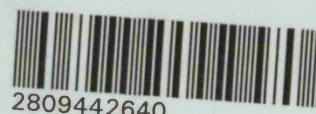


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LASER Doppler Perfusion

Imaging of the Normal and

Diseased Vulva

M.D.

Jamna Saravanamuthu

2007

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Abstract

Vulval lichen sclerosus (LS) and high-grade intraepithelial neoplasia (VIN 3) are two common and distressing diseases. Significant morbidity is caused by symptoms of persistent pruritus and surgical treatment of skin areas suspicious of malignancy. The risk of developing cancer in a background of LS and VIN 3 is poorly defined. The methods currently available for clinical assessment of the vulva are limited. There is abundant research on the application of the LASER Doppler technique - laser Doppler Flowmetry (LDF) - showing changes in perfusion within the small blood vessels of the skin as a useful parameter for more accurate disease classification. There is also research on immunohistochemical microvessel density (MVD) studies showing increases in blood supply in tissues prone to develop cancer or as a prognostic marker of cancer outcome. The Laser Doppler perfusion imager (LDPI) provides a rapid, real-time, non-invasive and non-contact method to measure skin blood flow in an area as opposed to a single point by the LDF, making the LDPI more suitable for application to the vulva. This thesis reports for the first time, the application of the LDPI to the vulva. Initially the LDPI was applied to the clinically normal vulva to study perfusion variance related to menstrual cycle, age and local skin temperature provocation. The application was then extended to vulval disease, LS and VIN 3, and validated against morphological differences in MVD. The LDPI and MVD studies suggest that in VIN 3 there is an actual increase in skin perfusion. In LS the situation is more complex and suggests that the LDPI measured perfusion at a greater depth than the MVD. Studies on base line perfusion variance of vulval LS to topical therapy show that there is no overall difference in baseline perfusion in spite of symptom improvement. Temperature provocation studies suggest differences in skin blood flow response in diseased compared to the normal vulva.

Acknowledgements

I am grateful to Ms Wendy Reid, Consultant Gynaecologist and Professor Allan MacLean for giving me the opportunity, encouragement and supervising the work presented in this thesis. The study of the application of the LDPI to the vulva is challenging and could not have been possible without the inspiration and guidance of Professor Alex Seifalian. Dr Chris Perrett, Non-Clinical Senior Lecturer provided invaluable supervision with the immunohistochemical studies. Dr David StGeorge, Consultant in Public Health, helped patiently with all the statistical analysis and graph presentation. I am also very grateful to Dr Julie Crow, Senior Lecturer and Consultant Pathologist, for assistance with the histological aspects of the immunohistochemical studies and selection of the photomicrographs presented in this thesis. Finally I would also like to thank Ms Anne Jackson, Dr Kerstin Rolfe, Dr Emmanuelle Ferraux, and Mrs Karen Gleeson. I am most of all indebted to all the women who volunteered for the studies presented in this thesis.

Contents		Page
Title page -----		1
Abstract -----		2
Acknowledgements -----		3
Contents -----		4
Figures -----		10
Tables -----		17
Abbreviations -----		19
1 Introduction -----		23
1.1 Introduction		24
1.2 Vulval disease		24
1.3 Background to thesis		25
1.4 Objective for this thesis		27
1.5 Hypothesis		27
2 The vulval skin -----		29
2.1 Anatomy, physiology and function of the vulva		30
2.1.1 Introduction		30
2.1.2 Anatomy		31
2.1.3 Physiology		32
2.1.3.1 Blood supply		32
2.1.3.2 Lymphatic supply		33
2.1.3.3 Nerve supply		33
2.1.3.4 Age and hormones		34
2.1.4 Function of the vulva		36
2.2 Structure of skin and organisation of the microvasculature		37
2.2.1 Histology		37
2.2.2 Skin blood supply		39
2.2.2.1 Papillary dermis		40
2.2.2.2 Reticular dermis		43
2.2.2.3 Regional differences		43
2.2.3 Application of optics to assess SBF		45
2.2.3.1 Spatial heterogeneity		45
2.2.3.2 Temporal heterogeneity		47
2.3 Conclusion		48

3	Review of vulval diseases - LS and high-grade VIN - and the assessment of the vulval skin -----	49
3.1	Introduction	50
3.2	Vulval lichen sclerosus (LS)	50
3.2.1	Presenting symptoms	52
3.2.2	Examination findings	52
3.2.3	Histological findings	57
3.2.4	Aetiology	60
3.2.4.1	Autoimmune disease	60
3.2.4.2	Hormonal dependence	61
3.2.4.3	Infective cause	61
3.2.4.4	Abnormal cell kinetics	62
3.2.5	Relationship between LS and vulval carcinoma (SCC)	63
3.2.6	Management	66
3.3	High grade vulval intraepithelial neoplasia (VIN 3)	69
3.3.1	Clinical profile	70
3.3.2	Clinical appearance	72
3.3.3	Clinical symptoms	74
3.3.4	Histopathology	74
3.3.5	Natural history of VIN	77
3.3.6	Treatment	81
3.4	Assessment of the vulval skin	83
3.4.1	History	83
3.4.2	Clinical examination	84
3.4.3	Investigation	85
3.4.3.1	Biopsy and histological examination	85
3.4.3.2	Percutaneous absorption	86
3.4.3.3	Transepidermal water loss	87
3.4.3.4	Stratum corneum water holding capacity	87
3.4.3.5	Sensitivity to irritant activity	88
3.4.3.6	Mechanical trauma	88
3.4.3.7	Skin blood flow	88
3.5	Conclusion	89

4	A critical review of biophysical methods for the assessment of vulval skin microcirculation -----	90
5	Method used to assess the vulval skin microcirculation -----	107
5.1	Introduction	108
5.2	Basic theory and operating principles of the LDPI	108
5.3	Instrumentation	113
5.4	Image analysis	118
5.5	Calibration	119
5.6	Validation of the LDPI with a blood-flow model	121
5.6.1	Aim	121
5.6.2	Materials and methods	121
5.6.2.1	LDPI	121
5.6.2.2	Blood-flow model	121
5.6.2.3	Experiments	123
5.6.2.3.1	Effect of distance between the laser detector and the measurement site on LDPI signal	123
5.6.2.3.2	Accuracy of LDPI reading measuring blood flow vs. true flow rate	123
5.6.2.3.3	The effect of reduction of RBC concentration by haemodilution on LDPI signal	124
5.6.2.3.4	Changes in the partial pressure of oxygen (Po ₂) on LDPI signal	124
5.6.2.3.5	Effect of movement of the imaging site	124
5.6.2.3.6	Assessment of geometric distortion	125
5.6.3	Data analysis	125

5.6.4	Results	126
5.6.4.1	Effect of distance between the laser detector and the measurement site on LDPI signal	126
5.6.4.2	Accuracy of LDPI reading measuring blood flow vs. true flow rate	127
5.6.4.3	The effect of reduction of RBC concentration by haemodilution on LDPI signal	128
5.6.4.4	Changes in the partial pressure of oxygen (Po ₂) on LDPI signal	129
5.6.4.5	Effect of movement of the imaging site on the LDPI signal	130
5.6.4.6	Assessment of geometric distortion of image generated by the LDPI	131
5.6.5	Discussion	132
6	Assessment of normal vulval skin microcirculation -----	136
6.1	Introduction	137
6.2	Aim	137
6.3	Materials and methods	138
6.3.1	Study population	138
6.3.2	Measurement procedure	139
6.3.3	Reproducibility	140
6.3.4	Cyclical change of vulval perfusion	141
6.3.5	Age related variations of vulval perfusion	141
6.3.6	Local skin temperature challenge on vulval perfusion	142
6.3.7	Image analysis	142
6.3.8	Statistical analysis	143
6.4	Results	144
6.4.1	Reproducibility	146
6.4.2	Cyclical change of vulval perfusion	148
6.4.3	Age related variations of vulval perfusion	151
6.4.4	Vulval perfusion after local skin temperature challenge	152
6.5	Discussion	153

7	Assessment of diseased vulval skin microcirculation -----	157
7.1	Introduction	158
7.2	Aim	158
7.3	Methods and materials	158
7.3.1	Study population	159
7.3.1.1	Normal women	159
7.3.1.2	Clinical profile of women with LS	159
7.3.1.3	Clinical profile of women with VIN 3	161
7.3.2	Reproducibility	162
7.3.3	Statistical analysis	162
7.4	Results	162
7.4.1	Reproducibility	167
7.4.2	Comparison between normal vulva and vulval lichen sclerosus	167
7.4.3	Comparison between normal vulva and VIN 3	169
7.5	Discussion	171
8	Vulval lichen sclerosus-perfusion variance to topical steroid therapy	174
8.1	Introduction	175
8.2	Aim	175
8.3	Materials and methods	176
8.3.1	Study population	176
8.3.2	Statistical analysis	177
8.4	Results	177
8.4.1	Perfusion changes in the control non-genital skin site (thigh) and the vulva during therapy.	181
8.4.1.1	Thigh	181
8.4.1.2	Clitoris	182
8.4.1.3	Clitoral hood	184
8.4.1.4	Labia minora	185
8.4.1.5	Inter-labial space	187
8.4.1.6	Perineum	189
8.4.1.7	Summary of results	190
8.5	Discussion	190

9	Diseased vulval skin – perfusion variance to local skin	
	temperature challenge -----	195
9.1	Introduction	196
9.2	Aim	196
9.3	Materials and methods	196
9.3.1	Study population	196
9.3.1.1	Clinical profile of women with LS	197
9.3.1.2	Clinical profile of women with VIN 3	198
9.3.2	Statistical analysis	199
9.4	Results	199
9.4.1	Vulval LS – PTS	199
9.4.2	Vulval LS – MPTS	201
9.4.3	VIN 3	203
9.5	Discussion	204
10	Normal and diseased vulva – Immunohistochemical microvessel	
	density (MVD) analysis -----	209
10.1	Introduction	210
10.2	Aim	210
10.3	Materials and methods	211
10.3.1	Archival tissue	211
10.3.2	Immunohistochemistry	213
10.3.3	Microvessel density (MVD)	214
10.3.4	Statistical analysis	215
10.4	Results	216
10.5	Discussion	225
11	Summary and future work -----	231
	Reference List -----	238

FIGURES

Figure 2.1	Structure of the vulva	30
Figure 2.2a	Photomicrograph of keratinised stratified squamous epithelium in the vulva (Magnification x40)	38
Figure 2.2b	Photomicrograph of stratified squamous epithelium of the mucous membrane from the vestibule. There is no keratinisation or granular zone (Magnification x40)	38
Figure 2.3	Diagram of the segmental arrangement of the microvasculature in the papillary dermis	41
Figure 2.4	Diagrammatic representation of computer reconstruction of vessels present in the papillary dermis	46
Figure 3.1a-d	Clinical photographs of vulval LS	53-54
Figure 3.2	Clinical photograph of vulval SCH	56
Figure 3.3	Photomicrograph of vulval LS	57
Figure 3.4	Photomicrograph of vulval SCH	59
Figure 3.5a-d	Clinical photographs of high-grade VIN (VIN 3)	73
Figure 3.6a-b	a) Photomicrograph of high grade VIN (VIN 3) – usual type (Magnification x20) b) Photomicrograph of high grade VIN (VIN 3) – usual type, showing abnormal mitosis (Magnification x60)	75
Figure 5.1	Laser light transmitted to skin, by the LDPI, is scattered by stationary tissue elements and by moving blood cells within the microvessels.	109

Figure 5.2	Block diagram of the LDPI	112
Figure 5.3	Colour-coded perfusion image of the hand	112
Figure 5.4a	The PC controls the optical scanner, processes the photocurrent from the detector to form a signal proportional to skin perfusion and generates a colour-coded image on the monitor	113
Figure 5.4b	The imaging system consists of a source of laser and a scanning head.	114
Figure 5.4c-d	The scanning head houses the stepping-motor which controls the mirrors moving laser beam over the measuring site, and a detector unit that collects the backscattered Doppler shifted light.	115
Figure 5.5	Colour coding of the LDPI image.	117
Figure 5.6	Blood-flow model	122
Figure 5.7	Mean LDPI reading vs. distance between the laser detector and the imaging site at the flow rate of $1.23 \mu\text{s}^{-1}$.	126
Figure 5.8	Relationship between blood flow determined by timed fluid collection and mean flux determined by the LDPI	127
Figure 5.9	Relationship between blood flow rate and LDPI reading with haemodilution of 10%, 20% and 30% by volume with physiological saline solution.	128

Figure 5.10	Response of the LDPI to changing pressure of oxygen supplied to the blood. Blood flow rate was constant at $4 \mu\text{ls}^{-1}$.	129
Figure 5.11	Effect of the speed of movement of the imaging site (blood flow model) on LDPI reading at constant blood flow of $3.4 \mu\text{ls}^{-1}$.	130
Figure 5.12	Diagrammatic representation of the image distortion by LDPI.	131
Figure 6.1a	Diagrammatic representation of the use of the LDPI on the vulva.	139
Figure 6.1b	Clinical photo of the assessment of vulval perfusion with the LDPI	139
Figure 6.2 a - b	Clinical photograph (Figure 6.2a) and LDPI image (Figure 6.2b) of a normal vulva in a 25y old. Note a region of relatively high blood flow in the clitoral area.	147
Figure 6.3 a	Percentage change in vulval SBF during week 2, 3 and 4 with reference to week 1 of the menstrual cycle	149
Figure 6.3b	Percentage change in vulval SBF during week 1, 3 and 4 with reference to week 2 of the menstrual cycle	149
Figure 6.3c	Percentage change in vulval SBF during week 1, 2 and 4 with reference to week 3 of the menstrual cycle	150
Figure 6.3d	Percentage change in vulval SBF during week 1,2 and 3 with reference to week 4 of the menstrual cycle	150

Figure 6.4	Comparison of vulval skin baseline perfusion in premenopausal, ‘young’ postmenopausal (< 70y) and ‘elderly’ postmenopausal (≥ 70 years) woman.	151
Figure 6.5	Change in normal vulval baseline perfusion in response to hot and cold provocation	152
Figure 7.1a - b	Clinical photograph (a) and LDPI (b) of normal vulva. There is a region of increased perfusion around the clitoris	164
Figure 7.2a - b	Clinical photograph (a) showing vulval LS. There is pallor, absorption of labia minora, partial phimosis and excoriation on the left labium minus. The LDPI image (b) shows a high perfusion area on both the right and left labia minora.	165
Figure 7.3a - b	Clinical photograph (a) showing a large area of VIN3 in the left anterior inter-labial space, anterior left labium minus, clitoral hood and the perineum. The LDPI image (b) shows high perfusion areas corresponding to regions of suspected VIN 3, which was later confirmed on biopsy.	166
Figure 7.4	Graph comparing the vulva perfusion in normal women, and in women with LS, before and after treatment with topical steroids.	168
Figure 7.5	Graph comparing the vulva perfusion in normal women and in women with VIN 3.	169

Figure 7.6	Graph comparing the vulva perfusion in adjacent areas of VIN 3 and normal skin in the same woman.	170
Figure 8.1 a-e	Clinical photograph (a) of vulval LS with corresponding LDPI images on the next page (b) before treatment, followed by serial images at four weeks (c) and eight weeks (d) after treatment with potent topical steroids - clobetasol propionate, and eight weeks (e) after treatment with moderately potent topical steroid - clobetasone butyrate.	178
Figure 8.2	Graph shows the change of mean base-line thigh (control skin area) skin blood flow with 95% confidence interval, during treatment of vulval LS.	181
Figure 8.3	Graph shows the change in mean base-line clitoral skin blood flow with 95% confidence interval, during treatment of vulval LS.	183
Figure 8.4	Graph shows the change in mean baseline clitoral hood skin blood flow with 95% confidence interval, during treatment of vulval LS.	184
Figure 8.5	Graph shows the change in mean baseline right labium minus skin blood flow with 95% confidence interval, during treatment of vulval LS.	186
Figure 8.6	Graph shows the change in mean baseline left labium minus skin blood flow with 95% confidence interval, during treatment of vulval LS.	186

Figure 8.7	Graph shows the change in mean baseline skin blood flow right inter-labia space with 95% confidence interval, during treatment of vulval LS.	188
Figure 8.8	Graph shows the change in mean baseline skin blood flow left inter-labia space with 95% confidence interval, during treatment of vulval LS.	188
Figure 8.9	Graph shows the change in mean baseline perineal skin blood flow with 95% confidence interval, during treatment of vulval LS.	189
Figure 9.1 and Figure 9.2	Graphs show the change in mean baseline perfusion with 95% confidence interval with local hot and cold provocation in vulval LS before (Figure 9.1) and after (Figure 9.2) the first phase of treatment with potent topical steroids – clobetasol propionate or Dermovate.	200
Figure 9.3 and Figure 9.4	Graphs show the change in mean baseline perfusion with 95% confidence interval with local hot and cold provocation in vulval LS before (Figure 9.3) and after (Figure 9.4) the second phase of treatment with moderately potent topical steroids – clobetasone butyrate or Trimovate.	202
Figure 9.5	Graph shows the change in mean baseline perfusion with 95% confidence interval with local hot and cold provocation in VIN 3 before treatment	203

Figure 10.1a	Photomicrograph of normal vulval skin sectioned at an angle showing normal distribution of microvessels within the superficial dermis and cross-section of the dermal papillary microvessels within epidermis, immunostained with anti-vWF (Magnification x200).	217
Figure 10.1b	Photomicrograph of vulval LS with a sparse number of microvessels immunostained with anti-vWF, within the superficial dermis. (Magnification x100).	218
Figure 10.1c	Photomicrograph of vWF immunostain in VIN 3. Microvessels are clearly denser within the sub-epithelial dermis compared to LS. (Magnification x100).	219
Figure 10.1d	Photomicrograph showing an invading tongue of vulval SCC surrounded by numerous microvessels in the stroma. (Magnification x100)	220
Figure 10.2	Scatter graph showing the overall median (●) values, comparing the distribution of the HVD per unit area for normal vulva, LS, VIN 3 and SCC.	221
Figure 10.3	Scatter graph showing the overall median (●) values, comparing the distribution of the AVD per unit area for normal vulva, LS, VIN 3 and SCC	222

TABLES

Table 3.1	Nonneoplastic epithelial disorders (NNED) of the vulval skin and mucosa	51
Table 3.2	The association of vulval LS with squamous cell carcinoma	64
Table 3.3	Vulval intraepithelial neoplasia	69
Table 3.4	Modified terminology for VIN	71
Table 3.5	Reported progression, recurrence and occult invasive disease rates for VIN	79
Table 4.1	Non-invasive methods for the clinical study of blood flow in small vessels	91
Table 4.2	Biophysical methods for measuring skin blood flow – Date of proposal and principle	92
Table 4.3	Biophysical methods for measuring skin blood flow – Methodology; units of measurement, direct or indirect measure of SBF and remarks	93 - 94
Table 4.4	Biophysical methods for measuring skin blood flow – Advantages and disadvantages	95 - 97
Table 4.5	Biophysical methods for measuring skin blood flow – Research and clinical application	98-100
Table 6.1	Sub-group analysis of the age (years \pm standard deviation), systolic blood pressure (sBP)(mmHg \pm SD), diastolic blood pressure (dBP) and mean arterial blood pressure (MAP).	145

Table 6.2	The mean Flux, standard deviation (SD) and coefficient of variability (CV) in normal vulva skin and non-genital skin	146
Table 7.1	The age, body temperature, skin temperature on the right labium majus, heart rate, arterial blood pressure (sBP=systolic blood pressure, dBP=diastolic blood pressure and MAP=mean arterial pressure) and body mass index (BMI) of all the subjects in study.	163
Table 7.2	<i>P values</i> for vulval flux of selected <i>regions of interest</i> (ROI) in normal vulva, untreated LS and treated LS.	168
Table 8.1	The number of patients in each treatment pair, the age and body mass index with observations of temperature, pulse rate and blood pressure	180
Table 10.1	The median MVD values (HVD and MVD) per unit area with lower and upper quartiles for each vulval tissue specimen.	223

ABBREVIATIONS

°C	degrees centigrade
3-D	3-dimensional
APES	aminopropyltriethoxysilane
AVA	arteriovenous anastomosis
A-V	arterio-venous
AC	alternating current
AVD	average vessel density
BMI	body mass index
CI	confidence interval
CIN	cervical intraepithelial neoplasia
CIN 1	mild grade cervical intraepithelial neoplasia
CIN 3	severe grade cervical intraepithelial neoplasia
CIS	carcinoma in-situ
CMBC	concentration of moving red blood cells
CO ₂	carbon dioxide
CP	clobetasol propionate
CV	coefficient of variation
CVI	chronic venous insufficiency
dBp	diastolic blood pressure
DC	direct current
DNA	deoxyribonucleic acid
dVIN	differentiated vulval intraepithelial neoplasia

F	Flux
F8RA	Factor 8 Related Antigen
FU	follow-up
h	hours
HPV	human papilloma virus
HVD	highest vessel density
He-Ne	helium-neon
H + E	haemotoxylin and eosin stain
H ₂ O ₂	hydrogen peroxide
IMS	Industrial methylated spirit
IR-TH	infrared thermography
ISSVD	International Society for the Study of Vulvovaginal Disease
L	lumbar
LASER	light amplification by stimulated emission of radiation
LDF	laser Doppler flowmetry
LDPI	laser Doppler perfusion Imager
LDV	laser Doppler velocimetry
LS	lichen sclerosis
LSC	lichen simplex chronicus
MAP	mean arterial pressure
min	minutes
MPTS	moderately potent topical steroids
MRBCC	mean red blood cell concentration

MRBCV	mean red blood cell velocity
mRNA	messenger ribonucleic acid
ms	millisecond
MVD	microvessel density
n	number of subjects
NNED	non-neoplastic epithelial disorder
NSBF	nutritive skin blood flow
O ₂	oxygen
PBS	ph buffered saline
PC	personal computer
PCR	polymerase chain reaction
P _a O ₂	arterial partial pressure of oxygen
PTS	potent topical steroids
PVD	peripheral vascular disease
PPG	photoplethysmography
RA	relative absorption
RBC	red blood cell
RBCV	red blood cell velocity
RFH	Royal Free Hospital
RFHSM	Royal Free Hospital School of Medicine
RFS	Reflectance spectrophotometry
ROI	region of interest
S	sacral

SBF	skin blood flow
sBP	systolic blood pressure
SCC	squamous cell carcinoma
SCH	squamous cell hyperplasia
SD	standard deviation
SH	Squamous hyperplasia
SPP	skin perfusion pressure
T	temperature
TEWL	transepidermal water loss
T _c PO ₂	transcutaneous partial pressure of oxygen
TSBF	total skin blood flow
UEA-1	<i>Ulex europaeus</i> agglutinin 1
VEGF	vascular endothelial growth factor
VIN 1	mild grade vulval intraepithelial neoplasia
VIN 2	moderate grade vulval intraepithelial neoplasia
VIN 3	high grade vulval intraepithelial neoplasia
VPF	vascular permeability factor
vWF	von Willebrand Factor
W	week
y	years

CHAPTER 1

Introduction

1.1 Introduction

The vulva is an area of specialised skin, associated with the openings of the urinary, genital and intestinal tract, and their various functions. The scientific investigation of the anatomy, physiology and pathology of the vulva is a challenge in view of the considerations associated with religion, ethnicity and embarrassment. The study of vulval pathology is also impaired by the differences in presentation compared to the same disease in better-investigated non-genital skin sites and by the confusing array of clinical terms used by the different specialities with an interest in the care of women presenting with vulval symptoms³³⁰.

During the past 30 years increasing interest in diseases of the vulva has led to the formation of specialist vulval clinics at several centres around the country, including the Royal Free Hospital (RFH)¹⁹⁸. The International Society for the Study of Vulvovaginal Disease (ISSVD), established in 1970, has facilitated standardisation of the nomenclature and stimulated discussion at an inter-disciplinary international level²⁵⁴.

1.2 Vulval disease

Vulval lichen sclerosus (LS) and high-grade vulval intraepithelial neoplasia (VIN) are two diseases frequently seen in a specialist vulval clinic and have an unclear potential for neoplastic progression. LS, classified as a non-neoplastic epithelial disorder of the vulva, is the most common dermatological disease in this specialised skin, accounting for as much as 23% of new patients seen in the vulval clinic^{198,254}. Vulval LS has a 3-5% reported risk of malignant transformation but histological analysis of squamous cell carcinoma (SCC) demonstrates LS in the adjacent skin in 65 to 75% of cases^{63,193}. High-grade VIN is less common than vulval LS but the reported risk of malignant

progression varies between 5% and 87%, depending on previous treatment^{151;193}.

Occult invasive carcinoma is present in up to 23% of high-grade VIN^{19;133;159;217;252;286}.

Histological studies report high-grade VIN in the adjacent skin in approximately 25% of women diagnosed with SCC¹⁹³. Clinically, there is concern that high-grade VIN is increasingly being diagnosed in young women, with implications of distortion or disfiguration from surgical treatment. This was previously thought to be a relative increase attributed to greater clinical acumen particularly as the incidence of SCC had been reported to have remained static^{125;139}. However sub-analysis of women under the age of 50y by Joura et al¹⁵⁵ indicated that these young women experienced a more than doubling of the incidence in invasive vulval carcinoma. More recently MacLean¹⁹⁴ also reported that the incidence of SCC showed the greatest increase in women under the age of 50y, which raises concern about a real increase in the incidence of high-grade VIN in young women.

1.3 Background to thesis

The aim of this thesis was to introduce a new physiological parameter to assess vulval disease. Advances in laser Doppler technology have provided a non-invasive and non-contact biophysical method for measuring skin blood flow (SBF) over a wide area such as the vulva, within several minutes. The ability to assess changes in SBF in vulval disease may pave the way towards developing more effective medical methods of treatment and to reduce the need for surgery, as well as identifying women with LS and high-grade VIN at greater risk of progression to cancer.

The significance of SBF as a potential biophysical parameter to assess disease with a risk of developing malignancy arises from immunohistochemical studies on histopathological tissue samples in a variety of cancers demonstrating a correlation

between an increase in the microvessel density (MVD) with metastatic disease and prognosis for - malignant melanoma¹¹⁰, breast carcinoma^{162:339}, non-small cell lung carcinoma¹⁹⁰ and prostatic adenocarcinoma³³⁸. This increase in MVD is due to angiogenesis, the formation of new blood vessels, an essential physiological process in embryonic development and wound healing; it is also believed to have a critical role in the growth of malignant tissue beyond the limits (1-2 mm) of simple diffusion of nutrients and oxygen^{96:97}. In human pre-cancerous lesions of the colon, 3-dimensional (3-D) reconstruction of microvascular architecture and vascular endothelial growth factor (VEGF) immunostaining also suggest that formation of new blood supply, termed angiogenesis, sets in long before the progress towards invasive phenotypes¹⁷¹. Indeed, increased vascularisation measured by immunostaining for various endothelial markers has been reported in pre-neoplastic disease in a wide range of tissues: breast intra-ductal carcinoma^{36:112:339}, cervical intraepithelial neoplasia^{80:291}, complex endometrial hyperplasia², latent prostatic carcinoma¹⁰⁴ and colorectal adenomas³⁷. The work by Dobbs et al⁸⁰ on the cervix, which relates to vulva demonstrated a progressive increase in MVD and VEGF expression from the normal cervix through mild cervical intraepithelial neoplasia (CIN 1) to high grade disease (CIN 3) and invasive SCC, providing further support for the hypothesis that an increase in blood supply by angiogenesis is an early event in pre-neoplastic disease. More recent work by Ozalp et al²³⁷ and Sotiropoulou et al²⁹⁵ also concluded that the onset of angiogenesis is an early event in cervical SCC, starting in the pre-invasive stage. Studies on the microvasculature in high-grade VIN show a significantly higher MVD using anti-von Willebrand factor (vWF) immunostain and a greater expression of VEGF compared to lower grade disease¹⁴. VEGF, a leading mediator in the induction of angiogenesis, is reported by MacLean et al¹⁹⁷ to be present in relatively fewer cases of VIN compared to vulval SCC: potentially these may be cases of VIN at risk of malignant progression.

Angiogenesis is a complex process and techniques to observe and quantify microvessels histologically in sections of tissue can, in addition to being invasive, only provide an estimate of the final product; physiological variables such as blood flow, vascular permeability and interstitial pressure, which influence the relationship of angiogenesis to malignant transformation, are very relevant but also much more complex to measure^{141:337}.

1.4 Objective for this thesis:

To study the SBF in the normal and diseased vulval skin.

1.5 Hypothesis

1. Does the laser Doppler perfusion imager (LDPI) allow assessment of the microcirculation of the vulva?
2. Is the use of the LDPI to measure vulval SBF acceptable to women with normal vulva? How constant is the SBF on the normal vulva?
3. Does the SBF in the normal vulva vary according to age and menstrual cycle? How does the SBF in the normal vulva respond to temperature provocation?
4. Is the use of the LDPI to measure vulval SBF acceptable to women with vulval lesions? How constant is the SBF on the vulva in the presence of vulval lesions?
5. Does SBF in vulval LS vary when symptomatic before treatment compared to when asymptomatic after treatment, and when compared to the normal vulva?

6. Does the SBF in vulval LS vary during the recommended therapeutic regimen of potent followed by moderately topical steroids phased down to maintenance therapy, compared to before treatment?
7. Is there any difference in SBF between high-grade VIN and normal vulval skin?
8. Does the use of temperature provocation explain more about the SBF in vulval LS and high-grade VIN?
9. Is there any correlation between the SBF measurements by the LDPI and MVD assessment by immunohistochemistry?

The terms SBF, skin microcirculation and skin perfusion refer to the same function and are interchanged in the thesis.

CHAPTER 2

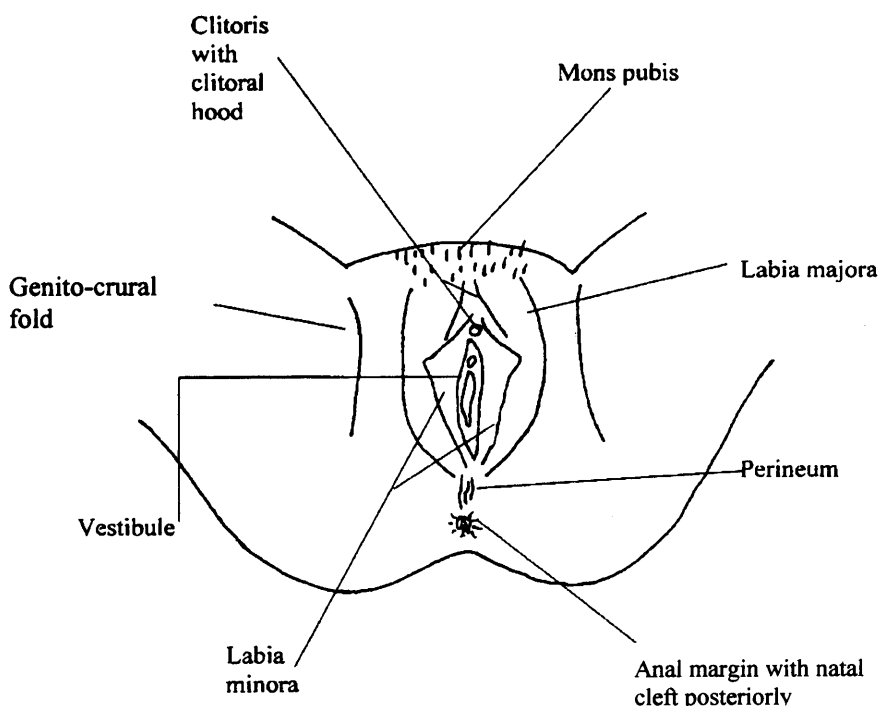
The Vulval Skin

2.1 Anatomy, Physiology and Function of the Vulva.

2.1.1 Introduction

The vulva forms the external female genitalia. In the lithotomy position it is bound anteriorly by the mons pubis, posteriorly by the anus, and laterally by the genito-crural folds (Figure 2.1). The vulval skin is comprised of the mons pubis, the labia majora and minora, the vestibule and the clitoris as shown in Fig. 2.1. In this thesis the area of study includes the inner labia majora (i.e. part of the inter-labial space and which is not covered with hair) and labia minora, clitoris, clitoral hood and perineum, which are affected by LS and VIN. The genito-crural fold and natal cleft are not studied in this thesis although LS, in particular, may extend laterally into these areas.

Figure 2.1 Structure of the vulva



2.1.2 Anatomy

The mons pubis is a rounded fibro fatty subcutaneous pad, covered with coarse hair, forming a cushion over the symphysis pubis^{205;219;340}. It extends over the perineum in an elliptical fashion to form the labia majora. The labia majora are two large, longitudinal folds of skin enclosing the vulval cleft and consisting of fibro fatty tissue attached to overlying skin by a smooth muscle network. Posteriorly the labia majora forms a horizontal fold of skin lying behind the fourchette, termed the posterior commissure. The outer-pigmented skin surface is covered by coarse hair and contains sebaceous and sweat glands while their moist internal aspect is smooth, hairless, gland-studded and usually slightly separated by protrusion of the labia minora.

The labia minora are richly innervated thin, delicate, smooth, pigmented fat-free and hairless skin with “free” sebaceous glands opening directly onto the skin surface. The core of each fold of labia minora consists of loose connective tissue permeated by a fine elastic network, some smooth muscle fibres and extensive vascular spaces forming erectile tissue. The labia minora lie between the labia majora and enclose the vestibule. Anteriorly the labia minora bifurcate into the prepuce and the frenulum, enclosing the clitoris. Posteriorly the labia minora fuse to form a transverse fold behind the vaginal opening, the fourchette.

The vestibule extends anteroposteriorly from the frenulum of the clitoris to the fourchette and laterally from the hymenal ring to Hart’s line on each labium minus.

Hart’s line is the junction between the smooth, nonkeratinised epithelium medially and the slightly papillated, keratinised epithelium of the more lateral labia minora.

Localised within the vestibule are the openings of the vagina, urethra, the ducts of the Bartholin’s glands and the minor vestibular glands.

The clitoris is an erectile body consisting of two corpora cavernosa beneath the clitoral hood and a glans distally, visible between the bifurcations of the labia minora.

The perineal skin lies between the fourchette and the anal margin while the natal cleft forms the skin fold posterior to the anus.

2.1.3 Physiology

2.1.3.1 Blood supply

The arterial supply to the vulva is from the internal iliacs by way of the internal pudendal artery and the external iliacs by way of the branches of the femoral artery provided by the superficial and deep external pudendal arteries^{205;219;340}. These vascular systems extensively communicate by anastomosis, which explains why ischaemia of the vulva is impossible even with bilateral blockage of the internal iliac arteries, and account both for its capacity to bleed profusely and to heal with remarkable ease. An extensive intercommunicating venous plexus leave the vulva accompanying the arterial supply. Venous drainage is mainly via the internal pudendal veins draining into the internal iliac veins and the external pudendal veins which pass to the great saphenous veins, to reach the femoral and subsequently the external iliac veins. The deep dorsal vein drains the clitoris, through the vesicle plexus and into the internal iliac veins.

2.1.3.2 *Lymphatic supply*

The major lymphatic drainage from the vulva is to the medial horizontal group of superficial inguinal lymph nodes^{205;219;340}. There is additional drainage by the superficial external pudendal lymphatic vessels, to the superficial femoral nodes. The region of the clitoris is drained by the deeper lymphatic nodes, which pass directly to the deep femoral nodes and to the external iliac nodes. The relevance of regional lymph drainage is in the management of malignant disease.

2.1.3.3 *Nerve supply*

The nerve supply of the vulva is derived from the sacral (S) and lumbar (L) plexuses, namely the pudendal nerve from S2, S3, S4, perineal branch of the posterior cutaneous nerve of thigh from S3, genital branch of the genitofemoral from L1, L2, the ilioinguinal from L1 and the anterior hypogastric branch of the iliohypogastric nerve from (thorax) T12-L1.

There are specialised nerve endings of myelinated and unmyelinated nerves in the labia majora and minora and the clitoris. Encapsulated nerve endings called Meissner's corpuscles and Merkel's discs subserve touch. Pacinian corpuscles abound and respond to pressure induced by venous engorgement. Ruffini corpuscles and Dogiel-Krause corpuscles vary greatly in size and shape and may respond to changes of temperature stimuli associated with sexual activity.

The cutaneous nerves convey all modalities of common sensation – touch, pain, itch, warmth and cold, as well as complex sensations such as wetness. In addition, these cutaneous nerves carry post-ganglionic sympathetic nerves that are motor to sweat glands, pilomotor units and the adventitia of the microvasculature. No parasympathetic

fibres participate in this cutaneous innervation²³³. The sensory component of the parasympathetic innervation of the perineum mediate the sensation of distension from the anal canal and the vagina while the motor component extensively innervates the vessels in the external genitalia and is responsible for vascular engorgement of the vaginal erectile tissue⁸⁸.

2.1.3.4 Age and hormones

The labia majora and minora undergo changes in relation to puberty and menopause. The labia majora are practically absent in the young child and then develop primarily by the deposition of fat with the heralding of puberty. The presence of pigmentation and hair on their lateral surfaces establishes the labia majora as well defined structures. The pigmentation of the labia minora also becomes more obvious during adolescence. After the menopause the labia majora becomes less prominent with gradual decrease in subcutaneous fat with increasing age and there is loss of hair follicles causing thinning of labial hair¹⁸.

Jones¹⁴⁷ examined the histology in normal vulva from postmortem tissue, using radial biopsies extending from the vaginal margin, through the labia minora, extending to the lateral margin of the labia majora. The keratin and epithelial layer are thin at birth, thicken after menarche reaching a maximum during reproductive years and finally start to thin after the menopause. The keratin layer increases in thickness from the medial to the lateral aspect of the vulva. The rete pegs change following the trend of the epithelial and keratin thickness, with their length and number reaching their maximum during the reproductive era and slowly reducing after the menopause. Harper and McNicol¹¹⁹ examined biopsies from the labium majus between the upper third and the lower two third, reported similar findings as Jones¹⁴⁷ on the rete pegs and epithelial thickness. In

contrast to Jones¹⁴⁷, Harper and McNicol¹¹⁹ noted very gradual thickening of the keratin layer throughout life. Erickson and Montagna⁸⁸ reported less well developed rete ridges in the labia majora compared with the labia minora and the most well developed long and slender rete ridges in the clitoris, suggesting the difference reflected the mechanical demands made in each area. There is a scarcity of information on the microanatomical structure of the vulva and comprehensive information including all structures of the vulva is limited to studies of cadaveric tissue specimens. The research reports^{88;119;147} described above are excellent but artefacts related to the method of collection of tissue specimen could be responsible for the differences in reports.

The vulval skin changes with puberty and menopause suggest steroid hormone influence on its development. This is however not supported by immunohistochemical study assessing the frequency of oestrogen receptors in the epidermis of the vulva, which show a low concentration, when compared to the vagina and to non-genital skin¹⁹⁵. Other studies comparing normal, dysplastic and malignant tissue also report a low concentration of oestrogen receptors in the vulval skin^{146;234}. Although the discrepancy between immunohistochemical hormone receptor studies compared with clinical physiological impression may be due to sensitivity and specificity of the assays used, these consistent research findings have implications for the use of topical oestrogen cream for the treatment of vulval conditions.

2.1.4 Function of the vulva

The general functions of this specialised skin are as with other skin, to maintain integrity of the body by providing a waterproof covering, detecting sensory stimuli, absorption and excretion of liquid, and acting as a barrier against microorganisms. In addition the vulva has specialised functions related to its role in reproduction. The vulva has erectile tissue in the labia minora, the clitoris and the vestibular bulbs on either side of the vaginal opening which are richly supplied with blood vessels forming a network of plexuses and anastomosis, as described in section 2.1.3.1. These vulval erectile tissue are capable of vasocongestion and resolution decongestion within minutes^{55,258}. Menopause and old age result in a gradual loss of this physiological response to sexual stimulation due mainly to loss of erectile tissue particularly in the labia minora.

2.2 Structure of skin and organisation of the microvasculature

2.2.1 Histology

All the structures of the vulva are cutaneous and consist mainly of keratinised stratified squamous epithelium (Figure 2.2a)^{341;347}. The vestibule is also cutaneous but the epithelial covering is not keratinised (Figure 2.2b).

Stratified squamous epithelium has four histological layers: - a basal layer or stratum germinativum resting on the basal lamina, a spinous or prickle-cell layer which forms the bulk of the epidermis, a granular layer and a horny/keratin layer or stratum corneum. The outer tough and protective keratin layer of the stratum corneum is continuously shed and replaced by progressive movement and maturation of cells produced by mitosis in the basal layer. These epithelial cells become flatter and broader, finally loosing their nucleus to form keratin.

Beneath the avascular epidermis is the dermis, which consists of the papillary and reticular parts. The papillary dermis projects upwards interdigitating with the epidermal rete ridges. The papillary connective tissue is composed of fine collagen fibres with reticular and elastic fibres, running at right angles to the surface²⁴⁰. The reticular dermis below the papillary dermis is composed of coarse collagen fibres lying parallel with the surface and thick elastic fibres that prevent the dermal collagen from being overstretched²⁴⁰. The exact position of the deep dermal boundary between the dermis and the subcutaneous fat beneath is less well defined¹⁴⁷.

Figure 2.2a: Photomicrograph of keratinised stratified squamous epithelium in the vulva (Magnification x40)

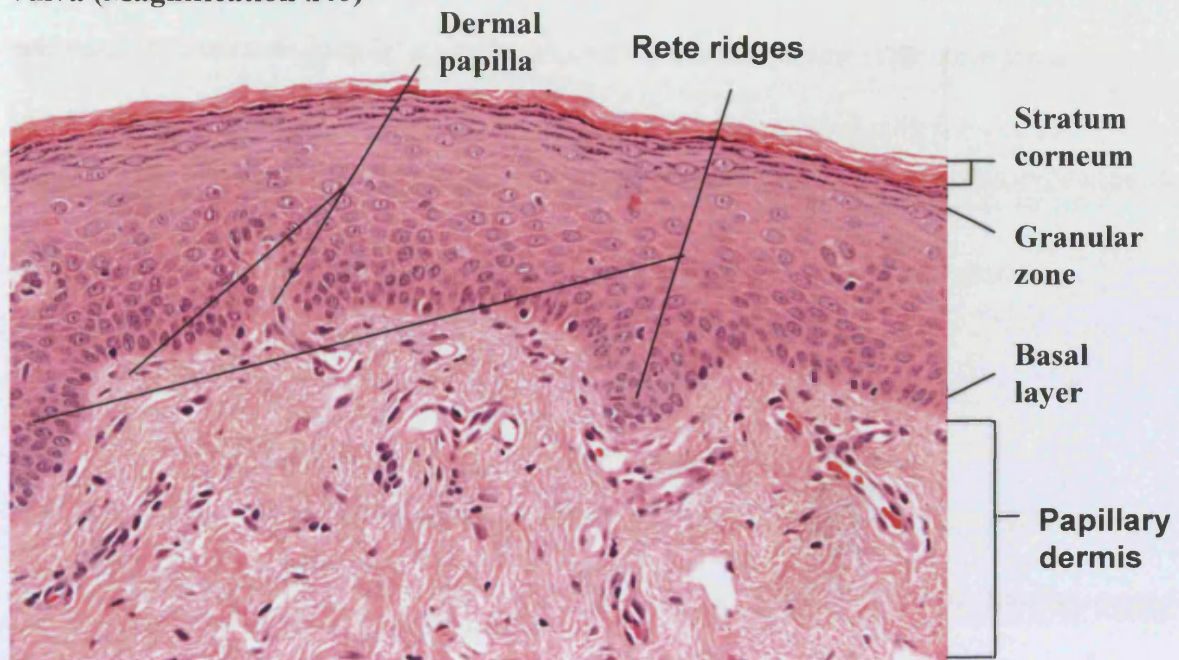
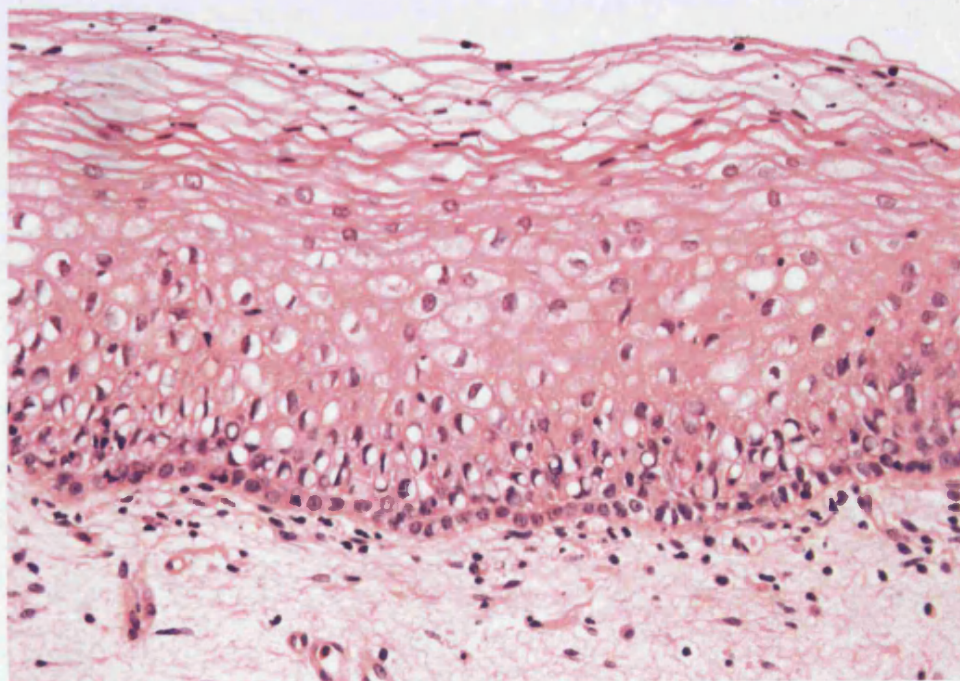


Figure 2.2b: Photomicrograph of stratified squamous epithelium of the mucous membrane from the vestibule. There is no keratinisation or granular zone (Magnification x40)



The skin appendages are very well vascularised in man compared to other primates¹⁹¹. The mons pubis, outer labia majora and the perineum are covered by hair bearing skin and contain sebaceous glands, apocrine and eccrine sweat glands. The inner labia majora and labia minora are non-hair bearing skin richly provided with sebaceous glands opening directly onto skin forming tiny elevations on the skin surface, referred to as Fordyce's spots. These areas are devoid of apocrine and eccrine sweat glands.

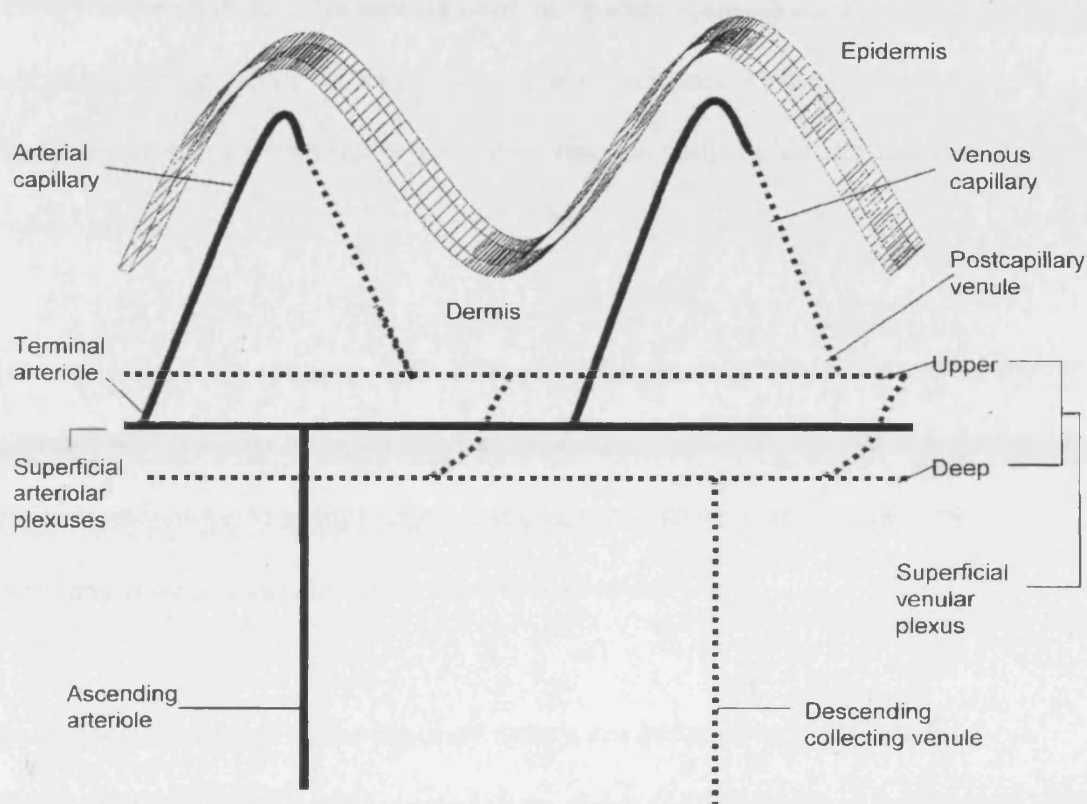
2.2.2 Skin blood supply

Musculocutaneous vessels branch off blood supply to the deep muscles and continue peripherally to form the main supply of the skin⁴⁰. A few direct cutaneous vessels perforate the muscle and go straight to the skin, travelling parallel to the surface of the skin. These major vessels perforate the subcutaneous fat and supply the arterioles and venules of the skin microcirculation to form two important plexus in the dermis: an upper horizontal network in the papillary dermis from which nutritive capillary loops of the dermal papilla arise and a lower horizontal plexus in the reticular dermis at the interface between the dermis and subcutaneous fat. The lower horizontal plexuses connect directly with the upper horizontal plexuses and also provide lateral tributaries that supply the hair bulbs and sweat glands. There are some interconnections among the ascending arterioles and descending venules within the dermis, but these two horizontal plexuses represent the physiologically important areas in the skin.

2.2.2.1 *Papillary dermis*

The microvessels in the mid to upper dermis or the papillary dermis vary in diameter from 10 to 35 μ m but most are in the 17 to 22 μ m range. The microvasculature between the ascending arteriole and the descending collecting venule could be divided into the following seven segments (Figure 2.3): arteriole of superficial plexus, terminal arteriole, arteriole and venous side of the capillary loop, postcapillary venule, venules of the superficial plexuses (upper and deep components interconnected by vertical shunts). Vessel classification by morphology of vessel wall is more reliable than measurement of vessel diameter, which tends to widely overlap between the different vascular segments and are influenced by the type of fixative used to prepare tissue^{45;127;351}.

Figure 2.3 Diagram of the segmental arrangement of the microvasculature in the papillary dermis (adapted from Higgins et al ¹²⁷)



The endothelial tube of the arterioles which most likely function as part of the resistance vessels in the skin is surrounded by two layers of smooth muscle cells – inner longitudinal and outer spiral, a subendothelial elastic lamina and a homogenous basement membrane. The diameter of the arterioles progressively decreases as it transcends to the beginning of the capillary bed by gradual disappearance of the two layers of smooth muscle cells to form the terminal arteriole. The terminal arteriole has a single-cell layer with less well-developed dense bodies and fewer filaments compared to the smooth muscle cells, and circumferential arms almost completely encircle the endothelial tube.

The next microvascular segment, the arterial capillary is surrounded by pericytes which replace the single cell, have contractile protein and form tight junctions with the endothelial cells through breaks in the homogenous basement membrane. As the capillary is traced around the hairpin loop the basement membrane progressively begins to develop lamella within its homogenous framework until a segment is reached in which the basement membrane in the entire vascular wall is multilaminated, forming the venous capillary.

The venous capillary connects with the postcapillary venule, whose external diameter progressively increases. The endothelial tube is surrounded by two to three layers of pericytes in contrast to a single layer in the venous capillary, and the basement membrane is multilaminated, as in the venous capillary.

The majority of vessels in the papillary dermis are postcapillary venules, the physiologically most reactive segment of the microcirculation. Here inflammatory cells migrate from the vascular space into the tissues, and endothelial cells often develop intercellular gaps that result in increased vascular permeability in response to acute inflammation.

2.2.2.2 *Reticular dermis*

The microvessel diameters in the reticular dermis in the mid to lower dermis are wider ranging from 40 to 50µm, although rarely a vessel as large as 100µm can be found⁴².

The walls of the arterioles and venules are thicker with smooth muscle cells or pericytes present as four to five layers in contrast to one to two layers in similar vessels of the superficial plexuses.

2.2.2.3 *Regional differences*

There are no structural differences in the vessel structure of the skin in different body regions, although there are regional differences in vessel density. The capillary density in the papillary dermis is significantly higher than in the reticular dermis²⁴⁰. There have been several studies comparing regional differences in capillary density, although none of these included the genital skin. Pasyk et al²⁴⁰ recorded the highest dermal capillary density on cadaver histological sampling in the head-face followed by chest/back area, upper extremities, abdomen/buttock and the lower extremities – thigh and lower leg. The calf and shin area had the lowest density. Park et al²³⁹ also reported a relative decrease in blood flow from the thorax/arm to the thigh/lower leg, measured using the laser Doppler flowmetry (LDF) in supine subjects. Previously, other workers in the late 1950's²²⁰ observed a correlation between the thickness of the epidermis and dermis, and alteration in papillary dermal vasculature. When the dermis was thin, as in the skin of the thigh and the calf, there were fewer capillaries and scant vascular bed, while areas with thicker epidermis and well-developed rete ridges, had capillaries present in each rete ridge. Although there have been no studies on the vulval skin capillary density, based on the information available on the blood supply to the vulva described in Section 2.1.3.1 above, it is likely that vulval skin has relatively higher blood supply compared to

the surrounding skin although the ratio of difference between vulval and non-vulval skin may change after the menopause.

Structures resembling arteriovenous (A-V) communications have only been identified within the skin microvasculature of the digits, nose and ears^{127;351}.

2.2.3 Application of optics to assess SBF

Current understanding of the biophysics of the cutaneous microvasculature is attributed to work by Braverman et al⁴¹, correlating LDF signals with histological examination.

There are two aspects to skin blood flow – the spatial and temporal heterogeneity.

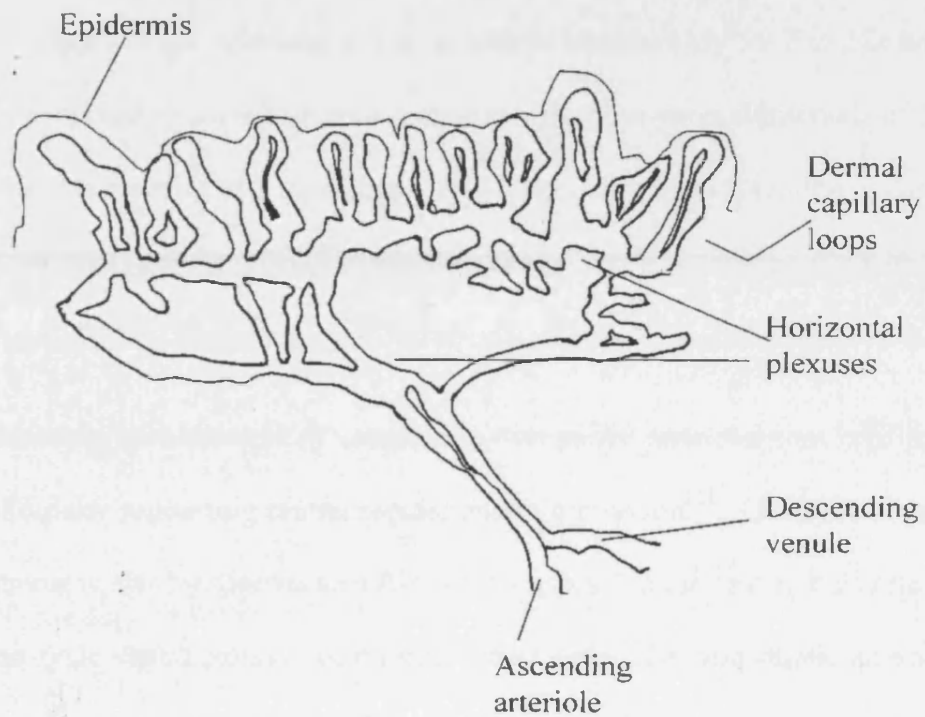
2.2.3.1 *Spatial heterogeneity*

The LDF gathers signals from a depth of only 1-1.5mm below the surface of the epidermis, therefore excluding the capillary blood supply to the sweat gland and hair follicles as these structures are 3-5mm below the skin surface. Most of the microvasculature contained in the papillary dermis is 1 to 2 mm below the epidermal surface. The LDF signal showing high amplitude red cell flux waves exhibiting vasomotion corresponded to an underlying ascending arteriole and low amplitude waves without vasomotor activity corresponded to venular predominance⁴³. Braverman et al⁴⁴ constructed separate topographic maps of LDF signal for flux and concentration of moving red blood cells (CMRBC), obtained simultaneously. Sites of high flux and high CMRBC indicated the presence of an underlying ascending arteriole and its paired descending venule. High flux, low-CMRBC sites indicated arteriolar predominance; low flux, high CMRBC sites indicated venular predominance; and low-flux, low-CMRBC sites denoted relative avascular areas. These LDF studies have demonstrated that the ascending arterioles are spaced randomly at intervals of 1.5-7mm, thus explaining the basis for spatial heterogeneity of LDF measurements⁴³.

Each ascending arteriole divides into four to five branches to form a portion of the upper horizontal plexus and is accompanied by a post capillary venule that is formed from the confluence of 8 to 10 branches within the upper plexuses. In 3-D the vascular unit resembles an umbrella (Figure 2.4): the paired ascending arteriole and descending

venule represent the handle. The arteriolar and venular branches form the umbrella proper, with the arterioles being predominant in the centre and the venules predominant in the periphery⁴¹.

Figure 2.4 Diagrammatic representation of computer reconstruction of vessels present in the papillary dermis (adapted from Braverman et al ⁴¹)



2.2.3.2 *Temporal heterogeneity*

The phasic (vasomotor) variation in red cell flux amplitude detected in the LDF signal is greatest over the ascending arteriole and its immediate branches, suggesting that these vessels are the morphological site for the generation of vasomotor activity in the skin⁴⁰. The putative sphincter, formed by the single cell with circumferential arms replacing the smooth muscle cells of the ascending arterioles, encircling the terminal arteriole, before transcending into the capillary, may be the origin of the vasomotion detected by the LDF. When a single arteriolar site is monitored continuously for 1 to 2 hours, the red cell flux changes from minimum to maximum and vice versa at intervals of 12 –20 minutes at some sites and 70-90 minutes at other sites, thus explaining in part the temporal heterogeneity of LDF measurements.

Simultaneous measurement of vasomotion over paired arteriolar sites confirms synchronicity suggesting central regulation of vasomotion²⁶⁸. Quantification of vasomotor waves by spectral analysis demonstrates that the beat-to-beat rate of the cardiac cycle varied around two means, one related to the sympathetic tone and the other to parasympathetic tone and respiratory rate. When a patient is tilted quickly from the supine to erect position the sympathetic tone is accentuated at the expense of the parasympathetic tone presumably because of baroreceptor generated increased sympathetic tone. Bernardi et al²³ demonstrated regional differences in sympathetic tone with significant reduction in responses in the human forearm.

2.3 Conclusion

The vulval skin has many histological similarities with skin in other sites and some differences related to its specialised physiology and function. More studies are required to clarify differences in histological features of the human vulval structures to variables such as age and cyclical hormone and, their influence on blood supply and flow.

Section 2.2.3 suggests that optical methods such as the LDF have the potential to further improve our understanding of vulval skin microvasculature in the presence and absence of disease, adding another dimension to the assessment of the vulval skin.

In the next chapter vulval LS and VIN, and their association with cancer are reviewed followed by the techniques currently available in clinical and research practise to assess the vulval skin.

CHAPTER 3

Review of vulval diseases -LS and
high-grade VIN and the assessment
of the vulval skin

3.1 Introduction

The aim of this chapter is to review vulval LS and high-grade VIN. The clinical presentation of these diseases with diagnosis, aetiology and the relationship with malignant disease are discussed. This is followed by a description of the methods used for assessment of the vulval skin in the clinical setting and the special techniques utilised in research.

3.2 Vulval Lichen sclerosus (LS)

LS is important because it is one of the most common dermatological diseases of the vulva and has a small but definite association with the development of squamous carcinoma of the vulva^{60;63;193;198;209}. It is an inflammatory disease of unknown cause and incompletely characterised pathogenesis. The definitive diagnosis of LS is made on histological examination of biopsy specimens¹²⁶.

The earliest description of this ‘persistent pruritic white vulval lesion’ was by Robert Weir in 1875. Henri Hallopeau coined the term ‘lichen sclerosus’ in 1887. The typical histological features of LS was first formally described in 1892 by J Darier^{192;256}. Since the first description of this disease a confusing array of clinical terminology has arisen which has impaired communication of research findings¹⁹². When the ISSVD was established in 1970 one of their main tasks was to standardise the nomenclature for vulval disease. The latest classification for nonneoplastic epithelial disorders is shown in Table 3.1²⁵⁵.

Table 3.1 **Nonneoplastic epithelial disorders (NNED) of the vulval skin and mucosa²⁵⁵**

Lichen Sclerosus

Squamous cell hyperplasia (formerly hyperplastic dystrophy) (SCH)

Other Dermatoses

The clinical and histopathological application of this classification is still lacking in practicality. In a study by Ambros et al⁵ and another by O’Keefe et al²³⁰ there was good agreement among pathologists on the histological diagnoses of LS, whereas the term squamous cell hyperplasia (SCH) was infrequently used and instead lichen simplex chronicus (LSC) was the more common rendered diagnosis. The authors dispute the position of SCH below LS in the classification because it is relatively rare in isolation. The symptom of pruritus in LS leads to chronic rubbing, which can cause superimposed LSC. To identify LSC separately from SCH in the absence of squamous cell carcinoma is problematic⁶³. Scurry et al²⁷⁴ confirmed the presence of SCH and indicated that the diagnosis should be with LS. However Scurry et al²⁷⁴ also admitted to difficulties in histological differentiation between LSC and SCH particularly with end-stage LS when the classical feature of oedematous-hyaline layer is lost.

3.2.1 Presenting symptoms

LS can affect both sexes at all ages and skin sites but the typical patient is a middle aged woman with anogenital involvement, who may have suffered the symptoms without accurate diagnosis for many years²⁵⁶. The most common complaint is severe and intractable itching (pruritus vulvae), followed by soreness, particularly during micturation. Superficial dyspareunia is also common and is caused by narrowing of the introitus, and splitting of the skin at the fourchette during sexual intercourse. A few women may complain of vulval pain (vulvodynia). In the detailed study by Lorenz et al¹⁸⁹ itching was the most frequent (98%) symptom followed by irritation (60.5%), then burning and dyspareunia (~25%), tearing (~14.8%), bleeding and fissuring (~9%).

Occasionally the patient is asymptomatic and is referred because of the incidental finding of a white lesion at routine clinical examination²⁴⁵. When young girls are affected, difficulty with defecation and micturition are common, in addition to itching²⁴.

3.2.2 Examination findings

LS lesions present as white or slightly pink polygonal papules that coalesce into plaques giving the appearance of being “splashed with whitewash” on fine “cigarette paper” wrinkling of the cutaneous surface of the vulva and perineal region (Figure 3.1a-d).

Figure 3.1a-d Clinical photographs of vulval LS

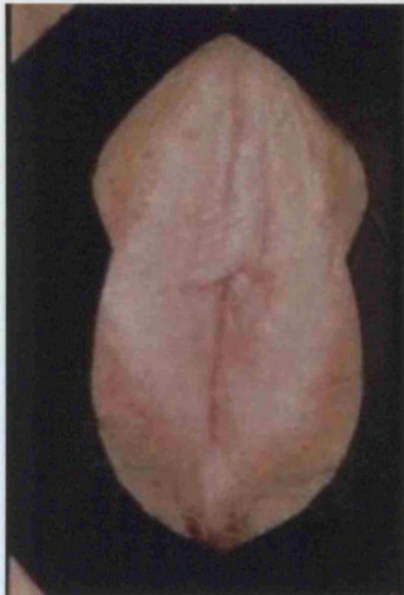


Figure 3.1a – This patient (72y) presented with a 20y history of vulval pruritus and soreness. On examination there was pallor with ‘cigarette paper’ crinkly appearance. Note the loss of labia minora (more on the left than right) with midline fusion partially occluding the clitoris. The adjacent “mask” has been positioned prior to LDPI.

Figure 3.1a

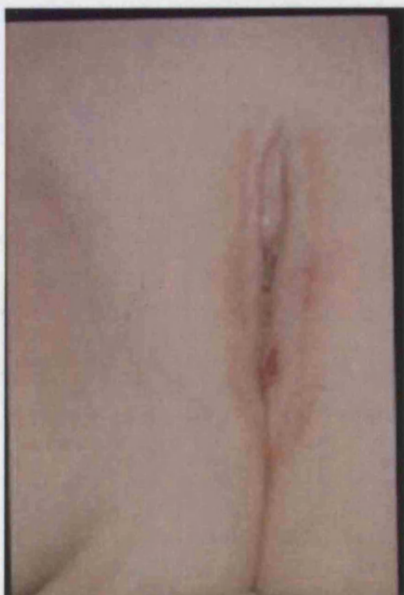


Figure 3.1b - This child (5y) presented with several months of vulval pruritus and painful micturition. Note the shiny smooth pallor with some ecchymosis.

Figure 3.1b

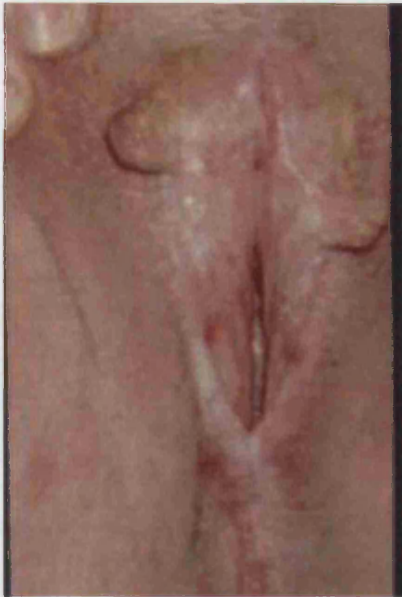


Figure 3.1c - This patient (54y) presented with a 2y history of vulval pruritus. There is partial resorption of labia minora and narrowing of introitus with midline fusion partially occluding the clitoris. There is scratch damage causing ecchymosis on right inner labium minus.

Figure 3.1c



Figure 3.1d - This patient (61y) presented with a long history of untreated LS associated with a short history of a non-healing ulcerated lesion on the left inner labium majus, which proved to be SCC on biopsy.

Figure 3.1d

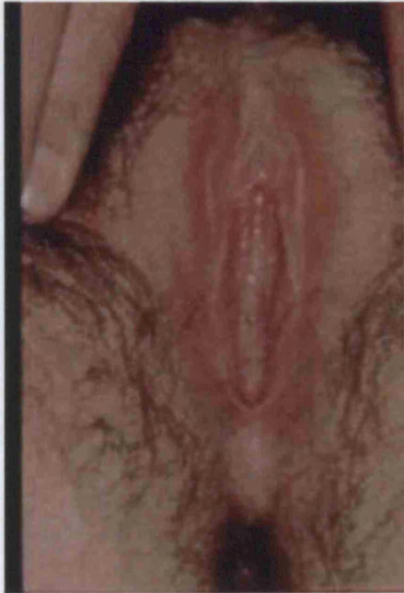
Some areas develop a chalky white, parchment-like hyperkeratosis of the vulval skin. The vulval architecture is modified in more advanced disease where there is absorption and loss of the labia minora, midline fusion burying the clitoral structures and eventually narrowing of the vaginal introitus. On gross morphology whiteness with crinkling are the most common features (87.7%) followed by thickening and fissuring (~30%), hyperaemia, phimosis, ecchymosis, erosions (~20%), thinning, agglutination, excoriations, tissue bridge (~13%), tearing and oedema (~4%)¹⁸⁹.

There is usually symmetrical involvement of the inner parts of the vulva - labia minora and inner aspect of labia majora (100%), and clitoris (70%)¹⁸⁹. In more extensive disease the perineal (68%) and perianal areas (32%), including natal cleft (32%) are affected giving the appearance of “figure 8” or “hourglass” distribution of the disease. Occasionally there may be extension of disease into the genito-crural folds (8.6%). The presence of associated extra-genital lesions varies between 3.7% and 18%^{145;174;208;209}.

During the symptomatic phase of the disease there is intense itching, usually associated with haemorrhagic bullae, telangiectases, erosions, purpura and oedema, which may be localised or generalised. During this active stage of the disease painful fissures also appear at the fourchette, perineal midline and in other natural skin creases. Fissures at the fourchette are precipitated or exacerbated by sexual intercourse.

SCH (Figure 3.2) is a difficult clinical diagnosis and is usually found in a background of LS. The skin surface appears thick with keratin overgrowth (hyperkeratosis) and fissured, with a dull red sheen as opposed to the glazed whiteness of LS.

Figure 3.2 Clinical photograph of vulval SCH

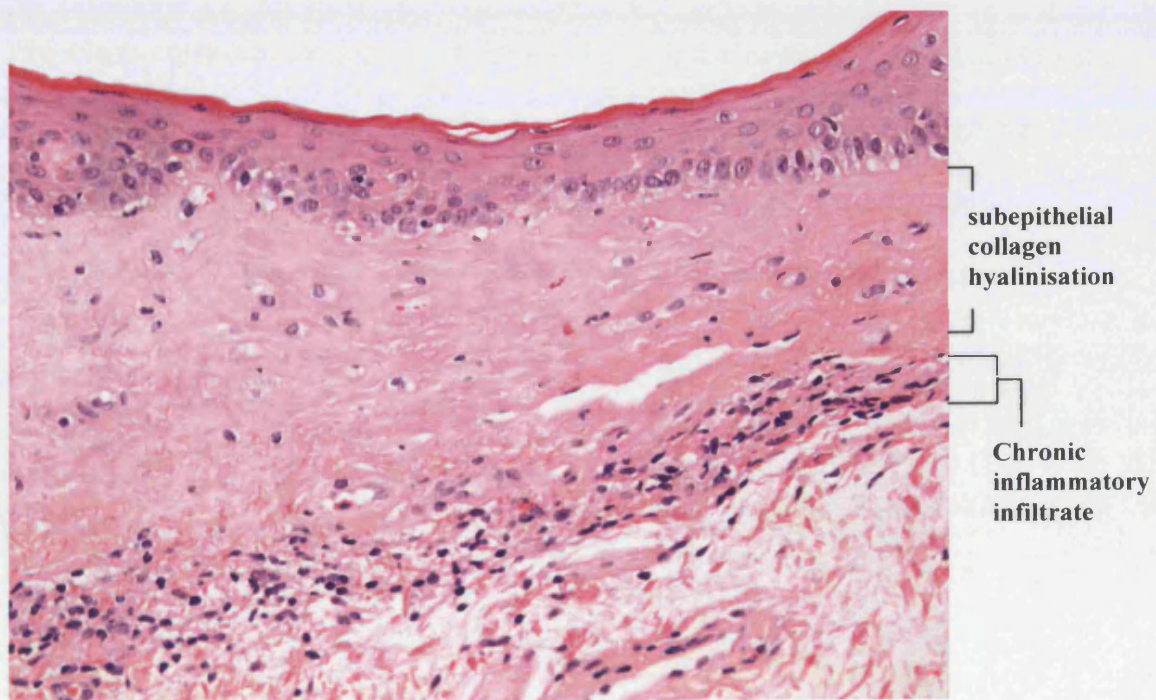


This patient (28y) presented with a 5y history of superficial dyspareunia and splitting at fourchette. Biopsy demonstrated squamous cell hyperplasia.

3.2.3 Histological findings

The epidermal layer in LS (Fig 3.3) is classically hyperkeratotic (thickening of the stratum corneum), thinned with shortening or even disappearance of the rete ridges such that the basal layer is arranged along a horizontal line. Hyperkeratosis is always present forming a thick multilayered keratin often greater in depth than the epidermis. Keratotic follicular plugging is also a common feature. The cells in the basal layer may show hydropic degeneration and when confluent form a subepidermal bulla. These epithelial changes are variable and not diagnostic^{126,145}.

Figure 3.3 Photomicrograph of vulval LS (Magnification x40)

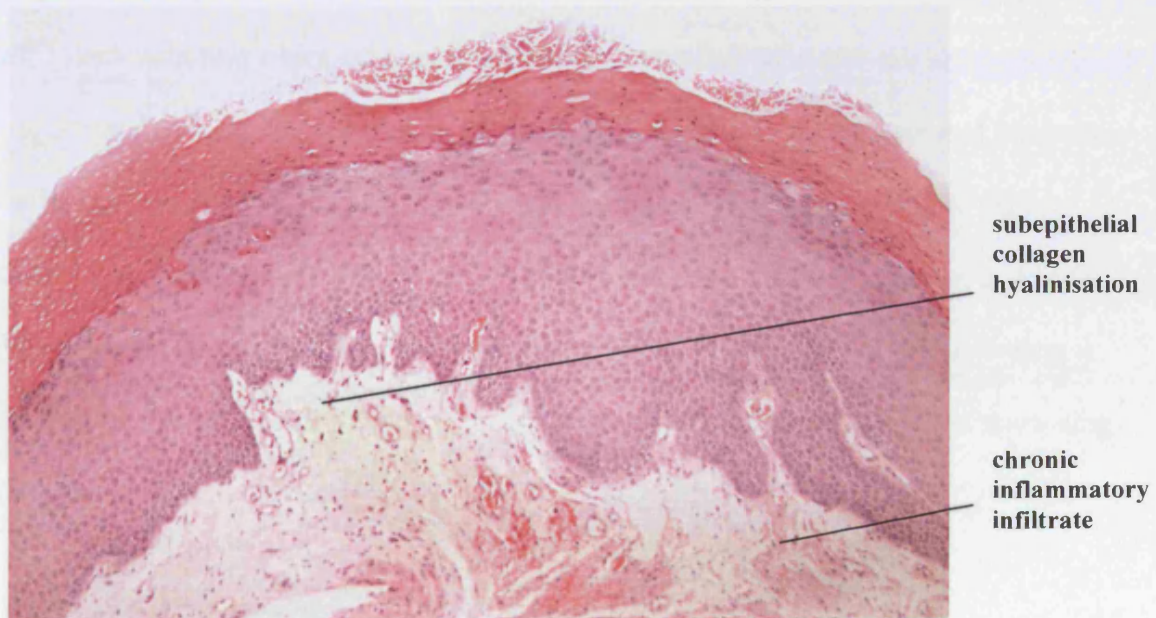


The more characteristic changes in this disease are seen in the dermis. There is a broad homogenous zone of subepithelial collagen hyalinisation with a deeper band of chronic inflammatory infiltrate. This diagnostic feature within the dermis of LS may be so scanty as to become invisible in places leading to false negative diagnosis when relying on a single punch biopsy¹²⁶.

Electron microscopy studies show that in early LS the elastin fibres are not destroyed but are pushed downwards by the subepidermal homogenous zone caused by deposition of acid mucopolysaccharide²¹⁴. Whereas in advanced disease elastin fibres are destroyed and replaced by a hybrid of elastin-like substance and microfilaments originating from collagen most likely produced by dysfunctional fibroblasts^{145;214}. The vulval dermal fibroblasts contain proteases, which are capable of inducing *in vivo* fragmentation of dermal elastic fibres¹⁰⁷. Computer-supported 3-D reconstruction of the papillary capillaries and superficial vascular plexus in non-genital LS show progressive disruption and disintegration of the capillary wall resulting in vessel reduction¹⁶⁷.

In SCH (Fig 3.4) there is hyperkeratosis, acanthosis (increase in depth of the prickle-cell layer) and parakeratosis (retention of nuclei within the horny layer). Marked acanthosis results in elongation and distortion of the architecture of the rete pegs forming clubbed and pointed configurations of the lower border of the epidermis. Parakeratotic thickening of the horny layer is more likely to be associated with the presence of VIN, than hyperkeratotic thickening¹²³. In the subepithelial dermis there is an inflammatory infiltrate consisting mainly of lymphocytes with some plasma cells and macrophages. The subepithelial collagen hyalinisation is less prominent beneath the thickened epidermis.

Figure 3.4 Photomicrograph of vulval SCH (Magnification x10)



3.2.4 Aetiology

The pathogenesis of LS is not clearly understood but several theories are being pursued.

3.2.4.1 *Autoimmune disease*

Autoimmune diseases such as thyroid disease, diabetes, vitiligo and alopecia areata have been linked with LS^{206,208}. These associations though established are weak and unrelated to the severity of the disease. Familial LS is well recognised. Genetically the HLA complex governs the susceptibility to inflammatory disease. In a group of 84 women with vulva LS there was an association with Class II antigen, in particular DQ7 and to a lesser extent DQ8 and DQ9, of the HLA system compared with controls²⁰². Carli et al⁶² found an increase in CD1a – Langerhan's cells at all stages of the disease suggesting an abnormality of the skin immunological system. The study by Marren et al²⁰³ demonstrating a lack of correlation between duration of symptoms and histological appearances, suggests a continuing inflammatory process in which activated Langerhan cells may be involved. Although the presence of an abnormality within the skin immune system, in particular a T-cell mediated response within the dermis, is disputed by some researchers^{62,272}, it is likely that the immunogenic profile in these women is the trigger the leads to development of LS following an innocent episode of scratching as a result of local irritants²⁷³.

Other genetic studies on cytokine receptors and inhibitors, which are thought to control inflammation, suggest that interleukin-1 receptor antagonist gene may relate to the severity of LS⁷⁰.

3.2.4.2 Hormonal dependence

Hormonal factors could be relevant because LS is ten times more common in women than men and the median age is about 50 years¹⁸¹. Friedrich and Kalra¹⁰¹ advocated the use of topical testosterone therapy based on low serum levels of dihydrotestosterone and testosterone found in these patients. A more recent study using immunohistochemical staining for hormone receptors in vulval tissue biopsy specimens show that in LS androgen receptors are greatly reduced when compared to normal vulval epithelium¹²⁹. In addition to this oestrogen receptors are also low in frequency on the vulva compared to the vagina¹⁹⁵. These findings support the use of neither topical oestrogen nor testosterone to treat vulval LS.

3.2.4.3 Infective cause

There is some interest in *Borrelia burgdorferi*, which has similarities with LS in the histopathologic features of the diseased tissue. Farrell et al⁹⁴ examined 16 specimens of vulval LS using light microscope and did not find any spirochete and, the coccoid bodies that were found appeared to be mast cells rather than bacilli. However detection of this spirochete, by DNA (deoxyribonucleic acid) amplification with PCR (polymerase chain reaction) suggests a geographic distribution in its association with LS^{76;102}.

3.2.4.4 Abnormal cell kinetics

Vulval LS appears to have a wide range of proliferative capacity and high levels have been associated with over-expression of wild type p53²⁹². When compared with normal vulval epithelium and non-genital LS, vulval LS appears to demonstrate altered p53 expression and epidermal cell proliferation³¹².

These studies suggest that in vulval LS there may be unknown factors triggering damage in DNA, which in turn increases the expression of p53. The mechanisms involved in the repair of damaged DNA, apoptosis when repair is not possible or the replacement of dead cells, may be impaired thereby triggering the pathway towards malignant transformation. Vulval LS also show abnormal expression of certain isoforms of epidermal CD44, which are designated as markers of tumour progression⁹³.

3.2.5 Relationship between LS and vulval carcinoma (SCC)

The association of vulval LS with squamous cell carcinoma (SCC) is clear from retrospective histological examination of the epithelial changes in the proximity of the cancer (see Table 3.2). Up to 75% of cases with vulval SCC are associated with LS/SCH. Many of the studies seem to suggest that SCH has a greater association compared to LS with SCC. As discussed in Section 3.2 above, the histopathological differentiation between LSC and SCH in the absence of cancer is problematic^{5;63;230;274}.

Table 3.2 reviews the literature on the reported relationship between LS and SCC. Differences in terminology and methodology used in these papers limits their comparison. For example, Leibowitch et al¹⁸² also included clinical evidence of epithelial disease in the vicinity of the cancer; to avoid under-reporting as in some cases histopathological examination alone was insufficient. The incidence of SCC in women prospectively followed up with vulval LS is in the range of 0-10% with an average rate of 3%¹²³. Hart et al¹²³ reported 4 cases of SCC in 107 women with LS at first examination and 1 case after 12 years in 92 long-term follow-up patients. Meyrick Thomas et al²⁰⁹ had 19 (5%) cases of SCC in 357 women with biopsy proven LS. In a prospective study with histologically confirmed LS in 211 women and with an age-matched control group Carli et al⁶⁰ found a cumulative risk of SCC of 14.8% (0.06% in the general female population) and a relative risk of 246.6.

Table 3.2 The association of vulval LS with squamous cell carcinoma

Author	No. of cases	Normal (%) epithelia	LS/SCH[?] (%)	#CIS (%)	Percentage with atypia
Buscema et al, 1980 ⁵⁶	98	2 (2%)	63 (64%)	33 (34%)	53%
Buscema et al, 1980 ⁵⁶ (revised *)	83	2 (2%)	63 (75%)	18 (21%)	44%
Zaino et al, 1982 ³⁵⁵	60	2 (3%)	39 (65%)	19 (32%)	72%
Leibowitch et al, 1990 ¹⁸²	78	6 (8%)	48 (61%)	24 (31%)	66%
Rueda et al, 1994 ²⁶³	64	7 (11%)	41 (64%)	16 (25%)	29%

CIS = high grade VIN (undifferentiated)

* Buscema et al⁵⁶ suggested excluding 15 cases of carcinoma in-situ (CIS), in which invasion was identified as microinvasive disease.

LS/SCH[?] includes the *dystrophies* described in the earlier papers.

The unanswered question is whether vulval LS is a premalignant disease. It is likely that the presence of LS rather than chronic tissue damage from itching and scratching is responsible for development of malignant disease, as other itchy conditions such as eczema and psoriasis do not develop cancer²⁷³. In an elaborate study by Carlson et al⁶³ DNA aneuploidy was present in 33% of vulval LS compared with other inflammatory conditions such as acute scars and lichen simplex chronicus, which were all diploid. DNA aneuploidy was also more likely to be found with LS associated with SCC compared to those without cancer. In the cases with DNA aneuploidy there was significant increase in epidermal thickness and p53 expression. This and the discussion in Section 3.2.4.4 above on cell kinetics provides some answers but more studies are required with better data collection and standardisation of terminology and histological definition to define the true impact of LS in the community, and on SCC.

There is concern expressed by some that the majority of patients with SCC in a background of LS were previously undiagnosed, suggesting that clinical surveillance may have an impact on reducing the risk of development of carcinoma¹⁸². The techniques currently available for clinical surveillance are limited, particularly as the studies suggest that the presence of SCH and/or atypia predict the risk of LS developing carcinoma^{193;355}. As LS often involves a large area of vulval skin, adequate tissue sampling to identify areas at risk of malignancy requires better techniques to localise these suspicious skin areas to avoid unnecessary excision of wide areas of the vulva.

3.2.6 Management

Effective treatment for LS has been a challenge, mainly because of the uncertainty concerning the aetiology and pathogenesis of this disorder. Therapy of vulval LS is usually protracted, providing symptomatic relief, without necessarily correcting the underlying disorder or architectural distortion.

The patient would greatly benefit from multidisciplinary input i.e. in a combined specialised vulval clinic with a dermatologist and gynaecologist, who have strong links with the genitourinary medicine and pathology departments. Confirmation with histological diagnosis is recommended. A 4 mm biopsy punch under local anaesthetic in the clinic can provide sufficient tissue for this purpose. An information leaflet containing general information about vulval LS, its small link to malignancy, the lack of a cure, the need for long-term follow-up and contact with support groups is helpful.

The management of vulval LS is based on controlling pruritus with the use of potent topical steroids followed by maintenance therapy. Maintenance therapy is recommended even if women are symptom-free. Clobetasol propionate is a highly effective synthetic fluorinated corticosteroid with a high degree of glucocorticoid but minimal mineralocorticoid activity. The drug is believed to contain anti-inflammatory, anti-pruritic and vasoconstrictive properties through the induction of phospholipase A2 inhibitory proteins called lipocortins¹⁸⁹. There is concern about contact dermatitis, increase predisposition to infection, steroid-induced atrophy, telangiectasia, stria and the suppression of the pituitary-adrenal axis. In the publications presented below in this section of this thesis contact dermatitis was the only occasional problem and none of the other side effects were reported.

Dalziel et al⁷³ prescribed Clobetasol propionate (CP) (0.5%) for 12 weeks twice daily application. On prospective review there was progressive and significant reduction of itch and soreness at 6 weeks and 12 weeks of therapy. Histological parameters did not show epidermal atrophy but there was significant reduction of hyperkeratosis and epidermal basal cell liquefaction. In the dermis there was significant reduction of the intensity of dermal inflammatory infiltrate and hyalinisation of collagen. On retrospective chart review by Lorenz et al¹⁸⁹ and on prospective observation by Sinha et al²⁹⁰ the complete/partial remission rate for symptoms was ~ 94% and no response rate ~ 5%. Gross morphology showed some improvement with complete remission in 31%, partial remission in 46% and no change in 22%¹⁸⁹. Previous studies have reported good response rates to topical testosterone but in view of the more recent comparative studies and unpleasant side-effects, this treatment is not recommended¹³. Bracco et al³⁹ have also shown significant improvement in symptoms, gross morphology and histological features with CP compared to testosterone, progesterone and a cream based placebo preparation. Topical testosterone causes elevation of serum levels and androgenic side-effects were reported in 4 of 10 patients - enlargement of clitoris (x3), alteration of voice (x2) and dramatic increase of libido (x1)¹⁵⁶.

The clinical impression is that CP does not cure vulval LS but controls symptoms of pruritus. Therefore in the long-term, after the initial “short and sharp” sustained treatment for 12 weeks with highly potent steroids, women are advised to continue with moderately potent steroids such as clobetasone butyrate. This is phased down to once or twice weekly application over a period of several weeks and maintained indefinitely to prevent recurrence of symptoms^{34;74;290}. Carli et al⁶¹ found modification of the immunohistochemical parameters activating the skin immune system and recommended the use of maintenance therapy in the hope that better disease control will reduce

scarring and discourage malignant change. Topical testosterone for long-term treatment is unsuccessful with a high rate of discontinuation of treatment due to exacerbation of symptoms^{34;59;67}.

Squamous hyperplasia is a controversial disease entity clinically and histologically^{5;230}. Clark et al⁶⁹ reported poorer symptom response with SCH (48%) compared with LS (62%) with graduated topical steroids and suggested that this disease may have a different underlying pathogenesis. Zorlu et al³⁵⁷ reported very high success rates of 100% for histological reversal and symptom relieve with topical fluorinated steroids. Cattaneo et al⁶⁶ also reported similarly high success rates for symptom relief and gross morphology. These extremely good results could be related to the pathological diagnostic dilemma between LSC and SCH^{5;230} as discussed in Section 3.2.

Other treatment options include topical or systemic retinoids and cyclosporins, which may be considered in women with refractory symptoms^{38;329}. Surgery to remove the diseased skin is not recommended as a rule because of a high recurrence rate and the associated morbidity³. However surgery does have a role in counteracting the effects of scarring such as dissection of buried clitoris, division of fused labia and enlargement of a narrowed introitus. The treatment of the narrowed introitus includes topical therapy and the use of dilators to prevent recurrence, in addition to surgery²⁴⁵.

3.3 High grade vulval intraepithelial neoplasia (VIN 3)

High-grade VIN (VIN 3) is an important disease as the rate of diagnosis has markedly increased particularly among young HPV (Human papilloma virus) - positive women^{15;72;75;125;137;163;215;282}. However the potential for increase in the vulval cancer incidence in young women is controversial^{137;139;149;207}. The overall incidence of vulval cancer in England is on the increase¹⁹⁴. MacLean¹⁹⁴ reports that although women over the age of 75y account for more than 50% of the cases with vulval cancer, women under the age of 50y have demonstrated a doubling of the percentage contribution from 6%(n=47) in 1979 to 12% (n=103) in 1998.

In 1986 the ISSVD defined two categories of VIN (Table 3.3)²⁵⁵.

Table 3.3 Vulval Intraepithelial Neoplasia²⁵⁵

Squamous

VIN 1 (mild)

VIN 2 (moderate)

VIN 3 (severe)

Non squamous

Paget's disease

Melanoma in-situ

Table 3.3 shows that the grade classification system is similar to CIN but unlike CIN, no biological continuum has been shown for VIN^{57;173}. Although there are vague suggestions of a continuum when comparing the mean age of diagnosis of VIN 1, VIN 2 and VIN 3 in the presence of HPV^{138;215}, we should be cautious against drawing parallels between VIN and CIN.

VIN is different from CIN. VIN is a symptomatic disease unlike CIN. VIN is found in only 20-30% of vulval SCC (Table 3.2) while CIN is present adjacent to >90% of cervical SCC¹⁴⁸. HPV has been identified in most cases of VIN 3, much less in vulval SCC and none in normal vulval biopsies in contrast to 80-90% of CIN 3 and invasive disease and in about 15% of normal cervical biopsies^{134;148}. The embryological origins of the squamous epithelium and the microenvironment of the cervix and the vulva are also very different³⁰⁸.

3.3.1 Clinical profile

Many reports define two cohorts of women with VIN 3 – the older and younger woman^{137;138;143}. The older (>40y) woman usually has unifocal disease and is HPV negative. The younger (<40y) HPV positive woman tends to be a cigarette smoker with multifocal and multicentric disease^{125;185;215;265;323}. Exogenous and in particular endogenous immunocompromise such as the use of medication in renal transplant and the presence of retrovirus disease, respectively, is associated with VIN 3 in even younger women which tends to be resistant to treatment^{172;179;331;349}.

The terminology for VIN was recently reviewed and modified to reflect the morphology of the disease rather than simply drawing parallels with CIN (Table 3.4).

Table 3.4 Modified terminology for VIN²⁸⁵

Main group	Sub-group	Previous category
Low grade VIN		VIN 1.
High grade VIN	Usual type <ul style="list-style-type: none"> • Warty • Basaloid • Mixed 	VIN 2 and 3.
	Differentiated	VIN of simplex type
	Unclassified	Pagetoid type or cannot be classified in the above categories.

Low grade VIN represents HPV effect and has considerable inter- and intra-observer variations, and so is not reproducible. High grade VIN has good interobserver histopathologic reproducibility. High grade VIN of the usual type occurs in young women and is associated with HPV and vulval cancer. High grade VIN of the differentiated type (dVIN) is less common, seen in older women in relation to LS and/or SCH and is also associated with vulval cancer. The high grade VIN of the usual type and differentiated type also reflect the clinical profile of the disease described above.

3.3.2 Clinical appearance

There is no pathognomonic clinical (Figure 3.5 a-d) or colposcopic feature for VIN 3 or for the presence of underlying early stromal invasion^{252;350}. High index of suspicion and histology is the key to diagnosis^{53;163;289}. The appearance of VIN 3 tends to vary with patient age, skin colour and location of the lesion within the vulval and perianal regions⁵⁸. The most common sites are the lower one third of the inner labia minora and fourchette⁷². The hairy area becomes involved usually as part of multifocal disease¹⁴³. On the keratinised vulval skin white lesions are the most frequently seen and are due to hyperkeratosis²¹⁵. On the mucosal surface subclinical macular lesions are best examined using a colposcope and 5% acetic acid. Recalcitrant and abnormal appearing condylomata acuminata may show VIN 3 on histology.

When VIN is suspected colposcopic examination with a magnification of at least 7.5x after application of acetic acid solution and directed biopsies is recommended to confirm diagnosis^{75;163}. Colposcopic examination of the vulva is far more complicated than examination of the cervix because it is impossible to see the subepithelial blood vessels especially in areas of the vulva covered by opaque keratinised epithelium¹⁹. The application of acetic acid is helpful to delineate multifocal disease. Vascular patterns are frequently absent within well-defined plaques of acetowhite epithelium caused in part by associated hyper- and para-keratosis. However the presence of abnormal vascular pattern, specifically widely spaced capillary punctation and rarely, mosaism, is highly suggestive of underlying invasive carcinoma¹⁴³.

Figure 3.5a-d Clinical photographs of high-grade VIN (VIN 3)



Figure 3.5a – The vulva (45y) is distorted by previous excision therapy for VIN 3. The raised red areas caused pruritus and biopsy showed VIN 3 on histology.

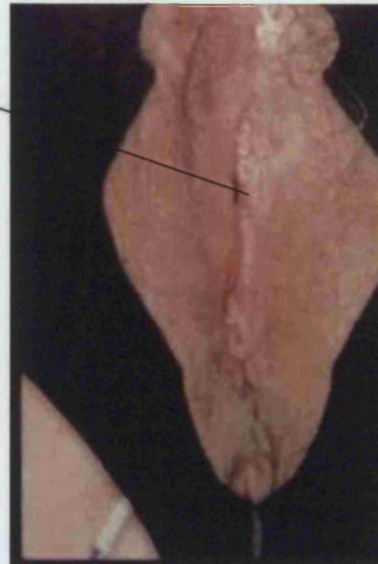


Figure 3.5b – (Age 59y) The left labia minora was previously excised for VIN 3. Recurrent pruritic symptoms presenting as a white area which on biopsy demonstrated VIN 3.



Figure 3.5c - (Age 48y) Previous excision of vulval warts. Presented with symptomatic pigmented areas, which confirmed VIN 3 on histology.

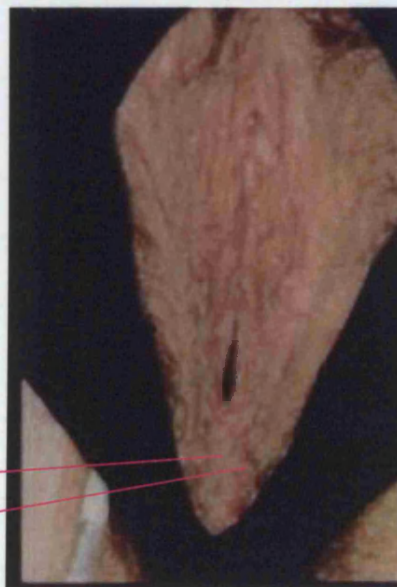


Figure 3.5d (Age 27y) Mildly symptomatic pigmented areas (VIN 3 on histology) in the perineum managed with topical steroids.

3.3.3 Clinical symptoms

VIN 3 is a symptomatic disease and the most common presenting symptom is vulval pruritus, followed by burning or pain and dysuria^{75;215;282}. About 20% of women diagnosed with VIN 3 are asymptomatic and the lesions are detected incidentally during clinical examination or other procedures. The number of asymptomatic women diagnosed with VIN 3 is reportedly higher in genito-urinary medicine clinics^{72;137;215}. The concept of vulval self-care is slow to evolve and even in the presence of symptoms there is delay in presentation.

3.3.4 Histopathology

The stratified layers of cutaneous squamous cells lose their normal orderly stratified architecture and show lack of progressive maturation from the basal layer to the more superficial layers (Figure 3.6a). Nuclear pleomorphism, hyperchromatism and pyknosis occur in conjunction with abundant mitotic activity. Abnormal mitotic figures can extend throughout the epithelium (Figure 3.6b). Hyperkeratosis, parakeratosis and dyskeratosis are also observed. Acanthosis and broadening of the rete pegs may be evident. Skin appendages specifically sweat glands and hair follicles are involved in more than 50% of cases.

Figure 3.6a Photomicrograph of high-grade VIN – usual type (VIN 3)
(Magnification x20)

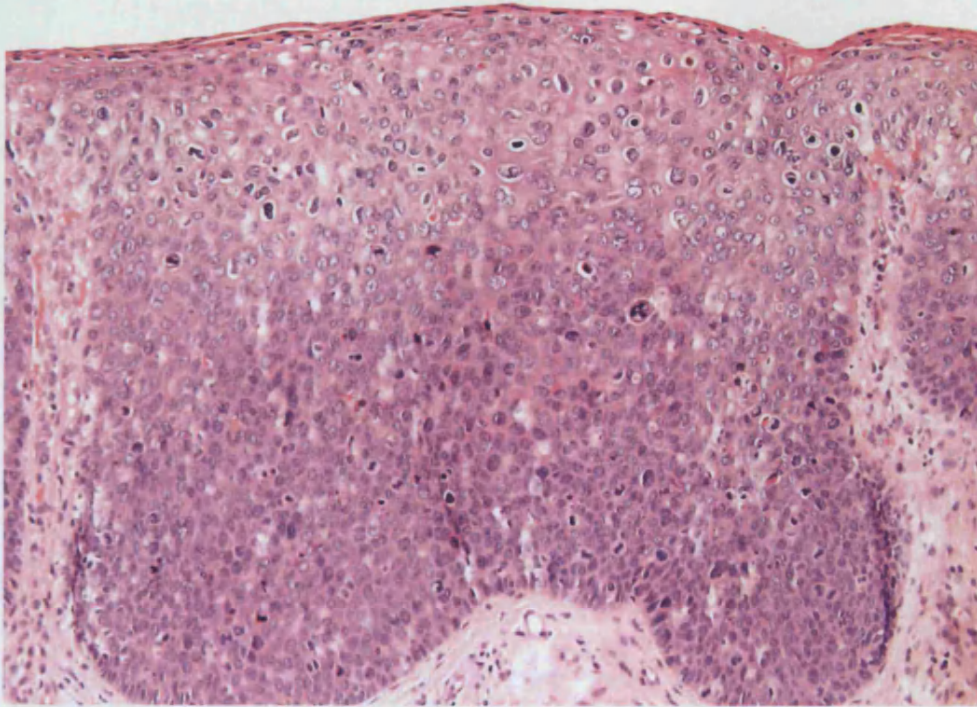
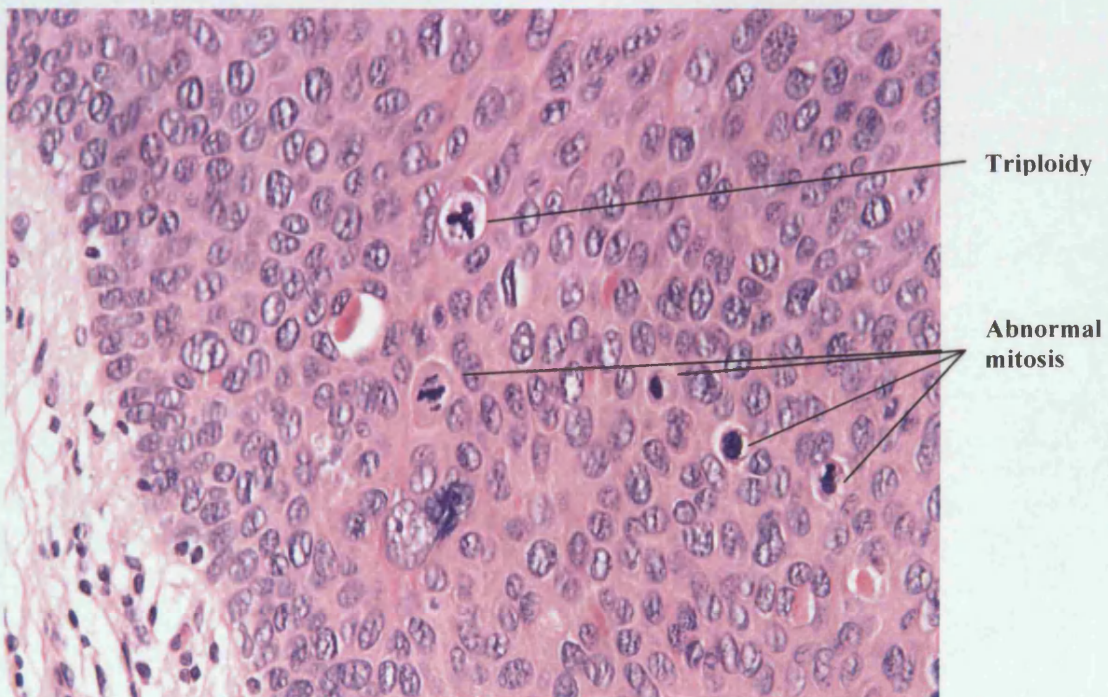


Figure 3.6b Photomicrograph of high-grade VIN – usual type (VIN 3) showing
abnormal mitosis (Magnification x60)



High-grade VIN is subdivided into three different categories based on histology – usual type (basaloid, warty, mixed), differentiated and unclassified - in the proposed modified categorisation of VIN²⁸⁵. The most common is the high grade usual type - basaloid, warty, mixed - variety with abnormal cells affecting the full thickness of the epithelium (VIN 3) and associated with HPV^{133;306}. Differentiated high-grade VIN is infrequently diagnosed as an isolated finding and is usually found adjacent to SCC, often in association with LS^{115;308}. Differentiated high-grade VIN has a low incidence of HPV, is possibly missed as the abnormal cells are found deep within the epithelial folds^{163;306}. The patients included in the investigations in this thesis had high-grade VIN of the usual type - basaloid, warty, mixed (VIN 3).

3.3.5 Natural history of VIN

The diagnosis of VIN 1 and 2 is variable but on the increase due either to a real increase in incidence or to greater awareness^{125;138;159}. These lesions, particularly VIN 1 are thought to be the result of reactive cytology associated with vulval inflammatory disorders^{19;53}. Immunohistochemical techniques could be developed to differentiate between hyperplastic change and dysplasia⁸⁹.

Angiogenesis, a phenomenon describing the formation of a new blood supply from existing blood vessels, is considered to be a key switch in tumour growth. In SCC there is a significant correlation between strong expression of the angiogenic factor VPF (vascular permeability factor) / VEGF (vascular epithelial growth factor) and a high microvessel count with poor survival rates²³². In VIN the presence of VEGF and MVD is significantly lower in VIN 1 and 2 compared to VIN 3¹⁴. This suggests that VIN 3 is the relevant precursor of invasive cancer and that VIN 1 and 2 are low-grade disease. It is of concern that VIN 2 and 3 tend to be classed as high-grade disease as in CIN because of histological difficulties in differentiating between these conditions²⁴⁶. Pathologists have rightly endeavoured to reduce this confusion by developing immunohistochemical techniques to better differentiate VIN 2 and VIN 3³²³. As recommended by Sarhanis et al²⁶⁵ and in view of the implications of treatment it is recommended that may be VIN 1 and 2 be considered low-grade disease. This suggestion however does contradict the recent proposed modified classification for VIN²⁸⁵ in Table 3.4.

The malignant potential of VIN 3 is not disputed but the risk of progression to invasive disease is controversial. Histological review of excised invasive vulval SCC specimens demonstrates the co-existence of VIN in 20-34% of cases (Table 3.2). Occult invasive

carcinoma is present in up to 23% of VIN 3 (Table 3.5). The previous discrepancy between the frequency of diagnosis of VIN 3 and the age-adjusted incidence of SCC^{125;139;282} is now showing the expected cohort effect of an increase in the number of women diagnosed with vulval cancer particularly in women under the age of 50y^{155;194}.

Table 3.5 Reported progression, recurrence and occult invasive disease rates for VIN

Study group	N	FU	Progression with treatment	Recurrence	Occult disease
*Sideri et al, 1999 ²⁸⁶	52	6m-7y			12%
Modesitt et al, 1998 ²¹⁷	59	7m-8y	2.3%		23.2%
Junge et al, 1997 ¹⁵⁹	61	22y	0%		10%
Herod et al, 1996 ¹²⁵	133	15y	7%	65% with laser 25% with excision	
Ben David et al, 1996 ²¹	102	12y		29%-excision 55.8% - laser vaporisation	
Hørding et al, 1995 ¹³³	73	23y	4%		16%
Jones et al, 1994 ¹⁵¹	75 - treated (8 - untreated)	6m-32y	5.3% treated (87.5% untreated)	21.3% - in treated group.	
*Babero et al, 1993 ¹⁶	58	31m	5%	11%	
Hoffman et al, 1992 ¹³⁰	18	6m-5y	5.5%	16%	
*Babero et al, 1990 ¹⁵	60			16% -surgical treatment – VIN 2,3 5% -medical treatment – VIN 1,2	
Shafi et al, 1989 ²⁸²	46	10y	6.5%		
*Basta, 1989 ¹⁹	71	>5y	7% - VIN 3	†16% - VIN 2 and 3	5.6%
Rettenmaier et al, 1987 ²⁵²	48	4m- 7.25y	2.6%	23% complete margins 32% incomplete margins	19%
Crum et al, 1984 ⁷²	41		10%	†22%	

*Includes VIN 1 and 2, as well as VIN 3

N = number of subjects

FU = Follow-up

† Radical surgery better cure rate compared to conservative surgery

The true natural history of high-grade VIN has not been systematically studied and therefore remains unanswered. The thought-provoking studies from the New Zealand experience reported by Jones and McLean in 1986 and, Jones and Rowan in 1994 show that the risk of progression of untreated VIN 3 to invasion is as high as 87% in the presence of multicentric disease in a background of HPV in the older woman^{150,311}. In view of these reports there are serious ethical issues impeding acceptance of a prospective study of VIN 3. As with cervical neoplasia, it is not possible to clinically differentiate lesions at increased risk of progression, although immunohistochemical studies such as the expression of VEGF may provide some pointers in the future¹⁹⁷. Compared to the cervix, repeat excision of the affected vulval region increases morbidity more significantly. There is therefore a great need for the development of techniques to allow more accurate diagnosis and follow-up, particularly if conservative medical therapy for VIN 3 is to be developed.

In the older woman wide excision is recommended because of higher progression rate and possible presence of occult invasive carcinoma¹⁶. In the very young (15-27y) asymptomatic women, particularly in the presence of pregnancy, spontaneous regression occurs and therefore observation with close follow-up should be the rule¹⁵². In young women, particularly if asymptomatic, with multifocal disease, observation with close follow-up is recommended to detect recurrence¹²⁵. In up to 10% of patients with VIN 3, invasive vulval cancer will develop despite adequate treatment and follow-up (Table 3.5). This underlies the need for close long-term surveillance.

3.3.6 Treatment

Treatment is usually indicated on confirmation of a diagnosis of VIN 3, particularly in the presence of vulval symptoms. There is now a trend towards less radical surgery with close follow-up to reduce morbidity by conservation of the vulval architecture and function^{133;163}. There are broadly two modes of treatment – the excisional and ablative methods.

Excisional treatment when compared to ablative techniques has the advantage of providing a specimen for histological examination to exclude microinvasive disease and for inspection of the margins. Accurate diagnosis of the depth of invasion in the presence of invasive disease is critical to determining the requirement for nodal dissection and cure rates.

Ablative techniques using CO₂ laser seem to have a higher recurrence compared to excisional methods^{21;125;282;286}. There is also concern about the depth of treatment in the presence of disease in the skin appendages, particularly hair follicles on the initial biopsy²². In non-hairy areas preservation of sebaceous glands is important to maintain lipid secretion, which acts as a water barrier and maintains skin suppleness, thus preventing dry skin and severe dyspareunia²⁸³. Therefore ablative methods after adequate biopsies to exclude early invasive disease is recommended for mucosal areas particularly the periurethral and periclitoral areas while excisional method for hairy areas because of the depth of destruction required and closure of defect is eased by the presence and mobility of subcutaneous tissue^{35;125;163}.

In-spite of careful choice of the combination of treatment modality, **recurrence of disease** is common and very variable – 64%²¹; 11% (VIN 1,2,3)¹⁶; 22%⁷², 16%¹³⁰, 16%(VIN 2,3)¹⁵. Young women with multifocal HPV positive disease have a higher recurrence rate which could be due to new disease or inadequate treatment^{21;159;217}. The status of the margin is an important factor in the rate of recurrence, with reports of a three fold increase in half the mean time when the margin is incomplete^{159;217;252}. Modesitt et al²¹⁷ also commented on the difficulty in obtaining negative margins due to microscopic disease extending beyond acetowhite changes. In these women development of disease at other lower genital tract sites is also frequently observed, and the future risk for lower genital tract invasive cancer is increased, so life-long vigilance is important²¹.

Medical treatment such as corticosteroid, testosterone, and β -interferon have been used to treat VIN 1 and VIN 2 particularly in young women^{15;16}. The cure rate with medical treatment for VIN 1 in young women is as high as 85%¹⁵. The results of medical treatment for VIN 3 are unreliable compared to surgery^{125;163}, although currently there is growing interest in imiquimod 5% cream which is an immune response modifier^{201;325}. Todd and Luesley³¹⁵ reviewed the literature and reported that although there was a pressing need for Nonsurgical interventions, none of the Nonsurgical treatments reviewed resulted in optimal outcomes. This may be partly due to the medical professional's anxiety of disease progression in the presence of high grade disease, as there is currently no clinical technique that can assess disease status reliably without the need for invasive investigation i.e. surgery.

3.4 Assessment of the vulval skin

In many societies the importance and frequency of vulval disease is underestimated because this subject is a source of embarrassment. Women with symptoms tend to delay seeking medical advice, sometimes suffering in silence for many years before presentation. As many women perceive the assessment of the vulva as a source of embarrassment, the examiner should do everything possible to minimise this understandable anxiety.

Multidisciplinary clinical input –from a closely linked team of gynaecologist, dermatologist and genitourinary physician greatly facilitates the consultation^{20:305}. A committed pathologist is invaluable to the team as accurate histopathological diagnosis is much more relevant to the vulval skin compared to other skin sites because of the confusing nature of the clinical presentation of vulval skin disease. Clinically, history followed by examination, are as always the preliminary investigative tools.

3.4.1 History

Turner and Marinoff³¹⁷ provide a good review on history taking for vulval disease. Pruritus is the most common complaint followed by pain, which may be associated with superficial dyspareunia. Occasionally the woman may have noticed a lesion on the vulval skin. The history should include information on the general health status of the patient and a complete gynaecological and obstetric review. The history provides clues to the site of disease and puts into context the presenting symptoms with other disease and lifestyle factors.

3.4.2 Clinical Examination

Privacy is essential, and the presence of an experienced nurse who can talk to the patient and allay anxiety during the examination is invaluable. Examination of the vulva is described in detail by MacLean and Reid¹⁹⁶ and includes inspection of other skin areas. Dermatological diseases of the vulva when compared to other skin sites are influenced by the location of this specialised skin in a constantly warm and moist area. Colposcopy with colpophotography of the vulva aided by application of 5% acetic acid and 1% toluidine blue helps demarcate areas of disease and follow-up examination^{50:170:250}.

Colposcopic examination of the subepidermal vessels in the vulva is more difficult compared to the cervix because the presence of keratin increases reflection and interference by hair. Not all areas of acetowhite epithelium represent neoplastic change, such as VIN. The acetic acid precipitates nucleoprotein to reflect more light giving the white appearance. Other causes of increased nucleoprotein include infection and tissue repair following scratching or coital friction. Toluidine blue is a nuclear stain, which is retained in any tissue where the more superficial cells are nucleated, for example in VIN^{350:71}. Although there are some criticisms of this technique, toluidine blue is useful to define the areas of abnormality that should be further assessed, including clear demarcation of areas requiring biopsy¹⁵⁷.

3.4.3 Investigation

The main investigative tool is histological examination of a skin biopsy. In research several non-invasive bioengineering techniques have been applied to study various parameters of the normal vulval skin. The same research parameters can be developed to assess diseased skin, such as is studied in this thesis, the application of a laser Doppler technique to measure vulval skin blood flow.

3.4.3.1 *Biopsy and histological examination*

Histological examination is usually needed to confirm diagnosis. Outpatient biopsy of vulval lesions can be performed after infiltration with local anaesthetic, using a 4 or 6 mm Stiefel disposable sterile biopsy punch²⁰⁴. 3% citanest (prilocaine 30mg/ml) plus octapressin (felypressin 0.03 iu/ml), contained together within a 2.2 ml cartridge is injected via a dental syringe and a 27-gauge needle. A healthy 70 kg adult can tolerate up to 600 mg of prilocaine (that is 20 ml) without developing features of toxicity. Once anaesthesia is established the punch is pushed with a twisting action through the skin, the resulting plug carefully moved out of the wound with fine dissecting forceps and the base of the plug cut with a scalpel blade or stitch cutter. Ferric sub-sulphate (Monsel solution) or a silver nitrate stick is applied to the hole to secure haemostasis. Larger areas of skin biopsy can be excised with scalpel under local or general anaesthesia depending on the extent of biopsy, and sutured to achieve haemostasis.

The skin biopsy is transferred to the pathology laboratory in a fixative such as formal saline. Specimens for immunofluorescence and electron microscopy require special transport medium, which need to be pre-arranged with the laboratory.

3.4.3.2 Percutaneous absorption

Percutaneous penetration of topical application of drugs is generally limited by the stratum corneum and differences in regional penetration may be as great as hundredfold. Labelling the topical drug such as ^{14}C -hydrocortisone and measuring the amount excreted in the urine can assess the percutaneous absorption. The absorption of ^{14}C hydrocortisone by the labia majora is 7.7% compared to 1.3% in the volar forearm and 6% on the face⁴⁹. This is however much less than the scrotal skin (42%), which is homologous to the labia majora. The absorption was higher in the premenopausal compared to the postmenopausal women for both the volar forearm and the vulva. The kinetics of percutaneous penetration in the vulva showed a rapid absorption followed by a slow decrease whereas in the volar forearm it was a plateau. Topical testosterone also showed a higher absorption rate on the vulva compared to the volar forearm but there was no age related difference²³⁵. These marked regional differences could be attributed to variations in the thickness of the stratum corneum or the number and size of hair follicles and other appendages or skin blood flow.

3.4.3.3 *Transepidermal water loss*

The stratum corneum provides an interface to retain water. Water diffuses from viable dermal tissues through the stratum corneum to the external environment as a result of the gradient that exists. The rate at which it diffuses across the stratum corneum is called transepidermal water loss (TEWL) – an index of the efficiency and integrity of the stratum corneum. Damage to the barrier by skin disease or physical or chemical trauma result in increased TEWL from baseline values. At baseline the vulva appears to be far more permeable to water than the forearm⁴⁷. However in some vulval studies there was periodic bursts of markedly increased water loss which was not demonstrated in the forearm⁸⁴. This is attributed to increased emotional sweating characteristic of the vulva due to the increased number of eccrine sweat glands on the external surface of the labia majora, at the site of these studies.

3.4.3.4 *Stratum corneum water-holding capacity*

Stratum corneum water content is a function of at least two properties: (1) hygroscopicity (i.e. ability to take up water) and (2) ability to retain water. The lipids within the stratum corneum of the vulva are relevant to barrier function and delipidisation increase TEWL equally in pre- and post-menopausal women²³⁶.

3.4.3.5 *Sensitivity to irritant activity*

Vulval skin was significantly more reactive than forearm to certain irritants⁴⁸. Increased subjective complaints of burning and stinging may be related to the nerve receptors in the genital region having lower threshold to stimuli, reflective of its role in sexual response. However other irritants which produce marked clinical signs of dermatitis on the forearm, only produce subtle sub-clinical changes on the vulva detectable only by modern bioengineering instruments such as relative capacitance⁸⁵. Therefore potential substances to be used on the vulva should be tested on the vulva rather than on the arm or the back.

3.4.3.6 *Mechanical trauma*

Stripping of the stratum corneum results in relatively less change in barrier function compared to the forearm, probably since the epidermal barrier is physiologically reduced at the vulva³⁴³. The post-traumatic regeneration of the epidermal barrier is accelerated at the vulva compared with the forearm. This suggests a higher basal epidermal cell turnover of vulval skin.

3.4.3.7 *Skin blood flow*

The mean basal blood flow through labia majora measured with the LDF was twice that of the forearm ($P < 0.003$)⁸³. Nicotinate-induced erythema is less intense and lasts shorter in the vulva compared to the forearm, probably due to dumping effect by higher basal blood flow⁸³.

3.5 Conclusion

Vulval LS and VIN 3 cause significant morbidity and risk mortality when there is development of carcinoma. There is a definite but poorly defined risk of progression to cancer. However the majority of women with these diseases do not develop cancer but will require careful monitoring and excision of suspicious lesions for diagnosis and treatment. Excision of large areas of vulval skin distorts the external genitalia causing significant morbidity. Currently there is no reliable non-invasive technique to monitor disease progress and to accurately identify malignant disease. If such a technique were to allow objective measurement of disease progress, in particular, to reliably detect the point as close as possible to the transition from pre-malignant to malignant disease, there would be greater clinician confidence in conservative monitoring and use of medical treatment, reducing the need for disfiguring vulval surgery.

Currently the only non-invasive technique for the assessment of the vulva comprises of the colposcope, particularly when pre-invasive disease is suspected, to study the morphology of the sub-epidermal blood vessels. The presence of keratin on vulval skin impedes the reliability of vulval colposcopy. The flow of blood within these sub-epidermal vessels could provide a further parameter in VIN 3 and LS to monitor serial changes in disease progress, including response to conservative medical therapy and to identify skin areas at risk of malignant invasion. In the next chapter the methods available to study SBF are reviewed.

CHAPTER 4

A critical review of biophysical
methods available for the assessment
of the vulval skin microcirculation

Interest in the measurement of the skin microcirculation started in the 1930's, when many of the principles underlying the techniques currently available were conceived. The advent of microprocessors and microcomputers in the 1960's and 1970's was the key to the rapid progress in technology during the past few decades. Table 4.1 shows the non-invasive methods for the clinical study of the skin microcirculation.

Table 4.1 Non-invasive methods for the clinical study of blood flow in small vessels

Blood flow parameter	Direct technique	Indirect technique
Temperature		<ul style="list-style-type: none"> • <i>Thermography</i> – temperature gradient • <i>Heat clearance</i>
Oxygen content		<ul style="list-style-type: none"> • <i>Tc PO₂</i> – O₂ diffusing through skin • <i>Reflectance Spectrophotometry</i> – haemoglobin or blood content and O₂ saturation
Physical movement of blood	<i>Capillaroscopy</i> – movement of RBC	<ul style="list-style-type: none"> • <i>Photoplethysmography</i> – blood volume flow • <i>LASER Doppler</i> – movement of RBC

RBC - red blood cell

Tc PO₂ –transcutaneous oximetry

O₂ - Oxygen

Invasive techniques include clearance of radioactive material and dye dilution such as dynamic fluorescent capillaroscopy. Table 4.2 (a-e) describes the features and application of these techniques in more detail. All these techniques have been extensively applied to measure SBF to study pathophysiology and/or to directly monitor skin disease or indirectly as a marker of systemic diseases such as diabetes.

Table 4.2 Biophysical methods for measuring skin blood flow – *Date of Proposal and Principle.*

Methodology	Date of proposal	Principle
Radioactive clearance	1948 ³⁵³	Rate of disappearance of a radioactive isotope in perfused skin is determined by the rate of SBF.
Heat clearance	1933 ²⁶²	Rate of change in temperature with time of a well-insulated probe on the skin surface is related to the rate of SBF.
Skin temperature - Thermography	1931 ¹¹¹	Heat generated by the body is dissipated as infra-red emission (heat) through the skin by changing skin perfusion ³¹⁸ .
Transcutaneous oximetry	1972 ²⁴⁴	Surplus oxygen from superficial papillary capillaries diffusing towards the skin surface is proportional to the rate of SBF.
Reflectance Spectrophotometry	1935 ⁷	The relative absorption (RA) value at specific wavelengths of white light projected onto the skin surface is proportional to the haemoglobin content in the tissue ¹⁶⁰ .
Capillaroscopy	1950's ¹²⁴	Microscopy used to directly observe capillaries (x5-x100) and the movement of RBC (x250-x1000) within capillaries in the papillary dermis.
Photoplethysmography	1937 ³⁴⁸	Near infra-red light radiated onto skin and the emerging amount of reflected light at the skin surface is inversely proportional to the blood volume ²⁷¹ .
Laser Doppler	1972 ²⁵⁹ 1975 ³⁰⁰	A monochromatic coherent laser light is shifted in frequency according to the Doppler effect when scattered by moving red blood cells in tissues. The Doppler broadening or change in frequency is proportional to the product of the red cell velocity and their concentration.

Table 4.3 Biophysical methods for measuring skin blood flow – *Methodology; Units of measurement, direct or indirect measure of SBF and remarks.*

Methodology	Method	Unit of measurement	Direct or indirect	Remarks
Radioactive clearance ²⁸¹	The radioactive isotope of an inert gas dissolved in saline solution introduced intradermally or by the epicutaneous technique. The amount of isotope remaining at the injection site is monitored using a scintillation counter.	Absolute units: ml/g/min*	Indirect	Isotope clearance curve is biexponential ³⁵³ .
Heat Clearance	A well-insulated probe with good skin contact using contact liquid is required. Several variations of the technique include <i>steady-state heat clearance</i> ^{132,212} in which time taken either for a heated probe or a disc with an annulus to reach thermal equilibrium is measured, <i>conductivity</i> ⁷⁹ in which the power to maintain a temperature differential between central disc and the annulus is measured and <i>transient heat clearance</i> ²²⁷ measures time taken for probe to reach equilibrium with skin temperature.	Absolute units: ml/g/min* Arbitrary units for thermal conductivity: 10 ⁻⁴ cal/cm°C.sec	Indirect	†The depth response depends on the sensor geometry ³¹³ .
Skin temperature - Thermography	Skin temperature is measured at a single point or a continuum of points over an extended body surface area displaying a thermal image (thermography) of the spatial distribution of temperature ¹¹¹ , which can be enhanced by thermostimulation for diagnostic purposes ⁷⁷ .	Qualitative analysis of right versus left side temperature differences: °C.	Indirect	†Thermographic changes can be result of disease in deeper organs ^{77,318} .
Transcutaneous oximetry ^{224,244}	The skin surface is heated to 44°C to reduce the barrier effect on oxygen diffusion and to arterialise the blood in the capillaries underneath the electrode. The oxygen solubility in blood is reduced and its disassociation curve is shifted to the right. An electrode reduces the oxygen diffusing through the skin surface and an electric current is generated, which is converted into a partial pressure measurement.	Arbitrary: mmHg	Indirect	Skin heating arterialises superficial capillaries ³¹⁹ .

†The depth of measurement from the skin surface is not clearly defined.

*Depends on the tissue-blood partition coefficient.

Table 4.3 (-continued) Biophysical methods for measuring skin blood flow – Methodology; Units of measurement, direct or indirect measure of SBF and remarks.

Methodology	Method	Unit of measurement	Direct or indirect	Remarks
Reflectance Spectro-photometry	The backscatter from white light projected onto skin is diffracted by a spectrometer and the RA at specific wavelengths provides a measure of the different skin elements ¹⁸⁴ . In the context of skin perfusion the RA of haemoglobin at wavelength 569µm in the visible region and 800µm in the near infrared region measures the microvascular red cell volume; and the RA of deoxygenated haemoglobin is used to measure oxyhaemoglobin ^{160;200;328} , an index of tissue oxygenation. Interference from melanin is mainly in the ultraviolet region, less in the visible region and non-existent in the near infra-red region ²²¹ .	Arbitrary units: RA units	Indirect	†The depth of measurement depends on the sensor wavelength and geometry.
Capillaroscopy ¹²⁴	White light with magnification and video recording facilities allow for real-time imaging and for retrospective analysis.	Qualitative and quantitative	Direct	Nutritive papillary capillaries – morphology and function studied.
Photoplethysmography	Infrared radiation from sensor collected by photoelectric detector either in reflection mode or transmission mode. 90% of the reflected light originates from the tissue substance, 10% from venous blood volume (quasi DC signal - slowly changing venous portion) [DC-direct current] and about 0.1% from arterial blood volume (AC signal - pulsating arterial signal) [AC-alternating current] ²⁷¹ .	Qualitative / quantitative analysis of waveform ²⁷¹ .	Indirect	Changes in blood volume in the cutaneous and subcutaneous vascular plexuses ²⁷¹ .
Laser Doppler	Monochromatic coherent laser light is transmitted to the skin and a photoelectric detector collects the backscatter containing the Doppler frequency information, which is converted to a voltage output, giving an arbitrary value of perfusion ^{90;91} .	Arbitrary: flux or perfusion units	Indirect	†The longer the wavelength the deeper the penetration of the laser light ^{41;142} .

†The depth of measurement from the skin surface is not clearly defined.

Table 4.4 Biophysical methods for measuring skin blood flow – *Advantages and Disadvantages*

Methodology	Advantages	Disadvantages
Radioactive clearance	<ul style="list-style-type: none"> • Quantitative measure if Kety's principle applied. • Calibration not required. • Reproducible⁹⁹. • Biexponential curve allows separate assessment of perfusion within the superficial papillary capillaries and the deep reticular vessels³⁵³. 	<ul style="list-style-type: none"> • Invasive, painful and time-consuming. • Repeatability limited by time taken for tissue stabilisation and therefore discontinuous and not real time. • Risk of radiation exposure. • Local changes in perfusion at injection site. • Requirements of Kety's principle not practical in complex tissue such as skin²¹².
Heat Clearance	<ul style="list-style-type: none"> • Quantitative measure if Kety's principle applied. • Capital cost and running expense is low. • Simple to use. • Allows serial readings from the same site. 	<ul style="list-style-type: none"> • Exact depth of measurement is not clear⁵¹. • Time consuming (~8 to 20 min) and therefore discontinuous and not real time. • Contribution to heat flow by tissue conduction cannot be separated from convection by blood flow. • Requirements of Kety's principle not practical in complex tissue such as skin²¹². • Skin contact required.
Skin temperature - Thermography	<ul style="list-style-type: none"> • Painless and well tolerated. • Non-contact technique. • Not influenced by skin pigmentation. • Large skin area analysed. • Telethermography can provide real time continuous measure. 	<ul style="list-style-type: none"> • Actual depth of skin contributing to the emitted heat is not clear but is likely to be large^{32;114}. • Time consuming with a total imaging time of up to 30 min¹¹¹. • Very expensive. • Only valuable for qualitative assessment of thermal gradients. • Thermostimulation may be needed to identify pathology⁷⁷. • Thermal diffusion to level out the temperature differences reduces sensitivity for assessment of spatial variation of superficial skin blood flow^{121;270;280}.

Table 4.4 (-continued) Biophysical methods for measuring skin blood flow – Advantages and Disadvantages

Methodology	Advantages	Disadvantages
Transcutaneous oximetry	<ul style="list-style-type: none"> • Capital cost and running expense is low. • Measurement depth is likely to be limited to the nutritive capillaries in the papillary dermis^{253;299}. 	<ul style="list-style-type: none"> • Calibration and data collection excessively delicate and time consuming²⁵³. • Maintenance tedious – re-site probe every 4h to 8h to avoid skin burns²⁴⁴. • Care with choosing skin site and application of probe to avoid false low or high values¹⁵⁸. • Many variables influencing measurement such as capillary density, skin metabolism and oxygen consumption, skin thickness, blood viscosity, haemoglobin concentration, haemoglobin affinity for oxygen and oxygen permeability of the vessel wall and skin, make interpretation of a single value complex^{224;296}. • Methodology alters the physiological state of the skin by heating the skin to 44⁰C and arterialising the papillary capillaries^{117;322}. • Skin contact required.
Reflectance Spectrophotometry	<ul style="list-style-type: none"> • Quantify haemoglobin content and oxygenation in the superficial dermis. • Real-time. • Fast response times (2ms) to obtain a single spectrum, ∴ relatively insensitive to movement artefact • Near-infra red spectroscopy is not influenced by melanin. 	<ul style="list-style-type: none"> • Exact depth of measurement is not clear. • Single point measurement so multiple readings required to assess spatial heterogeneity, which is time consuming. • Skin contact required.

Table 4.4 (-continued) Biophysical methods for measuring skin blood flow – Advantages and Disadvantages

Methodology	Advantages	Disadvantages
Capillaroscopy	<ul style="list-style-type: none"> • Real-time • Direct visualisation of nutritive capillaries in papillary dermis in-vivo under physiological conditions. • Expensive • Non-contact technique. 	<ul style="list-style-type: none"> • Limited access as skin surface perpendicular to microscope objective, with a large part of the capillary loop in the horizontal plane required, such as in the nail fold of finger. • Limited by skin translucency and pigmentation. • Increase in magnification reduces the observation area and the number of capillaries available for observation. • Depth of measurement is not clear. • Does not provide quantitative measure of capillary blood flow. • Repeatability depends on equipment performance, in particular its ability to produce uniform epi-illumination across the field of interest.
Photoplethysmography	<ul style="list-style-type: none"> • Not expensive • Rapid and clinically convenient method which can be used at the bedside and procedure takes 2 min to complete at each skin site¹⁸⁰. 	<ul style="list-style-type: none"> • Technique requires skin contact²⁹⁷ • Delay in detecting some blood flow changes such as venous obstruction when assessing the viability of venous flap³²⁶ • Reduced sensitivity for skin microcirculation as the signal reflects the changes in blood volume in the cutaneous and subcutaneous vascular plexuses.
Laser Doppler	<ul style="list-style-type: none"> • Simple to use • Real time measure with instant response to blood flow changes • LDF provides a continuous record at a single point. • LDPI can assess spatial heterogeneity in an area of skin without contact³³⁵. 	<ul style="list-style-type: none"> • Depth of measurement not clear. • LDF requires skin contact. • LDPI is expensive • When comparing LDPI output between different subjects measurement of relative changes to provocation tests is advocated rather than measuring absolute values.

Table 4.5 Biophysical methods for measuring skin blood flow – *research and clinical application*

Methodology	Research application	Clinical application
Radioactive clearance	<ul style="list-style-type: none"> • Previously used as gold standard against which new techniques studied. Now rarely used because of invasiveness and also improved understanding and advances of the other non-invasive techniques^{113;301}. • Used with other non-invasive techniques to study blood flow within the arterio-venous anastomosis (AVA), using the fast component of the biexponential curve to measure capillary blood flow²¹¹. 	<ul style="list-style-type: none"> • Viability of various types of venous flaps by Wolff et al³⁴⁶. • To determine level of amputation in critical limb ischaemia by Harrison et al^{120;122}.
Heat Clearance	<ul style="list-style-type: none"> • SBF in hand in Raynaud's Phenomenon by Roussel et al²⁶². • Age related physiological changes in blood flow within AVA by Midttum²¹⁰. • Pre and post-menopausal changes in regional SBF in the areola of normal breast by Nitzan et al²²⁷. 	<ul style="list-style-type: none"> • Thermal conductivity to differentiate between deep partial thickness and full thickness burn by Dittmar et al⁷⁹ • Regional SBF with pressure index to measure specific vascular resistance in diabetic and non-diabetic patients with peripheral vascular disease by Nitzan et al²²⁸
Skin temperature - Themography	<ul style="list-style-type: none"> • The dynamic balance between the thermoregulatory and haemodynamic processes during exercise by Zontak et al³⁵⁶. • Thermal inertia during application of local vasoconstrictor and vasodilator on the forearm by Huang & Togawa¹³⁶. 	<ul style="list-style-type: none"> • Static area and dynamic area telethermography in management of chronic orofacial pain, temporomandibular joint disorders and inferior alveolar nerve deficit¹¹¹. • Thermostimulation in the diagnosis of melanoma and identification of an irritant in allergic contact dermatitis⁷⁷. • Dermothermometry in conjunction with capillaroscopy for detection of subclinical changes within skin microvasculature in medical professionals exposed to ionising radiation⁵².

Table 4.5 (-continued) Biophysical methods for measuring skin blood flow – research and clinical application

Methodology	Research application	Clinical application
Transcutaneous oximetry	<ul style="list-style-type: none"> • Compare differences in SBF in adjacent undamaged skin and at ulcer edge in pressure ulcers²⁶⁹. • Used in conjunction with LDF to assess microvascular reserve in diabetes and connective tissue diseases such as systemic sclerosis and scleroderma by Valentini et al³²². 	<ul style="list-style-type: none"> • Used in neonatal intensive care units to monitor P_{aO_2}²⁵³. • To discriminate between fast and slow healing venous ulcers in conjunction with capillaroscopy by Steins et al²⁹⁹. • To discriminate patients in the intermediate group suffering intermittent claudication who may benefit from conservative therapy by using provocative tests such as total occlusion of circulation to measure reactive hyperaemia^{78;266}.
Reflectance Spectro-photometry	<ul style="list-style-type: none"> • To investigate the skin structure, monitoring of molecules in metabolic processes and the evaluation of skin diseases and therapeutic results¹⁸⁴. • Kakizoe et al¹⁶⁰ studied the contribution of alpha adrenoreceptor subtypes to skin microcirculation in rats. • Hanna et al¹¹⁷ compared $T_c Po_2$ with the spectrometer in patients with chronic venous insufficiency (CVI). 	<ul style="list-style-type: none"> • The pulse oximetry measures blood oxygenation and is widely used in clinical practise for quick determination of respiratory and circulatory status of the patient. • Harrison et al^{120;122} used haemoglobin oxygenation to replace their ‘gold standard’ radioisotope clearance in patients with critical limb ischaemia to determine the level of amputation.
Capillaroscopy	<ul style="list-style-type: none"> • Used to validate other techniques that are more amenable to clinical application such as Steins et al²⁹⁹ showed the presence of angiogenesis as the reason for improved $T_c Po_2$ measurements in fast ulcer healers. • Bull et al⁵⁴ studied skin areas predisposed to venous ulceration in healthy young adults. • Stucker et al³⁰³ studied a variety of microcirculatory disturbances in CVI. • Used with LDF to understand the differences in control of the microcirculatory subsystems during mental stress¹⁸³, reflex sympathetic dystrophy¹⁷⁷, systemic lupus erythematosus²⁸, peripheral vascular disease (PVD) and diabetes^{153;154}. 	<ul style="list-style-type: none"> • Ubbink et al^{319;320} discriminated the different patient groups with lower limb ischaemia using red blood cell velocity (RBCV) at peak reactive hyperaemia. • In women with postmastectomy ‘oedema’ capillaroscopy with fluorescein angiography revealed neovascularisation as the underlying cause²⁶⁰. • Capillary morphology in the superficial skin vessels has been analysed in several dermatological and general medical conditions^{29;124}.

Table 4.5 (-continued)

Biophysical methods for measuring skin blood flow – research and clinical application

Methodology	Research application	Clinical application
Photoplethysmography (PPG)	<ul style="list-style-type: none"> • Lee et al¹⁸⁰ in PVD to determine the optimum amputation level. • Klyszcz et al¹⁶⁹ studied arterial circulation in workers exposed to vibration. • Laan et al¹⁷⁸ studied vasocongestion of the vaginal wall to sexual stimuli. 	<ul style="list-style-type: none"> • Assessment of leg vein function, and disturbances of peripheral arterial circulation²⁷¹. • Postoperative radial forearm free flap surveillance by Stack et al²⁹⁷.
Laser Doppler	<ul style="list-style-type: none"> • LDF <ul style="list-style-type: none"> ○ In animal study to investigate reserve circulatory capacity in cerebral circulation by Barfod et al¹⁷. ○ In animal studies to detect early changes in circulatory failure in hypovolaemia³⁰⁹. ○ To detect inflammatory changes before naked eye and in dermatopharmacology^{91,267}. ○ Useful in diagnosis of fibromyalgia¹⁴⁴. ○ In aetiology of peripheral neuropathy in Type 1 diabetes and leprosy by Abbot et al¹. ○ In sepsis to demonstrate impaired hyperaemic response by Young and Cameron³⁵⁴ due to abnormal rheological properties of blood¹⁰. • LDPI <ul style="list-style-type: none"> ○ In patients with systemic sclerosis and Raynaud’s phenomenon by Seifalian et al²⁷⁷ and Clark et al⁶⁸. ○ In breast cancer by Seifalian et al²⁷⁵ and Parbhoo & Seifalian²³⁸. 	<ul style="list-style-type: none"> • LDF <ul style="list-style-type: none"> ○ To measure skin perfusion pressure (SPP) to assess severity in lower limb arterial disease^{65,95}. ○ Preferred to PPG for accurate measurement of SPP by Malvezzi et al¹⁹⁹. ○ To assess treatment of CVI with leg elevation by Abu-Own et al⁴. ○ To predict post-burn non-healing wounds by Atilas et al¹¹. ○ Healing of anastomosis site of low anterior resection for carcinoma¹¹⁶. • LDPI <ul style="list-style-type: none"> ○ For measurement of SPP by Tsai et al³¹⁶. ○ To assess burn depth by Niazi et al²²⁵. ○ In transplant surgery to measure blood flow over a wide area without contact to demonstrate the spatial heterogeneity, as a useful parameter of reperfusion injury^{9,82,216,276,278,279,321,342}. ○ At wound margins following total elbow replacement by Ljung et al¹⁸⁷. ○ To study ulnar artery ligation vs. resuture by Bornmyr et al³⁰ ○ For patch testing^{25,248}. ○ To study whole area of hyperperfusion in skin reactions caused by allergy and exposure to ultraviolet^{302,352}.

Invasive techniques such as radioisotope washout are not popular because they are cumbersome, lengthy and uncomfortable for the patient^{199;247}. Sodium fluorescence also requires an interval of up to 100-min between repeated measurements because of high background fluorescence, so limiting its use^{9;247}. Proano et al²⁴⁷ compared LDF, ¹³³Xe clearance and sodium fluorescence. The LDF was found to be the most convenient method because it is non-invasive and provides continuous real-time measurement, although perfusion is measured in vessels deeper than the nutritive capillaries. Braverman et al^{40;41} correlated LDF signals with histological examination of the organisation of the cutaneous microvasculature, and concluded that the LDF signal was generated mainly by the movement of RBC within the upper horizontal plexuses.

When LDF is compared to thermal clearance using probe designed to measure the same sample volume, the shape of the signal was the same but the absolute values were very different, with the LDF providing better-defined changes in perfusion due to its instantaneous nature of measurement^{249;310}. LDF has a low spatial resolution in the order of 1mm³, which is overcome by the LDPI, which has a high resolution allowing large skin area measurement. The LDPI used in this thesis can measure skin microcirculation in an area of 12mm². The advantage of this high resolution is demonstrated by the LDPI measuring a significantly higher skin microcirculation in early stage pressure sore compared to undamaged skin, while other methods with lower resolution – LDF, sodium fluorescence and skin temperature - failed to measure any significant difference in the two skin areas²⁷⁰. The LDF appears to compare well to ‘area’ measurement of temperature by Infra-Red Thermography (IR-TH). This is however believed to be due to similar changes in perfusion taking place in the different skin perfusion compartments¹³⁵.

The main limitation of the $T_c PO_2$ is the need for heating the skin to obtain a discernable result, which suppresses the local arteriolar vasoconstriction and thereby reducing its diagnostic capacity. Ubbink et al³¹⁹ could not detect a clear hyperaemic response in addition to noting a less pronounced postural constrictive response with $T_c PO_2$ when comparing asymptomatic and symptomatic patients with lower limb ischaemia. Kuvers et al¹⁷⁷ could not detect any differences between the different stages of reflex sympathetic dystrophy with $T_c PO_2$, although the LDF and capillaroscopy, differentiated these stages of disease by demonstrating differences in control of the microcirculatory subsystems.

Reflectance spectrophotometry (RFS) provides a non-invasive, rapid, portable and simple method for monitoring changes in haemoglobin content and oxygen saturation during disease and its treatment^{121;223;224}. The RFS and LDF measure different aspects of SBF. RFS provides a measure of haemoglobin content within the microvasculature of the skin, while the LDF measures the velocity or movement of RBC. Simonen et al²⁸⁸ reported good correlation between RFS and LDF at most anatomical sites, although the overall perfusion pattern was different. The SBF measurement decreased in the cranio-caudal direction with the RFS while the LDF was relatively uniform across the body, except the head and groin were higher and the abdomen was lower. When compared to the histological capillary density studies by Pasyk et al²⁴⁰ it is tempting to speculate that the signal from the RFS correspond to subepidermal papillae and the LDF, from the deeper microvascular plexuses of the reticular dermis. Interestingly Stücker et al³⁰⁴ applied the LDPI to a similar study and reported significantly higher blood flow on the face compared to the trunk and the lowest blood flow in the buttock and lower limb suggesting a cranio-caudal decrease similar to that reported with the

RFS. The measurement of the two techniques is influenced by the skin morphology, blood flow and content.

The photoplethysmography (PPG) signal reflects the changes in blood volume.

Lindberg et al¹⁸⁶ compared LDF with a modified micro PPG-laser to overcome the widely different optical arrangements. The LDF principle proved to be a more sensitive parameter for recording more superficial skin perfusion while the PPG principle was more sensitive with appropriate probe geometry for measuring blood volume change within the cutaneous and subcutaneous plexuses¹⁸⁶. In order to understand the pathophysiology of disease and to clinically monitor disease processes, limiting the sample volume as close as possible to the nutritive perfusion is valuable. The LDF when compared to the PPG measures a smaller microvascular volume within the superficial cutaneous plexuses and so meets this requirement more suitably.

The optical techniques discussed in this chapter – laser Doppler, spectroscopy and photoplethysmography – are influenced by the wavelength of light used and the skin optical properties^{8,184}. The skin optical characteristics are defined as the ability of skin to reflect, scatter and absorb light. For example in the epidermis melanin content and distribution plays a major but highly variable role in determining the transmission of optical radiation through skin. In the dermis scattering by collagen fibres is of major importance in determining the penetration of optical radiation. The presence of chromophores such as haemoglobin and bilirubin influence light absorption.

The vulva is an area of specialised skin, which unlike other skin areas is not easily accessible for examination, and as a result is rarely studied. Research of the vulval skin microcirculation requires the patient in the lithotomy position, which is embarrassing and uncomfortable. In elderly patients restricted movement at the hip joint is common

and reduces access to the vulval structures. The vulva consists of overlapping skin folds with varied morphology, as described in Section 2.1.3.4, and so the microcirculation in each fold of skin would be better assessed separately. This makes the process of studying the vulval skin microcirculation very delicate and precise with the important priority being that the technique selected should avoid lengthy examination periods.

There have been several studies on vulval skin microcirculation using the LASER Doppler technique. Wilhelm et al³⁴⁴ and Elsner et al⁸³ investigated the physiology of normal vulval skin using low resolution (1mm²) measurement with LDF on the labium majus at a single point to represent vulval skin perfusion. Jackson et al¹⁴⁰ used the LDF at several points on comparative sites of the vulva and so obtained a better representation of skin blood flow to demonstrate abnormal perfusion in vulva cancer. Bohm-Starke et al²⁷ used the LDPI to demonstrate significantly increased skin blood flow in the most painful part of the vestibular mucosa in women with vulval vestibulitis. This group was able to assess the whole vestibular mucosa in each patient within 3 minutes.

Therefore the technique most suitable for assessment of vulval skin microcirculation should be able to make rapid measurements in real-time over a representative area of skin. The depth of measurement should be known and adjustable to limit measurement to the nutritive papillary capillaries in the superficial dermis in the different areas of vulval skin. The technique should also be repeatable, non-invasive, affordable and portable.

The majority of the non-invasive techniques are able to make a rapid measurement in real time but tend to use a probe-based system, which requires direct contact with the

skin surface, and only allows measurement at one point, which in the LDF has a low resolution of 1mm^2 . The value of measuring blood flow at a single point to represent the whole area of vulval skin, particularly considering the varied morphology of the vulval skin folds, is doubtful. Comprehensive assessment of vulval skin perfusion with repeated single point measurements is excessively time-consuming and tedious for both the patient and the investigator. Therefore the Infra-red Thermography (IR-T) and the LASER Doppler perfusion imager (LDPI) that can assess the microcirculation in an area of skin such as the vulva, without direct contact with the skin surface and within a short period of time to produce a colour-coded image of skin perfusion are most suitable.

IR-T is dependent upon the skin temperature, which is influenced by both external and internal factors^{121;135;280}. Skin heating depends on heat delivery by blood and thermal conductivity of the subcutaneous structures while skin cooling or heat loss occurs by radiation from the surface, in addition to convection by blood and tissue conduction. Therefore it is important that the conditions, in particular the ambient temperature, are very closely controlled and defined. The signal of the IR-T is influenced by inflow from the superficial and deep layers of dermis and subcuticular tissue^{51;345}. A feature of skin temperature as a parameter of skin perfusion is thermal diffusion to level out the temperature differences between local adjacent areas of high and low flow resulting in a less sensitive measure of spatial differences in superficial skin perfusion²⁷⁰.

The LDPI applies the same principle as the LDF and measures the movement of red blood cells within the upper horizontal plexuses^{41;108;121}. The LDPI provides a sharper definition of spatial differences in perfusion compared to IR-T^{121;280;335}. As with other optical techniques, the LDPI does not easily lend itself to depth assessment because the

exact path taken by a light photon in the skin is difficult to determine and varies with morphology²²¹. Although more or less equally expensive to the IR-T, the LDPI is more compact and portable.

In conclusion the LDPI is the most suitable method for use on the vulval skin because SBF can be measured over a wide area (12mm²) within a short period of time. The advantage of representing blood flow over a specified area rather than at a single point is that the effect of large spatial and temporal fluctuations in tissue blood flow occurring in small areas is minimised. In the next chapter, Chapter 5, the methodology of the LDPI is discussed on greater detail and includes validation studies.

CHAPTER 5

Method Used To Assess The Vulval

Skin Microcirculation

5.1 Introduction

As discussed in Chapter 4 there are many methods available to assess SBF. The LASER Doppler perfusion Imager (LDPI) is a new and novel, non-invasive technique used for the first time on the vulva to measure perfusion²⁶⁴. The LDPI is able to map the spatial variation of the microcirculation in an area of skin and display the information as a two-dimensional colour-coded image on the computer monitor³³⁵. This task is performed without contact with the skin surface, thereby reducing the risk of spread of infection, which is relevant to assessing SBF on the external genital skin.

The aim of this chapter is to highlight the theoretical principles and instrumentation of the LDPI and to validate this instrument using a blood-flow model.

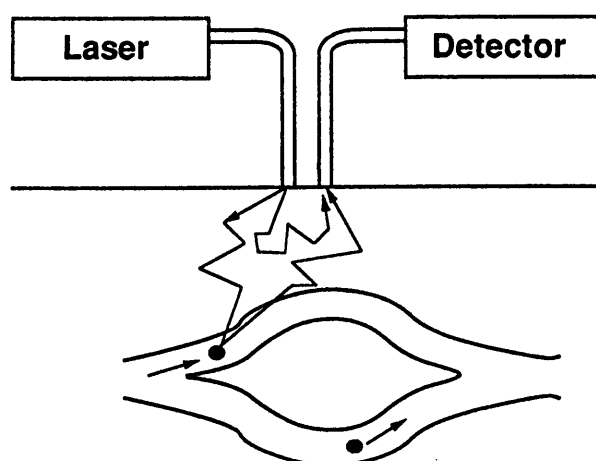
5.2 Basic theory and operating principles of the LDPI

LASER is an acronym for Light Amplification by Stimulated Emission of Radiation¹⁶¹. A laser beam is produced by excitation of certain materials known as active media, using an electrical current. The active media in the LDPI is a mixture of helium and neon gases (HeNe, $\lambda=633\text{nm}$). This is followed by amplification using optical feedback between a pair of mirrors located on either side of the active medium. The laser light is extracted by slightly reducing the reflectivity of one mirror, allowing transmission of part of the light impinging on it. LASER light has three special qualities compared to ordinary light - coherence, collimation and monochromaticity.

The principle of the laser Doppler technique is based on the Doppler effect first described by Christian Johann Doppler, an Austrian physicist and mathematician, in 1842²⁸⁴. This phenomenon describes the change in the observer's perceived frequency of waves (sound, light, water and radio waves) as a result of the relative motion of the source of the waves and/or the observer; the frequency increases as they approach each other and decrease as they move apart.

The laser beam incident on tissue is scattered in static structures as well as in moving red cells (Figure 5.1). Light beams scattered in moving red cells undergo a frequency shift according to the Doppler effect, while beams scattered in static tissue alone remain unshifted in frequency. A portion of the frequency shifted or broadened backscattered light is brought to impinge on the surface of a photodetector where beat notes produced by mixing waves scattered in different structures are formed.

Figure 5.1 Laser light transmitted to skin, by the LDPI, is scattered by stationary tissue elements and by moving blood cells within the microvessels.



The outermost layer of skin is the epidermis, which is free from blood vessels. The superficial dermis beneath the epidermal layer is supplied with a profuse system of capillaries which arise from the papillae of the corneum and return to the subpapillary plexus, as described in Section 2.2.2.1. The interaction between light and tissue involves a process of multiple scattering and absorption. The laser Doppler technique measures blood flow in the very small blood vessels of the microvasculature, such as the low speed flows associated with nutritional blood flow in capillaries close to the skin surface and flow in the underlying arterioles and venules involved in regulation of skin temperature. Detailed knowledge of the scattering process and depth of penetration is extremely difficult to obtain because factors affecting skin morphology such as pigmentation and changes in blood volume influence the penetration depth of light in tissue.

In theory, the shape of the power spectrum will for the main part be unaffected by variations in red cell concentration, if the velocity of the red cells and the microvascular bed geometry are fixed, while the magnitude of the Doppler signal increases with red cell concentration. If, on the other hand, the red cell concentration and the flow geometry are fixed, the energy of the power spectrum will be shifted towards higher frequencies when the mean value of the red cell velocity distribution is increased. Apart from the vascular bed geometry, red cell concentration and velocity, secondary parameters such as scattering in more than one moving red cell and changes in penetration depth due to variation in tissue morphology, may influence the power spectrum of the Doppler signal.

At each measuring point, a photo detector in the scanner head detects the scattered laser light. The resulting photocurrent has a DC component proportional to the total back-

scattered light intensity, and an AC component, the amplitude and frequency of which are dependent on the Doppler broadened laser light. An analog or digital processor convert the photocurrent measured by the laser Doppler into units of Flux.

$$F = \underline{V} \times \underline{C}$$

The flux (F) is given as the product of the average velocity of the moving red blood cell (V) by concentration of the moving red blood cell (C) in a given sampling tissue volume.

Traditionally the laser Doppler flowmetry (LDF) allowed continuous recording of temporal variations of perfusion at one point with time and facilitated measurement of perfusion at tissue sites not easily accessible to the laser beam such as internal organs during minimal access surgery. However, the low spatial resolution ($\sim 1\text{mm}^2$) of the LDF does not take into account of the large spatial and temporal fluctuations in tissue blood flow that occur over small areas of skin, which the LDPI can minimise⁴⁴. The LDPI is able to integrate tissue perfusion over a specified area and provide a valid measure of in vivo tissue perfusion⁹⁸. Other limitations associated with the LDF include limitation of some applications such as open wound sites and movement of the fibre optics during measurement resulting in artefacts that may make interpretation of results difficult.

The basic elements of the LDPI are shown schematically in figure 5.2 and a colour-coded image of the hand is shown in figure 5.3.

Figure 5.2 Block diagram of the LDPI

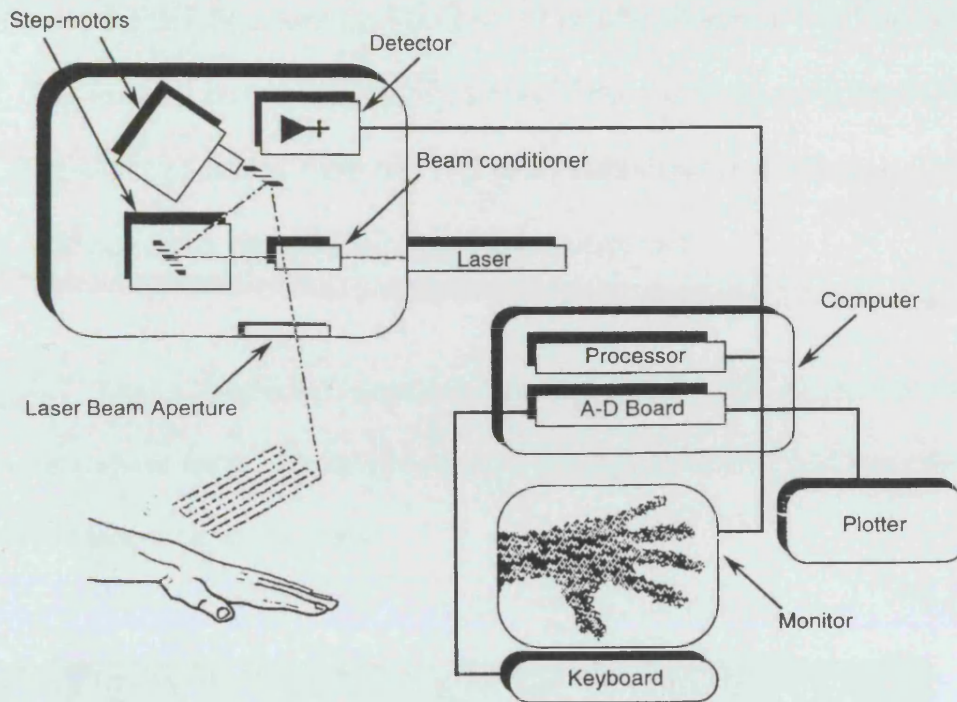
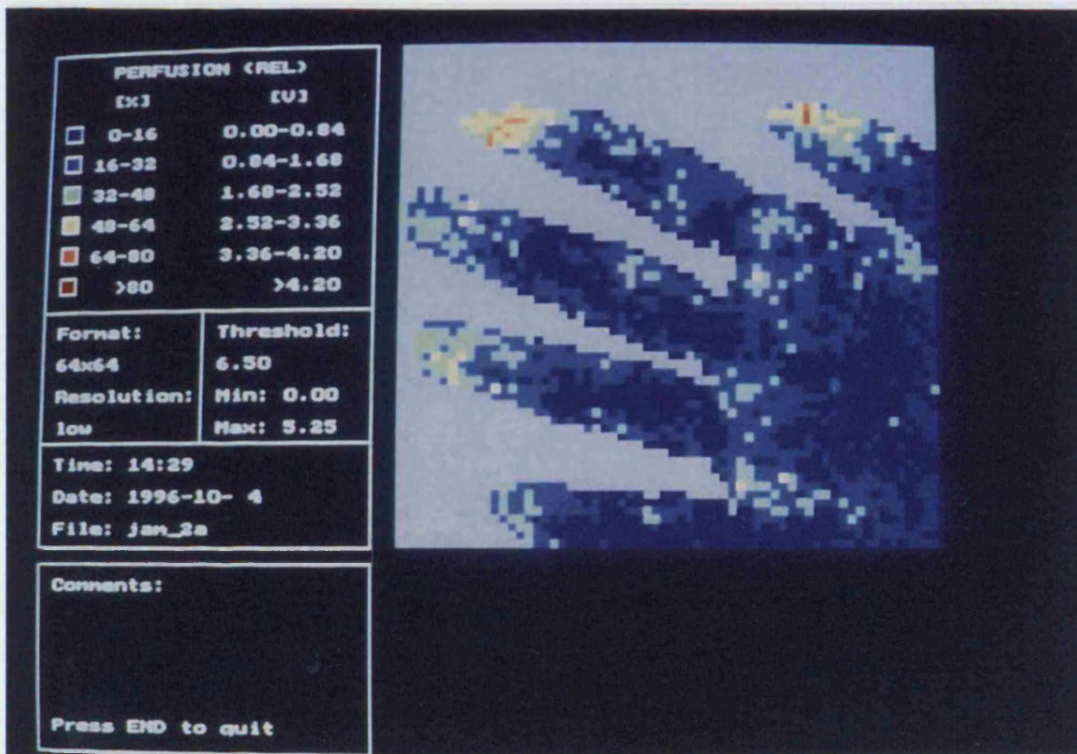


Figure 5.3 Colour-coded perfusion image of the hand.



5.3 Instrumentation

The LDPI used for this thesis was the PIM 1.0 by Lisca Development AB, Linköping, Sweden. This imaging system consists of a source of laser and a scanning head (Fig 5.4a-d). The source of laser is a low power (2 mW) HeNe laser ($\lambda = 633 \text{ nm}$). The scanning head houses the optical scanner and the detector unit.

Figure 5.4a The PC controls the optical scanner, processes the photocurrent from the detector to form a signal proportional to skin perfusion and generates a colour-coded image on the monitor.



The optical scanner consists of two mirrors controlled by two stepping motors, which direct the HeNe laser beam sequentially, in a raster fashion at up to 4,096 points. The area of tissue covered is $120 \times 120 \text{ mm}^2$, in a time period of about 4 min. This 4 min period includes the time required for the beam to remain stationary at each measurement point to calculate the perfusion parameter ($\sim 35 \text{ ms}$) and a certain time for movement ($\sim 15 \text{ ms}$) to the next measurement point. A Personal Computer (PC) controls the stepping motors.

Figure 5.4b The imaging system consists of a source of laser and a scanning head.

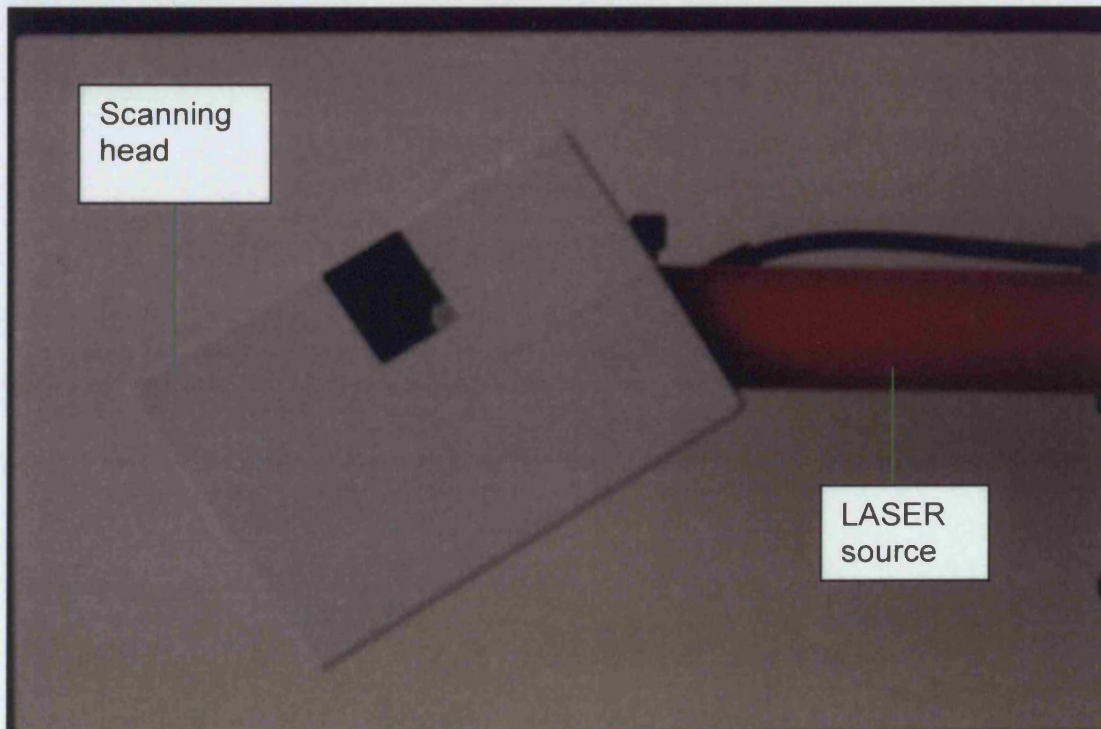


Figure 5.4c-d The scanning head houses the stepping-motor which controls the mirrors moving laser beam over the measuring site, and a detector unit that collects the backscattered Doppler shifted light.

Figure 5.4c

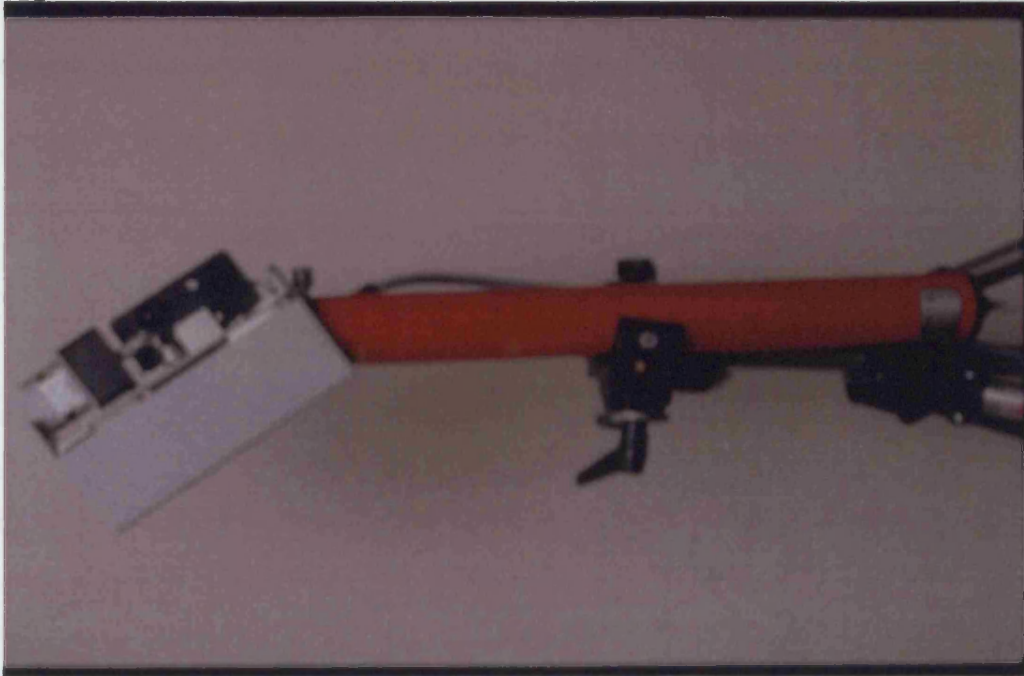
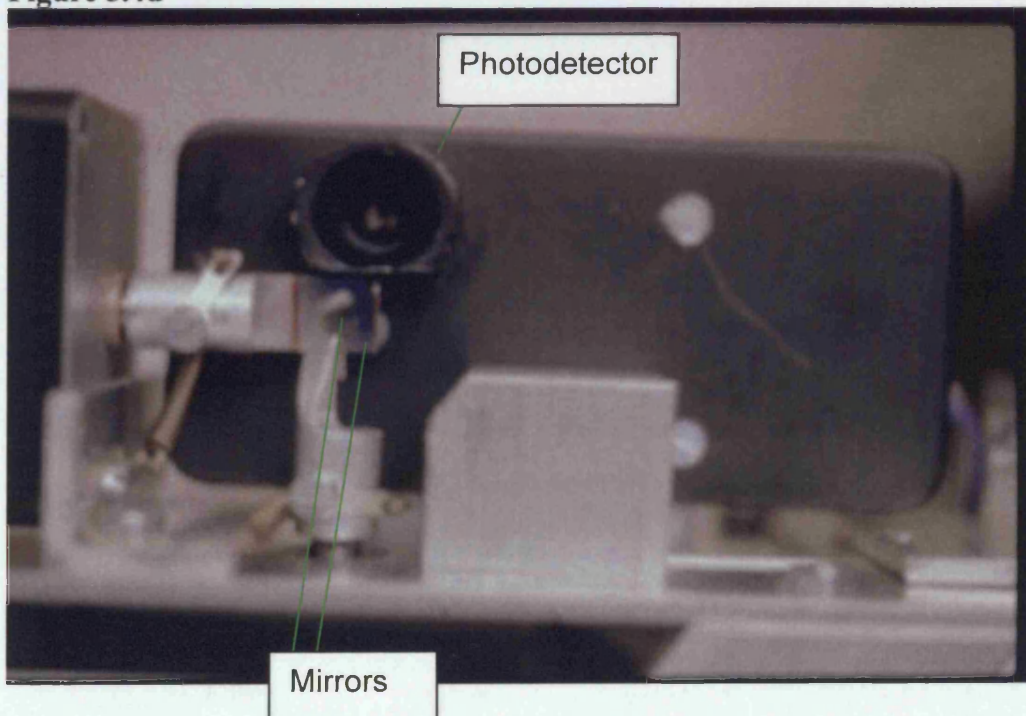


Figure 5.4d









The detector unit consists of a photodiode positioned at the same distance as the incident light from the measuring site, detecting the backscattered light. This photodetector neither senses the actual light wave frequency nor discriminates between the positive and negative frequency shifts. The detector only notes the absolute differences in frequencies between the frequency shifted beam and the portion of the unshifted light in the backscatter. An alternating electric signal is then produced at a frequency equal to the difference between the two incoming wave frequencies.

The PC controls the optical scanner, processes the photocurrent from the detector to form a signal proportional to skin perfusion and generates a colour-coded image on the monitor. The PC also provides an efficient means of data storage for retrospective analysis.

The colour-coded image is 64 by 64 pixels and the resolution for each pixel is $\sim 2 \text{ mm}^2$. The colour of each pixel on the image reflects the value of the perfusion at the corresponding measurement site. Six colours are used to give an overview of the perfusion and its spatial variation in the skin (Figure 5.5).

Figure 5.5 Colour coding of the LDPI image.

The highest of the captured perfusion values is set at a 100%, and the other values are scaled relative to highest value. A pixel coded in dark blue corresponds to a low perfusion value (less than 16% of the highest), while a pixel coded in red corresponds to a high perfusion value.

PERFUSION		
	[%]	[Volts]
	0 - 16	0.00 - 0.46
	16 - 32	0.46 - 0.93
	32 - 48	0.93 - 1.39
	48 - 64	1.39 - 1.86
	64 - 80	1.86 - 2.32
	> 80	>2.32

Light blue and dark blue colours represent low flux corresponding to low blood flow and red and orange represent high flux, which correspond to high blood flow. The colour coding procedure is performed in *relative or absolute mode*³³⁶. In *relative mode* the entire span of the perfusion values is divided into six intervals. The highest of the captured perfusion values is set at a 100%, and the other values are scaled relative to highest value (Figure 5.5). A pixel coded in light blue corresponds to a low perfusion value (less than 16% of the highest), while a pixel coded in red corresponds to a high perfusion value. The *relative mode* is preferable when the maximal dynamic range is desirable to compare adjacent areas within a single perfusion image. In *absolute mode* the highest and lowest perfusion values are selected *before* colour coding and presentation. The *absolute mode* is useful when different perfusion images are to be compared or when single extreme values are to be excluded in the presentation. All data collected are stored in ASCII code, which facilitates transfer of data to other systems

5.4 Image Analysis

Each pixel in the image generated by the LDPI contains a numerical value representing the mean blood flow within an area of $\sim 2 \text{ mm}^2$ of skin. The images are analysed using representative regions of interest (ROI) identified by means of markers and framed according to the chosen number of measurement sites to be analysed. In this thesis two software programmes were used for image processing – VIEW (Moor Instruments Ltd.) in MS-DOS for the validation studies on the LDPI and LDISOFT Version 1 (Lisca Development AB, Sweden) in a Microsoft[®] Windows program for the clinical studies. For the purpose of this thesis the main difference between the two programmes was the type of markers used to frame the ROI. The LDISOFT software, unlike the VIEW software, allowed markers to frame irregularly shaped ROI, which was necessary for

analysis of vulval skin perfusion images. The image analysis programme computed the mean within framed ROI, in *flux* units. The mean values were then exported to Excel (Microsoft Inc. USA) for further processing of data sets before statistical analysis by SPSS and ANOVA.

Black paper was used to localise or provide a mask for the measurement site to standardise the viewing area of the LDPI and to provide reference points on the image. As the black paper was strongly light absorbent it ensured zero background noise at the margins. Localising the measurement site in this way also reduced the scanning time at sites such as the vulva where the shape of the measurement site of interest on the skin does not fit neatly into the LDPI measurement-site-reduction-scales.

The images are converted to TIFF format and exported to Microsoft Word or PowerPoint® (Microsoft, USA) for printout or slide presentation. The images can also be captured directly from the computer monitor by camera for slide processing and by the LDISOFT software for printout.

5.5 Calibration

Currently there is no comparative gold standard technique to assess skin microcirculation. The validity of a universal calibration factor is questionable because of the presence of unknown variables such as RBC distribution within tissue and the variation in skin morphology affecting the optical properties of the skin or the pathway taken by light particles through skin. The LDPI system used in this thesis has been validated using a blood flow model, as described in Section 5.6. *In vitro* models are useful to study new devices under controlled conditions but are not analogous to human skin.

The LDPI technique provides excellent and reproducible resolution of spatial variation compared to visual assessment and standard LDF estimation as demonstrated in studies of trauma response^{298,302}. Temporal fluctuations in the microcirculation observed on the LDF monitor that occur as a result of periodic events such as vasomotion and, respiratory and heart synchronous wave patterns are eliminated by the LDPI^{44,334}. Temporal resolution of the LDPI may be limited by the scanning time period of the measurement site, which can be up to 4 minutes, although in a study of irritant trauma response Stücker et al³⁰² reported good temporal reproducibility. Temporal resolution with the LDPI can be improved by reducing the measurement site to produce shorter imaging times.

5.6 Validation of the LDPI with a blood-flow model

The LDPI apparatus was evaluated using a simple blood flow phantom simulating skin, which allowed independent control of a specific parameter while the other variables remain constant⁷⁻⁹. *In vitro* experiments was selected because *in vivo* evaluations are limited by the variations in the vascular geometry at different sites and the difficulty in controlling factors regulating changes of the perfusion parameter.

5.6.1 Aim

To investigate the LDPI signal response to the following variables which could affect the interpretation of the perfusion parameter.

- I.Distance between the laser detector and the measurement site
- II.Blood flow rate
- III.Haemodilution
- IV.Partial pressure of oxygen (P_{O₂}) of the circulating blood
- V.Movement of the measurement site
- VI.The degree of geometric distortion generated by the LDPI

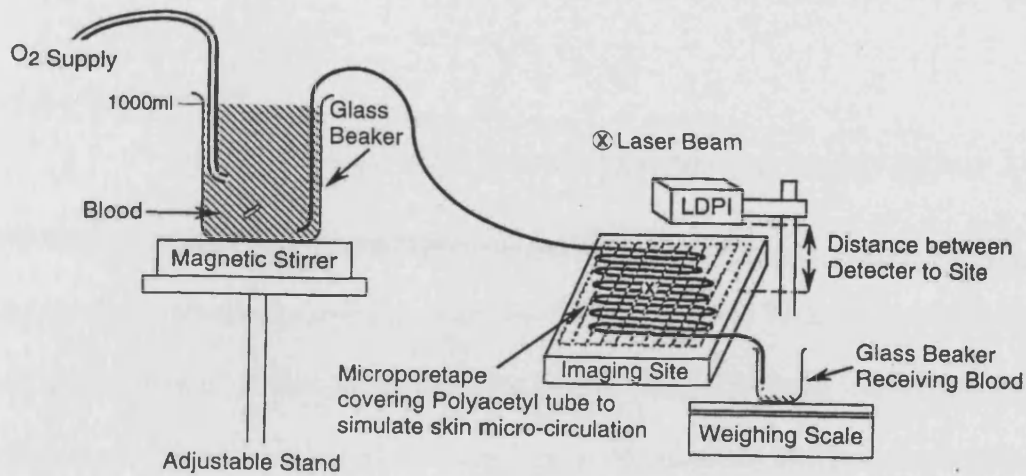
5.6.2 Materials and methods

5.6.2.1 LDPI – PIM 1.0 by Lisca Development AB, LinkÖping, Sweden

5.6.2.2 Blood-Flow Model

The flow model or perfusion fluid circuit (Figure 5.6) consisted of a glass beaker containing out-dated human blood placed on a shelf with adjustable height.

Figure 5.6 Blood flow Model



An anti-coagulating agent had been previously added to the blood. The blood was bubbled with oxygen (95%) and placed on a magnetic stirrer to ensure a supply of homogenous oxygenated blood, which was carried by a hydrostatic pressure gradient through a polyacetal tube to the imaging site. The imaging site consisted of polyacetal tube arranged in series and covered by micropore tape to simulate the optical properties of skin. The blood emptied into a small beaker placed on a weighing scale, which allowed measurement of blood flow rate by fluid collection. Flow measurements were performed over an area of $110 \times 40 \text{ mm}^2$, which was localised by a perimeter of strongly light-absorbent black-coloured material. This ensured zero background noise at the margins and that the same area was scanned on each occasion. For each experiment LDPI signal was recorded six times.

Although this blood-flow model simulates skin, it is not analogous with skin. In skin tissue the number of RBCs per unit volume is likely to vary according to the presence of

arterio-venous shunts (high RBC concentration), the orientation of the capillaries and the thickness of the avascular epidermis.

5.6.2.3 Experiments

5.6.2.3.1 *EFFECT OF DISTANCE BETWEEN THE LASER DETECTOR AND THE MEASUREMENT SITE ON LDPI SIGNAL*

The distance between the LDPI head and the imaging site was varied between 20 mm and 200 mm in steps of 20 mm by adjusting the height of the LDPI head relative to the imaging site. The flow rate was monitored by blood collection and this was constant at $1.23 \mu\text{s}^{-1}$ during the experiment.

The LDPI was positioned at a distance of 160 mm from the imaging site during the subsequent experiments as this was found to be the optimal distance. The results are described in section 5.6.4.1.

5.6.2.3.2 *ACCURACY OF LDPI READING MEASURING BLOOD FLOW VS. TRUE FLOW RATE*

The blood flow rate was varied by altering the height of the shelf supporting the beaker of blood (1 litre). The flow rate was measured by calculating the weight of blood collected in the glass beaker situated on the weighing scale, in micrograms per second (μgs^{-1}). This was converted to micro litres per second (μls^{-1}) by calculating the average weight of 1 ml of used blood, by sequentially adding each ml and recording the weight for up to 10 ml. The range of the blood flow rate validated was 0 to $5.89 \mu\text{ls}^{-1}$.

5.6.2.3.3 THE EFFECT OF REDUCTION OF RBC CONCENTRATION BY HAEMODILUTION ON LDPI SIGNAL

Replacing 10 %, 20 % and 30 % of volume of blood in the beaker with physiological saline solution diluted the red blood concentration. The LDPI reading was recorded for blood flow rates of 1.3, 2.0 and 3.4 μs^{-1} for each haemodiluted solution.

5.6.2.3.4 CHANGES IN THE PARTIAL PRESSURE OF OXYGEN (P_{O_2}) ON LDPI SIGNAL

The P_{O_2} of the blood in the delivering beaker was increased by gradually increasing the pressure of oxygen (95 %) bubbled into the blood. The range of P_{O_2} validated in this experiment was 10.7 kPa to 66 kPa. During the time of the experiment the blood flow rate was monitored by fluid collection and remained constant at flow rate of 3.9 μs^{-1} .

5.6.2.3.5 EFFECT OF MOVEMENT OF THE IMAGING SITE

The blood flow phantom was placed on a movable platform attached to a motor with a frequency regulator. This resulted in a regular and continuous vibratory type movement of the imaging site during the scanning procedure. The range of movement frequency validated was between 15 cycles per minute (0.3 Hz) to 78 cycles per minute (1.3 Hz). The blood flow rate was constant at 3.4 μs^{-1} .

5.6.2.3.6 ASSESSMENT OF GEOMETRIC DISTORTION

A sheet of white paper with black lines drawn across was found to be more appropriate for the assessment of geometric distortion generated by the LDPI because of the contrast produced by the complete absence of backscatter from the black lines compared to the small backscatter detected from the white surface. The black line was 2 mm thick and the gap (white) between the black lines was 15 mm broad. Total area scanned was about 9.5 mm². This sheet of paper was scanned with the direction of the laser beam initially moving along the black lines and then perpendicular to the black lines, to assess vertical and horizontal axis geometric distortion, respectively.

5.6.3 Data Analysis

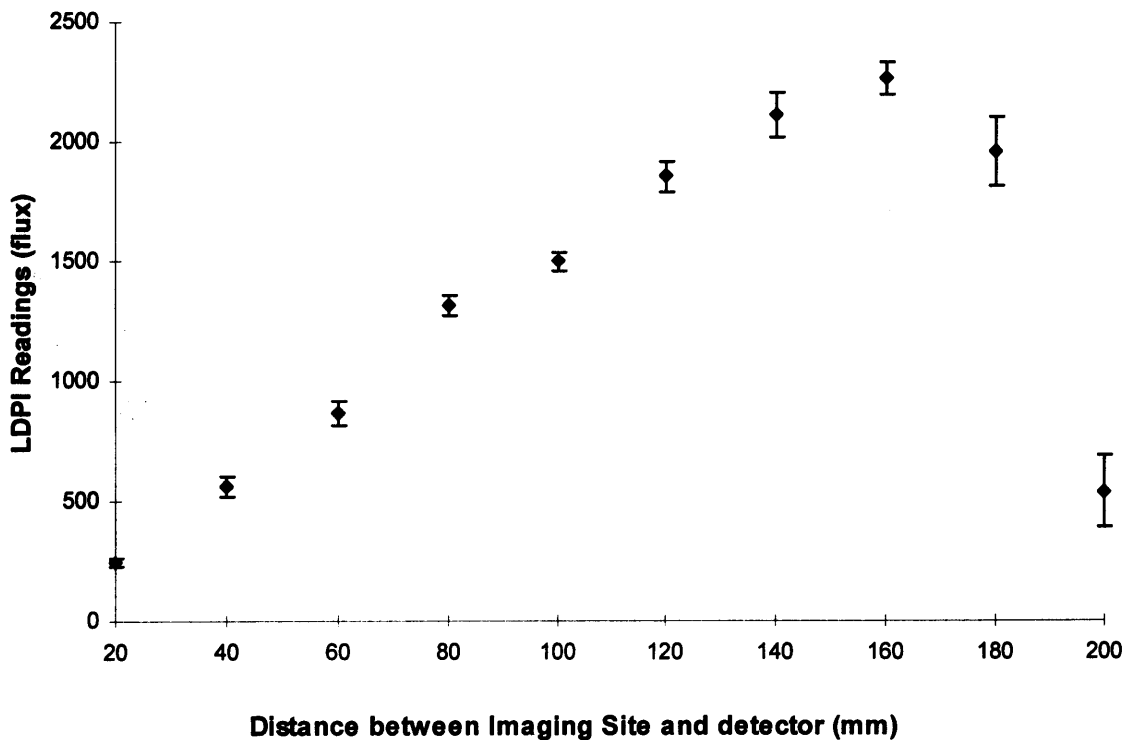
The VIEW software was used to calculate the mean LDPI value from one representative region (7 x 7 pixels) at an identical site on each image. Each experiment was repeated six times and the average of these readings were taken as the LDPI value. Statistical analysis was performed using the ANOVA: single factor; $p < 0.05$ was considered significant. To estimate the reproducibility of the LDPI measurements, the coefficient of variability (SD/mean) [SD - standard deviation] was calculated.

5.6.4 Results

5.6.4.1 Effect of distance between the laser detector and the imaging site on LDPI signal

Figure 5.7 shows a progressive increase in LDPI signal up to 160 mm distance between the laser head and the imaging site. There was approximately a 7% error in flux measurements within 10 mm of the 160 mm point. The percentage error was much greater with a further increase, compared to an equivalent decrease in distance from the 160 mm position.

Figure 5.7 Mean LDPI reading vs. distance between the laser detector and the imaging site at flow rate of $1.23 \mu\text{s}^{-1}$.

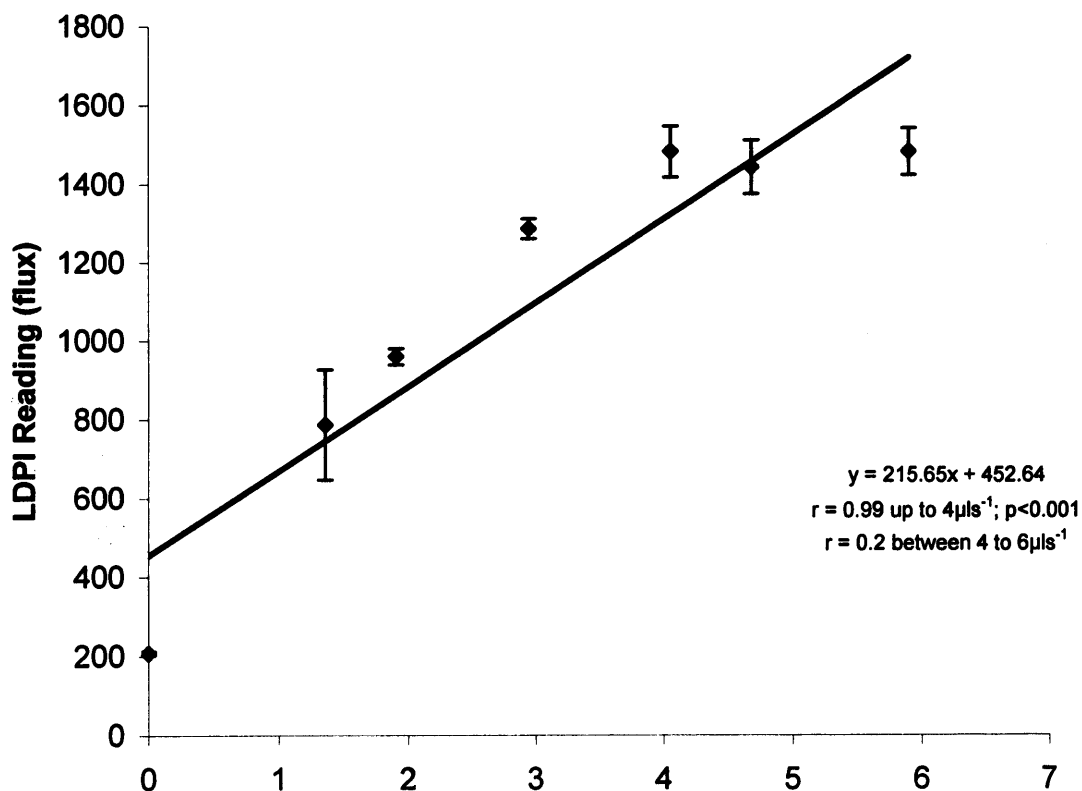


5.6.4.2 Accuracy of LDPI signal measuring blood flow vs. true flow rate

There was a significant correlation ($r=0.99$, $p<0.001$) between the actual flow rates using fluid collection, for the range of 0 to $4.89 \mu\text{s}^{-1}$, and the LDPI signal as shown in figure 5.8.

The best-fit straight line for the data is described by the equation: $Y = 215X + 452$, where Y is the LDPI signal in flux units and X is the fluid collection in μs^{-1} . The reproducibility of LDPI readings for flow rate had a coefficient of variability in the order of 2% to 5%.

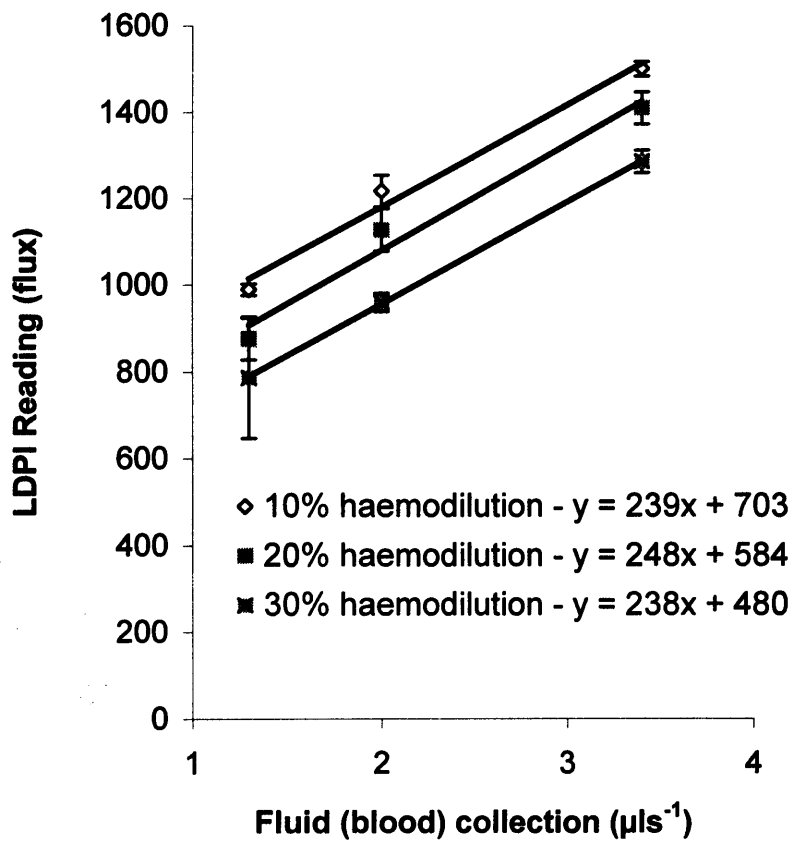
Figure 5.8 Relationship between blood flow rates determined by timed fluid collection and mean flux determined by the LDPI.



5.6.4.3 The effect of reduction of RBC concentration by haemodilution on LDPI signal

Figure 5.9 shows reduction of the LDPI signal with decreasing RBC concentration by dilution of 10 %, 20 % and 30 % of the blood volume with physiological saline solution at constant blood flow rates.

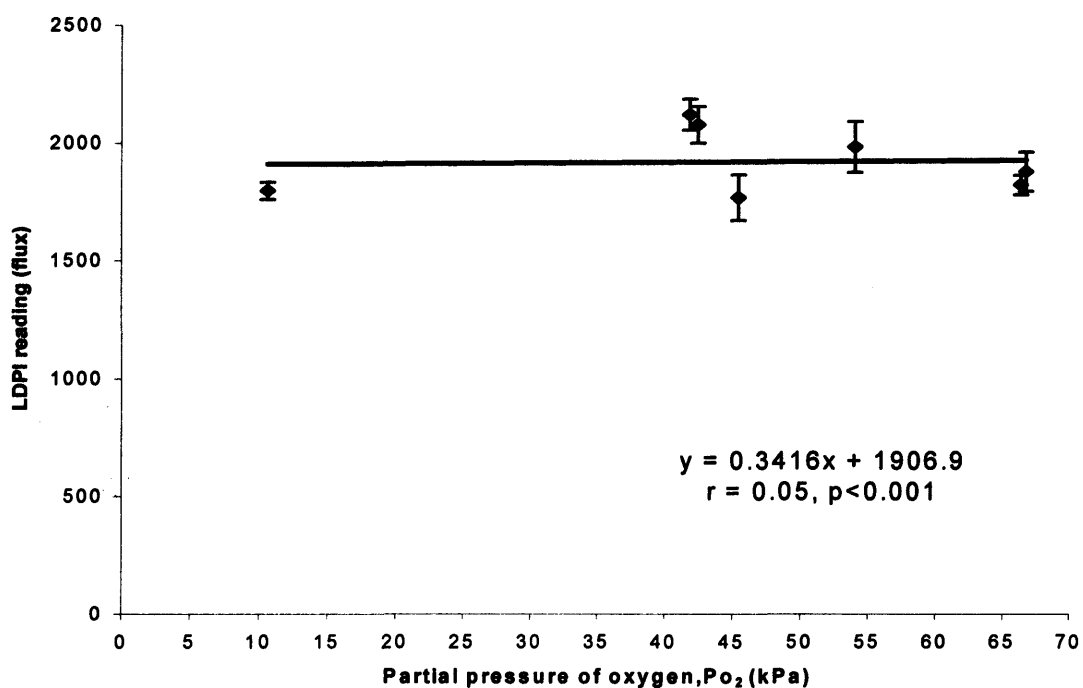
Figure 5.9 Relationship between blood flow rate and LDPI reading with haemodilution of 10%, 20% and 30% by volume with physiological saline solution.



5.6.4.4 Changes in the partial pressure of oxygen (P_{O_2}) on LDPI signal

The LDPI signal was independent ($r=0.05$, $p<0.001$) of changes in P_{O_2} of the blood circulating the flow phantom as shown in Figure 5.10.

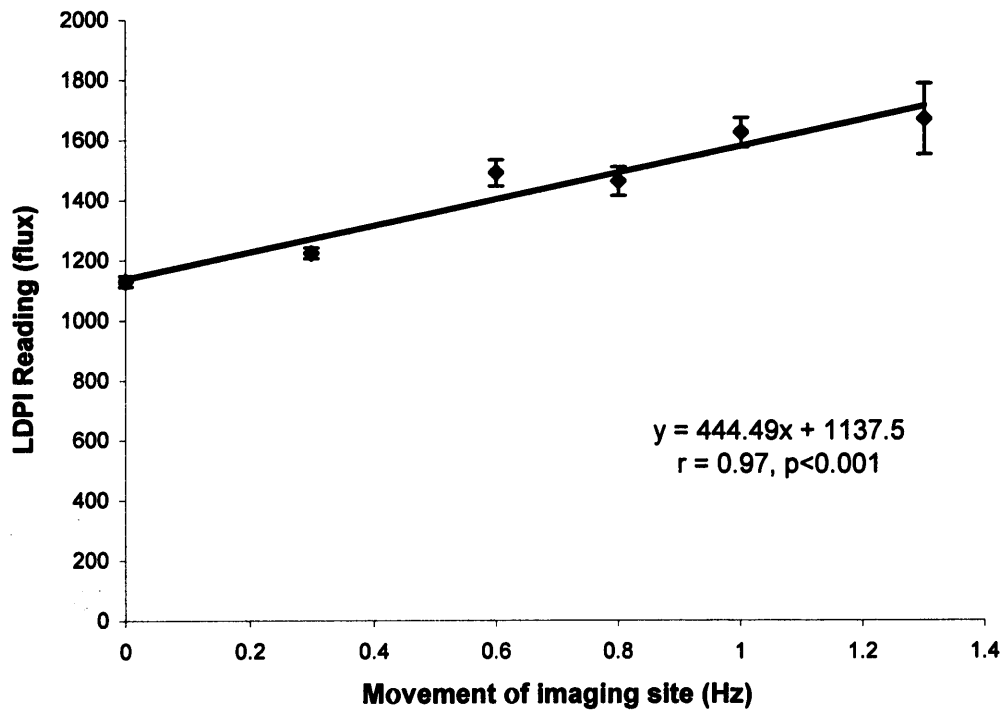
Figure 5.10 Response of LDPI to changing pressure of oxygen supplied to the blood. Blood flow rate was constant at $4\mu\text{s}^{-1}$.



5.6.4.5 Effect of movement of the imaging site on LDPI signal

In figure 5.11 movement of the imaging site resulted in a significant ($p < 0.001$) increase in the LDPI signal. At 15 cycles per minute or 0.3 Hz (respiratory movement) and 60 cycles per minute or 1 Hz (heart movement) the LDPI compounded the flow measurement by 8% and 44%, respectively.

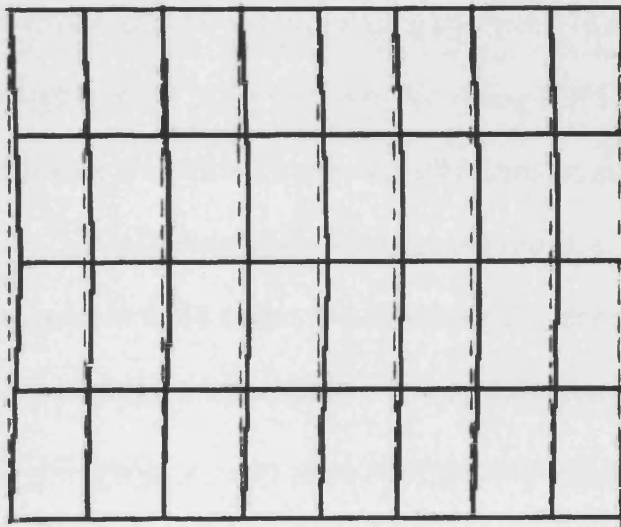
Figure 5.11 Effect of the speed of movement of the imaging site (blood-flow model) on LDPI reading at constant blood flow rate of $3.4 \mu\text{s}^{-1}$.



5.6.4.6 Assessment of geometric distortion of image generated by the LDPI

Figure 5.12 demonstrates the lack of geometric distortion in the horizontal axis and a small distortion ranging from 1.6 % to 3 % in the vertical axis of the image generated by the LDPI.

Figure 5.12 Diagrammatic representation of image distortion by LDPI – the vertical dotted lines indicate the actual position of the black lines imaged on the white sheet of paper. The solid vertical line shows the distortion in this axis arising as a result of the crescent shaped pathway taken by the laser beam during raster fashion scanning. There is no image distortion in the horizontal axis.



5.6.5 Discussion

The LDPI was able to map the spatial variation of perfusion over a wide area of this simple blood-flow model without surface contact and within a short period of time. The flow model simulates some of the optical properties of skin but is not an anatomical analogue of the skin microcirculation. With this limitation in mind the model was useful to evaluate the LDPI signal against several parameters influencing measurement of the microcirculation.

The manufacturer's recommended distance between the laser head and the imaging site is 150 mm to 200 mm. We found the optimal distance at 160 mm, with approximately a 7% error between 140 mm to 170 mm. The lower range of 140 mm is slightly lower than the manufacturer's recommendation of 150 mm. In this experiment a further increase in the distance between the detector and the imaging site from 170 mm to 200 mm resulted in a higher percentage increase in the error with decreasing LDPI signal. This could be due to a wider spread of the area of backscatter signalled from the moving RBCs, not detected by the photodiode. These findings differ from that of Kernick & Shore¹⁶⁶ who report a progressive increase in LDPI output with increasing distance up to 200mm. The reason for this variance is not clear but could be related to differences in the design of the flow-model or LDPI settings.

Clinically this difference in detection of the backscattered signal by the photodiode of the LDPI is not a problem when measuring perfusion from surfaces with a flat contour, such as the dorsum of the hand. In this clinical situation all measuring points on the skin surface are at the same distance from the detecting photodiode. If the skin surface had an irregular contour, such as the breast or the vulva, the investigator needs to be aware of the source of error introduced by the different distances between the measurement points and the LDPI

head. In this thesis, studying the vulval skin perfusion, care was taken to ensure that all measuring points on the imaging site was within 140mm to 170mm from the laser head, to keep any error in measurement to a minimum.

There was a significant correlation between the blood flow rate, measured by fluid collection, ranging from 0 to $5.89 \mu\text{s}^{-1}$, and the LDPI signal. The increase in LDPI signal with blood flow rate is due to the increase in the magnitude of the Doppler shift detected by the photodiode. However we observed a saturation limit for the detection of flow rate at $4.04 \mu\text{s}^{-1}$ (Fig. 5.8), which may be due to limitation of the manufacturer setting of the maximum detectable Doppler shift frequency²³¹. This does not cause a problem in clinical practice as the maximum blood flow in the microcirculation is within this range¹⁰⁶.

The LDPI signal is dependent on the concentration of the moving RBCs as well as its velocity. Reduction in the concentration of the RBCs by haemodilution was detected by the LPDI signal and there was a good correlation with flow rate measured by fluid collection.

LDPI readings were found to be independent of Po_2 in the range of 10.7 kPa to 66 kPa of the blood supplied to the flow phantom. We accept that the range of Po_2 investigated extends into that experienced during oxygen therapy. However we note that the LDPI readings were independent of the Po_2 over this wide range. The question of whether this could be extrapolated to the narrow range of Po_2 experienced in the clinical situation, between the capillary blood in the arterial end ($\text{Po}_2 = 12 \text{ kPa}$) and the capillary blood in the venous end ($\text{Po}_2 = 2 \text{ kPa}$), remains to be answered. Evaluation of the LDF signal for changes in the Po_2 in the range of 5.3 kPa to 16 kPa showed a minor but insignificant influence on flux measurements²²⁶.

Regular and continuous vibratory type movement of the imaging site significantly increased the LDPI signal during the scanning procedure. This is attributed to the photodiode detecting a Doppler shift from previously static measuring points. Clinically this is relevant when measuring superficial tissue blood flow during surgery from organs such as the heart or the liver. The heart moves regularly to an intrinsic rhythm set at 60 to 80 beats per min. and the liver moves with movement of the diaphragm during respiration. At 0.3 Hz (15 cycles per minute simulating respiratory movement) and 1 Hz (60 cycles per minute simulating heart movement) the increase in LDPI measurement was estimated at 8% and 44%, respectively. In fact any movement of measuring site on poorly stabilised tissue – intermittent, random or cyclical – increases the recorded LDPI signal. This also includes clinical situations involving young children and neonates.

There is a small degree of geometric distortion in the vertical axis but no distortion in the horizontal axis. This geometric distortion, which has never been reported, is most likely due to the crescent shaped pathway taken by the laser beam during scanning in a raster fashion. The resulting small image distortion does not affect measurement of the skin microcirculation and is unlikely to be of clinical significance.

The LDPI used in this thesis when validated with a blood-flow model was a reproducible, non-invasive technique that enabled mapping of the spatial variation of blood flow over a wide area, without surface contact, and within a few minutes. The LDPI signal is independent of tissue oxygenation in the range of 10.7 to 66 kPa and the image displayed on the computer monitor has only a very minor degree of image distortion in the vertical axis, which is unlikely to be of clinical significance. These results of the experiments to validate the LDPI demonstrate that this technique would be suitable for use on the vulval skin.

In the next chapter the LDPI is used on the normal vulva bearing in mind the effects of the distance between the laser head and the measuring site, and the movement of the measuring site during scanning on the LDPI signal.

CHAPTER 6

Assessment of normal vulval skin
microcirculation

6.1 Introduction

There is very limited information on the microcirculation of the specialised vulval skin. This is most likely due to anatomical access and the availability of suitable biophysical technology. The vulva also consists of several skin structures with varied morphology^{88;147}, each requiring individual representation for the study of skin blood flow. Several workers have successfully applied the LDF to study real time vulval perfusion^{83;140;344}. The LDF provides single point (1mm²) measurement and requires direct skin contact risking spread of infection. There are large spatial and temporal fluctuations of SBF within small areas, which can be minimised by measuring perfusion in an area of skin^{41;168;176;307}. The LDF can measure SBF in an area but a low resolution of 1mm² makes this technique time consuming. The LDPI however can measure skin perfusion over a specific area of interest without contact to produce a two-dimensional colour-coded image of the spatial variation of perfusion^{166;336}. These features of the LDPI are more suitable for measurement of vulval skin microcirculation compared to the LDF.

6.2 Aim

In this chapter the aim was to use the LDPI on women with normal vulva to find out if this technique was acceptable to women and to study the short-term reproducibility of vulval SBF. The study was then extended to observe the effect of age, menstrual cycle and recovery from local skin temperature change on vulval skin microcirculation.

6.3 Materials and methods

6.3.1 Study Population

This study was carried out in 28 healthy female volunteers, recruited from health care workers and patients admitted for routine surgery such as hysterectomy, hysteroscopy, diagnostic laparoscopy and sterilisation, all clinical conditions unrelated to diseases of the vulva. Care was taken to ensure that the women of reproductive age were not on hormonal contraception and that the postmenopausal women were not taking any hormone replacement therapy. 10 women with a mean age of 25.5 ± 5.23 years (mean \pm SD) were entered for the study on cyclical change in vulval skin perfusion, 10 women with a mean age of 59.6 ± 10.5 years were entered for the study on recovery of vulval perfusion from local skin temperature challenge and 19 women, including the latter group, were entered for the study on variations in vulval skin perfusion related to age. The women gave informed consent to the study, which was approved by the Ethics Committee at the RFHSM (Royal Free Hospital and School of Medicine).

In 12 other cases the procedure was unsuccessful because the subjects did not attend further assessment for cycle change, did not tolerate the lithotomy position for the duration of the investigation or the images acquired were inadequate for assessment.

6.3.2 Measurement procedure

The women were examined in the lithotomy position (Figure 6.1a + b).

Figure 6.1a Diagrammatic representation of the use of the LDPI on the vulva

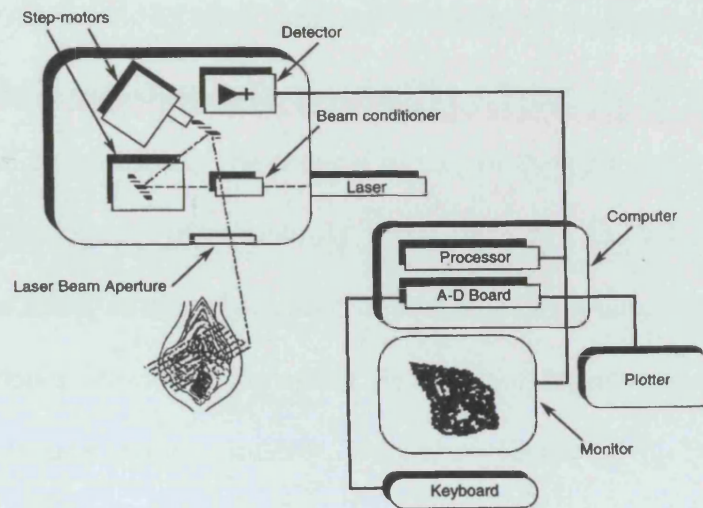
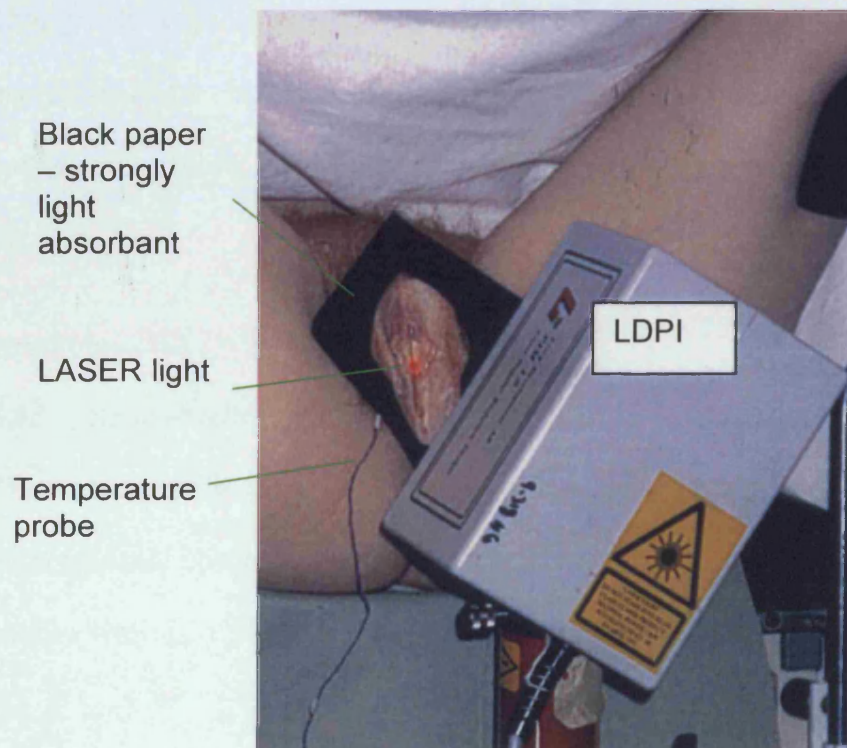


Figure 6.1b Clinical photo of the assessment of vulval perfusion with the LDPI



Each woman was acclimatised for 10 minutes in the examination room with ambient temperature at 22-23°C. The blood pressure, core (oral) temperature, skin temperature and pulse rate were measured with the subject in the lithotomy position and before the LDPI examination was commenced. The local skin temperature was recorded with a thermocouple attached on the external surface of the right labium majus, just outside the area of interest – the labia minora, inner surface of the labia majora, the clitoral hood and the clitoris, and the perineum. The external surface of the labia majora was excluded because the presence of hair interferes with imaging. The vulval area was localised with a perimeter of strongly light-absorbent black material, which in addition to providing a marker to identify and orientate the perfusion image, also reduces the total data acquisition time to approximately 2.5 minutes (Figure 6.1b). The LDPI head was placed approximately 160 mm from the measuring area, with care taken to ensure that the laser head was parallel with the skin as best as possible. This can be problematic considering the location and uneven topography of the vulva. A colour photograph of the vulval area was taken separately to correlate with the image, during analysis at a later date. An adjacent area of normal non-genital skin on the medial surface of the thigh, away from the vulva was also imaged, as a control area for the cycle study.

6.3.3 Reproducibility

The reproducibility was analysed by the sequential scanning mode to produce six and eight replicate images in two women with normal vulva. Non-genital skin was also studied with six replicate images of the medial surface of the thigh.

6.3.4 Cyclical change of vulval perfusion

10 women with a mean age of 25.6 ± 5.23 were imaged once a week throughout the cycle. The menstrual cycle was orientated retrospectively by recording the first day of the imaged cycle and that of the subsequent cycle with the assumption that the luteal phase is fixed at two weeks. In this group the menstrual cycle length was 28 ± 1.5 days. The volunteers in this group consisted of only health care workers who were more conveniently placed to provide the commitment of serial perfusion images. Volunteers from the general public were recruited unsuccessfully due to the required frequency of attendance. Image data set was complete for 8 volunteers and incomplete for two volunteers. One of these volunteers failed to attend on one and the other, on two occasions.

6.3.5 Age related variations of vulval perfusion

19 women were available for vulval skin perfusion imaging in this group. The women were arbitrarily divided into three groups – premenopausal under the age of 50 years, young postmenopausal between the ages of 50 to 70 years and old postmenopausal were aged more than 70 years. The study group consisted of six premenopausal women aged 32 ± 11 (mean \pm SD) years, six “young” postmenopausal women aged 60.3 ± 6.5 years and seven “elderly” postmenopausal women aged 75.8 ± 7 years. The use of antihypertensive drugs was prevalent in the postmenopausal group.

6.3.6 Local skin temperature challenge on vulval perfusion

In 10 women with a mean age of 59.6 ± 10.5 , the vulva was imaged at normal skin temperature and after provocation of local skin temperature change. The local skin temperature was first increased by gently applying a hot pack on the vulval surface for approximately 3 minutes. The hot pack consisted of disposable towels soaked in 'hand-hot' water and placed in a polythene bag to prevent wetting the skin surface while at the same time effectively conducting the heat to the skin. The skin temperature was cooled in a similar way using a cold pack which consisted of crushed ice wrapped in three layers of disposable towels, which was then placed in a polythene bag and held gently against the vulval skin. Care was taken to ensure that the skin temperature returned to the previous baseline after warming the skin and before starting to cool the skin. The procedure was well tolerated by the women. The change in temperature was recorded using a thermocouple and was between 4 and 6 °C above during hot provocation and below during cold provocation compared to the baseline temperature. The LDPI was performed after removal of the pack and there was a small drop ($\sim 1^\circ\text{C}$) in temperature during the 2.5 min scanning procedure.

6.3.7 Image analysis

The colour of each pixel on the image reflects the value of the perfusion at the corresponding measurement site. Six colours are used to give an overview of the perfusion and its spatial variation in the vulval skin. Light blue and dark blue colours represent low flux corresponding to low blood flow and red and orange represent a high flux, which correspond to high blood flow. The colour coding is described in detail in Chapter 5, Section 5.3 and Figure 5.5³³⁶.

The images were stored in a PC for later advanced analysis with an Image-processing software LDISOFT Version 1 (Lisca Development AB, Sweden) in a Microsoft® Window program. The images were manually correlated with the clinical photographs and *regions of interest* (ROI) were framed for calculation of the mean baseline skin blood flow in *flux* units for the following vulval areas: clitoral hood, clitoris, right and left labia minora, right and left inter labial space, and the perineum. A control area selected on the medial surface of the thigh was also imaged and analysed.

The mean skin blood flow of all framed *ROI* was recorded on Excel (Microsoft Inc. USA) spreadsheet and transferred to SPSS statistical software package for data set evaluations.

6.3.8 Statistical analysis

The results are expressed as mean \pm SD. The percentage differences for each week of the menstrual cycle with respect to the remaining three weeks were compared with Wilcoxon Signed Ranks Test. The same statistical test was used to analyse the variation in base-line perfusion after local skin temperature challenge. Probability values of less than 0.05 were regarded as significant. Short-term reproducibility of the LDPI measurement was determined by calculating the coefficient of variability (SD/Mean).

6.4 Results

Hypertension was the most commonly treated medical disorder in this study group particularly among the postmenopausal women. Table 6.1 shows the age and mean arterial blood pressure for all the subjects in this study.

Table 6.1 Sub-group analysis of the age (years \pm standard deviation), systolic blood pressure (sBP)(mmHg \pm SD), diastolic blood pressure (dBP) and mean arterial pressure (MAP)

	Cycle				Pre-menopausal ($< 50y$)	Young Post-menopausal (55-69y)	Old Post-menopausal ($\geq 70 y$)	Local skin temperature challenge
	Week 1	Week 2	Week 3	Week 4				
Subject numbers	10	10	9	8	6	6	7	10
Age (years)	25.6 \pm 5.23	25.6 \pm 5.23	26.2 \pm 5.1	26.8 \pm 5	32 \pm 11	60.3 \pm 6.5	75.75 \pm 7.14	59.6 \pm 10.5
Arterial blood pressure (mmHg)								
sBP	117 \pm 10.9	113.9 \pm 9	115 \pm 16	120.7 \pm 4.5	131 \pm 25	145 \pm 17.6	150 \pm 19.5	142 \pm 20
dBP	67.22 \pm 8	68.6 \pm 10	65.33 \pm 10	71.43 \pm 4.76	80 \pm 11	87.5 \pm 18.9	79.29 \pm 18.8	86 \pm 18
MAP	83.81 \pm 8.4	83.7 \pm 8.1	81.89 \pm 8.79	87.86 \pm 4.16	97 \pm 15	106.5 \pm 16.5	99.43 \pm 12.88	105 \pm 18

6.4.1 Reproducibility

The coefficient of variation for normal vulval baseline perfusion was between 5% and 7%, and that for non-genital skin was 1% (Table 6.2). A coefficient of variation of 10% or less is considered satisfactory⁸⁷.

Table 6.2 The mean Flux, standard deviation (SD) and coefficient of variability (CV) in normal vulval skin and non-genital skin (thigh)

	Normal vulva		Normal vulva		Thigh	
	Mean Flux	SD	Mean Flux	SD	Mean Flux	SD
Image 1	0.69	0.4	1.05	0.48	0.52	0.11
Image 2	0.71	0.45	0.98	0.43	0.53	0.12
Image 3	0.66	0.37	0.93	0.41	0.52	0.1
Image 4	0.75	0.38	0.91	0.39	0.54	0.13
Image 5	0.69	0.39	0.91	0.39	0.53	0.12
Image 6	0.72	0.38	0.86	0.36	0.53	0.11
CV	5%		7%		1%	

Figure 6.2 (a + b) is a photograph of a normal vulva and the colour-coded image of the skin perfusion.

Perineum



Clitoral region

Figure 6.2a

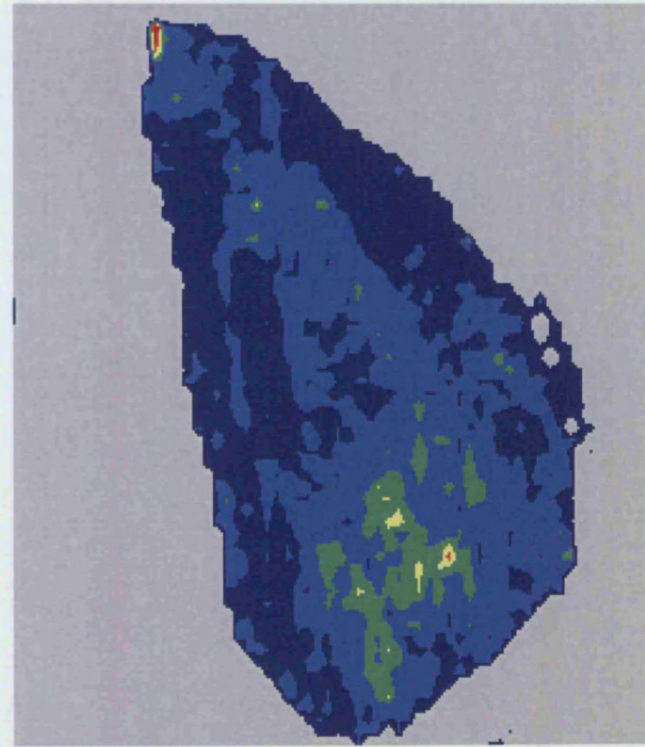
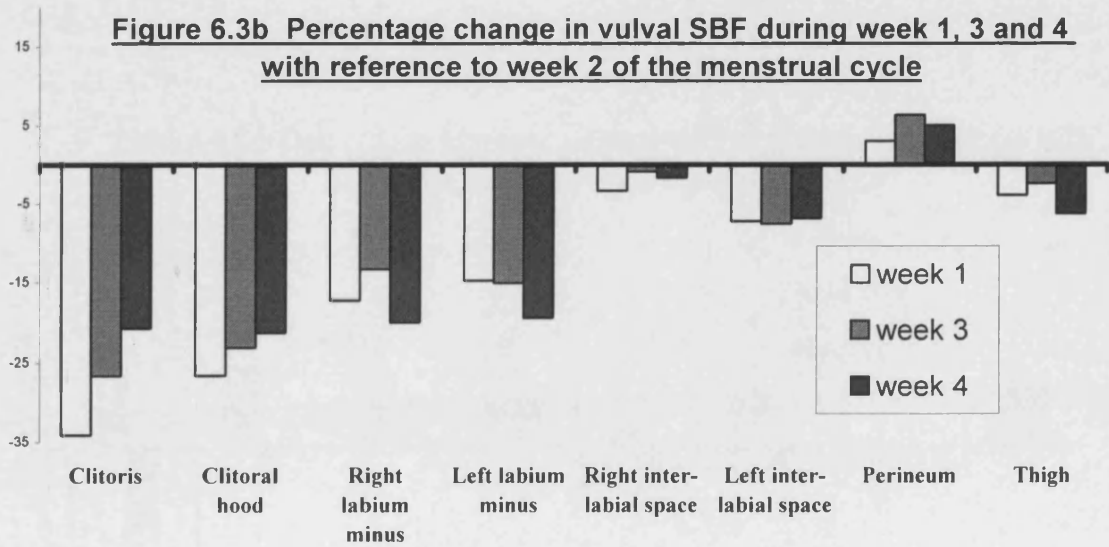
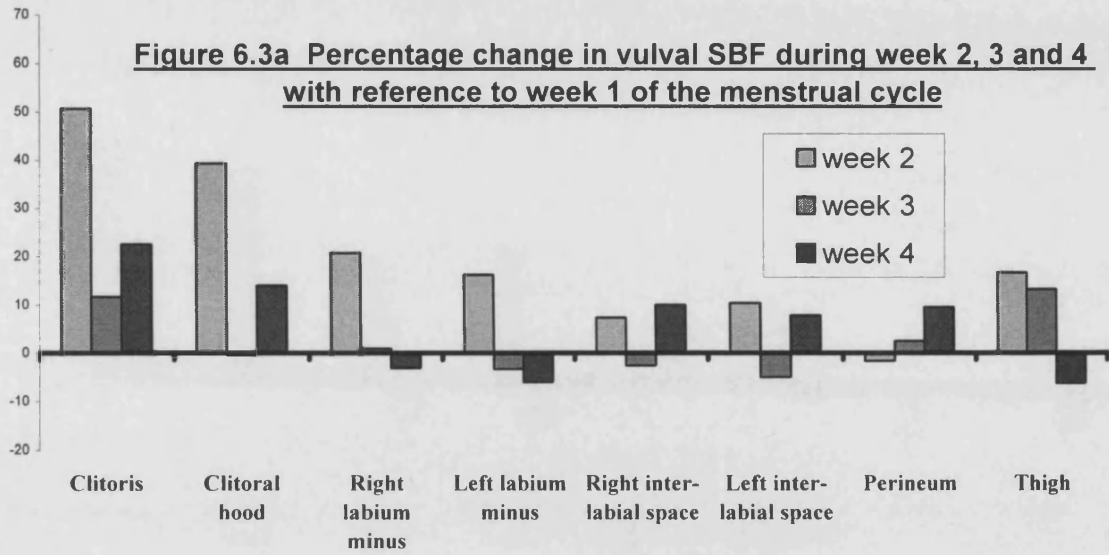


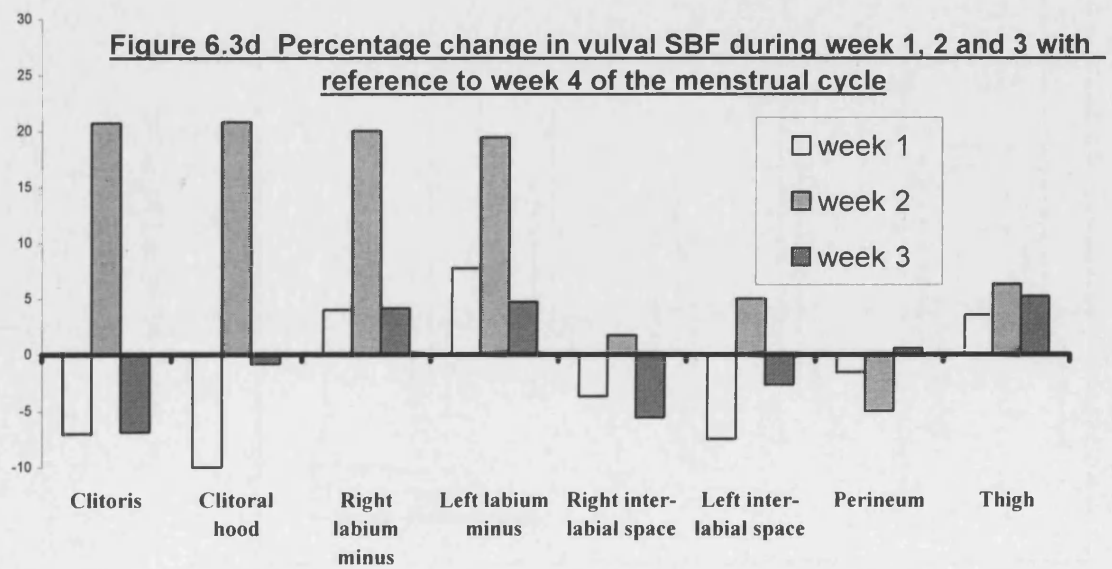
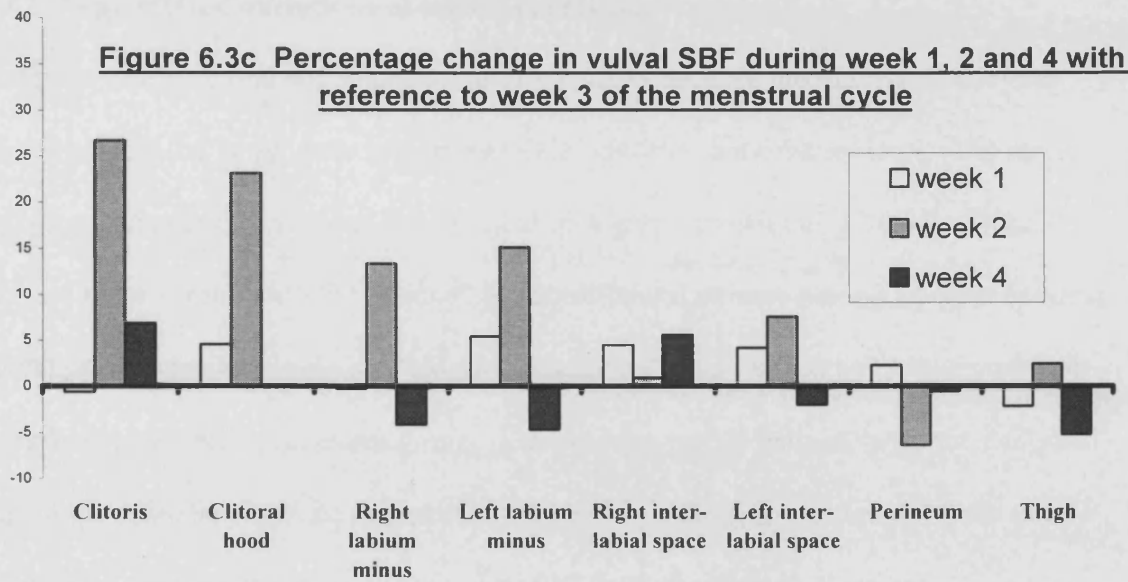
Figure 6.2b

Figure 6.2a-b Clinical photograph (Figure 6.2a) and LDPI image (Figure 6.2b) of a normal vulva in a 25y old. Note a region of relatively high blood flow in the clitoral area

6.4.2 Cyclical change of vulval perfusion

Cyclical change in perfusion was analysed by comparing the differences in perfusion for each week compared to the other three weeks. Wilcoxon signed ranks test did not show any significant cyclical difference in vulval skin perfusion during the menstrual cycle. However bar chart representation (Figure 6.3a-d) of differences in perfusion between weeks 1 to 4 of the menstrual cycle, show a possible increase in perfusion in week 2, during the follicular phase. In Figure 6.3b all areas of the vulva consistently showed a higher perfusion on week 2 compared to the other three weeks. The non-genital skin on the medial surface of the thigh also showed a similar perfusion pattern to the vulval skin.

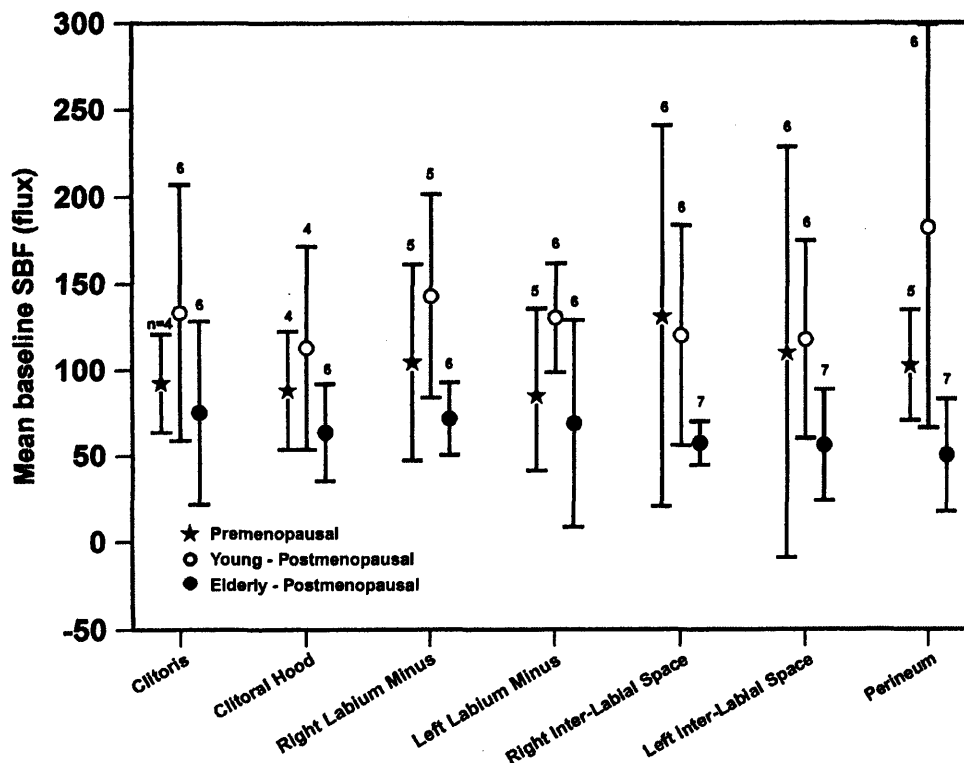




6.4.3 Age related variations of vulval perfusion

Statistical analysis was not done for this comparison because the number of subjects was small and the large variations in base-line perfusion between subjects. The mean values and standard deviations were plotted on a graph shown in figure 6.4. There appears to be a trend with the “young” postmenopausal women having a higher baseline vulval skin perfusion, while the “elderly” postmenopausal women had lower perfusion compared to the premenopausal group. The premenopausal women were not studied during the same week of the menstrual cycle as the findings in Section 6.4.2 did not show any significance difference in vulval SBF with the menstrual cycle.

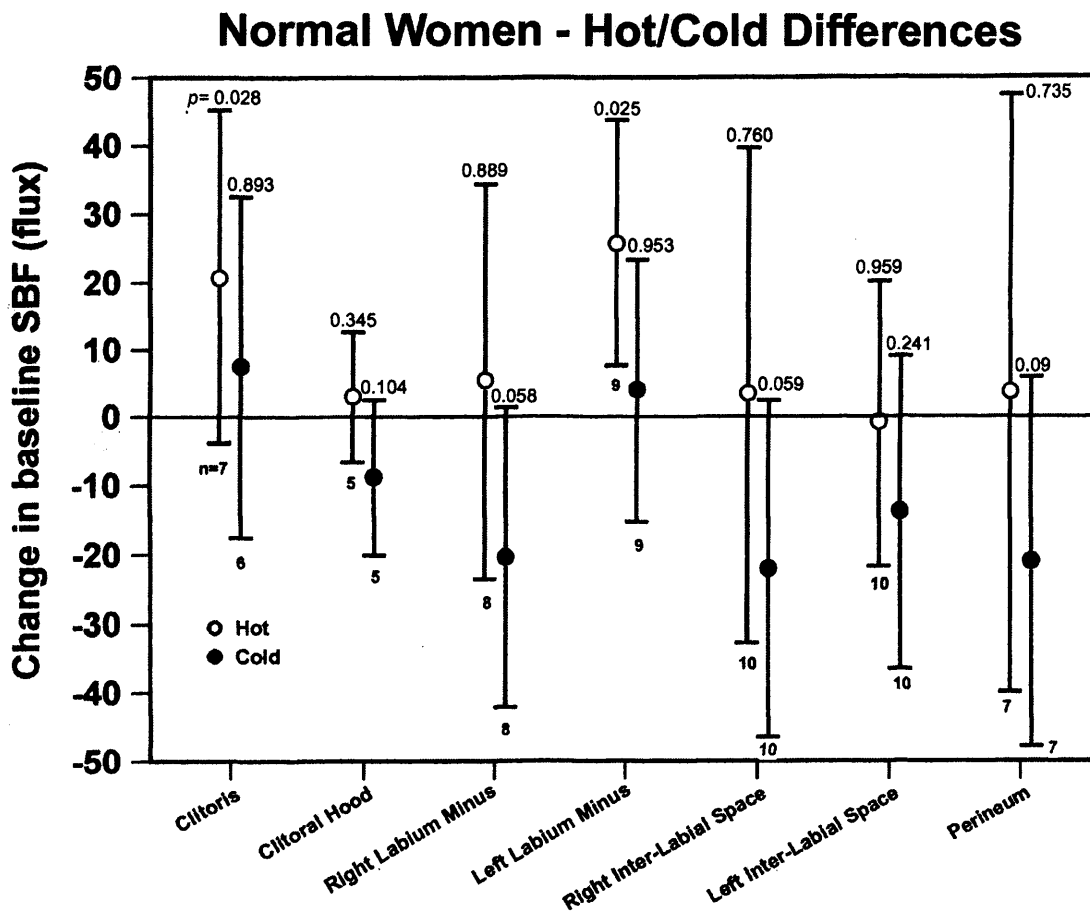
Figure 6.4 Comparison of vulval skin baseline perfusion in premenopausal, ‘young’ postmenopausal (< 70y) and ‘elderly’ postmenopausal (≥ 70 years) woman



6.4.4 Vulval perfusion after local skin temperature challenge

The response of vulval skin perfusion following local skin temperature challenge was analysed by comparing the differences in perfusion to the baseline. Figure 6.5 shows the mean value and 95% confidence interval of change in vulval skin perfusion measured after hot and cold provocation. Wilcoxon signed ranks test does not show significant differences in skin perfusion to warm and cold local temperature challenge in all areas of the vulva except for a minor difference in the left labium minus and clitoris. The 95% confidence interval is wide suggesting a wide variability of this parameter in the general population.

Figure 6.5 Change in normal vulval baseline perfusion in response to hot and cold provocation



6.5 Discussion

This preliminary study does not show significant differences in baseline vulval skin perfusion with age and the phase of the menstrual cycle. Local skin temperature challenge with hot and cold packs also does not show significant differences in the baseline perfusion during the recovery phase, in the normal vulva, except for a minor difference on the left labium minus and clitoris following hot provocation. The age and temperature challenge graphs (Figures 6.4 and 6.5) show a wide 95% confidence interval suggesting a large variation within the normal population. However there are subtle trends of vulval perfusion, which may prove to be significant with larger subject numbers. The reproducibility of the LDPI apparatus in the short term (within minutes) is good.

Age related changes in baseline skin perfusion are influenced by the thickness of the epidermis, density of the microvessels and the blood flow velocity. The keratin and epithelial layer of the vulva is at its maximum thickness during the reproductive years and gradually thinning after the menopause¹⁴⁷. This is attributed to depleting oestrogen levels with the menopause, although immunohistochemical studies show a very low frequency of oestrogen receptors suggesting that oestrogen may not be as influential in the vulval skin as is commonly believed¹⁹⁵. Capillaroscopic studies show a striking loss of dermal papillary loops but no quantitative change in the upper horizontal vessel plexus in the older woman compared to younger subjects in the forehead and the forearm¹⁶⁵. In contrast the LDF and LDPI did not show any difference in baseline perfusion with advancing age and this was attributed to the greater penetration of the laser beam, thus giving information from vessels deeper than the dermal papillary loops i.e. horizontal plexus and arteriovenous anastomosis (AVAs)^{165;288;304}. Heat washout studies however do show decreased blood flow rate in AVAs with increasing age in the

finger and toe pulps attributed to a combination of increasing stiffness of the AVAs, reduced metabolic rate and presumably endothelial changes²¹⁰. The microvessel structure, including the presence of AVAs in the vulval skin is not known and so, the above reported findings of age related changes in the non-genital skin microvasculature should be cautiously extrapolated to the specialised vulval skin. The trend of the data in Figure 6.4 could be the result of an initial decrease in epidermal thickness with ageing, thus increasing the depth of penetration of the laser beam in young postmenopausal women, followed by an actual decrease in microcirculation in the elderly postmenopausal women. Although none of the postmenopausal women in this group were on hormone replacement therapy, the effect of postmenopausal hormone therapy is difficult to predict as immunohistochemical evidence¹²⁸ suggest sparsity of oestrogen receptors in the vulval skin compared to vaginal skin. In general oestrogens protect against skin ageing by increasing the collagen content and skin thickness³²⁷.

The phase of the menstrual cycle does not alter the base line perfusion in the pulp of the big toe but significantly increases perfusion in the luteal phase in the vaginal and rectal mucosa⁸⁷. The vulval skin is unique compared to the regions studied by Emmanuel et al^{87;147;195}. Observation in this thesis of a higher (not significant) baseline vulval perfusion during the follicular phase (week 2) of the menstrual cycle is similar to that found in the cervix and the endometrium, and thus requires further study^{105;140}.

The measurement of skin perfusion over a wide area such as the vulva, in response to recovery from local skin temperature challenge is problematic because of the practical difficulties of maintaining a constant vulval skin temperature during the 2.5 minutes imaging time. The irregularity of the vulval skin surface may also cause different levels of exposure to temperature change as the pack is placed gently to avoid local pressure

altering the skin perfusion. A perfusion imager that is able to stabilise the skin temperature as well as measure perfusion would be valuable to perform this provocation test more reliably. Our results did not show significant differences in baseline vulval perfusion following local skin temperature challenge except during hot provocation in the left labium minus and clitoris. This difference in response in the left labium minus and clitoris compared to the remaining vulva is unclear but it is of note that on cold provocation the mean response also resulted in a higher SBF compared to the remaining of the vulva. It is of interest that the other vulval structures and the perineum showed a greater decrease in mean blood flow to cold compared to mean increase in SBF to hot provocation (Figure 6.5). A larger study is required to elaborate on these observations, which appear to suggest AVAs or a similar structure may be predominant in the vulva as contributors to the noticeable vasoconstriction during cold challenge¹⁷⁵. Cold challenge decreases heterogeneity of skin perfusion while hot challenge appears to have the opposite effect²⁴². Hot challenge tends to increase perfusion variable until 44 °C when the maximum increase is achieved⁹².

This preliminary work is limited by the small numbers of women recruited and the lack of standardisation for confounding variables such as smoking, eating, medical diseases: controlled hypertension, drug history including hormone replacement therapy and time of the day. The 95% confidence intervals were wide resulting in a large overlap of perfusion values. This wide variation may be a reflection of the degree of intrinsic heterogeneity of normal skin perfusion^{41,336} and the effect of confounding factors⁸⁶. This feature could also be attributed to the skin attempting to maintain normal perfusion within the narrow temperature challenge applied in this thesis. Future work with larger numbers and better standardisation could contribute valuable information on the microcirculatory physiology of this specialised area of skin.

The use of the LDPI apparatus to study vulval SBF is acceptable to women and has a good short-term reproducibility for assessment of vulval and non-genital skin perfusion.

The data suggests that menstrual cycle and age do not affect the perfusion parameter.

The response of vulval perfusion during recovery from local skin temperature challenge is more complex, and demonstrates a wide variation in this parameter.

In the next chapter the LDPI is used to study skin microcirculation in women with vulval LS and VIN 3.

CHAPTER 7

**Assessment of diseased vulval skin
microcirculation**

7.1 Introduction

Vulval LS and VIN 3 require long-term follow-up for symptom control and to provide surveillance for early detection of malignant change as described in Chapter 3.

Currently the available techniques for examination of the vulva as discussed in Chapter 3 are limited for early diagnosis of malignant change. In particular the colposcope, useful on the cervix, has limited application on the vulva. The presence of keratin on the vulva reduces the penetration of ordinary light necessary to study the morphology of the superficial vessel pattern, as seen in the cervix.

7.2 Aim

In this chapter the LDPI was used in vulval disease. The vulval skin perfusion in LS and in VIN 3 was compared with that in normal women. In women with LS the study differentiated women before and after treatment with successful symptom relieve. The purpose of this study is to develop a further parameter to objectively monitor women with LS and VIN 3, and perhaps pave the way to develop a new technique to identify cases at high risk of malignant change.

7.3 Methods and materials

The LDPI instrumentation is described in Section 5.3 and application to the vulva in Section 6.3.2. Vulval image analysis is discussed in Section 6.3.7.

7.3.1 Study population

The study population consisted of 31 women before and after treatment of vulval LS ($58 \pm 13.8y$), and 11 women with VIN 3 ($45 \pm 12y$) recruited from the Vulval Clinic based at the Royal Free Hospital. The women gave informed consent for the study, which was approved by Ethics Committee at the RFHSM.

7.3.1.1 Normal women

The control subjects ($n=19$; $50 \pm 20y$) were healthy women with normal vulval skin recruited from patients admitted for unrelated gynaecological surgery and from hospital staff as described in Chapter 6, Section 6.3.1.

7.3.1.2 Clinical profile of women with LS

The women with vulval LS were almost all new patients to the vulval clinic with symptoms between six months and 22 years. The main presenting symptoms were vulval pruritus and soreness, while many of the younger women also complained of superficial dyspareunia. Most of the women had received some form of treatment in the past – topical oestrogen cream, anti-fungal preparations, topical steroids of varying potency and duration while two patients also had had a simple vulvectomy. The courses of topical steroids used by these women were either of much lower potency and/or of inadequate duration compared to our regimen. These patients were diagnosed on clinical examination, although 23 patients had histological diagnosis in the past. We did not perform any biopsy for the purpose of this study, as this would have interfered with blood flow measurements.

20 women were referred by the family practitioner, five were in-house referrals and six, tertiary referrals.

The women in this study received potent topical steroids (clobetasol propionate 0.05%, fluocinolone acetonide or triamcinolone) for 8 to 12 weeks followed by moderately potent topical steroids (clobetasone butyrate 0.05%) gradually reduced to once or twice a week¹⁹⁸. The patients included in this study showed good response with significant, and often complete resolution of symptoms. Women with thick hyperkeratotic areas excluded themselves as their response to treatment was brief with repeated exacerbation of symptoms requiring excision biopsy. The vulval architecture at presentation appeared to vary with the duration of disease and intensity of symptoms. After commencing treatment and with resolution of symptoms, evidence of active disease due to pruritus resolved but the architectural changes related to resorption of the labia minora, obliteration of the interlabial sulci and phimosis of the clitoris remained the same as before treatment.

These women were seen and examined for the purpose of this study at referral and serially throughout their course of treatment. The women had not received any topical treatment for at least three weeks before the first pre-treatment examination of LDPI. The post-treatment examination of LDPI was done when patients were symptom free and at least two weeks into the second phase of treatment with moderately potent topical steroids. The results of serial examination during the two phases of treatment as measured by the LDPI are presented in Chapter 8.

7.3.1.3 Clinical profile of women with VIN 3

The women with VIN 3 consisted of only one patient referred by the family practitioner, while two were in-house referrals and eight, tertiary referrals. The duration of the disease varied between three and 11 years, with all the women having previously received excision or laser ablative treatment. The main presenting symptoms were pruritus, soreness, ulceration and superficial dyspareunia. These women were seen and examined for the purposes of this study only at referral. All these women had histological confirmation of the diagnosis.

In this study the women with vulval disease (LS & VIN 3) were not standardised for medical history, smoking or use of hormonal preparations for contraception (one woman with VIN 3) or replacement therapy. Anti-hypertensive therapy was prevalent in the older women in the LS group. Smoking (> 10 cigarettes a day) was prevalent in the women with VIN 3. Smokers were advised not to have a cigarette for at least three hours before the examination.

7.3.2 Reproducibility

The reproducibility was analysed by the sequential scanning mode to produce six and eight replicative images in two women with vulval LS and one, with normal vulva.

7.3.3 Statistical analysis

The results were expressed as mean \pm SD for each area of genital and non-genital skin. Differences between the mean values of the different groups of subjects for each skin area were assessed by Kruskal-Wallis' test (a non-parametric one way analysis of variance). Paired and unpaired data were compared with Wilcoxon signed ranks' test and Mann-Whitney *U* Test, respectively. Probability values of less than 0.05 were regarded as significant. Reproducibility of the LDPI measurement was determined by calculating the coefficient of variability (SD/Mean).

7.4 Results

Table 7.1 shows the age, body temperature, skin temperature on the right labium majus, heart rate, mean arterial blood pressure and body mass index of all the subjects in the study.

Table 7.1 The age, body temperature, skin temperature on the right labium majus, heart rate, arterial blood pressure (sBP=systolic blood pressure, dBP=diastolic blood pressure and MAP=mean arterial pressure) and body mass index (BMI) of all the subjects in study

	Normal	VIN 3	Lichen Sclerosus	
			<i>Pre-treatment</i>	<i>Post-treatment</i>
Subject number	19	11	31	31
Age (years)	50.1 ± 20.2	44.7 ± 12.26	58.5 ± 13.8	58.5 ± 13.8
Body temp. (°C)	36.6 ± 0.5	37 ± 0.2	36.45 ± 0.33	36.62 ± 0.22
Skin temp. (°C)	32.4 ± 0.5	32.44 ± 0.82	32.1 ± 1.14	32 ± 1.08
Heart rate (beats/min)	71 ± 12	62 ± 3	67.2 ± 6.5	67 ± 9.1
Arterial blood pressure (mmHg)				
sBP	142.4 ± 21.4	132 ± 19	145.94 ± 24.21	137.03 ± 20.67
dBP	81.1 ± 17.4	81 ± 10	86.87 ± 11.22	83.03 ± 14
MAP	101 ± 17	98 ± 3	106.46 ± 13.51	101.03 ± 14.74
BMI (kg/m ²)	25.14 ± 3.53	22.65 ± 3.34	27.83 ± 4.84	(not repeated)

Figure 7.1a is a clinical photograph of a normal vulva, with corresponding colour-coded perfusion output by the LDPI in Figure 7.1b. Figures 7.2 and 7.3 show vulval LS and VIN 3, respectively.

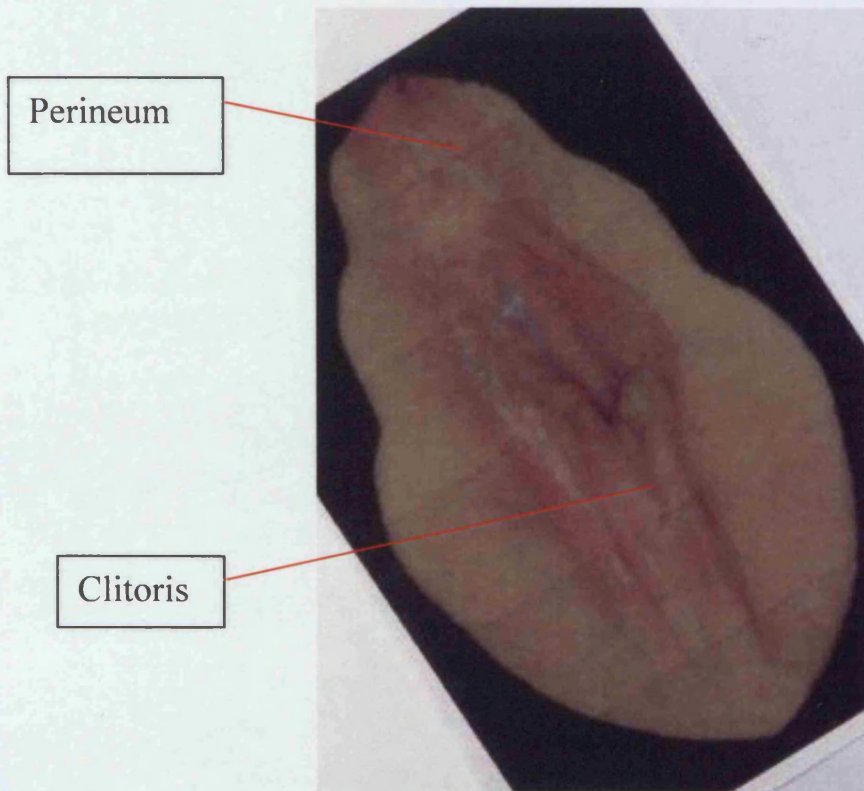


Figure 7.1a

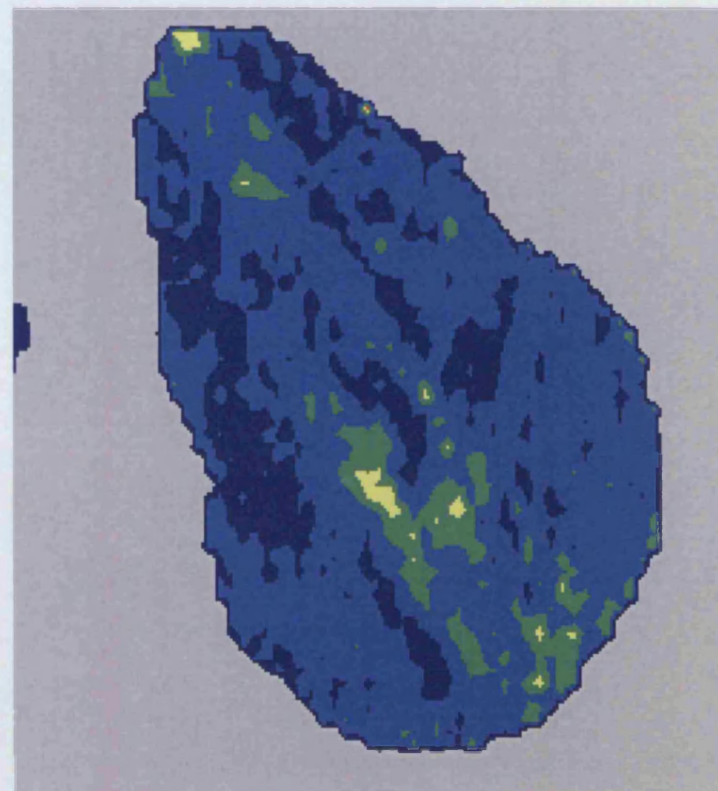


Figure 7.1b

Figure 7.1a-b Clinical photograph (Figure 7.1a) and LDPI of normal vulva (Figure 7.1b). There is a region of increased perfusion around the clitoris

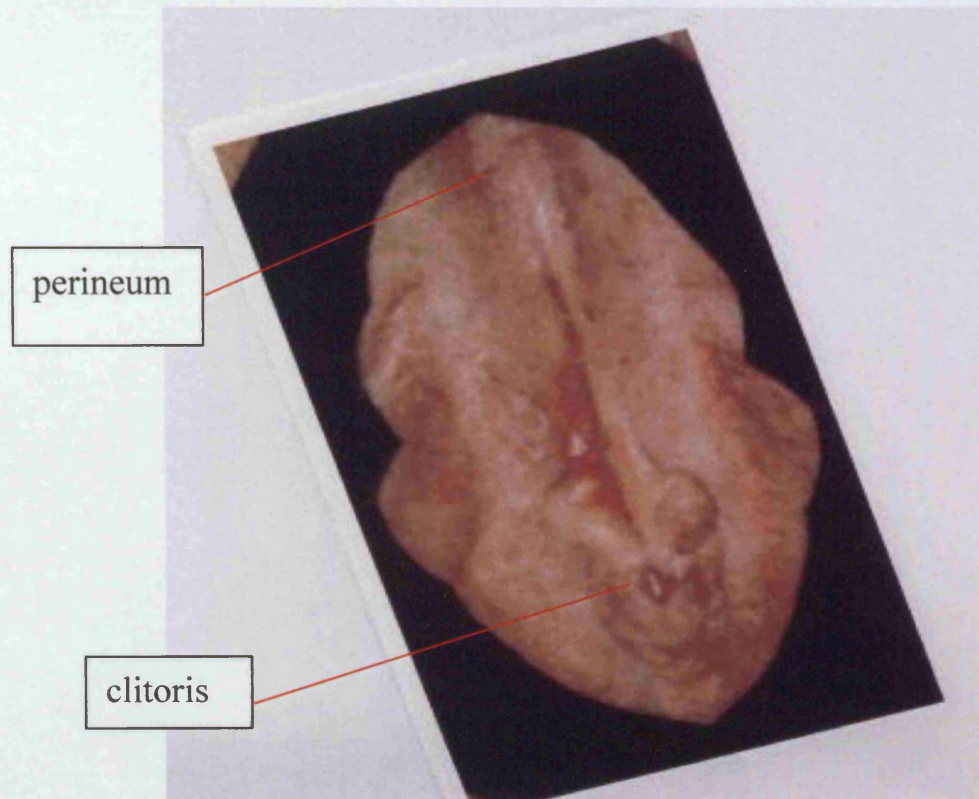


Figure 7.2a

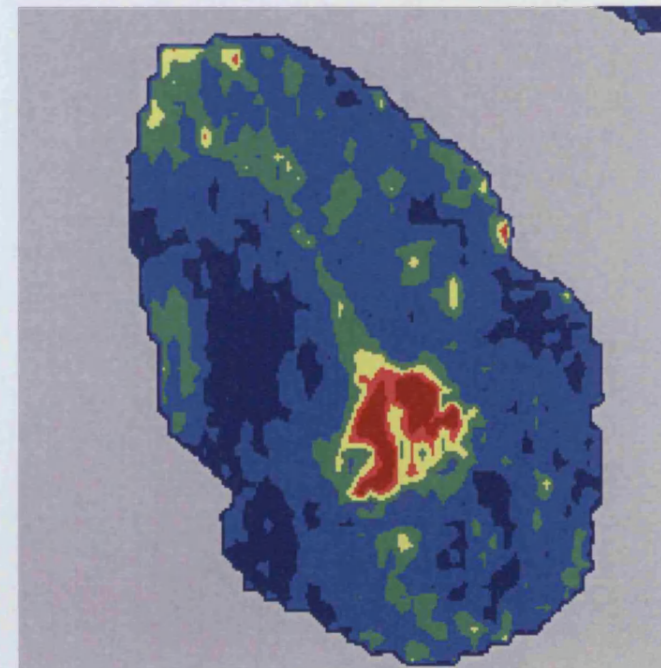


Figure 7.2b

Figure 7.2a-b Clinical photograph (Figure 7.2a) showing vulval LS. There is pallor, absorption of labia minora, partial phimosis and excoriation on the left labium minus. The LDPI image (Fig7.2b) shows a high perfusion area on both the right and left labia minora.

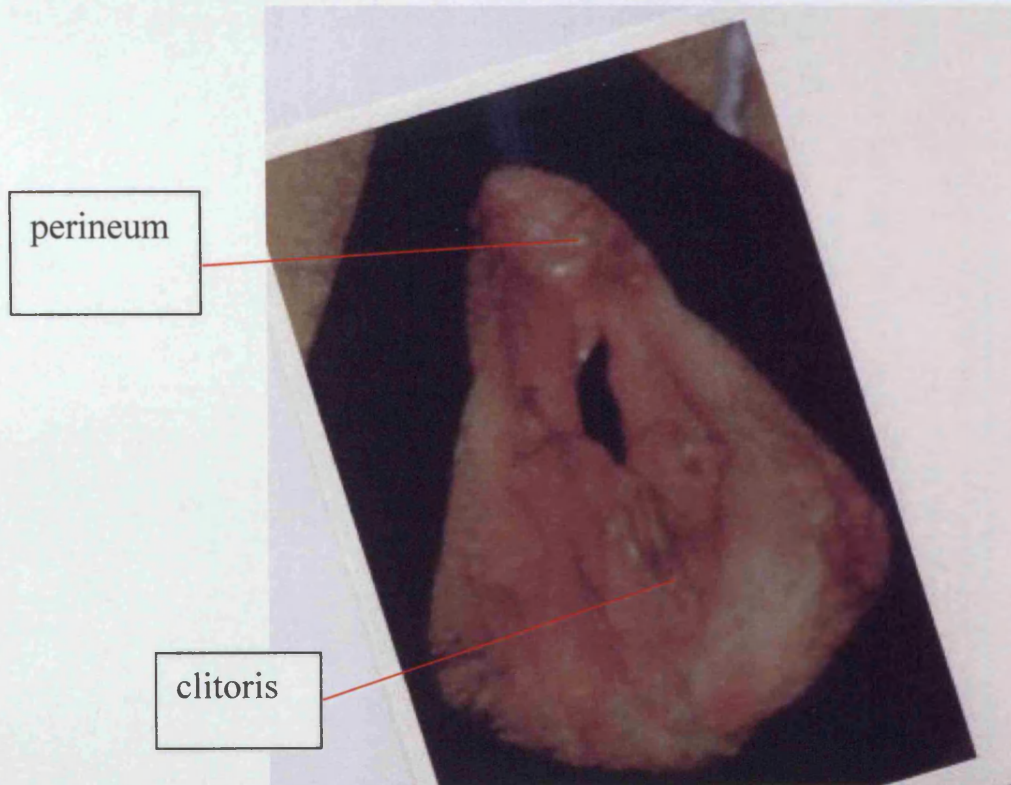


Figure 7.3a

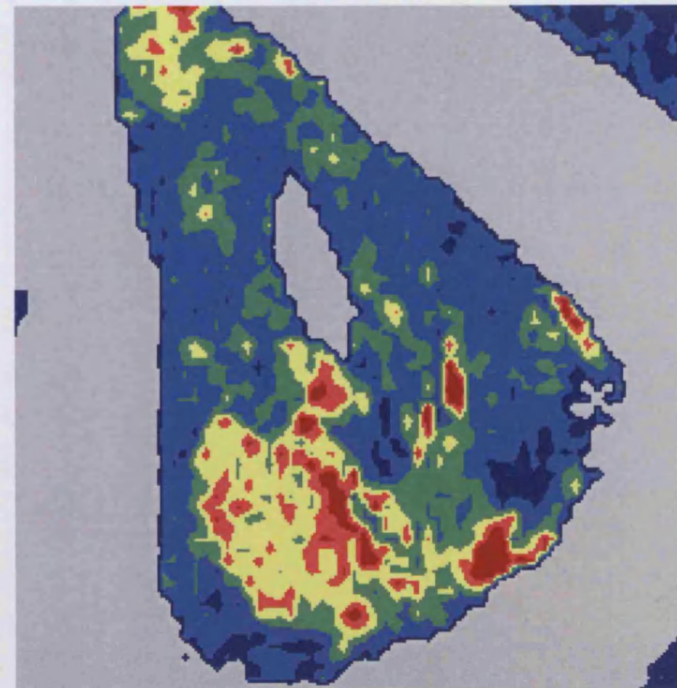


Figure 7.3b

Figure 7.3a-b Clinical photograph (Figure 7.3a) showing a large area of VIN3 in the left anterior inter-labial space, anterior left labium minus, clitoral hood and the perineum. The LDPI image (Figure 7.3b) shows high perfusion areas corresponding to regions of suspected VIN 3, which was later confirmed on biopsy.

7.4.1 Reproducibility

Vulva - The coefficient of variation for normal vulval baseline perfusion was 6% and for vulval LS between 1% and 4%. A coefficient of variation of 10% or less is considered satisfactory reproducibility⁸⁷.

Thigh - The total inter-patient variability for the control non-genital area on the thigh for the four groups was 5%. The intra-patient variability for patients with LS serially followed up during treatment with topical steroids was between 7% and 22%. This could be attributed to the area scanned on the thigh, which was not identical in location on each follow-up and the small number of images (n= 6 to 9 images in each of 8 patients) analysed compared to the inter-patient (n=71 images) variability group.

7.4.2 Comparison between normal vulva and vulval lichen sclerosus

The mean \pm SD of vulval skin perfusion (flux values) in normal women, and in women with LS before and after treatment is compared graphically in figure 7.4. There was no significant difference in perfusion values between the normal women and those with untreated LS for all the areas of the vulva and perineum. After treatment with topical steroids, vulval LS had significantly lower perfusion values compared to their pre-treatment values (except for the perineum). Post-treatment vulval LS also had significantly lower perfusion values compared to normal women (except for the perineum and clitoral hood). Table 7.2 shows the *P* values for these comparisons.

Figure 7.4 Graph comparing the vulval perfusion in normal women, and in women with LS, before and after treatment with topical steroids

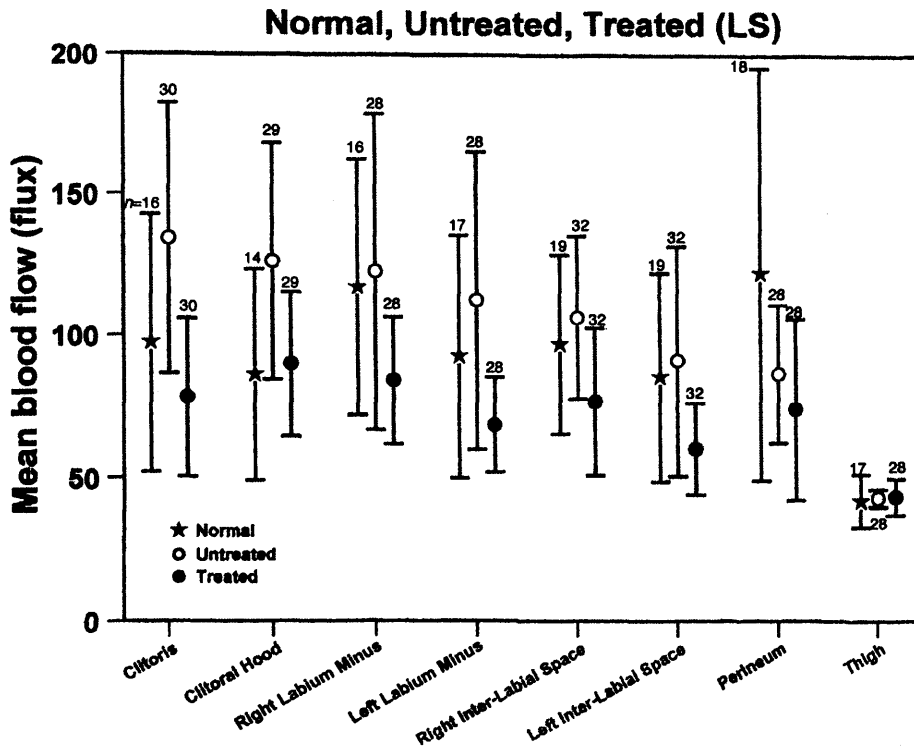


Table 7.2 P values for vulval flux of selected regions of interest (ROI) in normal vulva, untreated LS and treated LS.

ROI	Normal vulva vs Untreated LS vs. Treated LS (Kruskal-Wallis Test)	Normal vulva vs. Untreated LS (Mann-Whitney Test)	Normal vulva vs. Treated LS (Mann-Whitney Test)	Treated LS vs. Untreated LS (Wilcoxon Signed Ranks Test)
Clitoris	0.011	0.549	0.05	0.005
Clitoral-hood	0.014	0.195	0.182	0.005
Right labium minus	0.02	0.971	0.002	0.015
Left labium minus	0.005	0.944	0.007	0.005
Right inter-labial space	0.01	0.8	0.021	0.006
Left inter-labial space	0.001	0.495	0.002	0.001
Perineum	0.026	0.110	0.10	0.147
Thigh	0.729	0.649	0.444	0.646

7.4.3 Comparison between normal vulva and VIN 3

Figure 7.5 compares the perfusion values between normal women and VIN 3. The mean perfusion values were higher (not significant) for VIN 3 compared to the normal vulva (except for perineum). The comparison between these two groups was impaired by the distribution of VIN 3, which tends to be patchy, compared to vulval LS. Vulval LS usually affects the whole of the skin in the inner vulva. A separate comparison (Figure 7.6) between areas affected by VIN 3 and an adjacent normal area of the vulva in the same patient showed significantly higher perfusion in VIN 3.

Figure 7.5 Graph comparing the vulval perfusion in normal women and in women with VIN 3

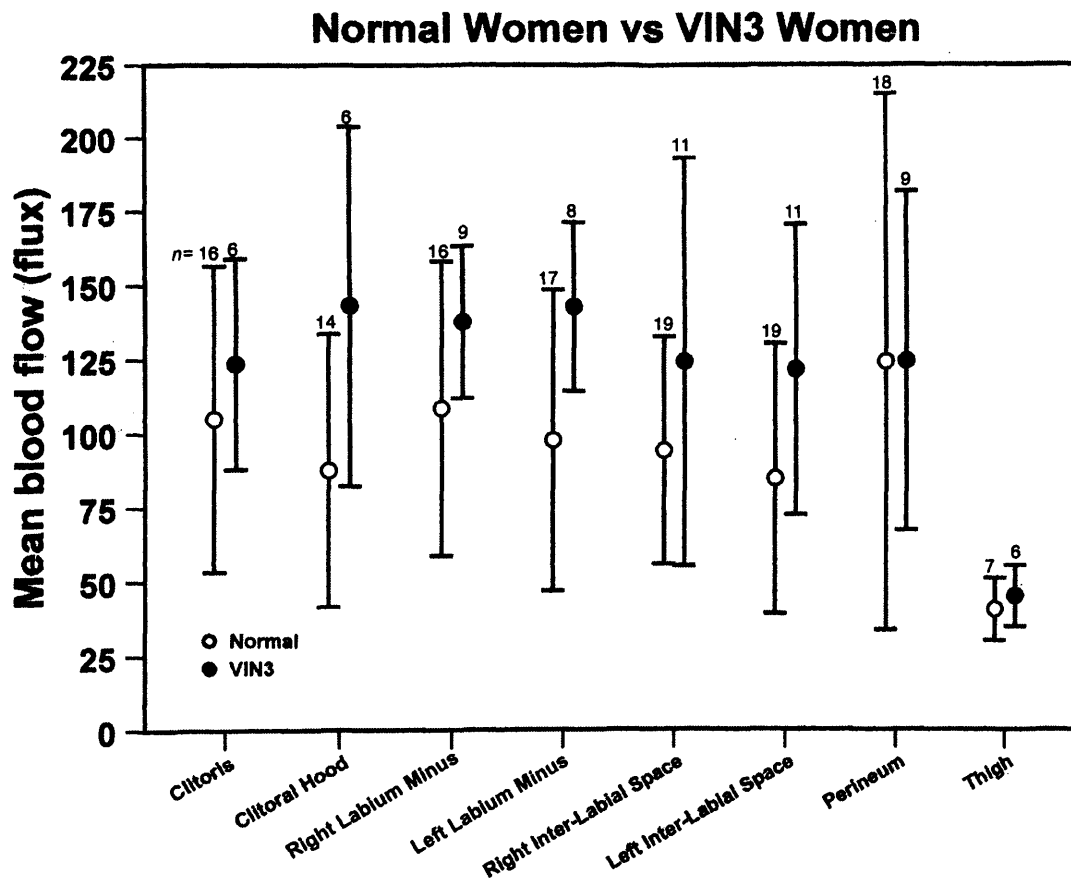
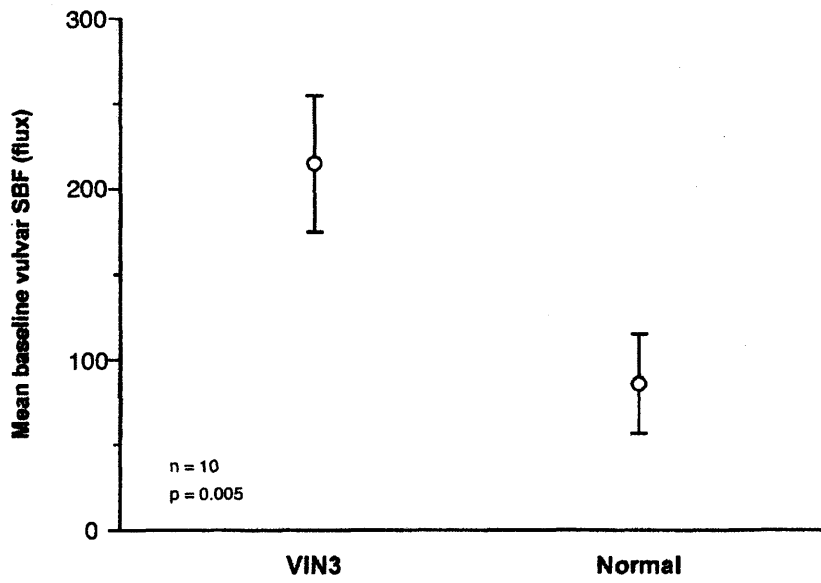


Figure 7.6 Graph comparing the vulval perfusion in adjacent areas of VIN 3 and normal skin in the same woman



7.5 Discussion

The reproducibility of the LDPI apparatus in the normal and diseased (LS) vulva using the 'mean of the total vulval flux' for each image was good-to-excellent (CV= 1-6%). The thigh was a good reference point for the study of variations in vulval skin perfusion as the reproducibility for all the women in the vulval disease group was 5% (n=71 images) and the skin is conveniently located. However it is interesting that the long-term intra-individual (n=6 to 9 images in each of 8 patients) temporal heterogeneity in skin perfusion for the thigh (non-genital control skin area) had a poor reproducibility (CV=7-22%). The latter result could be attributed to imaging a similar but not identical imaging site (32 by 32 pixels) on the thigh or to the small number of images analysed in the intra-patient variability group. This could perhaps be overcome in future studies by marking the skin area.

Preliminary data of the diseased vulva in this thesis show a significant reduction in skin perfusion with treatment of vulval LS with topical steroids. A short course of potent topical steroids on vulval LS reduces hyperkeratosis of the epidermis, in addition to reducing inflammation and hyalinisation of the dermal collagen⁷³. The laser Doppler technique is not sensitive to the iatrogenic blanching of the skin by topical steroids. This is due to venular rather than arterial vasoconstriction which cannot be recorded by flow-dependant techniques such as LDF and LDPI^{131,229}. Moreover the laser Doppler technique records blood flow deeper than the superficial papillary capillaries, which are not involved in the blanching effect. The depth of measurement may also be influenced by topical preparation increasing hydration of the stratum corneum and reducing the scattering of the laser beam¹³¹. The findings in this thesis therefore could be partly attributed to changes in the optical properties of vulval LS caused by differences in morphological features before and after treatment, in addition to a real decrease in the

microcirculation incited by reduced inflammation within the dermis by topical steroids. The morphological features influence the optical pathway and therefore the scatter volume of the LDPI output. A larger scatter volume measures a higher perfusion while a smaller volume, a lower perfusion.

When compared with the normal subjects, women with vulval LS had a higher mean base-line perfusion (not significant) before treatment which could be attributed to the persistent antigen-driven inflammation and thinning of the epidermis⁶⁴, which may result in a real increase in blood flow and an increase the scatter volume of the LDPI output, respectively. After good response to treatment a significantly lower perfusion was recorded by the LDPI in vulval LS compared to the normal vulva. As explained above this is likely to be the result of both morphological changes and an actual decrease in perfusion.

In women with VIN 3 the mean base-line perfusion was higher (not significant) than in normal women. The difference reached significance when symmetrical normal and diseased vulval areas were compared, as was found in vulval cancer by Jackson et al¹⁴⁰. Immunohistochemical studies show a significantly higher MVD with Factor VIII-related antigen (F8-RA) stain and vascular endothelial growth factor (VEGF) expression in VIN 3 compared to lower grade disease and adjacent normal epithelium¹⁴. Previously, pre-invasive disease of the vulva was thought to be an uncommon clinical problem but over the past two decades the number of women referred with the disease has significantly increased, with ~50% of women aged 40 years or younger¹²⁵. The natural history of the disease is however still poorly understood. The recommended treatment is local surgical excision or laser ablation with care to preserve the normal appearance and function of the vulva. Conservative management is only used in special

circumstances such as when the patient declines surgery or if the disease is asymptomatic, but there is no consensus on the multitude of medical therapies available¹²⁵.

The LDPI can be used to study vulval disease and is well tolerated by the patients. The SBF in the diseased vulva is also found to be constant as was the case in the normal vulval skin. The studies presented in this chapter do demonstrate differences in SBF between normal and diseased vulva, but the extent of these differences are not reliable enough for precise disease monitoring or for identification of invasive disease.

In the next chapter the serial changes in vulval skin perfusion is studied during treatment of LS with topical steroids.

CHAPTER 8

Vulval Lichen Sclerosus – Perfusion

Variance to Topical Steroid Therapy

8.1 Introduction

As described in Chapter 3, the management of vulval LS involves potent and moderately potent topical steroids to control the symptoms and probably disease progression^{73;74}. The examination of the vulva is subjective and mainly concerns identifying active disease characterised by evidence of pruritus and excluding malignant disease¹⁹⁶. As vulval LS is an inflammatory disease, changes within the microcirculation may provide another dimension for examination and monitoring of the disease process^{40;41}. In Chapter 7 data demonstrated that good symptom control with topical steroids resulted in a significant reduction in vulval skin perfusion compared to skin perfusion during active disease.

8.2 Aim

In this chapter the aim is to serially measure the changes in vulval skin perfusion in LS during topical steroid therapy using the LDPI. In contrast to Chapter 7 when perfusion was measured as soon as patient achieved good symptom control and while receiving daily moderately potent topical steroid therapy, this chapter follows the changes in perfusion through treatment, until topical therapy had been phased out to maintenance dose. The purpose of this study is to understand the changes in SBF during topical steroid treatment of vulval LS and to examine the potential role of vulval skin perfusion as a parameter to objectively assess the disease process.

8.3 Materials and methods

8.3.1 Study population

The study population consisted of 39 women (57±15 years) with symptomatic vulval LS, recruited from the Vulval Clinic based at the Royal Free Hospital, as described in Chapter 7. The age range was 23–82y, with 26% (n=10) under the age of 50y. Twenty-six women were referred by the family practitioner, seven were in-house referrals and six, tertiary referrals.

Twenty-two patients had histological diagnosis in the past and the remaining patients were clinically diagnosed¹⁹⁶. We avoided biopsy during the study to prevent iatrogenic increase in perfusion. Most of the women had received some form of topical therapy in the past, as described in Chapter 7, section 7.3.1.2. Four patients also had surgery - two had had simple vulvectomy, one laser ablation and one a Fenton's procedure.

We prescribed potent topical steroids (*Dermovate*, clobetasol propionate 0.05%, *Synalar*, fluocinolone acetonide 0.025% or *Tri-Adcortyl*, triamcinolone acetonide) nocté for 8 to 12 weeks followed by maintenance with moderately potent steroids (*Trimovate*, clobetasone butyrate 0.05%)¹⁹⁸. Clobetasol propionate therapy was the first-line potent topical steroid. Two women experienced irritation with clobetasol propionate but responded well to fluocinolone acetonide. Maintenance therapy with clobetasone butyrate was gradually phased-out to twice a week over a period of 12 weeks.

In this study we only included patients who showed good response with resolution of symptoms. Clinical features of disease improved, particularly erosions, purpura, erythema and 'whiteness'. There was no visible change in atrophy. These women were seen and examined for the purpose of this study at referral and serially during treatment.

The frequency of post-treatment examination (LDPI) varied between two and four weeks until twelve weeks after commencing clobetasone butyrate.

For the purpose of data analysis the LDPI measurements for the *same patient* was compared before and after treatment allowing between six and twenty-five pairs to be compared for each duration of treatment.

8.3.2 Statistical Analysis

The perfusion for each region of the vulva and the non-genital skin was expressed as mean \pm 95% CI (confidence interval). The paired data for each treatment group were compared using Wilcoxon's signed-rank's test. Probability values of less than 0.05 were regarded as significant.

8.4 Results

Figure 8.1 a and b show a clinical photograph with corresponding LDPI image of a woman with untreated vulval LS. Fig. 8.1(c-e) shows a series of perfusion images from the same woman (c) four weeks and (d) eight weeks after clobetasol propionate followed by (e) eight weeks after clobetasone butyrate therapy. The series of colour-coded perfusion images show a progressive reduction in vulval skin blood flow.

Table 8.1 shows the patient profile for each treatment pair.

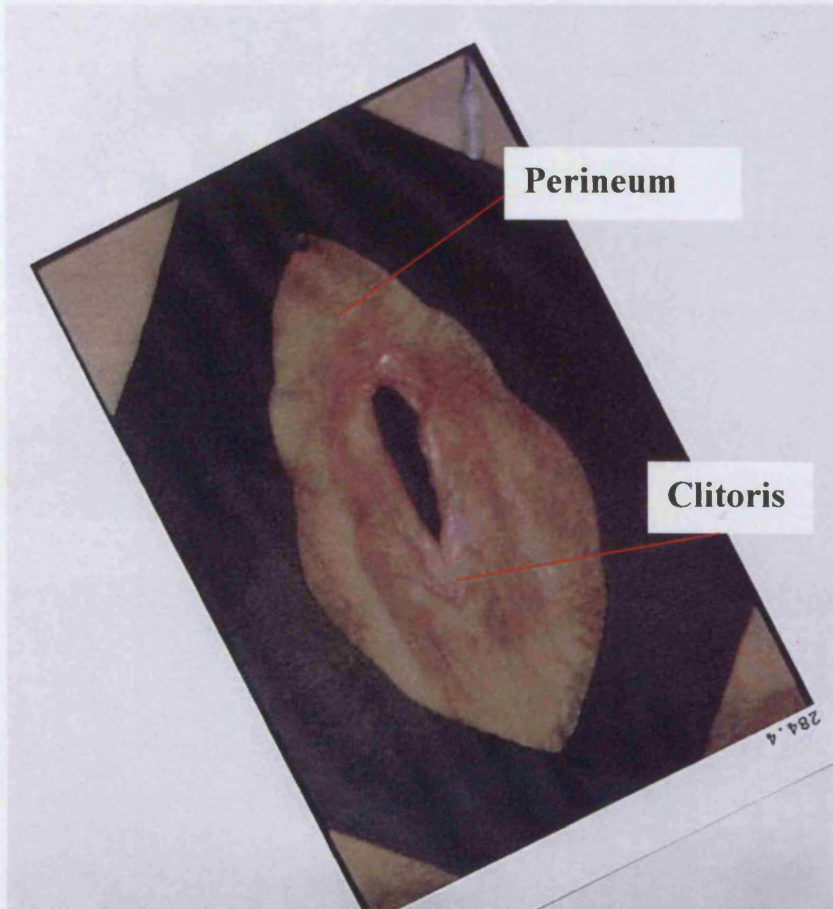


Figure 8.1a Photo of Vulval LS

Figure 8.1a-e

Clinical photograph (8.1a) of vulval LS with corresponding LDPI images on the next page (8.1b) before treatment followed by serial images at four weeks (8.1c) and eight weeks (8.1d) after treatment with potent topical steroids - clobetasol propionate, and eight weeks (8.1e) after treatment with moderately potent topical steroid - clobetasone butyrate.

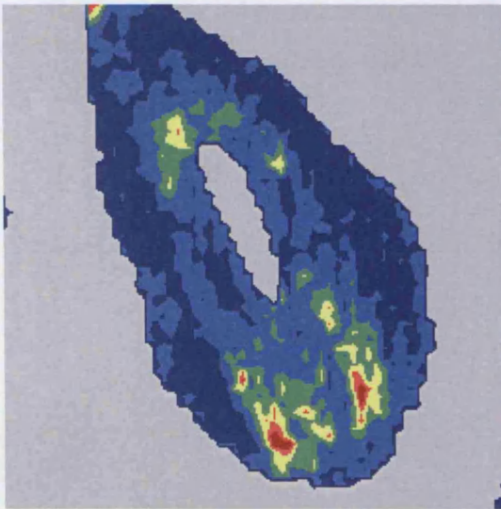


Figure 8.1b
LDPI image for Figure
8.1a before treatment

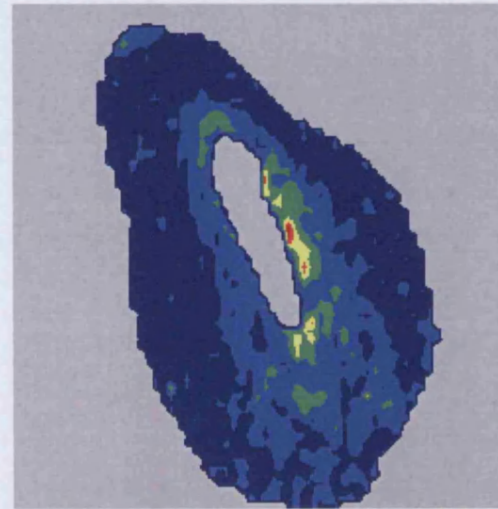


Figure 8.1c
LDPI image for Figure
8.1a after four weeks of
potent topical steroids

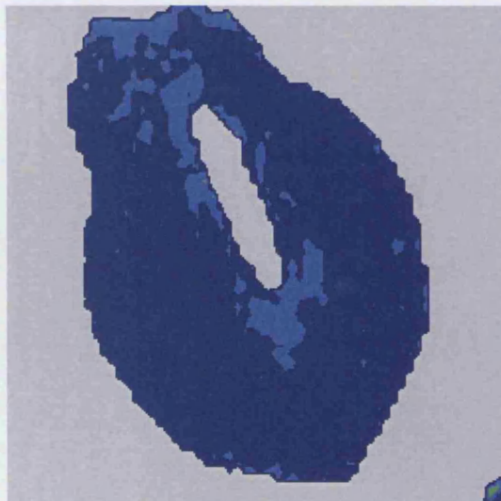


Figure 8.1d
LDPI image for Figure
8.1a after eight weeks of
potent topical steroids

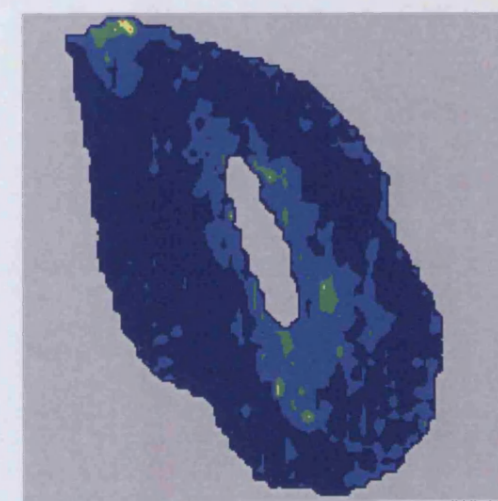


Figure 8.1e
LDPI image for Figure
8.1a after eight weeks of
moderately potent topical
steroids

Table 8.1 The number of patients in each treatment pair, the age and body mass index with observations of temperature, pulse rate and blood pressure

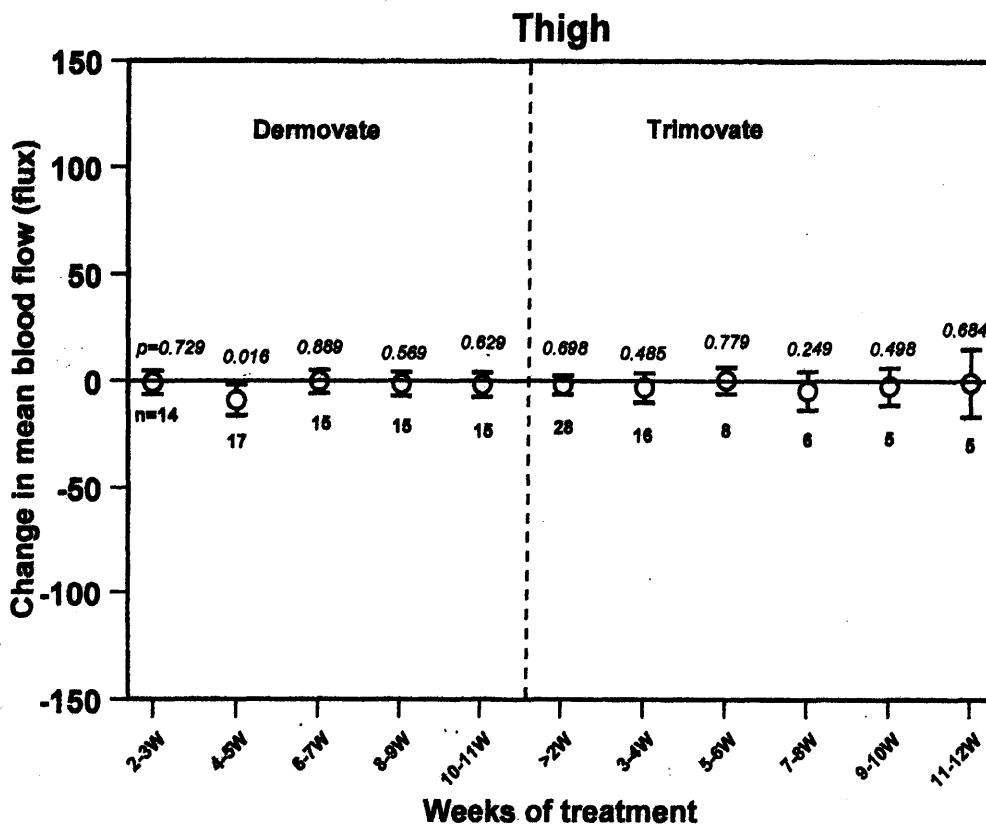
Treatment type	Duration (weeks)	Number of treatment pairs (n)	Age (years)	Body mass index (kg/m ²)	Body temperature (°C)		Skin temperature (°C)		Heart rate (beats per minute)		Systolic Blood Pressure		Diastolic Blood pressure		Mean arterial pressure	
					Pre-treatment	Post-treatment	Pre-treatment	Post-treatment	Pre-treatment	Post-treatment	Pre-treatment	Post-treatment	Pre-treatment	Post-treatment	Pre-treatment	Post-treatment
POTENT TOPICAL STEROID CREAM	2-3 w	23	57.09 ± 14.69	27.99 ± 4.7	36.58 ± 0.28	36.52 ± 0.22	32.28 ± 0.29	31.85 ± 1.07	68 ± 7	67 ± 7	139.52 ± 19.68	143.95 ± 16.95	83.57 ± 12.4	87.77 ± 8.9	102.22 ± 12.79	106.5 ± 10.6
	4-5 w	25	54.79 ± 15.02	27.71 ± 4.83	36.57 ± 0.38	36.52 ± 0.35	32.29 ± 1.26	31.46 ± 1.05	66 ± 7	65 ± 8	138.36 ± 20.57	143.63 ± 22.45	84.14 ± 11.46	86.17 ± 10.6	102.48 ± 13.63	105.32 ± 13.24
	6-7 w	19	56.22 ± 16.39	27.48 ± 4.82	36.54 ± 0.4	36.39 ± 0.33	32.29 ± 1.32	31.31 ± 1.21	68 ± 7	68 ± 7	150 ± 21.63	141.24 ± 28	88.5 ± 9.64	83.65 ± 9.27	109 ± 12.9	102.84 ± 15.1
	8-9 w	19	55.94 ± 14.11	27.85 ± 4.31	36.56 ± 0.31	36.66 ± 0.29	31.95 ± 1.23	31.37 ± 1.13	68 ± 6	66 ± 6	142 ± 20.91	136.47 ± 21.56	84.67 ± 12.42	81.12 ± 11.78	103.8 ± 13.42	99.57 ± 14.14
	10-11 w	18	57.35 ± 15.03	27.86 ± 4.89	36.53 ± 0.3	36.50 ± 0.27	32.15 ± 1.14	30.81 ± 0.69	68 ± 7	69 ± 8	147.8 ± 17.98	138.71 ± 19.85	87.69 ± 11.79	83.88 ± 11.1	107.73 ± 12.11	102.16 ± 12.62
MODERATELY POTENT TOPICAL STEROID CREAM	3-4 w	21	59.75 ± 14.6	27.45 ± 4.49	36.6 ± 0.24	36.52 ± 0.33	32 ± 1.2	31.92 ± 1.04	67 ± 6	67 ± 6	147.85 ± 24.44	138.25 ± 19.95	86.55 ± 10.97	82.55 ± 10.94	106.98 ± 14.87	101.12 ± 13.22
	5-6 w	9	56.75 ± 16.63	30.05 ± 6.01	36.54 ± 0.26	36.43 ± 0.37	31.74 ± 1.73	31.40 ± 1.19	69 ± 7	69 ± 10	144.38 ± 21.95	133.63 ± 18.58	88.13 ± 13.35	82 ± 9.68	106.88 ± 13.52	99.21 ± 11.54
	7-8 w	7	52 ± 12.96	25.15 ± 3.1	36.85 ± 0.19	36.73 ± 0.16	32.93 ± 0.85	32.83 ± 0.96	65 ± 5	66 ± 3	140.33 ± 20.41	136.67 ± 21.6	82.5 ± 7.58	79.17 ± 17.15	101.78 ± 11.17	98.33 ± 18.23
	9-10 w	6	58.8 ± 10.4	28.18 ± 4.36	36.52 ± 0.24	36.54 ± 0.32	31.93 ± 0.79	31.50 ± 0.95	65 ± 5	76 ± 18	153 ± 18.57	149.20 ± 14.18	97 ± 9.08	91 ± 11.4	115.67 ± 10.78	110.4 ± 11.48
	11-12 w	8	59.43 ± 12.46	29.30 ± 5.5	36.55 ± 0.19	36.53 ± 0.31	31.8 ± 0.62	32.72 ± 1.06	70 ± 4	64 ± 5	132.86 ± 17.29	110 ± 25.82	83.57 ± 12.82	73.75 ± 31.98	100 ± 13.84	85.83 ± 28.07

8.4.1 Perfusion changes in the control non-genital skin site (thigh) and the vulva during therapy.

8.4.1.1 Thigh (Figure 8.2)

There was no significant difference in perfusion in the thigh measured before and during the course of treatment, except during *week (W) 4 to 5 of clobetasol propionate therapy ($p=0.016$). During this period* perfusion in the thigh was significantly reduced following topical steroid therapy of the vulva. The reason is unclear but does indicate that data from the vulva during this period* should be interpreted with caution.

Figure 8.2 Graph shows the change in mean base-line thigh (control skin area) skin blood flow with 95% confidence interval, during treatment of vulval LS



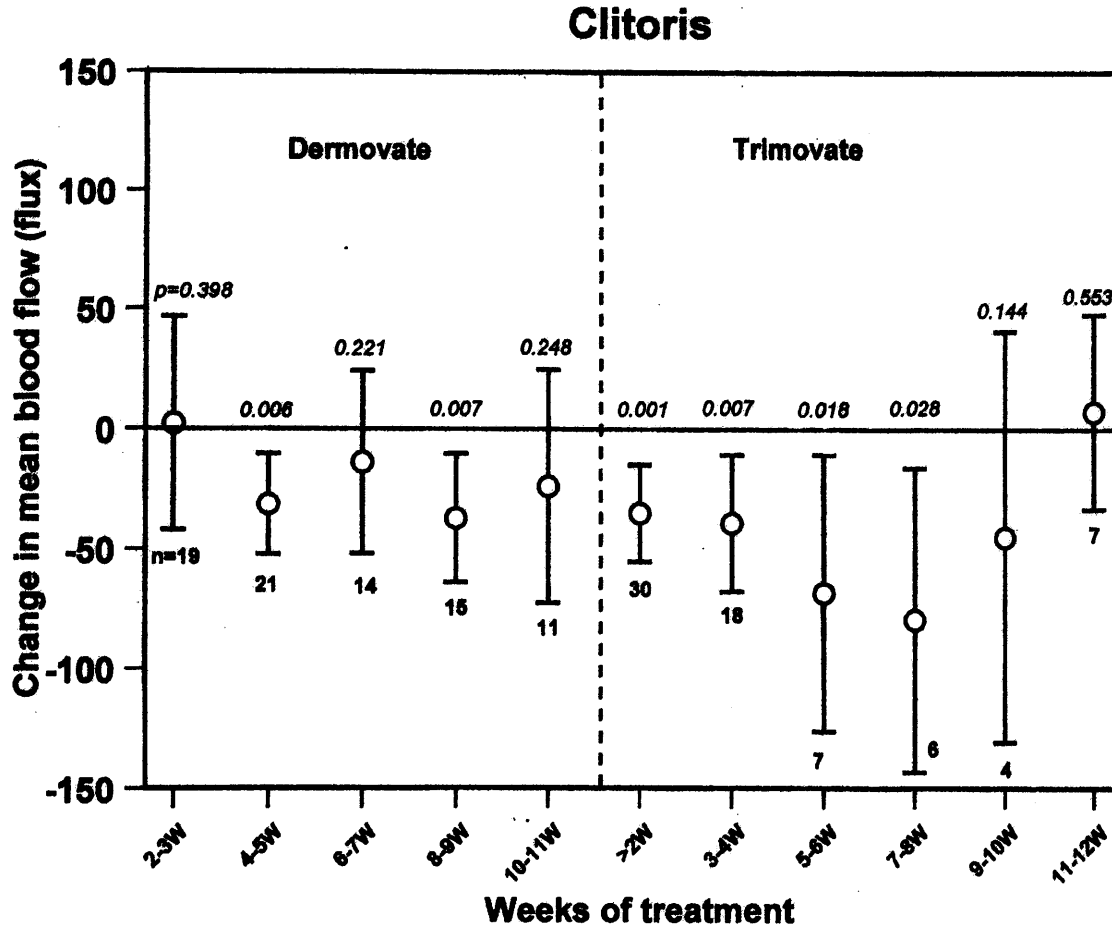
8.4.1.2 Clitoris (Figure 8.3)

During the course of clobetasol propionate therapy there was no change in perfusion 2-3 weeks following therapy. After this period there was a reduction in perfusion, although the levels were significant only during *W 4-5 ($p= 0.006$) and W 8-9 ($p=0.007$).

Clobetasone butyrate therapy showed a significant reduction in base-line blood flow until W7-8. Afterwards the perfusion gradually increased and returned to baseline at W 11-12, probably reflecting the gradual reduction in the effects of topical therapy.

The clitoral area was difficult to define and correlate with the clinical photograph because of its size, location and in some cases phimosis by disease.

Figure 8.3 Graph shows the change in mean base-line clitoral skin blood flow with 95% confidence interval, during treatment of vulval LS

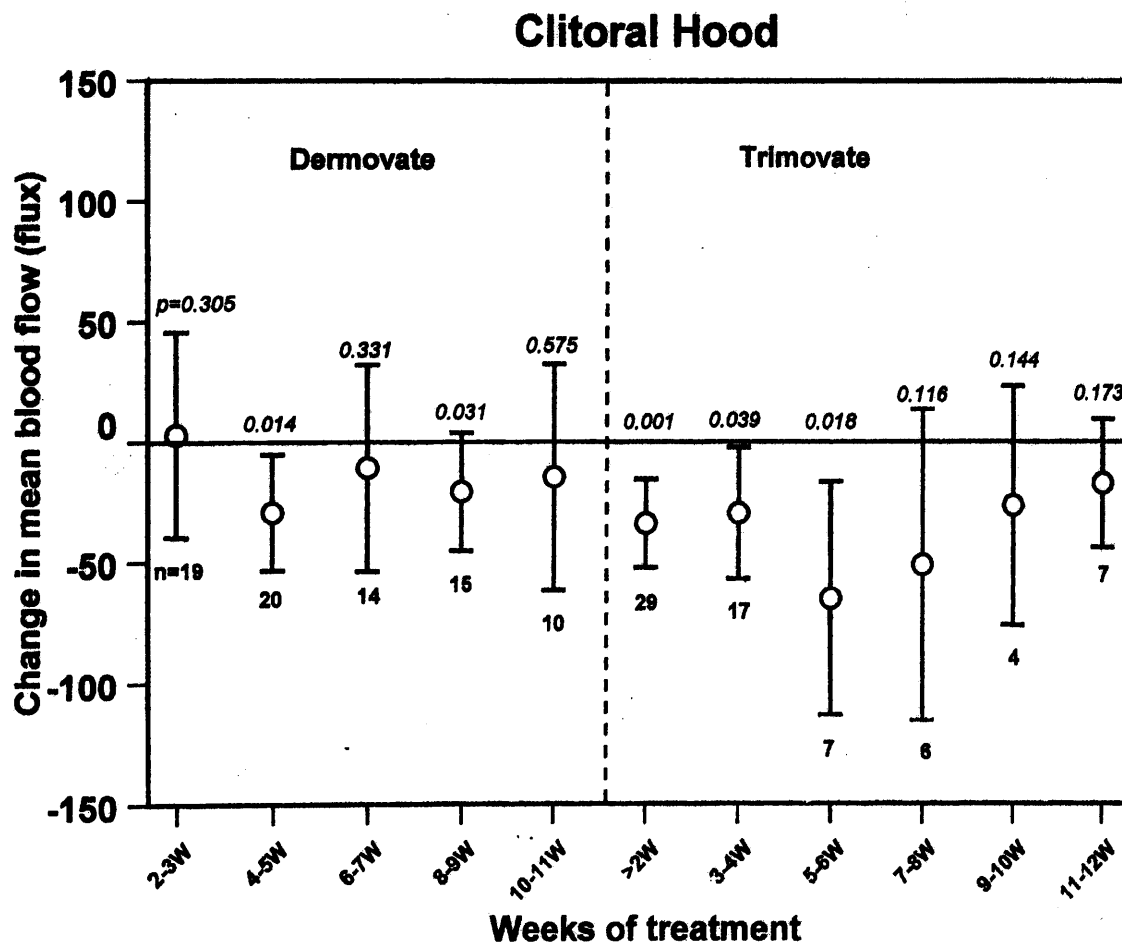


8.4.1.3 Clitoral hood (Figure 8.4)

During clobetasol propionate therapy the perfusion changes were similar to the clitoris, with a reduction in perfusion after W2-3. The levels were significantly reduced during *W 4-5 ($p=0.014$) and W 8-9 ($p=0.031$), as in the clitoris.

Topical therapy with clobetasone butyrate showed a significant reduction in base-line perfusion until W 5-6, after which there was a gradual rise to the pre-treatment base-line perfusion, as in the clitoris.

Figure 8.4 Graph shows the change in mean base-line clitoral hood skin blood flow with 95% confidence interval, during treatment of vulval LS



8.4.1.4 *Labia Minora (Figure 8.5 and 8.6)*

During clobetasol propionate therapy the right labium minus (Figure 8.6) showed a similar pattern of perfusion change to the clitoris and clitoral hood, with a reduction in perfusion after W 4-5*. The left labium minus did not show any change in perfusion until W 8-9 (Figure 8.5).

Therapy with clobetasone butyrate altered the base-line perfusion in a similar pattern for both the right and left labia minora. There was an initial reduction in perfusion until W 5-6 followed by a progressive rise to the pre-treatment base line by W11-12.

Figure 8.5 Graph shows the change in mean base-line right labium minus skin blood flow with 95% confidence interval, during treatment of vulval LS

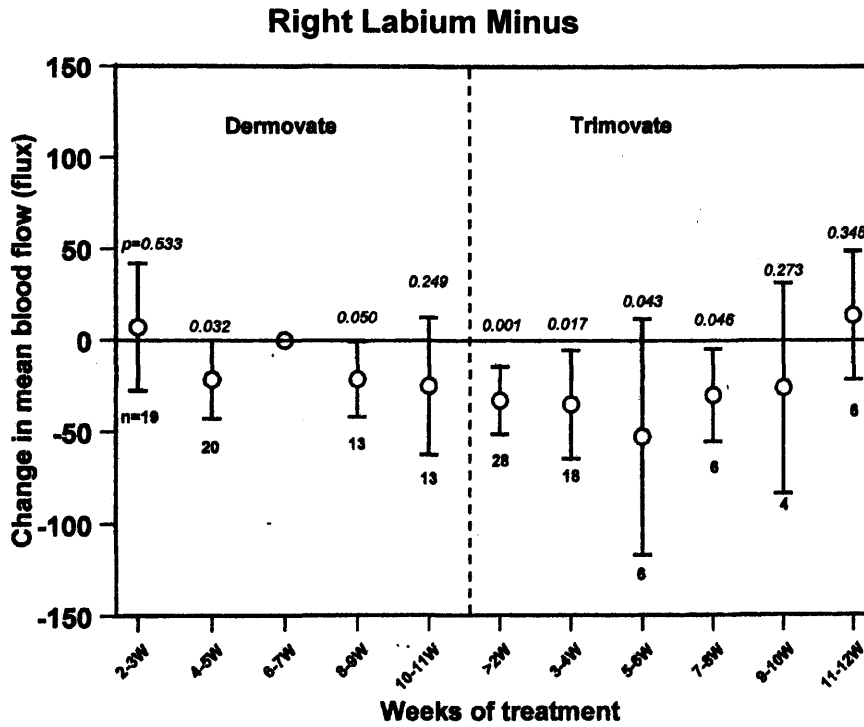
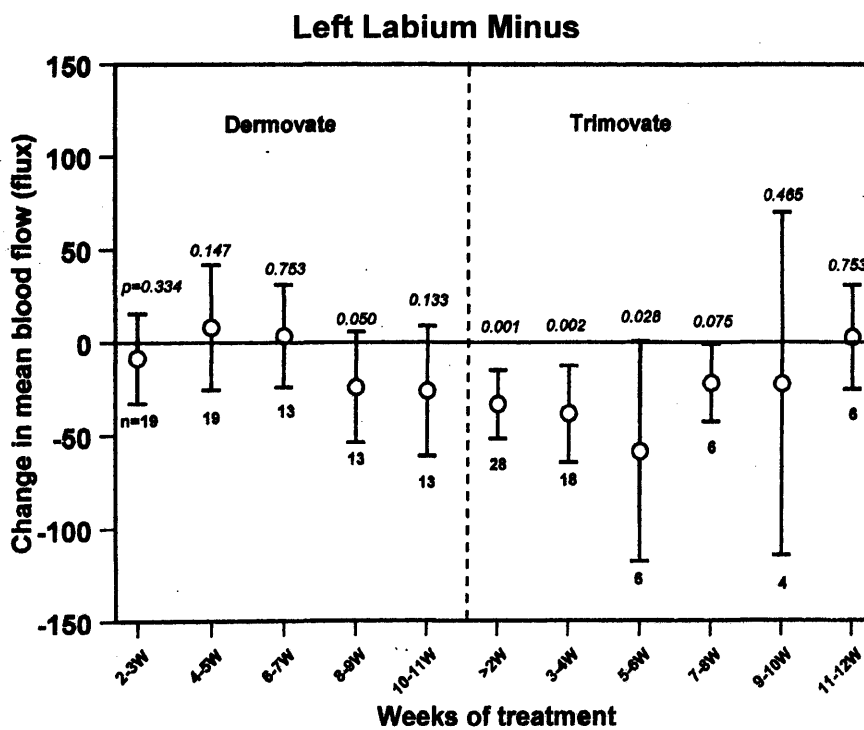


Figure 8.6 Graph shows the change in mean base-line left labium minus skin blood flow with 95% confidence interval, during treatment of vulval LS



8.4.1.5 *Inter-labial space (Figure 8.7 and 8.8)*

Therapy with clobetasol propionate showed some reduction in the base-line perfusion in the right inter-labial space at about *W 4-5. There was no change in perfusion in the left inter-labial space.

Therapy with clobetasone butyrate produced a similar pattern in perfusion change in the right and left inter-labial space. There was initially a significant reduction in base-line perfusion until W 3-4, followed by a return to baseline perfusion on W11-12.

Figure 8.7 Graph shows the change in mean base-line right inter-labial space skin blood flow with 95% confidence interval, during treatment of vulval LS

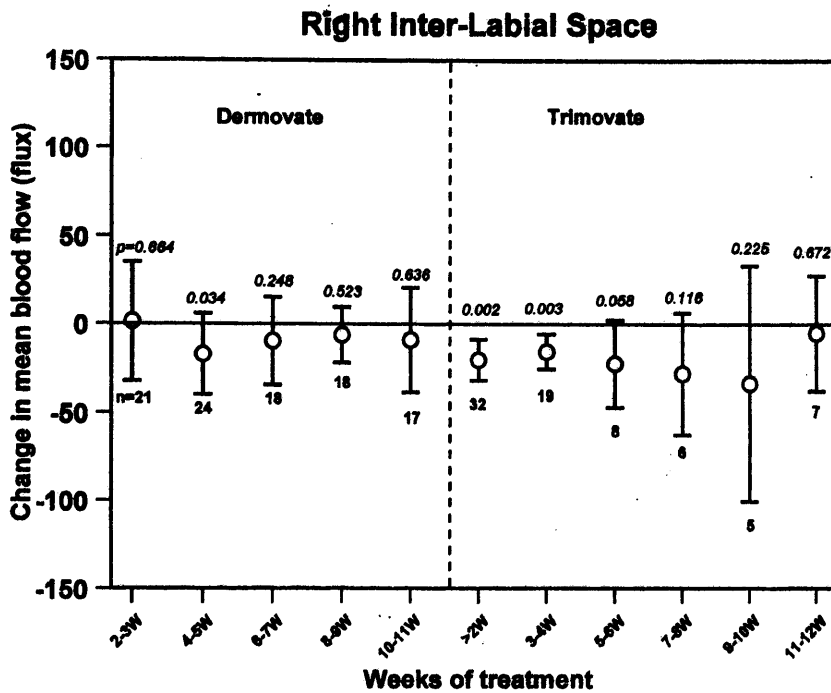
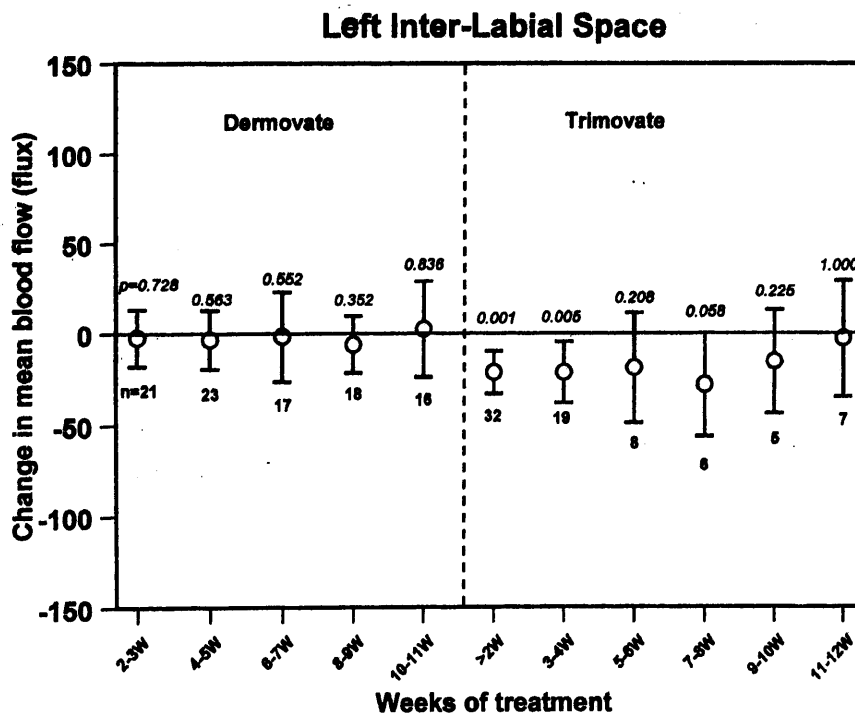


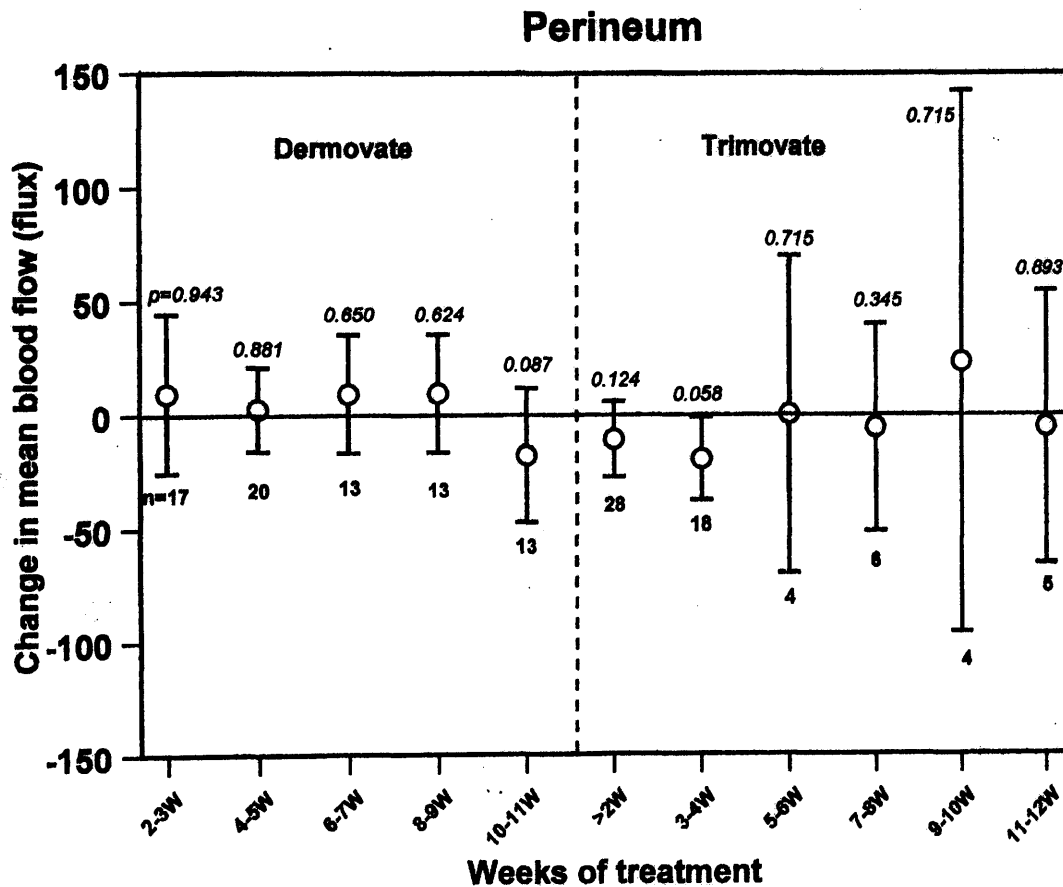
Figure 8.8 Graph shows the change in mean base-line left inter-labial space skin blood flow with 95% confidence interval, during treatment of vulval LS



8.4.1.6 *Perineum (Figure 8.9)*

Therapy with clobetasol propionate and clobetasone butyrate did not appear to produce any significant perfusion change in the perineal skin. However there is a trend towards a reduction approaching the end of the potent topical steroid phase which continued into the moderately potent steroid phase before return to base line with gradual phasing down of frequency of topical therapy.

Figure 8.9 Graph shows the change in mean base-line perineal skin blood flow with 95% confidence interval, during treatment of vulval LS



8.4.1.7 *Summary of results*

Potent topical steroids (clobetasol propionate 0.05%): The change in vulval skin perfusion during therapy with potent topical steroids is less predictable compared to moderately potent steroids. There is no change in perfusion for two to three weeks after commencing therapy with potent topical steroids. Afterwards there is a tendency towards a reduction in vulval skin perfusion.

Moderately potent topical steroids (clobetasone butyrate 0.05%): There is a significant decrease in perfusion for three to four weeks after commencing therapy in all the vulval areas, which continued until about W7-8. The base-line perfusion then returned to pre-treatment levels, coinciding with reduction in frequency of treatment.

8.5 **Discussion**

Topical steroids are now accepted as first line treatment of vulval LS³¹⁴. The treatment regime for vulval LS in this thesis is based on the efficacy of potent topical steroids shown by Bracco et al³⁹, Dalziel et al⁷³ and others^{34;67;189}. These workers reported significant improvement of symptoms of pruritus and soreness with a short course of potent steroids. Clinically there is reported reduction in erosions, hyperkeratosis, purpura and thickness of plaques, while histologically, hyperkeratosis, epidermal basal cell liquefaction, intensity of inflammatory infiltrate and hyalinisation of dermal collagen improved. Although potent topical steroids did not worsen epidermal atrophy, the improvement of this feature histologically in vulval LS is not as clear as the other features mentioned above. Subsequent long-term control of symptoms and disease activity is better managed with a small amount of moderately potent topical steroids⁷⁴.

The cause of vulval LS remains elusive but tissue metabolism is not decreased despite the severe atrophic appearance macro- and microscopically. Metabolic studies in the mid to late 1960's demonstrated an increase in glucose metabolism²⁵⁷, alkaline phosphatase levels and adenosine triphosphatase activity²¹⁸, while there was no decrease in radioactive phosphorus uptake¹⁶⁴ or nucleic acid activity¹⁰⁰. In the last decade the availability of immunohistochemical techniques has shown significantly increased expression of p53, a guardian genome protecting against DNA damage by regulating cell growth, in vulval LS compared to normal vulva and non-genital LS³¹². This finding suggests abnormalities in the regulation of epidermal proliferation, the nature of which is still being debated^{292;312}. The skin immune system is also altered with activation of the antigen presenting cells within the epidermis, perpetuating a continuous inflammatory response causing sclerosis within the dermis^{62;64;93}.

Clobetasol propionate is a highly effective synthetic fluorinated corticosteroid with a high degree of glucocorticoid activity, but minimal mineralocorticoid activity. There is generally a reluctance among clinicians to use potent topical steroids because of concern for the antiproliferative effects in a skin condition already characterised by epidermal atrophy¹¹⁸. In addition to this, there is evidence of high penetrance of topical hydrocortisone in the normal vulva compared to the forearm skin⁴⁹. Histological studies of vulval LS before and after treatment with potent topical steroids have not qualified these concerns^{39;73}. Other side effects such as sensitivity and candidal infection have not been a problem in this thesis or in the studies by Dalziel et al⁷³. Potent topical steroids are believed to reduce the number and activity of inflammatory infiltrates, in addition to preventing phagocytic cell mobilisation to the reaction site by inducing vasoconstriction¹⁰³. It is believed that the anti-inflammatory, antipruritic and vasoconstrictive properties of the drug are achieved through inhibition of phospholipase

A and interference with the arachidonic acid cascade. Topical steroids also block histamine release following antigenic stimulation and thus reducing their effect on peripheral target cells and tissues. These are valuable properties for treating vulval LS, which is characterised by inflammation⁶⁴.

In this thesis it is reasoned that if the base-line tissue metabolism is raised in untreated vulval LS, with inflammation being a part of the process, then therapy using topical steroids, by affecting a reduction in this inflammatory response also reduce the tissue metabolic rate which could translate into changes in tissue blood flow amenable to measurement by the LDPI. The laser Doppler technique is not sensitive to the iatrogenic blanching of the skin by topical steroids, as discussed previously in Chapter 7^{131;229}.

The inconsistent reduction in vulval skin blood flow during potent topical steroid therapy is attributed to variations in disease activity within our group of women at the start of treatment. This is followed by differences in response to therapy causing different degrees of alteration in tissue morphology, which affect the scattering properties of the incident laser beam. During the second phase of therapy with moderately potent steroids the findings were more consistent. This is attributed to the more homogenous clinically quiescent nature of disease sequel within the group of women at the start of this stage of treatment. Subsequent treatment with gradually phased-out moderately potent steroids produced skin perfusion changes corresponding to the frequency of therapy. It is of interest that the base-line blood flow returned to pre-treatment levels as the frequency of medication was reduced in spite of good symptom control, suggesting no permanent change in SBF in these cases with treated vulval LS.

The site of skin studied and the nature of the disease limit this study. The vulva has an irregular surface contour unique to each woman and the location is such that exposure depends on the degree of abduction in the lithotomy position. The nature of vulval LS is such that the disease causes varying degrees of resorption and fusion of the labia minora, and phimosis of the clitoris altering the surface contour of the vulva.

Histologically there is variation in the degree of hyperkeratosis, depth of the epidermis, extent of hyalinisation of the superficial dermis and intensity of the inflammatory infiltrate in the dermis. All these macroscopic and microscopic morphological differences influence the scattering properties of the incident laser beam of the LDPI. One of the major limitations of the LASER Doppler technique is the inability to determine the depth of tissue sampled³³⁶, which will vary with the microscopic morphology. Repeated tissue biopsy for histological studies during the course of treatment was avoided, as this would have interfered with the blood flow measurements by iatrogenically increasing the perfusion in the region of the biopsy.

In conclusion topical steroids decrease base-line vulval skin perfusion in LS while controlling the symptomatic and clinical sequel of the disease process. The data also show that reduction in the dose of therapy causes return of base-line skin perfusion to pre-treatment levels although the disease continues to remain quiescent. This is in concurrent with the clinical impression that topical steroids do not cure vulval LS but temporarily control the disease process by pruritic symptom control. This study shows that there is no overall permanent change in the skin perfusion parameter with topical steroid therapy relieving and controlling pruritic symptoms.

In the next chapter, Chapter 9, this investigation continues by studying the response of SBF in diseased vulval skin during recovery form local skin temperature challenge compared to findings in that of the normal vulval skin described in Chapter 6.

CHAPTER 9

Diseased vulval skin – perfusion

variance to local skin temperature

challenge

9.1 Introduction

In Chapter 6 the normal vulva did not show any significant difference in vulval skin perfusion from the baseline during recovery from local skin temperature provocation with the exception of the left labium minus and the clitoris. Further the 95% confidence interval in Figure 6.5 was widely distributed.

9.2 Aim

In this chapter the ability of vulval perfusion to recover from local skin temperature provocation in the diseased vulva, which include LS before and after treatment, and VIN 3 are investigated. The purpose of the study is to identify the presence and nature of any differences in skin perfusion response in these diseases compared to the findings in the normal vulval skin in Chapter 6.

9.3 Materials and methods

9.3.1 Study population

The study population consisted of 30 women with LS and 6 women with VIN 3 recruited from the Vulval Clinic at the Royal Free Hospital.

All women with LS were examined before treatment. Ten women were examined after completion of a course of potent topical steroids (PTS). The preparation used was clobetasol propionate 0.05% [Dermovate] while fluocinolone acetonide 0.025% [Synalar] and triamcinolone acetonide [Tri-Adcortyl] were reserved for patients with adverse reaction to clobetasol propionate 0.05% (one case). Twenty women were

examined after their course of moderately potent topical steroids (MPTS). The preparation used was clobetasone butyrate 0.05% or Trimovate.

Women with VIN 3 were examined only before treatment.

9.3.1.1 Clinical profile of women with LS

The referrals were received from the family practitioner (n=22), tertiary consultants (n=5) and in-house consultants (n=3). The mean age of the ten women examined after PTS was 57.1 ± 10.5 years and mean body mass index (BMI) of 27.1 ± 4.9 . In the twenty women examined after MPTS the mean age was 56.75 ± 15.8 years and mean BMI of 26.83 ± 4 . 73% (n=22) of women were postmenopausal. All the women had received various forms of topical treatment in the past, as described in Chapter 7, Section 7.3.1.2. Three women had also received surgery: - simple vulvectomy, laser ablation and a Fenton's procedure. 21 women had a histological diagnosis, while the other women were clinically diagnosed. Patients requiring tissue biopsy were excluded in this study because of iatrogenic increase in perfusion in the region of the biopsy. In the medical history there was a predominance of women on antihypertensive (n=4), thyroxin (n=2) and hypoglycaemic (n=5) therapy.

PTS noctè was prescribed for 8 to 12 weeks followed by maintenance with MPTS¹⁹⁸. Maintenance therapy with MPTS was gradually phased down to twice per week over a period of eight to twelve weeks. Only patients who showed good response with resolution of symptoms were included in the study. Clinical features of disease improved, particularly erosions, purpura, erythema and whiteness. There was no visible change in atrophy.

The women had not received any topical treatment for at least three weeks before the first pre-treatment LDPI examination. LDPI examination with temperature challenge as described in Section 6.3.6 was performed before commencing treatment, after completing the course of PTS (n=10) and MPTS (n=20). For the purpose of data analysis the LDPI measurements from the same patient was compared before and after treatment.

9.3.1.2 Clinical profile of women with VIN 3

The mean age of the women with VIN 3 was 42 ± 12.6 years and mean BMI of 22.65 ± 3.34 . They were all tertiary consultant referrals. The symptom profile for these women are as described in Chapter 7, Section 7.3.1.3. All cases had histological confirmation of VIN 3. Medical history in these women consisted of previous treatment for VIN 3 (n=5) and CIN (n=3). These women had multifocal VIN 3 mainly affecting the labia minora and interlabial sulci. These women were examined at referral, before excisional biopsy.

The laser Doppler perfusion imager (Section 5.3), measurement procedure (Section 6.3.2.) and colour-coding and image analysis (Section 6.3.7) have been described elsewhere.

9.3.2 Statistical analysis

The perfusion for each region of the vulva was expressed as mean \pm SD. The paired data for each treatment group were compared using Wilcoxon's signed-rank's test. Probability values of less than 0.05 were regarded as significant.

9.4 Results

The data is presented in Figures 9.1 to 9.5, showing the mean with 95% confidence interval of the variation in vulval skin perfusion from the baseline with hot (above the baseline) and cold (below the baseline) provocation.

9.4.1 Vulval LS – PTS

Figure 9.1 and 9.2 compares vulval skin perfusion in LS of paired samples from 10 women before and after good response to PTS. The differences in subject numbers (n) between Figures 9.1 and 9.2 are due to differences in image quality.

Hot provocation before treatment produced an increase in vulval skin perfusion with significant change in the right labium minus, left inter-labial space and the perineum. The 95% confidence interval was similar to that in the normal group described in Chapter 6, Section 6.4.4 and Figure 6.5, when the changes in the scale of the y-axis is taken into consideration. Post-treatment patients appear to have a more significant increase in blood flow to hot provocation compared to before treatment.

Cold provocation shows a narrower 95% confidence interval compared to hot provocation. The cold perfusion measurements appear more significantly reduced before treatment compared to after treatment.

Figure 9.1 and 9.2 Graphs show the change in mean baseline perfusion with 95% confidence interval to local hot and cold provocation in vulval LS before (Figure 9.1) and after (Figure 9.2) the first phase of treatment with potent topical steroids – Clobetasol Propionate or Dermovate

Figure 9.1

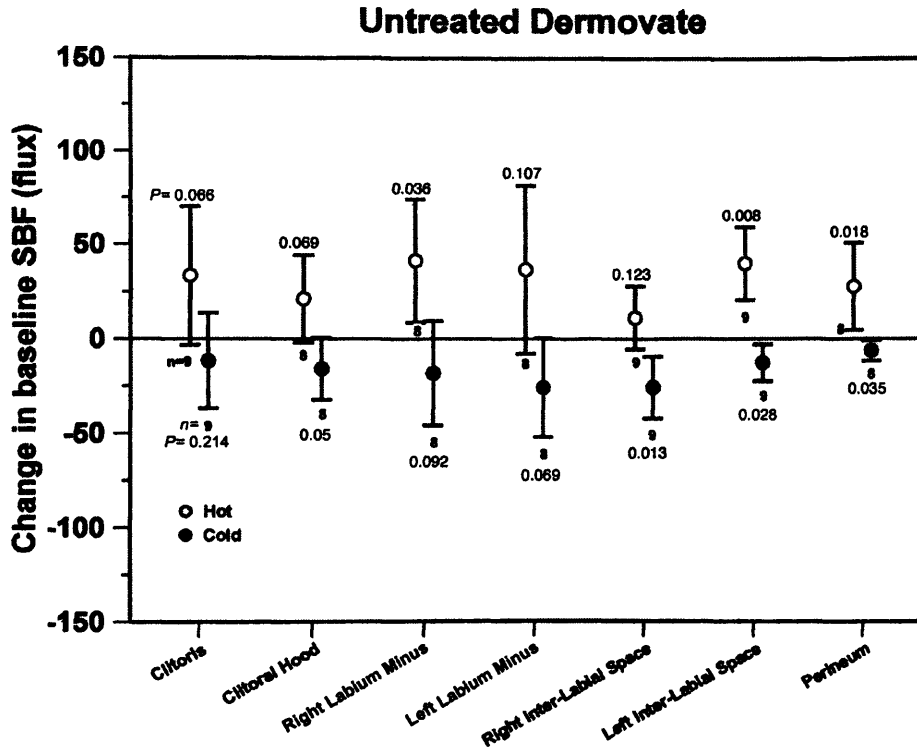
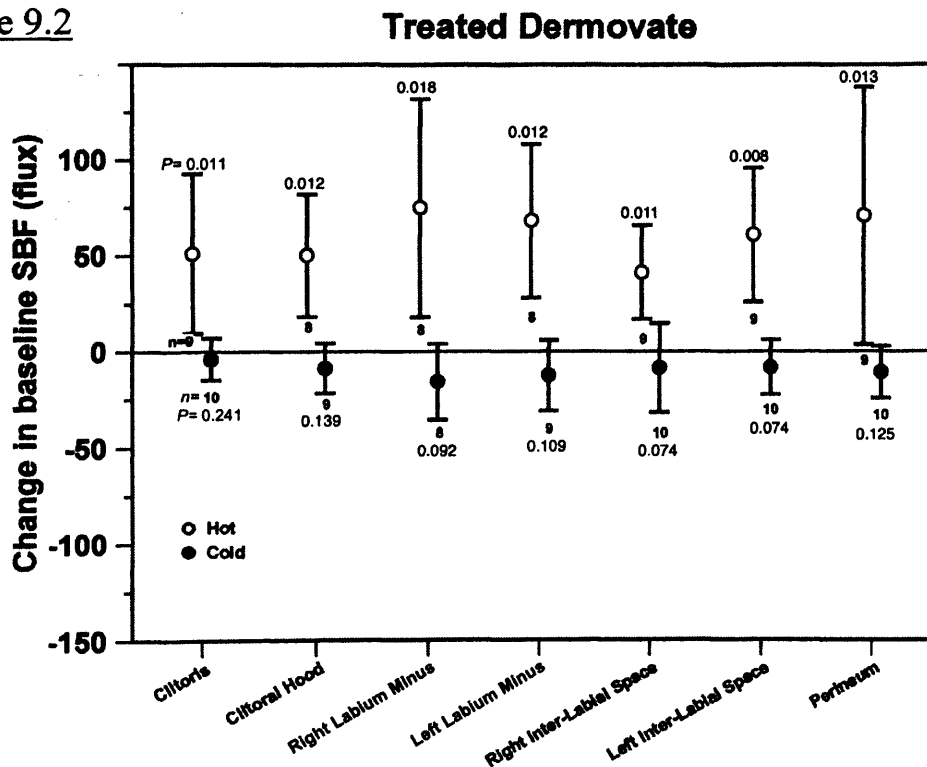


Figure 9.2



9.4.2 Vulval LS – MPTS

Figure 9.3 and 9.4 compare vulva skin perfusion in LS of paired samples from 20 women before and after their course of MPTS. The differences in subject numbers (n) between Figures 9.3 and 9.4 are due to differences in image quality.

Hot provocation resulted in a significant increase in perfusion with narrow 95% confidence intervals in both before and after treatment with MPTS. During cold challenge there is a significant decrease in perfusion with an even smaller 95% confidence interval, compared to the response with PTS.

There does not appear to be a difference in response to temperature provocation in vulval LS before and after good response to topical therapy.

Figure 9.3 and 9.4 show the change in mean baseline perfusion with 95% confidence interval with local hot and cold provocation in vulval LS before (Figure 9.3) and after (Figure 9.4) the second phase of treatment with moderately potent topical steroids – Clobetasone butyrate or Trimovate

Figure 9.3

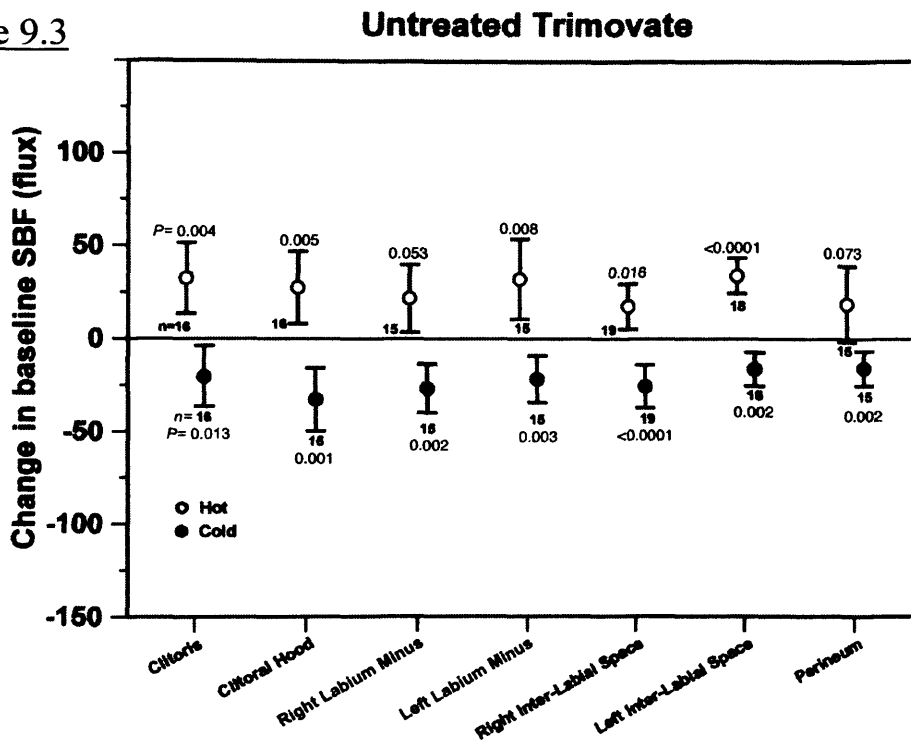
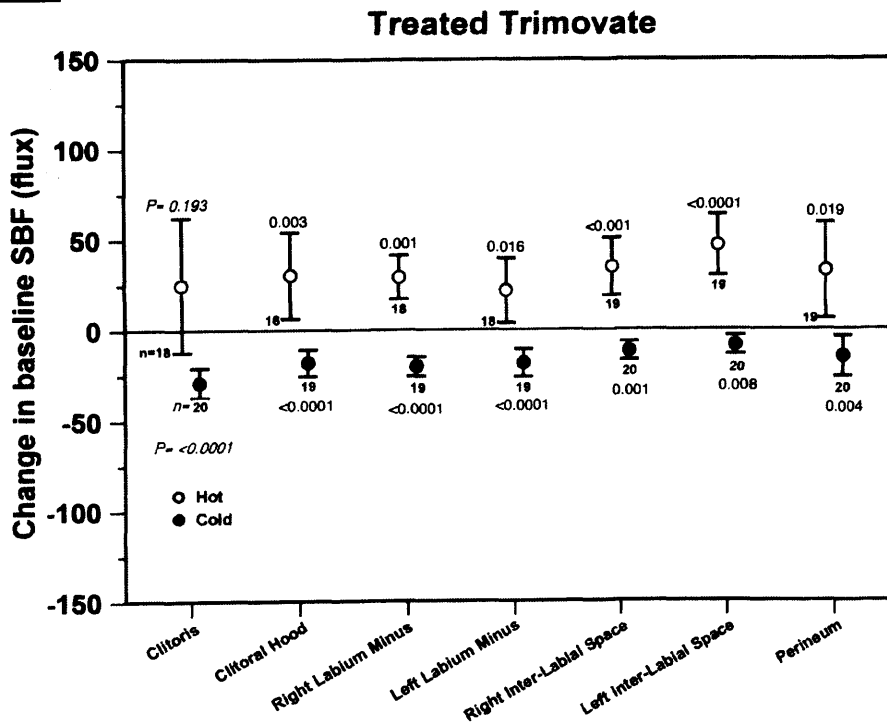


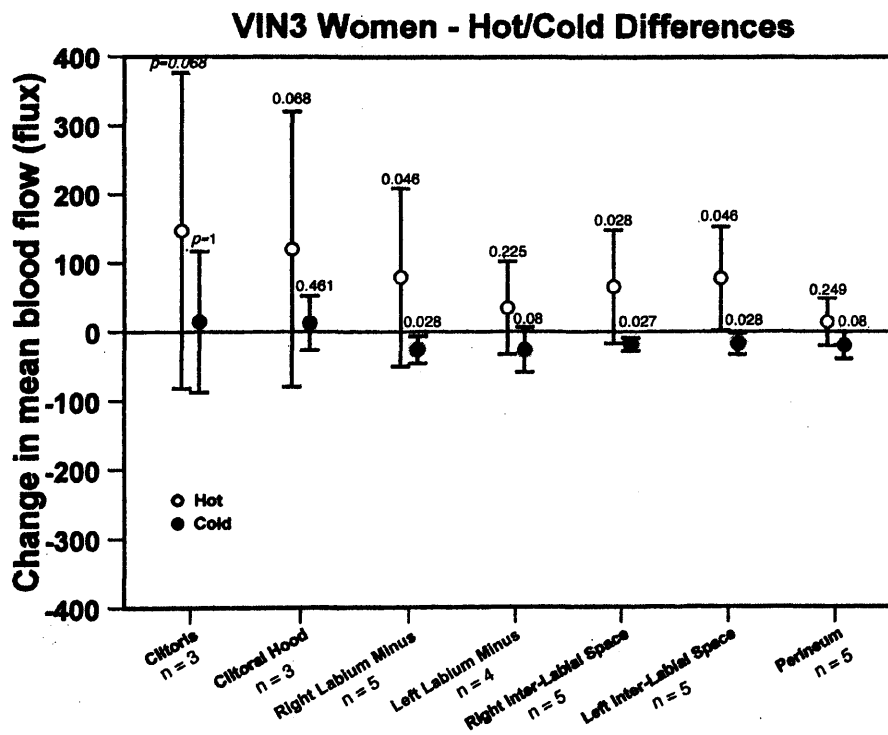
Figure 9.4



9.4.3 VIN 3

Figure 9.3 shows vulva skin perfusion in six women with VIN 3. There is a more significant change with cold provocation compared to hot provocation of the right labium minus and interlabial sulci. Although the number of women studied was small the 95% confidence interval was narrow, in particular with cold provocation.

Figure 9.5 Graph shows the change in mean baseline perfusion with 95% confidence interval with local hot and cold provocation in VIN 3 before treatment



9.5 Discussion

This chapter describes the recovery of vulval skin blood flow changes during local hot and cold temperature challenge of ~ 4 to 6 °C from baseline vulval skin temperature.

Women with LS showed a slower recovery in mean baseline perfusion compared to normal women, before and after clinical response to treatment. In women with VIN 3 there was a mixed response probably reflecting the patchy distribution of disease.

This study varies from other temperature studies in that the skin blood flow change was measured over an area of interest and only during recovery from temperature challenge, rather than during the challenge^{33;293;294;324}. The period of warming and cooling the vulval skin was also short (~3 minutes), compared to the 20 to 60 minute period used in other studies, which was not feasible, when in the lithotomy position. These other studies have also applied the LDF, which measures perfusion continuously over a single point (1mm²) during the period of local temperature change followed by recovery to baseline. The LDPI measures perfusion only once at each point (2 mm²) while scanning the area of interest over a period of ~ 2.5 minutes, so disregarding any rapid changes in perfusion. The perfusion perspective of the LDPI is therefore different from the LDF.

Increasing the local skin temperature increases skin blood flow but the degree of response depends on the final temperature of the skin surface. Song et al²⁹³ described an increase in skin perfusion varying between 2-fold at 35°C to almost 15-fold at 43°C by continuous measurement at a single point using the LDF on forearm skin. These workers also reported an early decline in blood flow in spite of maintaining a raised local skin temperature when the temperature rise was less than 43°C. This 'die away' phenomenon remains unexplained. During recovery of baseline temperature after warming, the skin perfusion decreased with decline in temperature until 32°C, at which

point there was a brief temporal rise in blood flow followed by further decline to baseline perfusion²⁹⁴.

The increase in skin blood flow during warming is attributed to dilatation of arterioles and recruitment of capillaries. The skin vasculature and the presence of special structures such as AVA varies at different body sites²⁹⁴. Although the vulva is functionally not a site for temperature regulation, there is potential for large changes in blood flow involving cavernous tissue within the labia minora, vestibule and clitoris.

Song et al²⁹⁴ also reported an increase in the amplitude of vasomotion in the warmed skin with a gradual return to baseline after the warming period attributed to contraction and relaxation of smooth muscles of the arteriolar vessel walls. In this thesis, study of the normal vulva, during the first few minutes after warming, an increase in amplitude of vasomotion may be contributing to the wide variation in measured perfusion. The normal vulva appears to be able to stabilise skin blood flow during small changes in local skin temperature in this study. In vulval LS reduced variation in perfusion could be attributed to impaired vasomotion as a result of disease affecting the walls of the arteriolar vessels. In VIN 3 the response was less marked compared to vulval LS probably reflecting the patchy distribution of disease and the small subject numbers. Abnormal skin perfusion sensitivity to warming in VIN 3 could also be attributed to abnormal vascular geometry and/or blood rheology in diseased areas with high baseline perfusion^{242;251}. Rheologic changes in disease include impaired red blood cell deformability, increased leukocyte aggregation and endothelial adherence¹⁰. These changes in rheological properties may contribute to abnormal perfusion by compromising the effective blood vessel cross-sectional area and red cell velocity, which would be relevant to the laser Doppler technique as it is flow-dependant.

Van den Brande et al³²⁴ continuously measured perfusion changes with the LDF during cold provocation on the normal skin of the calf in the lower limbs. During cooling, the workers reported an abrupt drop in skin blood flow followed by a rapid recovery ('cold vasodilatation') in spite of continued cooling. Vasomotion also followed a similar pattern with complete absence during the initial phase suggesting a reflex active vasoconstriction followed by a gradual increase in vasomotion with appearance of 'cold vasodilatation'. When cooling was stopped there was a hyperaemic response with more than doubling of perfusion above baseline during the initial period of recovery, before descent to the baseline. Van den Brande et al³²⁴ also reported an almost doubling of the vasomotion from baseline levels during this initial period of recovery when cooling was stopped.

In the study described in Chapter 6, section 6.4.4, Figure 6.5 the normal vulva recovered rapidly with a similar cooling period and with a similar temperature change compared to the findings described in this chapter. Increased vasomotion during the recovery phase of the normal vulva may be contributing to the wide variation in skin blood flow. In vulval LS, skin blood flow remained significantly reduced after the cooling period with marked narrowing of the variation (95% confidence interval) in perfusion. Abnormal cold reactivity in vulval LS could be attributed to the persistent absence of vasomotion and/or the development of abnormalities in blood rheology, such as deformity of the blood cells and stasis^{10;242}, affecting blood flow. In VIN 3 a similar response to vulval LS was noted after cold provocation. VIN 3 has a significantly higher baseline skin blood flow as measured by the LDPI (Chapter 7) and by immunohistochemistry with factor VIII related-antigen vascular stain¹⁴. In VIN 3 abnormal vasomotion activity,

vascular geometry and blood rheology, as in vulval LS, may also be involved in abnormal cold reactivity²⁴².

The role of local and central factors in the response of skin blood flow to local temperature change is controversial. It is generally believed that a local axon reflex from cutaneous receptors mediates changes in capillary blood flow to local temperature change²⁹³. There is also an efferent neural control of skin blood flow through sympathetic neural pathways, which have a vasoconstrictor and possibly a vasodilator system²⁴¹. Local metabolic and humoral factors may also affect the response of flow through microvessels in recovery from temperature provocation²⁹³. This study does not allow identification of the factors altering vulval skin vascular reactivity in disease and, the differences between these factors in vulval LS and VIN 3.

The comparison of the results in this study with previously published material is difficult, because to the best of current knowledge, there are no other studies measuring skin microvessel reactivity with the LDPI after temperature provocation in the vulva. Study of vulval skin perfusion has been greatly facilitated by the availability of the LDPI but is still restricted by the location and poor understanding of the morphology of the vulva, in particular of the vascular geometry. Improvements in techniques to change local vulval skin temperature over short periods and better facilities for image analysis will allow easier application of the LDPI to the vulva.

This study shows that in vulva LS before and after treatment, and in VIN 3 skin perfusion response during recovery from local skin temperature change is different from that of the normal vulva. This could not be simply attributed to differences in the subject numbers (*n*) because the subject numbers in the normal group (*n*=10) although

smaller than in the group with vulval LS –MPTS (n=20), was similar to vulval LS-PTS (n=10) and VIN 3 (n=6). The abnormal skin perfusion response in the presence of vulval disease could be attributed to abnormal vessel reactivity, which may be part of the disease pathogenesis. The persistence of the abnormal skin perfusion response after treatment of vulval LS suggests that disease pathogenesis is not altered by the treatment providing symptom relief and control.

In the next chapter the morphological differences in microvessel density (MVD) using immunohistochemical techniques in normal and diseased vulva are compared with LDPI measurements.

CHAPTER 10

Normal and diseased vulva –
immunohistochemical microvessel
density analysis

10.1 Introduction

The microvasculature can be quantified morphologically by MVD (microvessel density) using immunohistochemical-staining techniques on tissue biopsy samples to stain and locate these vessels. The vast majority of the studies on MVD have been in relation to the development and prediction of prognosis of pre-malignant and malignant disease, including in the vulva as discussed in Chapter 1. It is clear from the discussion in Chapter 3 that vulval LS (Table 3.2) and VIN 3 (Table 3.5) have a definite but poorly defined association with the development of malignant disease, thus requiring long term follow-up with a frequency to contain clinical work and a quality of reliability to detect early changes in disease process leading to malignancy which are more amenable to conservative treatment and preservation of vulval structure. The preliminary work presented in this thesis raises the possibility of further developing the LDPI as a new non-invasive and painless biophysical method to measure blood flow changes within the superficial skin microvasculature to improve the scope for objective assessment of these vulval skin diseases.

10.2 Aim

In this chapter the MVD in the normal vulva, vulval LS, VIN 3 and vulval cancer was studied, using anti-von Willebrand factor (vWF) or (Factor 8 Related antigen) F8-RA immunostain to compare the MVD in LS and VIN 3 with that of the normal vulva and vulval SCC. The morphological findings in LS and VIN 3 were then correlated with the biophysical findings in Chapter 7 to understand the LDPI output.

10.3 Materials and Methods

10.3.1 Archival tissue

Tissue specimens from cases with vulval LS, VIN 3, SCC and normal epithelium were obtained from the files of the Department of Histopathology at the Royal Free Hospital for the years 1990 to 1996. The tissue specimens were obtained from diagnostic Stiefel punch biopsy, wide excision biopsy and vulvectomy undertaken for investigation or treatment of vulval lesions. Ideally skin biopsy from women with LS and VIN 3 who were previously scanned would have provided a better study pool for comparison with LDPI findings. Unfortunately there was no ethical approval for vulval biopsy for the purpose of this study.

There are a number of methods for fixing tissue to preserve its morphology, depending on tissue type and which techniques will be used after sectioning. The most widely used fixatives in diagnostic hospital histology laboratories are formalin based, as formalin is a neutral salt employed to maintain tonicity. In this study 10% neutral buffered formalin (10% w/v formaldehyde in water) was used.

After fixation was complete the fixative was poured off and processed using an enclosed automatic processing system (VIP 2000F/300E) programmed with the following schedule: 10% neutral buffered formalin, 2h, 40°C; 70% industrial methylated spirit (IMS), 1h, 40°C; 90% IMS, 1h, 40°C; absolute IMS, 3h, 40°C; xylene, 4h, 40°C; paraffin wax, 3h, 60°C. Tissues were embedded in paraffin wax utilising the Tissue-Tek III. The cast blocks were then left at room temperature to allow the wax to become hard. After the wax hardened, the cast blocks were removed from embedding mould and stored in a dry place at room temperature until required.

The blocks were sectioned (5µm) using a microtome at room temperature. The sections were floated on warmed distilled water (45°C) to prevent creasing, mounted onto 2% 3-aminopropyltriethoxysilane (APES) coated slides (Sigma-Aldrich, Poole, Dorset, UK) and dried at 42°C for 30 min. The slides were placed in the incubator at 37°C for 2 days to firmly attach the sections to slides and were stored in a slide box and placed at 4°C until required. Each block had a corresponding haematoxylin and eosin (H&E) stained slide for histological evaluation. A gynaecological pathologist {J C Crow FRCPath} reviewed the H&E slides.

Thirty-one women with LS, 13 with VIN 3, 10 with vulval SCC and 11 with normal vulval epithelium were included in this investigation. The mean and standard deviation (mean±SD) of the age in years (y) of the women included in this study were:

LS: 55 ± 11y, VIN 3: 37 ± 10y, vulval SCC: 68 ± 9y and normal vulval epithelium: 49 ± 15y.

10.3.2 Immunohistochemistry

Five-micrometer thick paraffin wax-embedded sections were stained immunohistochemically for vWF antigen using the streptavidin-biotin-peroxidase complex technique. The sections were deparaffinised in xylene and rehydrated in different grades of ethanol up to distilled water. Endogenous peroxidase activity was quenched with a 3% v/v hydrogen peroxide (H₂O₂) and pre-treated with proteinase (bacterial protease type XXIV, P8038, Sigma-Aldrich, Poole, Dorset, 12.5mg in 100ml of phosphate-buffered saline [PBS], 37°C) for 15 min. To optimise immunoreactivity, antigen retrieval was also tested with microwaving (sodium citrate buffer – 0.01M sodium citrate pH 6.0 at 25°C) for 10 min at full power, protease digestion using different time intervals (10, 12, 15, 17 and 20 min) and without the antigen retrieval step. Results were compared for non-specific staining, preservation of tissue and overall appearance. The least background with best specific staining was obtained with 15 min incubation in protease solution. The tissue sections were then incubated with the primary antibody. The concentration of the primary antibody was compared at 1:20, 1:40, 1:60 and 1:80 dilutions. Monoclonal anti-human vWF antibody, 1:40 dilution (M0616, clone F8/86, Dako, Ely, Cambs) was found to be optimal and was used in this study. The duration of incubation was compared using several techniques - overnight at 4° C and at room temperature in a humidity chamber for 1h, 2h and 4h. The most specific staining with the least background was obtained with 1h incubation in a humidity chamber at room temperature. This was followed by application of secondary antibody (biotinylated rabbit anti-mouse immunoglobulin, E0354, Dako diluted 1:200 in PBS-SAB [phosphate buffered saline – bovine serum albumin] pH 7.4 with 10% normal human serum) for 45 min and then with streptavidin-biotin-horseradish peroxidase (K0492, Dako diluted 1:200 in Tris buffer Ph 7.6 [x10]) for 30 min. The chromogen,

3,3'-diaminobenzidine tetrahydrochloride solution (Sigma-Aldrich) was used.

Counterstaining of nuclei was performed with Mayers haematoxylin.

Staining consistency and reproducibility was confirmed by staining 3 different sections from the same block on separate days. There was 5% variation between sections.

Sections of human placenta were used as positive controls for each staining run. For negative controls the primary antiserum was replaced with PBS. vWF was selected as the immunohistochemical vascular endothelial marker because its high specificity provides good contrast between microvessels and other tissue components, as has been reported by Miettinen et al²¹³ and Weidner³³⁷ and more recently, confirmed by our group⁶.

10.3.3 Microvessel density (MVD)

Each section was examined by light microscopy at low magnification (x40 and x100) to identify areas with the greatest density of stained microvessels ('hot spots'). Three non-overlapping fields of vision with the highest number of hot spots were selected. The number of stained microvessels was quantified in each section at high magnification (x200) using an image analysis system (Seescan Imaging, Cambridge). The microvessels were point counted in frame-defined areas of the section. The areas were expressed as $10^4 \mu\text{m}^2$ of dermal tissue. The frame-defined areas that were examined varied in size between $1.2 \times 10^4 \mu\text{m}^2$ to $4.8 \times 10^4 \mu\text{m}^2$ (mean $3.8 \times 10^4 \mu\text{m}^2$). The MVD within the framed areas was divided by the area of the section and expressed as MVD/unit area, the unit area being $10^4 \mu\text{m}^2$.

In cases of VIN 3, LS and normal epithelium, the frame-defined areas were confined to the dermis, i.e. the epidermis was excluded because this structure is avascular and

varied in thickness between the 3 histologically different tissue types. The MVD in these sections was confined to the area within 200µm of the epidermo-dermal junction just beneath the basement membrane of the epidermis. In cases of vulval SCC, we excluded sclerotic, hypocellular areas and regions immediately adjacent to benign disease, when determining MVD.

Any brown-staining endothelial cell or endothelial cell cluster that was clearly separate from adjacent microvessels, tumour cells and other connective tissue elements was considered as a single countable microvessel³³⁷. Even those distinct clusters of stained endothelial cells that might be from the same vessel snaking its way in and out of the section were considered distinct and countable as separate microvessels. The presence of a vascular lumen was not relied upon to define a microvessel³³⁷. Macrovessels, characterised by thick muscular walls or a diameter greater than 50µm, were excluded from the count.

All sections were analysed by 3 independent observers (J C Crow FRCPath, Consultant Pathologist, K J Rolfe PhD, and myself). There was less than 5% variation between the observers.

10.3.4 Statistical Analysis

The highest vessel density (HVD) and average vessel density (AVD) per unit area for each tissue specimen were analysed. Non-parametric analysis was used because of the skewed data distribution. The Kruskal-Wallis test analysed the differences among the 4 groups of tissue specimens and then the Mann-Whitney *U*-Test was used for pairwise comparisons between the groups. Probability values less than 0.05 were considered significant.

10.4 Results

The anti-vWF antibody stained the vascular endothelial cells brown as shown in a typical field from cases of normal vulva (Figure 10.1a), LS (Figure 10.1b), VIN 3 (Figure 10.1c) and vulval SCC (Figure 10.1d). Tumour cells, as well as other normal constituents, were consistently unstained.

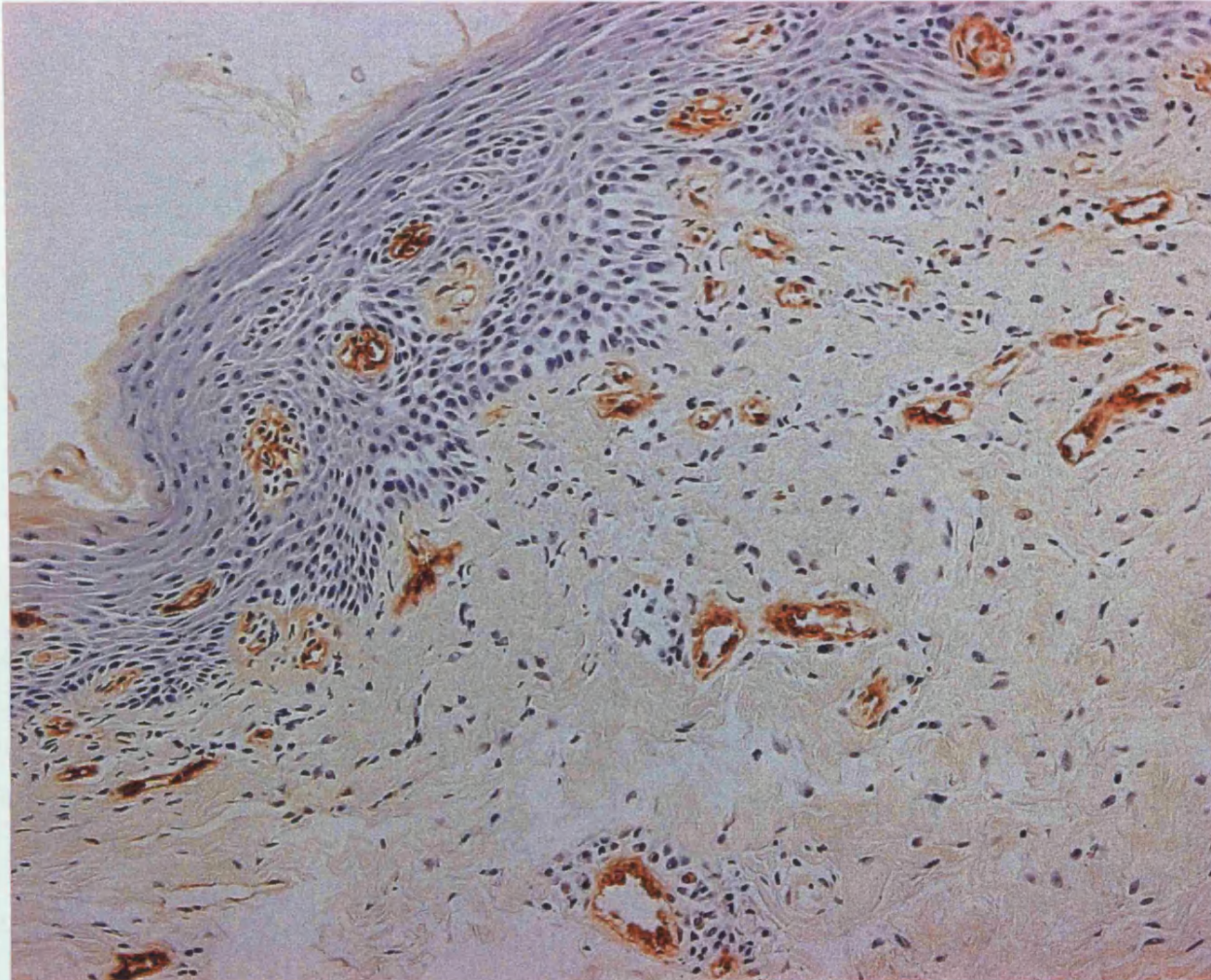


Figure 10.1a

Photomicrograph of normal vulval skin sectioned at an angle showing normal distribution of microvessels within the superficial dermis and cross-section of the dermal papillary microvessels within epidermis, immunostained with anti-vWF (Magnification x200).

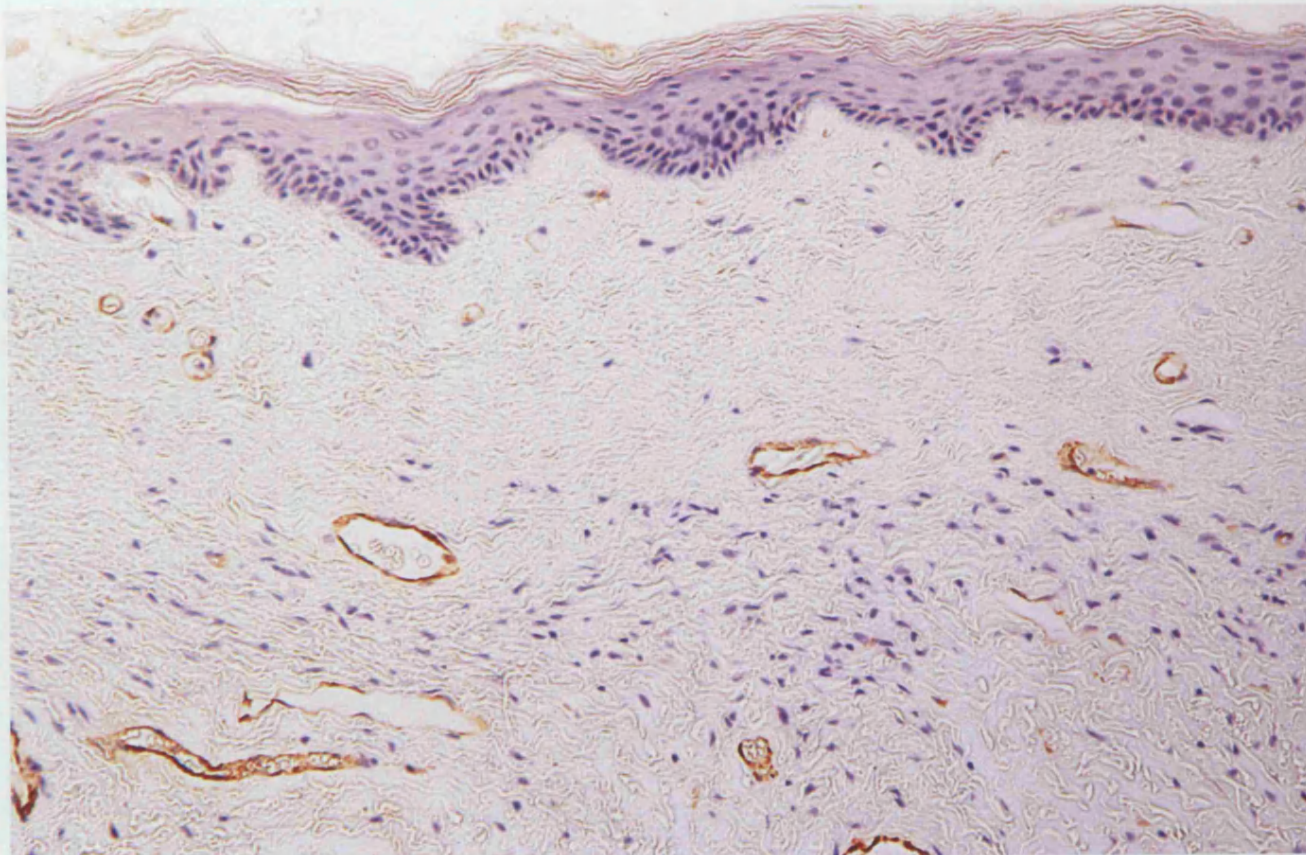


Figure 10.1b

**Photomicrograph of vulval LS
with a sparse number of
microvessels immunostained
with anti-vWF, within the
superficial dermis.**

(Magnification x100).

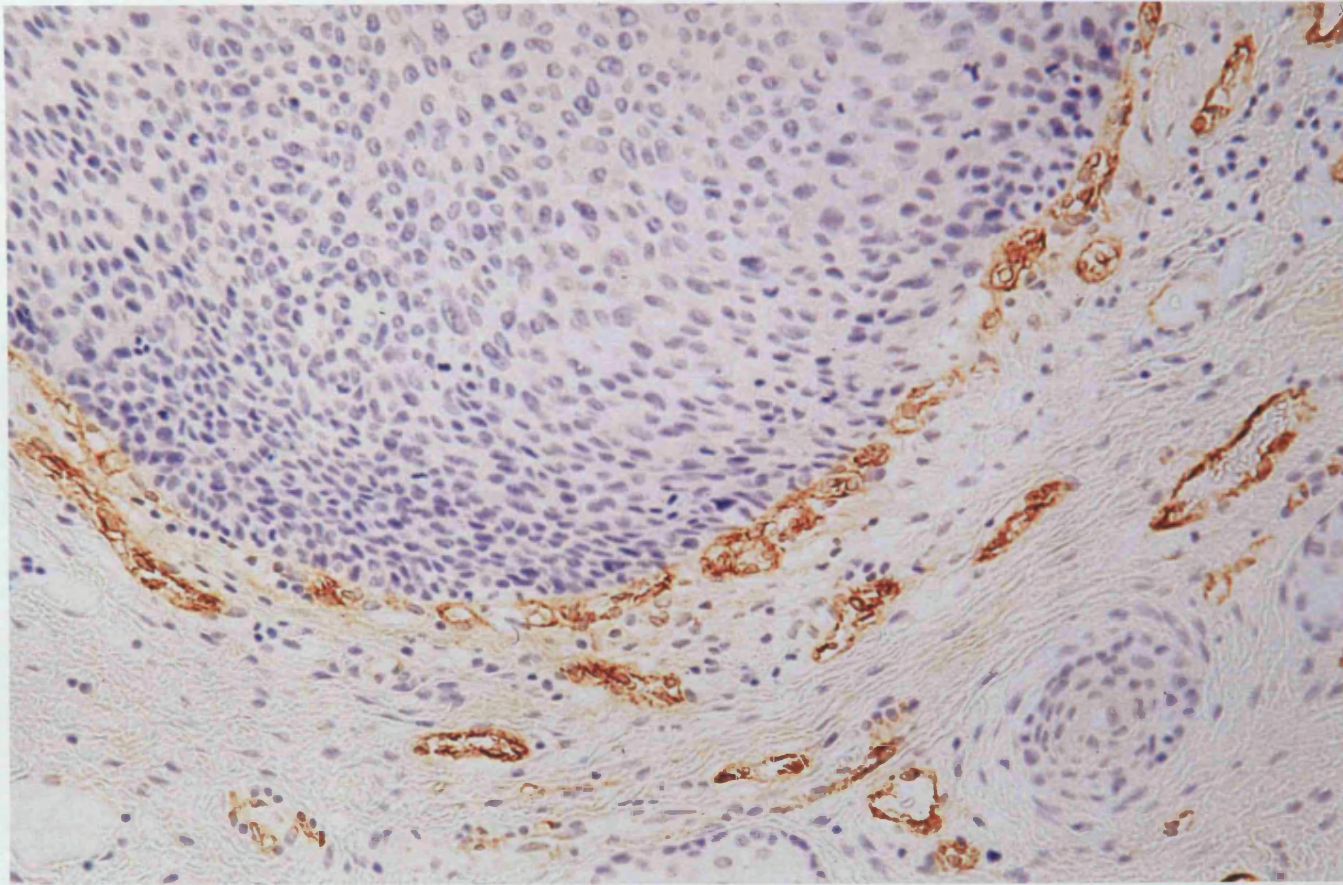


Figure 10.1c

Photomicrograph of vWF

immunostain in VIN 3.

**Microvessels are clearly denser
within the sub-epithelial dermis
compared to LS.**

(Magnification x100).

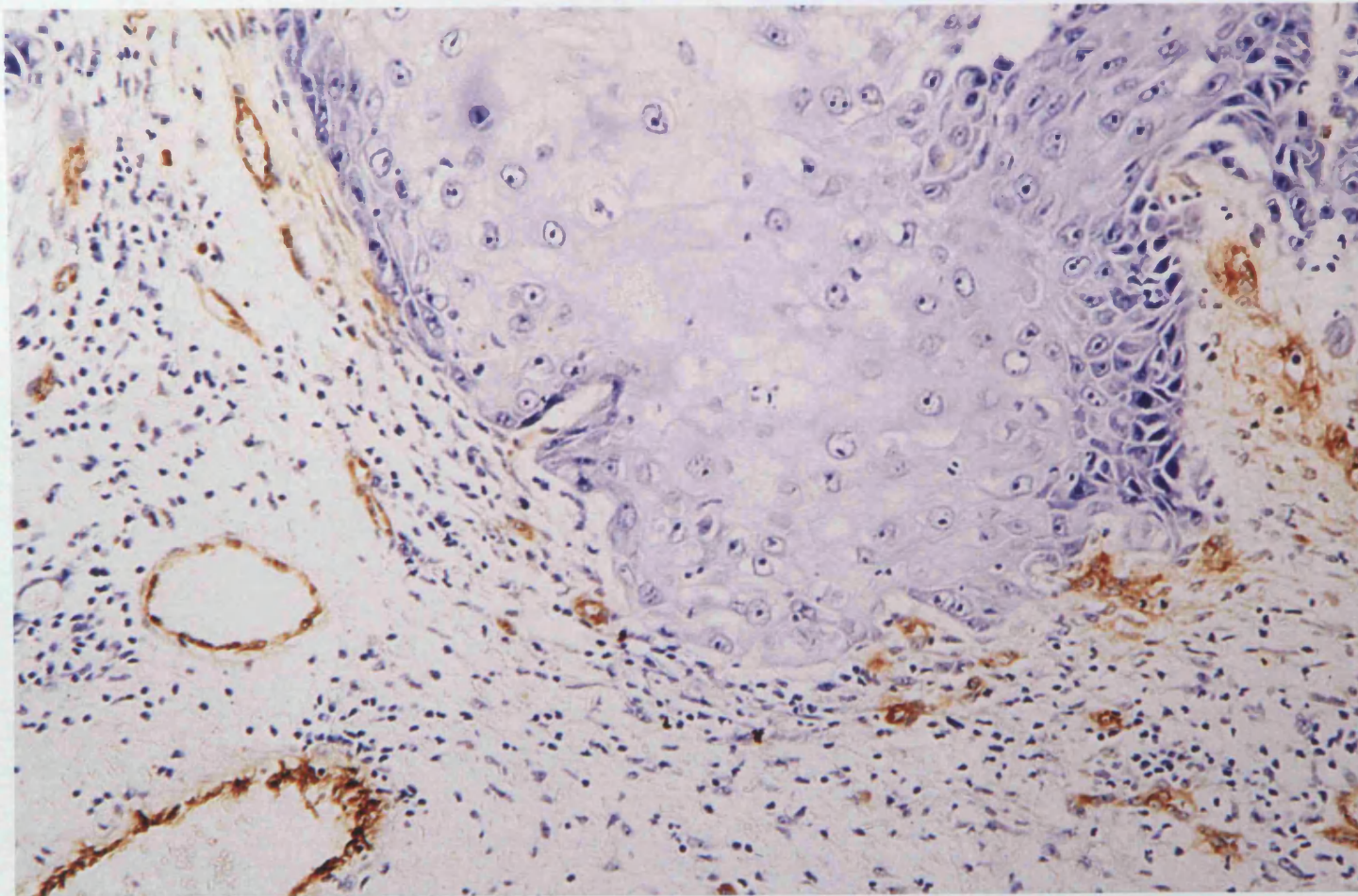


Figure 10.1d

Photomicrograph
showing an invading
tongue of vulval SCC
surrounded by
numerous microvessels
in the stroma.

(Magnification x100)

The distributions of the HVD and AVD for each type of vulval tissue specimen are compared as scatter graphs in Figure 10.2 (HVD) and 10.3 (AVD).

Figure 10.2 Scatter graph showing the overall median (●) values, comparing the distribution of the HVD per unit area for normal vulva, LS, VIN 3 and SCC

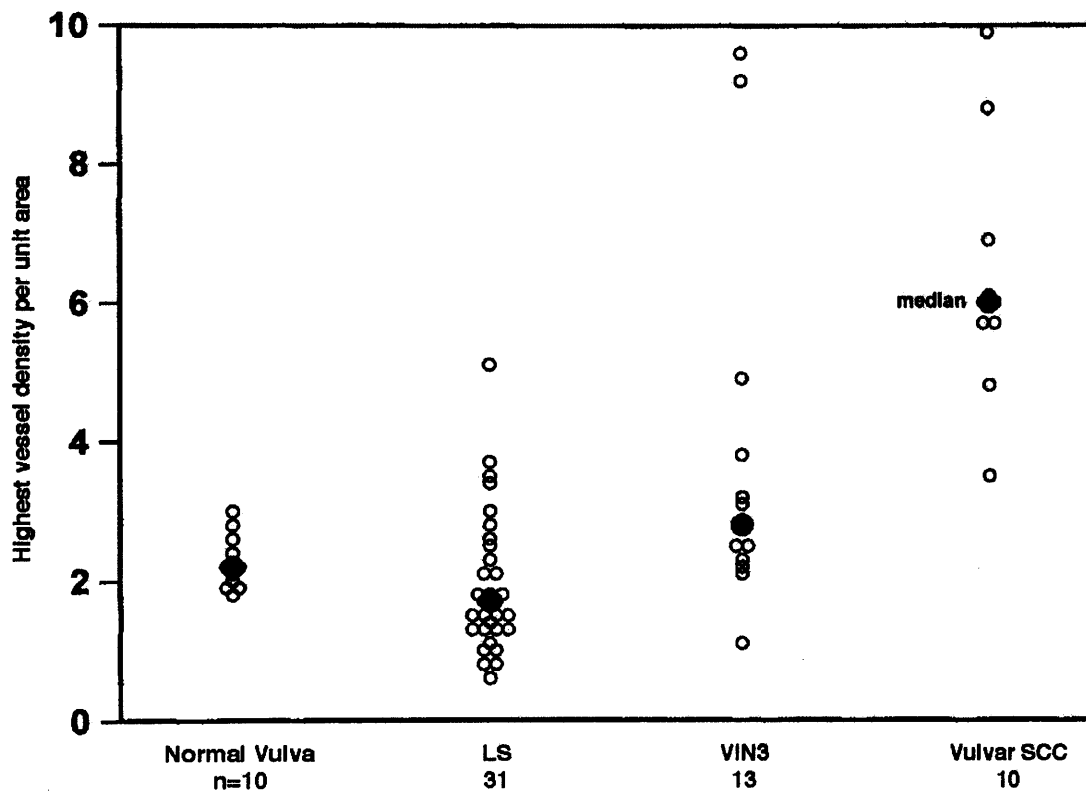
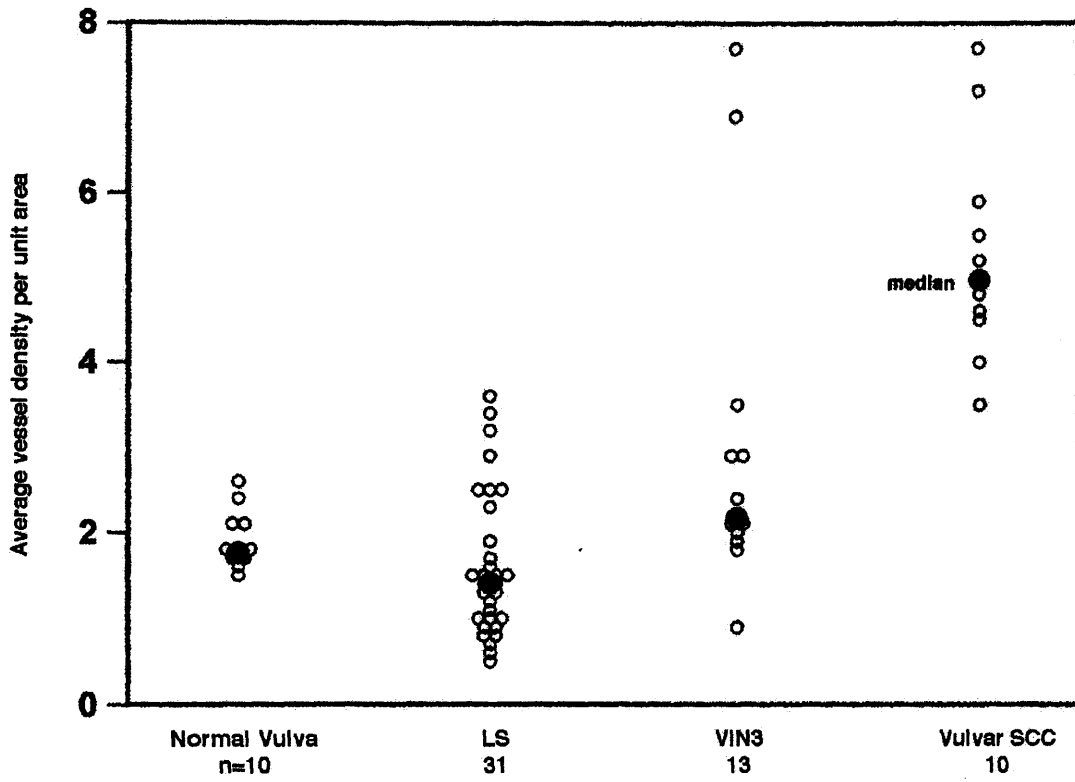


Figure 10.3 Scatter graph showing the overall median (●) values, comparing the distribution of the AVD per unit area for normal vulva, LS, VIN 3 and SCC



The Kruskal-Wallis test showed that the observed differences among the 4 groups of tissue specimens were highly significant as a whole ($p < 0.0005$). Table 10.1 shows the overall median for the HVD and MVD values with the lower and upper quartiles for each clinical group.

Table 10.1 The median MVD values (HVD and AVD) per unit area with lower and upper quartiles for each vulval tissue specimen

Vulval tissue specimen	HVD values			AVD values		
	Median	Lower Quartile	Upper Quartile	Median	Lower Quartile	Upper Quartile
Normal (n=11)	2.2	1.9	2.6	1.76	1.65	2.15
LS (n=31)	1.7	1.3	2.5	1.41	0.98	2.34
VIN 3 (n=13)	2.8	2.25	4.35	2.17	1.98	3.21
SCC (n=10)	6.0	5.48	7.38	4.98	4.36	6.22

n=number of cases.

The P values for the HVD* and AVD† per unit area for normal vs LS: 0.038*, 0.031†; normal vs VIN 3: 0.03*, 0.034†; normal vs SCC: <0.005*†; LS vs VIN 3: 0.002*, 0.004†; LS vs SCC: < 0.0005*†; VIN 3 vs SCC: 0.006*, 0.004†.

When the two outliers were removed in the VIN 3 group (n=11), the normal vs VIN 3 was no longer significant ($p = 0.08^*$, 0.09^\dagger); the LS vs VIN 3 comparison was less significant ($p = 0.013^*$, 0.021^\dagger) and the VIN 3 vs SCC comparison remained highly significant ($p < 0.005^*^\dagger$)

Vulval LS had significantly the lowest MVD per unit area within 200 μm of the subepithelial dermal tissue. Interestingly, the MVD in LS was also significantly lower than in normal vulval epithelium. The MVD continued to significantly increase from normal epithelium to VIN 3 and to vulval SCC. This can be seen in the photomicrography (Figures 10.1b-c) and is demonstrated in Table 10.1.

Morphologically there was a marked difference in the density of the subepithelial microvessels between LS and VIN 3.

It is worthy of note that the distribution of MVD in VIN 3 in the scatter graphs in Figures 10.2 and 10.3 showed 2 cases with a much higher HVD (9.16 and 9.61) and AVD (6.89 and 7.71) per unit area compared to the main cluster of 11 patients. Removing these 2 outliers made small differences in the results (Table 10.1)

10.5 Discussion

Immunostaining to visualise microvessels for the purpose of counting can be performed using various vascular endothelial cell markers such as vWF, ABH blood group antigens, CD31/PECAM-1, CD34, CD36 and *Ulex europaeus* agglutinin 1 (UEA-1)³³⁷. von-Willebrand factor (vWF), a multimeric protein synthesised by endothelial cells and present in the cytoplasm of these cells in blood vessels of all sizes, is considered the gold standard³³⁷. No endothelial cell marker is perfect and vWF is no exception. The vWF endothelial cell marker is identifiable to a varying degree in megakaryocytes, platelets and lymphatic endothelial cells, but it does not have the high cross reactivity with stromal fibroblasts, tumour cells and plasma cells found with the other markers. In fact Miettinen et al²¹³ and more recently Amis et al⁶ demonstrated the best contrast between microvessels and other tissue components with vWF, compared to the other endothelial cell markers such as CD31, CD34 and monoclonal antibody BNH9.

In this study VIN 3 had a significantly lower MVD compared to vulval SCC and a higher MVD compared to normal vulval epithelium, which is similar to the findings of Dobbs et al⁸⁰ on the cervix. In Chapter 3 the new classification for high-grade VIN is sub-categorised into the usual type or undifferentiated (VIN 2 and 3 – warty, bowenoid and mixed), differentiated (dVIN) and unclassified varieties. VIN 3 in the patients presented in this thesis refers to the undifferentiated VIN 3 or usual type – warty, bowenoid and mixed. The risk of high-grade VIN (undifferentiated VIN 3) progressing to malignant disease is influenced by treatment^{125;151;193}. The reported risk varies between about 5% for treated disease and 87.5% for untreated disease (see Table 3.5). The incidence of occult microinvasive disease has been reported to be as high as 23%²¹⁷. Microinvasive vulval disease can sometimes be visualised colposcopically by the presence of abnormal vasculature¹⁷⁰, as is frequently the case for microinvasive

cervical neoplasia^{287;291}, implying that changes in vascular morphology do occur during the process of malignant progression. These changes in vasculature in vulval SCC may in fact begin during the pre-neoplastic phase (e.g. VIN 3), as demonstrated by a study on MVD by Bancher-Todesca et al¹⁴ and, more recently, by a study on VEGF expression by MacLean et al¹⁹⁷. This study, showing MVD significantly higher in VIN 3 than in normal vulva but lower than in SCC, provides further support to this hypothesis. It is also worthy of note that 2 patients had a much higher MVD (both HVD and AVD) compared to the main cluster, as shown in Figures 10.2 and 10.3. It can be hypothesised that these patients could belong to a pool at greater risk of progression to malignant disease; this can only be demonstrated by long-term follow-up studies.

The LDPI findings in Chapter 7 reported a significantly higher perfusion in skin affected by VIN 3 compared to adjacent normal vulval skin in the same patient. However when normal women were compared to women with VIN 3 the perfusion was higher in VIN 3, although the difference did not reach significance, most likely due to the small number of subjects in the study. This morphological immunohistochemical study also demonstrated significantly increased MVD in VIN 3 compared to normal cases. These findings imply that the increased perfusion detected by the LDPI is more likely to be due to a real increase in blood flow, rather than an apparent increase caused by morphological features influencing photon pathway such as a greater depth of penetration resulting in a larger volume of tissue assessment, beyond the superficial microvessels.

LS is also believed to carry an increased risk of progression to malignancy but the MVD in this disease was significantly lower than in normal vulval epithelium. Computer-

supported 3-D reconstruction of the papillary capillaries and superficial vascular plexus in non-genital LS show progressive disruption and disintegration of the capillary wall resulting in vessel reduction¹⁶⁷. The findings in this immunohistochemical study support a similar process in vulval LS. The immunohistochemical results could also be attributed to the methodology, which only targeted microvessels within 200 µm of the epidermo-dermal junction. In classical vulval LS the superficial dermis consists of a band of acellular oedematous hyalinised tissue, which in this study was present in all cases. Although the methodology of selecting hot spots would have excluded frankly abnormal areas with a very low number of microvessels, overall this selected group with classical LS may be at lower risk of progression to carcinoma. Doldi et al⁸¹ reported great variability in the expression of VEGF mRNA (messenger ribonucleic acid) in a very small group of non-neoplastic vulval lesions (n=8) which included only 1 case of LS, while MacLean et al¹⁹⁷ showed no significant difference in immunohistochemical expression of VEGF between vulval LS (n=25) compared to normal vulval epithelium (n=10). Both this data and that of MacLean et al¹⁹⁷ suggest that angiogenesis is unlikely to be a feature of classical vulval LS. Other mechanisms such as p53 protein accumulation^{63;312}, alteration in the cytokeratin profile^{63;222} and presence of allelic imbalance^{63;243} may be the critical factors which are more relevant to the earlier detection of classical LS cases at high risk of malignant transformation. Rolfe et al²⁶¹ showed increased expression of cell cycle proteins (p53 and Ki67) in areas of squamous hyperplasia (SH) and dVIN in multiple sequential vulval biopsies taken from the same woman with LS who developed SCC. The work by Rolfe et al²⁶¹ suggests that tissue specimens selected from areas with SH and dVIN may have a higher MVD compared to those selected exclusively from areas of classical LS.

The interpretation of the biophysical findings in LS in Chapter 7 with respect to the immunohistochemical morphological findings in this chapter is more complex compared to VIN 3. The LDPI measured a higher perfusion in untreated LS compared to normal vulval epithelium, although this difference did not reach significance. After treatment, the skin perfusion in LS decreased significantly below that in normal women and untreated LS. Unlike VIN 3, which is an asymmetrical patchy disease, LS affects the whole of the vulval skin symmetrically preventing comparison of adjacent normal and abnormal vulval skin areas in the same patient with the LDPI. The LDPI measurements are affected by physiological and skin morphological variations between subjects and therefore comparison of symmetrical normal and abnormal areas in the patient reduces the effect of these confounding factors. The cases studied immunohistochemically were selected from the archive of samples available and is not a systematic study of cases before and after treatment of good responders as was done with the biophysical method. However it is possible that the cases studied immunohistochemically are more comparable to the untreated cases studied with the LDPI as it is a more usual clinical practice to take biopsies in symptomatic patients either before treatment or when resistant to treatment to confirm diagnosis. The morphological study showed a significantly lower MVD while the biophysical study appears to show a higher (not significant) perfusion in LS compared to normal. The difference in the morphological compared to the biophysical findings in untreated LS could be attributed to differences in the depth of tissue studied. The LDPI could be studying blood flow at a much greater depth than within 200 μ m of the epidermo-dermal junction as applied to the morphological study. Alternatively the MVD selectively measured the microvessels (<50 μ m) focused upon by identifying hot spots while the LDPI measures the blood flow in all the vessels with the scatter volume.

Although vWF is specific for staining endothelial cells lining blood vessels it does not differentiate between proliferating and mature microvessels. Recently, newer antibodies have become available which are able to detect activated/proliferating endothelial cells and to distinguish between newly formed immature vessels and those that are more established and mature^{332;333}. CD 105/Endodlin is a member of the transforming growth factor β_1 receptor complex that binds preferentially to proliferating endothelial cells that participate in tumour angiogenesis. In cancers of the breast and cervix endoglin is a more specific and sensitive microvessel marker than other panendothelial antibodies such as vWF^{26;46}. It is also worth noting that angiogenesis is a complex process and that techniques to observe and quantify microvessels histologically in sections of tissue only provide an estimate of the final product; physiological variables such as blood flow, vascular permeability and interstitial pressure, which influence the relationship of angiogenesis to malignant transformation, are more complex to measure^{141;337}. The study of changes in vasculature and blood flow in diseases at risk of malignant change increases not only our understanding of the underlying pathophysiology but also provides opportunities to develop new markers to identify high-risk cases and to design new therapies aimed at inhibiting angiogenesis or selectively destroying the 'new' vasculature, giving rise to the possibility of medical rather than surgical cure^{109;188}.

The LDPI has the potential for clinical applications for measurement of blood flow within microvessels, non-invasively, without contact and in real-time. The interpretation of comparative studies between morphological and biophysical methods could be improved by a greater understanding of the structure of the vulval microvasculature, of the photon pathway through vulval skin, physiological variables such as age related changes in perfusion both in the normal and in the presence of

disease. Morphological studies using 3-D computerised reconstruction of the vulval vasculature as shown by Auer et al¹² in describing changes in perfusion in psoriasis before and after treatment and by Konerding et al¹⁷¹ in colon pre-cancerous lesions, will greatly improve our understanding of the morphology of the vulval vasculature.

In conclusion the increase in perfusion detected by the LDPI in cases of VIN 3 is most likely due to a real increase in perfusion as demonstrated by the MVD findings. In vulval LS the biophysical and morphological comparisons are more complex.

CHAPTER 11

Summary and future work

In this thesis the LASER Doppler Perfusion Imager was used for the first time to study SBF in women with normal and diseased vulval skin. Previously Jackson et al¹⁴⁰ had used the LDF to show differences in SBF in vulval cancer compared to adjacent normal vulval skin. Mapping of SBF over a specific area (12mm²) within a short period of time (~3min) minimises the effect of large temporal and spatial variations compared to manually repeated single point (1mm²) measurement, which is also time consuming and tedious for both the patient and operator. In addition, as the methodology crucially involves the patient in the lithotomy position to obtain adequate exposure of the vulval skin, the technique should be as brief as possible. For these reasons the LDPI was found to be a suitable technique.

Response to hypotheses posed in Section 1.5

1. Yes, experiments with a blood-flow model demonstrated that the LDPI technique used in this thesis was reliable in measuring SBF on the vulva. These experiments showed that the LDPI readings were optimal at a distance of 160mm between the imaging site and the laser head with a 7% error within 10mm. The LDPI measurements correlated significantly with blood flow rate and haemodilution and were not influenced by the changes in the Po₂. Movement of the imaging site reduced the reliability of the LDPI measurements but this was not a problem at the vulval skin site. There was only minor geometric distortion in the vertical axis.
2. Yes, women with normal vulva found the use of the LDPI acceptable and the reproducibility of the measurement of vulval SBF was good (CV = 5-7%).

3. Age and menstrual cycle did not show any variation in SBF, although further studies with larger subject numbers are required to confirm these findings.
Recovery from local skin temperature challenge also showed little difference in change of baseline SBF, but the wide 95% confidence interval (CI) again suggests that larger subject numbers are required.
4. Yes, women with vulval lesions found the use of LDPI acceptable and the reproducibility of the measurement in the presence of skin disease was good.
5. Women with untreated vulval LS had a higher SBF compared to normal women, although the results did not reach significance. An intra-patient comparison between symmetrical normal and diseased skin sites was not possible in LS as the disease distribution is symmetrical, affecting all vulval structures. After treatment and with good symptom response, there was a significant decrease in baseline SBF.
6. Serial measurements of SBF in vulval LS at the commencement of treatment and during treatment with potent topical steroids demonstrated a gradual decrease in baseline SBF. As the women achieved good symptom control and progressed to moderately potent topical steroids, the vulval SBF remained significantly below the pre-treatment baseline values. However as the frequency of moderately potent topical steroid therapy was phased down to maintenance therapy the vulval SBF progressively increased to pre-treatment baseline levels in spite of good symptom relief.

7. In high-grade usual type VIN (undifferentiated VIN 3) the baseline SBF was higher compared to normal women but again did not reach significance. An intra-patient comparison was possible with high-grade VIN as the disease distribution is asymmetrical. When SBF in high-grade VIN (undifferentiated VIN 3) was compared in the same patient between 'normal' and diseased areas there was a significantly higher SBF in areas of high-grade VIN (undifferentiated VIN 3) compared to normal skin.

8. The response of vulval SBF to recovery from local skin temperature challenge demonstrates skin perfusion differences in normal and diseased skin. There is a significant increase and decrease in skin perfusion after hot and cold provocation compared to the response in normal vulval skin. This may be related to impaired skin vessel reactivity to changes in local skin temperature. This abnormality in skin perfusion persists after good symptom relief with treatment in vulval LS.

9. MVD in vulval LS was significantly lower than in the normal vulva whereas the LDPI showed a higher, though non-significant, SBF in women with untreated LS compared to normal women. This discrepancy between biophysical and morphological findings is attributed either to different depths of tissue assessment or alternatively to the LDPI measuring changes in SBF in blood vessels with diameter greater than 50 μ m while the MVD only focused on the blood vessels with diameter less than 50 μ m (microvessels). In high-grade VIN (undifferentiated VIN 3) there was good correlation between the MVD and LDPI studies in that both techniques showed significantly higher blood supply in diseased tissue.

An important clinical question for this thesis was whether the use of real-time SBF imaging of the vulva with LDPI had a role in improving identification of invasive disease within vulval LS and high-grade VIN. The majority of women with vulval LS and high-grade VIN do not develop malignant disease and therefore it would be of great assistance to the clinician if invasive disease could be diagnosed more reliably at an early stage to reduce the morbidity and mortality associated with treatment of vulval cancer¹⁹⁴. This question can only be answered by a prospective trial following-up patients with LS and VIN 3, clinically and with the LDPI, although logistically this would be a difficult study. During this period of study none of the women presented in this thesis developed cancer. The measurements made by the LDPI method require much refinement and a better way of measuring relative changes in blood flow, compared to temperature challenge used in this thesis, to more clearly differentiate the normal from the abnormal skin. However this preliminary work has made some contribution towards the study of the vulval skin.

The LDPI is suitable for use on the vulva. The technique is acceptable to women and the SBF of the vulva has a good reproducibility in the normal and diseased skin. There are changes in SBF in the presence of vulval disease but a much larger subject group is required to compare SBF between women with normal and diseased skin (e.g. vulval LS). In high-grade VIN this problem was overcome as the disease has an asymmetrical distribution allowing comparison of symmetrical abnormal and 'normal' regions in the same patient. The morphological studies using MVD support the LDPI finding in high-grade VIN. The situation with vulval LS is more complex and more information is required on the microanatomical changes within the skin microvasculature, the passage of light photons within the skin, and the influence of morphology on the light passing

through skin. LS is a heterogeneous disease and therefore it would be necessary to stratify subjects with different disease presentations to understand the different groups. The local skin temperature provocation studies suggest an abnormality within SBF in the diseased vulva. The pattern of data gives the impression that in the presence of vulval LS (irrespective of treatment) and in high-grade VIN the SBF is less able to maintain normal baseline perfusion suggesting the possibility of changes within the wall of the blood vessels; the nature of the exact pathophysiology is not revealed by these studies and is very likely to be different for each disease. More work is required before the LDPI could become part of routine clinical assessment of patients with LS and high-grade VIN.

These are questions for future work:

- What is the exact structure of the microvasculature in the different regions of the vulval skin? Although this is likely to be similar to other skin areas, it requires confirmation in view of the function of the vulva, which includes rapid changes in organ perfusion. 3D-computerised imaging has been helpful in the colon and for the study SBF changes in psoriasis^{12;171}.
- How do skin morphological factors influence the pathway of the light photons through the skin in the presence of disease and after iatrogenic intervention (e.g. after surgery or topical medical therapy)? This information could improve assessment of the exact depth of penetration of the laser beam.
- What is the correlation between abnormal vessel patterns seen on vulval colposcopy and SBF mapping by the LDPI? LDPI with digital photography will facilitate more accurate correlation of the blood flow image with the photograph,

allowing for a more precise and time-saving comparison between the perfusion and colposcopic image³¹. The measuring of perfusion in regions of the vulva such as the vestibular glands by Bohm-Starke et al²⁷ is greatly facilitated by generation of a digital photograph to correlate with the two-dimensional blood flow image.

- What is the correlation between 3-D computerised study of the skin microvasculature and LDPI in vulval LS? As in the study by Auer et al in psoriasis this type of correlation study will greatly improve our understanding of SBF changes within vulval LS¹².
- Other techniques such as infrared spectrophotometry, which is not influenced by skin pigmentation, can assess the microvascular haemoglobin content and tissue oxygenation, and therefore may prove more valuable in accurately predicting areas at risk of malignant progression.

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A very special Thank You
to
Professor Allan B MacLean
and
Professor Alexander M Scifalian