)
Afro-Caribbean	1 (1.6)
African	2 (3.1)
Oriental	2 (3.1)
Others	3 (4.7)
Pre-menopause: n (%)	33 (51.6)
No OCP	29 (90.9)
OCP	3 (9.1)
Post-menopause: n (%)	31 (48.6)
Onset ≤ 50 years	6 (19.4)
On HRT	11 (35.5)
No HRT	20 (64.5)

Table 7.2 Frequency of ACR criteria in 64 SLE patients:

Criteria	Number of patients	Percentage (%)
Malar rash	22	33.8
Discoid rash	3	4.7
Photosensitivity	25	39.1
Mucosal ulcers	25	39.1
Arthritis	60	93.8
Serositis: - Pleurisy - Pericarditis - Both	23 19 7 3	35.9 29.7 10.9 4.7
Renal involvement	10	15.6
Neurological disorders	6	9.4
Haematologic features - Low WBC - Lymphopenia - Haemolytoc anaemia -Thrombocytopenia	54 16 36 3 9	84.4 25.0 56.0 5.0 14.0
Immunologic features - Anti-dsDNA - Anti-CL - LAC - Anti-Sm	47 29 22 14 4	73.4 45.0 34.0 22.0 6.0
Antinuclear antibody	60	93.8

Table 7.3 Features of SLE patients with three ACR criteria:

Patient ID	SLE criteria	Additional clinical features	Additional lab features
1	Arthritis Haematological ANA	Raynaud's	Anti-RO antibody
2	Arthritis Immunological ANA	-	Anti-RO Anti-La Low complement C_4
3	Haematological Immunological ANA	Raynaud's Splenectomy for thrombo-cytopenia	-
4	Arthritis Haematological ANA	Raynaud's	Ant-RNP
5	Arthritis Haematological ANA	-	Ant-RNP
6	Haematological Immunological ANA	-	Anti-RO Anti-La Low complement

Table 7.4 Additional features in SLE patients:

	Patients (n)	Percentage (%)
Raynaud's	36	56.3
Alopecia	11	17.2
Hypocomplementaemia	18	29.5
APL syndrome	6	9.3
Sicca symptoms	6	9.3
Anti-Ro	22/62	35.5
Anti-La	10/62	16.1
Anti-RNP	13/62	21.0

7.7 Disease activity at the time of study:

7.7.1 SLEDAI:

The median (range) SLEDAI at the time of study was 2(0 – 12). Twenty six (41%) patients had inactive disease with a SLEDAI score of zero. Only nine (14%) patients had a SLEDAI of ≥ 6, reflecting active to moderately active disease (Figure 7.2).

7.7.2 BILAG index:

Thirteen (20.3%) patients had an A or B score category in at least one of the eight systems of the BILAG. The three A scores were in the mucocutaneous, musculoskeletal and neurology respectively. Seven patients had their B score in the musculoskeletal system. Of the 13 patients who scored A or B in the BILAG, nine (69%) also scored ≥ 6 in SLEDAI (Figure 7.3).

Figure 7.2 Frequency distribution of SLEDAI score in 64 SLE patients:

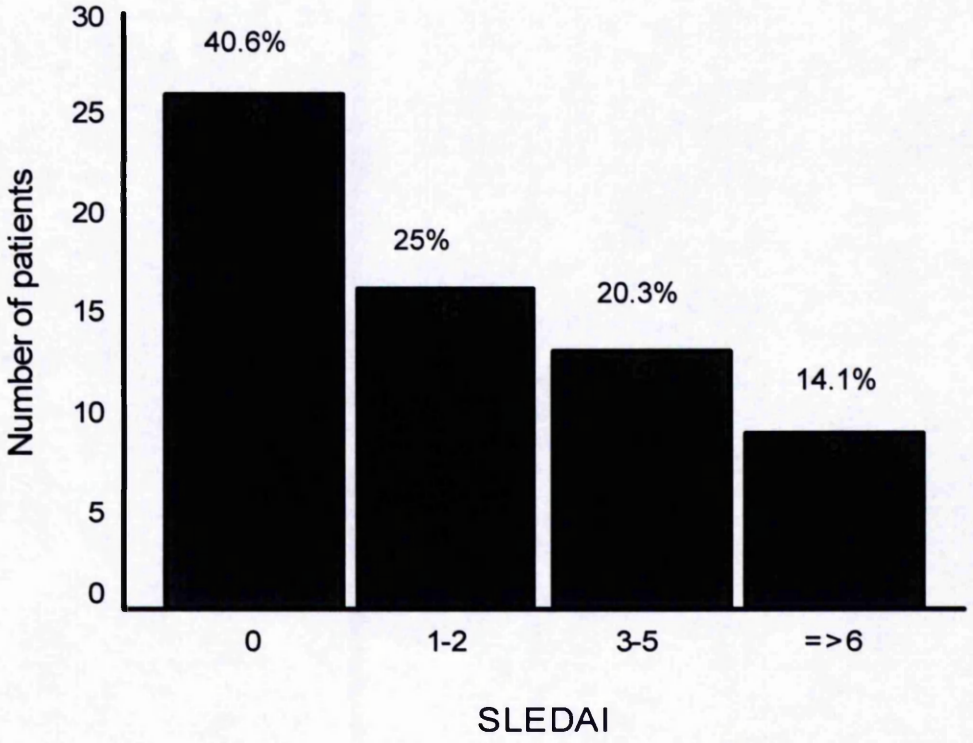
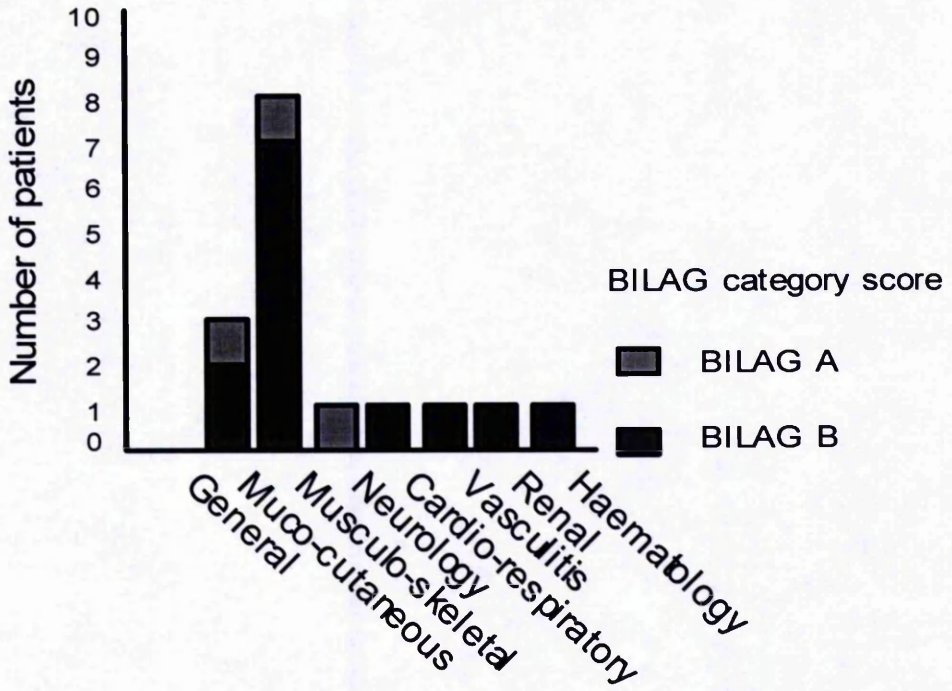


Figure 7.3 Frequency of A and B scores of the BILAG in 64 SLE patients:

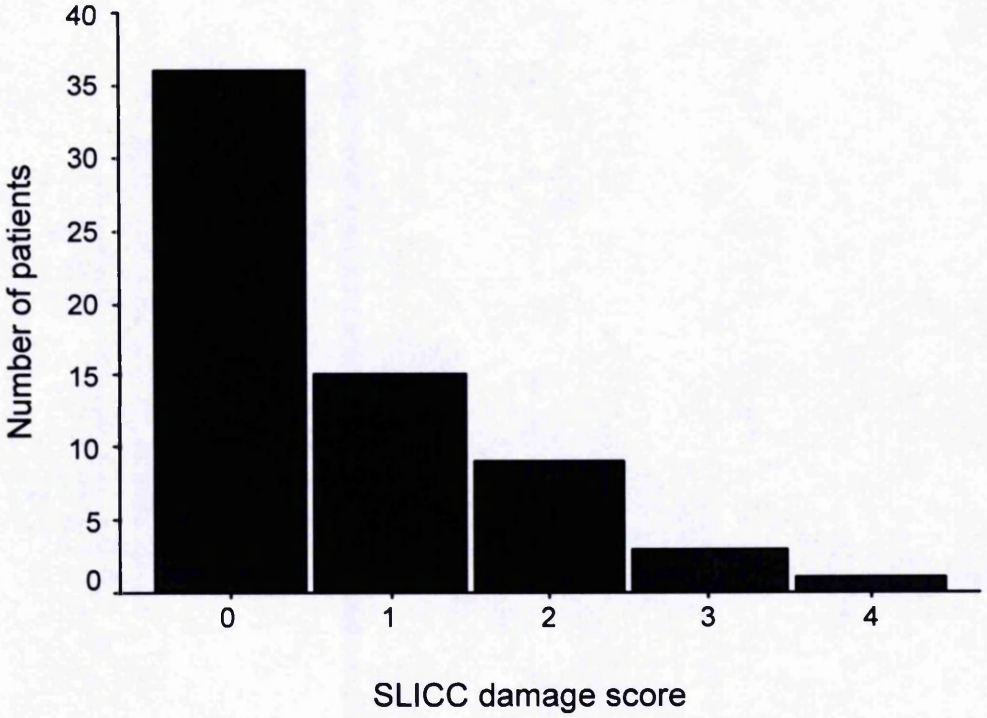


For the purpose of analysis, we defined the presence of active disease if a patient had either a SLEDAI ≥ 6, or BILAG score A or B or if they were taking ≥ 10 mg of prednisolone/day. By this definition, 21(34%) had active and 43 (66%) had inactive disease at study.

7.8 SLICC damage index (SDI):

The distribution of the SLICC damage index scores are displayed in table 7.5 and figure 7.4. The majority (58%) had a SDI of zero. The most common damage was in the neuropsychiatric system, which affected 13 patients and included CVA (8), cognitive dysfunction (4) and peripheral nerve palsy (common peroneal nerve) in one patient. The second most common damage was in the musculoskeletal system (8), seven of whom had deforming arthritis and one had avascular necrosis of both hip joints. Damage of the cardiovascular system was reported in three patients; two with MI and one with pericardial thickening (Table 7.5).

Figure 7.4 SLICC damage score in SLE patients:



7.9 Therapy:

7.9.1 Anti-malarial therapy:

In this cohort 16 (25%) patients were on no SLE-specific drugs at the time of study. Of the others, 32 (50%) were taking AM's. This was HCQ in 28 cases. Four other were taking chloroquine phosphphate. The median (range) daily dose of OHCQ was 200 (100 – 400) mg/day.

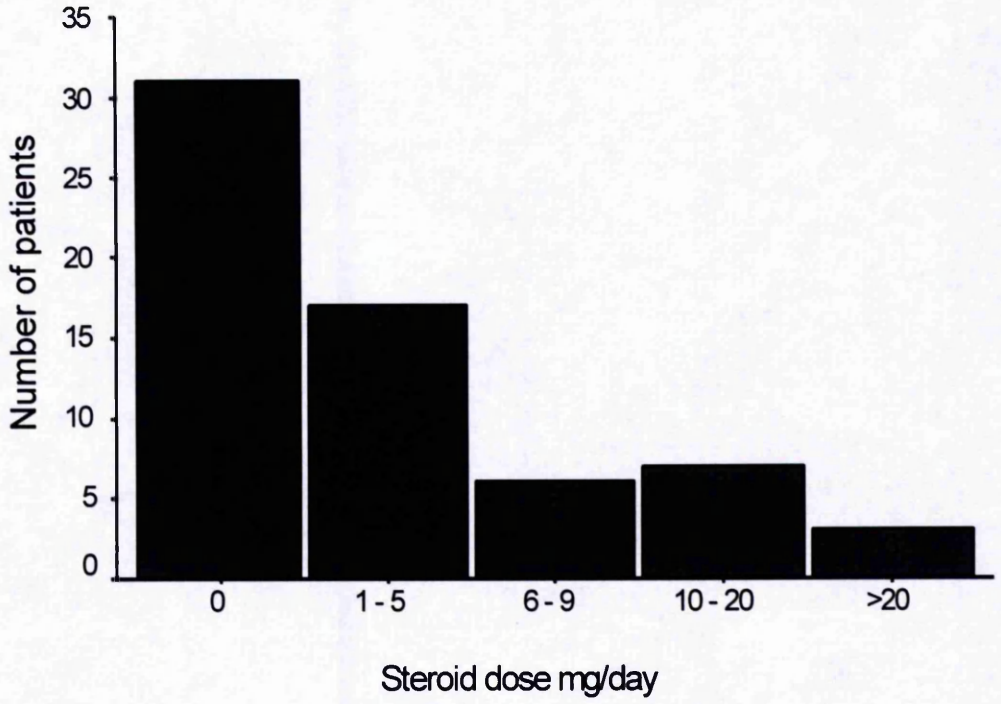
7.9.2 Steroid therapy:

Thirteen (20%) of our patients had never had steroid therapy for SLE. Of the 51(80%) who had taken steroids, 33 (52%) were taking them at the time of study. The median (range) of total steroid duration in these patients was 51 (1.0 – 216) months. Patients on steroid therapy were significantly younger than those not on steroids [median (range) age was 45 (21 – 65) vs 53 (24 – 73) years, $p = 0.010$]. In general, patients were on a low dose of steroids. The median (range) daily dose was 5 (1.3 – 30) mg/day. Ten patients were taking ≥ 10 mg prednisolone daily (Figure 7.5).

Table 7.5 Distribution of SLICC damage index scores in 64 SLE patients:

Damage	Number of patients n (%)
Ocular: - Cataract	 1 (1.6%)
Neuropsychiatric - CVA - Cognitive impairment - Common peroneal nerve palsy	13 (20.3%) 8 4 1
Renal - Proteinuria - Decreased GFR	2 (3.1%) 1 1
Pulmonary - Pulmonary hypertension - Pulmonary fibrosis - Pulmonary infarction	3 (4.7) 1 1 1
Cardiovascular - Myocardial infarction - Pericardial thickening	3 (4.7%) 2 1
Peripheral vascular - Minor tissue loss	3 (4.7%) 3
Gastrointestinal - Oesophageal stricture - Bowel resection - Splenectomy	3 (4.7%) 1 1 1
Musculoskeletal - Deforming or erosive arthritis - Avascular necrosis	8 (12.5%) 7 1
Skin - Skin ulceration	1 (1.6%)
Premature gonadal failure	1(1.6%)
Diabetes mellitus	3 (4.7%)
Malignancy - Adenocarcinoma of uterus - Breast cancer	3 (4.7%) 1 2

Figure 7.5 Current daily steroid dose in SLE patients:



7.9.3 Immunosuppressive therapy:

Forty-six (72%) patients were not on immunosuppressive therapy, 13 (20%) were on azathioprine, four (6.3%) were on methotrexate and one patient was on cyclosporine. No patients were on cyclophosphamide at the time of study.

7.9.4 Other drugs:

Additional therapies taken by the patients at the time of study are shown in table 7.5. Of the 31postmenopausal patients, 14 (42%) were taking hormonal replacement therapy and of 34 premenopausal patients three were taking combined contraceptive pills. Of note, only one patient was on lipid lowering therapy, eleven (17%) were on aspirin, nine (14%) were on non-steroidal anti-inflammatory drugs and six (9%) were on oral anticoagulants. Sixteen patients were on anti-hypertensive drugs.

Table 7.6 Other therapies taken by SLE patients:

Therapy	n (%)
Hormone replacement therapy	14 (21.8)
Oral contraceptives	3 (4.7)
Antihypertensive therapy	16 (25.0)
On one drug:	10
1- Diuretics	2
2- ACE inhibitors	3
3- Calcium blockers	3
4- Direct vasodilators	1
5-Angiotensin II receptor antagonist	1
6- B-blocker	0
On two drugs:	5
(1 and 4)	2
(1 and 6)	1
(3 and 4)	1
(2 and 3)	1
On three drugs:	
(2, 3 and 4)	1
Lipid lowering therapy	1(1.6)
Aspirin	11(17.2)
Warfarin	6 (9.4)
Non-steroidal anti-inflammatory drugs	9 (14.5)

7.10 Atherosclerosis:

7.10.1 Prevalence:

In view of the studies planned, we determined the prevalence of atherosclerotic complications and risk factors in our cohort. Two patients (3%), aged 73 and 67 years, had a history of MI at age 65 and 60 years respectively. The 67-year old had had SLE for seven years and had a past history of both MI and CVA at the age of 60 years. She had never received steroid therapy but was positive for ACL antibodies. The 73-year old patient had had SLE for 40 years and she had been on steroid therapy for approximately 20 years. She was negative for anti-cardiolipin antibodies. Eight (12%) patients had a history of CVA, their median (range) age was 55 (41 – 67) years. Four (50%) of these had positive ACL

antibodies and two were positive for both anti-cardiolipin antibodies and lupus anticoagulants. None of the 64 patients had a history of peripheral vascular disease.

7.10.2 Risk factors for CHD

Table 7.6 shows the definitions we employed and the prevalence of the CHD risk factors in our patients. According to these definitions, 60% of our patients had at least one abnormality in their lipid profile. The most prevalent risk factors in this group were hypertension (48%), raised TC (44%) and increased BMI (56%).

Thirteen (20) patients had a TGs level of >1.8 mmol/l and 20 (31%) patients also had a level of >1.5 mmol/l, eleven (17%) patients had HDL-C level of ≤ 1.0 mmol/l and 5 (8%) had HDL-C level of ≤ 0.9 mmol/l.

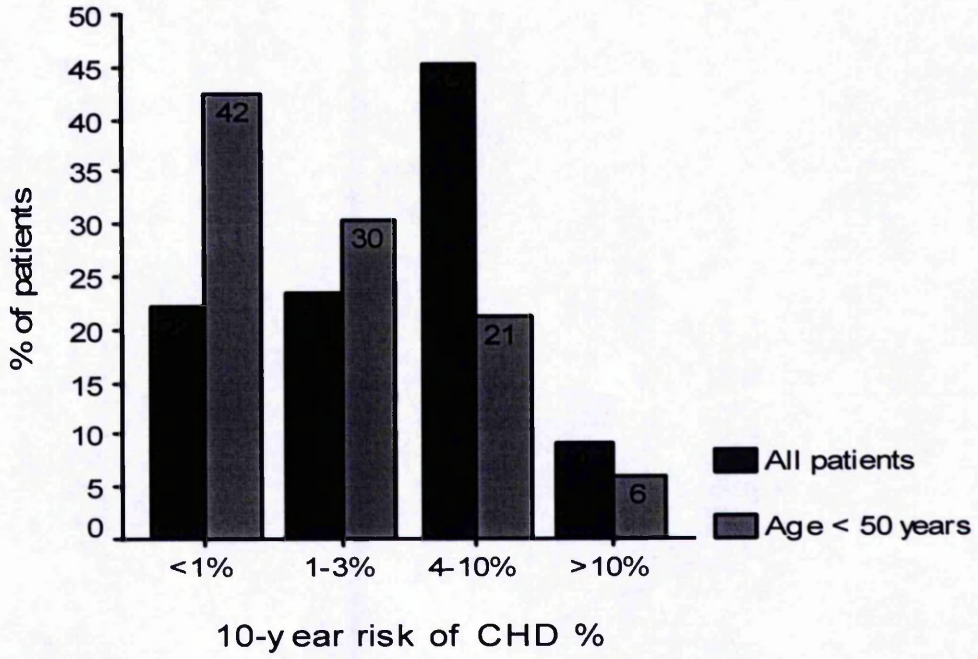
7.10.3 Assessment of percentage 10-year risk of CHD:

We used a computer program (Cardiac Risk Assessor Version 98.02) (Appendix 7) developed in conjunction with The Joint Recommendations (1998) of the British Cardiac Society, British Hyperlipidaemia Association, British Hypertension Society and British Diabetic Association on Prevention of Coronary Heart Disease in Clinical Practice. This was based on equations used for the calculation of CHD risk in the Framingham Heart Study (Anderson *et al* 1991). The program computes the risk of CHD and stroke as the percentage likelihood ratio of an event over a period of ten years. Data entered into the program are gender, age in years, systolic and diastolic blood pressure, total cholesterol, HDL-C, smoking status (current or non-smoker), diabetes status and left ventricular hypertrophy on ECG if available. We were unable to obtain a 12-lead ECG on all patients. The calculation therefore excluded this variable. The median (range) 10-year risk of CHD was 4.0 (0.0 – 15.8%). Twenty-nine (45%) patients had a <3% and six (9%) had a >10% 10-year risk of CHD. When we looked only at patients < 50 years old we found that the median (range) 10-year risk was 1.2% (0 – 12%). Two (6%) patients in subgroup had a 10-year risk of >10% (Figure 7.6).

Table 7.7 Definitions and frequency of CHD risk factors in SLE patients:

Risk factors	Number (%)
Hypertension: SBP > 140 or DBP > 90 mmHg or on anti-hypertensive therapy.	31 (48.4)
Diabetes mellitus FBG > 7.0 mmol/l or on anti-diabetic therapy.	3 (4.7)
Smoking: Current smokers Ex-smokers Non-smokers	 7 (10.9) 12 (18.8) 45 (70.3)
Dyslipidaemia: TC > 5.2 mmol/l TGs > 1.8 mmol/l LDL-C > 3.3 mmol/l HDL-C < 0.9 mmol/l	 28 (43.8) 13 (20.3) 15 (25.0) 4 (6.3)
Family history of CHD: CHD in a male first degree relative at age < 55 or In a female at age < 60 years.	15 (23.4)
Body mass index: BMI is derived by weight in kilograms divided by squared height in meters (Kg/M^2): Over weight BMI = 25 – 30 Obese BMI > 30	 24 (37.5) 12 (18.8)

Figure 7.6 10-year risk of CHD in all patients (n=64) and in patients of age < 50 years (n=33)



7.11 Summary

Generally our patients represent a mild SLE cohort characterized generally by:

- Older age.
- Higher proportion of postmenopausal women.
- Low disease activity.
- Low damage index.
- Low prevalence of clinical CHD.

Chapter 8

8 Study of endothelial function in SLE Flow-mediated dilation

8.1.1 Hypothesis:

Endothelial function is impaired in SLE. Factors other than the traditional Framingham risk factors for CHD may have an influence on endothelial function. Endothelial dysfunction is associated with established atheroma or markers of subclinical atherosclerosis.

8.1.2 Aim:

Our aim is to compare endothelial function in women with SLE without CHD and in healthy controls. Within SLE patients, we aim to determine the influence of disease and therapy related factors on endothelial function.

8.1.3 Patients:

- Consecutive Caucasian female SLE patients recruited from the Lupus Clinic in the Rheumatology Department at the Manchester Royal Infirmary with ≥ 4 of the ACR criteria 1982.
- No pregnancy or lactation within six months of the study.
- Patients have no active arthritis of the elbow or wrist joints and no fixed flexion deformities of the elbow joints.

8.1.4 Controls:

These were healthy females recruited from the secretarial and nursing staff at Manchester Royal Infirmary and from the staff at the ARC Epidemiology Research Unit in the Medical School at Manchester University. None of the controls had a history of CHD.

8.2 Methods:

8.2.1 Assessment of endothelial function:

We used a B mode doppler ultrasound (ATL HDI 5000 with a 7.4 MHz linear array transducer) to measure flow mediated endothelium-dependent dilation of the brachial artery in response to reactive hyperaemia and endothelium non-

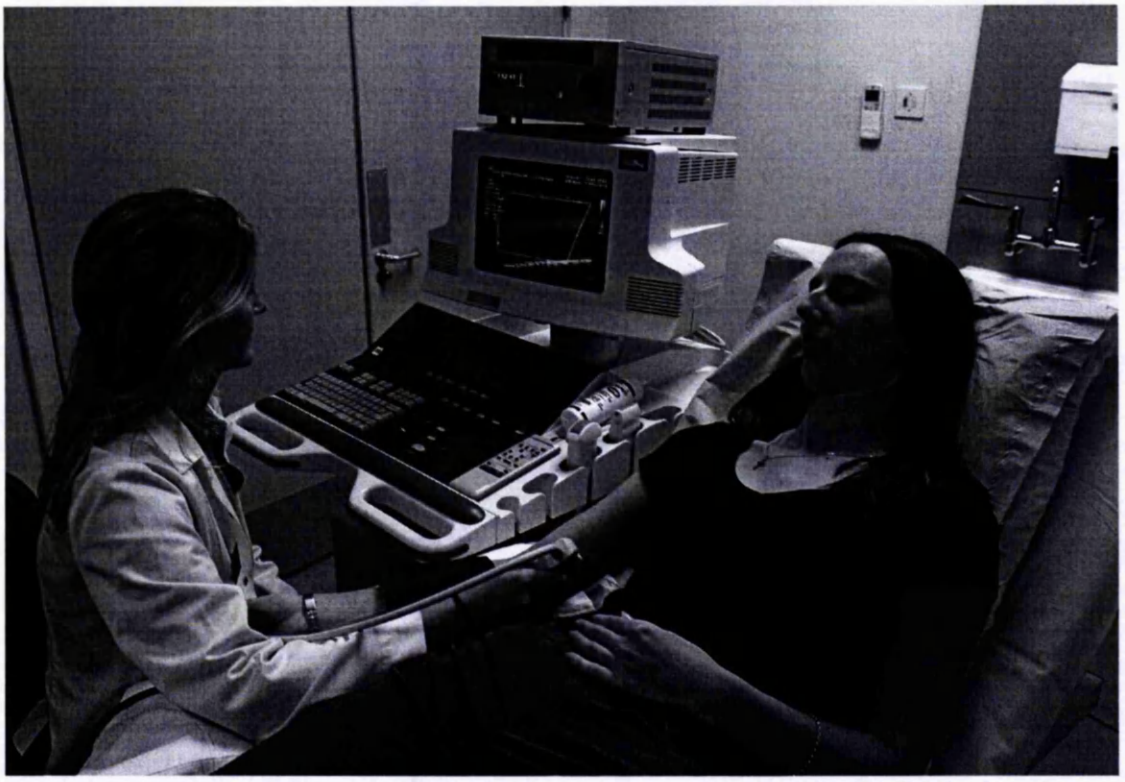
dependent dilation in response to sublingual spray of GTN. We used the protocol described by Celermajer *et al* (1992). All the patients and controls were studied in the morning between 8.00 and 11.00 am after an overnight fast. They were advised not to drink alcohol for 48 hours before and not to smoke in the morning before the study. Patients on antihypertensive therapy, including angiotensin converting enzyme inhibitors, β-blockers and calcium channel blockers, were asked to stop their medications for 24 hours before the study. The room was temperature-controlled with the temperature set at 23 °C. Patients rested on a couch for 15-20 minutes in the room prior to the study beginning. The forearm was rested on a pillow with the head of the couch raised to about 30°. The brachial artery was scanned longitudinally 5-15 cm above the antecubital fossa (Figures 8.1, 8.2). The optimal position of the artery was identified when the perpendicular beam gave a clear B-mode image of the anterior and posterior walls of the artery. The transducer position was marked on the skin and the limb kept in the same position for the whole study. Depth and gain settings were adjusted to obtain a clear image of the wall lumen interface and the images magnified using a resolution box. The scans were recorded on a super VHS videotape for later measurement of resting diameter and blood velocity.

A blood pressure cuff was inflated around the forearm to 300 mm Hg for 4.5 minutes. The cuff pressure causes temporary cessation of blood flow to the distal forearm. Immediately after the cuff was released, blood flow in the brachial artery increases by several folds, which induces high shear stress and stimulates endothelium dependent vasodilation. Video recording continued 30 seconds before to one minute after cuff release.

Measurement of the post deflation diameter was taken 45-60 seconds after cuff release (Figures 8.2, 8.3). After 15 minutes rest for the artery to recover to the baseline resting diameter, a further measurement was

Figure 8.1 Ultrasound measurement of flow mediated dilation.

This picture shows our technician performing a scan of the brachial artery above the elbow with the pressure cuff in place around the forearm:



taken at rest and at three minutes following sublingual spray of 400μg of GTN to assess endothelium independent vasodilation. All measurements were taken at the end of diastole, which coincides with the R-wave on an ECG monitor. Distance measured was from anterior to posterior M-lines (media-adventia interface) and every measurement was taken as an average of five consecutive cardiac cycles (Figure 8.4). The percentage FMD was calculated as the percentage change in diameter 45-60 seconds after cuff release compared to the resting diameter:

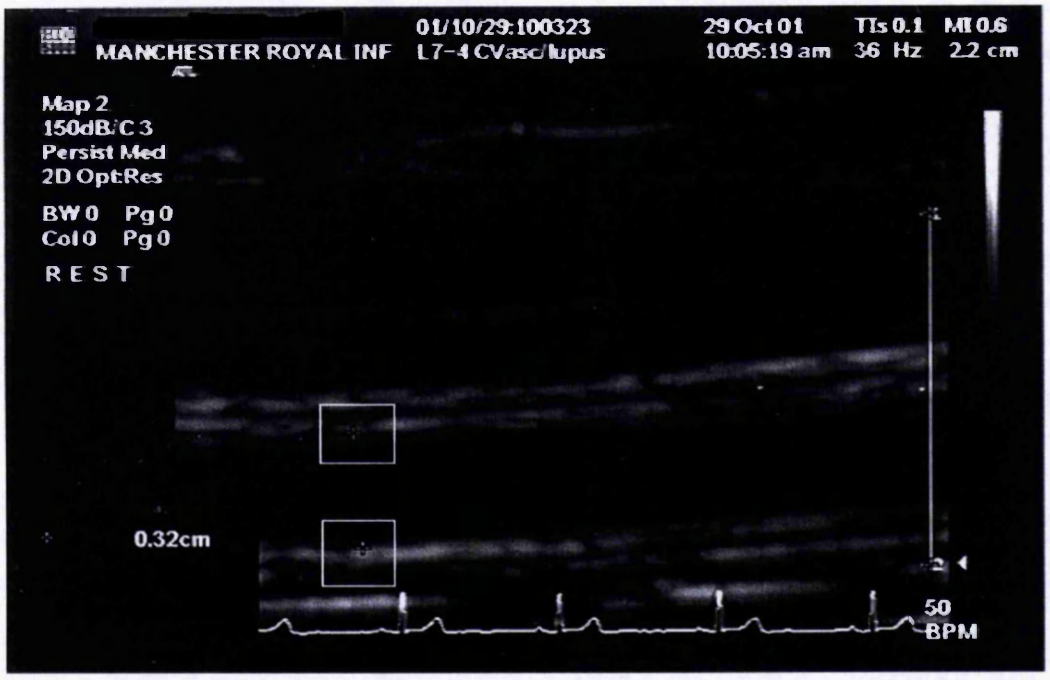
[post-deflation diameter – resting diameter] / [resting diameter] × 100%.

Blood flow was calculated using the formula:

$[\pi \times (\text{diameter/cm})^2 / 4 \times (\text{velocity cm/sec} \times 60) = \text{flow ml/min}]$.

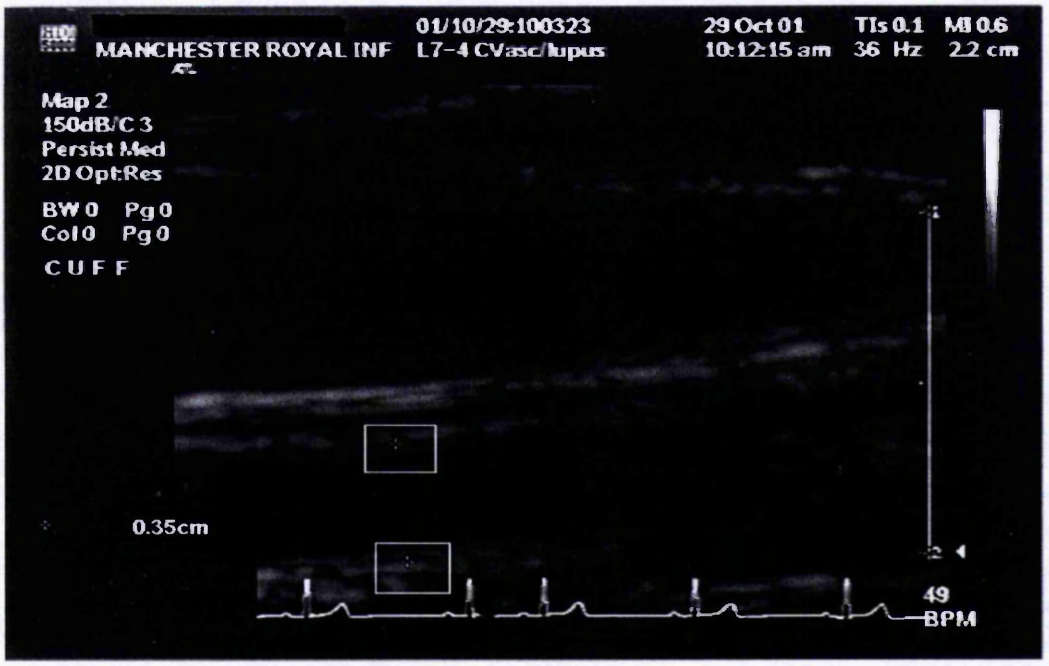
For calculation of resting flow, resting velocity and resting diameter were used in the formula. For maximum post deflation flow, maximum velocity was measured in the first 15 seconds after deflation and the diameter measured 30 seconds before deflation, and there were used to calculate the maximum post deflation flow.

Figure 8.2 Ultrasound image of the resting diameter.



Brachial artery diameter is measured from the media-adventia interface of the anterior and posterior walls (the cursors shown inside the white rectangles).

Figure 8.3 Ultrasound image of the maximum psotdeflation diameter



The cursors (shown inside the white rectangles) are in the media-adventia interface of the anterior and posterior walls.

8.2.2 Carotid intima-media thickness (IMT):

The measurement of the carotid IMT and determination of carotid plaque was done during the 15-minute resting time between FMD and GTN study. With the neck extended, the common carotid artery (CCA) was scanned longitudinally and the measurement was taken in the proximal part of the CCA, one centimetre proximal to the carotid bulb (Figure 8.4). The position of the bulb was defined when the far wall deviates from the long axis of the CCA. The IMT was defined as the maximum distance between the intima-lumen interface and adventia-media interface in areas without carotid plaque (Sidhu and Desai 1997) (Figure 8.5). Three measurements were taken from both the left and right CCA. The IMT was determined as the average of the six IMT measurements.

8.2.3 Statistical analysis:

We used version 10.1 of the SPSS statistical package in the analysis of the vascular measurements. Non-parametric tests were used. Differences between numeric variables between patients and controls were tested with Mann-Whitney's U-test. Correlation between variables was tested with Spearman's rank order correlation. The significance level was set at a probability value of ≤ 0.05. For comparison of categorical variables or percentages we used Chi-square and Fisher's exact tests. Linear regression analysis was used to test for independent association between %FMD and various factors.

Figure 8.4 Ultrasound image of the common carotid artery.

This ultrasound image shows the segment of the CCA 1 cm proximal to the carotid, bulb, carotid IMT measurements were taken from this segment:

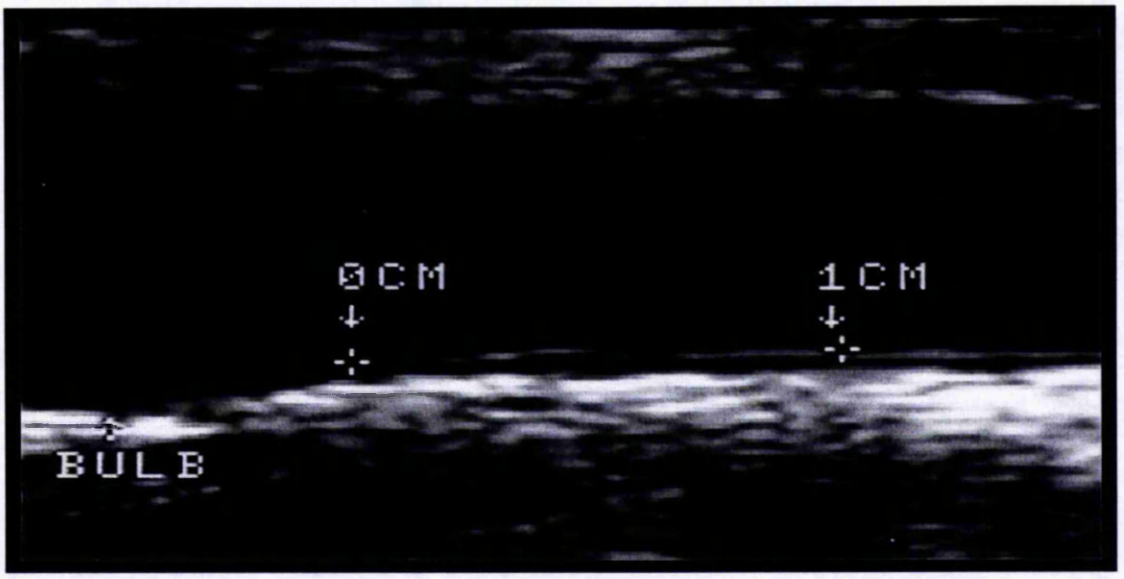
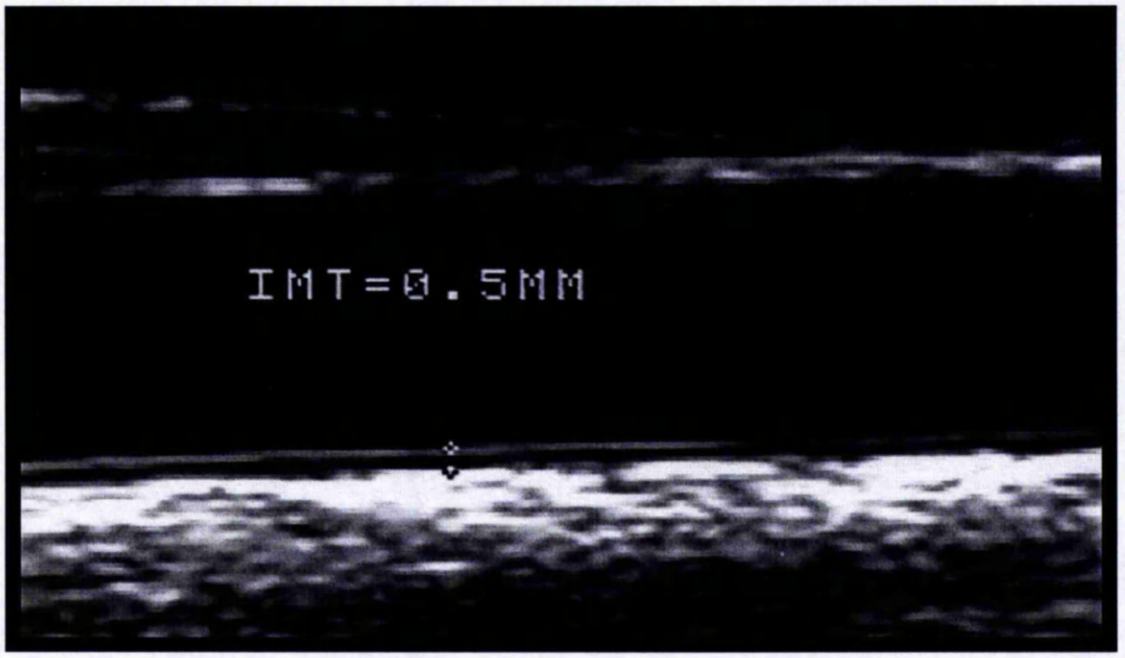


Figure 8.5 Ultrasound image showing carotid IMT measurement.



8.3 Preliminary studies of FMD on health volunteers:

From our preliminary studies on healthy volunteers we were able to demonstrate the time course changes in blood flow and diameter (Figure 8.6) that occur during measurement of FMD using the reactive hyperaemia technique. As described by Celermajer *et al* (1992), immediately after cuff release, there is a sudden increase in velocity and blood flow by several fold, which lasts for a few seconds and decreases rapidly. The change in diameter lags behind flow changes and maximum dilation occurs between 45 and 60 seconds after cuff release and continues for up to 90 seconds.

From repeated studies (n = 20) performed on one healthy volunteer over a period of two months, resting diameter showed a significant positive correlation with resting flow, r = 0.792; P <0.001 (Figure 8.7), and a significant negative correlation with %FMD r = - 0.757 P <0.001 (Figure 8.8). Maximum post deflation flow correlated with maximum post-deflation diameter r = 0.435 P = 0.056 (Figure 8.9). There was no significant correlation between percentage FMD and percentage change in flow from resting to maximum flow (r = 0.295, P 0.207).

Figure 8.6 A representative diagram of the time course changes in velocity, flow and diameter of brachial artery following cuff deflation during reactive hyperaemia (Healthy control).

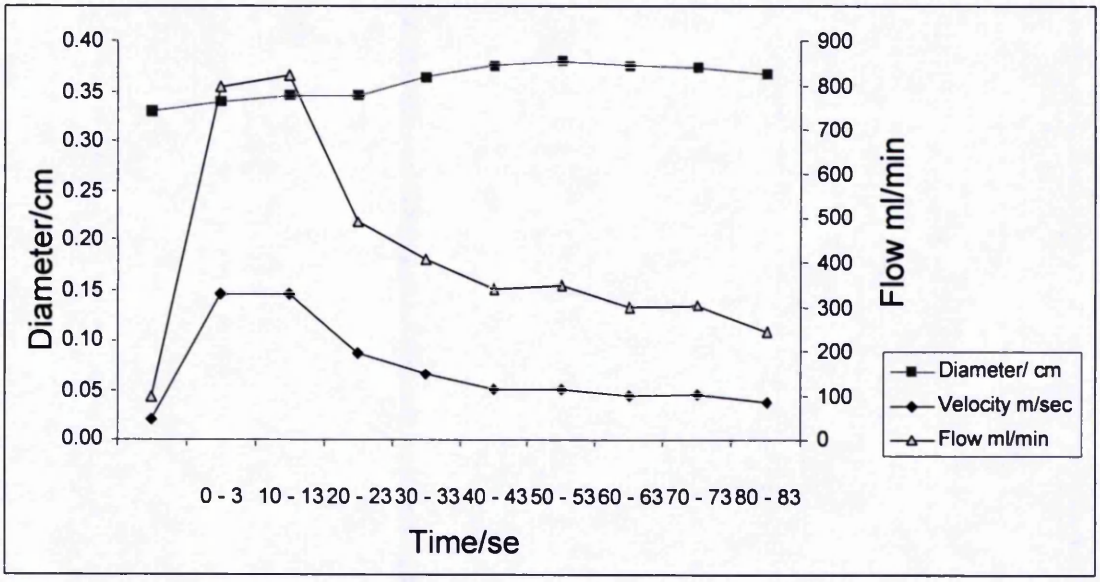


Figure 8.7 Relationship between resting diameter and resting flow from repeated studies on one subject:

(r = 0.792, P <0.001)

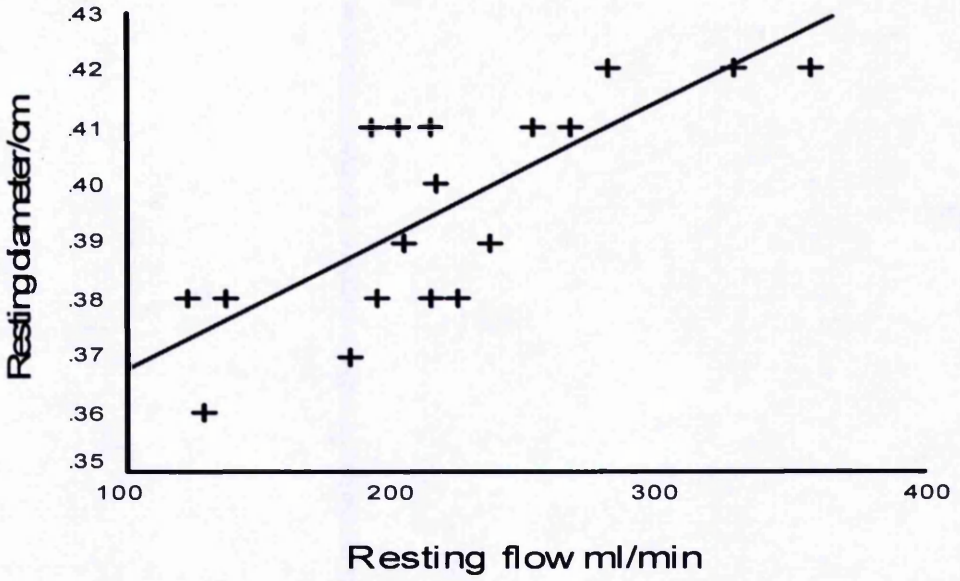


Figure 8.8 Relationship between resting diameter and %FMD diameter from repeated studies on one subject:

(r = - 0.757,P <0.001)

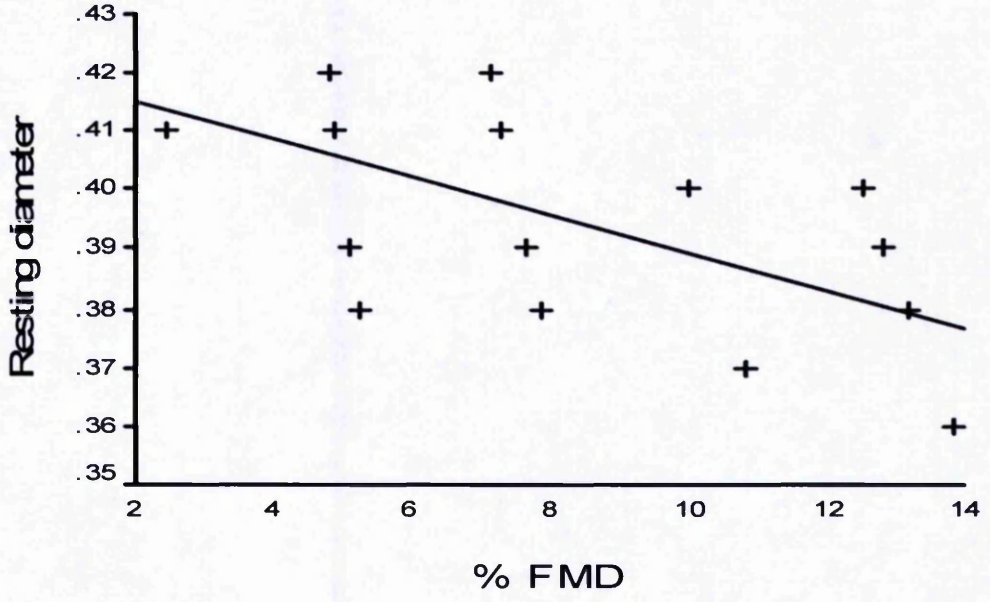
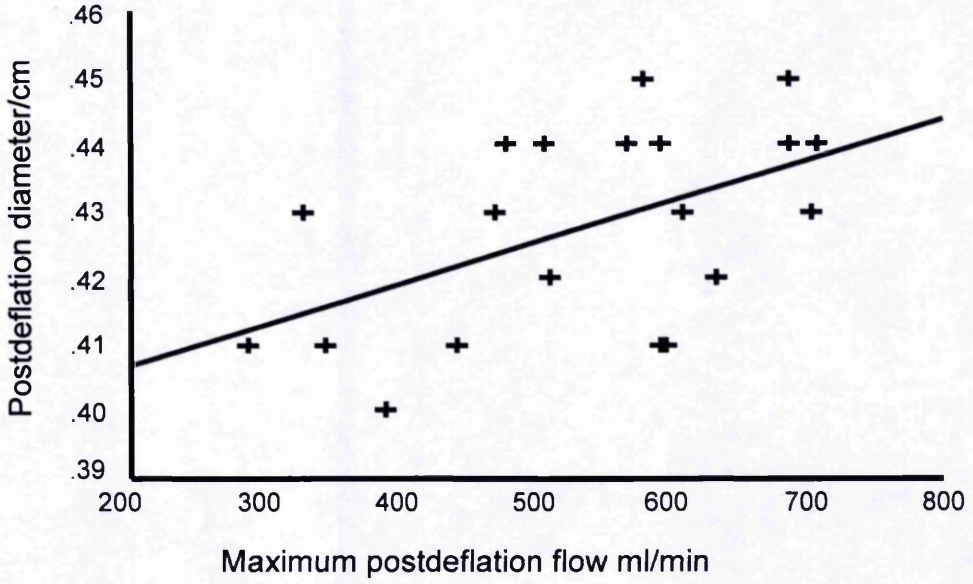


Figure 8.9 Relationship between maximum post deflation flow and maximum post deflation diameter from repeated studies on one subject:

(r = 0.435, P = 0.056)



8.4 Reproducibility of the technique:

In our preliminary testing of the technique, our vascular technician (HB) recorded scans of the brachial artery of one subject for ten times in one setting. Then the brachial artery diameters were measured (by HB) from videotape. After she had stabilized the cursors on the measurement points, a second observer read the results from the covered part of the screen.

Mean (SD) resting diameter of the ten measurements was 0.408 (0.011)

Coefficient of variance (CV%) = SD/mean x100 = 2.7%.

8.4.1 Intra- and inter-observer variability:

A random sample of 15 recorded scans from patients and controls who completed the study were re-measured by the same vascular technician (HB1 and HB2) and also once by myself (MM) to test for within observer (intra-observer) and between observers (inter-observer) variability respectively (Tables 8.1, 8.2).

8.4.1.1 Intra-observer variability

Table 8.1 shows the mean and standard deviation of resting diameter, FMD and percentage FMD and the absolute differences between the two measurements of one observer (HB1 and HB2). Figures 8.10, 8.11 show Bland-Altman plots for intra-observer variability in resting diameter and percentage FMD. The intra-class correlation coefficient for resting diameter and percentage FMD was 0.934 [95%CI (0.558 – 0.950)] and 0.820 [95%CI (0.300 – 0.965)] respectively.

Table 8.1 Measurements in 15 subjects in which the scan was read twice by one observer, HB1 and HB2:

	(HB1)	(HB2)	Mean HB1 & HB2	Mean Absolute difference HB1& HB2
Mean (SD) resting d /cm	0.34(0.04)	0.34(0.045)	0.34(0.04)	0.005(0.014)
Mean (SD) FMD (absolute)	.017(0.014	.017(0.013)	0.017(0.013)	- 0.0003(0.007)
Mean (SD) %FMD	5.2(4.6)	5.3(4.5)	5.2(4.3)	- 0.13(2.6)

Figure 8.10 Bland-Altman plot for first and second resting diameter measurements by one observer:

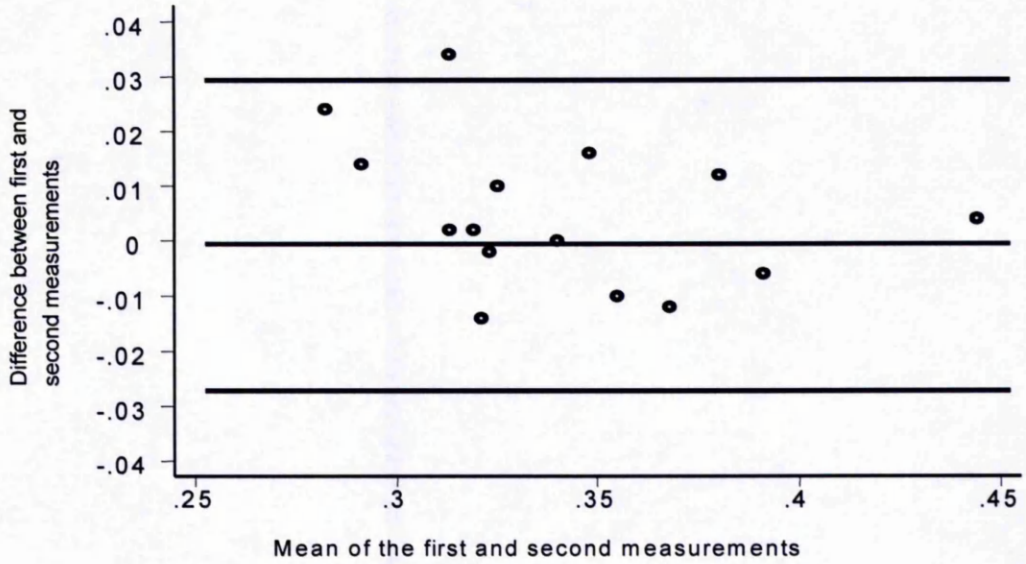
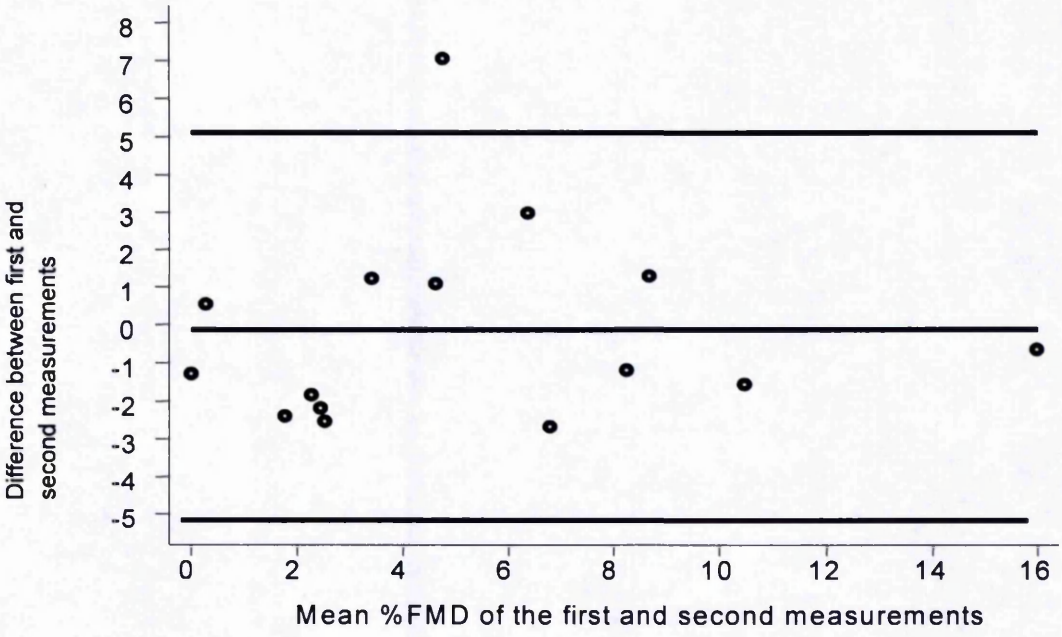


Figure 8.11 Bland-Altman plot for first and second %FMD measurements of one observer:



8.4.1.2 Inter-observer variability:

Table 8.2 shows the mean and standard deviation of resting diameter, FMD and percentage FMD and absolute differences between the two measurements by 2 observers (HB2 & MM). Figures 8.12, 8.13 show Bland-Altman plots for inter-observer variability in resting diameter and percentage FMD. Intra-class correlation coefficient for resting diameter and percentage FMD was 0.944 [95%CI (0.833 – 0.951)] and 0.229 [95%CI (- 1.29 – 0.741)] respectively.

Table 8.2 Measurements in 15 subjects where the scan was read by studies by two observers HB1 and MM:

	(MM)	(HB2)	Mean MM & HB2	Absolute diff. MM & HB2
Mean (SD) resting d /cm	0.33(0.04)	0.34(0.05)	0.33(0.04)	0.13(0.02)
Mean (SD) FMD (absolute)	0.019 (0.019)	.017(0.013)	-0.0015 (0.02)	- 0.0017(0.024)
Mean (SD) %FMD	5.7(6.2)	5.2(4.5)	5.5(4.6)	- 0.39(7.0)

Figure 8.12 Bland-Altman plot of resting diameter measurements by two observers, MM and HB2

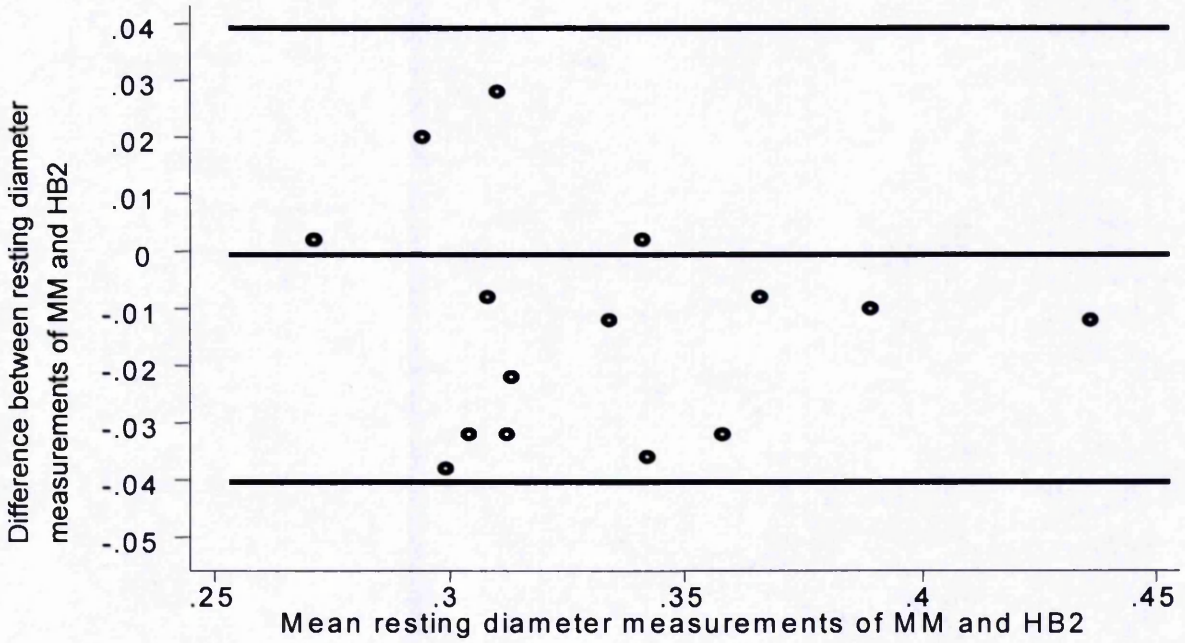
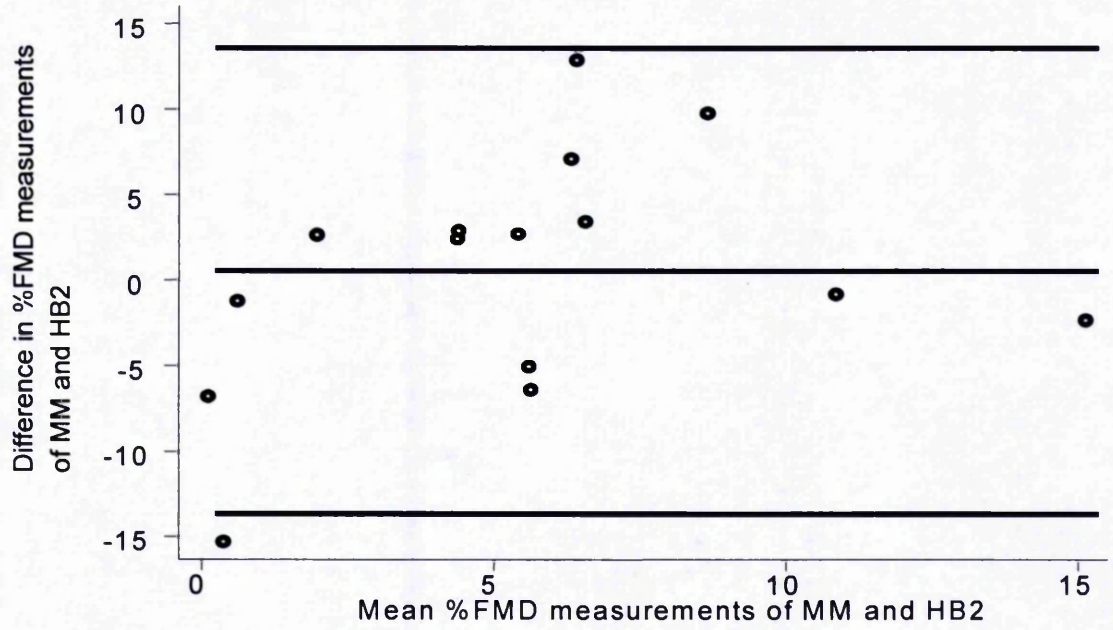


Figure 8.13 Bland-Altman plot of %FMD by two observers, MM and HB2:



8.4.2 Test of reproducibility:

For this, we studied resting diameter, FMD and percentage FMD in one healthy volunteer repeatedly over a period of two months. The scans and measurements were carried out by our vascular technician (HB) following the same protocol used for our study. Figures 8.14, 8.15 show scatter plots of 20 measurements of resting diameter and %FMD.

Mean (SD) of resting diameter for all studies = 0.40 (0.018)

Coefficient of variance (CV%) = 4.5%

Mean (SD) of FMD (absolute) for all studies = 0.03 (0.013)

Coefficient of variance (CV%) = 43%

Mean (SD) of %FMD for all studies = 8.3 (3.5)%.

Coefficient of variance (CV%) = 42%.

Figure 8.14 Scatter plot of resting diameter measurements in 20 studies on one subject:

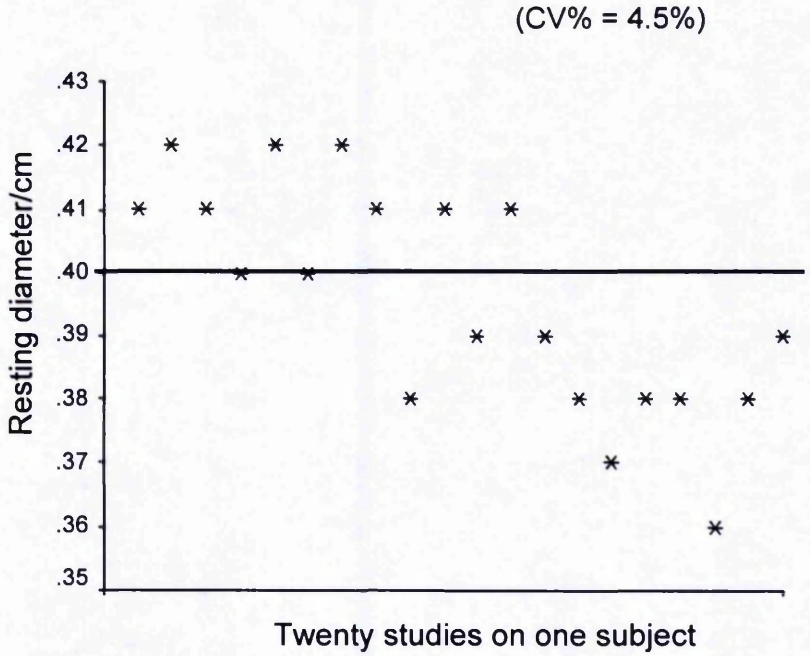
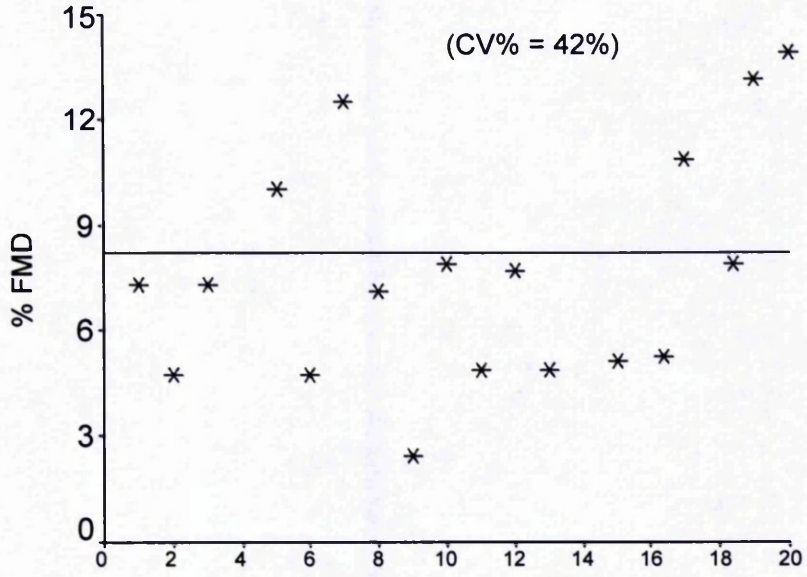


Figure 8.15 Scatter plot of %FMD in 20 studies on one subject:



8.5 Vascular studies in healthy controls:

Altogether we studied 38 healthy Caucasian women recruited from the nursing and secretarial staff at Manchester Royal Infirmary and the staff at the ARC Epidemiology Research Unit in the Medical School at University of Manchester.

In the first instance, to determine factors associated with percentage FMD, we analysed controls who were:

- Premenopausal.
- On no medication.
- No history of CHD or SLE.

Of the 13 others:

- 10 were postmenopausal (6 were on and 4 were not on hormone replacement therapy).
- One was taking anti-hypertensive therapy.
- Two were taking the combined oral contraceptive tablets.

This initial study therefore included 25 women. Their median (range) age was 41.5 (25 – 50) years. There were three current smokers, three ex-smokers and 19 non-smokers. Information about the time in the menstrual cycle on the day of the study

was available for only 11 controls, six were in the first half and five were in the second half of their menstrual cycles. Four had a family history of CHD. The clinical characteristics of this selected group are shown in table 8.3.

The median (range) resting diameter was 0.32 (0.26 – 0.41) cm, the percentage FMD was 9.7 (- 3.1 – 17.8)% and the percentage GTN dilation was 22.6 (11.8 – 31)% (Table 8.4). GTN responses were obtained for eleven (40%) of this initial group because of time constraints in the vascular laboratory as well as some of the controls declining to take GTN spray. There was no difference in percentage FMD of those in the first or second half of the menstrual cycle: 7.5 (0.0 – 12.5)% vs 7.1 (6.0 – 13.8)% respectively. Five controls (20%) had a percentage FMD ≤ 4.5%, a level generally felt to reflect poor endothelial function (Enderle *et al* 1998) (Table 8.4).

Table 8.3 Clinical features of 25 healthy controls:

	Median (range) n = 25
Age/yrs	41.5 (25 – 50)
Body mass index (BMI)	26.7 (17.1 – 34.6)
Waist/hip ratio	0.76 (0.68 – 0.89)
Systolic blood pressure/ mmHg	110 (90 – 140)
Diastolic blood pressure/ mmHg	70 (50 – 100)
Total cholesterol/ mmol/l	4.9 (2.6 – 7.0)
HDL-C/ mmol/l	1.6 (1.0 – 3.0)
Fasting glucose/ mmol/l	4.7 (3.8 – 5.4)
%10-year risk of CHD	0.6 (0.0 – 5.2%).

8.5.1 Factors influencing flow mediated dilation in healthy premenopausal controls:

Percentage FMD showed a significant negative correlation with resting diameter (r = -0.524, P = 0.007) (Figure 8.17). However, percentage FMD did not correlate with maximum post-deflation flow or fold increase in flow (r = 0.007, P = 0.972) and (-0.023, P =0.902) respectively. There was no correlation of percentage FMD with any of the standard Framingham CHD risk factors nor with the 10-year risk of CHD in this group (Tables 8.5, 8.6).

Table 8.4 FMD and GTN study measurements in 25 healthy controls:

	Healthy controls n = 25 Median (range)
Resting diameter/cm	0.32 (0.26 – 0.41)
Resting flow ml/min	29.7 (11.9 – 136)
Post-deflation flow ml/min	384 (162 – 719)
Fold increase from resting to maximum flow ml/min	13 (2.6 – 33)
Maximum post-deflation diameter/cm	0.34 (0.30 – 0.44)
Absolute FMD (cm)	0.03 (- 0.01 – 0.05)
%FMD	9.7 (- 3.1 – 17.8)
%GTN dilation*	22.6 (11.8 – 31)
No (%) of subjects with %FMD ≤ 4.5	5 (20)%

* %GTN study in 11 controls of this group of 25.

8.5.2 Factors influencing resting diameter in healthy controls:

Although percentage FMD was the main outcome in our studies of endothelial function, we also explored the resting diameter, which showed a strong positive correlation with resting flow (r = 0.595, P < 0.002) and a negative correlation with percentage FMD (r= – 0.524, P = 0.007) (Figures 8.16, 8.17). Compared to percentage FMD, resting diameter correlated more strongly with systolic and

diastolic blood pressure r = 0.690, P< 0.001 and r = 0.671, P< 0.001 respectively. Resting diameter also correlated significantly with TG (r = 0.425, P = 0.043), fasting glucose (r = 0.513, P = 0.012) and correlated weakly with %10-year risk of CHD (r = 0.295, P = 0.161) (Table 8.5, 8.6).

The relationship between resting blood flow and resting diameter might be affected by the functional state of endothelium. The shear stress of normal blood flow on endothelial cells is an important stimulus of basal NO production and thus may alter the resting diameter especially early in the process of endothelial dysfunction before development of structural changes in the arterial wall. However, the correlation between resting diameter, and resting flow was similar in a group of five controls who had percentage FMD of ≤ 4.5% (r = 0.894, p = 0.041) and in 20 controls with percentage FMD of > 4.5% (r = 0.678, P = 0.001).

Table 8.5 Correlations of Framingham risk factors for CHD with percentage FMD and resting diameter in 25 controls:

	Resting diameter		%FMD	
	r	P value	r	P value
Age	0.244	0.250	- 0.009	0.769
BMI	0.188	0.378	0.063	0.769
Waist/hip ratio	0.422	**0.040**	- 0.136	0.527
SBP mmHg	0.690	**< 0.001**	- 0.236	0.278
DBP mmHg	0.671	**< 0.001**	- 0.142	0.518
Fasting glucose	0.513	**0.012**	0.025	0.908
Total cholesterol	0.071	0.749	0.123	0.576
HDL-C	- 0.194	0.376	- 0.061	0.780
%10-year risk of CHD	0.295	0.161	0.142	0.508

Table 8.6 Correlation of percentage FMD and resting diameter with the metabolic factors and in 25 controls:

	Resting diameter		%FMD	
	r	P-value	r	P-value
Fasting glucose	0.513	**0.012**	0.025	0.908
Insulin	0.176	0.422	0.113	0.608
HOMA-S	- 0.262	0.227	- 0.151	0.491
HOMA-B	- 0.154	0.425	0.179	0.305
Triglycerides	0.425	**0.043**	- 0.354	0.097
VLDL-C	0.204	0.350	- 0.294	0.173
LDL-C	0.262	0.226	- 0.158	0.471
Oxidized LDL-C	0.328	0.126	- 0.343	0.109
Lipoprotein(a)	- 0.198	0.366	- 0.035	0.875
Paroxonase activity	- 0.039	0.860	- 0.104	0.636
Hs-CRP	- 0.089	0.678	0.249	0.164
IL-6	0.338	0.170	- 0.098	0.698

Figure 8.16 Relationship between resting diameter and resting flow in 25 controls.

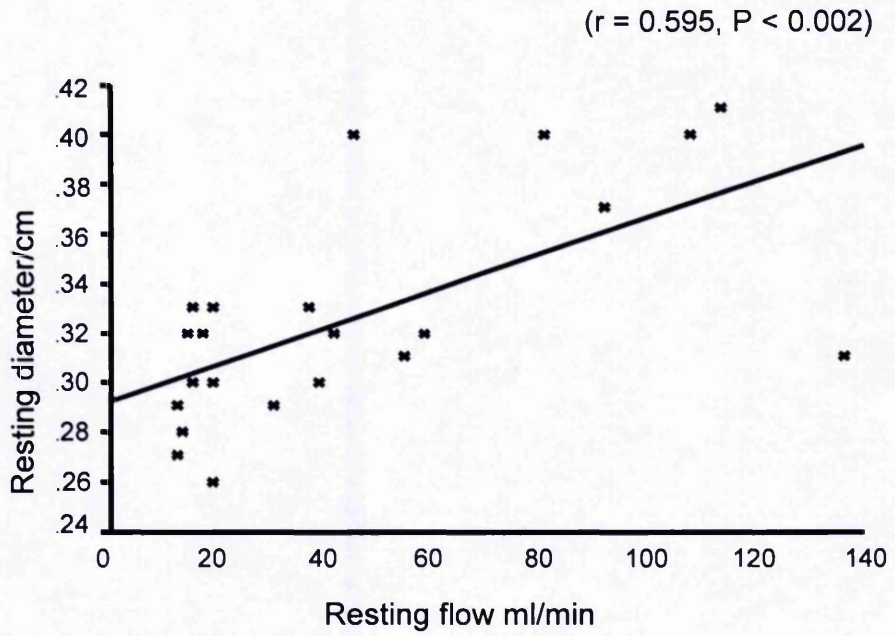
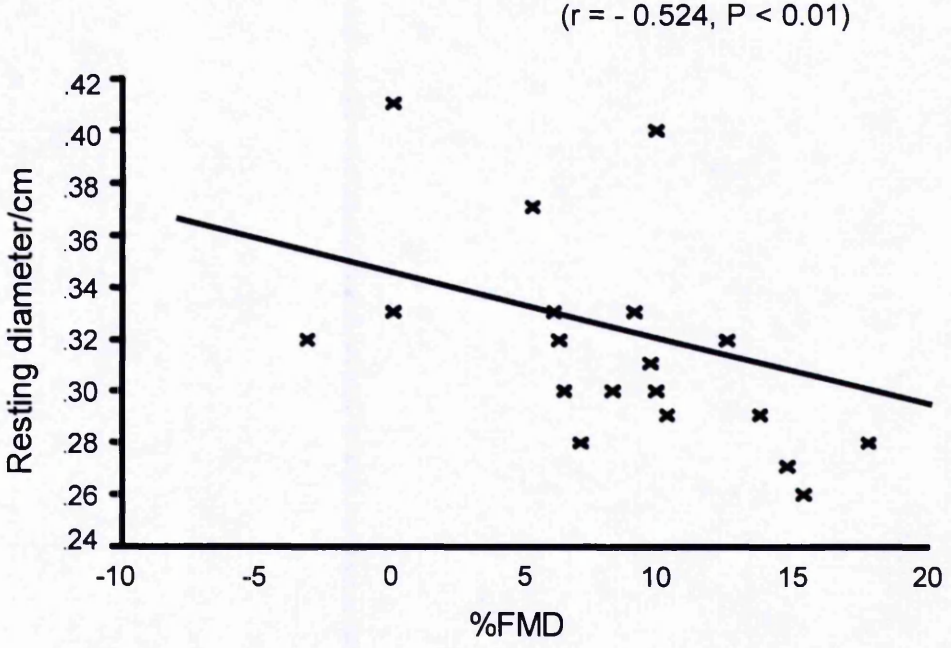


Figure 8.17 Relationship between resting diameter and percentage FMD in 25 controls:



8.6 Comparison of percentage FMD in SLE patients and healthy controls:

For our initial comparative study we studied 24 Caucasian women with SLE who were < 50 years old and 26 healthy women matched for age and ethnicity. The median (range) age of patients and controls was 40.5 (24 – 47) vs 40.5 (25 – 48) years respectively. Despite this, more patients were postmenopausal (five patients compared to one control). The patients and controls were of similar body mass index, but waist/hip ratio was higher in patients (P=0.04) (Table 8.7). In the patient group, the median (range) age at diagnosis and disease duration was 26.5 (11 – 45) and 11.5 (1 – 23) years respectively. Six (25%) patients had category A or B on the BILAG disease activity score in at least one organ system at the time of study. The median (range) SLEDAI was 0.0 (0 – 12), seven (29%) patients having a SLEDAI score of ≥ 6 on the day of the study. Sixteen (67%) patients were taking steroid therapy. For the whole group the median (range) steroid dose was 5.0 (0 – 30) mg/day. Fifteen (62.5%) patients were on anti-malarial therapy; 13 on hydroxychloroquine and two on chloroquine phosphate. Nine (37.5%) patients were on immunosuppressive therapy; eight were taking azathioprine and one was on methotrexate. Four postmenopausal patients and one postmenopausal control were on hormone replacement therapy. We did not collect information about time in menstrual the cycle on the day of study. We therefore re-contacted patients and controls to ascertain the point in their menstrual cycle on their day of study.

Table 8.7 Demographic features in Caucasian patients and controls:

	SLE n = 24 Median (range)	Controls n = 26 Median (range)	P-value
Age/years	40.5 (24 – 47)	40.5 (25 – 48)	0.922
BMI	25.3 (21 – 41)	25.6 (17 – 34)	0.961
Waist/hip ratio	0.82 (0.69 – 0.92)	0.77 (0.68 – 0.89)	**0.044**
Postmenopausal n (%)	5 (20.8)	1 (3.8)	0.093

There were 7/19 (37%) patients and 11/25 (44%) controls who had information available. Four out of seven (57%) patients and 6/11 (54%) controls were in the first half and 3/7 (43%) of patients and 5/11(46%) of controls were in the second half of their menstrual cycle.

8.6.1 Coronary heart disease risk factors in patients and controls:

Overall, the Framingham risk factors for CHD were more prevalent in the patients compared to controls (Table 8.8). Waist/hip ratio was significantly higher in patients compared to controls (P=0.044). Hypertension was present in seven (29.2%) patients and two (7.7%) controls. SLE patients had significantly higher TGs and lower HDL-C (Table 8.8). There was also a trend in the patients toward higher SBP and DBP (Table 8.8).

8.6.2 Vascular studies in SLE patients vs controls:

When we compared vascular function in patients and controls, the resting diameter was similar in SLE patients compared to controls. Resting and peak blood flow also did not differ (Table 8.9).

Absolute FMD was significantly lower in patients compared to controls and the percentage FMD was significantly lower in patients compared to controls [median (range) 4.2 (0.0 – 13.7)% vs 8.8 (- 6.6 – 17), P = 0.010]. The proportions of SLE patients and controls with percentage FMD of ≤ 4.5% were 52.2% vs 23% (P = 0.0344). When we compared only premenopausal patients and controls, SLE patients had significantly lower resting diameter and percentage FMD, Median (range) [0.30 (0.24 – 0.36) vs 0.32 (0.27 – 0.41), P = 0.045] and [4.1 (0 – 13.7)% vs 9.1 (- 3.1 – 17.8)%, p = 0.007] respectively. The median (range) percentage FMD in four patients on hormone replacement therapy was 4.3 (2.7 – 7.1). Interestingly, one of the controls who was on hormone replacement therapy showed a constrictive response with a percentage FMD of – 6.6%, she was 46 years old and a current smoker of 20 cigarettes/day for the last 10 years. Although her 10-year risk of CHD was <3%, she had a very high Lp(a), 53.6 mg/dl.

Table 8.8 Comparison of classic and novel risk factors in Caucasian patients and controls aged < 50years:

	SLE n = 24 Median (range) n (%)	Controls n = 26 Median (range) n (%)	P-value
Hypertension	7(29.2)	2 (7.7)	0.069
SBP mmHg	120 (96 – 170)	110 (90 – 140)	0.086
DBP mmHg	78 (60 – 110)	70 (50 – 100)	0.102
Diabetes mellitus	2 (8.3)	0.0 (0.0)	0.235
Fasting glucose mmol/l	4.4 (3.4 – 10.2)	4.6 (3.8 – 5.6)	0.341
Smoking: n (%) Current smokers Ex-smokers Non-smokers	 3 (12.5) 5 (20.8) 16 (66.7)	 4 (15.4) 3 (11.5) 19 (73.1)	0.663
TC mmol/l	5.0 (3.3 – 7.5)	5.5 (2.6 – 7.2)	0.232
LDL-C mmol/l	2.5 (0.3 – 4.1)	2.6 (0.6 – 4.0)	0.973
HDL-C mmol/l	1.4 (0.6 – 2.7)	1.7 (1.0 – 3.0)	**0.002**
TGs mmol/l	1.3 (0.4 – 3.9)	0.85 (0.4 – 3.1)	**0.020**
VLDL-C mmol/l	0.2 (0.06 – 0.85)	0.17 (0.01 – 2.0)	0.333
Lp(a) mg/dl	13 (0.5 – 52)	14.1 (0.3 – 81)	0.674
Ox-LDL-C mU/l	34 (13 – 59.4)	32 (11 – 43.8)	0.435
Paroxonase activity	254 (22 – 439)	197 (26 – 409)	0.074
10-year risk of CHD	1.2(0 – 11.9)%	0.6(0 – 5.2)%	0.178

The percentage GTN dilation response was assessed in 22 patients and 12 controls (Table 8.10). Both absolute and percentage GTN dilation were similar between the two groups and there was no difference in proportions of patients and controls with percentage GTN dilation of > 20%.

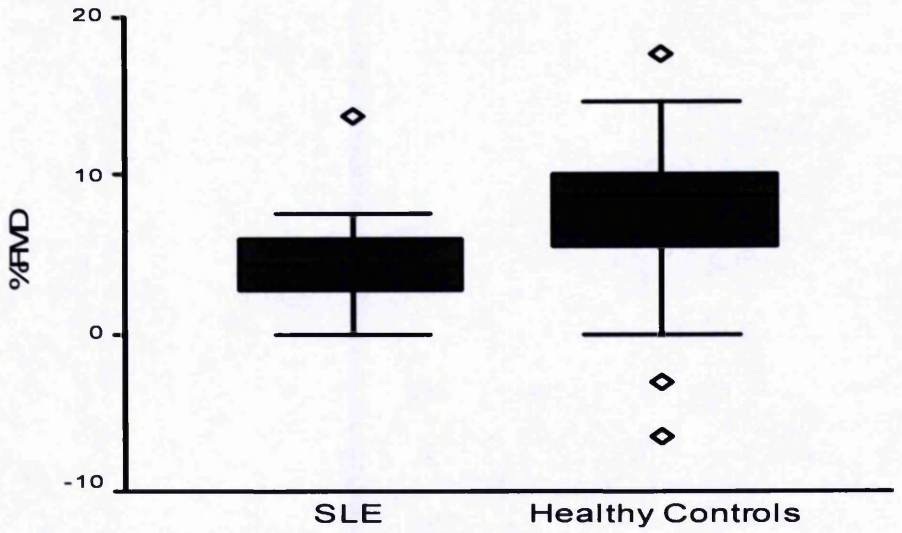
Table 8.9 Comparison of vascular function in SLE patients vs healthy controls only subjects< 50 years old of white Caucasian origin were included:

	SLE n = 24 Median (range)	Controls n = 26 Median (range)	P-value
Resting diameter /cm	0.30 (0.24 – 0.39)	0.32 (0.27 – 0.41)	0.201
Resting flow ml/min	27.7 (3.0 – 78)	20.1 (12 – 136)	0.896
Maximum flow ml/min	242 (175 – 583)	344 (114 – 594)	0.157
Fold increase in blood flow	11 (2.8 – 62)	13 (2.6 – 33)	0.845
Absolute FMD/cm	0.01 (0.0 – 0.04)	0.03 (- 0.02 – 0.05)	**0.010**
%FMD	4.2 (0.0 – 13.7)	8.75 (- 6.6 – 17)	**0.010**
FMD ≤4.5 % n (%)	12 (52.2)	6 (23.1)	**0.043**

Table 8.10 Endothelium non-dependent vasodilation in patients and controls:

	SLE n = 22 Median (range)	Controls n = 12 Median (range)	P-value
GTN resting diameter /cm	0.32 (0.25 – 0.43)	0.32 (0.26 – 0.45)	0.736
Percentage GTN dilation	21.7 (12.9 – 36.6)	22.9 (11.8 – 31)	0.901

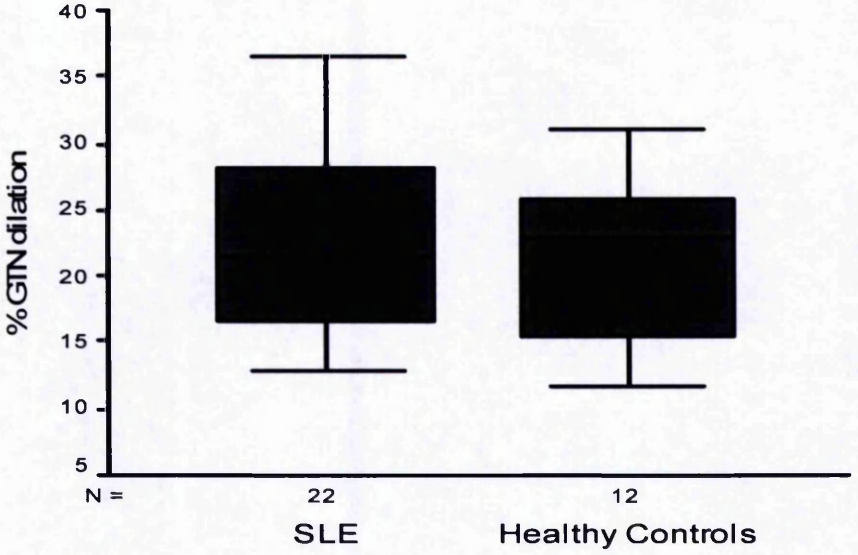
Figure 8.18 Percentage FMD in Caucasian SLE patients and controls aged < 50 year (P = 0.01)



Box plots diagram: The boxes represent interquartile range (IQR) and the horizontal line inside the box is the median value. The whiskers extend to the lowest value within 1.5 times IQR below the first quartile and the highest value within 1.5 times IQR above the third quartile. Points below and above the whiskers represent outlayier values. (SLE patients different from controls, Mann-Whitney test, p= 0.01).

Figure 8.19 Percentage GTN dilation in Caucasian SLE patients and controls:

(P = 0.901)



8.7 Is endothelial dysfunction in SLE due to differences in classic Framingham risk factors:

In the previous small groups, we noted there were important differences in the prevalence of classic CHD risk factors in patients vs controls. We were interested to know whether the endothelial dysfunction in SLE was explained by these differences.

In order to do this we extended our recruitment of patients and healthy controls to determine if SLE had an independent effect on endothelial dysfunction after adjustment for classic CHD risk factors: The risk factors studied were, age, ethnicity, post-menopause status, BMI, hypertension, blood pressure, fasting glucose, HDL-C, LDL-C and smoking. We could not ascertain presence of left ventricular hypertrophy. Since resting diameter is known to profoundly influence FMD, this was also included in our model.

Overall, 64 patients have participated in our study of endothelial function (see chapter 7). However, two patients were excluded because their scans were of a poor quality, which precluded accurate measurements. Therefore, for this study, I report the results for 62 patients and 38 controls (Tables 8.11, 8.12).

We included all 62 patients and 38 controls in a single group for linear regression analysis. We first assessed the association of the percentage FMD as a dependent variable by entering independent variables individually (one at a time) into the model (Table 8.13). This showed that the variables significantly associated with percentage FMD were systolic pressure, resting diameter, 10-year risk of CHD and SLE. The unstandardized regression coefficient (B) for resting diameter was - 22.9 ($P = 0.027$), systolic pressure - 0.083 ($P = 0.002$), 10-year risk of CHD - 0.27 ($P = 0.038$) and SLE - 3.35 ($P = 0.001$).

Multiple linear regression analysis was used to assess which variables were independently associated with percentage FMD after adjustment for the other factors. The variables, which showed a degree of association ($P < 0.200$) with percentage FMD in univariate analysis (Table 8.13), were entered in a multiple linear regression model. The variables included were resting diameter, age, ethnicity, menopausal state, SBP, BMI, fasting glucose, hypertension and 10-year risk of CHD.

Table 8.11 Comparison of demographic and classic risk factors for CHD in patients and controls:

	SLE n = 62 Median (range) n (%)	Controls n = 38 Median (range) n (%)	P-value
Age/years	48 (21 – 73)	43 (25 – 62)	0.062
Menopause Pre-menopausal Post-menopausal	 33 (53.2) 29 (46.8)	 27 (71.1) 11 (28.9)	0.094
HRT	10 (34.5)	6 (54.5)	0.600
BMI	25.8 (17.8 – 42)	27.4 (17.1 – 36.2)	0.281
Hypertension	21 (33.9)	3 (7.9)	**0.003**
Systolic BP mmHg	120 (90 – 170)	112 (90 – 140)	**0.010**
Diastolic BP mmHg	80 (40 – 110)	70 (50 – 100)	0.272
Diabetes mellitus	3 (4.7)	0.0 (0%)	0.286
Fasting glucose mmol/l	4.6 (3.6 – 10.9)	4.7 (3.8 – 5.6)	0.247
Smoking: current smokers ex-smokers non-smokers	 3 (4.9) 12 (19.7) 46 (75.4)	 4 (10.5) 9 (23.7) 25 (65.8)	0.467
TC mmol/l	5.1 (3.0 – 8.7)	5.1 (2.6 – 4.6)	0.731
LDL-C mmol/l	2.6 (0.33 – 5.0)	2.7 (0.64 – 3.5)	0.613
HDL-C mmol/l	1.4 (0.6 – 3.0)	1.7 (1.0 – 3.5)	**0.011**
10-year risk of CHD	3.8 (0.0 – 15.8)	1.5 (0.0 – 10.9)	**0.007**
Triglycerides mmol/l	1.4 (0.04 – 3.9)	0.9 (0.4 – 3.1)	**0.001**
VLDL-C mmol/l	0.23 (0.06 – 1.0)	0.18 (0.01 – 2.0)	0.543
Lipoprotein (a)	15.9 (0.5 – 139)	16.8 (0.3 – 81.8)	0.867
Oxidized LDL-C mU/l	32 (12.9 – 76.8)	35.8 (10.8 – 67.5)	0.402
Paroxonase activity	211 (22 – 564)	188 (26 – 445)	0.619

Table 8.12 Comparison of vascular function results in patients and controls:

	SLE n = 62 Median (range) n (%)	Controls n = 38 Median (range) n (%)	P-value
Resting diameter /cm	0.33 (0.24 – 0.43)	0.32 (0.26 – 0.45)	0.695
Absolute FMD /cm	0.01 (-0.02 – 0.04)	0.025 (-0.02 – 0.05)	**< 0.001**
%FMD	3.6 (-6.2 – 13.7)	6.9 (-6.6 – 17.8)	**0.001**
%GTN dilation	17.4 (2.8 – 44)	22.2 (8.9 – 31)	0.256

Table 8.13 Association of individual variables with percentage FMD in 62 SLE patients and 38 healthy controls as one group:

Independent variables	R^2 *	B**	p value	95%CI of B
Resting D/cm	0.049	- 22.9	**0.027**	- 43.2 – -2.70
Age	0.030	- 0.070	0.091	- 0.152 – 0.011
Menopause	0.024	- 1.51	0.126	- 3.40 – 0.43
Ethnicity ***	0.02	2.1	0.161	- 0.84 – 5.0
Smoking	0.000	0.137	0.865	- 1.45 – 1.72
Hypertension	0.029	- 1.90	0.091	- 4.12 – 0.310
SBP	0.095	- 0.083	**0.002**	- 0.14 – - 0.031
DBP	0.008	- 0.036	0.375	- 0.012 – 0.044
BMI	0.032	0.182	0.078	- 0.021 – 0.38
W/h ratio	0.012	- 8.7	0.276	- 24.5 – 7.10
FBG	0.021	0.634	0.167	- 0.270 – 1.54
Total cholesterol	0.000	-0.073	0.867	- 0.94 – 0.79
LDL-C	0.000	0.098	0.845	- 0.90 – 1.10
HDL-C	0.015	- 1.16	0.227	- 3.10 – 0.73
10-y risk of CHD	0.044	- 0.27	**0.038**	- 0.053 – - 0.016
SLE	0.155	- 3.35	**0.001**	- 5.21 – - 1.49

* Multiple coefficient of determination of this model (R^2)
* B = Unstandardized regression coefficient, (represent unit change in dependent variable %FMD associated with each unit change in the independent variable).

- Each one mm decrease in resting diameter is associated with a 2.29% lower FMD.
- Each 10mmHg increase in SBP is associated with a 0.95% lower FMD.
- SLE is associated with a 3.35% lower %FMD compared to controls.

*** White Caucasian or Non-white Caucasians

In stepwise multiple regression model, factors independently associated with percentage FMD were systolic pressure (P = 0.007), BMI (P = 0.024) and SLE (P = 0.049) (Table 8.14). After adjusting for systolic pressure and BMI, SLE patients had a 1.9% lower FMD compared to controls.

Table 8.14 Stepwise multiple regression analysis of factors associated with percentage FMD in 62 SLE patients and 38 healthy controls:

Predictors in the model	B*	Beta**	95%CI of B	P value
Systolic pressure	- 0.074	- 0.275	(- 0.128 – - 0.02)	**0.007**
BMI	0.220	0.223	(0.03 – 0.41)	**0.024**
SLE	- 1.872	- 0.198	(- 3.74 – - 0.006)	**0.049**

Excluded variables	Beta	P-value
Resting diameter	- 0.148	0.159
Age	- 0.082	0.448
Menopausal state	0.000	0.997
Ethnicity	0.127	0.201
Fasting glucose	0.140	0.174
Hypertension	0.087	0.766
10-year risk of CHD	0.014	0.911

* B = Unstandardized regression coefficient.
After adjuatment for other factors:

- Each increase of 10 mmHg in SBP is associated with 0.74% decrease in %FMD.
- SLE is associated with a 1.872 lower %FMD compared to controls.
- Each unit increase in BMI is associated with 0.22% higher %FMD.

** Beta = Standaradized regression coefficient, (a change of a standard deviation change in dependent variable with standard deviation change in the independent variable).
Multiple coefficient of determination of this model (R^2) = 0.18, P = 0.049.

8.8 Examination of SLE-related factors influence on vascular function in SLE patients:

In order to determine the influence of different aspects of SLE on endothelial function, we further analysed additional factors of interest in patients with SLE and their effect on endothelial function. These factors included:

1. Disease activity.
2. APL antibodies.
3. Raynaud's phenomenon.
4. Atheroma.
5. Additional novel atherosclerosis risk factors including:
    - Lp(a).
    - TGs.
    - VLDL-C.
    - Ox-LDL-C.
    - Paroxonase activity.
6. We will also explore insulin and genetic factors in later chapters.

8.8.1 Disease Activity:

Generally, disease activity in the patients we studied was low with a median (range) SLEDAI of 2 (0.0 – 12.0). Nine (15.3%) patients had a SLEDAI of ≥ 6. Thirteen (21%) patients had either A or B category of the BILAG index. Ten patients were on a prednisolone dose of ≥ 10 mg/day. As pointed out in (chapter 7) 21(34%) patients had active disease at study (Table 8.15). Patients with active disease had a significantly lower resting diameter and higher percentage FMD of borderline significance ($p = 0.053$) (Table 8.16) and (Figures 8.22, 8.23). There was no difference in percentage GTN dilation.

Using Spearman's correlation coefficient, SLEDAI showed a positive correlation with percentage FMD ($r = 0.342$, $P = 0.007$) and a negative correlation with resting diameter ($r = 0.329$, $p = 0.010$) (Figures 8.20, 8.21). When we looked at the individual components of our active disease definition we found that, the main differences were found in the group divided according to SLEDAI or steroid dose. In particular, percentage FMD was higher in patients with SLEDAI ≥ 6 ($p = 0.058$)

(Table 8.17). However, in the group with SLEDAI ≥ 6, the percentage FMD was still lower than our control group 5.5 (0.0 – 13.7%).

Figure 8.20 Relationship between SLEDAI and percentage FMD:

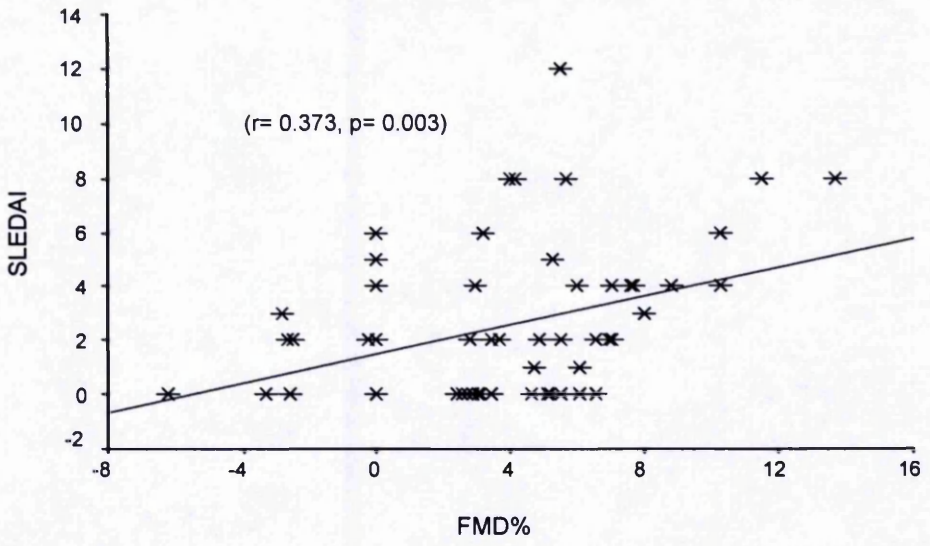


Figure 8.21 Relationship between SLEDAI and resting diameter:

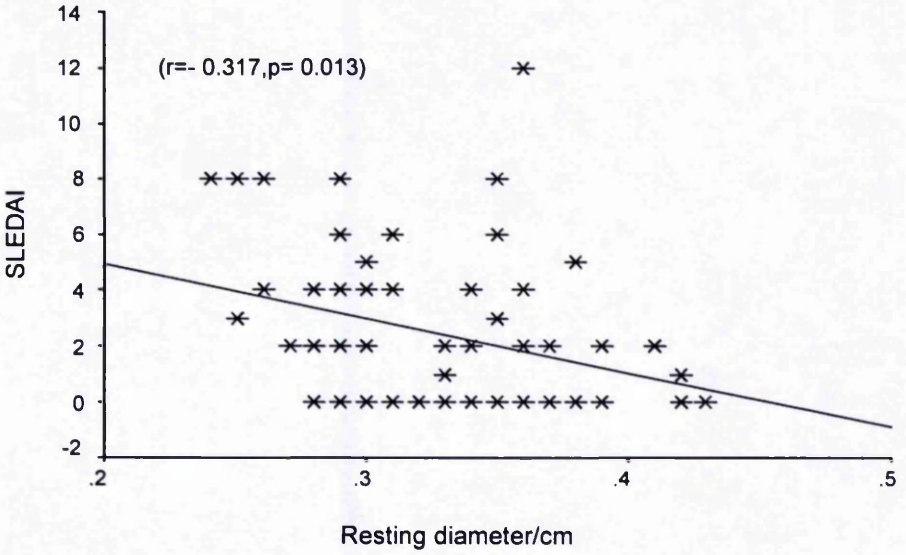


Table 8.15 Comparison of demographic and classic risk factors in patients with active and inactive disease:

	Patients with active disease n = 21 Median (range)	Patients with inactive disease n =40 Median (range)	P value
Age/years	45 (29 – 65)	50 (21 – 73)	0.288
Menopause Pre-menopausal Post-menopausal	 13 (62) 8 (38)	 19 (47.5) 21 (52.5)	0.419
BMI	25 (18.8 – 41.9)	26.5 (20.7 – 38)	0.222
Hypertension	5 (23.8)	16 (40)	0.263
SBP mmHg	120 (90 – 160)	129 (90 – 170)	0.056
DBP mmHg	80 (60 – 100)	77 (40 – 110)	0.859
Diabetes mellitus	2 (9.5)	1 (2.5)	0.270
Fasting glucose	4.5 (3.6 – 10.2)	4.5 (3.8 – 10.9)	0.600
Smoking: current smokers ex-smokers non-smokers	 1 (4.8) 4 (19) 16 (76.2)	 2 (5.0) 8 (20) 30 (70)	0.995
TC mmol/l	5.3 (3.0 – 6.7)	5.0 (3.2 – 8.7)	0.773
LDL-C mmol/l	2.7 (0.33 – 4.0)	2.6 (0.45 – 5.0)	0.883
HDL-C mmol/l	1.4 (0.6 –2.7)	1.4 (0.8 – 3.0)	0.837
10-year risk of CHD	2.0 (0.0 – 15.8)	1.2 (0.0 – 13.8)	0.335
TGs mmol/l	1.5 (0.4 – 3.9)	1.2 (0.4 – 3.0)	0.076
VLDL-C mmol/l	0.28 (0.06 – 0.85)	0.20 (0.06 – 1.0)	0.080
Ox-LDL-C mU/l	30.8 (12.8 – 61.6)	32.1 (14.1 – 76.8)	0.796
Paroxonase activity	251.9 (114 – 564)	208.8 (22 – 475)	0.071
Lp(a) mg/dl	9.7 (0.5 – 81.8)	20.4 (0.5 – 81.8)	**0.049**

Table 8.16 Comparison of vascular function in patients with active and inactive disease:

	Active disease n = 21 Median (range)	Inactive disease n =40 Median (range)	P value
Resting diameter /cm	0.29 (0.24 – 0.38)	0.34 (0.25 – 0.43)	**0.009**
FMD/cm (absolute)	0.02 (- 0.02 – 0.04)	0.01(- 0.01 – 0.03)	0.194
%FMD	5.7 (- 6.3 – 13.7)	3.1 (-3.3 – 8.0)	0.053
N (%) with FMD ≤ 4.5%	9 (43)	24 (60)	0.281
%GTN dilation	20 (5.7 – 34.7)	16.6 (2.8 – 44)	0.101

Table 8.17 Resting diameter and percentage FMD in patients with active and inactive disease:

Disease status	Number	Resting diameter (cms) Median (range)	% FMD Median (range)
SLEDAI >= 6	9	0.29 (0.24 - 0.36)	5.5 (0.0 - 13.7)
SLEDAI < 6	50	0.33 (0.25 - 0.43)	3.2 (-6.3 - 10.3)
P-value		0.083	0.058
BILAG A or B	13	0.33 (0.26 - 0.38)	5.5 (-6.3 - 13.7)
No BILAG A or B	48	0.33 (0.24 - 0.43)	3.6 (-3.3 - 11.5)
P-value		0.690	0.930
>=10mg prednisolone /day	10	0.30 (0.24 - 0.36)	5.5 (-6.3 - 13.7)
<10mg prednisolone /day	51	0.33 (0.25 - 0.43)	3.1 (-3.3 - 8.0)
P-value		0.116	0.145

Figure 8.22 Resting diameter in patients with active and inactive disease:

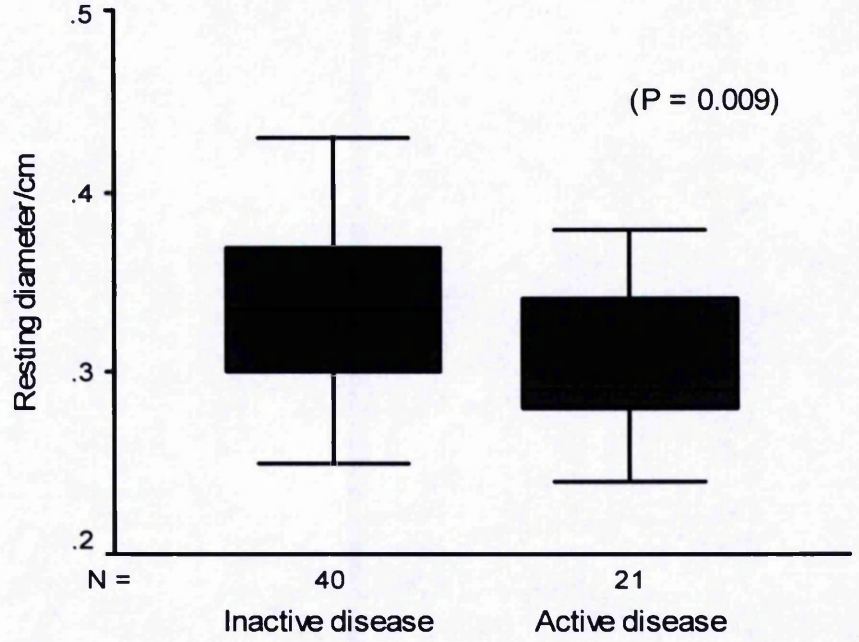
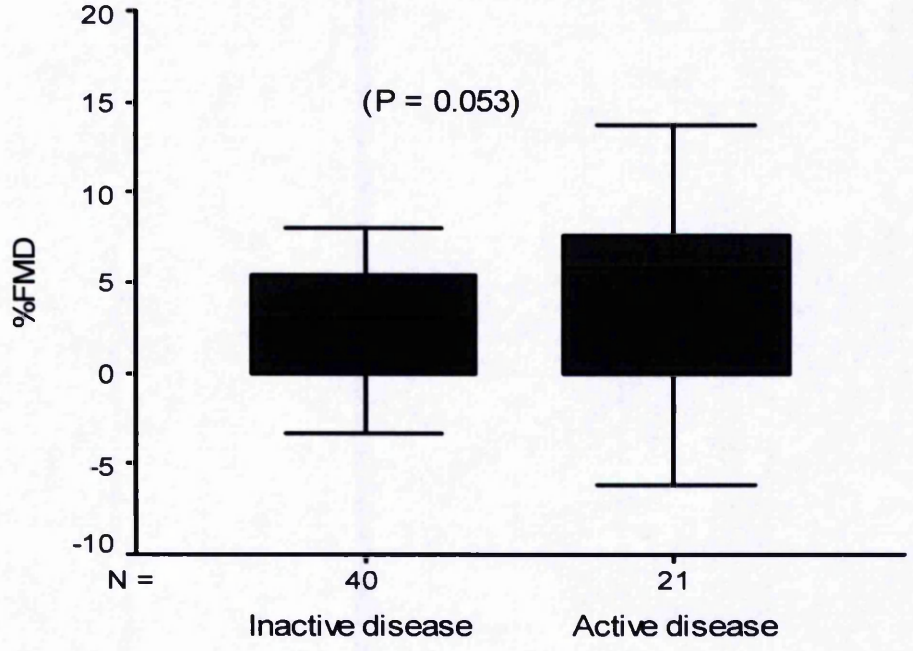


Figure 8.23 Percentage FMD in patients with active and inactive disease:



In view of changes associated with clinical disease activity, we examined this further by looking at other serological and biochemical markers of inflammation in this group. With regard to SLE we recorded hypocomplementaemia and ds-DNA antibodies. Patients with Hypocomplementaemia on the day of study tended to have higher percentage FMD [median (range) 5.5 (- 2.7 – 11.5) vs 3.1 (- 6.3 – 13.7), P = 0.055] and lower resting diameter [median (range) 0.30 (0.24 – 0.37) vs 0.33 (0.25 – 0.43), P = 0.095]. We also measured hs-CRP and IL-6, which will be outlined in detail in a subsequent chapter. In this section, we found no correlation between the percentage FMD and IgG ds-DNA antibodies (r = 0.183, P = 0.137), hs-CRP (r = 0.143, P = 0.270) or IL-6 (r = 0.128, P = 0.458).

8.8.2 Anti-phospholipid (APL) antibodies:

For the purpose of this study, we considered patients who either had a positive titre of IgG anti-cardiolipin antibody or lupus anticoagulant on the day of the study as positive for APL antibodies. Overall, we had 23 patients positive and 38 negative for APL antibodies. Both groups were of similar age, age at diagnosis and disease duration (Table 8.18).

As can be seen in table 8.18, there was a higher proportion of patients with active disease in the group with APL antibodies (48% vs 26%, p = 0.103). This was because of a higher number of APL antibody positive patients who had a BLAG A or B category in any system (Table 8.18). Interestingly less APL antibody positive patients were taking anti-malarial drugs (22% vs 68%, p =0.001). There were also important differences in the metabolic factors studied between the two groups. In particular APL antibody positive patients had higher concentration of fasting glucose, LDL-C, TGs, VLDL-C, ox-LDLC (Table 8.19). Paroxonase activity did not differ between groups.

As can be seen in Table 8.20, the vascular function did not differ significantly in the APL antibody positive compared to the negative group (Figures 8.24, 8.25).

Table 8.18 Disease features in anti-phospholipid antibody positive and negative SLE patients:

	APL antibody +ve n = 23 Median (range)	APL antibody - ve n = 35 Median (range)	P value
Disease duration/years	7.0 (1.0 – 23)	12.5 (2 – 35)	0.065
Age at diagnosis/years	36 (22 – 61)	31.5 (10 – 62)	0.065
Active disease n (%)	11 (47.8)	10 (26.3)	0.103
SLEDAI ≥ 6	4 (17.4)	5 (13.2)	0.718
BILAG A or B category	7 (30.4)	6 (15.8)	0.208
Prednisolone dose ≥ 10 mg/day	4 (16.7)	6 (15.8)	0.100
SLEDAI	3.0 (0 – 8)	0.5 (0 – 12)	**0.030**
No (%) on steroids	11 (47.8)	18 (51.4)	1.000
Current steroid dose in mg/day	0.0 (0.0 – 30)	3.5 (0.0 – 30)	0.583
Average steroid dose in last 6 months mg/day	2.0 (0.0 – 16)	5.0 (0.0 – 20)	0.565
No (%) on AM's	5 (20.8)	25 (67.6)	**0.001**

Table 8.19 Demographic, classic risk factors and metabolic factors in APL antibody positive and negative SLE patients:

	APL antibody +ve n = 23 Median (range)	APL antibody - ve n = 38 Median (range)	P value
Age/years	50 (26 – 67)	47 (21 – 73)	0.715
Menopause Pre-menopausal Post-menopausal	 20 (52.6) 18 (47.4)	 12 (52.2) 11 (47.8)	1.000
BMI	26.8 (18.6 – 41.9)	24.7 (18.8 – 38.1)	0.097
Hypertension	8 (34.8)	13 (34.2)	1.000
SBP mmHg	120 (100 – 160)	124 (90 – 170)	0.839
DBP mmHg	80 (60 – 100)	71 (40 – 110)	0.149
Diabetes mellitus	1.0 (4.3)	2 (5.3)	1.000
Fasting glucose mmol/l	4.8 (3.6 – 10.2)	4.4 (3.6 – 10.9)	**0.031**
Smoking: current smokers ex-smokers non-smokers	 2 (8.7) 5 (21.7) 16 (69.6)	 1 (2.6) 7 (18.4) 30 (78.9)	0.551
TC mmol/l	5.4 (3.3 – 5.0)	4.9 (3.0 – 7.5)	0.100
LDL-C mmol/l	3.1 (0.33 – 5.0)	2.5 (0.6 – 3.0)	**0.018**
HDL-C mmol/l	1.4 (0.8 – 2.7)	1.5 (0.6 – 3.0)	0.114
10-year risk of CHD	5.4 (0.0 – 15.8)	3.1 (0.0 – 14.2)	0.114
TGs mmol/l	1.6 (0.7 – 3.9)	1.1 (0.4 – 3.2)	**0.009**
VLDL-C mmol/l	0.28 (0.06 – 1.0)	0.20 (0.06 – 0.79)	**0.037**
Ox-LDL-C mU/l	44.8 (12.9 – 76.8)	27.5 (14 – 64.9)	**< 0.001**
Paroxonase activity	228 (22 – 439.5)	211 (44.5 – 564)	0.878
Lp(a) mg/dl	13.6 (1.8 – 129.6)	17 (0.5 – 139)	0.964

Table 8.20 Comparison of vascular function in APL antibody positive and negative patients:

	APL antibody +ve n = 23 Median (range)	APL antibody -ve n = 38 Median (range)	P value
Resting diameter /cm	0.32 (0.25 – 0.43)	0.33 (0.24 – 0.42)	0.383
FMD (absolute)	0.01 (- 0.02 – 0.03)	0.01 (- 0.01 – 0.03)	0.431
%FMD	4.7 (- 6.3 – 13.7)	3.3 (- 3.3 – 10.3)	0.366
n (%) with %FMD ≤ 4.5	11 (47.8)	22 (57.9)	0.597
%GTN	17.2 (2.8 – 44)	19.3 (4.9 – 36.6)	0.323

Figure 8.24 Percentage FMD in APL antibody positive and negative patients:

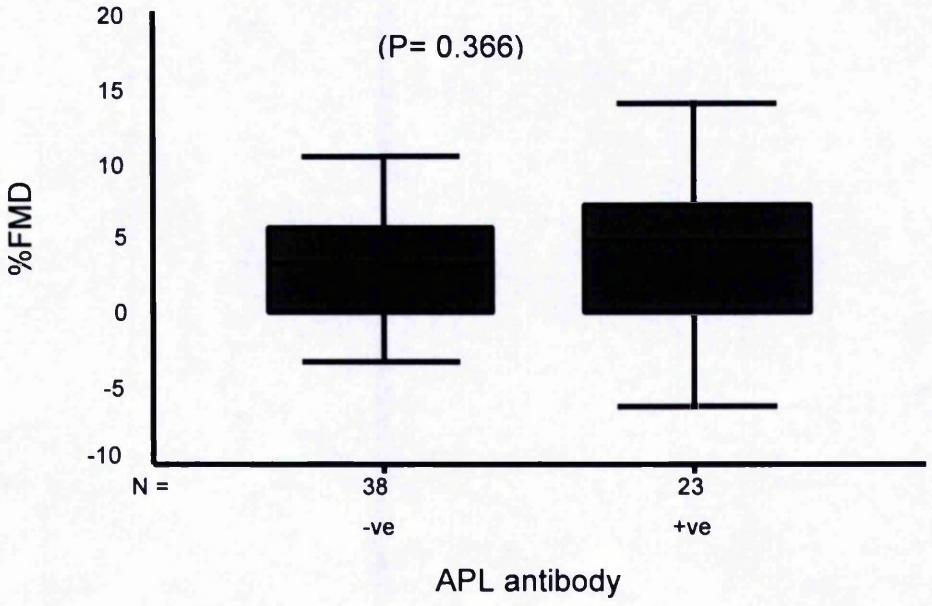
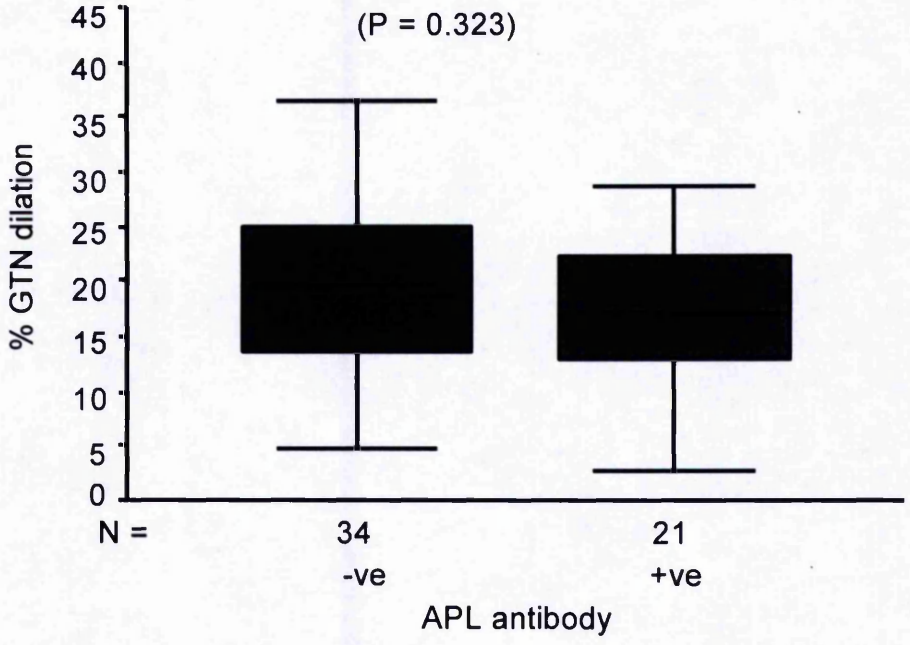


Figure 8.25 Percentage GTN dilation in APL antibody positive and negative patients:



8.8.3 Raynaud's phenomenon:

Thirty-four patients had a history of Raynaud's phenomenon with a 2 or 3 phase colour change in response to cold stress. Table 8.21 shows a comparison of patients with and without history of Raynaud's. There was no significant difference between those with and without a history of Raynaud's in resting diameter or percentage FMD and percentage GTN dilation (Figures 8.26, 8.27). There was also no difference in proportions of patients with %FMD ≤ 4.5% between the two groups (Table 8.22).

Table 8.21 Demographic, classic risk factors and metabolic factors in patients with and without history of Raynaud's:

	Raynaud's +ve n = 34 Median (range)	Raynaud's - ve n = 27 Median (range)	P value
Age/years	47 (21 – 73)	50 – 66)	0.833
Menopause Pre-menopausal Post-menopausal	 17 (50) 17 (50)	 15 (55.6) 12 (44.4)	0.797
BMI	24.5 (18.6 – 35.4)	26 (21.3 – 41.9)	0.016
Hypertension	11 (32.4)	10 (37.0)	0.789
SBP mmHg	122 (90 – 170)	120 (90 – 160)	0.942
DBP mmHg	78 (60 – 100)	80 (40 – 98)	0.912
Diabetes mellitus	2 (5.9)	1 (3.7)	1.000
Fasting glucose mmol/l	4.5 (3.6 – 10.9)	4.6 (3.8 – 10.2)	0.282
Smoking: current smokers ex-smokers non-smokers	 2 (5.9) 7 (20.6) 25 (73.5)	 1 (3.7) 5 (18.5) 21 (77.8)	1.000
TC mmol/l	5.2 (3.0 – 7.5)	5.0 (3.2 – 8.7)	0.379
LDL-C mmol/l	2.7 (0.33 – 4.2)	2.5 (3.21 – 8.7)	0.934
HDL-C mmol/l	1.5 (0.6 – 2.7)	1.4 (0.8 – 3.0)	0.230
10-year risk of CHD	3.5 (0.0 – 13.8)	5.3 (0.0 – 15.8)	0.601
TGs mmol/l	1.3 (0.4 – 3.2)	1.2 (0.4 – 6.9)	0.407
VLDL-C mmol/l	0.24 (0.06 – 0.83)	0.20 (0.06 – 1.0)	0.753
Ox-LDL-C mU/l	30.7 (12.9 – 76.8)	33.2 (14 – 71.5)	0.622
Paroxonase activity	249.9 (22 – 564)	166.4 (44 – 426)	0.160
Lp(a) mg/dl	15.9 (0.5 – 139)	13.9 (1.0 – 129.6)	0.257

Table 8.22 Comparison of vascular study on patients with and without history of Raynaud's phenomenon:

	History of Raynaud's		
	Yes (n =34)	NO (n =27)	p value
Resting diameter /cm	0.31(0.24 – 0.42)	0.33(0.25 – 0.43)	0.295
FMD (absolute)/cm	0.01(- 0.01 – 0.03)	0.01(- 0.02 – 0.04)	0.940
%FMD	3.6(-3.3 – 11.5)	4.7(- 6.3 – 13.7)	0.930
N (%) with FMD ≤ 4.5%	14(41.2)	14(52)	0.447
%GTN dilation	18(2.8 – 44)	17.6(4.7 – 36.6)	0.890

Figure 8.26 Resting diameter in patients with and without Raynaud's:

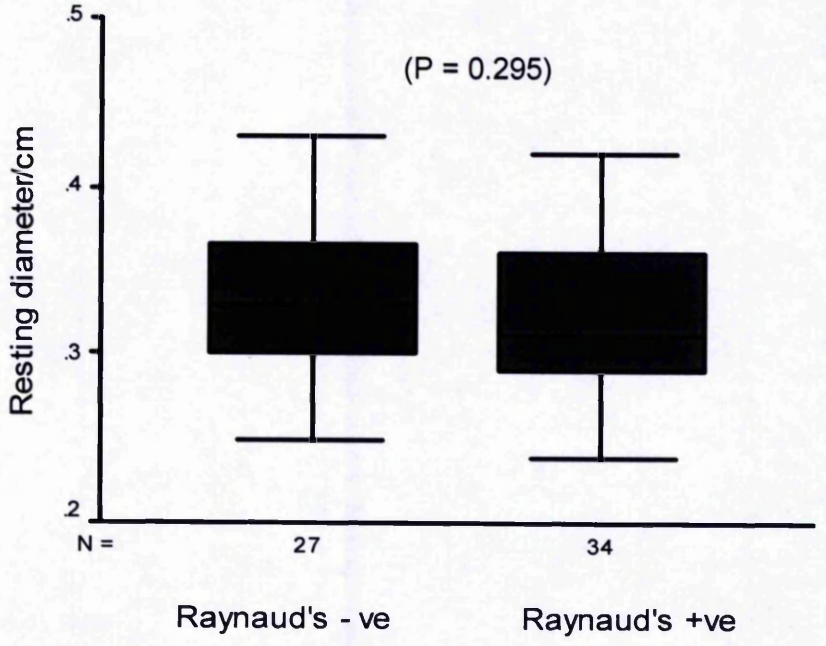
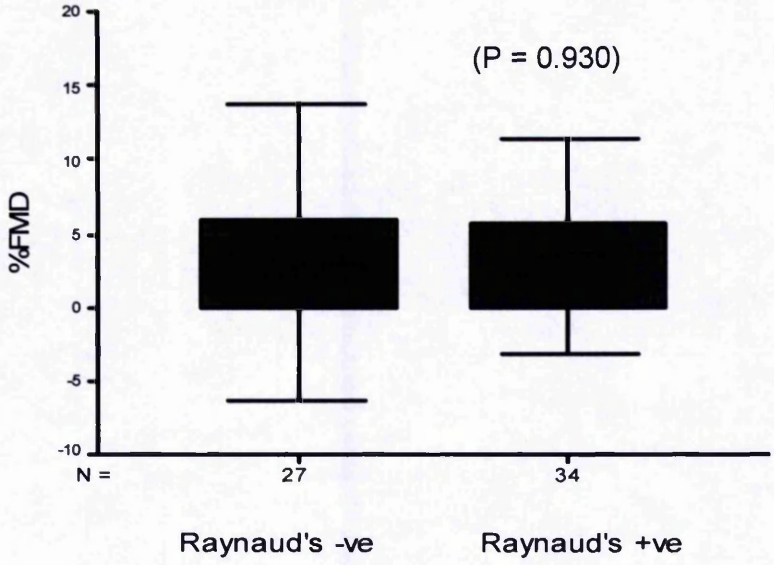


Figure 8.27 Percentage FMD in patients with and without Raynaud's:



8.9 Influence of atherosclerosis risk factors, %10-year risk of CHD, carotid intima media thickness and presence of carotid plaques on %FMD:

In this study the key measure of atherosclerosis was carotid IMT. In addition we assessed the presence of carotid atherosclerotic plaques as well as percentage 10-year risk of CHD.

Carotid IMT showed a positive correlation with resting diameter [r = 0.425, P = 0.001] as well as a negative correlation with percentage FMD (r = - 0.367, P= 0.003) (Figure 8.28) and percentage GTN dilation (r = - 0.440, P = 0.001) carotid IMT also correlated with age and SBP (r = 0.574, P < 0.001) and (r = 0.547, P < 0.001) respectively. Carotid IMT showed a tendency for a positive correlation with hs-CRP (r = 0.230, P = 0.072).

Carotid artery plaques were detected in 12 (19.4%) patients and four of whom were of age <50 years. Those with plaques had significantly higher resting diameter and lower percentage GTN dilation. There was also a trend to lower percentage FMD in those with carotid plaques (Table 8.23). The 10-year risk of CHD in SLE patients showed a positive correlation with resting diameter (r = 0.270, P = 0.036) and a negative correlation with percentage GTN dilation [-0.315, P = 0.019] but did not correlate with percentage FMD (r = 0.047, P =

0.719). The 10-year risk correlated with intima media thickness (r = 0.515, P < 0.001) and tended to correlate with hs-CRP (r = 0.231, P = 0.074).

There was no correlation between percentage FMD and the novel risk factors TGs, VLDL-C, ox-LDL-C, Lp(a) and paroxonase activity (Table 8.24). However, there was a negative correlation of resting diameter with TGs (r = - 0.305, P =0.017) and paroxonase activity (r = - 272, p = 0.008).

Figure 8.28 Relationship between carotid IMT and percentage FMD:

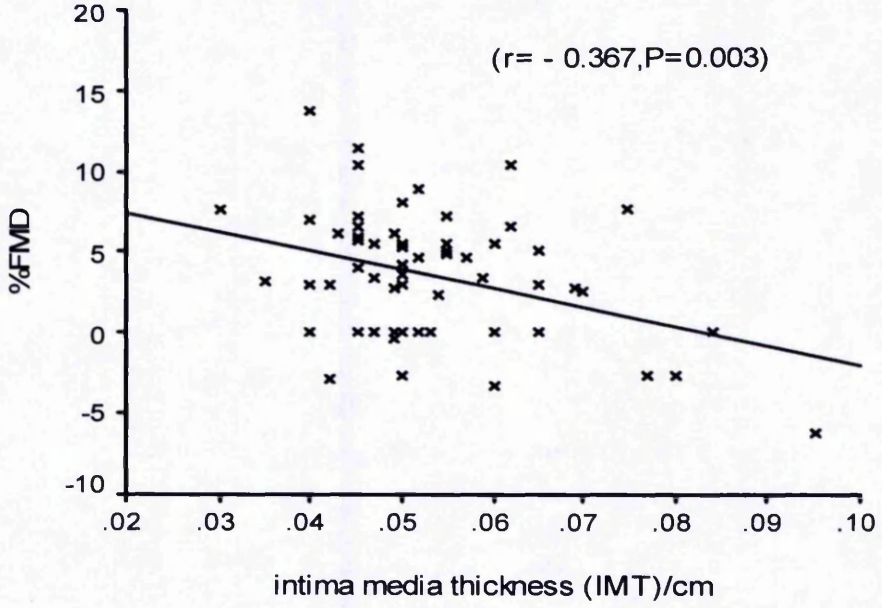


Table 8.23 Comparison of patients with and without carotid plaques:

	Carotid plaque +ve (n = 12)	Carotid plaque -ve (n = 50)	P value
Age/years	58.5 (41 – 67)	49 (21 – 73)	**0.005**
Resting diameter/cm	0.36 (0.28 – 0.43)	0.31 (0.24 – 0.39)	**0.014**
FMD/cm (absolute)	0.01 (- 0.02 – 0.02)	0.01 (- 0.01 – 0.04)	0.323
%FMD	2.7 (- 6.3 – 7.1)	4.2 (- 3.3 – 13.7)	0.096
%GTN dilation	11.9 (4.7 – 25.8)	19.4 (2.8 – 44)	**0.014**
%FMD<4.5% n(%)	8 (66.7)	25 (50%)	0.518

Table 8.24 Correlations of percentage FMD with novel risk factors:

	r	P-value
TGs	0.115	0.380
VLDL-C	0.089	0.515
Ox-LDL-C	0.124	0.342
Lp(a)	- 0.150	0.249
Paroxonase activity	0.051	0.700

We used linear regression analysis to assess the influence of different factors on percentage FMD within SLE patients. Table 8.26 shows univariate regression of different variables with percentage FMD as a dependent variable. In addition to our key factors we included SLEDAI because of the correlation reported earlier in the disease activity analysis. Although current steroid dose did not look significant in the disease activity analysis, we included it in this section to rule out any association. In univariate analysis factors associated with percentage FMD were resting diameter, SBP, SLEDAI and carotid IMT (Table 8.32). For multiple regression analysis, we entered into the model those variables, which showed a p-value of < 0.2 in univariate regression. These variables were resting diameter, carotid IMT, SBP, plaque, disease activity, SLEDAI, hypocomplementaemia, fasting glucose, LDL-C and BMI.

In stepwise multiple regression analysis, carotid IMT was independently associated with percentage FMD (P = 0.026). Each millimeter increase in IMT is associated with 0.92% decrease in percentage FMD. Table 8.25 summarizes the regression model.

Table 8.25 Stepwise multiple regression analysis for variables associated with %FMD:

Predictors in the model	B*	Beta**	95%CI of B	P value
Intima media thickness	- 92.3	- 0.305	(- 173.2 – - 11.3)	**0.026**

Excluded variables	Beta	P-value
Resting diameter	- 0.187	0.177
Systolic pressure	- 0.190	0.278
Carotid plaque	- 0.126	0.375
BMI	0.237	0.080
Fasting glucose	0.038	0.781
LDL	0.239	0.081
SLEDAI	0.209	0.173
Disease activity	0.127	0.354
Complement levels	0.216	0.114

* B = Unstandardized regression coefficient.
  - After adjustment for other variables each 0.1mm increase in IMT is associated with a 0.92% decrease in %FMD.

** Beta = Standaradized coefficient

Multiple coefficient of determination of this mode (R^2) = 0.093, P = 0.026.

Table 8.26 Associations of separate variables with %FMD in SLE using linear regression model:

Independent variables	R^2 *** value	B**	p	95%CI of B
Resting diameter	0.096	- 26.2	**0.014**	- 46.9 – - 5.4
Age	0.004	0.020	0.640	- 0.10 – 0.064
Ethnicity *	0.005	0.69	0.584	- 1.8 – 3.2
Menopause	0.007	0.656	0.511	- 1.30 – 2.60
Smoking	0.009	0.837	0.472	- 3.10 – 1.50
BMI	0.044	0.171	***0.103***	- 0.036 – 0.38
Waist/hip ratio	0.000	- 0.937	0.910	- 17.4 – 15.5
FBG	0.052	0.682	***0.084***	- 0.094 – 1.46
Hypertension	0.013	- 0.914	0.385	- 3.00 – 1.17
SBP	0.083	- 0.060	**0.024**	- 0.11 – - 0.08
DBP	0.002	- 0.015	0.711	- 0.09 – 0.065
Total cholesterol	0.008	0.301	0.492	- 0.569 – 1.17
LDL-C	0.039	0.713	***0.142***	- 0.25 – 1.67
HDL-C	0.022	- 1.17	0.256	- 3.20 – 0.871
Triglycerides	0.005	0.390	0.574	- 0.99 – 1.77
VLDL-C	0.010	- 1.18	0.454	- 2.9 – 6.50
Lipoprotein (a)	0.011	- 0.125	0.419	- 0.043 – 0.018
Paroxonase activity	0.000	1.86	0.879	- 0.007 – 0.009
Ox-LDL-C	0.010	0.025	0.438	- 0.043 – 0.018
Disease activity	0.050	1.81	***0.084***	- 0.25 – 3.87
SLEDAI	0.139	0.519	**0.003**	0.183 – 0.855
Hypcomplementaemia	0.049	1.86	***0.095***	- 0.33 – 4.10
Current steroid dose	0.023	0.088	0.244	- 0.06 – 0.240
Raynaud's	0.006	0.613	0.543	- 1.40 – 2.60
APL antibodies	0.013	0.900	0.400	- 1.20 – 3.00
%10-y risk of CHD	0.022	- 0.14	0.254	- 0.39 – 0.11
IMT	0.135	- 119	**0.003**	- 197 – - 41.1
Plaques	0.032	- 1.74	***0.165***	- 4.20 – 0.74

* White Caucasian or Non-white Caucasians

** B = Unstandardized regression coefficient:

- Each mm decrease in resting diameter is associated with a 0.965 decrease in %FMD.
- Each 10 mmHg increase in SBP is associated with a 0.835 decrease in %FMD..
- Each in increase in SLEDA by 1 unit is asscoited with a 0.139% higher %FMD.
- Each 0.1mm increase in IMT is associated with a 0.135% lower FMD%.

*** R^2 Coefficient of determination.

8.10 Summary:

Validation of our technique showed:

- Good reproducibility for resting diameter.
- Intra-class correlation coefficient for intra-observer variability for resting diameter and percentage FMD is similar to that reported in other studies.
- There is poor interobserver variability that precludes the use of multiple observers in reading the scans.

Endothelial dysfunction:

- SLE patients have evidence of impaired endothelial function compared to healthy controls.
- Classic risk factors, particularly systolic blood pressure, contribute to endothelial dysfunction.
- SLE is an independent predictor of endothelial dysfunction.

Within SLE:

- None of the disease associated factors were independently associated with endothelial dysfunction.
- Carotid IMT was the only independent predictor of endothelial dysfunction.

Chapter 9

9 Insulin Study

9.1 Introduction:

As discussed in the chapter on novel risk factors for CHD, hyperinsulinemia and insulin resistance are known risk factors for CHD in the general population. Adipose tissue is an important source of inflammatory cytokines. Increased adiposity is associated with increased basal inflammation and increased oxidant stress in the general population, and obesity is one of the components of the metabolic syndrome. SLE is an immuo-inflammatory disease associated with increased levels of inflammatory cytokines, which may also reduce insulin sensitivity and contribute further to the development of the metabolic syndrome. However, the prevalence, associations and the potential role of the metabolic syndrome in the development of premature CHD in SLE have not been explored.

9.1.1 Hypothesis:

SLE patients have decreased insulin sensitivity that is associated with the lipid profile abnormality of high TGs and low HDL-C frequently described in these patients.

9.1.2 Aim:

To measure insulin sensitivity and beta cell function in Caucasian SLE patients and in matched community controls.

9.1.3 Patients:

For this study we used consecutive women with SLE from our Lupus Clinic who had ≥ 4 of the revised 1997 ACR criteria (Hochberg 1997) for diagnosis of SLE. Women with three 3 criteria and no alternative diagnosis were also included (n=2). We excluded patients on any drugs that might affect glucose homeostasis other than steroid therapy. Of note we excluded patients on anti-malarial drugs. Because of the effect of the AM's on insulin action (prolongation

of the half-life of the active insulin-insulin receptor complex) and blood glucose, concerns were raised about the validity of the use of HOMA models in patients taking anti-malarial therapy (HCQ or chloroquine phosphate).

9.1.4 Controls:

Two sources of controls were employed and from this pool of controls we matched two healthy controls to each SLE case. Controls were matched for age within five years of their cases. The recruited controls were:

- Healthy Caucasian women from the secretarial and nursing staff at Manchester Royal Infirmary, and from the ARC Epidemiology Research Unit staff. These controls represent part of the subjects recruited for our concurrent study of endothelial function and CHD risk factors in SLE. All controls were free of CHD. One patient was on anti-hypertensive treatment (ACE inhibitors).
- Healthy Caucasian women from a recently completed cardiovascular study undertaken at Manchester Royal Infirmary. They were recruited from three different sources within Greater Manchester area; an agriculture machinery factory, a computer design company and a detergent factory. They had no history of any medical conditions, including diabetes and hypertension, no history of CHD and they were on no medications.

9.2 Methods:

The patients' assessment was summarized in Chapter 7. Plasma was separated and stores at –20°C until analysis. An aliquot of the fasting plasma sample was used to measure insulin. Fasting glucose was measured in the hospital laboratory using the glucose oxidase method, on the day of blood collection.

9.2.1 Measurement of plasma insulin:

Insulin was measured by sensitive delayed addition radioimmunoassay, a specifically modified method to enhance assay sensitivity in the normal range of 1.0 – 10mU/L. This assay has a lower limit of detection of 0.38 mU/L.

1. Aliquots of samples (100μl) were reacted with 100μl of anti-insulin antibody solution (polyclonal anti-porcine insulin raised in guinea pig (Diagnostics Scotland, Carluke, Scotland) and incubated overnight at 4.0 °C
2. After 24 hours, 100μl of radiolabelled insulin (^{125}I-Insulin; Biosource Europe from Qbiogene, Livingstone, Scotland) was added to each assay tube and the mixture incubated for a further 24 hours.
3. Bound and free insulin was then separated using the charcoal separation technique; the charcoal binds to free insulin and does not bind to antibody-bound insulin. Insulin in the sample competes with the labelled insulin for binding sites on the antibody. Thus, with more insulin in the sample more free radiolabelled insulin will be picked up by the charcoal and will give a higher rate on a gamma counter.
4. The amount of insulin in the sample was then calculated by comparison with a standard curve of human insulin, made up of a range of standards in confirmed hormone free plasma, and assayed in the same technique described above (WHO 1st international reference preparation).

The performance and reproducibility of the assay was monitored by the inclusion of quality control material, both internal and external (UKNEQAS), in every assay. The lower limit of detection using this assay was calculated to be less than 0.4 mU/L. Using this assay, the mean (SD) fasting plasma insulin level in a reference range of 154 healthy hospital employees was 5.06 (2.43) mU/L with a normal of 0.5 – 10.0 mU/L (Laing *et al* 1998).

9.2.2 Homeostasis Model Assessment:

Homeostasis Model assessment (HOMA) is a simple arithmetic way of deriving indices of pancreatic endocrine function (beta cell function HOMA-B) and peripheral tissue insulin sensitivity (HOMA-S) from fasting plasma samples (Matthews *et al* 1985). This model assumes that plasma glucose and insulin in the fasting state are controlled by a feed back loop between the pancreas, liver and insulin-sensitive and insulin-insensitive peripheral tissues. Differences between individuals can thus be expressed in terms of relative β-cell response to glucose and in terms of hepatic and peripheral sensitivity to insulin and

glucose. HOMA correlates well with, and is validated against, the gold standard methods of assessment of these functions, such as the euglycemic hyperinsulinaemic clamp technique (Bonora *et al* 2000, Hermans *et al* 1999).

9.2.3 Calculation of β-cell function and insulin sensitivity:

Paired fasting insulin and glucose were transformed using a mathematical model of glucose/insulin feedback system (HOMA) to give insulin sensitivity (HOMA-S) and pancreatic β-cell function (HOMA-B):

HOMA-S = 22.5 / [insulin (mU/l) × glucose (momol/l)]

HOMA-B = [20 × insulin (mU/l)] / [glucose (mmol/l) – 3.5)]

In an ideal reference group of healthy subjects HOMA-B and HOMA-S are maximum at 100% and 1 (arbitrary units) respectively.

9.3 Results

We studied 27 women with SLE and 50 healthy controls matched within five years of age, the median (range) age was 52 (26 – 67) and 47 (25 – 62) years respectively.

9.3.1 Disease features in the SLE patients group:

The median (range) disease duration of SLE patients was eight (1 – 32) years. Disease activity was low in this group of patients with median (range) SLEDAI of two (0 – 8). Ten (37%) patients had a SLEDAI score of 0.0 and only three (11%) patients had a score of ≥ 6. Ten (37%) patients were on steroid therapy of whom four were on a current dose of ≥ 10 mg/day. Six patients had a BILAG A or B category. Using our criteria for active disease described in chapter 7, eight (30%) patients had active or moderately active disease (Table 9.1).

9.3.2 Metabolic and inflammatory markers in SLE patients and healthy controls:

BMI did not differ between SLE patients and healthy controls. Although the waist circumference in SLE patients and healthy controls were comparable, waist/hip ratio tended to be higher in SLE patients (Table 9.2).

Compared to controls, SLE patients had significantly higher TGs and lower HDL-C levels. SBP and 10-year risk of CHD were also significantly higher in patients compared to controls (Table 9.2). There was a trend for hs-CRP to be higher in SLE patients compared to healthy controls. IL-6 levels were measured in 20 SLE patients and 27 healthy controls and were significantly higher in patients group. Ox-LDL-C did not differ between the groups (Table 9.2).

Table 9.1 Features of SLE patients not on anti-malarial therapy

Disease features	SLE patients n = 27 Median (range)
Age /years	52 (26 – 67)
Disease duration /years	8.0 (1 – 32)
Post-menopause n (%)	15 (56)
SLEDAI	2.0 (0 – 8)
Active disease n (%)	8 (30)
Current steroid dose treatment n (%)	10 (37)
Steroid dose ≥10 mg/day n (%)	4 (15)
Mean steroid dose in the last 6 months (mg/day)	0.0 (0.0 – 12)
Total steroid duration /months	16 (0 – 372)

9.3.3 Comparison of insulin parameters in SLE patients and healthy controls:

The FBG was similar in SLE patients and controls (Table 9.3). SLE patients had significantly higher fasting insulin (Figure 9.1, Table 9.3). Insulin sensitivity (HOMA-S) was significantly lower and β-cell function (HOMA-B) was significantly higher in SLE patients compared to healthy controls (Table 9.3 and Figures 9.2, 9.3).

Table 9.2 Traditional risk factors for CHD, lipid profile and inflammatory markers in SLE patients and healthy controls:

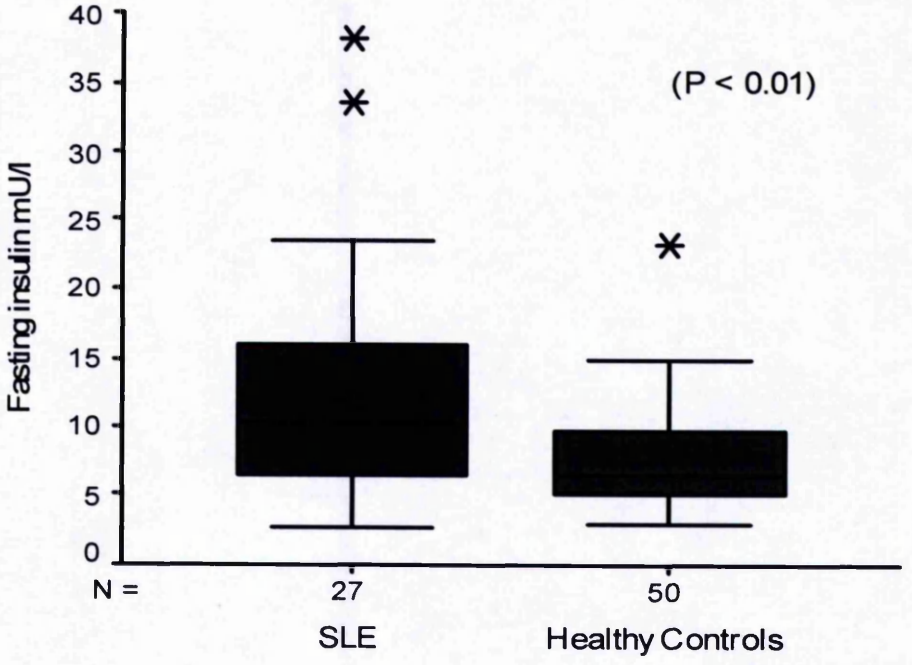
CHD risk factors	Women with SLE n = 27 Median (range)	Healthy women n = 50 Median (range)	P value
BMI	26.5 (18.6 – 41.9)	24.6 (17.1 – 36.2)	0.477
Waist/cm	80 (66 – 107)	80 (58 – 107)	0.687
Waist/hip ratio	0.80 (0.69 – 0.96)	0.76 (0.68 – 0.89)	0.085
TC mmol/l	5.3 (3.3 – 8.7)	5.1 (2.6 – 7.2)	0.514
HDL-C mmol/l	1.4 (0.8 – 2.6)	1.7 (1.0 – 3.0)	**0.003**
SBP mmHg	124 (96 – 160)	112 (90 – 140)	**0.006**
DBP mmHg	80 (56 – 98)	70 (50 – 100)	0.126
10-year risk of CHD %	5.7 (0 – 15.8)	1.4 (0 – 10.9)	**0.001**
TGs mmol/l	1.2 (0.7 – 3.9)	0.9 (0.4 – 3.1)	**0.008**
LDL-C mmol/l	2.8 (0.45 – 5.0)	2.6 (0.64 – 4.1)	0.212
VLDL-C mmol/l	0.22 (0.07 – 1.0)	0.17 (0.01 – 2.0)	0.325
hs-CRP mg/l	2.85 (0.36 – 22)	1.5 (0.17 – 22.5)	0.066
IL-6 pg/ml	2.3 (1.0 – 10)	1.0 (1.0 – 14.7)	**0.002**
Ox-LDL-C mU/l	36.1 (13 – 76.8)	35.8 (10.8 – 67.5)	0.386

Table 9.3 Comparison of fasting insulin and related parameters in SLE patients and healthy controls:

	Women with SLE n = 27 Median (range)	Healthy women n = 50 Median (range)	P-value
Fasting glucose mmol/L	4.7 (3.8 – 10.9)	4.7 (3.8 – 10.2)	0.757
Fasting insulin mU/L	10.2 (2.8 – 38)	6.6 (3.1 – 26.4)	**0.008**
HOMA-S*	0.45 (0.09 – 1.9)	0.73 (0.16 – 1.3)	**0.006**
HOMA-B**	185 (10.3 – 1566)	112.7 (28.2 – 653.3)	**0.018**

* HOMA-S = Insulin sensitivity. ** HOMA-B = Pancreatic β-cell function.

Figure 9.1 Fasting insulin levels in SLE patients and healthy controls:



Box plots diagram: The boxes represent interquartile range (IQR) and the horizontal line inside the box is the median value. The whiskers extend to the lowest value within 1.5 times IQR below the first quartile and the highest value within 1.5 times IQR above the third quartile. Points below and above the whiskers represent outlayier values. (SLE patients different from controls, Mann-Whitney test, p< 0.01).

Figure 9.2 Insulin sensitivity (HOMA-S) in SLE patients and healthy controls:

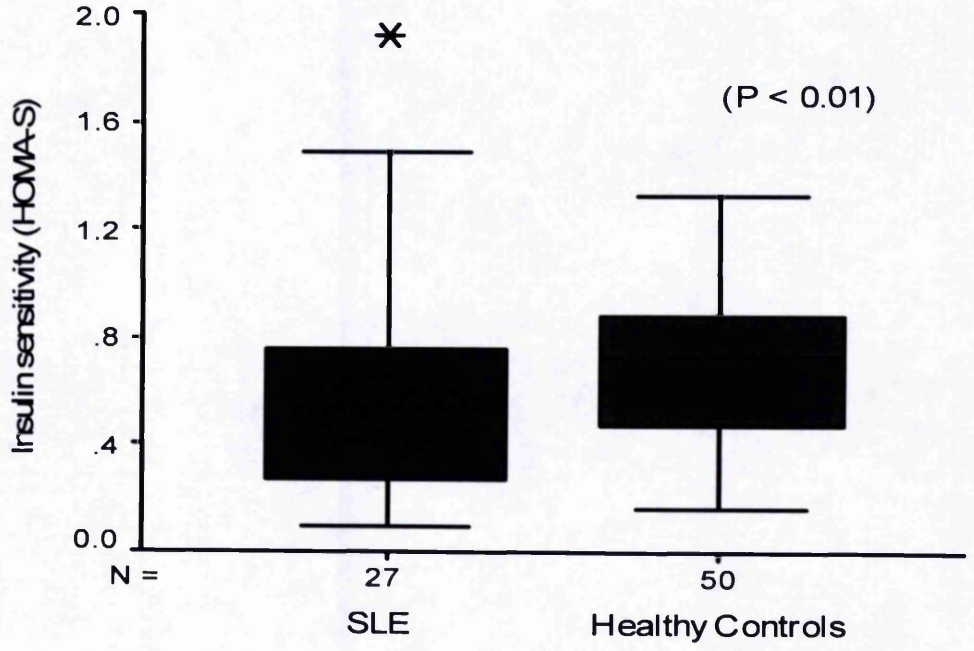
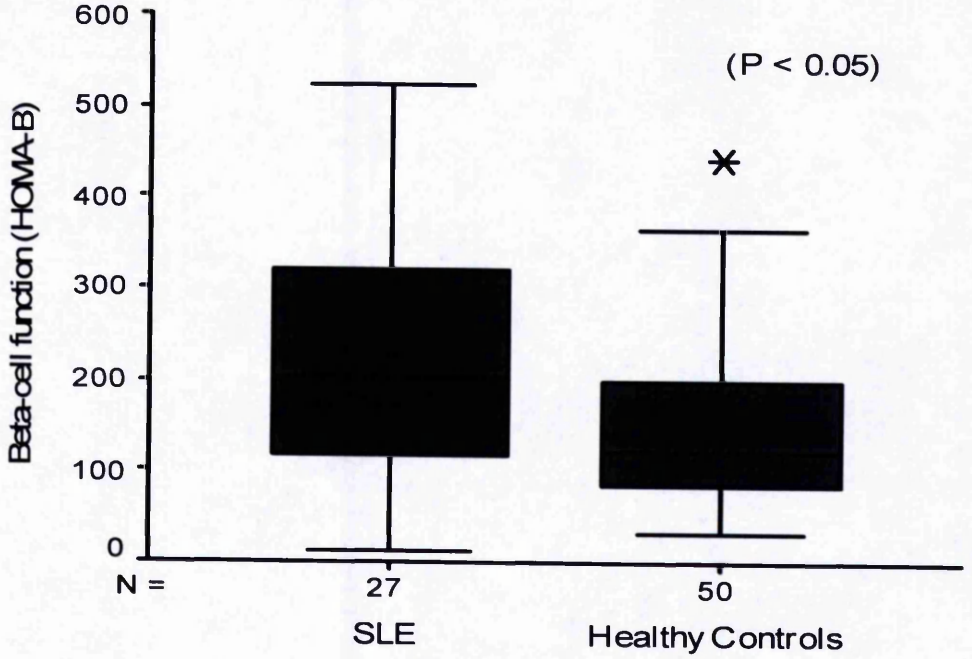


Figure 9.3 Pancreatic β-cell function (HOMA-B) in SLE patients and healthy controls:



9.3.4 Factors which influence fasting insulin in SLE patients:

In our group of 27 patients we examined factors associated with fasting insulin. Firstly with regard to steroid therapy, there was only a weak association between insulin and current or recent steroid dose (Table 9.4).

Similarly, there was no association with disease activity either using our categorical definition [median (range) 15 (6 – 17) vs 8.9 (2.8 – 38), P = 0.549], nor with SLEDAI as a continuous variable (Table 9.4).

In contrast, we found significant association with other metabolic parameters in this population (Table 9.5). In particular fasting insulin showed strong correlation with BMI (r = 0.518, P= 0.006) and other anthropomorphic measures such as waist circumference and waist/hip ratio (Table 9.5). There was also a strong correlation with TGs (r = 0.621, P =0.001) and HDL-C ((r = - 0.501, P = 0.008). The association with other lipid parameters was less pronounced (Table 9.5).

Interestingly, we also found a strong association with ox-LDL-C (r = 0.516, P = 0.006) but no association with hs-CRP or IL-6 levels (Figures 9.4 – 9.6).

Table 9.4 Correlations of fasting insulin with parameters of steroid therapy and disease activity:

	Fasting insulin	
	r	P value
Current steroid mg/d	0.204	0.308
Average steroid dose in last 6 months	0.230	0.248
Total steroid duration /months	0.043	0.830
SLEDAI	0.056	0.781

9.3.5 Correlations of fasting insulin in healthy controls:

In healthy controls, there was similar association with anthropomorphic measures especially BMI and waist circumference. Again, there were similar correlations with TGs (r = 0.443, P = 0.007) and HDL-C (r = -0.330, P = 0.05).

Table 9.5 Correlations of insulin in SLE patients and healthy controls:

	SLE patients		Healthy controls	
	r	P value	r	P value
Age/ years	0.055	0.784	0.028	0.849
BMI	0.518	**0.006**	0.538	**< 0.001**
Waist/ cm	0.389	**0.045**	0.557	**< 0.001**
Waist/hip ratio	0.406	**0.036**	0.357	**0.033**
Fasting glucose	0.197	0.325	0.165	0.259
HOMA-S	- 0.907	**< 0.001**	- 0.953	**< 0.001**
HOMA-B	0.746	**< 0.001**	0.774	**< 0.001**
TGs	0.621	**0.001**	0.443	**0.007**
TC	0.035	0.862	0.263	0.122
HDL-C	- 0.501	**0.008**	- 0.330	**0.050**
LDL-C	0.394	0.103	0.175	0.330
VLDL-C	0.369	0.083	0.264	0.138
Hs-CRP	0.279	0.159	0.387	**0.020**
IL-6	0.044	0.853	0.426	**0.027**
Ox-LDL-C	0.516	**0.006**	- 0.043	0.808

In controls group, the strength of association of insulin with hs-CRP was similar to the SLE patients, but reached statistical significance (Table 9.5). In contrast to SLE patients however, there was no correlation between insulin and ox-LDL-C, but there was a significant correlation with IL-6 levels (r = 0.426, P = 0.027). To explore this, we examined SLE patients further:

In SLE patients, IL-6 levels showed no correlation with TGs but a weak correlation with HDL-C (r = -0.376, P = 0.102). In contrast, IL-6 in healthy controls strongly correlated with TGs and HDL-C (r = 0.548, P = 0.003) and (r = - 0.503, P = 0.007) respectively (Table 9.6)

Table 9.6 Correlations of IL-6 in SLE patients and controls:

	SLE (n = 20)		Controls (n = 27)	
	r	P- value	r	P- value
SLEDAI	- 0.162	0.495		
BMI	0.431	0.058	0.571	**0.002**
Waist	0.328	0.313	0.589	**0.001**
W/hip ratio	0.036	0.881	0.252	0.206
TGs	- 0.088	0.712	0.548	**0.003**
HDL-C	- 0.376	0.102	- 0.503	**0.007**
Hs-CRP	0.744	**< 0.001**	0.538	**0.004**

Figure 9.4 Scatter plot of fasting insulin and hs-CRP in SLE patients and controls:

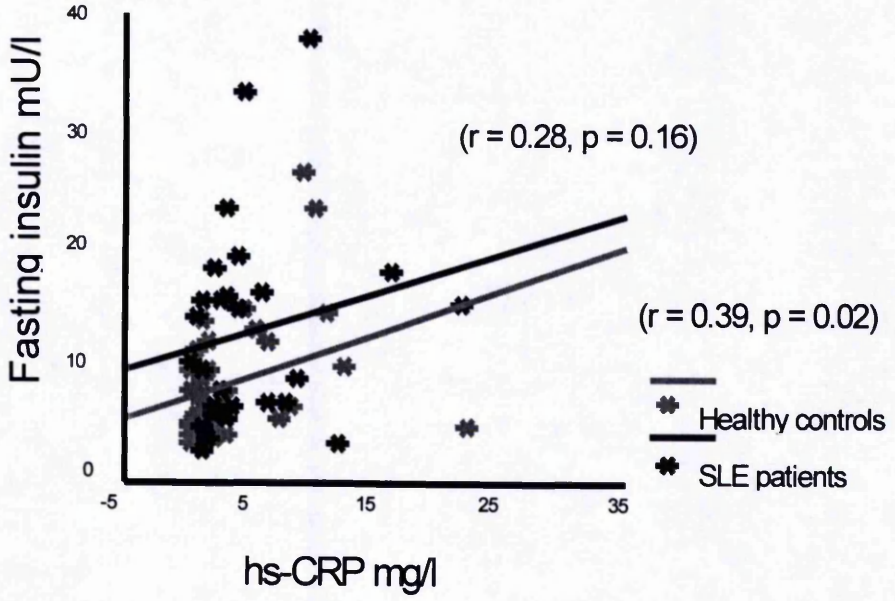


Figure 9.5 Scatter plot of fasting insulin and ox-LDL-C in SLE patients and controls:

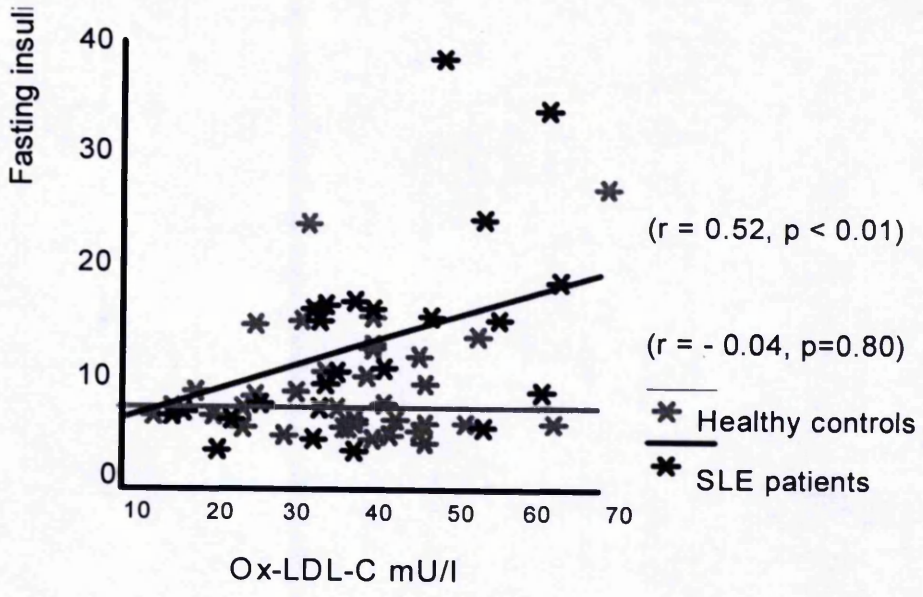
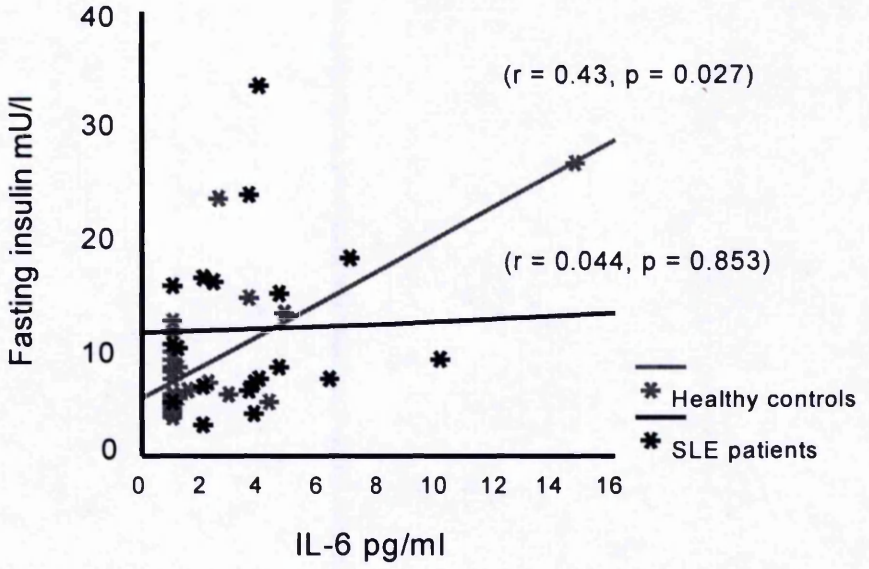


Figure 9.6 Scatter plot of fasting insulin and IL-6 in SLE patients and controls:



9.3.6 Effect of steroid therapy on homeostasis model assessment:

Although we found no correlation between fasting insulin levels and several parameters of steroid therapy, we were interested to explore further the effect of steroid therapy by comparing patients on or not on steroids at the time of study. As can be seen in table 9.7, fasting glucose was comparable in both groups. There was a trend towards higher fasting insulin and lower HOMA-S in those on steroids, although the wide range limited statistical power (Figures 9.7 – 9.9). However, even after adjusting for the effect of steroid therapy, fasting insulin levels remained higher and insulin sensitivity lower in patients compared to healthy controls [median (range) 8.9 (2.8 – 38) vs 6.6 (3.1 – 26.4) mU/l, P = 0.110] and [0.55 (0.09 – 1.9) vs 0.73 (0.16 – 1.3), P = 0.051] respectively.

We also compared other metabolic and inflammatory parameters according to steroid use. Again, although the numbers were small, steroid treated patients tended to have higher TGs, VLDL-C. In contrast, IL-6 levels tended to be lower in steroid treated patients (Table 9.8). Fasting insulin showed the same direction and strength of correlation with inflammatory markers in SLE patients on and not on steroids (Table 9.9).

Table 9.7 Comparison of insulin parameters in SLE patients on and not on steroids:

	On steroid n = 10	Not on steroids n = 17	P Value
Waist/hip ratio	0.81 (0.71 – 0.87)	0.80 (0.69 – 0.96)	0.902
FBG mmo/l	4.7 (3.8 – 10.2)	4.9 (4.0 – 10.9)	0.264
Insulin mU/l	15.5 (4.8 – 23.5)	8.9 (2.8 – 38)	0.238
HOMA-S	0.30 (0.15 – 1.0)	0.55 (0.09 – 1.9)	0.473
HOMA-B	275 (43 – 1567)	144 (10.3 – 477)	0.141

Table 9.8 Comparison of metabolic parameters in SLE patients on and not on steroids:

	On steroid n = 10	Not on steroids n = 17	P Value
TGs mmol/l	1.5 (0.7 – 3.9)	1.1 (0.7 – 2.8)	0.155
VLDL-C mmol/l	0.33 (0.1 – 1.0)	0.20 (0.07 – 0.83)	0.169
CRP mg/l	2.6 (0.76 – 7.8)	3.6 (0.36 – 22)	0.286
IL-6 pg/ml	1.5 (1.0 – 6.3)	3.8 (1.0 – 10)	0.082
Ox-LDL-C mU/l	34.7 (20.6 – 71.5)	36.1 (12.9 – 76.8)	0.749

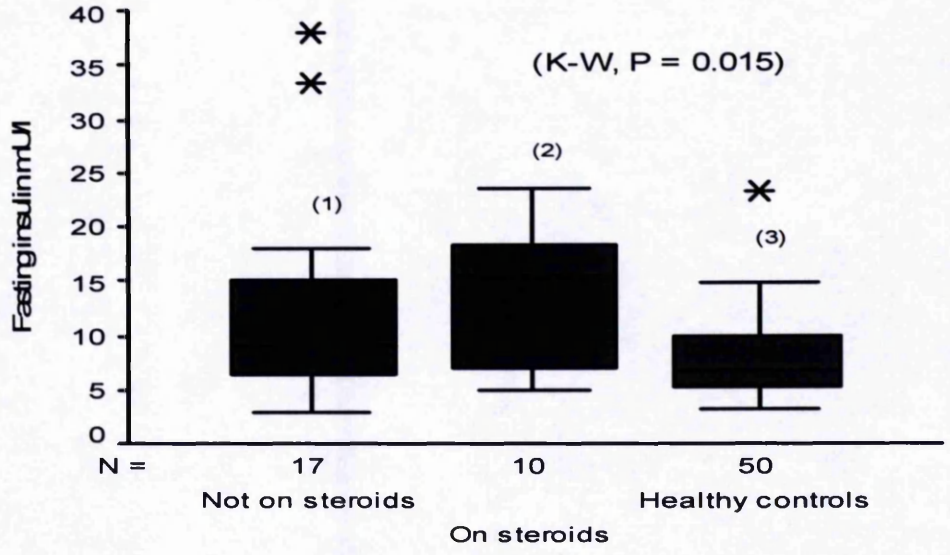
Table 9.9 Correlations of fasting insulin with inflammatory parameters in SLE patients on and not on steroids:

	On steroids n = 10		Not on steroids n = 17	
	r	P value	r	P value
Hs-CRP	0.322	0.364	0.419	0.094
IL-6	0.277	0.595	0.287	0.320
Ox-LDL-C	0.456	0.185	0.605	**0.010**
TGs	0.627	0.050	0.605	**0.010**
HDL-C	-0.477	0.085	-0.605	**0.010**

Table 9.10 Correlations of IL-6 with inflammatory parameters in SLE patients on and not on steroids:

	On steroids n = 6		Not on steroids n = 14	
	r	P value	r	P value
Waist/hip ratio	- 0.031	0.954	0.319	0.267
TGs	- 0.123	0.816	0.411	0.144
HDL-C	- 0.880	**0.021**	- 0.477	0.085
Hs-CRP	0.880	**0.021**	0.776	**0.001**
Ox-LDL-C	- 0.395	0.085	0.434	0.121
SLEDAI	- 0.092	0.862	- 0.056	0.850

Figure 9.7 Fasting insulin in patients on and not on steroid therapy and in healthy controls:

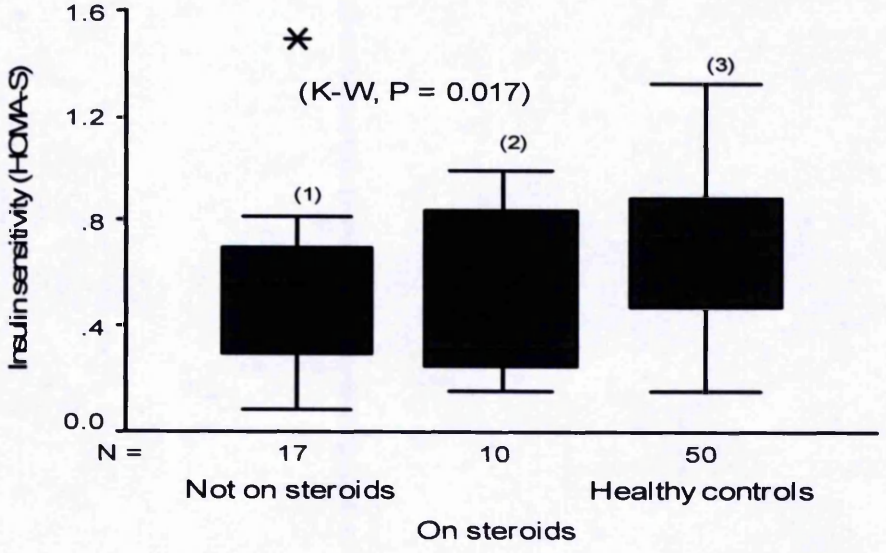


K-W : Kruskal-Wallis test of significance across groups.

The difference between the three groups (1), (2) and (3) using Mann-whitney test:

[(1) vs (2), P = 0.238], [(2) vs (3), P = **0.007**], [(1) vs (3), P = **0.110**]

Figure 9.8 Insulin sensitivity in patients on and not on steroid therapy and in healthy controls:

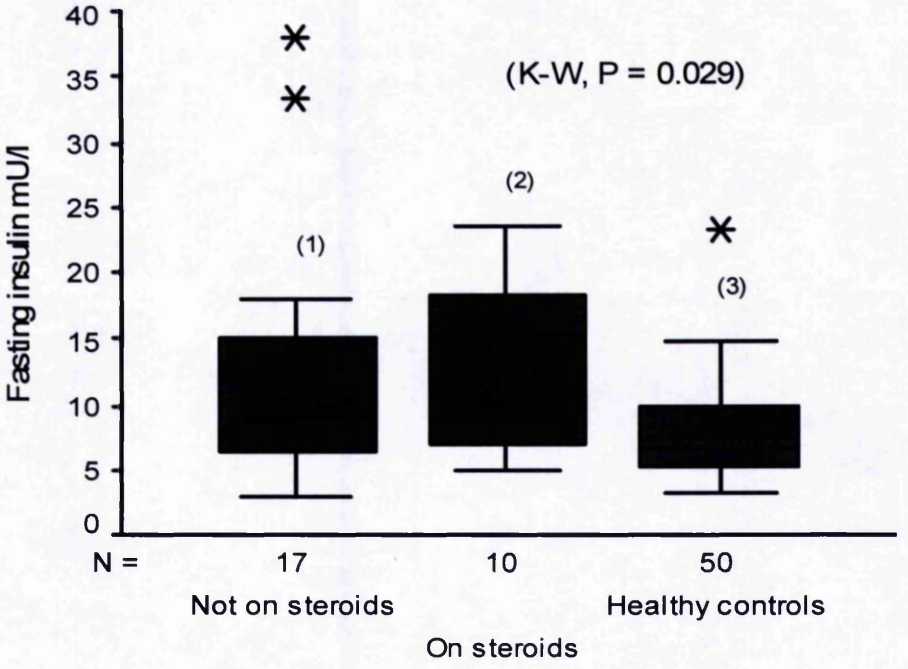


K-W : Kruskal-Wallis test of significance across groups.

The difference between the three groups (1), (2) and (3) using Mann-whitney test:

[(1) vs (2), P = 0.473], [(2) vs (3), P = **0.015**], [(1) vs (3), P = 0.051].

Figure 9.9 Pancreatic β-cell function in patients on and not on steroid therapy and in healthy controls:



K-W : Kruskal-Wallis test of significance across groups.

The difference between the three groups (1), (2) and (3) using Mann-whitney test:

[(1) vs (2), P = 0.14], [(2) vs (3), P = **0.017**], [(1) vs (3), P = 0.14]

9.4 The Metabolic syndrome:

In order to study this, we used the Adult Treatment Panel III (ATPIII) criteria to define the metabolic syndrome in our patients. These criteria are more practical than the WHO criteria, which require estimation of insulin resistance in subjects with normal glucose tolerance and estimation of the urinary albumin excretion rate. Metabolic syndrome is defined in the presence of ≥ 3 out of 5 criteria (waist circumference >88cm, TGs ≥ 1.69 mmol/l, HDL-C <1.29 mmol/l, blood pressure SBP ≥130 mmHg or DBP ≥ 85 mmHg and fasting glucose ≥ 6.1 mmol/l). We wanted to explore the clinical syndrome associated with insulin resistance in our whole group of SLE patients since this was not dependent on insulin calculations and therefore we could include patients on anti-malarial drugs.

9.4.1 Metabolic syndrome in healthy controls:

We examined the presence of the metabolic syndrome and its associated features in our 36 healthy controls. Only one of the controls had the metabolic syndrome. Twenty (56%) had none of the five criteria. The number of controls with the number of criteria, and also the frequency of each parameter contributing to the syndrome is outlined in (Figures 9.10, 9.11).

There was a significant decrease in insulin sensitivity with increasing number of metabolic syndrome criteria (Kruskal-Wallis test, $P < 0.001$) (Figure 9.12).

We compared controls with 0, 1 and ≥ 2 criteria (Table 9.11). There was no difference in age between the groups. There was a significant decrease in HOMA-S and increase in BMI, fasting insulin and HOMA-B with increase in number of metabolic syndrome criteria (Figures 9.13, 9.14). There was also a trend for inflammatory markers, hs-CRP and IL-6, to increase with the increase in the number of metabolic syndrome criteria, but there was no such a trend for ox-LDL-C (Table 9.11, Figures 9.15, 9.16).

Figure 9.10 Number of metabolic syndrome criteria in 36 healthy controls:

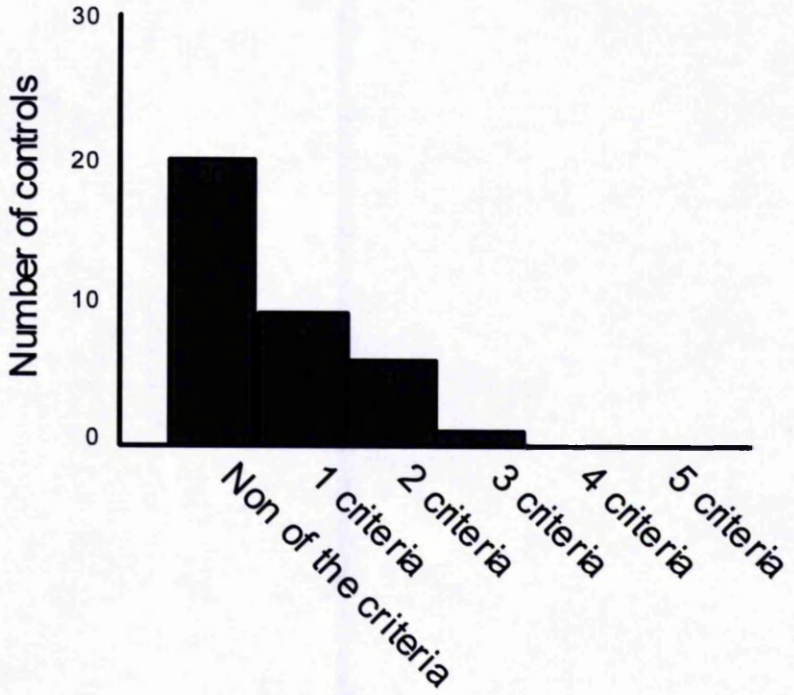


Figure 9.11 Frequency of each of the metabolic syndrome criteria in controls:

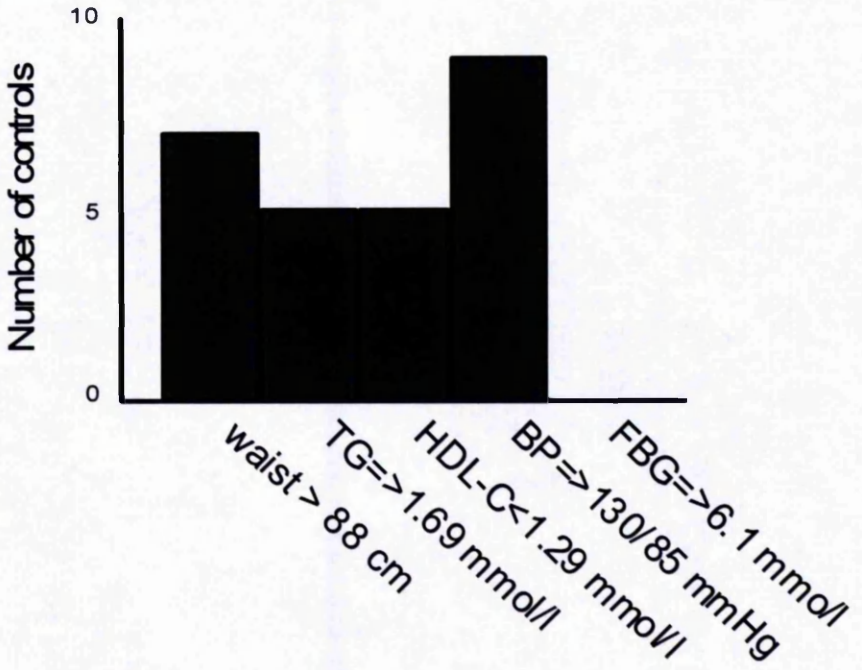
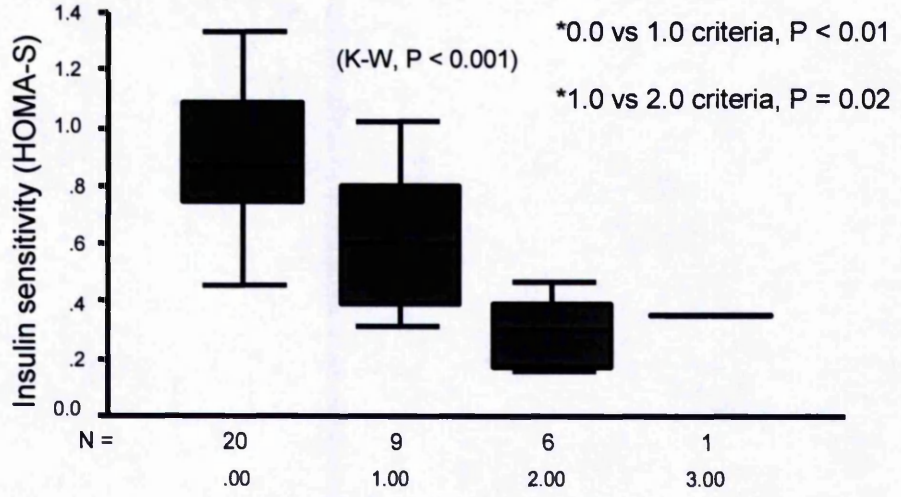


Figure 9.12 HOMA-S in healthy controls according to the number of metabolic syndrome criteria:



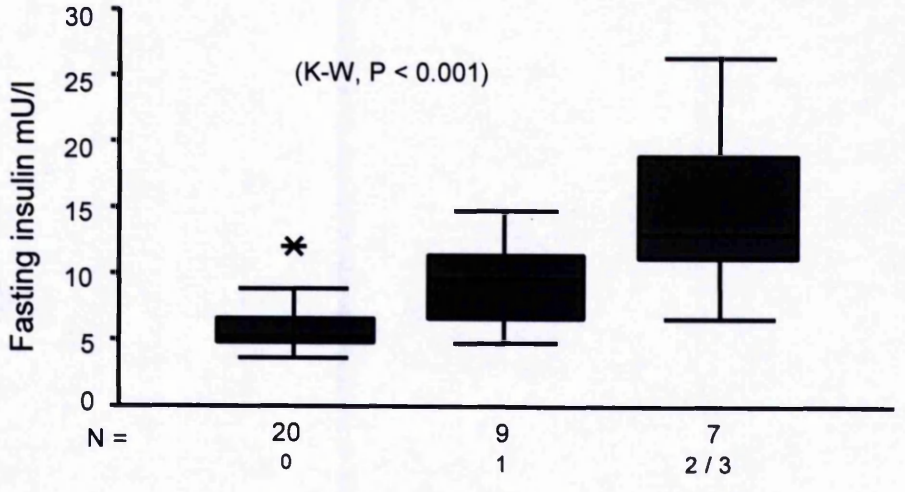
*Difference in HOMA-S according to metabolic syndrome criteria Using Mann-Whitney test. K-W : Kruskal-Wallis test of significance across the groups.

Table 9.11 Comparison of healthy controls with 0, 1 and ≥2 criteria of metabolic syndrome:

	0 criteria n =20	1 criteria n = 9	≥ 2 criteria n = 7	p-value*
Age	43 (25 – 56)	43 (25 – 62)	50 (28 – 56)	0.481
BMI	24.8 (17 – 30)	26 (19.6 – 33)	33 (30 – 36.2)	**0.001**
Insulin mUI/	5.3 (3.6 – 12)	9.6 (4.8 – 15)	13.7 (10 – 26)	**< 0.001**
HOMA-S	0.87(0.4 –1.3)	0.6 (0.3 – 1.0)	0.3(0.16 – 0.5)	**<0.001**
HOMA-B	96 (50 – 403)	160 (87 – 653)	194(152 –277)	**0.002**
VLDL-C mmol/l	0.13(0.01–0.5)	0.17(0.13-0.5)	0.24(.18 – 2.0)	0.066
Ox-LDL-C mU/l	36.8 (11– 76)	32 (13 – 43.8)	34.2 (18 – 67)	0.376
hs-CRP mg/l	1.5 (0.17 – 22)	1.2 (0.23 -4.2)	8.9 (0.5 – 12)	0.051
IL-6 pg/ml	1.0 (1.0 – 2.9)	1.3 (1.0 – 4.3)	3.6 (1.0 -14.7)	**0.012**

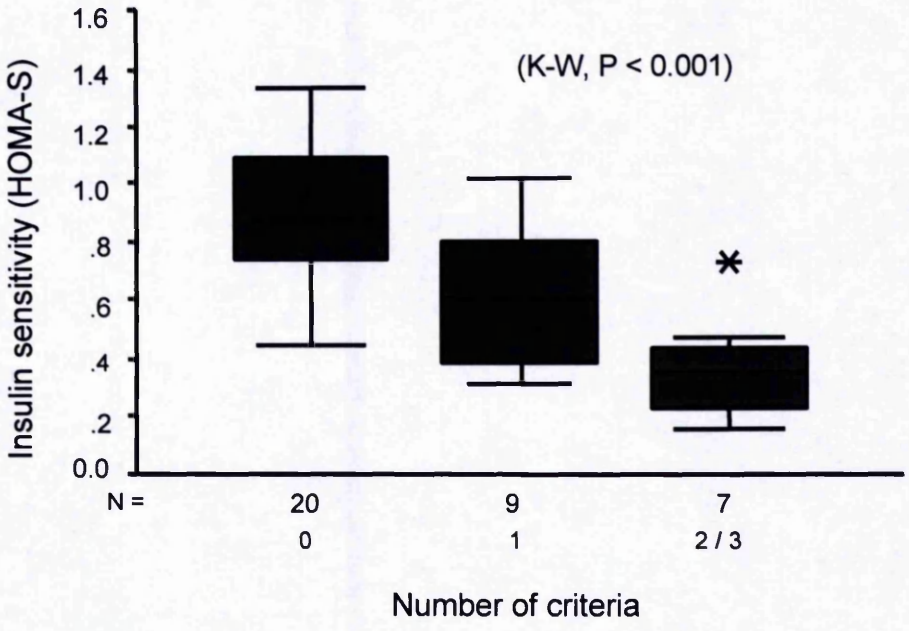
*Kruskal-Wallis test

Figure 9.13 Fasting insulin in healthy controls with 0, 1 and ≥2 criteria of metabolic syndrome:



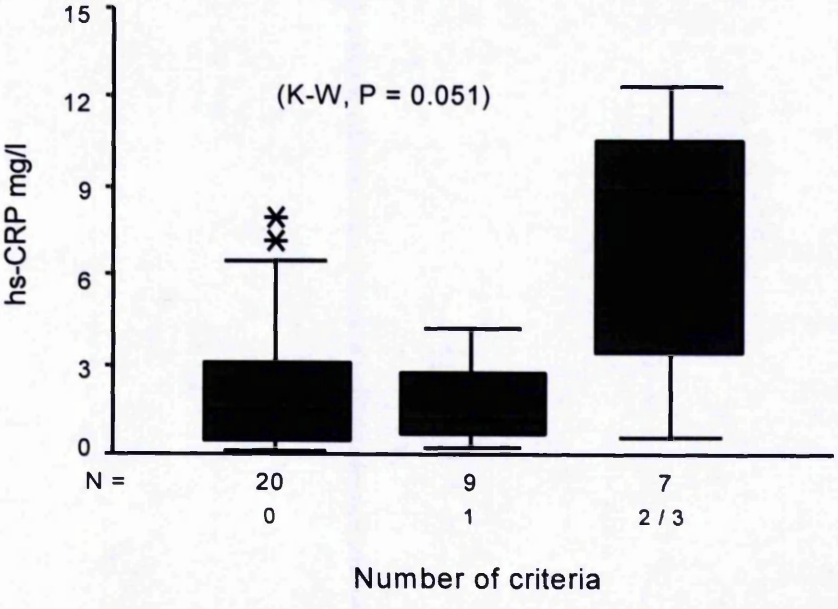
K-W : Kruskal-Wallis test of significance across the groups.

Figure 9.14 HOMA-S in healthy controls with 0, 1 and ≥2 criteria of metabolic syndrome:



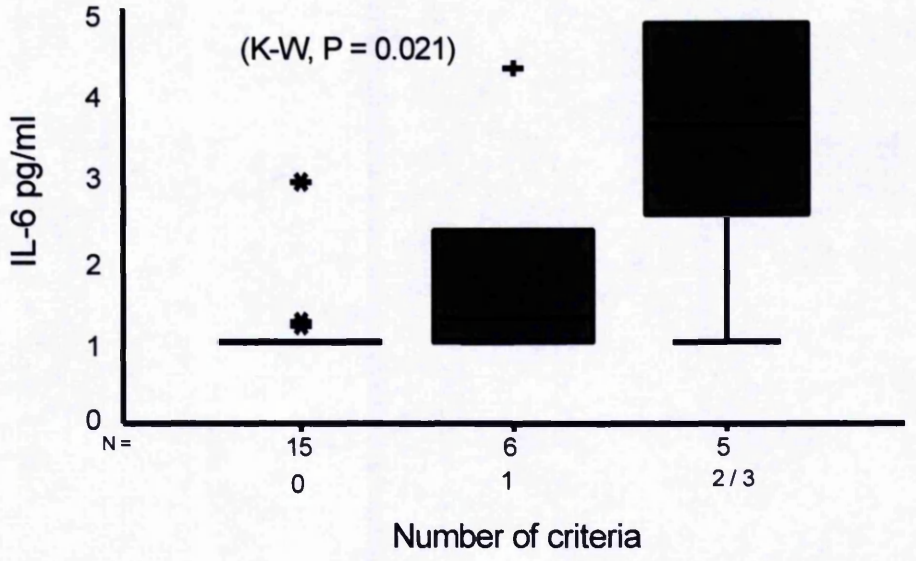
K-W : Kruskal-Wallis test of significance across the groups.

Figure 9.15 Hs-CRP in healthy controls with 0, 1, and ≥2 criteria of metabolic syndrome:



K-W : Kruskal-Wallis test of significance across the groups.

Figure 9.16 IL-6 in healthy controls with 0, 1, and ≥2 criteria of metabolic syndrome.



K-W : Kruskal-Wallis test of significance across the groups.

9.4.2 Metabolic syndrome in SLE patients:

For this part of the study a total of 61 women with SLE were studied, 51 (84%) were Caucasians and ten (14%) non-Caucasians. Two patients were known diabetics and one was found to have high fasting glucose (7.9 mmol/l) on the day of the study, therefore three (5%) had diabetes mellitus. We included these also. This group had a median (range) age and disease duration of 48 (21 – 73) years and 11 (1.0 – 35) years respectively.

Eleven (18%) patients had the metabolic syndrome. The number of patients with the number of criteria, and also the frequency of each parameter contributing to the syndrome is outlined in figures 9.17, 9.18. As can be seen, only ten (16%) patients had no criteria. The commonest criterion seen was increase in blood pressure (59%). There was an increase in fasting insulin with increase in the number of metabolic syndrome criteria (Figure 9.19). There was also significant increase in ox-LDL-C with increase in the number of metabolic syndrome criteria (P = 0.040) (Figure 9.20). Since eleven patients had the metabolic syndrome, we used categorical groups for comparison between those with and without the syndrome (Table 9.12). Both groups were of similar age, however patients with the syndrome had a trend towards shorter disease

Figure 9.17 Frequency of metabolic syndrome criteria in 61 patients:

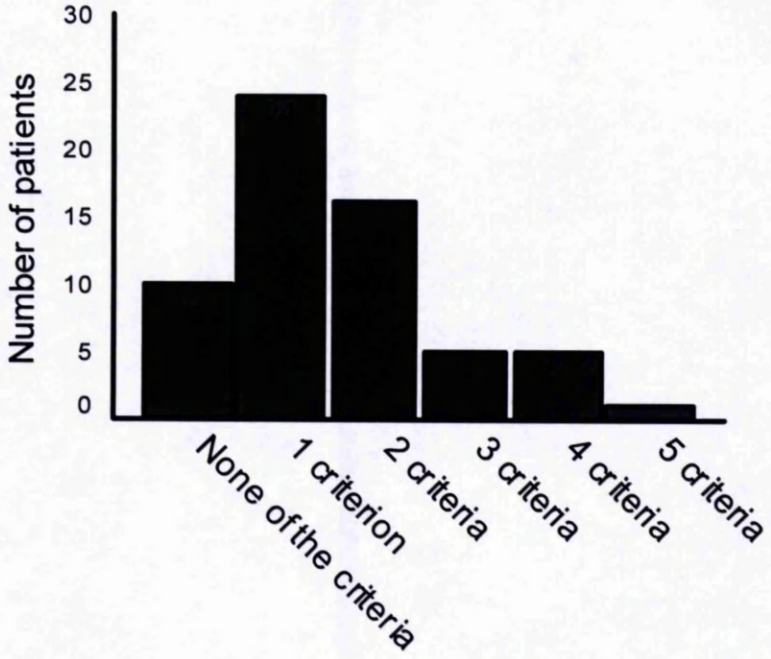
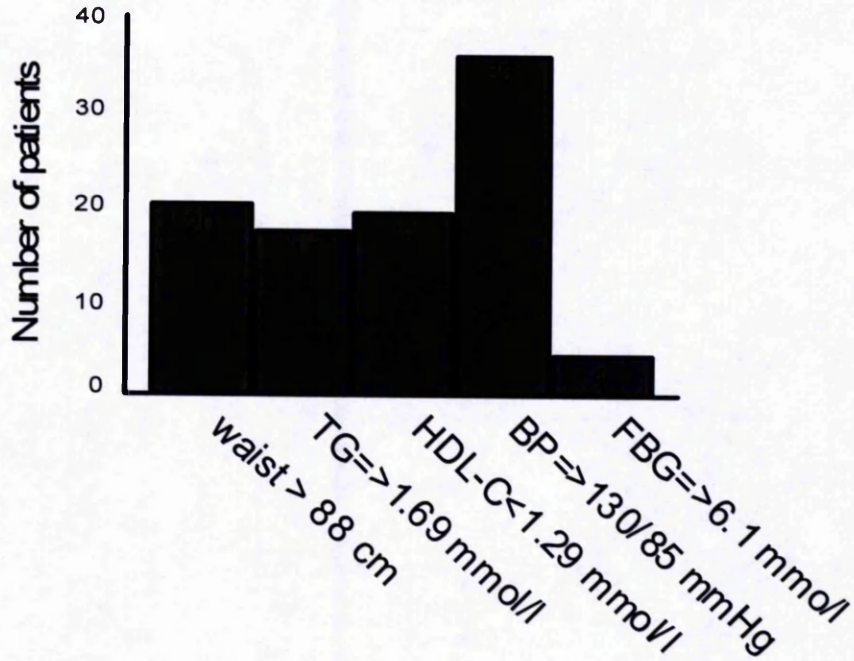


Figure 9.18 Frequency of each of the metabolic syndrome criteria in SLE patients:



duration at the time of study. They also had significantly higher fasting insulin levels, lower insulin sensitivity and higher ox-LDL-C (Table 9.12). There was no significant difference in the number of patients currently taking steroids or anti-malarial drugs (Table 9.13).

In addition, there was also no significant difference in the median current steroid dose or mean dose over the past six months and indeed if any thing patients with the metabolic syndrome had lower steroid exposure (Table 9.13). Those with the metabolic syndrome had significantly higher ox-LDL-C and a trend towards higher median hs-CRP and IL-6 levels. There was no significant difference in SLEDAI in patients with and without the syndrome or in proportion of patients with a BILAG category A or B in at least one organ system (Tables 9.12, 9.13).

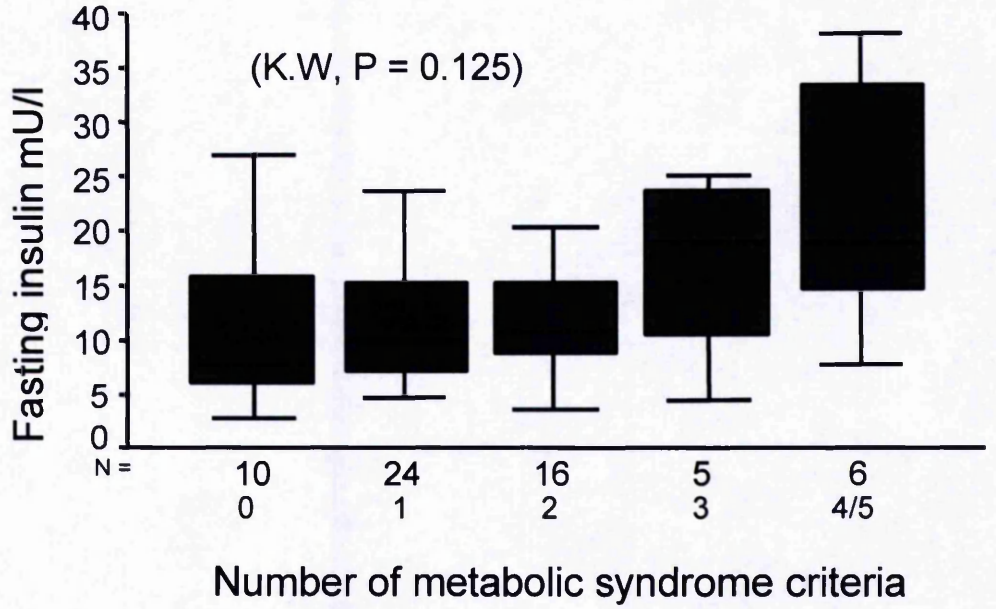
Table 9.12 Comparison of SLE patients with and without the metabolic syndrome:

	Metabolic syndrome YES n = 11	Metabolic syndrome NO n = 50	P value
Age	46 (26 – 73)	49.5 (21 – 67)	0.978
BMI	29 (24 – 34)	25 (19 – 42)	**0.005**
Disease duration/years	6 (1 – 29)	12 (1 – 32)	0.121
Fasting glucose mmoll	5.9 (3.8 – 7.9)	4.5 (3.6 – 10.9)	**0.031**
Fasting insulin mU/l	18.9 (4.5 – 38)	10 (2.8 – 40.7)	**0.013**
VLDL-C mmol/l	0.39 (0.09 – 0.83)	0.21 (0.06 – 1.0)	**0.014**
Ox-LDL-C mU/l	45.7 (18 – 61)	31.7 (13 – 77)	**0.017**
CHD 10-year risk %	5.8 (0 – 15.8)	3.3 (0 – 11.9)	0.167

Table 9.13 Therapy and inflammatory parameters in patients with and without the metabolic syndrome:

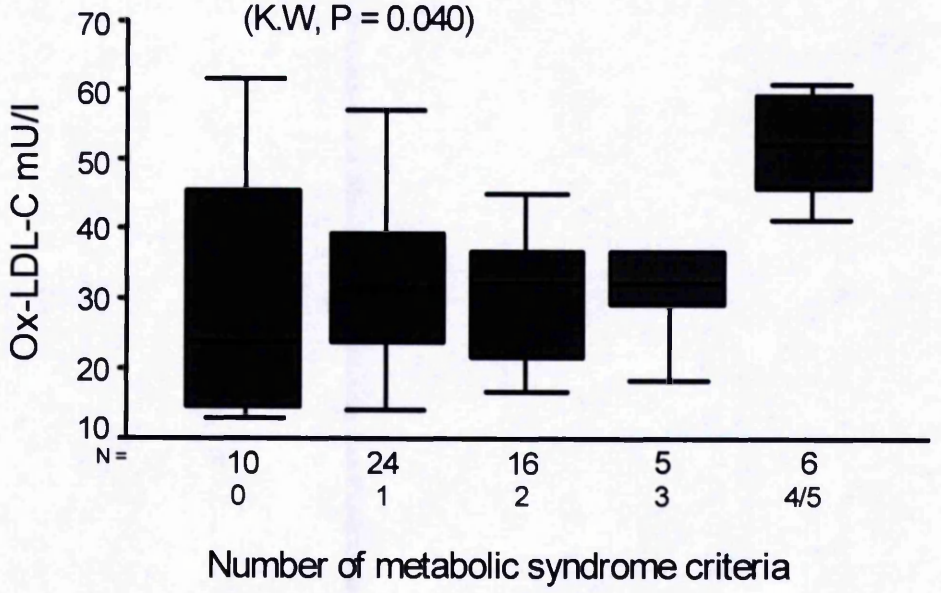
	Metabolic syndrome YES n = 11	Metabolic syndrome NO n = 50	P value
Number (%) on steroid therapy	4 (36.4%)	29 (58%)	0.317
Current steroid dose mg/day	0.0 (0 – 30)	4.0 (0 – 30)	0.289
Average steroid dose in the last 6 months	0.0 (0 – 10)	5.0 (0 – 20)	0.237
Total steroid duration/months	37 (0 – 372)	39 (0 – 264)	0.713
Number (%) on anti-malarial therapy	4 (36.4%)	25 (50%)	0.514
SLEDAI	2 (0 – 12)	2.0 (0 – 8)	0.877
Number (%) with BILAG category A or B	3 (27%)	9 (18%)	0.676
CRP mg/l	3.0 (1.8 – 16.8)	2.4 (0.15 - 31)	0.124
IL-6 pg/ml	4.6 (2.0 – 17.7)	2.3 (1.0 – 17.7)	0.091

Figure 9.19 Fasting insulin in SLE patients according to the number of metabolic syndrome criteria:



K-W : Kruskal-Wallis test of significance across the groups.

Figure 9.20 Ox-LDL in SLE patients according to the number of metabolic syndrome criteria:



K-W : Kruskal-Wallis test of significance across the groups.

9.5 Effects of anti-malarial therapy on fasting glucose, insulin and other metabolic parameters:

Several studies have highlighted the potential beneficial effects of anti-malarial therapy on fasting glucose and lipid profile in SLE patients (Wallace *et al* 1990 and Petri *et al* 1994). We therefore examined the influence of AM's on these parameters in our SLE group. Thirty-two (50%) were taking AM's at the time of study. 28 were on hydroxychloroquine (OHCQ) and four were on chloroquine phosphate. The median daily dose of OHCQ was 200 mg/day (range 100 – 400) mg/day. There was a higher proportion of patients taking steroids in the group treated with AM's (69% vs 41%, p = 0.040) (Table 9.14).

Table 9.14 Comparison of patients on and not on anti-malarial therapy:

	On AM's n = 32	Not on AM's n = 29	P value
Age/years	43 (21 – 66)	51.5 (21 – 73)	0.084
BMI	25 (20.7 – 41.9)	26.4 (18.6 – 33.9)	0.817
Waist/cm	80 (63 – 110)	79.5 (66 – 99)	0.613
Waist/hip ratio	0.80 (0.7 – 0.9)	0.82 (0.69 -0.96)	0.487
Fasting glucose mmol/l	4.4 (3.6 – 10.2)	4.7 (3.8 – 10.9)	**0.042**
Fasting insulin mU/l	10.8 (5.7 – 40.7)	11.0 (2.8 – 38)	0.629
Number (%) on steroids	20 (69%)	13 (40.6%)	**0.040**
Current steroid dose mg/day	5.0 (0.0 – 30)	0.0 (0.0 – 30)	0.052
Average steroid dose in last 6 months	6.5 (3.0 – 20)	7.5 (3.5 – 12)	0.870
SLEDAI	2.0 (0.0 – 12)	2.0 (0.0 – 8)	0.316

Despite this there was no difference in BMI, waist circumference or waist/hip ratio between the two groups. Also despite this, fasting blood glucose was significantly lower in patients on AM's. In contrast, insulin levels were similar in patients on and off AM's. Rahman *et al* (1999) has also suggested that the major influence of AM's on lipid profiles was in the presence of steroid therapy.

Table 9.15 Comparison of groups of patients according to anti-malarial and steroid therapy:

	AM's only n =12	AM's & steroids n = 20	Steroids only n = 13	No AM's No steroids n = 19	K-W p-value
Age	53 (24 – 66)	43.5 (22 – 65)	48 (21 – 61)	56 (33 – 73)	0.055
BMI	27.9 (20.7 – 38)	24.7 (21 – 41.9)	26.8 (18.6 – 34)	26.2 (18.8 – 31)	0.849
Waist/hip ratio	0.83 (0.73 – 0.9)	0.79 (0.7 – 0.86)	0.82 (0.72 – 0.8)	0.83 (0.7 – 0.96)	0.516
Fasting glucose	4.5 (3.9 – 5.7)	4.4 (3.6 – 10.2)	4.7 (3.8 – 4.8)	4.9 (4.0 – 10.9)	**0.045**
Fasting insulin	8.9 (5.7 – 21.3)	12.5 (6.3 – 40.7)	15.5 (4.8 – 27)	8.9 (2.8 – 38)	0.111
TC	5.1 (3.6 – 6.3)	5.0 (3.0 – 6.7)	4.9 (3.9 – 8.7)	4.5 (3.2 – 7.5)	0.646
LDL-C	2.5 (0.85 – 3.9)	2.3 (0.33 – 3.7)	2.6 (1.7 – 5.0)	2.8 (0.4 – 4.2)	0.333
HDL-C	1.4 (0.9 – 2.2)	1.5 (0.6 – 3.0)	1.4 (0.8 – 2.0)	1.4 (0.9 – 2.6)	0.476
TGs	1.1 (0.4 – 2.0)	1.4 (0.4 – 3.2)	1.5 (0.7 – 3.9)	1.1 (0.7 – 2.8)	0.277
VLDL-C	0.12 (0.06 – 0.5)	0.20 (0.06 – 0.8)	0.27 (0.10 – 1.0)	0.22 0.07 – 0.8)	0.109
IL-6	1.5 (1.0 – 5.7)	9.0 (1.0 – 17.7)	1.2 (1.0 – 17.7)	3.8 (1.0 – 10)	0.116
Hs-CRP	2.5 (0.9 – 47.6)	2.4 (0.47 – 17)	2.4 0.15 – 7.8)	3.0 (0.36 – 22)	0.578
Ox-LDL-C	35 (14.7 – 65)	26 (14 – 57)	32 (15.8 – 56)	36 (13 – 76.8)	0.134

K-W : Kruskal-Wallis test of significance across the groups.

We therefore compared our SLE patients according to the combination of anti-malarial and/or steroid therapy they were taking (Table 9.15). There was a significant difference in fasting glucose between the groups. In particular, patients on anti-malarials had lower fasting glucose than patients on steroid alone or on no therapy. Those on steroids and anti-malarials also had lower fasting glucose than those on steroids alone or on no therapy (P= 0.024). There was also a trend towards higher fasting insulin in the steroid only group, compared with intermediate levels in those taking anti-malarials and steroids.

9.6 Influence of insulin and metabolic syndrome on endothelial function in SLE:

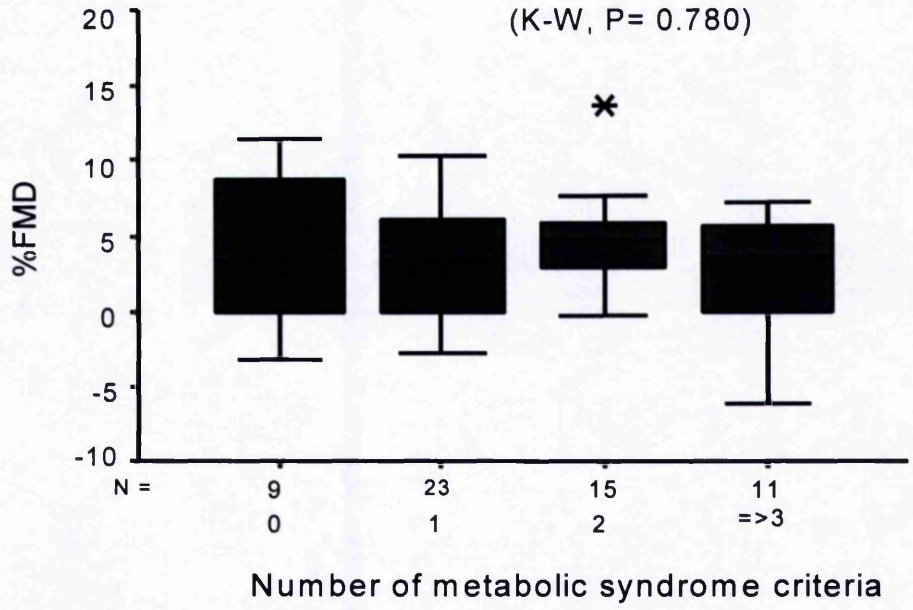
In our patients, fasting insulin did not correlate with percentage FMD, resting diameter or percentage GTN dilation (Table 9.15).

The number of metabolic syndrome criteria showed no association with percentage FMD, resting diameter or with percentage GTN dilation (Figure 9.21, Table 9.16)

Table 9.16 Correlations of insulin with endothelial function in 64 SLE patients:

	Insulin	
	r	P-value
Resting diameter	- 0.191	0.152
FMD (absolute)	0.108	0.424
Percentage FMD	0.132	0.322
Percentage GTN dilation	0.236	0.086

Figure 9.21 Percentage FMD according to the number of metabolic syndrome criteria:



K-W : Kruskal-Wallis test of significance across the groups.

Table 9.17 Comparison of resting diameter percentage FMD and GTN dilation according to the number of metabolic syndrome criteria:

Number of criteria	Resting diameter	%FMD	%GTN dilation
0 criteria n = 9	0.33 (0.26 – 0.35)	3.4 (- 3.3 – 11.5)	22.2 (5.7 – 29)
1 criteria n = 23	0.33 (0.25 – 0.42)	3.2 (-2.9 - 10)	17.2 (2.8 – 30)
2 criteria n = 15	0.33 (0.24 – 0.43)	4.6 (- 0.3 – 13.7)	15.4 (4.6 – 34.6)
=>3criteria n = 11	0.31 (0.28 – 0.38)	3.4 (- 6.3 – 7.1)	19.3 (13.3 – 44)
K-W p-value	0.722	0.780	0.780

9.7 Summary:

Insulin sensitivity:

- Euglycaemic SLE patients have evidence of decreased insulin sensitivity with increased fasting insulin and pancreatic β-cell function.
- Fasting insulin in SLE correlates with other components of the metabolic syndrome in SLE and healthy controls.
- Steroid theapy is not a primary factor that determines insulin sensitivity in SLE.
- Fasting insulin correlates with ox-LDL-C; a marker of oxidative stress.

With regard to metabolic syndrome:

- Eighteen % of SLE patients had the metabolic syndrome.
- No association with steroid therapy.
- SLE patients with the metabolic syndrome had higher ox-LDL-C and tended to have higher hs-CRP and IL-6.
- AM's significantly associated with lower fasting glucose. The hypoglycaemic effect of AM's is more evident in patients taking steroids.

With regard to vascular function:

- No association between fasting insulin or the metabolic syndrome and endothelial function in SLE.

Chapter 10

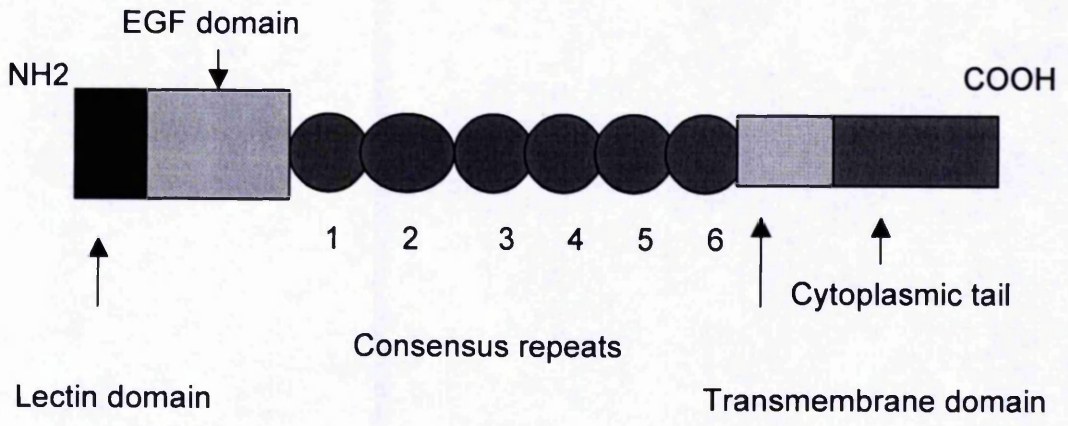
10 A study of E-selectin A561C gene polymorphism in SLE

10.1 Introduction:

Genetic factors play an important role in the pathogenesis of CHD as well as of SLE. Several genetic association studies have shown association of E-selectin gene (A561C) polymorphism and premature CHD (Wenzel *et al* 1996 & Ye *et al* 1999). In addition, functional studies of this polymorphism have shown that, it increases the number of rolling leukocytes over the endothelial surface endothelium, and enhances arrest and subsequently transmigration of leukocytes into the sub-endothelial space. Interestingly, the E-selectin gene is located within the linkage area for SLE, on the chromosome 1q 21-23.

Therefore, this polymorphism may be important to SLE, because arterial wall inflammation and inflammatory cell recruitment is common to the pathogenesis of both atherosclerosis and SLE.

Figure 10.1 Structure of E-selectin molecule:



A561C polymorphism in E-selectin gene results in exchange of Serine to Arginine in the EGF domain 128 (S128R) near the lectin binding site.

10.1.1 Aim:

Our first aim was to study the genotypes of E-selectin gene A561C polymorphism and to determine the C-allele frequency in 3 distinct populations of SLE and their respective controls. Our second aim was to study the association of the C allele with disease features and endothelial function in our population of 64 SLE patients.

10.1.2 Patients and controls:

Our first study was performed on stored DNA samples in the arc Repository of SLE patients and their ethnic matched controls from 3 different populations:

- Caucasian SLE patients and controls of British Isles descent from the North-West of England. The stored DNA were collected mainly from SLE patients from Hope Hospital, Salford, Blackburn Royal Infirmary and other hospitals in and other hospitals in Greater Manchester over the past 4-5 years. These cases were separate from our cohort.
- Caucasian SLE patients and controls of Spanish origin from Barcelona.
- SLE patients and controls of Turkish origin from Istanbul.

All patients fulfilled the 1987 American College of Rheumatology (ACR) revised criteria for SLE.

10.2 Methods:

10.2.1 DNA extraction:

This was carried out on blood samples, which were collected from our SLE patients during the study period in EDTA tubes and were stored in -20°C. DNA extraction from whole blood is done in two steps:

10.2.2 Lysis of blood samples to produce a white blood cell pellet:

After thawing the blood samples at room temperature, each sample was poured into a 50 ml centrifuge tube labelled with the sample number. Buffer lysis solution was then added to the centrifuge tubes up to a volume of 40 ml. After rotating the tubes for ten minutes on a rotator, they were centrifuged at 3000 rpm for ten minutes. The supernatant was then discarded into 2% virkon. The pellets in the tubes were then resuspended by adding 20 ml of lysis buffer,

followed by vigorous shaking to break up the pellet. The tubes were rotated once more for ten minutes and again centrifuged at 3000 rpm for ten minutes. After the supernatant was discarded a white blood cell pellet became visible. These pellets, which were still pink, were re-washed with lysis buffer until they became white.

10.2.3 DNA extraction using the phenol/chloroform method:

The clean white cell pellet was transferred to another tube and the following solutions were added to the tube; 3.5ml 6 $molL^{-1}$ GuHCl, 250µl 7.5$molL^{-1}$ NH_4Ac, 250µL 20% Na Sarcosyl and 50µL 10$mgmL^{-1}$ proteinase K. The tube was then vortexed until the pellet was dissolved, and incubated at 60°C for one hour. The tubes were vortexed again to ensure digestion was successful, cooled at room temperature and the contents were then transferred to a 15 ml centrifuge tubes. An equal volume of phenol/chloroform/isoamylalcohol (25:24:1) mixture was then added to the aqueous DNA in the tubes. The tubes were vortexed vigorously until a white emulsion was formed, left to stand for one minute and then centrifuged at 3000 rpm for 45 minutes. Only the clear upper aqueous layer containing DNA was then carefully transferred to another tube, avoiding the protein and chloroform lower layers. The DNA was then precipitated by adding the aqueous DNA to 10 ml of absolute ethanol in a 15 ml tube. To increase the yield the ethanol and aqueous DNA mix was stored at -20°C overnight. The tubes were then gently inverted 50 – 60 times to fully precipitate the DNA and then were centrifuged at 3000 rpm for 15 minutes to pellet the DNA. At this stage, DNA could be seen as a white precipitate at the bottom of the tube. The ethanol was poured off and the pellet was washed with 2ml of 70% ethanol again. The tube was centrifuged at 3000rpm for five minutes and the ethanol was again poured off. The tubes were left to air-dry for 15-20 minutes then the pellet was re-suspend in 300µl water Once the pellet had been resuspended, the DNA concentration was measured using PicoGreen® Assay.

10.3 Polymerase chain reaction:

This technique allows rapid exponential amplification of a target region or sequence of genomic DNA using a thermal cycler. This involves incubation of genomic DNA with:

- Heat-stable Taq polymerase, which is isolated from eubacterial microorganism Thermus aquaticus (Taq). This enzyme withstands repeated heating and cooling.
- Two primers (forward and reverse); these are short synthetic oligonucleotides, usually 15 – 30 nucleotide-long. They bind to single stranded DNA at a region that has a complementary sequence to that of the primer.
- Deoxynucleotide triphosphate nucleotides (dNTPs) solution, consisting of equal amount of each of the four dNTPs.
- Magnesium chloride and a buffer (either KCl or NH^{4-}).

The thermal cycles are as follows:

1. Denaturation of the double strand DNA by heating to 95°C. This separates the double stranded DNA into single strands.
2. Annealing of the primers to their complementary sequence on DNA single strands, which occurs at a lower temperature. The annealing temperature of the primers is optimised for each reaction and is related to proportions of the four nucleotides in the primers. The annealing temperature = T_m - 5°C, where T_m = 4 (G+C) + 2(A + T).
3. Synthesis or extension of the annealed primers by the Taq polymerase in a 5' to 3' direction. In this cycle the temperature is raised to 72°C.

The first extension produces, two double-strand DNA of the target segment. Repeated cycles of denaturation, annealing and extension results in rapid exponential accumulation of the amplified segment (Figure 10.2)

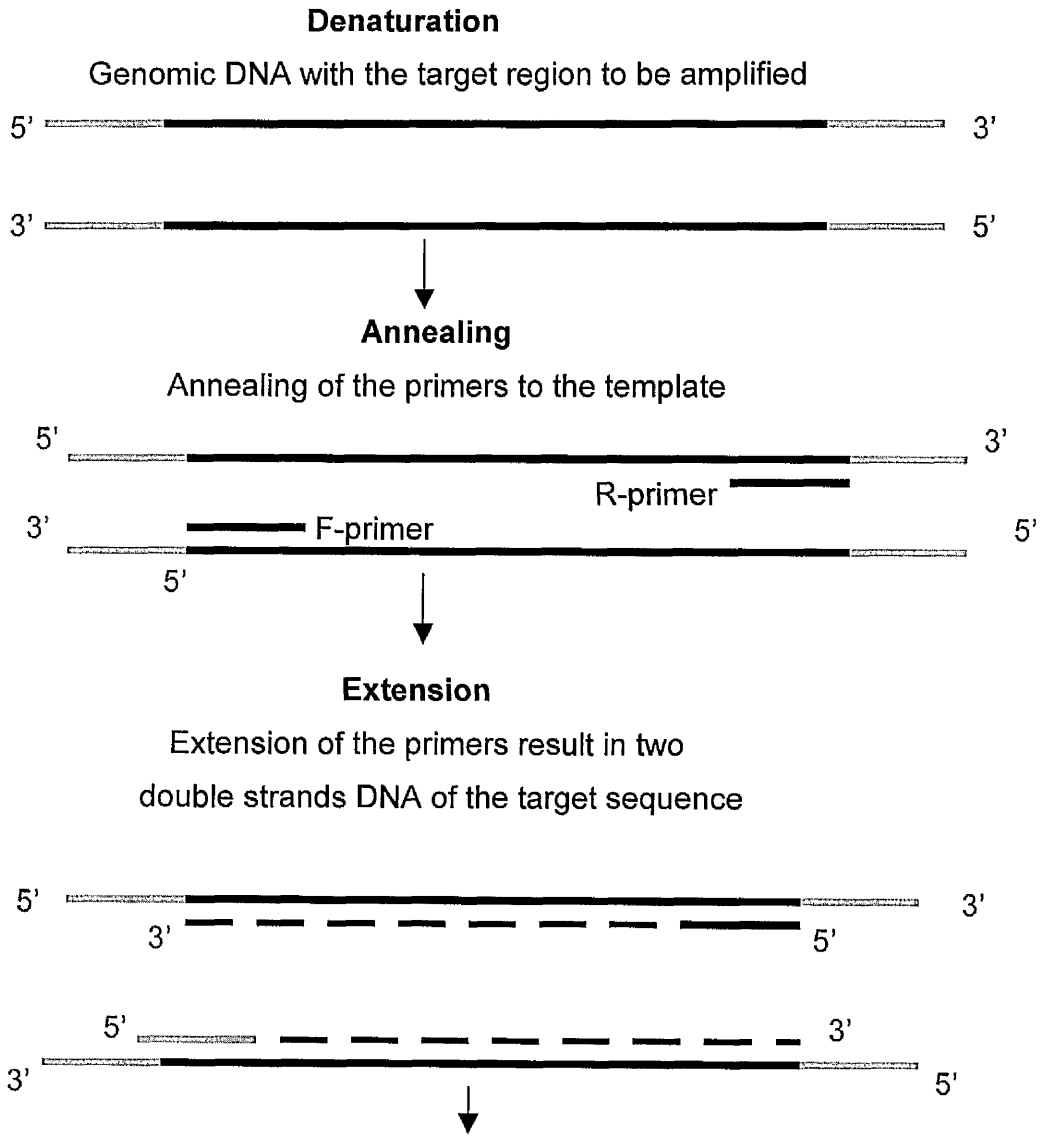
10.4 DNA gel electrophoresis:

This technique is used to separate DNA fragments according to their size utilizing the fact that, in the presence of voltage difference across a suitable medium, the negatively charged DNA moves toward the positive electrode. Gel electrophoresis is used to check for the specificity of PCR products and to

determine different alleles following digestion of PCR product by restriction enzymes. Smaller DNA segments move faster than larger segments.

For preparation of 2% agarose gel: 1 gm of agarose and 50 ml of 0.5X TBE were microwaved in a flask for two minutes to dissolve the agarose.

Figure 10.2 Schematic presentation of Polymerase chain reaction:



After a brief cooling, 12 μl of ethidium bromide was then added and the gel was then poured into a gel tray. The combs were placed in the tray and the gel left to cool for at least 20 minutes. The combs were then removed, and 0.5XTBE buffer was poured on the gel to fill the wells and cover the surface of the gel. 5μl of PCR sample or digestion product was then mixed with 5μl of loading buffer. The mixture was pipetted into the wells of the gel. A DNA size marker was run with every line of samples on the gel. The gel was run in electrophoresis at 160mA until the dye had migrated to a desirable distance. The gel was then placed in an UV box with a camera and photographs were obtained for analysis of the bands.

10.5 Study of E-selectin gene A561C polymorphism in three distinct populations:

We used a bioinformatic web site to identify the sequence of E-selectin gene (http://www.ncbi.nlm.nih.gov/Entrez/Genome/org.html). We used Primer3 version 0.2C software to define sequences of possible primers that could be used to amplify any selected segment of DNA. To amplify a 249 bp segment that contains the polymorphic site A652C. For genotyping of the E-selectin A561C polymorphism, we used polymerase chain reaction and restriction fragment length polymophism (PCR-RFLP) technique. The protocols for PCR and digestion of product by restrictive enzyme (*PstI)* are shown in tables 10.1, 10.2. The primers sequences were, forward (5' GTC TCA GCT CAC GAT CAC CA -3') and reverse (5'-CCG TAG CTG CCT GTA CCA AT-3').

PCR was carried out in a total volume of 25 μl and was optimised to an initial cycle of denaturation for two minute at 94°C followed by 35 cycles each consisting of denaturation for one min at 94°C, annealing for one minute at 55 °C and synthesis for one minute at 72 °C. A final extension cycle was for five minutes at 72 °C. Seven μl of the PCR product was then digested with four units of *PstI* restriction enzyme. These digestion products were then separated on a 3% gel electrophoresis. The A561C polymorphism inhibits the restriction site for *Pst*I enzyme therefore, in the homozygous AA genotype, both alleles will be digested at the restriction site and the product is seen as two fragments of 219

and 30 base pair (bp). The homozygous CC genotype appears as one band at 249 bp as there is no digestion. In heterozygous AC genotype, only the A allele is cut, which result in three bands at 249, 219 and 30 bp (Figure 10.3).

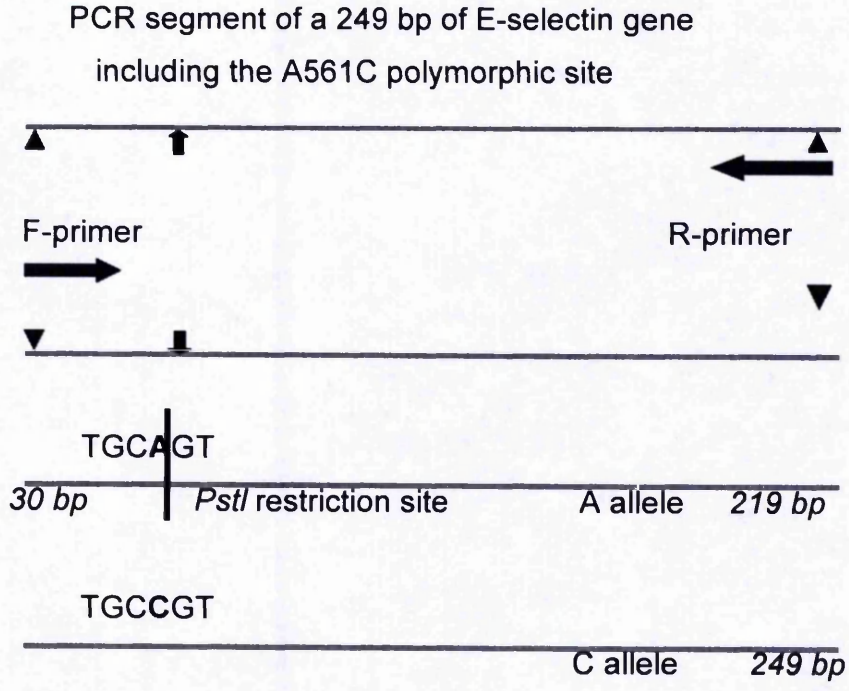
Table 10.1 PCR and digestion protocol used for genotyping E-selectin A561C:

PCR		Digestion	
Reagent	Vol. μl / sample	Reagent	vol. μl/ sample
R- primer (50pm/μl)	0.1	PCR product	7μl
F-primer (50pm/μl)	0.1	PstI enzyme	0.4
Taq (5 units/μl)	0.1	Buffer	1.5
Buffer (NH_4)	2.5	Water	6.1
dNTPs	2.5	Total	15
Mg^{++} (1.5mM)	1.25	Digestion was over night at 37°C	
Additive (Betain)	6.0		
Water	11.45		
DNS	1		
Total	25		

Table 10.2 PCR condition for E-selectin A561C polymorphism:

PCR cycle	DNA position	Condition	Number of cycles
Initiation	Initial denaturation	94°C for 5 minutes	1
Extension	Denaturation Primer annealing Primer extension	94°C for 1 minute 55°C for 1 minute 72°C for 1 minute	35
Final extension	Extension	72°C for 5 minutes	1

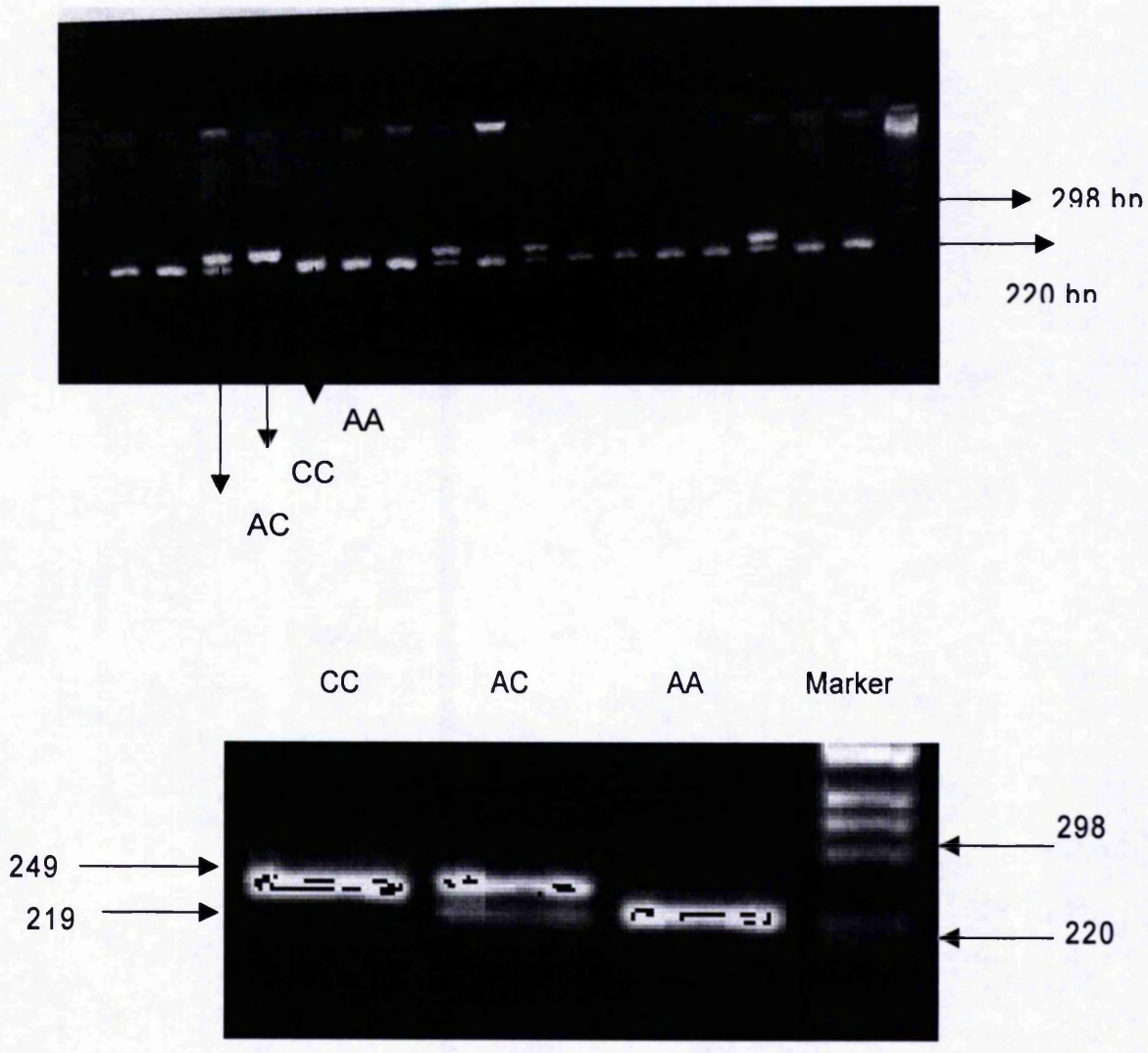
Figure 10.3 Schematic representation of PCR-RFLP technique used to genotype E-selectin gene for A561C polymorphism:



Genotypes:

1. AA genotype will appear on the gel as one band at 219 bp (both alleles are cut).
2. CC genotype will appear as one band at 249 bp (no cutting).
3. AC will appear as two band at 249 and 219 bp (only A allele is cut).

Figure 10.4 Gel electrophoresis showing three genotypes AA, AC, and CC resulting from a single nucleotide polymorphism (A561C) in E-selectin gene.



The presence of the A561C polymorphism inhibits the restriction site for PstI restriction enzyme. CC genotype is represented by one band, appearing as a 249 bp fragment, as there was no digestion of the product. In AA genotype, where the two alleles were digested resulting in two fragments; a larger fragment of 219 bp appears opposite to 220 bp on the DNA marker and smaller fragment of 30 bp, which is not shown. In the heterozygous AC genotype, the A

allele is cut and the C allele is not cut, which results in two bands at 249 bp (A allele) and 219 bp (C allele).

10.5.1 Statistical analysis:

We used version 6 of the Stata program to compare the frequency of the mutant C allele in relation to the normal A allele in patients and controls. Presence or absence of C allele represents exposure and no exposure in the typical contingency 2x2 table. Odd ratio with 95%CI is calculated for the frequency of C allele

	C allele	A allele
Patients	a	b
Controls	c	d

10.5.2 Results:

We genotyped 113 Caucasian patients and 148 controls from the UK, 145 Caucasian patients and 179 controls from Spain and 93 patients and 96 controls from Turkey. The genotype and allele frequency in the three groups is shown in tables 10.3 and 10.4. The AC genotype was more frequent in SLE patients compared to their respective controls in the Spanish (22% vs 14%) and the UK groups (19.5% vs 16.9%). In contrast, the AC genotype was not significantly more common in SLE patients of Turkish origin (20.8 % vs 17.2%) (Table10.1). The frequency of the C allele was significantly higher in the UK and Spanish patients compared to their respective controls, but not in the Turkish group (Table 10.4).

Table 10.3 Genotypes frequency of E-selectin A561C polymorphism in the three groups of SLE patients and controls:

	Genotypes					
	AA		AC		CC	
	n	%	n	%	n	%
UK						
Cases	85	75.2	22	19.5	6.0	5.3
Controls	122	82.4	25	16.9	1.0	0.7
Spanish						
Cases	110	75.8	32	22.1	3.0	2.1
Controls	153	85.4	25	14.0	1.0	0.6
Turkish						
Cases	74	79.6	16	17.2	3.0	3.2
Controls	75	78.1	20	20.8	1.0	1.0

Table 10.4 A and C allele frequency in the three groups of SLE patients and controls:

	A allele		C allele		OR	P-value
	n	%	n	%		
UK						
Cases	192	85.0	34	15	1.67 (1.03 – 3.0)	0.037
Controls	269	90.9	27	9.1		
Spanish						
Cases	252	86.9	38	13.1	1.84 (1.10 – 3.1)	0.019
Controls	331	93.0	27	7.0		
Turkish						
Cases	164	88.2	22	11.8	1.03 (0.55 – 1.97)	0.910
Controls	170	89.6	22	10.4		

10.6 Influence of E-selectin gene A561C polymorphism in SLE patients:

The second study of the E-selectin A561C polymorphism was performed on DNA samples from the 64 patients who participated in our vascular study. Our aim was to study associations of the C allele with SLE features, endothelial function and atherosclerosis. Eleven (17.2%) patients had the AC genotype and 53 (82.8%) had AA genotype. None of the patients had a CC genotype.

10.6.1 Influence on SLE disease expression:

Comparing patients with and without C allele, patients with the C allele tended to be younger at study ($p = 0.17$), but had a similar age at diagnosis (Table 10.5). With regard to disease characteristics, patients with the C allele had higher disease activity at time of study. There was however no association of the C allele with any system involvement and in particular, renal disease was no different in the C allele group. Serologically, there was a trend towards higher antiphospholipid antibody in the group with the C allele. There was no difference in SLICC damage score or Raynaud's phenomenon between each group.

10.6.2 Influence on cardiovascular risk factors and endothelial function:

When we looked at the cardiovascular risk factors, lipid profiles and the proportion of patients with hypertension were comparable in both groups. There was no association of the C allele with the vascular function and in particular %FMD did not differ in patients with the C allele (Tables 10.6, 10.7).

Table 10.5 Comparison of SLE features in AA and AC genotypes:

	AC n =11 Median (range)	AA n = 53 Median (range)	P value
Age /years	40 (21 – 62)	50 (12 – 73)	0.173
Age at diagnosis /years	32 (10 – 55)	34 (11 – 62)	0.527
SLEDAI	2 (0.0 – 12)	0.0 (0.0 – 8.0)	0.042
SLICC	0.0 (0.0 – 3.0)	0.0 (0.0 – 4.0)	0.667
APL antibodies n (%) - ACL antibody - Lupus anticoagulant	7 (63.7) 7 (63.6) 3 (27.3)	18 (34.0) 15 (28.3) 11 (20.8)	0.092 0.037 0.693
Raynaud's phenomenon	7 (63.6)	29 (54.7)	0.743
Renal involvement n (%)	3 (27.3)	7 (13.2)	0.356
Serositis n (%) - Pleurisy - Pericarditis - Both	6 (54.5) 4 (36.4) 4 (36.4) 2 (18.2)	17 (32.1) 15 (28.3) 3 (5.7) 1 (1.9)	0.182 0.719 0.014 0.074
Photosensitivity n (%)	2 (18.2)	32 (43.4)	0.178

Table 10.6 Lipid profile in patients with and without C allele:

	AC n = 11 Median (range)	AA n = 53 Median (range)	P value
Total cholesterol mmol/l	5.0 (3.3 – 7.3)	5.1 (3.0 – 8.7)	0.776
Triglycerides mmol/l	1.3 (0.7 – 3.2)	1.2 (0.4 – 3.9)	0.979
HDL-C mmol/l	1.6 (0.94 – 3.0)	1.4 (0.6 – 2.7)	0.618
LDL-C mmol/l	2.5 (1.7 – 4.1)	2.6 (0.33 – 5.0)	0.915
Ox-LDL-C mU/l	32 (13 – 57)	32 (14 – 77)	0.449
Hypertension n (%)	4 (36.4)	23 (43.4)	0.748

Table 10.7 Vascular study in patients with and without C allele:

	AC n = 11 Median (range)	AA n = 53 Median (range)	P-value
Resting diameter /cm	0.32 (0.24 – 0.39)	0.33 (0.25 – 0.43)	0.592
Resting flow ml/min	19.6 (8.0 – 63.6)	29.8 92.9 – 113)	0.036
FMD/cm	0.02 (- 0.01 – 0.03)	0.01 (- 0.02 – 0.04)	0.579
%FMD	5.5 (-2.6 – 11.5)	3.3 (- 6.3 – 13.7)	0.469
%GTN dilation	17.2 (2.8 – 44)	21.7 (13 – 30)	0.156

10.7 Summary:

- There is an association between SLE and E-selectin A561C gene polymorphism in the British and Spanish populations.
- We found no association between the mutant C allele and organ involvement or endothelial function.

Chapter 11

11 Discussion

11.1 Introduction:

Patients with SLE have an increased risk of CHD and with increased survival in SLE, CHD has emerged as a major cause of morbidity and mortality. In several large clinic series, the cumulative prevalence of CHD related events in SLE is 6 -10% and the mean age at first CHD event is 48 – 50 years (Bruce *et al* 2000a) In addition, several studies of sub-clinical atherosclerosis have detected carotid plaques or abnormal isotope myocardial perfusion in 30 – 40% of patients (Manzi *et al* 1999, Bruce *et al* 2000b). Classic Framingham risk factors do contribute to risk of CHD in SLE, however even after adjustment for these factors the risk remains 8 – 10 fold increased (Esdaile *et al* 2001). In the general population, endothelial dysfunction occurs early in the process of atherogenesis and it can be detected before other pathological changes. Measurement of FMD of the brachial artery is increasingly used, as a non-invasive method for assessment of endothelial function. Very little is known about endothelial function in SLE. As discussed earlier, we aimed to determine whether endothelial dysfunction occurs in SLE and whether it is related to classic risk factors and/or other markers of atherosclerosis in the context of SLE. In addition, we sought to explore some additional novel risk factors, which have emerged as potentially important in the general population to determine their prevalence and potential influence in the context of SLE.

In this section I will discuss several relevant aspects of the studies performed. In particular, I will discuss the patient cohort and its generalizability as well as considering in turn the important results of our studies on endothelial function, insulin sensitivity and E-selectin genetics.

11.2 Summary of the main results of the studies:

11.2.1 Endothelial function:

When we compared SLE patients and healthy controls, we found that, flow mediated dilation was significantly reduced in SLE. When we restricted our analysis to Caucasian patients < 50 years old and matched controls, FMD was

also significantly lower in SLE patients. In contrast, endothelium-independent dilation did not differ between patients and controls (Tables 8.9, 8.10)

We then used linear regression analysis in a group that included 62 patients and 38 controls. In univariate analysis, factors associated with FMD were SLE (P = 0.001), resting diameter (P = 0.027), systolic blood pressure (P = 0.002) and 10-year risk of CHD (P = 0.038) (Table 8.13). In stepwise multiple regression, factors independently associated with FMD were systolic pressure (P = 0.007), BMI (P = 0.024) and SLE (P = 0.049) (Table 8.14). We also used linear regression analysis to assess the influence of different factors within SLE patients, in univariate analysis, factors associated with percentage FMD were resting diameter, systolic pressure, carotid IMT and SLEDAI (Table 8.26). Again in a stepwise multiple regression model, carotid IMT alone was independently associated with the FMD (P = 0.026) (Table 8.25).

11.2.2 Insulin sensitivity:

In the first study we found that although the fasting glucose was similar in SLE and control subjects, SLE patients had significantly lower insulin sensitivity, higher fasting insulin and higher pancreatic β-cell function. SLE patients also had significantly higher triglycerides, hs-CRP, IL-6 and lower HDL-C (Tables 9.2, 9.3).

Within SLE patients, fasting insulin showed no correlation with SLEDAI nor with IL-6 and hs-CRP levels. There was however a strong positive correlation with BMI (r = 0.518, P = 0.006), waist/hip ratio (r= 0.406, P = 0.036), triglycerides (r= 0.621, P = 0.001), and ox-LDL-C (r = 0.516, P = 0.006) and a negative correlation with HDL-C (r = - 0.501, P = 0.008) (Table 9.5). While fasting insulin was higher in patients taking steroids (Figure 9.7), this did not reach statistical significance and there was no correlation of the fasting insulin with current steroid dose or with the mean daily dose of steroids over the previous six months (Table 9.4).

In healthy controls, fasting insulin showed similar correlation with BMI, waist/hip ratio, TGs and HDL-C. The strength of association with hs-CRP was similar to the SLE patients. In contrast to SLE however, fasting insulin did not correlate with ox-LDL-C, but there was a significant positive correlation with IL-6.

In our second study, we found that 11(18%) patients had the metabolic syndrome. There was no difference in the current or mean steroid dose over the past six months and also no difference in disease activity in those with the syndrome. The metabolic syndrome was associated with higher fasting insulin, ox-LDL-C, and a trend towards higher hs-CRP and IL-6 levels (Tables 9.12, 9.13).

11.2.3 E-selectin study:

We studied the E-selectin gene A561C polymorphism in three different ethnic groups. The frequency of the C allele was significantly higher in UK and Spanish Caucasians patients compared to their respective controls; this was not seen in those of Turkish origin (Table 10.4). We also performed genotyping of DNA samples in the patients who participated in our vascular study. Eleven (17.2%) patients had AC genotype. There was no association of the C allele with organ involvement nor with endothelial function.

11.2.4 SLE cohort:

It is important when considering SLE studies to determine the generalizability of the cohort studied. We therefore compared our patients with several clinic cohorts in the published literature form Birmingham UK, Europe and North America. The mean age of our patients was comparable to other studies (Table 11.1). Ethnic and socio-economic variation is known to have effects on disease course and expression. Caucasians represent a majority of our study group as well as in the Toronto cohort. In Baltimore African-Americans represent, nearly 50% and they have higher prevalence and more severe renal disease compared to the Caucasians.

Disease duration in our patients was similar to that in the Toronto study, but longer compared to that in other studies. However, a study of 300 patients attending a clinic in London (Moss *et al* 2002) showed mean (range) disease duration of 13 (1.0 – 37), which is quite similar to our group.

Anti-ds-DNA antibodies were positive in 45% of our patients, which is comparable to other UK studies, for example 54% in Nottingham (Hopkinson *et al* 1994) and 55% in London (Worrall *et al* 1990)

The frequency of renal involvement in our patients was 17%, which is low compared to most of other studies e.g 22% in Nottingham (Hopkinson *et al* 1994) and 26% and 33% in two studies from London (Worrall *et al* 1990 and Moss *et al* 2002). A low frequency of renal involvement 20% was reported in a study from Iceland, which captured all patients with SLE in the Iceland. (Gudmundsson and Steinsson 1990). Also in a multi-centre study of 1000 patients from different European countries, 22.2% had renal involvement. (Cervera *et al* 1999). Our cohort was drawn principally from the Rheumatology Department at Manchester Royal Infirmary with no formal link with the nephrologists was established prior to this study getting underway. This may explain the low prevalence of renal disease. Conversely, the severity of SLE, using renal disease as a surrogate, was comparable to the Iceland study and this might suggest less referral bias in our cohort than occurs in other major centres.

Generally disease activity was low in our group. Twenty-six (41%) had a SLEDAI score of zero and only one patient had a score of 12. The mean SLICC/DI score and percentage of patients with a SLICC score of ≥1 (42%) was lower in our group compared to some other North American and European studies (60% and 70% respectively) (Zonana-Nacash *et al* 2000, Gilboe *et al* 2001). However, it is comparable the Birmingham UK cohort. In accord with most studies, the most frequent damage was seen in the musculo-skeletal and neuro-psychiatric systems. However, our group had a low prevalence of cardio-respiratory damage. The reason for this is not known, but may reflect generally milder disease and /or better control of disease activity. The low prevalence of damage in renal system in our group, and also in the Birmingham group may reflect low renal system involvement by the disease process. Importantly, it may also be related to selection bias due to missing a proportion of patients with nephritis seen by renal physicians only.

The prevalence of CHD in our group was 3%, compared to 6 – 10% in the large North American cohorts Toronto, Baltimore and Pittsburgh. One patient in our clinic who had an MI at a younger age refused to participate in the study, including this patient would raise the prevalence to 5%, which is closer the lower end of the reported prevalence. The cross sectional nature of our study

increases the chance of missing CHD cases especially angina. Also in the North American cohorts, the 6-10% prevalence was from prospective studies and represented a cumulative prevalence, which included also those who died of CHD. The prevalence of storke or transient ischemic attacks in our group was 12.5%. This is similar to the reported in some other studies (3% and 15%) (Badui *et al* 1985, Futrell *et al* 1989).

We compared CHD risk factors in our group to a large recently reported case control study of CHD risk factors from the Toronto SLE cohort (Bruce *et al* 2003) (Table 11.2). This group of 250 patients was comparable to our group in age and ethnic origin, with a majority being Caucasians, males were excluded. This study also used similar definitions for hypertension, diabetes, and cut-off points for abnormalities in lipid profile. Our group was older and therefore more likely to be postmenopausal. This may in part explain the higher prevalence of hypertension and obesity. We did however have less smokers and less patients with low HDL-C. Importantly however, compared to their respective controls, both SLE populations were more likely to have hypertension and diabetes, higher TGs and VLDL-C.

Taken together, our patients are broadly comparable, although perhaps older and with increased disease duration with milder diasease than some other cohorts reported in UK and North America. The overall levels of disease activity and damage were low, suggesting inclusion of milder SLE patients. These factors clearly need to considered when interpreting our results and generalizing them. The milder disease observed may therefore have made it more difficult to detect any differences in the factors studied especially if they are disease activity and therapy related. Conversely, longer disease duration may increase the chances of developing changes that reflect CHD risks.

Table 11.1 Principle SLE characteristics of our group and in other SLE cohorts

	Our study	Birmingham † 348	Toronto *	Baltimore ***	Norway ****
Age	48 (21 – 73)	41(18 – 82)	44.5 (12.0)	Mean (SD) 38 (12.1)	45.5 (18 – 82)
Ethnicity Caucasian	83%	60%	77%	44%	100%
Disease duration/ys	11 (1.0 – 40)	7 (1 – 43)	13.7 (9.7)	8.1 (6.9)	6.1 (0.0 – 31)
SLEDAI	2.4 (2.3)	-	4.5 (4.5)	-	6.5 (5.7)
SLICC/ACR-DI					
SLICC ≥1	42%	44.8%		60% **	70%
Median (range)	0 (0 – 4)	0 (0 – 12)		1.0 (0.0 – 11)	
Mean (SD)	0.72 (0.98)		1.49 (1.6)	1.51 (1.97)	1.82 (1.99)
Main systems affected					
Neuropsycaitic	20%	14%		20%	23%
Musculoskeletal	13%	17%		22%	34%
Cardio-respiratory	5%	18%		16%	38%
Renal	5%	5%		15%	8%

*Bruce *et al* (2003). ** Zonana-Nacash *et al* (2000). *** Petri *et al* (1992). **** Gilboe *et al* (2001). †Yee *et al* (2003)

Table 11.2 Comparison of CHD risk factors in our patients and in a study from the Toronto cohort:

	Our cohort (n = 64)	Toronto* (n = 250)
	%	%
Atherosclerosis: MI CVA	 3.1 12.5	 **
Age /years mean (SD)	47.8 (12.3)	44.5 (12)
BMI > 27.0	38.0	28.0
Waist/hip ratio > 0.85	22	15.6
Hypertension	48.0	33.0
Diabetes	4.7	5.0
TC > 5.2 mmom/l > 6.7 mmol/l	 43.8 9.4	 34.0 6.7
LDL-C > 3.4 mmol/l	1.7	3.8
HDL-C < 0.9 mmom/l	6.3	13.0
TGs > 2.5 mmol/l	9.4	6.3
VLDL-C > 0.92 mmol/l	1.7	6.3
Current smoking	10.9	17.0
Post-menopausal	48.0	36.0
Family history of CHD	23.4	20.0

* Bruce *et al* (2003). ** CHD cases excluded.

11.3 Assessment of FMD:

Our first task was to establish the technique of measuring FMD of the brachial artery. To validate our technique, we initially assessed within-subject variability of resting diameter measurements. The within-subject variability is affected by two factors; physiological variability and intra-observer variability. The latter depends on the accuracy of the operator and the inherent "noise" in the machine. In order to eliminate the physiological variability, our technician measured the resting diameter of one subject in a single investigation ten times from a single video recording. The CV% was 2.7% compared to 4.5% when the resting diameter of the same subject was measured 20 times over a period of two months. This suggests that, 60% of the variability in resting diameter may be due to inaccuracy of measurements and 40% due to physiological variability. Other investigators have reported the CV% of within subject variability of the resting diameter to be between 1.5% and 6% (de Ross *et al* 2003).

We also assessed intra- and inter-observer variability of resting diameter and percentage FMD by randomly re-measuring 15 recorded studies by the same observer and once by another observer. As shown in table 8.1, the mean (SD) absolute difference in resting diameter between the first and second measurements by one observer was 0.005 (0.014) cm. As the machine can only detect diameter changes of ≥ 0.01 cm, the mean difference would be rounded up to 0.01(0.01) cm, which represent 2.9% of mean resting diameter. Lima *et al* (2002) have used the same protocol we used and reported intra-observer variability that was comparable to our finding [mean (SD) difference of 0.008 (0.016) cm]. The intra-class correlation coefficient for intra-observer variability of resting diameter and percentage FMD was good 0.934 [95%CI (0.558 – 0.950)] and 0.820 [95%CI (0.300 – 0.965)] respectively (Table 8.1). This was also comparable to intra-class coefficients of 0.81 and 0.90 that have been reported by Lima *et al* (2002) and Welsch *et al* (2002) respectively. In our study, there was a good correlation between the first and the second 15 measurements of percentage FMD by one observer (r = 0.750), this is similar to that reported by Welsch *et al* (2002) after 13 scans were read twice by one observer (r = 0.740). However, we found poor inter-observer variability for percentage FMD (Table

8.2). This is likely to be because the second observer had no past experience in vascular ultrasound.

To assess the reproducibility of FMD over time, we studied one subject 20 times over a period of two months. The mean (SD) percentage FMD of all the studies was 8.3 (3.5) % with a CV% of 42%. The mean (SD) of the difference from the mean percentage FMD of all the studies was 0.085 (3.5%). High variability in percentage FMD has also been reported by Hardie *et al* (1997). In their study of 19 subjects on two separate occasions, the mean (SD) of the difference between the two occasions was 0.57 (6.83)%, while the mean (SD) percentage FMD of all the studies was 3.0 (2.7)%. De Roos *et al* (2003) studied 13 subjects on six occasions and also reported a high CV% (50)% of percentage FMD. However, some groups reported a low within subject variability in percentage FMD between studies with a CV% as low as 1.8% (Celermajer *et al* 1992) and 2.3% (Sorensen *et al* 1995). However, Woodman *et al* (2001) commented on these as inappropriately low as they were a result of expressing FMD as percentage of the baseline diameter rather than percent change, the appropriate CV% would be 22% and 28% respectively. Larger mean percentage FMD appears to result in smaller CV%. For example Liang *et al* (1998) reported a CV% of 10.8% in a group of 30 subjects with a high mean percentage FMD of 10.8%. The CV% for resting diameter is usually 10 to 20 fold lower than the CV% of percentage FMD. For example, in a study to determine reproducibility with a mean (SD) resting diameter of 0.40 (0.01) cm and a mean (SD) percentage FMD of 10% (2.5%), the CV% for resting diameter and percentage FMD will be 2.5% and 25% respectively. Our high CV% for percentage FMD may not be completely out of keeping with other studies. There are inherent problems with the technique and the limits of resolution for the ultrasound probe makes the variability of percentage FMD quite large. Several studies have used computer software to increase the accuracy of diameter measurements and to reduce variability in FMD. Recently, Sonka *et al* (2002) introduced an automated method for analysis of brachial artery ultrasound images. In this method the sonographer identifies the initial region of interest of an artery. All the subsequent analyses are fully automated. These involve automated learning of vessel wall properties, border detection in image

sequence, border quality control in individual frames, temporal diameter function quality control and calculation of indices of vascular function. Vessel diameter is imaged continuously on line for 10-20 seconds for resting diameter and for two minutes after cuff deflation for maximum dilation. The images are digitized at a resolution between 0.03 – 0.06 mm/pixel. Using the automated method for repeated diameter measurements, the mean difference was close to zero and 95% of the differences were within a range of ± 1.5 pixels, which correspond to about 3% of a mean diameter. For the manual method, the mean difference was – 1.42 pixels and 95% of the differences were within a range of -1.5 to + 2.4 pixels or about – 11% to + 5% of a mean diameter. This new technique reduces variability, increases accuracy, decreases inter and intra-observer as well as variability between institutions and significantly reduced the analysis time. This is currently employed in a large cross sectional study as part of the Framingham study, which involve more than 3,300 subjects (Benjamin *et al* 2001). There is also evidence from several studies that FMD can vary considerably in response to several factors, such as fasting state, time of the day, level of exercise, smoking, alcohol and mental stress. Our protocol sought to minimize these sources of variability. What these show however is that endothelial function is very sensitive to many factors. It may be impossible to fully control for all sources of variability and this also likely contributes to some of the variance others and we have found.

We therefore, established the technique of assessment of endothelial function and our validity results are comparable to those in other published studies. For FMD measurement, one experienced technician should record scans and take measurements, as a second observer clearly introduces excessive variability. In a cross-sectional study such as this, it may introduce excessive noise and increase the likelihood of missing important differences.

11.4 Endothelial dysfunction in SLE:

We found impaired endothelial function in SLE. Two other studies have reported similar findings of impaired endothelial function in SLE patients. In a study from Sao Paulo, Lima *et al* (2002) found that the mean (SD) percentage FMD in SLE was 5.0 (5.0)% compared to healthy controls. In this study, postmenopausal

women and subject with CHD risk factors were excluded. Piper *et al* (2002) in a UK cohort found that SLE patients had a median (IQR) percentage FMD of 5.6 (3.1 – 7.2)% compared to 8.0 (6.3 – 9.3)% in controls. The differences in subject selection and variation in the technique used will clearly influence the absolute values between studies. Nevertheless, these studies and our own have confirmed endothelial function is impaired in SLE. The role of classic risk factors for CHD was not clear from these previous studies. Piper *et al* (2002) found a significant negative correlation between total cholesterol and percentage FMD ($r = -0.442$). In contrast Lima *et al* (2002) found no association with cholesterol or blood pressure, although, it should be noted that this study excluded patients with hypertension and hyperlipidaemia and therefore may be limited in its ability to detect an association with these factors. For our study we did not strictly match patients and controls. A recent large cohort control study found that SLE patients have a high prevalence of several key cardiovascular risk factors that are likely to reflect population characteristics, which need to be taken into consideration when assessing CHD risk and atherogenesis in SLE. In particular, SLE patients were more likely to have hypertension and to be postmenopausal at a particular age. They also had more risk factors per patient compared to controls (Bruce *et al* 2003 in press). These finding are similar to our results. In our population, patients also had lower HDL-C. This has also been found by others when comparing patients and controls and probably reflects the reduction in HDL-C associated with inflammatory states as this has also been observed in rheumatoid arthritis patients (Borba and Bonfa 1997, Heldenberg *et al* 1983). Therefore, in order to adjust for these differences, we performed a multivariate analysis on all patients and controls, in which we included demographic and classic Framingham risk factors. Of the classic risk factors, systolic blood pressure was the most strongly associated with reduced percentage FMD. We estimated that each 10 mmHg increase in systolic pressure was associated with a 0.89% reduction of percentage FMD. Several other studies in the general population also have shown that blood pressure is strongly associated with endothelial dysfunction (Panza *et al* 1990, Li *et al* 1997). In addition, we also found that BMI was positively associated with percentage FMD. This association was seen in

multivariate but not in univariate analysis. Most studies suggest that increased body mass index and central obesity are associated with impaired endothelial function. This may therefore represent a chance finding. Alternatively since the majority of this population had a BMI within normal range (< 30kg/m^2) and also had SLE, higher BMI may reflect better overall nutritional status associated with this inflammatory disease.

Importantly however, we found an independent effect of SLE on endothelial function. Other studies have suggested an influence of CHD risk factors (Piper *et al* 2000) or despite attempts at close matching have still noted significant differences in risk factor status between SLE patients and controls (Lima *et al* 2002). Therefore, it remains an open question whether SLE exerts an effect on endothelial function independent of the classic risk factors. However, our finding that SLE is an independent predictor of endothelial dysfunction reflects the clinical observation by Esdaile *et al* (2001) that, classic risk factors do not explain all the risk of CHD, and a diagnosis of SLE by it self is a significant risk factor.

As discussed in chapter 4 (page 70), normal endothelial function is maintained by the balance between basal NO and O^-_2 anion, increased production of O^-_2 anion reduces bioavailability of NO that results in impairment of EDD, which is used as a surrogate for endothelial dysfunction. The shift in the balance between NO and O^-_2 also switches-off the anti-atherogenic properties of the endothelium and enhances proinflammatory, proliferative and procoagulant properties which promotes progression of atherosclerosis. The presence of endothelial dysfunction allows other risk factors for atherosclerosis to exert their effects in a pro-atherogenic environment. This hypothesis explains why certain individuals do not develop atherosclerotic complications in the presence of several CHD risk factors. There is evidence that many of the risk factors such as hypercholesterolaemia, hypertension, hyperhomocysteinaemia, diabetes and smoking are associated with increased oxidant stress and excessive production of O^-_2. Increased oxidant stress is believed to be a major mechanism in the pathogenesis of endothelial dysfunction, and is also a common final pathway for the effect of several risk factors on the endothelium. Increased oxidant stress has been observed in SLE patients. Iuliano *et al* (1997) found enhanced urinary

excretion of isoprostanes, which is especially pronounced in patients with APL antibodies. More recently, Nuttall *et al* (2003) have found a highly significant increase in concentrations of lipid peroxides; a marker of oxidative damage, as well as higher levels of the small dense LDL-C sub-fraction in SLE. These small dense LDL-C particles are more susceptible to oxidation. Several studies have found increased NO production in SLE in association with disease activity (Belmont *et al* 1997, Gilkeson *et al* 1999). NO reacts with O^-_2 to produce peroxynitrite, a highly reactive molecule, which mediates oxidative damage associated with inflammatory conditions through nitration of tyrosine residues on proteins. Nitrotyrosine levels were found in significantly higher levels in patients with diabetes mellitus (Ceriello *et al* 2001) and in patients with CHD (Shishehbor *et al* 2003) compared to controls. After adjustment for the Framingham risk factors and CRP, nitrotyrosine levels in the upper quartiles were associated a relative risk for CHD of 4.4 95%CI (1.8 – 10.6), nitrotyrosine levels were reduced by 25% after treatment with statins for 12 weeks (Shishehbor *et al* 2003). In SLE patients, levels of nitrotyrosine level were significantly higher than in controls and higher in patients with active disease (Gilkeson *et al*, Oates *et al* 1999).

In our study, patients with APL antibodies had significantly higher levels of ox-LDL-C, LDL-C and triglycerides levels (Table 8.19). However, we found no correlation between FMD and the levels of ox-LDL as a measure of oxidant stress. Ox-LDL may not be the best measure of oxidant stress as it strongly correlates with LDL-C levels ($r = 0.700$, $P < 0.001$). Other markers such as isoprostanes or nitrotyrosine alone or in combination may be a better alternative to reflect oxidative stress in SLE and are worthy of further study.

Within SLE patients only, univariate analysis showed that percentage FMD correlated negatively with resting diameter, systolic pressure, IMT and positively with SLEDAI. A multivariate analysis showed that IMT was independently associated with percentage FMD. The negative association of carotid IMT confirms our initial hypothesis that endothelial dysfunction is associated with markers of atherosclerosis. Twelve of our patients had evidence of carotid plaque and in this group there was also a trend towards lower percentage FMD compared to patients without carotid plaque. They also had a significantly lower

GTN dilation (P = 0.014). Within our SLE patients, we also found a negative correlation between percentage GTN dilation and IMT. Impaired GTN dilation has been noted in patients with established atherosclerosis in the general population. Adams *et al* (1998) have also shown that smooth muscle dysfunction occur in asymptomatic subjects with impaired endothelial function.

Patients with carotid plaque also had a significantly higher resting diameter. Celermajer *et al* (1992) noted larger resting diameter in subjects with atherosclerosis. In a study of 378 women with chest pain, Holubkov *et al* (2002) also found that a larger resting brachial artery diameter was an independent predictor of significant CHD after adjustment for age, body size and other CHD risk factors. Women with a resting diameter of >4.1 mm had a 3.6-fold (95%CI 1.8 – 7.1, $p < 0.001$) increased risk of significant CHD compared to women with resting diameter of ≤ 3.6 mm.

Our findings in multivariate analysis as well as in patients with carotid plaque confirm that in the context of SLE, endothelial dysfunction is associated with other markers of atheroma development. Piper *et al* (2001) noted that CHD risk factors contribute to endothelial dysfunction in SLE. Carotid IMT will, however, represent the final common pathway of several other risk factors and will make it difficult for any single risk factor to remain in a multivariate analysis. When we excluded carotid IMT from our analysis of SLE patients, systolic pressure was alone independently associated with percentage FMD (P = 0.028).

Within our SLE patients, disease activity, assessed by SLEDAI, was positively associated with percentage FMD and negatively with resting diameter. Patients with a SLEDAI ≥ 6 had a tendency for lower resting diameter (P = 0.083) and higher percentage FMD (P = 0.058) compared to patients with SLEDAI < 6. This finding of enhanced endothelial function in inflammatory disease was also noted in a study of patients with primary systemic necrotizing vasculitis (SNV) (Bruce *et al* 1997). This observation is however controversial, and recently Raza *et al* (2000) found that patients with primary SNV showed evidence of impaired FMD, which improved following specific therapy with IV cyclophosphamide. The authors hypothesised that inflammation associated with primary SNV induced stunning and impaired function of the endothelium. Lima *et al* (2002) found no correlation between disease activity and FMD or GTN dilation.

There is evidence, that SLE patients have increased iNOS expression and this may also influence endothelial responses and reduce vascular tone. Lima *et al* (2002) found a weak positive association between resting diameter and SLEDAI. On the other hand, there is also evidence of reduced eNOS enzyme activity and increased oxidant stress in inflammatory states, will reduce NO bioavailability, may alter the vascular tone balance toward constriction and hence lower resting diameter. This lower resting diameter paradoxically may lead to a spuriously increase in percentage dilation for any given absolute FMD change. Our results suggest this since SLEDAI correlated negatively with resting diameter and positively with percentage FMD, but adjusting for resting diameter in our multivariate analysis eliminated the effect of SLEDAI in this univariate finding. It should also be noted that this is a cross sectional study and a high proportion of patients had a low SLEDAI score. Also it is important to note that while there was a correlation between SLEDAI and FMD, patients with SLEDAI ≥ 6 still had impaired endothelial function [median (range) 5.5 (0.0 – 13.7)%]. We also noted that no difference in the proportion of patients with percentage FMD ≤ 4.5% between active and inactive disease groups 43% vs 60% respectively (Tables 8.16, 8.17). Therefore, these results may not suggest that endothelial function is "improved" during active disease. A prospective study of patients with high disease activity before and after treatment would however be important to further study the effect of disease activity on vascular responses.

With recent evidence that inflammation plays an important role in initiation, progression and clinical presentation of atherosclerosis in the general population, in the context of SLE, chronic inflammation is hypothesised to be the key additional factor that contributes to the risk of atherosclerosis and CHD events. Although inflammatory markers hs-CRP and IL-6 were higher in SLE patients, we found no correlation between these markers and FMD in our cohort.

Endothelial function in an individual may be viewed as an integrated index of all the cardiovascular risks and vasculo-protective mechanisms including yet unknown metabolic and genetic factors. Endothelial dysfunction may represent a final common pathway, which translates the cardiovascular risk into

atherogenesis and a predisposition to vascular events. Our finding that SLE predicts endothelial dysfunction independent of the classic risk factors, suggests that additional factors associated with SLE are also likely to impair endothelial function. Other mechanisms may be important for example; increased oxidant stress and other metabolic abnormalities such as hyperhomocysteinaemia. Asymmetric dimethyl arginine (ADMA), an inhibitor of eNOS enzyme activity, which plays an important role in the regulation of NO bioavailability, has recently been found to be raised in patients with hypertriglyceridaemia, insulin resistance, hyperhomocysteinaemia, and chronic renal disease (Lundman *et al* 2001, Stuhlinger *et al* 2002 and 2001, Cooke *et al* 1998). Pathways such as these require further investigation in the context of SLE. Altogether therefore in SLE, classic risk factors are likely to contribute to endothelial dysfunction, but are not sufficient in themselves to completely explain the occurrence of endothelial dysfunction. This point is of relevance when considering interventions to improve endothelial function and/or reduce CHD risk in SLE.

Intervention to improve endothelial function can be considered in three broad categories:

1- Control of classic risk factors, particularly, hypertension and dyslipidaemia with the use of lipid lowering agents (statins) and ACE inhibitors:

Improvement in endothelial function has been suggested to explain the early and substantial reductions in major cardiovascular events associated with cholesterol lowering in the large statin trails. There is *in vitro* evidence that statins can directly up-regulate eNOS (Laufs *et al* 1998), and in a dose dependent effect, statins reduce the ability of human monocytes to modify LDL-C by oxidation (Giroux *et al* 1993). In a study on normocholesterolaemic rabbits with spontaneous hypertension, treatment with atorvastatin for 30 days significantly improved endothelial function, reduced production of oxygen free radicals and reduced systolic pressure (Wassmann *et al* 2001). Following induction of atherosclerosis in 32 monkeys with a high cholesterol diet for two years, a lipid lowering diet was then started with or without pravastatin. Using quantitative angiography, although, there was no change in plaque size, the

group receiving pravastatin however showed improved coronary dilation in response to acetylcholine. Pathologically the plaques contained less inflammatory cells and appeared more stable. In contrast, the untreated group showed coronary constriction to acetylcholine and more inflammatory changes in their plaques (Williams *et al* 1998a). Clinical trials in humans also showed improvement in endothelial function with statins in patients with CHD (Treasure *et al* 1995) and in IDDM and NIDDM independent of their lipid lowering effect (O'Driscoll *et al* 1997, 1999).

Angiotensin II has been implicated in several mechanisms leading to endothelial dysfunction mainly increased O^{-}_{2} production via activation of the pro-oxidant enzymes NADPH oxidase (Hanna *et al* 2002) and up-regulation of pro-inflammatory molecules such as NF-κB and MCP-1 (Hernandez-Presa *et al* 1997). There is evidence that the long term benefit of ACE inhibitors may be related to reduction in oxidant stress and inflammation. In the HOPE study, the significant reduction in cardiovascular morbidity and mortality with ramipril was achieved without an accompanying anti-hypertensive effect (Yusuf *et al* 2000).

In the TREND study, normotensive patients with angiographically documented CHD treated with quinapril, showed significant improvement in coronary endothelial function despite no change in blood pressure (Mancini *et al* 1996). Also in a study of 19 patients with mild atherosclerosis, Prasad *et al* (1999) showed a significant improvement of EDD of the coronary arteries in response to inalprilat.

2- Identification of novel risk factors and to manage them accordingly:

Homocycteine is raised in SLE patients compared to healthy controls (Bruce et al 2003 and Petri et al 1996). Hyperhomocysteinaemia is a novel factor and is associated with increased risk of CHD. Chambers et al (2000) found that Hyperhomocysteinaemia is independent risk factor for CHD in Europeans and Indian Asians. Each 5 μmol/l increase in fasting plasma homocysteine is associated with OR (95%CI) of CHD of 1.3 (1.1 – 1.6) and 1.2 (1.0 – 1.4) for Europeans and Indian Asians respectively (Chambers et al 2000). Hyperhomocysteinaemia is associated with increased O^{-}_{2} production, decreased bioavailability of NO and increased formation of $OONO^{-}$ in the

arterial wall (Ungvari 2002). Hyperhomocysteinaemia impairs endothelial function, and in a study by Woo et al (1997) it predicted impaired FMD independent of age, BMI and blood pressure. Woo et al (2002) also showed in a study of 29 adults with hyperhomocysteinaemia that long term supplementation with folic acid significantly improved endothelial function and reduced homocysteine levels. SLE patients taking methotrexate should also receive folic acid.

3- Treatment of endothelial dysfunction on its own merits, for example use of omega-3 fatty acid, antioxidants vitamin C and vitamin E, aspirin and NO donors.

Several studies have shown that omega-3 polyunsaturated fatty acids in the form of oily fish or supplements of fish oil (eicosapentanoic and docosahexaenoic acids) have recently been recommended by the American Heart Association for their cardio-protective effects (Kris-Etherton *et al* 2003). There is strong evidence from epidemiological studies and randomized controlled trials that omega-3 fatty acids have a beneficial effect on cardiovascular disease either in primary or secondary prevention. Kris-Etherton *et al* (2002) reviewed this evidence and the proposed mechanisms of action of omega-3 fatty acids, which include lowering triglycerides level by 20 – 40%, improvement of endothelial dysfunction, suppression of inflammation, decreased risk of thrombosis and decrease in progression of atherosclerosis. Omega-3 fatty acids are of interest in the context of SLE, as they have shown to improve disease activity due to their anti-inflammatory and immuno-modulatory (Duffy *et al* 2003).

Vitamin C is an antioxidant agent, which has been shown to improve abnormal coronary endothelial function in hypertensive patients with patent coronary arteries (Solzbach *et al* 1997). Vitamin C improved FMD in patients with congestive heart failure when given either acutely intra-arterial infusion or after four weeks of oral therapy. The degree of inhibition of FMD by LNMMA was increases after acute as well as chronic use of vitamin C, which indirectly suggest that vitamin C increased bioavailability of NO (Hornig *et al* 1998).

Similarly, vitamin C improved EDD in a group of patients with type II diabetes (Ting *et al* 1996).

Aspirin use is associated with a significant reduction in cardiovascular events. In addition to its anti-platelet effect, which results from irreversible inhibition of cyclooxgenase in platelets, recent studies have highlighted other possible mechanisms for its cardioprotective effects. Grosser and Schroder (2003) showed that pre-incubation of endothelial cells with aspirin protected them from the O^{-}_{2} mediated toxicity. It enhanced eNOS activity and increased intracellular cyclic GMP. L-NMMA inhibited the cytoprotective effect of aspirin, which suggest that the effect of aspirin involves NO/cyclic GMP pathway. This cytoprotective effect of aspirin was not shared by salicylate or other non-steroidal anti-inflammatory drugs. Steer *et al* (1997) showed both *in vitro* and *in vivo* that aspirin significantly reduced LDL-C peroxidation.

Xanthine oxidase is a pro-oxidant enzyme and an important source of reactive oxygen species O^{-}_{2}, H_2O_2 and hydroxyl group as a by-product of a reaction that converts hypoxanthine into uric acid. Inhibition of xanthine oxidase with allopurinol and oxypurinol improved FMD in patients with hypercholesterolaemic and patients with type II diabetes respectively (Cardillo *et al* 1997 and Butler *et al* 2000).

11.5 Insulin sensitivity in SLE:

In recent years, insulin resistance and hyperinsulinaemia have been increasingly recognised to have a significant impact on the risk of CHD in the general population. Several cross-sectional studies of SLE cohorts have suggested that diabetes mellitus may occur more frequently in this population than expected. For example, Petri *et al* (1992) found 7% of patients with SLE had diabetes mellitus and that 10% of patients with SLE had glucose intolerance (Petri *et al* 1994a). More recently, a cohort control study found diabetes to be significantly more common in patients with lupus than in a population control sample (Bruce *et al* 2003).

In view of the chronic inflammatory nature and the lipid profile abnormalities in SLE, which frequently resemble the atherogenic dyslipidaemia associated with insulin resistance syndrome (high TGs and low HDL-C levels), and since

increased systemic inflammation is associated with metabolic syndrome, the possible role of insulin resistance in SLE needed to be studied. As far as we are aware, this is the first study that has systematically examined insulin sensitivity in a cohort of SLE patients. One study included a small number of patients with SLE as part of broader study of insulin handling in inflammatory rheumatic diseases (Paolisso *et al* 1991).

In the first part of this study, we found significantly reduced insulin sensitivity in a group of lupus patients compared to population controls. The median HOMA-S in the patient group is similar to the degree of insulin sensitivity seen in newly diagnosed type II diabetes mellitus and in women with polycystic ovary syndrome (I Laing personal communication). Patients with SLE however maintain their euglycaemic state by significantly increasing insulin secretion from pancreatic beta cells. In keeping with decreased insulin sensitivity in SLE being part of the overall "metabolic syndrome", we found a strong correlation between fasting insulin and several components within the insulin resistance cluster, especially BMI, waist/hip ratio, TGs and HDL-C. In SLE patients, there was also a strong positive correlation between levels of fasting insulin and ox-LDLC (Table 9.5). Fasting insulin in healthy controls showed similar correlations with BMI, wais/hip ratio, TG and HDL-C, but had no correlation with ox-LDL-C. There was no difference in paroxonase activity between patients and controls (Table 9.5). With regard to the factors that may promote the development of reduced insulin sensitivity in lupus, we found only a modest effect of corticosteroids in this context. There was no significant correlation between fasting insulin and current or mean dose over the past six months (Table 9.4). Patients currently taking steroids did have higher fasting insulin, β-cell function and lower insulin sensitivity that were not statistically significant (Table 9.7). However, even after adjustment for steroid therapy, SLE patients had a tendency for higher fasting insulin ($P = 0.110$) and lower insulin sensitivity ($P = 0.051$) compared to healthy controls (Figures 9.7, 9.8). While lack of statistical power in this study needs consideration, it appears likely that steroid therapy may not be the sole factor driving decreased insulin sensitivity in SLE. Although disease activity in our group was generally low there was no correlation between fasting insulin and SLEDAI (Table 9.4), nor was there a difference in

fasting insulin when we compared patients with active and inactive disease according to our categorical definition (P = 0.549).

Increased basal inflammation is a strong risk factor for CHD in the general population and is included among the risk factor cluster of the insulin resistance syndrome. Baseline levels of hs-CRP are highly predictive for future CHD (Ridker *et al* 1997). Also have prognostic value in patients presenting with acute coronary syndromes (Liuzzo *et al* 1994). In the Physician Health Study, aspirin treatment was associated with significant reduction in CRP and the best risk reduction achieved was in subjects with the highest quartile of baseline hs- CRP (Ridker *et al* 1997). Similarly, benefit from pravastatin in the Cholesterol and Recurent Events (CARE) where mainly observed in those with higher levels of hs-CRP. Also in this study, statin therapy was associated with significant reduction in CRP levels independent of the lipid lowering effect (Ridker *et al* 1999). This suggests that statins may have an important anti-inflammatory effect. There is also experimental evidence that CRP may not be just a marker of CHD risk, but itself promotes atherogenesis and thrombogenesis. CRP reduces expression of eNOS (Venugopal *et al* 2002, Verma *et al* 2002). Incubation of human endothelial aortic cells with CRP resulted in a dose and time dependent increase in the expression and activity of tPAI-1 (Devaraj *et al* 2003). CRP has been shown to activate monocytes and increase their adhesion to endothelial cells (Woollard *et al* 2002).

In this study, we found a trend toward higher hs-CRP in SLE patients compared to controls (P = 0.066). The difference reached statistical significance when we compared all our patients and controls [median (range) 2.6 (0.15 – 47.6) vs 1.52 (0.17 – 22.5) mg/l, P = 0.046]. IL-6 was also significantly higher in SLE patients compared to controls [3.6 (1.0 – 17.7) vs 1.0 (1.0 – 14.7) pg/ml, P = 0.002].

We found a similar correlation between hs-CRP and IL-6 in SLE patients and healthy controls (r = 0.518) and (r = 0.535) respectively. However, the inflammatory markers hs-CRP and IL-6 showed a positive correlation with fasting insulin in healthy controls but not in SLE patients, particularly IL-6. Interestingly, however, IL-6 also showed strong correlations with components of insulin resistance syndrome in healthy controls but not in SLE patients (Table

9.5), this may suggest that excessive production of IL-6 in SLE is chiefly derived from inflammatory cells and that adipose tissue contributes less to the circulating IL-6 levels in SLE. In our controls it is likely that adipose tissue represents a major source of IL-6.

There is evidence that the molecular basis of insulin resistance may be explained by the link between cytokines and insulin action. Several cytokines such as TNF-α, IL-6, IL-1 and interferon-γ have been shown in vitro to interfere with insulin action and cause a dose dependent decrease in insulin sensitivity (Hotamisligril *et al* 1994). In vitro studies suggest that TNF-α may be the key mediator of insulin resistance. TNF-α expression is increased in adipose tissue from animals and humans with insulin resistance and correlates with obesity and hyperinsulinaemia (Hotamisligil *et al* 1993, 1995). TNF-α suppresses insulin-induced tyrosine phosphorylation of the insulin receptor and its substrate and thus reduces insulin-stimulated glucose uptake (Hotamisligril *et al* 1994). In studies on human adipose tissue, Lofgren *et al* (2000) found a strong negative correlation between secretion of TNF-α from adipose tissue and maximum insulin-stimulated glucose transport independent of age and BMI or fat cell size. As much as one third of the variation in glucose transport is related to variation in TNF-α secretion. However, in vivo studies on humans showed conflicting results regarding the relationship between circulating TNF-α levels and insulin sensitivity. Infusion of anti-TNF-α antibodies did not improve insulin sensitivity in humans (Paquot *et al* 2000). This may suggest that the effect of TNF-α on insulin action is mediated locally in a paracrine fashion. Mohammed-Ali *et al* (1997) found no difference in TNF-α concentration between veins draining adipose tissue and peripheral veins suggesting minimal or no mobilization of TNF-α from adipose tissue.

Recent studies also suggest an important role of IL-6 in insulin resistance and CHD. Muller *et al* (2002) found that serum IL-6 was significantly higher in subjects with diabetes compared to those with impaired glucose tolerance (IGT) and higher in those with IGT than in non-diabetic controls. In contrast, TNF-α and its receptors were not raised in subject with IGT compared to controls. IL-6 is produced by a variety of cells including activated leukocytes adipocytes and

endothelial cells. In animal and human studies infusion of IL-6 resulted in hyperglycaemia and hyperinsulinaemia (Stith and Luo 1994, Tsigos *et al* 1997). Baseline levels of IL-6 and CRP also predicted development of type II diabetes independent of BMI, family history of diabetes and exercise (Pradhan *et al* 2001). Recently, Rotter *et al* (2003) showed in human adipose cells, that IL-6 and TNF-α have different effects on phosphorylation of insulin receptor substrates IRS-1. Both reduce expression of IRS-1 and GLUT-4 and both decrease insulin-stimulated glucose uptake. They also reported a marked increase in IL-6 in response to TNF-α from adipose cells. IL-6 and TNF-α gene expression was also markedly increased in adipose cells from non-obese but insulin resistant subjects. In a prospective study of 1293 subjects followed for 4.6 years in the Rural Health Study, Harris *et al* (1999) showed that elevated basal levels of IL-6 predicted cardiovascular and total mortality independent of prevalent CVD and traditional risk factors.

SLE is characterized by enhanced basal production of several inflammatory cytokines. In particular, IL-6 and TNF-α are implicated in the pathogenesis of SLE and both could equally be implicated in the development of insulin resistance in SLE. Therefore, insulin resistance in the context of SLE may represent a link between inflammation and risk of atherosclerosis, and may explain in part the increased risk of CHD in SLE.

Hypertriglyceridaemia is a common lipid abnormality in SLE patients. Increased lipolytic activity in adipose tissue leads to increased delivery of FFA to liver and excess production of VLDL-C from the liver. This mechanism underlies the dyslipidaemia of metabolic syndrome (Ginsberg *et al* 2000). In the general population hypertriglyceridaemia is an independent risk factor for CHD. Hokanson and Austin (1996) showed in their meta-analysis that a rise in TGs levels of 1 mmol/l is associated with a 14% to 37% higher incidence of cardiovascular disease after adjustment for other risk factors. Elevated TGs is associated with hyperinsulinaemia, insulin resistance, low HDL-C, higher small dense fraction of LDL-C, increased oxidative stress and central obesity (Grundy 1997). The finding that hypertriglyceridaemia is an independent risk factor suggest that some of the triglyceride rich lipoproteins (TRL's) are atherogenic. Recently, TRL's, which include VLDL-C, intermediate density lipoprotein (IDL),

chylomicrons and chylomicrons remnants have been implicated in the risk of CHD. Levels of both TRL's and HDL-C are mainly controlled by the activity of lipoprotein lipase, which facilitate their removal from the circulation (Huttunen *et al* 1976). Reichlin *et al* (2002) reported presence of anti-lipoprotein lipase antibodies in about 50% of SLE patients, and a strong correlation between the antibody levels and TGs.

Triglycerides as well as other atherogenic lipid particles are carried on non-HDL-C, which calculated simply by substraction of HDL-C from total cholesterol. Non-HDL-C has been found to be a strong risk for CHD. It appears to even stronger than LDL-C (Cui *et al* 2001). Several studies have shown that high TGs impairs endothelial function (Bae *et al* 2000 and Lundman *et al* 2001). Short term hypertriglyceridaemia induced in healthy subjects by infusion of TGs that doubled the level of free fatty acids and raised TGs level by 4-fold, decrease FMD significantly from 7.1% to 1.6% (Lundam *et al* 1997). In a study of subjects with elevated TGs without other risk factors, Capell *et al* (2003) showed a significant improvement in endothelial function after 14 days treatment with fenofibrate. There is evidence that free fatty acids (FFA's) play a direct and important role in endothelial dysfunction and insulin resistance syndrome. Release of FFA's at the endothelial surface by the action of endothelial lipoprotein lipase on TRL's was proposed to explain the effect of FFA's on endothelial function and is supported by the observation that subjects with severe hypertriglyceridaemia due to lipoprotein lipase deficiency do not have impaired endothelial dysfunction. Several studies have shown that FFA's induce a state of insulin resistance. Carpentier *et al* (1999) showed that both acute and chronic elevation of FFA's induces insulin resistance. FFA's also increases TPAI-1 levels independent of insulin, Kerbs *et al* (2003) showed a 2.5-fold increase in TPAI-1 levels in healthy subjects in response to TGs and heparin infusion to raise FFA's, during the test, insulin level were maintained constant by somatostatin infusion.

As mentioned previously there is some controversy over the independent effect of insulin over and above the risk factors associated with insulin resistance. Whether hyperinsulinaemia is just a marker of insulin resistance or it has a separate effect on atherogenesis is controversial. An independent effect of

insulin on the CHD risk was reported earlier in several prospective studies, e.g the Helsinki Policemen Study (Pyorala *et al* 1985) and the Paris Prospective Study ((Fontbonne and Eschwege 1991). The results from subsequent prospective studies have however been contradictory. Some recent studies show an independent effect of fasting insulin after adjusting for TGs, HDL-C and other risk factors (Despres *et al* 1996), while others showed no association or loss of independent association after adjustment for other risk factors. A recent meta-analysis of 17 studies a weak positive association of fasting insulin with cardiovascular disease with a RR (95% CI) of 1.18 (1.08 – 1.29) for a difference in fasting insulin of 50 pmol/l or equivalent to the difference between 75th and 25th percentiles. The RR risk in studies of white populations was 1.42 (0.23 – 1.65). However, in this meta-analysis, none of the studies measured insulin sensitivity, therefore, the effect of insulin independent of insulin resistance could not be assessed (Ruige *et al* 1998).

There is evidence from several studies that in patients with CHD without obesity or any other risk factors also show a degree of insulin resistance. Bressler *et al* (1996) quantified insulin-mediated glucose disposal and measured insulin sensitivity using euglycaemic clamp technique in 13 patients with documented CHD and ten age and weight matched controls. Both the group were normotensive, not obese, and with normal glucose tolerance, LDL-C and HDL-C. They found that, Patients with CHD had mild to severe insulin resistance and hyperisulinaemia and there was correlation between insulin sensitivity and extent of CHD ($r = 0.48$, $p < 0.05$). The amount of insulin resistance resembles that seen hypertension, diabetes, obesity and NIDDM and the authors suggested inclusion of CHD in the cluster of disorders that comprise insulin resistance syndrome. Another study of 15 patients with significant coronary occlusion and age matched controls. Both groups had no diabetes, hypertension, obesity or hyperlipidaemia and had a normal glucose tolerance. Insulin sensitivity was significantly lower and AUC for fasting glucose and insulin was higher in patients compared to controls (Ariza *et al* 1997). These studies may suggest that CHD and insulin resistance may have a common aetiology.

In the second part of this study, we examined the presence of the metabolic syndrome in our SLE group. Eighteen percent had the metabolic syndrome. We again found that this syndrome was not directly related to current steroid use or the dose of steroid employed. Indeed patients with the metabolic syndrome had less exposure to steroids. It is difficult to conceive however that steroids do not play a role and it may be that an individuals' metabolic response to any given steroid dose may be a more important factor. Therefore, the development of insulin resistance in steroid treated patients may be at least partly under individual genetic control. There may also be a threshold dose above which the metabolic effects of steroids are enhanced with little or no effect below it. This threshold effect has been described for steroids-induced osteoporosis, where doses > 7.5 mg/day increases bone loss (Sambrook and Jones 1995). Although, steroid therapy has been implicated as a risk factor for CHD, it is difficult to separate the effect of steroid therapy from the effect of disease activity. Patients with higher disease activity and a longer duration of active disease tend to be on a higher steroid dose. With regard to the role of inflammation, we found that in patients who have the metabolic syndrome there was no significant difference in disease activity reflected in the BILAG or SLEDAI. Patients with the metabolic syndrome had significantly higher ox-LDL-C, which reflects higher oxidant stress in the group as described in other states of insulin resistance. There was also a tendency for fasting insulin and ox-LDL to increase with increase in the number of metabolic syndrome criteria (Figures 9.19, 9.20). Interestingly, patients with the metabolic syndrome tended to have higher levels of hs-CRP and IL6 compared to patients without the syndrome. Hs-CRP correlated significantly with fasting insulin ($r = 0.606$, $P = 0.048$) in patients with the metabolic syndrome but not in patients without ($r = 0.059$, $P = 0.682$). This may mean that in the presence of the additional risk factors such as abdominal obesity low-grade inflammation is enhanced. About 30% of the circulating IL-6 is secreted from the adipose tissue (Mohamed-Ali et al 1997), and omental fat cells secrete more IL-6 than subcutaneous fat cells by about 2-3 times (Fried *et al* 1998). This observation may explain why intra-abdominal fat is more linked to insulin resistance than subcutaneous fat.

Within our SLE group, we compared patients who were on and not on anti-malarials drugs, those on anti-malarials had significantly lower fasting glucose despite this group containing a higher proportion of patients taking steroids. This confirms that our decision to exclude patients on antimalarials in the first study was appropriate. The glucose lowering effect of the anti-malarials was more pronounced in patients who were also taking steroids (Table 9.14). Similarly, fasting insulin in those on steroid and anti-malarials was intermediate between those on steroids only and those on anti-malarials only or not taking either. We found no association between anti-malarials and total cholesterol as reported in studies from the Baltimore and Toronto SLE cohorts (Petri *et al* 1994b and Rahman *et al* 1999).

There was no correlation between FMD and fasting insulin nor with the number of metabolic syndrome criteria in our SLE patients.

The metabolic syndrome has recently been recognised in the Adult Treatment Panel (ATP) III guidelines. In the general population, management of metabolic syndrome has two objectives:

1- Targeting the predisposing factors obesity and physical inactivity.
2- Treatment of associated lipid and non-lipid risk factors.

Weight reduction is the first line for both lipid and non-lipid risk factors, and is associated with improvement in all the risk factors of the metabolic syndrome.

Physical inactivity is a risk factor for CHD and aggravates other risk factors in the metabolic syndrome. In contrast regular physical activity such as walking, running or swimming decreases the risk of CHD, diabetes, osteoporosis, obesity and depression. The recommendations of the Centres for Disease Control and Prevention and the American College of Sports Medicine state that individuals should have a 30-minutes or more of moderate intensity aerobic physical activity on most days and preferably on all days of the week (Pate *et al* 1995). Physical exercise improves peripheral and coronary endothelial function (Hambrecht *et al* 2000, 2003).

Instituting such regimes in SLE therefore would be relevant. However, several conditions associated with SLE such as fatigue, decreased muscle strength, avascular necrosis, depression and muscle atrophy may limit patients' ability to have and maintain regular physical exercise. Tench *et al* (1998) have reported

that the aerobic fitness level in SLE patients is 65% of that expected in healthy age-matched controls. Fatigue is common in SLE patients and its cause is not known. In a study of 93 SLE patients and 41 sedentary controls, Tench *et al* (2002) found that SLE patients had significantly reduced levels of aerobic fitness and reduced exercise capacity (10.4 vs 13.1 minutes). They also had reduced muscle strength and poorer resting lung function, more fatigue, depression and poor sleep quality. Severity of fatigue in SLE is associated with poor quality of life as measured by the SF-26 questionnaire, it does not appear to correlate with disease activity (Bruce *et al* 1999a). Exercise in SLE can be increased, Datlroy et al (1995) assessed in the effectiveness of a prescribed unsupervised exercise programme of stationary cycling. Compared to controls, patients on exercise showed improvement (although not significant) in exercise tolerance, fatigue and depression. In a recent study of 93 patients with inactive disease, Tench *et al* (2003) showed a significant improvement in fatigue after 12 weeks of graded exercise compared to relaxation therapy or the control groups.

With regard to lipid and non-lipid risk factors in SLE, it is important in the context of CHD risk assessment and management to consider SLE as a CHD equivalent condition such as diabetes. Since, currently there are no special guidelines of management of CHD risk factors in SLE, the ATP III guidelines for the category, which include CHD equivalent should be followed. The LDL-C target in this group is < 2.6 mmol/l. Management of moderately high TGs (1.7 – 2.25 mmol/l) especially in the presence of the metabolic syndrome is by weight reduction and increase in physical exercise. In the presence high TGs (> 2.25 mmol/l) non-HDL-C is a secondary target of therapy and is set at 3.4 or 0.8 mmol/l higher than that for LDL-C. Drug therapy for high TGs is approached first by intensifying therapy for LDL-C and if inadequate, fibrates and nicotinic acid can be added.

Our group has recently proposed guidelines for classic risk factors management in SLE. The proposed "ideal" targets for blood pressure is <130/80 mmHg, LDL-C <2.6 mmol/l and BMI < 25 kg/cm^2. Aspirin is indicated for any SLE patient with one additional risk factor, positive ACL antibodies or lupus anticoagulants and patients with prevalent CVD. ACE inhibitors would be the preferred second

agent for hypertension and also indicated in patients with known with CVD, diabetes or left ventricular hypertrophy. In addition to classical risk factor modification, control of disease activity with judicious adjustment of steroids and use of steroid sparing drugs including a low threshold for commencing AM's and also life style changes to maintain ideal body weight are important measures to reduce CHD risk in SLE population.

There have been no trials as yet of interventions to reduce CHD risk in SLE. These are clearly necessary and require large-scale multi-centre studies. The ideal candidates to test may be statins and /or ACE inhibitors for all SLE patients in the first instance. The of other agents e.g glitazones and/or omega-3 fatty acids, which reduce triglycerides and/or would also be of interest.

11.6 E-selectin gene A561C polymorphism:

Several polymorphisms have been described within the selectin gene cluster on the long arm of chromosome-1 (1q 22-25). Linkage studies in murine models and in human SLE have located a susceptibility region on the syntenic region of chromosome 1 (1q 21-42) (Tsao *et al* 1997, Moser *et al* 1998). This region contains several relevant genes including several immunomodulatory, complement, autoantigens, and apoptosis related genes. Several polymorphism have been described in the E-selectin gene, which is located on chromosome 1q 21-23. The best characterized is A561C or Serine 128 Arginine, in which a single nucleotide polymorphism from adenine to cytosine, results in amino acid exchange of uncharged serine with a positively charged arginine at position 128 in epidermal growth factor domain of E-selectin molecule (Wenzel *et al* 1996). Expression of E-selectin on endothelial cells is tightly regulated by inflammatory cytokines, mainly IL-1 and TNFα. On cytokine activation, E-selectin appears within one hour, reaches maximum at six hours and then gradually returns to basal level (Wellicome *et al* 1990). This polymorphism is of particular interest because it has been found to be associated with premature atherosclerosis. Wenzel et al (1994) found a higher frequency of C allele (16% vs 9%) in 97 patients aged $\leq$ 50 years with severe coronary or peripheral vascular disease compared to controls. This was confirmed in a second study, which showed frequencies of 21.6%, 15.7% and 8.7% of C allele in two groups of patients with

premature severe atherosclerosis in patients aged ≤ 50 (n = 50) and ≤ 40 years (n = 40) compared to 100 healthy controls respectively (Wenzel et al 1996). In 82 young patient with > 50% coronary artery stenosis, Ye SQ *et al* (1999) found a C allele frequency of 20% compared to 11% in controls. This polymorphism alters the E-selectin function. Revelle *et al* (1996) showed that the C allele alters the binding specificity to carbohydrate molecules on the leukocytes and increases leucocyte binding by 2-3 fold compared to the wild allele type gene. Rao *et al* (2000 and 2002a) tested the function of the mutant C allele under flow condition, and found that it increases rolling and tethering over the endothelium, and in the presence of β2 integrin it enhances firm adhesion and transmigration. The number of recruited leukocytes was significantly higher with the C allele than the A allele. Leukocyte recruitment is a cell-specific process, and the C allele has been shown in recent work by the same group to extend the range of recruited lymphocytes and to allow significant accumulation of Th0 and Th2 but not Th1 lymphocytes compared to the wild A allele (Rao *et al* 2002b). This observation may suggest a link between expression of C allele and increased incidence of inflammation and atherosclerosis. Other clinical studies have also highlighted the importance of the E-selectin polymorphism. In a study of 193 patients who underwent coronary angioplasty, the frequency of C allele was significantly higher in the group who subsequently developed re-stenosis compared to the group who did not develop re-stenosis 16.6% vs 5.32%. In multivariate analysis the C allele was an independent predictor of re-stenosis (Rauchhaus *et al* 2002). Using electron beam computed tomography, Ellsworth *et al* (2001) found that in women aged < 50 years, that coronary artery calcification was detected in higher frequency 27% vs 6.7% in those with the C allele. Whereas, in women age > 50 years, the frequency of coronary calcification was similar 38% vs 39% in those with and without the C allele.

The prevalence of C allele in healthy UK population has been reported in two studies. Gbadegesin *et al* (2002) found a prevalence of 7.4%, in 170 subjects in Manchester. McLaren *et al* (1999) reported a prevalence of 12.4% in 101 subjects in Oxford. In Belfast Ireland, the frequency of C allele is 9.0% and in France 10.4% (Herrmann *et al* 1998). In our study, the prevalence of C allele in controls was 9.1%.

We have found an association between SLE and the E-selectin A561C polymorphism in two independent populations. The strength of the association, with odds ratios of 1.6-1.8, is also consistent with this polymorphism being one of several genetic markers that are likely to contribute to disease susceptibility in SLE in a particular population. The lack of association in the Turkish patients confirms previous observations that genetic associations in SLE vary with ethnicity. This polymorphism may be directly implicated in the disease susceptibility or it may be in linkage disequilibrium with another polymorphism in the vicinity of the E-selectin gene. The pivotal role of E-selectin in leucocyte-endothelial cell interactions may increase susceptibility to vascular inflammation and therefore may contribute directly to the pathogenesis of SLE.

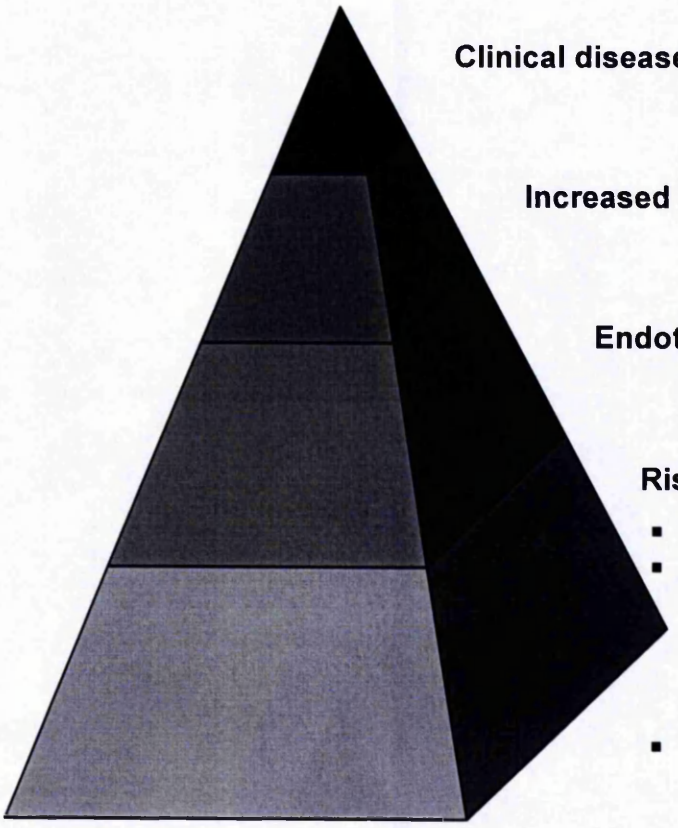
Since enhanced recruitment of inflammatory cells is a common mechanism for both SLE and atherosclerosis, this finding may provide an explanation for the observed associations of premature atherosclerosis with SLE.

In the second study therefore, we examined the association of C allele in our group of 64 patients. Although patients with the C allele tended to be younger, there was no effect of the C-allele on age at disease onset. Patients with the C allele had higher disease activity compared to those without the C allele. However, age may be a confounder in this respect, since age correlated negatively with SLEDAI ($r = 0.344$, $P = 0.005$). The group with the C allele had more history of pericarditis and a trend towards higher positive APL antibodies. We found no effect of the C allele on the damage index. To assess the effect of the C allele on disease activity, longitudinal rather than cross sectional studies would be better. There was no association of the C allele with the vascular function and in particular, FMD did not differ between the two groups.

This exploratory study has however several limitations. Firstly, the small number of patients limited its ability to detect association of C allele with different disease aspects in SLE, and also to examine the association of the C allele with prevalent CHD, which needs a large prospective study. It also truly dose not affect FMD. The effect of the C allele may be more towards accelerating plaque development or alternatively in facilitating early onset of the atherosclerosis.

In summary, CHD risk in SLE bears the analogy of an iceberg with clinical events MI and angina represent the exposed tip. The base of the iceberg is formed by a collection of classic and novel metabolic risk factors as well as SLE related factors and also yet unknown metabolic, immune and genetic factors. This basal risk leads to endothelial dysfunction, which leads to subclinical disease represented by increased carotid IMT (Figure 11.1).

Figure 11.1 The iceberg of atherosclerosis in SLE:



Clinical disease: MI, angina (6 %– 10%)

Increased intima media thickness (IMT)

Endothelial dysfunction

Risk factors:

- Classical CHD risk factors.
- Novel metabolic risk factors:
  - Homocysteine.
  - **Triglycerides**
  - **Insulin resistance**
  - Other factors?
- SLE factors:
  - APL antibodies.
  - Inflammation.
  - Steroid therapy
  - Oxidant stress?
  - **E-selectin polymorphism.**
  - Unknown factors?

11.7 Future directions:

Our study has clearly demonstrated the presence of endothelial dysfunction in SLE and has shown that SLE influences endothelial function independently of classic CHD risk factors. We have also found that within SLE, there is a significant association between early marker atherosclerosis carotid IMT and endothelial dysfunction. We have also confirmed the observation of others that there is a high variability in assessment of endothelial function that needs to be born in mind when further utilizing this technique. There are nevertheless several further studies that this work suggests. Firstly, it would be important to undertake a larger prospective study using edge tracking software to decrease variability. Key questions that need to be addressed including:

- Follow-up measurement of FMD in selected groups from patients who had normal and severe impairment of FMD, to determine the range of variability in FMD in each group.
- Assess the effect of disease activity and therapy over time.
- Measurement of other novel risk factors such as ADMA, nitrotyrosine and isoprostanes and study their association with endothelial function in SLE.
- Conduct intervention studies with ACE inhibitors, statins, omega-3 fatty acids and anti-oxidants.
- To test the hypothesis that endothelial dysfunction may be part of SLE phenotype, for this we may determine the endothelial function in all newly diagnosed patients and their first-degree relatives.

With regard to our finding of association of SLE with decreased insulin sensitivity, a larger study is underway to enable us to understand the interplay of steroids, inflammation and adiposity in development of metabolic syndrome. The prevalence of the metabolic syndrome in SLE should also be compared to that in a larger group of community controls.

We also need to understand more about lipoprotein sub-fractions and lipid metabolism in SLE. In particular, to understand whether the elevated TGs are mainly secondary to the metabolic syndrome or if they are primary to SLE phenotype and promote both insulin resistance and risk of CHD. With regard to free fatty acids levels, the role of lipoprotein lipase activity and its genetic polymorphisms should be explored. Genetic polymorphisms in LpL gene have

been described. Although, homozygous deficiency is very rare, heterozygosity for LpL deficiency is common. In particular, two polymorphisms, Asp-9-Asn and Asn-291-ser have been shown in a meta-analysis to have a significant association with atherogenic lipid profile of high TGs and low HDL-C and a borderline significant increase in risk of CHD (Wittrup *et al* 1999). A polymorphism in IL-6 (G174C) is also associated with higher TGs. However, a study in Germany showed no association of this polymorphism with SLE (Schotte *et al* 2001).

Leptin is a hormone produced in adipose tissue and is related to adiposity and insulin resistance in patients with type II diabetes (Wauters *et al* 2003). In a recent study Garcia-Gonzalez *at al* (2003) found elevated serum leptin in female SLE patients compared to age and BMI matched healthy controls. In multivariate analysis, SLE and BMI were independently associated with elevated leptin levels. Another study also reported elevated leptin in SLE (La Cava *et al* 2003). The LMNA gene, is located on chromosome 1q 21-23 within the linkage area for SLE and encodes for nuclear envelope proteins lamin A and C. Rare mutation in this gene are related to familial partial lipodystrophy, a condition associated with insulin resistance. More common polymorphism C1908T has been associated with high leptin levels and human subcutaneous abdominal adipocyte size (Hegele *et al* 2000). This polymorphism was found to be associated with insulin resistance and dyslipidaemia in the Japanese (Murase *et al* 2002). Therefore, LMNA gene polymorphisms may predispose to insulin resistance and dyslipidaemia and being within the linkage area for SLE would be interesting to examine its association with SLE.

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13 Appendices

13.1 Appendix. 1 The 1982 revised criteria for classification of SLE*

(Tan E *et al* 1982). Subsequently revised in 1997 (Hochberg 1997)

Criterion	Definition
Malar rash	Fixed erythema, flat or raised, over the malar eminences, tending to spare the nasolabial folds.
Discoid rash	Erythematous raised patches with adherent keratotic scaling and follicular plugging, atrophic scarring may occur in older lesions.
Photosensitivity	Skin rash as a result of unusual reaction to sunlight, patient history or physician observation.
Oral ulcers	Oral or nasopharyngeal ulceration, usually painless, observed by a physician.
Arthritis	Non errosive arthritis involving two or more peripheral joints, characterised by tenderness, swelling or effusion.
Serositis	Pleuritis, convincing history of pleuritic chest pain or rub heard by a physician or evidence of pleural effusion or Pericarditis, documented by ECG or rub or evidence of pericardial effusion.
Renal disorder	Persistent proteinuria >0.5gm/day or > 3+ if quantification not performed or Cellular casts either red cell, haemoglubin, granular, tubular or mixed.
Neurological disorder	Seizures or psychosis in the absence of offending drugs or known metabolic derangements; e.g uraemia, ketoacidosis or electrolyte disturbance.
Haematologic disorder	Haemolytic anaemia with reticulcytosis Leucopenia < 4000/mm^3 on two or more occasions Lymphopenia <1500/mm^3 on two or more occasions Thrombocytopenia < 100,000/mm3 in the absence of offending drugs.
Immunological disorder	**Positive LE cell preparation **** Anti-DNA antibody to native DNA in abnormal titer. Anti-Sm, antibody to Sm nuclear antigen **False positive serological tests for syphilis known to be positive for the at least six months.****
Antinuclear antibody	Abnormal titer of antinuclear antibody by immunoflourescence or an equivalent assay at any point in time and in the absence of drugs known to be associated with drug-induced lupus syndrome.

* A diagnosis of SLE requires fulfilling four or more of the eleven criteria simultaneously or serially during any interval of observation.

** In the 1997 ACR revised criteria, these are replaced by positive IgG or IgM anti-cardiolipin antibodies and positive lupus anticoagulants.

13.2 Appendix. 2 Patients data collection forms

The British Isles Lupus and Atherosclerosis Study

CENTRE NAME: _____ PATIENT INITIALS:_____

CENTRE NO: _____
(Assigned by coordinating centre)

PATIENT I.D. NO: _____
(Assigned by submitting centre using consecutive numbers i.e., 001, 002 etc.)

DATE OF ASSESSMENT:__ / __ / __
d m y

DEMOGRAPHIC DATA		
Date of Birth: __ / __ / __ (d m y)		Date of Diagnosis of SLE: __ / __ / __ (d m y)
ACR Criteria for Diagnosis (✓):		
☐ Malar rash	☐ Arthritis	☐ Haematologic disorder
☐ Discoid rash	☐ Photosensitivity	☐ Immunologic disorder
☐ Oral ulcers	☐ Renal disorder	☐ ANA
☐ Serositis	☐ Neurologic disorder	
Marital Status: (circle)		
1 = single	4 = divorced	
2 = married	5 = separated	
3 = widowed	6 = common law	
Education:		
Number of years prior to college or university ___		Number of years at college or university ___
Occupation: (specify) _____		

FAMILY HISTORY AND LIFESTYLE			
Alcohol consumption ____ ounces/week or ___ ml/week			
Cigarette Smoking	- current	N Y	# cigarettes/day ___
	- ex-smoker	N Y	# years smoking ___
	Date stopped	__ / __ / __ (d m y)	
Ethnicity, Physical Activity and Family History Questionnaires to be completed by patient interview.			

CLINICAL DATA

Height _____cm

Weight (shoes and coat off) ___ ___ ___kg

Waist / hip ratio _____ / _____ cm/cm

Blood pressure (xxx/xxx) systolic/diastolic _____/_____

Antihypertensive Therapy

- current N Y If yes – Type (circle)
  0 = Diuretics
  1 = Adrenergic inhibitors
  2 = Central α agonists
  3 = β blockers
  4 = Direct vasodilators
  5 = Calcium antagonists
  6 = Angiotensin-converting enzyme inhibitors
  7 = Other
  8 = Combination

- in the past N Y If yes – Type (circle)
  0 = Diuretics
  1 = Adrenergic inhibitors
  2 = Central α agonists
  3 = β blockers
  4 = Direct vasodilators
  5 = Calcium antagonists
  6 = Angiotensin-converting enzyme inhibitors
  7 = Other
  8 = Combination

Myocardial Infarction

In the past: N Y If yes, date(s): ___ ___ ___ (y m d) ___ ___ ___ (y m d)

Angina

Current: N Y

In the past: N Y Date of Diagnosis: ___ ___ ___ (y m d)

Congestive Heart Failure

Current: N Y

In the past: N Y If yes, date(s): ___ ___ ___ (y m d) ___ ___ ___ (y m d)

Angioplasty

Ever: N Y If yes, date(s): ___ ___ ___ (y m d) ___ ___ ___ (y m d)

Bypass Surgery:

Ever: N Y If yes, date: ___ ___ ___ (y m d)

PREVIOUS SLE CARDIAC MANIFESTATIONS

Pericarditis N Y If yes, date(s) ___ ___ ___ (y m d) ___ ___ ___ (y m d)

Myocarditis N Y If yes, date(s) ___ ___ ___ (y m d) ___ ___ ___ (y m d)

Endocarditis N Y If yes, date(s) ___ ___ ___ (y m d) ___ ___ ___ (y m d)

PERIPHERAL VASCULAR

Intermittent claudication

Current: N Y

In the past: N Y If yes, date(s): ____ ____ ____ y m d ____ ____ ____ y m d

CEREBROVASCULAR

Transient Ischemic Attack:

Current: N Y

In the past: N Y If yes, date(s): ____ ____ ____ y m d ____ ____ ____ y m d

Stroke:

Current: N Y

In the past: N Y If yes, date(s): ____ ____ ____ y m d ____ ____ ____ y m d

Type (if known): 1 = hemorrhagic 2 = Thrombotic

HYPERLIPIDEMIA THERAPY:

Current: N Y Current therapy (circle)

0 = None 4 = Fibrates
1 = Statins 5 = Cominations
2 = Sequestrants 6 = Other
3 = Nicotinic Acid

In the past: N Y Therapy in the past (circle)

0 = None 4 = Fibrates
1 = Statins 5 = Cominations
2 = Sequestrants 6 = Other
3 = Nicotinic Acid

HORMONAL FACTORS

Ovarian function (circle all that apply)

1 = menstruating 5 = pre-menopausal hysterectomy
2 = premenarche 6 = post-menopausal hysterectomy
3 = postmenopausal 7 = pre-menopausal hysteroophorectomy
4 = amenorrhea

Oral contraceptive:

- current N Y
- in the past N Y

If yes, number of years: _____

Hormone Replacement Therapy:

- current N Y

Current course start date: ____ ____ ____ y m d

Current Type (circle):

1 = estrogen (specify) _____
2 = estrogen + progesterone
3 = progesterone only
4 = other (specify) _____

- in the past N Y

Past Type (circle)

1 = estrogen (specify) _____
2 = estrogen + progesterone
3 = progesterone only
4 = other (specify) _____

Pregnant now: N Y

Gravida / Para: _____/_____ miscarriage (No): _____

ENDOCRINE

Hypothyroidism
- current N Y
- ever N Y

Diabetes N Y Date diagnosed: ____ ____ ____ (y m d)

Type I: N Y

Type II: N Y

Requiring chronic insulin > 3 months N Y

RENAL

Active nephritis:
- current N Y
- in the past N Y

Nephrotic syndrome:
- current N Y
- in the past N Y

THERAPY

Steroids:

Current: N Y

Current course start date: ____ ____ ____ (y m d) Current course average daily dose ____mg./day

In the past: N Y

No. of previous courses: ____ Average daily dose previous course(s): ____ mg.

Antimalarials:

Current: N Y

Current course start date: ____ ____ ____ (y m d) Current daily dose: ____ mg./day

Current type (circle): 1 = chloroquine 2 = hydroxychloroquine
3 = atabrine 4 = other (Specify)____

In the past: N Y

Past type (circle): 1 = chloroquine 2 = hydroxychloroquine
3 = atabrine 4 = other (Specify)____

Immunosuppressives:

Current: N Y

Current Type (circle) Current daily dose: ____ mg./day
1 = imuran 4 = cyclosporin
2 = cyclophosphamide 5 = mycophenolic acid
3 = methotrexate 6 = Other (Specify:____)

In the past: N Y

Past type. (circle)
1 = imuran 4 = cyclosporin
2 = cyclophosphamide 5 = mycophenolic acid
3 = methotrexate 6 = Other (Specify: ____)

13.3 Appendix.3 Systemic Lupus Erythematosus Disease Activity Index (SLEDAI)

Study No.: _____ Patient Name: _____ Visit Date: ____ ____ ____
dd mo yr

(Enter weight in SLEDAI-2K Score column if descriptor is present at the time of the visit or in the preceding 10 days.)

SLEDAI 2K Weight	SCORE	Descriptor	Definition
8	_____	Seizure	Recent onset, exclude metabolic, infectious or drug causes.
8	_____	Psychosis	Altered ability to function in normal activity due to severe disturbance in the perception of reality. Include hallucinations, incoherence, marked loose associations, impoverished thought content, marked illogical thinking, bizarre, disorganized, or catatonic behavior. Exclude uremia and drug causes
8	_____	Organic brain syndrome	Altered mental function with impaired orientation, memory, or other intellectual function, with rapid onset and fluctuating clinical features, inability to sustain attention to environment, plus at least 2 of the following: perceptual disturbance, incoherent speech, insomnia or daytime drowsiness, or increased or decreased psychomotor activity. Exclude metabolic, infectious, or drug causes.
8	_____	Visual disturbance	Retinal changes of SLE. Include cytoid bodies, retinal hemorrhages, serous exudate or hemorrhages in the choroid, or optic neuritis. Exclude hypertension, infection, or drug causes.
8	_____	Cranial nerve disorder	New onset of sensory or motor neuropathy involving cranial nerves.
8	_____	Lupus headache	Severe, persistent headache; may be migrainous, but must be nonresponsive to narcotic analgesia.
8	_____	CVA	New onset of cerebrovascular accident(s). Exclude arteriosclerosis.
8	_____	Vasculitis	Ulceration, gangrene, tender finger nodules, periungual infarction, splinter hemorrhages, or biopsy or angiogram proof of vasculitis.
4	_____	Arthritis	≥ 2 joints with pain and signs of inflammation (i.e., tenderness, swelling or effusion).
4	_____	Myositis	Proximal muscle aching/weakness, associated with elevated creatine phosphokinase/aldolase or electromyogram changes or a biopsy showing myositis.
4	_____	Urinary casts	Heme-granular or red blood cell casts.
4	_____	Hematuria	>5 red blood cells/high power field. Exclude stone, infection or other cause.
4	_____	Proteinuria	>0.5 gram/24 hours
4	_____	Pyuria	>5 white blood cells/high power field. Exclude infection.
2	_____	Rash	Inflammatory type rash.
2	_____	Alopecia	Abnormal, patchy or diffuse loss of hair.
2	_____	Mucosal ulcers	Oral or nasal ulcerations.
2	_____	Pleurisy	Pleuritic chest pain with pleural rub or effusion, or pleural thickening.
2	_____	Pericarditis	Pericardial pain with at least 1 of the following: rub, effusion, or electrocardiogram or echocardiogram confirmation.
2	_____	Low complement	Decrease in CH50, C3, or C4 below the lower limit of normal for testing laboratory
2	_____	Increased DNA binding	Increased DNA binding by Farr assay above normal range for testing laboratory.
1	_____	Fever	>38° C. Exclude infectious cause.
1	_____	Thrombocytopenia	<100,000 platelets / x10^9/L, exclude drug causes.
1	_____	Leukopenia	< 3,000 white blood cells / x10^9/L, exclude drug causes.
TOTAL SCORE	_____		

13.4 Appendix.4 British Isles Lupus Assessment Group (BILAG) index:

BLIPS BILAG ASSESSMENT FORM

Patient ID	Patient Name	Date Of Birth
Clinic	Assessed By	Assessment Date

SCORES: 1 = IMPROVING ; 2 = SAME ; 3 = WORSE ; 4 = NEW

GENERAL		
1. Pyrexia (documented)		
2. Weight loss - unintentional > 5%		
3. Lymphadenopathy/splenomegaly		
4. Fatigue/malaise/lethargy		
5. Anorexia/nausea/vomiting		
MUCOCUTANEOUS		
6. Maculopapular eruption - severe, active (or		
7. Maculopapular eruption - mild		
8. Active discoid lesions - generalised/extensive		
9. Active discoid lesions - localised include. lupus		
10. Alopecia (severe, active)		
11. Alopecia (mild)		
12. Panniculitis (severe)		
13. Angio-oedema		
14. Extensive mucosal ulceration		
15. Small mucosal ulcers		
16. Malar erythema		
17. Subcutaneous nodules		
18. Pernionic skin lesions		
19. Peri-ungual erythema		
20. Swollen fingers *Y/N*		
21. Sclerodactyly *Y/N*		
22. Calcinosis *Y/N*		
23. Telangiectasia *Y/N*		
NEUROLOGICAL		
24. Deteriorating level of consciousness		
25. Acute psychosis, delerium, confused state		
26. Seizures		
27. Stroke or stroke syndrome		
28. Aseptic meningitis		
29. Mononeuritis multiplex		
30. Ascending or transverse myelitis		
31. Peripheral or cranial neuropathy		
32. Disc swelling/cytoid bodies		
33. Chorea		
34. Cerebellar ataxia		
35. Headache severe, unremitting		
36. Organic depressive illness		
37. Organic brain syndrome incl. pseudotumor cerebri		
38. Episodic migrainous headaches		
MUSCULOSKELETAL		
39. Definite myositis (Bohan & Peter)		
40. Severe polyarthritis - with loss of function		
41. Arthritis		
42. Tendonitis		
43. Mild chronic myositis		
44. Arthralgia		
45. Myalgia		
46. Tendon contractures and fixed deformity *Y/N*		
47. Aseptic necrosis		

CARDIOVASCULAR + RESPIRATORY		
48. Pleuropericardial pain		
49. Dyspnoea		
50. Cardiac failure		
51. Friction rub		
52. Effusion (pericardial or pleural)		
53. Mild or intermittant chest pain		
54. Progressive cxr changes-lung fields *Y/N*		
55. Progressive cxr changes-heart size *Y/N*		
56. ECG evidence of pericarditis or myocarditis *Y/N*		
57. Cardiac arrhythmias incl. tachy-cardia > 100. no fever		
58. Pulmonary function fall by > 20% *Y/N*		
59. Cytohistological evidence of inflammatory lung disease		
VASCULITIS		
60. Major cutaneous vasculitis incl.ulcers		
61. Major abdominal crisis due to vasculitis		
62. Recurrent thromboembolism (excluding strokes)		
63. Raynaud's		
64. Livido reticularis		
65. Superficial phlebitis		
66. Minor cutaneous vasculitis		
(nailfold vasculitis, digital vasculitis, purpura,		
67. Thromboembolism (excl. stroke) - 1st episode *Y/N*		
68. Systolic blood pressure mm Hg *value*		
69. Diastolic blood pressure (5th phase) mm Hg *value*		
70. Accelerated hypertension *Y/N*		
71. Dipstick (- = 0, + = 1, ++ = 2, +++ = 3) *value*		
72. 24 hour urinary protein (g) *value*		
73. Newly documented proteinuria of > 1g / 24hrs *Y/N*		
74. Nephrotic syndrome *Y/N*		
75. Creatinine (plasma/serum) *value*		
76. Creatinine clearance/GFR ml/min *value*		
77. Active urinary sediment *Y/N*		
78. Histological evidence of active nephritis - within 3		
HAEMATOLOGY		
79. Haemoglobin g/dl *value*		
80. Total white cell count x 10^9/l *value*		
81. Neutrophils x 10^9/L *value*		
82. Lymphocytes x 10^9/L *value*		
83. Platelets x 10^9/L *value*		
84. Evidence of active haemolysis *Y/N*		
85. Coomb's test positive *Y/N*		
86. Evidence of circulating anticoagulant *Y/N*		
TREATMENT MEDICATION		
SEE PAGE 2		
LIST OF OTHER DRUGS TAKEN		
SEE PAGE 2		

13.5 Appendix.5 Lupus International Collaborating Clinics (SLICC/ACR) damage index.

SLICC Assessment Form

	SCORE	
OCULAR (either eye, by clinical assessment)		
Any cataract ever	1	
Retinal change *OR* Optic atrophy	1	
NEUROPSYCHIATRIC		
Cognitive impairment (e.g. memory deficit, difficulty with calculation, poor concentration, difficulty in spoken or written language, impaired performance level) *OR* Major psychosis	1	
Seizures requiring therapy for 6 months	1	
Cerebravascular accident ever (score 2 if > 1)	1	(2)
Cranial or peripheral neuropathy (excluding optic)	1	
Transverse myelitis	1	
RENAL		
Estimated or measured glomerular filtration rate < 50%	1	
Proteinuria ≥3.5gm/24h	1	
OR End-stage renal disease (regardless of dialysis or transplantation)	3	
PULMONARY		
Pulmonary hypertension (right ventricular prominence, or loud P2)	1	
Pulmonary fibrosis (physical and radiograph)	1	
Shrinking lung (radiograph)	1	
Pleural fibrosis (radiograph)	1	
Pulmonary infarction (radiograph)	1	
CARDIOVASCULAR		
Angina *OR* Coronary artery bypass	1	
Myocardial infarction ever (score 2 if > 1)	1	(2)
Cardiomyopathy (ventricular dysfunction)	1	
Valvular disease (diastolic, murmur, or systolic murmur >3/6)	1	
Pericarditis for 6 months, *OR* Pericardiectomy	1	
PERIPHERAL VASCULAR		
Claudication for 6 months	1	
Minor tissue loss (pulp space)	1	
Significant tissue loss ever (eg loss of digit or limb) (score 2 if > 1 site)	1	(2)
Venous thrombosis with swelling, ulceration *OR* Venous stasis	1	
GASTROINTESTINAL		
Infarction or resection of bowel below duodenum, spleen, liver, or gall bladder ever, for cause any (score 2 if > 1 site)	1	(2)
Mesenteric insufficiency	1	
Chronic peritonitis	1	
Stricture *OR* Upper gastrointestinal tract surgery ever	1	
Chronic pancreatitis	1	
MUSCULOSKELETAL		
Muscle atrophy or weakness	1	
Deforming or erosive arthritis (including reducible deformities, excluding avascular necrosis)	1	
Osteoporosis with fracture or vertebral collapse (excluding avascular necrosis)	1	
Avascular necrosis (score 2 if > 1)	1	(2)
Osteomyelitis	1	
Tendon rupture	1	
SKIN		
Scarring chronic alopecia	1	
Extensive scarring or panniculum other than scalp and pulp space	1	
Skin ulceration (excluding thrombosis for > 6 months)	1	
PREMATURE GONADAL FAILURE	1	
DIABETES (regardless of treatment)	1	
MALIGNANCY (exclude dysplasia) (score 2 if > 1 site)	1	(2)

13.6 Appendix. 6 International consensus on preliminary classification criteria for definite antiphospholipid syndrome

(Sapporo 1999).

Clinical criteria:

(1) Arterial, venous or microvascular thrombosis

At least one clinical episode in any tissue or organ, (except superficial venous thrombosis) confirmed by imaging studies, Doppler, or histology (without significant parietal inflammation)

(2) Complications of pregnancy:

At least one instance of unexplained fetal loss (≥10 weeks of gestation) without fetal abnormalities detectable by ultrasonography or visual inspection, or

At least one instance of premature delivery (<34 weeks of gestation of a morphologically normal baby, related to eclampsia, preeclampsia, or severe placental insufficiency, or

At least three unexplained, spontaneous consecutive abortions (<10 weeks of gestation) not related to anatomic or hormonal abnormalities in the mother or to chromosomal aberrations.

Biological criteria (confirmed after 6 weeks)

(1) IgG and /or IgM anticardiolipin antibodies, in moderate or high titers, by a standard ELISA for β2-GP1-dependent anticardiolipin antibodies.

(2) Lupus anticoagulant detected in plasma according to the recommendations of the International Society on Thrombosis and Haemostasis.

Phospholipid-dependent increase in a clotting time detected by a screening test: activated partial thromboplastin time (APTT), Koalin clotting time (KCT), dilute Russell viper venom test (dRVVT), dilute thromboplastin time (TTd), textarin time.

Abnormal screening test not corrected by addition of normal platelet-dependent plasma.

Abnormal screening test completely or partially corrected by addition of a phospholipid excess.

Exclusion of other clotting disorders, related for instance to heparin therapy or factor VIII inhibitor.

Definite antiphospholipid syndrome is present if the patient has at least one clinical and one biological criterion

13.7 Appendix. 7 The Cardiac risk assessor Excel program

The cardiac risk assessor Excel programme

This programme is accessible via the British Hyperlipidaemia Society web site "www.heartuk.org.uk"

CARDIAC RISK ASSESSOR

V98.02

For use with the Joint Recommendations of the British Cardiac Society, British Hyperlipidemia Association, British Hypertension Society and British Diabetic Association on Prevention of Coronary Heart Disease in Clinical Practice.

Heart 1998; 80 Suppl 2:1 - 28

Continue

CARDIAC RISK ASSESSOR

Based on the equations in the following publication:

Anderson, K.M., Odell, P.M., Wilson, P.W.F., Kannel, W.B. Cardiovascular disease risk profiles. Am. Heart J. 1990;121:293-8.

The programme computes CHD and stroke risk as the percentage likelihood of an event over a period of 10 years. e.g. a risk of 30 means that there is a 30 in 100 chance of an event in the next 10 years.

CARDIAC RISK ASSESSOR

Risk Factors

Move through RISK FACTOR boxes to enter & amend data.
Use cursor keys to move through boxes.

			CHD risk % over 10 years	Stroke risk % over 10 years
Female?(yes=1,no=0)	0	SBP	#NUM!	#NUM!
Age(years)	0			
SBP (mmHg)	0	DBP	#NUM!	#NUM!
DBP (mmHg)	0			
Smokes?(yes=1,no=0)	0			
Total - C (mmol/l)	0			
HDL - C (mmol/l)	0		Print	
Diabetes(yes=1,no=0)	0			
Known to have ECG-LVH? (yes=1,no=0)	0			
Period of predicted risk (years)	10		Exit	