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Merci to my husband Eng Tin who brings out the best in me

And to my father and mother who believe in me

'Philosophy is only a recognition of the abysses which lie on each side of the path that the vulgar follow with the serenity of sleepwalkers.'

Georges Sorel



Improving the Skin Barrier Function in Atopic Dermatitis

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Abstract

Atopic dermatitis, AD (synonym eczema) is a chronic inflammatory skin disease. It affects between 10 to 20% of children and 1 to 3% of adults worldwide. It is an important cause of morbidity and is estimated to cost £465 million per annum to the UK. AD is part of a family of Th-2 driven diseases and is often the first of these atopic diseases to manifest. The development of AD is often followed by asthma and allergic rhinitis later in life (a phenomenon known as the 'atopic march').

Up to 50% of moderate to severe AD cases have been associated with genetic mutations affecting the epidermal barrier protein filaggrin. Filaggrin aggregates keratin filaments during terminal keratinocyte differentiation, allowing normal epidermal stratification. The role of filaggrin in maintaining a functional skin barrier is further supported by a clinical study conducted by ourselves. This is the first clinical study on a European cohort (58 participants) which showed that *FLG* mutations were associated with experimentally demonstrable defects of skin barrier function (increased baseline transepidermal water loss), more so following exposure to a chemical irritant. However, the majority of patients with AD, especially the milder cases, do not have *FLG* mutations. Some of the wild-type patients in our study were noticed to have accumulation of the large filaggrin proprotein and a lack of filaggrin monomers, indicating defective proteolysis of profilaggrin into the functional monomers. Our study also found disproportionately raised protease inhibitory activities amongst the AD participants. This inappropriately raised protease inhibition may interfere with profilaggrin proteolysis, leading to the development of AD in some wild-type patients.

Having demonstrated that deficiency of filaggrin monomers is associated with a defective skin barrier, we focused on the function of filaggrin in the skin and attempted to improve the skin barrier function. In addition to keratin aggregation, filaggrin constitutes the natural moisturizing factors in the epidermis following its natural breakdown into amino acids. We note that filaggrin is disproportionately rich in amino acid histidine, implying that this amino acid may have a particular significance in maintaining a functional epidermal barrier. Using an in-house skin-equivalent model, we have shown that by increasing the histidine content in the cell culture media, we could increase the expression of filaggrin monomers and reduce the penetration of a fluorescent dye into the skin-equivalents. The latter indicates improved barrier function. Finally, we conducted a pilot human study which showed that histidine, when applied to mechanically damaged skin in AD and healthy participants, was associated with a faster recovery of the skin barrier function.

These studies suggest that histidine is of therapeutic benefits in AD. A histidine-based treatment may be developed as an alternative to current anti-inflammatory and immunosuppressive agents used to treat AD.

Author's Declaration

This is to certify that that the work contained within has been composed by me and is entirely my own work. No part of this thesis has been submitted for any other degree or professional qualification.

Signature: _____

Date:

Acknowledgement

There are many people to whom I owe my deepest thanks for making the past three years the most enjoyable learning experience that I could have ever wanted. Without their guidance, assistance, encouragement or sheer presence, my journey as a PhD student would have been less challenging and fun. I would like to record my appreciation to them here.

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Table of Contents

<u>Contents</u>	<u>Page</u>
Title Page	I
Abstract	II
Author's Declaration	III
Acknowledgement	IV
Table of Contents	VI
List of Figures	XI
List of Tables	XVI
List of Abbreviations	XVII
The Thesis: An Introduction	XIX
Novel Contributions	XX
List of Publications	XXI
Chapter 1: Atopic Dermatitis	1-1
1.1 Introduction	1-2
1.2 Clinical presentation	1-3
1.2.1 Diagnostic criteria	1-4
1.3 Epidemiology	1-7
1.4 Pathogenesis of Atopic Dermatitis	1-9
1.5 Treatment	1-16
1.5.1 Restoration of skin barrier function	1-16
1.5.2 Allergen avoidance	1-16
1.5.3 Anti-inflammatory	1-17
1.5.4 Antiseptics / antibiotics	1-19
1.5.5 Others	1-19
1.6 Social and financial implications	1-21
1.7 Chapter 1 Conclusion	1-22
1.8 Chapter 1 References	1-23
Chapter 2: The Effects of Filaggrin Gene Mutations on Skin Barrier Function in Patients with Atopic Dermatitis	2-30
2.1 Introduction: Filaggrin	2-31
2.2 Chapter 2 Results	2-35
2.2.1 Demographic data of AD participants	2-35
2.2.2 Genotype	2-35
2.2.3 Baseline TEWL and skin capacitance	2-36
2.2.4 Disease severity	2-37
2.2.5 Presence of other atopic conditions	2-37
2.2.6 Family history of atopic conditions	2-38
2.2.7 IgE level and number of contact allergies	2-39
2.2.8 Relationships between age with:	
2.2.8.1 Baseline TEWL	2-41
2.2.8.2 Baseline skin capacitance	2-42
2.2.8.3 Disease severity (SASSAD score)	2-43
2.2.9 Relationships between gender with:	
2.2.9.1 Baseline TEWL	2-44
2.2.9.2 Baseline skin capacitance	2-45
2.2.9.3 Disease severity (SASSAD score)	2-46

2.2.10	Relationships between baseline TEWL and skin capacitance with:	
2.2.10.1	Co-existing presence of other atopic conditions	
2.2.10.1.1	Dry skin	2-47
2.2.10.1.2	Asthma	2-49
2.2.10.1.3	Hay fever	2-51
2.2.10.2	Family history of atopic conditions	
2.2.10.2.1	AD	2-53
2.2.10.2.2	Asthma	2-55
2.2.10.2.3	Hay fever	2-57
2.2.10.2.4	Atopic conditions (any of AD, asthma or hay fever)	2-59
2.2.10.3	Number of contact allergies	2-60
2.2.11	Relationships between SASSAD disease severity score with:	
2.2.11.1	Subjective scoring of disease severity	2-61
2.2.11.2	Baseline TEWL and skin capacitance	2-61
2.2.11.3	Presence of other atopic conditions	
2.2.11.3.1	Dry skin	2-63
2.2.11.3.2	Asthma	2-64
2.2.11.3.3	Hay fever	2-64
2.2.11.4	Family history of atopic conditions	
2.2.11.4.1	AD	2-66
2.2.11.4.2	Asthma	2-67
2.2.11.4.3	Hay fever	2-68
2.2.11.4.4	Atopic conditions (any of AD, asthma or hay fever)	2-68
2.2.11.5	IgE level and number of contact allergies	2-69
2.2.12	Correlations between IgE level with:	
2.2.12.1	Baseline TEWL and skin capacitance	2-70
2.2.12.2	Number of contact allergies	2-72
2.2.13	Correlations between susceptibility to physical insult by tape-stripping with:	
2.2.13.1	Age and gender	2-72
2.2.13.2	Genotype	2-74
2.2.13.3	Baseline TEWL and skin capacitance	2-75
2.2.13.4	Disease severity (SASSAD score)	2-77
2.2.13.5	Presence of other atopic conditions	
2.2.13.5.1	Dry skin	2-78
2.2.13.5.2	Asthma	2-79
2.2.13.5.3	Hay fever	2-80
2.2.13.6	Family history of atopic conditions	
2.2.13.6.1	AD	2-81
2.2.13.6.2	Asthma	2-82
2.2.13.6.3	Hay fever	2-83
2.2.13.6.4	Atopic conditions (any of AD, asthma or hay fever)	2-84
2.2.13.7	IgE level	2-85
2.2.14	Chemical irritancy study	
2.2.14.1	Clinical score of the skin post-SDS application	2-86
2.2.14.2	Change in TEWL	2-87
2.2.14.3	Changes in skin fold thickness	2-87
2.3	A summary of the main findings	2-88
2.4	Chapter 2 Discussion	2-89
2.5	Chapter 2 References	2-93

2.6	Chapter 2 Materials and Methods	
2.6.1	Participant recruitment	2-96
2.6.2	Genotyping	2-96
2.6.3	Laboratory test	2-97
2.6.4	Measurements of TEWL and skin capacitance	2-97
2.6.5	Tape-stripping experiment	2-98
2.6.6	SDS-irritancy study	2-99
2.6.7	Statistical analysis	2-100
	Chapter 3: Protease-Antiprotease Imbalances and Atopic Dermatitis in Patients with a Normal Filaggrin Gene	3-101
3.1	Introduction: It is not all about the Gene	3-102
3.1.1	The role of epidermal proteases and the importance of protease-antiprotease balance	3-106
3.1.2	Chapter 3 Hypothesis	3-108
3.2	Chapter 3 Results	3-109
3.2.1	Demographic data of participants	3-109
3.2.2	Genotype	3-109
3.2.3	Protein concentrations of suction-induced blister fluids and blister skin cap extracts	3-110
3.2.4	SDS-PAGE and Western-blot for filaggrin in suction-induced skin caps	3-110
3.2.5	Immunohistochemistry double staining for filaggrin and cytokeratin	3-112
3.2.6	Cryopreservation studies	3-113
3.2.7	Protease activity assays	3-114
3.2.8	Protease inhibition assays	3-114
3.2.9	Quantitation of known epidermal protease inhibitors	3-116
3.3	Chapter 3 Discussion	3-117
3.4	Chapter 3 References	3-121
3.5	Chapter 3 Materials and Methods	3-126
3.5.1	Participant recruitment	3-126
3.5.2	Genotyping	3-126
3.5.3	Development of suction-induced blisters	3-126
3.5.4	Protein extraction from blister skin caps	3-127
3.5.5	Determination of protein concentration	3-127
3.5.6	SDS-PAGE and Western-blot	3-127
3.5.7	Immunohistochemistry double-staining	3-127
3.5.8	Determination of proteases activity of suction-induced blister fluid	3-128
3.5.9	Determination of proteases inhibitory activity of suction-induced blister fluid	3-128
3.5.10	Measurement of secretory leukocyte protease inhibitor (SLPI) and elafin	3-129
3.5.11	Statistical analysis	3-129
	Chapter 4: Development of an In-house Organotypic Skin-equivalent Model	4-130
4.1	Introduction: Organotypic skin-equivalent model	4-131
4.2	Chapter 4 Results	4-133
4.2.1	The expression of early and late keratinocyte differentiation markers in the epidermis of a healthy human volunteer	4-133
4.2.2	Organotypic skin-equivalent models	4-134
4.2.2.1	Method 1: The 'Transwell Technique'	4-134
4.2.2.2	Method 2: The 'Disc Technique'	4-136
4.2.2.3	Method 3: The 'Ring Technique'	4-139

4.2.2.4	Method 4: The 'Grid Technique'	4-143
4.2.3	Assessment of the barrier function of skin-equivalent model (the 'Grid Technique') using Lucifer Yellow dye penetration assay	4-147
4.2.4	Organotypic skin-equivalent model (the 'Grid Technique') in comparison to human epidermis	4-148
4.3	Chapter 4 Discussion	4-149
4.4	Chapter 4 References	4-152
4.5	Chapter 4 Materials and Methods	4-154
4.5.1	Development of suction-blisters	4-154
4.5.2	Organotypic skin-equivalent models	4-154
4.5.2.1	Method 1: The 'Transwell Technique'	4-154
4.5.2.2	Method 2: The 'Disc Technique'	4-154
4.5.2.3	Method 3: The 'Ring Technique'	4-155
4.5.2.4	Method 4: The 'Grid Technique'	4-155
4.5.3	Lucifer Yellow penetration assay	4-156
4.5.4	Immunohistochemistry	4-156
Chapter 5: Improving the Barrier Property of Organotypic Skin-equivalent Model		5-157
5.1	Introduction: It is not all about keratin aggregation	5-158
5.2	Chapter 5 Results	5-164
5.2.1	Effects of amino acids L-histidine, L-lysine and L-serine on Lucifer Yellow dye penetration into skin-equivalent models	5-164
5.2.2	Effects of metabolites of histidine, D-histidine and cytokine modulation on Lucifer Yellow dye penetration into skin-equivalent models.	5-164
5.2.3	Effects of varying the pH of the culture media on Lucifer Yellow dye penetration into skin-equivalent models	5-166
5.2.4	Immunohistochemistry for keratinocyte differentiation markers in skin-equivalent models	5-167
5.2.4.1	Effects of amino acids and their metabolites, cytokine modulation and pH on keratin 10 protein expression and distribution	5-167
5.2.4.2	Effects of amino acids and their metabolites, cytokine modulation and pH on involucrin protein expression and distribution	5-167
5.2.4.3	Effects of amino acids and their metabolites, cytokine modulation and pH on filaggrin protein expression and distribution	5-168
5.2.4.4	Effects of amino acids and their metabolites, cytokine modulation and pH on loricrin protein expression and distribution	5-168
5.3	Chapter 5 Discussion	5-173
5.4	Chapter 5 References	5-176
5.5	Chapter 5 Materials and Methods	5-178
5.5.1	Organotypic skin-equivalent model	5-178
5.5.2	Immunohistochemistry	5-178
5.5.3	Statistical analysis	5-179
Chapter 6: Exogenous Amino Acids Altering the Expression of Epidermal Barrier Proteins in Human Keratinocyte Cell Line HaCaT		6-180
6.1	Introduction: What makes a brick wall?	6-181
6.2	Chapter 6 Results	6-186
6.2.1	(pro)Filaggrin expression of HaCaT cells vs human-derived keratinocytes	6-186
6.2.2	Raised extracellular free amino acids and the expression of epidermal barrier proteins in HaCaT monolayer cell cultures	6-188
6.2.2.1	The effects of L-lysine, L-histidine and D-histidine on the	6-188

	expression of filaggrin	
	6.2.2.2 The effects of L-lysine, L-histidine and D-histidine on the expression of loricrin	6-190
	6.2.2.3 The effects of L-lysine, L-histidine and D-histidine on the expression of involucrin	6-190
6.2.3	Histidine metabolites (histamine and urocanic acid) and the expression of epidermal barrier proteins in HaCaT monolayer cell cultures	6-191
	6.2.3.1 The effects of histamine (with and without histamine antagonists) on filaggrin	6-191
	6.2.3.2 The effects of histamine (with and without histamine antagonists) on loricrin and involucrin	6-193
	6.2.3.3 The effects of <i>cis</i> -urocanic acid on filaggrin	6-195
	6.2.3.4 The effects of <i>cis</i> -urocanic acid on loricrin	6-196
	6.2.3.5 The effects of <i>cis</i> -urocanic acid on involucrin	6-196
6.2.4	L-serine and the expression of filaggrin in HaCaT monolayer cell cultures	6-197
6.2.5	pH of cell culture media and the expression of epidermal barrier proteins in HaCaT monolayer cell cultures	6-198
	6.2.5.1 The effects of varying the pH of cell culture media on filaggrin	6-198
	6.2.5.2 The effects of varying the pH of cell culture media on loricrin	6-199
	6.2.5.3 The effects of varying the pH of cell culture media on involucrin	6-199
6.2.6	Summary of results	6-200
6.3	Chapter 6 Discussion	6-200
6.4	Chapter 6 References	6-205
6.5	Chapter 6 Materials and Methods	6-208
	6.5.1 HaCaT monolayer cell cultures	6-208
	6.5.2 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western-blotting	6-208
	6.5.3 Statistical analysis	6-209
Chapter 7: A Pilot Human Study and Future Works		7-210
7.1	Introduction: Transdermal drug delivery	7-211
7.2	Chapter 7 Results	7-214
	7.2.1 AD participant	7-214
	7.2.2 Healthy control	7-217
7.3	Chapter 7 Discussion	7-219
7.4	Potential future works	7-221
	7.4.1 Potential future work (1)	7-221
	7.4.2 Potential future work (2)	7-222
	7.4.3 Potential future work (3)	7-222
	7.4.4 Potential future work (4)	7-223
	7.4.5 Potential future work (5)	7-224
7.5	Chapter 7 References	7-226
7.6	Chapter 7 Materials and Methods	7-228
	7.6.1 Pilot human study	7-228
	7.6.2 Statistical analysis	7-228
Chapter 8: Conclusion- From bedside to bench and back		8-229
Appendix		9-232
	Proforma for clinical study (Chapter 2)	9-233
	Conference publications	9-237
	Journal publication	9-243

List of Figures

<u>Figures</u>	<u>Page</u>
<u>Chapter 1</u>	
Figure 1-1 Atopic dermatitis (photographs)	1-4
Figure 1-2 Corneodesmosomes	1-11
Figure 1-3 A hypothetical model of AD	1-15
<u>Chapter 2</u>	
Figure 2-1 The structure and function of filaggrin protein	2-32
Figure 2-2 Baseline TEWL of WT-AD and <i>FLG</i> -AD	2-36
Figure 2-3 Baseline capacitance of WT-AD and <i>FLG</i> -AD	2-37
Figure 2-4 Prevalence of other concurrent atopic diseases	2-38
Figure 2-5 Genotype and family history of AD, asthma and hay fever	2-38
Figure 2-6 Genotype and family history of atopy (any of AD, asthma or hay fever)	2-38
Figure 2-7 Genotype and total serum IgE	2-39
Figure 2-8 Genotype and number of contact allergies	2-40
Figure 2-9 Correlations between age and baseline TEWL in (a) WT-AD (b) <i>FLG</i> -AD	2-41
Figure 2-10 Correlations between age and TEWL of all participants	2-41
Figure 2-11 Correlation between age and baseline skin capacitance in (a) WT-AD (b) <i>FLG</i> -AD	2-42
Figure 2-12 Correlation between age and baseline skin capacitance of all participants	2-42
Figure 2-13 Correlation between age and disease severity in (a) WT-AD (b) <i>FLG</i> -AD	2-43
Figure 2-14 Gender and baseline TEWL, based on genotype	2-44
Figure 2-15 Gender and baseline TEWL of all participants	2-44
Figure 2-16 Gender and skin Capacitance, based on genotype	2-45
Figure 2-17 Gender and baseline skin capacitance of all participants	2-45
Figure 2-18 Gender and disease severity (SASSAD), based on genotype	2-46
Figure 2-19 Gender and disease severity (SASSAD) of all participants	2-46
Figure 2-20 Baseline TEWL and co-existing presence of dry skin, based on genotype	2-47
Figure 2-21 Baseline TEWL and co-existing presence of dry skin in all participants	2-47
Figure 2-22 Baseline skin capacitance and co-existing presence of dry skin, based on genotype	2-48
Figure 2-23 Baseline skin capacitance and co-existing presence of dry skin in all participants	2-48
Figure 2-24 Baseline TEWL and co-existing asthma, based on genotype	2-49
Figure 2-25 Baseline TEWL and co-existing asthma in all participants	2-49
Figure 2-26 Baseline skin capacitance and co-existing asthma, based on genotype	2-50
Figure 2-27 Baseline skin capacitance and co-existing asthma in all participants	2-50
Figure 2-28 Baseline TEWL and co-existing hay fever, based on genotype	2-51
Figure 2-29 Baseline TEWL and co-existing hay fever in all participants	2-51
Figure 2-30 Baseline skin capacitance and co-existing hay fever	2-52
Figure 2-31 Baseline skin capacitance and co-existing hay fever in all participants	2-52
Figure 2-32 Baseline TEWL and family history of AD, based on genotype	2-53
Figure 2-33 Baseline TEWL and family history of AD amongst all participants	2-53
Figure 2-34 Baseline skin capacitance and family history of AD, based on genotype	2-54
Figure 2-35 Baseline skin capacitance and family history of AD amongst all participants	2-54

Figure 2-36	Baseline TEWL and family history of asthma, based on genotype	2-55
Figure 2-37	Baseline TEWL and family history of asthma amongst all participants	2-55
Figure 2-38	Baseline skin capacitance and family history of asthma, based on genotype	2-56
Figure 2-39	Baseline skin capacitance and family history of asthma amongst all participants	2-56
Figure 2-40	Baseline TEWL and family history of hay fever, based on genotype	2-57
Figure 2-41	Baseline TEWL and family history of hay fever amongst all participants	2-57
Figure 2-42	Baseline skin capacitance and family history of hay fever, based on genotype	2-58
Figure 2-43	Baseline skin capacitance and family history of hay fever amongst all participants	2-58
Figure 2-44	Baseline TEWL and family history of atopic condition(s) , based on genotype	2-59
Figure 2-45	Baseline TEWL and family history of atopic condition(s) amongst all participants	2-59
Figure 2-46	Baseline skin capacitance and family history of atopic condition(s), based on genotype	2-60
Figure 2-47	Baseline skin capacitance and family history of atopic condition(s) amongst all participants	2-60
Figure 2-48	Relationship between SASSAD score and self-perception of disease severity in (a) WT-AD (b) <i>FLG</i> -AD	2-61
Figure 2-49	Relationship between SASSAD score and TEWL in (a) WT-AD (b) <i>FLG</i> -AD	2-62
Figure 2-50	Relationship between SASSAD and skin capacitance in (a) WT-AD (b) <i>FLG</i> -AD	2-62
Figure 2-51	SASSAD and co-existing presence of dry skin, based on genotype	2-63
Figure 2-52	SASSAD and co-existing presence of dry skin in all participants	2-63
Figure 2-53	SASSAD and co-existing asthma, based on genotype	2-64
Figure 2-54	SASSAD and co-existing asthma in all participants	2-64
Figure 2-55	SASSAD and co-existing hay fever, based on genotype	2-65
Figure 2-56	SASSAD and co-existing hay fever in all participants	2-65
Figure 2-57	SASSAD and family history of AD, based on genotype	2-66
Figure 2-58	SASSAD and family history of AD in all participants	2-66
Figure 2-59	SASSAD and family history of asthma, based on genotype	2-67
Figure 2-60	SASSAD and family history of asthma in all participants	2-67
Figure 2-61	SASSAD and family history of hay fever, based on genotype	2-68
Figure 2-62	SASSAD and family history of hay fever in all participants	2-68
Figure 2-63	SASSAD and family history of atopic condition(s), based on genotype	2-69
Figure 2-64	SASSAD and family history of atopic condition(s) in all participants	2-69
Figure 2-65	Correlation between SASSAD and total serum IgE in (a) WT-AD (b) <i>FLG</i> -AD	2-70
Figure 2-66	Correlation between total serum IgE and TEWL in (a) WT-AD (b) <i>FLG</i> -AD	2-70
Figure 2-67	Correlation between total serum IgE and skin capacitance in (a) WT-AD (b) <i>FLG</i> -AD	2-71
Figure 2-68	Correlation between total serum IgE and the number of contact allergies in (a) WT-AD (b) <i>FLG</i> -AD	2-72
Figure 2-69	Correlation between susceptibility to physical insult and age in (a) WT-AD (b) <i>FLG</i> -AD	2-73
Figure 2-70	Correlation between susceptibility to physical insult and age (all participants)	2-73
Figure 2-71	Susceptibility to physical insult and gender, based on genotype	2-74
Figure 2-72	Susceptibility to physical insult and gender amongst all participants	2-74
Figure 2-73	Susceptibility to physical insult and genotype	2-75
Figure 2-74	Relationship between susceptibility to physical insult and baseline TEWL(all participants)	2-75
Figure 2-75	Relationship between susceptibility to physical insult and baseline skin capacitance(all participants)	2-76

Figure 2-76	Relationship between susceptibility to physical insult and SASSAD in (a) WT-AD (b) <i>FLG</i> -AD	2-77
Figure 2-77	Relationship between susceptibility to physical insult and SASSAD (all participants)	2-77
Figure 2-78	Susceptibility to physical insult and co-existing presence of dry skin, based on genotype	2-78
Figure 2-79	Susceptibility to physical insult and co-existing presence of dry skin amongst all participants	2-78
Figure 2-80	Susceptibility to physical insult and co-existing asthma, based on genotype	2-79
Figure 2-81	Susceptibility to physical insult and co-existing asthma amongst all participants	2-79
Figure 2-82	Susceptibility to physical insult and co-existing hay fever, based on genotype	2-80
Figure 2-83	Susceptibility to physical insult and co-existing hay fever amongst all participants	2-80
Figure 2-84	Susceptibility to physical insult and family history of AD, based on genotype	2-81
Figure 2-85	Susceptibility to physical insult and family history of AD amongst all participants	2-81
Figure 2-86	Susceptibility to physical insult and family history of asthma, based on genotype	2-82
Figure 2-87	Susceptibility to physical insult and family history of asthma amongst all participants	2-82
Figure 2-88	Susceptibility to physical insult and family history of hay fever, based on genotype	2-83
Figure 2-89	Susceptibility to physical insult and family history of hay fever amongst all participants	2-83
Figure 2-90	Susceptibility to physical insult and family history of atopic condition(s), based on genotype	2-84
Figure 2-91	Susceptibility to physical insult and family history of atopic condition(s) amongst all participants	2-84
Figure 2-92	Correlation between susceptibility to physical insult and total serum IgE in (a) WT-AD (b) <i>FLG</i> -AD	2-85
Figure 2-93	Correlation between susceptibility to physical insult and total serum IgE amongst all participants	2-85
Figure 2-94	Skin reaction to increasing concentrations of SDS	2-86
Figure 2-95	Change in TEWL post-SDS application	2-87
Figure 2-96	Change in skin fold thickness post-SDS application	2-87
Figure 2-97	Measurement of TEWL	2-97
Figure 2-98	Measurement of skin capacitance	2-98
Figure 2-99	Tape-stripping	2-99
Figure 2-100	SDS-irritancy study	2-100
 <u>Chapter 3</u>		
Figure 3-1	Degradation of filaggrin into free amino acids	3-105
Figure 3-2	Comparison of (pro)filaggrin protein expression between HC with wild type <i>FLG</i> and AD participants by Western-blotting	3-111
Figure 3-3	Immunohistochemical staining of suction-induced blister skin caps for filaggrin and keratin	3-112
Figure 3-4	Cryopreservation of neutrophil elastase activity by sucrose, mannitol and polyethylene glycol-400 (PEG-400), compared to control (Tris HCL buffer)	3-113
Figure 3-5	Comparison of the protease inhibition profile between HC and AD	3-115
Figure 3-6	Silver staining of proteins in SDS-polyacrylamide gradient gels (6-15%)	3-116

Chapter 4

Figure 4-1	Representative images of epidermal samples obtained from one healthy human volunteer by suction-blistering technique	4-133
Figure 4-2	Calcium is required for keratinocyte differentiation	4-134
Figure 4-3	Diagrammatic representation of the 'Transwell Technique' and photographs demonstrating the technique	4-135
Figure 4-4	Immunohistochemistry (transverse sections) of organotypic skin-equivalent model (the 'Disc Technique')	4-137
Figure 4-5	A representative image of a transverse section of the skin model (the 'Disc Technique')	4-138
Figure 4-6	Diagrammatic representation of the 'Disc Technique'	4-138
Figure 4-7	x10 magnification of transverse sections of skin-equivalent models (Day 7, 13 and 17, the 'Ring Technique')	4-140
Figure 4-8	x100 magnification of transverse sections of skin-equivalent model (the 'Ring Technique')	4-141
Figure 4-9	The 'Ring Technique'	4-142
Figure 4-10	Haematoxylin and eosin staining (transverse sections) of organotypic skin-equivalent model (the 'Grid Technique')	4-143
Figure 4-11	Keratinocyte differentiation markers at Day 12 (the 'Grid Technique')	4-144
Figure 4-12	Keratinocyte differentiation markers at Day 19 (the 'Grid Technique')	4-144
Figure 4-13	Keratinocyte differentiation markers at Day 26 (the 'Grid Technique')	4-145
Figure 4-14	The 'Grid Technique'	4-146
Figure 4-15	Lucifer Yellow dye penetration assay (photograph)	4-147
Figure 4-16	Lucifer Yellow dye penetration assay	4-147

Chapter 5

Figure 5-1	The amino acid composition of filaggrin vs the average composition in a protein	5-159
Figure 5-2	Histidine	5-160
Figure 5-3	The putative 'beneficial' and 'detrimental' effects of urocanic acid, UCA in the skin	5-161
Figure 5-4	Comparison of the effects of free amino acids L-histidine, L-lysine and L-serine vs controls on penetration of Lucifer Yellow dye into the skin models	5-164
Figure 5-5	Comparison of the effects of histidine metabolites, D-histidine and cytokines on penetration of Lucifer Yellow dye into the skin models	5-165
Figure 5-6	Comparison of the effects of different pHs on Lucifer Yellow dye penetration into the skin models	5-166
Figure 5-7	Representative images comparing the effects of extracellular, free amino acids and their metabolites, cytokine modulation and pH on keratin 10 expression in organotypic skin-equivalent model	5-167
Figure 5-8	Representative images comparing the effects of extracellular, free amino acids and their metabolites, cytokine modulation and pH on the involucrin expression in organotypic skin-equivalent model	5-168
Figure 5-9	Representative images comparing the effects of extracellular, free amino acids and their metabolites, cytokine modulation and pH on on filaggrin expression in organotypic skin-equivalent model	5-168
Figure 5-10	Representative images comparing the effects of extracellular, free amino acids and their metabolites, cytokine modulation and pH on loricrin expression in organotypic skin-equivalent model	5-168

Chapter 6

Figure 6-1	Differences in (pro)filaggrin expression between a human-derived sample and keratinocyte cell line HaCaT	6-187
Figure 6-2	Comparisons of the effects of L-lysine, L-histidine and D-histidine on the expression of profilaggrin and filaggrin	6-189
Figure 6-3	Comparisons of the effects of exogenous, free amino acids L-lysine, L-histidine and D-histidine on loricrin protein expression	6-190
Figure 6-4	Comparisons of the effects of exogenous, free amino acids L-lysine, L-histidine and D-histidine on involucrin protein expression.	6-191
Figure 6-5	The effects of 1 μ M histamine on filaggrin protein expression	6-192
Figure 6-6	The effects of 1 μ M histamine, with and without H1+H2 receptor antagonists (pyrilamine maleate and cimetidine), on filaggrin protein expression	6-192
Figure 6-7	The effects of 1 μ M histamine on loricrin and involucrin protein expression.	6-193
Figure 6-8	The effects of histamine, with and without H1+H2 receptor antagonists (pyrilamine maleate and cimetidine), on loricrin protein expression	6-193
Figure 6-9	The effects of histamine, with and without H1+H2 receptor antagonists (pyrilamine maleate and cimetidine), on involucrin protein expression	6-194
Figure 6-10	The effects of <i>cis</i> -urocanic acid, <i>cis</i> –UCA, on filaggrin protein expression	6-195
Figure 6-11	The effect of <i>cis</i> -urocanic acid on loricrin protein expression	6-196
Figure 6-12	The effect of <i>cis</i> -urocanic acid on involucrin protein expression	6-196
Figure 6-13	The effect of increasing concentrations of exogenous, free serine on filaggrin protein expression	6-197
Figure 6-14	Comparisons of the effects of different pHs of the cell culture media on filaggrin protein expression	6-198
Figure 6-15	Comparisons of the effects of different pHs of the cell culture media on loricrin protein expression	6-199
Figure 6-16	Comparisons of the effects of different pHs of the cell culture media on involucrin protein expression	6-199

Chapter 7

Figure 7-1	Comparison of the effects of different topically applied amino acids on skin barrier recovery following disruption of the physical barrier by tape-stripping in a single AD participant	7-216
Figure 7-2	Comparison of the effects of different topically applied amino acids on skin barrier recovery following disruption of the physical barrier by tape-stripping in a single healthy control	7-218
Figure 7-3	Application of amino acid solutions onto the skin	7-228

Chapter 8

-

List of Tables

<u>Tables</u>		<u>Page</u>
<u>Chapter 1</u>		
Table 1-1	The Hanifin and Rajka Diagnostic Criteria for AD	1-5
Table 1-2	The U.K. Working Party's Diagnostic Criteria for Atopic Dermatitis	1-6
Table 1-3	Differential diagnosis for AD	1-7
Table 1-4	Examples of topical steroids of different potencies	1-18
Table 1-5	Treatment options for AD	1-20
<u>Chapter 2</u>		
Table 2-1	Demographic data of AD participants	2-35
Table 2-2	Frequency of <i>FLG</i> null alleles amongst the study participants	2-35
Table 2-3	Number of contact allergies and the corresponding IgE level of WT-AD and FLG-AD	2-40
Table 2-4	Main findings from the clinical study: Comparisons between WT-AD and FLG-AD	2-88
Table 2-5	The U.K. Working Party's Diagnostic Criteria for Atopic Dermatitis	2-96
Table 2-6	Visual scoring system for the skin reaction to SDS solution	2-100
<u>Chapter 3</u>		
Table 3-1	Skin disorders caused by protease-antiprotease imbalances	3-107
Table 3-2	Genotypes of study participants	3-109
Table 3-3	Comparison of overall epidermal skin cap thickness and filaggrin layer, of HC and AD participants	3-113
Table 3-4	Comparison of the levels of protease inhibitors between HC and AD participants	3-116
<u>Chapter 4</u>		
Table 4-1	Comparisons between skin model (the 'Grid Technique') and native human epidermis	4-148
<u>Chapter 5</u>	-	
<u>Chapter 6</u>		
Table 6-1	Chemical composition of natural moisturising factor (NMF)	6-183
Table 6-2	Summary of results	6-200
<u>Chapter 7</u>		
Table 7-1	Calculations for 'Percentage correction to baseline TEWL'	7-216
<u>Chapter 8</u>	-	

List of Abbreviations

Greek Notations

α	alpha
ε	epsilon

Units

$^{\circ}$	Degree
$^{\circ}\text{C}$	Celcius
cm	Centimetre
μm	Micrometre
mm	Millimetre
%	Percent

Acronyms

2D	Two-dimensional
3D	Three-dimensional
AD	Atopic dermatitis
AU	Arbitrary unit
CE	Cornified cell envelope
EDC	Epidermal differentiation complex
FH	Family history
<i>FLG</i>	Filaggrin gene
<i>FLG-AD</i>	Atopic dermatitis patients with <i>FLG</i> mutations
<i>FLG +/-, -/-</i>	<i>FLG-AD</i> who are heterozygous(+/-) or homozygous (-/-) for <i>FLG</i> mutations
HaCaT	Human adult low-calcium high temperature keratinocytes (cell line)
HC	Healthy controls
HDF	Human dermal fibroblasts
HDFa	Adult human dermal fibroblasts
HF	Hay fever
IV	Ichthyosis vulgaris
K-S	Kolmogorov-Smirnov
LEKTI	Lymphoepithelial Kazal-type-related inhibitor

M	Mean
n	Number of participants involved
NMF	Natural moisturizing factors
n.s.	Not significant
OD	Optical density
OSCORAD	Objective score for atopic dermatitis
PEG	Polyethylene glycol
PBS	Phosphate-buffered saline
SASSAD	Six Area, Six Sign Atopic Dermatitis Severity Score
SCCE	Stratum corneum chymotryptic enzyme
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SFT	Skin fold thickness
SLPI	Secretory leukocyte protease inhibitor
TEWL	Transepidermal water loss
TGases	Transglutaminases
TS	Tape-stripping
UCA	Urocanic acid
UVR	Ultraviolet radiation
WT	Wild-type
WT-AD	Wild type patients with no known <i>FLG</i> mutations.

The Thesis: An Introduction

Atopic dermatitis, AD, is a chronic inflammatory skin disease. It affects up to 30% of children worldwide and is a significant source of morbidity. Yet, for such a common and important disease, it is hard to believe that we are yet to uncover its exact pathogenesis. Is it complex beyond our ability to discover and comprehend or is it because it is rarely a cause of mortality?

In AD, the skin no longer serves as an effective mechanical and chemical barrier which is meant to keep the outside world out and the inside world in. The thesis is dedicated to improving our understanding on how AD may develop and to propose ways to improve the skin barrier function.

Thesis Scope

The fact that AD rarely kills should not make it less attractive from research funding point of view. It is important to those with the disease because of the incurred medical, financial and social burden. It is also important to those not directly affected by the disease because AD is common and is becoming increasingly so, thus posing a huge burden to the healthcare system. To appreciate the significance of the disease, a general understanding of its clinical presentation, epidemiology, pathogenesis, prognosis and treatment is required (Chapter 1).

Genetic mutations of the epidermal barrier protein filaggrin are found to be strongly associated with the disease. This is an important finding because it brings us a step closer to understanding the cause of the disease and therefore, better treatment methods. It is important to know the clinical implications of having such mutations because a genetic abnormality that bears no clinical significance is probably not a priority for research, especially in such economic climate. Much effort has therefore been spent over the last years in examining the relationships between filaggrin genotype with phenotypic traits of patients in order to understand the physiological significance of having such mutations (Chapter 2). The results were presented at the 2009 European Society for Dermatological Research Annual Meeting by the Lead Investigator of this clinical study, Dr S Abdul Ghaffar, who led the project under the supervision of Consultant Dermatologist Dr R Weller.

My participation in the clinical study and increased understanding of the filaggrin protein led me to seek other potential modifying factors that are involved in the clinical manifestations of the disease (Chapter 3). The delicate balance of the protease-antiprotease system in the skin is important in facilitating epidermal physiology and skin barrier function. In relation to AD, I am interested in the proteolytic processing of the filaggrin proprotein into functional filaggrin peptides. This is the focus of Chapter 3. In a recent publication in the British Journal of Dermatology, we highlighted abnormal proteolysis as a potential cause of AD in patients with a normal filaggrin gene.

AD, as we know it, is characterised by a defective skin barrier. The remaining chapters are dedicated to improving the skin barrier function. In Chapter 4, I describe the setting-up of an in-house organotypic skin-equivalent model, which allows us to ethically perform pharmacological intervention studies. This is followed by results of various experimental studies aimed at improving the barrier function of our skin model, such as manipulation of the amino acid composition and pH of the cell culture media (Chapter 5).

Development of a competent skin barrier is dependent on the appropriate expression of keratinocyte differentiation proteins such as filaggrin, loricrin and involucrin at the right time and place. Chapter 6 is an extension of Chapter 5, where we investigated if the pharmacological interventions shown to have improved the barrier function of our skin model directly affect the expression of these differentiation proteins. Based on the results of our *in-vitro* studies, we identified several factors which may lead to improvement in epidermal barrier function.

As a medical doctor, I am naturally inclined to incorporate elements of translational medicine into my PhD project. My team and I sought to obtain a proof of principle, by conducting a pilot human study, for the effectiveness of our proposed 'treatment' aimed at improving skin barrier function (Chapter 7). The encouraging results highlight the potential for commercialisation of this 'treatment'. Larger clinical studies are, however, required before a definitive conclusion can be made regarding its efficacy.

Finally, I provide a general overview of all the chapters in this thesis in Chapter 8, summarising the key findings or discussion points of each chapter.

Novel contributions to the field of atopic dermatitis

1. The first clinical study in a European population which showed significant differences in skin barrier function between wild-type and filaggrin-related AD patients (Chapter 2).
2. Defective proteolysis of profilaggrin in some AD patients with a normal filaggrin gene was demonstrated. This provides new pathomechanistic insights into the development of the disease in wild-type AD patients (Chapter 3).
3. Imbalances in the skin protease-antiprotease system in AD patients were demonstrated. Abnormally raised protease inhibitor activities in AD patients were found (Chapter 3). This may interfere with the proteolytic processing of (pro)filaggrin.
4. The potential benefits of amino acid histidine in improving the barrier function of an in-house organotypic skin model was demonstrated (Chapter 5). This finding is of potential therapeutic significance and may, in the future, be developed as a viable alternative to current treatment methods of AD.

List of Publications

Conference publications

1. Tan, S.P., Brown, S.B., Weller, R. A pilot study on skin proteases and their inhibitors. *Br. J. Dermatol.* **160**: 917-8 (2009)
2. Abdul Ghaffar, S., Tan, S.P., Campbell, L.E., Lee, L.H., Sergeant, A., McLean, W.I., Weller, R. The effects of filaggrin mutations on barrier function in eczema patients. *J Invest Dermatol.* **129**: S17 (2009)
3. Tan, S.P., Abdul Ghaffar, S., Campbell, L.E., McLean, I., Weller, R., Brown, S.B. An imbalance of protease and antiprotease activities in the skin of eczema patients. *J Invest Dermatol.* **129**: S19 (2009)
4. Tan, S.P., Weller, R., Brown, S.B. Protease–antiprotease imbalance: a potential confounder in the pathogenesis of eczema. *Br. J. Dermatol.* **162**: 909–50 (2010)
5. Tan, S.P., Weller, R., Brown, S.B. Histidine increases the expression of filaggrin protein and improves barrier function. *J Invest Dermatol.* **131**: S39 (2011)
6. Abdul-Ghaffar, S. *et al.* Filaggrin mutations are associated with altered epidermal barrier and antigen presenting cell immunophenotypes in atopic eczema patients. *J Invest Dermatol.* **131**: S54 (2011)

Journal publications

1. Tan, S.P., Abdul-Ghaffar, S., Weller, R. B. & Brown, S. B. Protease-antiprotease imbalance may be linked to potential defects in profilaggrin proteolysis in atopic dermatitis. *Br. J. Dermatol.* **166**, 1137–1140 (2012)
2. Tan, S.P. *et al.* Beneficial effects of oral L-histidine in atopic dermatitis. (manuscript in preparation)
3. Abdul-Ghaffar, S. *et al.* Impaired barrier function and elevated immuno-competent dendritic cells found in healthy skin of filaggrin null-mutation carriers. (manuscript in preparation)

Chapter 1

Atopic Dermatitis

Atopic dermatitis is a chronic inflammatory skin disease with a rising incidence worldwide. It has significant medical, financial and social implications to the patients, their families and the healthcare providers. Here, a brief overview of this common disease is given. We discuss its clinical presentation, epidemiology, pathogenesis and current management.

1.1 Introduction

Atopic dermatitis, AD^{1,2} is a chronic inflammatory skin condition characterised by intense pruritus (itch), erythematous lesions, increased water loss through the skin and xerosis (dry skin). It affects between 10 to 20% of children and 1 to 3% of adults³ worldwide and has adverse medical, financial and social impacts on the patients and their families. 85% of affected children develop the disease before the age of 5 years (60% before the age of 1)^{4,5}. Although many eventually grow out of the condition (improvement during puberty is a common phenomenon), it may persist into or manifest for the first time in adulthood². There are at least two types of AD: extrinsic and intrinsic⁶. 70-80% of AD patients are said to have the former which is associated with IgE-mediated sensitisation. It has been linked to immune hyperactivity and is characterised by raised serum IgE level. The other 20-30% of patients do not, at least initially, have raised IgE level.

AD is part of a family of Th-2 driven atopic diseases and is often the first of these disorders to manifest itself. 50% of patients with childhood AD develop asthma and two-thirds develop allergic rhinitis at a later stage^{7,8}. This phenomenon is known as the 'atopic march'. Patients with AD are also more likely to develop irritant and allergic contact dermatitis than the general population. The word 'atopy' was introduced by Coca and Cooke in 1923⁹, referring to an abnormal level of sensitiveness, the mechanism of which is uncertain. It originates from the Greek word 'atopia' which means 'unusualness or strangeness'⁹. The original definition 'atopy' by Coca and Cooke included only asthma and allergic rhinitis; AD was later added onto the definition. AD has been mentioned repeatedly throughout the history of medicine. One of the first documented sufferers of AD is the Roman emperor Octavianus Augustus, described by Suetonius in *De Vita Caesarum* ('The Twelve Caesars'). He was described to suffer from '*extremely itchy skin, seasonal rhinitis and tightness of the skin.*' Centuries of dermatological research have brought significant advances to the understanding and treatment of the disease. However, the cause of the disease has remained somewhat elusive. It is generally regarded as the result of complex interactions between genetics, environment, skin barrier dysfunction, immunological defects and recurrent cutaneous infections.

1.2 Clinical Presentation

The clinical picture of AD varies widely from minor xerosis to a severe, acute erythrodermic rash (Figure 1) or the lichenification commonly seen in chronic disease. Nonetheless, there are several major features by which AD can be identified. The classical picture of AD is one that consists of dry, pruritic, lack-lustre skin, with chronic or relapsing lesions that have a typical distribution and appearance².

Pruritus

If it is not itchy, it is not atopic dermatitis.

-R. Weller

Pruritus is seen in virtually all AD patients and is easily triggered by, for example, stress, sweating, presence of allergens and changes in the humidity². It is often worse in the evening or at night, though it may occur throughout the entire day². Scratching and picking, over prolonged period of time, result in lichenification and/or prurigo nodularis.

Acute, subacute and chronic erythematous lesions

Acute and subacute lesions are more commonly seen in younger children. Here, erythematous papulovesicles and signs of excoriations are seen. The skin weeps serous exudates. Some patients may not display papulovesicular lesions; however, histological examinations will reveal spongiosis. In long-standing cases, signs of excoriations and lichenification due to repetitive scratching are seen, instead of red, weepy, erythematous papules.

Distribution

The distribution and skin reaction pattern of AD are influenced by the disease activity and the patient's age². In infancy, AD typically affects the head (face and scalp) and extensor surfaces of the hands and legs. Beyond infancy, AD has the tendency to affect the flexural folds of the extremities. In adults, the distribution of the skin lesions may be rather patchy in nature. Many adults have hand dermatitis as their primary complaint.

Recurrent skin infections

AD patients suffer from recurrent, sometimes severe bacterial and viral infections of their skin^{10,11}. Eczema herpeticum (caused by herpes simplex virus) is known to occur primarily in AD patients¹². 80-100% of AD patients have colonization with *Staphylococcus aureus* on their skin (which often consists of a heterogenous mixture¹³), compared to only 5-30% of the normal population^{14,15}. This may escalate rapidly into severe bacterial superinfection as more than 50% of *Staphylococcus aureus* isolates from AD patients were found to produce one or more superantigenic toxins¹³. *Staphylococcus aureus* colonization and infection influence the chronicity and exacerbation of AD by

producing strong activating signals to T-cells and macrophages, augmenting the inflammatory response in the skin¹⁶⁻²⁰. Studies have shown that in those with infected AD, treatment with glucocorticoid and topical antibiotics for *Staphylococcus aureus* yields better results, compared to using glucocorticoid alone²¹. Reducing the bacterial count on the skin also leads to significant improvement in the clinical severity of the disease²².

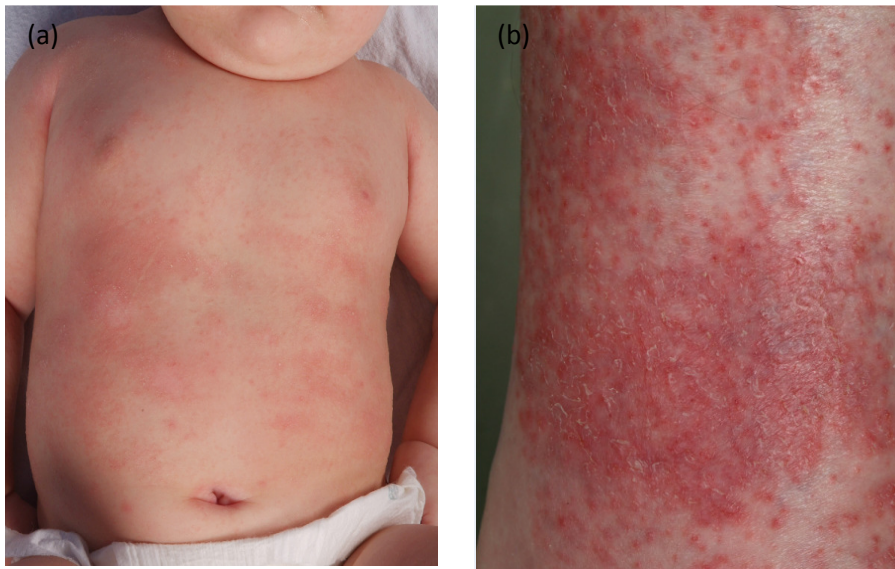


Figure 1.
Atopic dermatitis
(photographs courtesy
of Dr R. Weller):
Pruritic, erythematous
lesions with exudates
affecting the (a) trunk
of an infant (b) lower
leg of a child.

1.2.1 Diagnostic criteria

AD is a disease diagnosed based on the clinical presentation rather than laboratory tests. An experienced clinician looks for: 1) the essential features of AD, i.e. a pruritic dermatitis 2) typical features, such as a classical distribution pattern 3) features frequently associated with AD such as a strong family history of atopy and 4) Other less common but known associated features such as infraorbital darkening²³. Skin prick tests or specific IgE measurements have questionable sensitivity and specificity and are not used for diagnostic purposes. An experienced clinician may have no difficulty in identifying AD. This may not be the case for less experienced doctors or lay persons. A set of criteria which helps to define AD is therefore needed. It also confers a degree of uniformity required to facilitate effective communication within the scientific community. This will allow comparisons to be made and conclusions drawn from population studies consisting of heterogeneous groups of patients from different countries or continents.

It is, however, an extremely challenging task to draw a set of criteria to define a disease which shows such significant variability in time, morphology and distribution, in the absence of a laboratory reference standard²⁴. AD is not an all-or-nothing phenomenon. Symptoms vary widely between individuals. Some individuals may have no significant skin problem at all, yet a high level of

total serum IgE; some may have pruritus as the only complaint; others may have a dry skin with occasional cutaneous infections. There is no absolute cut-off point between diseased and non-diseased states.

Based on the 24 commonest clinical signs and symptoms of AD, Hanifin, Lobitz and Rajka proposed a set of major and minor diagnostic criteria for AD^{1,25,26} (Table 1, adapted from 'Atopic Dermatitis: The Epidemiology, Causes and Prevention of Atopic Eczema'²⁴).

Table 1

The Hanifin and Rajka Diagnostic Criteria for AD

Must have three or more of the following major features:

- Pruritus
- Typical morphology or distribution: facial and extensor involvement in children; flexural involvement in adults.
- Personal or family history of atopic diseases (AD, asthma or allergic rhinitis)
- Chronic or chronically relapsing dermatitis

Plus three or more of the following minor features:

- Early age of onset
 - Xerosis
 - Ichthyosis/ palmar hyperlinearity/ keratosis pilaris
 - Sweat-related pruritus
 - Raised serum IgE
 - Type I skin hypersensitivity reaction
 - Intolerance to wool and lipid solvents
 - Food intolerance
 - Recurrent cutaneous infections
 - Non-specific hand and foot dermatitis
 - Recurrent conjunctivitis
 - Dennie-Morgan infraorbital fold
 - Infraorbital darkening
 - Keratoconus
 - Anterior subcapsular cataracts
 - Facial pallor or erythema
 - Cheilitis
 - Nipple eczema
 - Perifollicular accentuation
 - Pityriasis alba
 - Anterior neck fold
 - White demographism or delayed blanch to cholinergic agents
 - Course of disease influenced by emotional or environmental factors
-

While this set of diagnostic criteria has a high specificity for AD in hospital settings and represents a major step forward in defining the disease, it is unsuitable for population-based studies or as a diagnostic aid in primary care²⁷⁻³¹. This is partly due to the fact that the features included in the criteria were drawn from predominantly white hospital in-patients. Some of these features used to define AD are rather poorly defined themselves³², such as pityriasis alba; some features are relatively rare, for example keratoconus^{33,34} while many are non-specific to the disease, for example, food intolerance. In addition, the list is too long to be remembered easily. Its usage is also limited by a high degree of intra- and inter-observer variation.

However, this seminal work by Hanifin, Lobitz and Rajka served as a foundation stone for Williams HC *et al* who formulated a much simplified set of diagnostic criteria for AD known as The U.K. Working Party's Diagnostic Criteria for Atopic Dermatitis³⁵⁻³⁷ (Table 2). This set of diagnostic criteria has been shown to have an acceptable sensitivity of 85% and specificity of 96% in an independent validation study conducted on 114 consecutive children attending out-patient dermatology clinics³⁷. It is easy to use, takes less than 2 minutes to perform and patients are not required to undress³⁷. In short, it is a reasonable good diagnostic aid for AD in primary care and out-patient settings.

Table 2

The U.K. Working Party's Diagnostic Criteria for Atopic Dermatitis³⁵

Must have: An itchy skin condition in the last 12 months

Plus three or more of the following:

- Onset under the age of 2 years*
- History of flexural involvement
- History of a generally dry skin
- Personal history of other stopic disease(s)**
- Visible flexural dermatitis as per photographic protocol

* *not used in children under 4 years*

***in children aged under 4 years, history of atopic disease(s) in a first degree relative may be included*

An alternative diagnosis should be sought if the lesions do not fit the diagnostic criteria or when a patient does not respond to the standard treatment given. There are many diseases that may present in a similar manner to AD (Table 3). If in doubt, a skin biopsy should be considered.

Table 3

Differential diagnosis for AD (adapted^{2,23})

Chronic dermatoses

- Examples: contact (allergic or irritant) dermatitis, seborrhoeic dermatitis, psoriasis, ichthyosis vulgaris

Infections and infestations

- Examples: scabies, fungal infections, human immunodeficiency virus-associated dermatitis.

Malignancies

- Examples: cutaneous T-cell lymphoma (Sézary Syndrome), Letterer-Siwe disease

Immunodeficiencies:

- Examples: Wiskott-Aldrich, Hyper-IgE Syndrome, severe combined immunodeficiency.

Immunological diseases:

- Examples: dermatitis herpetiformis, dermatomyositis, pemphigus foliaceus.

Metabolic disorders:

- Examples: phenylketonuria, zinc deficiency.

Congenital disorders:

- Examples: familial keratosis pilaris, Netherton's Syndrome
-

1.3 Epidemiology

Over the last 40 years, the incidence of AD has risen, perhaps by 2 to 3 folds, in developed or industrialised nations³⁸⁻⁴³ while remaining low in agricultural nations. The prevalence also appears to be higher in the urban areas, compared to rural in developed nations and more common amongst those from higher social classes⁴⁴. Many of the earlier works on the prevalence of AD were done in Northern Europe, UK, Australia and New Zealand, hence giving the impression that it is a disease that mainly affects developed nations with cooler climates⁴⁵, due to the so-called 'western lifestyle' (small family size, higher income and level of education and better access to healthcare)⁴⁶.

In 1991, the International Study of Asthma and Allergies in Childhood (ISAAC), an epidemiological research programme, was established amidst worries that asthma, allergic rhinoconjunctivitis and AD were increasing significantly in prevalence and severity. Yet, the global scale of the disease especially that of developing nations, remained unknown. It was an enormous undertaking carried out over a decade, involving many developed and developing nations.

In ISAAC Phase One⁴⁷, a total of 700,000 children from 156 centres in 56 countries were recruited into the study. It revealed a 1-year prevalence ranging from less than 5% in Eastern Europe and Central Asia and higher than 15% in Northern and Western Europe, Australia, the Baltics and urban Africa. A slight female preponderance especially in countries with the highest prevalence of AD was noted, with an overall female to male ratio of 1.3: 1.0. The high prevalence in developed

countries such as Japan, United Kingdom and New Zealand was not surprising. However, the high prevalence in African cities such as Addis Ababa showed that AD is a major public health problem in developing countries as well. There were also wide variations found in the prevalence of AD both within and between countries inhabited by genetically similar people, i.e. people of similar ethnicity, for example, between East and West Germany⁴⁸. The data from this study showed that AD is a major public health problem globally and suggest that environmental factors, such as economic development, climate, dietary factors may be critical in the driving the disease expression.

In ISAAC Phase Two⁴⁹, the investigators attempted to identify the causes for the differences in prevalence rates in different countries by studying selected populations in 30 centres from 22 countries. A questionnaire coupled with clinical examination and several measurements of physiological variables including skin prick and blood tests for IgE analyses and genotyping were done. Genetic-environment interactions were examined; household endotoxins and house dust mite antigen measurements were also performed. Phase Two found little evidence to support the effects of genetic variations on asthma symptoms in worldwide populations⁵⁰. It also showed that most asthma, hay fever and AD have a non-allergic basis⁵¹⁻⁵⁴.

ISAAC Phase Three^{55,56}, a repetition of ISAAC Phase One, was done 5-10 years after the initial survey to look at the changing global trend in the prevalence of the disease. It was hoped that by revealing the geographical variations in the changing prevalence of these allergic diseases, the causes for AD may be identified. Williams *et al*⁵⁶ reported on the comparison of the prevalence of AD between ISAAC Phase One and Phase Three, which involved 187,943 children aged 6 to 7 years from 64 centres in 35 countries and 302,159 children aged 13 to 14 years from 105 centres in 55 countries. Globally, a significant increase in the prevalence of AD over the 5-10 years was found, more so in children aged 6-7 than those in the 13-14 category. Several hypotheses were put forward to explain this, including better treatment over time in the older children or earlier pubescence (it is known that AD sometimes gets better upon reaching puberty)⁵⁶. The study also found that although the prevalence of the disease in most countries has increased over the 5-10 years, especially amongst the 6-7 year olds, some countries with previously very high disease prevalence such as the United Kingdom were showing signs of plateau. A prevalence plateau of 20% was seen in the study, raising the rather reassuring possibility that there is a finite pool of people within any population that can develop AD given the 'right' (or wrong) conditions. There is perhaps a genetic predisposition, when coupled with appropriate environmental triggers, leads to the manifestation of the disease.

1.4 Pathogenesis of Atopic Dermatitis

Despite the great amount of attention received by this extremely common disease, the pathogenesis of AD has remained somewhat unclear. Whether AD is a condition, a disease or a syndrome is still very much a debatable topic.

There are two major schools of thought when it comes to the pathogenesis of AD: 1) The 'Outside-Inside' Hypothesis and 2) The 'Inside-Outside' Hypothesis.

The 'Outside-Inside' Hypothesis proposes that a defective skin barrier is the primary event in the development of AD. The defective barrier allows easier penetration of antigens and microbes which trigger the immune system. The resultant inflammation in turn causes worsening of the barrier function and increases the severity of the disease. Other atopic diseases (asthma, allergic rhinitis, chronic AD) may occur at a later stage as a secondary event due to hypersensitization of the immune system, possibly precipitated by other forms of allergens.

The 'Inside-Outside' Hypothesis proposes otherwise. The abnormally 'sensitive' immune system mounts a disproportionately high response to what is usually an insignificant level of allergens. The severe inflammation leads to the skin being unable to function as an effective barrier, which in turn allows more antigens and microbes to penetrate through the skin into the body, perpetuating the immune system activation.

Skin barrier defects

Historically, immune hyperactivity has been regarded as the primary event in AD. Nonetheless, it is known that many AD patients have normal IgE level and do not display hyperactivity of the immune responses (intrinsic AD). This prompts scientists to look for causes beyond allergy. Elias⁵⁷ and Taieb⁵⁸ were one of the first to suggest that skin barrier defect(s) may be the primary cause of AD in the late 1990s. Xerosis and defective barrier facilitate interactions between antigen presenting and immune effector cells in the skin with environmental allergens. This may explain why 80% of the initially non-atopic AD patients subsequently develop raised IgE level and other atopic diseases^{59,60}. Experimentally-induced barrier dysfunction has been shown to lead to cytokines production, such as IL-1 α ⁶¹ and tumour necrosis factor- α ⁶².

A defective skin barrier can be caused by many factors. Deficiency of the epidermal barrier protein filaggrin due to genetic mutations is one of them. This has been mentioned earlier and will be extensively explored in subsequent chapters. Exogenous proteases from house dust mite Der p 1 and Der p 2 and those from the colonizing *Staphylococcus aureus* can degrade the corneodesmosomes, the protein structures which lock the keratinocytes together by binding to intracellular keratin filaments of adjacent cells (Figure 2). There are also endogenous skin proteases

such as the human kallikrein-related peptidases that can degrade the corneodesmosomes - the key one being stratum corneum chymotryptic enzymes (SCCE, kallikrein-7). Genetic variations of the SCCE gene⁶³, as will be mentioned below, may lead to inappropriately increased activity of the enzyme. Secondary proteases produced by cells from the inflammatory infiltrate can do the same, such as mast cell chymase, a chymotrypsin-like serine protease found in abundance in lesional AD skin⁶⁴.

Under physiological condition, kallikrein peptidases including the SCCE hydrolyse the corneodesmosin and desmocollin 1 within the corneodesmosomes to facilitate physiological desquamation. Desquamation allows the cells at the surface of the epidermis to be shed and replaced from underneath by keratinocytes undergoing proliferation and differentiation. The activity of these proteases is tightly regulated by pH (serine proteases work optimally in a slightly alkaline environment) and protease inhibitors such as elafin and lymphoepithelial Kazal-type-related inhibitor (LEKTI). If there is an imbalance between removal of the 'old' keratinocytes and keeping the epidermis physically intact, derangement of the skin barrier function occurs. The use of soap and detergent affect this delicate balance by increasing the natural pH of the skin from 5.5 to around 7.5. The natural pH of the skin on the lower arm of a healthy adult Caucasian male ranges from 5.4 to 5.9⁶⁵. This 'acid mantle' is maintained by endogenous pathways, such as the generation of urocanic acids from the breakdown of filaggrin and fatty acids from phospholipid hydrolysis. The low pH is not only optimal for maintaining permeability barrier homeostasis, it is also antimicrobial⁶⁶, reduces colonisation by pathogens^{66,67} and facilitates the binding of non-pathogenic bacteria to the skin⁶⁸. In the UK, £76 million was spent on personal use of soap or detergent while 11L of water per day was spent for personal washing from 1960 to 1981. In comparison, £453 million (inflation adjusted) was spent on soap or detergent and 51L of water per day was used for personal washing from 1995 to 2001⁶⁹. Excessive usage of soap and detergent not only create an alkaline environment suitable for the degradation of corneodesmosomes by SCCE, they also remove the ceramides and other natural moisturising factors which maintain skin hydration. The final result is a dry, barrier-defective skin with enhanced water loss.

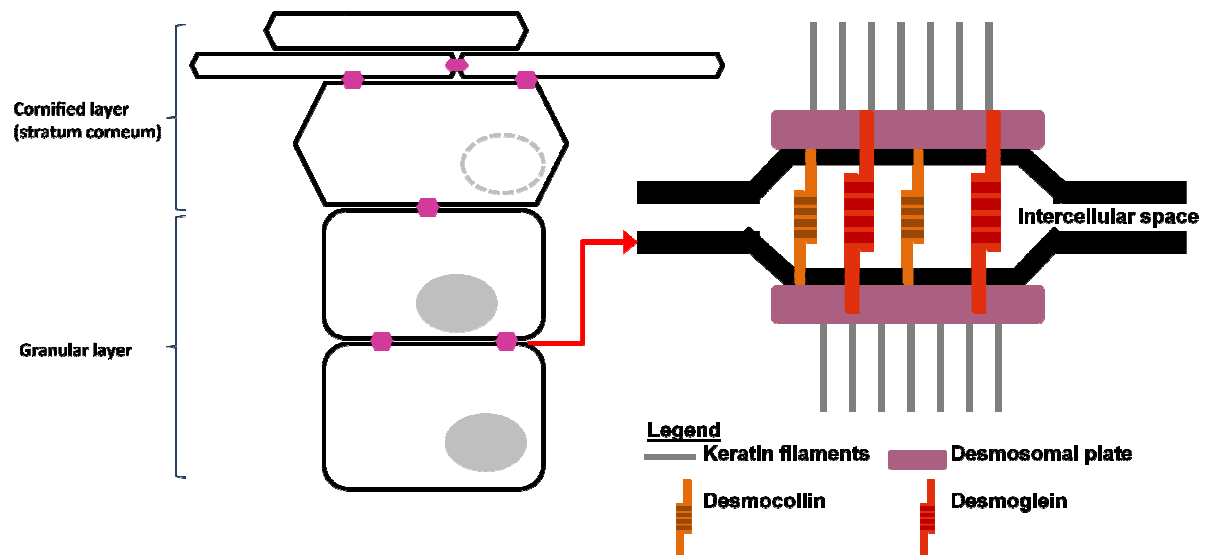


Figure 2. Corneodesmosomes. Corneodesmosomes are cellular junctions that maintain keratinocyte cohesion. They are composed of calcium-dependent transmembrane glycoproteins desmogleins and desmocollin, which are members of the cadherin superfamily. These bind keratin filaments of adjacent cells via the desmosomal plate consisting of plakophilin, plakoglobin and desmoplakin proteins. Keratinocytes in the granular layer also secrete another desmosomal protein known as corneodesmosin. Corneodesmosin is a 52kDa glycoprotein expressed only in epithelial cells undergoing keratinisation. Following secretion into the extracellular space, corneodesmosin localises to the transitional zone between the granular and cornified layers and is then incorporated into the extracellular component of the desmosomes. It is said to play a regulatory role in the stratum corneum cohesion. Physiological desquamation (exfoliation of keratinocytes accumulated at the skin surface) requires proteolytic degradation of corneodesmosomes by proteases such as SCCE. This process is regulated by factors including the presence of inhibitors, pH, humidity and the state of stratum corneum hydration.

Immunological

AD has been viewed by many to be the result of primary immune defects due to abnormal cutaneous hypersensitivity to environmental agents. The immunological basis of AD is supported by the finding that those with primary immunological disorders often display eczema-like lesions. The clearance of eczematous lesions in Wiskott-Aldrich Syndrome (an X-linked recessive disorder characterised by immune deficiencies, eczematous eruptions and thrombocytopenia) following bone marrow transplantation⁷⁰ provides further evidence that the immune dysfunction has a direct role in the development of AD.

The immunopathology of AD is very complex and involves many different types of inflammatory cells and pathways⁷¹. In acute or subacute AD, a Th2-predominant immune response is seen. Th-2 cytokines such as IL4 and IL13 promote IgE secretion from mast cells and sensitization to allergens which are normally harmless. As with any inflammatory process, lesional skin of AD patients displays infiltration of inflammatory cells. In particular, increased numbers of CD4 cells are

seen. Upregulation of chemokines including eotaxin, monocyte chemoattractant protein-4, pulmonary and activation-regulated chemokine (PARC) and cutaneous T cell-attracting chemokine is seen, all of which serve to recruit eosinophils, macrophages and T-cells to the site of injury or inflammation^{72,73}. T-cells are also recruited by IL-16 secreted by Langerhans cells in the skin^{74,75}.

In chronic AD, there is a change in the Th-1-Th-2 balance. There are less IL-4 and IL-13 cytokines but increased IFN- γ , GM-CSF, IL-5, IL-12 which favour Th1 inflammation^{76,77}. As with other chronic inflammatory states, skin re-modelling is observed and fibrosis takes place⁷⁸. The level of profibrotic cytokines such as IL-11 and IL-17 are increased⁷⁸. IL-11 also encourages collagen deposition.

It is interesting to note that patients with AD, but not psoriasis (another chronic inflammatory skin disease) have frequent skin infections. AD patients are found to have downregulation of the genes responsible for innate immune defense system, such as human β defensin-2 and inducible nitric oxide synthetase. This has been suggested to be related to the abundance of Th-2 cytokines⁷⁹. It is therefore useful to improve our understanding of the key cytokines involved in the development of AD for further elucidation of the disease process. New anticytokine therapies will provide an alternative to steroids in regulating inflammation and restoring our innate immune defense system.

Whether it is due to barrier or immunological dysfunction (or both), AD is strongly influenced by genetic and environmental factors.

Genetics

Genetic imprinting is thought to occur in atopic diseases such as AD. Many previous studies looking at the parent-of-origin effects suggest that atopy in a child is more strongly associated with maternal than paternal atopy.⁸⁰⁻⁸² Having said this, there are studies which have suggested otherwise, such as that by Wadonda-Kabondo *et al* which found no parent-of-origin effect in AD.⁸³ Compared to allergic asthma and rhinitis, parental AD carries a higher transmission risk to offspring². As a general rule, if one parent has AD, the child has a 20% chance of getting the disease. This is increased to 50% if both parents have or had AD. The concordance rate for AD among monozygotic twins is 77% compared to 15% of dizygotic twins⁸⁴. This evidence suggests the presence of AD-specific genes. Genome-wide scans revealed several chromosomal regions as potentially of pathophysiological relevance in AD^{85,86}. The area of highest linkage is found to be on chromosome 1q21 which contains a collection of epithelium-related genes known as the epidermal differentiation complex⁸⁷. Others include chromosome 5q31-33 which contains a cluster of Th-2 cytokine genes (IL-3, 4, 5 13 and GM-CSF)⁸⁸

and chromosome 11q12-13 which carries the gene encoding the β -subunit of high-affinity IgE receptor⁸⁹. Other studies have found an association between polymorphisms of IL-4 promoter region (linked to increased activity of the gene promoter) and predisposition to AD⁹⁰. Chromosome 3q21⁹¹ (encoding co-stimulatory molecules CD80 and CD86), a gain-of-function polymorphism in the α -subunit of IL-4 receptor on chromosome 16q12 and variants of IL-13² have also been implicated. Overall, there is a genetically determined predisposition to favour the generation of Th-2 responses.

Most of the studies mentioned above involved patients with raised IgE level, i.e. those with extrinsic AD. Until recently, the genetic basis of those with normal IgE level, who possibly have skin barrier dysfunction as their primary event, remained elusive. Two important works which shed light into the genetic basis of skin barrier dysfunction in AD are hereby discussed. The first is the finding by Vasilopoulos *et al*⁶³ which suggests an association between variations in the SCCE gene and AD. A 4-base pair (AACC) insertion in the SCCE gene may lead to increased enzyme activity or changes to its natural lifespan. The second, perhaps the most important discovery in the field of AD in recent decades is the identification of loss-of-function mutations of the epidermal barrier protein filaggrin as a major predisposing factor for AD⁹². *FLG*, the gene encoding filaggrin is one of those found on the epidermal differentiation complex on chromosome 1q21. Up to 50% of patients with moderate to severe AD have been found to have one or more of these mutations. The study has been replicated in multiple studies worldwide with similar results found. This topic will be discussed in subsequent chapters in the thesis.

Other genes which have been mentioned in the literature are mast cell chymase gene and genes encoding protease inhibitors. Variants of the former have been associated with AD in children⁹³. Polymorphism within the promoter region of this gene has also been associated with high serum IgE level⁹⁴. Maternally inherited mutations in the *SPINK5* gene which encodes LEKTI, a protease inhibitor, have been linked to AD, although the evidence is not strong⁹⁵⁻⁹⁷. *SPINK5* mutations are, however, a known cause for Netherton Syndrome⁹⁸⁻¹⁰⁰, an autosomal recessive disorder in which sufferers exhibit atopic manifestations and severe skin barrier dysfunction. Reduced protease inhibition by LEKTI leads to abnormally increased corneodesmosomal cleavage and poor intercorneocyte adhesion. Mutations in the gene encoding cystatin A, a cysteine protease inhibitor have also been associated with AD. Lesional skin in AD patients has been found to express reduced levels of cystatin A protein¹⁰¹, compared to control individuals with no AD. Cystatin A inhibits endogenous and exogenous cysteine proteases such as those released by house dust mite Der p1. Transgenic mice with a null mutation in their cystatin M/E gene have severe barrier dysfunction and die shortly after birth¹⁰².

Environmental

Many argue that the rapid increase in the incidence of AD and the fact that it appears to be associated with urbanisation and higher social classes are strong indicators that environmental factors are the driving force behind the disease. It is not possible for genetic changes to happen within such a short span of time. In very simplified terms, allergic responses are Th2-mediated¹⁰³ while infections are Th1-driven. It is thought that over the last century, the development of antibiotics, declining family size and improved household amenities and personal hygiene (seen especially amongst the higher social classes) have reduced childhood infections which are important for proper maturation of the immune system. This has probably dampened Th-1 immunological programming, which confers protection against Th-2-mediated allergic responses¹⁰⁴. This is known as the '*hygiene hypothesis*', which was first proposed by David Strachan¹⁰⁵ in 1989 who observed that the prevalence of hay fever was inversely related to the number of children (especially older siblings) in the household. The '*hygiene hypothesis*' is one of the most controversial topics in the world of immunology. There has been a large number of population based studies, many of which support the hypothesis¹⁰⁶, for example, those exposed to farm animals early in life were found to have a lower incidence of atopic diseases¹⁰⁷⁻¹¹². Nonetheless, there are also many studies which have produced equivocal results, some even showing that certain infections can exacerbate or increase one's risk of atopic diseases¹¹³⁻¹¹⁸. Other environmental factors that have been associated with exacerbation of the disease include temperature, humidity, stress, infections and the presence of irritants or allergens¹¹⁹.

Autoallergens: Patients with severe AD are often found to have IgE antibodies against endogenous human proteins¹²⁰. These are usually intracellular proteins released during tissue damage and can trigger IgE or T-cell mediated responses. This suggests that even though the initial immune responses in AD may be triggered by environmental allergens, the resultant tissue damage may lead to the release of endogenous antigens which perpetuate the inflammatory state².

Aeroallergens: In AD patients who are sensitised (i.e. those with specific IgE), inhalation of aeroallergens can lead to dermatitic lesions and pruritus¹²¹. Reducing the level of exposure to house dust mites has been suggested to have beneficial effects on the skin condition of AD patients^{122,123}. The data on this matter is, however, inconsistent.

Food allergens: In animal models, following oral sensitisation, dermatitic skin lesions can be elicited on subsequent food challenges¹²⁴. 40% of children with moderate to severe AD patients are said to have concomitant food (e.g. peanuts, milk and eggs) allergies which when ingested, induce a rash, urticarial reaction or flare-up of their AD^{2,125}. A raised serum IgE, food-specific T cells¹⁷ or positive skin tests to certain foods may be found¹²⁷. Nonetheless, avoidance of food allergens correlates

poorly with clinical improvement, rendering these blood and skin tests unhelpful and of limited diagnostic value.

Recurrent skin infections

As mentioned earlier, AD patients are often subjected to recurrent, sometimes severe bacterial and viral infections. Eczema herpeticum (caused by herpes simplex virus) is known to occur primarily in AD patients¹². The skin of most AD patients is colonized with *Staphylococcus aureus*. Skin infection, inflammation and the related scratching exposes collagens and fibronectin, to which *Staphylococcus aureus* strongly binds. Studies have shown that such binding is greater at sites with Th-2 mediated inflammation as IL-4 increases the expression of fibronectin^{11,128}. This is supported by the finding that treatments aiming to suppress the inflammatory state using topical steroids¹²⁹ and tacrolimus¹³⁰ also reduce the bacterial count on the skin. The skin of AD patients is also less able to protect itself against colonization by bacteria due to the lack of endogenous antimicrobial peptides such as cathelicidins and beta-defensins¹³¹. Although skin infection(s) may not be the primary cause of the disease, it certainly contributes towards the chronicity of the inflammation.

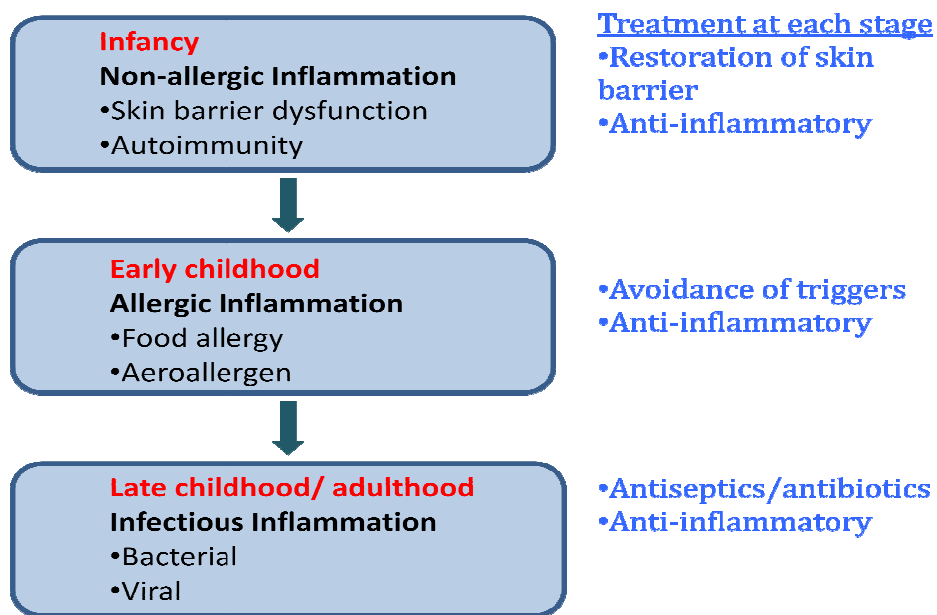


Figure 3. A hypothetical model of AD. An adapted diagram¹³² showing the three different phases (non-allergic, allergic and infectious) in a hypothetical model of AD and the targeted management for each phase.

1.5 Treatment

A multipronged approach is required in the treatment of AD. The aims are to relieve symptoms (e.g. pruritus), reduce inflammation and the frequency of flare-ups and fight infection. The management of AD therefore involves 1) restoration of skin barrier function, 2) reduction of inflammation, 3) identification and elimination of triggering or exacerbating factors and 4) antiseptics or antibiotics. Educating the patient regarding the chronic nature of the disease, triggers for exacerbation, treatment options and management plan is central in ensuring compliance to treatment and reducing the psychological burden of the disease. Patients should also be made aware of self-help groups or support organizations where they can obtain more information and support.

Intensity of the treatment offered is determined by the severity of the disease, potential side effects from the treatment itself and impact of the disease or its treatment on the quality of life of the patient and his/her family.

1.5.1 Restoration of skin barrier function

Liberal usage of emollients (moisturizers) at least twice a day is encouraged for all AD patients. These may be in the form of creams, lotions, ointments or bath oils. Frequent usage is recommended even in the absence of symptoms. Emollients restore skin barrier function and prevent the development of painful cracks or fissures due to dryness. Gentle body washes or moisturizing bath oils should be used instead of regular soaps. Long, hot baths should be avoided as this will remove the natural moisturizing factors in the skin; water contact should be kept to a minimum and immediate application of emollients after baths or showers is advised to lock in the moisture¹³³.

Sedating oral antihistamines are particularly useful at night in reducing further damage to the skin caused by scratching. Non-sedating ones have limited benefits. Antihistamines have a good safety profile and doses higher than that recommended by the manufacturers can be used if required. Peripheral histamine is not involved; hence topical antihistamines are ineffective.

1.5.2 Allergen avoidance

Efforts to minimise exposure to known agents that trigger the itch-scratch cycle or precipitate a flare-up can be attempted. These may include aeroallergens such as house dust mites, animal danders, pollens and food allergens. This is easier said than done as it is almost impossible to avoid exposure to common environmental agents such as house dust mite. The sensitivity and specificity of skin-prick and blood tests for specific IgE level aiming to identify the 'culprit' allergens are also highly questionable. Skin-prick testing has a very high false positive rate and is, therefore, not routinely used. Blood test for specific IgE is only used if there is a strong clinical suspicion and not as

a general measure. Other known triggers may include emotional stress, irritants (e.g. soaps, wool) and changes in environmental temperature or humidity. Stress is not a primary cause of AD but chronic stress has been linked to suppression of Th-1 cytokines and enhancement of Th-2 mediated humoral immune response¹³⁴. This Th-1-to-Th-2 shift may increase susceptibility to AD or exacerbate the disease.

1.5.3 Anti-inflammatory

The anti-inflammatory agents most commonly used in AD patients are the topical corticosteroids. Where fungal or bacterial infection is present, a combination of corticosteroids and antimicrobials can be considered. Examples of preparations consisting of steroids and antimicrobials include Betnovate-C, Betnovate-N, FuciBET, Locoid C, Synalar-C and Synalar-N. Otherwise, the evidence for the advantages of routine usage of steroids combined with antimicrobials over steroids alone is poor. Topical antibiotics can cause cutaneous sensitization and should not be used on a regular basis. Topical corticosteroids are classified according to their potencies, determined by their degree of anti-inflammatory action and the amount of vasoconstriction induced (Table 4). There are four general classes of potencies: mild, moderately potent, potent and very potent. Their usage should be tailored individually according to the severity of the disease, the site(s), total surface area involved and the age of the patient. Generally, the weakest steroid that provides effective control of the disease should be used. Some dermatologists prefer to use the step-up approach, i.e. starting from weaker and gradually increasing the strength of the steroid used to bring the disease under control; others prefer the opposite. Guidelines issued by the British Association of Dermatologists recommend the usage of once or twice daily corticosteroids for up to 1 week in acute cases and up to 6 weeks to control chronic disease. This is followed by steroid-free/ emollients-only period. However, there is evidence that intermittent usage of topical steroids for maintenance when the disease is clinically inactive can effectively reduce the number of flare-ups and in the long run, the total steroid usage¹³⁵.

The choice of steroid is not only dictated by the severity of the disease but the affected area as well. High-potency corticosteroids should be avoided in infants and at the following areas where the skin is thinner and more sensitive: face, eyelids, genitalia and intertriginous areas²³. Very potent topical steroids should generally be used only for a short period of time or on thick, lichenified skin. This is because the main concern for AD patients using topical steroids is the risk of irreversible skin atrophy (thinning of the skin) after prolonged exposure. Signs of atrophy include increased transparency of the skin, striae and telangiectasia. Prolonged usage on eyelids has been associated with the development of glaucoma. Other potential side effects from the systemic absorption of topical preparations include growth restriction through suppression of pituitary-adrenal axis. Very

potent corticosteroids are therefore contraindicated in infants and should be used with extreme care in younger children. The use of growth chart is advisable for children on moderate to potent steroids; all patients on such steroids should be followed up regularly. The risk of systemic absorption through the skin is correlated to the degree of surface area involved, amount applied and potency of the preparation, frequency of application and the concomitant use of occlusive dressing. During a flare-up, patients can be temporarily switched to a stronger preparation or given a short course of oral steroid. With oral steroids, dramatic improvement may be seen, although one may experience an equally dramatic rebound flare following cessation of the oral therapy.

Table 4

Examples of topical steroids of different potencies (Source: British National Formulary¹³⁶).

<u>Mild</u>	<u>Moderately Potent</u>	<u>Potent</u>	<u>Very Potent</u>
<ul style="list-style-type: none"> • Hydrocortisone 0.1-2.5% 	<ul style="list-style-type: none"> • Betnovate-RD 	<ul style="list-style-type: none"> • Betamethasone valerate 0.1% 	<ul style="list-style-type: none"> • Dermovate
With antimicrobials:	<ul style="list-style-type: none"> • Eumovate 	<ul style="list-style-type: none"> • Betnovate 	<ul style="list-style-type: none"> • Clarelux
<ul style="list-style-type: none"> • Canesten HC • Daktacort • Fucidin H 	<ul style="list-style-type: none"> • Haelan • Ultralanum Plain • Modrasone • Synalar 1 in 4 	<ul style="list-style-type: none"> • Bettamousse • Betacap • Elocon • Diprosone • Locoid (Crelo) • Nerisone • Propaderm • Metosyn • Synalar 	<ul style="list-style-type: none"> • Halciderm Topical • Nerisone Forte
			With antimicrobials
			<ul style="list-style-type: none"> • Dermovate-NN

Tacrolimus and pimecrolimus are members of a class of immunosuppressants known as calcineurin inhibitors. Pimecrolimus is weaker than tacrolimus and is not often effective in more severe cases of AD. Calcineurin inhibitors reduce inflammation by suppressing the activity of T-lymphocytes and have been shown to be effective in relieving symptoms and reducing the number of flare-ups. As they have a different mechanism of action from steroids, these drugs do not cause skin atrophy and are therefore suitable for facial and eyelid AD. The commonest adverse effect associated with calcineurin inhibitors is local skin irritation, especially with tacrolimus. Other effects include increased sensitivity to hot and cold, alcohol intolerance, folliculitis and viral infections. The National Institute of Health and Clinical Excellence (NICE)¹³⁷ recommends for topical tacrolimus to be used only in moderate and severe AD which is intolerant or show inadequate response to adequate usage of maximum potency of topical corticosteroids suitable for the area(s) treated and the age of the

patient involved. They should not be used where infection is on-going or suspected. As these immunomodulators are relatively new drugs compared to steroids, their long term effects are yet to be determined. There are concerns over potential long term effects of local immunosuppression, such as skin malignancies and infections. This is one of the main reasons why calcineurin inhibitors are not recommended for mild AD or as a first-line treatment for AD of any severity.

1.5.4 Antiseptics/antibiotics

A sudden worsening of the skin condition accompanied by crusting, pustulation or surrounding erythema is suggestive of infected AD. Swabs should be sent off for cultures. A 7-day course of oral flucloxacillin is usually sufficient to cover for staphylococcal infection. Phenoxymethyl penicillin can be given concomitantly if streptococcal infection is suspected or proven on culture. The use of long term topical antibiotics is generally not recommended as it may lead to bacterial resistance or cutaneous sensitization. Viral infections such as eczema herpeticum should be treated with antivirals.

1.5.5 Others

Wet dressing and occlusion

Wet dressing, especially when used in combination with steroids, is effective in severe AD in children. This comprises of wrapping the skin in wet bandages after applying emollients (with or without steroids), followed by dry bandages. This is left overnight. It has a soothing effect and provides an occlusive barrier against scratching.

Phototherapy

Patients often report that their AD improves during summer times or while on holidays. Natural sunlight is beneficial for AD, possibly due to its immunosuppressive effect. In resistant or severe AD, ultraviolet therapy can be given to bring the disease under control, followed by maintenance steroid therapy.

Immunosuppressants

In a small group of patients with extremely severe AD that has not responded to steroids, immunosuppressive agents such as methotrexate, azathioprine, mycophenolate mofetil and cyclosporine can be considered although its potential benefits should be weighed up carefully against the side effects.

Biological therapy

Biological agents are protein-based therapies used to modulate the immune response. These include recombinant interferon, intravenous immunoglobulin, tumor necrosis factor- α inhibitors (infliximab, etanercept and adalimumab) and recombinant humanized monoclonal antibodies targeting the immunoglobulin-E / interleukin-5 pathway (omalizumab and mepolizumab). Other agents include

rituximab which acts on CD20 of B-lymphocytes and alefacept which selectively inhibits T-cell activation and reduces memory T-cells.¹³⁸

Tar

Tar preparations have been used for hundreds of years to treat pruritic and inflamed skin although the evidence for its efficacy is limited. It contains chemicals which soothe the skin with relatively few side effects. It may induce sun sensitivity and should not be applied to acutely inflamed AD lesions as it can act as an irritant. Its popularity is limited by the fact that it is smelly, messy to apply and stains skin and clothings. There are now newer, refined coal tar preparations which can be purchased over the counter. These are more cosmetically acceptable to patients.

Hospitalisation

In rare occasions such as in severe widespread erythrodermic AD, hospitalisation may be considered for aggressive medical treatments in addition to avoiding environmental allergens and providing intensive patient education.

Table 5

Treatment options for AD (source: National Institute of Health and Clinical Excellence¹³⁹)

Mild AD	Moderate AD	Severe AD
<ul style="list-style-type: none"> • Emollients • Topical corticosteroids (mild potency) 	<ul style="list-style-type: none"> • Emollients • Topical corticosteroids (moderate potency) • Topical calcineurin inhibitors • Bandages 	<ul style="list-style-type: none"> • Emollients • Topical corticosteroids (potent or very potent) • Topical calcineurin inhibitors • Bandages • Phototherapy • Systemic therapy (oral steroids or immunosuppressants)

More information regarding the guidelines on managing AD is available on the National Institute of Health and Clinical Excellence (NICE) and British Association of Dermatologists official websites.

1.6 Social and Financial Implications

Many patients with AD find the stigma associated with the visibility of the disease just as, if not more distressing than the physical symptoms of the disease itself. AD has significant impacts not only to the patients but their families as well. Meltzer LJ *et al* conducted a systemic review of 19 studies investigating the prevalence, causes and consequences of sleep disruptions in parents of children with chronic illnesses. Those with children with AD were the most severely affected, with a prevalence of sleep disruptions of 15-86% due to night-time caregiving, stresses relating to the illness and overnight monitoring. This resulted in poor quality sleep in both children and their parents, increased anxiety, depression¹⁴⁰, absences from school and social activities, having fewer friends, issues with intimacy, employment loss¹⁴¹ and negatively affecting the quality of life of the patients and their families. Caring for children with moderate or severe AD has been said to be more stressful to the family than caring for children with Type 1 diabetes mellitus¹⁴². Having AD or having had AD predisposes an individual to multiple contact sensitivities. AD sufferers are therefore often encouraged to steer clear from certain career paths including becoming a florist or hairdresser.

The financial burden of AD is known to be high and is said to be comparable to that of asthma¹⁴³, with half the cost being met by the family¹⁴⁴. Multiple studies have been conducted worldwide to measure the cost of this important disease^{142,144-147}. However, these studies, done in different parts of the world, varied in their methodologies. The prevalence of AD varied from one country to another, making direct comparison between these studies virtually impossible.¹⁴³ Even studies within the same country yielded significantly different results. Mancini *et al*¹⁴⁸ carried out a systematic review attempting to quantify the direct and indirect costs of atopic dermatitis in the United States. Out of the 59 papers reviewed in detail, 4 studies met their inclusion criteria and only 1 study measured the indirect costs of AD. The overall direct costs were found to have ranged from USD364 million to USD3.8 billion per year. In the UK, a study on the cost of AD was done by dermatologists at The University of Edinburgh on 150 patients in the community over a period of 2 months in 1995¹⁴⁴. The study revealed a mean personal cost of £25.90 (spent on, for example, clothing and laundry), and a mean cost of £16.20 to the health service. Majority of the latter was spent on treatment such as emollients, bath additives, steroids and bandages. A loss of 58 working days and 17 school days were calculated. A further cohort of 10 patients with severe AD was found to have spent, on average, £325 each in 2 months and costed the NHS £415 each in the same period. Extrapolations revealed an annual personal cost of £297 million across UK and £125 millions to the NHS. Annual cost to the society due to lost working days was estimated at £43 millions. This brought the total cost of AD to £465 million per annum to the UK. To put things into perspective, the annual

per capita cost of treating AD was calculated at £7.38, compared to that of treating venous ulceration at £6.73¹⁴⁹ while the per capita cost of treating benign prostatic hypertrophy was £1.53¹⁵⁰.

1.7 Chapter 1 Conclusion

Atopic dermatitis is an extremely common skin disease with rising incidence worldwide. It bears significant medical, social and financial implications. It is a disease of complex aetiology. The exact pathogenesis is still unclear. Historically, immune dysfunction has been viewed as the primary cause of AD. Topical steroids which suppress the local immune responses are therefore one of the main treatments for the disease. The discovery of genetic association between skin barrier protein filaggrin and AD in 2006 represents one of the most significant breakthroughs in recent decades. This seminal work supports the hypothesis that skin barrier dysfunction is the primary event in the development of AD; a defective barrier allows easier penetration of allergens into the body, thus triggering the cascading sequence of immunological events. This concurs with the observation that 50% of AD patients develop other atopic diseases such as asthma at a later stage (the '*atopic march*'). All four factors (genetic, environmental, barrier defects or immune dysfunction) are involved in the development of AD, each exerting a different degree of influence at different stages of the disease. This is also influenced by the patient's age. Much work is yet to be done in improving our understanding of the various phenotypes of AD and the genetics and immunological abnormalities that predispose one to the disorder. Such new knowledge will be expected to be followed by pharmacological advancement which oversees the development of more effective treatments targeting particular subtypes of AD. It is hoped that in the future, highly effective treatments will minimise symptoms and reduce relapses, improving the quality of life of the patients. Perhaps, they may even halt the '*atopic march*'.

1.8 Chapter 1 references

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Chapter 2

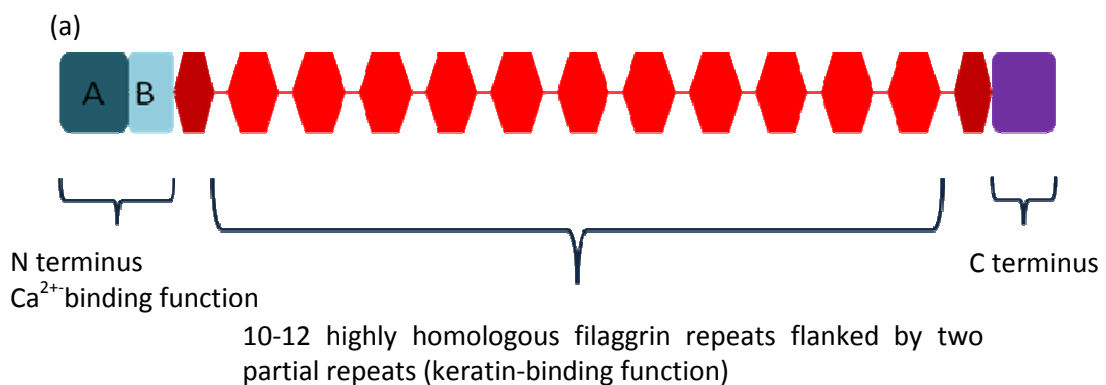
The Effects of Filaggrin Gene Mutations on Skin Barrier Function in Patients with Atopic Dermatitis

Filaggrin is one of the key epidermal barrier proteins.

Since common loss-of-function mutations in the filaggrin gene (FLG) have been found to be strongly associated with atopic dermatitis (AD), the focus of much research has shifted from immunological abnormalities to barrier dysfunction as the primary cause in the pathogenesis of AD. In this chapter, I examine the relationships between genotype and the phenotypic traits of AD patients. Comparison of the skin barrier function of filaggrin-related AD (FLG-AD) and wild-type AD (WT-AD) allows us to understand the physiological significance of having such mutations.

2.1 Introduction: Filaggrin

Filaggrin (**filament aggregating protein**), being one of the key proteins involved in epidermal barrier formation, has long been thought to be an important player in the pathogenesis of AD. Profilaggrin (Figure 1), its precursor protein, is the major component of keratohyalin granules in the granular layer of the skin¹. Filaggrin is thought to have two major functions in the skin. During terminal differentiation of keratinocytes, filaggrin is thought to play a transient role in aggregating keratin intermediate filaments into macrofibrils which are aligned in parallel², thus collapsing the keratinocytes to form flattened squames of the cornified envelope. Filaggrin is subsequently degraded by proteases into hygroscopic amino acids and other derivatives such as urea, pyrrolidone carboxylic acid and urocanic acids. Proteolytic processing of (pro)filaggrin will be explored further in the next chapter. All of these highly hygroscopic elements are collectively known as the natural moisturizing factors (NMF). In addition to being the main humefactant of the stratum corneum, the NMF is said to play a key role in conferring ultraviolet protection, regulation of enzymatic events, maintaining the optimal pH gradient within the stratum corneum and protection against microbes^{1,3,4} (Figure 1). Together with lipids and other proteins in the cornified layer, they form a tough physical barrier which protects the internal bodily physiology from the harsh external environment.



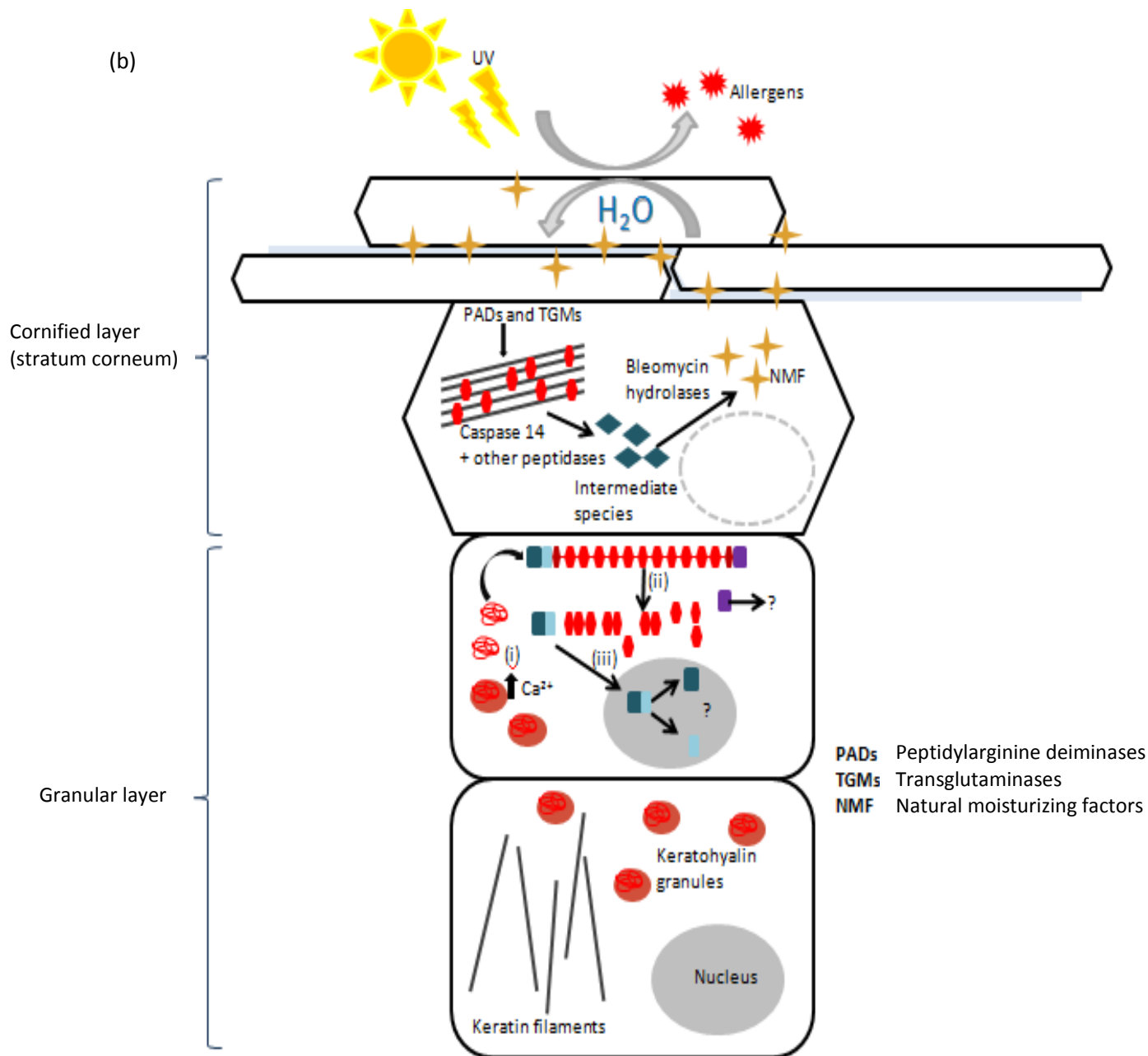


Figure 1. The structure and functions of filaggrin protein. Diagram adapted from Sandilands et al¹ showing (a) Structure of the (pro)filaggrin. (b) Filaggrin in the skin. Profilaggrin processing occurs during terminal differentiation of keratinocytes. (i) A rise in Ca²⁺ level causes the keratohyalin granules to degranulate and release the inactive 400-kDa profilaggrin. (ii) Profilaggrin is dephosphorylated and proteolysed in multiple steps to release the 37-kDa filaggrin monomers (see Chapter 3 for details). (iii) The N terminus translocates into the nucleus where it is further degraded into the A and B domains. The former consists of two Ca²⁺-binding regions are similar to the EF-hands of the S100p family. The exact functions of the N and C terminal domains are not well-understood. Filaggrin aggregates keratin filaments. This is followed by a series of proteolytic activities which breaks down the protein into natural moisturizing factors which protect the skin.

The gene encoding the filaggrin protein (*FLG*) lies on chromosome 1q21, within a cluster of genes known as the epidermal differentiation complex (EDC). The EDC is known to be a susceptibility locus for AD and psoriasis. Despite advances in molecular genetics, establishing *FLG* mutations as a key player in the pathogenesis of AD had been challenging due to the repetitive elements of the gene. Most of the profilaggrin protein is encoded by the third and final exon of *FLG* which consists of 10-12 highly homologous *FLG* repeats (Figure 1a). In 2006, Smith *et al* successfully adapted the long-range polymerase chain reaction technique used in cloning to sequence the *FLG*. This stroke of ingenuity led to the identification of 2 *FLG* mutations (R501X and 2282del4) as a major cause for ichthyosis vulgaris, the commonest inherited disease of keratinisation^{5,6}. As atopic dermatitis is a common feature of ichthyosis vulgaris, this swiftly led to the discovery of *FLG* mutations as a major predisposition factor for moderate-severe AD by Palmer *et al*⁷. This pivotal work is one of the most important breakthroughs in the understanding this disease in recent decades. It suggests that genetically driven skin barrier dysfunction is a key driver of the disease.

To date, approximately 40 different *FLG* mutations have been identified⁸⁻¹⁰. The association between *FLG* mutations with AD has been validated by numerous association studies which have also shown these mutations to be population or family-specific^{11,12}. This includes studies done on North Americans¹³ and European and Asian¹⁴ populations (including Ireland⁷, Scotland⁷, England^{15,16}, Denmark⁷, Sweden¹⁷, Germany¹⁸⁻²¹ and Japan^{22,23}). These nonsense or frameshift mutations^{24,25}, which reduce or prevent the expression of the protein, occur in 15-55% of AD patients. In moderate-severe forms of AD, they are thought to be inherited in an autosomal semidominant manner with incomplete penetrance⁷. Nonetheless, there are several studies that argue against *FLG* mutations being a major risk factor for AD. For example, Giardina *et al* have shown that R501X and 2282del4 *FLG* mutations do not confer susceptibility to AD amongst Italian patients²⁶. Furthermore, only a small percentage of Italian AD patients have *FLG* mutations²⁷. This may be because Italians and Northern Europeans are of different ethnic origins. *FLG* mutations may also confer a selective advantage to Northern Europeans. *FLG* mutations are not found in the majority of patients with mild AD, in whom they appear to follow an autosomal recessive mode of inheritance¹⁵. Heterozygotes have a 60% probability of developing the disease which increases to 90% in homozygotes²⁸. The incomplete penetrance of *FLG* mutations and the absence of such mutations in half of the AD population indicate the presence of other modifying factors which may have an impact on the protein expression and clinical manifestation. Recently, wild-type AD patients (WT-AD) have been found to have reduced filaggrin protein compared to healthy controls (HC). This is thought to be caused, in part, by the overexpression of Th-2 cytokines²⁹.

Following the renewed interest in this barrier protein, several studies have been conducted to characterise the phenotypic nature of *FLG*-associated AD (*FLG*-AD). Barker *et al* have shown that those with *FLG* null alleles have a poorer prognosis. They are more likely to have an early-onset AD that starts in early infancy and a disease that is more likely to persist into adulthood¹⁶. Having *FLG* mutations predisposes to allergic rhinitis, dry skin and palmar hyperlinearity (a shared clinical feature of AD and ichthyosis vulgaris). Interestingly, *FLG* mutations are associated with asthma that occurs in the context of AD^{19,21,30-32} but not with asthma *per se*. *FLG* mutations also confer an increased risk of developing irritant and allergic contact sensitization, especially to nickel^{31,33}. This has led to suggestions that *FLG* mutations may confer susceptibility to hand dermatitis³³.

While the phenotype of *FLG*-AD has been relatively well-studied, there is less evidence for *FLG*-associated defects in physiological barrier function. TEWL and skin capacitance (a reflection of stratum corneum hydration) are measurable parameters for skin barrier function. Increased TEWL and reduced skin capacitance are recognised features of AD^{34,35}. The skin of AD patients has also been shown to allow easier penetration of chemicals including polyethylene glycol³⁶ and sodium dodecyl sulphate (SDS)³⁷, as compared to that of healthy individuals. A recent study done in a Japanese AD cohort confirmed that compared to HC, WT-AD patients have a higher TEWL while the *FLG*-AD have a reduced level of hydration and increased thickness of the stratum corneum. In the same study, the Objective Score of AD (OSCORAD), a clinical index for disease severity, was found to be positively correlated with TEWL and stratum corneum thickness and negatively correlated with skin hydration in the *FLG*-AD patients. Such correlations were not seen in the WT-AD patients³⁸.

As with Nemoto-Hasebe *et al*, our group supports the hypothesis that skin barrier dysfunction, caused by genetically driven deficiency of filaggrin, is the initial event in the development of AD. In this chapter, the results of our cohort study investigating *FLG*-associated physiological defects in skin barrier function are presented. The cohort consisted of patients, predominantly Caucasians, with mild-moderate AD. This is representative of the majority of AD sufferers in the UK. All participants were genotyped for the 4 commonest *FLG* mutations found in the Caucasian population. Their baseline skin barrier function was evaluated through measurements of TEWL, skin capacitance and skin fold thickness³⁹. Correlations between skin barrier function and disease severity, concomitant atopic diseases, family history of atopic conditions and total serum IgE level were examined. The effects of physical and chemical challenges on the skin of *FLG*-AD participants were compared to that of wild-types.

2.2 Chapter 2 Results

2.2.1 Demographic data of AD participants

58 participants with AD, diagnosed according to the United Kingdom Working Party's Diagnostic Criteria for Atopic Dermatitis⁴⁰, were recruited into the study. Age and gender distribution of the WT-AD and FLG-AD groups were shown in Table 1.

An unpaired, two-tailed t-test was conducted to compare the age of the participants in both groups. There was no significant difference in the age (in years) between WT-AD ($M=35.27$, $SD=13.71$) and FLG-AD ($M=36.00$, $SD=12.52$) groups; $t(56)=0.17$, $p=0.86$. This suggests that the two groups were comparable in terms of age. *Note: M=mean; SD=standard deviation*

Table 1

Demographic Data of Participants: Comparison of the Age and Gender Ratio in WT-AD and FLG-AD

	WT-AD, n=45		FLG-AD, n=13		
	<u>Mean (Range)</u>	<u>SD</u>	<u>Mean (Range)</u>	<u>SD</u>	
Age (years)	35.27 (18-76)	13.71	36.00 (22-57)	12.52	p= 0.86 (n.s.)
Female: Male ratio	1.81		1.60		

2.2.2 Genotype

All 58 participants screened for the 4 commonest European FLG null variants (2282del4, R501X, R2447X and S3247X). There were 45 WT-AD and 13 FLG-AD participants. The latter includes 2 compound heterozygotes (2282del4/R501X; 2282del4/S3247X) and 1 homozygote (R501X) (Table 2). Amongst the 13 FLG-AD, 10 had 2282del4, 3 had R501X and 2 had S3247X FLG null alleles.

Table 2

Frequency of FLG Null Alleles Amongst All Study Participants

Genotype	Number of AD participants, n
AA	45
Aa	10
aa	3
<i>Total</i>	58

13 of the 58 participants had FLG null variants.

'AA'- Wild-type. 'Aa'- heterozygous genotype for one of the FLG variants screened. 'aa'- compound heterozygous or homozygous for any of the screened FLG null variants.

2.2.3 Baseline TEWL and skin capacitance

Measurements of the baseline (resting) TEWL and skin capacitance of all participants were done on uninvolved areas of their flexor forearm skin.

The WT-AD data on baseline TEWL (measured in $\text{g m}^{-2} \text{hour}^{-1}$) deviated significantly (** $p=0.01$) from normality when one-sample Kolmogorov-Smirnov (K-S) normality testing was applied; the *FLG*-AD data passed the normality testing ($p>0.10$, n.s.). The WT-AD data did not appear to be normally distributed; some outliers were evident (Figure 2). Mann Whitney test was, therefore, used to compare the baseline TEWL of the participants in the both groups. The baseline TEWL of *FLG*-AD participants was slightly higher ($M=8.44$, $SD=2.00$, range 5.67-13.23) than that of WT-AD participants ($M=8.15$, $SD=5.68$, range 4.07-40.03), with a p -value that approaches significance ($p=0.05$, n.s.) (Figure 2).

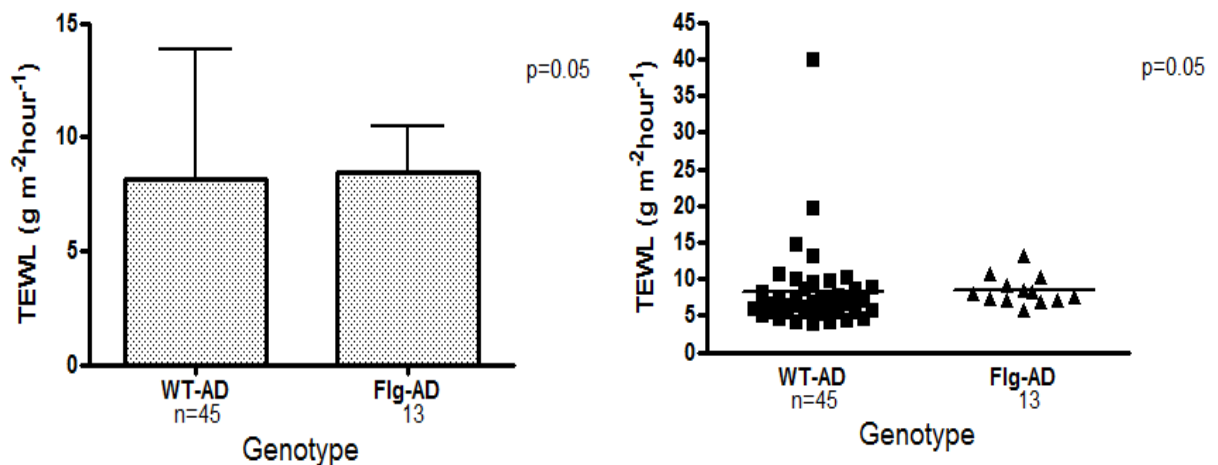


Figure 2. Comparison of the baseline transepidermal water loss (TEWL) in those without filaggrin mutations (WT-AD) and those with filaggrin mutations (*FLG*-AD). The data are represented in a bar graph, showing mean and SD, and a vertical scatter plot, showing individual data points. WT-AD group showed deviation from Gaussian distribution when Kolmogorov-Smirnov normality testing was applied (** $p=0.01$). Mann Whitney U test was used to compare between the groups ($p=0.05$, n.s.).

Unpaired, two-tailed t -test was conducted to compare the baseline skin capacitance (measured in arbitrary unit, AU) of both groups. There was no significant difference in the skin capacitance between WT-AD ($M=58.99$, $SD=17.83$) and *FLG*-AD ($M=58.29$, $SD=17.68$) groups; $t(56)=0.13$, $p=0.90$, n.s. This suggests that the two groups were comparable in terms of baseline skin capacitance (Figure 3).

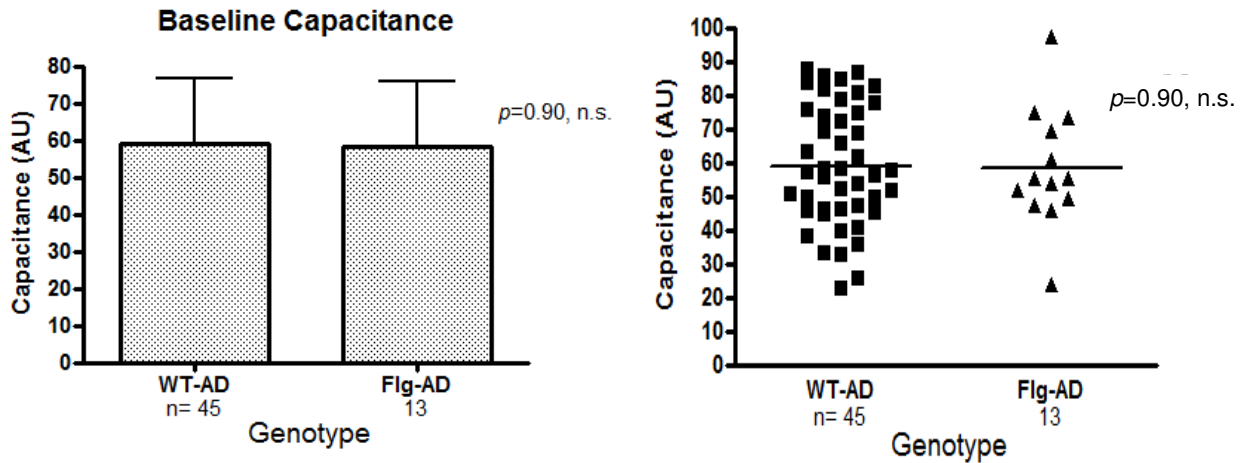


Figure 3. Comparison of the baseline capacitance in those without filaggrin mutations (WT-AD) and those with filaggrin mutations (*FLG*-AD). The data are represented in a bar graph showing mean and SD, and a vertical scatter plot, showing individual data points. Unpaired, two-tailed t-test was conducted to compare between the groups ($p=0.90$, n.s.).

2.2.4 Disease Severity

The disease activity of participants was measured using the Six Area, Six Sign Atopic Dermatitis (SASSAD) severity score⁴¹ which measures six clinical features (erythema, lichenification, dryness, exudation, excoriation and cracking) at six areas (head and neck, trunk, hands, feet, arms and legs). Each feature is scored on a scale of 0 (absent), 1 (mild), 2 (moderate), 3 (severe) with a maximum total score of 108. SASSAD is a widely used severity score to assess response to treatment in clinical trials. In addition to being simple and quick to perform in a clinical setting, the inter-observer variation is reduced by scoring symptoms and signs separately and only lesional sites are scored⁴². Using SASSAD, all participants were found to have mild or moderate form of AD. Unpaired, two-tailed t-test was conducted to compare the total SASSAD scores of both groups. There was no significant difference in the SASSAD scores of between WT-AD ($M=11.64$, $SD=9.32$) and *FLG*-AD ($M=11.38$, $SD=7.53$) groups; $t(56)=0.09$, $p=0.93$, n.s. This suggests that the two groups were comparable in terms of disease severity at the beginning of the study.

2.2.5 Presence of other atopic conditions

The filaggrin genotype was not associated with a higher prevalence of concurrent asthma ($p=1.00$, Fisher's Exact test) or self-reported dry skin in the last 12 months ($p=1.00$, Fisher's Exact test) amongst the participants. The prevalence of hay fever was, however, significantly higher amongst the *FLG*-AD participants (84.62%), as compared to the wild types (48.89%) ($*p=0.03$, Fisher's Exact test) (Figure 4). Note: The statistical analysis was done using three independent 2x2 analyses (dry skin, asthma and hay fever) with a significance found only in the hay fever group.

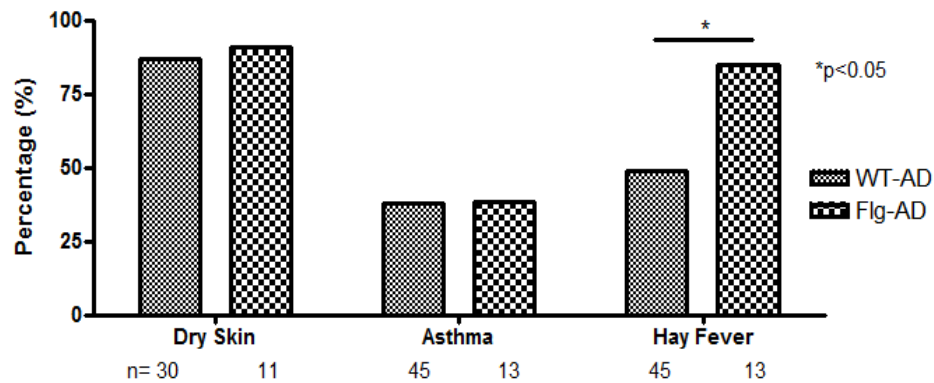


Figure 4. Comparison of the prevalence of other concurrent atopic diseases (self-reported dry skin in the last 12 months, asthma or hay fever) in those without filaggrin mutations (WT-AD) and those with filaggrin mutations (*FLG*-AD). Only hay fever was found to be significantly more prevalent amongst the *FLG*-AD (* $p < 0.05$, Fisher's Exact test).

2.2.6 Family history of atopic conditions

Figure 5 showed the percentages of participants in the WT-AD and *FLG*-AD groups who self-reported having one or more family member(s) with AD, asthma or hay fever.

Figure 6 showed the percentages of participants in the WT-AD and *FLG*-AD groups who self-reported having one or more family member(s) with atopic disease(s) (any of AD, asthma and/or hay fever)

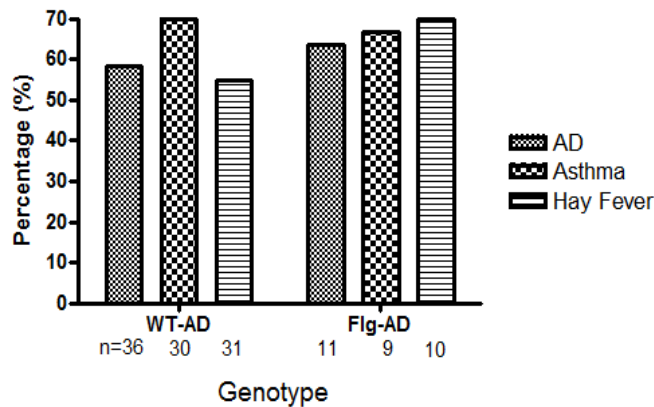


Figure 5. Comparison of the percentages of participants with family history of AD, asthma and hay fever in those without filaggrin mutations (WT-AD) and those with filaggrin mutations (*FLG*-AD).

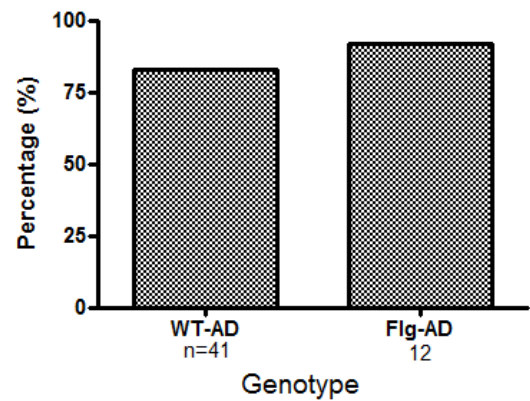


Figure 6. Comparison of the percentages of participants with family history of atopy (any of AD, asthma or hay fever) in those without filaggrin mutations (WT-AD) and those with filaggrin mutations (*FLG*-AD).

2.2.7 IgE level and number of contact allergies

The WT-AD data on IgE (in IU/ml) deviated significantly ($***p=0.0001$) from normality when one-sample Kolmogorov-Smirnov (K-S) normality testing was applied; the *FLG*-AD data passed the normality testing ($p>0.05$, n.s.). The WT-AD data did not appear to be normally distributed; some outliers were evident (Figure 7). Mann Whitney test was, therefore, used to compare the IgE level of the participants in the both groups. There was no significant difference ($p=0.63$, n.s.) in the total serum IgE level of WT-AD ($M=690.00$, $SD=1387.00$) from *FLG*-AD ($M=420.10$, $SD=858.20$) (Figure 7). The large standard deviations seen in both groups were noted.

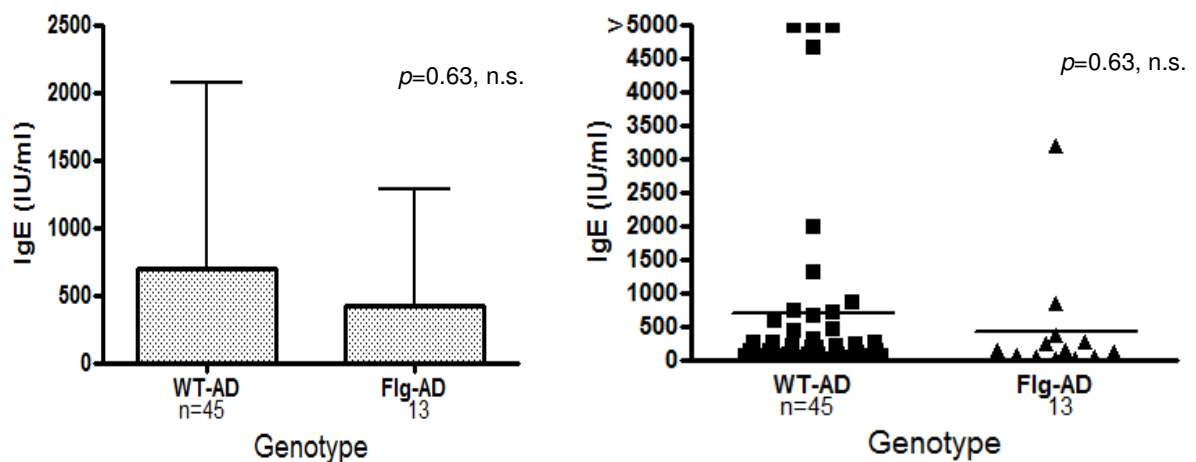


Figure 7. Comparison of the total serum IgE in those without filaggrin mutations (WT-AD) and those with filaggrin mutations (*FLG*-AD). The data are represented in a bar graph, showing mean and SD, and a vertical scatter plot, showing individual data points. WT-AD group showed deviation from Gaussian distribution when Kolmogorov-Smirnov normality testing was applied ($***p=0.0001$). Mann Whitney U test was used to compare between the groups ($p=0.63$, n.s.).

34 participants were patch-tested using the European Standard Battery.

An unpaired, two-tailed t-test with Welch's correction was conducted to compare the number of contact allergies of the participants in WT-AD ($M=2.33$, $SD=2.17$) and *FLG*-AD ($M=4.71$, $SD=6.16$) groups; $t(6)=1.01$, $p=0.35$, n.s. This suggests that the number of contact allergies, although higher in the *FLG*-AD group, was not significantly different between the two groups (Figure 8).

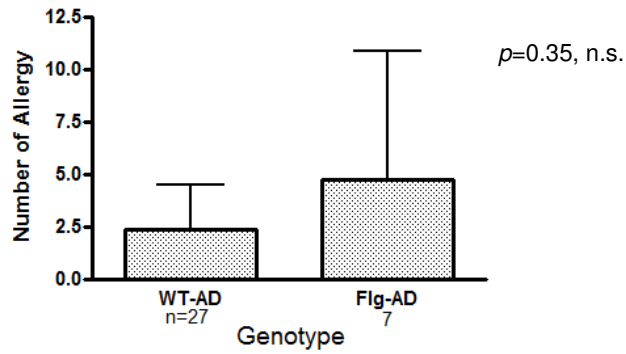


Figure 8. Comparison of the number of contact allergies in those without filaggrin mutations (WT-AD) and those with filaggrin mutations (*FLG*-AD). Data are represented in a bar showing mean and SD. Unpaired, two-tailed t-test with Welch's correction was used to compare between the groups ($p=0.35$, n.s.).

Table 3 compared the number of contact allergies of WT-AD with *FLG*-AD, and the corresponding IgE levels.

Both Figure 8 and Table 3 suggest that *FLG* genotype may be associated with a higher number of contact allergies. Future works are needed to confirm these apparent differences.

Table 3

Number of Contact Allergies and the Corresponding IgE Level of WT-AD and *FLG*-AD

WT-AD		FLG-AD	
IgE (IU/ml)	Number of Contact Allergies	IgE (IU/ml)	Number of Contact Allergies
739	5	64.7	15
664	4	837	0
140	0	238	1
74.2	9	134	0
4661	4	140	0
>5000	3	40.6	6
13.5	0	3180	11
472	3		
93.1	0		
103	0		
183	0		
65.6	1		
278	2		
307	3		
433	2		
239	1		
7.7	0		
223	4		
143	1		
19	1		
43.6	1		
14.1	1		
711	2		
3.7	5		
10.3	5		
128	4		
>5000	2		

2.2.8 Relationships between age with:

2.2.8.1 Baseline TEWL

There was a negative but non-significant correlation between age and TEWL in both WT-AD (Spearman $r(45)=-0.12$, $p=0.43$, n.s.) and *FLG*-AD (Pearson $r(13)=-0.28$, $p=0.36$, n.s.) groups, with r^2 of 0.08 (Figure 9).

Similarly, a negative but non-significant correlation (Spearman $r(58)=-0.13$, $p=0.32$, n.s.) between age and TEWL of all participants, regardless of *FLG* status, was seen (Figure 10).

Pearson correlation was used when data passed the K-S normality test. Nonparametric correlation (Spearman) was used when the data did not pass the K-S normality test (i.e. non-Gaussian).

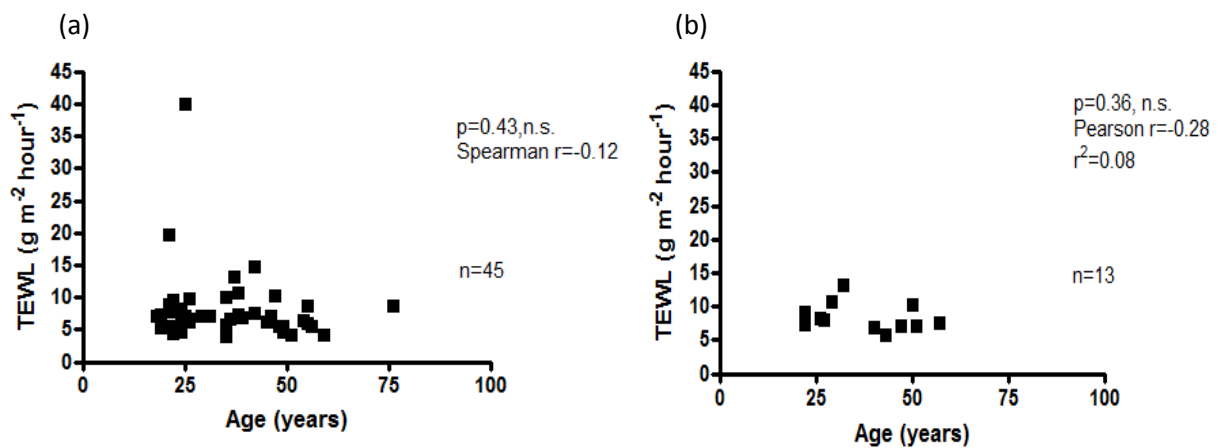


Figure 9. Correlation between age and baseline transepidermal water loss (TEWL) in (a) those without filaggrin mutations, WT-AD (Spearman $r(45)=-0.12$, $p=0.43$, n.s.) and (b) those with filaggrin mutations, *FLG*-AD (Pearson $r(13)=-0.28$, $p=0.36$, n.s., with r^2 of 0.08).

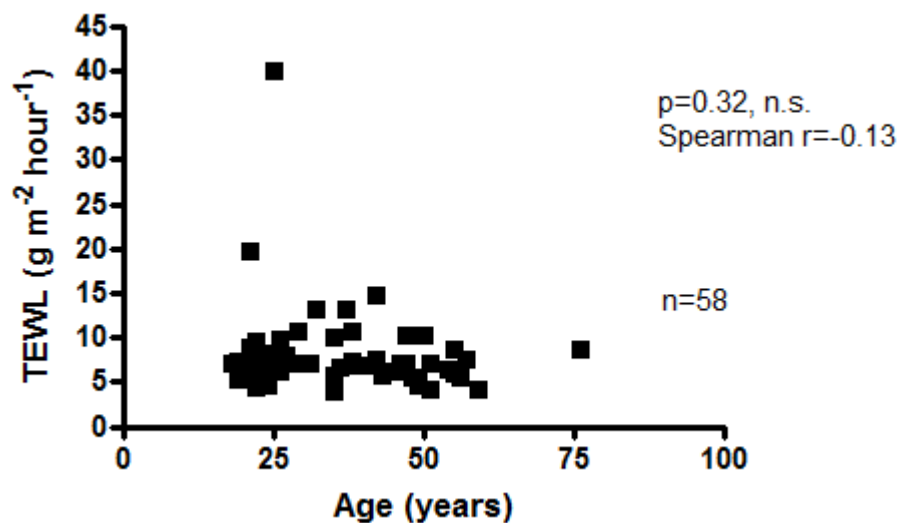


Figure 10. Correlation between age and baseline transepidermal water loss (TEWL) of all participants (Spearman $r(58)=-0.13$, $p=0.32$, n.s.).

2.2.8.2 Baseline skin capacitance

Age was found to be positively correlated with baseline skin capacitance in the WT-AD group (Pearson $r(45)=0.41$, $p<0.01$ with r^2 of 0.17, Figure 11a). In the *FLG*-AD group, a positive but non-significant correlation was also seen (Pearson $r(13)=0.21$, $p=0.49$, n.s., Figure 11b). Overall, there was a significantly positive correlation between age and baseline skin capacitance amongst all participants, regardless of genotype (Pearson $r(58)=0.37$, $p<0.01$ and r^2 of 0.14, Figure 12). Pearson correlation was used as the data passed the normality test, i.e. assumed to be sampled from a Gaussian population.

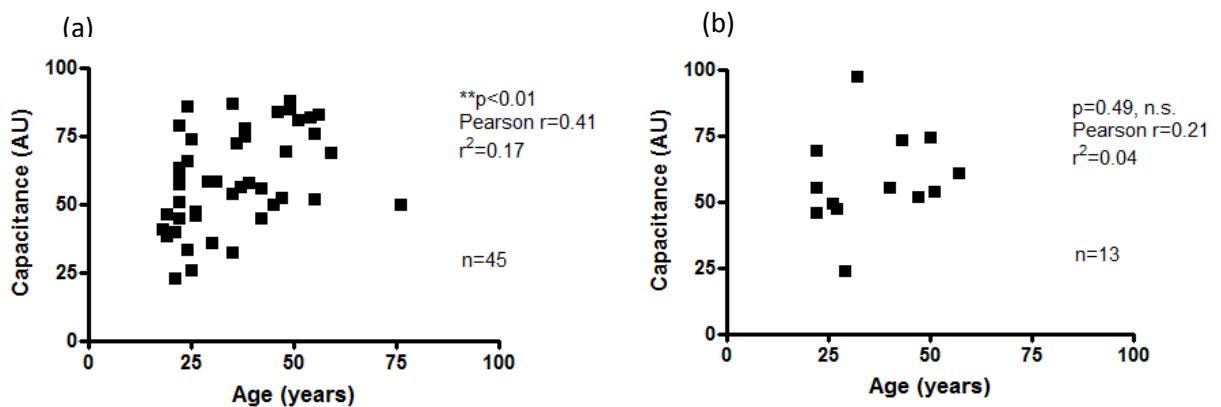


Figure 11. Correlation between age and baseline skin capacitance in (a) those without filaggrin mutations, WT-AD (Pearson $r(45)=0.41$, $p<0.01$ with r^2 of 0.17) and (b) those with filaggrin mutations, *FLG*-AD (Pearson $r(13)=0.21$, $p=0.49$, n.s.).

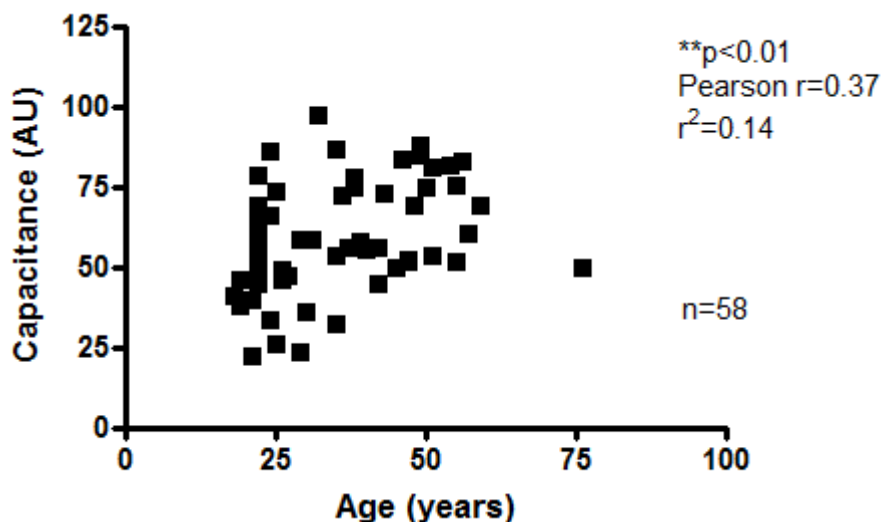


Figure 12. Correlation between age and baseline skin capacitance of all participants (Pearson $r(58)=0.37$, $p<0.01$ and r^2 of 0.14).

2.2.8.3 Disease severity (SASSAD score)

There was no significant correlation between age and disease severity in both groups ([WT-AD: Pearson $r(45)=0.05$, $p=0.75$, n.s., Figure 13a], [*FLG*-AD: Pearson $r(13)=0.08$, $p=0.79$, n.s., Figure 13b]). Pearson correlation was used as the data passed the normality test, i.e. assumed to be sampled from a Gaussian population.

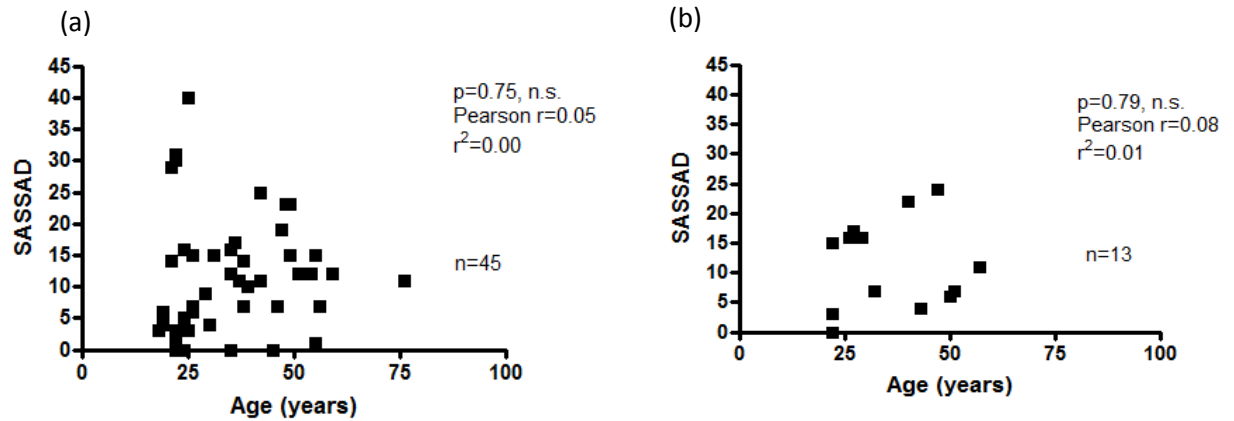


Figure 13. Correlation between age and disease severity (indicated by SASSAD score) in (a) those without filaggrin mutations, WT-AD (Pearson $r(45)=0.05$, $p=0.75$, n.s.) and (b) those with filaggrin mutations, *FLG*-AD (Pearson $r(13)=0.08$, $p=0.79$, n.s.).

2.2.9 Relationships between gender with:

2.2.9.1 Baseline TEWL

A two-way ANOVA found no significant difference in the baseline TEWL between the two genders which yielded an F ratio of $F(1,54)=1.10$, $p=0.30$, n.s., in both the WT-AD and FLG-AD groups ($F(1,54)=0.00$, $p=1.00$, n.s.) and no significant interaction between gender and filaggrin status, $F(1,54)=0.31$, $p=0.58$, n.s. (Figure 14).

The data on baseline TEWL (measured in $\text{g m}^{-2} \text{hour}^{-1}$) of male participants deviated significantly ($*p=0.03$) from normality when one-sample Kolmogorov-Smirnov (K-S) normality testing was applied; the data on females passed the normality testing ($p>0.10$, n.s.). The data on males did not appear to be normally distributed; some outliers were evident (Figure 15). Mann Whitney test was, therefore, used to compare the baseline TEWL of the male and female participants, regardless of FLG status. There was no significant difference in the baseline TEWL between males ($M=9.64$, $SD=7.33$) and females ($M=7.41$, $SD=3.02$), $p=0.12$, n.s. (Figure 15).

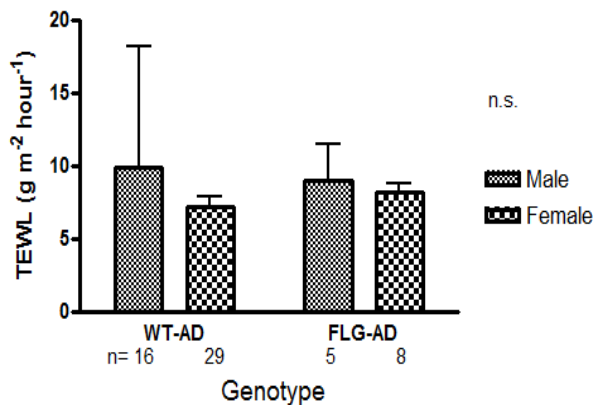


Figure 14. Comparison of the baseline transepidermal water loss (TEWL) of males and females in those without filaggrin mutations (WT-AD) and those with filaggrin mutations (FLG-AD). The data are represented in a bar graph showing mean and SD. Two-way ANOVA was applied, revealing no significant effect of gender ($p=0.30$, n.s.), FLG status ($p=1.00$, n.s.) or interaction between gender and genotype ($p=0.58$, n.s.) on TEWL.

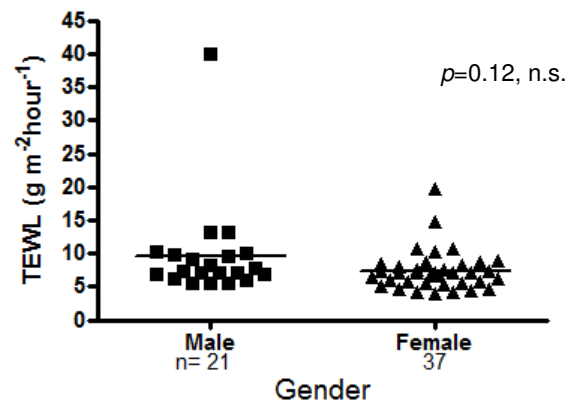


Figure 15. Comparison of the baseline transepidermal water loss (TEWL) of male vs female participants, irrespective of genotype. The data are represented in a vertical scatter plot, showing individual data points. Male group showed deviation from Gaussian distribution when Kolmogorov-Smirnov normality testing was applied ($*p=0.03$). Mann Whitney U test was used to compare between the groups ($p=0.12$, n.s.).

2.2.9.2 Baseline skin capacitance

A two-way ANOVA found no significant difference in the baseline skin capacitance between the two genders, which yielded an F ratio of $F(1,54)=0.18$, $p=0.68$, n.s., in both the WT-AD and *FLG*-AD groups ($F(1,54)=0.00$, $p=0.97$, n.s.) and no significant interaction between gender and *FLG* status, $F(1,54)=0.45$, $p=0.51$, n.s. (Figure 16).

Unpaired, two-tailed t-test was used to compare the baseline skin capacitance of both male ($M=59.04$, $SD=16.71$) and female ($M=58.72$, $SD=18.36$) groups, regardless of *FLG* status. There was no significant difference between the groups, $t(56)=0.07$, $p=0.95$, n.s (Figure 17).

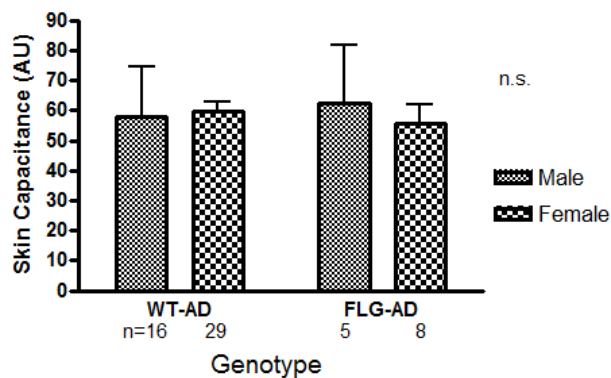


Figure 16. Comparison of the baseline skin capacitance of males and females in those without filaggrin mutations (WT-AD) and those with filaggrin mutations (*FLG*-AD). The data are represented in a bar graph showing mean and SD. Two-way ANOVA was applied, revealing no significant effect of gender ($p=0.68$, n.s.), *FLG* status ($p=0.97$, n.s.) or interaction between gender and genotype ($p=0.51$, n.s.) on skin capacitance.

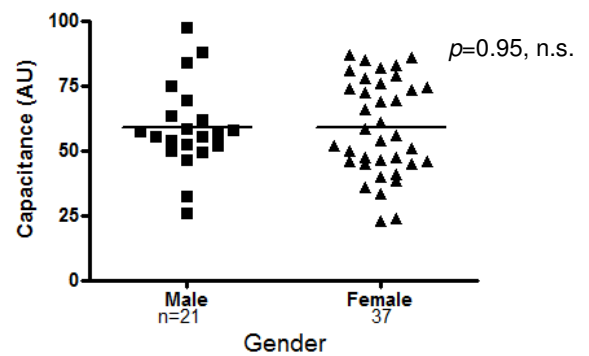


Figure 17. Comparison of the baseline skin capacitance of male vs female participants, irrespective of genotype. The data are represented in a vertical scatter plot, showing individual data points. Unpaired, two-tailed t-test was used to compare between the groups ($p=0.95$, n.s.).

2.2.9.3 Disease Severity (SASSAD score)

A two-way ANOVA found no significant difference in the SASSAD scores between the two genders, which yielded an F ratio of $F(1,54)=0.10$, $p=0.75$, n.s., in both the WT-AD and *FLG*-AD groups ($F(1,54)=0.06$, $p=0.82$, n.s.) and no significant interaction between gender and *FLG* status, $F(1,54)=0.28$, $p=0.60$, n.s. (Figure 18).

Unpaired, two-tailed t-test was used to compare the SASSAD of both male ($M=12.71$, $SD=10.25$) and female ($M=10.95$, $SD=8.09$) groups, regardless of *FLG* status. There was no significant difference between the groups, $t(56)=0.73$, $p=0.47$, n.s. (Figure 19).

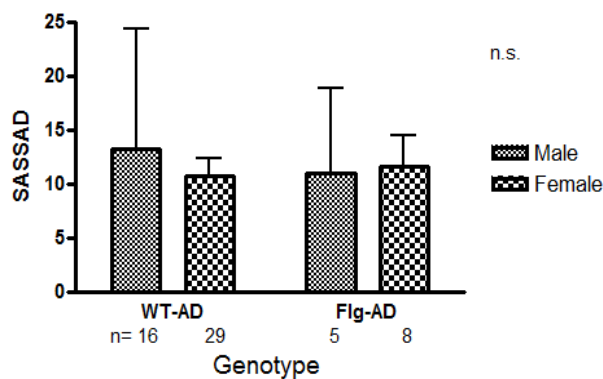


Figure 18. Comparison of the disease severity (indicated by SASSAD score) of males and females in those without filaggrin mutations (WT-AD) and those with filaggrin mutations (*FLG*-AD). The data are represented in a bar graph showing mean and SD. Two-way ANOVA was applied, revealing no significant effect of gender ($p=0.75$, n.s.), *FLG* status ($p=0.82$, n.s.) or interaction between gender and genotype ($p=0.60$, n.s.) on disease severity.

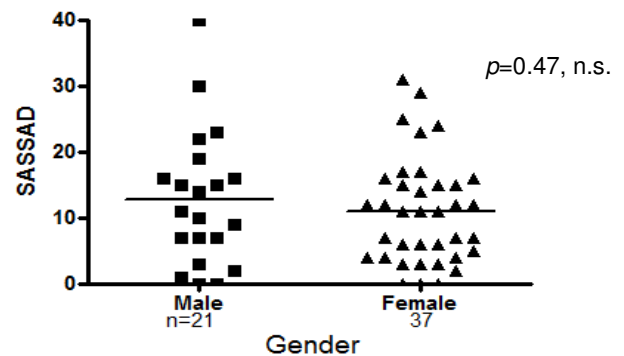


Figure 19. Comparison of the disease severity (indicated by SASSAD score) of male vs female participants, irrespective of genotype. The data are represented in a vertical scatter plot, showing individual data points. Unpaired, two-tailed t-test was used to compare between the groups ($p=0.47$, n.s.).

2.2.10 Relationships between baseline TEWL and skin capacitance with:

2.2.10.1 Co-existing presence of other atopic conditions

2.2.10.1.1 Dry skin

A two-way ANOVA found no significant difference in the baseline TEWL between those reported having dry skin and those denied having dry skin, which yielded an F ratio of $F(1,37)=0.01$, $p=0.93$, n.s., in both the WT-AD and *FLG*-AD groups ($F(1,37)=0.11$, $p=0.74$, n.s.) and no significant interaction between having dry skin and filaggrin status, $F(1,37)=0.15$, $p=0.70$, n.s. (Figure 20).

The data on baseline TEWL (measured in $\text{g m}^{-2} \text{hour}^{-1}$) of those reported having dry skin deviated significantly ($**p=0.01$) from normality when one-sample Kolmogorov-Smirnov (K-S) normality testing was applied; the data on those denied having dry skin passed the normality testing ($p>0.10$, n.s.). The data on those with dry skin did not appear to be normally distributed; some outliers were evident (Figure 21). Mann Whitney test was, therefore, used to compare the baseline TEWL of participants from both groups--with and without dry skin, regardless of *FLG* status. There was no significant difference in the baseline TEWL between dry skin ($M=8.65$, $SD=6.11$) and no dry skin ($M=8.16$, $SD=1.78$), $p=0.50$, n.s. (Figure 21).

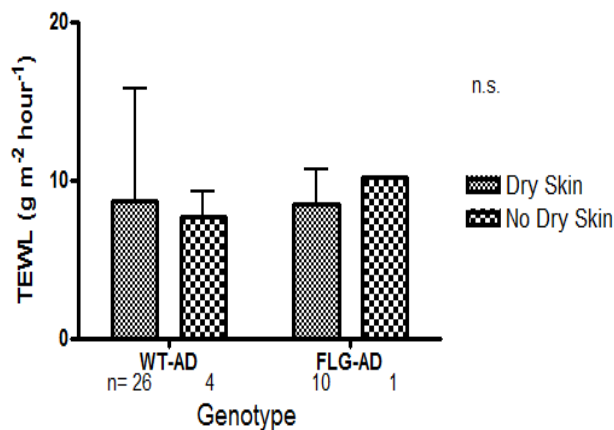


Figure 20. Comparison of the baseline transepidermal water loss (TEWL) of those who self-reported having dry skin and those who denied so, in participants without filaggrin mutations (WT-AD) and participants with filaggrin mutations (*FLG*-AD). The data are represented in a bar graph showing mean and SD. Two-way ANOVA was applied, revealing no significant effect of dry skin/no dry skin ($p=0.93$, n.s.), *FLG* status ($p=0.74$, n.s.) or interaction between dry skin/no dry skin and genotype ($p=0.70$, n.s.) on TEWL.

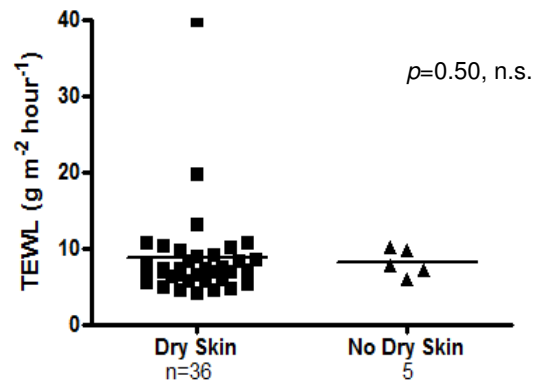


Figure 21. Comparison of the baseline transepidermal water loss (TEWL) in those who self-reported having dry skin and those who did not, irrespective of genotype. The data are represented in a vertical scatter plot, showing individual data points. 'Dry skin' group showed deviation from Gaussian distribution when Kolmogorov-Smirnov normality testing was applied ($**p=0.01$). Mann Whitney U test was used to compare between the groups ($p=0.50$, n.s.).

A two-way ANOVA found no significant difference in the baseline skin capacitance between those reported having dry skin and those denied having dry skin, which yielded an F ratio of $F(1,37)=0.63$, $p=0.43$, n.s., in both the WT-AD and *FLG*-AD groups ($F(1,37)=1.04$, $p=0.31$, n.s.) and no significant interaction between having dry skin and filaggrin status, $F(1,37)=0.66$, $p=0.42$, n.s. (Figure 22). Unpaired, two-tailed t-test was used to compare the baseline skin capacitance of both 'dry skin' ($M=55.24$, $SD=19.01$) and 'no dry skin' ($M=58.46$, $SD=12.70$) groups, regardless of *FLG* status. There was no significant difference between the groups, $t(39)=0.37$, $p=0.72$, n.s. (Figure 23).

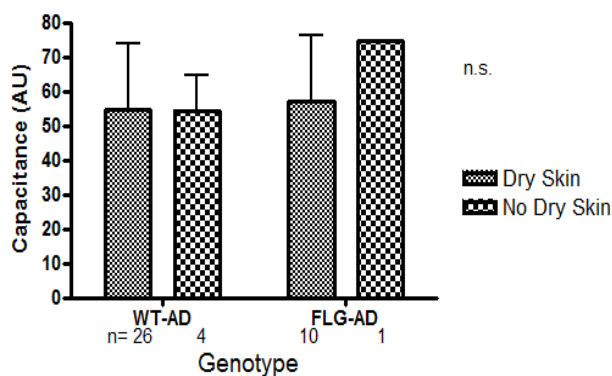


Figure 22. Comparison of the baseline skin capacitance of those who self-reported having dry skin and those who did not, in participants without filaggrin mutations (WT-AD) and participants with filaggrin mutations (*FLG*-AD). The data are represented in a bar graph showing mean and SD. Two-way ANOVA was applied, revealing no significant effect of dry skin/no dry skin ($p=0.43$, n.s.), *FLG* status ($p=0.31$, n.s.) or interaction between dry skin/no dry skin and genotype ($p=0.42$, n.s.) on skin capacitance.

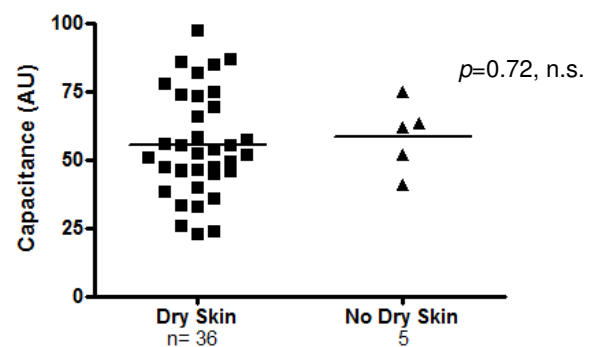


Figure 23. Comparison of the baseline skin capacitance in those who self-reported having dry skin and those who did not, irrespective of genotype. The data are represented in a vertical scatter plot, showing individual data points. Unpaired, two-tailed t-test was used to compare between the groups ($p=0.72$, n.s.).

These results suggest that the presence of dry skin (as reported by participants themselves) was not associated with worse TEWL or skin capacitance.

2.2.10.1.2 Asthma

A two-way ANOVA found no significant difference in the baseline TEWL between those with co-existing asthma and those without, which yielded an F ratio of $F(1,54)=0.09$, $p=0.76$, n.s., in both the WT-AD and *FLG*-AD groups ($F(1,54)=0.00$, $p=0.99$, n.s.) and no significant interaction between having co-existing asthma and filaggrin status, $F(1,54)=0.60$, $p=0.44$, n.s. (Figure 24).

The data on baseline TEWL (measured in $\text{g m}^{-2} \text{hour}^{-1}$) of those reported having co-existing asthma deviated significantly (** $p=0.01$) from normality when one-sample Kolmogorov-Smirnov (K-S) normality testing was applied; the data on those without asthma passed the normality testing ($p>0.10$, n.s.). The data on those with asthma did not appear to be normally distributed; some outliers were evident (Figure 25). Mann Whitney test was, therefore, used to compare the baseline TEWL of participants with and without asthma, regardless of *FLG* status. There was no significant difference in the baseline TEWL between those with asthma ($M=8.97$, $SD=7.62$) and those without ($M=7.76$, $SD=2.55$), $p=0.71$, n.s. (Figure 25).

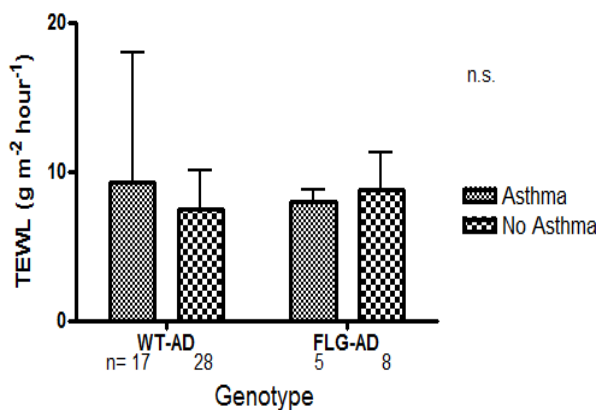


Figure 24. Comparison of the baseline transepidermal water loss (TEWL) of those with and without co-existing asthma, in participants without filaggrin mutations (WT-AD) and participants with filaggrin mutations (*FLG*-AD). The data are represented in a bar graph showing mean and SD. Two-way ANOVA was applied, revealing no significant effect of co-existing asthma ($p=0.76$, n.s.), *FLG* status ($p=0.99$, n.s.) or interaction between the presence of co-existing asthma and genotype ($p=0.44$, n.s.) on TEWL.

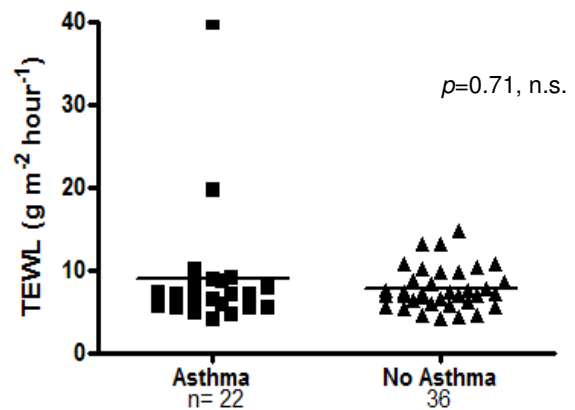


Figure 25. Comparison of the baseline transepidermal water loss (TEWL) of those with and without co-existing asthma, irrespective of genotype. The data are represented in a vertical scatter plot, showing individual data points. The 'asthma' group showed deviation from Gaussian distribution when Kolmogorov-Smirnov normality testing was applied (** $p=0.01$). Mann Whitney U test was used to compare between the groups ($p=0.71$, n.s.).

A two-way ANOVA found no significant difference in the baseline skin capacitance between those with co-existing asthma and those without, which yielded an F ratio of $F(1,54)=0.68$, $p=0.41$, n.s., in both the WT-AD and *FLG*-AD groups ($F(1,54)=0.02$, $p=0.89$, n.s.) and no significant interaction between having co-existing asthma and filaggrin status, $F(1,54)=0.01$, $p=0.94$, n.s. (Figure 26).

Unpaired, two-tailed t-test was used to compare the baseline skin capacitance of those with co-existing asthma ($M=56.02$, $SD=20.18$) and those without ($M=60.56$, $SD=15.94$), regardless of *FLG* status. There was no significant difference between the groups, $t(56)=0.95$, $p=0.35$, n.s. (Figure 27).

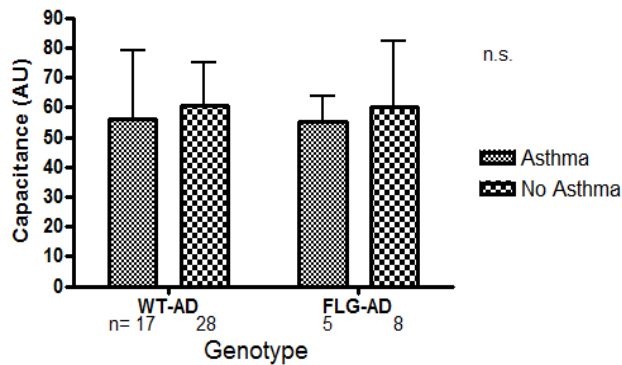


Figure 26. Comparison of the baseline skin capacitance of those with and without co-existing asthma, in participants without filaggrin mutations (WT-AD) and participants with filaggrin mutations (*FLG*-AD). The data are represented in a bar graph showing mean and SD. Two-way ANOVA was applied, revealing no significant effect of co-existing asthma ($p=0.41$, n.s.), *FLG* status ($p=0.89$, n.s.) or interaction between the presence of co-existing asthma and genotype ($p=0.94$, n.s.) on skin capacitance.

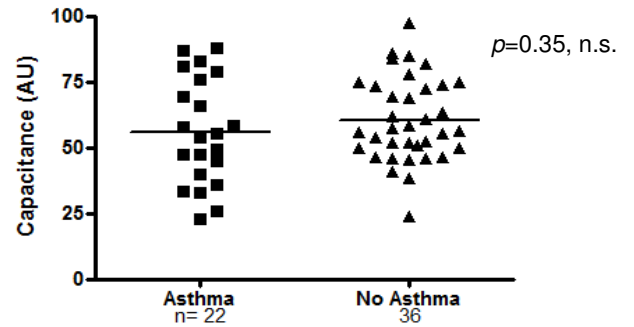


Figure 27. Comparison of the baseline skin capacitance of those with and without co-existing asthma, irrespective of genotype. The data are represented in a vertical scatter plot, showing individual data points. Unpaired, two-tailed t-test was used to compare between the groups ($p=0.35$, n.s.).

These results suggest that co-existing asthma was not associated with worse TEWL or skin capacitance.

2.2.10.1.3 Hay fever

A two-way ANOVA found no significant difference in the baseline TEWL between those with co-existing hay fever and those without, which yielded an F ratio of $F(1,54)=0.52$, $p=0.47$, n.s., in both the WT-AD and *FLG*-AD groups ($F(1,54)=0.01$, $p=0.93$, n.s.) and no significant interaction between having co-existing hay fever and filaggrin status, $F(1,54)=0.37$, $p=0.54$, n.s. (Figure 28).

The data on baseline TEWL (measured in $\text{g m}^{-2} \text{hour}^{-1}$) of those reported having co-existing hay fever deviated significantly ($*p=0.02$) from normality when one-sample Kolmogorov-Smirnov (K-S) normality testing was applied; the data on those without hay fever passed the normality testing ($p>0.10$, n.s.). The data on those with hay fever did not appear to be normally distributed; some outliers were evident (Figure 29). Mann Whitney test was, therefore, used to compare the baseline TEWL of participants with and without hay fever, regardless of *FLG* status. There was no significant difference in the baseline TEWL between those with hay fever ($M=9.20$, $SD=6.40$) and those without ($M=6.91$, $SD=1.87$), $p=0.09$, n.s. (Figure 29).

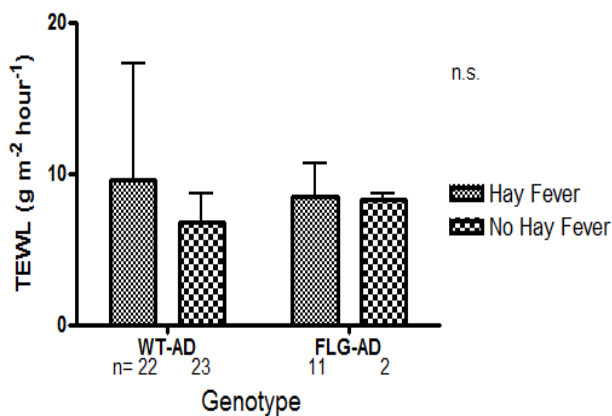


Figure 28. Comparison of the baseline transepidermal water loss (TEWL) of those with and without co-existing hay fever, in participants without filaggrin mutations (WT-AD) and participants with filaggrin mutations (*FLG*-AD). The data are represented in a bar graph showing mean and SD. Two-way ANOVA was applied, revealing no significant effect of co-existing hay fever ($p=0.47$, n.s.), *FLG* status ($p=0.93$, n.s.) or interaction between the presence of co-existing hay fever and genotype ($p=0.54$, n.s.) on TEWL.

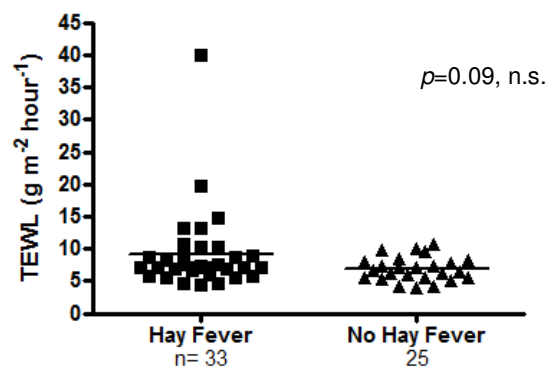


Figure 29. Comparison of the baseline transepidermal water loss (TEWL) of those with and without co-existing hay fever, irrespective of genotype. The data are represented in a vertical scatter plot, showing individual data points. The 'hay fever' group showed deviation from Gaussian distribution when Kolmogorov-Smirnov normality testing was applied ($*p=0.02$). Mann Whitney U test was used to compare between the groups ($p=0.09$, n.s.).

A two-way ANOVA found no significant difference in the baseline skin capacitance between those with co-existing hay fever and those without, which yielded an F ratio of $F(1,54)=0.39$, $p=0.54$, n.s., in both the WT-AD and *FLG*-AD groups ($F(1,54)=0.54$, $p=0.47$, n.s.) and no significant interaction between having co-existing hay fever and filaggrin status, $F(1,54)=1.54$, $p=0.22$, n.s. (Figure 30).

Unpaired, two-tailed t-test was used to compare the baseline skin capacitance of those with co-existing hay fever ($M=57.92$, $SD=18.81$) and those without ($M=60.04$, $SD=16.26$), regardless of *FLG* status. There was no significant difference between the groups, $t(56)=0.45$, $p=0.65$, n.s. (Figure 31).

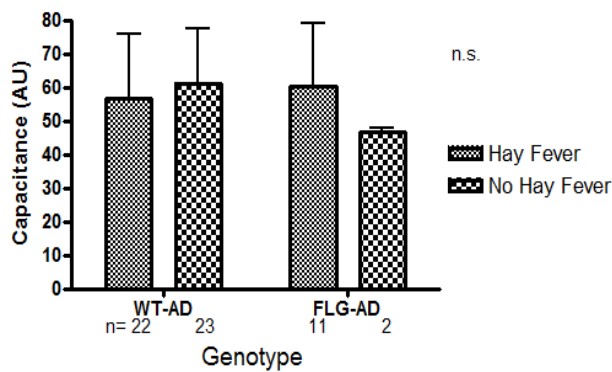


Figure 30. Comparison of the baseline skin capacitance of those with and without co-existing hay fever, in participants without filaggrin mutations (WT-AD) and participants with filaggrin mutations (*FLG*-AD). The data are represented in a bar graph showing mean and SD. Two-way ANOVA was applied, revealing no significant effect of co-existing hay fever ($p=0.54$, n.s.), *FLG* status ($p=0.47$, n.s.) or interaction between the presence of co-existing hay fever and genotype ($p=0.22$, n.s.) on skin capacitance.

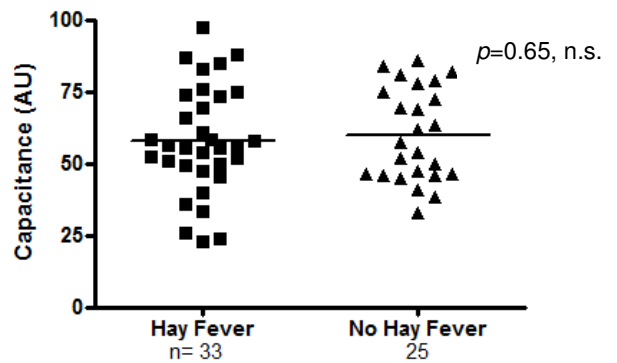


Figure 31. Comparison of the baseline skin capacitance of those with and without co-existing hay fever, irrespective of genotype. The data are represented in a vertical scatter plot, showing individual data points. Unpaired, two-tailed t-test was used to compare between the groups ($p=0.65$, n.s.).

These results suggest that hay fever was not associated with worse TEWL or skin capacitance.

2.2.10.2 Family history of atopic conditions

2.2.10.2.1 AD

A two-way ANOVA found no significant difference in the baseline TEWL between those with family history of AD and those without, which yielded an F ratio of $F(1,43)=0.46$, $p=0.50$, n.s., in both the WT-AD and FLG-AD groups ($F(1,43)=0.01$, $p=0.90$, n.s.) and no significant interaction between having family history of AD and filaggrin status, $F(1,43)=0.23$, $p=0.64$, n.s. (Figure 32).

The data on baseline TEWL (measured in $\text{g m}^{-2} \text{hour}^{-1}$) of those reported having family history of AD deviated significantly (** $p=0.01$) from normality when one-sample Kolmogorov-Smirnov (K-S) normality testing was applied; the data on those without family history of AD passed the normality testing ($p>0.10$, n.s.). The data on those with family history of AD did not appear to be normally distributed; some outliers were evident (Figure 33). Mann Whitney test was, therefore, used to compare the baseline TEWL of participants with and without family history of AD, regardless of FLG status. There was no significant difference in the baseline TEWL between those with family history of AD ($M=9.50$, $SD=6.83$) and those without ($M=7.65$, $SD=1.82$), $p=0.63$, n.s. (Figure 33).

These results suggest that having a family history of AD was not associated with worse baseline TEWL.

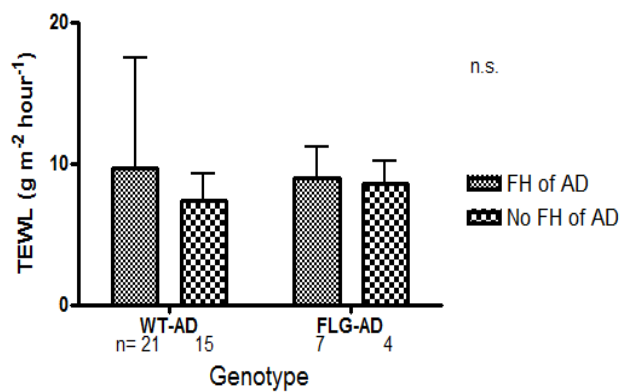


Figure 32. Comparison of the baseline transepidermal water loss (TEWL) of those with and without family history (FH) of AD, in participants without filaggrin mutations (WT-AD) and participants with filaggrin mutations (FLG-AD). The data are represented in a bar graph showing mean and SD. Two-way ANOVA was applied, revealing no significant effect of having FH of AD ($p=0.50$, n.s.), FLG status ($p=0.90$, n.s.) or interaction between having FH of AD and genotype ($p=0.64$, n.s.) on TEWL.

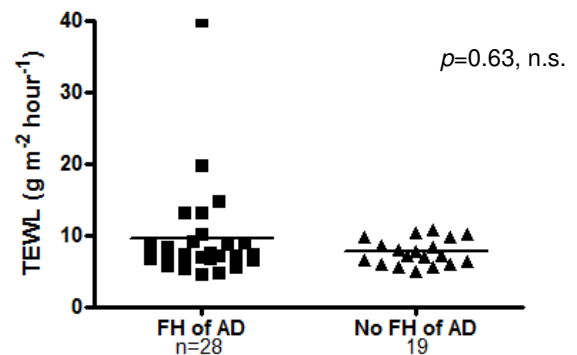


Figure 33. Comparison of the baseline transepidermal water loss (TEWL) of those with and without family history (FH) of AD, irrespective of genotype. The data are represented in a vertical scatter plot, showing individual data points. The 'FH of AD' group showed deviation from Gaussian distribution when Kolmogorov-Smirnov normality testing was applied (** $p=0.01$). Mann Whitney U test was used to compare between the groups ($p=0.63$, n.s.).

A two-way ANOVA found increased baseline skin capacitance amongst those with a family history of AD compared to those without, with an F ratio of $F(1,43)=5.58$, $*p=0.02$. Here, genotype was found to have no effect on the baseline skin capacitance ($F(1,43)=0.16$, $p=0.69$, n.s.) and no significant interaction between having family history of AD and filaggrin status, $F(1,43)=1.87$, $p=0.18$, n.s., was found (Figure 34).

Further analysis using unpaired, two-tailed t-test was used to compare the baseline skin capacitance of those with a family history of AD ($M=61.21$, $SD=19.70$) with those without a family history of AD ($M=51.05$, $SD=14.23$), irrespective of the *FLG* status. There was no significant difference between the groups, $t(45)=1.93$, $p=0.06$, n.s. (Figure 35).

These results suggest that having a family history of AD may be associated with worse baseline skin capacitance. Further works are needed to confirm this finding.

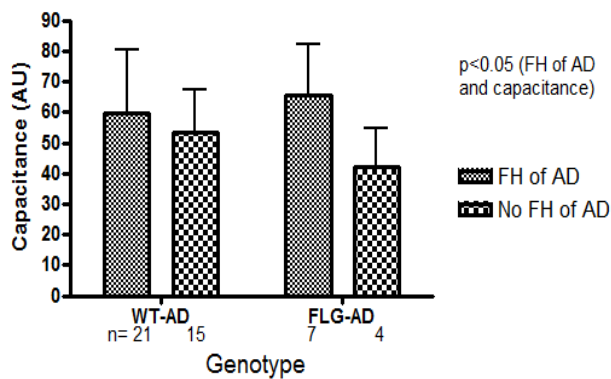


Figure 34. Comparison of the baseline skin capacitance of those with and without family history (FH) of AD, in participants without filaggrin mutations (WT-AD) and participants with filaggrin mutations (*FLG*-AD). The data are represented in a bar graph showing mean and SD. Two-way ANOVA was applied, revealing higher skin capacitance amongst those with FH of AD ($*p=0.02$). *FLG* status had no effect on skin capacitance ($p=0.69$, n.s.), nor did interaction between having FH of AD and genotype ($p=0.18$, n.s.).

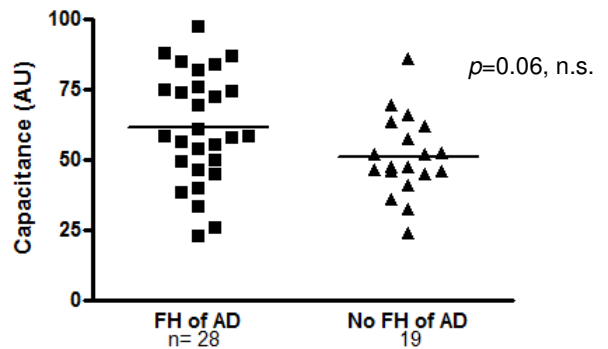


Figure 35. Comparison of the baseline skin capacitance of those with and without family history (FH) of AD, irrespective of genotype. The data are represented in a vertical scatter plot, showing individual data points. Unpaired, two-tailed t-test was used to compare between the groups ($p=0.06$, n.s.).

2.2.10.2.2 Asthma

A two-way ANOVA found no significant difference in the baseline TEWL between those with family history of asthma and those without, which yielded an F ratio of $F(1,35)=0.03$, $p=0.87$, n.s., in both the WT-AD and *FLG*-AD groups ($F(1,35)=0.05$, $p=0.83$, n.s.) and no significant interaction between having family history of asthma and filaggrin status, $F(1,35)=0.42$, $p=0.52$, n.s. (Figure 36).

The data on baseline TEWL (measured in $\text{g m}^{-2} \text{ hour}^{-1}$) of those reported having family history of asthma deviated significantly (** $p=0.01$) from normality when one-sample Kolmogorov-Smirnov (K-S) normality testing was applied; the data on those without family history of asthma passed the normality testing ($p>0.10$, n.s.). The data on those with family history of asthma did not appear to be normally distributed; some outliers were evident (Figure 37). Mann Whitney test was, therefore, used to compare the baseline TEWL of participants with and without family history of asthma, regardless of *FLG* status. There was no significant difference in the baseline TEWL between those with family history of asthma ($M=8.69$, $SD=6.91$) and those without ($M=8.28$, $SD=1.94$), $p=0.20$, n.s. (Figure 37).

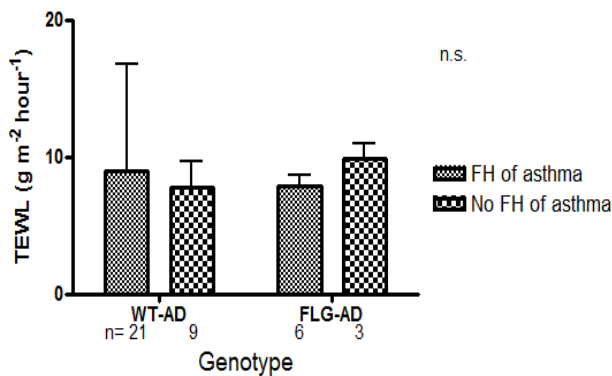


Figure 36. Comparison of the baseline transepidermal water loss (TEWL) of those with and without family history (FH) of asthma, in participants without filaggrin mutations (WT-AD) and participants with filaggrin mutations (*FLG*-AD). The data are represented in a bar graph showing mean and SD. Two-way ANOVA was applied, revealing no significant effect of having FH of asthma ($p=0.87$, n.s.), *FLG* status ($p=0.83$, n.s.) or interaction between having FH of asthma and genotype ($p=0.52$, n.s.) on TEWL.

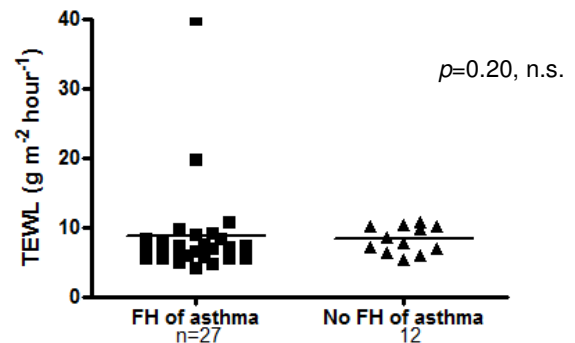


Figure 37. Comparison of the baseline transepidermal water loss (TEWL) of those with and without family history (FH) of asthma, irrespective of genotype. The data are represented in a vertical scatter plot, showing individual data points. The 'FH of asthma' group showed deviation from Gaussian distribution when Kolmogorov-Smirnov normality testing was applied (** $p=0.01$). Mann Whitney U test was used to compare between the groups ($p=0.20$, n.s.).

A two-way ANOVA found no significant difference in the baseline skin capacitance between those with family history of asthma and those without, which yielded an F ratio of $F(1,35)=1.39$, $p=0.25$, n.s., in both the WT-AD and *FLG*-AD groups ($F(1,35)=0.20$, $p=0.66$, n.s.) and no significant interaction between having family history of asthma and filaggrin status, $F(1,35)=0.03$, $p=0.86$, n.s. (Figure 38). Unpaired, two-tailed t-test was used to compare the baseline skin capacitance of those with family history of asthma ($M=59.45$, $SD=19.72$) and those without ($M=49.62$, $SD=14.93$), regardless of *FLG* status. There was no significant difference between the groups, $t(37)=1.54$, $p=0.13$, n.s. (Figure 39).

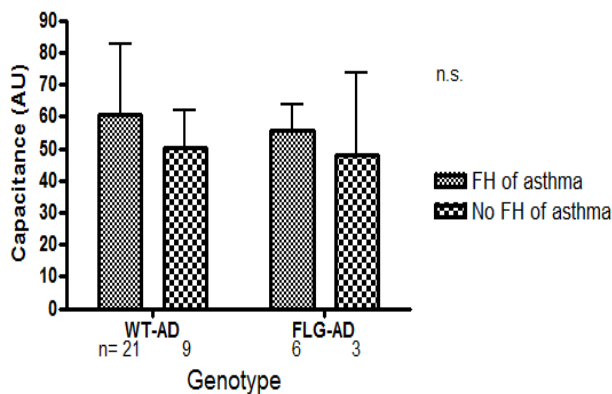


Figure 38. Comparison of the baseline skin capacitance of those with and without family history (FH) of asthma, in participants without filaggrin mutations (WT-AD) and participants with filaggrin mutations (*FLG*-AD). The data are represented in a bar graph showing mean and SD. Two-way ANOVA was applied, revealing no significant effect of having FH of asthma ($p=0.25$, n.s.), *FLG* status ($p=0.66$, n.s.) or interaction between having FH of asthma and genotype ($p=0.86$, n.s.) on skin capacitance.

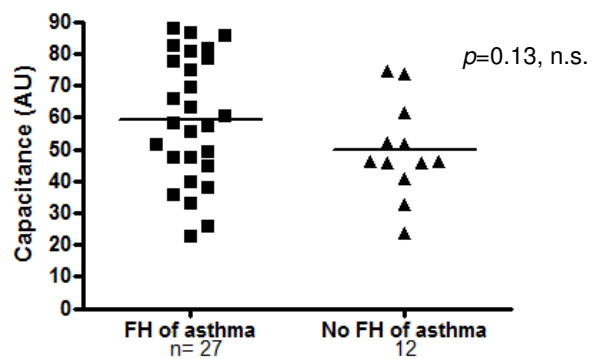


Figure 39. Comparison of the baseline skin capacitance of those with and without family history (FH) of asthma, irrespective of genotype. The data are represented in a vertical scatter plot, showing individual data points. Unpaired, two-tailed t-test was used to compare between the groups ($p=0.13$, n.s.).

These results suggest that having a family history of asthma was not associated with a worse baseline TEWL or skin capacitance.

2.2.10.2.3 Hay fever

A two-way ANOVA found no significant difference in the baseline TEWL between those with family history of hay fever and those without, which yielded an F ratio of $F(1,37)=0.01$, $p=0.93$, n.s., in both the WT-AD and *FLG*-AD groups ($F(1,37)=0.01$, $p=0.92$, n.s.) and no significant interaction between having family history of hay fever and filaggrin status, $F(1,37)=0.60$, $p=0.45$, n.s. (Figure 40).

The data on baseline TEWL (measured in $\text{g m}^{-2} \text{hour}^{-1}$) of those reported having family history of hay fever deviated significantly (** $p=0.00$) from normality when one-sample Kolmogorov-Smirnov (K-S) normality testing was applied; the data on those without family history of hay fever passed the normality testing ($p>0.10$, n.s.). The data on those with family history of hay fever did not appear to be normally distributed; some outliers were evident (Figure 41). Mann Whitney test was, therefore, used to compare the baseline TEWL of participants with and without family history of hay fever, regardless of *FLG* status. There was no significant difference in the baseline TEWL between those with family history of hay fever ($M=9.17$, $SD=7.26$) and those without ($M=8.08$, $SD=1.84$), $p=0.47$, n.s. (Figure 41).

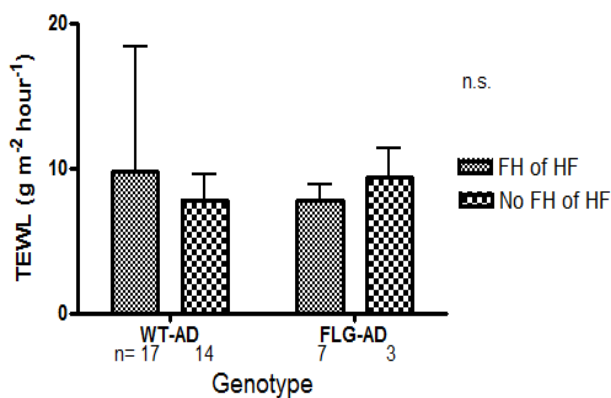


Figure 40. Comparison of the baseline transepidermal water loss (TEWL) of those with and without family history (FH) of hay fever (HF), in participants without filaggrin mutations (WT-AD) and participants with filaggrin mutations (*FLG*-AD). The data are represented in a bar graph showing mean and SD. Two-way ANOVA was applied, revealing no significant effect of having FH of HF ($p=0.93$, n.s.), *FLG* status ($p=0.92$, n.s.) or interaction between having FH of HF and genotype ($p=0.45$, n.s.) on TEWL.

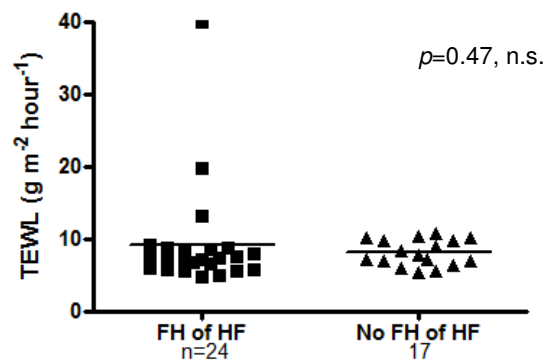


Figure 41. Comparison of the baseline transepidermal water loss (TEWL) of those with and without family history (FH) of hay fever (HF), irrespective of genotype. The data are represented in a vertical scatter plot, showing individual data points. The 'FH of HF' group showed deviation from Gaussian distribution when Kolmogorov-Smirnov normality testing was applied (** $p=0.00$). Mann Whitney U test was used to compare between the groups ($p=0.47$, n.s.).

A two-way ANOVA found no significant difference in the baseline skin capacitance between those with family history of hay fever and those without, which yielded an F ratio of $F(1,37)=0.72$, $p=0.40$, n.s., in both the WT-AD and *FLG*-AD groups ($F(1,37)=0.01$, $p=0.91$, n.s.) and no significant interaction between having family history of hay fever and filaggrin status, $F(1,37)=0.06$, $p=0.81$, n.s. (Figure 42).

Unpaired, two-tailed t-test was used to compare the baseline skin capacitance of those with family history of asthma ($M=56.74$, $SD=17.11$) and those without ($M=51.99$, $SD=16.21$), regardless of *FLG* status. There was no significant difference between the groups, $t(39)=0.89$, $p=0.38$, n.s. (Figure 43)

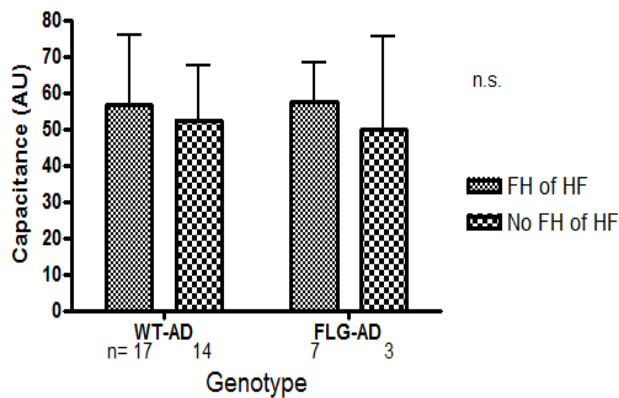


Figure 42. Comparison of the baseline skin capacitance of those with and without family history (FH) of hay fever (HF), in participants without filaggrin mutations (WT-AD) and participants with filaggrin mutations (*FLG*-AD). The data are represented in a bar graph showing mean and SD. Two-way ANOVA was applied, revealing no significant effect of having FH of HF ($p=0.40$, n.s.), *FLG* status ($p=0.91$, n.s.) or interaction between having FH of HF and genotype ($p=0.81$, n.s.) on skin capacitance.

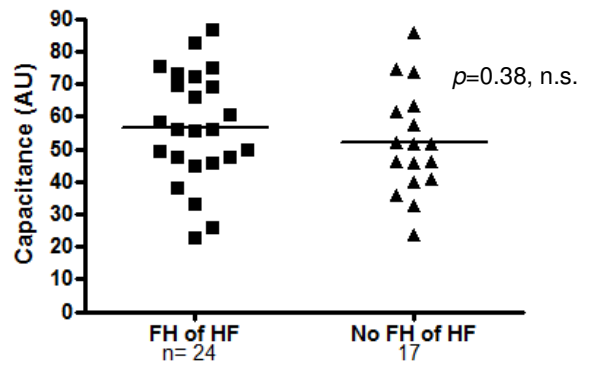


Figure 43. Comparison of the baseline skin capacitance of those with and without family history (FH) of hay fever (HF), irrespective of genotype. The data are represented in a vertical scatter plot, showing individual data points. Unpaired, two-tailed t-test was used to compare between the groups ($p=0.38$, n.s.).

These results suggest that having a family history of hay fever was not associated with a worse baseline TEWL or skin capacitance.

2.2.10.2.4 Atopic conditions (any of AD, asthma or hay fever)

A two-way ANOVA found no significant difference in the baseline TEWL between those with family history of atopy and those without, which yielded an F ratio of $F(1,49)=0.12$, $p=0.73$, n.s., in both the WT-AD and *FLG*-AD groups ($F(1,49)=0.16$, $p=0.69$, n.s.) and no significant interaction between having family history of atopy and filaggrin status, $F(1,49)=0.21$, $p=0.65$, n.s. (Figure 44).

The data on baseline TEWL (measured in $\text{g m}^{-2} \text{ hour}^{-1}$) of those reported having family history of atopy deviated significantly (** $p=0.00$) from normality when one-sample Kolmogorov-Smirnov (K-S) normality testing was applied; the data on those without family history of atopy passed the normality testing ($p>0.10$, n.s.). The data on those with family history of atopy did not appear to be normally distributed; some outliers were evident (Figure 45). Mann Whitney test was, therefore, used to compare the baseline TEWL of those with and without family history of atopy, regardless of *FLG* status. There was no significant difference in the baseline TEWL between those with family history of atopy ($M=8.49$, $SD=5.61$) and those without ($M=8.53$, $SD=1.95$), $p=0.20$, n.s. (Figure 45). These results suggest that having other family member(s) with atopic condition(s) was not associated with increased baseline TEWL.

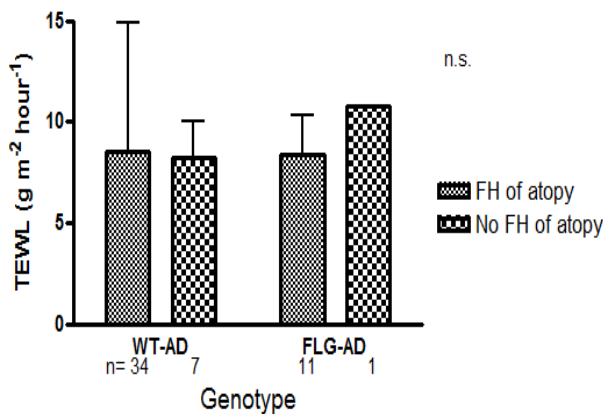


Figure 44. Comparison of the baseline transepidermal water loss (TEWL) of those with and without family history (FH) of atopy, in participants without filaggrin mutations (WT-AD) and participants with filaggrin mutations (*FLG*-AD). The data are represented in a bar graph showing mean and SD. Two-way ANOVA was applied, revealing no significant effect of having FH of atopy ($p=0.73$, n.s.), *FLG* status ($p=0.69$, n.s.) or interaction between having FH of atopy and genotype ($p=0.65$, n.s.) on TEWL.

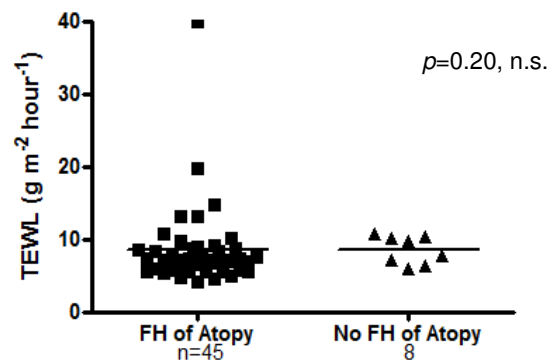


Figure 45. Comparison of the baseline transepidermal water loss (TEWL) of those with and without family history (FH) of atopy, irrespective of genotype. The data are represented in a vertical scatter plot, showing individual data points. The 'FH of Atopy' group showed deviation from Gaussian distribution when Kolmogorov-Smirnov normality testing was applied (** $p=0.00$). Mann Whitney U test was used to compare between the groups ($p=0.20$, n.s.).

A two-way ANOVA found increased baseline skin capacitance amongst those with a family history of atopy compared to those without, with an F ratio of $F(1,49)=7.16$, $*p=0.01$. Here, genotype was found to have no effect on the baseline skin capacitance ($F(1,49)=1.46$, $p=0.23$, n.s.) and no significant interaction between having family history of atopy and filaggrin status, $F(1,49)=1.44$, $p=0.24$, n.s., was found (Figure 46).

Further analysis using unpaired, two-tailed t-test was used to compare the baseline skin capacitance of those with a family history of atopy ($M=61.75$, $SD=18.08$) with those without a family history of atopy ($M=44.35$, $SD=11.94$), irrespective of the *FLG* status. A significantly higher baseline skin capacitance was found in those with a family history of atopic condition(s), $t(51)=2.61$, $*p=0.01$. (Figure 47).

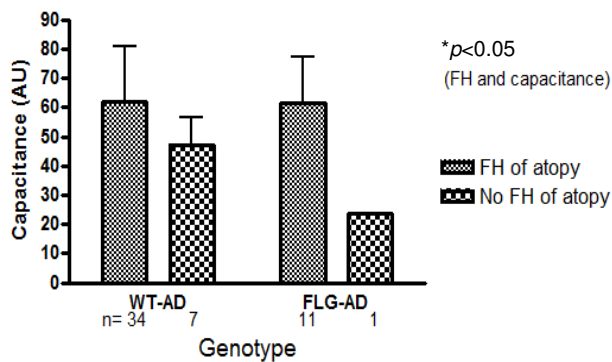


Figure 46. Comparison of the baseline skin capacitance of those with and without family history (FH) of atopy, in participants without filaggrin mutations (WT-AD) and participants with filaggrin mutations (*FLG*-AD). The data are represented in a bar graph showing mean and SD. Two-way ANOVA was applied, revealing higher skin capacitance in those having FH of atopy ($*p=0.01$). *FLG* status had no effect on skin capacitance ($p=0.23$, n.s.), nor did interaction between having FH of atopy and genotype ($p=0.24$, n.s.).

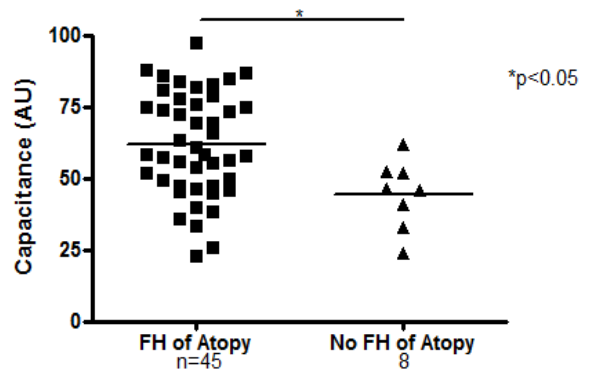


Figure 47. Comparison of the baseline skin capacitance of those with and without family history (FH) of atopy, irrespective of genotype. The data are represented in a vertical scatter plot, showing individual data points. Unpaired, two-tailed t-test showed higher baseline skin capacitance in those with FH of atopy ($*p=0.01$).

2.2.10.3 Number of contact allergies

No significant correlation was found between baseline TEWL with the number of contact allergies of participants in both WT-AD (Pearson $r(27)=0.22$, $p=0.28$, n.s.) and *FLG*-AD (Pearson $r(7)=-0.19$, $p=0.68$, n.s.) groups.

No significant correlation was found between baseline skin capacitance with the number of contact allergies of participants in both WT-AD (Pearson $r(27)=-0.03$, $p=0.88$, n.s.) and *FLG*-AD (Pearson $r(7)=-0.31$, $p=0.50$, n.s.) groups.

2.2.11 Relationships between SASSAD disease severity score with:

2.2.11.1 Subjective scoring of disease severity

Participants were asked to score the severity of their disease on the scale of 0 to 10 (0 - negligible; 10 - extremely severe). This was followed by a clinical examination during which the severity of their disease was objectively scored using the SASSAD system. Interestingly, linear regression analysis showed no relationship between the participants' perception of their disease severity and the SASSAD scores objectively determined by the investigator (in both WT-AD [$F(1,35)=4.11$, $p=0.05$, n.s., $r^2=0.11$, $n=37$] and *FLG*-AD [$F(1,8)=0.00$, $p=0.98$, n.s.; $r^2=0.00$, $n=10$] groups) (Figure 48).

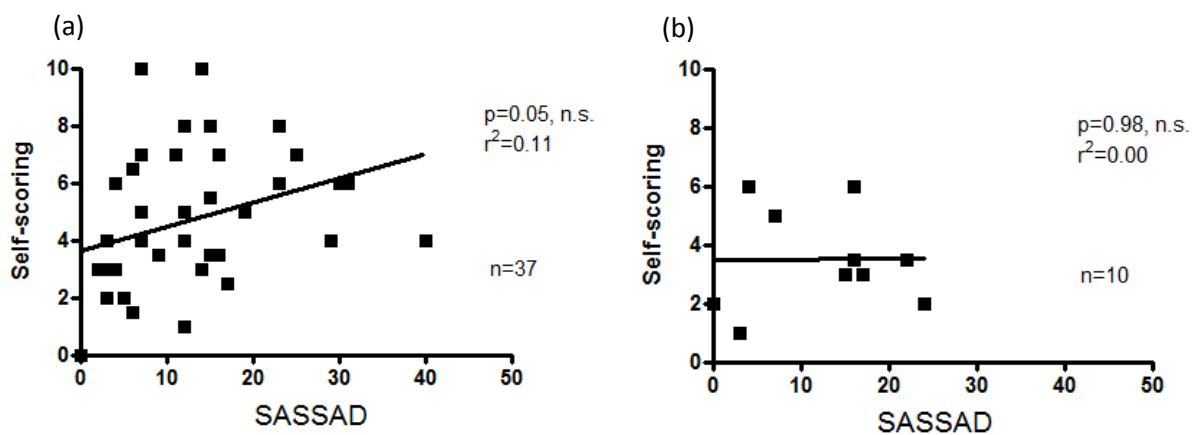


Figure 48. Relationship (linear regression analysis) between disease severity (indicated by SASSAD score) and self-perception of the disease severity in (a) WT-AD, $p=0.05$, n.s., as compared to (b) *FLG*-AD, $p=0.98$, n.s.

2.2.11.2 Baseline TEWL and skin capacitance

Linear regression analysis showed that higher TEWL was associated with increasing disease severity (represented by SASSAD score) in the WT-AD group ($F(1,43)=12.81$, $***p<0.00$; $r^2=0.23$, $n=45$, Figure 49a). This relationship was not seen in the *FLG*-AD group, potentially due to the smaller sample size ($F(1,11)=0.61$, $p=0.45$, n.s.; $r^2=0.05$, $n=13$, Figure 49b).

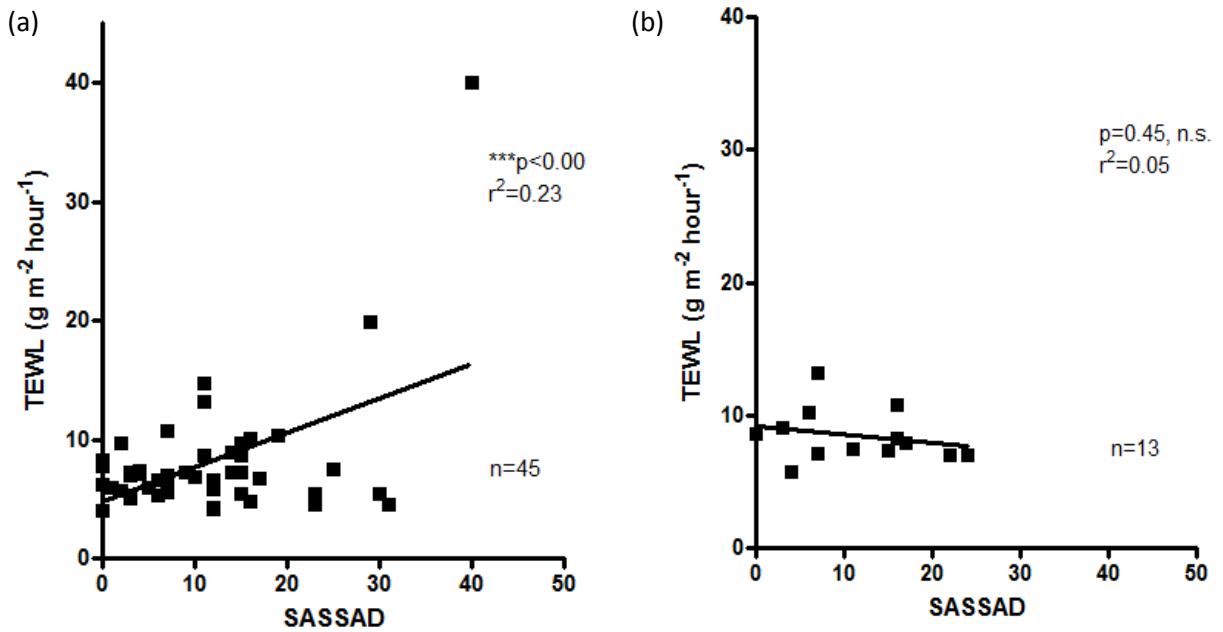


Figure 49. Relationship between disease severity (as indicated by SASSAD score) and baseline transepidermal water loss (TEWL) in (a) WT-AD, which showed higher TEWL with increasing disease severity ($***p < 0.00$), and (b) FLG-AD, which showed no significant relationship between TEWL and SASSAD ($p = 0.45$).

Linear regression analysis showed that in both WT-AD and FLG-AD groups, decreasing capacitance (i.e. skin hydration) was seen with increasing disease severity (SASSAD). This relationship was, nonetheless, not statistically significant ([WT-AD: $F(1,43) = 1.77$, $p = 0.19$, n.s., $r^2 = 0.04$, $n = 45$, Figure 50a], [FLG-AD: $F(1,11) = 1.29$, $p = 0.28$, n.s., $r^2 = 0.10$, $n = 13$, Figure 50b]).

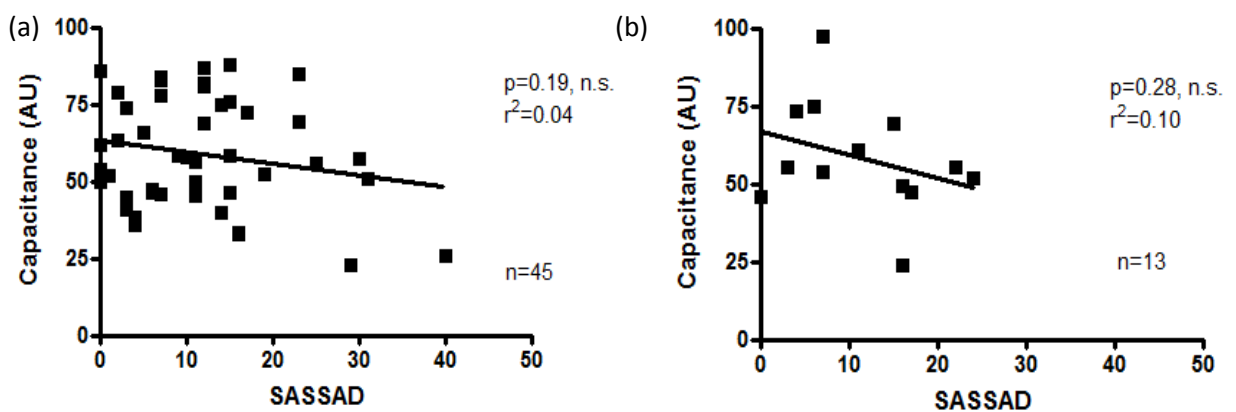


Figure 50. Relationship between disease severity (as indicated by SASSAD score) and baseline skin capacitance in (a) WT-AD, $p = 0.19$, n.s., as compared to (b) FLG-AD, $p = 0.28$, n.s.

2.2.11.3 Presence of other atopic conditions

2.2.11.3.1 Dry skin

Despite an apparent difference, two-way ANOVA analysis revealed no significant increase in the disease severity score (SASSAD) in those who reported having a dry skin compared to those who did not ($F(1,37)=2.59$, $p=0.12$, n.s., Figure 51). This is unsurprising given the small sample size of those who denied having dry skin (5/41; 1 in the *FLG*-AD group), which affects the degree of freedom in the analysis. Genotype had no effect on disease severity ($F(1,37)=0.09$, $p=0.77$, n.s.) and no significant interaction between having dry skin and genotype, $F(1,37)=0.24$, $p=0.63$, n.s., was found (Figure 51). Unpaired t-test with Welch's correction showed that amongst all participants, those who reported having dry skin had a higher SASSAD score ($M=13.17$, $SD=9.97$) than those who did not ($M=2.40$, $SD=2.30$), $t(29)=5.51$, $***p<0.0001$, Figure 52.

Note: Skin dryness, objectively assessed by the investigator, forms a part of the SASSAD score in these analyses. The 'dry skin' here refers to the presence of skin dryness in the last 12 months, as reported by the participants themselves. Patients' perception of their disease and the objectively determined SASSAD score can differ, as shown in Figure 48.

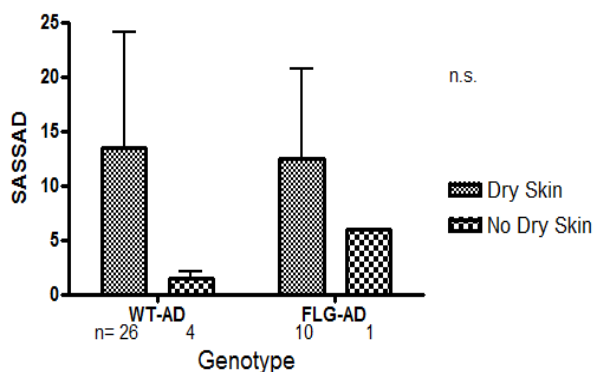


Figure 51. Comparison of the disease severity (indicated by SASSAD score) of those with and without self-reported skin dryness, in participants without filaggrin mutations (WT-AD) and participants with filaggrin mutations (*FLG*-AD). The data are represented in a bar graph showing mean and SD. Two-way ANOVA was applied, revealing no significant effect of having dry skin (as reported by participants themselves), ($p=0.12$, n.s.), *FLG* status ($p=0.77$, n.s.) or interaction between having dry skin and genotype ($p=0.63$, n.s.) on disease severity.

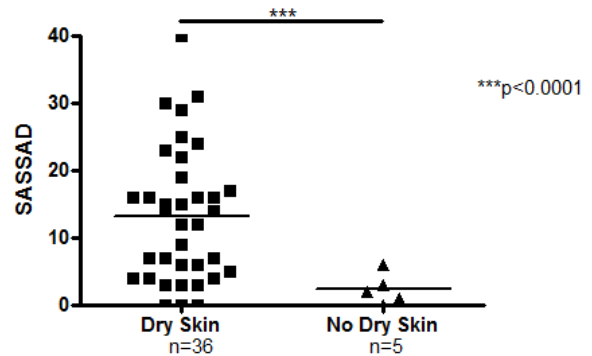


Figure 52. Comparison of the disease severity (indicated by SASSAD score) of those with and without self-reported skin dryness, irrespective of genotype. The data are represented in a vertical scatter plot, showing individual data points. Unpaired, two-tailed t-test with Welch's correction showed more severe disease in those who reported having dry skin ($***p<0.0001$).

2.2.11.3.2 Asthma

A two-way ANOVA found no significant difference in the disease severity (indicated by SASSAD score) between those with co-existing asthma and those without, which yielded an F ratio of $F(1,54)=0.11$, $p=0.74$, n.s., in both the WT-AD and *FLG*-AD groups ($F(1,54)=0.02$, $p=0.89$, n.s.) and no significant interaction between having co-existing asthma and filaggrin status, $F(1,54)=0.05$, $p=0.83$, n.s. (Figure 53).

Unpaired, two-tailed t-test was used to compare the disease severity (indicated by SASSAD score) of those with co-existing asthma ($M=12.41$, $SD=8.86$) and those without ($M=11.08$, $SD=8.99$), regardless of *FLG* status. There was no significant difference between the groups, $t(56)=0.55$, $p=0.59$, n.s. (Figure 54)

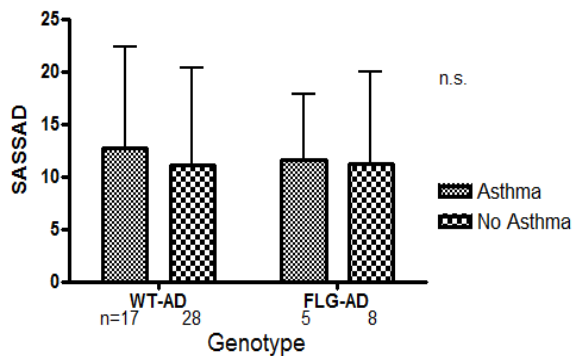


Figure 53. Comparison of the disease severity (indicated by SASSAD score) of those with and without asthma, in participants without filaggrin mutations (WT-AD) and participants with filaggrin mutations (*FLG*-AD). The data are represented in a bar graph showing mean and SD. Two-way ANOVA was applied, revealing no significant effect of having co-existing asthma ($p=0.74$, n.s.), *FLG* status ($p=0.89$, n.s.) or interaction between having asthma and genotype ($p=0.83$, n.s.) on disease severity.

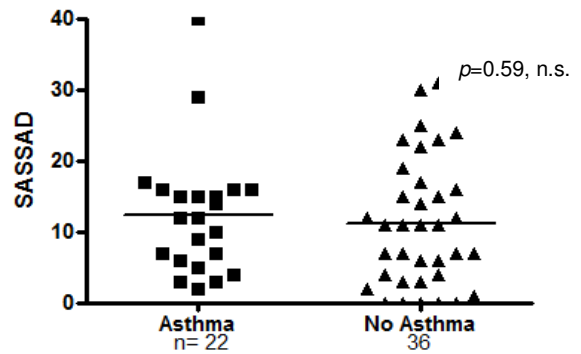


Figure 54. Comparison of the disease severity (indicated by SASSAD score) of those with and without asthma, irrespective of genotype. The data are represented in a vertical scatter plot, showing individual data points. Unpaired, two-tailed t-test was used to compare between the groups ($p=0.59$, n.s.).

2.2.11.3.3 Hay Fever

Similar to Section 2.2.11.3.1, two-way ANOVA analysis revealed no significant increase in the disease severity score (SASSAD) in participants who have co-existing hay fever, compared to those who do not ($F(1,54)=2.02$, $p=0.16$, n.s.). Genotype was also found to have no effect on disease severity ($F(1,54)=0.18$, $p=0.67$, n.s.) and no significant interaction between having co-existing hay fever and filaggrin status, $F(1,54)=0.21$, $p=0.65$, n.s., was found (Figure 55).

Further analysis using unpaired, two-tailed t-test found that amongst all participants, those with co-existing hay fever had a significantly higher SASSAD score ($M=14.00$, $SD=8.80$) than those without ($M=8.40$, $SD=8.11$), $t(56)=2.48$, $*p=0.02$, Figure 56.

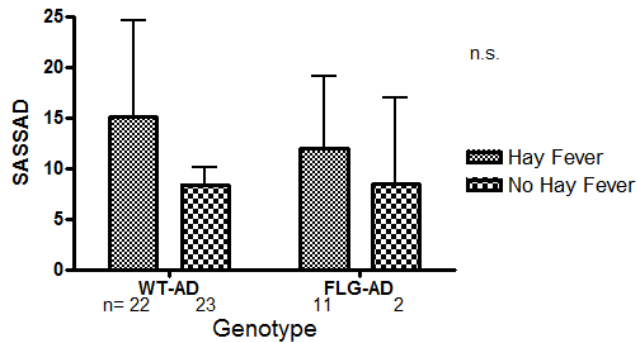


Figure 55. Comparison of the disease severity (indicated by SASSAD score) of those with and without hay fever (HF), in participants without filaggrin mutations (WT-AD) and participants with filaggrin mutations (FLG-AD). The data are represented in a bar graph showing mean and SD. Two-way ANOVA was applied, revealing no significant effect of having co-existing HF ($p=0.16$, n.s.), FLG status ($p=0.67$, n.s.) or interaction between having HF and genotype ($p=0.65$, n.s.) on disease severity.

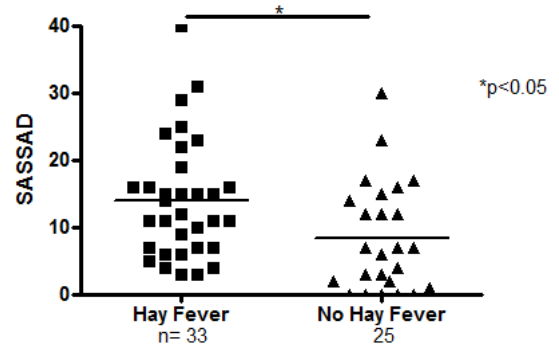


Figure 56. Comparison of the disease severity (indicated by SASSAD score) of those with and without hay fever (HF), irrespective of genotype. The data are represented in a vertical scatter plot, showing individual data points. Unpaired, two-tailed t-test showed more severe disease in those who reported having HF ($*p<0.05$).

2.2.11.4 Family history of atopic conditions

2.2.11.4.1 AD

A two-way ANOVA found no significant difference in the disease severity (indicated by SASSAD score) between those with family history of AD and those without, which yielded an F ratio of $F(1,43)=0.00$, $p=0.99$, n.s., in both the WT-AD and *FLG*-AD groups ($F(1,43)=0.01$, $p=0.92$, n.s.) and no significant interaction between having family history of AD and filaggrin status, $F(1,43)=2.77$, $p=0.10$, n.s. (Figure 57).

Unpaired, two-tailed t-test was used to compare the SASSAD score of those with family history of AD ($M=12.82$, $SD=7.86$) and those without ($M=10.05$, $SD=9.49$), regardless of *FLG* status. There was no significant difference between the groups, $t(45)=1.09$, $p=0.28$, n.s. (Figure 58)

These results suggest that having a family history of AD was not associated with a significant increase in disease severity (indicated by SASSAD score) amongst the participants.

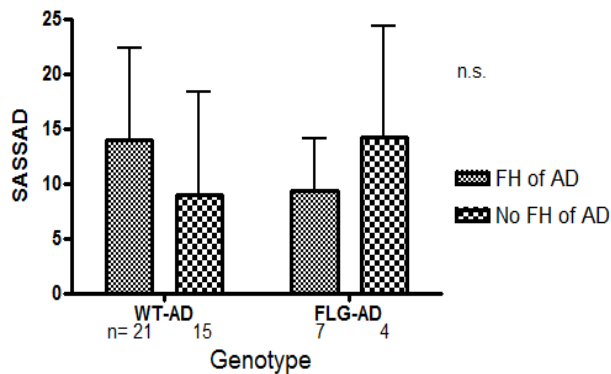


Figure 57. Comparison of the disease severity (indicated by SASSAD score) of those with and without family history (FH) of AD, in participants without filaggrin mutations (WT-AD) and participants with filaggrin mutations (*FLG*-AD). The data are represented in a bar graph showing mean and SD. Two-way ANOVA was applied, revealing no significant effect of having FH of AD ($p=0.99$, n.s.), *FLG* status ($p=0.92$, n.s.) or interaction between having FH of AD and genotype ($p=0.10$, n.s.) on disease severity.

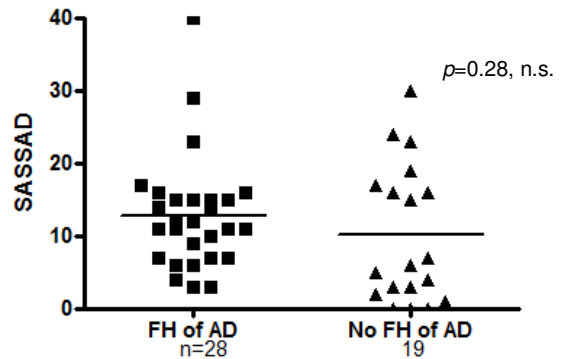


Figure 58. Comparison of the disease severity (indicated by SASSAD score) of those with and without family history (FH) of AD, irrespective of genotype. The data are represented in a vertical scatter plot, showing individual data points. Unpaired, two-tailed t-test was used to compare between the groups ($p=0.28$, n.s.).

2.2.11.4.2 Asthma

A two-way ANOVA found no significant difference in the disease severity (indicated by SASSAD score) between those with family history of asthma and those without, which yielded an F ratio of $F(1,35)=2.14$, $p=0.15$, n.s., in both the WT-AD and *FLG*-AD groups ($F(1,35)=0.10$, $p=0.76$, n.s.) and no significant interaction between having family history of asthma and filaggrin status, $F(1,35)=0.19$, $p=0.67$, n.s. (Figure 59).

Unpaired, two-tailed t-test was used to compare the SASSAD score of those with family history of asthma ($M=12.19$, $SD=9.61$) and those without ($M=7.67$, $SD=6.96$), regardless of *FLG* status. There was no significant difference between the groups, $t(37)=1.46$, $p=0.15$, n.s. (Figure 60)

These results suggest that having a family history of asthma was not associated with a significant increase in disease severity (indicated by SASSAD score) amongst the participants.

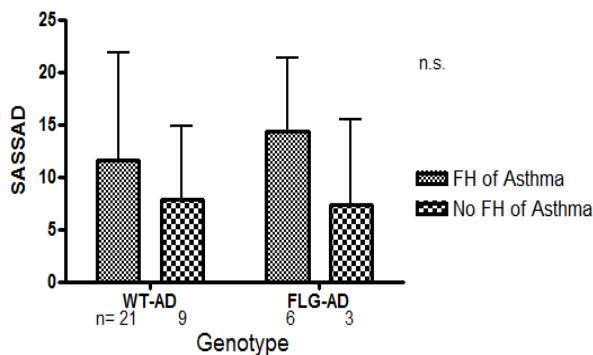


Figure 59. Comparison of the disease severity (indicated by SASSAD score) of those with and without family history (FH) of asthma, in participants without filaggrin mutations (WT-AD) and participants with filaggrin mutations (*FLG*-AD). The data are represented in a bar graph showing mean and SD. Two-way ANOVA was applied, revealing no significant effect of having FH of asthma ($p=0.15$, n.s.), *FLG* status ($p=0.76$, n.s.) or interaction between having FH of asthma and genotype ($p=0.67$, n.s.) on disease severity.

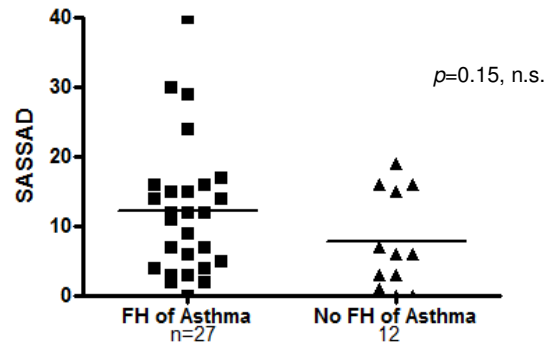


Figure 60. Comparison of the disease severity (indicated by SASSAD score) of those with and without family history (FH) of asthma, irrespective of genotype. The data are represented in a vertical scatter plot, showing individual data points. Unpaired, two-tailed t-test was used to compare between the groups ($p=0.15$, n.s.).

2.2.11.4.3 Hay fever

A two-way ANOVA found no significant difference in the disease severity (indicated by SASSAD score) between those with family history of hay fever and those without, which yielded an F ratio of $F(1,37)=0.00$, $p=0.99$, n.s., in both the WT-AD and *FLG*-AD groups ($F(1,37)=0.05$, $p=0.82$, n.s.) and no significant interaction between having family history of hay fever and filaggrin status, $F(1,37)=2.80$, $p=0.10$, n.s. (Figure 61).

Unpaired, two-tailed t-test was used to compare the SASSAD score of those with family history of hay fever ($M=13.04$, $SD=9.32$) and those without ($M=9.77$, $SD=9.05$), regardless of *FLG* status. There was no significant difference between the groups, $t(39)=1.12$, $p=0.27$, n.s. (Figure 62).

These results suggest that having a family history of hay fever was not associated with a significant increase in disease severity (indicated by SASSAD score) amongst the participants.

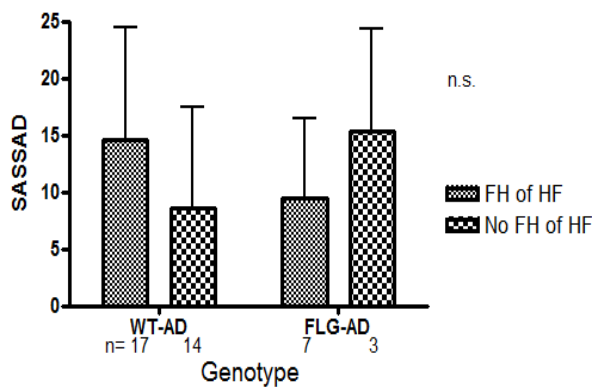


Figure 61. Comparison of the disease severity (indicated by SASSAD score) of those with and without family history (FH) of hay fever (HF), in participants without filaggrin mutations (WT-AD) and participants with filaggrin mutations (*FLG*-AD). The data are represented in a bar graph showing mean and SD. Two-way ANOVA was applied, revealing no significant effect of having FH of HF ($p=0.99$, n.s.), *FLG* status ($p=0.82$, n.s.) or interaction between having FH of HF and genotype ($p=0.10$, n.s.) on disease severity.

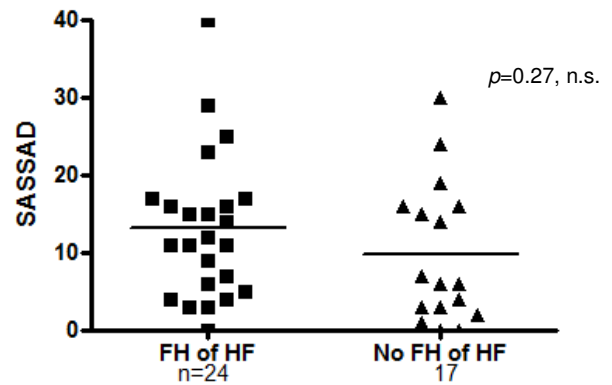


Figure 62. Comparison of the disease severity (indicated by SASSAD score) of those with and without family history (FH) of hay fever (HF), irrespective of genotype. The data are represented in a vertical scatter plot, showing individual data points. Unpaired, two-tailed t-test was used to compare between the groups ($p=0.27$, n.s.).

2.2.11.4.4 Atopic conditions (any of AD, asthma or hay fever)

45 out of 53 participants had a family history of atopic condition(s) (AD, asthma or hay fever).

A two-way ANOVA found no significant difference in the disease severity (indicated by SASSAD score) between those with family history of atopy and those without, which yielded an F ratio of $F(1,49)=0.06$, $p=0.81$, n.s., in both the WT-AD and *FLG*-AD groups ($F(1,49)=0.26$, $p=0.61$, n.s.) and no

significant interaction between having family history of atopy and filaggrin status, $F(1,49)=1.00$, $p=0.32$, n.s. (Figure 63).

Unpaired, two-tailed t-test was used to compare the SASSAD score of those with family history of atopy ($M=11.78$, $SD=8.66$) and those without ($M=9.63$, $SD=7.71$), regardless of *FLG* status. There was no significant difference between the groups, $t(51)=0.66$, $p=0.51$, n.s. (Figure 64)

These results suggest that those with a family history of atopic condition(s) were no more severe, in terms of their AD, than those without a family history of atopic condition(s).

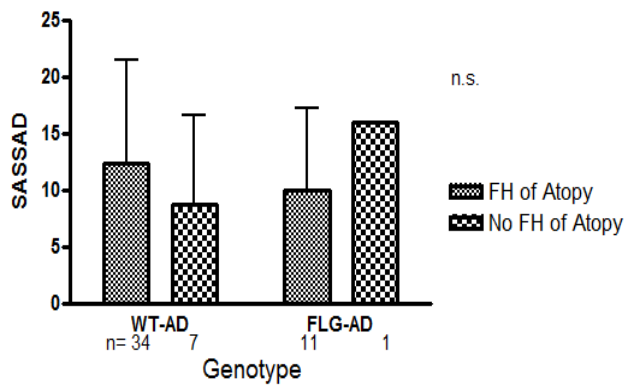


Figure 63. Comparison of the disease severity (indicated by SASSAD score) of those with and without family history of atopy, in participants without filaggrin mutations (WT-AD) and participants with filaggrin mutations (*FLG*-AD). The data are represented in a bar graph showing mean and SD. Two-way ANOVA was applied, revealing no significant effect of having FH of atopy ($p=0.81$, n.s.), *FLG* status ($p=0.61$, n.s.) or interaction between having FH of atopy and genotype ($p=0.32$, n.s.) on disease severity.

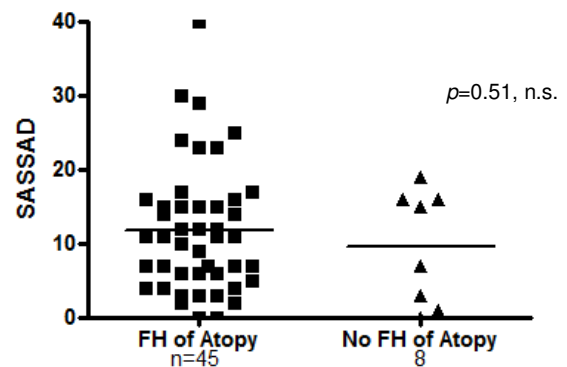


Figure 64. Comparison of the disease severity (indicated by SASSAD score) of those with and without family history of atopy, irrespective of genotype. The data are represented in a vertical scatter plot, showing individual data points. Unpaired, two-tailed t-test was used to compare between the groups ($p=0.51$, n.s.).

2.2.11.5 IgE level and number of contact allergies

There was no significant correlation between the number of contact allergies amongst participants of both groups with the severity of their disease ([WT-AD: Pearson $r(27)=-0.11$, $p=0.60$, n.s., and r^2 of 0.01]; [*FLG*-AD: Pearson $r(7)=-0.21$, $p=0.65$, n.s. and $r^2=0.04$]).

Disease severity (SASSAD score) was found to be positively correlated with the level of total serum IgE in the WT-AD group (Pearson $r(45)=0.39$, $p<0.01$ with r^2 of 0.15, Figure 65a). This relationship was not seen in the *FLG*-AD group, possibly due to the much smaller sample size (Pearson $r(13)=-0.06$, $p=0.84$, n.s. and r^2 of 0.00, Figure 65b).

Pearson correlation analysis was used as the data passed the normality test, i.e. assumed to be sampled from a Gaussian population.

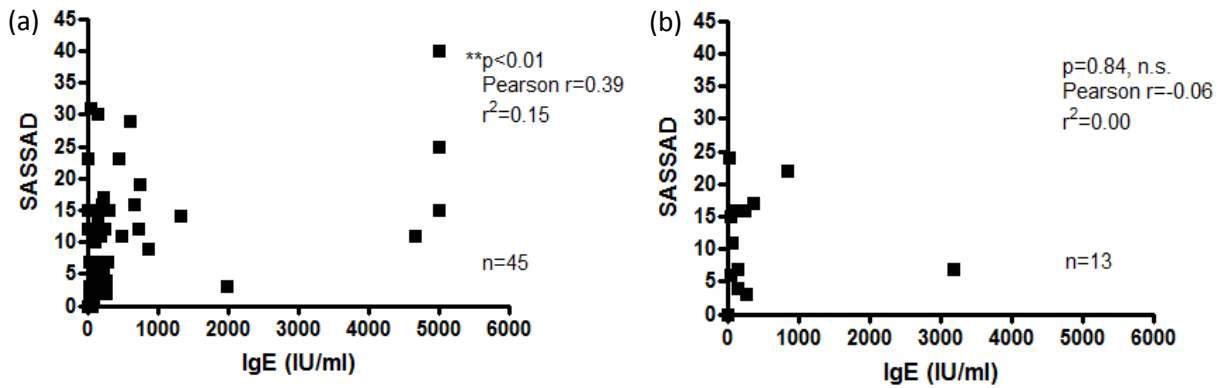


Figure 65. Correlation between total serum IgE level and disease severity (indicated by SASSAD score) in (a) those without filaggrin mutations, WT-AD (Pearson $r(45)=0.39$, $p<0.01$ with r^2 of 0.15), and (b) those with filaggrin mutations, FLG-AD (Pearson $r(13)=-0.06$, $p=0.84$, n.s. and r^2 of 0.00).

2.2.12 Correlations between IgE level with:

2.2.12.1 Baseline TEWL and skin capacitance

TEWL was found to be positively correlated with the level of total serum IgE of the WT-AD participants (Spearman $r(45)=0.38$, $p<0.01$, Figure 66a). This was not observed amongst FLG-ADs, possibly due to the smaller sample size (Pearson $r(13)=-0.25$, $p=0.41$, n.s. and r^2 of 0.06, Figure 66b). Nonparametric correlation (Spearman) was used for the WT-AD as the data failed the normality test (non-Gaussian). Pearson correlation was used for the FLG-AD as the data passed the normality test.

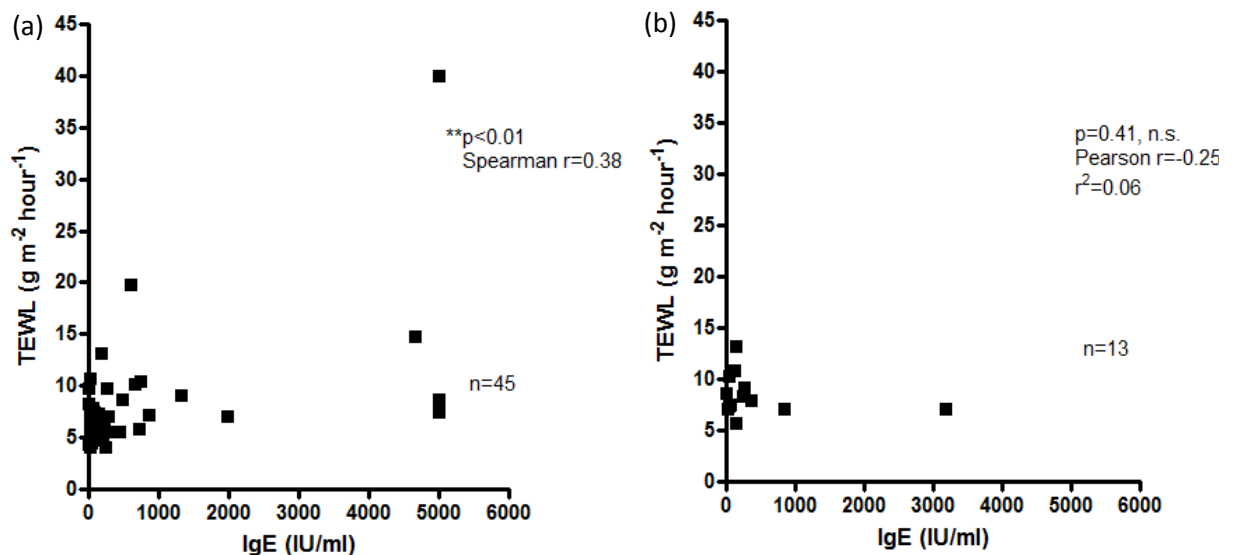


Figure 66. Correlation between total serum IgE level and baseline transepidermal water loss (TEWL) in (a) those without filaggrin mutations, WT-AD (Spearman $r(45)=0.38$, $p<0.01$), and (b) those with filaggrin mutations, FLG-AD (Pearson $r(13)=-0.25$, $p=0.41$, n.s. and r^2 of 0.06).

Correlation analysis showed a non-significant negative correlation between total serum IgE and skin capacitance was seen in both the WT-AD (Pearson $r(45)=-0.16$, $p=0.31$, n.s. and r^2 of 0.02, Figure 67a) and *FLG*-AD groups (Pearson $r(13)=-0.11$, $p=0.72$, n.s. and r^2 of 0.01, Figure 67b).

Pearson correlation was used for the *FLG*-AD as the data passed the normality test.

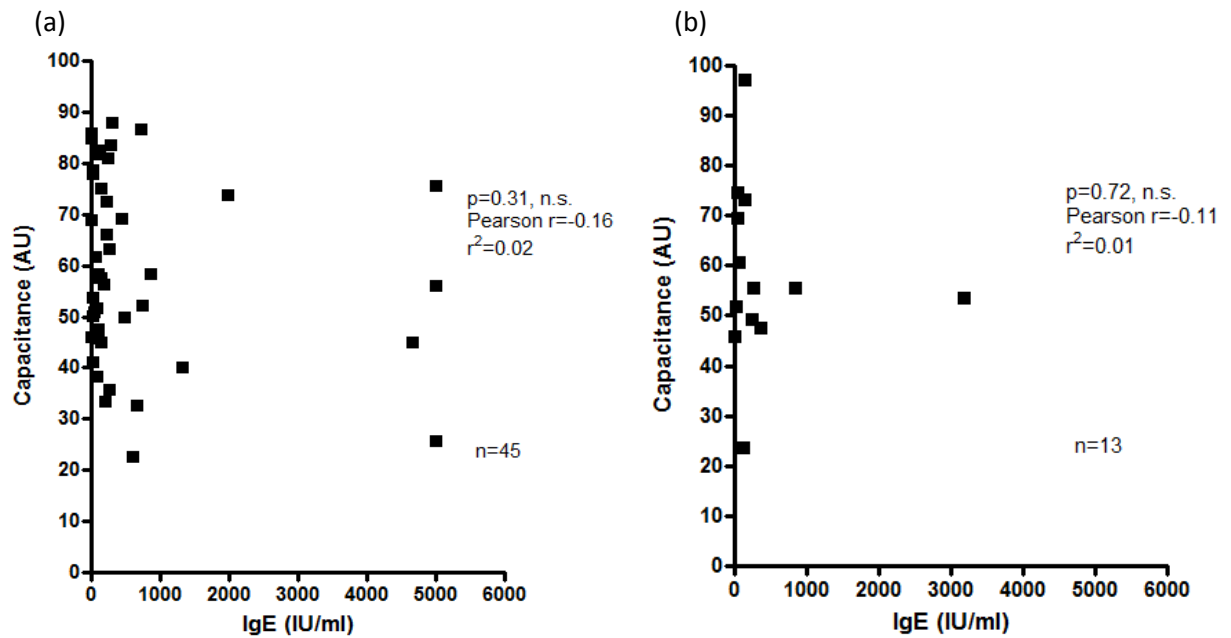


Figure 67. Correlation between total serum IgE level and baseline skin capacitance in (a) those without filaggrin mutations, WT-AD (Pearson $r(45)=-0.16$, $p=0.31$, n.s. and r^2 of 0.02), and (b) those with filaggrin mutations, *FLG*-AD (Pearson $r(13)=-0.11$, $p=0.72$, n.s. and r^2 of 0.01).

2.2.12.2 Number of contact allergies

There was a positive, though not significant, correlation between the level of total serum IgE and the number of contact allergies of participants (identified by patch-testing using the European Standard Battery) in both WT-AD (Pearson $r(27)=0.14$, $p=0.49$, n.s. and r^2 of 0.02) and *FLG*-AD (Pearson $r(7)=0.35$, $p=0.45$, n.s. and r^2 of 0.12) groups (Figure 68).

Pearson correlation analysis was used as the data passed the normality test, i.e. assumed to be sampled from a Gaussian population.

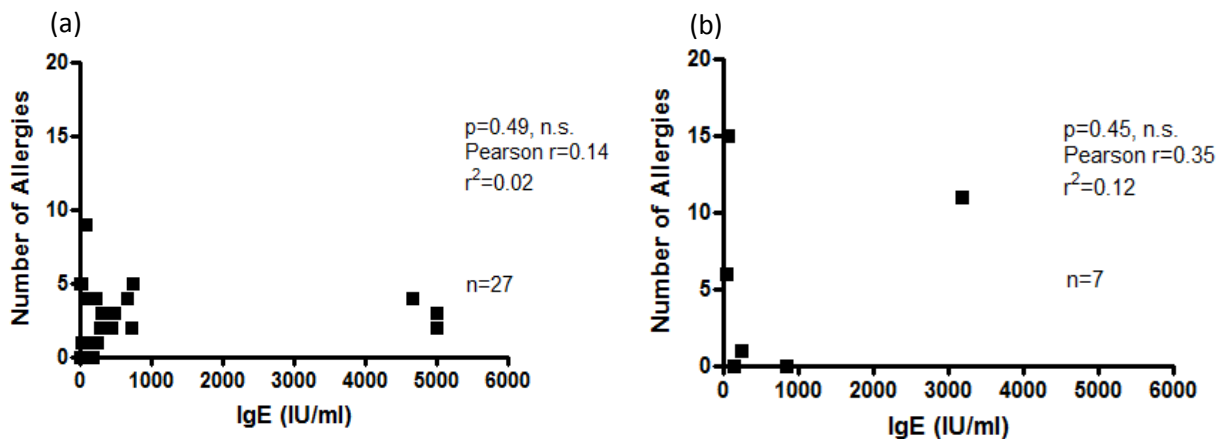


Figure 68. Correlation between total serum IgE level and the number of contact allergies in (a) those without filaggrin mutations, WT-AD (Pearson $r(27)=0.14$, $p=0.49$, n.s. and r^2 of 0.02), and (b) those with filaggrin mutations, *FLG*-AD (Pearson $r(7)=0.35$, $p=0.45$, n.s. and r^2 of 0.12).

2.2.13 Correlations between susceptibility to physical insult by tape-stripping with:

The tape-stripping (TS) method was used to mechanically disrupt the skin barrier function. The number of TS required to abrogate the permeability barrier (defined as $TEWL > 20 \text{g m}^{-2} \text{ hour}^{-1}$) in 58 participants was recorded. A low number of TS used suggests a high level of susceptibility to physical disruption.

2.2.13.1 Age and gender

Correlation analysis showed no significant correlation between age and susceptibility to physical insult, as indicated by the number of TS required to reach $TEWL > 20 \text{g m}^{-2} \text{ hour}^{-1}$, in both WT-AD (Pearson $r(45)=0.05$, $p=0.75$, n.s. with r^2 of 0.00) and *FLG*-AD (Pearson $r(13)=0.11$, $p=0.72$, n.s. and r^2 of 0.01) groups (Figure 69).

Further correlation analysis also showed no correlation between age and the number of TS required in all participants, regardless of their genotype (Pearson $r(58)=0.05$, $p=0.73$, n.s. and r^2 of 0.00, Figure 70).

Pearson correlation analysis was used as the data passed the normality test, i.e. assumed to be sampled from a Gaussian population.

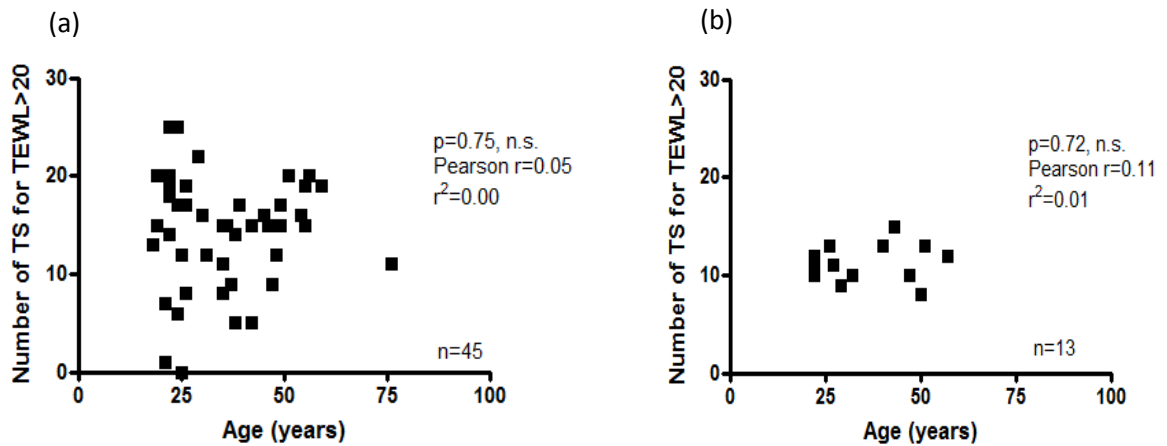


Figure 69. Correlation between age and susceptibility to physical insult (indicated by the number of tape-strippings [TS] required to reach transepidermal water loss [TEWL] $>20\text{g m}^{-2}\text{ hour}^{-1}$) in (a) those without filaggrin mutations, WT-AD (Pearson $r(45)=0.05$, $p=0.75$, n.s. with r^2 of 0.00), and (b) those with filaggrin mutations, FLG-AD (Pearson $r(13)=0.11$, $p=0.72$, n.s. and r^2 of 0.01).

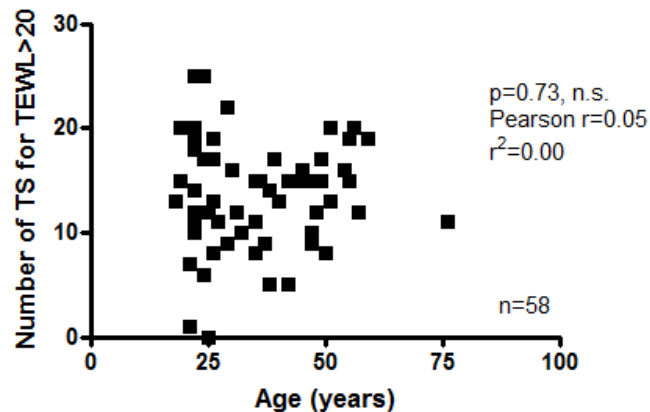


Figure 70. Correlation between age and susceptibility to physical insult of all participants (indicated by the number of tape-strippings [TS] required to reach transepidermal water loss [TEWL] $>20\text{g m}^{-2}\text{ hour}^{-1}$). Pearson $r(58)=0.05$, $p=0.73$, n.s. and r^2 of 0.00.

A two-way ANOVA found no significant difference in the number of TS required to reach TEWL of more than $20\text{g m}^{-2}\text{ hour}^{-1}$ between males and females, which yielded an F ratio of $F(1,54)=0.23$, $p=0.63$, n.s., in both the WT-AD and FLG-AD groups ($F(1,54)=2.79$, $p=0.10$, n.s.) and no significant interaction between gender and filaggrin status, $F(1,54)=0.03$, $p=0.85$, n.s. on the number of TS required (Figure 71).

Unpaired, two-tailed t-test was used to compare the number of TS required of males ($M=13.90$, $SD=5.40$) and females ($M=13.32$, $SD=5.14$), regardless of *FLG* status. There was no significant difference between the groups, $t(56)=0.41$, $p=0.69$, n.s. (Figure 72).

These results suggest that gender has no effect on the skin's susceptibility to physical insult.

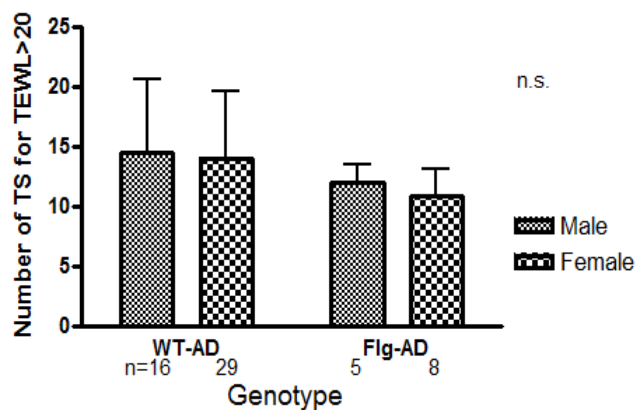


Figure 71. Comparison of the susceptibility to physical insult (indicated by the number of tape-strippings [TS] required to reach transepidermal water loss [TEWL] $>20\text{g m}^{-2}\text{ hour}^{-1}$) of males and females in those without filaggrin mutations (WT-AD) and those with filaggrin mutations (*FLG*-AD). The data are represented in a bar graph showing mean and SD. Two-way ANOVA was applied, revealing gender ($p=0.63$, n.s.) and *FLG* status ($p=0.10$, n.s.) had no effect on the number of TS required. No interaction between gender and genotype ($p=0.85$, n.s.), which could have influenced the number of TS, was found.

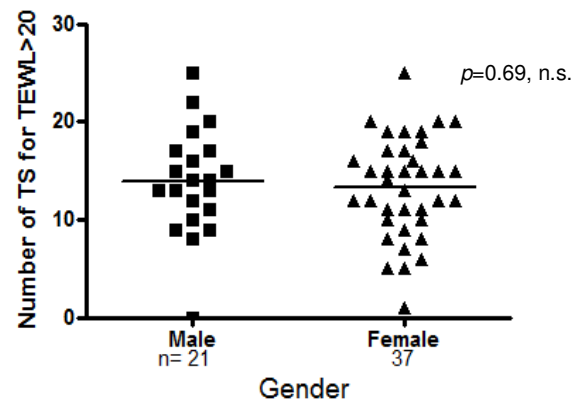


Figure 72. Comparison of the susceptibility to physical insult (indicated by the number of tape-strippings [TS] required to reach transepidermal water loss [TEWL] $>20\text{g m}^{-2}\text{ hour}^{-1}$) of male vs female participants, irrespective of genotype. The data are represented in a vertical scatter plot, showing individual data points. Unpaired, two-tailed t-test was used to compare between the groups ($p=0.69$, n.s.).

2.2.13.2 Genotype

Unpaired, two-tailed t-test with Welch's correction showed that the number of TS required to reach TEWL of more than $20\text{g m}^{-2}\text{ hour}^{-1}$ in the *FLG*-AD group ($M=11.31$, $SD=1.93$) was significantly less than the WT-AD group ($M=14.18$, $SD=5.67$), indicating an increased susceptibility to physical insult amongst the *FLG*-AD participants ($t(54)=2.87$, $p<0.01$) (Figure 73).

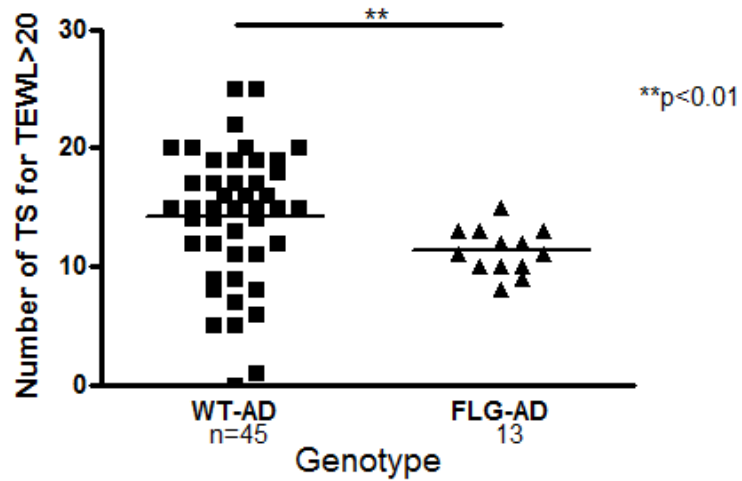


Figure 73. Comparison of the susceptibility to physical insult (indicated by the number of tape-strippings [TS] required to reach transepidermal water loss [TEWL] $>20\text{g m}^{-2}\text{ hour}^{-1}$) in those without filaggrin mutations (WT-AD) and those with filaggrin mutations (FLG-AD). Unpaired, two-tailed t-test with Welch’s correction was used, showing reduced number of TS required by the FLG-AD group, $p<0.01$.

2.2.13.3 Baseline TEWL and skin capacitance

Linear regression analysis showed that a lower number of TS was required to abrogate skin permeability barrier when the baseline TEWL was higher ($F(1,55)=39.13$, $***p<0.0001$, $r^2=0.42$, $n=58$, Figure 74).

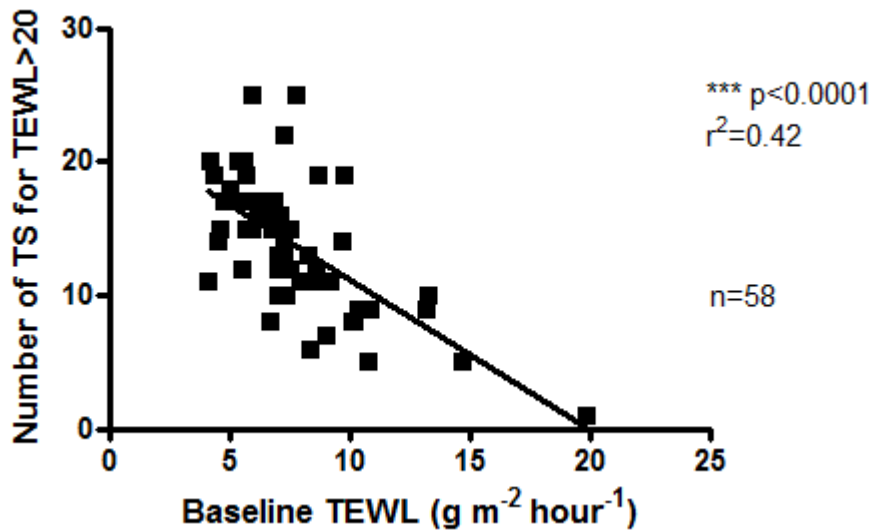


Figure 74. Relationship between baseline transepidermal water loss (TEWL) and susceptibility to physical insult (indicated by the number of tape-strippings [TS] required to reach $\text{TEWL}>20\text{g m}^{-2}\text{ hour}^{-1}$) in all study participants. Less TS was required when the baseline TEWL was higher ($***p<0.0001$).

Linear regression analysis also showed that a higher number of TS was required to abrogate skin permeability barrier when the baseline skin capacitance was higher ($F(1,56)=5.18$, $*p<0.05$, $r^2=0.08$, $n=58$, Figure 75).

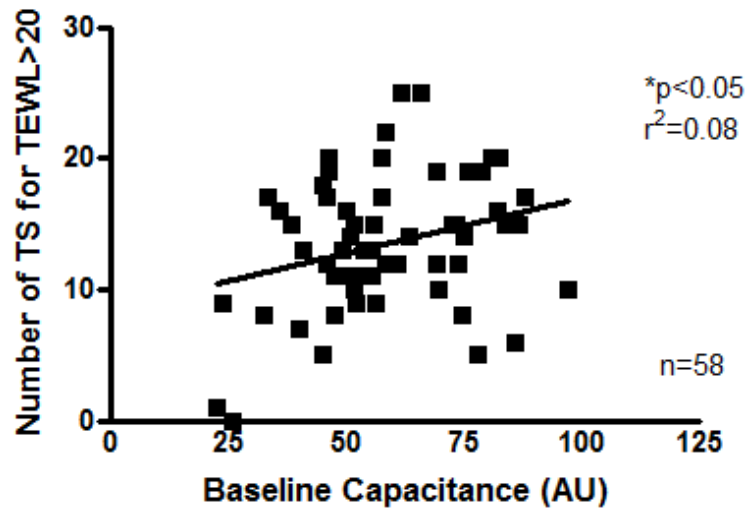


Figure 75. Relationship between baseline skin capacitance and susceptibility to physical insult (indicated by the number of tape-strippings [TS] required to reach transepidermal water loss [TEWL] $>20\text{g m}^{-2}\text{ hour}^{-1}$) in all study participants. More TS was required when the baseline skin capacitance was higher ($*p<0.05$).

2.2.13.4 Disease severity (SASSAD score)

Linear regression analysis showed that, in the WT-AD group, less TS were needed to abrogate skin permeability barrier when the disease was more severe (indicated by higher SASSAD score) ($F(1,43)=4.80$, $*p<0.05$, $r^2=0.10$, $n=45$, Figure 76a). Such relationship was not found in the *FLG*-AD group ($F(1,11)=0.22$, $p=0.65$, n.s., $r^2=0.02$, $n=13$, Figure 76b). This is, again, likely due to the small sample size.

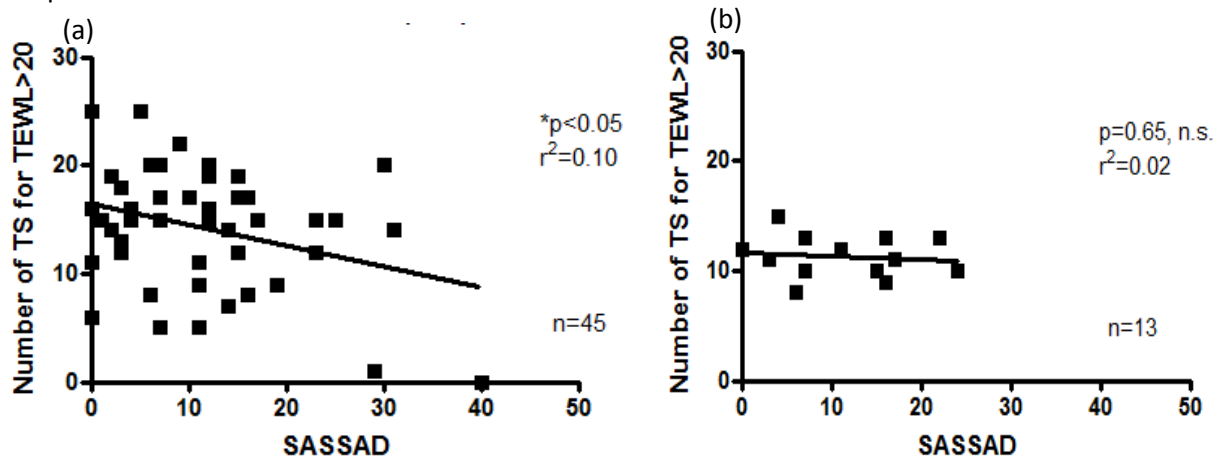


Figure 76. Relationship between disease severity (as indicated by SASSAD score) and susceptibility to physical insult (indicated by the number of tape-strippings [TS] required to reach transepidermal water loss [TEWL] $> 20 \text{ g m}^{-2} \text{ hour}^{-1}$) in (a) WT-AD (where less TS was required with increasing SASSAD, $*p < 0.05$), as compared to (b) *FLG*-AD ($p = 0.65$, n.s.).

In all 58 participants, regardless of *FLG* status, less tape-strippings were needed to abrogate skin permeability barrier when the disease was more severe (higher SASSAD score), as shown by linear regression analysis ($F(1,56)=4.99$, $*p < 0.05$, $r^2=0.08$, $n=58$, Figure 77).

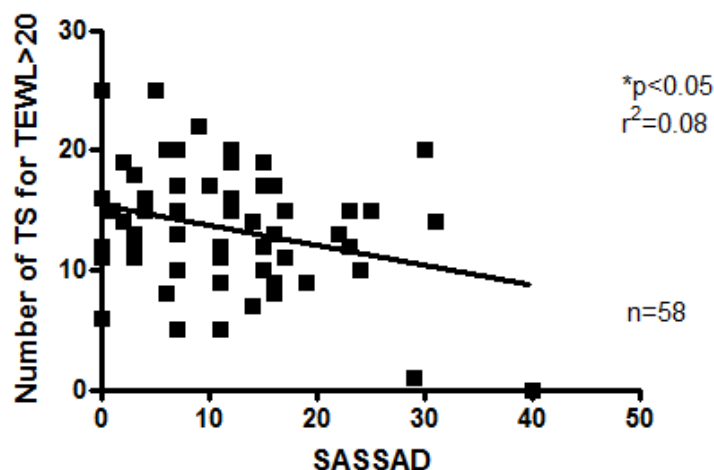


Figure 77. Relationship between disease severity (as indicated by SASSAD score) and susceptibility to physical insult (indicated by the number of tape-strippings [TS] required to reach transepidermal water loss [TEWL] $> 20 \text{ g m}^{-2} \text{ hour}^{-1}$) in all study participants. Less TS was required when the disease was more severe ($*p < 0.05$).

2.2.13.5 Presence of other atopic conditions

2.2.13.5.1 Dry skin

An overwhelming majority of participants (36 out of 41) complained of dry skin (Figure 79).

A two-way ANOVA found no significant difference in the susceptibility to physical insult between those who complained of dry skin and those who did not ($F(1,37)=0.00$, $p=0.99$, n.s.) in both the WT-AD and *FLG*-AD groups ($F(1,37)=2.72$, $p=0.11$, n.s.). There was no significant interaction between having dry skin and filaggrin status ($F(1,37)=1.14$, $p=0.29$, n.s.) which could have influenced the number of TS required (Figure 78).

Unpaired, two-tailed t-test was used to compare the number of TS required in those who complained of having dry skin ($M=12.75$, $SD=5.45$) and those who did not ($M=15.00$, $SD=6.21$), regardless of *FLG* status. There was a small but non-significant reduction in the number of TS required to abrogate skin permeability barrier in those who complained of dry skin ($t(39)=0.85$, $p=0.40$, n.s. Figure 79).

These results suggest that those who complained of dry skin were not more susceptible to physical or mechanical insult to their skin.

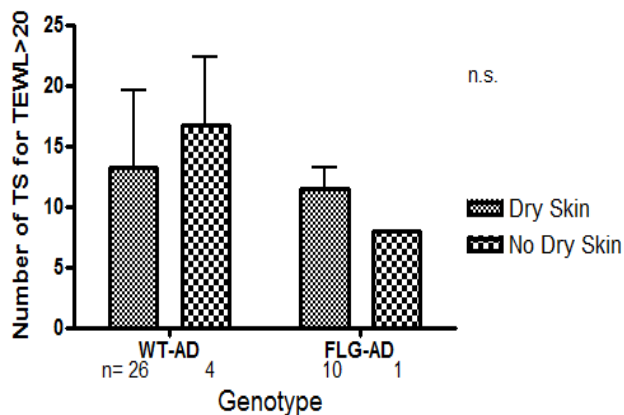


Figure 78. Comparison of the susceptibility to physical insult (indicated by the number of tape-strippings [TS] required to reach transepidermal water loss [TEWL] > 20g m⁻² hour⁻¹) of those who self-reported having dry skin and those who did not, in participants without filaggrin mutations (WT-AD) and participants with filaggrin mutations (*FLG*-AD). The data are represented in a bar graph showing mean and SD. Two-way ANOVA was applied, revealing no significant effect of having dry skin ($p=0.99$, n.s.), *FLG* status ($p=0.11$, n.s.) or interaction between having dry skin and genotype ($p=0.29$, n.s.) on the number of TS required.

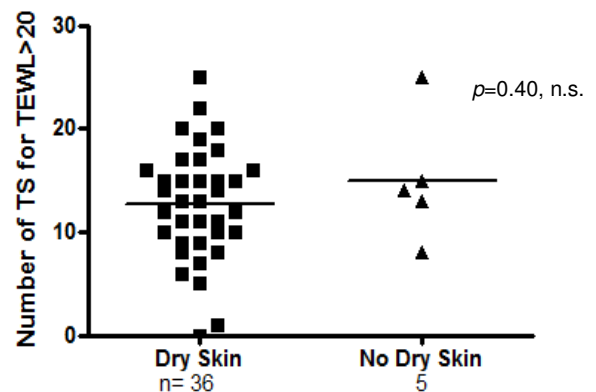


Figure 79. Comparison of the susceptibility to physical insult (indicated by the number of tape-strippings [TS] required to reach transepidermal water loss [TEWL] > 20g m⁻² hour⁻¹) in those who self-reported having dry skin and those who did not, irrespective of genotype. The data are represented in a vertical scatter plot, showing individual data points. Unpaired, two-tailed t-test was used to compare between the groups ($p=0.40$, n.s.).

2.2.13.5.2 Asthma

A two-way ANOVA found no significant difference in the susceptibility to physical insult between those with co-existing asthma and those without ($F(1,54)=0.13$, $p=0.72$, n.s.) in both the WT-AD and *FLG*-AD groups ($F(1,54)=3.00$, $p=0.09$, n.s.). There was no significant interaction between having co-existing asthma and filaggrin status ($F(1,54)=0.01$, $p=0.93$, n.s.) which could have influenced the number of TS required (Figure 80).

Unpaired, two-tailed t-test with Welch's correction was used to compare the number of TS required in those with co-existing asthma ($M=13.95$, $SD=6.46$) and those without ($M=13.28$, $SD=4.33$), regardless of *FLG* status. There was no significant difference between the groups ($t(32)=0.44$, $p=0.67$, n.s., Figure 81).

These results suggest that those with co-existing asthma were not more susceptible to physical or mechanical insult to their skin.

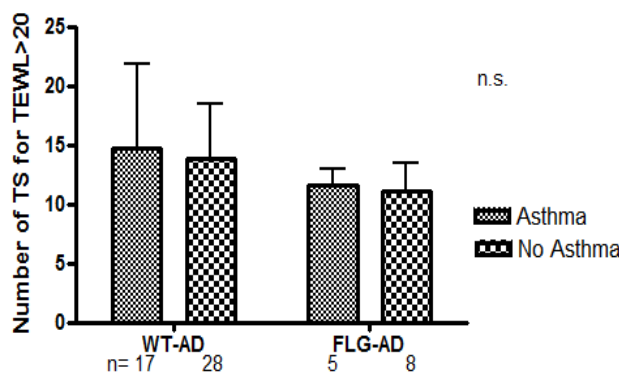


Figure 80. Comparison of the susceptibility to physical insult (indicated by the number of tape-strippings [TS] required to reach transepidermal water loss [TEWL] $>20\text{g m}^{-2}\text{ hour}^{-1}$) of those with and without asthma, in participants without filaggrin mutations (WT-AD) and participants with filaggrin mutations (*FLG*-AD). The data are represented in a bar graph showing mean and SD. Two-way ANOVA was applied, revealing no significant effect of having co-existing asthma ($p=0.72$, n.s.), *FLG* status ($p=0.09$, n.s.) or interaction between having asthma and genotype ($p=0.93$, n.s.) on the number of TS required.

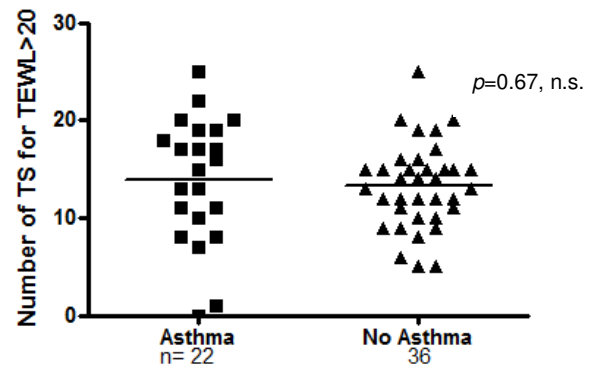


Figure 81. Comparison of the susceptibility to physical insult (indicated by the number of tape-strippings [TS] required to reach transepidermal water loss [TEWL] $>20\text{g m}^{-2}\text{ hour}^{-1}$) in those with and without asthma, irrespective of genotype. The data are represented in a vertical scatter plot, showing individual data points. Unpaired, two-tailed t-test with Welch's correction was used to compare between the groups ($p=0.67$, n.s.).

2.2.13.5.3 Hay fever

A two-way ANOVA found no significant difference in the susceptibility to physical insult between those with co-existing hay fever and those without ($F(1,54)=0.36$, $p=0.55$, n.s.) in both the WT-AD and *FLG*-AD groups ($F(1,54)=1.74$, $p=0.19$, n.s.). There was no significant interaction between having co-existing hay fever and filaggrin status ($F(1,54)=0.25$, $p=0.62$, n.s.) which could have influenced the number of TS required (Figure 82).

Unpaired, two-tailed t-test was used to compare the number of TS required in those with co-existing hay fever ($M=12.42$, $SD=5.37$) and those without ($M=15.00$, $SD=4.67$), regardless of *FLG* status. There was a small but non-significant reduction in the number of TS required to abrogate skin permeability barrier in those with hay fever ($t(56)=1.91$, $p=0.06$, n.s., Figure 83).

These results suggest that those with co-existing hay fever were not more susceptible to physical or mechanical insult to their skin.

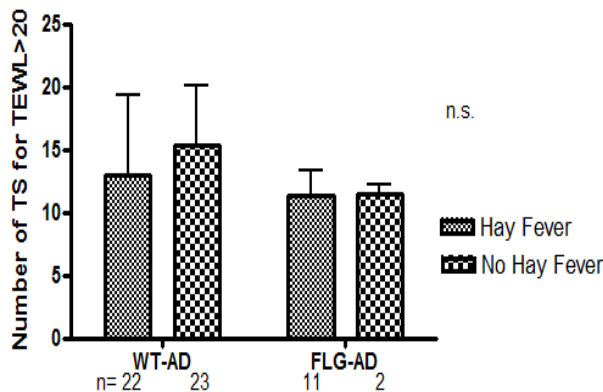


Figure 82. Comparison of the susceptibility to physical insult (indicated by the number of tape-strippings [TS] required to reach transepidermal water loss [TEWL] $>20\text{g m}^{-2}\text{ hour}^{-1}$) of those with and without hay fever (HF), in participants without filaggrin mutations (WT-AD) and participants with filaggrin mutations (*FLG*-AD). The data are represented in a bar graph showing mean and SD. Two-way ANOVA was applied, revealing no significant effect of having co-existing HF ($p=0.55$, n.s.), *FLG* status ($p=0.19$, n.s.) or interaction between having HF and genotype ($p=0.62$, n.s.) on the number of TS required.

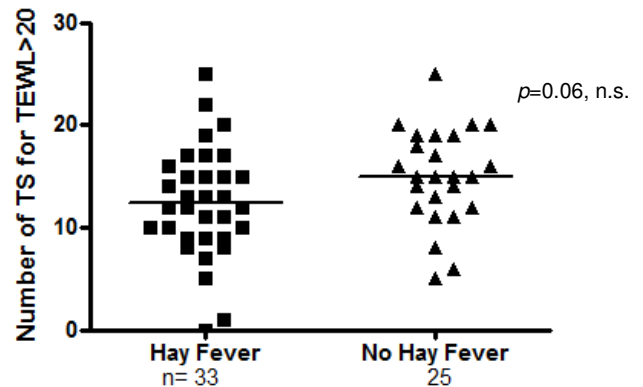


Figure 83. Comparison of the susceptibility to physical insult (indicated by the number of tape-strippings [TS] required to reach transepidermal water loss [TEWL] $>20\text{g m}^{-2}\text{ hour}^{-1}$) in those with and without hay fever, irrespective of genotype. The data are represented in a vertical scatter plot, showing individual data points. Unpaired, two-tailed t-test was used to compare between the groups ($p=0.06$, n.s.).

2.2.13.6 Family history of atopic conditions

2.2.13.6.1 AD

A two-way ANOVA found no significant difference in the susceptibility to physical insult (indicated by the number of TS required to achieve TEWL more than $20\text{g m}^{-2}\text{ hour}^{-1}$) between those with family history of AD and those without, which yielded an F ratio of $F(1,43)=0.15$, $p=0.70$, n.s., in both the WT-AD and FLG-AD groups ($F(1,43)=3.06$, $p=0.09$, n.s.) and no significant interaction between having family history of AD and filaggrin status, $F(1,43)=0.43$, $p=0.52$, n.s., which could have influenced the number of TS required (Figure 84).

Unpaired, two-tailed t-test was used to compare the number of TS required by those with family history of AD ($M=12.54$, $SD=5.17$) and those without ($M=14.05$, $SD=5.54$), regardless of FLG status. There was a small but non-significant reduction in the number of TS required to abrogate the skin barrier in those who had other family member(s) with AD, compared to those without $t(45)=0.96$, $p=0.34$, n.s. (Figure 85)

These results suggest that those with a family history of AD were not more susceptible to physical or mechanical insult to their skin.

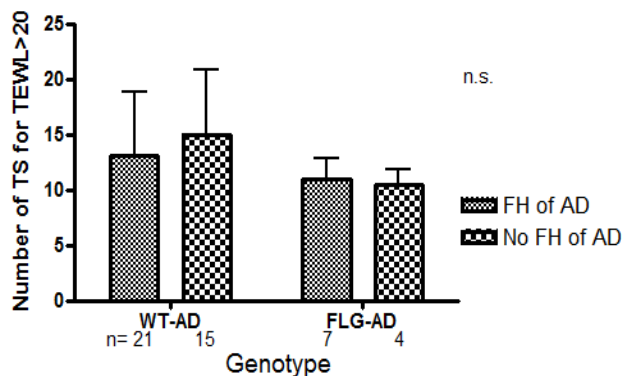


Figure 84. Comparison of the susceptibility to physical insult (indicated by the number of tape-strippings [TS] required to reach transepidermal water loss [TEWL] $>20\text{g m}^{-2}\text{ hour}^{-1}$) of those with and without family history (FH) of AD, in participants without filaggrin mutations (WT-AD) and participants with filaggrin mutations (FLG-AD). The data are represented in a bar graph showing mean and SD. Two-way ANOVA was applied, revealing no significant effect of having FH of AD ($p=0.70$, n.s.), FLG status ($p=0.09$, n.s.) or interaction between having FH of AD and genotype ($p=0.52$, n.s.) on the number of TS required.

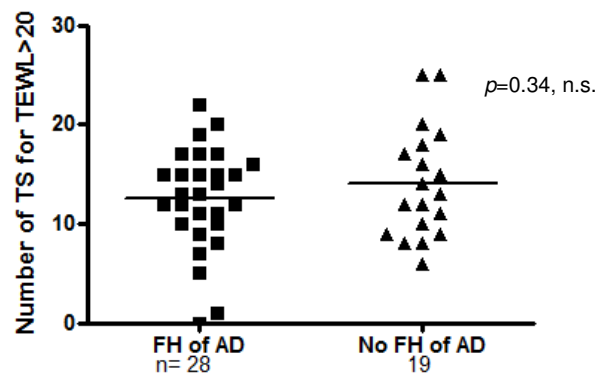


Figure 85. Comparison of the susceptibility to physical insult (indicated by the number of tape-strippings [TS] required to reach transepidermal water loss [TEWL] $>20\text{g m}^{-2}\text{ hour}^{-1}$) in those with and without family history of AD, irrespective of genotype. The data are represented in a vertical scatter plot, showing individual data points. Unpaired, two-tailed t-test was used to compare between the groups ($p=0.34$, n.s.).

2.2.13.6.2 Asthma

A two-way ANOVA found no significant difference in the susceptibility to physical insult (indicated by the number of TS required to achieve TEWL more than $20\text{g m}^{-2}\text{ hour}^{-1}$) between those with family history of asthma and those without, which yielded an F ratio of $F(1,35)=0.00$, $p=0.96$, n.s., in both the WT-AD and FLG-AD groups ($F(1,35)=3.20$, $p=0.08$, n.s.) and no significant interaction between having family history of asthma and filaggrin status, $F(1,35)=0.34$, $p=0.56$, n.s., which could have influenced the number of TS required (Figure 86).

Unpaired, two-tailed t-test was used to compare the number of TS required by those with family history of asthma ($M=13.41$, $SD=6.21$) and those without ($M=13.92$, $SD=5.42$), regardless of FLG status. There was no significant difference between the groups, $t(37)=0.25$, $p=0.81$, n.s. (Figure 87) These results suggest that those with a family history of asthma were not more susceptible to physical or mechanical insult to their skin.

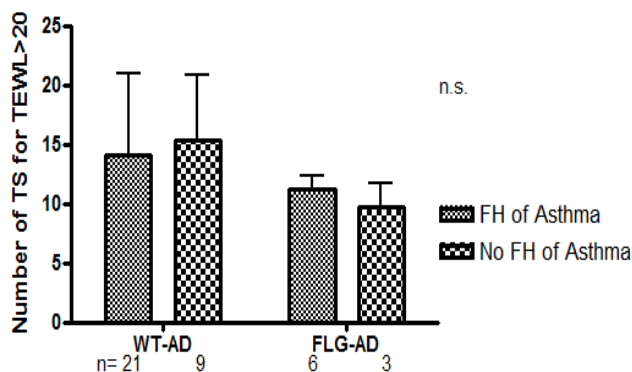


Figure 86. Comparison of the susceptibility to physical insult (indicated by the number of tape-strippings [TS] required to reach transepidermal water loss [TEWL] $>20\text{g m}^{-2}\text{ hour}^{-1}$) of those with and without family history (FH) of asthma, in participants without filaggrin mutations (WT-AD) and participants with filaggrin mutations (FLG-AD). The data are represented in a bar graph showing mean and SD. Two-way ANOVA was applied, revealing no significant effect of having FH of asthma ($p=0.96$, n.s.), FLG status ($p=0.08$, n.s.) or interaction between having FH of asthma and genotype ($p=0.56$, n.s.) on the number of TS required.

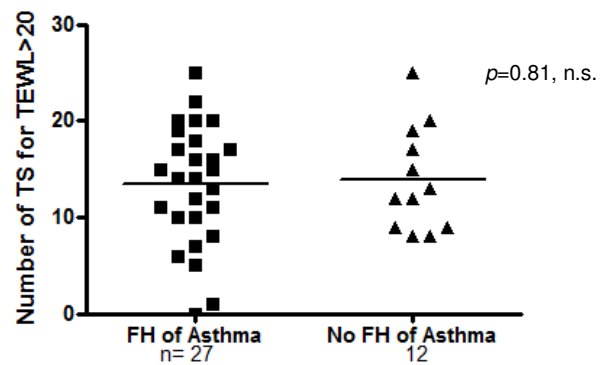


Figure 87. Comparison of the susceptibility to physical insult (indicated by the number of tape-strippings [TS] required to reach transepidermal water loss [TEWL] $>20\text{g m}^{-2}\text{ hour}^{-1}$) in those with and without family history of asthma, irrespective of genotype. The data are represented in a vertical scatter plot, showing individual data points. Unpaired, two-tailed t-test was used to compare between the groups ($p=0.81$, n.s.).

2.2.13.6.3 Hay fever

A two-way ANOVA found no significant difference in the susceptibility to physical insult (indicated by the number of TS required to achieve TEWL more than $20\text{g m}^{-2}\text{ hour}^{-1}$) between those with family history of hay fever and those without, which yielded an F ratio of $F(1,37)=0.34$, $p=0.56$, n.s., in both the WT-AD and FLG-AD groups ($F(1,37)=2.80$, $p=0.10$, n.s.) and no significant interaction between having family history of hay fever and filaggrin status, $F(1,37)=0.65$, $p=0.43$, n.s., which could have influenced the number of TS required (Figure 88).

Unpaired, two-tailed t-test was used to compare the number of TS required by those with family history of hay fever ($M=13.33$, $SD=5.70$) and those without ($M=13.41$, $SD=5.49$), regardless of FLG status. There was no significant difference between the groups, $t(39)=0.04$, $p=0.97$, n.s. (Figure 89) These results suggest that those with a family history of hay fever were not more susceptible to physical or mechanical insult to their skin.

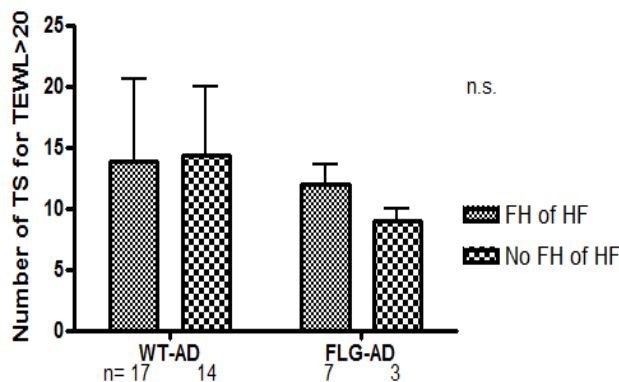


Figure 88. Comparison of the susceptibility to physical insult (indicated by the number of tape-strippings [TS] required to reach transepidermal water loss [TEWL] $>20\text{g m}^{-2}\text{ hour}^{-1}$) of those with and without family history (FH) of hay fever (HF), in participants without filaggrin mutations (WT-AD) and participants with filaggrin mutations (FLG-AD). The data are represented in a bar graph showing mean and SD. Two-way ANOVA was applied, revealing no significant effect of having FH of HF ($p=0.56$, n.s.), FLG status ($p=0.10$, n.s.) or interaction between having FH of HF and genotype ($p=0.43$, n.s.) on the number of TS required.

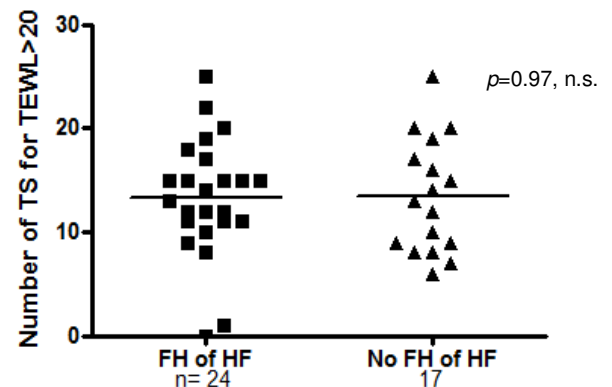


Figure 89. Comparison of the susceptibility to physical insult (indicated by the number of tape-strippings [TS] required to reach transepidermal water loss [TEWL] $>20\text{g m}^{-2}\text{ hour}^{-1}$) in those with and without family history of hay fever (HF), irrespective of genotype. The data are represented in a vertical scatter plot, showing individual data points. Unpaired, two-tailed t-test was used to compare between the groups ($p=0.97$, n.s.).

2.2.13.6.4 Atopic conditions (any of AD, asthma or hay fever)

A two-way ANOVA found no significant difference in the susceptibility to physical insult (indicated by the number of TS required to achieve TEWL more than $20\text{g m}^{-2}\text{ hour}^{-1}$) between those with family history of atopy and those without, which yielded an F ratio of $F(1,49)=0.03$, $p=0.86$, n.s., in both the WT-AD and FLG-AD groups ($F(1,49)=2.07$, $p=0.16$, n.s.) and no significant interaction between having family history of atopy and filaggrin status, $F(1,49)=0.36$, $p=0.55$, n.s., which could have influenced the number of TS required (Figure 90).

Unpaired, two-tailed t-test was used to compare the number of TS required by those with family history of atopy ($M=13.27$, $SD=5.32$) and those without ($M=14.38$, $SD=5.88$), regardless of FLG status. There was no significant difference between the groups, $t(51)=0.53$, $p=0.60$, n.s. (Figure 91)

These results suggest that those with a family history of atopic conditions (any of AD, asthma or hay fever) were not more susceptible to physical or mechanical insult to their skin.

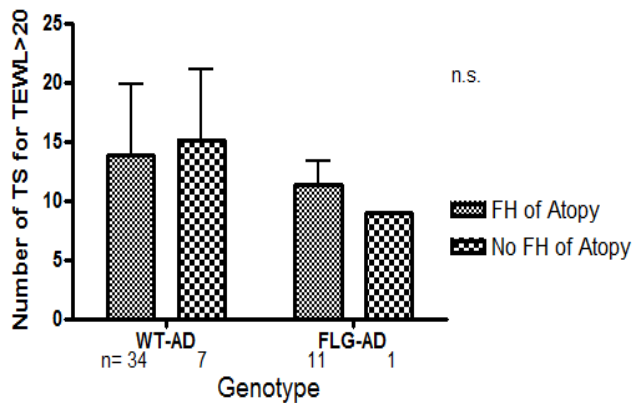


Figure 90. Comparison of the susceptibility to physical insult (indicated by the number of tape-strippings [TS] required to reach transepidermal water loss [TEWL] $>20\text{g m}^{-2}\text{ hour}^{-1}$) of those with and without family history of atopy, in participants without filaggrin mutations (WT-AD) and participants with filaggrin mutations (FLG-AD). The data are represented in a bar graph showing mean and SD. Two-way ANOVA was applied, revealing no significant effect of having FH of atopy ($p=0.86$, n.s.), FLG status ($p=0.16$, n.s.) or interaction between having FH of atopy and genotype ($p=0.55$, n.s.) on the number of TS required.

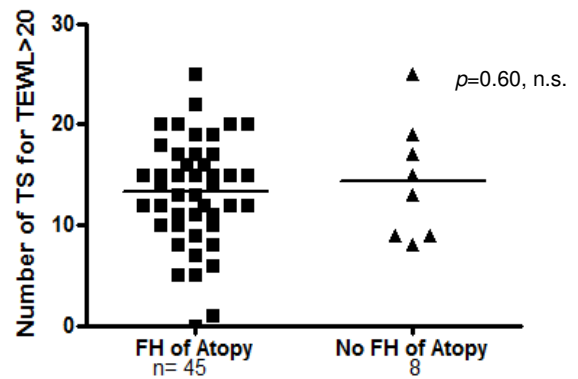


Figure 91. Comparison of the susceptibility to physical insult (indicated by the number of tape-strippings [TS] required to reach transepidermal water loss [TEWL] $>20\text{g m}^{-2}\text{ hour}^{-1}$) in those with and without family history of atopy, irrespective of genotype. The data are represented in a vertical scatter plot, showing individual data points. Unpaired, two-tailed t-test was used to compare between the groups ($p=0.60$, n.s.).

2.2.13.7 IgE level

The number of TS required to abrogate skin permeability barrier was negatively correlated to total serum IgE level in the WT-AD group (Pearson $r(45)=-0.29$, $p<0.05$, n.s. and r^2 of 0.09, Figure 92a). Such correlation was not observed in the *FLG*-AD group (Pearson $r(13)=0.35$, $p=0.25$, n.s. with r^2 of 0.12, Figure 92b) and when the participants' *FLG* status was not taken into account (Pearson $r(58)=-0.23$, $p=0.08$, n.s. and r^2 of 0.05, Figure 93).

Pearson correlation analysis was used as the data passed the normality test, i.e. assumed to be sampled from a Gaussian population.

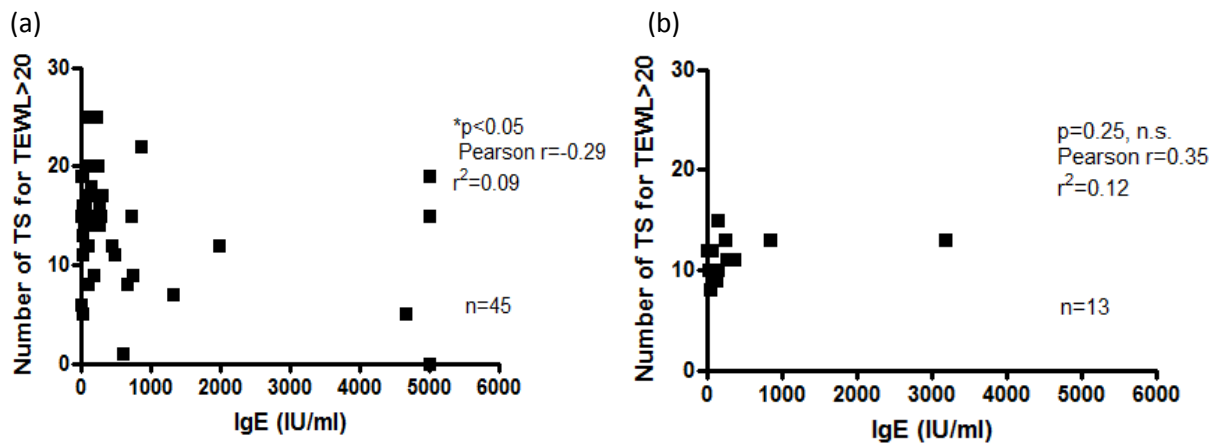


Figure 92. Correlation between total serum IgE level and susceptibility to physical insult (indicated by the number of tape-strippings [TS] required to reach transepidermal water loss [TEWL] $>20\text{g m}^{-2}\text{ hour}^{-1}$) in (a) those without filaggrin mutations, WT-AD, Pearson $r(45)=-0.29$, $p<0.05$, n.s. and r^2 of 0.09 and (b) those with filaggrin mutations, *FLG*-AD, Pearson $r(13)=0.35$, $p=0.25$, n.s. with r^2 of 0.12.

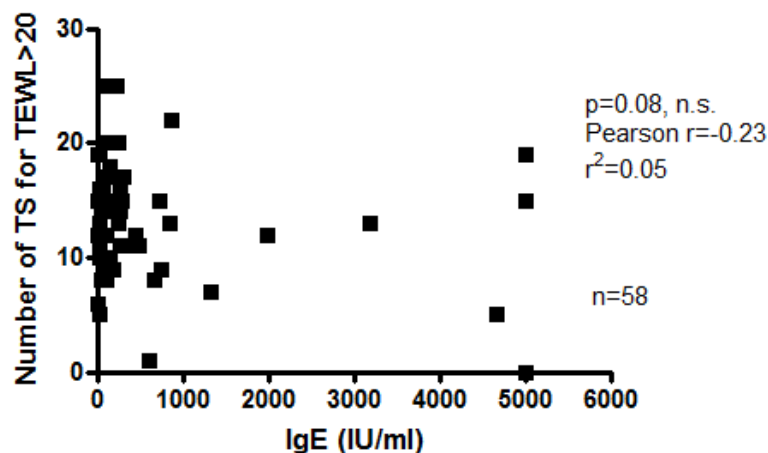


Figure 93. Correlation between total serum IgE level and susceptibility to physical insult of all participants (indicated by the number of tape-strippings [TS] required to reach transepidermal water loss [TEWL] $>20\text{g m}^{-2}\text{ hour}^{-1}$), Pearson $r(58)=-0.23$, $p=0.08$, n.s. and r^2 of 0.05.

2.2.14 Chemical irritancy study:

The effect of sodium dodecyl sulphate application to the skin

SDS of varying concentration (0.06% - 4.00%) was applied to 8 different sites on the uninvolved flexor forearm of 25 participants for 24 hours. The skin was clinically examined for signs of irritation or inflammation 24 hours post-SDS application. TEWL and skin fold thickness of all sites was measured before and 24 hours after SDS application, as described in Materials and Methods. Non-linear comparison of fits (one-site binding parabola) was used to compare between the curves--whether one curve suffices for all data sets or if the individual curves are statistically distinguishable ('does the best-fit value of a selected parameter differ between data sets?'). Null hypothesis ('one curve for all data sets') is rejected if the p-value is <0.05.

2.2.14.1 Clinical score of the skin post-SDS application

The reaction of the skin to SDS was clinically scored and found to be increasingly severe, as expected, with increasing concentrations of the solutions. *FLG*-AD participants displayed a more severe skin reaction to the chemical irritant than the WT-AD participants, although the difference was not statistically significant ($F(2,220)=1.13$, $p=0.33$, n.s., Figure 94).

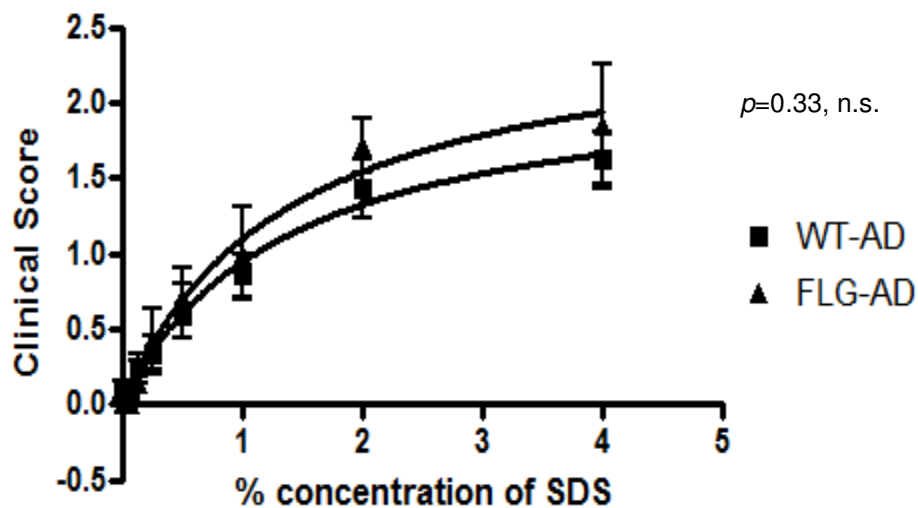


Figure 94. Comparison of the skin reaction (indicated by clinical score) to increasing concentrations of sodium dodecyl sulphate (SDS) in those without filaggrin mutations (WT-AD) and those with filaggrin mutations (*FLG*-AD). No significant difference between WT-AD and *FLG*-AD was seen, $F(2,220)=1.13$, $p=0.33$, n.s.

2.2.14.2 Change in TEWL

TEWL measurements showed a dose-dependent rise in TEWL 24 hours post-application of SDS. This rise was significantly greater in the *FLG*-AD group than in the WT-AD ($F(2,196)=10.07$, $*** p<0.0001$, Figure 95).

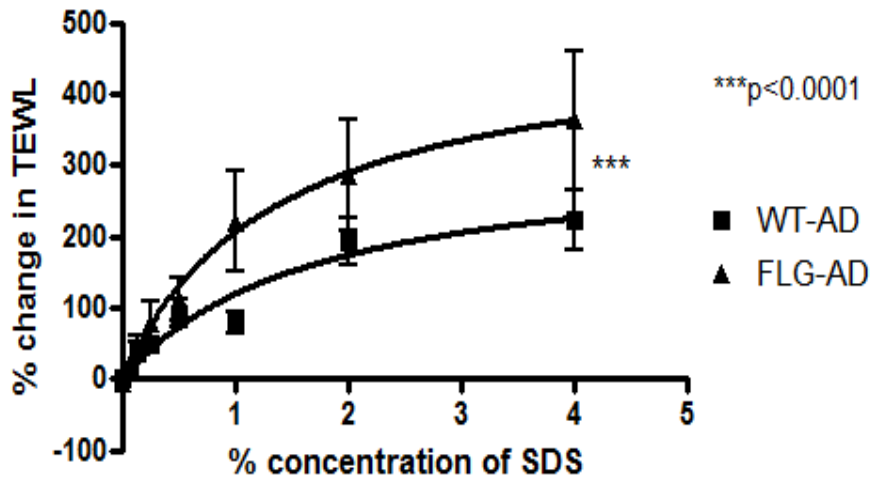


Figure 95. Comparison of the percentage change in transepidermal water loss (TEWL) in response to increasing concentrations of sodium dodecyl sulphate (SDS) in those without filaggrin mutations (WT-AD) and those with filaggrin mutations (*FLG*-AD). *FLG*-AD showed significantly greater rise in TEWL post SDS application, compared to WT-AD, $F(2,196)=10.07$, $***p<0.0001$.

2.2.14.3 Changes in skin fold thickness

A dose-dependent rise in skin fold thickness was seen 24 hours post SDS application. However, there was no significant difference in the change in skin fold thickness between both groups ($F(2,196)=0.94$, $p=0.39$, n.s., Figure 96).

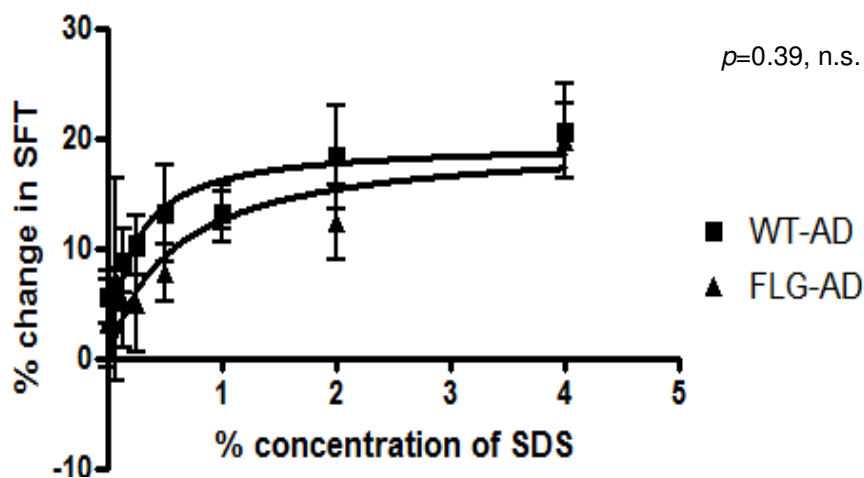


Figure 96. Comparison of the percentage change in skin fold thickness (SFT) in response to increasing concentrations of sodium dodecyl sulphate (SDS) in those without filaggrin mutations (WT-AD) and those with filaggrin mutations (*FLG*-AD). No significant difference between WT-AD and *FLG*-AD was seen, $F(2,196)=0.94$, $p=0.39$, n.s.

2.3 A summary of the main findings

Table 4 provides an overview of the main findings from this clinical study. Comparisons between the WT-AD with the *FLG*-AD participants were given.

Table 4

Main Findings from the Clinical Study: Comparisons Between WT-AD and FLG-AD.

	WT-AD	FLG-AD	P-value	Reference
Baseline TEWL		(+)	0.05	Figure 2
Baseline skin capacitance			n.s.	Figure 3
Associations with other atopic diseases:				
• Dry skin			n.s.	Figure 4
• Asthma			n.s.	Figure 4
• Hay fever		+	<0.05	Figure 4
Susceptibility to physical insult		++	<0.01	Figure 73
Increase in TEWL with increasing concentration of chemical irritant SDS		+++	<0.0001	Figure 95
Correlations between:				
			<u>P-value</u>	<u>P-value</u>
• Age and baseline capacitance	++		<0.01	n.s.
• Disease severity and total serum IgE	++		<0.01	n.s.
• Disease severity and susceptibility to physical insult	+		<0.05	n.s.
• Susceptibility to physical insult and total serum IgE	+		<0.05	n.s.
• TEWL and total serum IgE	++		<0.01	n.s.
<hr/>				
Other Associations	Family history of atopy	Reference	Susceptibility to physical insult	Reference
Baseline TEWL	n.s.	Figure 45	+++ (p<0.0001)	Figure 74
Baseline capacitance	+ (p<0.05)	Figure 47	- (p<0.05)	Figure 75

2.4 Chapter 2 Discussion

Our understanding of atopic dermatitis has been, in my opinion, rather lacking for a disease that imposes such enormous economic, health and social burdens on the society. The aetiology of the disease has often been loosely described as '*complex interactions between genetic and environmental factors, the skin barrier and immunological disturbances*⁴³'. Without adequate knowledge on the pathogenesis of the disease, treatments for AD have generally been directed at alleviating the symptoms, not targeting the cause. The identification of *FLG* as, to date, the most important causative gene for AD⁷, undoubtedly has shed new light on the pathogenesis of AD. The association has been universally validated and to date, no negative association has been found in European cohorts. Evidence of such association should be supported by some evidence of the functional significance of the defective gene. The main aim of this study is to demonstrate if *FLG* mutations are associated with changes in clinical parameters, such as increased TEWL or reduced skin capacitance. These will suggest a defective barrier which may allow easier penetration of environmental chemicals, allergens or irritants, which lead to skin inflammation, immune activation and possible culminating in the clinical manifestations of AD. Only when such evidence is seen should treatments of a disease targeting the causative gene be actively pursued.

TEWL, skin capacitance and stratum corneum thickness are experimentally demonstrable markers of the skin barrier function. Using these parameters, many previous studies have demonstrated a defective barrier function in AD patients compared to healthy controls^{34,35}. Penetration of SDS through the stratum corneum has been shown to be increased in eczematous compared to non-eczematous skin³⁷. These studies, many of which were done prior to the identification of the *FLG* mutations, will have included a heterogeneous mixture of wild-types and *FLG*-AD patients. This study attempts to relate functional measurements of the skin barrier with the genotype and to demonstrate the phenotypic traits of *FLG*-AD vs WT-AD. The outcome of such study may allow stratification of phenotype based on the genotype, if the defective gene is found to be functionally significant. As seen from the graphs presented in the Results section, there are apparent outliers for disease severity (SASSAD score), IgE level, number of contact allergies, baseline TEWL and susceptibility to physical insults (i.e. the number of tape strips required to achieve TEWL more than 20 gm⁻² hour⁻¹). These outliers were different individuals, except for one WT-AD with IgE above 5000IU/ml who also had a high SASSAD score of 40 and high baseline TEWL of 40 gm⁻² hour⁻¹, therefore requiring no tape-stripping to be done. They did not appear to share similar characteristics or have unusual phenotypes, for example, they were not of a particular genotype or had similar areas affected by AD at the time of the study. Although these outliers may represent experimental artefacts, they have not been excluded from our statistical analysis. Molin *et al* suggest that *FLG*

mutations confer susceptibility to chronic hand dermatitis³³ due to an increased risk of irritant and allergic sensitization. Indeed, *FLG* null mutations have been associated with hand eczema, palmar hyperlinearity and dry fissures in the hands⁴⁴⁻⁴⁶.

Although this study is not the first clinical study looking at *FLG* phenotype-genotype relationships, it is the first to show significant *FLG*-associated functional defects in the skin barrier in a European population. It has yielded interesting results, some of which are consistent with previous studies done elsewhere, others are in complete contrast to findings in other studies. *FLG*-AD participants in this study had a marginally higher baseline TEWL than WT-AD. This indicates a physical loss of barrier function in filaggrin-deficient AD patients and thus, lends support to the hypothesis that *FLG* mutations cause a physiological defect in the skin. A previous study on a French cohort by Hubiche *et al* also showed that *FLG*-AD patients had a slightly higher, though not significantly different, TEWL than WT-AD⁴⁷. In that study, *FLG* mutations were shown to have no significant influence on the overall disease severity. Hubiche *et al* involved 99 participants with AD in their study, including 59 children less than 15 years. This difference in the age of participants between their study and ours may account for the differences in results found. As shown in our study, there is a positive correlation between age and hydration state of the skin (capacitance). Another study conducted by Nemoto-Hasebe *et al*³⁸ on a small Japanese cohort of AD patients (24 AD participants, 12 healthy controls) found that the stratum corneum hydration of *FLG*-AD participants was lower than that of healthy controls ($p < 0.01$) and WT-AD, though the data on the latter was not statistically significant. Paradoxically the WT-AD had a higher TEWL than the *FLG*-AD. The study also found that, in the *FLG*-AD group (but not in the WT-AD group), TEWL and stratum corneum thickness was positively correlated with the disease severity. A negative correlation was seen between disease severity and the stratum corneum hydration. These are in contrast with the results presented in this chapter, where no significant correlation was seen between disease severity and TEWL or stratum corneum hydration in both WT-AD and *FLG*-AD groups. In addition, Nemoto-Hasebe *et al* also found significant positive correlations between disease severity and specific IgEs for mite allergen, house dust and cat dander in the *FLG*-AD group but not in the wild-types. In our study, we measured total serum IgE and not allergen-specific IgEs. We found significant positive correlations between disease severity and TEWL with total serum IgE in the wild-types ($p < 0.01$) but not in the *FLG*-AD group. These apparently contradictory results could be due to their smaller ($n=24$) sample size and the fact that the *FLG* mutations found in the Japanese population (p.Ser2554X, c.3321del, p.Ser2889X and p.Ser3296X) are different to those commonly found in the Europeans. Asian populations have a larger variety of *FLG* mutations as compared to Caucasians⁴⁸. Future studies may shed light on the differing effects of race-specific *FLG* mutations on phenotypic traits

and barrier function. Another possible explanation for the differences seen between this study and the others^{38,47} lies in our participant selection process. Our participants were required to avoid antihistamines and all topical treatments including emollients and corticosteroids for one week prior to the study. This is to avoid the confounding effects of such treatments on the measurements. Consequently, the AD cohort in this study consisted of those who had milder diseases, which is more typical of the general UK population with the disease. 13 of our 58 participants had *FLG* mutations. This falls within the expected frequency of *FLG* mutations in the milder spectrum of the disease¹⁵. The age, gender and disease severity of our *FLG*-AD participants were comparable to that of the WT-AD group.

In addition to correlating the genotype with the disease severity and other functional measurements, we also examined the relationships between concomitant atopic diseases and family history of such conditions with the skin barrier function and genotype. We found that compared to the WT-AD, the prevalence of hay fever, but not asthma, was significantly higher in the *FLG*-AD group. We also found that the disease was more severe in AD participants with concomitant hay fever ($p < 0.05$) and in those complaining of dry skin ($p < 0.0001$). Surprisingly, no increase in family history of atopic conditions (AD, asthma and hay fever) was found amongst the *FLG*-AD participants. In fact, those who have a family history of atopy were surprisingly found to have a higher baseline skin capacitance in our study (both WT-AD and *FLG*-AD groups). Nonetheless, the data on family history was based solely on the participants' own account and not on a comprehensive pedigree study.

Perhaps the most interesting finding in our study is the response of the skin of *FLG*-AD vs WT-AD when challenged with physical and chemical insults. The difference in baseline TEWL between *FLG*-AD and WT-AD was slight. Nonetheless, when challenged with SDS of varying concentrations, a marked difference in TEWL was seen between the two groups. Compared to the WT-AD group, the *FLG*-AD group showed a significantly higher increase in TEWL, which rose in a dose-dependent manner ($p < 0.0001$). This suggests that filaggrin-deficient skin is less able to cope with irritant stresses. Subjective clinical scoring for erythema was done post-SDS application because this was simple and fast to perform and comparable to the pre-SDS clinical scoring of erythema (which was done as part of the initial SASSAD scoring). In retrospect, an objective scoring method for erythema, such as using a colorimeter or laser-Doppler flowmetry⁴⁹, is perhaps a better idea. This could have increased the sensitivity of the testing and improved the significance of the post-SDS results. There are also variations between (and within) anatomical sites with regards to the response to topical applications. These variations within an individual can be related to differences in skin permeability due to variations in skin properties at different anatomical sites such as pH, stratum corneum

thickness, hair follicle density and skin hydration^{50,51}. The impact of these variations (which can be expected to occur in all participants, irrespective of their *FLG* status) on our results was reduced by the equal treatment of all participants, i.e. similar areas were treated with the same concentrations of SDS in both groups, under standardised conditions.

Nemoto-Hasebe *et al* showed that the stratum corneum of *FLG*-AD patients was significantly thicker than that of WT-AD ($P < 0.05$) and healthy controls ($p < 0.01$). The natural moisturising factor (NMF), which consists mainly of filaggrin breakdown products, serves to fill the interstices between corneocytes and hold them in position. If there is reduction in the quality or quantity of this 'cement' due to filaggrin deficiency, the corneocytes may be hypothesized to be less densely packed or adhere less well to one another, thus allowing easier removal of the cells by tape stripping. This is reflected in our finding that *FLG*-AD group required significantly less number of tape-stripping to reach TEWL of more than $20\text{g m}^{-2}\text{ hour}^{-1}$, the level of which permeability barrier is deemed abrogated. Those with a more severe disease and poorer skin barrier function (reflected by a higher baseline TEWL and lower skin capacitance) were also found to require a lesser number of tape-strippings ($p < 0.05$; $p < 0.0001$; $p < 0.05$ respectively). This result should ideally be followed up by an experiment to quantitate and compare the amount of stratum corneum being removed by the tape-stripping process. A larger amount of stratum corneum being removed per tape, if found in the *FLG*-AD participants, would confirm poorer inter-corneocyte adherence.

Our human data correlate closely with the murine data from Fallon *et al*⁵² which showed that topically applied ovalbumin caused a significant increase in the TEWL of homozygous flaky tail (ft/ft) *FLG*-null mice. Similar treatment caused no change in the TEWL of wt/ft and wt/wt mice. Both human and murine data point to a *FLG*-associated predisposition to mount an exaggerated cutaneous inflammation in response to environmental allergens, followed by enhanced cutaneous allergen priming. A significant proportion of patients with *FLG*-associated ichthyosis vulgaris (IV) have AD or other atopic diseases^{5,53}. We suggest that the minority without AD have perhaps not been subjected to a second, inflammation-inducing exposure encountered by the *FLG*-AD patients. We therefore concur with the common view that AD is the result of '*complex interactions between genetic and environmental factors, the skin barrier and immunological disturbances*⁴³'.

In summary, this study confirms that *FLG* mutations have functional significance. The defective gene is associated with experimentally measurable defects in the integrity of the epidermal barrier, particularly so following exposure to chemical irritants. It is the first European study that has shown significant differences in the skin barrier function between *FLG*-AD and WT-AD and thus, lends support to the hypothesis that skin barrier dysfunction may be the initial event in the pathogenesis of AD.

2.5 Chapter 2 References

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2.6 Chapter 2 Materials and Methods

2.6.1 Participant recruitment

58 participants with mild-moderate disease according to the United Kingdom Working Party's Diagnostic Criteria for Atopic Dermatitis⁴⁰ (Table 5) were recruited from the outpatient and patch test clinics at the Department of Dermatology, The Royal Infirmary of Edinburgh and from The University of Edinburgh Medical School. Exclusion criteria: Patients under 18 or above 60 years of age; those with active inflammation or infection on the flexor surfaces on their forearm and those unable to give informed consent. No selection of male/ female ratio was made. Experiments were approved by the Scotland A Research Ethics Committee (Ref: 07/MRE00/109)⁵⁴ and complied with principles of the Helsinki Accord. All participants gave written, informed consent.

Table 5

The U.K. Working Party's Diagnostic Criteria for Atopic Dermatitis⁴⁰

Must have: An itchy skin condition in the last 12 months

Plus three or more of the following:

- Onset under the age of 2 years*
- History of flexural involvement
- History of a generally dry skin
- Personal history of other atopic disease(s)**
- Visible flexural dermatitis as per photographic protocol

* *not used in children under 4 year*

***in children aged under 4 years, history of atopic disease(s) in a first degree relative may be included*

Clinical history

Clinical history (See Appendix: Chapter 2 Proforma for clinical study) was obtained from participants. For participants who have previously undergone patch-testing in clinics, the number of contact allergies and types of allergens were recorded.

2.6.2 Genotyping

Genotyping was conducted by our collaborators at Epithelial Genetics Group (led by Professor W.H. Irwin McLean), Colleges of Life Sciences and Medicine, Dentistry and Nursing, University of Dundee, using previously described techniques⁵⁵. All participants were screened for the four commonest *FLG* mutations in the European populations (2282del4, R501X, R2447X and S3247X) using TaqMan allelic discrimination assays (96-well ABI 7900HT sequence detection systems, Applied Biosystems, Foster

City, CA). Standard TaqMan conditions for Applied Biosystems Universal PCR master mix were used. Reaction volumes were 10 μ l. Probes and primers were as described elsewhere⁵⁵.

2.6.3 Laboratory test

Blood taken from each participant was stored at -20^oC prior to analysis of the total serum IgE level by the blood transfusion service at the Royal Infirmary of Edinburgh.

2.6.4 Measurements of TEWL and Capacitance

Measurements of TEWL and capacitance were done on uninvolved flexor forearm skin (4cm below the antecubital fossa) under standardised conditions (room temperature 20-22^oC; humidity 40-60%). Participants were instructed to stop oral anti-histamines and the application of all topical treatments, including emollients, for one week prior to the experiments. Prior to the measurements being performed, participants were rested and given 10-15 minutes to adapt to the room conditions without covering the measurement site. Measurements were conducted in an open top box to limit air convection currents and condensation (Figure 97a) and were done in accordance with previously published guidelines⁵⁶.

TEWL

TEWL was measured using the Tewameter TM300 (Courage and Khazaka, Cologne, Germany) with an open chamber probe (Figure 97b). TEWL is calculated from the difference between the humidity of incoming and outgoing air passing over the test area measured by the hygrometer.

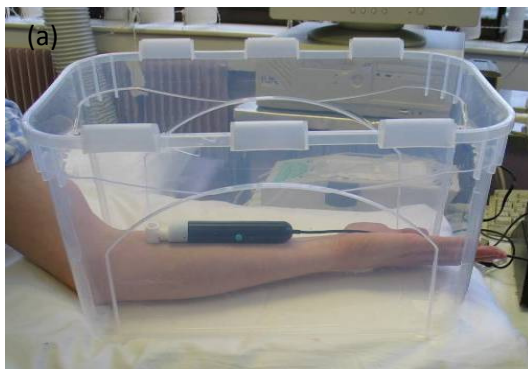
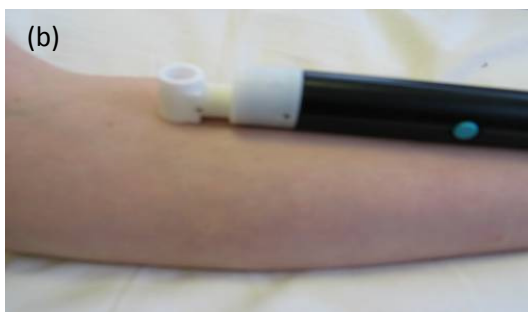


Figure 97. Measurement of TEWL.
(a) An open top box was used to limit air convection currents and condensation.



(b) A Tewameter being used to measure the TEWL on the flexor forearm of a participant.

Skin capacitance (reflective of the state of hydration in the stratum corneum)

Corneometer CM 825 (Courage and Khazaka, Cologne, Germany) was used to measure skin capacitance (Figure 99). Refer to manufacturer's website (<http://www.courage-khazaka.de/index.php/en/products/scientific/55-corneometer>) for further information regarding Corneometer CM825 and the measuring principle. Capacitance is dependent on the dielectric property of the medium involved. Water has a high dielectric constant (dielectric constant of water= 80 at 20⁰C; vacuum=1). Changes in the level of hydration in the skin will affect the measured value of skin capacitance. Skin capacitance is, therefore, reflective of the state of hydration in the skin^{57,58}. The higher the capacitance, the more hydrated the skin.

The corneometer measures capacitance in the superficial 10-20 µm of the stratum corneum; measurement is therefore independent of the underlying capillary blood flow.

All measurements were performed in three repeats for each test area.



Figure 98. Measurement of skin capacitance. Corneometer CM825 being used in a participant to measure skin capacitance, which is reflective of the level of skin hydration.

2.6.5 Tape-stripping experiment

Tape-stripping experiment was done using 14mm D-Squame tape discs (CuDerm Corporation, Dallas, TX). Using a cylindrical weight (Figure 99a), a defined pressure of 225 g/cm² was applied for 10 seconds before the tapes were peeled off unidirectionally using a forcep⁵⁹ (Figure 99b). The number of tape strips required to abrogate the permeability barrier (defined as TEWL >20g/m²/hour) were recorded⁶⁰.



Figure 99. The tape-stripping process. (a) The cylindrical weight was used to apply a defined pressure of 225 g cm^{-2} on the tapes for 10 seconds before (b) the tapes were peeled off in a unidirectional manner.

2.6.6 SDS-irritancy study

Experiment was done at eight different sites on of the participants (Figure 100). On Day 1, the baseline TEWL and skin fold thickness of the test areas were measured. Skin fold thickness was measured using a Harpenden skin fold calliper (*Comment: Removal of one spring to minimise compression was not done*). $20\mu\text{l}$ of SDS solutions of various concentrations (0%, 0.0625%, 0.125%, 0.25%, 0.5%, 1%, % and 4%) were applied onto round filter papers (8mm in diameter) which were placed within small aluminium discs (known as Finn Chambers). The Finn Chambers, which are commonly used for patch-testing purposes in clinical settings, were secured onto the skin with a Scanpor tape. On Day 2, following 24 hours of occlusion, the Finn Chambers (and the SDS-containing filter papers) were removed. On Day 3, measurements of TEWL and skin fold thickness were repeated at the SDS pre-treated test areas^{61,62}.

The skin reaction to SDS was visually scored in Table 6.

Table 6

Visual scoring system for the skin reaction to SDS solution⁶³

0	no erythema
1	very slight erythema (barely perceptible)
2	well-defined uniform erythema
3	moderate to severe erythema
4	severe erythema to slight eschar formation
5	severe erythema with oedema

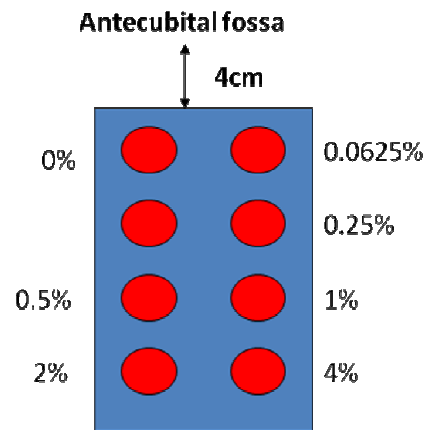
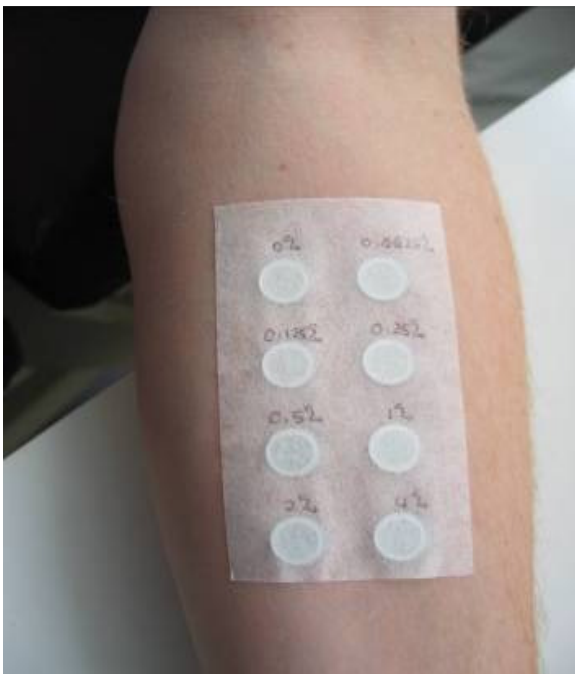


Figure 100. The sodium dodecyl sulphate (SDS)-irritancy study. SDS solutions of various concentrations were applied for 24 hours onto the uninvolved flexor forearm skin of a participant, using Finn Chambers secured with a Scanpor tape.

2.6.7 Statistical analysis

Statistical analysis was performed using Microsoft Office Excel 2007 and GraphPad Prism version 4.00 for Windows, GraphPad software, San Diego California USA, www.graphpad.com. Where stated in the text, Kolmogorov-Smirnov goodness-of-fit test was used for normality testing, i.e. testing whether the distribution of a data set is Gaussian. Unpaired, two-tailed t-test (parametric) and Mann-Whitney U test (non-parametric) were used to compare variables between the WT-AD and the *FLG*-AD groups. P-values of less than 0.05 were considered as significant. Data are reported as mean \pm standard deviation (SD) unless stated otherwise.

Chapter 3

Protease-antiprotease imbalances and atopic dermatitis in patients with a normal filaggrin gene.

Although filaggrin protein deficiency is associated with atopic dermatitis (AD), most AD patients have a normal filaggrin gene (FLG). A multi-step proteolysis of profilaggrin, its non-functional precursor protein, is central for the formation of the keratin-binding filaggrin monomers. In this chapter, we explore the possibility that local imbalances in the protease-antiprotease system may lead to accumulation of the proprotein and reduced expression of the functional filaggrin monomers.

Our data offer a pathomechanistic insight into the development of AD, especially in wild-type FLG patients.

3.1 Introduction: It is not all about the Gene

Knowing the importance of filaggrin in maintaining epidermal hydration and that xerosis is the central feature of atopic dermatitis (AD), the discovery of genetic associations between filaggrin gene (*FLG*) mutations and AD merely confirms a long-standing hypothesis that filaggrin is implicated in the pathogenesis of AD. Indeed, as shown in Chapter 2, *FLG* mutations predispose to a higher transepidermal water loss, increased likelihood of polysensitization and sensitivity to chemical allergens.

Despite this, only half of all children with moderate to severe AD have *FLG* mutations¹. The association is even less distinct in milder cases of AD². This means that most AD patients, the majority of whom have milder forms of the disease, do not have an apparent *FLG* mutation. It is possible that there are other forms of *FLG* mutations that have yet to be identified. Interestingly, *FLG* mutations are predisposing factors which do not necessarily lead to AD. Mutations in *FLG* are found in approximately 9% of healthy population³ without the disease. One therefore needs to consider the existence of other modifying factors which can affect the expression or functioning of the 37kDa protein and thus, influence the clinical outcomes. A good example for this is the study by Howell *et al* which showed the ability of Th-2 inflammatory cytokines in causing a reduction in the filaggrin protein expression⁴. Other possible modifying factors include variations in proteolytic processing of (pro)filaggrin into filaggrin, which will be discussed next.

As mentioned in Chapter 2, filaggrin is initially synthesized as a large 400-kDa precursor protein profilaggrin, which is an insoluble, highly-phosphorylated, histidine-rich polyprotein found in the epidermal granular layer. The extensive phosphorylation, a process facilitated by casein kinase-2⁵, confers insolubility to the proprotein and induces conformation changes in the protein structure which may prevent premature proteolytic processing of the proprotein by rendering the cleavage sites inaccessible to proteases⁶. This prevents premature association of filaggrin with keratin filaments⁷. Profilaggrin consists of an S100 calcium-binding amino terminal, a B-domain, followed by 10-12 highly homologous filaggrin repeats flanked by two partial repeats and a carboxy terminal domain⁸. Profilaggrin is unable to bind keratin until it is dephosphorylated by phosphatases and undergoes a series of poorly understood site-specific proteolytic changes within the cytoplasm to release the functional 37kDa filaggrin monomer⁹. The intracellular processing of profilaggrin occurs as the keratinocytes undergo transition from being a granular cell to a cornified cell. Degranulation of keratohyalin granules, which occurs in response to a rise in intracellular calcium level, releases profilaggrin into the cytoplasm. This is swiftly followed by dephosphorylation of the proprotein by protein phosphatases (PPases), such as acid phosphatases and a PP2A-type enzyme¹⁰. The N- and C-

terminal domains are cleaved off the profilaggrin, the former undergoes nuclear translocation and is further cleaved into two sections (the A- and B- domains)¹¹. Their exact function is unknown.

Profilaggrin-to-filaggrin processing is said to be mediated by several proteases, including profilaggrin endopeptidase-1¹², calcium-dependent serine proteases furin and PACE4¹³ and calcium-dependent cysteine protease μ -calpain¹⁴. Recent experiments using knockout mice have identified several other proteases involved in this profilaggrin-to-filaggrin processing. These include serine protease matriptase, prostasin (CAP1/*Prss 8*) and 12R-lipoxygenase (12R-LOX). Deficiency of matriptase/MT-SP1, a Type II transmembrane serine protease with extracellular catalytic domain¹⁵, has been shown to cause accumulation of profilaggrin and filaggrin intermediate products in the stratum corneum and loss of processed filaggrin monomers¹⁶. Prostasin, a channel-activating transmembrane serine protease with extracellular catalytic domain, is said to be able to influence intracellular profilaggrin processing by activating the epithelial sodium channel (ENaC). The latter leads to calcium influx through a voltage-gated calcium channel and consequently activating intracellular calcium-dependent proteases involved in profilaggrin processing¹⁷. Prostasin (CAP1/*Prss 8*) knockout mice¹⁸ showed accumulation of certain filaggrin intermediates and barely detectable filaggrin monomers, again indicating defective profilaggrin-to-filaggrin proteolysis. In mice, deficiency of 12R-LOX, which catalyses the dioxygenation of fatty-acid substrates, led to a complete absence of filaggrin monomers and increased levels of other filaggrin intermediates. This was associated with a severe skin barrier defect characterised by increased transepidermal water loss and altered stratum corneum lipid composition¹⁵. In contrast, *SPINK5* deficient mice (*SPINK5* codes for lympho-epithelial Kazal type inhibitor [LEKTI], a serine protease inhibitor) showed an accelerated processing of profilaggrin, resulting in accumulation of filaggrin monomers^{19,20}. Mutations of *SPINK5* in human result in Netherton Syndrome, a severe autosomal recessive disorder characterised by severe skin barrier dysfunction and a specific bamboo hair defect²¹. These are features similar to that seen in the *SPINK5* deficient mice. LEKTI co-localises with matriptase and ablation of matriptase in LEKTI-deficient mice can restore epidermal function²². This indicates that LEKTI, also found in the extracellular compartment, is an inhibitor of matriptase. Overall, multiple extracellular and intracellular factors (mainly proteases and their inhibitors/antiproteases) are involved in profilaggrin-to-filaggrin processing. Inadequate or excessive proteolysis of the proprotein due to imbalances of the protease-antiprotease system will have deleterious effects on the proper formation of the stratum corneum (Table 1).

Degradation of filaggrin monomers into free amino acids (which may be triggered by stratum corneum dehydration) probably begins with the action of peptidylarginine deiminases (PADs) 1 and 3 which convert the arginine residues in filaggrin to citrulline. This process, known as

deimination²³⁻²⁵, changes the net charge of filaggrin from basic to nearly neutral. Deimination disrupts the filaggrin-keratin ionic interaction and promotes their dissociation²⁶, rendering the free filaggrin susceptible to degradation by epidermal proteases. Calpain 1 has been shown to be able to proteolyse deiminated filaggrin into small peptides of different masses which can be subsequently broken down into free amino acids by bleomycin hydrolase²⁷. Another protease shown to be able to degrade deiminated filaggrin is caspase-14. In a study involving caspase-14-knockout mice, accumulation of a small filaggrin fragment of 12-15kDa was found. Increased transepidermal water loss and reduced stratum corneum hydration were also seen²⁸. This suggests that caspase-14 is involved in the degradation of filaggrin into free amino acids and their derivatives such as pyrrolidone carboxylic acid (PCA) which constitute the natural moisturizing factors (NMF). *Trans*-urocanic acid (UCA) is an important derivative of the histidine-rich filaggrin, generated from the amino acid histidine through the action of histidase²⁹. It is photoisomerized to *cis*-urocanic acid by UVB radiation. The beneficial or detrimental effects of UCA in the skin have been heavily argued. Caspase-14 deficient mice, which presumably have less UCA due to impaired degradation of the filaggrin monomers, have shown increased sensitivity to UVB²⁸. While some champion the role of *trans*-UCA as a natural sunscreen that absorbs UV radiation and protects against thymine dimer formation in keratinocytes, other are concerned about the role of *cis*-UCA as a key mediator of photoimmunosuppression and how it may increase photocarcinogenic risk³⁰. This will be further discussed in subsequent chapters.

Caspase-14's involvement in the process of keratinocyte differentiation makes it a unique member of the caspase family, a group of proteases that are mainly involved in apoptosis and inflammation. As caspases can only cleave after an aspartate residue, it is unlikely that caspase-14 directly releases free amino acids from the filaggrin fragments. Two potential mechanisms of action for caspase-14 have been proposed (Figure 1): 1) caspase-14 is indirectly involved in the proteolysis of filaggrin monomers by either activating the proteases responsible for such reaction or inactivating the inhibitor(s) of these proteases 2) caspase-14 may cleave the filaggrin monomers and expose cleavage sites that are recognizable to other proteases which will further proteolyse the filaggrin fragments²⁸.

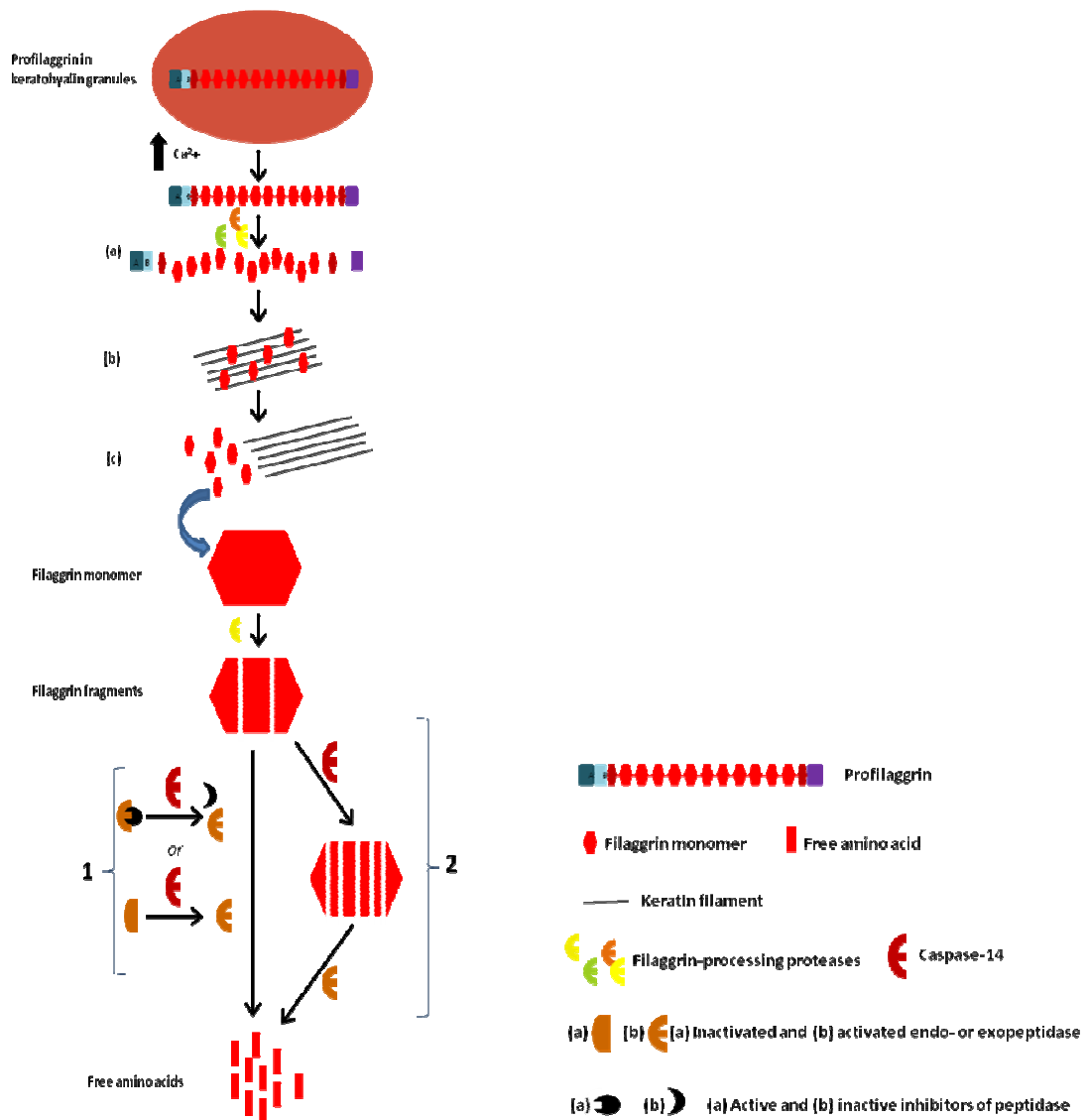


Figure 1. Degradation of filaggrin into free amino acids. Diagram adapted from Denecker *et al*²⁸ showing (pro)filaggrin processing into free amino acids. Increased intracellular calcium triggers the degranulation and release of profilaggrin from the keratohyalin granules. (a) Dephosphorylation, followed by cleavage of profilaggrin by filaggrin-processing proteases, release free filaggrin monomers (b) Transglutaminases facilitate the binding of filaggrin to keratin (c) Deimination, a process facilitated by peptidylarginine deiminases where arginine residues in the filaggrin monomers are converted to citrulline, triggers filaggrin-keratin dissociation. In response to reduced stratum corneum hydration, caspase-14 and other proteases facilitate the breakdown of filaggrin into free amino acids which constitute approximately 40% of the natural moisturizing factors found in the stratum corneum. As caspase-14 is unlikely to directly release free amino acids from the filaggrin fragments, two mechanisms of action for caspase-14 have been proposed: 1) Caspase-14 activates the proteases responsible for such reaction or inactivating the inhibitor(s) of such proteases 2) Caspase-14 cleaves the filaggrin fragments and exposes cleavage sites recognizable to other proteases which breakdown the filaggrin fragments into free amino acids.

3.1.1 The role of epidermal proteases and the importance of protease-antiprotease balance

Degradation of proteins, such as the proteolytic activation of profilaggrin, has long been considered to be the primary function of proteases. Cleavage of peptide bonds by proteases occurs via a general acid-base hydrolysis following nucleophilic attack on the carbonyl carbon³¹. There are six major groups of proteases (serine, cysteine, aspartate, threonine, metallo- and glutamic acid proteases). Proteases are classified based on the mechanism through which the nucleophile is generated at the active site^{32,33}, for example a serine protease has a serine residue at the active site which contains an –OH group that acts as a nucleophile. Another useful example of proteolytic activation of a proprotein is the activation of transglutaminase-1 by cathepsin D, an aspartic protease. During keratinocyte differentiation, transglutaminase-1 catalyzes the cross-linking of cornified envelope proteins filaggrin, loricrin and involucrin via formation of a ϵ -(γ -glutamyl)lysine isodi-peptide bonds³⁴. The covalent ester binding of ω -hydroxyceramide to involucrin³⁵ is also said to be catalyzed by transglutaminase-1. Prior to this occurring, the large precursor protein of transglutaminase-1 (an inactive zymogen of 106kDa) has to be cleaved at two sites to release the enzymatically active transglutaminase fragments^{34,36,37} (67,33,10 kDa) that have 100-fold higher specific activity^{34,36}. This reaction is said to be catalyzed by cathepsin-D³⁸. Cathepsin-D deficient mice were found to have a reduced transglutaminase-1 activity, reduced levels of involucrin and loricrin and abnormal stratum corneum morphology³⁸ similar to that seen in human lamellar ichthyosis.

However, the role of proteases in the skin extends well beyond this 'housekeeping' function. Proteases are involved in various physiologic functions relating to the regulation of epidermal homeostasis including antigen presentation³⁹, matrix resorption⁴⁰, (pro)enzyme activation, post-translational modification of proteins, tissue re-modeling and as mediators of apoptosis^{41–43}. An example of the role of proteases, apart from hydrolysis of peptide bond, is in bone matrix resorption. Metalloproteinases and interstitial collagenase appear to regulate the initiation of bone resorption while cathepsin K, an efficient collagenolytic cysteine protease, is predominantly involved in the solubilization of bone matrix⁴⁰. Abnormalities of these proteases are associated with bone-related disorder, for example the upregulation of metalloproteinases is said to be associated with loosening of prosthesis^{44–46}, osteoarthritis^{47–49} and cancer-related osteolysis^{50–53} while mutations of the cathepsin K gene is associated with pycnodysostosis^{54,55}, a rare osteopetrotic disease. The relative importance of a protease also varies depending on the physiological context. For example, the effects on long bones caused by a deficiency of metalloproteinase-9 are different from that on skull⁴⁰.

As enzymes, the activity of proteases is regulated by factors such as pH, temperature and most importantly, the presence of cognate protease inhibitors (antiproteases)^{56,57}. Numerous skin

disorders, including AD, have been attributed to protease-antiprotease imbalances occurring at the wrong location and time⁵⁸. Examples of such disorders are shown in Table 1.

Table 1

Skin Disorders caused by Protease-Antiprotease Imbalances (adapted from Zeeuwen, 2004⁵⁸)

Protease-antiprotease imbalance	Manifestations	Reference(s)
<i>Excess proteolytic activity</i>		
Overexpression of stratum corneum chymotryptic enzyme (SCCE) ^{59,60}	Upregulation of SCCE may lead to increased cleavage of plakoglobin and corneodesmosin, thus affecting normal stratum corneum desquamation. This has been proposed to be an underlying mechanism for atopic dermatitis.	Hansson <i>et al</i> , 2002 Vasilopoulos <i>et al</i> , 2004
Deficiency of LEKTI (Netherton Syndrome) ^{21,61,62}	Mutations in <i>SPINK5</i> encoding serine protease inhibitor LEKTI lead to Netherton Syndrome characterized by atopic dermatitis, congenital ichthyosiform erythroderma and trichorrhexis invaginata (bamboo hair shaft defect).	Chavanas <i>et al</i> , 2000 Bitoun <i>et al</i> , 2002 Descargues <i>et al</i> , 2004
<i>Inadequate proteolytic activity</i>		
Cathepsin C mutations (Papillon-Lefevre Syndrome) ^{63,64}	Palmoplantar keratoderma with epidermal hyperkeratosis and periodontal destruction.	Toomes <i>et al</i> , 1999 Hart <i>et al</i> , 1999
Cathepsin C mutations (Haim-Munk Syndrome) ⁶⁵	A variant of Papillon-Lefevre Syndrome with additional features including atrophic changes of the nails, arachnodactyly and acro-osteolysis.	Hart <i>et al</i> , 2000
Caspase-14 deficiency ^{28,66}	Increased sensitivity to UV radiation, higher transepidermal water loss and abnormal (pro)filaggrin proteolytic processing are seen in caspase-14 deficient mice.	Denecker <i>et al</i> , 2007 Denecker <i>et al</i> , 2008
Cathepsin D deficiency ³⁸	Reduced transglutaminase activity, altered expression of cornified envelope proteins and stratum corneum morphology are seen, similar to lamellar ichthyosis in human.	Egberts <i>et al</i> , 2004
Cathepsin L deficiency (<i>Furless</i> mice) ⁶⁷	Acathosis, hyperkeratosis, epidermal hyperplasia and periodic hair loss.	Roth <i>et al</i> , 2000
Matriptase/MT-SP1 deficiency (mice) ¹⁶	Accumulation of profilaggrin and filaggrin intermediates, with absence of proteolytically processed filaggrin monomer. Abnormal epidermal lipid matrix, corneocyte morphology and stratum corneum desquamation are seen.	List <i>et al</i> , 2003
Prostasin deficiency (mice) ¹⁸	Severely impaired skin barrier function with abnormal stratum corneum lipid composition, (pro)filaggrin processing and corneocyte morphogenesis leading to rapid and fatal postnatal dehydration.	Leyvraz <i>et al</i> , 2005

3.1.2 Chapter 3 hypothesis

On the basis of the importance of proteolytic processing of profilaggrin and the observation that WT-AD patients often have a reduced expression of filaggrin monomers, we hypothesised that the deranged proteolysis of profilaggrin into functional filaggrin monomers may be the underlying mechanism for the clinical manifestations of AD in those with normal *FLG*. Although processing of profilaggrin into filaggrin is thought to be an intracellular event, extracellular proteases such as matriptase^{15,16} and prostasin, as mentioned above, have been shown to influence the expression of the filaggrin monomers. The mechanism by which this occurs is not entirely clear. As will be discussed later in this chapter in Discussion, a potential target of matriptase is the transmembrane protease-activated receptor-2 (PAR-2)⁶⁸. Inhibition of matriptase or other extracellular proteases (for example, by extracellular protease inhibitors such as LEKTI) may affect PAR-2, the activation of which is shown to influence cornification and skin barrier function⁶⁹. These extracellular proteases may also be channel-activating proteases which activate epithelial sodium and calcium channels on the cell membrane. The consequent influx of calcium is essential for the proteolytic processing of filaggrin¹⁷. It therefore follows from our main hypothesis that the intracellular processing of profilaggrin could be regulated by extracellular proteolytic events.

In this pilot study, we set out to obtain a proof of principle that AD patients have defective processing of profilaggrin and to determine if the relative activity of extracellular proteases and/or antiproteases in skin interstitial fluid was deranged in AD patients. Most of the data presented in this chapter have recently been published by ourselves in British Journal of Dermatology⁷⁰.

3.2 Chapter 3 Results

3.2.1 Demographic data of participants

A total of 11 HC (age: mean, $M=23.5$, standard deviation, $SD=6.4$ years; female: male ratio 7:4) with no history of inflammatory skin conditions and 10 mild-moderate AD participants (age: $M=27.8$, $SD=11.5$ years; female:male ratio 8:2) were recruited into the study (Table 2).

In the AD group, the disease severity score (SASSAD) of the participants ranged from 3-16 for the WT-AD and 0-24 for the *FLG*-AD.

3.2.2 Genotype

1 out of the 11 HC was heterozygous for the *FLG* 2282del4 mutation. 4 out of the 10 AD participants were heterozygous for *FLG* 2282del4, of whom one was a compound heterozygote (2282del4/R501X) (Table 2).

Table 2

Genotypes of Study Participants (a) Healthy Controls (b) AD Participants

(a)			<i>FLG</i> genotype			
Code	Age (years)	Gender	2282del4	R2447X	R501X	S3247X
N1	34	Female	Wild Type	Wild Type	Wild Type	Wild Type
N2	20	Female	Wild Type	Wild Type	Wild Type	Wild Type
N3	25	Female	Wild Type	Wild Type	Wild Type	Wild Type
N5	18	Female	Wild Type	Wild Type	Wild Type	Wild Type
N6	20	Male	Wild Type	Wild Type	Wild Type	Wild Type
N7	20	Female	Wild Type	Wild Type	Wild Type	Wild Type
N8	21	Male	Wild Type	Wild Type	Wild Type	Wild Type
N9	38	Male	Wild Type	Wild Type	Wild Type	Wild Type
N10	19	Female	Wild Type	Wild Type	Wild Type	Wild Type
N11	25	Female	Wild Type	Wild Type	Wild Type	Wild Type
N4	20	Male	Heterozygous	Wild Type	Wild Type	Wild Type

(b)			<i>FLG</i> genotype			
Code	Age (years)	Gender	2282del4	R2447X	R501X	S3247X
S17	21	Female	Wild Type	Wild Type	Wild Type	Wild Type
S21	27	Male	Wild Type	Wild Type	Wild Type	Wild Type
S22	19	Female	Wild Type	Wild Type	Wild Type	Wild Type
S23	26	Female	Wild Type	Wild Type	Wild Type	Wild Type
S24	20	Male	Wild Type	Wild Type	Wild Type	Wild Type
S26	19	Female	Wild Type	Wild Type	Wild Type	Wild Type
S16	47	Female	Heterozygous	Wild Type	Wild Type	Wild Type
S18	27	Female	Heterozygous	Wild Type	Wild Type	Wild Type
S25	22	Female	Heterozygous	Wild Type	Wild Type	Wild Type
C9	50	Female	Heterozygous	Wild Type	Heterozygous	Wild Type

3.2.3 Protein concentrations of suction-induced blister fluids and blister skin cap extracts

Insufficient skin caps were obtained from 3 HC and 1 *FLG*-AD participants. Blister fluid samples from all participants were not used in every experiment in the study due to small amount of fluids obtained from some participants.

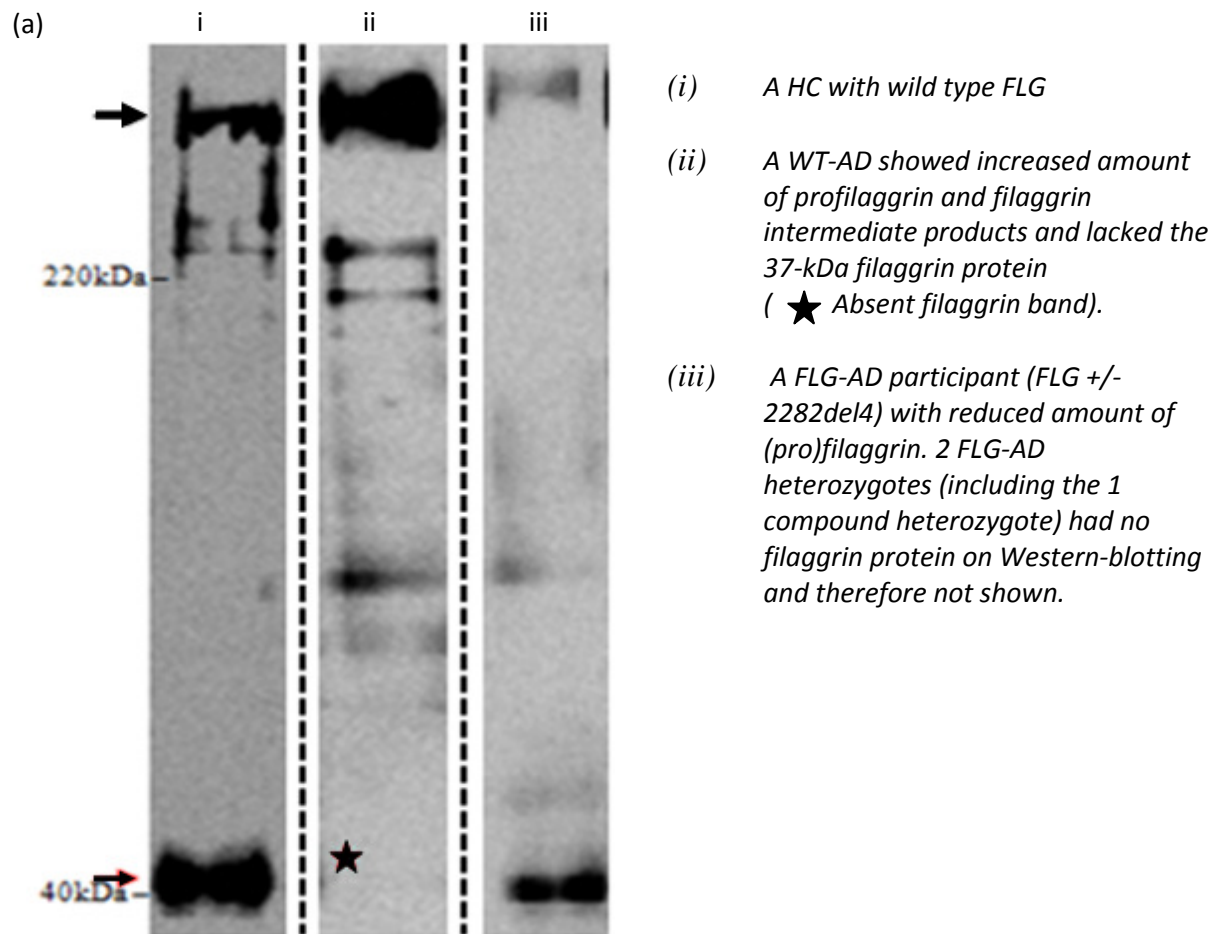
Suction-induced blister fluids and protein extractions from blister skin caps were found to have average protein concentrations of 62.4 mg/ml ($SD=8.9$, $n=18$) and 484.8 mg/ml ($SD=18.4$, $n=19$) respectively.

3.2.4 SDS-PAGE and Western-blot for filaggrin in suction-induced skin caps.

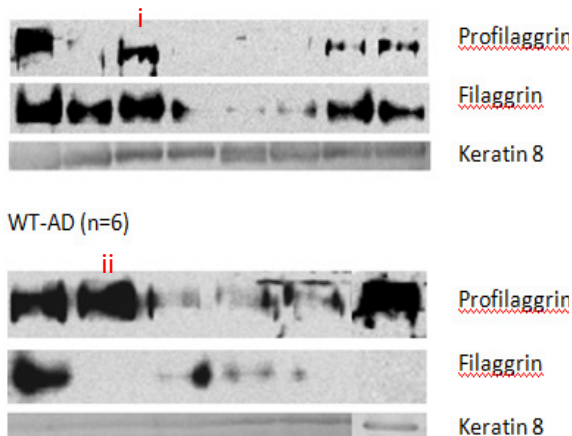
SDS-PAGE using gradient gels (6-15%) was performed using proteins extracted from the blister skin caps, as described in Materials and Methods. This was followed by Western-blotting for (pro)filaggrin. Intensity of the (pro)filaggrin bands were normalised to that of a housekeeping protein, keratin 8.

Western-blot band densitometric analysis showed that the 6 WT-AD participants had, on average, higher amount of profilaggrin compared to 8 HC with wild-type *FLG* ($M=192.5$, $SD=173.6$ vs $M=26.0$, $SD=17.9$, Figure 2c). WT-AD participants also had, on average, a higher profilaggrin: filaggrin ratio, as compared to HC with wild-type *FLG* ($M=2.3$, $SD=2.2$ vs $M=0.8$, $SD=0.3$) (Figure 2).

The higher profilaggrin:filaggrin ratio may imply accumulation of profilaggrin (Figure 2a_{ii}) due to reduced proteolytic processing of the proprotein. Despite the relatively small sample size, the data provide a proof of principle to our hypothesis.



(b) HC (wild-type, n=8)



(c)

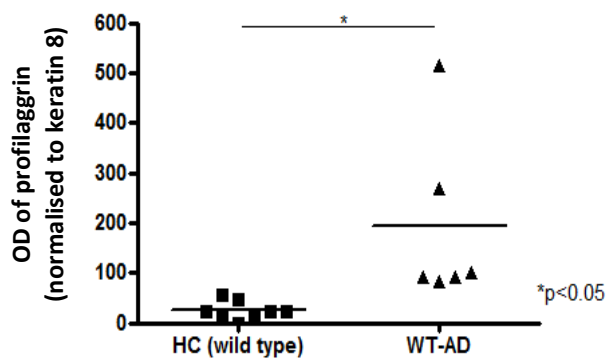


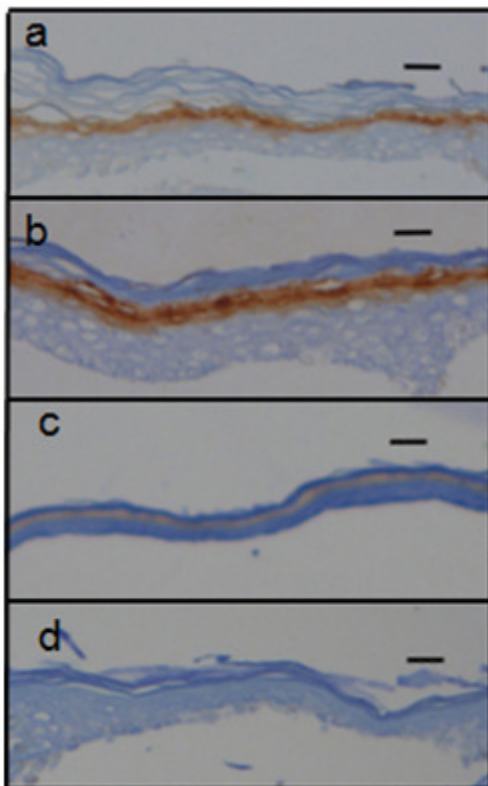
Figure 2. Comparison of (pro)filaggrin protein expression between HC with wild type *FLG* and AD participants by Western-blotting. (a) Representative Western-blots showing profilaggrin (thick arrow, >220kDa) and filaggrin (thin arrow, ~40kDa) (b) WT-AD exhibited an overall increased profilaggrin and reduced filaggrin monomers in comparison with HC with wild type *FLG*. Keratin 8 was used as loading controls. (c) Densitometric band analysis of the Western-blots, followed by unpaired, two-tailed t-test, showed accumulation of the profilaggrin in WT-AD (* $p < 0.05$). Marker ladder (Benchmark Protein Ladder) was not visualised here using a chemiluminescent substrate as it is non-biotinylated. The marker ladder was non-permanently visualised via Ponceau Red staining and hand-marked prior to application of primary antibodies. These markings were superimposed onto the images shown here to ascertain the size of the protein bands seen.

3.2.5 Immunohistochemistry double staining for filaggrin and keratin

Immunohistochemical staining for filaggrin and keratin (Figure 3) was performed using suction-induced skin caps of the 6 WT-AD and 8 HC. Keratin staining allowed convenient visualisation of the entire skin cap.

The results were compared to that of skin caps from the 3 *FLG*-AD 2282del4 heterozygotes (compound heterozygote excluded). 2 of these heterozygotes showed an apparent reduction in their filaggrin staining (Figure 3c). In one *FLG*-AD 2282del4 heterozygote, no filaggrin staining was observed (Figure 3d).

Quantitation by Image-Pro Plus on the entire length of the skin caps showed that the 6 WT-AD had higher average thicknesses of the skin cap ($M=55.8$, $SD=10.8$ vs $M=28.4$, $SD=10.4$ μm) and filaggrin layer ($M=10.0$, $SD=3.5$ vs $M=2.6$, $SD=2.3$ μm) than the 3 *FLG*-AD 2282del4 heterozygotes (Table 3). The intense filaggrin staining in WT-AD (Figure 3b) may be due to the fact that the primary antibody used detects not only the filaggrin monomers but unproteolysed profilaggrin and filaggrin intermediates as well. However, the average thicknesses of the skin cap and filaggrin layer of the 6 WT-AD were similar to that from the 8 HC who were wild-types for *FLG* (Figure 3b, Table 3). The thicknesses of the skin cap and filaggrin layer for the latter were on average 57.1 μm ($SD=21.2$) and 10.1 μm ($SD=4.4$) respectively.



Scale bar=20 μm

Figure 3. Immunohistochemical staining of suction-induced blister skin caps for filaggrin and keratin.

Skin caps from 9 AD participants (6 WT-AD, 3 FLG heterozygotes for 2282del4 mutation) and 8 HC with wild type FLG were stained for filaggrin (brown) and keratin (blue).

(a) A HC with wild type FLG.

(b) A WT-AD participant.

(c) 2 FLG-AD heterozygotes (FLG +/- 2282del4) showed reduced filaggrin staining.

(d) 1 FLG-AD heterozygote (FLG +/- 2282del4) showed no filaggrin staining.

Table 3

Comparison of Overall Epidermal Skin Cap Thickness and Filaggrin Layer, of HC and AD participants¹

	HC		AD participants			
	WT		WT-AD		FLG-AD	
	<i>M</i>	<i>SD</i>	<i>M</i>	<i>SD</i>	<i>M</i>	<i>SD</i>
Total skin cap thickness (μm)	57.1	21.2	55.8	10.8	28.4	10.4
Thickness of filaggrin layer (μm)	10.1	4.4	10.0	3.5	2.6	2.3
Percentage (filaggrin/ total)	17.6		17.9		9.2	

¹Measurements were done using Image-Pro Plus software. *M*=mean, *SD*=standard deviation.

3.2.6 Cryopreservation studies

Assay conditions were established for commercially available, purified proteases where enzyme activity satisfied linear kinetics and at substrate concentrations of 1mM. Our measured *K_m* values for their respective substrates were *M*=0.4, *SD*=0.3mM for human leukocyte elastase, *M*=0.7, *SD*=0.1mM for papain, *M*=0.5, *SD*=0.2mM for trypsin and *M*=0.6, *SD*=0.1mM for chymotrypsin.

We compared the abilities of 2M sucrose, 2M mannitol and 2.5M polyethylene glycol-400 (PEG-400) to preserve the activity of neutrophil elastase following one freeze-thawing cycle at Day 0, 1 and 4 by measuring the time-dependent hydrolysis of a chromogenic substrate. Compared to sucrose and mannitol, PEG-400 was the best in maintaining the activity of neutrophil elastase, with no significant loss of proteolytic function detected at 1 and 4 days after freezing at -20°C [*V₀* remained the same as on Day 0 (0.04 AU/min) on Day 1 and 4, Figure 4].

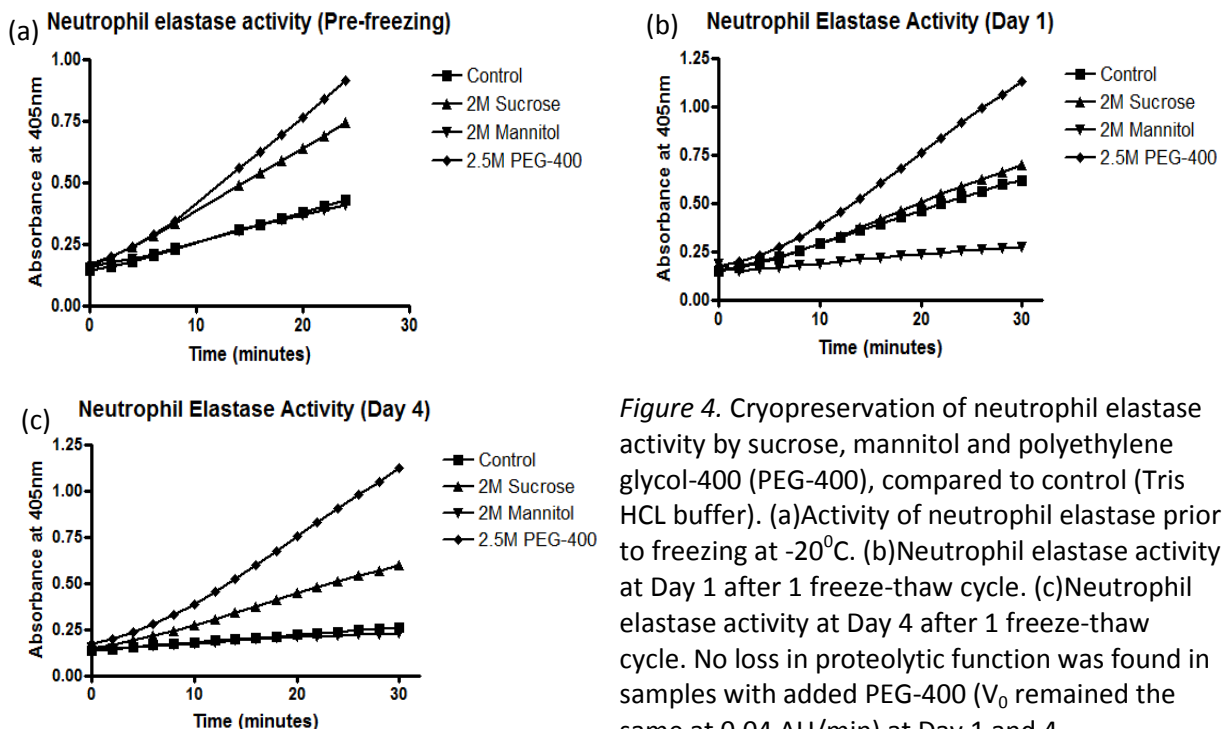


Figure 4. Cryopreservation of neutrophil elastase activity by sucrose, mannitol and polyethylene glycol-400 (PEG-400), compared to control (Tris HCL buffer). (a)Activity of neutrophil elastase prior to freezing at -20°C. (b)Neutrophil elastase activity at Day 1 after 1 freeze-thaw cycle. (c)Neutrophil elastase activity at Day 4 after 1 freeze-thaw cycle. No loss in proteolytic function was found in samples with added PEG-400 (*V₀* remained the same at 0.04 AU/min) at Day 1 and 4.

3.2.7 Protease activity assays

Using chromogenic and fluorogenic peptides, no significant elastolytic, chymotryptic, tryptic, or papain-like activity was detected within the suction-induced blister fluid obtained from all AD participants and HC which had been stored at -20°C . Prior experiments using freshly obtained, unfrozen blister fluid samples ($n=3$) detected no significant protease activity in those samples either. The lack of protease activity within freshly recovered or stored blister fluid indicated the potential presence of one or more potent inhibitors within the blister fluid.

3.2.8 Protease inhibition assays

Suction-induced blister fluid was analyzed for inhibitory activity against serine (elastase, trypsin, chymotrypsin) and cysteine (papain) proteases. As mentioned, the average protein concentration of blister fluids from study participants (AD and HC) was 62.4mg/ml with a standard deviation of 8.9mg/ml . In view of the acceptable variance amongst the participants, an equal volume of blister fluid from each participant was used for the protease inhibition assays.

When compared to HC, AD participants showed higher anti-trypsin and anti-papain activity (Figure 5). Blister fluid from AD participants resulted in a higher inhibition of trypsin ($M=25$, $SD=9\%$ vs $M=18$, $SD=4\%$, $t(16)=2.13$, $*p<0.05$, $n=9[\text{HC}]$, $n=9[\text{AD}]$), papain ($M=69$, $SD=5\%$ vs $M=62$, $SD=8\%$, $t(16)=2.38$, $*p<0.03$, $n=8[\text{HC}]$, $n=10[\text{AD}]$) and, perhaps, elastase ($M=40$, $SD=21$ vs $M=25$, $SD=21\%$, $t(16)=1.48$, $p<0.16$, n.s., $n=8[\text{HC}]$, $n=10[\text{AD}]$) (Figure 5a). No significant difference in the anti-chymotrypsin activity of blister fluid was observed between AD and HC found (AD: $M=4$, $SD=4$ vs HC: $M=6$, $SD=4\%$, $t(16)=0.88$, $p<0.39$, n.s., $n=9[\text{HC}]$, $n=9[\text{AD}]$).

While PEG-400 was observed to preserve proteases activity following freezing at -20°C , it also serendipitously precipitated proteins larger than 80kDa (as observed by Silver staining of the protein gels, Figure 6). Clarified supernatant of PEG-treated blister fluid consisting of predominantly lower-molecular weight inhibitors was therefore tested. These fractionated samples from AD participants were found to have higher anti-elastase ($M=75$, $SD=9$ vs $M=54$, $SD=21\%$; $t(15)=2.72$, $*p<0.02$, $n=8[\text{HC}]$, $n=9[\text{AD}]$), anti-trypsin ($M=52$, $SD=3$ vs $M=49$, $SD=2\%$, $t(17)=2.36$, $*p<0.03$, $n=10[\text{HC}]$, $n=9[\text{AD}]$) but lower anti-papain ($M=46$, $SD=5$ vs $M=57$, $SD=9\%$, $t(15)=3.33$, $**p<0.005$, $n=8[\text{HC}]$, $n=9[\text{AD}]$) activities compared to HC (Figure 5b). Again, no significant difference in chymotrypsin inhibition between AD and HC was found (AD: $M=23$, $SD=2$ vs HC: $M=21$, $SD=2\%$, $t(16)=1.78$, $p<0.09$, n.s., $n=9[\text{HC}]$, $n=9[\text{AD}]$).

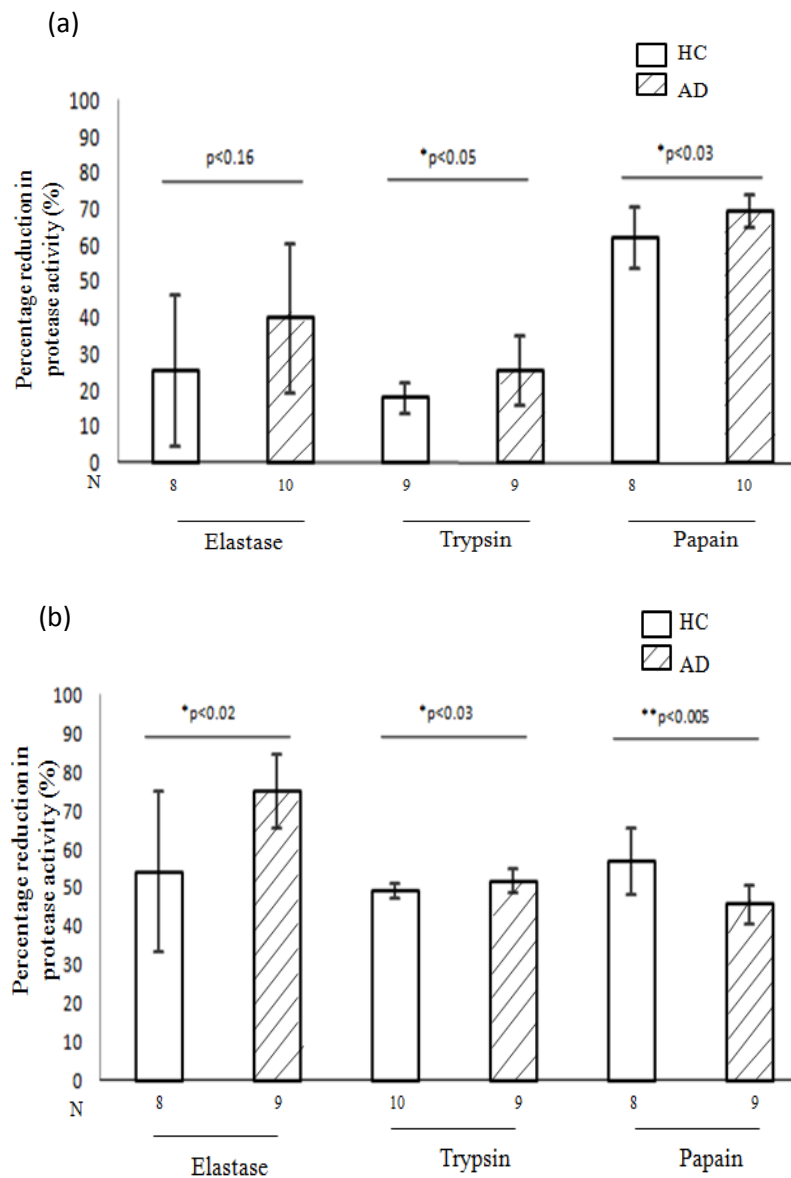


Figure 5. Comparison of the differences in the protease inhibition profile between HC and AD. (a) Percentage reduction in the initial enzymatic reaction velocity (V_0) caused by the addition of blister fluid. AD participants showed higher anti-trypsin ($*p < 0.05$) and anti-papain ($*p < 0.03$) activities. (b) Percentage reduction in the initial enzymatic reaction velocity (V_0) caused by the addition of partially purified blister fluid which contained predominantly lower-molecular weighted inhibitors. Fractionated samples from AD participants were found to have higher anti-elastase ($*p < 0.02$), anti-trypsin ($*p < 0.03$) but lower anti-papain ($**p < 0.005$) activities compared to HC. Unpaired, two-tailed t-test was used for these analysis.

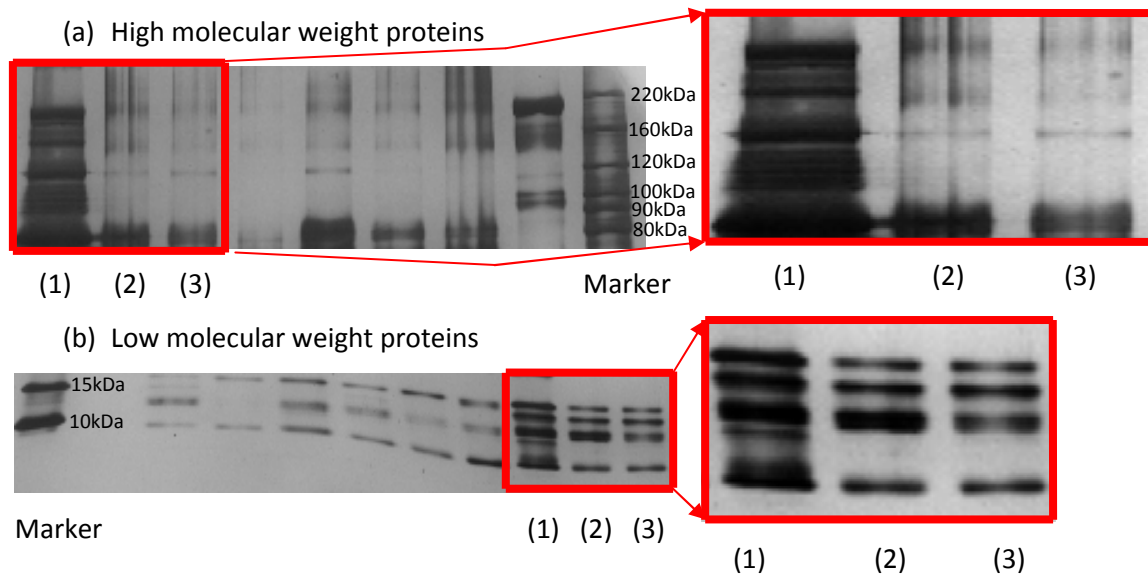


Figure 6. Silver staining of protein bands in SDS-polyacrylamide gradient gels (6-15%). Sample in Lane 1 was not pre-treated with polyethylene glycol-400 (PEG-400). Samples in Lane 2 and 3 were pre-treated with PEG-400. PEG-400 removed most proteins larger than 80kDa (a) while lower molecular weight proteins were relatively unaffected (b).

*Total protein concentration (measured using BCA Protein Assay Reagent, as described in Materials and Methods) was used to determine loading volume in each well. No other loading control was performed here.

3.2.9 Quantitation of known epidermal protease inhibitors.

ELISA assay, followed by analysis using unpaired, two-tailed t-test, found no significant difference in the quantity of secretory leukocyte protease inhibitor (SLPI) (HC: $M=45473$, $SD=9361$ pg/ml vs AD: $M=37207$, $SD=10251$ pg/ml, $t(16)=1.79$, $p<0.09$, n.s., $n=9$ [HC], $n=9$ [AD]) and elafin (HC: $M=29040$, $SD=15594$ pg/ml vs AD: $M=30207$, $SD=18847$ pg/ml, $t(16)=0.14$, $p<0.89$, n.s., $n=9$ [HC], $n=9$ [AD]) between HC and AD participants (Table 4). Western-blot revealed no difference in the expression of α -1-antitrypsin (HC: $M=1908$, $SD=569$ vs AD: $M=1815$, $SD=293$, $t(8)=0.39$, $p=0.70$, n.s., $n=7$ [HC], $n=9$ [AD]) between HC and AD participants (Table 4). Western-blot did not detect any significant quantity of SerpinE2 (protease nexin-1) and LEKTI in the blister fluid, indicating that these inhibitors were not responsible for the observed protease inhibition by blister fluid.

Table 4

Comparison of the Levels of Protease Inhibitors between HC and AD Participants.

	Method	HC		AD (WT, FLG)		p-value
		M	SD	M	SD	
SLPI (pg/ml)	ELISA	45473	9361	37207	10251	0.09, n.s.
Elafin (pg/ml)	ELISA	29040	15594	30207	18847	0.89, n.s.
α 1-antitrypsin (OD)	WB	1908	569	1815	293	0.70, n.s.
SerpinE2/protease nexin-1	WB	ND	ND	ND	ND	-
LEKTI	WB	ND	ND	ND	ND	-

OD=optical density of Western blot, M=Mean, SD=standard deviation, ND=not detected.

3.3 Chapter 3 Discussion

The keratinocyte granular layer expresses at least 3387 genes, including proteases and protease inhibitors, many of which are essential in cornification and desquamation^{3,71}. Increased chymotryptic and tryptic-like protease activities are associated with increased monomeric filaggrin and decreased larger precursors, indicating enhanced proteolytic processing of profilaggrin⁶². This prompts the question if the accumulation of profilaggrin in our participants with an apparently normal *FLG* (Figure 2) is caused by a reduction in the proteolysis of the proprotein, perhaps due to increased inhibition of the proteases responsible for the proteolytic pathway. The inability to break down the large proprotein may explain its apparent accumulation and absence of filaggrin monomer band seen in Figure 2a(ii). Although the sample size of this pilot study is insufficient to draw a relation between individual genotype and protease inhibitory activity, the disproportionately raised inhibition against proteases seen in our AD participants (Figure 5) cannot be ignored. It raises important questions regarding the importance of protease-antiprotease balance in the pathogenesis of the disease, especially among the WT-AD. The reversed observation seen in anti-papain like activities following the removal of predominantly higher molecular-weighted inhibitors suggests that more than one imbalance against cysteinyl proteases is involved. In the process of identifying the responsible skin protease inhibitors, we have excluded several candidates including anti-elastases elafin and SLPI, α 1-antitrypsin, LEKTI and serpinE2. In our Western-blotting, anti-pan cytokeratin primary antibody was used due to availability of resources and of the three keratin bands detected (keratin 5 [58kDa], keratin 6 [56kDa] and keratin 8 [52kDa]), keratin 8 was chosen as the housekeeping protein. This was because keratin 8 was observed to be reasonably comparable and most abundantly expressed in all samples, allowing easy, cost-effective visualisation using 4-chloro-1-naphthol, followed by quantitation using the VersaDoc Imaging System. Although keratin 8 has been previously used by others as loading control⁷², one may raise concerns regarding the limitations of using this protein for this purpose. Using a differentiation marker (keratin) as loading control for another (filaggrin) is perhaps not ideal, especially when we have not conclusively demonstrate that factors that affect filaggrin expression do not affect keratin as well. Nonetheless, keratin expression occurs much earlier than filaggrin (as will be demonstrated in subsequent chapters), making this less of a concern. In addition to using keratin 8 as loading control, the total protein concentration of all samples was also measured in order for similar amount of protein from each sample to be loaded.

Imbalance of proteases and protease inhibitors has been linked to numerous skin disorders^{58,60}. For example, disturbance in the level of cystatin M/E (a small 14-kDa cysteine protease inhibitor), cathepsins (partly regulated by cystatin M/E) and transglutaminase 3 has been described

in lesional AD by Cheng *et al.* At the transcriptional and protein levels, cystatin M/E and cathepsin V were decreased; while transcriptional levels of cathepsin L and transglutaminase-3 were both increased⁷³. This study highlighted the importance of protease-antiprotease imbalance in the pathogenesis of AD and is in line with our findings that lower-molecular weight inhibitors in AD participants had lower cysteine protease inhibitory activity.

In AD patients whose primary problem is the inadequate processing of profilaggrin due to excessive inhibition, downregulating the abnormally high protease inhibition may be the way forward. Raised protease inhibition in the interstitium of AD patients, as shown in this study, may affect extracellular protease activities such as matriptase, which is known to influence intracellular processing of profilaggrin. Nonetheless, little is known of the mechanism by which the latter occurs. A potential target of matriptase is the protease-activated receptor-2 (PAR-2)⁶⁸. The inhibition of matriptase or other extracellular serine proteases may affect PAR-2, the activation of which is shown to influence cornification (of which filaggrin expression is an essential component) and skin barrier function⁶⁹. There are also suggestions these extracellular matriptase and prostatic, as mentioned earlier, are channel activating proteases which can lead to activation of epithelial sodium and calcium channels on the cell membrane. The influx of calcium is essential for the proteolytic processing of filaggrin¹⁷. Future works should be directed at the activity of matriptase or prostatic and their specific inhibitors (such as α 1-antitrypsin⁷⁴ for matriptase and hepatocyte growth factor activator inhibitor-1, HAI-1⁷⁵ for both matriptase and prostatic) in AD patients.

Some readers may question the validity of the suction blister technique used in this study to obtain extracellular/ interstitial fluid. Our literature review revealed that suctioning induces a non-traumatic split at the level of lamina lucida in the basement membrane and is a validated, well-established technique used to obtain extracellular/ interstitial fluid⁷⁶⁻⁷⁸. Many important epidermal protease inhibitors such as elafin, SLPI and LEKTI are secreted extracellularly into the interstitial space. As the focus of this study is on the potential detrimental effects of extracellular protease-antiprotease imbalances (such as that of matriptase) on profilaggrin processing, the suction blister approach which obtain extracellular fluids is the best-suited method. The authors acknowledge the limitations of this method. For example, many protease inhibitors are themselves substrates for transglutaminases^{79,80}, a group of enzymes that are essential in keratinocytes cross-linking during terminal differentiation. Our assays may thus have underestimated the true levels of anti-proteases present in the epidermis. In addition, suction-induced blister fluid may yield antiproteases of diverse origin. Proteins can diffuse from the serum into the blisters and despite being generally regarded as a non-traumatic technique, suctioning may potentially generate a small amount of secondary inflammation and influx of antiproteases to the test sites. Suctioning time was therefore kept to a

minimum to minimise generating secondary inflammatory response. Care was taken in this study to ensure standardisation of the procedure, such as ensuring all the recruited AD participants had only mild-moderate form of the disease according to SASSAD classification (a significant degree of variation was, however, present within the group, with SASSAD scores ranging from 0 to 24), all participants were treated equally and the procedure was performed on uninvolved skin at which no sign of inflammation was observed. The time taken for the blisters to form differed significantly from one participant to another and did not appear to be influenced by AD or *FLG* status. For standardisation, suctioning time was limited in all participants (AD and HC) to 1 hour 45 minutes or until a 7-mm blister was formed. Activities of endogenous proteases were preserved by adding PEG-400 to the blister fluids upon collection. PEG-400 has been shown to fully preserve the activity of lactate dehydrogenase following free-thaw cycle⁸¹. The absence of detectable protease activity, even with PEG-400, was surprising and to our best knowledge, not previously demonstrated by others. It is, however, arguable that this was a result of poor experimental sensitivity. In a similar study of antiprotease activity in uninvolved skin of psoriatic patients, reduced anti-elastase but no change in anti-trypsin and anti-chymotrypsin properties was observed⁸². As psoriasis and AD are both chronic inflammatory skin disorders, this study may indicate that our observations are AD-specific, rather than representing a non-specific inflammatory response to a traumatic stimulus. It is also arguable that suctioning provides protease inhibitors which are present within the extracellular space at the basal layer, which may be different from those present in the more superficial layers of the epidermis.

FLG mutations are thought to be inherited in an autosomal semi-dominant manner⁸³ and may be expressed, though in a reduced amount or be completely absent, in heterozygotes (as in Figure 3c,d). Our immunohistochemistry results suggest that reduced or absent filaggrin protein in *FLG*-AD may be associated with a reduction in the overall thickness of the epidermis. This concurs with the current literature on the role filaggrin as an important structural protein which facilitates keratinocyte differentiation and desquamation^{1,3,83,84}. Our WT-AD participants, however, had macroscopically normal epidermal structure. The thickness of the epidermis of WT-AD participants was comparable to that from HC and higher than the epidermal thickness of *FLG*-AD participants. This is rather surprising because if some of these WT-AD participants develop the disease due to defective profilaggrin processing as hypothesized, they would be expected to have abnormal skin structure and function, similar to those who have reduced or absent filaggrin protein due to genetic mutations. The accumulated profilaggrin in WT-AD is said to have no keratin-binding function⁹. Unless the proprotein has other unknown structural function in the epidermis, this discrepancy

could be due to the small sample size of our study and highlights the need for a larger population study before any postulation can be made.

In conclusion, this study supports an imbalance in the extracellular protease-antiprotease activities as a possible pathomechanistic insight into the development and propagation of AD. While previous studies have suggested that excessive proteolysis in the skin is causally linked to skin barrier dysfunction^{60,85-89}, this study suggests that abnormally high protease inhibition could lead to barrier dysfunction by interfering with the processing of profilaggrin into functional filaggrin monomers. This is, however, a preliminary study based on a small sample size to explore potential associations between extracellular protease-antiprotease imbalances to intracellular processing of profilaggrin. Any association shown between raised antiprotease activities and the degradation of profilaggrin does not necessarily imply causation. The results seen here aim to provoke thoughts on the pathomechanism of this complex disease and open doors for future studies, such as one involving a larger sample size, or perhaps a proteomic study to identify the responsible protease inhibitors and the extracellular-intracellular pathways involved. Future works can also be directed at investigating the 9% of the healthy population who have *FLG* mutations but do not develop AD. It would be of great academic interest to know if these people have a relatively normal level of filaggrin protein expression and if so, whether this is due to an enhanced ability to proteolyse profilaggrin. Such information may have potential therapeutic applications.

3.4 Chapter 3 References

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3.5 Chapter 3 Materials and Methods

3.5.1 Participant recruitment

10 participants aged 27.76 ± 11.54 years with mild-moderate AD according to the UK criteria⁹⁰ (disease severity scored using the Six Area, Six Sign Atopic Dermatitis [SASSAD]⁹¹ system) were recruited. SASSAD score <21 is considered mild AD, $21-40$ is considered moderate and >40 is considered severe. 11 HC aged 23.47 ± 6.45 years with no history of inflammatory skin condition were also recruited. Exclusion criteria: Those with active inflammation or infection on the flexor surfaces on their forearm (where blisters were induced) and those unable to give informed consent. Experiments were approved by the Scotland A Research Ethics Committee (Ref: 07/MRE00/109)⁹² and complied with principles of the Helsinki Accord. All participants gave written, informed consent.

3.5.2 Genotyping

Genotyping for 4 common *FLG* polymorphisms found in Caucasian populations (2282del4, R2447X, R501X, S3247X) were performed as described elsewhere⁹³.

3.5.3 Development of suction-induced blisters

PTC3300 VAC Vacuum Unit (InnoKas Medical Oy, Kempele, Finland) was used to induce 10 blisters of 6-7mm in diameter and 3-4mm in height on the flexural surfaces of left arm (≈ 5 cm above and below the antecubital fossa), as per manufacturer's instructions using previously described techniques⁷⁶. The left arm was covered loosely with a cotton towel to keep the skin warm. This has been suggested to expedite the development of the blisters.

Since its development by Kiistala⁷⁶, the suction blister model has been widely used in dermatological works and is an established way of obtaining interstitial fluid⁷⁷. Those with active disease or signs of infection or active inflammation on the left arm were excluded from the study. Suctioning was performed at a vacuum pressure of 160-240 mBar for 1 hour 15 minutes to 1 hour 45 minutes or until a 7-mm blister was formed.

Blister fluid samples from all participants (AD participants and HC) were removed with a sterile 1-ml insulin syringe and polyethylene glycol (PEG) 400 (Fluka, Gillingham, UK) was added in a 50:50 ratio to all the blister fluid samples before immediate storage at -20°C until subsequent analysis. This procedure, done to preserve existing protease activity, also resulted in significant protein precipitation of larger molecular weight proteins on thawing that was removed by centrifugation but which dissolved readily on resuspension in an aqueous assay buffer. This partial fractionation provided an opportunity to assess the lower molecular weight proteins, typically less than 80 kDa in size, that remained in suspension.

Blister skin caps were removed with sterile forceps and treated under the following conditions: (1) Fixed in neutral buffered 10% formalin (Sigma, Poole, UK) and embedded in paraffin for immunohistochemistry or (2) Stored without fixatives in -20°C for Western-blot analysis.

3.5.4 Protein extraction from blister skin caps

100 µL of extraction buffer (8M urea; 50mM TRIS HCL pH7.6; 100mM dithiothreitol; 0.13M 2-mercaptoethanol; 100µg/ml phenylmethylsulfonylfluoride) was added for every mg of skin. Samples were left to stand in 4°C for 16 hours before the skin samples were discarded. Extracted protein samples were stored in -20°C.

3.5.5 Determination of protein concentration

Total protein concentration of blister fluid and skin extracts was determined with BCA Protein Assay Reagent (Thermo Scientific, IL, USA) as per manufacturer's instructions. Measurements were performed in either duplicates or triplicates.

3.5.6 SDS-PAGE and Western-blot

SDS-PAGE was performed using acrylamide gels (6-15% gradient gels for filaggrin from skin cap extracts, 9% gels for α 1-antitrypsin and serpinE2 from blister fluid), electroblotted overnight onto nitrocellulose membrane at 0.2 mA/cm² of membrane. Benchmark Protein Ladder [10747-012] (Invitrogen, Paisley, UK) was used as the size marker. For the protease inhibitor blots, the blister fluid samples (with added PEG400, 50:50) were first spun at 13,000g for 5 minutes. The supernatants which contained proteins typically smaller than 80kDa were then removed for SDS-PAGE analysis. Primary antibodies against the following antigens were used: filaggrin (goat polyclonal antibody [SC-25897] from Santa Cruz, CA, USA), pan-cytokeratin (mouse monoclonal antibody [C1801] from Sigma, Poole, UK), SerpinE2/PN1 (mouse monoclonal antibody [MAB2980] from R&D Systems, Minneapolis, USA). Alpha-1-antitrypsin antibody was a gift from Dr Jason King, University of Edinburgh. Quantitative densitometric analysis of the protein bands, visualised using SuperSignal West Femto chemiluminescent substrate [34094] (Thermo Scientific, Hemphstead, UK), was done using the VersaDoc Imaging System (Bio-Rad) and all bands normalised to keratin 8.

3.5.7 Immunohistochemistry double-staining.

Using general double immunohistochemical staining techniques, the 3-µm formalin-fixed, paraffin-embedded transverse sections of skin caps obtained from suctioned-induced blisters were incubated at 4°C overnight with the primary antibodies raised against: filaggrin (mouse monoclonal antibody [15C10] from Novocastra, Newcastle, UK) and pan-cytokeratin (mouse monoclonal antibody [C1801] from Sigma, Poole, UK). Samples were then incubated with the appropriate secondary antibodies for

40 minutes at room temperature. Diaminobenzidine (DAB) and Vector blue alkaline phosphatase were used to visualise the filaggrin layer (brown colour) and keratins (blue colour) respectively. Keratin, widely present in the epidermis, allowed convenient visualisation of the entire skin caps. The total thickness of the epidermal skin caps and the filaggrin layer were measured and averaged across the entire length of the skin caps using Image-Pro Plus.

3.5.8 Determination of proteases activity of suction-induced blister fluid

The Synergy HT Multi-Mode Microplate Reader (Biotek, UK) was used to detect time-dependent hydrolysis of fluorogenic (chymotrypsin, trypsin, and papain assays) and chromogenic (elastase assay) substrates caused by endogenous proteases in the blister fluid. Enzymatic reactions were measured in a 96-well plate. In view of the acceptable standard deviation for protein concentration of blister fluids, as determined using the BCA Protein Assay Reagent (Thermo Scientific, IL, USA), a standard volume of blister fluid was loaded into each well. 20 μ L Blister fluid (with added PEG400, 50:50) was added to 20 μ L commercially purchased enzyme substrates (final concentration of 1mM) and 60 μ L stock buffers. Reagents used were as detailed next. Measurements were done at 37 $^{\circ}$ C over 30 minutes in 2-minute intervals.

3.5.9 Determination of proteases inhibitory activity of suction-induced blister fluid

Inhibitory activity was calculated as the percent reduction in the initial enzyme reaction velocity(V_0) following the addition of a fixed volume of blister fluid. V_0 of enzymatic reactions without blister fluid (and its inhibitory activity) was used as the denominator. V_{max} and K_m of each enzyme at the concentration used in this study was calculated using Lineweaver-Burk plot. Synergy HT Multi-Mode Microplate Reader (Biotek UK) was used to detect changes in absorbance at 405nm (for elastase assay) and fluorescence at 360/460nm for the chymotrypsin, trypsin, and papain assays. Experiments were conducted in 96-well plates at 37 $^{\circ}$ C for 30 minutes in 2-minute intervals. As mentioned earlier, a standard volume of blister fluid was loaded into each well. In each well, there were 50 μ L of stock buffers, 20 μ L of stock substrates (final concentration of 1mM), 20 μ L of the commercially purchased enzymes and 10 μ L of blister fluid (with added PEG400, 50:50). For the controls, the 10 μ L of blister fluid was replaced with an additional 10 μ L of stock buffer. Note: 1 in 4 dilution of the blister fluid samples was used for the elastase assay due to high elastase inhibitory activity.

The reagents used are as followed:

Enzymes and final concentration within reaction mixture: human leukocyte elastase (Cat.#E8140 Sigma, Poole, UK) 0.004 units/ assay, chymotrypsin from bovine pancreas (Cat.#230834 Calbiochem,

Darmstadt, Germany) 0.2mg/ml, trypsin from bovine pancreas (Cat.#650200 Calbiochem, Darmstadt, Germany) 0.13mg/ml, papain (Cat.#5125 Calbiochem, Darmstadt, Germany) 0.02mg/ml. Refer to manufacturers for specific activity of individual enzymes. Stock substrates: 5mM N-ms-Ala-Ala-Pro-Val-p-nitroanilide for elastase assay (Sigma, Gillingham, UK), 5mM Glutaryl-Glycine-Glycine-Phenylalanine- β -naphthylamide for chymotrypsin assay (Bachem, Weil am Rhein, Germany), 5mM N α -benzoyl-DL-arginine- β -naphthylamide hydrochloride for trypsin and papain assays (Sigma, Schnelldorf, Germany). Stock buffers: 0.1M TRIS HCL; 0.5M NaCl pH 7.4 for elastase assay, 0.1M TRIS HCL; 0.1M CaCl₂ pH8 for chymotrypsin assay, 0.05M TRIS HCL; CaCl₂ 0.0115M pH8.1 for trypsin assay, 1mM TRIS HCL; 3mM NaCl; 0.0115M CaCl₂ pH6.5 for papain assay.

3.5.10 Measurement of secretory leukocyte protease inhibitor (SLPI) and elafin.

Human SLPI Quantikine ELISA kit (R&D Systems, Minneapolis, USA) and Human Trappin-2/Elafin DuoSet kit (R&D Systems, Minneapolis, USA) were used as per manufacturer's instructions for quantitation of SLPI and elafin.

3.5.11 Statistical analysis

Student's t-test was used for comparisons between the two groups in all experiments, except for the immunohistochemistry data where one-way ANOVA was employed. *p*-values<0.05 were considered as significant. Data were presented as mean, *M* \pm standard deviation, *SD*.

Chapter 4

Development of an In-house Organotypic Skin-equivalent Model

The study of skin barrier function and morphological structure is best done using a 3-dimensional model which approximates a native human epidermis. Organotypic skin-equivalent models can be used as an alternative to animal models. Such models are commercially available but they are costly. Here, we describe the development of an in-house organotypic skin-equivalent model which can be easily established in any laboratory with basic cell culture facilities.

4.1 Introduction: Organotypic skin-equivalent models

Advances in techniques for culturing human keratinocytes in recent decades have led to the development of skin-equivalent models that bear functional and morphological characteristics of a native human epidermis¹⁻⁴. There are a number of commercially available skin-equivalent models, such as EpiDerm™ (MatTek, Massachusetts, USA) and Episkin™ (L'ORÉAL, Clichy Cedex, France). Skin-equivalent models are now widely used to investigate keratinocyte differentiation and function, gene expression^{5,6}, epidermal-dermal interaction, wound healing^{7,8}, percutaneous absorption, toxicity and metabolism of topically applied products⁹, transdermal drug delivery and skin grafting^{10,11}. 3D skin-equivalent models are a suitable alternative for animal models and are advantageous over monolayer cultures¹². Monolayer cell cultures enable the study of gene transcription, translation and protein synthesis but do not adequately reflect the complex cell-cell (for example, between keratinocytes and fibroblasts) and cell-matrix interactions. The study of skin barrier function and morphological structure, which is our main interest, is best done using a 3-dimensional model.

A good skin-equivalent model is one that closely resembles the native human epidermis in terms of structure and function. Having a reliable and reproducible skin model will provide us a means of performing ethical pharmacological intervention studies, as will be described in the next chapter. Our process of developing the skin model is based on the basic principles of co-culturing human keratinocytes with primary human dermal fibroblasts (HDF) at an air-liquid interface. Co-culturing with fibroblasts has been shown to stimulate the growth of keratinocytes and lead to a thicker, more organised epithelium¹. The thickness of the 'epithelium' correlates with the number of fibroblasts within the gel. The aim is to obtain a well-stratified artificial skin, with the air-exposed side consisting of terminally differentiated keratinocytes while the liquid (culture media)-exposed side being the 'basal', undifferentiated layer of cells.

Instead of using primary human keratinocytes, we opted for keratinocyte cell line HaCaT (human adult low-calcium high-temperature keratinocytes) for our model. HaCaT is the first permanent human-derived keratinocyte cell line that retains full differentiation capacity¹³. These cells were derived from a histologically normal, full-thickness skin sample taken at the distant periphery of a melanoma located on the back of a 62-year old male patient in 1980s. The isolated keratinocytes were initially cultured in low calcium media (0.2mM) and at an elevated temperature of 38.5°C. While a high-calcium environment favours keratinocyte differentiation, a low calcium environment is said to be optimal for long-term growth in primary cultures, probably due to a reduction in terminal differentiation and increase in proliferative activity. One of the subcultures continued to proliferate indefinitely with no obvious crisis observed even after 140 passages. Upon

karyotyping, these cells were found to be aneuploids and possess unique marker chromosomes denoting a monoclonal origin. Despite the unlimited growth potential and chromosomal abnormalities, HaCaT cells are non-tumourigenic. Cells subcutaneously injected or transplanted onto nude mice were found to form a structured and differentiated epidermis and express differentiation markers including keratins, involucrin and filaggrin. The resulting spontaneously immortalised cell line was named HaCaT, denoting its origin¹³. Unlike primary keratinocytes (normally derived from human foreskin samples), the supply of HaCaT keratinocytes is limitless and of standardised quality which is independent of donor variations.

Schoop *et al*¹ has shown that HaCaT cells, cultured at an air-liquid interface on various matrixes serving as dermal-equivalents, can be used to generate highly differentiated organotypic skin-equivalent structures *in-vitro*. These matrixes may be a porous membrane^{10,14,15}, de-epidermized and de-vitalised dermis¹⁶⁻¹⁸ or collagen gel²⁻⁴ populated with fibroblasts. Within 1 week of HaCaT cells being seeded on top of a collagen structure containing HDF, Schoop *et al* detected a thin and disorganized 'epithelium'¹. Early markers of keratinocyte differentiation, including keratin 10 and involucrin, were found at this stage. After 2-3 weeks, a relatively well-organized, multi-layered, stratified squamous epithelium with a near-normal basement membrane was seen. Basal layer consisting of cuboidal cells, distinct spinous and granular layers and a parakeratotic stratum corneum were seen. Structures including hemidesmosomes, anchoring filaments and a nearly continuous lamina densa were found. Markers of late keratinocyte differentiation including filaggrin and loricrin were also detectable at this stage. By adapting the method used by Schoop *et al*¹, we attempted to develop a cost-efficient way of creating a functional skin-equivalent model which can be easily set up in any laboratory with basic cell culture facilities. Over a period of 6 months, a variety of techniques were utilised and improved on in establishing our skin-equivalent model. These different methods were sequentially numbered and represent different stages of optimisation. We started with using Transwell culture plates to establish our model (Method 1: the 'Transwell Technique') and we finally settled on a method that uses plastic grids to create the air-liquid interface (Method 4: the 'Grid Technique').

4.2 Chapter 4 Results

4.2.1 The expression of early and late keratinocyte differentiation markers in the epidermis of a healthy human volunteer

Suction-blistering, as described in Chapter 3, was employed to obtain human epidermal samples to be used as a benchmark for our skin-equivalent model.

Suction-induced blister skin caps obtained from one healthy human volunteer with no history of inflammatory skin disease were stained for markers of early (keratin 10 and involucrin) and late (filaggrin and loricrin) keratinocyte differentiation.

Keratin 10 was predominantly found in the suprabasal layers of the epidermis (Figure 1a). Involucrin was found in abundance throughout the entire skin caps (Figure 1b) while filaggrin and loricrin were found suprabasally in the terminally differentiated stratum corneum (Figure 1c,d).

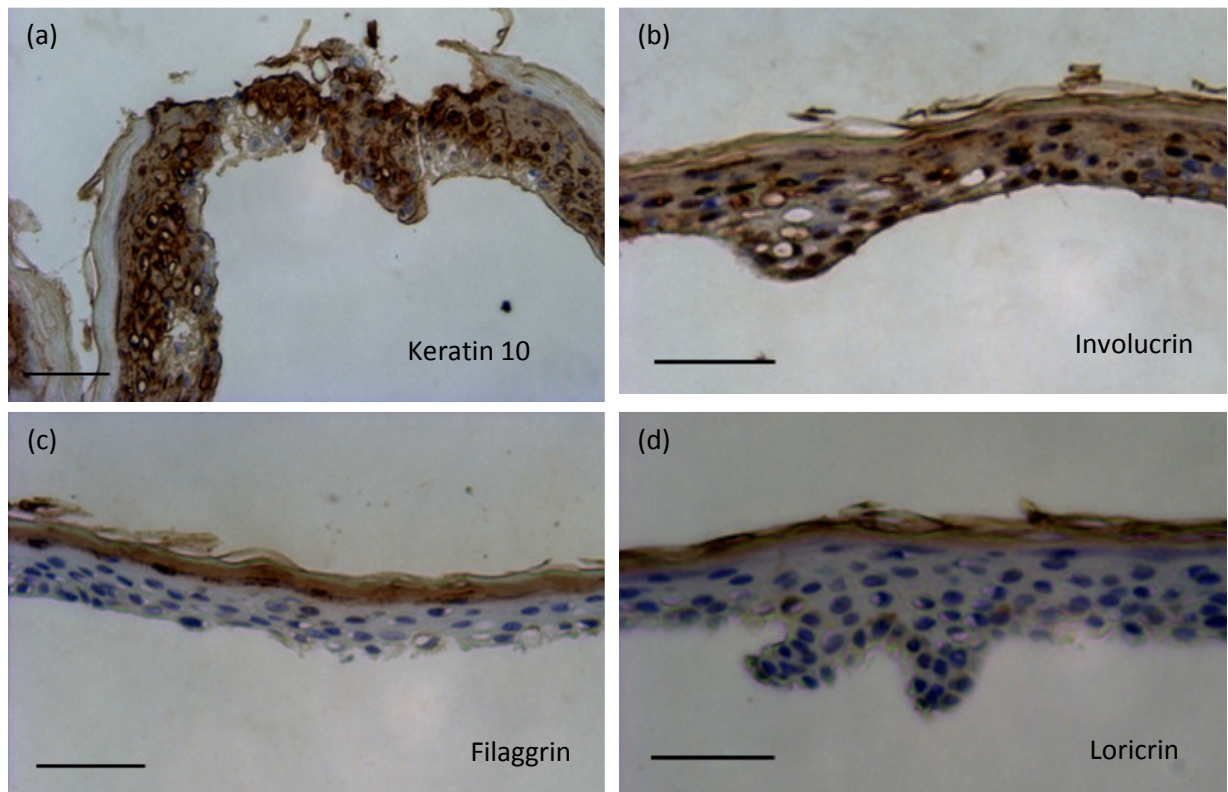


Figure 1. Representative images of epidermal samples obtained from one healthy human volunteer by suction-blistering technique. Blister skin caps were stained (brown) for early (keratin 10, involucrin) and late (filaggrin, loricrin) keratinocyte differentiation markers. (a) Keratin 10 was found in abundance, particularly in the upper epidermal layers consisting of more differentiated cells. (b) Involucrin was found in abundance throughout the entire skin cap. (c) Filaggrin and (d) loricrin were predominantly seen in the terminally differentiated stratum corneum. Scale bar=50 μ m

4.2.2 Organotypic skin-equivalent models

4.2.2.1 Method 1: The 'Transwell Technique'

Using Method 1, isolated foci of differentiated HaCaT cells could be detected at Day 5 (n=6, Figure 2a), in the presence of 1.4mM calcium. No islet of differentiated keratinocytes was seen in skin models grown in calcium-depleted media (n=6, Figure 2b).

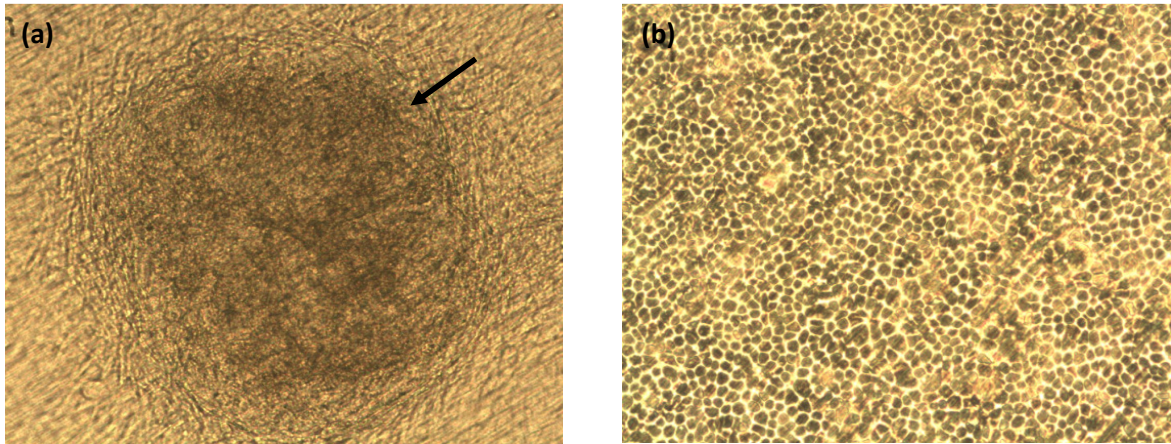


Figure 2. Calcium is required for keratinocyte differentiation. (a) Islets of differentiated HaCaT cells (black arrow) were seen in skin models grown in calcium-enriched (1.4mM) media at Day 5 (b) No islet of differentiated keratinocytes was seen in models grown in calcium-free media at Day 5. Pictures shown are of x10 magnification, courtesy of Dr Catherine Wright, Glasgow Caledonian University [Pollock et al., 2009 (submitted)].

Method 1: the 'Transwell Technique' (Figure 3) is an adaptation of that developed by Dr C. Wright, Glasgow Caledonian University. Schematic representation of this method is shown in Figure 3. For detailed description of the method, see Materials & Methods.

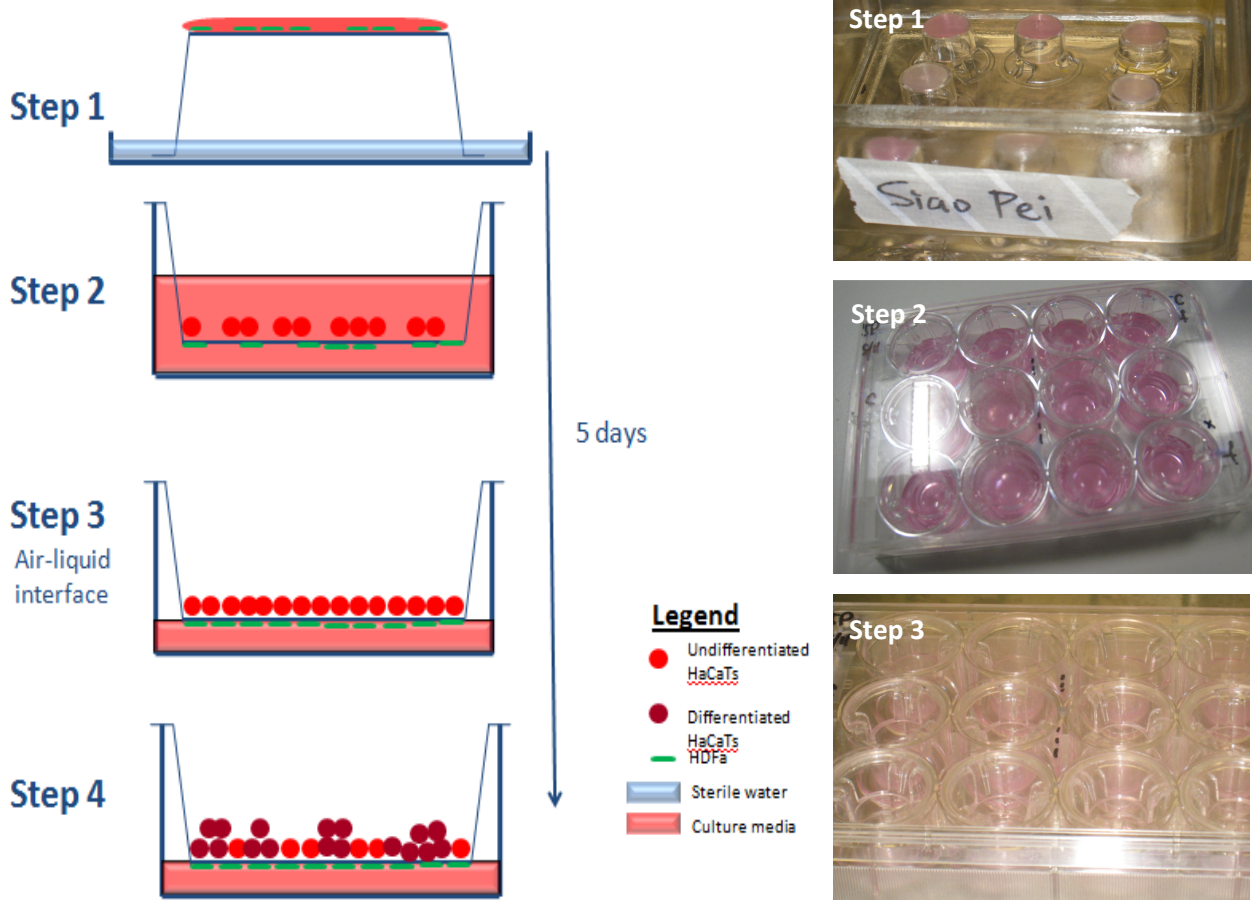


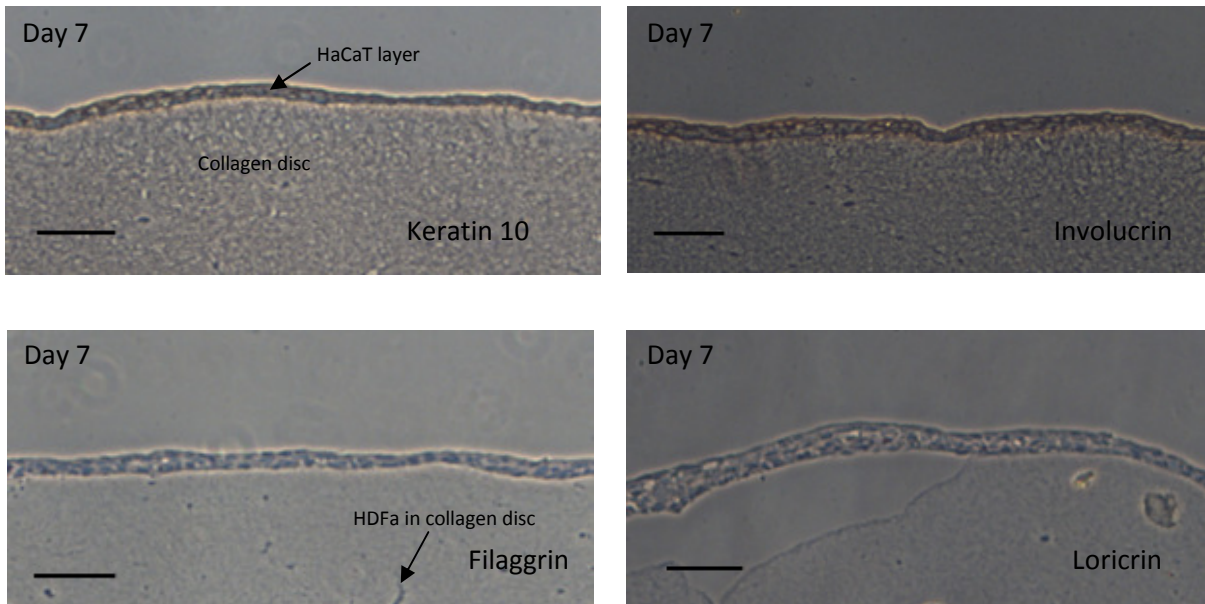
Figure 3. Diagrammatic representation of the ‘Transwell Technique’ (left) and photographs demonstrating the technique (right). A Transwell culture plate is used to suspend the keratinocytes (seeded in the wells) in an air-liquid interface and keeping them segregated from the primary human dermal fibroblasts (HDF) which are seeded onto the underside of the collagen-coated PTFE membrane. (Step 1) HDF were seeded onto the underside of a Transwell culture plate and left to adhere overnight. (Step 2) HaCaT cells were seeded directly into the upper Transwell chamber. Cell culture media was placed in both upper and lower chambers and replaced daily until a confluent monolayer of HaCaT cells was obtained (usually occurring after 24 hours). (Step 3) At confluency of the HaCaT cells, culture media was removed from the upper chamber, exposing the HaCaT cells to air. (Step 4) Isolated foci of differentiated HaCaT cells were detectable at Day 5.

4.2.2.2 Method 2: The 'Disc Technique'

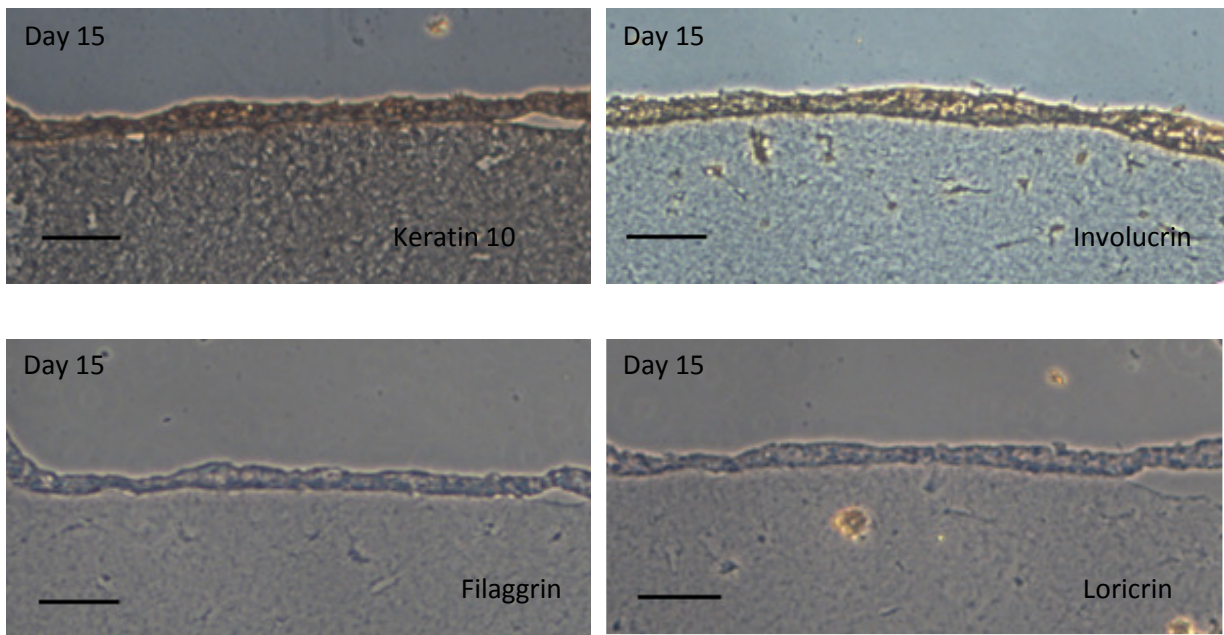
Compared to the 'Transwell Technique', a uniformly thin layer (averaging 2 to 3 cells in thickness) of HaCaT keratinocytes was found on top of the collagen discs at Day 7 (n=1, Figure 4a). The skin model at Day 15 appeared to be thicker than that of Day 7 (n=1, Figure 4b). The thickness of the keratinocyte layer appeared to continue to increase (though minimally) at Day 21 (n=1, Figure 4c). At Day 21, the cells were noted to be detaching from the collagen discs.

Staining intensity of early markers of keratinocyte differentiation (keratin 10 and involucrin) appeared to increase from Day 7 to Day 21. No significant staining of late keratinocyte differentiation markers (filaggrin and loricrin) was found at all stages (Figure 4).

(a)



(b)



(c)

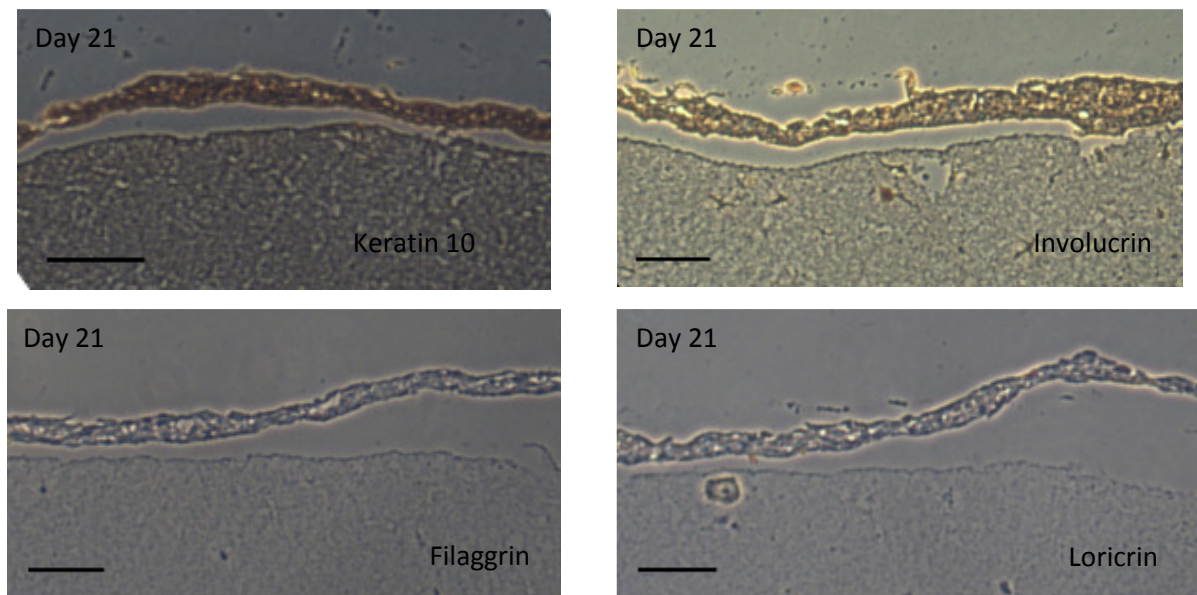


Figure 4. Immunohistochemistry (transverse sections) of organotypic skin-equivalent model (the 'Disc Technique'). (a) Day 7: A uniformly thin layer of HaCaT cells was seen at the apical aspect of the collagen discs. HaCaT cells were positive for early markers of keratinocyte differentiation (keratin 10 and involucrin, brown staining). (b) Day 15: A layer of HaCaT cells thicker than that of Day 7 was seen on the apical aspect of the discs. Increased positive (brown) staining for early markers of keratinocyte differentiation (keratin 10 and involucrin) was seen. (c) Day 21: A layer of HaCaT keratinocytes (minimally thicker than that of Day 15) was seen on the apical aspect of the collagen discs. Positive (brown) staining for early markers of keratinocyte differentiation (keratin 10 and involucrin) was seen. No obvious staining for filaggrin and loricrin (late markers of keratinocyte differentiation) was seen at all stages. Scale bar=100µm *HDFa: Adult human dermal fibroblasts

The thickness of the HaCaT layer was found to be greater at the peripheral edges of the collagen discs than at the apical aspect of the discs (Figure 5: black arrow).

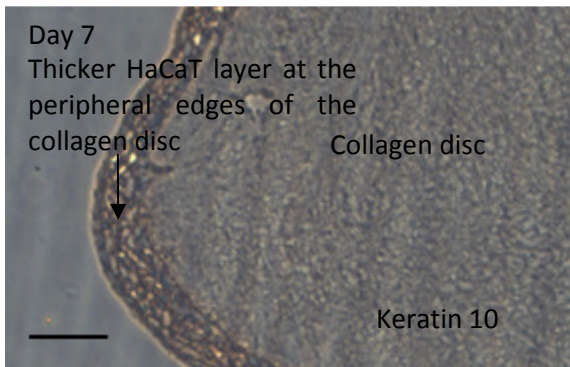


Figure 5. A representative image of a transverse section of the skin model (the 'Disc Technique'). The HaCaT layer appeared to be thicker at the peripheries than at the apical aspect of the disc. Scale bar=100µm

Schematic representation of the 'Disc Technique' is shown in Figure 6. For detailed description of the method, see Materials & Methods.

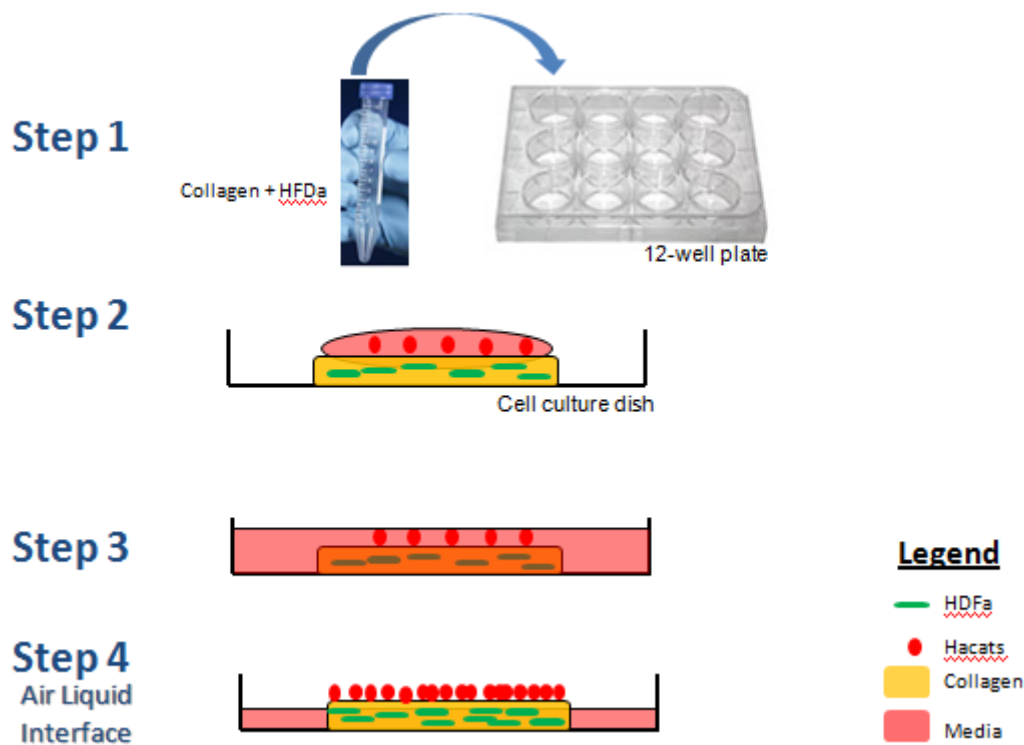
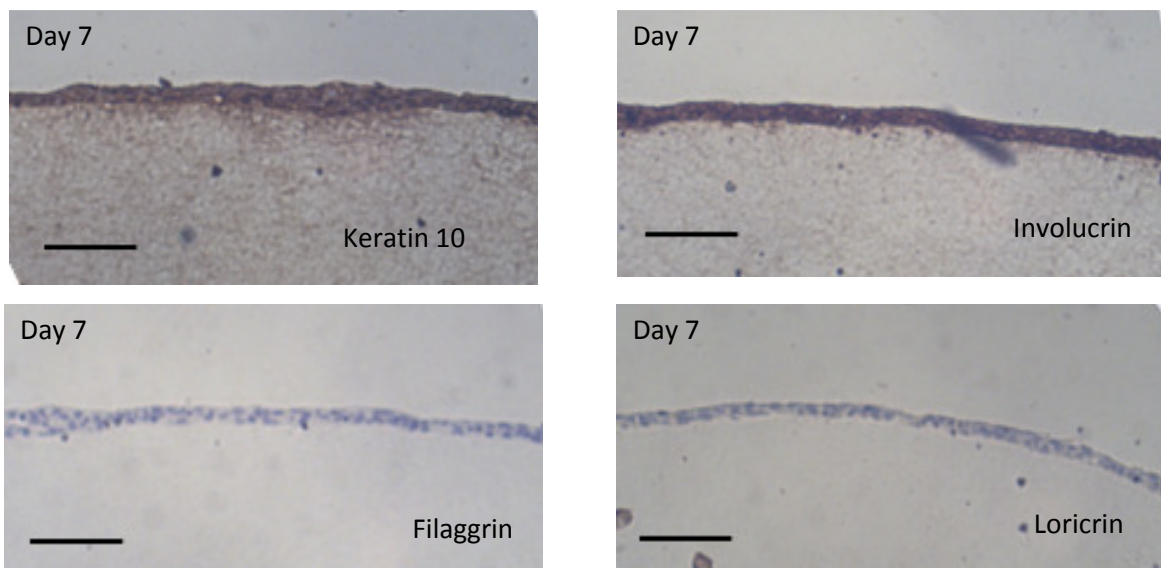


Figure 6. Diagrammatic representation of the 'Disc Technique'. (Step 1) Adult human dermal fibroblasts (HDFa) suspended in 100% fetal bovine serum was mixed with collagen and left to set in standard 12-well cell culture plates. (Step 2) Upon gelling, each collagen structure was removed from the 12-well plate and placed in a 100x20mm standard cell culture dish. HaCaT cells were seeded on top of the gels. (Step 3) The discs were fully submerged in culture media until the keratinocytes achieved confluency. (Step 4) Upon confluency, the level of the culture media was reduced to slightly below the height of the collagen discs, creating an air-liquid interface. Skin models were maintained without further passaging for up to 21 days.

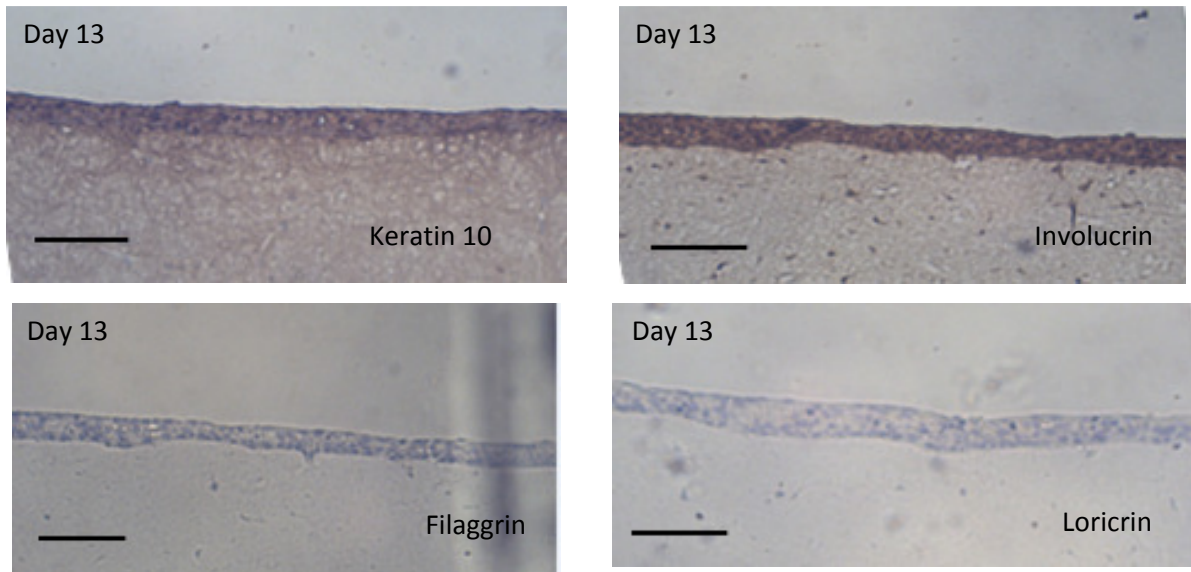
4.2.2.3 Method 3: The 'Ring Technique'

At Day 7, a uniformly thin layer (2 to 3 cells thick) of HaCaT cells was seen on the apical aspect of the collagen ring structure (n=1, Figure 7a). The thickness of the skin model appeared to be increased from Day 7 to Day 13 (n=1, Figure 7b) and minimally increased from Day 13 to Day 17 (n=1, Figure 7c). Compared to the Day 21 samples of the 'Disc Technique' (Figure 4c), no gross detachment of keratinocytes from the collagen structure was seen at Day 17 using the 'Ring Technique' (Figure 7c). Keratin 10 and involucrin were found from Day 7 onwards (Figure 7, brown staining). Under x10 magnification, no significant staining of filaggrin and loricrin was seen at Day 7 (Figure 7a). Weak, positive brown staining representative of these late markers of keratinocyte differentiation was seen at Day 13 under x10 magnification (Figure 7b). This was more apparent under x100 magnification (Figure 8b). At Day 17, filaggrin and loricrin were seen under x10 magnification (Figure 7c) and this was again more apparent under x100 magnification (Figure 8c).

(a)



(b)



(c)

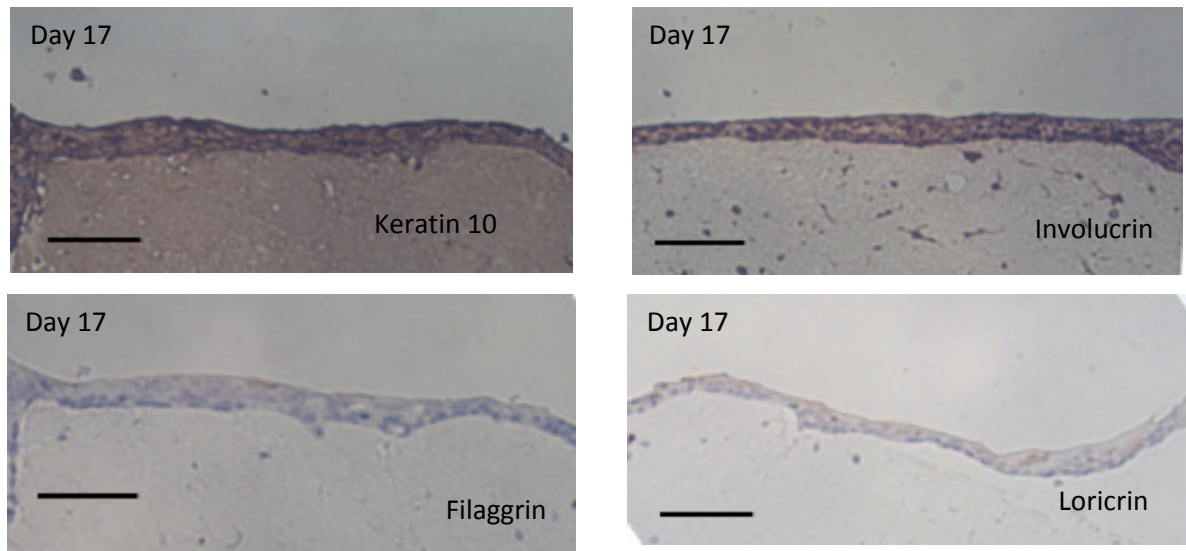
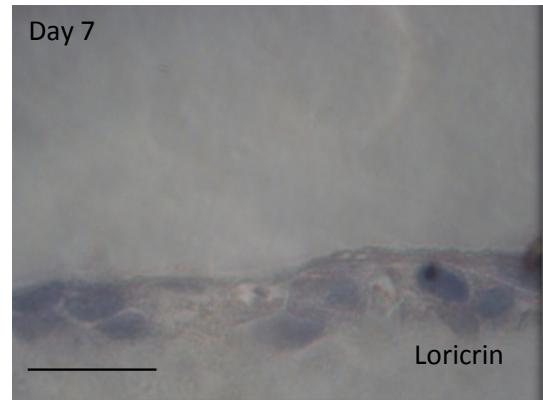
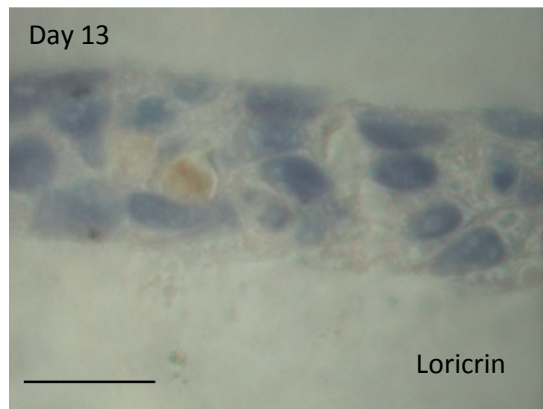
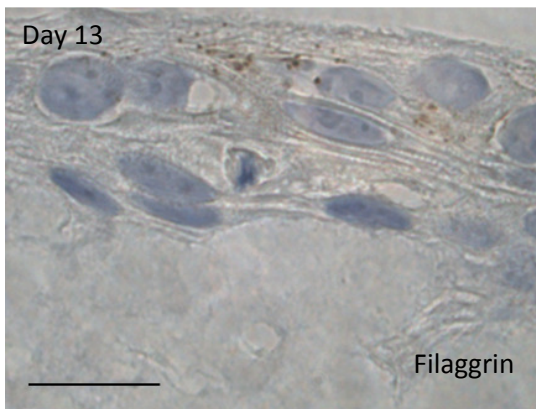


Figure 7. x10 magnification of transverse sections of skin-equivalent models (n=1 for Day 7, 13 and 17, the 'Ring Technique'). (a)Day 7: A thin layer of HaCaT cells was seen apical to the supporting collagen ring structure. HaCaT cells were positive for keratin 10 and involucrin (brown staining). No obvious staining for filaggrin and loricrin was seen. Nuclei of all cells were stained blue with haematoxylin. (b)Day 13: A thicker layer of HaCaT cells (as compared to that of Day 7) was seen apical to the collagen structure. Positive (brown) staining for keratin 10 and involucrin was seen. Weak positive staining for filaggrin and loricrin was seen. (c)Day 17: A layer of HaCaT cells (possibly slightly thicker than that of Day 13) was seen. Positive (brown) staining for keratin 10, involucrin, filaggrin and loricrin was seen. Scale bar=100 μ m

(a)



(b)



(c)

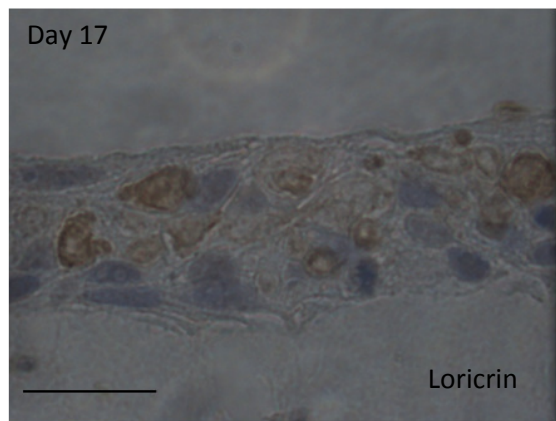
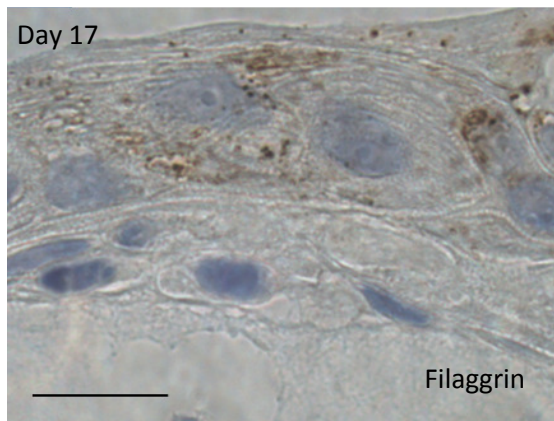


Figure 8. x100 magnification of transverse sections of skin-equivalent model (the 'Ring Technique') (a) Day 7: A thin layer of HaCaT cells (2-3 cells thick) was seen at Day 7. No obvious positive (brown) staining for filaggrin and loricrin was seen. (b) Day 13: A thicker layer of HaCaT cells (4-5 cells thick) was seen. Positive brown staining for filaggrin and loricrin was seen. (c) Day 17: Positive brown staining for filaggrin and loricrin was clearly visible at Day 17. Scale bar=25 μ m

Schematic representation of the 'Ring Technique' is shown in Figure 9. For detailed description of the method, see Materials & Methods.

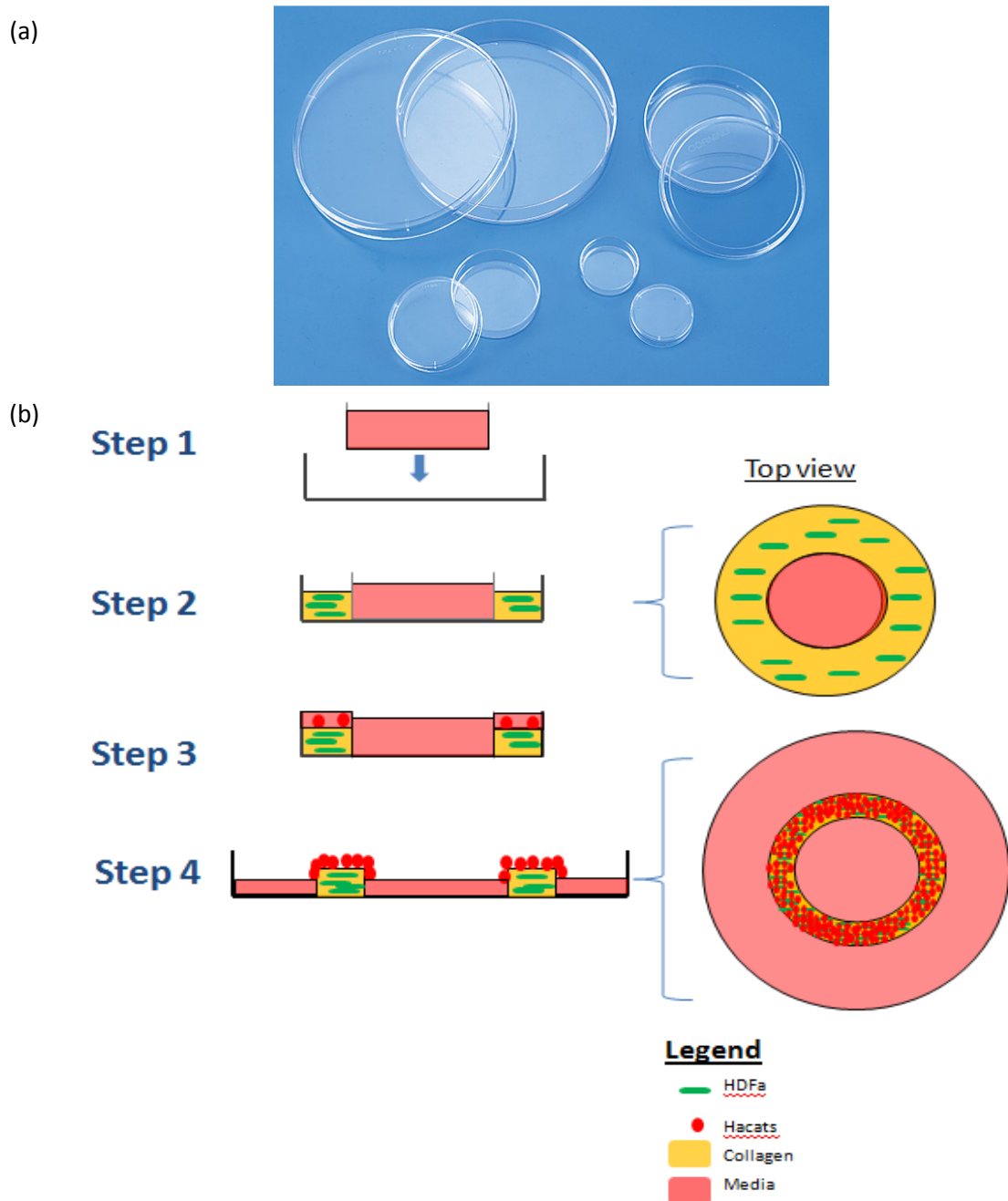


Figure 9. The 'Ring Technique' involved the usage of (a) cell culture dishes of varying sizes to create the collagen ring structures. (b) (Step 1) A 35x10mm cell culture dish was inserted into a 60x15mm cell culture dish. (Step 2) HDFa suspended in fetal bovine serum was mixed with collagen and left to set in the space between the two cell culture dishes. (Step 3) Upon gelling, HaCaTs were seeded on the apical aspect of the gel. (Step 4) Upon confluency of the HaCaT cells, the collagen ring was placed in a 100x20mm cell culture dish. The level of the culture media was adjusted to slightly below the height of the collagen structure, creating an air-liquid interface. Skin models were maintained without further passaging for up to a total of 17 days.

4.2.2.4 Method 4: The 'Grid Technique'

At Day 12, a layer of HaCaT cells (3-4 cells thick) was seen. A thicker layer (5-6 cells thick) of HaCaT cells was seen at Day 19. HaCaT cells at the basal layer (Figure 10, labelled B) appeared to be roundish in shape while those at the apical layer of the skin model (Figure 10, labelled T) appeared to be slightly flattened. At Day 26, disintegration of the skin model, characterised by detachment of the cells from one another and from the collagen structure, was seen (Figure 10).

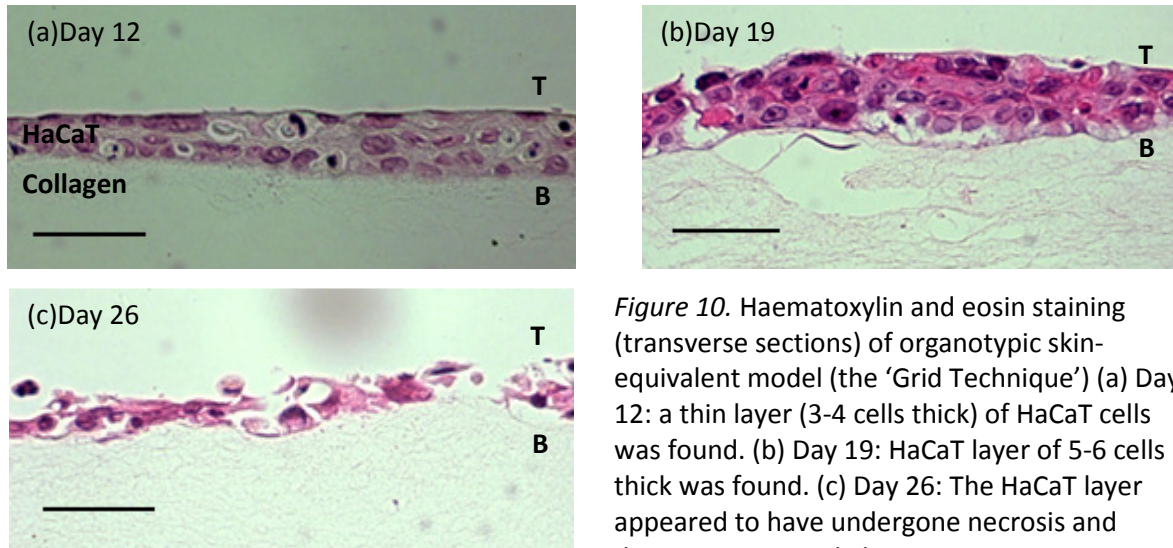


Figure 10. Haematoxylin and eosin staining (transverse sections) of organotypic skin-equivalent model (the 'Grid Technique') (a) Day 12: a thin layer (3-4 cells thick) of HaCaT cells was found. (b) Day 19: HaCaT layer of 5-6 cells thick was found. (c) Day 26: The HaCaT layer appeared to have undergone necrosis and disintegration. Scale bar= 50µm

*T=apical surface; B=basal surface

At Day 12, early markers of keratinocyte differentiation (keratin 10 and involucrin) were found (Figure 11, brown staining). Keratin 10, usually found at the outer, highly differentiated layers of the stratum corneum in human epidermis (Figure 1), was seen suprabasally in the skin-equivalent model. No obvious filaggrin and loricrin (late markers of keratinocyte differentiation) staining was seen on Day 12 (Figure 11).

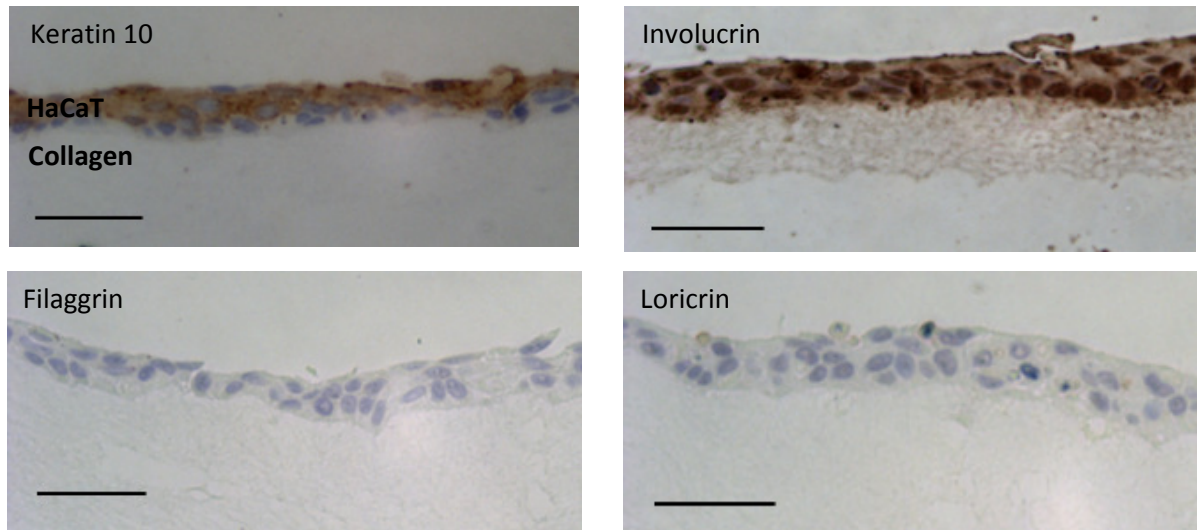


Figure 11. Keratinocyte differentiation markers at Day 12: Skin-equivalent model (3-4 cells thick) was positive for keratin 10 and involucrin (brown staining). Keratin 10 was found at the suprabasal layers. No obvious staining for filaggrin and loricrin was seen. Scale bar=50 micron

At Day 19, intense staining for keratin 10 and involucrin was seen (Figure 12, brown staining). As with Day 12, keratin 10 was found at the suprabasal layers of the model. Positive (brown) staining for filaggrin and loricrin was seen at the suprabasal layers on Day 19. The basal cells were larger in size and roundish in shape, as compared to those at the suprabasal layers which appeared flattened (Figure 12). This bears remarkable similarity to a native human epidermis (Figure 1).

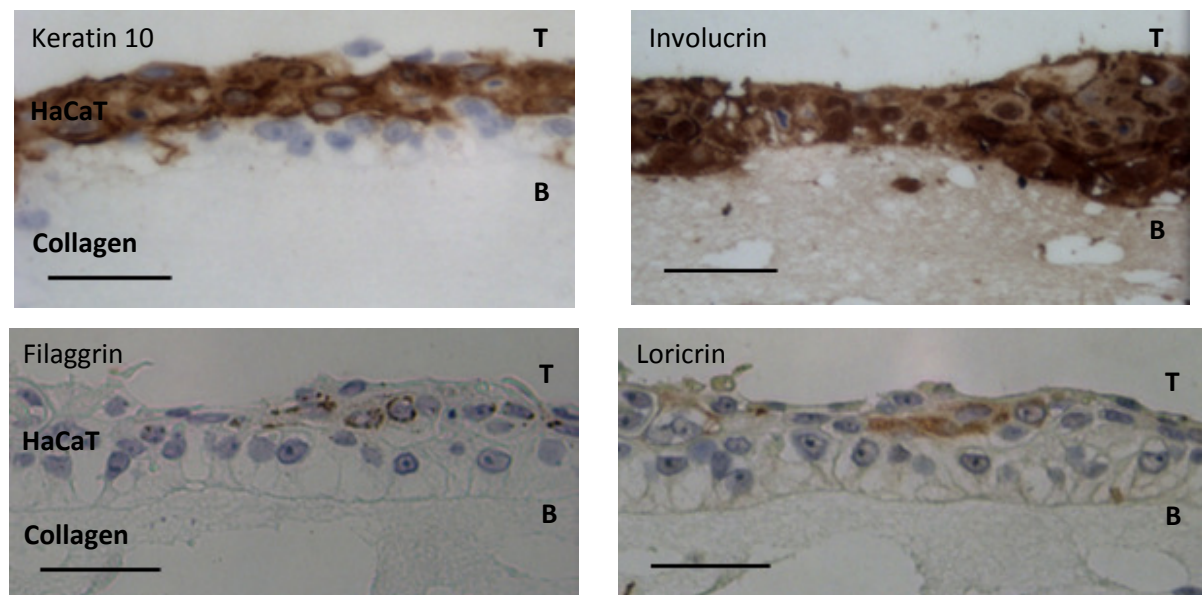


Figure 12. Keratinocyte differentiation markers at Day 19: Skin-equivalent model (5-6 cells thick) was strongly positive for keratin 10 and involucrin (brown staining). Keratin 10 was seen at the suprabasal layers. Positive staining for filaggrin and loricrin was seen suprabasally. HaCaT cells appeared to be larger and roundish in shape in the basal layer (B) and appeared flattened at the top layer of the skin-equivalent model (T). Scale bar=50 micron

At Day 26, the HaCaT layer appeared to be grossly abnormal (Figure 13). The HaCaT layer was thin (1-3 cells thick), with poor cell-cell and cell-matrix (collagen) adhesion. Discontinuity and gaps were seen within the HaCaT layer (Figure 13, black arrow). Vacuolization was observed in numerous HaCaT cells (Figure 13, red arrow).

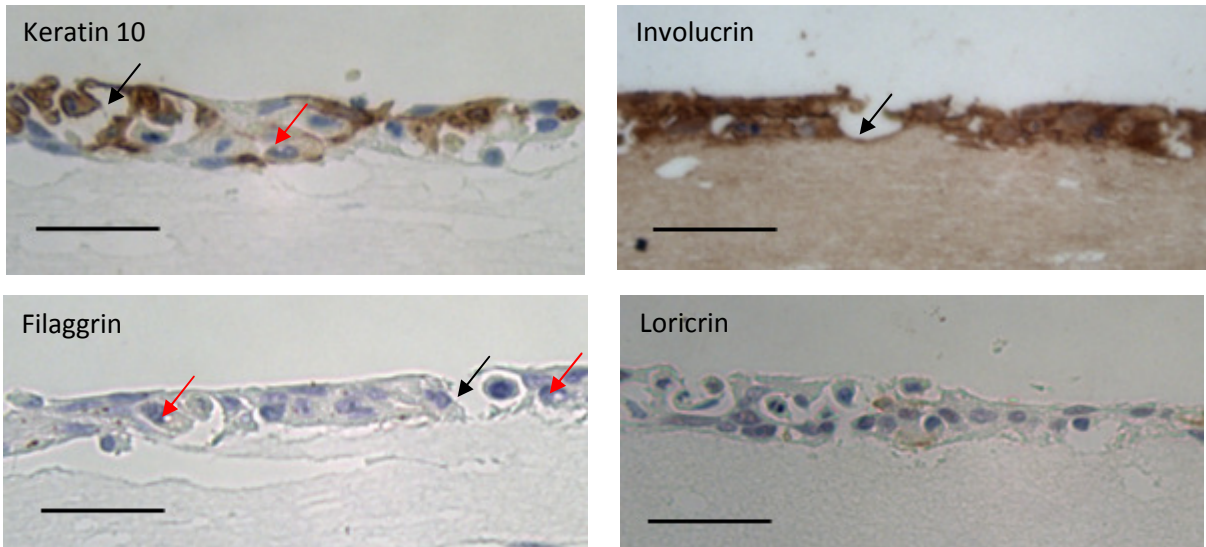


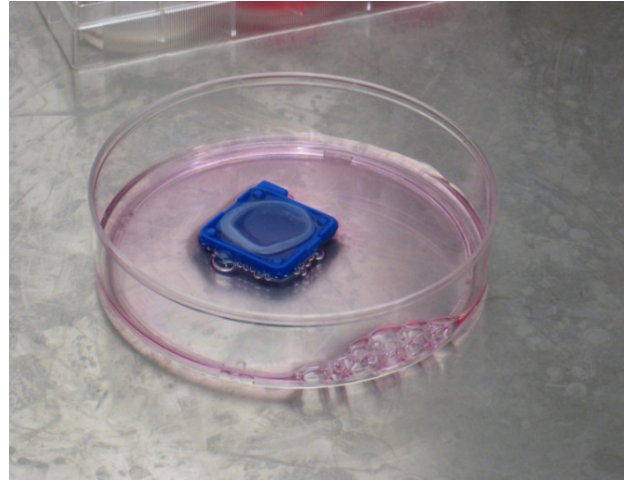
Figure 13. Keratinocyte differentiation markers at Day 26: Skin-equivalent model appeared grossly abnormal. Discontinuity and gaps were seen within the thin HaCaT layer (black arrow) and vacuolization (red arrow) was observed in many HaCaT cells. Scale bar=50 micron

Schematic representation of the 'Grid Technique' is shown in (Figure 14). For detailed description of the method, see Materials & Methods.

(a)



(b)



(c)

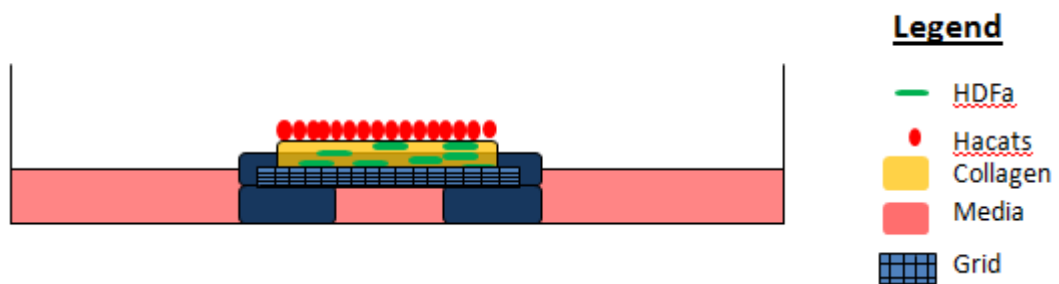


Figure 14. The 'Grid Technique' (a) Plastic grids used in the 'Grid Technique' (b) Photograph showing an organotypic skin-equivalent model on a plastic grid (c) Schematic representation of the skin-equivalent. HDFa was mixed with rat tail collagen and left to set in a 6-well cell culture plate. Upon gelling, HaCaT cells were seeded on the apical aspect of the collagen gel. The collagen structure was left to undergo natural shrinkage for 1-2 days. The shrunken structure was placed on the top of the grid and maintained for up to 26 days without further passaging.

4.2.3 Assessment of the barrier function of skin-equivalent model (the 'Grid Technique') using Lucifer Yellow dye penetration assay

Barrier function of two Day 19 skin-equivalent models established using the 'Grid Technique' was assessed using the Lucifer Yellow dye penetration assay (Figure 15).

After 5 minutes, minimal penetration of 5mM Lucifer Yellow fluorescent dye into the skin-equivalent model was seen (n=1, Figure 16a). After 80 minutes, the dye has penetrated through almost the entire thickness of the skin-equivalent model (n=1, Figure 16b).

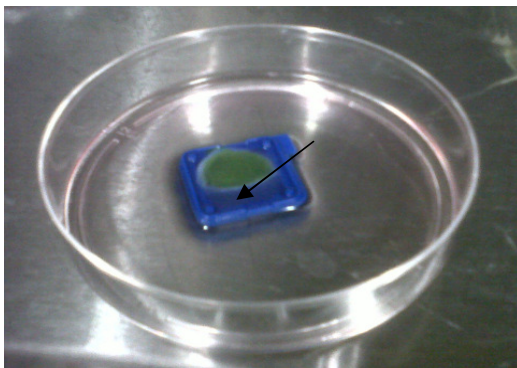


Figure 15. Lucifer Yellow dye penetration assay (photograph). 50 μ L of 5mM Lucifer Yellow CH dipotassium dye (black arrow) applied onto the 'well' structure of the organotypic skin-equivalent model grown on a plastic grid.

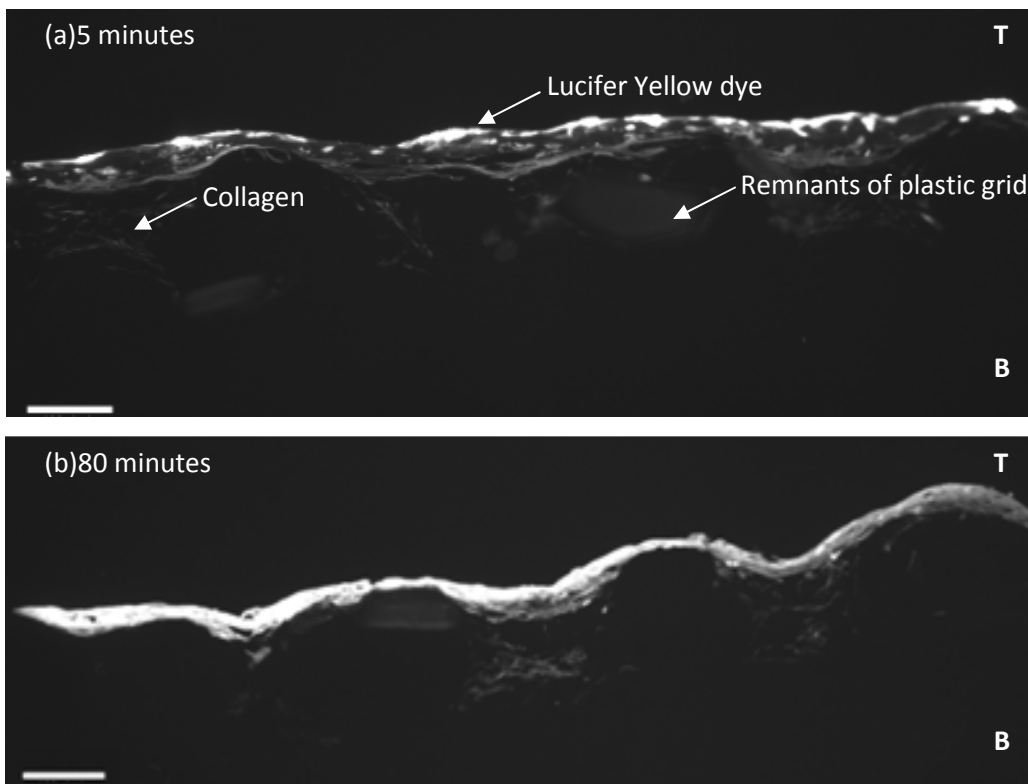


Figure 16. Lucifer Yellow dye penetration assay. Dye penetration after (a) 5 minutes was minimal, as compared to that after (b) 80 minutes, at which the dye has almost completely penetrated the entire thickness of the skin-equivalent model. Scale bar= 100 μ m (T=Top; B=Bottom, of the skin model).

4.2.4 Organotypic skin-equivalent model (the 'Grid Technique') in comparison to human epidermis

Comparisons between the Day 19 organotypic skin-equivalent model established using the 'Grid Technique' and the native human epidermis are given in Table 1.

Table 1

Comparisons between Skin Model (the 'Grid Technique') and Native Human Epidermis

	Native human epidermis¹⁹	Skin-equivalent Model
Thickness	Varies from 0.1mm to 1mm	5-6 cells in thickness
Layers (from basal to outer layers)	Basal layer (consisting of a single layer of columnar cells), prickle, granular and horny (flattened, anucleated squames) layers	Larger, roundish cells at the basal layer; flattened cells at the outermost layer.
Epidermal transit time (from basal layer to the surface)	30-60 days	Optimal : 19 days
Distribution of differentiation markers		
• Keratin 10	Suprabasal ^{20,21}	Suprabasal
• Involucrin	Suprabasal	Widespread
• Filaggrin	Granular and horny layer	Suprabasal
• Loricrin	Granular and horny layer	Suprabasal

4.3 Chapter 4 Discussion

Schoop *et al*¹ has shown that HaCaT cells can be used instead of primary keratinocytes for establishing organotypic skin-equivalent models. Organotypic co-cultures by Schoop *et al*¹, however, exhibited some deviations from native human epidermis, particularly at the ultrastructural level. These include a reduction in the amount of keratohyalin granules, incompletely degraded organelles and the presence of lipid droplets in the stratum corneum which indicates dysfunctional lipid metabolism. Despite these subtle abnormalities, organotypic co-cultures using HaCaT cells provide a valuable functional model for understanding the molecular mechanisms regulating keratinocyte differentiation, epidermal barrier formation and the effects of pharmacotherapy on epidermal structure and function.

By understanding the basic principles of establishing an organotypic skin-equivalent model and adapting the techniques of Schoop *et al*¹, we have constructed our own skin model using available resources in our laboratory, an effort which took 6 months. We started with Method 1 (the 'Transwell Technique'), using both calcium-depleted and calcium-enriched enriched cell culture media. In the absence of calcium, the keratinocytes remained as a monolayer. In the presence of calcium, HaCaTs formed islets of differentiation upon achieving confluency. Even though a low extracellular calcium environment is said to be associated with higher proliferative activity, it has been shown that after approximately 10 passages, proliferation of HaCaT cells becomes independent of extracellular calcium concentration and temperature¹³. Our HaCaT keratinocytes (passages 35-41) proliferated well even in the presence of calcium. We therefore chose to use non-calcium depleted media. The main issue with the 'Transwell Technique' was that the differentiation process failed to occur uniformly across the entire layer(s) of skin model. This model consisted of a largely undifferentiated monolayer of keratinocytes with isolated foci of differentiation, which is inadequate for assessment of barrier function as it deviates significantly from native human epidermis. The reason for this is unclear but it may be due to the transwell PTFE-membrane not being a suitable dermal-equivalent matrix for the keratinocytes.

The transwell was replaced by a self-constructed collagen disc structure in Method 2 (the 'Disc Technique'). Using this technique, the skin model displayed a more uniformly differentiated keratinocyte layer. However, the thickness of the HaCaT layer was noted to be greater at the peripheral edges of the collagen disc. This may be due to a better contact between HaCaT cells with the culture media at the edges. There was, therefore, a need to improve the contact between HaCaT keratinocytes in the inner aspect of the collagen disc with the culture media. In addition, detachment of HaCaT keratinocytes from the collagen disc at Day 21 was seen. This indicates that

prolonged culture period, though may be associated with improved keratinocyte differentiation, may also be associated with cellular distress.

In our third attempt, we constructed a ring structure instead of a disc to increase the surface area that was in contact with the media. Although the expression of filaggrin and loricrin appeared to have increased using this technique, the thickness of the HaCaT cell layer, was again greater at the peripheries of the collagen structure. Further improvement of the contact between HaCaT cells in the inner aspect of the collagen matrix with the culture media was needed. Here, the total length of the culture period was reduced from 21 days to 17 days. This may have led to the reduction in the detachment of HaCaT cells from the collagen matrix seen on Day 17, as compared to the 21-day model of Method 2. Prolonged keratinocyte culture may, therefore, be associated with cellular distress and death. This risk has to be balanced with the need to maximise the culture period. The latter is important in encouraging stratification of the 'epithelium'.

In subsequent organotypic cultures (Method 4: the 'Grid Technique'), the length of the culture period was set at 12, 19 and 26 days. We attempted to improve the contact between the HaCaTs and culture media by further reducing the concentration of the collagen used and suspending the organotypic skin-equivalent model on a grid. By reducing the collagen concentration, we produced a structure that was more readily yielding which would be expected to facilitate easier penetration of the culture media. Using the 'Grid Technique', the basal cells were found to appear larger in size and roundish in shape while those at the apical aspect of the skin model appeared flattened. This is not dissimilar to the native human epidermis. Involucrin was found to be widely expressed throughout the entire HaCaT layer of the skin model and keratin 10 was expressed suprabasally. These findings were again similar to the human samples obtained from suction-blistering, suggesting that this model bears resemblance to a native human epidermis. The expression of filaggrin and loricrin were, however, less intense than that observed in our human samples. This may be due to the insufficient length of our culture period which was limited by apoptosis or necrosis of the skin model, as seen on Day 26. Although it was not histochemically demonstrated, it is likely that many of these cells were undergoing the process of apoptosis or necrosis, as suggested by the grossly abnormal appearance of the skin model at Day 26.

We therefore propose that amongst our 4 different methods, the 'Grid Technique' is the best. It is an extremely cost-efficient method which can be used to set up a skin-equivalent model in any laboratory with basic tissue-culturing facilities. Both HDFa and HaCaT keratinocytes should be seeded at a concentration of 0.5×10^6 cells/ml. The concentration of rat tail collagen used should not be more than 2.9mg/ml. The D-MEM/F-12 with GlutaMax culture media + 10% fetal bovine serum +

1% penicillin/streptomycin (pH 7.4) is a suitable culture media for the skin model. The total culture period should not be longer than 19 days.

One of the main functions of the skin is to act as a physical and chemical barrier which keeps the outside world out and the inside world in. Barrier function of our models was assessed through the ability of Lucifer Yellow CH fluorescent dye^{22,23} to penetrate and bind to the skin-equivalents. At the end of the study, samples were washed twice in phosphate-buffered saline to remove any excess dye which has not bound to the skin-equivalents. Lucifer Yellow ($C_{13}H_9K_2N_5O_9S_2$; Mr 521.57) is a water-soluble fluorescent disulfonic acid anionic dye, frequently used to study neuronal morphology. The carbonylhydrazone (CH) group enables it to covalently bind to surrounding biomolecules. Lucifer Yellow has also been used to assess the permeability barrier of mice²⁴⁻²⁷ and skin-equivalent models²⁸. It is, however, less frequently used (its validity is therefore questionable) in studies involving human epidermis. Lucifer Yellow has been shown to penetrate the human stratum corneum poorly²⁹. When injected into the epidermis, the transmission of the dye was limited to a small number of neighbouring cells adjacent to the injection site³⁰. The dye has been suggested to pass intercellularly rather than through the keratinocytes themselves^{29,30}. Our Day 19-organotypic skin model constructed under the conditions mentioned above exhibited an ability to function as a reasonable physical barrier, evident by the poor penetration of dye into the skin model at 5 minutes.

In conclusion, we have demonstrated that a functional, in-house skin-equivalent model can be easily constructed by following several basic principles. This involves culturing keratinocytes at an air-liquid interface on a scaffold which serves as a dermal equivalent, such as a de-epidermized and devitalised dermis or in our case, fibroblast-populated collagen matrix. Having established a functional skin-equivalent model, we can now investigate the effects of pharmacotherapy on the 'epidermal' structure and barrier function, represented by the degree of Lucifer Yellow dye penetration through the skin model.

4.4 Chapter 4 References

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4.5 Chapter 4 Materials and Methods

4.5.1 Development of suction-blisters

As described in Chapter 3 Materials and Methods.

4.5.2 Organotypic skin-equivalent models

4.5.2.1 Method 1: The 'Transwell Technique'

The 'Transwell Technique' is an adaptation of that employed by Dr C. Wright, Department of Biological and Biomedical Sciences, Glasgow Caledonian University, based on mouse organotypic models previously developed by that department^{31,32}. Primary human dermal fibroblasts, HDF, of passages 3-5 (harvested and maintained according to previously published technique³¹) were seeded at a concentration of 0.5×10^6 cells/ml onto the underside of a 12mm collagen-coated PTFE membrane (pore size= 0.4 μm) of a Transwell culture plate (Sigma, Dorset, UK). The porous membrane allows nutrients and fibroblast growth factors to reach the keratinocytes. Fibroblasts were left to adhere for 12 hours surrounded by sterile water to prevent the culture media containing the fibroblasts from drying up. To form the organotypic epidermis, HaCaT keratinocytes (gift from Dr J. Wood, Dundee) were seeded at 1×10^6 cells/ml directly into the upper Transwell chamber. Epilife media (Cascade Biologicals, Invitrogen, Paisley, U.K.) with 10% fetal bovine serum and 1% penicillin/streptomycin was placed in both upper and lower chambers and changed daily until a confluent monolayer of HaCaT cells was obtained (usually occurring after 24 hours). When HaCaT keratinocytes are fully confluent, the culture media was removed from the upper chamber, exposing the HaCaT cells to air in order to facilitate stratification of the keratinocytes. Culture media in the lower chamber was changed every 2 days for a total of 14 days

4.5.2.2 Method 2: The 'Disc Technique'

Adult human dermal fibroblasts, HDFa (Invitrogen, Paisley, UK) of passages 3-5, suspended in 100% fetal bovine serum, were mixed with Collagen I, Rat Tail (Gibco, Paisley, UK) and left to set in a standard 12-well cell culture plate. The collagen was made up as per manufacturer's guidelines. The final concentrations of HDFa and collagen in the mixture were 0.5×10^6 cells/ml and 3.3mg/ml respectively. Upon gelling (approximately 20-30 minutes), the collagen discs were removed and placed in individual 100x20mm cell culture dishes (Corning, Poole, UK). HaCaT cells of passages 35-41 suspended in standard cell culture media (D-MEM/F-12 with GlutaMAX [Gibco, Paisley, UK] plus 10% fetal bovine serum and 1% penicillin/streptomycin) were seeded at a concentration of 0.5×10^6 cells/ml on the top of each disc, and left to adhere overnight at 37.5°C. Following 18 hours of incubation, the discs were fully submerged in culture media until the HaCaT cells formed a confluent

monolayer. Upon confluency (approximately 48-72 hours), the level of the culture media was reduced to slightly below the height of the collagen discs. In doing so, the keratinocytes were exposed to air while remaining in constant contact with the media nutrients which diffused through the collagen structure. Skin models were maintained without further passaging for up to 21 days, with the culture media changed every 2-3 days, to facilitate stratification of the keratinocytes. The skin models were washed twice with PBS, fixed in formalin solution, neutral buffered, 10% (Sigma, Dorset, UK) and embedded in paraffin.

4.5.2.3 Method 3: The 'Ring Technique'

This is an adaptation of the 'Disc Technique'. Instead of a collagen disc, a ring was created to maximise the contact between HaCaT cells and the culture media. Cell culture dishes of various sizes (Corning, Poole, UK) were used in the creation of this organotypic model. A 35x10mm cell culture dish filled with culture media (or sterile water) was inserted into a 60x15mm cell culture dish. The culture media (or sterile water) in the smaller cell culture dish functioned as a weight. As in the 'Disc Technique', HDFa were suspended in 100% fetal bovine serum (final concentration of 0.5×10^6 cells/ml) and mixed with Collagen I, Rat Tail (final concentration of 3.3mg/ml) and left to set in the space between the two cell culture dishes. Upon gelling (usually within 20-30 minutes), HaCaT cells suspended in D-MEM/F-12 with GlutaMax culture media (Gibco, Paisley, UK) plus 10% fetal bovine serum and 1% penicillin/streptomycin were seeded at a concentration of 0.5×10^6 cells/ml on the top of the gels. Upon confluency of the HaCaT cells (usually between 48-72 hours), the collagen ring was gently lifted off and placed in a 100x20mm cell culture dish. The level of the culture media (within and outside of the ring) was adjusted to slightly below the height of the collagen structure, creating an air-liquid interface. The skin model was maintained without further passaging for up to a total of 17 days with the culture media changed every 2-3 days.

4.5.2.4 Method 4: The 'Grid Technique'

The plastic grids used in this technique are available in most histology departments. They are often used to hold small samples intended for paraffin embedding.

Adult human dermal fibroblasts, HDFa, (Invitrogen, Paisley, UK) of passages 3-5, suspended in 100% fetal bovine serum, were mixed with Collagen I, Rat Tail (Gibco, Paisley, UK) and left to set in a standard 6-well cell culture plate. The collagen was made up as per manufacturer's guidelines. The final concentrations of HDFa and collagen in the mixture were 0.5×10^6 cells/ml and 2.9mg/ml respectively. Upon gelling (usually within 20-30 minutes), the gels were washed twice with PBS. 2 ml of HaCaT cells suspended in D-MEM/F-12 with GlutaMax culture media (Gibco, Paisley, UK) plus 10% fetal bovine serum and 1% penicillin/streptomycin were seeded at 0.5×10^6 cells/ml on top of the gels. An additional 1 ml of culture media was added to each of the 6 wells one hour later. The collagen

structures, fully submerged in culture media, were left to undergo natural shrinkage for 24-48 hours during which HaCaT cells continued to proliferate and achieved confluency (usually occurring within 48-72 hours).

The shrunken collagen structures formed a 'well' with rolled-up edges, partly due to the reduced concentration of collagen used which made the structures more yielding. Each skin-equivalent model was placed on the top of a grid and maintained for up to 26 days. During this period, cell culture media was changed every 2-3 days and adjusted to reach the bottom of the grid.

4.5.3 Lucifer Yellow penetration assay

On Day 19, 50 μ L of 5mM Lucifer Yellow CH dipotassium salt (Sigma, Dorset, UK) was applied onto the apical surface (HaCaT side) of two organotypic skin-equivalent models established using Method 4 for 5 minutes and 80 minutes respectively. In the latter, 50 μ L of 5mM Lucifer Yellow was re-applied every 5 minutes. Following that, the samples were washed twice in PBS, fixed in formalin solution, neutral buffered, 10% (Sigma, Dorset, UK) and embedded in paraffin. Dewaxed and hydrated 3- μ m transverse sections were visualised using fluorescence at 455-495/505-555 nm.

4.5.4 Immunohistochemistry.

Peroxidase-based EnVision™+ kits (Dako, Cambridgeshire, UK) for the following mouse and rabbit primary antibodies were used on dewaxed and hydrated 3- μ m formalin-fixed, paraffin-embedded transverse sections from the organotypics, as per manufacturer guidelines: (pro)filaggrin (mouse monoclonal; Novocastra, Newcastle, UK), involucrin (mouse monoclonal; Sigma, Dorset, UK), loricrin (rabbit polyclonal; Sigma, Dorset, UK) and keratin 10 (rabbit monoclonal; Abcam, United States). Samples were visualised using conventional light microscope.

Chapter 5

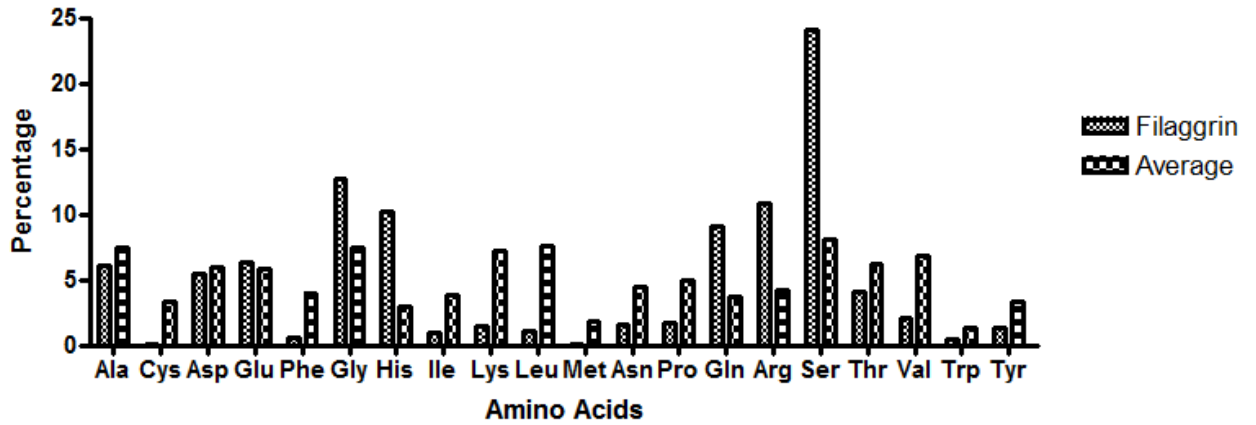
Improving the Barrier Property of Organotypic Skin-equivalent Model

The disproportionate abundance of histidine in the filaggrin protein, deficiency of which is associated with barrier dysfunction and atopic dermatitis, is surprising and indicates that this amino acid may have a particular significance in maintaining the epidermal barrier function. Using our in-house organotypic skin-equivalent model, we investigated the effects of raised extracellular, free amino acid histidine and its metabolites on the barrier function of this 'artificial' skin. The effects of manipulating the Th1-Th2 cytokine balance and varying the pH of the culture media using various buffers were also investigated.

5.1 Introduction: It is not all about keratin aggregation.

Though keratin aggregation is generally thought to be the primary function of filaggrin, it has been shown that this process can occur in the absence of filaggrin¹. In addition, the absence of filaggrin does not render keratins more susceptible to extraction by urea². These evidences argue against the importance of filaggrin in keratin aggregation. Filaggrin is the main source of hygroscopic amino acids and amino acid derivatives which constitute the natural moisturizing factor (NMF) found in the stratum corneum. Those with a reduced expression of filaggrin protein were, perhaps not surprisingly, found to have a reduced amount of NMF³, which are important in reducing transepidermal water loss and maintaining stratum corneum hydration. It is therefore possible that the major function of filaggrin may not be that of filament aggregation but in providing the matrix substance of the epidermis, i.e. forming the NMF following enzyme-catalysed breakdown. An interesting observation in favour of this argument is the rising incidence of AD after World War 2 in countries undergoing rapid economic changes⁴. Environmental factors such as increased usage of harsh cleaning products (soaps and shampoos) which can solubilise and remove the NMF are more likely to explain the phenomenon than defective keratin aggregation due to genetic mutations. Genetic changes are unlikely to have taken place over a period of several decades and be solely responsible for the rise in the incidence of AD. This is particularly true in places that have not seen significant population migration which may be associated with significant changes in the gene pool. Environmental factors may, however, drive the manifestations of the disease in genetically predisposed individuals.

Our examination of the amino acid composition of filaggrin revealed unusually high levels of histidine and serine, in addition to a low level of lysine (Figure 1), as compared to the average amino acid composition of a protein⁵. The latter is calculated based on the frequency of amino acids among 5492 residues in 53 vertebrate polypeptides, such as insulin, cytochrome c, haemoglobin and immunoglobulin light chain⁵.



Amino Acid	Percentage in Filaggrin (%)	Percentage in an average protein (%) ⁵
Ala	6.1	7.4
Cys	0.0	3.3
Asp	5.5	5.9
Glu	6.3	5.8
Phe	0.6	4.0
Gly	12.8	7.4
His	10.2	2.9
Ile	0.9	3.8
Lys	1.4	7.2
Leu	1.1	7.6
Met	0.1	1.8
Asn	1.5	4.4
Pro	1.7	5.0
Gln	9.0	3.7
Arg	10.8	4.2
Ser	24.1	8.1
Thr	4.1	6.2
Val	2.0	6.8
Trp	0.5	1.3
Tyr	1.3	3.3

Figure 1. The amino acid composition of filaggrin vs the average composition in a protein⁵

Histidine ($C_6H_9N_3O_2$, Mr 155.15) is an amino acid and consists of a positively charged imidazole functional group (Figure 2a). The pKa of its imidazole sidechain is 5.97 and the overall pKa of histidine is approximately 6.5. Its solubility in water is 4.19g/100g at 25⁰C⁶. It is an essential amino acid in human⁷ and was first isolated by Albrecht Kossel, a German biochemist, in 1896 from protamines occurring in fish. Histidine exists in two forms of optical isomers, L-histidine and D-histidine. Natural proteins are made from the L-isomer. The major catabolism pathways of histidine are shown in Figure 2. The imidazole sidechain of histidine allow it to both accept and donate protons. The unprotonated form is nucleophilic and can act as a base while protonated form can act as a weak acid⁸. This allows histidine to participate in general acid-base catalysis. Histidine is therefore present in the active sites of many enzymes.

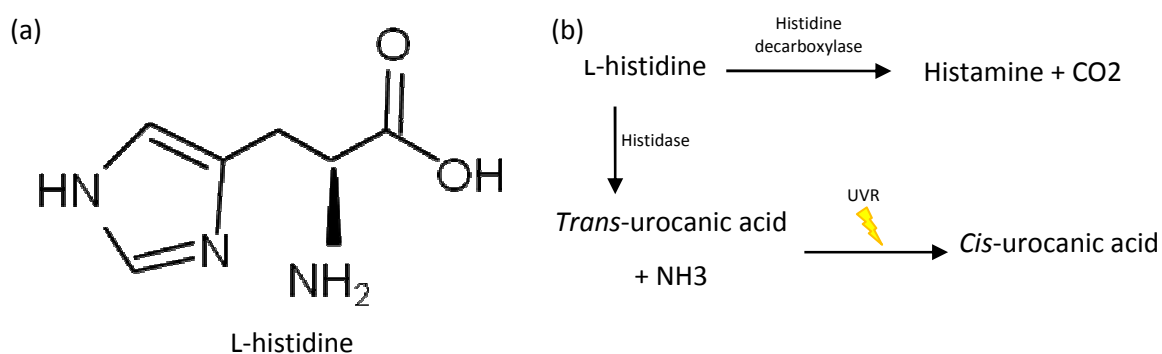


Figure 2. Histidine (a) The structure of L-histidine (b) Major pathways of histidine catabolism. Histidine is catabolised to histamine (by enzyme histidine decarboxylase) and *trans*-urocanic acid (by enzyme histidase/histidine ammonia lyase). *Trans*-urocanic acid is photoisomerized to the more biologically active *cis*-urocanic acid⁹ by ultraviolet radiation (270-320nm), indicated by UVR. (Diagram adapted from Taylor et al., 1991a¹⁰)

Histidine is converted into histamine by histidine decarboxylase^{11,12} while histidase (also known as histidine ammonia lyase) converts histidine into *trans*-urocanic acid (UCA)¹³ (Figure 2b). UCA was first isolated from a dog's urine in 1874 by Jaffé. Nonetheless, its presence in the epidermis was only confirmed in the 1950s. In the stratum corneum, histidase converts filaggrin-derived histidine into *trans*-UCA, which is later converted by UV-radiation to the more biologically active *cis*-UCA¹⁴. Deficiency of filaggrin is associated with reduced epidermal UCA; the normal levels of which range from 4 to 34 nM/cm² and is independent of age, sex, skin type and stratum corneum thickness¹⁵. Whether UCA is beneficial or detrimental to the skin remains an issue of dispute¹⁶. *Trans*-UCA is said to act as a natural sunscreen and protects against UV-induced thymine dimers formation¹⁷. Acidification of the intracellular environment caused by *cis*-UCA has been shown to inhibit the growth of melanoma and promoting tumour cell death¹⁸. On the other hand, *cis*-UCA is

said to be able to induce formation of intracellular reactive oxygen species and initiate the translation of genes associated with local and systemic immunosuppression^{19–22}. It is, therefore, not surprising that many believe *cis*-UCA plays a role in photocarcinogenesis, negating any beneficial photoprotective effect. Figure 3 described the putative ‘beneficial’ and ‘detrimental’ effects of UCA.

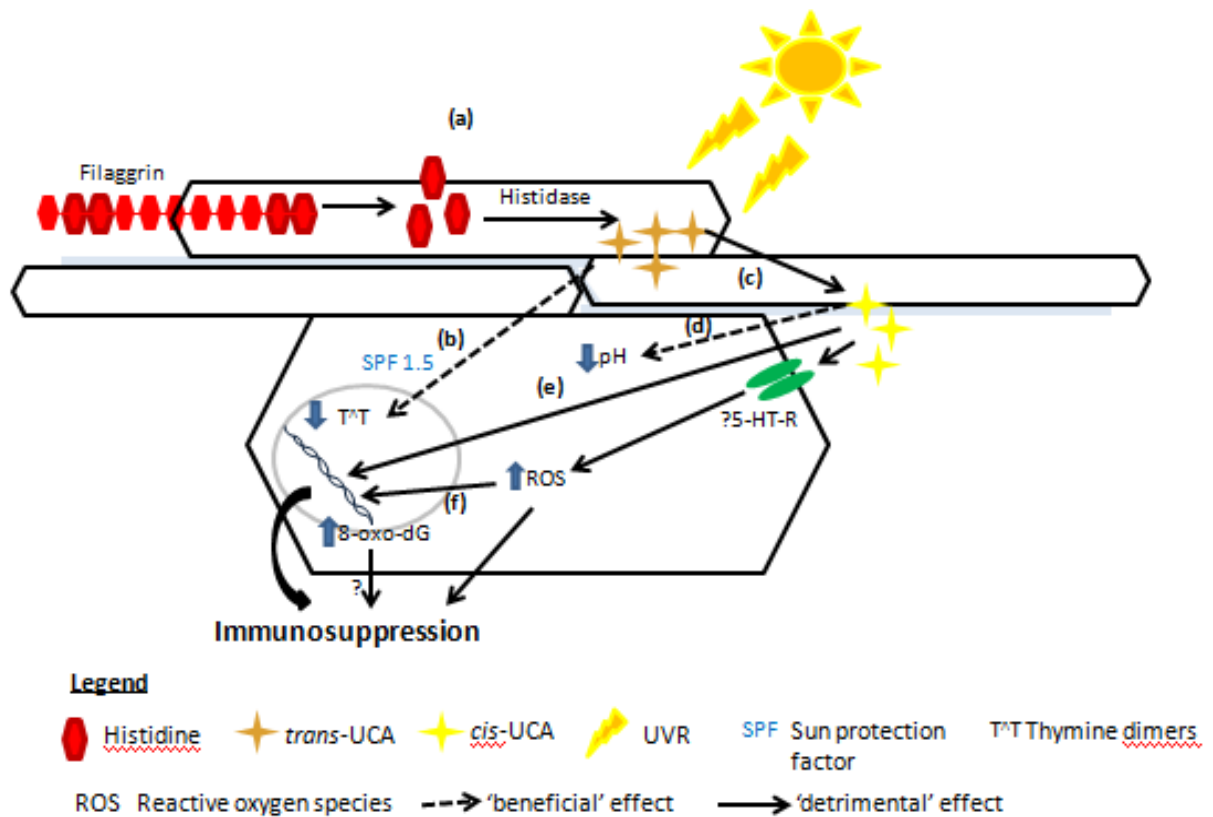


Figure 3. The putative ‘beneficial’ and ‘detrimental’ effects of urocanic acid, UCA in the skin. (a) Histidine within filaggrin is broken down by histidase into *trans*-UCA. (b) Exogenous *trans*-UCA acts as a natural sunscreen by absorbing UVR and protects against thymine dimers formation¹⁷. (c) *Trans*-UCA is photoisomerized to the more biologically active *cis*-urocanic acid⁹ by ultraviolet radiation (270–320nm), indicated by UVR. (d) In tumour cells, *cis*-UCA has been shown to facilitate proapoptotic intracellular acidification by transporting protons into the cells¹⁸. (e) Through direct signalling and ROS formation, *cis*-UCA triggers the translation of genes regulating immunosuppression. (f) *cis*-UCA, acting via membrane receptors, leads to the formation of ROS and hence oxidative DNA damage (raised 8-oxo-dG) (diagram adapted from Gibbs and Norval¹⁶).

Deficiency of histidase leads to histidinaemia¹⁰. This relatively common autosomal recessive disorder has an incidence similar to that of phenylketonuria (approximately 1: 11000 in England)²³. It is characterised by a decreased level of urocanic acid and raised levels of histidine in the body and histidine metabolites in the urine^{10,23,24}. Though early reports of histidinaemia documented increased incidence of speech delay and mental retardation, subsequent studies have shown that it is a benign metabolic disorder which does not require treatment^{10,23,24}. Histidinaemic mice have been shown to

have an increased sensitivity to UV-induced skin damage. In human, however, there is no conclusive evidence to suggest that histidinaemia leads to any significant dermatological condition.

The theory of evolution suggests that organisms arise and are propagated through selection of desirable characteristics and advantageous genes^{5,25}. Some molecular biologists and protein chemists went a step further by proposing the occurrence of natural selection at the molecular and genotypic level⁵. Smith states²⁶:

'One of the objectives of protein chemistry is to have a full and comprehensive understanding of all the possible roles that the 20 amino acids can play in function and conformation. Each of these amino acids must have a unique survival value in the phenotype of the organism—the phenotype being manifested in the structures of the proteins. This is as true for a single protein as for the whole organism.'

We hypothesized that the highly disproportionate abundance of histidine in the filaggrin protein, deficiency of which is associated with epidermal barrier dysfunction, indicates that this amino acid may have a particular importance in determining either the structure or function of filaggrin. It follows from our hypothesis that the hygroscopic histidine with its pKa of 6.5 may improve or maintain skin barrier by contributing towards the amount of the NMF in the skin or by affecting the expression of the filaggrin protein. In evolutionary terms, the abundance of histidine in filaggrin may be the result of a positive selection. On the other hand, filaggrin is a lysine-poor protein. Perhaps the high pKa (10.5) of this strongly basic amino acid renders it an unsuitable component of the NMF. The reduced amount of lysine in filaggrin may, therefore, be the result of a negative selection.

The normal skin pH ranges from 4-6 and is known to rise in diseased states, such as in atopic dermatitis²⁷. Maintaining the optimal skin pH is essential for lipid metabolism, epidermal barrier function, desquamation and antimicrobial activities²⁷. The skin pH is maintained by sodium-proton ion exchangers of the lamellar bodies, fatty acids found in sebum or sweats and breakdown products of phospholipids and filaggrin²⁷⁻²⁹. We therefore also investigated the effects of varying the pH of the cell culture media on barrier function of the skin-equivalent model. This is to (1) validate our skin model as an approximation to native human epidermis by demonstrating normal or improved barrier in an acidic environment and impaired barrier function in an alkaline environment (2) determine if any effects seen with histidine and/or its metabolites are pH-related observations.

Th-2 cytokines are known to induce atopic responses and AD skin is characterized by overexpression of IL4 and IL-13³⁰⁻³³. A previous study by Howell *et al* has shown that Th-2 cytokines may reduce the expression of filaggrin protein in patients with wild-type *FLG*³⁰, potentially affecting

the skin barrier integrity. We investigated the effects Th-1 and Th-2 cytokines on the barrier function of our skin-equivalent model and if 'neutralisation' of the Th-2 cytokines can improve the cytokine-induced barrier dysfunction.

The organotypic skin-equivalent models used here were established using the 'Grid Technique', as described in the previous chapter but with a lower collagen concentration of 2.5mg/ml (see Materials and Methods). The amino acids used for the study were L-histidine (and its metabolites histamine and *cis*-urocanic acids), D-histidine, L-lysine and L-serine. D-histidine is a biologically inactive isomer of L-histidine and was used as a negative chemical control. Our main objective was to demonstrate if the barrier function of our skin model can be improved through manipulation of the amino acid composition of the culture media and extracellular culture environment.

5.2 Chapter 5 Results

5.2.1 Effects of amino acids L-histidine, L-lysine and L-serine on Lucifer Yellow dye penetration into skin-equivalent models

One-way ANOVA analysis ($F(3,16)=14.13$) showed that an additional 5mM L-histidine in the cell culture media from day 13 to 19 led to a significant reduction in the penetration of Lucifer Yellow fluorescent dye into the skin models at Day 19, as compared to controls ($p<0.01$, $n=5$, Figure 4). No significant effect was found with 5mM L-lysine and L-serine (Figure 4).

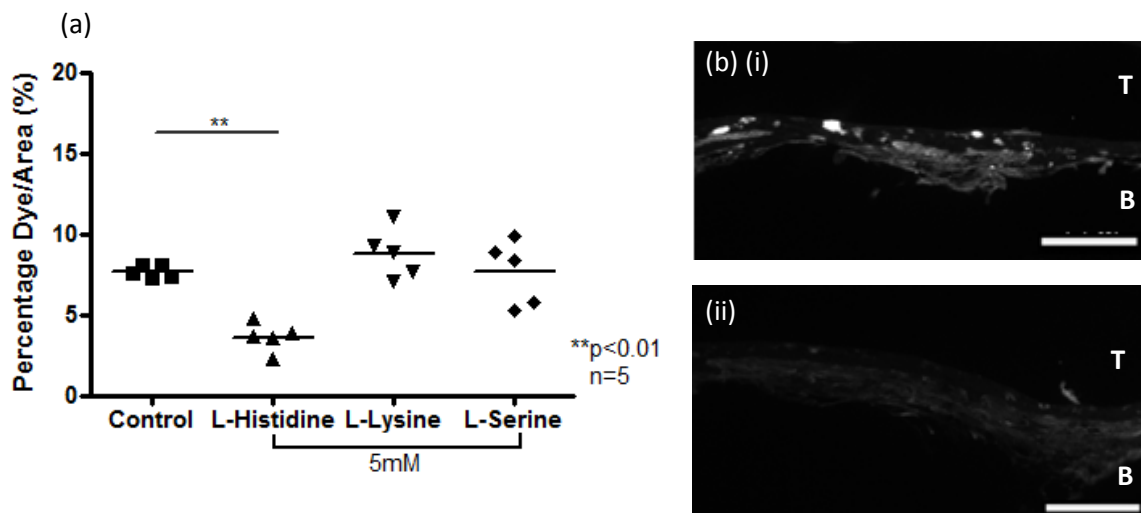


Figure 4. Comparison of the effects of free amino acids L-histidine, L-lysine and L-serine vs controls on penetration of Lucifer Yellow dye into the skin models. Vertical scatter plots showing the percentage of cross-sectional area of HaCaTs stained positive for dye per total area of skin model. One-way ANOVA was used. (a) 5 mM L-histidine led to significantly reduced penetration of the Lucifer Yellow dye ($p<0.01$). No significant effect was seen with L-lysine and L-serine. (b) Representative images of (i) control and (ii) L-histidine skin models. Lucifer Yellow dye is seen here as bright staining. Scale bar= 100 μ m. (T=Top; B=Bottom, of the skin model)

5.2.2 Effects of metabolites of histidine, D-histidine and cytokine modulation on Lucifer Yellow dye penetration into skin-equivalent models

One-way ANOVA analysis ($F(7,32)=12.06$) showed that 1 μ M histamine dihydrochloride, added to cultures from day 13 to 19, led to a reduced penetration of Lucifer Yellow dye into the skin models ($p<0.01$, $n=5$), even in the presence of H1+H2 receptor antagonists (10 μ M pyrilamine maleate and 10 μ M cimetidine) ($p<0.05$, $n=5$) [Figure 5].

100 μ g/ml *cis*-UCA ($n=5$) had no significant effect on the penetration of Lucifer Yellow dye into the skin-equivalent models (Figure 5).

5 mM D-histidine ($n=5$) had no significant affect on the penetration of Lucifer Yellow dye (Figure 5).

From day 13 to 19, 50ng/ml of IL-4 or 5ng/ml of IFN- γ was added to the culture media. Compared to controls, skin-equivalent models grown in the presence of 50ng/ml IL-4 showed increased Lucifer Yellow dye penetration ($p < 0.05$, $n = 5$) while 5ng/ml IFN- γ had no significant effect (Figure 5).

In the 'cytokine-neutralisation' study (Figure 5, labelled G), skin-equivalent models, following a 6-day treatment with 50ng/ml IL-4, were treated with 5ng/ml IFN- γ for a further 24 hours. A small but non-significant reduction in the dye penetration was seen ($M = 14.3$, $SD = 1.7\%$ vs $M = 15.1$, $SD = 2.4\%$, $n = 5$), as compared to treatment with IL-4 alone.

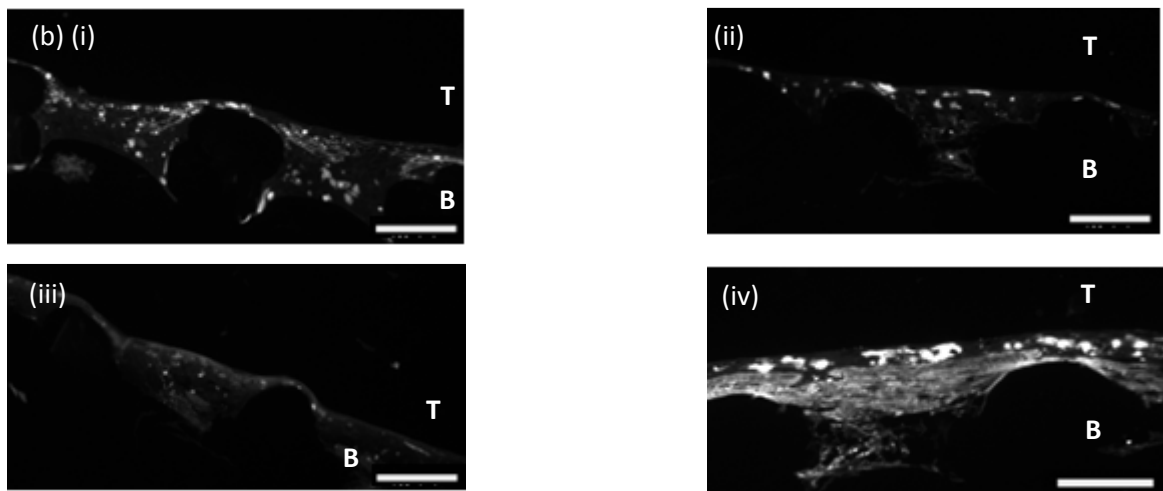
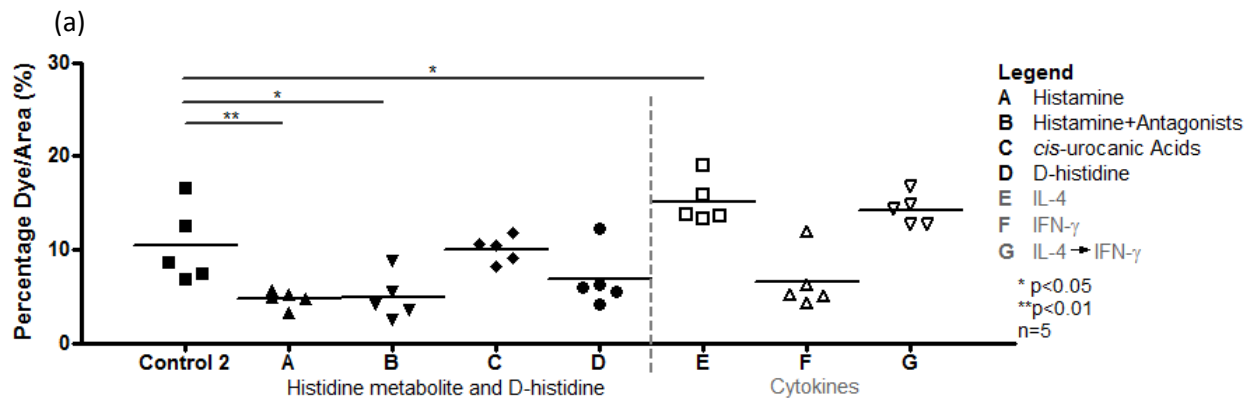


Figure 5. Comparison of the effects of histidine metabolites, D-histidine and cytokines on penetration of Lucifer Yellow dye into the skin models. One-way ANOVA was applied. (a) 1 μ M histamine dihydrochloride led to a significantly reduced penetration of Lucifer Yellow fluorescent dye into the skin-equivalent models ($p < 0.01$), even in the presence of H1+H2 histamine receptor antagonists (10 μ M pyrilamine maleate and 10 μ M cimetidine) ($p < 0.05$). 100 μ g/ml cis-UCA and 5mM D-histidine had no significant effect on the penetration of the fluorescent dye into the models. 50ng/ml IL-4 (label E) led to a statistically significant increase ($p < 0.05$) in Lucifer Yellow dye penetration. No significant effect was seen with 5ng/ml IFN- γ (label F). In the 'cytokine-neutralisation' study (label G), a small but non-significant reduction in the dye penetration was seen, as compared to treatment with IL-4 alone. (b) Representative images of (i) Control 2 (ii) A: Histamine (iii) B: Histamine+Antagonists (iv) E: IL-4. Lucifer Yellow dye is seen here as bright staining. Scale bar= 100 μ m. (T=Top; B=Bottom, of the skin model)

5.2.3 Effects of varying the pH of the culture media on Lucifer Yellow dye penetration into skin-equivalent models

One-way ANOVA analysis ($F(2,14)=11.96$) showed that adjusting the pH of the culture media to 8.1 led to a significantly increased penetration of the dye into the models ($p<0.01$, $n=5$, Figure 6).

Adjusting the pH of the culture media to 6.8 had no significant effect on the penetration of Lucifer Yellow fluorescent dye into the skin-equivalent models ($n=6$, Figure 6).

Extremes of pH can render cellular proteases/ enzymes ineffective and have direct toxicity effects on the cells. Assays to detect apoptosis or necrosis were not performed. The keratinocytes were, however, macroscopically observed to appear normal in both pHs 6.8 and 8.1 with no signs of detachment from one another or from the cell culture plates, which is often seen in HaCaTs cell death.

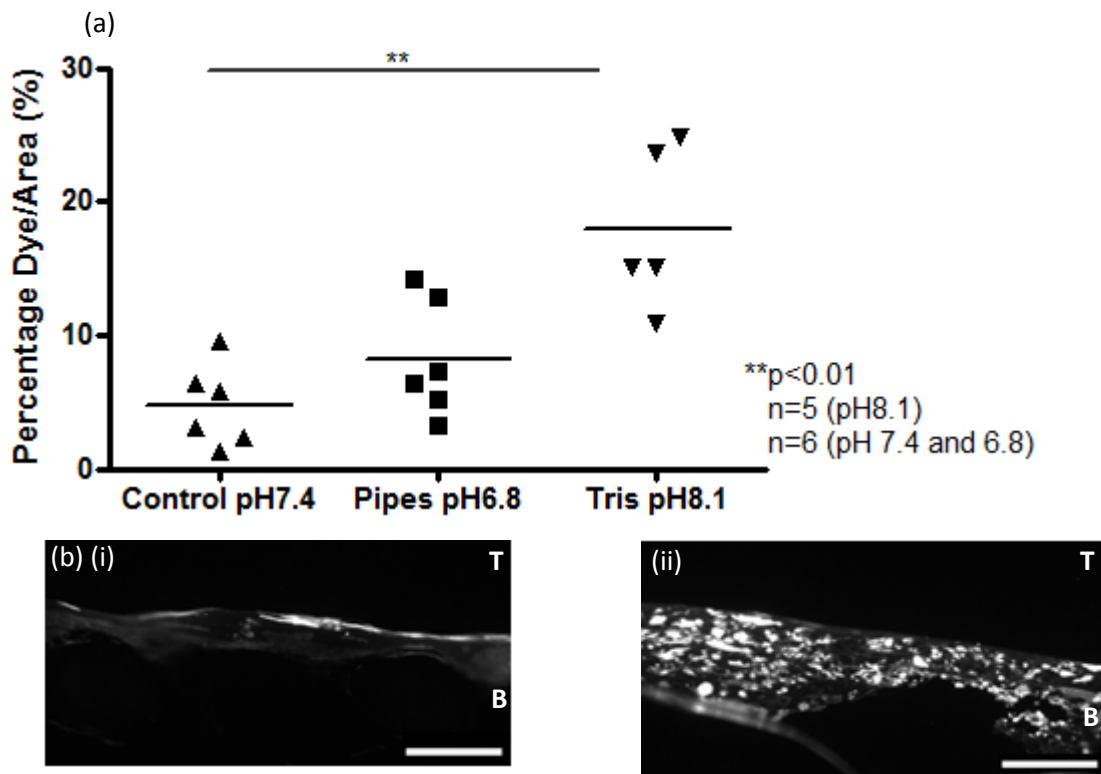


Figure 6. Comparison of the effects of different pHs on Lucifer Yellow dye penetration into the skin models. One-way ANOVA was applied. (a) Adjusting the culture media's pH to 8.1 with Tris buffer from day 13 to 19 led to a statistically significant increase ($p<0.01$) in the penetration of Lucifer Yellow fluorescent dye into the skin-equivalent models while pH of 6.8 (adjusted using Pipes buffer) showed no significant difference from controls (pH 7.4) (b) Representative images of (i) Control pH7.4 (ii) Tris pH8.1. Lucifer Yellow dye is seen here as bright staining. Scale bar=100 μ m. (T=Top; B=Bottom, of the skin model)

5.2.4 Immunohistochemistry for keratinocyte differentiation markers in skin-equivalent models

Given the differences seen with the Lucifer Yellow dye penetration assay, we next attempted to determine if the amino acids, cytokine modulation and different pHs are associated with significant morphological and phenotypic changes to our skin model. In particular, we examined for potential changes in the expression and distribution of keratinocyte differentiation markers (keratin 10, involucrin, filaggrin and loricrin) in the skin models used above, in response to the different interventions.

5.2.4.1 Effects of amino acids and their metabolites, cytokine modulation and pH on keratin 10 protein expression and distribution (a qualitative comparison)

In native human epidermis, keratin 10, an early marker of terminal differentiation, is found in the outer layers (granular and horny) of the epidermis.

Compared to the control skin-equivalents in which keratin 10 was found in the suprabasal layers, models grown in cell culture media with a pH of 8.1 and those supplemented with 5ng/ml IFN- γ appeared to have abnormal spatial distribution of keratin 10, where the protein was expressed throughout the entire thickness of the skin-equivalents (brown staining, Figure 7).

No obvious difference was seen with all other conditions.

(see page 169 for Figure 7)

Figure 7. Representative images comparing the effects of extracellular, free amino acids and their metabolites, cytokine modulation and pH on keratin 10 expression in organotypic skin-equivalent model. As in native human epidermis, keratin 10 (stained brown) was found in the suprabasal layers in the control models. High pH of 8.1 and IFN- γ (and possibly *cis*-UCA) led to keratin 10 being abnormally expressed throughout the entire thickness of the skin-equivalents. Each image is 100 μ m in horizontal length.

5.2.4.2 Effects of amino acids and their metabolites, cytokine modulation and pH on involucrin protein expression and distribution (a qualitative comparison)

Involucrin, another early marker of keratinocyte differentiation, was seen throughout the entire thickness of the epidermis obtained from a healthy human volunteer with no history of inflammatory skin condition (Figure 1b of Chapter 4). A similar finding was seen in the control skin equivalent models (Figure 8).

No obvious difference in the expression and distribution of involucrin in skin equivalents grown in the presence of additional amino acids, amino acid metabolites (histamine and *cis*-UCA), IL-4, IFN- γ and different pHs, (Figure 8).

(see page 170 for Figure 8)

Figure 8. Representative images comparing the effects of extracellular, free amino acids and their metabolites, cytokine modulation and pH on the involucrin expression in organotypic skin-equivalent model. As in native human epidermis, involucrin (stained brown) was found throughout the entire thickness of the skin-equivalent models. No significant effect by histidine, lysine, serine, histidine metabolites, varying pHs, IL-4 and IFN- γ was observed. Each image is 100 μ m in horizontal length.

5.2.4.3 Effects of amino acids and their metabolites, cytokine modulation and pH on filaggrin protein expression and distribution (a qualitative comparison)

Filaggrin, a late marker of keratinocyte differentiation, is seen in the granular and horny layers of native human epidermis. In our organotypic skin-equivalent models, its expression was found to be patchy and mainly concentrated in the suprabasal layers of the control skin models (Figure 9).

With this difference in mind, no obvious effect on filaggrin expression was seen in skin-equivalents grown in the presence of additional amino acids, amino acid metabolites (histamine and *cis*-UCA), IFN- γ and different pHs, as compared to the control skin models (Figure 9). A possible reduction in filaggrin protein expression was seen in skin-equivalents grown in the presence of 50ng/ml IL-4 (Figure 9).

(see page 171 for Figure 9)

Figure 9. Representative images comparing the effects of extracellular, free amino acids and their metabolites, cytokine modulation and pH on filaggrin expression in organotypic skin-equivalent model. In terminally differentiated human epidermis, filaggrin is widely distributed in the granular and horny layers. By contrast, a patchy distribution of filaggrin (stained brown) was found in the suprabasal layers of the skin-equivalent models. A possible reduction in filaggrin protein expression in models grown in 50ng/ml IL-4 was seen. No significant effect by histidine, lysine, serine, histidine metabolites, varying pHs and IFN- γ was seen. Each image is 100 μ m in horizontal length.

5.2.4.4 Effects of amino acids and their metabolites, cytokine modulation and pH on loricrin protein expression and distribution (a qualitative comparison)

Loricrin, a late marker of keratinocyte differentiation, is commonly seen in the granular and horny layers of native human epidermis. In our control skin-equivalent models, its expression was found to be patchy and less specific in terms of localization within the models (Figure 10).

No obvious effect on loricrin expression was seen in skin-equivalents grown in the presence of additional amino acids, amino acid metabolites (histamine and *cis*-UCA), IL-4, IFN- γ and different pHs, as compared to controls (Figure 10).

(see page 172 for Figure 10)

Figure 10. Representative images comparing the effects of extracellular, free amino acids and their metabolites, cytokine modulation and pH on loricrin expression in organotypic skin-equivalent model. Loricrin (stained brown) was found in a patchy and non-specific distribution within the skin-equivalent models. No significant effect by histidine, lysine, serine, histidine metabolites, varying pHs, IL-4 and IFN- γ was observed. Each image is 100 μ m in horizontal length.

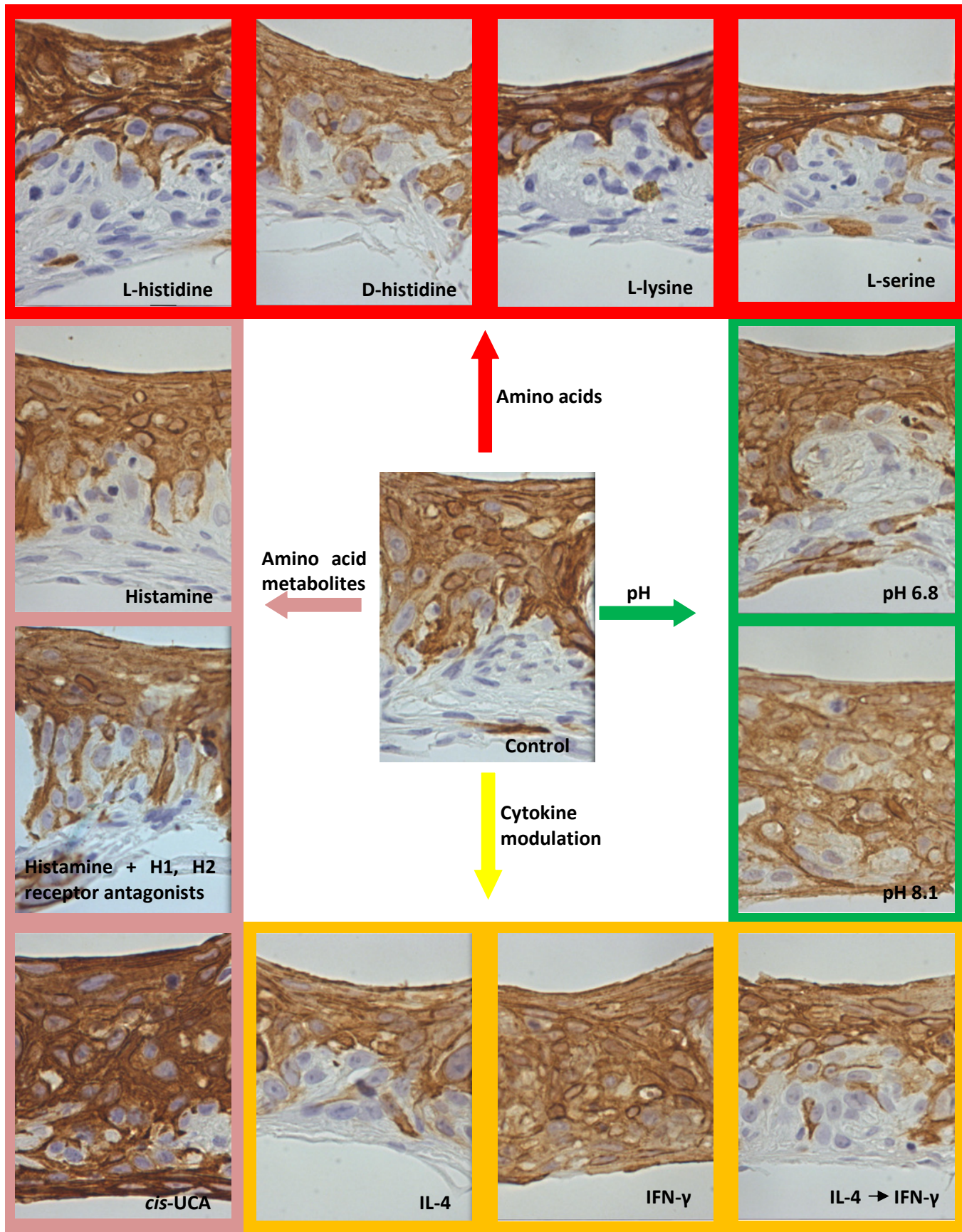


Figure 7

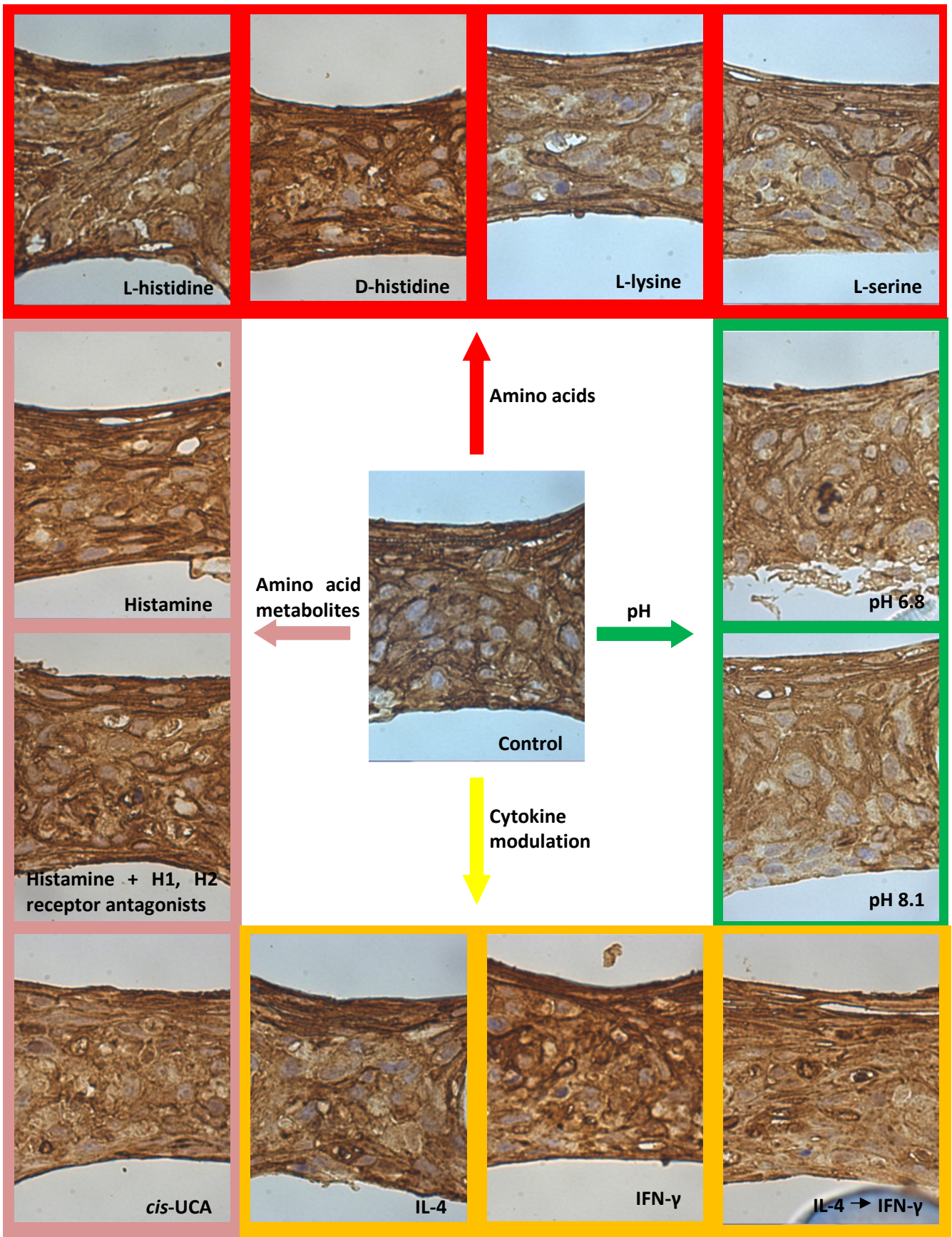


Figure 8

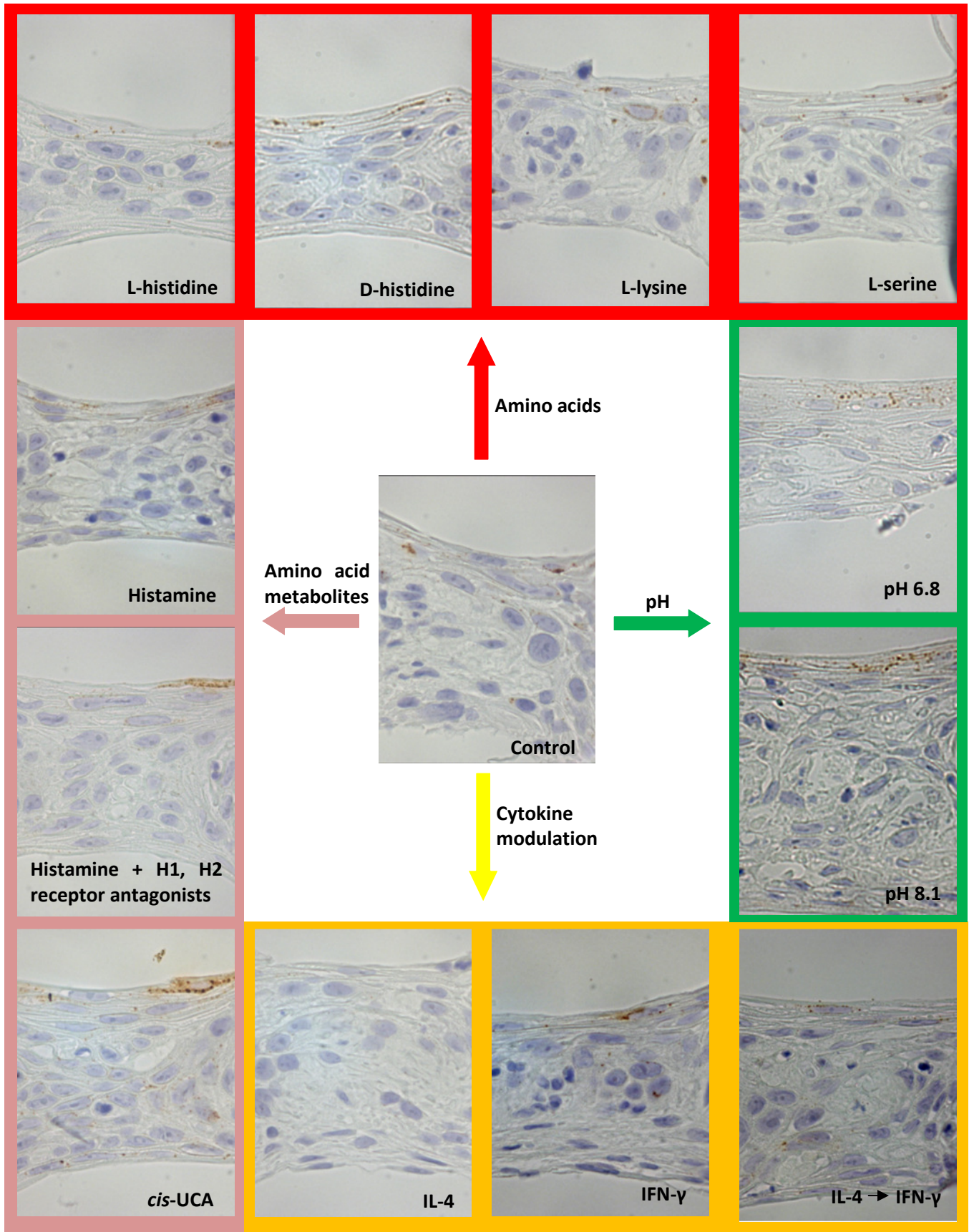


Figure 9

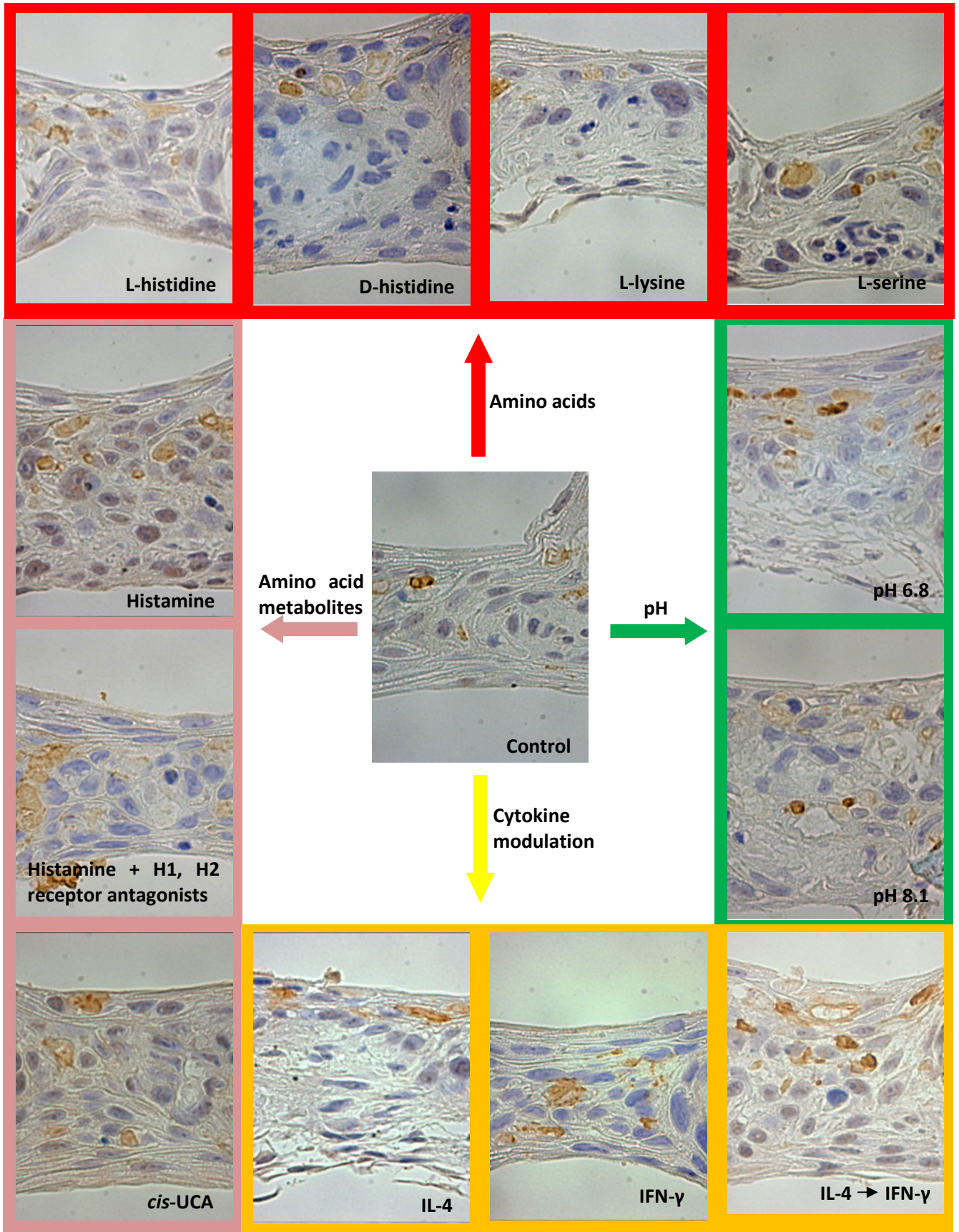


Figure 10

5.3 Chapter 5 Discussion

Using the 'Grid technique' with a lower collagen concentration of 2.5mg/ml, a significant increase in the thickness of the epidermal layer was seen in our skin-equivalent model. Compared to that described in Chapter 4, greater differences between the basal cells (larger, roundish in shape) and anucleated, flattened keratinocytes at the apical aspect of the skin model were also noted. These observations indicate an improved approximation of our model to native human epidermis. Our skin-equivalents grown in L-histidine-enriched media were more resistant to penetration by Lucifer Yellow fluorescent dye, suggesting an improved barrier function. To our best knowledge, this is a novel finding. As the pKa of L-histidine is 6.5, one may argue that this histidine-associated improvement in barrier function is a pH-induced effect. We have, however, shown that low pH of 6.8 did not lead to significant improvement in the barrier function of our skin-equivalent models. This suggests that the effect seen with L-histidine is unlikely to be attributed to pH alone. In addition, the final pH of histidine-enriched media used in our study ranged from 7.29 to 7.92, which was not much different from the non-enriched, base media (D-MEM/F-12 with GlutaMAX plus 10% fetal bovine serum and 1% penicillin/streptomycin, average pH 7.4). L-histidine, with its imidazole side chain, is a hygroscopic amino acid. Such physical property makes histidine an important component of the NMF. One may argue that the observed L-histidine-associated improvement in skin barrier function may simply be a chemical effect attributed to histidine's physical ability attract and hold on to water molecules. If this was indeed the case, D-histidine, the biologically inactive isomer of L-histidine with a similar chemical structure, should theoretically result in a similar degree of improvement in the barrier function of our skin-equivalents. D-histidine, used as a chemical control in our study for L-histidine, led to non-significant reduction in the degree of penetration of Lucifer dye into the skin-equivalents. We therefore propose that while the physical effects of L-histidine cannot be dismissed, it is possible that L-histidine may have some unknown biological effects on the keratinocytes which led to the barrier improvement seen, especially when L-histidine is known to be a common participant in enzymatic reactions⁸. A study done in 1978 (prior to the term 'filaggrin' being coined in 1981³⁴) has shown that radiolabeled histidine, injected subcutaneously into newborn rat epidermis, was subsequently incorporated into a histidine-rich protein '*assumed to be a molecular marker of the differentiation of a granular into a cornified keratinocyte*'³⁵. This histidine-rich protein is now known as filaggrin. In the next chapter, we investigated the biological effects of histidine and its metabolites on the expression of filaggrin and other differentiation markers.

As discussed earlier in this chapter, histidine is metabolised into UCA and histamine. We found no significant change in the permeability barrier of our skin models grown in *cis*-UCA-enriched media. This suggests that *cis*-UCA does not have significant short term effects on the epidermal

barrier function. *Trans*-UCA was not tested as it was not commercially available when our experiments were conducted. Compared to *trans*-UCA, *cis*-UCA is the biologically more reactive molecule.

Improvement in the permeability barrier was, however, seen in skin models grown in histamine-enriched media. This finding is surprising as histamine, by acting on histamine receptors, is a key mediator of inflammatory response and has been suggested to play a key role in the pathogenesis of AD³⁶⁻³⁹. Histamine is said to cause contraction of endothelial cells, forming intercellular gaps and resulting in plasma macromolecular extravasation due to increased vascular permeability⁴⁰⁻⁴³. Histamine can, however, restore endothelial barrier function by affecting cell-matrix and cell-cell adhesion⁴⁴. It is, perhaps, the overall balance between the pro-inflammatory and pro-resolution roles which determines its final effect. Histamine-associated improvement in barrier function seen in our skin-equivalents was found to occur even in the presence of H1- and H2-receptor antagonists. This potentially suggests the involvement of an unknown histamine-related but H1-,H2-receptors-independent mechanism. Future studies should involve other histamine receptors, in particular H4 receptor which has been shown to be involved in mast cell chemotaxis and mobilization of intracellular calcium stores.

The deficiency of lysine in filaggrin suggests that this amino acid is not an important component of the NMF. Lysine has an ϵ -amino group which has a very high pKa of 10.5. This would render lysine highly basic and unsuitable as a component of the NMF. In our skin model, raised level of lysine led to a slightly worse barrier function (though this result was not statistically significant). This was indicated by an increased penetration of the Lucifer Yellow dye into the skin models, as compared to controls. On the other hand, serine is an amino acid which, like histidine, is found in abundance in the filaggrin protein. One would presume that increased level of serine will lead to the opposite effect to that of lysine on the barrier property of our skin model. This was, however, not found. Raised level of serine did not lead to significant changes to the barrier function of our model, as compared to controls. The effects of serine on skin barrier function and keratinocyte protein expression were further tested using different systems and discussed in the subsequent chapters.

TH-2 cytokines such as IL-4 and IL-13, which are known to predominate in atopic dermatitis, can cause a reduction in filaggrin protein expression³⁰. Expectedly, our skin equivalents grown in IL4-enriched media showed increased dye penetration, suggesting a compromised epidermal barrier. Skin-equivalent models maintained in culture media with a high pH of 8.1 showed poorer barrier function, as demonstrated by the significantly increased penetration of Lucifer Yellow dye. This is in line with the observation that skin pH rises in diseased states and concurs with the current view that in order for a competent epidermal barrier to be formed, an acidic environment (pH5.5) is required⁴⁵.

Immunohistochemistry performed on skin models grown in a high pH of 8.1 revealed abnormal spatial distribution of keratin 10 (a marker of terminal differentiation found in the outer layers of the stratum corneum). This suggests that high pH may interfere with normal keratinisation process in the epidermis. The results of our Lucifer Yellow dye penetration assay in both the cytokine and pH studies indicate that our organotypic skin-equivalent model is a reliable tool for permeability barrier studies. Having said this, our skin-equivalents showed several obvious deviations from native human epidermis, as detailed in Table 1 of Chapter 4 and Figures 9 and 10 of this chapter. These include the reduced expression and patchy, non-specific distribution of late keratinocyte differentiation markers (filaggrin and loricrin) and reduced stratification of the artificial skin as compared to a native epidermis. This may be rectified if necrosis or apoptosis of the HaCaT keratinocytes following a prolonged culture period can be somehow avoided, thus permitting a longer culture time.

A qualitative, instead of quantitative, analysis was performed for the immunohistochemical staining of keratinocyte differentiation markers. For keratin 10, the normal distribution of the protein was limited to terminally differentiated keratinocytes at the suprabasal layers and any abnormality would be obvious. A quantitative analysis was therefore deemed unnecessary. For example, keratin 10 was abnormally expressed throughout the entire thickness of the skin-equivalents grown in high pH 8.1 and IFN- γ -enriched media, instead of being limited to the suprabasal layers, indicating abnormal keratinisation. Involucrin, an early marker of keratinocyte differentiation, was abundantly expressed throughout the entire thickness of all skin-equivalent models. Quantitative analysis in this case is unlikely to yield useful information. A possible reduction in the expression of filaggrin was seen in skin-equivalents grown in the presence of IL-4. However, it is difficult to quantitate this as the primary filaggrin antibody used is specific to neither the non-functional profilaggrin, filaggrin intermediates nor the functional filaggrin monomers.

In summary, this study highlights the potential benefits of amino acid histidine in improving skin barrier function. Histidine-enriched moisturiser has been used in the cosmetic industry although its mechanism of action is not understood. Our study showed that histidine metabolite histamine, but not UCA, appeared to have beneficial effects in a skin-equivalent model. In the 1990s, *cis*-UCA, a common ingredient in cosmetic products then, was found to be the mediator of the immunosuppressive effects of UV radiation in the skin¹⁹⁻²². This may be beneficial in immune-driven inflammatory skin diseases, such as AD but widespread concerns regarding its carcinogenicity due to presumed suppression of immunological rejection of tumours^{16,46} warranted the mass removal of UCA-containing products from the shelves. Future studies are therefore needed to improve our understanding on how histidine and its metabolites may lead to an improvement in skin barrier function before histidine compounds can be licensed for therapeutic usage in AD patients.

5.4 Chapter 5 References

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5.5 Chapter 5 Materials and Methods

5.5.1 Organotypic skin-equivalent model

Adult human dermal fibroblasts, HDFa, (Invitrogen, Paisley, UK) of passages 3-5, suspended in fetal bovine serum, were mixed with Collagen I, Rat Tail (Gibco, Paisley, UK) and left to set in a standard 6-well cell culture plate. The collagen was made up as per manufacturer guidelines. Final concentrations of HDFa and collagen in the mixture were 0.5×10^6 cells/ml and 2.5mg/ml respectively. Upon gelling (approximately 20-30 minutes), HaCaTs of passages 35-41 were seeded at 0.5×10^6 cells/ml on top of the collagen gels within the 6-well plate. Upon confluency of the HaCaTs (approximately 48-72 hours), the collagen gels were gently lifted off the plates and placed onto plastic grids with HaCaTs exposed to air and culture media (D-MEM/F-12 with GlutaMAX (Gibco, Paisley, UK) plus 10% fetal bovine serum and 1% penicillin/streptomycin, average pH 7.4) in constant contact with the bottom of the gels, creating an air-liquid interface. Care was taken to ensure that no air bubble was trapped under the grids. Skin models were maintained without further passaging for a total of 19 days (20 days for the 'cytokine-neutralisation' study). From day 13-19, 5mM of amino acids (L-lysine, L-serine, L-histidine, D-histidine), 1 μ M histamines (with and without H1+H2 antagonists [10 μ M pyrillamine maleate and 10 μ M cimetidine]) or 100 μ g/ml *cis*-UCA (Sigma, Dorset, UK) was added to the media. For the pH study, RPMI1640 media (PAA, Pasching, Austria) plus 10% fetal bovine serum, 1% penicillin/streptomycin and 1% glutamine was used instead of D-MEM/F12 with Glutamax. From day 13-19, pH of RPMI1640 media was adjusted using Pipes (pKa6.8) and Tris (pKa8.1) buffers (20mM respectively; Sigma, Dorset, UK). For the cytokine study, recombinant human IL-4 (50ng/ml; R&D Systems, Abingdon, UK) or IFN- γ (5ng/ml; Peprotech, London, UK) was added from day 13-19. In the 'cytokine-neutralisation' study, skin models were cultured in the presence of 50ng/ml IL-4 from day 13-19. On day 19, models were washed in PBS and 5ng/ml IFN- γ was added to the new media and the model was cultured for a further 24 hour. On day 19 (or day 20 for the 'cytokine-neutralisation' study), 50 μ L of 5mM Lucifer Yellow CH dipotassium salt (Sigma, Dorset, UK) was applied onto the surface of the all skin models (HaCaT side) at 5-minute intervals for a total of 10 minutes. Samples were washed twice in PBS, fixed in formalin and embedded in paraffin. Dewaxed and hydrated 3- μ m transverse sections were visualised using fluorescence at 455-495/505-555 nm for the fluorescent dye.

5.5.2 Immunohistochemistry.

As in Chapter 4 Materials and Methods

5.5.3 Statistical analysis

Bartlett's test for equal variances and one-way ANOVA with Dunnett's post test (95% confidence intervals) were used for comparisons between groups of our quantitative measurements. These were performed using GraphPad Prism version 4.00 for Windows, GraphPad software, San Diego California USA, www.graphpad.com.

Data are shown as mean, M values \pm standard deviation, SD . p -values <0.05 were considered as significant.

Chapter 6

Exogenous Amino Acids Altering the Expression of Epidermal Barrier Proteins in Human Keratinocyte Cell Line HaCaT

In Chapter 5, we have demonstrated that raised levels of free amino acid histidine and its metabolite histamine are associated with significant improvement in the epidermal permeability barrier of our organotypic skin model. One of the key determinants in stratum corneum integrity and barrier formation is the appropriate expression of the various epidermal barrier proteins. In this chapter, we investigate whether free amino acids histidine, lysine and serine affect the protein expression of three major epidermal barrier proteins filaggrin, loricrin and involucrin.

6.1 Introduction: What makes a brick wall?

Skin barrier dysfunction is the hallmark feature of common inflammatory skin disorders such as atopic dermatitis (AD). The epidermal stress response to this barrier perturbation plays a significant role in the progression of the disease. Barrier disruption, experimentally induced using chemical irritant or tape-stripping, leads to a cytokine cascade (for example, production of IL-1 α , granulocyte-monocyte colony stimulating factor and tumour necrosis factor- α) which maintains or aggravates the inflammatory state¹⁻³. Filaggrin-induced barrier disruption has been suggested to allow easier penetration of environmental allergens which interact with the local antigen presenting cells and immune effector cells, giving rise to a pro-inflammatory state with raised IgE levels⁴. A study in mice has shown that cutaneous sensitization to allergens can augment airway hyperreactivity, a characteristic feature of asthma⁵. It is therefore not unreasonable to suggest that protecting and improving the skin barrier, especially in early stages of AD, may halt the 'atopic march', i.e. the development of other atopic diseases such as asthma later in life.

In order to improve or maintain the barrier property of the skin, detailed understanding of the structure of the epidermal barrier is required. The epidermis consists of four distinct layers: basal, prickle, granular and the outermost horny layer⁶. The horny layer is also known as the stratum corneum. Cells within the stratum corneum, known as corneocytes, represent keratinocytes in the terminal phase of differentiation. Human stratum corneum consists of 20 corneocyte layers, on average. These flattened squames have no nuclei or organelles and contain densely packed keratin filaments⁷. The corneocytes are interlocked together by modified desmosomes called corneodesmosomes, which provide tensile strength for the stratum corneum to resist against shearing forces⁴. Surrounding the corneocytes is water-resistant lipid lamellae which accounts for 14% of the dry weight of stratum corneum⁸ and consists of ceramides, fatty acids, cholesterol and cholesterol esters⁹. This intercellular lipid matrix reduces water loss from the skin, impedes penetration of water-soluble materials and ensuring that the stratum corneum is as tight as possible while retaining a degree of flexibility⁴. It is synthesized during the formation of the corneocytes as granular cells spill the contents of their lamellar granules into the extracellular space¹⁰. The stratum corneum can therefore be visualised as a brick wall: the corneocytes are the bricks, the lipid lamellae is the mortar and the corneodesmosomes are the iron rods that are passed through the bricks.

The quality of the 'bricks' will determine the barrier function of the epidermis. A terminally differentiated corneocyte contains densely packed bundles of keratin filaments aligned in parallel to the skin surface. These keratin filaments make up 80-90% of its total mass and are encapsulated within an extremely tough, insoluble proteinaceous structure known as the cornified cell envelope (CE)¹¹. The CE, which contributes to the biomechanical properties of stratum corneum, makes up

approximately 7% of the corneocyte's total mass¹². Formation of the CE starts just below the cytoplasmic membrane in the most superficial granular cells, gradually increasing in thickness and density as the cell membrane disintegrates and finally becoming a uniform layer approximately 15nm in thickness encasing the dead cornified cells¹¹. It consists of two parts: the protein envelope (10nm thick) and the lipid envelope (5nm thick)¹¹. The protein envelope consists of specialized structural proteins, such as involucrin, loricrin and small proline rich proteins covalently cross-linked by disulfide and N^ε-(γ-glutamyl)lysine isopeptide bonds¹³ formed by transglutaminases (TGases). Involucrin is a glutamine-rich protein^{14,15}, thereby serving as an amine acceptor and is the preferred substrate of transglutaminase-1¹⁶. Due to its elongated shape, long length and circumferentially distributed glutamine residues which can interact with lysine residues of other membranous proteins, involucrin is an ideal intermolecular cross-bridge in the keratinocyte cornified envelope¹⁷. Similar to filaggrin, the gene encoding involucrin is located within the epidermal differentiation complex on chromosome 1q21. Loricrin is another major structural protein, contributing 70% of the mass of the cornified envelope¹⁸. As with involucrin and filaggrin, the gene encoding loricrin is found within the epidermal differentiation complex on chromosome 1q21¹⁹⁻²¹. This 26kDa glycine-serine-cysteine rich cationic protein is expressed late in epidermal differentiation in the stratum granulosum of human epidermis.^{22,23} It is first accumulated in the L-granules (different from the F-granules of filaggrin) before being crosslinked to other envelope proteins by transglutaminases via γ-glutamyl-lysyl-isopeptide bonds.^{22,23} It contains tandem repeats of glycine-rich peptide sequences which is thought to form 'loops', structures which confer flexibility to the CE²⁴. These 'glycine loops' are flanked by glutamine-rich sequences which are target sites of transglutaminase-catalysed crosslinking²⁵. The abundance of cysteine in loricrin suggests that it also forms extensive disulfide bridges²³.

This protein envelope is surrounded by a monomolecular layer of ω-hydroxyceramides which is covalently bounded by ester bonds to the structural proteins (mainly involucrin, periplakin and envoplakin²⁶), forming a lipid envelope on the exterior of the protein envelope^{8,11,12}. The ester bond between involucrin and ω-hydroxyceramides is said to be formed by transglutaminase-1²⁷. It has been suggested that this lipid envelope facilitates the organisation of the intercellular lipid matrix²⁸.

Another main component of the stratum corneum which functions as a barrier to transepidermal water loss is the natural moisturizing factor (NMF). This has been repeatedly mentioned in previous chapters. Most NMF agents, derived from the complete proteolysis of filaggrin protein, are found within the corneocytes, shielded by the protein and lipid envelopes but others such as lactate, hyaluronic acid and sugars are found extracellularly²⁹. The chemical composition of the NMF, of which 40% are free amino acids, is as detailed in Table 1 (adapted from Rawlings and Harding³⁰).

These hygroscopic agents are very effective humectants. They absorb water from the atmosphere while remaining soluble in their own water of hydration, thereby maintaining hydration in the outermost layers of the stratum corneum^{30,31}. By maintaining free water in the stratum corneum, the NMF also facilitates biochemical activities including the regulation of protease activities involved in the generation of NMF itself³⁰.

Table 1

Chemical Composition of Natural Moisturising Factor (NMF)³⁰

<u>Chemical compound</u>	<u>Percentage composition (%)</u>
Free amino acids	40
Lactate	12
Pyrrolidone carboxylic acid	12
Sugars	8.5
Urea	7
Chloride	6
Sodium	5
Potassium	4
Calcium	1.5
Glucosamine, ammonia, uric acid and creatinine	1.5
Magnesium	1.5
Citrate and formate	0.5
Phosphate	0.5

In Chapter 5, we have shown that the free amino acid L-histidine and its metabolite histamine led to improvements in the barrier function of our organotypic skin-equivalent model. To our best knowledge, this is a novel finding and it raises several important questions, such as the mechanism by which this may have occurred or the component of the epidermal barrier that is involved in this improvement. The observed beneficial effect may be attributed to the hygroscopic nature of L-histidine, an important component of the NMF. However, as discussed in Chapter 5, D-histidine, a structurally similar molecule, did not lead to similar finding. This argues against the L-histidine's effect being a purely chemical one and implies potential biological effects. Investigating if L-histidine exerts biological effects on keratinocytes which may lead to alterations in the skin barrier function is the main aim of this chapter.

Amino acids, including histidine, have been shown to regulate their own production by inhibiting the enzyme(s) involved in their generation in a negative feedback manner³². It is, therefore, intriguing to speculate that raised level of L-histidine, a common participant in enzymatic reactions, may reduce its own generation by inhibiting the enzymatic breakdown of histidine-rich filaggrin into its constituent amino acids. A corresponding increase in the level of filaggrin monomers may

therefore be expected. This may be associated with increased efficiency of keratin aggregation during the formation of the corneocyte squames and account for the improved barrier. It follows from this hypothesis that histidine may also influence the enzyme-related proteolysis of profilaggrin into filaggrin itself. The dephosphorylation and proteolysis of profilaggrin into filaggrin is not fully understood although kinases and several other enzymes are thought to be involved³³.

Radiolabeling studies have shown that histidine, either given orally or injected subcutaneously in animal models is soon incorporated into filaggrin protein within the epidermis^{34,35}. Free amino acids can be transported into the cells via several channels^{36,37} and changes in the levels of amino acids, including histidine, have been shown to regulate gene expression and transcription³⁸⁻⁴¹. It is also intriguing to note that the transcriptional control of filaggrin and loricrin appears to be closely coordinated⁴². There are multiple possibilities and limited evidence with regard to the mechanism(s) by which histidine may improve skin barrier function. Any factor in the transcription, translation and/or post-translational modification of epidermal barrier proteins may potentially be involved.

Chapter hypothesis

Improvement in barrier function seen in skin model: An intracellular biological or extracellular physical effect

We hypothesize that increased levels of free amino acid histidine and its metabolites in keratinocyte cell culture media is associated with alterations in the protein expression of (pro)filaggrin in the epidermis. This can be hypothesized to involve the imidazole ring of histidine, which may enable its participation in enzymatic reactions which drive the generation of histidine from (pro)filaggrin. Based on the reasons and results already mentioned in Chapter 5, we have also investigated the effects of other free amino acids (serine and lysine) and different pHs on the protein expression of involucrin and loricrin, even though some of these factors have not been shown to have significant effects on the barrier function of our skin models. Both serine and lysine do not have an imidazole ring and may therefore be used as potential negative controls. Involucrin and loricrin, like filaggrin, are important components of the cornified cell envelope; changes in their expression may influence the skin barrier function.

In testing our hypothesis, we used HaCaT keratinocytes cultured in standard 6-well plates, instead of the organotypic co-cultures described in Chapter 5. This is to optimise standardisation of our process by excluding confounding factors potentially incurred by co-culturing with human dermal fibroblasts and the usage of rat collagen. Keratinocyte monolayer cultures grown under suitable conditions will, in time, form islets consisting of differentiated cells. This is because mitosis continues to occur, to a certain extent, in monolayer cultures that have reached confluency⁴³. Cells undergoing differentiation are selectively expelled from the mitotically active basal layer⁴³. Similar to a native human epidermis or an organotypic skin model, changes in keratinocyte size, shape, structure and contents are seen as the cells differentiate and migrate outward from the monolayer. This simpler model is used here in this chapter. Organotypic models are not required as investigation of barrier function is not the main objective of this study.

6.2 Chapter 6 Results

6.2.1 (pro)Filaggrin expression of HaCaT cells vs human-derived keratinocytes.

The intermediaries in the proteolytic pathway of profilaggrin (~400kDa) into filaggrin (37kDa) of human epidermis-derived keratinocytes were different in size and quantity from those of keratinocyte cell line HaCaT (Figure 1).

Profilaggrin, its intermediates and filaggrin monomers extracted from a human epidermal sample (the sample was taken from the study presented in Chapter 3) are shown in Figure 1a.

In HaCaTs, heavy cross-linking with other proteins and variable extractability of (pro)filaggrin led to filaggrin bands of variable sizes. This may explain the 'ladder' effect seen on Western-blotting, which prevented reliable analysis of the pro-protein band (Figure 1b,c).

In view of the reliable expression of a 120kDa filaggrin intermediate within the HaCaT model, this intermediate was, therefore, used for comparison with the 37kDa monomers in subsequent experiments in this study. The same 120kDa filaggrin intermediate has been previously described by others who attempted to extract (pro)filaggrin from keratinocyte cell line N-TERT⁴⁴.

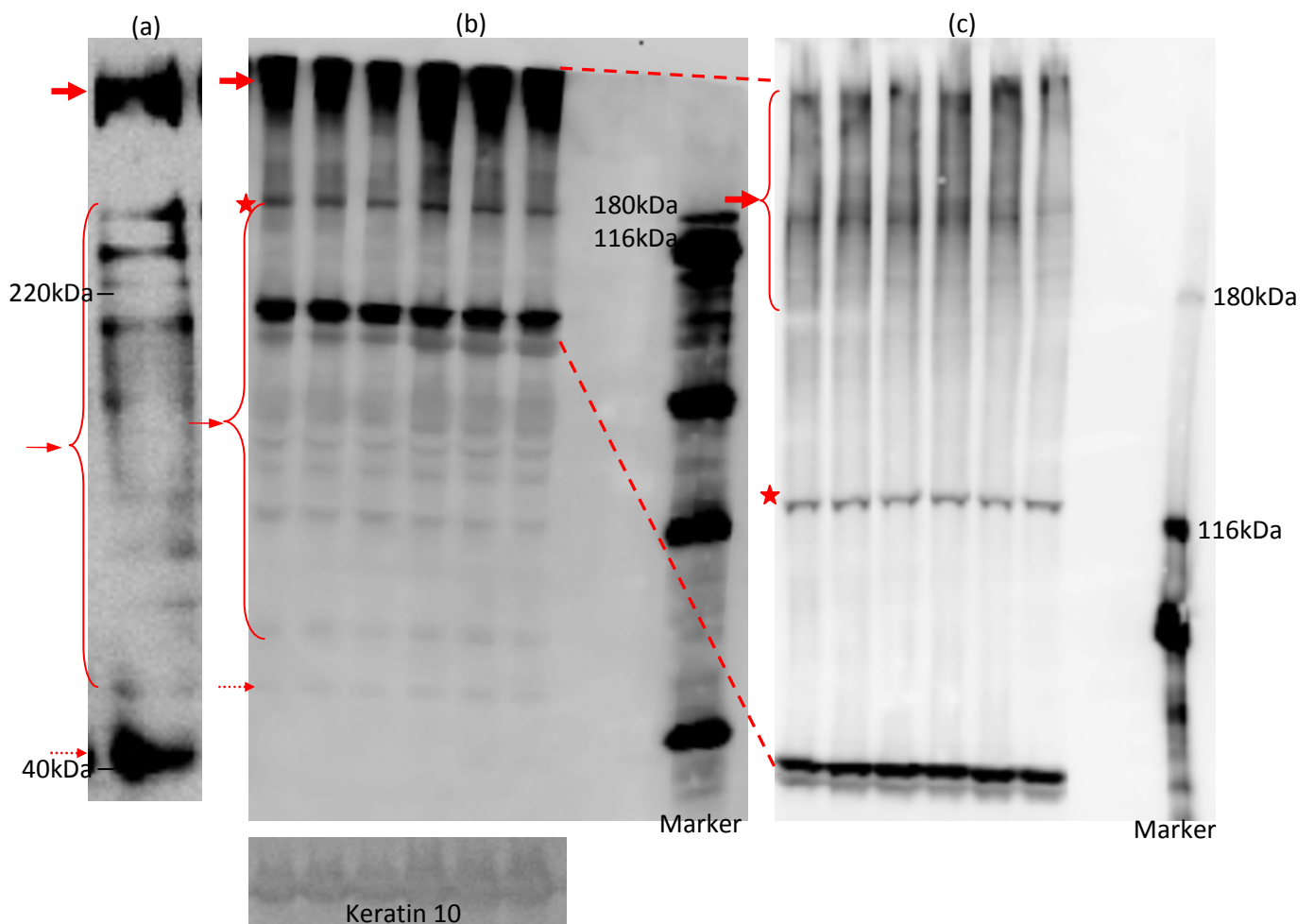


Figure 1. Differences in (pro)filaggrin expression between a human-derived sample and keratinocyte cell line HaCaT. Western-blotting of (a) gradient 6-15% SDS-PAGE showing profilaggrin, filaggrin intermediates and filaggrin monomers extracted from human epidermis. Marker ladder (Benchmark Protein Ladder) was not visualised here using a chemiluminescent substrate as it is non-biotinylated. The marker ladder was non-permanently visualised via Ponceau Red staining and hand-marked prior to application of primary antibodies. These markings were superimposed onto the images shown here to ascertain the size of the protein bands seen. Keratin 8, visualised using 4-chloro-1-naphthol, was used as the loading control (not shown here, refer to Chapter 3: Figure 2b-WT-AD Lane 1) (b) 9% SDS-PAGE showing heavily cross-linked profilaggrin, the 120kDa intermediate, other filaggrin intermediates and filaggrin monomer in keratinocyte cell line HaCaTs of 6 control samples. Keratin 10 was used as the loading control. (c) 6% SDS-PAGE showing heavily cross-linked profilaggrin in HaCaT cells of 6 control samples. This blot was done to qualitatively demonstrate the ladder effect in the high-molecular weight profilaggrin region using a 6% gel; a loading control was not used.

Legend

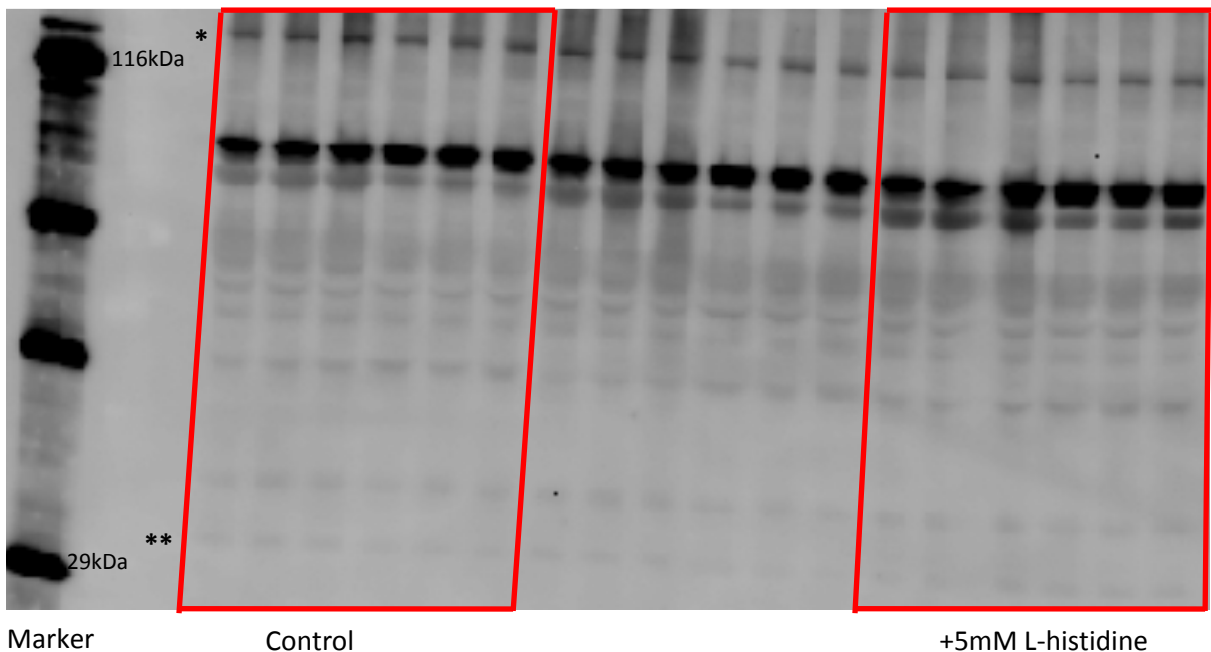
- ➔ Profilaggrin (~400kDa)
- ➔ Various intermediates
- (★120kDa intermediate)
- ➔ Filaggrin monomer (~37kDa)

6.2.2 Raised extracellular free amino acids and the expression of epidermal barrier proteins in HaCaT monolayer cell cultures

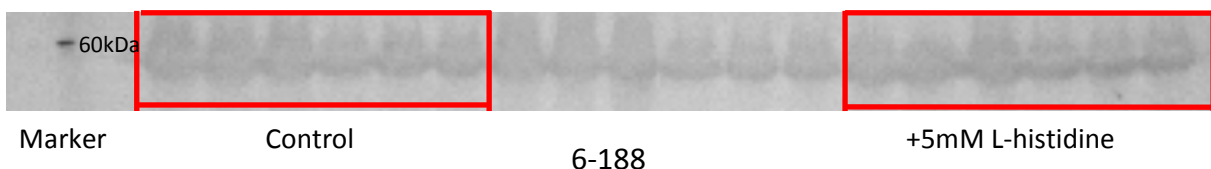
6.2.2.1 The effects of L-lysine, L-histidine and D-histidine on the expression of filaggrin

Western-blotting of HaCaTs from the 21-day monolayer cell cultures were used, with all bands standardised to housekeeping protein keratin 10 for loading control. Two-way ANOVA ($F(2,45)=5.09$) and Bonferroni post test were applied to the analysis, which showed that HaCaTs grown in L-histidine-enriched media (5mM) exhibited increased 37kDa filaggrin monomers and a corresponding reduction in a large 120kDa filaggrin intermediate, causing increased 37kDa:120kDa ratio (** $p<0.01$, $n=6$, Figure 2a, c). Linear regression analysis showed a dose-dependent increase in the 37kDa:120kDa ratio ($F(1,12)=14.11$, ** $p=0.00$, $r^2=0.54$, $n=6$ [controls and 5mM], $n=1$ [1mM, 2mM], Figure 2b) in HaCaTs grown in L-histidine-enriched media. Increased 37kDa:120kDa ratio represents increased expression of the filaggrin monomers in relative to the 120kDa intermediate. Such effect was not seen in HaCaTs grown in media enriched with 5mM D-histidine and L-lysine (Figure 2c). D-histidine, a biologically inactive isomer of histidine, and L-lysine were used as negative controls. The relative optical density of controls, grown in standard cell culture media, was scaled to 1 to allow inter-experimental comparisons to be made. All bands were standardised to housekeeping protein keratin 10 for loading control.

(a) (i) Western-blotting for (pro)filaggrin

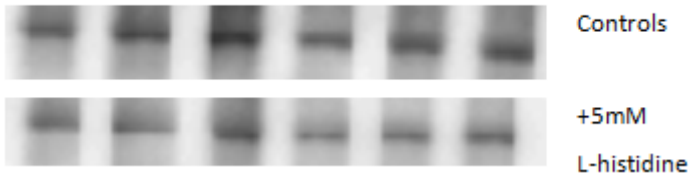


Western-blotting for keratin 10 (loading control)

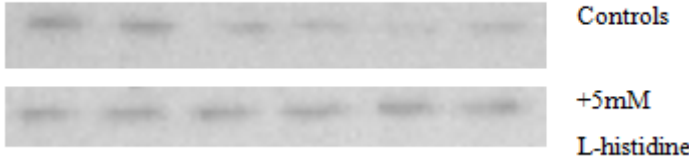


(ii)

*** 120kDa Filaggrin Intermediate**



**** 37kDa Filaggrin monomers**



Note: Quantitation of band density for the purpose of statistical analysis was performed using the unmodified image above. The images on the left are enlarged, cropped and modified (in terms of contrast and brightness) version of the above image.

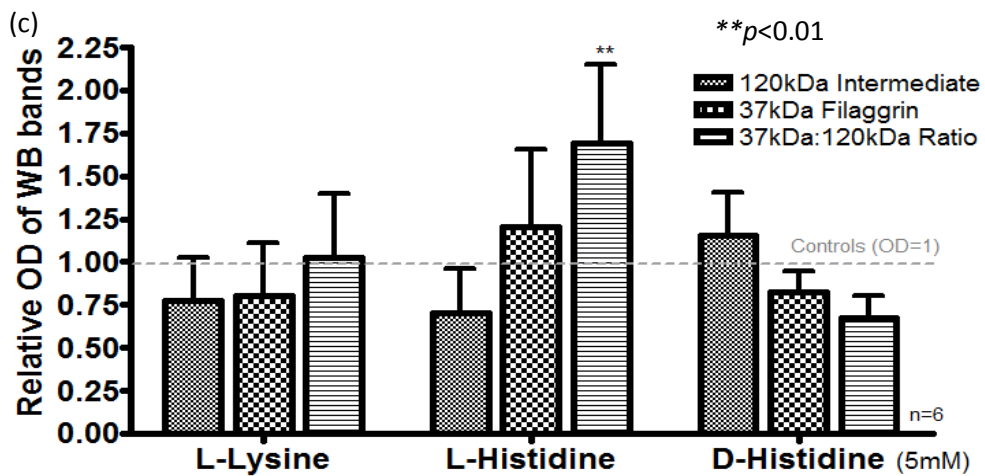
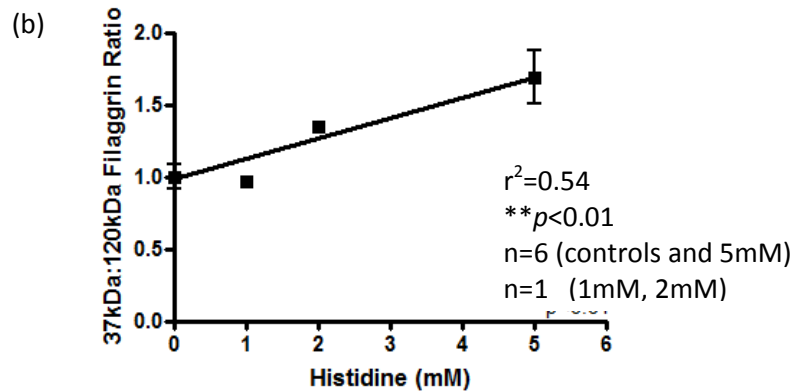


Figure 2. Comparisons of the effects of L-lysine, L-histidine and D-histidine on the expression of profilaggrin and filaggrin. (a) A reduction in the Western-blot band density of a 120kDa filaggrin intermediate and an increase in the 37kDa monomers were seen in HaCaTs grown in L-histidine-enriched media (i) Unmodified images of Western-blot for (pro)filaggrin (developed using enhanced chemiluminescent substrate) and loading control keratin 10 (developed from the same blot but using 4-chloro-1-naphthol) (ii) Enlarged, cropped and modified (in terms of contrast and brightness) images of (i). (b) Linear regression analysis showed a dose-dependent increase in the 37kDa:120kDa filaggrin ratio was seen in HaCaTs grown in L-histidine enriched media (** $p < 0.01$). (c) The effects of 5mM exogenous, free amino acids L-lysine, L-histidine and D-histidine on filaggrin protein expression were shown. Two-way ANOVA was used. L-histidine led to an increase in the 37kDa:120kDa filaggrin ratio (** $p < 0.01$). Bands were standardised to keratin 10 for loading control.

6.2.2.2 The effects of L-lysine, L-histidine and D-histidine on the expression of loricrin

One-way ANOVA with Dunnett's post test, with F ratio of $F(2,15)=1.65$, was used for data on L-lysine and L-histidine. Unpaired, two tailed t-test was used for data on D-histidine, which was obtained from another experiment with a different set of controls.

We have shown that, in relative to their controls, the addition of 5mM L-lysine ($p>0.05$, n.s., n=6), L-histidine ($p>0.05$, n.s., n=6) and D-histidine ($t(10)=1.28$, $p=0.23$, n.s., n=6) into the cell culture media had no significant effect on the protein expression of loricrin in HaCaT monolayer cultures (n=6, Figure 3). This suggests that the effect of 5mM L-histidine on filaggrin protein expression is real.

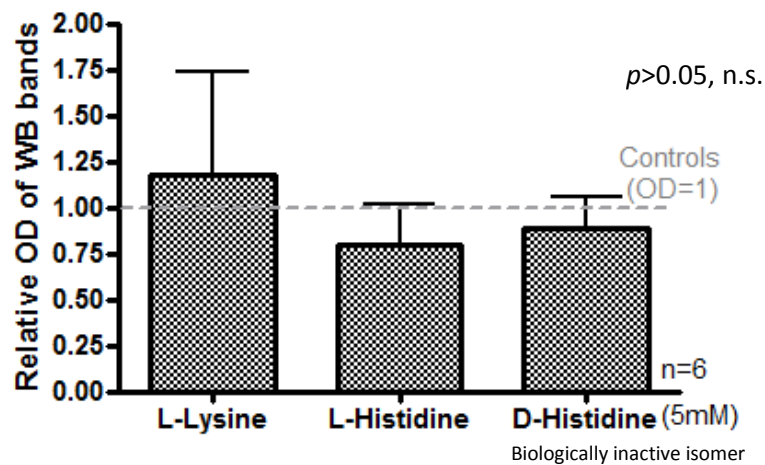


Figure 3. Comparisons of the effects of exogenous, free amino acids L-lysine, L-histidine and D-histidine on loricrin protein expression. One-way ANOVA was used for the L-Lysine ($p>0.05$, n.s.) and L-histidine data ($p>0.05$, n.s.); unpaired, two-tailed t-test was used for the data on D-histidine ($p=0.23$, n.s.).

6.2.2.3 The effects of L-lysine, L-histidine and D-histidine on the expression of involucrin

One-way ANOVA with Dunnett's post test, with F ratio of $F(2,15)=7.35$, was used for data on L-lysine and L-histidine. Unpaired, two tailed t-test was used for data on D-histidine, which was obtained from another experiment with a different set of controls.

In relative to their controls, L-lysine ($*p<0.05$) and L-histidine ($**p<0.01$), but not D-histidine ($t(10)=0.45$, $p=0.67$, n.s.), reduced the protein expression of involucrin in HaCaT monolayer cultures (n=6, Figure 4).

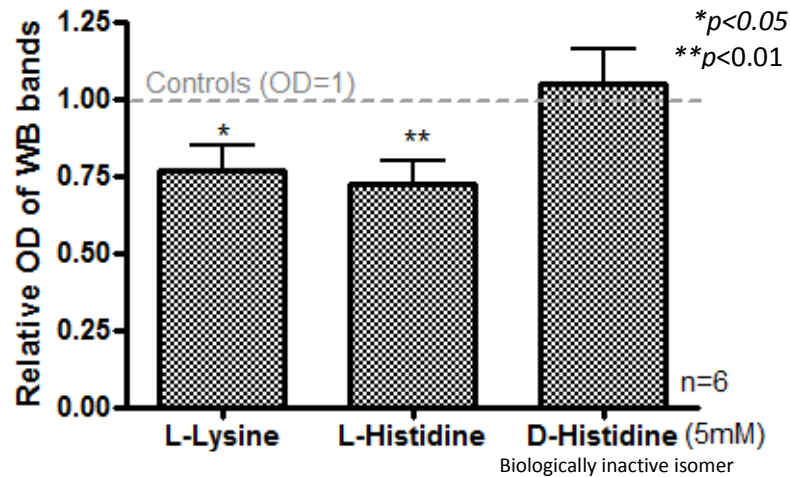


Figure 4. Comparisons of the effects of exogenous, free amino acids L-lysine, L-histidine (** $p < 0.01$) and D-histidine on involucrin protein expression. One-way ANOVA was used for the L-Lysine ($p < 0.05$) and L-histidine data (** $p < 0.01$); unpaired, two-tailed t-test was used for the data on D-histidine ($p = 0.67$, n.s.).

6.2.3 Histidine metabolites (histamine and urocanic acid) and the expression of epidermal barrier proteins in HaCaT monolayer cell cultures

As shown in Chapter 5, histamine, with and without H1+H2 antagonists, led to significant reduction in the penetration of Lucifer Yellow dye into the skin models, suggesting an improved barrier function ($p < 0.05$ and $p < 0.01$ respectively). Here, we investigate the effects of histamine on the expression of filaggrin, loricrin and involucrin.

The relative optical density of controls was scaled to 1 to allow inter-experimental comparisons to be made. All bands were standardised to housekeeping protein keratin 10 for loading control.

6.2.3.1 The effects of histamine (with and without histamine antagonists) on filaggrin

Western-blotting, followed by two-way ANOVA and Bonferroni post test ($F(1,30) = 17.79$), of HaCaTs from the monolayer cultures showed that $1 \mu\text{M}$ histamine, a key histidine metabolite, increased the expression of the 37-kDa filaggrin monomers (** $p < 0.001$, $n = 6$) and the 37kDa:120kDa filaggrin ratio ($p > 0.05$, n.s.), while having no effect on the expression of the 120kDa intermediate ($p > 0.05$, n.s., $n = 6$, Figure 5).

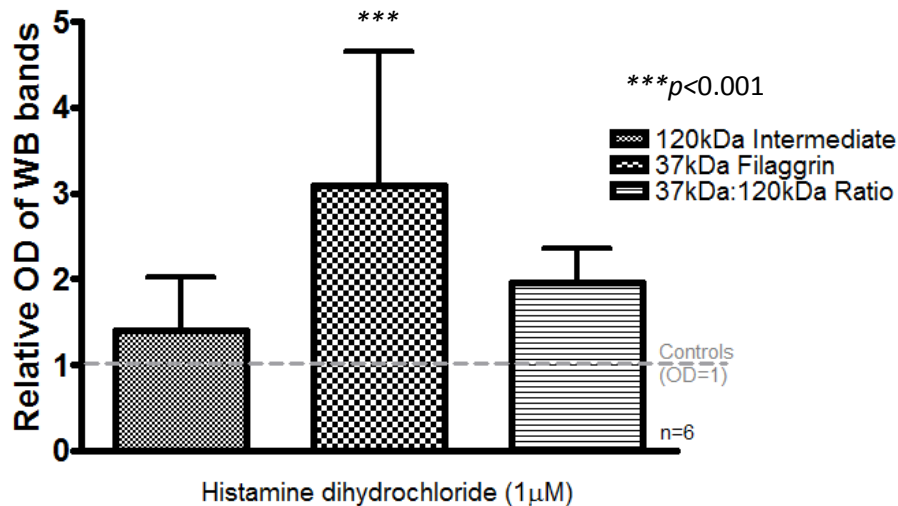


Figure 5. The effects of 1µM histamine on filaggrin protein expression. Two-way ANOVA showed no effect on the 120kDa filaggrin intermediate ($p>0.05$, n.s.), increased 37kDa filaggrin monomers ($***p<0.001$) and a non-significant increase in the 37kDa:120kDa ratio ($p>0.05$, n.s.).

In repeated experiments, two-way ANOVA with Bonferroni post test, $F(2,45)=16.59$, showed similar (though less than that of Figure 5) increase in the 37-kDa filaggrin monomers ($**p<0.01$, $n=6$) and the 37kDa:120kDa filaggrin ratio ($**p<0.01$, $n=6$) was noted with 1µM histamine, even in the presence of histamine-1 and -2 receptor antagonists (10µM pyrilamine and 10µM cimetidine), $***p<0.001$ [37-kDa filaggrin monomers] and $**p<0.01$ [37kDa:120kDa filaggrin ratio] (Figure 6).

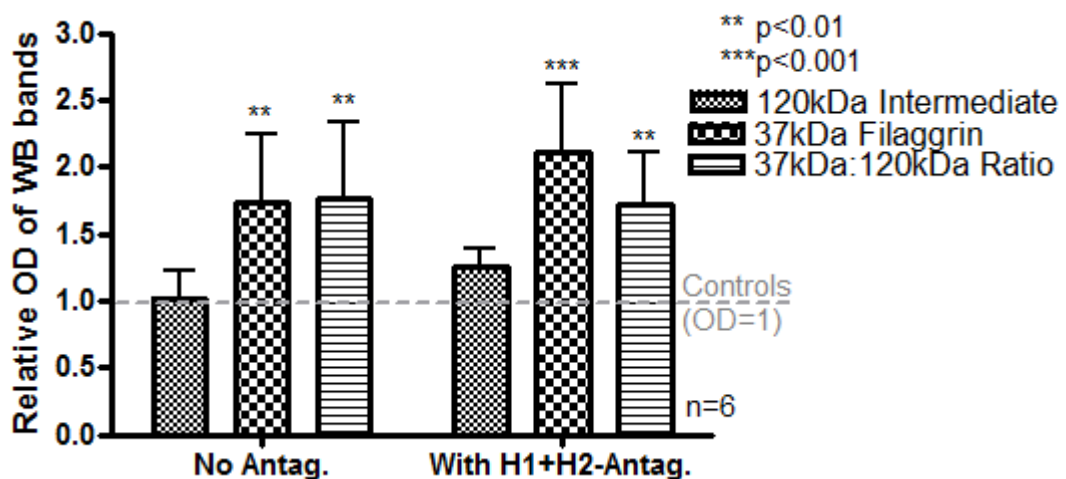


Figure 6. The effects of 1µM histamine, with and without H1+H2 receptor antagonists (pyrilamine maleate and cimetidine), on filaggrin protein expression. Two-way ANOVA was applied, showing significant increase in 37kDa filaggrin monomers with ($***p<0.001$) and without ($**p<0.01$) histamine antagonists and increase in the 37kDa:120kDa ratio with ($**p<0.01$) and without ($**p<0.01$) histamine antagonists.

6.2.3.2 The effects of histamine (with and without histamine antagonists) on loricrin and involucrin

Unpaired, two-tailed t-tests showed that 1 μ M histamine had no significant effect on the protein expression of loricrin ($t(9)=0.48$, $p=0.65$, n.s., $n=6$) and involucrin ($t(10)=1.90$, $p=0.09$, n.s., $n=6$) when compared to controls, Figure 7. The effects of histamine on loricrin and involucrin expression were investigated in two separate experiments, each with its own set of controls.

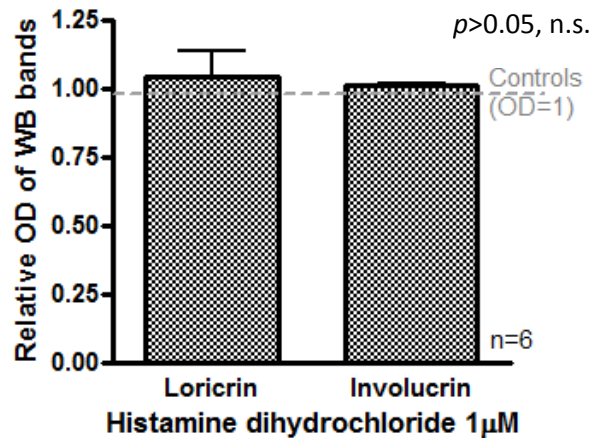


Figure 7. The effects of 1 μ M histamine on loricrin and involucrin protein expression. Unpaired two-tailed t-tests showed no difference between controls and the histamine group for both loricrin and involucrin expression, $p>0.05$, n.s.

In repeated experiments analysed with one-way ANOVA (Kruskal-Wallis and Dunn's post test), 1 μ M histamine again showed no effect on the expression of loricrin, with and without H1+H2 histamine receptor antagonists (10 μ M pyrilamine and 10 μ M cimetidine) when compared to control samples ($p>0.05$ for both with and without antagonists, n.s., $n=6$, Figure 8).

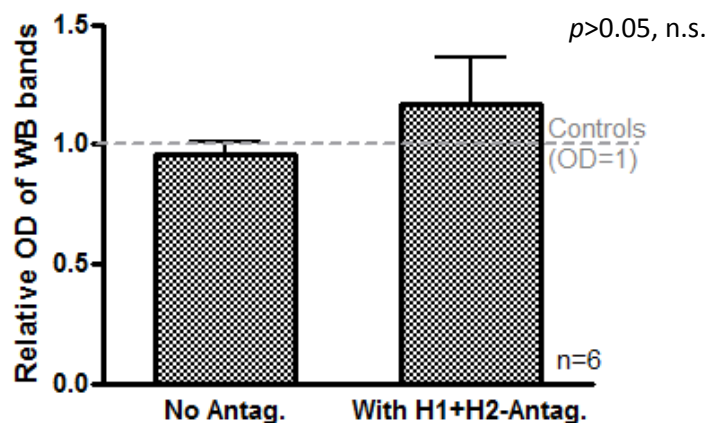


Figure 8. The effects of histamine, with and without H1+H2 receptor antagonists (pyrilamine maleate and cimetidine), on loricrin protein expression. One-way ANOVA showed no effect of histamine on loricrin expression. $p>0.05$, n.s.

In repeated experiments analysed with one-way ANOVA and Dunnett's post test ($F(2,15)=2.62$), $1\mu\text{M}$ histamine again showed no effect on the protein expression of involucrin, with and without H1+H2 histamine receptor antagonists ($10\mu\text{M}$ pyrilamine and $10\mu\text{M}$ cimetidine) ($p>0.05$ for both with and without antagonists, n.s., $n=6$, Figure 9).

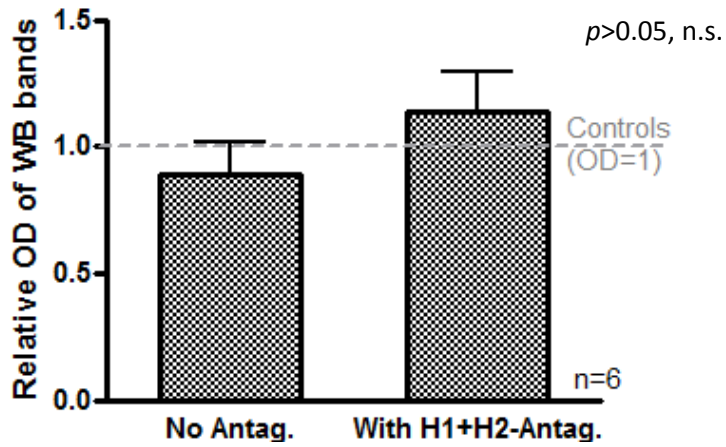


Figure 9. The effects of histamine, with and without H1+H2 receptor antagonists (pyrilamine maleate and cimetidine), on involucrin protein expression. One-way ANOVA showed no effect of histamine on involucrin expression. $p>0.05$, n.s.

6.2.3.3 The effects of *cis*-urocanic acid on filaggrin

Compared to controls (standardised OD of 1), linear regression analysis showed that *cis*-urocanic acid, *cis*-UCA, (another key histidine metabolite) increased the 120kDa filaggrin intermediate ($F(1,7)=18.20$, $**p=0.00$, $r^2=0.72$, $n=3$, Figure 10a) while having no significant effect on the expression of the 37kDa filaggrin monomers ($F(1,7)=0.02$, $p=0.89$, n.s., $r^2=0.00$, $n=3$, Figure 10b). This led to an overall dose-dependent reduction in the 37kDa:120kDa filaggrin ratio ($F(1,7)=18.34$, $**p=0.01$, $r^2=0.72$, $n=3$, Figure 10c), an effect which is opposite that of histamine.

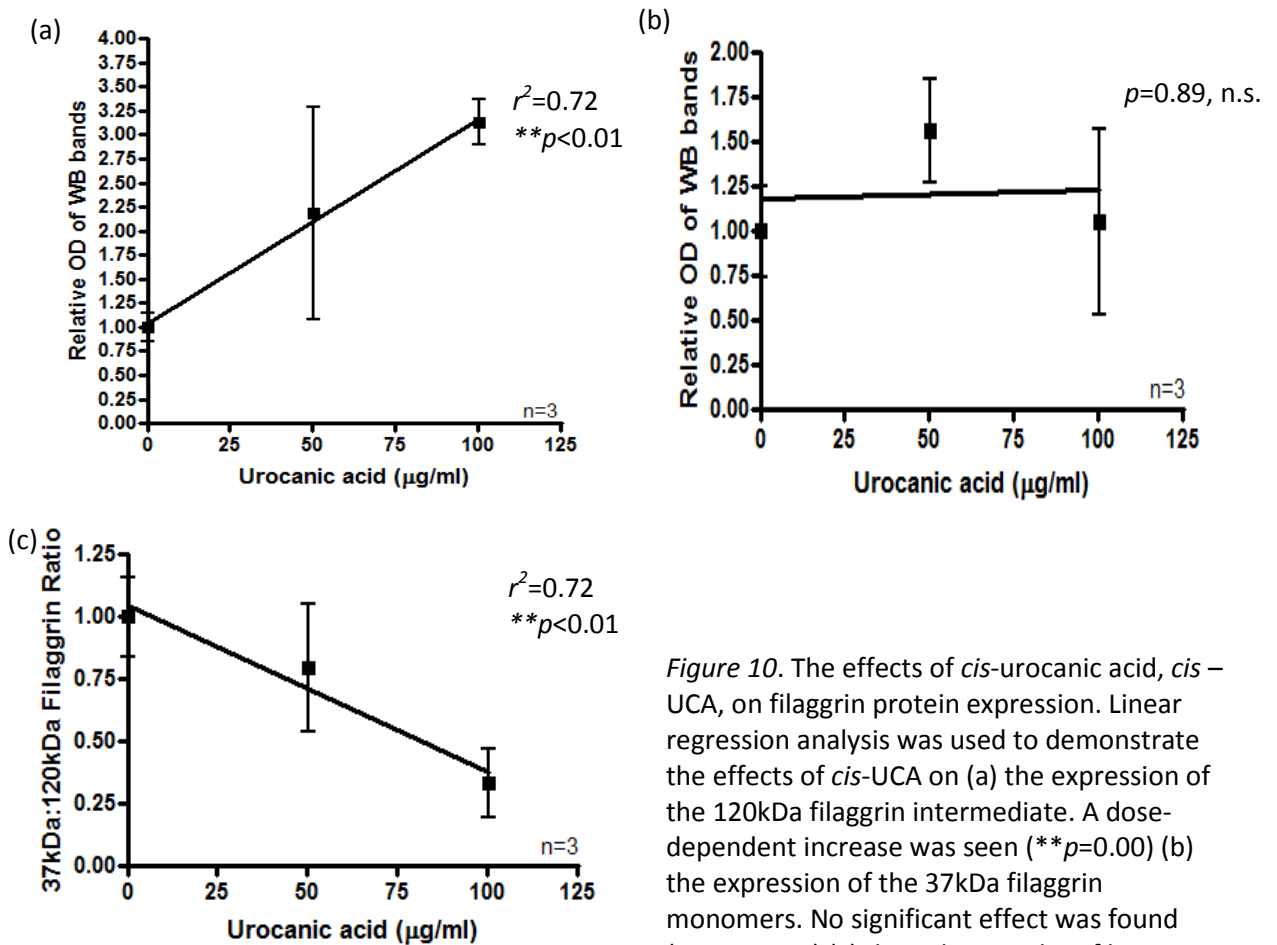


Figure 10. The effects of *cis*-urocanic acid, *cis* – UCA, on filaggrin protein expression. Linear regression analysis was used to demonstrate the effects of *cis*-UCA on (a) the expression of the 120kDa filaggrin intermediate. A dose-dependent increase was seen ($**p=0.00$) (b) the expression of the 37kDa filaggrin monomers. No significant effect was found ($p=0.89$, n.s.) (c) the 37kDa:120kDa filaggrin ratio. A dose-dependent decrease was seen ($**p=0.00$).

6.2.3.4 The effects of *cis*-urocanic acid on loricrin

Compared to controls (standardised OD of 1), linear regression analysis showed that *cis*-UCA increased loricrin protein expression in a dose-dependent manner ($F(1,7)=22.15$, $**p=0.00$, $r^2=0.76$, $n=3$, Figure 11).

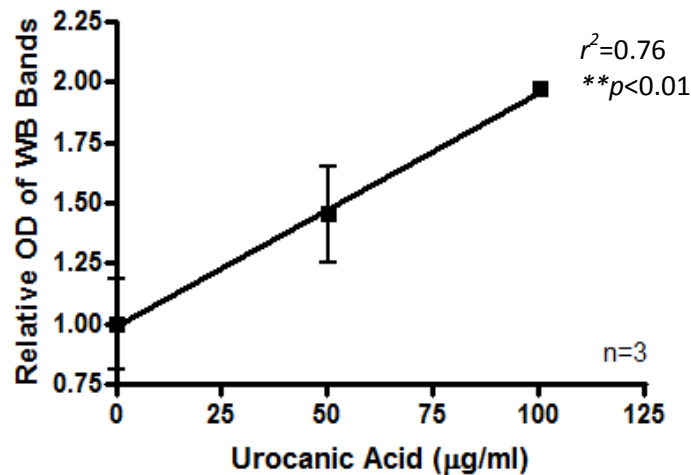


Figure 11. The effect of *cis*-urocanic acid on loricrin protein expression. Linear regression analysis was used. A dose-dependent increase was seen ($**p=0.00$).

6.2.3.5 The effects of *cis*-urocanic acid on involucrin

Linear regression analysis showed that *cis*-UCA had no significant effect on the protein expression of involucrin ($F(1,7)=1.55$, $p=0.25$, $r^2=0.18$, $n=3$, Figure 12).

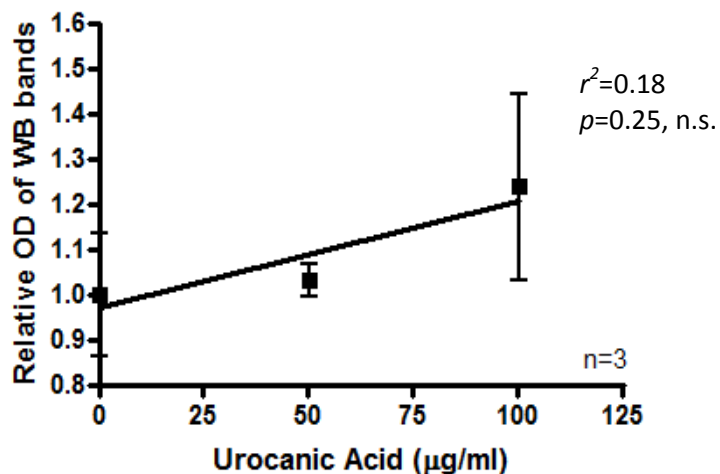


Figure 12. The effect of *cis*-urocanic acid on involucrin protein expression. Linear regression analysis was used. No significant effect was found ($p=0.25$, n.s.).

6.2.4 L-serine and the expression of filaggrin in HaCaT monolayer cell cultures

L-serine was investigated as a potential negative control for histidine as, similar to histidine, this amino acid is found in abundance in the filaggrin protein. L-serine, however, does not have the imidazole ring of histidine.

The relative optical density of controls was scaled to 1 to allow inter-experimental comparisons to be made. All bands were standardised to housekeeping protein keratin 10 for loading control.

Linear regression analysis showed a dose-dependent reduction in the expression of the 120kDa filaggrin intermediate was seen in HaCaTs grown in L-serine-enriched media ($F(1,7)=10.55$, $*p=0.01$, $r^2=0.60$, $n=6$ for controls, $n=1$ for 1,2,5mM, Figure 13a), with corresponding increase in the expression in the 37kDa filaggrin monomers ($F(1,7)=9.25$, $p=0.02$, $r^2=0.57$, $n=6$ for controls, $n=1$ for 1,2,5mM, Figure 13b). HaCaTs grown in media supplemented with 1,2 and 5mM serine therefore showed a dose-dependent increase in the 37kDa:120kDa filaggrin ratio ($F(1,7)=122.6$, $p<0.0001$, $r^2=0.95$, $n=6$ for controls, $n=1$ for 1,2,5mM, Figure 13c).

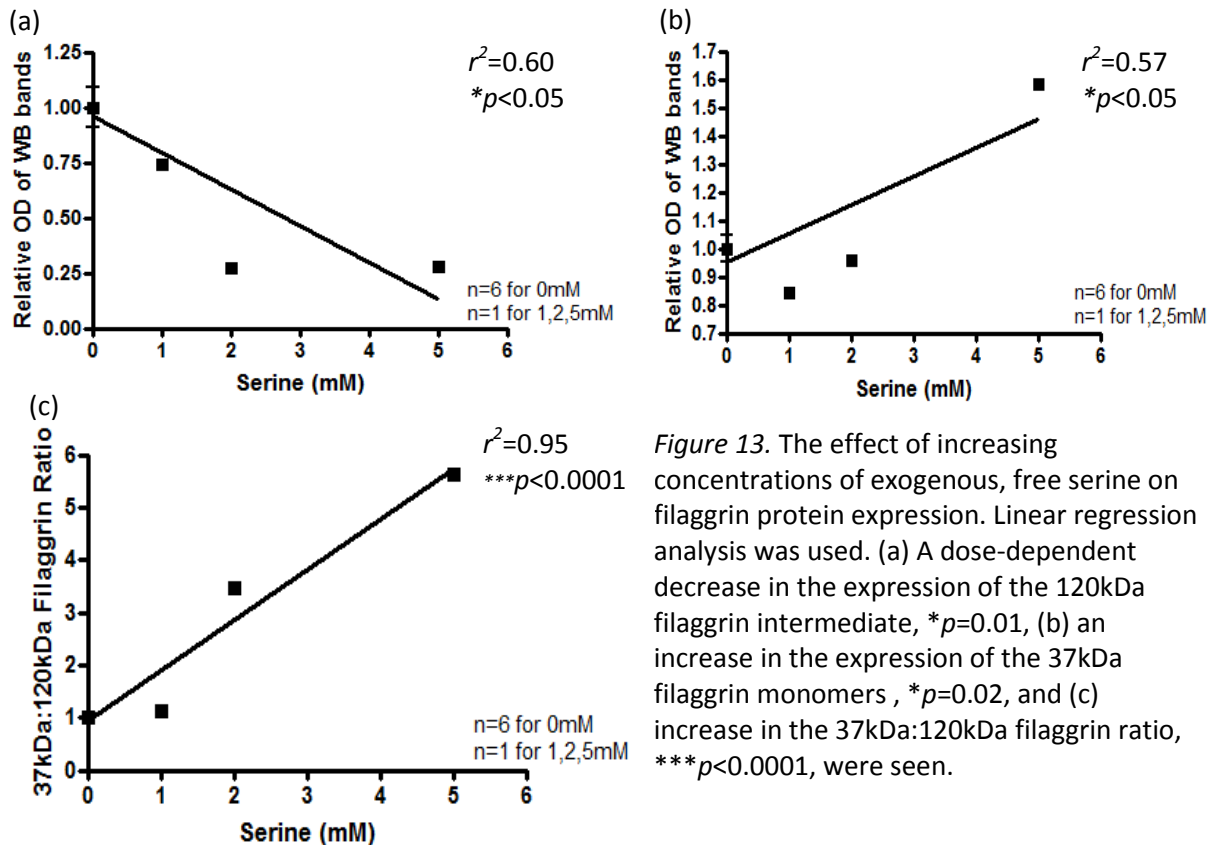


Figure 13. The effect of increasing concentrations of exogenous, free serine on filaggrin protein expression. Linear regression analysis was used. (a) A dose-dependent decrease in the expression of the 120kDa filaggrin intermediate, $*p=0.01$, (b) an increase in the expression of the 37kDa filaggrin monomers, $*p=0.02$, and (c) increase in the 37kDa:120kDa filaggrin ratio, $***p<0.0001$, were seen.

6.2.5 pH of cell culture media and the expression of epidermal barrier proteins in HaCaT monolayer cell cultures.

In Chapter 5, we have shown that compared to controls (pH7.4), high pH of 8.1 was associated with increased penetration of Lucifer Yellow dye into our skin models ($p < 0.01$) while low pH of 6.8 made no significant difference. Here, we investigate if the observed changes are associated with changes in the protein expression of filaggrin, loricrin and involucrin.

The relative optical density of controls was scaled to 1 to allow inter-experimental comparisons to be made. All bands were standardised to housekeeping protein keratin 10 for loading control.

6.2.5.1 The effects of varying the pH of cell culture media on filaggrin

Controls were grown in culture media with an average pH of 7.4.

Western-blotting, followed by analysis using two-way ANOVA and Bonferroni post test, showed that reducing the pH of the cell culture media to an average of 6.8 with 20mM Pipes buffer led to an increase in the expression of the 37kDa filaggrin monomers in relative to the 120kDa intermediate, as indicated by a rise in the 37kDa:120kDa ratio ($n=6$, Figure 14). The result was, however, not statistically significant.

The opposite effect was seen when the pH of the cell culture media was raised to an average of 8.1 with 20mM Tris buffer. A small reduction in the 37kDa:120kDa ratio was observed ($n=6$, Figure 14). This result was, again, not statistically significant.

These results suggest that varying pHs have no significant affect on filaggrin protein expression, $F(2,45)=0.09$, $p=0.92$, n.s.

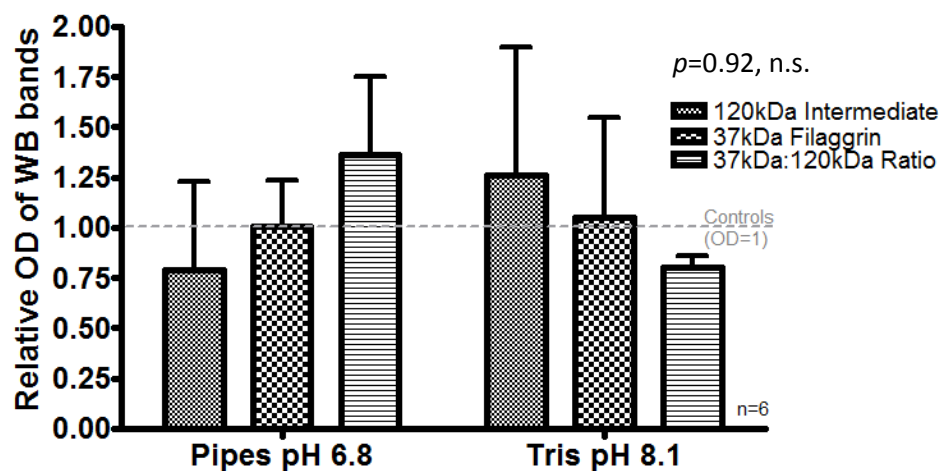


Figure 14. Comparisons of the effects of different pHs of the cell culture media on filaggrin protein expression. pH of the controls was 7.4. Two-way ANOVA showed that pH 6.8 and 8.1 had no significant effect on the protein expression, $p=0.92$, n.s.

6.2.5.2 The effects of varying the pH of cell culture media on loricrin

One-way ANOVA with Dunnett's post-test showed that changing the pH of the cell culture media to pH6.8 with 20mM Pipes and to pH8.1 with 20mM Tris had no significant effect on the protein expression of loricrin, as compared to controls (pH 7.4), $F(2,15)=1.53$, $p=0.25$, n.s., Figure 15.

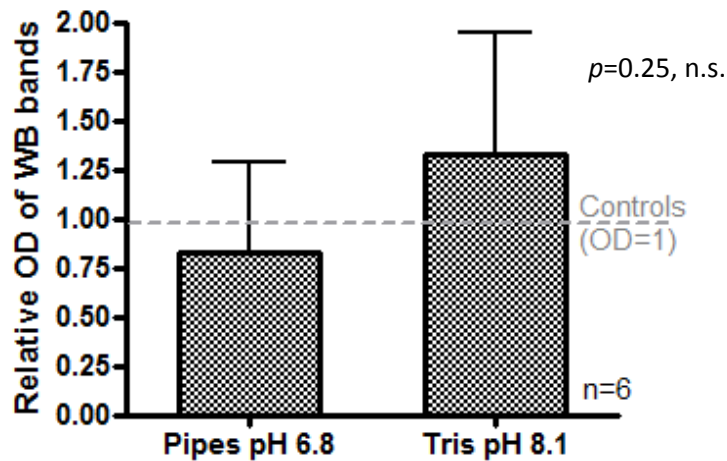


Figure 15. Comparisons of the effects of different pHs of the cell culture media on loricrin protein expression. pH of the controls was 7.4. One-way ANOVA showed that pH 6.8 and 8.1 had no significant effect on the protein expression, $p=0.25$, n.s.

6.2.5.3 The effects of varying the pH of cell culture media on involucrin

Similarly, one-way ANOVA with Dunnett's post-test showed that changing the pH of the cell culture media to pH6.8 with 20mM Pipes and to pH8.1 with 20mM Tris had no significant effect on the protein expression of involucrin, as compared to controls (pH7.4), $F(2,15)=0.74$, $p=0.49$, Figure 16.

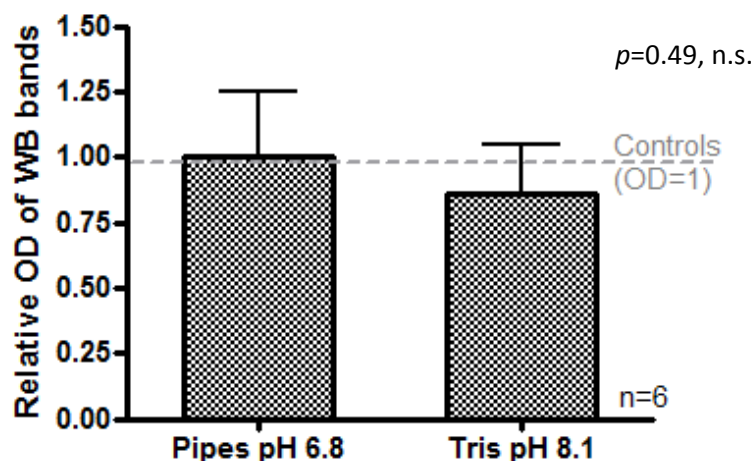


Figure 16. Comparisons of the effects of different pHs of the cell culture media on involucrin protein expression. pH of the controls was 7.4. One-way ANOVA showed that pH 6.8 and 8.1 had no significant effect on the protein expression, $p=0.49$, n.s.

6.2.6 Summary of results

Table 2

Summary of Results

Experiments	Effects of Epidermal Barrier Proteins Expression				
	Filaggrin			Loricrin	Involucrin
	120kDa Intermediate	37kDa monomers	37kDa:120kDa filaggrin ratio		
<u>Amino Acids</u>					
• L-lysine	n.s.	n.s.	n.s.	n.s.	↓(p<0.05)
• L-histidine	n.s.	n.s.	↑↑(p<0.01)	n.s.	↓↓(p<0.01)
• D-histidine	n.s.	n.s.	n.s.	n.s.	n.s.
• L-serine	↓(p<0.05)	↑(p<0.05)	↑↑↑(p<0.0001)	-	-
<u>Histamine</u>					
• Without H1+H2 antagonists	n.s.	↑↑(p<0.01)	↑↑(p<0.01)	n.s.	n.s.
• With H1+H2 antagonists	n.s.	↑↑↑(p<0.001)	↑↑(p<0.01)	n.s.	n.s.
<i>cis</i> -UCA	↑(p<0.01)	n.s.	↓(p<0.01)	↑(p<0.01)	n.s.
<u>pH</u>					
• pH 6.8	n.s.	n.s.	n.s.	n.s.	n.s.
• pH8.1	n.s.	n.s.	n.s.	n.s.	n.s.

n.s.- Not significant

6.3 Chapter 6 Discussion

In Chapter 5, we demonstrated that L-histidine and histamine were associated with improved barrier property of our organotypic skin-equivalent model. Here in our HaCaT monolayer cultures, we have shown that L-histidine and histamine were associated with significantly increased expression of the 37kDa filaggrin monomers in relative to the 120kDa filaggrin intermediate. This effect was seen (in both organotypics and monolayer cultures) even in the presence of histamine-1 and -2 receptor antagonists, suggesting a histamine-related but H1 and H2 receptors-independent mechanism. These effects were, however, not seen with organotypics and monolayer cultures grown in media enriched with D-histidine, which is a structurally similar but biologically inactive isomer of L-histidine. The D-histidine experiment suggests that our findings were due to biological, not chemical, events occurring within the keratinocytes which were triggered by L-histidine and/or its metabolites.

Cis-UCA, the other metabolite of histidine, on the other hand, increased the expression of the 120kDa filaggrin intermediate while having no effect on the expression 37kDa monomers in the

monolayer cultures. This is mirrored by the results of our organotypic models which showed no improvement in barrier property associated with raised *cis*-UCA. *Cis*-UCA is known to initiate the transcription of numerous genes in primary human keratinocytes, including that of serpin peptidase inhibitor (SERPINB2)⁴⁵. SERPINB2 (also known plasminogen activator inhibitor-2, PAI2) is located intracellularly at the suprabasal layer of the keratinocytes⁴⁶. Like many serpins, it has a broad inhibitory profile; it is yet unknown all the proteases it regulates⁴⁶. It is possible that the upregulation of SERPINB2 by *cis*-UCA may have impacted on the proteolysis of filaggrin, resulting in the accumulation of the 120kDa filaggrin intermediate.

Results from both our organotypic models and monolayer cultures suggest that increased expression of the 37kDa filaggrin monomers may be associated with improvement in skin barrier function. The increase in the expression of the 37kDa monomers may be, as suggested here, due to increased proteolytic degradation of the 120kDa filaggrin intermediate. This may lead to improved keratin-aggregation. One must, however, bear in mind that the 37kDa filaggrin monomers are not the final end product of profilaggrin proteolysis. The filaggrin monomers are further degraded into smaller filaggrin fragments by proteases including calpain-1⁴⁷ and caspase-14. Caspase-14 deficient mice have been shown to have accumulation of 15-25kDa filaggrin fragments⁴⁸. These fragments are finally degraded by bleomycin hydrolase⁴⁷ into free amino acids which constitute the NMF. In view of the fact that the final end-products of profilaggrin proteolysis are these free amino acids, any increase in the expression of the 37kDa monomers may also represent a reduction in its breakdown into smaller peptides and amino acids. In short, the 37kDa monomer is simply another intermediate product, with a rather important function, in profilaggrin metabolism. Future works are needed to improve our understanding on whether the histidine-associated increase in the expression of the 37kDa filaggrin was due to increased degradation of the 120kDa intermediate or caused by a reduction in its own metabolism into free amino acids (or both). These include quantitating and comparing the amount of smaller filaggrin peptides and free amino acids to the amount of 37kDa monomers and 400kDa profilaggrin expressed by the keratinocytes using Western-blotting, MALDI-TOF MS or amino acid analyser. Increased 37kDa filaggrin monomers may also lead to increased amino acid breakdown products, which constitute a major part of the NMF. If that were the case, one may argue that the improvement in barrier function seen with our organotypic models may be due to either 1) improvement in keratin-aggregating function due to increased functional filaggrin monomers or 2) increased level of NMF in the skin models due to increased substrate availability (i.e. more filaggrin monomers) for the proteolytic degradation.

In our experiment, we have shown that L-serine (an amino acid which is found in abundance in filaggrin protein) also led to a significant increase in the expression of the 37kDa filaggrin

monomers, with corresponding decrease in the 120kDa filaggrin intermediate. The dose-dependent effect was supported by simple linear regression analysis which revealed a p-value less than 0.0001 (and r^2 of 0.95) for the 37kDa:120kDa filaggrin ratio, suggesting that observation is real despite the low n number ($n=1$ for experiments involving 1, 2 and 5mM serine). This result seems to refute our hypothesis that the ability of histidine to influence filaggrin protein expression is due to its imidazole ring, which serine does not have. Having said this, an imidazole ring is not an absolute requirement for participation in end-product inhibition of an enzymatic reaction; serine, valine and lysine are end-products which have been shown to be able to inhibit the initial enzyme reactions which result in their production³². No improvement in the barrier property of the skin model was, however, seen with raised serine in Chapter 5. These two results, put together, suggest that increasing the expression of the 37kDa monomers alone is not sufficient to improve skin barrier function significantly. The physical or chemical effect of the imidazole ring of histidine, on its own, is also not sufficient to improve the barrier function significantly, as the biologically inactive D-histidine did not lead to barrier improvement in the skin model. Perhaps a synergistic interaction between both biological (increased filaggrin monomers expression) and chemical (hygroscopicity due to imidazole ring) effects are needed for a significant improvement in skin barrier function to be seen.

We have shown that *cis*-UCA significantly increased the protein expression of loricrin in a dose-dependent manner. *cis*-UCA is known to regulate the genetic transcription of both Th-1 and Th-2 cytokines in primary human keratinocytes⁴⁵. Cytokines have been shown to alter the gene and protein expression of loricrin⁴⁹. The expression of loricrin may therefore be subjected to immunoregulation, indirectly influenced by *cis*-UCA. It is also reasonable to suggest that the effects of UCA on loricrin and filaggrin expression seen here may be due to the acidic pH of UCA. UCA has been said to be one of the major acid-base regulators in the epidermis⁵⁰. The pH of UCA-enriched media used here in this study was not measured. Nonetheless, the amount of UCA added to the media was small (50-100 $\mu\text{g/ml}$) and not observed, to the naked eye, to cause any significant change in the colour of the media. The colour of the media is pH-dependent. Amino acids (in the media) are known to be good pH buffers. We have also shown that acidic pH of 6.8 did not have significant effects on the expression of loricrin and filaggrin. Despite the apparent significance of loricrin in the CE, loricrin-knockout mice exhibit only transient shiny, translucent skin with erythematous background which disappears 4-5 days after birth. Epidermal barrier function of these loricrin-deficient mice is not impaired and they have normal transepidermal water loss. This is thought to be due to a compensatory increase in the expression of other cornified envelope proteins such as repetin and small proline-rich protein¹⁸. In short, changes to the level of loricrin protein expression alone do not seem to have any significant impact on the overall barrier function. We have previously

shown that *cis*-UCA, despite being associated with a possible increase in loricrin expression as shown here, had no significant impact on the barrier function of our skin model. Interestingly, there appears to be a link between the transcriptional control of loricrin and filaggrin. The mRNA levels of filaggrin and loricrin were found to change in parallel in response to different culture conditions⁴². Yoneda *et al* have suggested that the filaggrin N-terminal domain, which undergoes nuclear localization following cleavage from main profilaggrin protein, interacts with loricrin and keratin 10, possibly playing a regulatory role in the formation of the CE⁵¹. In our study, *cis*-UCA which increased loricrin protein expression, was associated with an increased level of 120kDa filaggrin intermediate but caused no change to the expression of the 37kDa filaggrin monomers. Other factors such as L-histidine and histamine which led to changes in the filaggrin protein expression did not lead to any significant change in the loricrin protein expression in our cultures. Our results should not be compared to that from previous studies which showed relations between the transcriptional control, i.e. mRNA levels of filaggrin and loricrin as it was the post-translational proteolytic processing of filaggrin protein that was being examined here. The filaggrin N-terminal domain was also not examined in this study.

L-histidine and L-lysine were found to significantly reduce the protein expression of involucrin. The apparent reduction in involucrin protein expression may represent reduced extractability due to increased incorporation into matured CE. Increased water content has been shown to promote transglutaminase-mediated maturation of the CE in the stratum corneum⁵². Free amino acids histidine and lysine may lead to an increased level of skin hydration. It is proposed that involucrin is the first protein to be deposited in the cornified envelope and serves as a scaffold for other envelope proteins⁵³. It is also the main substrate for the attachment of ceramides to the cornified envelope²⁶. Despite earlier conclusion that it is an essential component of the cornified envelope, it has been shown that the absence of involucrin alone does not lead to barrier compromise. Involucrin knockout mice are viable, reproduce normally, possess normal-looking cornified envelope, normal epidermis and hair-follicle⁵⁴. This is rather surprising and has led to the suggestion that the absence of involucrin is compensated by the increased synthesis of other components of the cornified envelope, as with the case of loricrin. The reduction in the protein expression of involucrin, due to L-histidine and L-lysine seen in our experiments, may therefore have no significant effect on skin barrier property. In Chapter 5, we have indeed shown that the barrier function of our skin models grown in lysine-enriched media was not significantly different from that of controls. The significant improvement in barrier property seen with L-histidine in our skin model was probably related to the effect of L-histidine on filaggrin protein expression rather than involucrin.

The human stratum corneum has an acidic mantle of pH 4-6⁵⁵. It is known that neutralisation of the acid mantle of the stratum corneum affects its integrity by activating serine proteases which deactivate lipid-processing enzymes and degrade corneodesmosomes⁵⁶. Skin pH also influences antimicrobial activities of the stratum corneum⁵⁷. In previous chapter, we have shown that an alkaline environment is associated with poorer barrier function and abnormal spatial distribution of keratin 10, suggesting a negative effect on the keratinocyte differentiation process. Here, we have shown that altering the pH had no significant effect on the protein expression of loricrin and involucrin. For filaggrin, low pH 6.8 reduced the 120kDa filaggrin intermediate while high pH8.1 had the opposite effect although these results are not statistically significant. These results suggest that the pH-associated perturbation of skin barrier is not associated with changes in the expression of the epidermal barrier proteins examined in this study.

In conclusion, we have shown that altering the composition of the free amino acids in the cell culture media can affect the expression of epidermal barrier proteins in keratinocytes, although changes to the protein expression alone may not have any significant impact on the overall skin barrier function. In particular, we have shown that free L-histidine and histamine increased the expression of the 37kDa filaggrin monomers, in relative to the 120kDa filaggrin intermediate. Bearing in mind the importance of the 37kDa filaggrin monomers, this may have an important effect on the permeability barrier function of the stratum corneum.

6.4 Chapter 6 References

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6.5 Chapter 6 Materials and Methods

6.5.1 HaCaT monolayer cell cultures

HaCaT keratinocytes of passages 33-41 (gift from Dr J Woods, Dundee) were seeded at 0.5×10^6 cells/ml into standard 6-well plates and maintained without further passaging for 21 days in standard cell culture media (D-MEM/F-12 with GlutaMAX [Gibco, Paisley, UK] plus 10% fetal bovine serum and 1% penicillin/streptomycin). The HaCaTs achieved confluency within 48-72 hours. From day 15-21, the culture media was supplemented with additional 1-5mM of amino acids (L-lysine, L-serine, L-histidine, D-histidine), 1 μ M histamine dihydrochloride (with or without 10 μ M pyrilamine maleate and 10 μ M cimetidine), 50 μ g/ml and 100 μ g/ml *cis*-UCA (Sigma, Dorset, UK).

For the pH study, HaCaTs were grown in RPMI1640 media (PAA, Pasching, Austria) plus 10% fetal bovine serum, 1% penicillin/streptomycin and 1% glutamine. From day 15-21, pH of culture media was adjusted to 6.8 and 8.1 using 20mM Pipes (pKa6.8) and 20mM Tris (pKa8.1) buffers respectively (Sigma, Dorset, UK). On Day 21, HaCaT cells were trypsinised and spun at 280g for 5 minutes to form a pellet. The supernatant was discarded and the cell pellet lysed with x2-x4 in-house Laemmli buffer (400 μ l per pellet derived from 1 well). The whole cell lysates were spun through NucleoSpin filters (violet ring; Macherey-Nagel, Duren, Germany) at 11000g for 1 minute. The flow-through was stored at -20 $^{\circ}$ C, to be used for protein gel electrophoresis.

6.5.2 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western-blotting

Total cellular protein from whole cell lysates prepared from HaCaT monolayers were resolved by 9% SDS-PAGE with individual proteins identified through Western-blotting following overnight transfer onto nitrocellulose membrane at 0.2 mA/cm² of membrane. Primary antibodies against the following antigens were used: filaggrin (goat polyclonal antibody; Santa Cruz, CA, USA), involucrin (mouse monoclonal; Sigma, Dorset, UK), loricrin (rabbit polyclonal; Sigma, Dorset, UK) and cytokeratin 10 (rabbit monoclonal; Abcam, United States).

Cytokeratin 10 bands on the Western-blot were visualised using 4-chloro-1-naphthol: A small amount of 4-chloro-1-naphthol was dissolved in 2-3 drops of methanol. 45ml of dddH₂O and 5ml of 100mM Tris-HCL (pH7.4) were added to the 4-chloro-1-naphthol, followed by 50 μ l of hydrogen peroxide. The mixture was added to the membrane and left for 5-10 minutes for the bands to appear. The reaction can be stopped by washing the membrane with H₂O. The membrane was then left to air-dry.

Filaggrin, loricrin and involucrin bands on the Western-blot were visualised using SuperSignal West Femto chemiluminescent substrate (Thermo Scientific, IL, USA).

The VersaDoc Imaging System (Bio-Rad) was used to capture the bands on the Western-blot. Quantitative densitometric analysis of the protein bands was also done using the VersaDoc Imaging System. All bands were background-corrected and normalised to cytokeratin 10 for equal loading. The average optical density of control bands in each experiment was standardised to 1 to allow for inter-experimental comparisons.

6.5.3 Statistical analysis

Unpaired, two tailed t-test was used when comparing between two groups. Unless otherwise stated, one-way analysis of variance, ANOVA, with Dunnett's post-test was used when more than two groups were involved (except when data involved more than one filaggrin intermediate per category on the x-axis, in which case a two-way ANOVA with Bonferroni post test was used). Simple linear regression analysis was used for the UCA experiments and those involving amino acids in different concentrations. These were performed using GraphPad Prism version 4.00 for Windows, GraphPad software, San Diego California USA, www.graphpad.com. Data were presented as mean, $M \pm$ standard deviation, SD . p-values of less than 0.05 were considered as significant. All images were back-ground corrected.

Chapter 7

A Pilot Human Study and Future Works

Good medical research is followed by clinical application of basic discoveries. Here, we present the preliminary data from our pilot human study which suggests that topical application of histidine can accelerate the repair of damaged skin barrier. This provides a proof of principle to our hypothesis that histidine is beneficial to skin barrier function in human and sets the foundation for larger clinical trials in the future.

7.1 Introduction: Transdermal drug delivery

A great advantage of dermatological research is that the skin is essentially an epithelium on the outside. Given its accessibility and large surface area, the skin offers an excellent route for drug delivery and testing. Direct observation and clinical measurements of the resulting effects can be easily carried out. Transdermal drugs have the potential to reach a wider population as they can usually be self-administered and are therefore ideal in areas lacking medical personnel, for example in developing countries. They are generally less painful and cheaper than those administered by oral, intravenous, intramuscular or subcutaneous routes and can be released over a prolonged period of time; therefore compliance with the treatment is also higher. First-pass metabolism by the liver, a problem seen with many oral preparations, is less of an issue with transdermal drugs. The lack of hypodermic waste, which is associated with increased risk of disease-transmission through re-using needle, is another added benefit in developing nations¹. In addition, transdermally delivered drugs generate a stronger immune response through targeted delivery to the antigen presenting cells in the skin, making this an attractive alternative delivery route for vaccines¹⁻⁴. Last but not least, it makes good sense to treat a skin condition with a topical preparation, directly delivering the drug to where its highest concentration is required and reducing any unwanted systemic side effects. As demonstrated in previous chapters, skin models grown in histidine-enriched culture media showed improved barrier function, possibly associated with an increase in the expression of filaggrin protein. Can we, therefore, improve the barrier function of human skin by targeted delivery of histidine to the granular layer in the epidermis where filaggrin is found?

To do this, one has to overcome the physical and chemical barrier conferred by the stratum corneum. This is the outermost layer of human epidermis which is 10-20 μm in thickness. The stratum corneum is located on top of several inner layers of viable but avascular epidermis measuring 50-100 μm in thickness. Underneath this avascular epidermis is the highly vascular dermis which contains a network of capillaries, below the dermal-epidermal junction. These capillaries allow systemic absorption of drugs. Drug penetration through the skin occurs through three routes: transcellular, intercellular and through the eccrine sweat glands and hair follicles (appendageal)⁵. One of the main challenges in transdermal drug delivery is to overcome the lipid bilayer found in the intercellular space in the stratum corneum. Much research has gone into overcoming this lipid barrier in order to optimize controlled delivery of drugs via the skin to the right target area and at the right concentration. This has resulted in a large variety of topical preparations (creams, ointments and some 20 FDA-approved transdermal systems) currently available to treat or enhance the functionality of the skin¹. These transdermal systems can be categorized into three generations of development¹.

The first generation transdermal drugs are mainly lipophilic, small molecules (up to several hundred Daltons) that are effective at low doses (in milligrams per day)¹. Examples of these are the nicotine, oestrogen and fentanyl patches. They rely on diffusion through the lipid bilayer surrounding the corneocytes. Lipophilic molecules are able to diffuse via the lipid tails while the hydrophilic ones through the lipid head regions. There are a limited number of drugs which can be delivered via this mechanism. This has led to the development of second generation transdermal drugs, where reversible, molecular-scale disruption of the stratum corneum is carried out to enhance the drug delivery¹. This can be done using non-cavitation ultrasound (oscillating pressure wave at a high frequency which disrupts the lipid structure and leads to increased permeability)⁶, iontophoresis (application of low-dose current to facilitate movement of charged molecules across the stratum corneum)⁷ and chemical enhancers^{5,8-10}. An example of the latter is the insertion of amphiphilic molecules into the lipid bilayers. While the second generation transdermal systems enhance the delivery of small molecules, it bears negligible impact on the delivery of macromolecules such as proteins and DNA. The disruption is also not localised to the stratum corneum and can cause pain, skin irritation, heat generation and damage to deeper tissues. This has led to the development of the third generation transdermal systems, where the molecular or micron-scale disruption of the skin barrier is strictly localised to the stratum corneum¹. This allows more aggressive disruption to be performed and enables the penetration of macromolecules including proteins, DNA and vaccines. Examples of these include thermal ablation (controlled heating of the skin surface to hundreds of degrees for micro- to milliseconds leading to perforation of the stratum corneum without damaging deeper structures)⁴, microneedles (micron-scale piercing through the epidermis)^{3,11,12}, removal of the stratum corneum by abrasion (microdermabrasion or by using sandpaper)^{2,13} and cavitation ultrasound (ultrasound-induced formation, oscillation and collapse of bubbles at the skin surface which result in liquid microjet and shock waves targeted at the stratum corneum)^{6,14}.

In our experiments, in order to deliver histidine to the stratum granulosum, repeated tape-stripping was used to overcome the barrier conferred by stratum corneum. This was done on myself and a well-informed fellow dermatology doctor/ researcher who has inactive AD. A diseased skin often has poor barrier function. Even in the absence of symptoms, the skin barrier of a susceptible individual may be suboptimal. Asymptomatic patients with healed or inactive atopic dermatitis were found to have a higher baseline transepidermal water loss, TEWL and reduced level of skin hydration than those with no history of skin diseases¹⁵. Drug penetration through diseased skin may arguably be easier. As mentioned in earlier chapters, skin barrier is considered abrogated when the measured TEWL is above $20\text{ g m}^{-2}\text{ hour}^{-1}$. Following abrogation of the skin barrier, histidine is topically applied

and reapplied every 24 hours (as detailed in Materials and Methods) with measurements of TEWL taken every 48 hours for 4 days. One may argue that in this experiment, we were comparing the effects of histidine on the reparative function of the skin (healthy individual vs inactive AD), rather than on the improvement of barrier function *per se*. Nonetheless, the aim of this pilot study is to provide a proof of principle that histidine is beneficial to the human skin barrier. If this is indeed the case, the study will set the foundation for a future clinical trial. We shall, therefore, not shift the focus from the complex to the miniscule.

7.2 Chapter 7 Results

Here, the results of a pilot double-blinded human study involving the topical application of amino acid-enriched solutions aimed at facilitating skin barrier repair are presented. 5 rows of test areas, spanning from proximal to distal arm of a single AD participant and a single healthy control, were identified and marked. No active disease was evident at all test areas. Each row, labelled Row A to Row E, consisted of 5 individual test areas (see Figure 3 in Materials and Methods). The baseline TEWL was measured (in triplicates) in all 25 test areas, followed by tape stripping process to raise the TEWL to between 20 to 30 g m⁻² hour⁻¹ in these areas. Phosphate-buffered saline, PBS, was applied to test areas in Row A (to function as controls); L-lysine, L-histidine, L-serine and D-histidine were applied to Rows B, C, D and E respectively. TEWL was re-measured at Day 2 and 4 (see Materials and Methods).

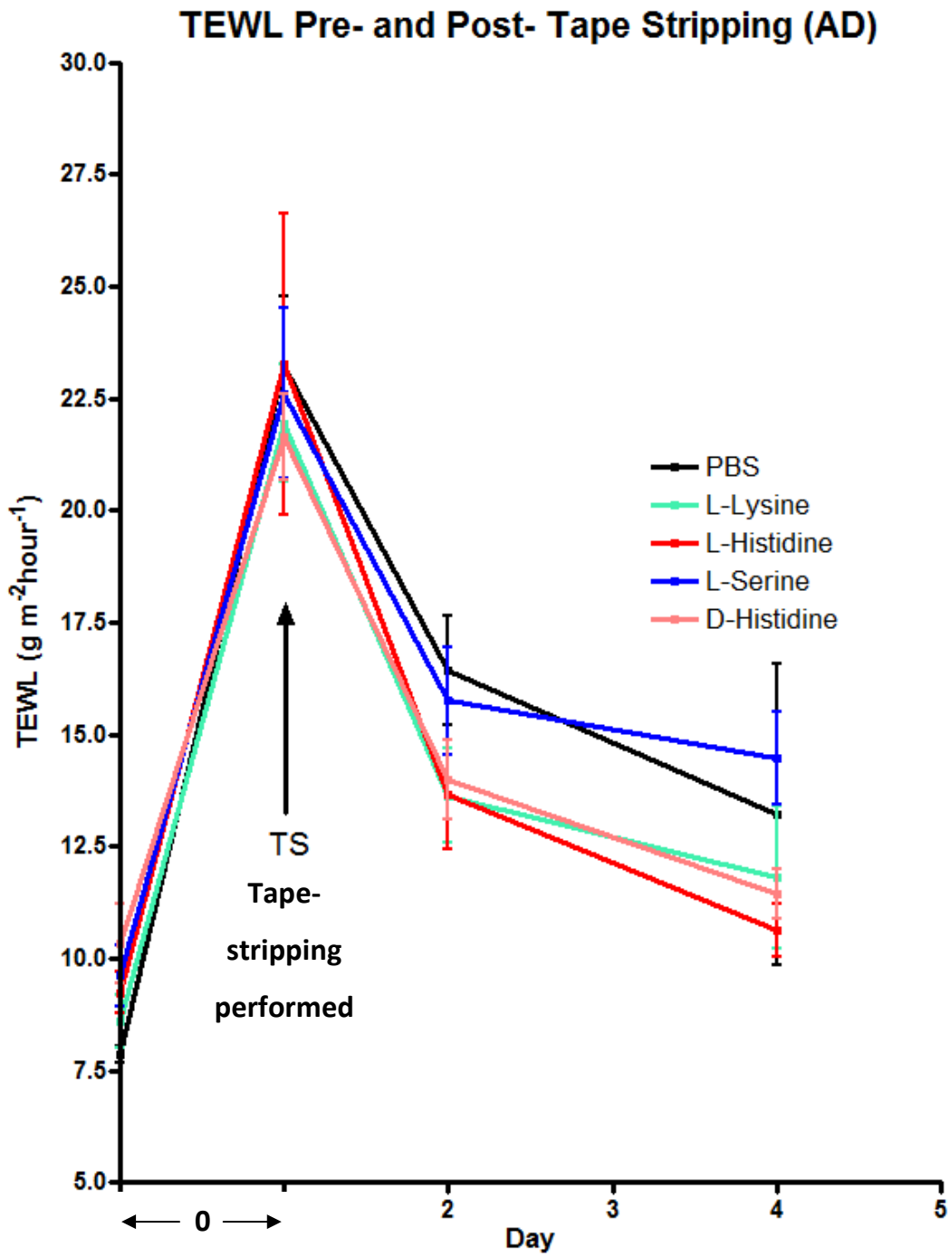
7.2.1 AD participant

The baseline TEWL (prior to tape-stripping) of these 25 test areas was mean, $M=9.3$, standard deviation, $SD=1.2$ g m⁻² hour⁻¹. One test area in Row A (PBS control) was excluded from analysis as its mean TEWL post-tape stripping was 30.03 g m⁻² hour⁻¹ (target TEWL post-tape stripping was 20-30 g m⁻² hour⁻¹). The number of tape-strips required to abrogate the skin barrier (to achieve TEWL >20g m⁻² hour⁻¹) in the AD participant was $M=8.5$, $SD=2.0$.

Figure 1a showed the raw TEWL data presented as $M \pm SD$. As the baseline and post-stripping TEWL readings differed from one area to another, the results were also presented as 'percentage correction to baseline TEWL' (presented as $M \pm SD$ in Figure 1b) for the convenience of the reader. Table 1 provides an example detailing how this 'percentage correction to baseline TEWL' was calculated for each group. This is representative of the recovery rate of post-stripping TEWL back to its pre-tape stripping baseline.

Non-linear regression analysis (one site binding hyperbola) showed that compared to carrier buffer phosphate-buffered saline, PBS, topical application of 5mM L-histidine ($F(2,23)=18.22$, $***p<0.0001$) and D-histidine ($F(2,23)=18.07$, $***p<0.0001$) to areas where the skin barrier were abrogated led to significantly faster recovery of TEWL to baseline, as indicated by TEWL measurements on Day 2 and 4 (Figure 1). Compared to PBS control, application of L-lysine also caused a significantly faster recovery to baseline TEWL ($F(2,23)=4.97$, $*p=0.02$, Figure 1), although to a lesser extent than histidine. No obvious skin irritation was detected during and 1 week after the completion of the study. No significant difference between L-serine and control areas was found, $F(2,23)=1.28$, $p=0.30$, n.s.

(a)



(b)

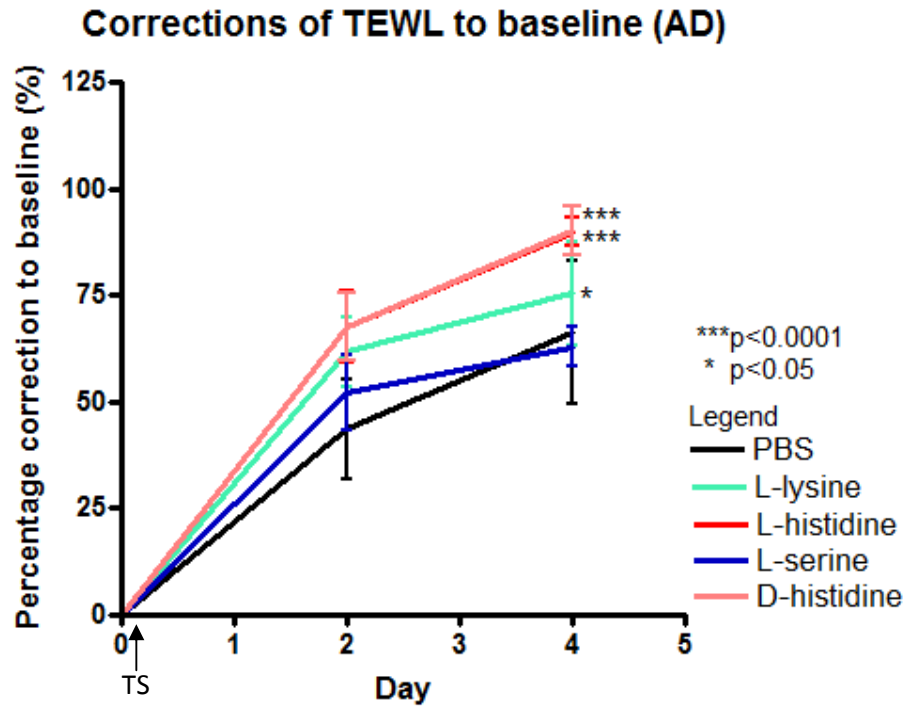


Figure 1. Comparison of the effects of different topically applied amino acids (5mM) on skin barrier recovery following disruption of the physical barrier by tape-stripping in a single AD participant. (a) TEWL (mean, $M \pm$ standard deviation, SD) measured on Day 0 (pre- and post-tape stripping) and on Days 2 and 4. (b) The same data were presented as 'percentage correction to baseline' (presented as $M \pm$ SD, see Table 1 for the calculations). Non-linear regression analysis showed that compared to other amino acids and PBS control, histidine (both L- and D-isomers) and L-lysine led to faster recovery of TEWL to baseline in the AD participant (*** $p < 0.001$, * $p < 0.05$).

Table 1

Calculations for 'Percentage correction to baseline TEWL'

Example: Area A3 required 9 tape-strippings to achieve TEWL between 20-30 $\text{g m}^{-2} \text{ hour}^{-1}$

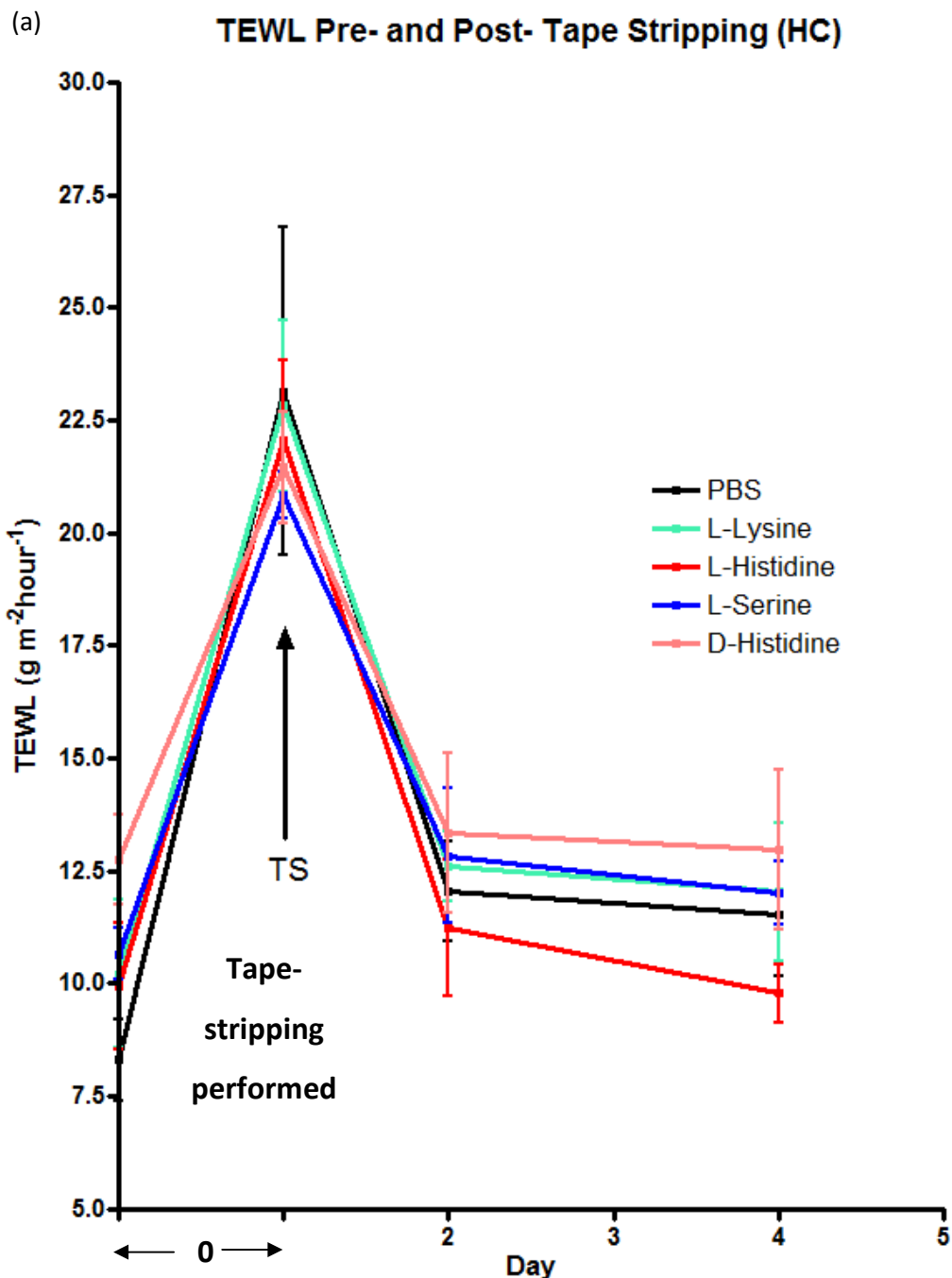
Baseline TEWL (pre-tape stripping)	TEWL post-tape strippings	TEWL measured at Day 2	Calculated percentage correction to baseline
7.9	22.5	18.1	$[(22.5 - 18.1) / (22.5 - 7.9)] \times 100 = 30.1$

7.2.2 Healthy control

The baseline TEWL of 25 test areas on the arm of a single age-, race-matched healthy control with no history of inflammatory skin condition was $M=10.4$, $SD=1.8$ $\text{g m}^{-2} \text{hour}^{-1}$. The number of tape-strips required to abrogate the skin barrier (to achieve $\text{TEWL} > 20 \text{g m}^{-2} \text{hour}^{-1}$) was $M=6.1$, $SD=1.9$.

Compared to PBS control, topical application of 5mM L-histidine ($F(2,26)=16.37$, $***p<0.0001$) and D-histidine ($F(2,26)=6.34$, $**p<0.01$) in the healthy participant led to significantly faster recovery of TEWL to baseline (Figure 2). No significant difference between control areas with areas treated with L-lysine ($F(2,26)=1.11$, $p=0.35$, n.s.) and L-serine ($F(2,26)=2.13$, $p=0.14$, n.s.) was seen.

No obvious skin irritation was detected during and 1 week after the completion of the study.



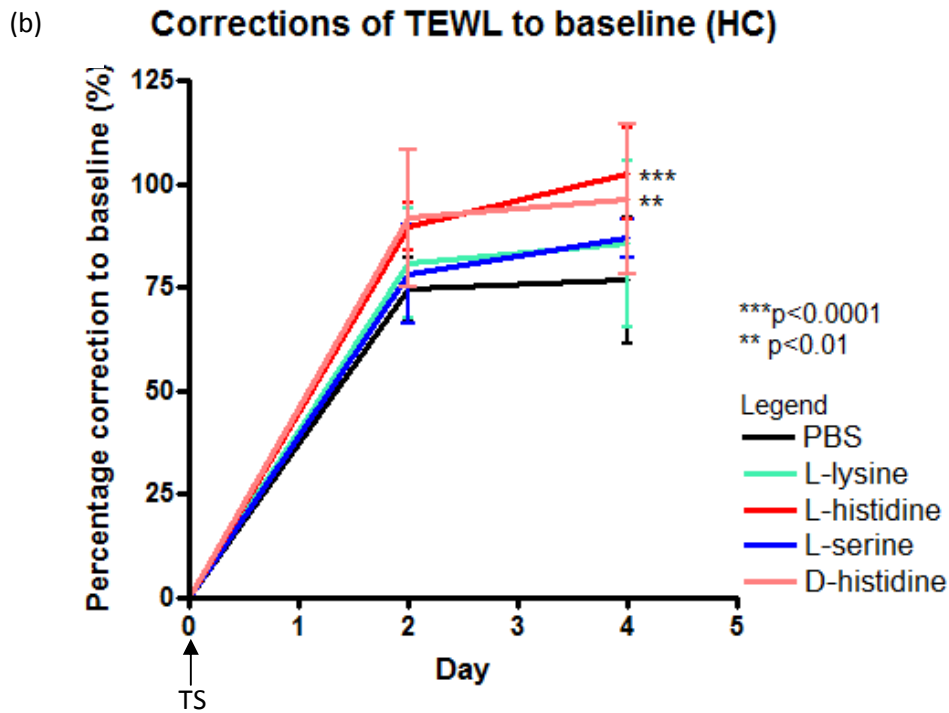


Figure 2. Comparison of the effects of different topically applied amino acids (5mM) on skin barrier recovery following disruption of the physical barrier by tape-stripping in a single healthy control. (a) TEWL (mean, $M \pm$ standard deviation, SD) measured on Day 0 (pre- and post-tape stripping) and on Days 2 and 4. (b) The same data were presented as 'percentage correction to baseline' (presented as $M \pm$ SD, see Table 1 for the calculations). Non-linear regression analysis showed that compared to other amino acids and PBS control, both L- and D-isomers led to significantly faster recovery of TEWL to baseline ($***p < 0.0001$, $**p < 0.01$). The healthy control also showed an overall faster recovery of TEWL to baseline than the AD participant.

Overall, and regardless of the treatment regime, the recovery of skin barrier following physical disruption by tape-stripping was faster in the healthy control (Figure 2) than in the AD (Figure 1) participant.

7.3 Chapter 7 Discussion

TEWL can vary significantly between and within an individual, depending on the number of sweat glands, skin hydration, regional skin temperature, epidermal thickness, usage of emollients and the presence or absence of disease. The baseline TEWL of our AD participant was within the expected range for the condition¹⁶ (refer to Figure 2 in Chapter 2). The baseline TEWL for the healthy control with no history of inflammatory skin condition was higher than expected, although as mentioned above, intra- and inter-individual variations¹⁷⁻¹⁹ are often seen. Similar explanation can be given for the lower-than-average number of tape-strips required to abrogate the skin barrier in both participants, when compared to the data in Chapter 2 (Figure 75). The reliability of TEWL as a stable indicator of barrier function depends on the intra-individual variation being smaller than the inter-individual variation. Our study compared 25 test areas on the upper arm of an AD patient with those of a healthy control. Bearing in mind the potential confounding effects of intra-individual TEWL variations, a randomised block design could have been used to improve our power of testing. Having said this, TEWL in the medial and lateral aspects of the upper arm has been shown to not differ significantly¹⁹. In our study, the abrogation of skin barrier by tape-stripping was stopped once the measured TEWL was above $20 \text{ g m}^{-2} \text{ hour}^{-1}$. Those areas where the post-stripping TEWL was more than $30 \text{ g m}^{-2} \text{ hour}^{-1}$ were excluded from the study. This was to ensure standardization of the 'starting point', in addition to allowing deeper penetration of the topical preparations by partially removing the stratum corneum. Setting an upper limit for post-stripping TEWL may reduce potential confounding effects of secondary inflammation due to the physical damage incurred. Only one test area (in Row A:PBS control of the AD participant) was excluded from our statistical analysis for this reason. Even if the TEWL readings of this test area were to be included in the analysis, the recovery rate (to baseline TEWL) of areas treated with histidine and lysine remain statistically faster than controls.

Despite being a preliminary study consisting of only two age- and race-matched participants, this study yielded interesting positive results. It is, however, important to view these results as a proof of principle which should now be followed by an application for ethical approval to conduct a larger clinical trial. The statistical significance found here should not be over-emphasized. Unfortunately, the *FLG* genotypes of both participants are unknown. Recovery of the skin barrier, indicated by percentage improvement of TEWL back to baseline, was rapid in the first 2 days but the rate appeared to be levelling off at Day 4. Dehydration due to impaired skin barrier initiates the hydrolysis of (pro)filaggrin, which in turn may trigger keratinocyte maturation²⁰. Improvement in skin barrier may result in reducing amount of histidine molecules that are able to penetrate into the stratum granulosum and affect filaggrin protein expression. In future studies, one should consider

performing skin biopsy and immunohistochemical staining for the 37-kDa filaggrin monomers to look for changes in its expression before and following the application of histidine. It also appeared that the recovery to baseline TEWL of the healthy control was much more rapid as compared to the AD participant. At Day 4, the histidine-treated areas of the healthy control have almost approached pre-test baseline while the AD have not. This may indicate a reduced ability amongst AD patients to respond to damage in their skin barrier.

In terms of barrier repair, L-lysine appeared to have beneficial effects in our AD participant while D-histidine, a biologically inactive isomer, appeared to have positive effects in both the AD and healthy participants. These findings are inconsistent with the results obtained from our organotypic skin models. As shown in Chapters 5 and 6, D-histidine had no significant effect on the barrier function of our skin models and on the expression of the loricrin, involucrin and filaggrin monomers in our monolayer cultures. One explanation is that the human skin is much more complex with constant dynamic interactions between components of the epidermis (including Langerhan's cells and melanocytes), dermis and the internal physiology. It is, therefore, not a huge surprise that the results of our human study differ from our *ex-vivo* models in some aspects. It is also possible that the effect seen here with D-histidine is purely a chemical one, attributed to its hygroscopicity. Lysine is a known substrate of transglutaminases, the enzymes that perform most of the cross-linking in the maturing keratinocytes. Increased concentration of L-lysine may have a positive effect on the activity of these enzymes.

Overall, our data suggests that histidine may have therapeutic benefits in barrier dysfunction, with no observable side effects during and after the completion of the study. Our findings may potentially have a huge significance on the management of numerous skin conditions involving skin barrier dysfunction, including atopic dermatitis. Our attempt to apply for a patent for the usage of histidine in improving skin barrier function was aborted upon finding out that the research group led by Dr Neil Gibbs (University of Manchester) has recently filed for a similar one involving the usage of orally ingested histidine in treating inflammatory skin diseases²¹. A collaborative effort between the Universities of Edinburgh and Manchester is now in place. The pilot double-blinded, placebo-controlled, crossover study in adult AD patients led by Dr Gibbs has so far demonstrated reduced clinician-scored disease activity by 22%, patient-scored disease activity by 25% and TEWL by 50% in AD patients who were given 4 weeks of daily oral 4.0g histidine, as compared to placebo (unpublished data, joint manuscript in preparation)²¹. Their results lend weight to the data obtained from our pilot study. One may say that our study has brought in a new player into the management of skin barrier dysfunction and opened doors for future studies, some of which are as suggested next.

7.4 Potential future works

7.4.1 Potential future work (1)

Background

As presented earlier in this chapter, our pilot study has shown that following abrogation of the skin barrier by tape-stripping, topical L-histidine application was potentially associated with an increased recovery rate of TEWL back to baseline. Our earlier cell culture works involving the usage of HaCaT keratinocytes suggest that L-histidine increases the protein expression of filaggrin monomers. These encouraging data should be followed up by a larger clinical trial.

Hypothesis

Following mechanical damage, topical L-histidine application leads to enhancement of epidermal reparative function in human. This is associated with increased expression of the filaggrin monomers in the epidermis. The beneficial effect may be less marked in those with *FLG* mutations who are unable to synthesize filaggrin monomers.

Objectives

- 1) To compare the recovery rate of AD participants vs healthy controls, with and without topical L-histidine application.
- 2) To correlate the response to L-histidine with *FLG* genotype in both AD participants and healthy controls.
- 3) To demonstrate changes in the distribution and amount of filaggrin monomers expressed in the epidermis, comparing between areas treated with topical L-histidine with areas treated with PBS carrier buffer, in both AD participants and healthy controls.

Study design

This study is a propagation of our pilot study, described here in this chapter. AD participants aged 18-40 years with mild-moderate disease and race-, age-matched controls are to be randomised into two groups, receiving either topical L-histidine or control PBS carrier buffer. The group receiving L-histidine should be further randomised to receive either 5mM or 10mM of topical L-histidine. Only uninvolved areas with no clinically obvious disease activity are tested. Genotyping should be performed on all participants. Similar methods used in the pilot study are to be applied, in addition to obtaining punch biopsies at the start and end of the study from both histidine- and PBS-treated areas. The biopsy samples will be used for immunohistochemistry works and filaggrin protein extraction. Quantitation of filaggrin monomers may be done by either ELISA or western-blotting.

7.4.2 Potential future work (2)

Background

Fish, soy protein, cheese and egg are examples of foods that are high in histidine content.

Hypothesis:

A histidine-rich diet is associated with improvement in skin barrier function in adult patients with atopic dermatitis.

Objectives:

- 1) To determine if a histidine-rich diet leads to measurable clinical and symptomatic improvement in skin barrier function in AD patients.
- 2) To formulate a recommended histidine-rich diet for AD patients.

Study design

This is a clinical study involving adult patients with moderate-severe AD who are aged 18-40 years and are otherwise healthy. Participants are randomised into three groups: 1) Control group taking the recommended daily dietary intake of histidine. 2) Histidine group A: Participants taking 50% more than the recommended daily dietary intake of histidine. 3) Histidine group B: Participants taking 100% more than the recommended daily dietary intake of histidine. The study will require a dietitian's input in formulating the diet for each group. (There are also various over-the-counter oral preparations of L-histidine which can be used as an alternative to histidine-rich diet for this study). Pre-study blood test for renal and liver functions is required. Strict compliance to the diet is to be strongly emphasized and participants will be asked to keep a food diary. Genotyping of the participants may be considered. Improvements are gauged by weekly subjective self-assessment by patients of their clinical signs and symptoms using Patient-Oriented Eczema Measurement (POEM) questionnaire, fortnightly assessment of their disease severity using the clinical scoring system- Scoring Atopic Dermatitis (SCORAD) and objective measurement of TEWL using a tewameter at week 2, 4, 6 and 8. Measurements of SCORAD and TEWL should be carried out by a blinded researcher. A cross-over of participants at week 8 may be considered.

7.4.3 Potential future work (3)

Background

Inflammatory processes in the skin such as chronic AD or psoriasis can lead to reduced filaggrin protein expression, regardless of the *FLG* status. We have previously demonstrated a potential association between L-histidine and increased expression of filaggrin monomers.

Hypothesis

Topical application of L-histidine may be associated with the resolution of inflammatory processes in the skin of AD patients, as indicated by restoration of the filaggrin protein level. Clinical improvement of the disease may be seen.

Objectives

- 1) To demonstrate potential improvements in disease signs and symptoms by topical application of L-histidine onto active AD lesions.

Study design

AD participants with active, on-going lesions in both antecubital fossa are recruited. 5mM L-histidine is to be applied twice daily to the antecubital fossa of one arm while control PBS buffer onto the other, in each of the participant. Skin barrier is defective in areas with active disease; adequate penetration of L-histidine may therefore be expected. Both participants and researchers are to be blinded to the topical preparations. Improvements are gauged by daily self-assessment by participants of their clinical signs and symptoms using Patient-Oriented Eczema Measurement (POEM) questionnaire, weekly assessment of disease severity using the clinical scoring system- Scoring Atopic Dermatitis (SCORAD) by researcher and weekly objective measurement of TEWL using a tewameter on both arms. The study is terminated when lesions at any one of the antecubital fossa show signs of deterioration (more than 20% change in SCORAD) on 3 consecutive days or at week 8 (whichever is sooner). Participants should therefore inform the researcher when any significant change in their symptoms is noted for an earlier assessment, if needed.

7.4.4 Potential future work (4)

Background

Histidine is a hygroscopic molecule and is an important component of the NMF.

Hypothesis

Topical histidine can improve the baseline TEWL of normal human skin and can be a useful constituent of moisturizers, whether it is used for diseases such as AD or for cosmesis. D-histidine, the biologically inactive isomer, will lead to improvement in TEWL due to a chemical effect, though to a lesser extent than L-histidine which exerts both biological and chemical effects.

Objectives

- 1) To demonstrate and compare the effects of topical L- and D-histidine on TEWL in healthy participants with no history of inflammatory skin condition.

Study design

Healthy participants are randomised into three groups: 1) Controls receiving PBS buffer. 2) Those receiving 5mM L-histidine. 3) Those receiving 5mM D-histidine. Topical preparations are applied

twice a day for 7 days, with TEWL measurements taken every 48 hours and compared to the baseline TEWL at the start of the study. Histidine is a small molecule and can be expected to penetrate the stratum corneum to a certain extent. Otherwise, methods such as microneedles, microdermabrasion or chemical enhancers which do not lead to damage to deeper structures or barrier dysfunction need to be considered. At Day 7, the topical application is to be stopped although TEWL continues to be measured every 48 hours for the next 7 days. This is to demonstrate any carry-over of potential beneficial effects conferred by histidine and provide a rough guidance on the optimal frequency of application of a histidine-based moisturizer.

7.4.5 Potential future work (5)

Background

For trial purposes and future commercial viability, we should aim at formulating a topical preparation that is cheap, can be easily self-applied, does not require intensive or invasive monitoring and one that causes minimal inconveniences or side effects to our participants. Our current method of applying L-histidine through filter papers held within Finn chambers that are secured to the skin by Scanpor tape is messy, expensive, labour-intensive and painful for the participants as the tape needs to be removed and reapplied every 24 hours (this is comparable to daily waxing). The small molecular weight of L-histidine (155.16 Da) may, by itself, allow sufficient penetration of the molecule into the epidermis to a clinically useful extent. Trial without pre-test tape-stripping is therefore not unreasonable.

Objectives

- 1) To incorporate L-histidine into a cream-based preparation which is cheap, easy to apply and efficacious.

Methods

The solubility of L-histidine in water is 4.19g/100g at 25°C²². This is increased in an acidic medium. To make up 15-20mM of L-histidine, incremental amount of lactic acid may be added (if needed) until complete dissolution of L-histidine in sterile water is observed. This can be followed by titration of the pH of the mixture to approximately 5 using urea. Alternatively, a small amount of DMSO can be used to dissolve the L-histidine prior to mixing with water. The 15-20mM histidine mixture is then blended at room temperature on a 50% weight basis with conventional aqueous cream, resulting in a cream composition.

The method described above is purely suggestive and is heavily subjected to modifications as needs arise. A higher concentration of L-histidine than that previously used in our *in-vitro* studies is proposed as this may be required for adequate penetration of the stratum corneum without first performing tape-stripping. A negative result may indicate insufficient penetration of the amino acid.

This may be resolved by increasing the concentration of L-histidine in the mixture (perhaps through incremental usage of DMSO) or by using suitable chemical penetration enhancers, such as glycols, fatty acids or surfactants^{5,8-10}. Other permeation-enhancing techniques such as electroporation and microporation are expensive and probably not practical in AD where large areas of skin may be involved.

7.5 Chapter 7 References

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7.6 Chapter 7 Materials and Methods

7.6.1 Pilot human study

TEWL of uninvolved skin (25 different areas on flexural aspect of the arm) of 2 age-, race-matched participants (1 AD; 1 healthy control) was measured using Tewameter TM300 (Courage and Khazaka, Cologne, Germany) under standardised conditions (room temperature 20-22°C; humidity 40-60%), as per manufacturer's guidelines. Readings were done in triplicates. This was followed by abrogation of the skin permeability barrier using 14mm D-Squame tape discs (CuDerm Corporation, Dallas, TX) by the tape-stripping technique, as described elsewhere²³. Skin barrier was considered abrogated when TEWL was $>20\text{g/m}^2/\text{hour}^{24}$. Areas where TEWL (post-stripping) was $>30\text{g/m}^2/\text{hour}$ were excluded. 20 μl of control PBS and 5mM L-lysine, L-serine, L-histidine and D-histidine (Sigma, Dorset, UK except D-histidine which was from Fluka, Gillingham, UK) made up in PBS and titrated to within a pH range of 7.03-7.12, were applied to the barrier-deficient areas (5 different areas for each solution, 25 areas in total) using round filter papers (8mm in diameter) placed within aluminium Finn Chambers mounted on a Scanpor tape (Figure 3). Solutions were re-applied every 24 hours. Measurements of TEWL were done on Day 2 and 4 and percentage improvement to baseline TEWL was calculated.



Figure 3: Application of amino acid solutions onto the skin following tape-stripping using round filter papers placed within aluminium Finn Chambers discs, secured with Scanpor tapes.

7.6.2 Statistical analysis

Non-linear regression analysis (one site binding hyperbola) was used to compare the amino acid groups with the PBS control. This was done using GraphPad Prism version 4.00 for Windows, GraphPad software, San Diego California USA, www.graphpad.com.

Data are shown as mean values, $M \pm$ standard deviation, SD . p -values <0.05 were considered as significant.

Chapter 8

Conclusion

Conclusion: From bedside to bench and back.

Atopic dermatitis, AD, is a complex disease. To date, its pathogenesis is still not fully understood. It is not wrong to suggest that the disease is the result of a complex interplay between skin barrier abnormality, immune dysfunction and chronic infection. The relative significance of each of these factors, however, varies from patient to patient according to age, genetic make-up and environmental influence. It is important for clinicians to know which factor plays what role in which type of individuals so that treatment can be better customized to individual cases.

The discovery of *FLG* mutations in up to half of all cases with moderate to severe AD in 2006 is undoubtedly one of the greatest leaps forward in understanding the aetiology of the disease in recent decades. It supports the outside-inside hypothesis, which proposes that barrier dysfunction (potentially as a consequent of genetically driven filaggrin protein deficiency), not immune abnormalities, is the primary cause of AD. However, association alone does not necessarily indicate causation. Despite multiple studies showing an unequivocal association between *FLG* mutations and AD, the functional implications of the genetic defects on skin barrier have not been well-demonstrated. The question here is, what do the mutations mean clinically in terms of the functionality of the skin? In Chapter 2 ('The Effects of *FLG* mutations on Skin Barrier Function'), the author presented the results of the first clinical study on a European cohort (58 participants) which showed that *FLG* mutations were associated with experimentally demonstrable defects of barrier function (increased baseline TEWL), more so following exposure to a chemical irritant. *FLG* mutations were also found to be associated with an increased tendency to develop multiple contact allergies.

However, the majority of patients with AD do not have *FLG* mutations. Some of our wild-type study participants were noticed to have accumulation of the large filaggrin proprotein and a lack of filaggrin monomers. Chapter 3 ('Protease-antiprotease imbalances and atopic dermatitis in patients with a normal *FLG*') focuses on the significance of protease-antiprotease imbalances in the proteolytic processing of profilaggrin. The study found disproportionately raised antiprotease activities amongst the AD study participants. This led to the suggestion that inappropriately raised protease inhibition may interfere with the proteolysis of profilaggrin, leading to an apparent deficiency of functional filaggrin monomers. A lack of functional filaggrin monomers may have resulted in the clinical manifestations of AD in some, if not all, of our wild-type participants who were shown to have accumulated profilaggrin and little filaggrin monomers.

Having demonstrated that filaggrin deficiency (whether genetically linked or proteolysis-related) is associated with functional barrier impairment, we shifted our focus to the function of the protein in the epidermis, of which keratin-aggregation is said to be most important. Nonetheless,

this process can occur even in the absence of filaggrin. We thus explored the less ‘celebrated’ role of filaggrin--as a major contributor to the natural moisturizing factors, NMF, pool in the epidermis. Filaggrin is disproportionately rich in amino acid histidine, implying that this amino acid has a particular significance in skin barrier function. In Chapters 4 and 5, the author described the set-up of a functional skin model, followed by data which show that histidine led to a reduced level of dye penetration into the skin model, indicative of improved barrier function.

It follows naturally for us to attempt to explore the mechanism(s) by which histidine may improve barrier function. In Chapter 6 (‘Exogenous amino acids altering the expression of epidermal barrier proteins’), we showed that histidine and its derivative histamine increased the expression of the 37kDa filaggrin monomer, in relative to a larger 120kDa filaggrin intermediate. The increased filaggrin monomers may be hypothesized to lead to improved keratin-aggregation function or an increased level of NMF in the epidermis. Obviously, more in-depth studies are required to test these hypotheses.

Finally in Chapter 7 (‘A pilot human study and future works’), the author described the principles of transdermal drug delivery systems and the data from a preliminary human study which show that histidine, when applied to physically damaged skin in both AD and healthy study participants, was associated with a significantly faster recovery of the skin barrier. The general recovery rate was also noted to be much higher in the healthy control than in the AD participant. This study highlights the therapeutic potentials of histidine and should be followed up by larger clinical trials and as mentioned above, more in-depth studies to investigate the potential mechanism(s) by which histidine improves skin barrier. The author concluded the chapter by proposing a cream-based formulation for histidine.

In summary, this is a good example of a from-bedside-to-bench-and-back project. We identified a clinical problem which bears huge medical, financial and social implications; improved the understanding of the pathomechanism of the disease through scientific experiments, established an *ex-vivo* model to test the solution to the problem and made an attempt to translate the knowledge obtained from research done in the laboratory into a new way to treat the disease.

Appendix

Chapter 2: Proforma for clinical study

Patient Identification No : _____ Date of study: _____

Name:
DOB:
Address:
Tel no:

Last topical application on area to be studied : _____

Any visible eczema on area to be studied : _____

Ethnic origin

Parents' Ethnic origin : _____

Grandparents' Ethnic origin : _____

Atopy/eczema details

Does patients has: Yes/No Details:

Childhood or past history of eczema/flexural dermatitis		
Personal history of dry skin in the last 12 months		
Family history of eczema		
Family history of asthma		
Family history of hayfever		
Personal history (current or past) of asthma		
Personal history (current or past) of hayfever		
Current eczema		No of flare ups over 1 year: Previous UVB/systemics/admissions: Current treatment:
Current medications		

Severity of current eczema: SASSAD

	Erythema	Exudation	Excoriation	Dryness	Cracking	Lichenification	Total
Head & Neck							
Trunk							
Hands							
Feet							
Arms							
Legs							
Total							

Visible flexural dermatitis: Yes/No

Total body score: _____

Blood measurements

IgE level: _____

Phadiatop: _____

Filaggrin Mutation: Yes / No

Details: _____

Contact allergies

Allergen(s)	Reaction score	Relevance

Performance tests

Humidity : _____
 Temperature : _____

	Reading 1	Reading 2	Reading 3	Average
Baseline				
TEWL				
Capacitance				
After x stripping				
TEWL				
Capacitance				
After x stripping				
TEWL				
Capacitance				
After x stripping				
TEWL				
Capacitance				
After x stripping				
TEWL				
Capacitance				
After x stripping				
TEWL				
Capacitance				

Number of strips to reach TEWL>20: _____

Irritancy study

Pre-SLS

	TEWL1	TEWL2	TEWL3	Average TEWL	% change TEWL
0%					
0.0625%					
0.125%					
0.25%					
0.5%					
1.0%					
2.0%					
4.0%					

	Skin Fold Thickness 1	Skin Fold Thickness 2	Skin Fold Thickness 3	Average Skin Fold Thickness	% change Skin Fold Thickness
0%					
0.0625%					
0.125%					
0.25%					
0.5%					
1.0%					
2.0%					
4.0%					

	Colorimeter 1	Colorimeter 2	Colorimeter 3	Colorimeter 4	% change Colorimeter
0%					
0.0625%					
0.125%					
0.25%					
0.5%					
1.0%					
2.0%					
4.0%					

Post-SLS

	Clinical score
0%	
0.0625%	
0.125%	
0.25%	
0.5%	
1.0%	
2.0%	
4.0%	

	TEWL1	TEWL2	TEWL3	Average TEWL	% change TEWL
0%					
0.0625%					
0.125%					
0.25%					
0.5%					
1.0%					
2.0%					
4.0%					

	Skin Fold Thickness 1	Skin Fold Thickness 2	Skin Fold Thickness 3	Average Skin Fold Thickness	% change Skin Fold Thickness
0%					
0.0625%					
0.125%					
0.25%					
0.5%					
1.0%					
2.0%					
4.0%					

	Colorimeter 1	Colorimeter 2	Colorimeter 3	Colorimeter 4	% change Colorimeter
0%					
0.0625%					
0.125%					
0.25%					
0.5%					
1.0%					
2.0%					
4.0%					

Conference Publications

1. Tan, S.P., Brown, S.B., Weller, R. A pilot study on skin proteases and their inhibitors. *Br. J. Dermatol.* **160**: 917-8 (2009)

A pilot study on skin proteases and their inhibitors

S.P. Tan, S.B. Brown and R. Weller¹

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Activity of skin proteases can be inhibited by inhibitors such as α 1-antitrypsin and human secretory leucocyte protease inhibitor (SLPI). We have set up an on-going pilot study looking at skin proteases and their inhibitors. The study will be extended to involve patients with eczema whom we hypothesize to have an abnormal permeability barrier caused by an imbalance in their proteases and inhibitors.

Ten suction blisters were induced (using PTC Vac 3300 machine) on five volunteers. Polyethylene glycol 400 (2.5 mol L⁻¹) was added to the blister fluids prior to storage at -20 °C. The proteolytic activity of the blister fluid was measured by adding the samples to known substrates of elastase, trypsin and papain (N-methoxysuccinyl-Ala-Ala-Pro-Val-p-NA and N α -benzoyl-DL-arginine β -naphthylamide HCl). The inhibitory activity was measured by the reduction in the rate of activity following addition of blister fluid to individual enzyme assays. Measurements were done using a BioTek microplate reader (absorbance at 405 nm for elastase activity; fluorescence at excitation/emission of 360/460 nm for trypsin and papain). Identification of inhibitors, SLPI and α 1-antitrypsin was by enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Abingdon, U.K.) and Western blotting.

We demonstrated that suction blister fluid obtained from volunteers had no significant proteolytic activity against known substrates of elastase, trypsin and papain. No significant inhibition was demonstrated on our trypsin assay (n = 4). There was a mean 93% (range 85–98%) reduction in proteolytic rates in our elastase assay (n = 5), and 38% (range 34–42%) in the papain assay (n = 4). Our ELISA and Western blots revealed the presence of SLPI and α 1-antitrypsin.

Unlike most previous studies which looked at protease activities, we are looking at protease inhibitors and have utilized a noninvasive technique for obtaining these inhibitors.

2. Abdul Ghaffar, S., Tan, S.P., Campbell, L.E., Lee, L.H., Sergeant, A., McLean, W.I., Weller, R. The effects of filaggrin mutations on barrier function in eczema patients. *J Invest Dermatol.* **129**: S17 (2009)

The effects of filaggrin mutations on barrier function in eczema patients

Abdul Ghaffar S¹, Tan SP¹, Campbell LE², Lee LH¹, Sergeant A¹, McLean WI¹, Weller R¹ ¹The University of Edinburgh, UK ²University of Dundee, UK

Mutations in filaggrin are strongly associated with atopic dermatitis (AD). A functional barrier defect is thought to be a key mechanism underlying eczema. Our aim was to determine whether filaggrin mutations correlate with altered skin barrier function in AD. 58 mild to moderate AD patients were recruited and screened for the common European filaggrin null variants. 45 were wild type; 12 were heterozygous carriers (9x 2282del4, 3x R501X, 1x S3247X); and 1 was a compound heterozygote (2282del4/R501X). Age, sex and SASSAD scores were similar between the filaggrin wild-type and heterozygote groups (11.6 ± 9.3 vs 11.4 ± 7.5). Hay fever (22/45 versus 11/13) was significantly higher in heterozygotes but not asthma. Functional measurements were performed on uninvolved flexor forearm skin. Trans-epidermal water loss (TEWL) was slightly elevated in heterozygotes (8.4 ± 0.6) versus wild-type (8.1 ± 0.9 g/m²/h $p < 0.05$). The number of tape strips required to reach TEWL > 20 gm⁻² per hour was significantly less in the filaggrin-related AD group (13.9 ± 5.7) versus wild-type (11 ± 1.7 , $p = 0.002$). TEWL rose in a dose-dependent manner following 24 hour application of SLS (0.06 to 4%) and this was significantly more marked in heterozygotes than wild-type AD patients at concentrations of SLS above 0.5%. In a cohort of patients patch tested to the European standard allergen battery, 1 of 25 wild-type subjects developed > 5 positive reactions compared to 3 of 7 heterozygotes ($p < 0.05$). The results support an underlying functional skin barrier defect in the pathogenesis of filaggrin-related AD. Filaggrin mutations are particularly associated with defects in the integrity of the epidermal barrier at rest and appear to enhance breakdown in response to irritants.

3. Tan, S.P., Abdul Ghaffar, S., Campbell, L.E., McLean, I., Weller, R., Brown, S.B. An imbalance of protease and antiprotease activities in the skin of eczema patients. *J Invest Dermatol.* **129**: S19 (2009)

An imbalance of protease and antiprotease activities in the skin of eczema patients

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Compromised skin barrier function in about 40% of severe eczema patients can be explained by truncating mutations in the filaggrin gene. We hypothesized that the remaining 60% of eczema cases are caused by downregulation of protease inhibitors, such that an excess of proteolytic activity within the skin was responsible for barrier dysfunction. We therefore tested for differences in skin protease inhibition between eczema and healthy subjects which we correlated to their filaggrin status. Suction blisters were induced in 10 eczema subjects and 11 healthy controls. Inhibitory activities of blister fluid against cysteine and serine proteases were measured using enzyme kinetic assays. The anti-elastases (elafin and secretory leukocyte protease inhibitor, SLPI) were quantified using ELISA. Blister cap sections were stained for filaggrin, CD1a, and CD68. Subjects were screened for filaggrin gene mutations (2282del4, R501X, S3247X, R2447X). 4 of 10 eczema subjects and 1 of 11 healthy controls had an abnormal filaggrin gene. Interestingly, compared to controls, eczema subjects showed higher inhibitory activity towards trypsin ($p < 0.05$), papain ($p < 0.03$), and possibly elastase ($p < 0.16$). No difference in chymotrypsin inhibition was found. Following precipitation and removal of high molecular weight proteins from the blister fluid, higher elastase ($p < 0.02$) and trypsin ($p < 0.03$), but lower papain inhibition ($p < 0.005$) were demonstrated in the eczema subjects. Apart from a higher elastase inhibition ($p < 0.03$) seen in the heterozygotes, we detected no other correlation between filaggrin genotype and protease inhibitor activities. Eczema subjects, irrespective of filaggrin status, had a reduced average level of SLPI in their skin ($p < 0.09$). Our pilot data confirmed an altered protease: antiprotease balance in the skin of eczema patients and may offer pathomechanistic insights into the aetiology of eczema.

4. Tan, S.P., Weller, R., Brown, S.B. Protease–antiprotease imbalance: a potential confounder in the pathogenesis of eczema. *Br. J. Dermatol.* **162**: 909–50 (2010)

Protease–antiprotease imbalance: a potential confounder in the pathogenesis of eczema

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Proteolytic processing of proflaggrin (pflg) into flaggrin (flg) is important in skin barrier formation. Compared with healthy controls (n = 8), we found that patients with eczema (n = 6) with normal flg gene had increased expression (P = 0.02) and possibly reduced processing (P = 0.08) of pflg. We hypothesized that some patients have defective processing of pflg due to imbalances in their protease–antiprotease activities, thus leading to the disease. We tested for differences in protease activities between patients with eczema and healthy subjects and observed increased antiprotease activities in the former. This might explain the elevated levels and reduced processing of pflg in our eczema samples. Inhibitory activities against cysteine and serine proteases of suction-induced blister fluid from eczema and healthy controls were measured using enzyme kinetic assays. The antiproteases [serpinE1, serpinE2, alpha-1-antitrypsin, elafin and secretory leukocyte protease inhibitor (SLPI)] were quantified using ELISA and Western blot. Blister skin caps were stained for flg. Subjects were screened for FLG mutations (2282del4, R501X, S3247X, R2447X). Compared with controls, subjects with eczema showed higher inhibitory activity towards trypsin (P < 0.05), papain (P < 0.03), and possibly elastase (P < 0.16). No difference in chymotrypsin inhibition was found. Apart from a higher elastase inhibition (P < 0.03) seen in the heterozygotes, we detected no other correlation between FLG genotype and protease inhibitor activities. No raised serpinE1, serpinE2, alpha-1-antitrypsin, elafin or SLPI was found. Although the responsible inhibitors have yet to be identified, we found an altered protease–antiprotease balance in the skin of patients with eczema. Our data may offer pathomechanistic insights into the aetiology of eczema in patients with a normal FLG.

5. Tan, S.P., Weller, R., Brown, S.B. Histidine increases the expression of filaggrin protein and improves barrier function. *J Invest Dermatol.* **131**: S39 (2011)

Histidine increases the expression of filaggrin protein and improves barrier function

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Filaggrin deficiency is associated with reduced level of natural moisturizing factor and skin barrier dysfunction. The unusually high level of histidine in filaggrin may indicate a prominent role for this amino acid in maintaining stratum corneum hydration. Our early human data found that following abrogation of skin barrier by tape-stripping, topically applied L-histidine improved the skin recovery at a faster rate than control PBS ($p < 0.01$). Here we describe improved barrier function in skin-equivalent models grown in histidine-enriched media and the effects of histidine on filaggrin protein expression of keratinocyte line HaCaT. Cell culture media was supplemented with 1, 2 and 5mM of L-histidine, D-histidine and L-lysine. Media pH was adjusted using Pipes (pKa 6.76) and Tris (pKa 8.08). Barrier function of skin-equivalents was assessed with penetration of Lucifer-Yellow dye. Skin-equivalents grown in L-histidine-enriched media showed reduced penetration by Lucifer-Yellow than controls ($p < 0.05$), implying improved barrier function. Western-blot analysis showed that L-histidine reduced the amount of a large 120kDa filaggrin intermediate and increased the 37kDa filaggrin monomers, causing a dose-dependent increase in the 37kDa:120kDa ratio ($p < 0.01$). Histidine metabolites urocanic acid reduced ($p < 0.01$) while histamine (with or without H_1+H_2 -receptor antagonists) increased ($p < 0.01$) the 37kDa:120kDa filaggrin ratio. No effect was noted in the controls (D-histidine and L-lysine) or under different pH conditions. The expression of loricrin, another late differentiation marker, was unchanged. This study suggests that histidine and its metabolite histamine may improve skin barrier function by increasing the expression of filaggrin monomers via a H_1+H_2 -receptors-independent pathway, implying a possible positive feedback mechanism.

6. Abdul-Ghaffar, S. *et al.* Filaggrin mutations are associated with altered epidermal barrier and antigen presenting cell immunophenotypes in atopic eczema patients. *J Invest Dermatol.* **131**: S54 (2011)

Filaggrin mutations are associated with altered epidermal barrier and antigen presenting cell immunophenotypes in atopic eczema patients

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Filaggrin null mutations (FLG) are associated with atopic dermatitis (AD) and skin barrier defects. We assessed the role of FLG on the skin barrier phenotype and dendritic cell (DC) subtypes. 94 subjects were genotyped for common European FLG variations (R501X and 2282del4) and phenotyped; 62 subjects with AD (13 FLG heterozygotes, 2 homozygote), 32 without AD (8 FLG heterozygotes). We measured trans-epidermal-water-loss (TEWL), hydration, number of tape-strips to raise TEWL>20g/h/m², SLS irritation and epidermal DC immunophenotypes by flow-cytometry. TEWL was highest in FLG AD 8.4±0.6; WT AD 8.1±0.8; FLG non-AD 7.9±0.7; WT non-AD 7.2±0.4 (p<0.05 for FLG vs. WT and FLG AD vs. WT AD). No. tape-strips to reach TEWL>20g/h/m² was lowest in FLG AD 11.3±1.9; WT AD 14.2±5.7; FLG non-AD 15.3±6.2; WT non-AD 18.9±5.9 (p<0.05 for FLG vs. WT, AD vs. non-AD and FLG AD vs. WT AD). The amount of protein removed per strip was greatest in FLG AD 31.2±8.27; WT AD 18.58±6.38; FLG non-AD 3.78±1.01 and WT non-AD 4.04±1.9mg.cm⁻². TEWL rose dose-dependently following 24hr application of SLS (0.06-4%); and significantly higher in FLG AD vs. WT AD patients at 1% and 2%. FLG AD subjects had a significantly higher proportion of IDEC and mature DC in their epidermal samples than WT AD subjects. This data infers a complex aetiology of AD where non-AD FLG carriers appear not to develop significant defects. In AD, our study supports the role of filaggrin mutations in defective corneocyte adhesion and epidermal barrier integrity, and a resultant alteration in antigen presenting cells.

Journal Publication

1. Tan, S.P., Abdul-Ghaffar, S., Weller, R. B. & Brown, S. B. Protease-antiprotease imbalance may be linked to potential defects in profilaggrin proteolysis in atopic dermatitis. *Br. J. Dermatol.* **166**, 1137–1140 (2012)

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Conflicts of interest: none to declare.

Protease–antiprotease imbalance may be linked to potential defects in profilaggrin proteolysis in atopic dermatitis

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MADAM, Most recent studies on atopic dermatitis (AD) have focused on the importance of the keratin-binding protein filaggrin in skin barrier function. Loss-of-function mutations of the filaggrin gene *FLG* are the major predisposing genetic factor for AD.¹ Nonetheless, the majority of patients with AD have an apparently normal *FLG*. Some patients with wild-type AD have reduced filaggrin protein expression,² implying the presence of other modifying factors. Profilaggrin, the 400-kDa precursor protein, is unable to bind keratin until it is proteolytically released to release the functional 37-kDa filaggrin monomer.³ Knowing the importance of profilaggrin processing, we conducted a small study to determine whether defective profilaggrin proteolysis may be found in patients with AD with wild-type *FLG*. Although profilaggrin processing is presumed to occur intracellularly, extracellular proteases such as matrilysin^{4,5} also influence the expression of filaggrin monomers.

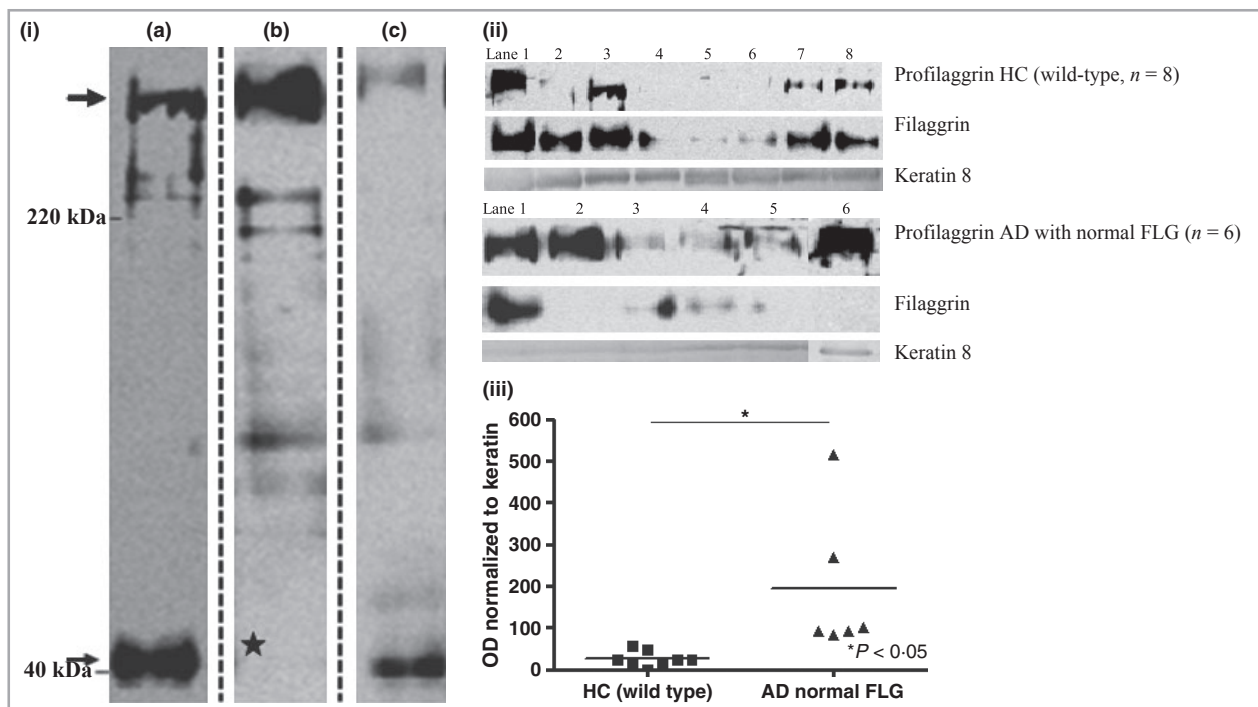


Fig 1. Differences in (pro)filaggrin protein expression between HC with normal *FLG* and patients with AD by Western blotting. (i) Representative Western blots showing profilaggrin (thick arrow, > 220 kDa) and filaggrin (thin arrow, ~40 kDa). (a) HC with normal *FLG*; (b) AD with normal *FLG* showed increased amount of profilaggrin and filaggrin intermediate products and lacked the 37-kDa filaggrin protein (★, absent filaggrin band). (c) AD with *FLG* mutation (heterozygous for 2282del4 mutation) with reduced amount of (pro)filaggrin. Two *FLG* heterozygotes (including the one compound heterozygote) had no filaggrin protein on Western blotting and therefore are not shown. (ii) AD with normal *FLG* exhibited an overall increased profilaggrin and reduced filaggrin monomers in comparison with HC with normal *FLG*. (iii) Densitometric band analysis of the Western blots showed accumulation of the profilaggrin in AD with normal *FLG* ($P < 0.05$). HC, healthy controls; AD, atopic dermatitis.

We further investigated whether the extracellular/interstitial protease–antiprotease balance is deranged in patients with AD.

We recruited 11 healthy controls (HC; age 23.5 ± 6.5 years; seven females) with no inflammatory skin conditions and 10 patients with mild-to-moderate AD (age 27.8 ± 11.5 years; eight females). In the AD group, the disease severity score (Six Area, Six Sign Atopic Dermatitis, SASSAD) ranged from 3 to 16 for the wild type and from 0 to 24 for those with FLG mutations. Genotyping for common caucasian FLG polymorphisms (2282del4, R2447X, R501X, S3247X) was performed as described elsewhere.⁶ One of the 11 HC was heterozygous for the FLG 2282del4 mutation. Four of the 10 patients with AD were heterozygous for FLG 2282del4, of whom one was a compound heterozygote (2282del4/R501X).

With a PTC3300 VAC Vacuum Unit (InnoKas Medical Oy, Kempele, Finland), we induced suction blisters on the flexural surfaces of our volunteers' arms. We excluded those with active disease or inflammation at the test area and kept the suctioning to the minimum to reduce any secondary inflammation. Suction blistering has been validated as a way of obtaining epidermal protease inhibitors such as elafin, SLPI and LEKTI, which are secreted into the interstitium.⁷ Western-blot band densitometric analysis using the epidermal skin caps (normalized to housekeeping protein keratin 8) showed that in two of the six patients with wild-type AD (Fig. 1ii, lower panel; lanes 2 and 6), no filaggrin monomers were apparent despite a significant presence of profilaggrin (see also Fig. 1ib). The six patients with AD with wild-type FLG had a higher amount of profilaggrin compared with eight HC (192.52 ± 173.61 vs. 25.96 ± 17.87 , Fig. 1iii). Patients with wild-type AD had, on average, a higher profilaggrin : filaggrin ratio, compared with HC (2.25 ± 2.20 vs. 0.75 ± 0.33) (Fig. 1). Skin caps were not obtained from three HC and one AD with FLG mutation. The higher profilaggrin : filaggrin ratio in patients with wild-type AD, especially that of lanes 2 and 6, suggests defective profilaggrin processing. The absence of pro- and processed filaggrin in some lanes (both HC and wild-type AD) may imply other filaggrin mutations not screened for in this study. It is also known that some people with filaggrin mutations do not develop AD. Suction-induced blister skin caps were stained for (pro)filaggrin and cytokeratin using general double immunohistochemical staining technique with primary antibodies raised against filaggrin (mouse monoclonal antibody from Novocastra, Newcastle, UK) and pan-cytokeratin (mouse monoclonal antibody from Sigma, Poole, UK). Representative images using samples obtained from HC with wild-type FLG, wild-type AD and AD patients with FLG mutations were shown in Fig. 2.

Blisters fluid (protein concentrations 62.4 ± 8.9 mg mL⁻¹, measured with BCA Protein Assay Reagent; Thermo Scientific, Barrington, IL, U.S.A.) was mixed with polyethylene glycol (PEG-400; Fluka, Gillingham, U.K.) in a 50 : 50 ratio before storage at -20 °C. PEG-400, added to preserve existing protease activity, also resulted in precipitation of larger-molecular-weight proteins on thawing that were removed by centrifugation (as observed by Coomassie Blue staining of the

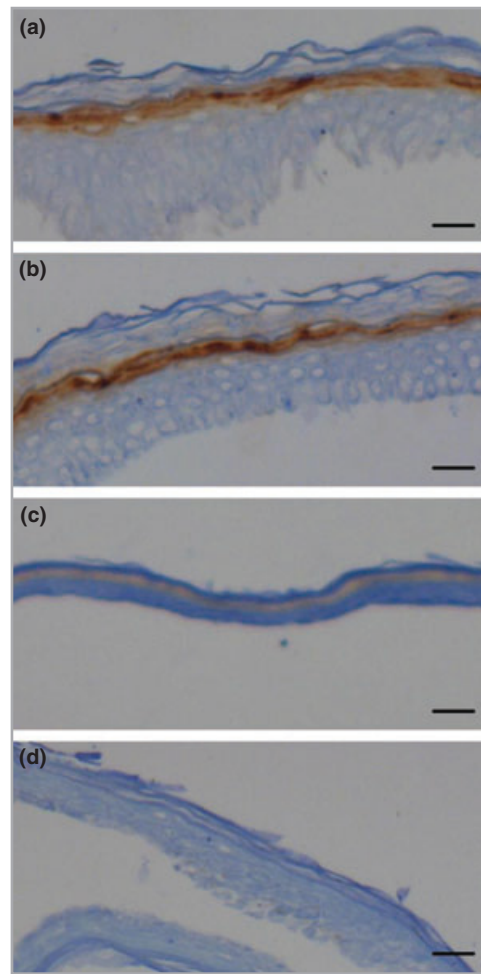


Fig 2. Immunohistochemical staining of suction-induced blister skin caps for (pro)filaggrin and cytokeratin. Suction-induced blister skin caps from 9 AD patients (6 WT-AD, 3 FLG heterozygotes for 2282del4 mutation) and 8 HC with wild-type FLG were stained for (pro)filaggrin (brown) and cytokeratin (blue). (a) A wild-type HC. (b) A WT-AD patient. Note: The thicknesses of skin cap and (pro)filaggrin layers of WT-AD were comparable to HC with wild-type FLG. (c) A FLG-AD heterozygote (FLG +/- 2282del4) showing reduced filaggrin staining. (d) A FLG-AD heterozygote (FLG +/- 2282del4) showed no obvious filaggrin staining. Scale bar = 20 μ m.

protein gels). This partial fractionation allowed assessment of the lower-molecular-weight proteins (typically < 80 kDa in size) that remained in suspension. Using chromogenic and fluorogenic peptides, we detected no significant elastolytic, chymotryptic, tryptic or papain-like activity within the blister fluid samples, indicating the presence of potent inhibitors. Blister fluid was therefore analysed for inhibitory activity against commercially available serine (elastase, trypsin, chymotrypsin) and cysteine (papain) proteases.

Compared with HC, blister fluid from patients with AD resulted in a higher inhibition of trypsin ($25 \pm 9\%$ vs. $18 \pm 4\%$, $P < 0.05$), papain ($69 \pm 5\%$ vs. $62 \pm 8\%$, $P < 0.03$) and, perhaps, elastase ($40 \pm 21\%$ vs. $25 \pm 21\%$, $P < 0.16$) (Fig. 3a). Partially fractionated samples consisting of predominantly

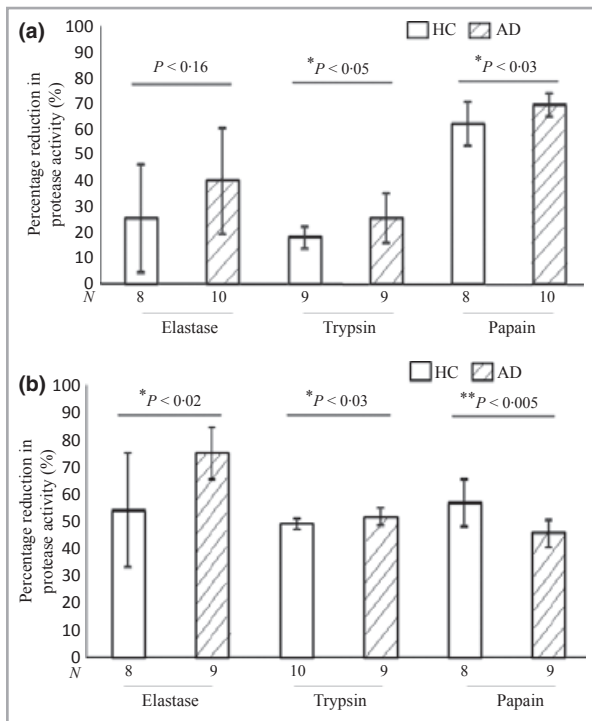


Fig 3. Differences in the protease inhibition profile between HC and AD. (a) Percentage reduction in the initial enzymatic reaction velocity caused by the addition of blister fluid. Patients with AD showed higher antitrypsin and antipapain activities. (b) Percentage reduction in the initial enzymatic reaction velocity caused by the addition of partially purified blister fluid that contained predominantly lower-molecular-weighted inhibitors. Fractionated samples from patients with AD were found to have higher anti-elastase and antitrypsin, but lower antipapain activities compared with HC. Data were presented as mean \pm SD. HC, healthy controls; AD, atopic dermatitis.

lower-molecular-weight inhibitors from patients with AD were found to have higher anti-elastase ($75 \pm 9\%$ vs. $54 \pm 21\%$, $P < 0.02$) and antitrypsin ($52 \pm 3\%$ vs. $49 \pm 2\%$, $P < 0.03$), but lower antipapain ($46 \pm 5\%$ vs. $57 \pm 9\%$, $P < 0.005$) activities compared with HC (Fig. 3b). No difference in the antichymotrypsin activity of blister fluid between AD and HC was found.

While previous studies suggest that excessive proteolysis in the skin is causally linked to skin barrier dysfunction,^{8–10} we propose that abnormally raised protease inhibition demonstrated in the interstitium of patients with AD could be linked to the clinical manifestations of AD, possibly by affecting proflaggrin processing through inhibition of proteases such as matriptase. Our findings are indirectly consistent with a previous study suggesting an association between decreased larger precursors and increased flaggrin monomers with increased protease activities.¹¹ Although our small sample size does not allow a robust relation between genotype and individual antiprotease activity to be drawn, the disproportionately raised protease inhibition and defective proflaggrin processing provide potentially useful pathomechanistic insights into the aetiology of AD.

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Successful management of severe infant bullous pemphigoid with omalizumab

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MADAM, Bullous pemphigoid (BP) is exceptional during childhood.¹ Autoantibodies directed against the hemidesmosomal proteins BP180 and BP230 are found in most adult patients with BP (review in reference²). In infants with BP (IBP), as in adult patients, topical corticosteroids are usually effective for disease control,³ but sometimes may require additional treatments^{4,5} in the most severe form.

A 5-month-old male infant was referred to our hospital (considered as day 0) for the management of IBP that failed to respond to oral prednisolone 2.5 mg kg⁻¹ daily. At 4 months, IBP had been diagnosed (Fig. 1) and confirmed by histopathological examination showing a subepidermal blister with a dermal infiltrate of eosinophils and a few lymphocytes. Direct immunofluorescence (IF) showed linear IgG, C3 deposits on the basement membrane zone (BMZ), and a few linear IgA deposits. No IgE deposits were observed on the BMZ. The blood cell count showed major eosinophilia ($11.5 \times 10^9 \text{ L}^{-1}$). The total IgE serum level was elevated (636 KU L⁻¹), as were the anti-BP180 and anti-BP230 serum antibody enzyme-linked immunosorbent assay (ELISA) values (Fig. 2). IgG2 and IgG3 circulating anti-BMZ antibodies were detected by indirect IF on normal human salt-split skin. No circulating anti-BP180 or anti-BP230 IgE antibodies were detected by immunoblot on human epidermal extract, or by indirect IF on normal or 1 mol L⁻¹ NaCl-split human skin. Despite increasing the prednisolone dose to 3 mg kg⁻¹ daily, and administering three intravenous (IV) pulses of methylprednisolone 120 mg, topi-

cal betamethasone 0.05%, dapsone 2 mg kg⁻¹ daily and azithromycin 10 mg kg⁻¹ daily, the disease remained uncontrolled. The patient was then treated with a 100-mg subcutaneous injection of omalizumab (calculated from the asthma chart) on day 17. During the following days, the number of new blisters and urticarial lesions decreased dramatically. Disease control was achieved by day 25. Omalizumab injections were continued every 2 weeks for a 3-month period and were then administered monthly for 4 months. After a 7-month follow-up period, no clinical relapse had occurred (Fig. 1). Anti-BP180 (but not anti-BP230) antibody ELISA values decreased after treatment (Fig. 2).

Some experimental and clinical data suggest that eosinophils and IgE may play an important role in BP pathophysiology. Up to 86% of adult patients with BP have anti-BP180 IgE autoantibodies.⁶ In an animal model using nude mice grafted with human skin, it was shown that injection of purified IgE isolated from patients with BP induced similar clinical and histological lesions.⁷ Omalizumab is approved by the U.S. Food and Drug Administration in severe atopic asthma management. It is a recombinant humanized monoclonal antibody that binds to the Cε3 domain of IgE and prevents free IgE from binding to the basophils and the mast cell high-affinity IgE receptor.⁸

Given the successful use of omalizumab in a previous case of adult BP⁹ and our patient's high total IgE level, elevated eosinophil blood count and dermal eosinophilic infiltrate, we hypothesized that blocking IgE–eosinophil pathways could be a potential alternative to immunosuppressive agents. We made this assumption before knowing that the patient did not have anti-BP180 or anti-BP230 IgE autoantibodies.

The mechanisms by which omalizumab was effective in this case remain unclear. It does not seem to involve the specific IgG2 and IgG3 humoral response, as BP180 autoantibodies were still detectable 4 months after the first injection.² The efficacy of omalizumab is paradoxical as no anti-BMZ IgE or IgG4 antibodies were detected. Although two techniques failed to detect anti-BMZ IgE antibodies, we cannot rule out that anti-BMZ IgE antibodies were present but not detected for technical reasons. We therefore hypothesize that omalizumab might interfere with cells involved in the BP-specific immune response, such as T lymphocytes or eosinophils. Indeed, *in vitro* experiments have shown that omalizumab is able to induce eosinophil apoptosis and downregulation of proinflammatory cytokines

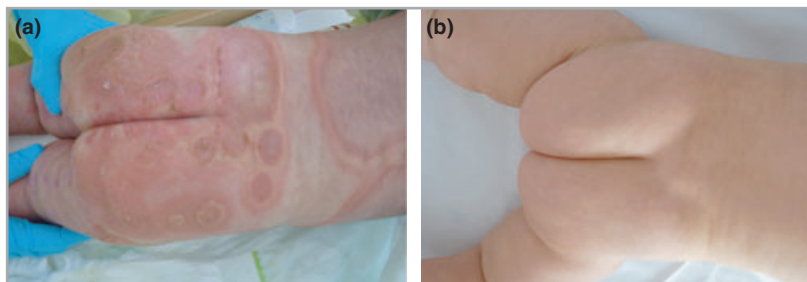


Fig 1. (a) Skin lesions in a 5-month-old boy suffering from pemphigoid bullous; large urticarial areas and bullae are spread over the back and buttocks. (b) The same infant at 10 months of age after seven injections of omalizumab.