

**UNIVERSITY OF SOUTHAMPTON**

**FACULTY OF HEALTH SCIENCES**

**Investigation of the underlying mechanisms leading to the  
development of incontinence-associated dermatitis**

by

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## **ABSTRACT**

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### **INVESTIGATION OF THE UNDERLYING MECHANISMS LEADING TO THE DEVELOPMENT OF INCONTINENCE-ASSOCIATED DERMATITIS**

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Incontinence-associated dermatitis (IAD) is an inflammatory response caused by the prolonged contact of the skin to urine and/or faeces. Although several theories exist to explain the pathophysiology of IAD, most are based on animal models, or derived from studies on the related condition of diaper dermatitis ('nappy rash') that occurs in babies. Thus, their relevance to IAD in adults is limited and the underlying mechanisms of IAD remain poorly understood. The motivation for this novel work was to develop and validate both *in vivo* and *in vitro* techniques to simulate IAD to provide empirical evidence on the biophysical and biochemical disruption of skin in IAD.

The integrity of the skin was evaluated by measuring changes in skin blood flow, transepidermal water loss (TEWL), hydration of the stratum corneum (SC) and skin surface pH. Synthetic-urine (s-urine) and a proteolytic solution were used to simulate the effects of urinary- and faecal-incontinence, respectively. The release of inflammatory biomarkers was investigated *in vivo* by two distinct sampling techniques, namely microdialysis and Sebutape™. *In vitro* investigations were also conducted with human keratinocytes to support *in vivo* studies, with cell viability and the secretion of inflammatory cytokines serving as output measures.

Results of the current study showed that while urine disrupts the integrity of intact skin, causing a significant increase in skin blood flow, TEWL, SC hydration and skin surface pH ( $p<0.05$ ), its effects are not pH dependent. In contrast, exposure of the skin to the proteolytic solution led to an enhanced increase in these biophysical parameters. This was also supported by *in vitro* keratinocyte experiments which indicated that the proteolytic solution is more deleterious than urine, as revealed by a decreased cell viability. Of particular importance is the finding that the permeability and susceptibility of the skin to urine depends on skin condition, with chemically-irritated skin, exacerbated by frequent cleansing activities, presenting an increased risk of IAD. Increased skin permeability was also associated with an elevated skin surface pH.

In this respect, in vitro investigations highlighted that pH is an important regulator for the function of keratinocytes and consequently the maintenance of skin barrier function. Additionally, it was shown that the effects of urinary pH are time-dependent. Importantly, exposure of skin to urine and the proteolytic solution resulted in an increase in the release of pro-inflammatory biomarkers and indeed during exposure there was a time-dependent accumulation of biomarkers in the interstitium. In particular, a significant increase was estimated in the ratio of IL-1 $\alpha$ /IL-1RA ( $p<0.05$ ) following exposure to s-urine. Additionally, results demonstrated that s-urine resulted in an increase in IL-1 $\alpha$ , whereas the proteolytic solution caused a marked increase in TNF- $\alpha$ . The increase in pro-inflammatory cytokines following exposure to urine was further supported by the in vitro studies. In these studies, an increase in the pH upregulated the release of inflammatory biomarkers, more particularly IL-1 $\alpha$ . These data propose that in future studies, IL-1 $\alpha$  and TNF- $\alpha$  have the potential to serve as responsive markers of skin damage caused by incontinence.

The findings presented in this thesis extend current knowledge on IAD, with noteworthy implications for the direction of future IAD research and the development of targeted clinical interventions. Additionally, the data add to the scientific body of evidence and propose that the damage caused by frequent cleansing activities and the release of inflammatory biomarkers are two mechanisms implicated in the pathophysiology of IAD. Additionally, an internal pH mechanism for keratinocytes function is also proposed, which needs to be further examined. Overall, this work establishes the methods to investigate IAD in an experimental setting, which can be translated to clinical studies.

# Table of Contents

<b>List of Figures .....</b>	<b>vii</b>
<b>List of Tables .....</b>	<b>xv</b>
<b>Academic Thesis: Declaration of Authorship.....</b>	<b>xvii</b>
<b>Dissemination .....</b>	<b>xviii</b>
<b>Acknowledgements.....</b>	<b>xix</b>
<b>Definitions and Abbreviations.....</b>	<b>xxi</b>
<b>Chapter 1:      Introduction .....</b>	<b>1</b>
1.1    Structure of the skin .....	1
1.1.1    Epidermis .....	2
1.1.2    Dermal-Epidermal Junction.....	3
1.1.3    Dermis .....	3
1.2    The Skin as a Barrier .....	4
1.3    Maintenance of skin barrier integrity .....	5
1.3.1    Desquamation .....	5
1.3.2    The cornified envelope .....	6
1.3.3    Epidermal cytokines.....	7
1.3.4    Transepidermal Water Loss (TEWL) .....	8
1.3.5    The acid mantle .....	9
1.4    Moisture-associated skin damage .....	10
1.5    Incontinence-associated dermatitis (IAD) .....	10
1.5.1    Risk factors .....	11
1.5.2    Prevalence and Incidence of IAD.....	12
1.5.3    Prevention and management of IAD .....	13
1.6    Differentiating IAD from pressure ulcers .....	14
1.7    Problems in IAD research .....	15
1.8    The Research Problem.....	16
1.9    Chapter Summary .....	17
<b>Chapter 2:      The aetiology and pathophysiology of IAD .....</b>	<b>19</b>

2.1	Introduction.....	19
2.2	Methods .....	19
2.2.1.	Search strategy.....	19
2.2.2.	Search outcome .....	20
2.3	Theoretical models of IAD pathophysiology .....	22
2.4	Aetiological factors .....	26
2.4.1	Moisture source .....	26
2.4.2	Frequent Cleansing.....	32
2.4.3	Duration and Frequency of exposure.....	32
2.4.4	Other factors .....	33
2.4.5	Inflammatory response of the skin in IAD .....	34
2.5	Aim of the study.....	37
2.5.1	Research Hypotheses .....	37
2.6	Chapter summary .....	37
<b>Chapter 3:</b>	<b>Methodology .....</b>	<b>39</b>
3.1	Introduction.....	39
3.2	Biophysical measurements to investigate the functional characteristics of skin	39
3.2.1	Skin Barrier assessment methods .....	39
3.2.2	Methods to compromise skin integrity .....	56
3.2.3	Methods for analysis of skin inflammation .....	63
3.3	Ethical considerations .....	73
3.4	Chapter summary .....	75
<b>Chapter 4:</b>	<b>The effect of synthetic-urine and its inherent pH on the functional characteristics of intact skin.....</b>	<b>77</b>
4.1	Introduction.....	77
4.2	Aim of the study.....	79
4.3	Objectives .....	79
4.4	Participants.....	79
4.5	Methods .....	80
4.6	Experimental protocol .....	80

4.7	Data Analysis.....	82
4.8	Results .....	83
4.8.1	Effect of s-urine solutions on the functional characteristics of healthy skin .....	83
4.8.2	The buffering capacity of the skin .....	91
4.8.3	The effects of water exposure and occlusion on the skin barrier function .....	91
4.9	Discussion .....	93
4.10	Chapter summary.....	94
<b>Chapter 5: The susceptibility and permeability of intact and compromised skin to synthetic-urine .....</b>		<b>97</b>
5.1	Introduction .....	97
5.2	Aim of the study.....	98
5.3	Objectives .....	98
5.4	Participants .....	98
5.5	Methods.....	99
5.6	Experimental protocol .....	100
5.7	Data Analysis.....	102
5.8	Results .....	102
5.8.1	The permeability of intact and compromised skin to s-urine.....	102
5.8.2	Susceptibility of intact skin to s-urine .....	104
5.8.3	Susceptibility of physically-irritated skin to s-urine .....	107
5.8.4	Susceptibility of chemically-irritated skin to urine.....	111
5.8.5	The temporal effects of tape stripping and SLS on blood perfusion	114
5.9	Discussion .....	116
5.10	Chapter summary.....	119
<b>Chapter 6: The inflammatory response triggered in the skin upon exposure to synthetic-urine, and a proteolytic-model of faecal-incontinence .....</b>		<b>121</b>
6.1	Introduction .....	121
6.2	Aim of the study.....	121
6.3	Objectives .....	121

6.4	Participants.....	122
6.5	Methods .....	122
6.5.1	Construction of microdialysis fibres.....	123
6.5.2	Efficiency of microdialysis.....	124
6.6	Experimental protocol .....	129
6.6.1	Sebutape™ sample preparation.....	131
6.6.2	Normalizing for total protein .....	132
6.6.3	Multiplex electrochemiluminescence immunoassays .....	132
6.7	Data Analysis .....	134
6.8	Results .....	135
6.8.1	Skin Blood Flow .....	135
6.8.2	TEWL.....	137
6.8.3	SC Hydration .....	138
6.8.4	Skin pH .....	140
6.8.5	Release of inflammatory mediators.....	142
6.9	Discussion .....	152
6.10	Chapter summary .....	155
<b>Chapter 7:</b>	<b><i>In vitro</i> investigations .....</b>	<b>157</b>
7.1	Introduction.....	157
7.2	Aim .....	158
7.3	Objectives.....	158
7.4	Methods .....	158
7.4.1	Cell culture .....	158
7.4.2	Cell stimuli.....	160
7.4.3	Measurement of cell viability .....	161
7.4.4	Quantification of the release of inflammatory cytokines .....	163
	Multiplex electrochemiluminescence immunoassays .....	163
7.5	Data analysis.....	163
7.6	Results .....	164
7.6.1	The optimum concentration of HaCaT cells .....	164

7.6.2	The effects of LPS on keratinocytes .....	165
7.6.3	The effects of pH on keratinocytes .....	165
7.6.4	The effects of various concentrations of s-urine and proteolytic-solution on keratinocytes over time .....	166
7.6.5	The effects of urinary pH on keratinocytes over time .....	168
7.6.6	Cytokine release .....	170
7.7	Discussion .....	173
7.8	Chapter summary.....	176
<b>Chapter 8:</b>	<b>General Discussion.....</b>	<b>177</b>
8.1	Addressing the research aim and hypotheses.....	177
8.2	Critical Analysis of Techniques and Results.....	182
8.2.1	<i>In vivo</i> studies .....	182
8.2.2	<i>In vitro</i> investigations.....	185
8.3	Future Directions.....	185
8.3.1	Clinical Validation .....	185
8.3.2	Further characterization of the damage at the cellular level .....	185
8.3.3	Other inflammatory mediators involved in the inflammatory response following exposure to urine and faeces .....	186
8.3.4	The effect of urine and faeces on SC serine proteases .....	188
8.3.5	The role of occlusive conditions caused by incontinence products in the development of IAD.....	188
8.3.6	The effect of skin care regimens on the release of inflammatory mediators .....	188
8.4	Implications for IAD research/clinical practice .....	189
8.5	Contribution of the thesis to the current state of knowledge .....	189
<b>Appendix A</b>	<b>Ethics Application.....</b>	<b>193</b>
A-1:	Reviewers' Comments and requested revisions .....	193
A-2:	Response to reviewer's comments.....	196
<b>Appendix B</b>	<b>Approved documents.....</b>	<b>203</b>
B-1:	ERGO application form .....	203

B-2: Recruitment Poster .....	212
B-3: Participant Information Sheet .....	213
<b>Appendix C      Typical standard curves for cytokines.....</b>	<b>217</b>
<b>Appendix D      Cytokine Concentrations in Sebutape and microdialysis samples</b>	<b>221</b>
D-1: Sebutape data .....	221
D-2: Microdialysis data.....	224
<b>References .....</b>	<b>233</b>

# List of Figures

<b>Figure 1.1. Skin structure and composition.</b> The skin is composed of the epidermis and the dermis. Figure adapted from Voegeli, 2012 .....	1
<b>Figure 1.2. Structure of the epidermis.</b> The different layers and cell types of the epidermis are shown. Figure based on Tortora and Derrickson, 2014. ....	3
<b>Figure 1.3. The structure of the stratum corneum.</b> Corneocytes are held together by intercellular lamellar lipids, and are arranged in a “brick and mortar” model. The figure is based on Voegeli, 2012 .....	5
<b>Figure 1.4. Skin desquamation.</b> At the final stage of keratinocytes differentiation, desquamation occurs at which cells in the SC are replaced by newly formed corneocytes. Figure adapted from (Milstone, 2004).....	6
<b>Figure 1.5. Cytokine network in human keratinocytes.</b> Several cytokines are released by keratinocytes. Cytokine receptors are also present on keratinocytes surface. Based on (Gröne, 2002) .....	8
<b>Figure 1.6. The clinical appearance of IAD.</b> IAD is present as erythema and inflammation of the skin. Figure adapted from Beeckman <i>et al.</i> , 2011 .....	11
<b>Figure 1.7. Histopathological appearances of grade 1 pressure ulcers and IAD lesions. a)</b> An ischaemic pattern was evident in pressure ulcer samples; <b>b)</b> IAD lesions were associated with an inflammatory pattern, evident by partial loss of the epidermis, dilated vessels with some swelling of the endothelium, oedema of the dermis and presence of inflammatory cells. Figure is based on the work by Houwing and colleagues (2007) .....	15
<b>Figure 2.1. Flow chart showing the results from the systematic search of the literature.</b> .....	21
<b>Figure 2.2. Conceptual model of perineal dermatitis development.</b> Based on the work of Brown and Sears (1993) .....	22
<b>Figure 2.3. The pathophysiology of IAD.</b> Based on the work of Jeter and Lutz (1996).....	23
<b>Figure 2.4. The aetiology and pathophysiology of IAD.</b> Figure adapted from Gray and colleagues (2007). .....	24
<b>Figure 2.5. The multifactorial nature of IAD.</b> Prolonged exposure to moisture leads to a compromised skin barrier function and an increased pH. As a consequence, the permeability of the skin is disrupted and becomes susceptible to secondary skin infections. All these lead to skin breakdown and IAD development. Figure adapted from the work of Beeckman and colleagues (2009), which is based on Jeter & Lutz (1996) and Newman and colleagues (2007). .....	25
<b>Figure 2.6. Skin response to different synthetic urine solutions, varying ammonium hydroxide concentration, with an alkaline pH.</b> The erythema that was caused was proportional to the concentration of ammonium hydroxide. a) The redness that was caused upon exposure to the different synthetic urine solutions was assessed using a visual scoring system, and b) changes in blood flow (perfusion units) using LDI also confirmed this erythema production. Figure taken from the work by Larner and colleagues (2015) .....	28
<b>Figure 2.7. Potential inflammatory response in IAD.</b> .....	36
<b>Figure 3.1. Open-chamber system for TEWL measurement.</b> In the open chamber method, a cylinder is used which is open at both ends and contains sensors for temperature (T) and relative humidity (RH). Based on Imhof <i>et al.</i> , 2009b.....	41
<b>Figure 3.2. The Tewameter® instrument for measurement of TEWL.</b> a) The first model to be launched was the TM210, b) the TM300 probe, c) the newly developed TM300 wireless probe,	

and **d**) when the probe is connected to a computer, results are displayed using the manufacturer's software. A graph is created showing TEWL over time. At the end of the measurement, the average value is calculated and shown on the left bottom corner (picture obtained during testing). .... 41

**Figure 3.3. Closed-chamber systems for TEWL measurement.** **a**) The condenser-chamber system and **b**) the unventilated chamber method. Figure adapted from Imhof *et al.*, 2009b..... 42

**Figure 3.4. Bland Altman plots to assess the agreement between instruments.** **a**) Vapometer Vs TM300 open-chamber: a good agreement exists between the two for average values up to 55 g/h/m<sup>2</sup>. Then, the Vapometer values are systematically higher than TM300. The mean difference is 1.2 g/h/m<sup>2</sup>, and the lower and upper agreement limits are -35.8 g/h/m<sup>2</sup> and 38.3 g/h/m<sup>2</sup> respectively, and **b**) TM300 open-chamber Vs TM300 closed-chamber: there is a good agreement between the two, the mean difference is 1.3 g/h/m<sup>2</sup>, and the lower and upper agreement limits are -5.4 g/h/m<sup>2</sup> and 8.2 g/h/m<sup>2</sup> respectively, and Figure adapted from Steiner *et al.*, 2011.... 43

**Figure 3.5. Measuring principle of the Corneometer®.** Adapted from Courage & Khazaka ..... 49

**Figure 3.6. The Corneometer® CM825.** **a**) Wired probe as part of the MP9 system, and **b**) Wireless probe. Figure adapted from Courage & Khazaka. .... 49

**Figure 3.7. Intra- and inter- observer reliability of the Corneometer CM825 on burn scars.** **a**) Good agreement between two repeated measurements obtained by Observer A, with the mean difference= -1.64, and **b**) good agreement between measurements obtained by two observers, mean difference= -0.03. Figure adapted from Anthonissen *et al.*, 2015..... 51

**Figure 3.8. SEM Scanner Model 200.** **a**) The electrode at the back of the SEM Scanner is applied on the skin, and **b**) average and maximum readings are displayed. Figure adapted from Bruin Biometrics ..... 52

**Figure 3.9. Skin-pH-Meter® 905.** **a**) Measuring principle, **b**) wired probe, and **c**) wireless battery-operated device. Pictures taken from Courage & Khazaka and during testing (c)..... 55

**Figure 3.10. The tape stripping procedure.** An adhesive tape is placed on the skin surface for the removal of the SC. Figure adapted from (Pailler-Mattei *et al.*, 2011) ..... 57

**Figure 3.11. Tape stripping at different anatomical locations.** TEWL value increases with the number of strips. The backside presented the higher and more dramatically increase in TEWL compared to the other anatomical locations. Figure adapted from Gao *et al.*, 2013..... 58

**Figure 3.12. Principle of LDF and LDI for the measurement of blood flow in the microcirculation.** **a**) LDF and **b**) LDI systems. Figure taken from Moor Instruments..... 64

**Figure 3.13. Microdialysis technique for the *in vivo* recovery of substances.** A probe is inserted superficially and perfused with sterile saline. Based on diffusion, substances released in the interstitial space are collected in the dialysate. Figure adapted from Schnetz and Fartasch, (2001) ..... 69

**Figure 3.14. Traditional ELISA for biomarker research.** **a**) The traditional sandwich ELISA, in which a capture antibody is coated at the bottom of the plate. Then the sample is added, followed by the addition of a detection antibody which is not labelled, and thus a secondary enzyme-conjugated detection antibody is required. **b**) in direct ELISA the detection antibody is enzyme conjugated, and there is no need for a secondary antibody, and **c**) in indirect ELISA the sample is first added to the plate, followed by the addition of an unlabelled primary antibody, specific to the sample. At the final step, an enzyme conjugated secondary antibody, specific to the primary antibody, is added. Figure taken from (ThermoFisher Scientific, 2017) ..... 72

**Figure 4.1. Application of synthetic-urine.** **a**) Six skin sites were marked on both the volar forearms, and **b**) 0.5ml s-urine was impregnated into HillTop chambers and applied on the skin, and medical tape was used to keep them in place. A sixth site served as the untreated control.81

<b>Figure 4.2. Box and whiskers plot for skin blood flow at baseline and following exposure to s-urine solutions.</b> All s-urine solutions caused a significant increase to baseline ( $p<0.05$ ), however there were no significant differences between the s-urine solutions ( $p>0.05$ ). A non-significant increase ( $p>0.05$ ) in blood flow is also observed at the control site, and this is possibly due to changes in blood perfusion affecting adjacent sites. Significance is displayed with (*). ....	83
<b>Figure 4.3. Box and whiskers plot for TEWL measurement at baseline and following exposure to s-urine solutions.</b> All the s-urine solutions caused a significant rise in TEWL, and a consequent disruption of the skin barrier, compared to baseline values ( $p<0.05$ ). However, there were no significant differences between the s-urine solutions ( $p>0.05$ ). Significance is displayed with (*). ....	85
<b>Figure 4.4. Box and whiskers plot for SC hydration at baseline and following exposure to s-urine solutions.</b> Generally, there is an increase in the hydration status of the SC compared to baseline following exposure to varying pH s-urine solutions. There were no significant differences between the s-urine solutions ( $p>0.05$ ). Significance is displayed with (*). ....	87
<b>Figure 4.5. Box and whiskers plot for skin surface pH at baseline and following exposure to s-urine solutions.</b> In some of the volunteers there was an increase in pH compared to baseline values following exposure to s-urine. No significant effect was found. ....	89
<b>Figure 4.6. Measurement of pH over time.</b> The skin's buffering capacity was investigated after exposure to different s-urine solutions (pH 5.0, 6.0 and $8.0 \pm 0.7$ ). After an initial increase in pH in all skin sites, skin pH returns to baseline values within 5 minutes post-application. ....	91
<b>Figure 4.7. Box and whiskers plot for TEWL at baseline and following exposure to water, occlusion and s-urine, varying pH.</b> ....	92
<b>Figure 5.1. Flow diagram of the initial study of the current investigation.</b> ....	101
<b>Figure 5.2. Desorption curves of TEWL of intact and compromised skin exposed to s-urine.</b> Mean baseline TEWL was subtracted from post-challenge TEWL, and plotted against time. There is an initial increase in TEWL following removal of urine patches; however the TEWL value decreases with time, as skin surface water is evaporating. It is evident from the graph that chemically-irritated skin presents an increased susceptibility to s-urine penetration. ....	103
<b>Figure 5.3. Box and whiskers plot showing the amount of skin surface water loss for intact and compromised skin following exposure to urine.</b> The amount of SSWL for chemically-irritated skin is significantly higher compared to both intact and mechanically-irritated skin ( $p<0.05$ ). There is no significant difference between intact and mechanically-irritated skin ( $p>0.05$ ). Significance is displayed by (*). ....	103
<b>Figure 5.4. The effect of s-urine on blood perfusion of intact skin. a)</b> Box and whiskers plot of skin blood flow of intact skin. Exposure to s-urine leads to increased blood perfusion, compared to both baseline and control values ( $p>0.05$ ). Percentage change compared to baseline is also shown, <b>b)</b> Generated perfusion images of an individual at baseline, control and following exposure to synthetic-urine (baseline subtracted). ....	104
<b>Figure 5.5. Box and whisker plot showing the effect of s-urine on skin barrier function of intact skin.</b> Exposure to s-urine caused a significant increase in TEWL compared to both baseline and control values ( $p>0.05$ ). Percentage change compared to baseline is also shown. Significance is displayed with (*). ....	105
<b>Figure 5.6. Box and whisker plot showing the effect of s-urine on SC hydration of intact skin.</b> S-urine causes a significant ( $p<0.05$ ) increase in skin hydration, compared to both baseline and control values, as indicated by (*). Percentage change compared to baseline is also shown. ....	106
<b>Figure 5.7. Box and whisker plot showing the effect of s-urine on the skin acid mantle of intact skin.</b> Exposure of the skin to urine led to a small increase (1%) in skin pH from baseline, but this was not significant ( $p>0.05$ ). ....	106

**Figure 5.8. The effect of s-urine on blood perfusion of physically-irritated skin. a)** A box and whisker plot of skin blood flow of physically-irritated skin. Following tape stripping, there was a significant increase in blood flow, compared to both baseline and control values ( $p<0.05$ ). However, exposure to s-urine led to a significant decrease in blood flow compared to tape stripping ( $p<0.05$ ), but blood perfusion was still higher than basal values ( $p<0.05$ ). There was also a significant rise in blood flow at the control site ( $p<0.05$ ). Significance is displayed with (\*). Changes compared to baseline values are also expressed as percentages. **b)** Images from the LDI measurement, showing blood perfusion at baseline, the control site, and following tape stripping and urine challenges. Baseline image has been subtracted from both tape stripping and s-urine images..... 107

**Figure 5.9. The effect of tape stripping on the skin barrier function.** Tape stripping causes a disruption of the skin barrier function, evidenced by the increase in TEWL compared to baseline values (tape 0). ..... 108

**Figure 5.10. Inter-individual variability in tape stripping the skin.** The inter-individual variation ranges between 30-75%. o= males, □= females ..... 108

**Figure 5.11. Box and whisker plot showing the effect of s-urine on skin barrier function of physically-irritated skin.** Tape stripping and the following exposure to s-urine caused a significant increase in TEWL compared to both baseline and control values ( $p>0.05$ ). Changes compared to baseline values are also expressed as percentages. Synthetic urine also caused a significant increase ( $p<0.05$ ) in TEWL compared to tape stripping, marked with (\*). ..... 109

**Figure 5.12. Box and whisker plot showing the effect of s-urine on SC hydration of physically-irritated skin.** SC hydration increases with both tape stripping and exposure to synthetic-urine, and this is significant compared to both baseline and control values ( $p<0.05$ ). % change compared to baseline values is also shown. Significance is indicated with (\*). ..... 110

**Figure 5.13. Box and whisker plot showing the effect of s-urine on the acid mantle of physically-irritated skin.** Both tape stripping and s-urine did not cause any significant change ( $p>0.05$ ) in skin surface pH. Changes compared to baseline are also expressed as %. ..... 110

**Figure 5.14. The effect of s-urine on blood perfusion of chemically-irritated skin. a)** Box and whisker plot of skin blood flow of chemically-irritated skin. Both SLS and s-urine exposure caused a significant increase ( $p<0.05$ ) in skin blood flow compared to baseline, 92% and 110% respectively, and control values. S-urine exposure also caused a further increase in blood flux compared to SLS ( $p<0.05$ ). **b)** Images from LDI measurement. Baseline image has been subtracted from both SLS and s-urine post-exposure images. Significance is displayed with (\*). ..... 111

**Figure 5.15. Box and whisker plot showing the effect of s-urine on skin barrier function of chemically-irritated skin.** Exposure to SLS led to a disrupted skin barrier, reflected by the significant increase in TEWL compared to both baseline and control values. However, following exposure to urine, TEWL was still significantly higher than baseline, but significantly lower compared to SLS ( $p<0.05$ ). % change compared to baseline values is also shown. Significance is marked with (\*). ..... 112

**Figure 5.16. Box and whisker plot showing the effect of s-urine on SC hydration of chemically-irritated skin.** Both the SLS and urine caused a significant increase in skin hydration compared to baseline and control values ( $p<0.05$ ), and indeed exposure to s-urine led to a significant decrease ( $p<0.05$ ) in hydration levels. Interestingly, 24 hours following SLS treatment, SC hydration further decreased. Change (%) compared to baseline is also shown. Significance is marked with (\*). 113

**Figure 5.17. The effects of s-urine on the acid mantle of chemically-irritated skin.** Both the SLS and urine led to a significant increase ( $p<0.05$ ) in skin pH compared to both baseline (+5%) and control values, however there was no significant difference between these two ( $p>0.05$ ). Significance compared to baseline and control values is indicated with (\*). ..... 113

**Figure 5.18. The temporal effects of tape stripping and SLS on blood perfusion.** Tape stripping causes a transient increase in blood perfusion compared to baseline values; however this returns back to basal levels and control values, within 30 minutes post-challenge. SLS causes a prolonged increase compared to baseline values, evident after 2 ½ hours post-challenge.....114

**Figure 5.19. Perfusion images of tape stripping and SLS over time.** It is clear that SLS causes a prolonged increase in blood perfusion. On the contrary, tape stripping initially causes an increase in blood perfusion, but following 30 minutes post-challenge it returns back to baseline and control levels. Baseline images have been subtracted from all post-challenge images.....115

**Figure 6.1. Construction of microdialysis fibres.** A stainless steel wire was inserted into the fibres and then attached to the polyethene tubing. The joint was sealed with a smooth sealant. In case of a damaged joint, Loctite instant adhesive was used for repair.....124

**Figure 6.2. No-net-flux method for the determination of microdialysis efficiency.** **a)** A microdialysis fibre was passed through the holes of a glass beaker and sealed. **b)** the bath was then filled with 5ml diluent containing known concentration of the analytes, and **c)** the fibre was perfused with different concentrations of analytes of interest (Table 6.1) at a flow rate of 5 $\mu$ l/min for 90 minutes. Dialysates were collected in vials in 30 minutes intervals and subsequently analysed using the electrochemiluminescence assay.....126

**Figure 6.3. Efficiency of microdialysis for IL-1 $\beta$ , IL-6, IL-8 and TNF- $\alpha$ .** For each of the analyte, mean data were plotted and the efficiency was determined by the slope of the regression line. Microdialysis performed relatively poor, and the efficiency was between 3-7%. .....127

**Figure 6.4. Protocol to investigate the inflammatory response in the skin.** **a)** Integrity of the fibres was checked, **b)** EMLA was applied for 90 mins, **c)** three sites were marked, **d)** needles were inserted superficially at a length of 20mm, **e)** fibres were inserted through the needles and the needles removed, **f)** baseline dialysate was collected for 30 mins, **g)** baseline Sebutapes were collected, and **h)** challenges were applied and dialysate was collected for 2 hours .....130

**Figure 6.5. Sample collection timings.** Microdialysis samples were collected throughout the study, as indicated by the blue arrows, whilst the red arrows correspond to the collection points of Sebutapes.....131

**Figure 6.6. Box-and-whisker plots for skin blood flow at baseline and after each challenge.** Tape stripping caused the highest average increase in blood perfusion. Both s-urine and the proteolytic solution led to increased blood perfusion, but the highest increase was observed with the latter. No significant differences were found between challenges on blood perfusion ( $p>0.05$ ). However, when assessing pre- and post- values, a significant increase was detected following tape stripping and exposure to s-urine and the proteolytic solution ( $p<0.05$ ). A significant increase was also observed at the control site compared to baseline values ( $p<0.05$ ). Significance is displayed with (\*). .....136

**Figure 6.7. Box-and-whisker plots for TEWL at baseline and after each challenge.** SLS caused the highest increase in TEWL and a severe disruption of the skin barrier function. Both s-urine and the proteolytic solution caused a significant increase in TEWL compared to baseline values, however no significant difference was found between them ( $p>0.05$ ). Data also revealed a significant increase in TEWL following each challenge compared to baseline values ( $p<0.05$ ). Statistical significance is displayed with (\*). .....137

**Figure 6.8. Box-and-whisker plots for SC hydration at baseline and after each challenge.** All challenges caused an increase in SC hydration compared to baseline, but no significant differences were found between challenges ( $p>0.05$ ). Pre- and post-data revealed a significant increase in SC hydration following tape stripping and treatment with s-urine and the proteolytic solution ( $p<0.05$ ). Significance is shown with (\*). .....139

**Figure 6.9. Box-and-whisker plots for skin surface pH at baseline and after each challenge.** SLS, s-urine and proteolytic solution caused a significant increase ( $p<0.05$ ) in skin pH from baseline,

however no differences were found between challenges ( $p>0.05$ ). There was no significant effect of tape stripping on skin pH ( $p>0.05$ ). Significance is shown with (\*). ..... 141

**Figure 6.10. Box and whiskers plots of cytokine ratio changes to baseline (n=10, except n=9 for tape stripping).** a) S-urine caused the highest median increase in IL-1 $\alpha$ , b) Highest increase in IL-1RA was also observed with s-urine, c) IL-1 $\beta$  results varied and there was a tendency to decrease, d) most of the data for IL-6 were below the detection limit of the assay, and no meaningful data could be extracted, e) All challenges caused a an increase in IL-8, except the proteolytic solution. S-urine caused a significant increase in IL-8 levels compared to the proteolytic solution and f) the proteolytic solution caused a high increase in TNF- $\alpha$  , with a median ratio change to baseline of 7.25. Significance is marked with (\*). ..... 144

**Figure 6.11. Box-whisker plot of IL-1 $\alpha$ /IL-1RA ratio at baseline and following each challenge.** All challenges caused an increase in the ratio of IL-1 $\alpha$ /IL-1RA, except tape stripping. However, this was only significant on the s-urine treated site ( $p<0.05$ ). ..... 145

**Figure 6.12. Relationship between cytokine groups.** a) a significant correlation was found between IL-1 $\alpha$  and IL-1RA, b) a significant correlation was found between IL-1A and IL-1 $\beta$ ... 146

**Figure 6.13. Cytokine levels at T0, T4 and T5 following exposure to SLS.** There is a general increase in cytokines at T5 compared to T4, however this was not significant ( $p>0.05$ ). A significant increase is only observed for IL-6 at T5 compared to baseline (T0) levels ( $p<0.05$ ). Significance is marked with (\*). ..... 147

**Figure 6.14. Cytokine levels at T0, T1 and T4 at the tape-striped site.** A number of cytokines (IL-1RA, IL-6, IL-8) were increased at T4 compared to T1, whilst others (IL-1 $\alpha$ , TNF- $\alpha$ ) presented a decrease at T4, two hours after the challenge. However, no significant differences were found ( $p>0.05$ ). ..... 148

**Figure 7.1. Trypan Blue exclusion assay.** Cells are counted at the four corner boxes and the average is calculated. Then cell density and the % viability are calculated. Figure adapted from Louis and Siegel, 2011..... 160

**Figure 7.2. The optimum cell density of HaCaT for cell viability assessment.** The optimum cell density of HaCaT corresponds to  $0.99 \times 10^5$  cells/ml. (n=6) ..... 164

**Figure 7.3. The effects of LPS on the growth inhibition of keratinocytes: a dose-response curve.** Growth in keratinocytes is inhibited over 50% when concentration of LPS is above 15.6  $\mu$ g/ml. (n=6) ..... 165

**Figure 7.4. The effect of varying pH solutions on the growth inhibition of keratinocytes.** There was a significant effect ( $p<0.05$ ) of pH on keratinocytes' growth. In particular, at pH 8.0 there is a significant reduction ( $p<0.05$ ) in the growth inhibition of keratinocytes, compared to the other pH solutions. Significance is displayed with (\*). (n=6) ..... 166

**Figure 7.5. The effects of varying s-urine and proteolytic-solution concentrations on the growth inhibition of keratinocytes over time.** The effects of s-urine (a) and the proteolytic-solution (b) on keratinocytes are time- and dose- dependent. However, this is more evident with s-urine, which showed a significant increase in growth inhibition after 24 hours of exposure ( $p<0.05$ ). (n=6) 167

**Figure 7.6. The effects of a low and a high s-urine concentration, varying pH, on the growth inhibition of keratinocytes.** Keratinocytes were treated with varying pH s-urine solutions at two concentrations for 24 hours, and based on the growth inhibition of cells it was revealed that the effects of urinary pH are more evident with a low urine concentration (10%). However, no significant differences were found ( $p>0.05$ ). (n=6) ..... 168

**Figure 7.7. The effects of urinary pH on the growth inhibition of keratinocytes over time.** There is a time-dependent relationship between urinary pH and growth inhibition of keratinocytes. Exposure for 24 and 72 hours leads to a significant increase ( $p<0.05$ ) in growth inhibition compared to the other timings. (n=6) ..... 169

<b>Figure 7.8. Cytokine release following stimulation with various LPS concentrations.</b> Results showed that cytokine production increases with increasing LPS concentration. No significant differences were found ( $p>0.05$ ) .....	170
<b>Figure 7.9. Cytokine release following exposure to varying pH s-urine solutions at different incubation times.</b> There was a significant effect of exposure time and pH on the release of pro-inflammatory cytokines. Significant differences between the different pH solutions are marked with (*). .....	172
<b>Figure 8.1. The study design to address the research hypotheses.</b> .....	177
<b>Figure 8.2. Proposed mechanism of PGE<sub>2</sub> and the production of erythema in IAD.</b> Based on (Welss <i>et al.</i> , 2004; Nicolaou, 2013) .....	187
<b>Figure 8.3. Contribution of this thesis to current knowledge.</b> Based on existing theoretical frameworks on IAD pathophysiology, this thesis investigated the proposed mechanisms in IAD development (displayed in blue circles). Empirical evidence provided by this research is marked in red line dotted boxes. .....	190



# List of Tables

<b>Table 1.1. Pro- and anti- inflammatory cytokines in the skin.</b> Table is based on Corwin 2000, Zhang and An, 2007 .....	7
<b>Table 1.2. Differences between infant, adult and geriatric skin.</b> Diaper dermatitis should be considered as a distinct condition to IAD due to differences in the skin functional and morphological characteristics with age.....	16
<b>Table 2.1. The search terms used in this systematic review of the literature using PubMed, CINAHL, Medline and Embase via OvidSP.</b> .....	19
<b>Table 3.1. Commercially available instruments for quantification of TEWL.</b> Based on Imhof <i>et al.</i> , 2009b .....	40
<b>Table 3.2. Key characteristics of open- and closed- chamber systems for TEWL measurement.</b> .....	44
<b>Table 3.3. Available methods for measurement of SC hydration.</b> Based on Girard <i>et al.</i> , 2000.....	47
<b>Table 3.4. The different instruments available for skin hydration measurement.</b> .....	48
<b>Table 3.5. Corneometer values and their meaning in terms of skin type.</b> Based on Heinrich <i>et al.</i> , 2003 .....	50
<b>Table 3.6. Chemical composition of s-urine.</b> Formulation is based on Mayrovitz and Sims (2001), and was prepared in 50ml of distilled water.....	62
<b>Table 3.7. Chemical composition of the proteolytic solution.</b> Formulation is based on Andersen <i>et al.</i> , 1994 and Mugita <i>et al.</i> , 2015. Prepared in 50ml of phosphate buffered saline.....	62
<b>Table 3.8. A critical analysis of microdialysis.</b> .....	70
<b>Table 3.9. Characteristics of methods for skin sampling.</b> .....	70
<b>Table 3.10. A critique of HPLC, ELISAs and multiplex assays.</b> .....	73
<b>Table 3.11. Inclusion and exclusion criteria for volunteer recruitment.</b> .....	74
<b>Table 3.12. The methods employed in <i>in vivo</i> studies with human volunteers</b> .....	75
<b>Table 4.1. Percentage change in blood flow following exposure to s-urine, compared to baseline values.</b> .....	84
<b>Table 4.2. Percentage change in TEWL following exposure to s-urine, compared to baseline values.</b> .....	86
<b>Table 4.3. Percentage change in SC hydration following exposure to s-urine, compared to baseline values.</b> .....	88
<b>Table 4.4. Percentage change in skin pH following exposure to s-urine, compared to baseline values.</b> .....	90
<b>Table 4.5. Percentage change in TEWL following exposure to water, occlusion and s-urine, varying pH, compared to baseline values.</b> .....	92
<b>Table 6.1. Investigation of efficiency of microdialysis for the recovery of analytes of interest.</b> .....	125
<b>Table 6.2. Detection limit and dynamic range for each of the cytokines investigated.</b> .....	133
<b>Table 6.3. Percentage change in blood flow from baseline at the control site and following exposure to s-urine, tape stripping, SLS and a proteolytic solution.</b> The highest mean increase in blood perfusion was observed with tape stripping. Exposure to S-urine and the proteolytic solution caused a milder increase in perfusion compared to both tape stripping and SLS. ....	136

<b>Table 6.4. Percentage change in TEWL from baseline at the control site and following exposure to s-urine, tape stripping, SLS and a proteolytic solution. It is evident that SLS caused a significantly higher TEWL compared to the other treatments (p&lt;0.05). ....</b>	138
<b>Table 6.5. Percentage change in SC hydration from baseline at the control site and following exposure to s-urine, tape stripping, SLS and a proteolytic solution. All challenges increased the hydration status of the skin barrier, with s-urine and the proteolytic solution having a similar effect. No significant differences were found (p&gt;0.05). ....</b>	140
<b>Table 6.6. Percentage change on skin surface pH from baseline at the control site and following exposure to s-urine, tape stripping, SLS and a proteolytic solution. All the challenges, except tape stripping, caused an increase in skin pH, with the highest increase caused by the SLS treatment. Mean percentage change for s-urine and proteolytic solutions was very similar, 11% and 13% respectively. ....</b>	141
<b>Table 6.7. Ratio to baseline values of cytokines following each challenge. Data is expressed as median (IQR). ....</b>	143
<b>Table 6.8. Ratio changes to baseline of cytokine concentrations at the control site (n=2) ...</b>	149
<b>Table 6.9. Ratio changes to baseline of cytokine concentrations at the SLS-treated site (n=8).150</b>	
<b>Table 6.10. Ratio changes to baseline of cytokine concentrations at the tape-striped site (n=1). ....</b>	150
<b>Table 6.11. Ratio changes to baseline of cytokine concentrations at the s-urine treated site (n=9).....</b>	151
<b>Table 6.12. Ratio changes to baseline of cytokine concentrations at the proteolytic-solution treated site (n=8). ....</b>	151
<b>Table 6.13. A comparative critique of dermal microdialysis and Sebutape™ absorption method .....</b>	153

# **Academic Thesis: Declaration of Authorship**

I, Sofoklis Koudounas, declare that this thesis and the work presented in it are my own and have been generated by me as the result of my own original research.

## **Investigation of the underlying mechanisms leading to the development of incontinence-associated dermatitis**

I confirm that:

1. This work was done wholly or mainly while in candidature for a research degree at this University;
2. Where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;
3. Where I have consulted the published work of others, this is always clearly attributed;
4. Where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;
5. I have acknowledged all main sources of help;
6. Where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;
7. None of this work has been published before submission

Signed:.....

Date:.....

# Dissemination

## Presentations:

1. **Paper (Oral) presentation**, Koudounas S., Voegeli D., Bader D.L., "Effect of surface liquid pH on the functional characteristics of healthy skin". 3<sup>rd</sup> EPUAP Scientific Focus Meeting 2016, Berlin, Germany
2. **Paper (Oral) presentation**, Koudounas S., Voegeli D., Bader D.L., "The permeability of intact and compromised skin to synthetic-urine". 19<sup>th</sup> EPUAP Annual Meeting 2017, Belfast, Northern Ireland
3. **Paper (Oral) presentation (accepted)**, Koudounas S., Bader D.L., Voegeli D., "The inflammatory response in IAD". 20<sup>th</sup> EPUAP Annual Meeting 2018, Rome, Italy

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## Definitions and Abbreviations

ACE= Angiotensin-converting-enzyme

AD= Atopic dermatitis

AU= Arbitrary units

BCA= Bicinchoninic acid

BSA = Bovine serum albumin

CE= Cornified envelope

CV= Coefficient of variation

DEJ= Dermal-epidermal junction

DMSO = Dimethyl sulfoxide

EB= Epidermolysis bullosa

EGF= Epidermal growth factor

ELISA= Enzyme-linked Immunosorbent Assay

FBS= Foetal bovine serum

GLOBIAD= Ghent Global IAD Categorisation Tool

HCl= Hydrochloric acid

HPLC= High-performance liquid chromatography

HSP = Heat shock protein

IAD= Incontinence-associated dermatitis

ICC= Intraclass correlation

ICD= Irritant contact dermatitis

IFN- $\gamma$ = Interferon gamma

IL= Interleukin

IL-1RA= Interleukin - 1 receptor antagonist

IL-1RI= Interleukin-1 receptor I

IQR = Interquartile range

kDa = Kilo Daltons

KGM= Keratinocyte growth medium

KLK= Kalikrein

LB= Lamellar bilayer

LDF= Laser Doppler Flowmetry

LDI= Laser Doppler Imaging

LLOD= Lower Limit of Detection

LPS= Lipopolysaccharide

LSCI= Laser Speckle Contrast Imaging

MASD= Moisture-associated skin damage

MEM= Minimum essential medium

NF-κB= Kappa-light-chain-enhancer of activated B cells

NH<sub>4</sub>OH= Ammonium hydroxide

NMF= Natural moisturizing factor

NO= Nitric oxide

NSAIDS= Nonsteroidal anti-inflammatory drugs

OTTER= Opto-thermal transient emission radiometry

PBS= Phosphate buffered saline

PGE<sub>2</sub>= Prostaglandin E<sub>2</sub>

PU= Pressure ulcer

RH= Relative humidity

ROI= Region of interest

RT-PCR= Real time polymerase chain reaction

SC= Stratum corneum

SEM= Subepidermal moisture

SLS= Sodium lauryl sulphate

SSWL= Skin surface water loss

S-urine = Synthetic-urine

T= Temperature

TAP= Transdermal Analyses Patch

TEWL= Transepidermal water loss

TGF- $\beta$ = Transforming growth factor beta

TNF- $\alpha$ = Tumour necrosis factor alpha

VEGF= Vascular endothelial growth factor

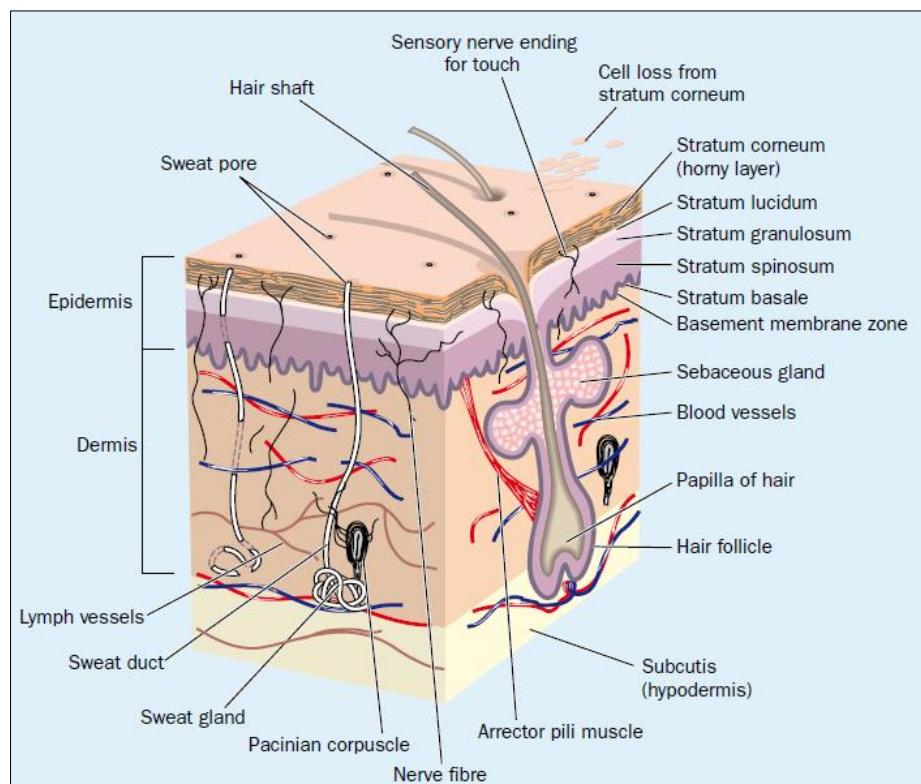


# Chapter 1: Introduction

Chapter 1 provides an introduction to the skin, the barrier function and the critical components involved in its maintenance. Then, the damaging effects of incontinence and the development of incontinence-associated dermatitis (IAD) are discussed. At the end of this chapter, the problems associated with IAD research are highlighted leading to the research problem addressed in the present study.

## 1.1 Structure of the skin

The skin is the largest organ of the human body composed of two layers, the epidermis and the dermis, as shown in Figure 1.1, and is responsible for multiple functions, including the protection of the body against foreign threats by forming a barrier against the external environment and the control of homeostasis, such as the regulation of body temperature (Sibbald *et al.*, 2003; Elias, 2005; Tortora and Derrickson, 2014).



**Figure 1.1. Skin structure and composition.** The skin is composed of the epidermis and the dermis. Figure adapted from Voegeli, 2012

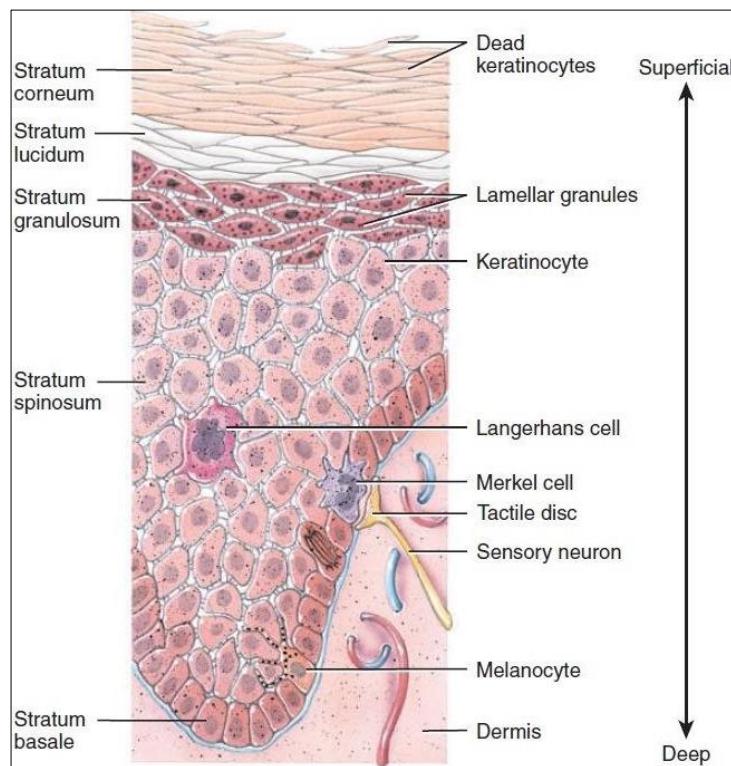
### 1.1.1 Epidermis

The epidermis is about  $100\mu\text{m}$  thick, avascular and is separated into four distinct layers, namely:

- 1) The stratum basale (basal layer) which is the deepest layer
- 2) The stratum spinosum
- 3) The stratum granulosum and
- 4) The stratum corneum (SC), which is the uppermost layer.

In thick skin areas, such as the palms of the hands and the soles of the feet, an additional layer, the stratum lucidum is also present between the stratum granulosum and the SC.

The cell population of the epidermis is composed of keratinocytes, melanocytes, intraepidermal macrophages (Langerhans cells) and Merkel cells. Keratinocytes are abundant in the epidermis and synthesize keratin, the main structural protein of the epidermis (Tortora and Derrickson, 2014). Primarily, it was believed that keratinocytes were just serving as passive barriers between the internal and external environment. However, pioneering work in the late 1970's discovered that keratinocytes secrete T-cell stimulating factors, establishing their importance in the skin immune system (Bos and Kapsenberg, 1993). Indeed, these cells contribute to the initiation, regulation and modulation of skin inflammation (Pastore *et al.*, 2006). Upon disruption of skin integrity, a dysregulated intercellular communication between keratinocytes is evident along with the release of inflammatory mediators, including interleukin-(IL) 1 alpha (IL-1 $\alpha$ ), tumour-necrosis factor alpha (TNF- $\alpha$ ), IL-6 and IL-8 (Köck *et al.*, 1990; Barker *et al.*, 1991; Coquette *et al.*, 2000). With respect to the other cell types of the epidermis, melanocytes are responsible for the synthesis and distribution of melanin for photoprotection, Langerhans cells are dendritic cells of the skin and are involved in the immune response by presenting antigens to T-cells, whilst Merkel cells are responsible for touch sensation. The different layers and cell populations of the epidermis are illustrated in Figure 1.2 (Tortora and Derrickson, 2014).



**Figure 1.2. Structure of the epidermis.** The different layers and cell types of the epidermis are shown. Figure based on Tortora and Derrickson, 2014.

### 1.1.2 Dermal-Epidermal Junction

The dermal-epidermal junction (DEJ) is found between the two principle layers of the skin, and is composed of the basal cell plasma membrane, the lamina lucida, the basal lamina and the sub-basal lamina. The DEJ tightly connects the epidermis to the dermis, but also serves as a barrier to the exchange of cells and molecules across this junction. Therefore, the DEJ is critical for skin integrity and any abnormalities are associated with the development of several skin disorders, that belong to a group of conditions termed epidermolysis bullosa (EB), and are characterized by increased skin fragility (Briggaman and Wheeler Jr, 1975; Burgeson and Christiano, 1997).

### 1.1.3 Dermis

The dermis is composed of two layers, the papillary (upper) and the reticular layer, which are responsible for the supply of oxygen and nutrients to the epidermis respectively (Pringle and Penzer, 2002). It also provides physical and structural support of the epidermis, due to the presence of matrix components, namely, collagen and elastin (Tortora and Derrickson, 2014).

## Chapter 1

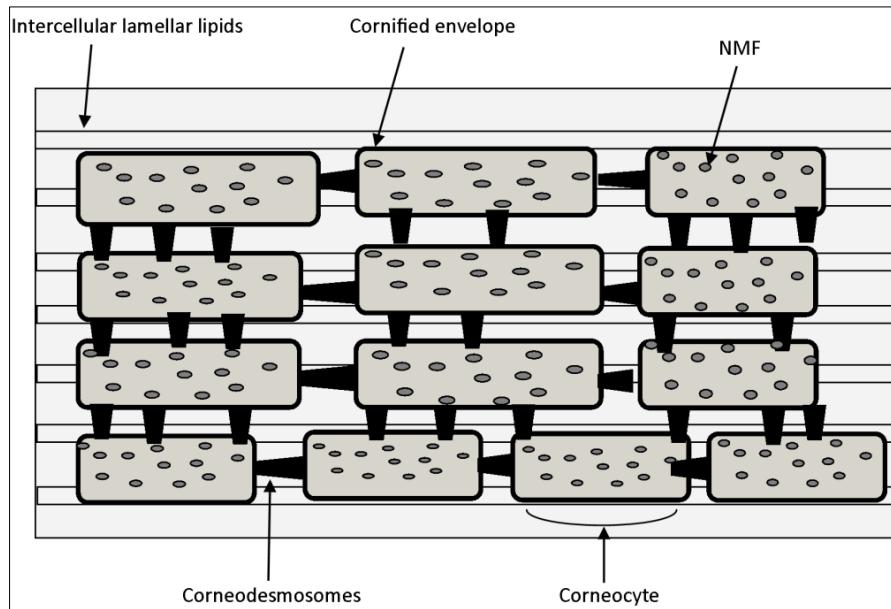
Blood and lymph vessels, nerve endings, hair follicles and sweat glands are also present in the dermis (Timmons, 2006). The dermis is composed of three major cell types: fibroblasts, macrophages and mast cells (Tortora and Derrickson, 2014).

Below the dermis, there is the subcutaneous tissue which is mainly composed of adipose and connective tissue (Benbow, 2009), and is responsible for: 1) controlling the thermal loss from the body by acting as an insulator, 2) the storage of lipids that can be used as an energy source in the case of emergency, and 3) the protection against mechanical damage, such as pressure, as the adipose tissue helps to evenly distribute the force applied (Agache and Humbert, 2004; Holloway and Jones, 2005). Nevertheless, it should be noted that the subcutaneous tissue is not considered as part of the skin (Tortora and Derrickson, 2014).

### 1.2 The Skin as a Barrier

As mentioned above (section 1.1), an important function of the skin is the formation of an effective barrier against the environment. This barrier function of the skin resides in the SC. Traditionally, the SC has been considered to be an inactive region (Scheuplein and Blank, 1971); however more recent studies have identified it to play a critical role in mechanisms associated with health and disease. Important functions of the SC include the protection against mechanical and physical loading (Madison, 2003), regulation of water diffusion from the skin, a process known as transepidermal water loss (TEWL), and the penetration of substances (Tregear, 1966; Blank, 1969; Scheuplein and Blank, 1971). These functions of the SC are achieved by the formation of the skin barrier, that begins with the differentiation (also known as cornification) and migration of keratinocytes from the stratum basale towards the SC (Elias, 2005). As the cells move upwards to the different layers, they receive less nutrition, become more keratinised and eventually become dead, anucleated, flattened cells in the SC, termed corneocytes (Kligman and Christophers, 1963; Tortora and Derrickson, 2009). The structure of the SC, with a thickness ranging between 10-40  $\mu\text{m}$ , can be considered as a two-component system traditionally reflected as a 'bricks and mortar' arrangement, with the corneocytes bricks held together by a lipid-rich (ceramides, cholesterol, fatty acids) matrix mortar, as shown in Figure 1.3 (Michaels *et al.*, 1975; Bohling *et al.*, 2014). Corneocytes contain a group of substances collectively known as natural moisturizing factor (NMF), which is responsible for attracting water from the atmosphere and retaining the skin in a hydrated state, as it contains humectant substances such as urea (Rawlings and Harding, 2004; Cork and Danby, 2009). Maintaining skin hydration is important for corneocytes' turgidity and shape, but also for the overall flexibility and elasticity of the skin (Rawlings and Harding, 2004; Voegeli, 2012a). In addition, the lipids in the SC are organized into lamellar bilayers (LB) and are important for the permeability of the skin barrier (Elias, 2012).

Specialized desmosomes, termed corneodesmosomes, are also present in the SC and are responsible for its adhesion properties and tensile strength (Harding, 2004; Menon *et al.*, 2012). Particularly, they are responsible in retaining the lipids and corneocytes together prior to desquamation, an important process which will be described later (Egelrud, 2000; Simon *et al.*, 2001; Harding, 2004).



**Figure 1.3. The structure of the stratum corneum.** Corneocytes are held together by intercellular lamellar lipids, and are arranged in a “brick and mortar” model. The figure is based on Voegeli, 2012

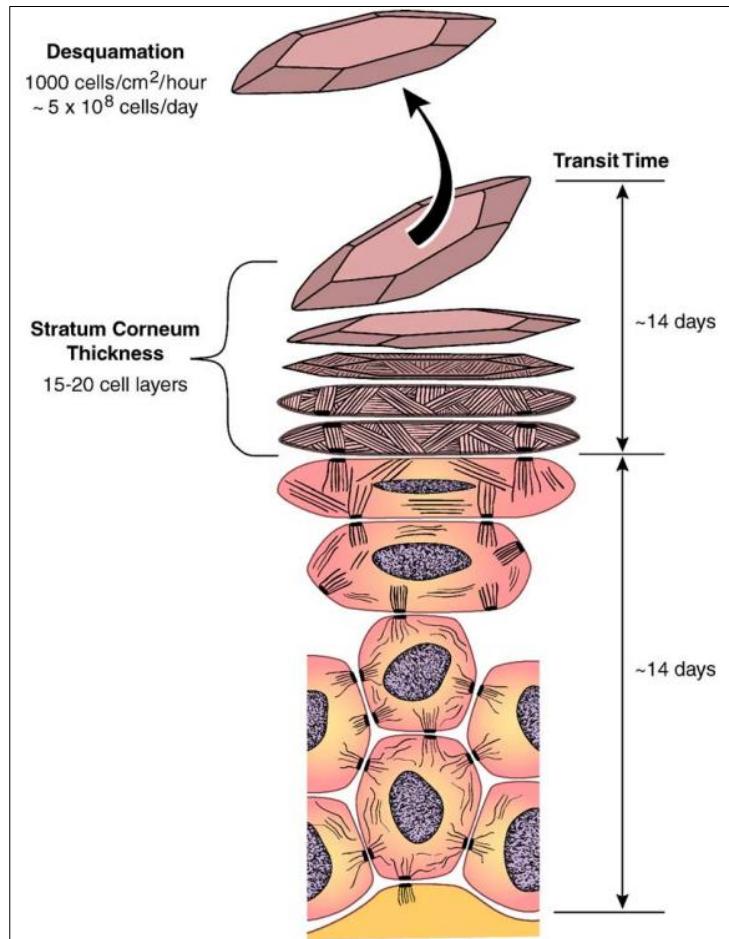
### 1.3 Maintenance of skin barrier integrity

Healthy skin is defined as the skin that has the ability to complete all its functions without affecting the individual's quality of life (Penzer and Finch, 2001). Important components of skin integrity include desquamation, the formation of the cornified envelope, epidermal cytokine network, the regulation of water loss and the maintenance of the acidity of the skin surface, known as the acid mantle. These will be discussed below.

#### 1.3.1 Desquamation

Desquamation refers to the natural shedding of the outermost layer of the skin so it can be replaced by newly formed corneocytes from the subsequent epidermal layers (Figure 1.4). This process takes about 14-28 days to complete and is important for SC thickness, and therefore should be balanced and tightly controlled (Jackson *et al.*, 1993; Egelrud, 2000; Harding, 2004).

Accordingly, any abnormalities lead to an accumulation of corneocytes in the SC, manifested as rough and dry skin. How this process is regulated has not been fully elucidated yet but it has been proposed that proteolytic enzymes in the SC are important, including the kalikrein (KLK) 5 and 7, also known as stratum corneum tryptic and chymotryptic enzymes (Egelrud, 2000; Prassas *et al.*, 2015).



**Figure 1.4. Skin desquamation.** At the final stage of keratinocytes differentiation, desquamation occurs at which cells in the SC are replaced by newly formed corneocytes. Figure adapted from (Milstone, 2004)

### 1.3.2 The cornified envelope

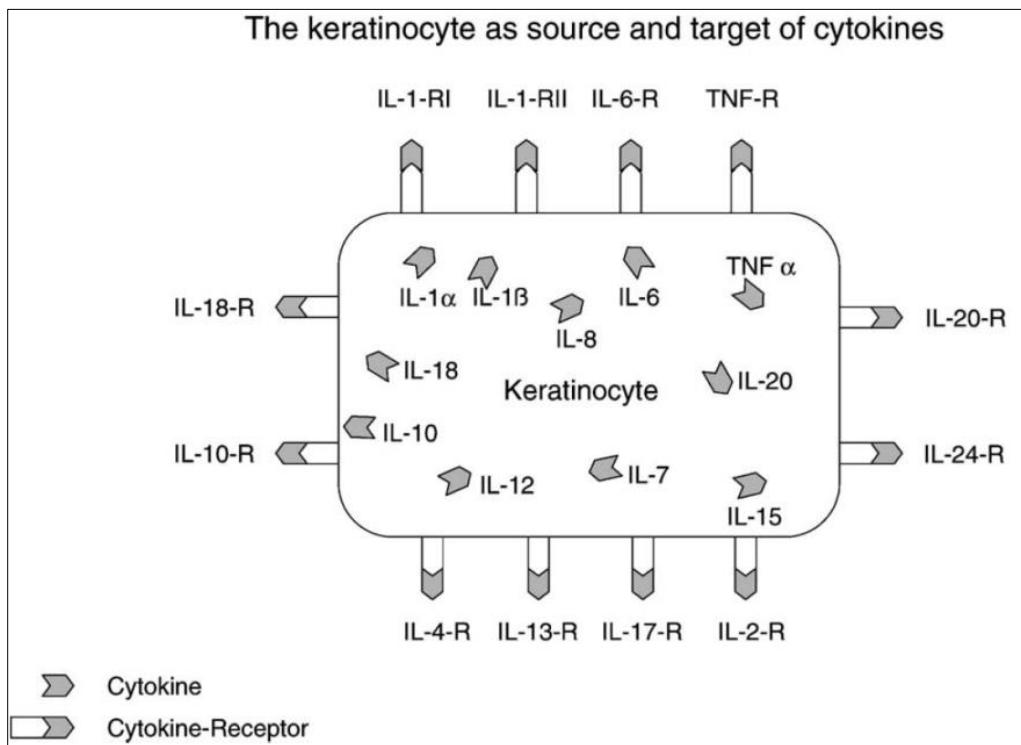
Corneocytes are protected from external damage by the cornified envelope (CE) that surrounds them, which is composed of a 10 nm thick layer of proteins and a 5 nm thick layer of ceramide lipids (Kalinin *et al.*, 2001; Harding, 2004). The protein component of the CE includes involucrin and loricrin, and is responsible for its biomechanical properties, and together with the lipid component prevent the movement of water and electrolytes (Harding, 2004; Candi *et al.*, 2005).

### 1.3.3 Epidermal cytokines

Cytokines are low molecular weight (between 8–30 kDa) proteins which are responsible for maintaining SC homeostasis, by influencing the growth and proliferation of keratinocytes, and also the interactions between epidermal cells (Zhang and An, 2007). Cytokines are also involved in the inflammatory response triggered in the skin, having both pro-inflammatory and anti-inflammatory effects, examples of which are shown in Table 1.1 (Dinarello, 1996; Czermak *et al.*, 1998; Coondoo, 2011). Accordingly, it has been reported that following injury cytokine levels are rapidly increased (Dinarello, 1999; Gabay and Kushner, 1999). Indeed, their levels are also increased in several skin inflammatory conditions, including atopic dermatitis (AD) (Hanel *et al.*, 2013). Once released, cytokines bind to specific receptors on target cells altering their function, and in some cases this leads to the production of additional cytokines (Corwin, 2000; Zhang and An, 2007). In the skin, keratinocytes express various cytokine receptors on their surface and represent a major source of cytokines in the epidermis, as illustrated in Figure 1.5 (Barker *et al.*, 1991).

**Table 1.1. Pro- and anti- inflammatory cytokines in the skin.** Table is based on Corwin 2000, Zhang and An, 2007

Pro-inflammatory	Anti-inflammatory
IL-1 ( $\alpha$ and $\beta$ )	IL-4
TNF- $\alpha$	IL-6
IL-6	IL-10
IL-8	IL-1 receptor antagonist (IL-1RA)
Interferon gamma (IFN- $\gamma$ )	Transforming growth factor beta (TGF- $\beta$ )



**Figure 1.5. Cytokine network in human keratinocytes.** Several cytokines are released by keratinocytes. Cytokine receptors are also present on keratinocytes surface. Based on (Gröne, 2002)

### 1.3.4 Transepidermal Water Loss (TEWL)

Transepidermal water loss can be defined as the water vapour flux that diffuses from the deeper layers of the dermis and the epidermis to the skin surface, and until the mid 1950's was primarily known as insensible perspiration (Rothman, 1954b; Kottner *et al.*, 2013). As the SC is responsible for regulating TEWL, any damage to the SC will inevitably result in an increase in TEWL and an associated impairment of its function (Fluhr *et al.*, 2006b). Several parameters are involved in limiting TEWL, including desquamation and the production of a lipid layer on the SC surface from sebum (Ersser *et al.*, 2005). Additionally, keratinocytes produce lamellar granules which in turn release a water-repellent sealant responsible for preventing water loss from the skin (Tortora and Derrickson, 2014). Quantitative measurements of TEWL are dated nearly half a century ago (Ray Bettley and Grice, 1965), and is now considered to be one of the most important parameters for the assessment of healthy skin. This was demonstrated in a seminal study by Fluhr and colleagues (2006) in which TEWL was measured after disruption of the skin barrier, using both human and murine models. In particular, TEWL was measured using three commercial available devices and barrier integrity was compromised by tape stripping.

Results showed that all devices could detect significant changes in TEWL values between baseline (prior challenge) and after tape stripping, establishing TEWL value as an indicator of the barrier function of the skin (Fluhr *et al.*, 2006b). This was also validated *in vitro*, in which cadaver skin was treated with tritiated water, and its penetration rate was associated with TEWL measurements (Elkeeb *et al.*, 2010). Although there is not a generally accepted TEWL value for normal skin, it has been proposed that low values of TEWL ( $\leq 10$ ) correspond to a healthy barrier function (Imhof *et al.*, 2009b).

### 1.3.5 The acid mantle

The acidity of the skin is another key factor for skin integrity. This was discovered in 1892 and highlighted nearly four decades later with the use of the term “acid mantle” (Schade and Marchionini, 1928). Indeed, the skin surface exhibits a pH between 4.2 and 6.1 (Braun-Falco and Korting, 1986), and varies between both the skin layers (Schreml *et al.*, 2010) and the internal value in the body which is normally at a pH of 7.4 (Rippke *et al.*, 2002). How the skin generates this acidic nature is not well-understood yet, but different active and passive mechanisms have been proposed. These include: 1) proton pump secretion via ion pumps located in the epidermis, 2) the generation of free fatty acids from phospholipids by phospholipases, and 3) the generation of cis-urocanic acid (Behne *et al.*, 1999; Krien and Kermici, 2000; Fluhr *et al.*, 2001). Traditionally, this acidic pH was believed to act as a protective mechanism against pathogenic species but recent studies revealed that it is also important for skin health, and any changes in pH are associated with disrupted SC integrity and cohesion, as well as with a disturbance of SC functions, such as the maintenance of homeostasis and protection (Fluhr *et al.*, 2001; Hachem *et al.*, 2003; Gunathilake *et al.*, 2009). In particular, several studies showed that key lipid-processing enzymes, such as  $\beta$ -glucocerebrosidase, which are critical for barrier synthesis, are active in an acidic environment (Rippke *et al.*, 2002; Hachem *et al.*, 2003; Hachem *et al.*, 2005). A study also examined the recovery rate of the skin barrier function, that is the return of TEWL to basal values, after induced-damage, and reported to occur faster in an acidic environment (pH 5.5) (Mauro *et al.*, 1998). Any shifts in pH will also hinder normal skin microbiota, which composes of viruses, fungi, mites and several bacteria, including the common commensal *Staphylococcus epidermidis* (coagulase-negative staphylococci), as their growth is optimal at an acidic pH (Grice and Segre, 2011; Weyrich *et al.*, 2015). In addition, the production of nitric oxide (NO), by specific nitric oxide synthases that metabolize L-arginine and/or by the action of bacterial nitrate reductase that sequentially transform sweat nitrate to nitrite and then NO, represents another protective mechanism against the growth of pathogens, such as *Candida albicans* and *Escherichia Coli*.

## Chapter 1

Indeed, a seminal study reported that its production is increased in acidic conditions, as the conversion of nitrite to NO is enhanced (Weller *et al.*, 1996). As a consequence, an increase in pH will inhibit NO normal production and promote the growth of pathogenic bacteria (Leyden *et al.*, 1979; Korting *et al.*, 1990; Weller *et al.*, 1996).

All these characteristics highlight the critical importance of maintaining an intact SC, not only to ensure the overall mechanical property of the skin but to maintain the skin in a healthy state.

### 1.4 Moisture-associated skin damage

Prolonged exposure of the skin to moisture can cause chronic inflammation and erosion of the skin that ultimately leads to skin breakdown (Gray *et al.*, 2011). A general term, moisture-associated skin damage (MASD), was coined by a panel of experts to better define and distinguish the effects of moisture from various sources, such as urine, stool, perspiration and wound exudate on the skin (Gray *et al.*, 2011). MASD covers four distinct conditions depending on moisture source, namely: 1) Incontinence-associated dermatitis (IAD), 2) Peristomal moisture-associated dermatitis, 3) Intertriginous dermatitis caused by perspiration trapped in skin folds and secondary skin infection and 4) Periwound moisture-associated skin damage due to exposure to wound exudate (Gray *et al.*, 2011). This research work is focused on IAD, and therefore this condition will be discussed further.

### 1.5 Incontinence-associated dermatitis (IAD)

IAD is caused by the persistent exposure of the skin to urine and/or faeces (Black *et al.*, 2011; Gray *et al.*, 2011). Until recently, it was believed that IAD was a type of irritant contact dermatitis (ICD) (Black *et al.*, 2011), however, a more recent histological study using an animal model suggested that IAD is different from ICD in terms of the mechanisms involved, as the tissue damage in IAD is deeper and in fact it reaches the dermal layer (Mugita *et al.*, 2015). Erythema and inflammation of the skin are the two most evident manifestations upon skin damage from urine/faeces, as shown in Figure 1.6, and if left untreated can lead to swelling and blister formation (Voegeli, 2016). In addition, affected skin areas may feel warmer and firmer compared to the surrounding unaffected skin. Secondary infections, mainly fungal infections, may also be developed due to increased susceptibility of the skin to pathogens, with one study reporting that 32% of IAD patients had a fungal infection (Campbell *et al.*, 2014). IAD also causes significant pain, discomfort, burning and itching which can eventually lead to a loss of independence and reduced quality of life (Beeckman *et al.*, 2015; Van Damme and Vanryckeghem, 2015; Beeckman *et al.*, 2016).

While the exact mechanisms that lead to the development of IAD have not been clarified yet, it has been suggested that in urinary incontinence, exposure to urine leads to an overhydrated epidermis and because of its chemical constituents, such as urea, skin pH is shifted to alkali levels (Black *et al.*, 2011). Overhydration of the epidermis leads to swelling of corneocytes and disruption of the barrier function leading to skin maceration (Ichikawa-Shigeta *et al.*, 2014), and to increased susceptibility to mechanical damage such as pressure, shear and friction (Beeckman *et al.*, 2015). In a similar manner, in faecal incontinence the skin damage is mainly occurring due to the detrimental effects of faeces and liquid stool, as they contain proteolytic enzymes that again raise the skin pH to alkaline and thus disrupting the acid mantle (Beeckman *et al.*, 2009; Beeckman *et al.*, 2015).



**Figure 1.6. The clinical appearance of IAD.** IAD is present as erythema and inflammation of the skin. Figure adapted from Beeckman *et al.*, 2011

### 1.5.1 Risk factors

Incontinence, the involuntary passing of urine (urinary), faeces (faecal) or both (double-incontinence), is considered the main risk factor for skin breakdown and the development of IAD (Beeckman *et al.*, 2009; Black *et al.*, 2011). It has been suggested that the rate of individuals that are affected by urinary incontinence is 50% in nursing homes and 10-35% in community settings (Newman *et al.*, 2007). Additionally, it is estimated that around 423 million people worldwide suffer from urinary incontinence (Irwin *et al.*, 2011).

Likewise, 23-66% of nursing home residents have been reported with faecal-incontinence, and it is anticipated that these figures will rise in the upcoming years (Newman *et al.*, 2007; Markland *et al.*, 2010). As described by Gray and colleagues (2002) the four factors that are mainly responsible for skin breakdown, especially in incontinent patients, include: 1) moisture, 2) skin surface pH, 3) microorganisms and 4) mechanical factors (friction) (Gray *et al.*, 2002). Other factors that also contribute to IAD development include use of occlusive products, mobility, poor personal hygiene, nutrition and medication, such as steroids and antibiotics (Beeckman *et al.*, 2015). It should be noted here that most of the risk factors are correlated with increasing age; however a recent study proposed that age is not a risk factor for the development of IAD (Kottner *et al.*, 2014).

### **1.5.2 Prevalence and Incidence of IAD**

Prevalence and incidence are two epidemiological terms that are often confused. Therefore, for clarification reasons, it should be mentioned here that prevalence refers to the proportion of a population (expressed as a percentage) that suffers from IAD at a given time, whilst incidence is the rate of new cases of people who develop IAD during a period of time (e.g. month, year) (Gray *et al.*, 2007). As reported in the literature, prevalence rates for IAD vary from 5.6% to 50%, while the incidence rates range from 3.4% to 25% (Bale *et al.*, 2004; Bliss *et al.*, 2006). However, prevalence figures may not be representative due to the lack of a validated assessment tool, the confusion between MASD and superficial pressure ulcers (PUs), as well as the lack of accepted clinical criteria for the diagnosis of IAD (Borchert *et al.*, 2010; Beeckman *et al.*, 2014). Recently, a panel of experts developed a simple IAD severity categorisation tool, known as the Ghent Global IAD Categorisation Tool (GLOBIAD), to inform clinical practice and to serve as a guide for research studies. This instrument included three categories for assessing IAD, namely: 1) no redness and skin intact (at risk, category 0), 2) red but skin intact (signs of oedema) (category 1), and 3) red with skin breakdown (signs can include vesicles, denudation and/or skin infection) (category 2) (Beeckman *et al.*, 2015). Recent published work examined the reliability and validity of the GLOBIAD in an international sample of 823 health professionals and results showed that this tool is characterized by increased sensitivity in diagnosing between intact but erythematous skin and skin loss. Although the clinical signs of infection were difficult to be determined, as signs of infection cannot be assessed based merely on photographs, this work is a step forward towards the development of an internationally accepted IAD categorization tool. Future work is required to assess its reliability and also to examine whether it improves prevention and treatment of IAD (Beeckman *et al.*, 2018).

### 1.5.3 Prevention and management of IAD

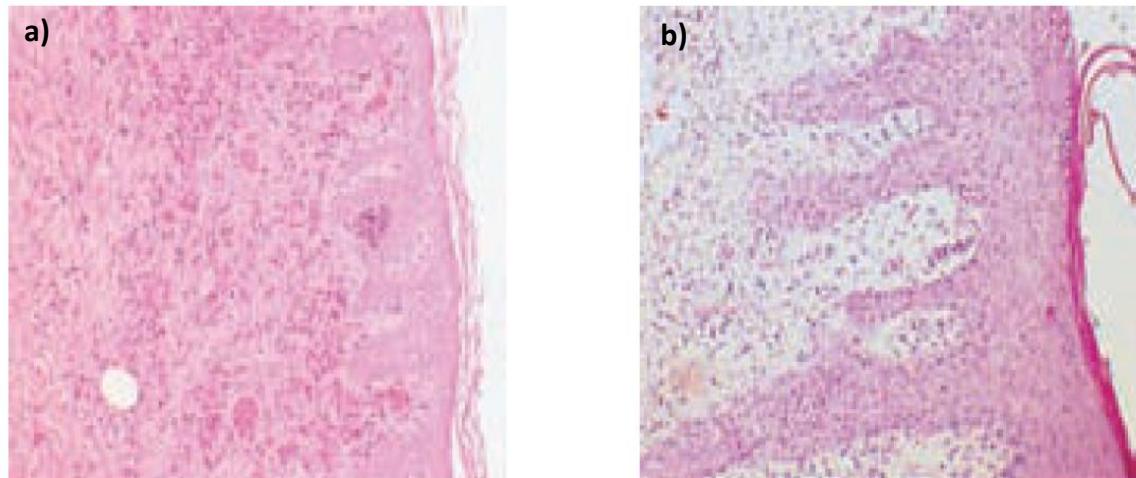
Considering the number of patients suffering from IAD, the prevention and management of IAD impose a heavy financial burden for both hospital and community settings. In particular, in 2014 in England, barrier products were prescribed at an estimated cost of £3.27 million, according to figures (Health and Social Care Information Centre., 2014). The primary preventive measure against the development of IAD is to minimize the exposure of skin to incontinence. For this reason, it has been suggested that four main strategies should be adopted, namely:

- i. the use of a standardized skin care regimen to cleanse the skin
- ii. the use of products that absorb moisture from the skin,
- iii. the removal of the source of excessive moisture and
- iv. the treatment of any secondary infections (Gray *et al.*, 2011)

Skin care regimens have been developed and adopted to protect the skin from the damaging effects of urine/faeces and to maintain the skin acid mantle. Skin regimens usually include the use of soap and water to cleanse the area at risk and the use of a moisturizer and/or a skin protectant (Gray *et al.*, 2007; Ali and Yosipovitch, 2013). Different studies have explored the efficacy of these skin care regimens, and interesting findings were shown, as no statistical differences were found between the different regimens in promoting skin health and in protecting against IAD (Lewis-Byers and Thayer, 2002; Bliss *et al.*, 2007, Beeckman *et al.*, 2016). Indeed, a very recent Cochrane review explored the effectiveness of various skin care products and procedures to prevent and treat IAD (Beeckman *et al.*, 2016). It included thirteen small randomised controlled trials, involving 1316 participants over 18 years of age, which compared skin care products and the procedures/methods and their frequency of use. All participants were incontinent for urine, stool, or both and lived in nursing homes or were hospitalised. Two of these randomized trials showed that washing with soap and water is not very effective against the development of IAD, and suggested that a skin cleanser/washcloth with cleansing, moisturising and protecting properties may perform better. Furthermore, findings from the other 11 trials proposed that a skin care product should be used in treating and preventing IAD. Nonetheless, the authors of the review concluded that there is no substantive evidence that one skin care product performs more effectively than others, and highlighted the need for larger, long-term and well-performed trials (Beeckman *et al.*, 2016). In another study, 6 washing and drying techniques were compared, and it was shown that washing with soap and water, and drying using a towel damages the skin barrier (Voegeli, 2008). Nevertheless, skin care regimens are important, and further research is required in order to establish a more structural approach to prevent the development of IAD (Gray *et al.*, 2007).

## 1.6 Differentiating IAD from pressure ulcers

Clinical evidence suggests that IAD is often confused with either stage I or stage II pressure ulcers (Mahoney *et al.*, 2013). These two conditions have a number of risk factors in common, and indeed IAD is a risk factor for the development of PUs, however, from a clinical perspective it is critical to distinguish these two conditions to enable appropriate treatment and prevention strategies for the affected individual and also for accurate documentation and quality reporting (Gray, 2004; Defloor *et al.*, 2005; Beeckman *et al.*, 2015). Clearly, if the patient is not incontinent then the condition is not IAD (Beeckman *et al.*, 2015), although there is some ongoing debate whether we can actually differentiate these two conditions when the patient is both incontinent and bedridden. It is important to consider that in IAD the damage begins on the surface of exposed skin, whilst in PUs the damage is initiated in the underlying soft tissue (Berlowitz and Brienza, 2007; Kottner *et al.*, 2009). In a clinical study by Houwing and colleagues (2007), skin biopsies were taken from patients with both grade 1 pressure ulcers and IAD, and were histologically examined showing two distinct histopathologies. It was revealed that pressure ulcers were associated with an ischaemic pattern (Figure 1.7a), that is the restricted blood supply to the tissue, while in IAD samples an inflammatory pattern was evident, characterized by partial loss of epidermis, dilated vessels with some swelling of the endothelium, oedema of the dermis and presence of inflammatory cells (Figure 1.7b) (Houwing *et al.*, 2007). However, samples from IAD patients who were not bedridden were not examined. While these findings suggest that these two conditions are different in regards to the underlying mechanisms involved, it is important to acknowledge that sometimes these two conditions co-exist, and particularly when the patient is both incontinent and bedbound.



**Figure 1.7. Histopathological appearances of grade 1 pressure ulcers and IAD lesions.** **a)** An ischaemic pattern was evident in pressure ulcer samples; **b)** IAD lesions were associated with an inflammatory pattern, evident by partial loss of the epidermis, dilated vessels with some swelling of the endothelium, oedema of the dermis and presence of inflammatory cells. Figure is based on the work by Houwing and colleagues (2007)

## 1.7 Problems in IAD research

In spite of the increasing interest in this condition, mainly from a clinical perspective; an exact definition of IAD is not listed in the World Health Organization's International Classification of Diseases (ICD-10) (World Health Organization., 2010). Certainly, it contains a detailed description of diaper dermatitis, but there is no reference to IAD (Kottner and Beeckman, 2015). This constitutes a major problem in the clinical field as diaper dermatitis occurs in infants and should be clearly distinguished from IAD that occurs in adults and specifically in the geriatric population (Beeckman, 2017), due to: 1) significant differences in skin barrier function and other related parameters, as illustrated in Table 1.2, and 2) bigger skin area that is affected in IAD compared to the perineum affected in diaper dermatitis (Berg *et al.*, 1994; Farage *et al.*, 2007; Gray *et al.*, 2007; Newman *et al.*, 2007; Telofski *et al.*, 2012). Other terms that are used to describe IAD also include: irritant dermatitis, perineal dermatitis, perineal rash and moisture lesions (Beeckman *et al.*, 2015). Overall, the lack of a common terminology of IAD additionally justifies the absence of significant progress regarding elucidating the underlying mechanisms of IAD (Beeckman *et al.*, 2015).

**Table 1.2.Differences between infant, adult and geriatric skin.** Diaper dermatitis should be considered as a distinct condition to IAD due to differences in the skin functional and morphological characteristics with age.

Skin Barrier Constituents						
Characteristics	Infant	Adult	Geriatric		Reference	
<b>Corneocytes size</b>	Small	< Large	< Large	(Germann <i>et al.</i> , 1980; Stamatias <i>et al.</i> , 2010)		
<b>Epidermis thickness</b>	Thin	< Thick	> Thick	Thin	(Chiou and Blume-Peytavi, 2004; Stamatias <i>et al.</i> , 2010; Voegeli, 2012b)	
<b>Water content of SC</b>	High	> Low	< Low	Low	(Nikolovski <i>et al.</i> , 2008; Voegeli, 2012b)	
<b>TEWL</b>	High	> Low	< Low	Low	(Ghadially <i>et al.</i> , 1995; Nikolovski <i>et al.</i> , 2008)	
<b>NMF</b>	Low	< High	> High	Low	(Nikolovski <i>et al.</i> , 2008; Voegeli, 2012b)	
<b>Skin pH</b>	High	> Low	< Low	High	(Yosipovitch <i>et al.</i> , 2000; Hoeger and Enzmann, 2002; Farage <i>et al.</i> , 2008)	

## 1.8 The Research Problem

IAD represents a major challenge in clinical practice and to a significant financial burden on healthcare systems. Although the damaging effects of urine and faeces on the skin are well accepted, the exact mechanisms leading to IAD remain poorly understood. To date research has focused on prevention and treatment strategies for IAD. As a consequence, theoretical models related to diaper dermatitis (in infants) and findings from animal studies have been adapted to describe the aetiology and pathophysiology of IAD, and are described in detail in chapter 2. However, as stated in section 1.6, these theories are flawed and should not be used to explain IAD pathophysiology in clinical practice. Therefore, gaps in the current knowledge of IAD are highlighted and consequently the research aim and hypotheses of the present research are clearly stated.

## 1.9 Chapter Summary

The skin is the largest organ of the human body, and is responsible for forming a barrier against the external environment. Accordingly the skin barrier function, related to the outermost layer of the epidermis, the SC, is highly impermeable and prevents both the loss of water out of skin (TEWL) and the entry of harmful microorganisms or irritants. Prolonged exposure to incontinence will inevitably compromise the integrity of the skin, leading to IAD. However, the exact mechanisms underlying the development of IAD have not been elucidated yet, mainly because of the lack of a consistent terminology used by clinicians/researchers and its confusion with diaper dermatitis. Indeed what is known about the pathophysiology of IAD is derived from either subjective clinical experience, studies related to diaper dermatitis or from animal models, and therefore robust empirical evidence is lacking. A systematic review of the literature was performed on the aetiology and pathophysiology of IAD and this will be discussed in Chapter 2.



## Chapter 2: The aetiology and pathophysiology of IAD

### 2.1 Introduction

As discussed in section 1.7, the exact mechanisms leading to the development of IAD have not been clarified yet. However, there are several theoretical frameworks, based on diaper dermatitis and clinical observations, which try to explain the pathophysiology of IAD and the factors involved in its development. Therefore, in this chapter a systematic review was performed to identify and summarize relevant literature on the aetiology and pathophysiology of IAD. Critical gaps in knowledge are highlighted and the research hypotheses that were investigated in the current research are clearly stated.

### 2.2 Methods

#### 2.2.1. Search strategy

A systematic search was performed in April 2018 using the databases of PubMed, the Cumulative Index to Nursing and Allied Health Literature (CINAHL), Medline, and Embase via OvidSP (from 1996 to 2018) and the search terms specified in Table 2.1. The Boolean operators “OR” and “AND” were used.

**Table 2.1. The search terms used in this systematic review of the literature using PubMed, CINAHL, Medline and Embase via OvidSP.**

Search Terms	
Incontinence-associated dermatitis <b>OR</b> incontinence associated dermatitis	<b>AND</b> aetiology pathophysiology pathology mechanisms inflammation

### **2.2.2. Search outcome**

The initial search retrieved 1408 records: 70 in PubMed, 580 in CINAHL, 710 in Medline, and 48 in Embase via OvidSP (Figure 2.1). Articles without the full text available were excluded, reducing the number of articles to 1041. The search was further filtered to show the articles published in English, leaving a total of 1031 articles. There was no publication date restriction. After screening of titles and abstracts, 135 eligible articles were identified on the aetiology and pathophysiology of IAD. After duplicate removal, 13 studies and review articles were included in this systematic review, including an additional 33 from their reference lists.

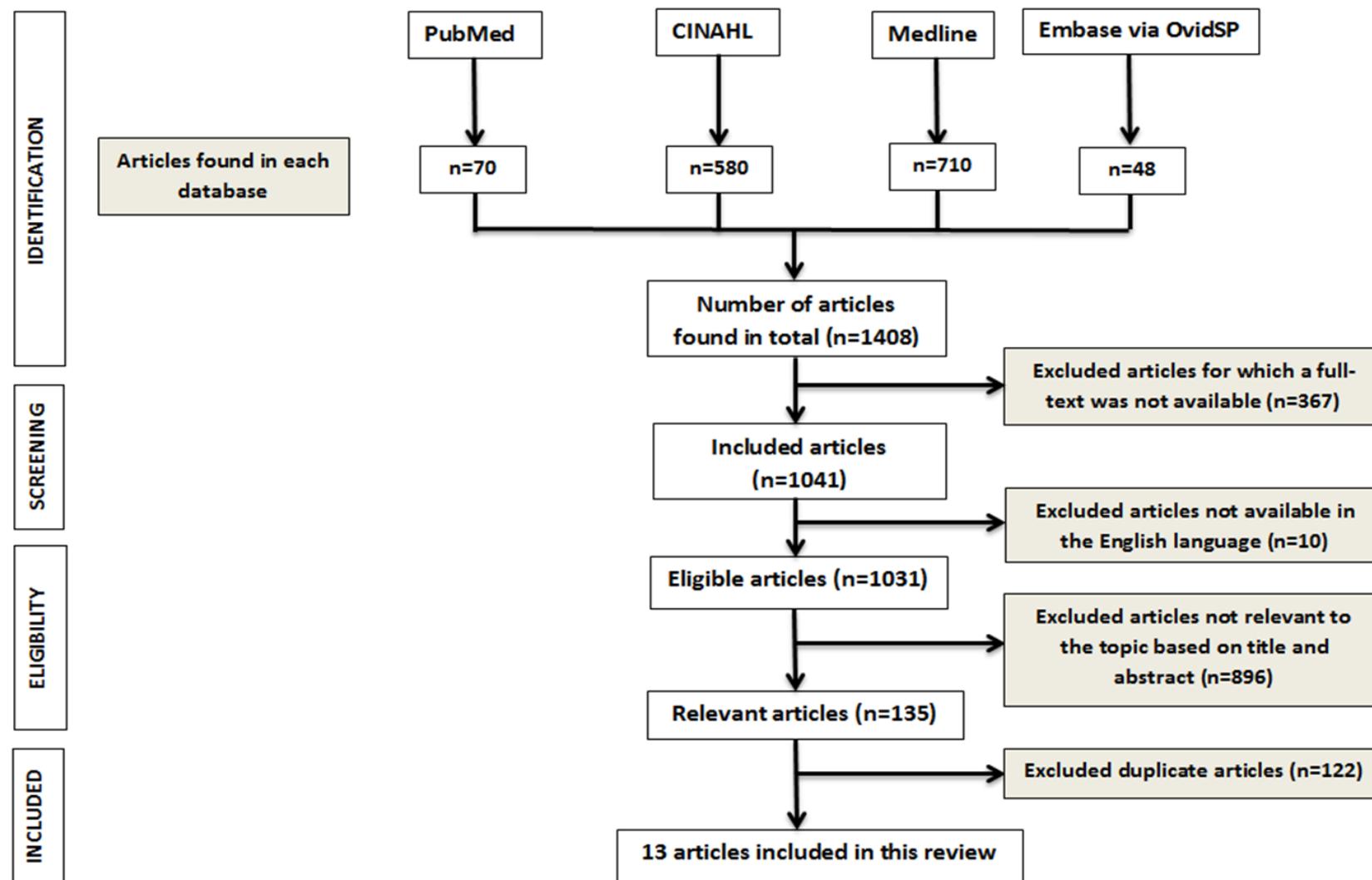
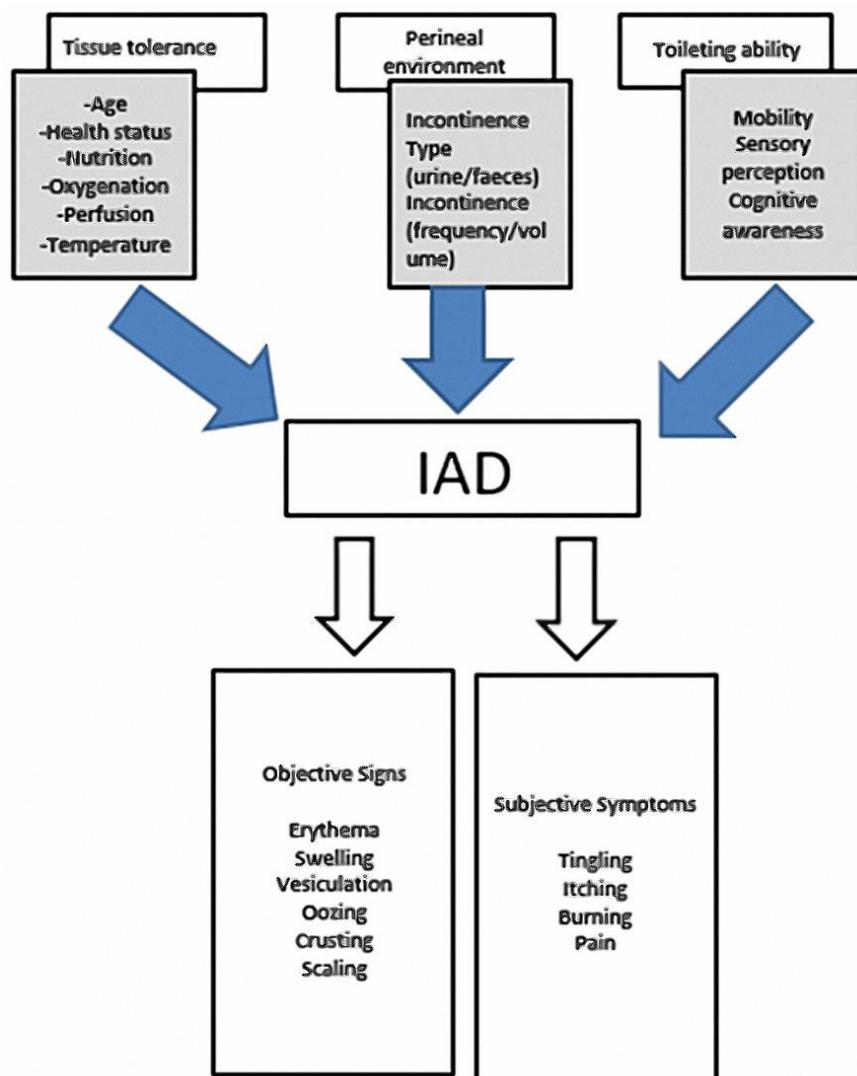


Figure 2.1. Flow chart showing the results from the systematic search of the literature.

### 2.3 Theoretical models of IAD pathophysiology

As stated in section 1.7, diaper dermatitis that occurs in the paediatric population is commonly confused with IAD. As a consequence, early theoretical models of diaper dermatitis have been adapted to describe and explain the pathophysiology of IAD. The first conceptual model on diaper dermatitis is dated 25 years back, and shows limited understanding of the clinical problem since only tissue tolerance, type of incontinence and toileting ability are identified as risk factors for the development of IAD (Brown and Sears, 1993) (Figure 2.2).



**Figure 2.2. Conceptual model of perineal dermatitis development.** Based on the work of Brown and Sears (1993)

A few years later, the role of skin pH, and specifically its shift to alkaline levels by the action of faecal enzymes and ammonia production was highlighted by Jeter and Lutz (1996). Additionally, in their theoretical framework of IAD development (Figure 2.3) they suggested that frequent cleansing regimens lead to physical and chemical irritation of the skin, hence contributing to the development of IAD. Additionally, in their theoretical framework, IAD was described as a risk factor for the development of PUs as a consequence of increased susceptibility of the skin to shear and friction (Jeter and Lutz, 1996).

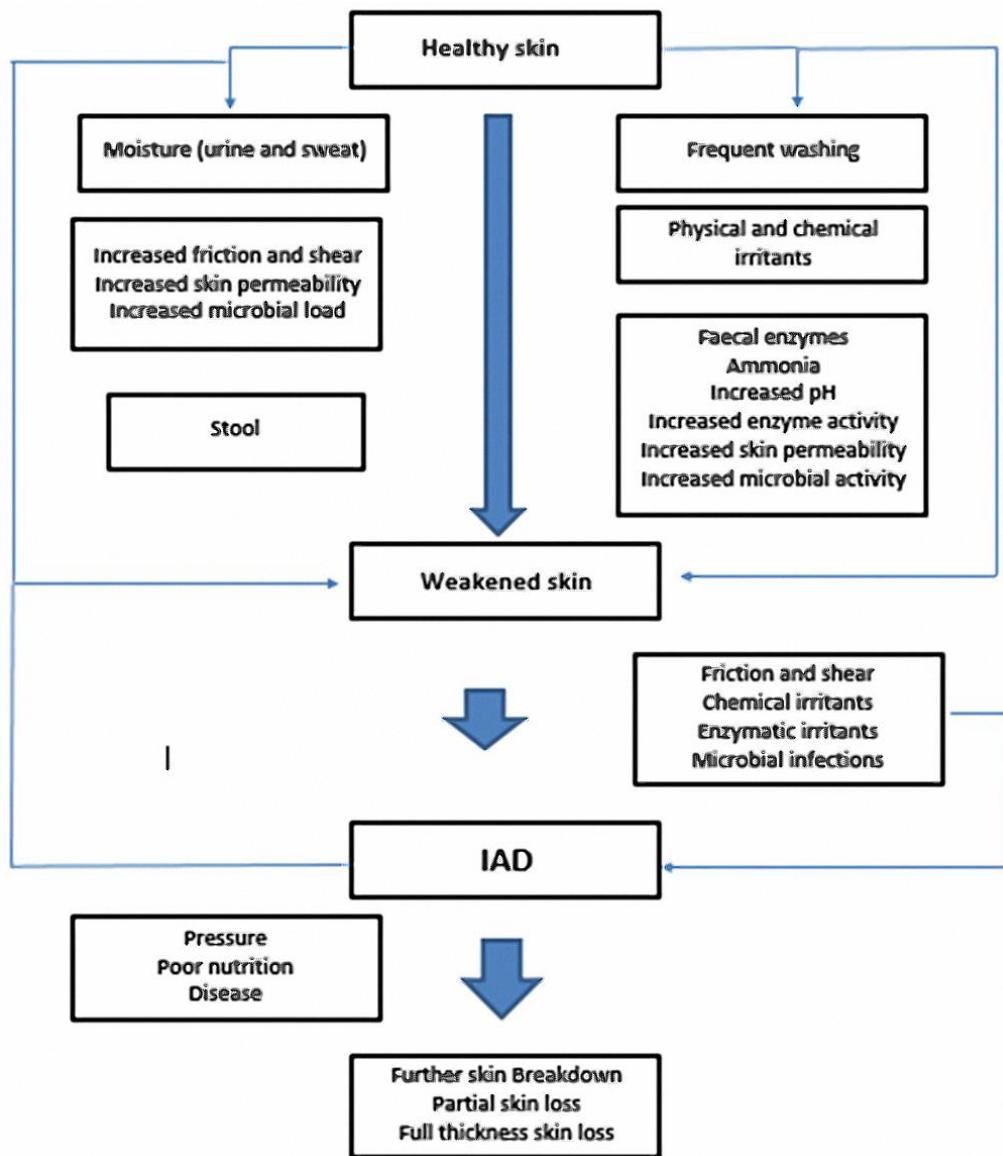
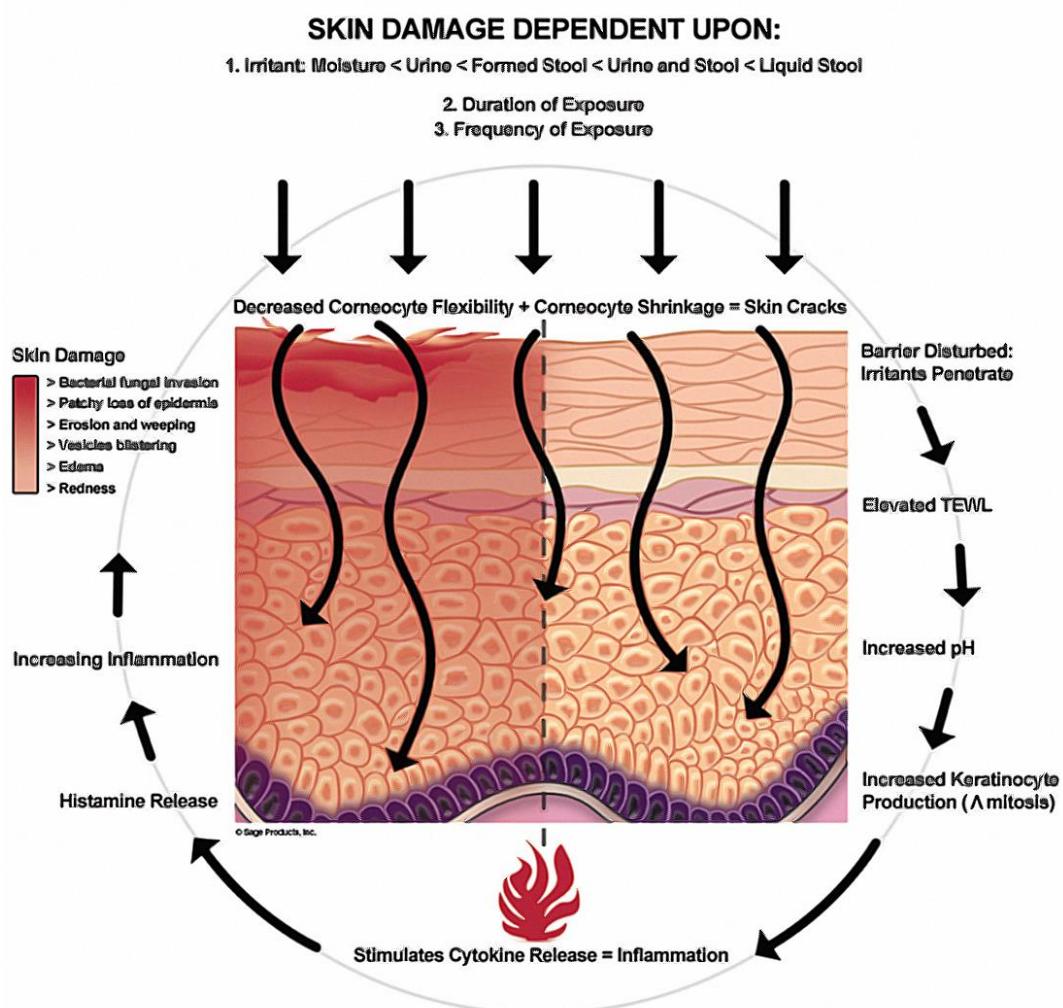


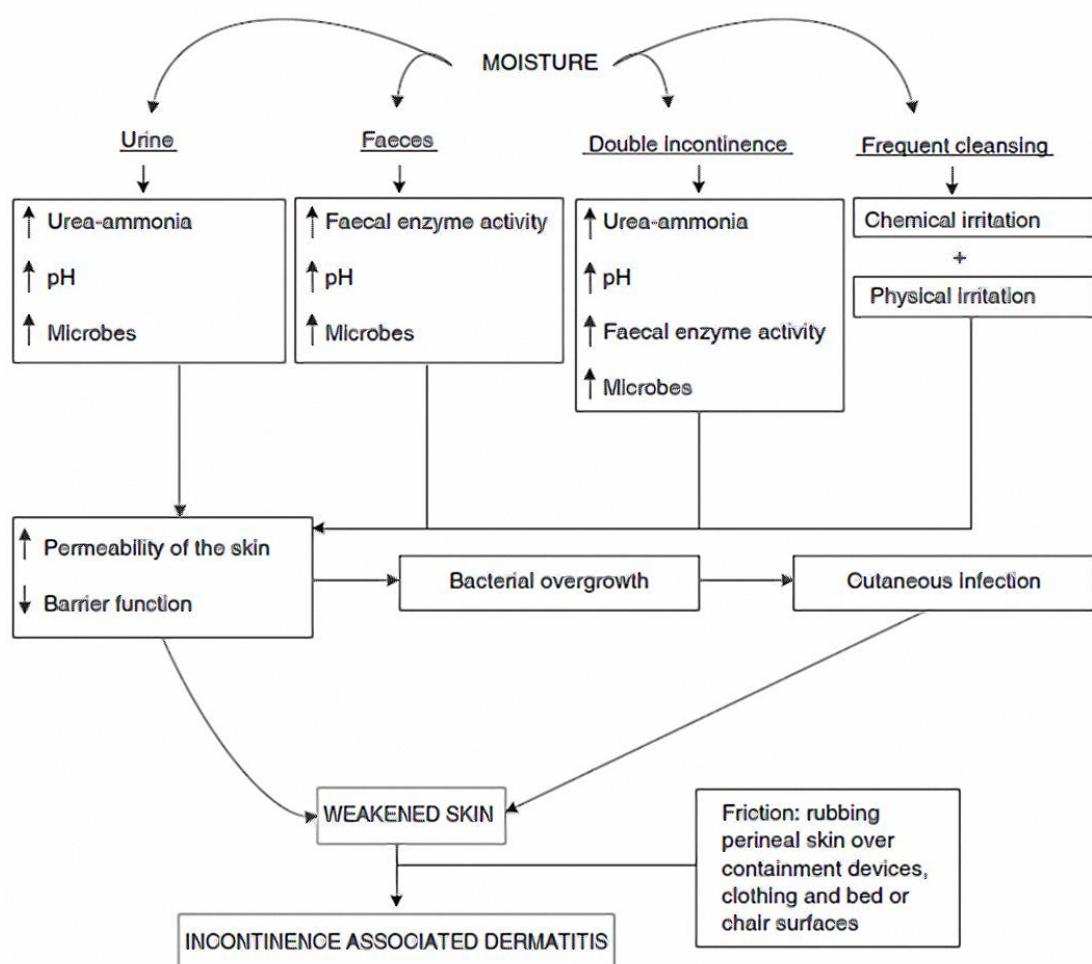
Figure 2.3. The pathophysiology of IAD. Based on the work of Jeter and Lutz (1996).

More recent models, which are merely based on clinical experience, highlight that the prolonged exposure to urine and/or faeces is critical for the development of IAD, but other factors are involved, including skin surface pH and mechanical irritation, highlighting thus the multifactorial nature of this condition (Gray *et al.*, 2007; Newman *et al.*, 2007; Beeckman *et al.*, 2009). However, these models are lacking good empirical evidence to support these hypotheses. The model developed by Gray and colleagues (2007), which is based on the work of other groups (Brown, 1994; Ghadially, 1998), gives more details about the physiological response of the skin to prolonged moisture from incontinence. As illustrated in Figure 2.4, it is proposed that following exposure to irritants there is an increase in TEWL, an indicator of skin barrier integrity (Fluhr *et al.*, 2006b), and pH, leading to a vicious circle of increased inflammation, evidenced by increased cytokine and histamine release, and eventually skin breakdown. Interesting to note, this model also postulated that the frequency and duration of exposure is a contributing factor to IAD development (Gray *et al.*, 2007).



**Figure 2.4. The aetiology and pathophysiology of IAD.** Figure adapted from Gray and colleagues (2007).

In addition to the model by Gray and colleagues (2007), work by Beeckman and colleagues (2009) proposed that apart from incontinence, IAD development is also dependent on the frequency of skin cleansing regimens (Figure 2.5). In particular, it has been suggested that chemical and physical irritation from cleansing activities also leads to an increased permeability of the skin and to a disruption of its barrier function and subsequently its protective acidic milieu. Other pathophysiological events have also been proposed, including bacterial colonization and secondary infections, that ultimately lead to skin breakdown and IAD (Beeckman *et al.*, 2009). In spite the fact that this model is descriptive around the pathophysiological events triggered in IAD, it is limited as there is no report of the inflammatory events triggered in the skin.



**Figure 2.5. The multifactorial nature of IAD.** Prolonged exposure to moisture leads to a compromised skin barrier function and an increased pH. As a consequence, the permeability of the skin is disrupted and becomes susceptible to secondary skin infections. All these lead to skin breakdown and IAD development. Figure adapted from the work of Beeckman and colleagues (2009), which is based on Jeter & Lutz (1996) and Newman and colleagues (2007).

## 2.4 Aetiological factors

Based on the recent theoretical models on IAD development, several aetiological factors contribute to the development of IAD, namely: 1) moisture source, type of incontinence, 2) frequent cleansing, 3) duration and frequency of exposure, 4) inflammatory response triggered and 5) other factors such as mechanical irritation and secondary infections. Each of these factors will be described in detail.

### 2.4.1 Moisture source

Incontinence (urinary, faecal or double) is the main risk factor for the development of IAD, and accordingly the severity of the skin damage depends on the moisture source and its chemical constituents. As a consequence, the risk of IAD is further increased in faecal and double incontinence than urine alone (Gray *et al.*, 2011; Beeckman *et al.*, 2015).

#### a) Urine

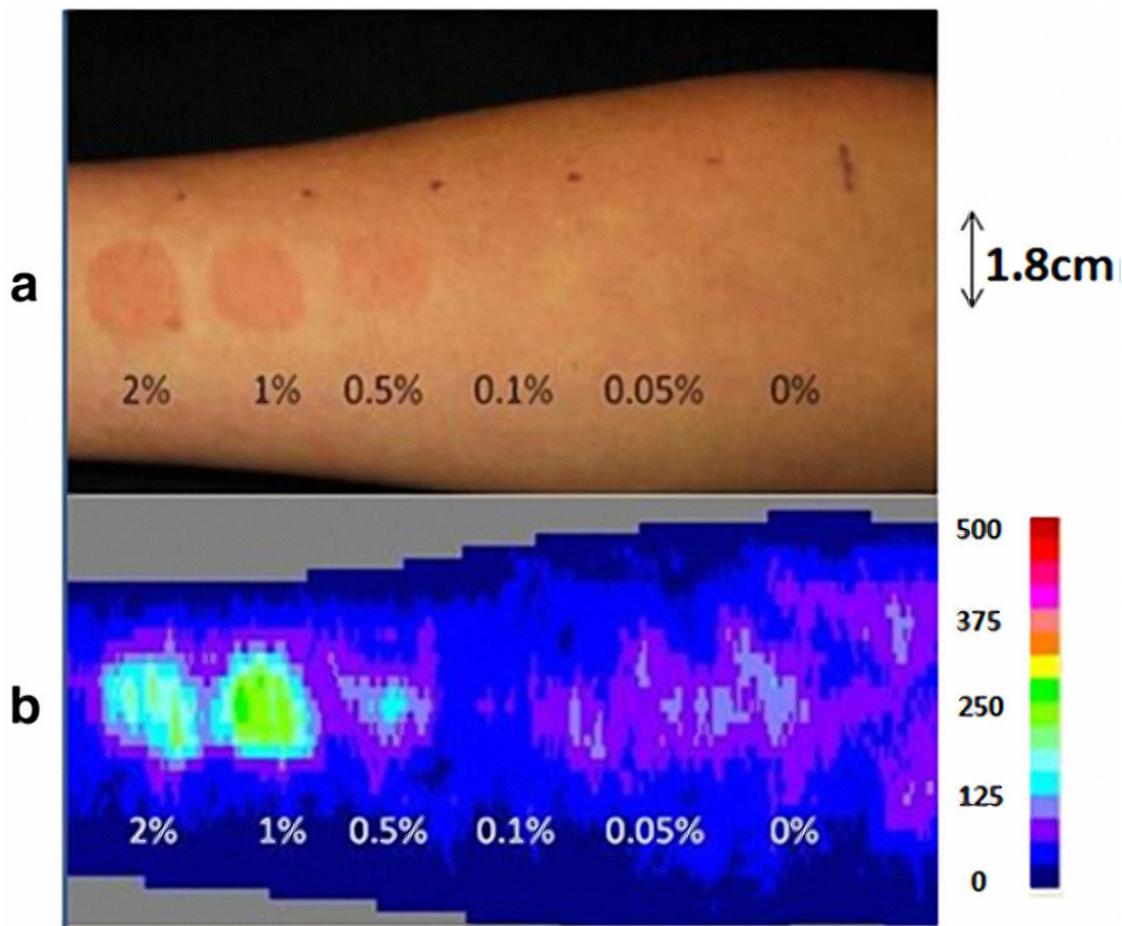
Urine is mainly composed of water (95%), urea, and several organic/inorganic compounds. Of interest, its composition varies between individuals, influenced by several factors including: 1) ethnicity, 2) diet, 3) physical exercise, 4) environmental conditions, 5) time of the day, and 6) the presence of any disease and drug metabolites, if any medication (steroids or chemotherapeutic agents) is taken (Shiu *et al.*, 2013). Physiological human urine has also a pH value ranging between pH 4.8-8.0 (Putman, 1971; Curhan *et al.*, 2001; Taylor and Curhan, 2007; Rose *et al.*, 2015).

The mechanisms underlying skin barrier disruption following urine exposure have not been clarified yet (Gray *et al.*, 2011), but it has been proposed that it leads to an overhydrated epidermis, swelling of corneocytes and eventually skin maceration (Ichikawa-Shigeta *et al.*, 2014; Beeckman *et al.*, 2015; Voegeli, 2016). As described by Minematsu and colleagues (2011a), skin maceration is a functional disorder of the barrier function, occurring due to structural alteration of the epidermal lipids and junctions between keratinocytes caused by exposure to irritants. The damage is also aggravated in the presence of skin bacteria that convert urea in urine into ammonia, shifting thus the skin pH to alkali levels (Black *et al.*, 2011). Evidence about the damaging effects of increased pH derives from studies related to diaper dermatitis, that investigated the effects of diapers on infant skin, showing that diapers produced a significant increase in TEWL (mean TEWL=10.70) and pH (mean pH= 5.90) compared to undiapered skin (mean TEWL=5.00, mean pH= 5.30). Accordingly, these changes were correlated with the severity of diaper dermatitis as determined by a visual grading scale (Jordan *et al.*, 1986; Berg *et al.*, 1994).

However, it should be acknowledged that these studies were performed in infants and any direct comparison with IAD should be avoided (Table 1.2).

With respect to IAD, a few studies attempted to investigate the role of urine and its inherent pH. In a study conducted by Farage and colleagues (2014), infant urine was applied for three days on adult skin on three anatomical sites, two on the arm and one on the back. Urine was applied on normal skin sites and on sites where the skin barrier was compromised by tape stripping, excessive hydration or both. Saline and sodium lauryl sulphate (SLS) also served as the negative and positive controls respectively. Results from the study reported that urine produced a higher degree of erythema compared to saline, but less than that caused from SLS treatment. On the contrary, results showed that urine led to a significant increase in skin pH compared to both control and SLS, which was not dependent on the skin state (Farage *et al.*, 2014). In spite of these findings, TEWL measurement was not obtained by the authors to assess the skin barrier function following exposure to urine. Additionally, the use of infant biological material on adult skin, clearly limits the physiological and clinical relevance of the study, due to differences in urine composition between infants and adults, an example of which is the less concentrated urea in infant urine (McCance and Young, 1941; Putman, 1971). Due to the similar responses between infants and adults, the authors claimed that both can be used in diaper dermatitis or IAD research. However, this is certainly problematic due to their differences in skin characteristics (Table 1.2) and in the susceptibility to irritants.

In another recent study, an experimental model of IAD was developed using synthetic-urine (s-urine), as first described by Mayrovitz and colleagues (2001), and ammonium hydroxide (% w/v) was used to adjust its pH to alkali levels, ranging between pH 7.9-10.7 (Lerner *et al.*, 2015). In the preliminary investigation, human volunteers (n=6) had both their forearms exposed to different s-urine solutions for six hours, and an erythematous skin response was observed (Figure 2.6), as determined by both a visual scoring system (Figure 2.6a) and changes in blood flow (Figure 2.6b), measured by a Laser-Doppler Imaging (LDI) system. In addition, the degree of erythema and the severity of skin barrier disruption, as measured by TEWL, were associated with increasing concentrations of ammonium hydroxide and accordingly pH. On the contrary, no significant effect of s-urine solutions was found on skin surface pH.



**Figure 2.6. Skin response to different synthetic urine solutions, varying ammonium hydroxide concentration, with an alkaline pH.** The erythema that was caused was proportional to the concentration of ammonium hydroxide. a) The redness that was caused upon exposure to the different synthetic urine solutions was assessed using a visual scoring system, and b) changes in blood flow (perfusion units) using LDI also confirmed this erythema production. Figure taken from the work by Larner and colleagues (2015)

Afterwards, the forearms of six volunteers were exposed to an s-urine solution, with a pH of 10.3, for six hours daily for a period of five days, and subsequently a visible erythema and a significant increase in blood flow ( $p<0.05$ ) were observed compared to saline control sites. Indeed, this erythema persisted up to three days post-exposure. Assessment of skin barrier function also showed an increase in TEWL compared to baseline values, obtained on the day prior application, but was only significant ( $p<0.05$ ) after the fourth day of exposure. Again, there were no significant effects on skin pH and as the authors stated this is possibly attributed to the physiological buffering capacity of the skin that does not allow any changes in pH (Zhai *et al.*, 2009; Larner *et al.*, 2015). However, this was not further examined.

Informative results were obtained from this study; however, this model is not representative of the physiological and clinical conditions that lead to the development of IAD, as:

- Biological urine has a pH value ranging between pH 4.8 and pH 8.0 and the urinary pH values used were beyond those levels
- The exposure time of 6 hours is longer than the frequency at which incontinent patients are checked for wetness in clinical practice, and which is normally two hours (Voegeli, 2008)
- The effects of wetness, water per se, and occlusion were not considered and distinguished from the effects of s-urine
- It is limited to urinary incontinence

In view of the limitations of both studies the exact relationship of urine and its inherent pH on IAD development needs to be elaborated in further studies.

### **Ammonia**

The production of ammonia by faecal bacteria was first demonstrated in a study conducted several years ago, in which it was shown that *Brevibacterium ammoniagenes*, a bacterium found in faeces, produces ammonia from urea, and when applied on the skin it causes an erythema (Cooke, 1921). This was also supported by another study, in which application of urine, strong-smelling of ammonia, for four hours on the buttock skin of infants produced an erythematous response (Rapp, 1955). Hence, ammonia was considered to be involved in the development of diaper dermatitis and subsequently adapted in IAD pathophysiology (Rook *et al.*, 1972). However, years later, the role of ammonia on diaper dermatitis was challenged in a study by Leyden and colleagues (1977), who reported that ammonia concentration was higher in infants with no diaper dermatitis (n=63) compared with infants suffering from the condition (n=18), although it should be noted that the numbers of infants recruited were not equal. Subsequently, they showed that when infant buttock skin was challenged (24 hours) with adult urine (pH 8.0), treated with urease to produce ammonia, no erythema could be observed. This was further demonstrated on adult forearm skin which was exposed to the same urine solution but with ammonium hydroxide added at various concentrations and the pH value adjusted to 8.0 using hydrochloric acid. Results showed that mild erythema is only evident after prolonged exposure to urine (Leyden *et al.*, 1977). Afterwards, the authors examined the effects of urine with low and high concentrations of ammonia, produced by urease, on adult skin that experienced skin barrier disruption by a scarification technique (Frosch and Kligman, 1976). Results showed an increased erythema on damaged skin following urine exposure compared to the saline control site.

Therefore, the authors asserted that the effects of ammonia are only evident on damaged skin, suggesting a secondary role of ammonia in the development of diaper dermatitis (Leyden *et al.*, 1977). The main limitation of that study is that urine samples were filtered before application and this might have influenced the results.

**b) Faeces**

Faeces are composed of about 75% water, protein, undigested fats and food residues, polysaccharides and bacteria. As with urine, their composition varies depending on diet such as fibre and protein intake, and moisture content (Eastwood, 1973; Snyder *et al.*, 1975; Schouw *et al.*, 2002; Achour *et al.*, 2007). Their pH ranges between pH 5.0 and 8.0 (Mai *et al.*, 2009). In faecal-incontinence the skin damage is more severe compared to urine per se (Bliss *et al.*, 2000; Beeckman *et al.*, 2015). In particular, clinical evidence reported that liquid stool is more damaging than formed stool, due to their rich composition in proteolytic enzymes (lipases and proteases) with an alkaline pH, compared to solid formed stool that have a neutral pH and less metabolically active enzymes (Gray *et al.*, 2007; Beeckman *et al.*, 2015). Additionally, it has been reported that faecal enzymes are highly active in an alkaline environment, so any changes in pH increase the severity of damage (Beeckman *et al.*, 2015). This was demonstrated by Andersen and colleagues (1994), who studied the metabolic activities of faecal enzymes at different pH levels. In that study, the back of healthy volunteers (n=11) was exposed to four different combinations of enzymes and bile salts solutions, prepared in buffers of pH 6.5 and 8.0. As positive and negative controls, SLS (0.25% w/v in water) and phosphate buffer (pH 8.0) were used respectively, together with an untreated site. Solutions were applied for 21 days and measurements of TEWL and skin pH, and visible assessment of erythema were taken at days 5, 12 and 19 of exposure, at which treatments were removed and re-applied. Results showed that after the fifth day of exposure, all enzyme solutions and the SLS caused a visible erythema that was increasing until day 19, with the latter also producing the highest degree of irritation. With respect to the enzyme preparations, the one that was composed of high amounts of digestive enzymes presented to be the most irritant, as determined by the increase in TEWL and visible erythema. The enzyme solution containing lipase and trypsin was also very irritating but not evident until day 12. By contrast, the solutions containing elastase, chymotrypsin and trypsin, were less irritating but their activity was pH-dependent, and increased in an alkaline buffer solution (pH 8.0) leading to a shift in skin pH to alkali levels (pH>7). Therefore, the authors concluded that these enzymes are activated in an alkaline environment; however, they acknowledged that this might be attributed to skin barrier disruption from the alkaline buffer, which is supported by a previous study (Aly *et al.*, 1978; Andersen *et al.*, 1994).

The main limitation of the study is that the enzymes and bile salts used corresponded to the concentrations in infant faeces and were tested in adults, and this does not reflect the clinical reality of IAD.

In another study, the effect of faecal material was investigated on the skin functional characteristics of both infants and adults. Briefly, faecal material was obtained from infants (n=16) by their mothers, who also participated in the study, and applied for 4 hours on two skin sites on the buttocks of infants and on two skin sites on the forearm of adults. An extra two sites on each anatomical site were also left untreated. Results showed that exposure to faecal material produced an erythema in both infants and adults compared to the untreated sites, and to a significant increase in TEWL and skin pH ( $p<0.05$ ). Subsequently, exposed sites were compromised by tape stripping (n=10) that led to a further increase in erythema, which was visually assessed, but not in TEWL or skin pH, suggesting that faecal exposure increases the susceptibility of the skin to other irritants. In spite of these results, infant material was applied on adult skin and any comparison is tenuous. Additionally, the faecal composition varies between infants and adults, including moisture content (Schouw *et al.*, 2002). Another limitation of that study is that each infant/mother was treated with the child's own biological material and hence each subject received a different treatment (Farage *et al.*, 2014).

Faeces are also composed of intestinal bacteria that can penetrate the skin, producing inner tissue damage. This was demonstrated in a recent study by Mugita and colleagues (2015), in which murine models had their dorsal skin exposed for four hours to: 1) a known maceration model of agarose gel containing proteases (Minematsu *et al.*, 2011) and 2) to a model of faecal-incontinence, of an agarose gel composed of a proteolytic solution, containing trypsin and chymotrypsin, corresponding to physiological adult concentrations. An untreated control group was also included. Then, treatments were removed and air dried for 30 minutes, followed by measurements of TEWL and skin hydration. Results showed a high increase in both parameters in the proteolytic-treated group compared to the macerated and untreated sites. Macroscopic analysis only revealed an erythematous response, resembling punctate bleeding, from the proteolytic solution after 24 hours post exposure. Subsequently, bacterial suspension, including *Pseudomonas aeruginosa*, which is found in patients with incontinence (Kudo *et al.*, 2003), was applied for 30 minutes to murine skin pre-treated with the proteolytic solution, and to an untreated skin site. Results showed an increased visible erythema on the proteolytic-treated skin but not on the untreated skin. Immunohistochemical analysis also revealed the invasion of bacteria and the presence of inflammatory cells, including epidermal Langerhans cells and macrophages, in the deeper areas of the dermis.

Consequently, the authors concluded that the tissue damage in IAD, and specifically in faecal-incontinence, is distinct from ICD, which is restricted to the DEJ (Mugita *et al.*, 2015). While this is a pioneer work, the use of animal models clearly limits its physiological relevance due to skin barrier differences between animals and humans. As such, additional future investigations are required, involving human volunteers, to explore the effects of this proteolytic model of faecal-incontinence, and to examine the inflammatory response triggered.

### **c) Double incontinence**

In double incontinence, the severity of the skin damage increases compared to that caused by urine or faeces alone. This can be explained by the fact that faeces break down urea into ammonia, leading to an alkaline pH that consequently activates digestive enzymes (Beeckman *et al.*, 2015). This is also supported by the study by Berg and colleagues (1986), who examined the effects of prolonged exposure to a combination of urine and faeces in hairless mice, and showing that this leads to a high degree of erythema (Berg *et al.*, 1986). Taken together, these findings suggest that there is a synergistic role of urine and faeces in the disruption of skin integrity.

#### **2.4.2 Frequent Cleansing**

Skin cleansing of incontinent patients is essential in clinical settings to protect the skin from potential irritants and excessive moisture. However, it may on its own be damaging to the skin, especially when frequent episodes of incontinence occur. This is supported by the study by Voegeli (2008), who showed that repeated cleansing of the skin, with soap and water, leads to an increased TEWL and skin pH, and consequently compromises skin integrity. In addition, Beeckman and colleagues (2009) proposed that frequent cleansing leads to increased skin permeability; even though there is no solid evidence to support this hypothesis. In a similar manner, physical irritation, such as friction, towel drying, and shear from clothing and incontinence pads, is also considered a contributing factor to IAD (Atherton, 2001; Beeckman *et al.*, 2009; Beeckman *et al.*, 2015).

#### **2.4.3 Duration and Frequency of exposure**

In addition to moisture source, duration and frequency of exposure are critical for the development of IAD. The exact exposure time required for the development of IAD has not been determined yet, however different studies reported that the median time for IAD development is 13 days in nursing homes, 13.5 in long-term care facilities and 4 days in intensive care settings (Bliss *et al.*, 2007; Bliss *et al.*, 2011; Long *et al.*, 2012).

In research settings, studies exposed the skin to urine and/or faecal material for various timings, ranging between 6 hours to 21 days, and contradictory results were obtained (Berg *et al.*, 1986; Andersen *et al.*, 1994; Mayrovitz and Sims, 2001; Farage *et al.*, 2014; Larner *et al.*, 2015). In particular, a recent study reported that exposure of the skin to alkaline s-urine solutions ( $>\text{pH}$  10.0) for up to 6 hours is adequate to cause a visible erythema, however, a previous study showed that exposure to s-urine ( $\text{pH}=7.9$ ) for the same time period caused a decrease in blood flow (Mayrovitz and Sims, 2001; Larner *et al.*, 2015). This discrepancy may be attributed to the different pH values of the s-urine solutions used in each study, which implies that the duration of exposure is pH-dependent. On the contrary, studies reported that exposure to urine/faeces for several days leads to a visible erythema and to the disruption of the skin barrier (Berg *et al.*, 1986; Andersen *et al.*, 1994; Farage *et al.*, 2014; Larner *et al.*, 2015). Yet again, the use of different skin models, the source of urine/faeces, and the various anatomical sites tested in those studies limit the extrapolation of the findings to IAD.

#### 2.4.4 Other factors

Despite the fact that incontinence is the main risk factor for skin breakdown, it should be acknowledged that IAD is a multifactorial condition. Indeed, it has been suggested that moisture alone cannot cause skin breakdown and IAD, and that its development is dependent on: 1) occlusion from absorptive products, 2) mechanical damage, and 3) infection from pathogens such as *Staphylococcus aureus* and *Pseudomonas aeruginosa* (Gray *et al.*, 2012). With respect to occlusion, it has been reported that the damaging effects of urine and/or faeces are aggravated under occlusive conditions created by absorbent products used to contain incontinence (Junkin and Selekov, 2007; Gray *et al.*, 2012). Evidence about the damaging effects of occlusion *per se* on skin barrier constituents is provided by several studies, which reported that occlusion leads to an increased skin hydration and skin pH (Aly *et al.*, 1978; Hartmann, 1983b; Zhai and Maibach, 2001). However, a study by Jungersted and colleagues (2010) reported that occlusion for a week does not have a significant effect on healthy skin, and in fact it makes the skin less susceptible to irritation (Jungersted *et al.*, 2010). Results from the various studies are conflicting, but it is clear that in IAD research the effects of occlusion need to be determined and distinguished from the effects of urine/faeces. Exposure to urine has also been linked with increased susceptibility to mechanical damage such as pressure, shear and friction (Beeckman *et al.*, 2015). This was established in the study by Mayrovitz and colleagues (2001), who studied the effects of pressure loading on the skin of healthy individuals exposed to s-urine ( $\text{pH}$  7.9) for six hours. Results showed that pressure loading on wet skin, exposed to urine and water, resulted in decreased tissue hardness, temperature and blood flow compared to dry skin (Mayrovitz and Sims, 2001).

Additionally, excess moisture on the skin surface increases the risk to secondary infections, such as *Staphylococcus aureus* and *Pseudomonas aeruginosa* (Dorko *et al.*, 2003; Newman *et al.*, 2007; Beeckman *et al.*, 2015). Indeed, a study reported that 32% of IAD patients had a fungal infection (Campbell *et al.*, 2014).

#### **2.4.5 Inflammatory response of the skin in IAD**

It is well-accepted now that IAD is an inflammatory skin condition. This was demonstrated in both human and animal studies (Houwing *et al.*, 2007; Mugita *et al.*, 2015). In a clinical study by Houwing and colleagues (2007), skin biopsies taken from patients with IAD were histologically examined and an inflammatory pattern was identified, as illustrated in Figure 1.7b (Houwing *et al.*, 2007). It has also been proposed that increased TEWL and pH lead to an increased proliferation of epidermal keratinocytes and to elevated levels of inflammatory mediators, such as histamine and cytokines (Gray *et al.*, 2007). However, scientific studies, at present, are still sparse, and therefore the current knowledge at the events occurring at the cellular level is still very limited. Only two studies were found in the literature that investigated the release of IL-1 $\alpha$  in healthy infants, in diapered (upper outer quadrant of the buttock) and undiapered (abdomen and upper leg) sites following different diaper care regimens. Both studies showed that the levels of IL-1 $\alpha$  were significantly ( $p<0.05$ ) higher in the diapered area (Garcia Bartels *et al.*, 2012; Garcia Bartels *et al.*, 2014), yet the fact that this study is associated with diaper dermatitis and not IAD directly should be taken into consideration and findings should not be extrapolated for IAD. Even though studies related to other skin conditions, including AD and psoriasis, suggest that cytokine release is responsible for the clinical features of the conditions, such as itch and redness, the inflammatory milieu of IAD has not been clarified yet. For instance, IL-1 $\alpha$  is the principle cytokine responsible for the initiation of the inflammatory cascade, and was in fact the first cytokine to be detected in the skin (Luger *et al.*, 1982). In the skin, and under physiological conditions, IL-1 $\alpha$  is removed by desquamation but following skin irritation its levels are rapidly increased. This was demonstrated in several studies involving animal and human models (Wood *et al.*, 1992; Nickoloff and Naidu, 1994; De Jongh *et al.*, 2007a; De Jongh *et al.*, 2007b). Upon its release, IL-1 $\alpha$  binds to IL-1 receptor I (IL-1RI) and triggers the expression of further pro-inflammatory mediators, such as IL-6 and IL-8, which are responsible for keratinocyte proliferation and the infiltration of inflammatory cells, respectively (Mohamadzadeh *et al.*, 1994; Yen *et al.*, 1996; Corsini and Galli, 1998; Sawamura *et al.*, 1998; Sugawara *et al.*, 2001). Additionally, IL-1 $\alpha$  induces the release of pro-inflammatory TNF- $\alpha$ , which modulates the production of several cutaneous and endothelial adhesion molecules (Köck *et al.*, 1990; Groves *et al.*, 1995; Corsini *et al.*, 1996).

Indeed, studies reported increased levels of TNF- $\alpha$  in samples of patients suffering from psoriasis and following barrier disruption (Wood *et al.*, 1992; Bonifati *et al.*, 1994). This can be explained by the fact that TNF- $\alpha$  induces the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF- $\kappa$ B), hence promoting inflammation (Pasparakis, 2012). Considering the above, it is clear that the inflammatory response in the skin is dependent on IL-1 $\alpha$  pathway, as schematically presented in Figure 2.7. In physiological conditions, this cascade of events (Figure 2.7) is inhibited by the binding of IL-1RA to IL-1RI, serving thus as an important anti-inflammatory mechanism (Hirao *et al.*, 1996; Corsini and Galli, 2000). Therefore, the balance between IL-1 $\alpha$  and IL-1RA is critical (Robinson *et al.*, 2003; Jensen, 2010), evidenced by several studies that reported an increased IL-1 $\alpha$ /IL-1RA ratio in various skin conditions and in experimental models of barrier disruption (Terui *et al.*, 1998; Robinson *et al.*, 2003; De Jongh *et al.*, 2006).

To the best of the author's knowledge, there are no studies in the literature, involving able-bodied volunteers, that examined the release of inflammatory mediators following exposure to urine/faeces, and this was the primary deliverable of the current research study that adds to existing knowledge of IAD. Indeed, the clinical translation of results will lead to more preventive and treatment strategies and open the doors for a whole new research area and the development of new therapeutic modalities.

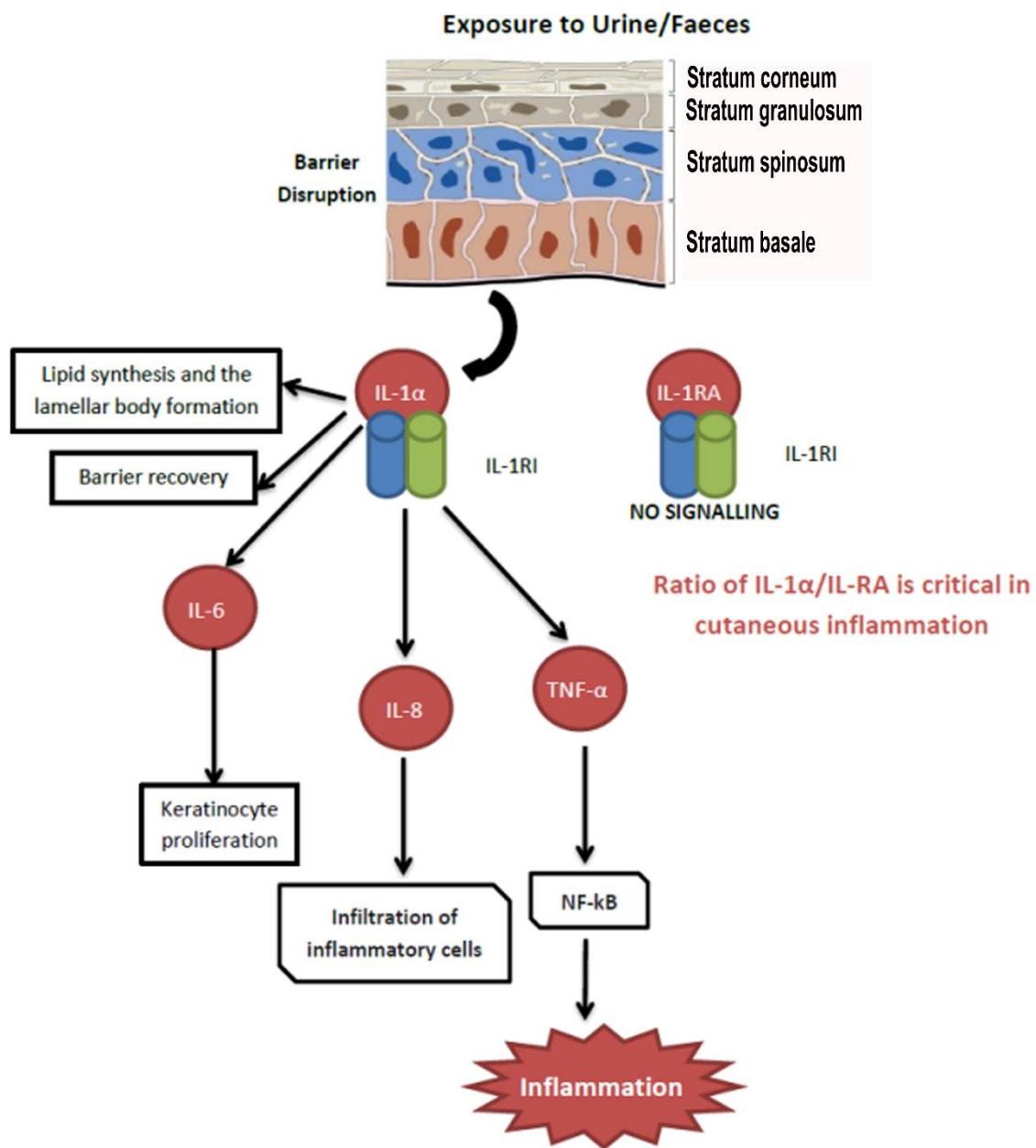


Figure 2.7. Potential inflammatory response in IAD.

## 2.5 Aim of the study

Despite the increasing interest in IAD, mainly from a clinical perspective, research directly investigating the underlying pathophysiological mechanisms of IAD remains sparse. This represents a clear gap in knowledge, where further research is required. Therefore the overarching aim of this research study was to investigate the mechanisms of IAD pathophysiology including a mix of *in vivo* and *in vitro* skin models, allowing *in vitro*-to-*in vivo* correlation and importantly providing a better representation of biological responses associated to IAD. Accordingly, based on recent theoretical frameworks of IAD (Figures 2.4 and 2.5), specific hypotheses were developed and tested to address the main aim of the study, and are outlined below.

### 2.5.1 Research Hypotheses

- 1) An increase in urinary pH disrupts stratum corneum function leading to an increase in TEWL and skin surface pH, thereby compromising the integrity of the skin
- 2) Frequent skin cleansing activities increase the permeability of the skin and consequently its susceptibility to urine
- 3) Exposure of the skin to urine and faeces leads to a release of inflammatory mediators
- 4) At the cellular level, increased pH is associated with an increased proliferation of keratinocytes and the release of inflammatory cytokines

## 2.6 Chapter summary

In this chapter, a systematic review of the literature was conducted to identify and critically appraise the results of published studies on the aetiology and pathophysiology of IAD, and highlight the gaps in current knowledge. PubMed, CINAHL, Medline and Embase via OvidSP were searched for relevant studies published up to April 2018. Theoretical frameworks have been developed to describe IAD aetiology and pathophysiology, but these are mainly based on clinical experience, animal studies and on studies related to diaper dermatitis. Indeed, there is a lack of strong empirical evidence to support the pathophysiology of IAD and therefore a considerable number of gaps in knowledge. In addition, there is a necessity for research studies involving human participants.

## Chapter 2

Therefore, the overarching aim of the current research was to investigate the underlying mechanisms leading to the development of IAD. To address this aim, specific hypotheses were generated based on the theoretical frameworks from Gray and colleagues (2007) and by Beeckman and colleagues (2009), and intended at elucidating: 1) the role of urine and its inherent pH on skin integrity, 2) the permeability and susceptibility of the skin to moisture following frequent cleansing activities and 3) the inflammatory response triggered following exposure to urine and faeces. In Chapter 3, the methods available in dermatological research are critiqued leading to those selected to address the project aims.

# Chapter 3: Methodology

## 3.1 Introduction

This chapter provides an outline of the biophysical, bioengineering and biochemical methods that are widely used in dermatological research, their advantages and limitations, leading to the justification of the methods chosen in the current study. At the end of this chapter, the ethical issues associated with the study are described. In particular, information on the ethical approval is provided, in combination with the inclusion/exclusion criteria for the study and how the participants were recruited.

## 3.2 Biophysical measurements to investigate the functional characteristics of skin

A diverse range of quantitative approaches, including biophysical and biochemical methods, are widely used in dermatological research. Indeed, some of these methods have the potential to be implemented in clinical practice. The methods can be conveniently divided into methods for skin barrier assessment, methods to compromise the integrity of the skin and methods to investigate skin inflammation.

### 3.2.1 Skin Barrier assessment methods

#### 1) Transepidermal Water Loss (TEWL)

Measurement of TEWL represents the gold-standard method to assess the status of the skin barrier, the irritant potential of various insults, and the effectiveness of cosmetic products (Fluhr *et al.*, 2006b). TEWL is a flux density and is expressed in grams of water per hour per square meter of skin ( $\text{g}/\text{h}/\text{m}^2$ ) (Kottner and Vogt, 2017). As stated in the literature, there is no optimum TEWL value for healthy skin, however low values of TEWL ( $\leq 10 \text{ g}/\text{h}/\text{m}^2$ ) correspond to limited transport of water movement in the SC and therefore an intact barrier (Imhof *et al.*, 2009b; Menon and Kligman, 2009). To date, no methods exist that measure TEWL directly, and available methods only quantify the water vapour flux density in the air above the SC (Imhof *et al.*, 2009b). Nonetheless, the measurements have been reported in the literature for many decades (Nilsson, 1977). Currently, three main instruments are available for TEWL measurement, namely: 1) an open-chamber system, 2) an unventilated closed-chamber system, and 3) a condenser closed-chamber system.

## Chapter 3

The open-chamber system is the most favoured choice in the majority of the studies (De Paepe *et al.*, 2005; Fluhr *et al.*, 2006b; Clark *et al.*, 2010; Fluhr and Darlenski, 2014). Commercially available instruments are summarized in Table 3.1.

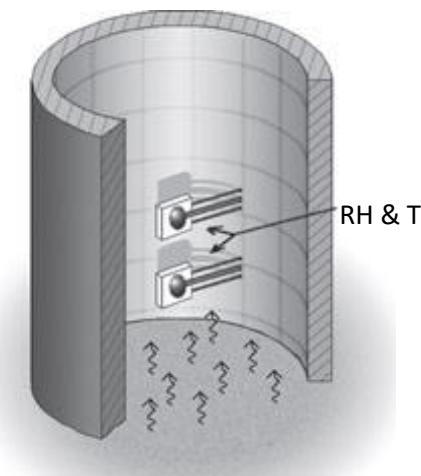
**Table 3.1. Commercially available instruments for quantification of TEWL.** Based on Imhof *et al.*, 2009b

Instrument	Method	Manufacturer
<b>AquaFlux</b>	Condenser closed-chamber	Biox Systems Ltd, London, UK
<b>DermaLab</b>	Open-chamber	Cortex Technology, Hadsund, Denmark
<b>Tewameter<sup>®</sup></b>	Open-chamber	Courage & Khazaka, Kohn, Germany
<b>Vapometer<sup>®</sup></b>	Unventilated closed-chamber	Delfin Technology, Kuopio, Finland

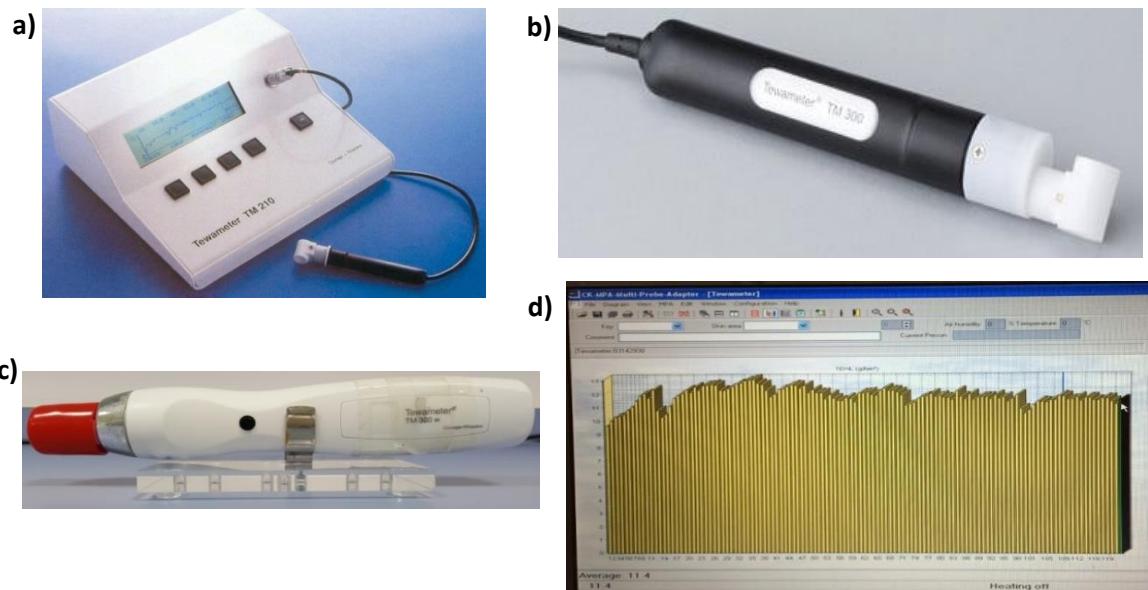
### Open chamber systems

The open-chamber method (Figure 3.1) is based on Fick's law of diffusion (Miteva *et al.*, 2006). It consists of a small hollow cylinder, used as the measuring head, containing two separate sensors, each of which continuously monitors temperature (T) and relative humidity (RH). TEWL is then calculated based on the readings from these sensors (Nilsson, 1977; Shah *et al.*, 2005; Imhof *et al.*, 2009b). Both ends of the cylinder are open, with the lower end applied to the skin and the other one serving as an exhaust, to allow evaporation of water flux into the ambient temperature (Imhof *et al.*, 2009b). An early model of an open chamber-system (TM210, Tewameter<sup>®</sup>) was characterized by certain limitations, involving frequent and time-consuming calibrations (Figure 3.2a). Therefore, a newer version of the device was launched (TM300) with a weight of 90 g, a probe length of 153mm with a cable length of 1.3m, a measuring chamber of 20 mm with a diameter of 10 mm covering a surface area of 7.9 mm<sup>2</sup> (Figure 3.2b) (Fluhr *et al.*, 2006b). The probe is connected to an adaptor and the readings are recorded using the manufacturer's software. Each sensor samples data at 0.5 Hz, and one TEWL measurement is recorded every 1.26 seconds. A TEWL curve is produced with time, and the average and standard deviation values are obtained, as shown in Figure 3.2d (Shah *et al.*, 2005). Recently, a wireless version of the TM300 (Weight: 150g, length without the measuring head: 202mm, diameter: 41 mm, transmission: via radio at 3.4 GHz, Courage and Khazaka) was developed, as shown in Figure 3.2c.

Advantages of the open-chamber method include its ability to provide continuous and reproducible readings, and also its sensitivity to detect small changes in TEWL. The main limitation of these systems is that they are influenced by air movements and application pressure. However, this issue can be resolved as the system can be converted to a closed-chamber system by using a ring, to cover the upper end of the measuring head (Tagami *et al.*, 2002; Imhof *et al.*, 2009b). It is important to note that according to the manufacturer (Courage and Khazaka) the measurement uncertainty of the TM300 is  $\pm 0.5 \text{ g/h/m}^2$  and  $\pm 1.0 \text{ g/hm}^2$  for relative humidities above 30% and below 30%, respectively.



**Figure 3.1. Open-chamber system for TEWL measurement.** In the open chamber method, a cylinder is used which is open at both ends and contains sensors for temperature (T) and relative humidity (RH). Based on Imhof *et al.*, 2009b.

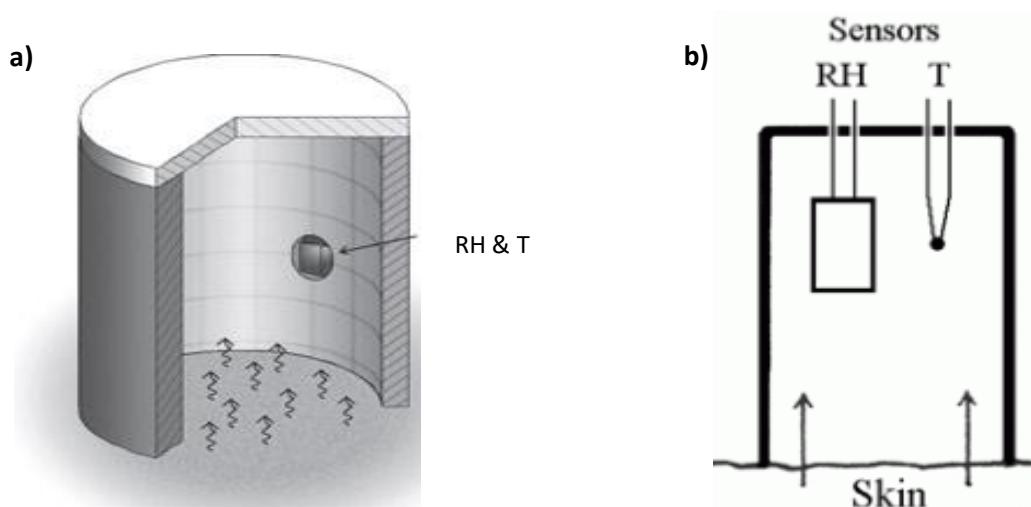


**Figure 3.2. The Tewameter® instrument for measurement of TEWL.** a) The first model to be launched was the TM210, b) the TM300 probe, c) the newly developed TM300 wireless probe, and d) when the probe is connected to a computer, results are displayed using the manufacturer's software. A graph is created showing TEWL over time. At the end of the measurement, the average value is calculated and shown on the left bottom corner (picture obtained during testing).

In a comparative study, Rosado and colleagues (2005) compared two devices (TM210 and TM300) from the same manufacturer, by measuring TEWL at both baseline and following application of an occlusive patch for 24 hours, on contralateral sites of the forearms of fifteen subjects. They reported that the TM300 requires less time to reach equilibrium (15 seconds) compared to the TM210 (300 seconds). The latter system also recorded higher corresponding TEWL values, although the differences were not statistically significant ( $p<0.05$ ). In spite of this, the authors concluded that studies should be conducted with the same device (Rosado *et al.*, 2005).

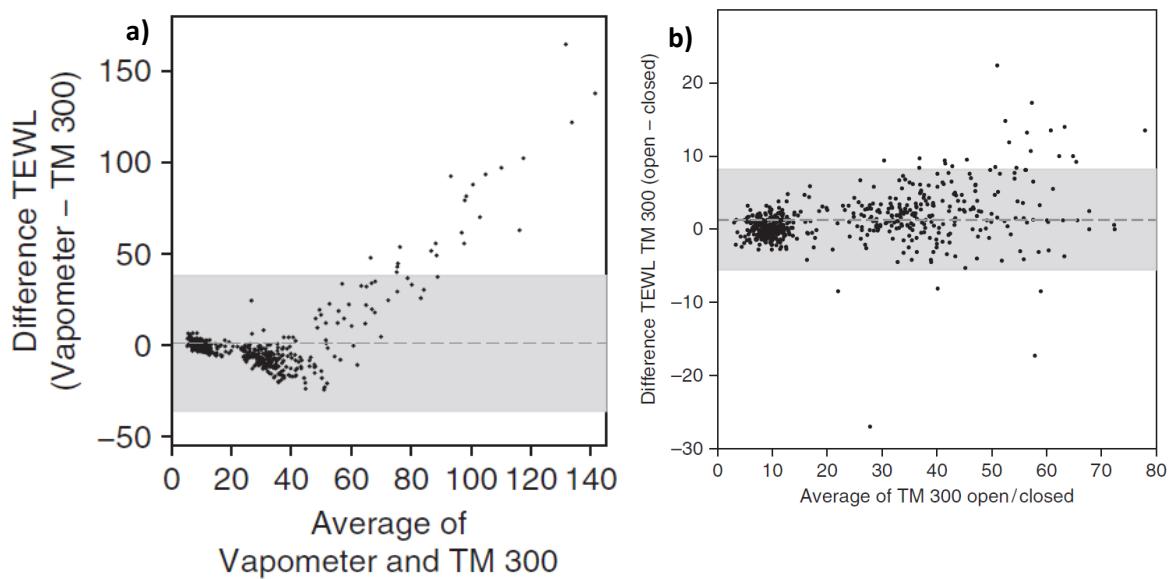
### Closed-chamber systems

Although developed many years ago, closed chamber systems have gained recent attention (Wallihan, 1964; Imhof *et al.*, 2009b). They can be conveniently divided into condenser-type and unventilated-type chambers, as shown in Figure 3.3 (Tagami *et al.*, 2002; Nuutinen *et al.*, 2003). In the condenser method (Figure 3.3a), a small cylinder serves as the measurement head, with its upper end closed with a condenser and its lower end in contact with the skin (Imhof *et al.*, 1999; Imhof, 2002; Imhof *et al.*, 2009a). TEWL measurements are based on RH and T sensors inside the cylinder (Imhof *et al.*, 2009b). By implication, the main advantage of this continuous measurement system is that its measurements are independent of any fluctuations of ambient air conditions (Imhof *et al.*, 1999; Imhof, 2002; Imhof *et al.*, 2009a; Kottner and Vogt, 2017). However, it represents a high cost system. By contrast, the unventilated method (Figure 3.3b), in which the water vapour collected from the skin surface is trapped in the chamber, requires lifting off of the skin between measurements (Imhof *et al.*, 2009b).



**Figure 3.3. Closed-chamber systems for TEWL measurement.** a) The condenser-chamber system and b) the unventilated chamber method. Figure adapted from Imhof *et al.*, 2009b

Several studies have compared the open- and closed- chamber instruments on healthy and damaged skin (De Paepe *et al.*, 2005; Shah *et al.*, 2005; Fluhr *et al.*, 2006b; Zhai *et al.*, 2007; Farahmand *et al.*, 2009; Elkeeb *et al.*, 2010; Steiner *et al.*, 2011). In a study involving 17 volunteers, the Tewameter® TM300 (open- and closed- systems) and the Vapometer® were used to measure TEWL on the dorsum and palm of both hands at baseline and following application of skin care products (Steiner *et al.*, 2011). Results revealed a high correlation between instruments, for low- and mid- range values. By contrast, at high range values ( $TEWL > 55 \text{ g/h/m}^2$ ), the Vapometer® presented an increased variability and recorded higher values as confirmed by Bland-Altman plots (Figure 3.4a). In addition, the Tewameter® was able to detect small changes in TEWL compared to the Vapometer®. Findings from the Tewameter® TM300 also revealed a high correlation between the values obtained from both the open- and closed- chamber systems, also supported by Bland-Altman plots (Figure 3.4b), with a near zero mean difference at mean TEWL values of up to  $20 \text{ g/h/m}^2$ . Results reported excellent reliability between the TM300 open- and closed-chamber systems, with an intraclass correlation (ICC) of 0.98. In addition, an ICC value of 0.70 was obtained between the Vapometer® and the TM300 open-chamber, also showing a good reliability. No such comparisons were performed between the closed-chamber system of the TM300 and the Vapometer® systems.



**Figure 3.4. Bland Altman plots to assess the agreement between instruments.** a) Vapometer Vs TM300 open-chamber: a good agreement exists between the two for average values up to  $55 \text{ g/h/m}^2$ . Then, the Vapometer values are systematically higher than TM300. The mean difference is  $1.2 \text{ g/h/m}^2$ , and the lower and upper agreement limits are  $-35.8 \text{ g/h/m}^2$  and  $38.3 \text{ g/h/m}^2$  respectively, and b) TM300 open-chamber Vs TM300 closed-chamber: there is a good agreement between the two, the mean difference is  $1.3 \text{ g/h/m}^2$ , and the lower and upper agreement limits are  $-5.4 \text{ g/h/m}^2$  and  $8.2 \text{ g/h/m}^2$  respectively, and Figure adapted from Steiner *et al.*, 2011.

Zhai and colleagues (2007) examined the effects of tape stripping on TEWL values measured using both open- and closed- chamber systems. There were no statistical significant differences between the instruments and, indeed, following the 10<sup>th</sup> tape strip the relative change in TEWL compared to baseline was the same in both systems. However, after 20 tape strips, the closed-chamber system presented a higher inter-individual variation, as reflected by the coefficient of variation (CV) which was 47.8%, compared to the TM210 (Zhai *et al.*, 2007) .Considering the results from the various comparative studies, Table 3.2 below summarizes the key characteristics for the open- and closed- chamber systems.

**Table 3.2. Key characteristics of open- and closed- chamber systems for TEWL measurement.**

Characteristics	Open-chamber	Closed-chamber
	systems	systems
<b>Small and handy</b>	(wireless probe)	✓
<b>High cost</b>		✓
<b>Reliable, accurate and reproducible readings</b>	✓	✓
<b>Continuous measurements</b>	✓	(condenser only)
<b>Able to detect small changes in TEWL</b>	✓	
<b>Influenced by air-movements</b>	✓	
<b>Inter-individual variation</b>		✓

The open-chamber measurement method, involving the Tewameter® (TM300, Courage and Khazaka, Germany), was used in this research work as it allows continuous measurements (Shah *et al.*, 2005), is sensitive to detect small changes in TEWL (De Paepe *et al.*, 2005) and is associated with lower variations between able-bodied individuals compared to the closed chamber (Zhai *et al.*, 2007). It is interesting to note that preliminary TEWL measurements were also taken by converting the Tewameter® into a closed-chamber system, using a black ring supplied by the manufacturer (Courage and Khazaka), and no differences were observed in measurements between the open- and closed-chamber Tewameter®. Subsequently, measurements were performed with the open-chamber system.

### Guidelines for TEWL measurement

All the available methods for TEWL quantification are characterized by specific inherent limitations, which need to be minimized. Sources of inter-individual variability with TEWL can include age, gender, race and anatomical site (Pinnagoda *et al.*, 1990). As an example, some studies report a decrease in TEWL values with age (Leveque *et al.*, 1984; Thune *et al.*, 1988), while others report no such correlation (Rougier *et al.*, 1988; Tupker *et al.*, 1989). In addition, it is well-documented that in infancy TEWL values are considerably lower than with adult skin (Nikolovski *et al.*, 2008). With respect to gender, a number of studies reported no difference in TEWL (Rougier *et al.*, 1988; Oestmann *et al.*, 1993; Reed *et al.*, 1995), whilst other studies reported that the skin of males yielded higher TEWL values (Conti *et al.*, 1995; Chilcott and Farrar, 2000; Firooz *et al.*, 2012). There are also reported variations in TEWL between anatomical sites (Rougier *et al.*, 1988; Conti *et al.*, 1995; Schnetz *et al.*, 1999a; Chilcott and Farrar, 2000). For example, TEWL was found to be higher in the forehead and lower in the forearm, with the differences being attributed to corneocyte size, with larger corneocytes corresponding to lower TEWL (Rougier *et al.*, 1988). Therefore, it is important only to compare data from the same anatomical location (Björnberg, 1968). Additionally, a recent study stated that the forearm presents an accessible site to measure TEWL, as it is smoother compared to other sites, such as the hip (Fader *et al.*, 2011). The variations across this site are well-accepted, with inter- and intra-individual CVs reported to be between 35-48% and 18%, respectively (Blichmann and Serup, 1987; Agner and Serup, 1990a). In addition, a higher variation has been reported in sites closest to the elbow and the wrist (Leveque, 1989; Treffel *et al.*, 1994; Barel and Clarys, 1995a; Chilcott and Farrar, 2000; De Paepe *et al.*, 2005). Thereupon, these two areas should be avoided for TEWL measurements (Pinnagoda *et al.*, 1989b; Panisset *et al.*, 1991).

In light of these variations, specific guidelines have recommended a standardized methodology for TEWL measurement (Rogiers, 2001). These involve an acclimatization period of at least 30 minutes prior to measurements, and that, temperature ( $22^{\circ}\text{C} \pm 3^{\circ}\text{C}$ ), pressure and humidity (40%-60%) should be controlled along with physical, thermal and emotional sweating (Pinnagoda *et al.*, 1989b; Rogiers, 2001; De Paepe *et al.*, 2005; Fader *et al.*, 2010; Fader *et al.*, 2011). Additionally, it is reported that measurements are affected by the basal temperature of the researcher and the application pressure of the probe (Barel and Clarys, 1995b). Therefore, special gloves should be worn and a special tool must be used to hold the probe horizontal and maintain a constant pressure (Pinnagoda *et al.*, 1989a; Pinnagoda *et al.*, 1990; Rogiers, 2001; Fader *et al.*, 2011).

Moreover, it should be highlighted that TEWL is not the only evaporation flux, as this can also be attributed to skin surface water loss (SSWL) and perspiration. Consequently, to ensure that what is actually measured is TEWL, the tested area should be pat dried using filter papers (Imhof *et al.*, 2009b). It has also been recommended that the application of moisturizers and other cosmetic products should be avoided prior to testing as these were reported to influence TEWL (Held *et al.*, 1999; Buraczewska *et al.*, 2007; Lodén, 2012).

The aforementioned guidelines for accurate TEWL measurements have been fulfilled in all the *in vivo* studies described in this thesis. First, testing was performed in a controlled laboratory environment, temperature of  $22 \pm 3$  °C and relative humidity of  $40 \pm 5\%$ , and all participants were left to acclimatize in these ambient conditions for 30 minutes prior to testing. During testing, the researcher recording the measurements was wearing gloves and a metal stand was used to hold the TEWL probe horizontally, thus maintaining a constant applied pressure on the skin. Furthermore, following exposure to treatments and prior to TEWL measurement, the skin was gently dried with filter paper to ensure that TEWL represented the only evaporation flux. Participants involved in the studies were also instructed not to apply any moisturizers or any other cosmetic products at least 12 hours prior to testing.

## **2) Stratum corneum (SC) hydration**

SC hydration is another important parameter when considering skin health. Measurement methods, commonly employed in dermatological research, provide either a direct, semi-direct or indirect measurement, as summarized in Table 3.3.

**Table 3.3. Available methods for measurement of SC hydration.** Based on Girard *et al.*, 2000

Method (studies)	Principle	Measurement of SC hydration
<b>1. Near-Infrared Spectroscopy</b>  (De Rigal <i>et al.</i> , 1993; Martin, 1993)	Absorption of near infrared light by water	Direct
<b>2. Nuclear Magnetic Resonance Spectroscopy</b>  (Girard <i>et al.</i> , 2000)	Measurement of resonance of hydrogen atoms under the magnetic field	Direct
<b>3. Transient-thermal transfer measurement</b>  (Berardesca <i>et al.</i> , 1990)	Measurement of the effect of hydration on the biophysical properties of skin	Semi-direct
<b>4. Bioengineering probes</b>  (Leveque and Derigal, 1983)	Electrical properties of skin	Indirect

### Opto-thermal transient emission radiometry (OTTER)

The OTTER technique was developed several years ago and it represents a method of assessing the hydration levels of the SC. It is an infrared remote sensing technique based on the spectral properties of water (Bindra *et al.*, 1994). In this method, a pulsed laser is used to heat up the skin, with excitation and thermal wavelengths absorbed by the superficial layer of the SC, and the resulting radiation signal is sensed by an infrared detector. The detection depth of SC hydration is reported to be 20µm. In addition, it has been reported that this technique can also assess the distribution of the water content in the SC (Xiao and Imhof, 1996). The main advantages of this technique are its non-invasive nature requiring no physical contact with skin, and that it is relatively quick. In a comparative study, involving different methods to assess SC hydration, Xiao and colleagues (2010) reported that OTTER was able to detect increased hydration levels following application of an occlusive wet patch, and that there was a good correlation with the other measurements (Xiao *et al.*, 2010).

## Chapter 3

It is now generally accepted that with a change in the water content of the skin there will be a change in its electrical properties (Tagami, 1995; Berardesca, 1997; Clarys *et al.*, 2012). Therefore, methods that measure either the capacitance (the ability of a body to store an electric charge), impedance (resistance to an electrical current), or conductance (the ease with which an electric current passes through a material) of the skin layers provide a simple means to measure skin hydration. Some of the commercial instruments for measuring the electrical properties of the skin are shown in table 3.4. The Corneometer® and the SEM Scanner Model 200 will be further discussed.

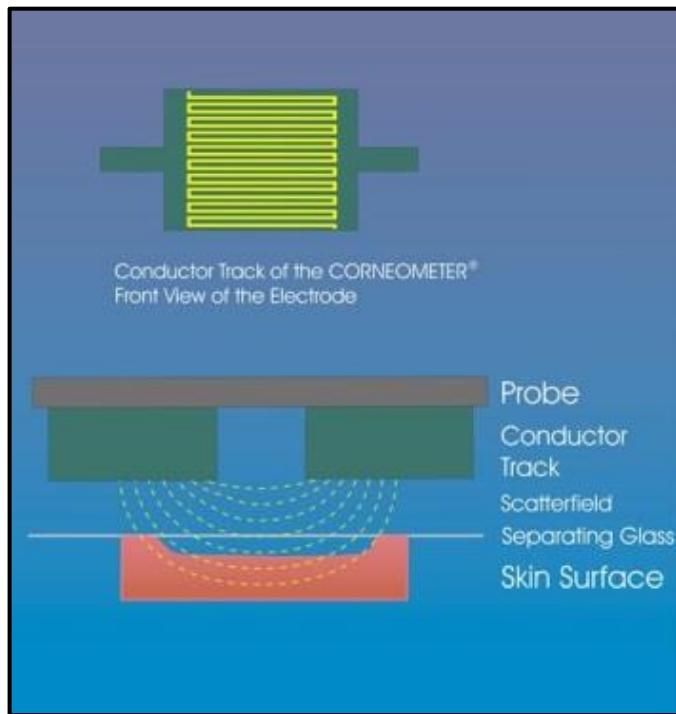
**Table 3.4. The different instruments available for skin hydration measurement.**

Instrument	Manufacturer	Measuring principle
<b>Corneometer®</b>	Courage & Khazaka, Germany	Capacitance
<b>DermaLab</b>	Cortex Technology, Hadsund, Denmark	Conductance
<b>SEM Scanner Model 200</b>	Bruin Biometrics, USA	Capacitance
<b>Skicon-200EX®</b>	I.B.S., Hamamatsu-shi, Japan	Conductance

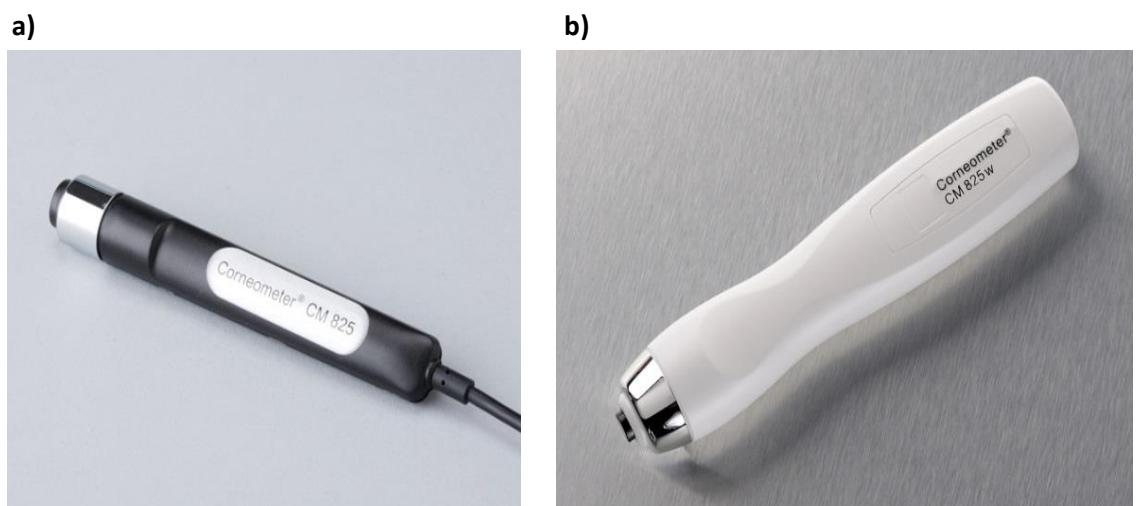
### **Corneometer®**

The Corneometer®, manufactured by Courage & Khazaka Electronics, contains an interdigital grid of golden electrodes, with different electrical charges, which are covered by a low dielectric material. In combination with the epidermal layer of the skin, this acts as a capacitor, and detects changes in the dielectric constant of the skin, as illustrated in Figure 3.5 (Barel and Clarys, 1997). The measurement depth of the Corneometer® is generally restricted to the SC (10-20µm), however this was challenged in a recent study using several sheets of plastic foil (thickness: 15µm), and the depth of measurement was estimated to be 45µm (Courage, 1994; Clarys *et al.*, 2012). The current model (CM 825) uses a digital sensor technology to provide continuous measurements, and incorporates a spring in the probe head to maintain a constant applied pressure, to ensure reproducible measurements (Heinrich *et al.*, 2003).

Indeed, the Corneometer® proved easy to use with a short measuring time (1s) and does not require any galvanic contact between the probe and the tested area (Barel and Clarys, 1995c, 1997; Fluhr *et al.*, 1999b; O'goshi and Serup, 2005; Clarys *et al.*, 2012). This probe is available as either part of the MP9 system (length: 110mm, cable length: 1.3m, measuring surface: 49mm<sup>2</sup>, weight: 41g, measurement frequency: 0.9-1.2 MHz) or as a wireless battery-operated probe, indicated in Figure 3.6.



**Figure 3.5. Measuring principle of the Corneometer®.** Adapted from Courage & Khazaka



**Figure 3.6. The Corneometer® CM825.** a) Wired probe as part of the MP9 system, and b) Wireless probe. Figure adapted from Courage & Khazaka.

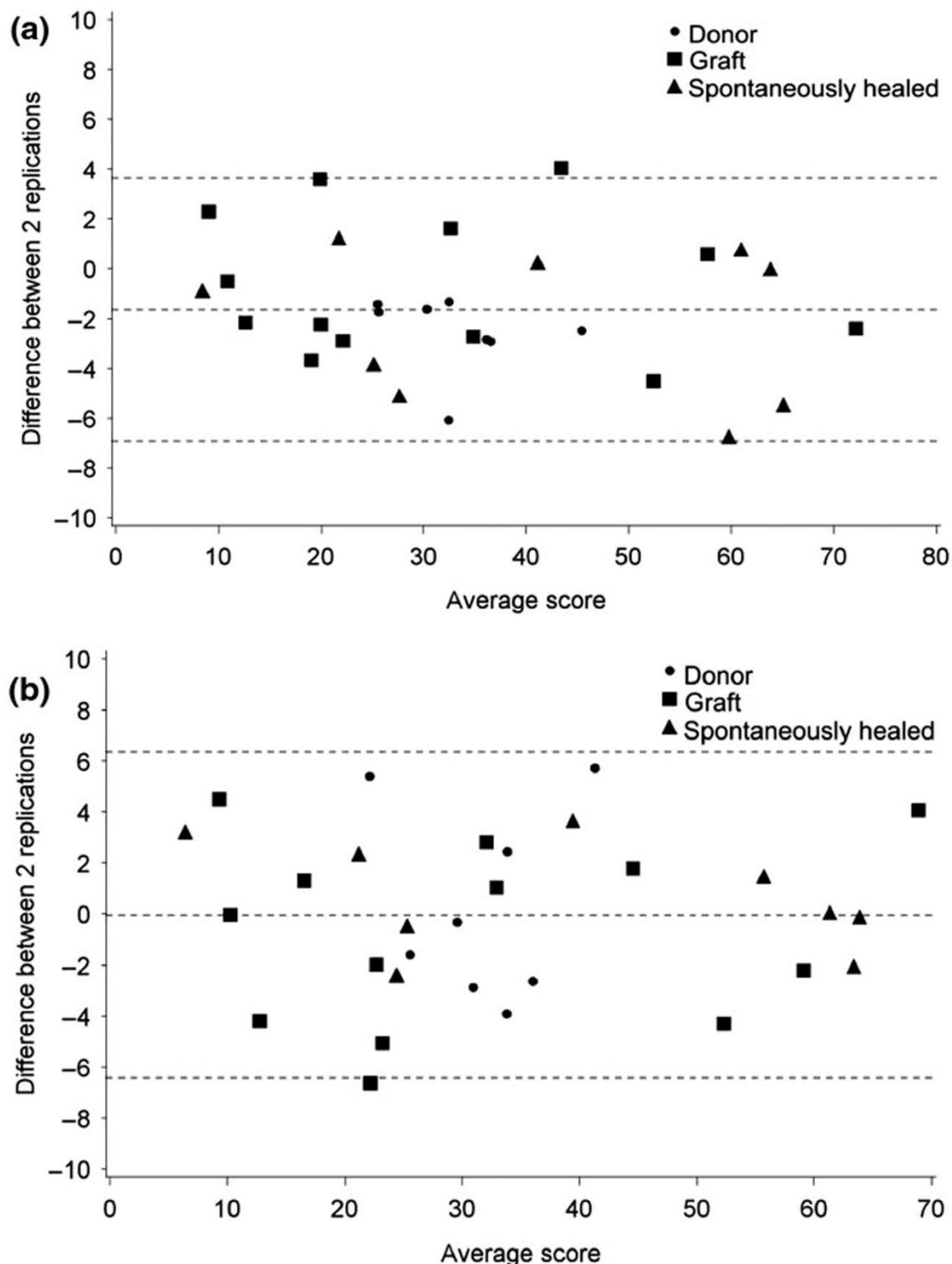
## Chapter 3

However, the system only provides a relative measure of SC hydration, expressed in arbitrary units (AU) ranging between 0-120 (Courage, 1994; Barel and Clarys, 1997; Berardesca, 1997). Despite this limitation, the system was characterized in a large multicentre study, with respect to skin type, as indicated in Table 3.5 (Heinrich *et al.*, 2003). According to the manufacturer, the reported uncertainty in the relative SC hydration values is  $\pm 3\%$ .

**Table 3.5. Corneometer values and their meaning in terms of skin type.** Based on Heinrich *et al.*, 2003

Skin Type	Corneometer values (AU)
Very dry	<30
Dry	30-40
<b>Normally moist skin, well hydrated</b>	<b>&gt;40</b>

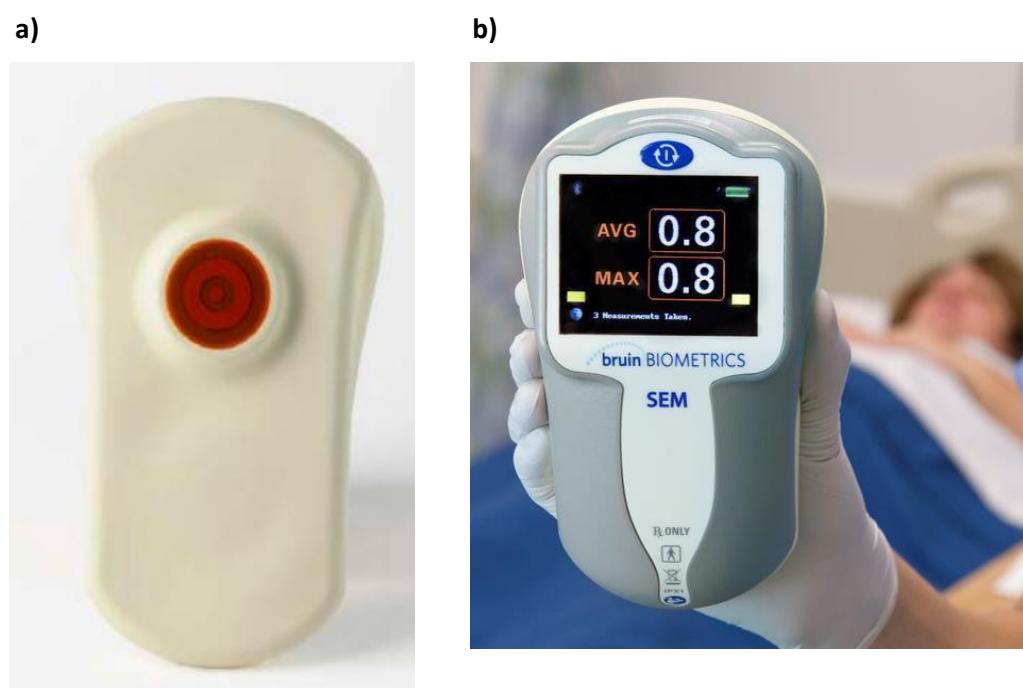
In a recent study on post-burn scars, the performance of the Corneometer® (Model CM825) was examined (Anthonissen *et al.*, 2015). It revealed both a good agreement between two sets of measurements, as indicated by the Bland-Altman analysis (Figure 3.7a) and a good agreement between observers (Figure 3.7b), with a mean difference close to zero (bias=-0.03). The corresponding ICC values for intra- and inter- observer reliability were 0.98 and 0.85, respectively.



**Figure 3.7. Intra- and inter- observer reliability of the Corneometer CM825 on burn scars. a)** Good agreement between two repeated measurements obtained by Observer A, with the mean difference= -1.64, and **b)** good agreement between measurements obtained by two observers, mean difference= -0.03. Figure adapted from Anthonissen *et al.*, 2015

### SEM Scanner Model 200

A recent advancement in this field is the development of the SEM Scanner Model 200 (Bruin Biometrics, US). This hand-held portable device is intended for use by healthcare professionals to identify early indications of tissue damage. The system purports to measure the water content of the extracellular space below the surface of target tissues, referred to as subepidermal moisture (SEM), based on electrical capacitance (Clendenin *et al.*, 2015). It is composed of a single coaxial electrode and an integrated pressure sensor, as shown in Figure 3.8. The device which makes contact with the skin at a controlled pressure provides an output in arbitrary units, ranging from 0.5 to 7.0, with a manufacturer reported accuracy of  $\pm 0.4$  SEM units. A recent study involving 31 volunteers examined the reliability of this device, in which SEM readings were obtained at four different skin sites by three independent researchers using three independent devices. Findings revealed a good agreement between devices and operators, with ICC values higher than 0.80, also supported by Bland-Altman plots (Clendenin *et al.*, 2015). However, a recent study performed within the host research group revealed high variability between the SEM readings, even within the same anatomical region.



**Figure 3.8. SEM Scanner Model 200.** a) The electrode at the back of the SEM Scanner is applied on the skin, and b) average and maximum readings are displayed. Figure adapted from Bruin Biometrics

In the studies described within this thesis, the hydration level of the SC was measured by corneometry with a Corneometer® (CM 825, Courage & Khazaka, Germany) as it provides continuous measurements, and has been used in previous studies within the research group and shown to produce reproducible readings.

#### **Guidelines for measurement of SC hydration using the Corneometer®**

Variation in measurements with respect to SC hydration has been attributed to age and anatomical site. It is well accepted that SC hydration decreases with age (Rogiers *et al.*, 1990; Verdier-Sevrain and Bonte, 2007), with infant skin presenting a higher SC hydration (Nikolovski *et al.*, 2008). It has been additionally reported that hydration levels depend on the local thickness of SC, with the forehead and palms presenting increased hydration values compared to other sites. By contrast, conflicting results are reported in the literature in regards to SC hydration levels between genders (Rogiers *et al.*, 1990; Man *et al.*, 2009).

Specific guidelines have been proposed when measuring the SC hydration, to improve the accuracy and reproducibility of results. These are most conveniently listed.

- Clean the measuring probe between measurements and ensure the test site is dry (Rogiers *et al.*, 1990; Berardesca, 1997)
- Apply a slight and constant pressure when taking measurements.
- Avoid sites with hair as they influence readings (Loden *et al.*, 1995)
- A refractory period of 5s should be adopted between measurements to avoid creating an occlusive effect that will lead to increased values (Clarys *et al.*, 2012)
- Perform at least 3 measurements on the same anatomical site
- Conduct measurements at an ambient temperature of between 20°C and 22°C and relative humidity of between 40% and 60%

Subjects should acclimatize for at least 30 minutes prior to testing (Moseley *et al.*, 1985).

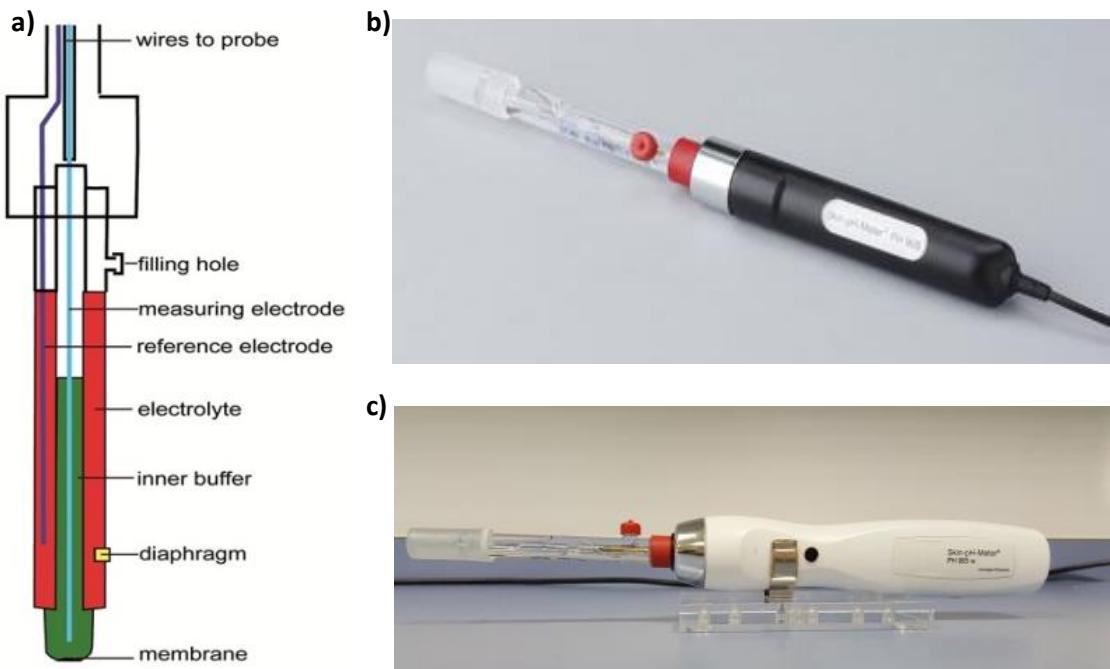
Given the popularity of using the forearm as a test site, it is important to note the variable findings across this anatomical area (Serup and Blichmann, 1986; Tagami, 2008).

All the aforementioned guidelines were followed in the current study. In a similar manner to TEWL, measurements were performed in a temperature and humidity controlled laboratory at an ambient temperature of  $22 \pm 3^\circ\text{C}$  and relative humidity of  $40 \pm 5\%$ . An acclimatization period of 30 minutes was also enforced to all participants prior to measurements. To obtain SC hydration measurements, the Corneometer® was gently applied to the skin by the researcher and 5 repeated measurements were taken at each anatomical location.

After each measurement, a refractory period of 5 seconds was enforced to minimise occlusion which would have resulted in elevated values. Before transferring to the next skin site, the probe was cleaned using a soft tissue to remove excessive dirt and the testing site was gently dried using filter paper. Sites with hair were avoided in all participants as these have been reported in the literature to influence hydration readings (Loden *et al.*, 1995).

### **3) Skin surface pH**

Skin surface pH is critical for the maintenance of an intact skin barrier, as highlighted in section 1.3.5, and it is commonly measured in dermatological research. In its traditional form, measurement of skin pH was achieved by applying a chemical dye on the skin and using a colorimetric procedure to detect any changes in colour and pH (Dikstein and Zlotogorski, 1989). In recent times, more sophisticated methods involving a glass electrode sensitive to ion concentration and a potentiometer to measure the difference in electric potential have been developed (Blank, 1939; Welzel, 1995). Currently, flat planar electrodes are used, which contain both a glass hydrogen ion sensitive electrode and a reference electrode (Figure 3.9a). When applied to the skin, the electrodes detect the concentration of hydrogen ions that are present, released from the fatty acid lipids (Welzel, 1995; Stefaniak *et al.*, 2013). Thus what is actually being measured represents the apparent skin surface pH, which might differ from the internal pH (Parra and Paye, 2003; Agache, 2004). Different commercial electrode instruments are available, examples of which include the Skin-pH-Meter® 900 or 905 (Figures 3.9b and 3.9c) (Courage & Khazaka, Germany), the pH-meter 1140 (Mettler Toledo Greisensee, Switzerland) and the pH meter (Radiometer, Copenhagen, Denmark). Performance of the former system has revealed rapid, accurate and reproducible values (Yosipovitch *et al.*, 1998; Fluhr *et al.*, 2002; Parra and Paye, 2003; Agache, 2004), with an uncertainty of  $\pm$  0.1 pH units (Ehlers *et al.*, 2001b). Accordingly, in the current research study the Skin-pH-Meter® 905 (Courage & Khazaka, Germany) was used to measure skin surface pH.



**Figure 3.9. Skin-pH-Meter® 905.** a) Measuring principle, b) wired probe, and c) wireless battery-operated device. Pictures taken from Courage & Khazaka and during testing (c).

In the in vivo studies described, skin surface pH was measured using the Skin-pH-Meter® 905, as preliminary testing has shown that it can provide accurate and reproducible readings, thus supporting previous studies within the host research group.

### Guidelines for skin pH measurement

The measurement of pH can be influenced by:

- 1) Endogenous factors, such as the anatomical site, gender, ethnicity and age (Zlotogorski, 1987; Berardesca *et al.*, 1998; Ehlers *et al.*, 2001b; Kobayashi and Tagami, 2004)
- 2) Exogenous factors, such as washing and occlusion (Voegeli, 2008; Moldovan and Nanu, 2010)
- 3) Environmental factors, such as ambient temperature and humidity (Abe *et al.*, 1980)

It is well-established that skin surface pH varies not only between anatomical sites but also between areas of the same anatomical region (Kobayashi and Tagami, 2004; Kim *et al.*, 2006; Marrakchi and Maibach, 2007; Kleesz *et al.*, 2012). In particular, it has been reported that in the forearm, sites close to the elbow should be avoided as they generally produce higher pH values (Braun-Falco and Korting, 1986; Fluhr *et al.*, 2006a). Additionally, it has been reported that the dominant arm presents a lower skin pH than the non-dominant arm (Treffel *et al.*, 1994).

## Chapter 3

In addition, some studies have reported lower pH values in male subjects (Jacobi *et al.*, 2005; Kim *et al.*, 2006; Man *et al.*, 2009; Luebberding *et al.*, 2013). By contrast, another study reported the reverse finding, whereas others reported no differences in skin pH values between genders (Zlotogorski, 1987; Wilhelm *et al.*, 1991b; Ehlers *et al.*, 2001a). It is also well-established that post-partum skin develops until a so-called acid mantle is attained. This corresponds to a reduction in pH from infant to adult skin, although in old age this change is not evident (Yosipovitch *et al.*, 2000; Farage *et al.*, 2008). In light of these variations, specific guidelines have been published concerning the measurement of skin pH in experimental settings (Parra and Paye, 2003; Stefaniak *et al.*, 2013), namely:

1. An acclimatization period of at least 20 minutes at an ambient temperature between 20°C and 22°C prior to testing
2. Humidity should be controlled at between 40% and 60%
3. Pressure application of the electrode to the skin should be minimal, just for optimal contact
4. Repeated measurement should be taken at each location
5. Measurements should be performed by the same researcher using a standard protocol

These were all fulfilled in the in vivo studies, and all measurements followed a standardized protocol. To review briefly, following an acclimatization period of 30 minutes at ambient laboratory conditions (temperature:  $22 \pm 3^\circ\text{C}$ , relative humidity:  $40 \pm 5\%$ ), skin surface pH was measured by applying the probe on the skin with minimal pressure. In a similar way to the Corneometer®, five repeat measurements were recorded on the same anatomical location and a mean value estimated.

### 3.2.2 Methods to compromise skin integrity

Irritant models are often employed in dermatological studies to investigate a number of issues including the:

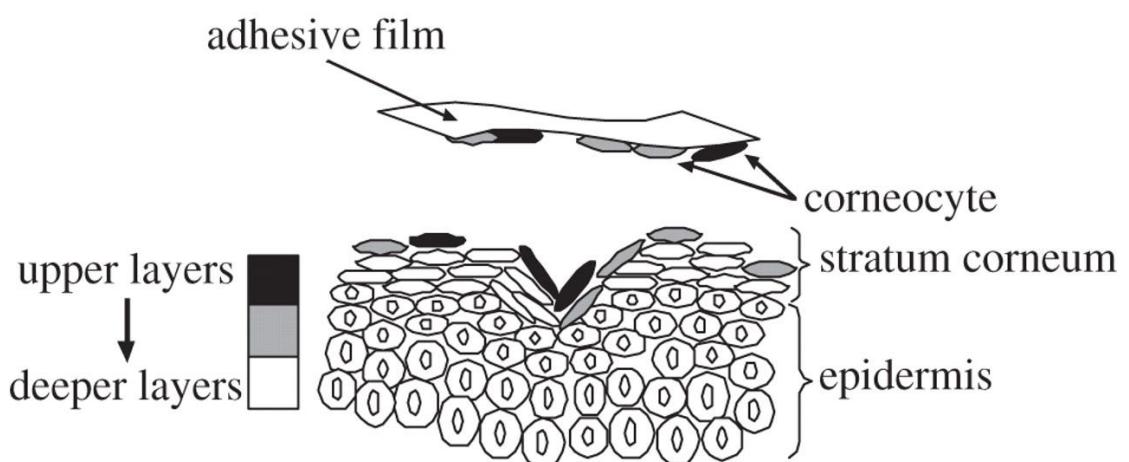
- physiology of the SC
- penetration of biomolecules
- recovery of barrier function
- release of inflammatory mediators from the skin
- effectiveness of skin products in restoring skin integrity

The models generally divide into those mimicking physical or chemical irritation.

## 1) Physical irritation model

### Tape stripping

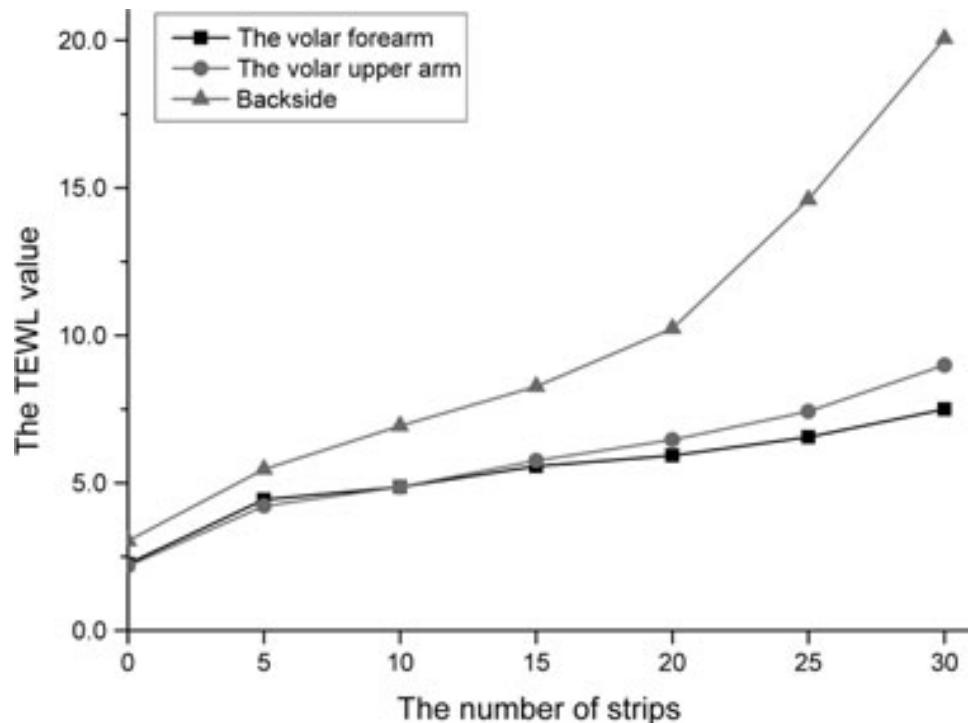
Tape stripping has been widely used in skin research. It is an efficient method to remove layers of the SC (Wolf, 1939; Pinkus, 1951) and consequently compromise skin integrity, as shown in Figure 3.10 (Lademann *et al.*, 2009). It represents a simple and non-invasive method, that simulates mechanical trauma, such as the application of frictional forces (Wilhelm *et al.*, 1991a). As a consequence, a number of interventional methods to restore the skin barrier and evoke a wound healing response can be examined (Dick *et al.*, 1997; Kondo *et al.*, 1998; Fluhr *et al.*, 1999a).



**Figure 3.10. The tape stripping procedure.** An adhesive tape is placed on the skin surface for the removal of the SC. Figure adapted from (Pailler-Mattei *et al.*, 2011)

As an example, tape stripping was performed thirty times at three different anatomical sites, and skin barrier function was assessed by TEWL measurement at baseline and on six subsequent increments (Gao *et al.*, 2013). Results, as seen in Figure 3.11, revealed that TEWL values increase with strip number, particularly at the backside, which supports an earlier finding (Löffler *et al.*, 2004). A possible explanation for this is that the backside represents an area with a thinner SC and accordingly decreased cell layers. Differences between sites can also be attributed to the biomechanical properties of anatomical sites that may resist to the tape application pressure (Löffler *et al.*, 2004). Although this study provided informative results on TEWL and tape stripping, no inclusion criteria were set for the subjects and this represents a major limitation. In another seminal study, the release of inflammatory mediators was investigated following tape stripping of the SC on the upper back of a cohort of able-bodied volunteers 6 hours after tape stripping (Dickel *et al.*, 2010).

Small curettage biopsies were performed and the tissue analysed by real time polymerase chain reaction (RT-PCR), to quantify mRNA expression of cytokines (Giulietti *et al.*, 2001). Results revealed a significant upregulation of biomolecules, TNF- $\alpha$ , IL-33, heat shock protein (Hsp) 70, Hsp 90 and IL-8/CXCL8, on the tape stripped area when compared to untreated controls ( $p<0.05$ ). Limited removal of the stratum corneum also yielded an upregulation of some of these inflammatory cytokines, thus confirming results of another study (De Jongh *et al.*, 2007b).



**Figure 3.11. Tape stripping at different anatomical locations.** TEWL value increases with the number of strips. The backside presented the higher and more dramatically increase in TEWL compared to the other anatomical locations. Figure adapted from Gao *et al.*, 2013

The main limitation of tape stripping is that it is affected by various intrinsic and extrinsic factors, including:

- The tape brand (Jui-Chen *et al.*, 1991b; Bashir *et al.*, 2001)
- The local properties of the SC such as thickness, and the size and distribution of corneocytes (Schwindt *et al.*, 1998; Black *et al.*, 2000)
- Anatomical site (Schwarb *et al.*, 1999; O'Goshi *et al.*, 2000)
- Age (Rougier *et al.*, 1987)
- The pressure used to apply the tape (Ghadially *et al.*, 1995)
- The removal process (Ghadially *et al.*, 1995; Löfller *et al.*, 2004)

Some of these parameters were examined in a seminal study (Löffler *et al.*, 2004). The authors affirmed the importance of employing a number of methodologies to assess the effectiveness of the tape stripping process to assess the amount of SC removed, and hence the disruption of skin integrity. These methods included:

1. Gravimetric (weighing) (Marttin *et al.*, 1996)

Although the traditional method it is time consuming and not accurate due to absorption and desorption of moisture from the tape (Bommannan *et al.*, 1990; Jui-Chen *et al.*, 1991a; Marttin *et al.*, 1996).

2. Spectroscopic (Weigmann *et al.*, 1999)

3. Colorimetric (Dreher *et al.*, 1998; Dreher *et al.*, 2005)

Involves the use of sodium hydroxide to dissolve the tape and its content, followed by a protein assay

4. Histological methods (Jenkins and Tresise, 1969).

The common haematoxylin and eosin staining can be used to stain the corneocytes attached to the tape and hence visualize the SC (Jenkins and Tresise, 1969).

In the present study, tape stripping was chosen as it represents a simple, efficient and less invasive technique to disrupt the integrity of the skin, resembling physical irritation. Additionally, tape stripping was performed following a standard protocol and the skin was stripped 20 times using equally-sized tapes from the same manufacturer (Sellotape™, UK). To ensure a constant application pressure, tapes were applied on the skin using sterile blunt tweezers and a roller was used back and forth 10 times. All the tapes were then rapidly removed.

## 2) Chemical irritation model

### **Sodium lauryl sulphate (SLS)**

Sodium lauryl sulphate is a surfactant and an emulsifier, found in many cosmetic and pharmaceutical products (Lee and Maibach, 2006), that has been extensively used as an experimental model to study contact dermatitis and subsequently the susceptibility of individuals to irritation. Indeed, its safe use in research was demonstrated in several studies, and as reported: 1) it is not carcinogenic, 2) does not impose any risk to volunteers, 3) it is not a sensitizer and 4) its chemical composition is well-defined (Kligman, 1966).

## Chapter 3

Various SLS concentrations have been used in experimental studies, ranging between 0.1% - 10%, while exposure times have varied from 4 hours up to several days (Lammintausta *et al.*, 1988; Loden and Andersson, 1996; Tupker *et al.*, 1997). However, in order to cause an erythematous response SLS should be used on the skin for at least 24 hours. Nonetheless, in the presence of underlying skin diseases SLS is contraindicated as its use on some anatomical sites, typically the area next to the wrist (Tupker *et al.*, 1997). Traditionally, visual scoring was used to assess the degree of skin irritation following exposure to SLS (Bettley, 1972; Frosch and Kligman, 1979), although more recent studies have employed TEWL measurements as the most appropriate method to quantify SLS-induced damage (Aramaki *et al.*, 2001). Other biophysical methods can also be used, including measurements of blood flow and SC hydration, although the latter is characterized by high intra-individual variation (Wilhelm *et al.*, 1989; Agner and Serup, 1990a; Agner and Serup, 1990b). Many authors agree that skin exposure to SLS produce features which resemble the clinical symptoms of contact dermatitis (Scheuplein and Ross, 1970; Elias, 1983; Van Der Valk *et al.*, 1984).

Several studies have investigated the release of inflammatory mediators from skin exposed to SLS (Hunziker *et al.*, 1992; Perkins *et al.*, 2001; De Jongh *et al.*, 2006; De Jongh *et al.*, 2007a; De Jongh *et al.*, 2007b; Koppes *et al.*, 2017). In a very recent study, the release of inflammatory mediators in the SC was investigated following exposure to SLS (2%) compared to known allergens, such as nickel, chromium, paraphenylenediamine and methylchloroisothiazolinone. After 48 hours exposure and following 24 hours post patch removal, tape stripping was performed to collect sample from the skin, and analysed using a chemiluminescence assay. Results showed that there is no significant difference between SLS and the allergens, indicating similar inflammatory mechanisms. Increased levels of both IL-8 and TNF- $\alpha$  were reported, confirming previous studies (Corsini and Galli, 2000; Steinhoff and Luger, 2004). However, for other cytokines, e.g. IL-1 $\alpha$ , IL-1 $\beta$  and IL-1RA, there was a downregulation compared to a petrolatum-treated control. These differences might be attributed to the specific protocol in which samples were taken 24 hours following removal of the patches. This would not have captured the temporal release profile of some cytokines.

In another study cytokine release was investigated by collecting interstitial fluid from the skin, 16-18 hours post SLS treatment, following a single- and repeated- exposure to SLS. Results showed increased levels of IL-1 $\alpha$ , IL-1RA and vascular endothelial growth factor (VEGF) in both states. However, the ratio between IL-1 $\alpha$  and IL-1RA, which is critical in the inflammatory response (Steinhoff and Luger, 2004), was only increased following repeated application of SLS. The levels of IL-6 were also decreased, whilst no change was observed in the levels of TNF- $\alpha$  (De Jongh *et al.*, 2007a).

In another study, cytokine levels at different parts of the SC were investigated following repeated exposure to SLS for 3 weeks, and subsequent tape stripping was used to obtain sample from the skin four days post SLS treatment (De Jongh *et al.*, 2007b). Tape stripping (n=30) was performed four days post SLS treatment, and the first eight tapes corresponded to the upper part of the SC, the second eight tapes to the intermediate part of the SC and the remaining tapes were referred to as the lower part of the SC. There were no significant differences ( $p>0.05$ ) in cytokine levels (IL-1 $\alpha$ , IL-RA, IL-8) for untreated skin across all layers of the SC, but for SLS-exposed skin lower levels of IL-1 $\alpha$  and IL-1RA ( $p<0.05$ ) were detected in the lowest part and in the upper layer of the SC, respectively. IL-8 was also found to be increased in the intermediate layer of the SC ( $p>0.05$ ) (De Jongh *et al.*, 2007b).

It is important to acknowledge that the contradicting results in these studies might be attributed to the time of collection of skin sample, and also there is no report of an equilibrium period to allow the skin to recover following the creation of micropores and prior to fluid collection (De Jongh *et al.*, 2007a).

In the present study, SLS was used to compromise the integrity of the skin, simulating the effects of chemical-irritation. A concentration of 0.5% SLS and an exposure time of 24 hours was chosen, as this is reported to represent the optimum time for the development of an erythematous response (Tupker *et al.*, 1997).

### **3) Experimental models of urinary- and faecal- incontinence**

#### **Synthetic-urine (s-urine)**

Synthetic-urine was used as a moisture irritant source, as experienced by patients with urinary-incontinence, and which has been used in different studies (Aurora *et al.*, 1980; Rodgers and Wandt, 1991; Mayrovitz and Sims, 2001; Sonsma *et al.*, 2006; Larner *et al.*, 2015; Matar *et al.*, 2017). It was firstly used in clinical research by Mayrovitz and Sims, 2001, to investigate the effects of pressure loading on wet skin after prolonged exposure to s-urine. The formulation ( $\text{pH}=7.9$ ) used by Mayrovitz and Sims (2001) was adapted and used in this study, and its composition is summarized in Table 3.6.

**Table 3.6. Chemical composition of s-urine.** Formulation is based on Mayrovitz and Sims (2001), and was prepared in 50ml of distilled water

Chemical constituent	Weight (g)	Concentration (w/v)
<b>Urea</b>	1.25	2.5%
<b>Sodium chloride</b>	0.45	0.9%
<b>Ammonium chloride</b>	0.15	0.3%
<b>Sodium sulphite</b>	0.15	0.3%
<b>Anhydrous disodium hydrogen orthophosphate</b>	0.125	0.25%
<b>Creatinine</b>	0.10	0.20%

### Proteolytic solution

As stated earlier (section 2.4.1b), faeces contain several proteolytic enzymes, such as trypsin,  $\alpha$ -chymotrypsin and lipase, as well as bile acids (Smith *et al.*, 1971; Buckingham and Berg, 1986; Andersen *et al.*, 1994). A combination of these enzymes and bile salts was firstly reported by Andersen and colleagues (1994) as a model of faecal-incontinence, and used to investigate its cumulative effects on skin barrier function, assessed by TEWL and pH. This model was also utilized in a very recent study; however the exclusion of bile salts clearly limits its physiological relevance (Mugita *et al.*, 2015). In the current investigation, a proteolytic-solution was also developed, based on previous studies, to investigate the mechanisms of skin inflammation upon exposure to faeces, and its composition is summarized in Table 3.7 below (Andersen *et al.*, 1994; Mugita *et al.*, 2015). The enzymes and bile salts concentrations represent adult physiological faecal concentrations.

**Table 3.7. Chemical composition of the proteolytic solution.** Formulation is based on Andersen *et al.*, 1994 and Mugita *et al.*, 2015. Prepared in 50ml of phosphate buffered saline

Chemical constituent	Weight (g)	Concentration (w/v)
<b>Trypsin</b>	0.125	0.25%
<b><math>\alpha</math>-chymotrypsin</b>	0.10	0.20%
<b>Lipase</b>	0.04	0.08%
<b>Bile salts</b>	5.00	10%

### 3.2.3 Methods for analysis of skin inflammation

#### 1) Measurement of blood flow in the microcirculation

The microcirculation is composed of small arteries, arterioles, capillaries and venules and is critical for tissue oxygenation and the exchange of nutrients (Rizzoni *et al.*, 2011; Eriksson *et al.*, 2014). Accordingly, direct monitoring of blood flow in the microcirculation is important to detect early signs of inflammation and to evaluate the effects of treatments on various organs, including the skin (Seifalian *et al.*, 1994; Choi and Bennett, 2003; Humeau *et al.*, 2007). This can be achieved by methods that assess either morphological parameters or endothelial function. The former involves vessel density and diameter, blood flow velocity, blood cell concentration and rate of perfused cells, which can be assessed by imaging methods, such as videomicroscopy and laser Doppler methods (Swain and Grant, 1989; Eriksson *et al.*, 2014). These will be discussed below.

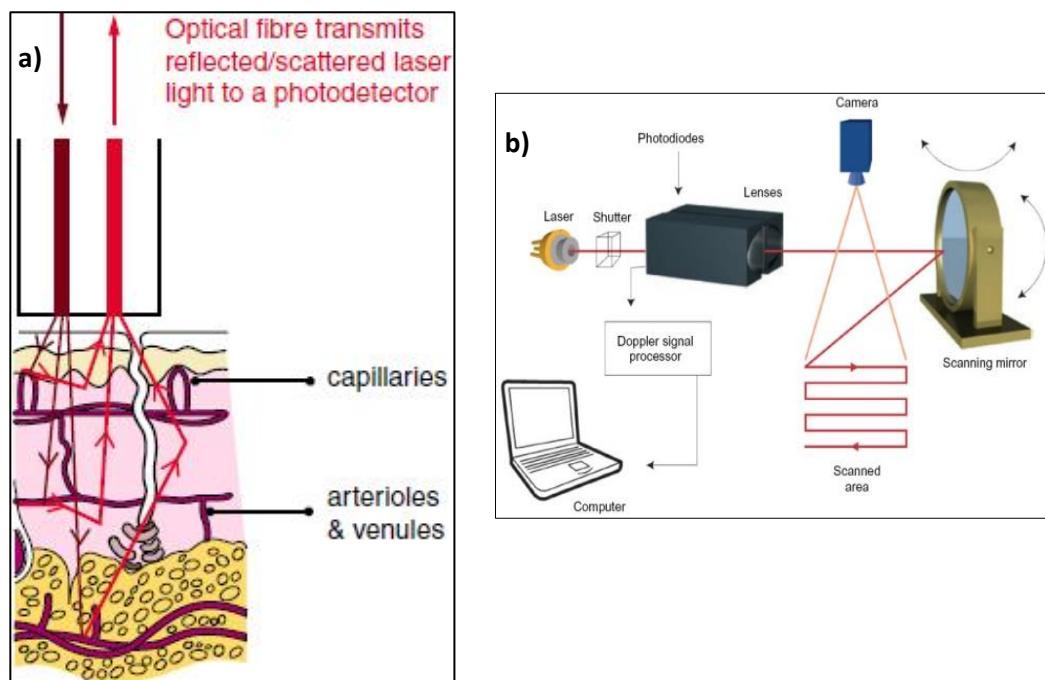
#### Videomicroscopy

This method was firstly described in a seminal paper by Groner and colleagues (1999), in which an orthogonal polarization spectral imaging system is incorporated in a hand-held device for the direct visualization of the microcirculation. Recently, the orthogonal polarization spectral was replaced by sidestream darkfield imaging (Eriksson *et al.*, 2014). The underlying principle is that polarized light is depolarized upon tissue penetration, and then collected by an orthogonal polarizer and recorded by a video camera (Groner *et al.*, 1999). The main advantage of this technique is that an image of the microcirculation can be obtained in real time, with the red blood cells appearing dark. However, this technique is characterized by certain limitations, including: 1) the increased sensitivity to motion and pressure artefacts, 2) the inability to measure high blood flow velocities and 3) long time required for analysis (Lindert *et al.*, 2002; De Backer *et al.*, 2010; Nilsson *et al.*, 2014).

#### Laser-Doppler Flowmetry/Imaging

It is now more than 40 years ago since Laser Doppler Flowmetry (LDF) was used to assess blood flow in the microcirculation (Riva *et al.*, 1972). This represents a non-invasive technique based on the Doppler Effect, which measures the speed and concentration of red blood cells, referred to as perfusion or flux (Holloway and Watkins, 1977; Leahy *et al.*, 1999; Leahy *et al.*, 2003; Humeau *et al.*, 2007). LDF systems consist of a 2.5mW laser and transmit light (785nm  $\pm$  10) to the skin surface via optical fibres in the probe head, as shown in Figure 3.12a. Transmitted light that is reflected and backscattered by moving red blood cells is collected by a separate optical fibre on the skin surface, and processed by a photodetector (Choi and Bennett, 2003).

The depth of measurement is 1mm, although it depends on the laser wavelength, skin pigmentation and probe type (Essex and Byrne, 1991; Schabauer and Rooke, 1994). Although used extensively, LDF systems present certain limitations, namely: 1) the direct contact with the skin, 2) the wide variation between sites, even on the same anatomical location, as it is a single-point measurement, and 3) the restricted measurement area ( $1\text{mm}^3$ ), which does not reflect the regional blood flow (Worsley and Voegeli, 2013). However, advancements led to the development of Laser Doppler imaging (LDI) systems, in which the probe is replaced by a system of mirrors and the optical fibres by light-collecting lenses. Reflected light is also collected by a photodetector, however, this is placed above the skin surface (Figure 3.12b), and consequently avoiding contact with the skin (Essex and Byrne, 1991; Choi and Bennett, 2003). Additionally, the LDI does not itself affect local blood flow, is able to provide a continuous read-out of dermal perfusion and can be used to detect sudden changes in blood flow. Accordingly, LDI instruments are particularly suited for the assessment of relative changes in skin perfusion at a given site in response to a standard stimulus. The built-in software allows the creation of colour-coded perfusion and photo images (Essex and Byrne, 1991; Worsley and Voegeli, 2013).



**Figure 3.12. Principle of LDF and LDI for the measurement of blood flow in the microcirculation. a) LDF and b) LDI systems.** Figure taken from Moor Instruments

Several studies in the literature compared the LDF and LDI systems on blood flow measurement (Seifalian *et al.*, 1994; Rajan *et al.*, 2009; Petersen, 2013). In a recent study, using an experimental model of skin irritation (SLS), it was shown that both systems were able to detect the changes in blood flow following induced-damage, and indeed a good correlation ( $r=0.79$ ) was found between the two instruments, as well as compared to conventional clinical grading. Reliability of methods was also assessed using the ICC analysis (single measures- two-way random) both for consistency (ICC value= 0.67) and absolute agreement (ICC value=0.57). Bland-Altman plots (data not shown by authors) also revealed a systematic bias between the methods possibly due to their different dynamic range (LDF=0-1000 AU, LDI=0-5000 AU) (Petersen, 2013). The main drawback of LDI, is the lack of a biological zero, since even when blood flow is arrested, a perfusion value is still measured by the system (Clinton *et al.*, 1991). Evidence in the literature suggests that this is attributed to the Brownian motion of molecules in the vasculature and the red blood vasomotion (Banic *et al.*, 1990; Clinton *et al.*, 1991; Kernick *et al.*, 1999). Despite the fact that LDI is not susceptible to pressure artefacts as its predecessor LDF, it is still prone to movement artefacts (Rajan *et al.*, 2009; Worsley and Voegeli, 2013). Also, both LDF and LDI instruments give values of perfusion in AU, and therefore are regarded as indirect measures of blood flow (Worsley and Voegeli, 2013).

LDI is the preferred method to measure blood flow in clinical research, and has been extensively used in numerous studies and in clinical practice, investigating burn depth, wound healing and a range of cutaneous inflammatory reactions, including psoriasis (Belcaro *et al.*, 1994; Clough *et al.*, 2001; Choi and Bennett, 2003; Khan and Newton, 2003; Murray *et al.*, 2005; Sainsbury, 2008).

### **Laser Speckle Contrast Imaging**

Laser Speckle Contrast Imaging (LSCI) is the more recent development of LDI, enabling high-resolution video of blood flow (Worsley and Voegeli, 2013). Several studies in the literature have used this technique both in human and animal models, and in healthy and diseased conditions, showing excellent reproducibility (Stewart *et al.*, 2005; Choi *et al.*, 2006; Roustit *et al.*, 2010; Du *et al.*, 2011; Cordovil *et al.*, 2012). In a similar manner to the LDI, limitations of this technique include: 1) its susceptibility to movement artefacts, 2) the arbitrary units of measurements, and 3) the lack of a biological zero (Eriksson *et al.*, 2014). In a recent comparative study in the literature, blood flow was assessed in healthy volunteers using both the LSCI and LDI systems, before and after the induction of local heating at different temperatures using specialized probes. The effect of biological zero on data analysis was also investigated, and this was recorded by applying a pressure cuff above the tested arm. Results showed a higher biological zero with the LSCI, and when subtracted from baseline it led to an increased variation between subjects for both systems.

## Chapter 3

Results also showed a good correlation between LSCI and LDI, which did not change after subtraction of the biological zero, but it was weakened due to increased variation. Agreement between methods was also assessed by Bland-Altman plots, showing a systematic bias, with the LDI recording higher values (Millet *et al.*, 2011). In terms of inter-individual variation, this was found to be higher with the LDI, possibly due to the higher measurement depth of LDI (Binzoni *et al.*, 2013).

Other techniques for measuring skin blood flow include: radionuclide techniques, thermography, capillaroscopy, photoplethysmography and optical coherence tomography. Nonetheless, imaging techniques are preferred in research as they are non-invasive, non-contact, results are obtained simultaneously over a large tissue area and can be easily interpreted and communicated to clinicians (Allen and Howell, 2014).

In the current research, a laser Doppler imaging system (LDI, Moor Instruments Ltd, Axminster, UK) was used to determine changes in blood flow in the microcirculation as it represents a non-invasive technique and does not come in contact with the skin.

### **Guidelines for measurement of blood flow in the microcirculation**

Research studies indicate that measurements of blood perfusion are independent of age, sex and ethnicity (Sundberg, 1984; Huether and Jacobs, 1986; Berardesca and Maibach, 1988; Agner, 1991). However, there is variation between anatomical sites with, for example, distal sites at the forearm present higher blood perfusion values than proximal sites (De Boer *et al.*, 1988). Accordingly, specific guidelines have been published to improve accuracy in measurements with the LDI systems, to minimize variation. Therefore, it is recommended that prior to any measurements (Bircher *et al.*, 1994; Fullerton *et al.*, 2002):

- 1) the test subject should be left to acclimatize for 20-30 minutes,
- 2) the temperature in the test room should be controlled, between 20°C and 22°C
- 3) the skin area to be scanned should be marked with ink, so that a region of interest (ROI) could be easily identified post-measurement,
- 4) the ambient light level in the test room should be low
- 5) the distance between the scanner and the skin area is approximately 300 mm to avoid saturation of the photo image

In addition, during measurements individuals should be comfortable and not be subjected to any interference (Fullerton *et al.*, 2002).

Accordingly, before testing commenced, an acclimatization period of 30 minutes was allowed for the participants to adjust to the ambient conditions in the laboratory (temperature:  $22 \pm 3^{\circ}\text{C}$ , relative humidity:  $40 \pm 5\%$ , low light level). Subsequently, each test site of dimensions, 20mmx20mm, was marked with a black marker pen and the distance of the forearm from the scanner was adjusted to 300mm using a ruler. During measurements, the forearm of participants was supported by a cushion to minimise movement.

## 2) Release of biomarkers

A biomarker can be defined as 'a characteristic that is objectively measured and evaluated as an indication of normal biologic processes, pathogenic processes, or pharmacological responses to a therapeutic intervention' (Definitions Working, 2000). Accordingly, their presence is now commonly considered as a primary endpoint measurement in clinical research (Stribu and Tavel, 2010). Cytokines and other mediators involved in the inflammatory response can be considered biomarkers. They are involved in the maintenance of an intact barrier function and several studies have reported their release following its damage (Nickoloff and Naidu, 1994; De Jongh *et al.*, 2007a; De Jongh *et al.*, 2007b; Dickel *et al.*, 2010). As a consequence, their release is commonly investigated in cutaneous research, by utilizing well-established sampling methods, involving tape stripping, skin suction blisters, skin biopsies and dermal microdialysis.

### Tape stripping

By compromising the barrier function, tape stripping facilitates the release of inflammatory mediators. In this method, a tape is applied on the skin for a short time to collect SC contents, followed by extraction in solvent. A range of cytokine and other markers of interest can be subsequently determined using specific immunoassays (Perkins *et al.*, 2001). Various commercial tapes are available to sample the skin, examples of which include the Sebutape™ (Cuderm Corporation, Dallas, TX, USA), D-squame® (Cuderm Corporation, Dallas, TX, USA) and the Corneofix® (Courage and Khazaka, Koln, Germany). Tape stripping has several advantages, as it is inexpensive, well-tolerated by participants, it is a rapid and convenient method, and more importantly it is non-invasive (Dupuis *et al.*, 1984; Paliwal *et al.*, 2013). Several studies in the literature have utilized tape stripping to investigate the release of cytokines, in healthy and diseased conditions, establishing the effectiveness of this technique in investigating the inflammatory response (Perkins *et al.*, 2001; Perkins *et al.*, 2002; De Jongh *et al.*, 2006; De Jongh *et al.*, 2007b).

## Chapter 3

Recently, the Transdermal Analyses Patch (TAP, FibtoTX, Tallinn, Estonia) was developed, that allows the detection of mediators directly from the skin. It is based on microarrays printed on the tape containing specific capturing antibodies that can be analysed using spot-ELISA (Orro *et al.*, 2014). Although research is limited, a recent study reported that TAP proved to be a sensitive method to measure the levels of IL-1 $\alpha$  and IL-1RA in irritated skin (Falcone *et al.*, 2017).

### **Skin Suction Blisters**

A blister is defined as a small pocket of body fluid within the upper layers of the skin, which can be caused by exposure to friction, heat, cold or chemicals (Uchinuma *et al.*, 1988). In skin research, a blister can be formed by applying a low vacuum suction pressure (200-300 mm Hg) thereby separating the epidermis from the dermis (Kiistala, 1968). Fluid is collected by aspiration and the top of the blister is then removed with sterile scissors (Semper *et al.*, 2003). The resulting skin suction blisters have been adopted in many research studies (Deleuran *et al.*, 1991; Salazar *et al.*, 2003; Saunders *et al.*, 2016; Koskela *et al.*, 2017). Despite the wide use of this technique, the main limitations of this method are: 1) its invasive nature, 2) the discomfort and pain caused to the volunteers, and 3) the long healing times of the skin (Benfeldt *et al.*, 1999). Also, the procedure per se might affect the release of inflammatory mediators (Anderson *et al.*, 1994).

### **Skin Biopsies**

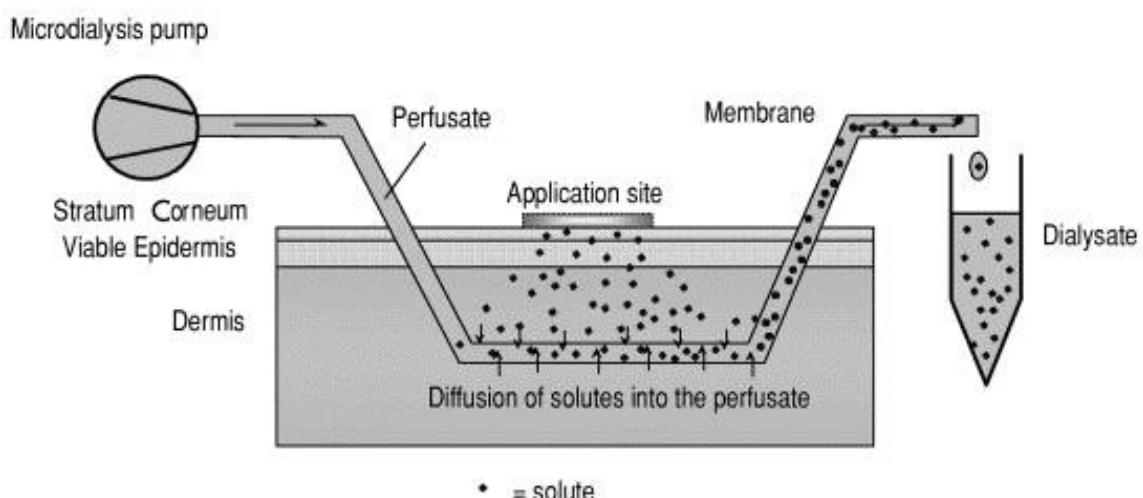
The most common method to sample the skin involves biopsy, in which a sample of tissue is removed, processed, and examined under a microscope. This approach can involve punch, shave or excision biopsies (Herkenne *et al.*, 2008), following a topical application of lidocaine, to anaesthetize the local skin. This method has been adapted in several studies to analyse the release of cytokines in healthy and damaged skin, as well as for the histopathological examination of IAD lesions (Nickoloff and Naidu, 1994; Houwing *et al.*, 2007; Dickel *et al.*, 2010). Clearly, the technique is invasive in nature and provides volunteers with the potential for scarring. Another limitation involves potential degradation of the tissue during its handling and processing (Nischal *et al.*, 2008).

### **Microdialysis**

The collection of biomolecules from the interstitial tissues using microdialysis has been widely used for the analysis of many tissues and organs (Bito *et al.*, 1966; Petersen *et al.*, 1994; Clough, 1999). Indeed, over 14000 papers have been published since its first application (Shukla *et al.*, 2014). Microdialysis represents a minimally invasive method to measure the concentration of endogenous and exogenous solutes in the extracellular space of the dermis based on passive diffusion (Clough and Noble, 2003).

It is a promising diagnostic tool, and has been used to investigate the inflammatory mechanisms in various skin conditions (Clough *et al.*, 2007; Salgo *et al.*, 2011; Sjögren *et al.*, 2012; Quist *et al.*, 2016). In this technique, following application of a topical anaesthetic, a microdialysis fibre is inserted superficially to the skin, serving as an artificial vessel, and perfused with sterile saline and the solutes pass through the fibre into the perfusate, and collected in a vial located at the skin surface (Schnetz and Fartasch, 2001; Clough and Noble, 2003). This is illustrated schematically in Figure 3.13. Despite the effectiveness of the anaesthetic to reduce pain, an increase in blood flow has been reported following fibre implantation, and therefore an equilibrium period of at least 120 minutes should be allowed prior to sampling (Groth and Serup, 1998). A critique of the microdialysis technique is provided in Table 3.8.

## Linear Microdialysis



**Figure 3.13. Microdialysis technique for the *in vivo* recovery of substances.** A probe is inserted superficially and perfused with sterile saline. Based on diffusion, substances released in the interstitial space are collected in the dialysate. Figure adapted from Schnetz and Fartasch, (2001)

**Table 3.8. A critical analysis of microdialysis.**

Advantages	Limitations
Minimally-Invasive	Long recovery times
Kinetic profile of analytes of interest	Sensitive analytical methods are required due to small volume collected and low concentration of analyte
No pain and scarring	High inter- and intra- individual variation
Clinically adapted technique	Surgical skills are required for fibre implantation
Sampling in several sites simultaneously	Relative recovery of analytes, calibration required
Well-tolerated by volunteers	Recovery is inversely correlated to perfusate flow rate
Combined with biophysical measurements of TEWL, LDI etc.	High cost of commercial fibres
Delivery of substances to target tissues	
Used in penetration studies	

Microdialysis has also been adapted in clinical practice, for example to monitor glucose levels in patients in intensive care and lactate levels during cardiac surgery (Rooyackers *et al.*, 2010; Lenkin *et al.*, 2017). Indeed it offers many benefits compared to other sampling methods in examining inflammatory responses in the skin, as summarized in Table 3.9.

**Table 3.9. Characteristics of methods for skin sampling.**

Characteristics	Tape stripping	Skin suction blisters	Skin biopsies	Microdialysis
<b>1. Invasive</b>		✓	✓	✓
<b>2. Tolerated by volunteers</b>	✓			✓
<b>3. Sample</b>	SC only	Interstitial fluid and epidermal layers	Down to subcutaneous tissue	Interstitial fluid
<b>4. Discomfort, pain, scarring</b>		✓	✓	
<b>5. Detailed time profile</b>				✓
<b>6. Sampling in multiple sites</b>				✓

In the current investigation, tape stripping was chosen for the recovery of biomarkers as it represents a less invasive method of sampling the skin, compared to skin suction blisters and skin biopsies, and it has the advantage of allowing the collection of multiple samples from the same subjects over time. Tape stripping was performed using the Sebutape™. Considering also the advantage of microdialysis to sample at multiple sites simultaneously and the expertise in microdialysis within the research group, this technique was employed to sample the interstitial fluid under the skin. Given the sampling depth of each technique, different cytokine profiles were assessed and results allowed a direct comparison between the two techniques which sampled at the skin surface and within the skin tissues.

### **3) Analytical methods**

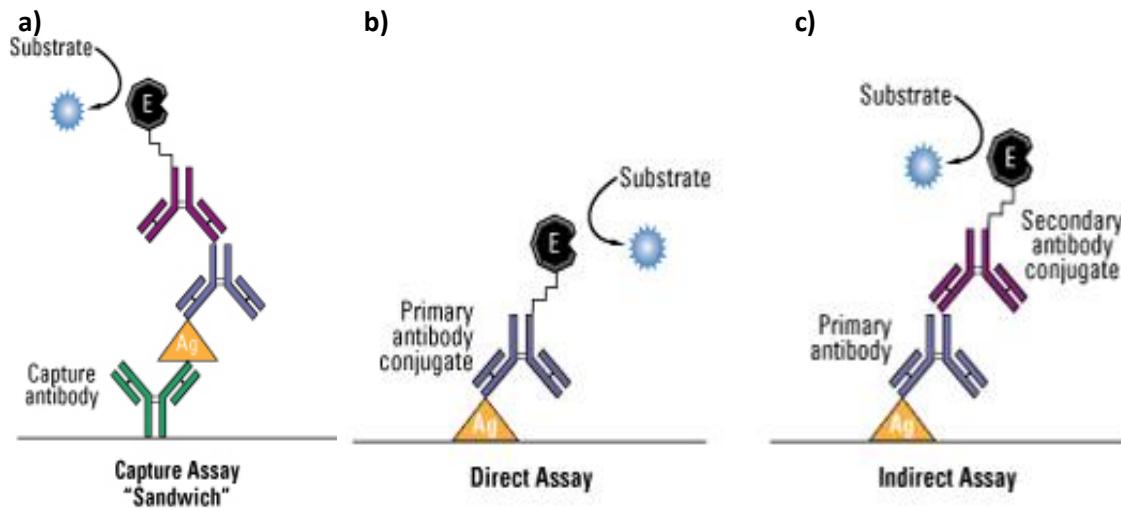
Following *in vivo* sampling of the skin, robust analytical methods are required to determine the release of biomarkers, examples of which include high performance liquid chromatography (HPLC) and Enzyme-Linked Immunosorbent assays (ELISA) (Schnetz and Fartasch, 2001; Shippenberg and Thompson, 2001; Schmidt *et al.*, 2008).

#### **High Performance Liquid Chromatography (HPLC)**

HPLC is a biochemical method that can be used to separate, identify and quantify the analytes in a solution. It involves pressurizing liquid solvent, containing the test solution, passed through a column phase, thus interacting with absorbent material, causing their separation based on different flow rates (Gerber *et al.*, 2004; Malviya *et al.*, 2010). Due to its high sensitivity, HPLC is used in pharmaceutical industry and in clinical practice, for example in the analysis of blood samples (Shah *et al.*, 1990; Bounine *et al.*, 1994; Sundstrom *et al.*, 2013). However, due to high costs and its complex nature the use of HPLC in research is limited (Keustermans *et al.*, 2013).

#### **Enzyme-Linked Immunosorbent assays (ELISA)**

ELISA is a plate-based technique that was developed several years ago, and is now considered the gold-standard method in biomarker research (Crowther, 2001). This technique involves the attachment of a primary antibody, with both antigen capture and immune specificity, to the bottom of the plate, prior to the addition of sample. At the final step, a secondary detection antibody is added to generate a colorimetric signal which is amplified by a substrate, as shown in Figure 3.14 (Leng *et al.*, 2008). The traditional assay is known as sandwich ELISA, as the sample is between the two antibodies, as shown in Figure 3.14a. Alternatives to this method include direct and indirect ELISA, in which less antibodies are used, as illustrated in Figure 3.14b and 3.14c respectively (ThermoFisher Scientific, 2017).



**Figure 3.14. Traditional ELISA for biomarker research.** **a)** The traditional sandwich ELISA, in which a capture antibody is coated at the bottom of the plate. Then the sample is added, followed by the addition of a detection antibody which is not labelled, and thus a secondary enzyme-conjugated detection antibody is required. **b)** in direct ELISA the detection antibody is enzyme conjugated, and there is no need for a secondary antibody, and **c)** in indirect ELISA the sample is first added to the plate, followed by the addition of an unlabelled primary antibody, specific to the sample. At the final step, an enzyme conjugated secondary antibody, specific to the primary antibody, is added. Figure taken from (ThermoFisher Scientific, 2017)

Conventional ELISA is well suited for clinical research, as there are numerous commercially available kits for all analytes of interest and it can provide high quantitative and reproducible results. Nevertheless, the performance and consequently the results of these assays are dependent on antibody quality, the researcher's skills and the kit manufacturer. In addition, only one cytokine/analyte can be examined each time. As a consequence, recent advances in the field led to the development of multiplex arrays which are based on flow cytometry, chemiluminescence and electrochemiluminescence technology. These allow the investigation of several cytokines in the same sample at the same time. Other advantages also include: 1) the time- and cost- effectiveness, 2) small sample volume required and 3) the broad dynamic range and 4) its high sensitivity (Leng *et al.*, 2008; Keustermans *et al.*, 2013). An example of a multiplex system is manufactured by Mesoscale Discovery and utilizes an electrochemiluminescence technology, in which each bead on an electric-wired plate corresponds to a specific capture antibody, and signal is generated by electrical stimulation and with the use of a labelled-detection antibody (MesoScale Discovery, 2017). Table 3.10 provides a critical analysis of the various analytical techniques.

**Table 3.10. A critique of HPLC, ELISAs and multiplex assays.**

Method	Advantages	Limitations
<b>HPLC</b>	<ul style="list-style-type: none"> <li>• Quick method</li> <li>• High sensitivity</li> </ul>	<ul style="list-style-type: none"> <li>• High cost</li> <li>• Complexity</li> <li>• Coelution of compounds with similar structure</li> </ul>
<b>ELISA</b>	<ul style="list-style-type: none"> <li>• Numerous kits are available for a wide range of analytes</li> <li>• Reproducibility</li> </ul>	<ul style="list-style-type: none"> <li>• One analyte each time</li> <li>• Large sample volume</li> <li>• Narrow dynamic range</li> <li>• Complex protocols</li> <li>• Slow read times</li> <li>• Depends on antibody quality</li> </ul>
<b>Multiplex assays</b>	<ul style="list-style-type: none"> <li>• Quick method</li> <li>• Detection of multiple cytokines simultaneously</li> <li>• Low sample volume</li> <li>• High specificity and sensitivity</li> <li>• Time- and cost- effective</li> <li>• Reproducibility</li> </ul>	<ul style="list-style-type: none"> <li>• Proteins in samples may influence the results</li> <li>• High cost of equipment</li> <li>• Less robust when using serum &amp; plasma samples</li> </ul>

Given the high cost of HPLC, the inability of traditional ELISA systems to detect more than one analyte each time and the analytical sensitivity of multiplex immunoassays, multiplex electrochemiluminescence assays were purchased to quantify the biomarkers recovered from microdialysis and the Sebutape™.

### 3.3 Ethical considerations

Ethical approval was obtained from the Faculty of Health Sciences research ethics committee (Project ID: 26040) to recruit up to 20 healthy volunteers to participate in the current study. Following initial ethical application, reviewers from the ethics committee suggested a number of revisions (Appendix A, A-1) and these were satisfied to improve clarity, as detailed in the response to reviewers comments section (Appendix A, A-2). The approved ethics application is shown in Appendix B-1. After securing ethical approval, volunteers were recruited from the staff and student populations of the University of Southampton via word of mouth and a study poster (Appendix B, B-2). Recruitment was based on inclusion and exclusion criteria (Table 3.11) which were developed to ensure the safety of the participants, particularly involving skin integrity, as reported in a previous study (Voegeli, 2008). No incentives for participation were offered.

## Chapter 3

Following recruitment, each participant was given a participant information sheet (Appendix B, B-3) a minimum of 48 hours and maximum of one week prior to the study, explaining the study aims and procedures, and making clear the voluntary nature of study participation. Participants were also encouraged to ask further questions or to seek clarification about any aspect of the study before their decision. All participants provided written, informed consent prior to the start of the study and a copy of the consent form was given to each.

**Table 3.11. Inclusion and exclusion criteria for volunteer recruitment.**

Inclusion criteria	Exclusion criteria
<ul style="list-style-type: none"><li>• Aged 18-65 years</li><li>• No active skin disease</li><li>• No previous history of skin diseases</li></ul>	<ul style="list-style-type: none"><li>• Pregnancy</li><li>• Pre-existing medical condition that is known to affect the dermal vasculature (e.g. diabetes mellitus, peripheral vascular disease, Raynaud's phenomenon; pregnancy)</li><li>• Treatment with any vasoactive medication (e.g. beta-blockers, nitrates, calcium channel blockers, angiotensin-converting-enzyme (ACE) inhibitors, antihistamines; nonsteroidal anti-inflammatory drugs (NSAIDS); steroids)</li><li>• Pre-existing dermatological condition</li><li>• Inability to give informed written consent</li></ul>

### 3.4 Chapter summary

Chapter 3 has discussed the various methods that are widely used in dermatological research and justified those chosen to assess the functional characteristics of the skin following exposure to experimental models of incontinence and to investigate the release of inflammatory mediators. These are summarized in Table 3.12.

**Table 3.12. The methods employed in *in vivo* studies with human volunteers**

Methods	Instrument	Manufacturer
<b>Skin Barrier assessment</b>		
Skin barrier function	Tewameter® TM300	Courage and Khazaka
SC hydration	Corneometer® CM1825	Courage and Khazaka
Skin surface pH	Skin-pH-Meter® 905	Courage and Khazaka
<b>Compromise skin integrity</b>		
Tape stripping	Adhesive tapes	Sellotape™
SLS		Fischer Scientific
<b>Experimental models of incontinence</b>		
S-urine		
Proteolytic-solution		
<b>Analysis of blood flow in the microcirculation</b>		
Skin blood perfusion	Laser Doppler Imaging	Moor Instruments Ltd
<b>Release of Biomarkers-sampling the skin</b>		
Tape stripping	Sebutape™	Cuderm Corporation
Microdialysis	CMA 400 syringe pump	CMA, Sweden
<b>Quantification of biomarkers</b>		
Multiplex immunoassays	96-well plates	Mesoscale Discovery



# Chapter 4: The effect of synthetic-urine and its inherent pH on the functional characteristics of intact skin

## 4.1 Introduction

In the UK, it is estimated that approximately 3 to 6 million people suffer from urinary incontinence, and consequently are at high risk of IAD (Irwin *et al.*, 2006). It has been reported that in urinary incontinence prolonged exposure of the skin to urine leads to swelling of corneocytes, an overhydrated epidermis, and eventually skin maceration (Ichikawa-Shigeta *et al.*, 2014). As discussed by Beeckman and colleagues (2009) in their theoretical framework of IAD pathophysiology, this can lead to a compromised skin barrier function, reflected by increased TEWL, and in the presence of bacteria/faecal material urea in urine is converted into ammonia, shifting thus the skin pH away from the protective acid mantle (Beeckman *et al.*, 2009). In this respect, a recent study in the literature, by Larner and colleagues (2015), showed that urine at an alkaline pH is associated with a severe disruption of skin barrier (Larner *et al.*, 2015). However, the study is characterized by limited physiological and clinical relevance as: 1) the urinary pH values used did not represent the physiological pH levels which range from 4.8 to 8.0, and 2) the exposure time is longer (6 hours) than the actual frequency at which incontinent patients are checked for wetness in clinical practice, which is typically every 2 hours (Voegeli, 2008). In view of these limitations, the exact mechanism of urinary pH in IAD development remains unknown. Therefore, the current study examined the hypothesis that an increase in urinary pH disrupts stratum corneum function leading to an increase in TEWL and skin surface pH, thereby compromising the integrity of the skin.

As it is well known, the skin surface pH is acidic in nature hence contributing to the physiological function of the skin. Accordingly, the skin has an inherent buffering capacity that tightly controls pH, and any changes are only short-lived (Agache, 2004; Levin and Maibach, 2008). The underlying mechanisms responsible for this buffering capacity are not well understood, however, a number of possible mechanisms have been suggested, involving: 1) sebum, 2) the water-soluble components of the skin, 3) sweat, 4) keratin and 5) SC thickness (Dünner, 1950; Vermeer *et al.*, 1951; Rothman, 1954a; Spier and Pascher, 1955; Wilhelm *et al.*, 1991b). Early studies suggested that sebum protects the epidermis against the influence of alkali and acids by slowing their penetration into the skin. It has also been proposed that fatty acids in sebum play a crucial role as they serve as a buffer.

However, later experiments neglected this hypothesis as delipidized skin presented a faster neutralization compared to untreated skin (Dünner, 1950; Mackenna *et al.*, 1950; Vermeer, 1950).

Another possible mechanism that has been proposed to contribute to the skin's buffering capacity involves the water-soluble components of the epidermis. This mechanism is based upon the fact that when these constituents were extracted, by soaking in water, the buffering capacity is reduced (Schmidt, 1941; Vermeer *et al.*, 1951). Furthermore, another study suggested that sweat components, such as lactic acid and amino acids also contribute to this buffering capacity of the skin, since in a comparative study involving sweating and non-sweating individuals it was demonstrated that amino acids are crucial players in the buffering capacity (Vermeer *et al.*, 1951). Keratin has also been involved in the skin's buffering capacity, and this can be explained by its inherent ability to neutralize acids and alkalis *in vitro* (Burckhardt, 1935; Burckhardt and Baumle, 1951). However, its exact role remains to be determined. Differences between with respect to the buffering capacity have also been reported and attributed to the thickness of the SC (Rothman, 1954a). This hypothesis also offers a possible explanation to the fact that decreased SC thickness in the elderly is associated with diminished buffering capacity (Wilhelm *et al.*, 1991b). In spite the fact that these mechanisms still need to be determined, the buffering capacity of the skin is critical for a healthy skin, as indeed individuals with a diminished buffering capacity are prone to irritation from external insults (Green and Behrendt, 1971). With respect to IAD, to clarify the mechanism by which urine leads to IAD development, its effects on the buffering capacity of the skin were investigated. To the best of the author's knowledge, this has never been reported.

It is important to note that when examining the irritant potential of a solution, the effects of water and occlusion need to be evaluated and distinguished from the irritant source (Tsai and Maibach, 1999). In particular, water is regarded as an irritant, as it gradually denatures keratin and removes SC lipids, altering thus the water-holding capacity of the skin (Fulmer and Kramer, 1986). Additionally, water leads to an overhydrated epidermis, swelling of corneocytes and eventually increased skin permeability (Van Der Valk *et al.*, 1984; Warner *et al.*, 2003). It has also been reported that prolonged exposure and/or immersion to water leads to an increased TEWL and pH, and hence increasing the risk of skin dermatoses, eczema and dry skin (Willis, 1973; Lammintausta and Kalimo, 1981; Agner and Serup, 1993; Tsai and Maibach, 1999; Firooz *et al.*, 2015). A possible mechanism for the effects of water can be attributed to its neutral pH which compared to normal skin pH (4.2 to 6.0) is alkali and along with over hydration may cause the growth of pathogens (Aly *et al.*, 1978; Rajka *et al.*, 1980; Schieferstein and Krich-Hlobil, 1982). In a similar manner, it has been shown that occlusion can also be irritating as several studies have shown that it leads to increased TEWL and SC hydration (Ryatt *et al.*, 1988; Bucks *et al.*, 1991; Bucks and Maibach, 1999).

Additionally, prolonged occlusion of the skin can disrupt the acid mantle. Evidence for this was provided in an early study which reported that a 3-day occlusion led to a rise in skin pH from 4.9 to 7.1, and that this was restored back to basal levels 24-hours after removal of occlusion (Hartmann, 1983a). This increase in skin pH was also supported in another study (Aly *et al.*, 1978). Nevertheless, the effects of occlusion are still debatable, as a recent study examined the effects of occlusion on healthy and damaged skin, treated with SLS and tape stripping, and reported that occlusion does not have any significant effect on healthy skin (Jungersted *et al.*, 2010). Considering these findings, and to fully comprehend the effects of urine, in the current investigation the effects of water exposure and occlusion were determined.

## 4.2 Aim of the study

- To investigate the effects of exposure to s-urine at varying pH on the functional characteristics of healthy skin

## 4.3 Objectives

- Determine an optimum s-urine solution, at which a skin response is triggered, to be used in subsequent studies
- Investigate the skin's buffering capacity
- Define the effects of occlusion and water exposure on the skin barrier function

## 4.4 Participants

The study was approved by the local university ethics committee (FHS no: 9349), and was completed in three stages. The number of participants required for this study was informed by a previous power calculation within the host research group, involving study methods to assess skin barrier function (Voegeli, 2008). In particular, given a statistical significance at  $p<0.05$  level and a required power of 80%, a minimum of 15 participants was required to detect a change of 25% in measurements. For the initial stage, sixteen healthy volunteers (mean age  $\pm$  SD:  $35.06 \pm 12.10$ , 7 males, 9 females) were recruited, who met the inclusion and exclusion criteria (Table 3.11), to investigate the effects of s-urine solutions of varying pH on the functional characteristics of the skin. In the two subsequent stages, five healthy volunteers (mean age  $\pm$  SD:  $44 \pm 12.30$ , 2 males, 3 females) participated in each study, each of whom had participated in the first study. In the second stage the buffering capacity of the skin was investigated, whilst the effects of occlusion and water exposure on the skin barrier function were determined in the final stage. Informed consent was obtained from all volunteers upon arrival to the laboratory and prior to testing.

Participants were required not to apply any cosmetic products at least 12 hours prior to the studies, as these have been reported to influence biophysical measurements (Held *et al.*, 1999; Plessis *et al.*, 2013). The studies were conducted in the bioengineering laboratory, within the Faculty of Health Sciences at University Hospital Southampton, at an ambient temperature ( $22 \pm 3^\circ\text{C}$ ) and humidity ( $40 \pm 5\%$ ). All participants were left to acclimatize for 30 minutes prior commencement of the studies.

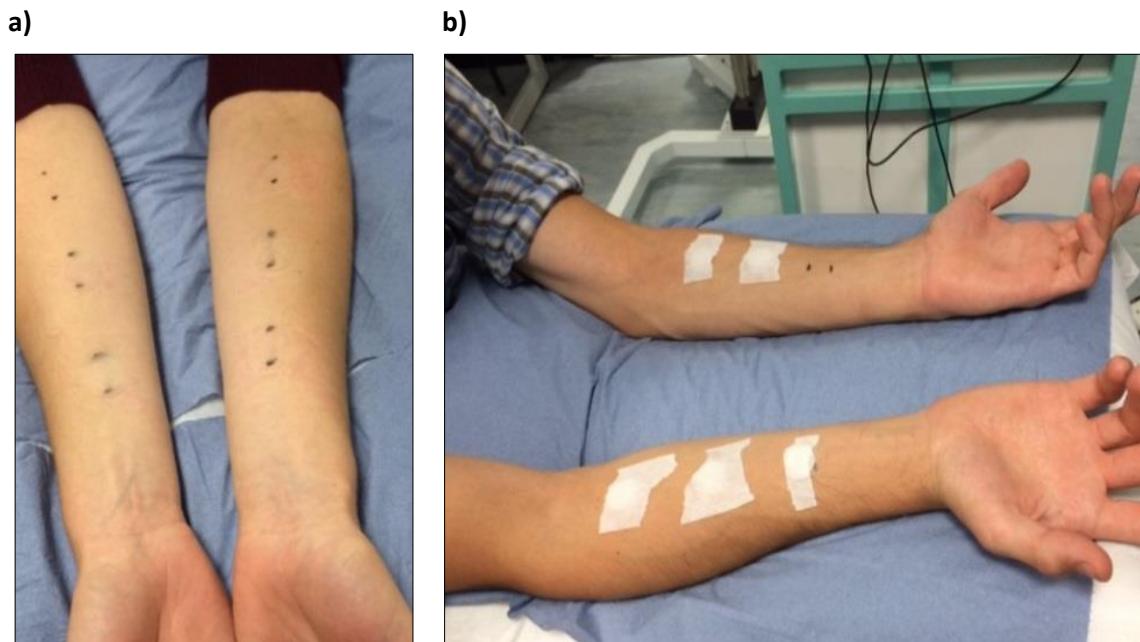
## 4.5 Methods

The methods used in this study are identical to those detailed in Chapter 3. Briefly, s-urine was used as a moisture irritant source to compromise skin integrity, as experienced by patients with urinary-incontinence. The formulation (pH=7.9) used by Mayrovitz and Sims (2001) was adapted and used in this study, and its composition is summarized in Table 3.6. 1M Hydrochloric acid (HCl) and 1M ammonium hydroxide ( $\text{NH}_4\text{OH}$ ) were used to adjust its pH, within the range between pH 5.0- 9.0. corresponding to the values of physiological urine, between 4.8 to 8.0 (Rose *et al.*, 2015). Treatments were applied to the skin using HillTop chambers (25mm, HillTop Labs, Miamiville, USA), soaked with 500 $\mu\text{l}$  solution, and secured in place with medical tape (3M, Transpore tape, UK) for two hours. To examine the effects of water exposure, distilled water ( $\text{dH}_2\text{O}$ ) was used and applied on the skin as described above. An empty HillTop chamber was also included to examine the effects of occlusion per se. Changes in skin blood flow were determined using a laser Doppler imaging system (LDI, Moor Instruments Ltd, Axminster, UK) that detects the movement of red blood cells in the microcirculation, a method which has been successfully adopted in various areas of clinical research. TEWL measurement is considered as a reliable indicator for skin barrier assessment (Fluhr *et al.*, 2006b), and was obtained using an open-chamber system (Tewameter<sup>®</sup> TM 300, MP 9, Courage & Khazaka). The hydration state of the SC was also determined by measuring the electrical capacitance of the skin using the Corneometer<sup>®</sup> CM 825 (MP 9, Courage & Khazaka), and a surface pH probe (Skin-pH-Meter<sup>®</sup> 905, MP 9, Courage & Khazaka) was used to measure skin pH. All measurements were taken at baseline and following each challenge.

## 4.6 Experimental protocol

Volunteers attended the laboratory on three separate study visits. In the initial stage (visit 1), baseline measurements of LDI, TEWL, SC hydration and skin pH were taken on six areas (20mmx20mm) on the volar aspects of both forearms (three sites on each forearm). Each test area was separated by a minimum distance of 40 mm, determined using a ruler (Figure 4.1a).

To eliminate potential selection bias the test areas were randomized using an online software package (Randomization., 2007). Then, the different s-urine solutions were applied on five sites, with the remaining site serving as the untreated control, as illustrated in Figure 4.1b. After an exposure time of two hours, the s-urine chambers were removed, and any excess moisture was removed by pat drying the skin with filter papers to ensure that what is measured is TEWL and not wet skin. Then biophysical measurements were repeated at all six sites. In the subsequent stage (visit 2), and to investigate the buffering capacity of the skin, baseline skin pH measurements were obtained on four areas in both forearms, and two acidic (pH 5.0 and pH 6.0) and one alkaline (pH 8.0) s-urine solutions were applied for two hours on the skin, with the remaining site serving as the untreated control. Following that, s-urine chambers were removed and the skin was pat dried, as previously. Skin surface pH was recorded immediately and then every 5 minutes for a total period of 40 minutes. In the final stage (visit 3), baseline measurements of TEWL were obtained on three areas (20x20mm, 40mm apart) on the volar aspect of the non-dominant arm, namely the left forearm. Following randomization, distilled water was applied on one site and a second site was occluded using an empty HillTop chamber, for 2 hours. The third site represented an untreated control site, as in previous stages. After exposure time, treatments were removed, the skin was pat dried with filter papers to avoid measuring wet skin, and measurements of TEWL were repeated at all sites.



**Figure 4.1. Application of synthetic-urine.** a) Six skin sites were marked on both the volar forearms, and b) 0.5ml s-urine was impregnated into HillTop chambers and applied on the skin, and medical tape was used to keep them in place. A sixth site served as the untreated control.

## 4.7 Data Analysis

For each biophysical measurement, data are presented at baseline (BL) and after each challenge as boxplots showing the median (horizontal line in box), difference between 25th and 75th percentile (length of box) and data range (whiskers), unless stated otherwise. Tables showing the percentage change compared to baseline values are additionally shown. Analysis of the LDI measurements was conducted using the manufacturer's software (mLDI Main version 5.3, Moor Instruments Ltd, Axminster, UK), and the following steps were performed:

1. The marked skin site on each volunteer's forearm was visible in the photo image from each scan. A ROI (20x20mm, 3300 pixels) was selected, covering exposed area, and saved to be used in all volunteers. Then, the mean blood flux was obtained for each volunteer using the statistics function in the software.
2. The mean baseline flux was calculated for all volunteers, and served as a threshold level.
3. Baseline images were subtracted from post-challenge images, using the image-subtraction function.
4. Using a "cut below" function the threshold value (step 2) was set, so that only the flux above this threshold was shown.
5. Mean blood flux was then obtained again for each volunteer using the statistics option in the software, as described above.

Post-processing of LDI images for a particular volunteer (P15) was problematic as the data deviated markedly as opposed to the other volunteers, presenting a -100% change to baseline with all s-urine solutions, and therefore the data obtained from that participant was excluded from further analysis.

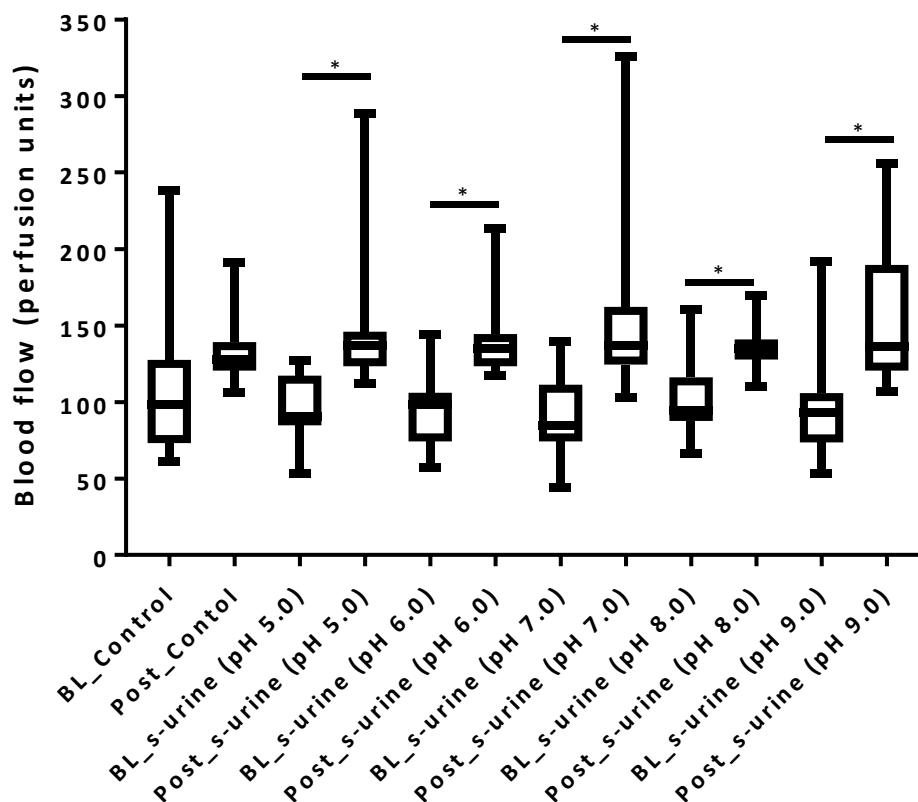
For the statistical analysis, IBM SPSS version 21.0 (SPSS Inc., Chicago, IL, USA) was used. Due to the small sample size (n=15) a non-parametric distribution of the data was assumed and data obtained at baseline and after each challenge were compared by Wilcoxon signed-rank tests. Then, differences between the various s-urine solutions were investigated using the Friedman test (non-parametric repeated measures ANOVA). If a significant difference was observed, the Wilcoxon signed-rank test was performed for pairwise comparisons. Statistical significance was set at  $p<0.05$ .

## 4.8 Results

### 4.8.1 Effect of s-urine solutions on the functional characteristics of healthy skin

#### Skin Blood flow

As illustrated in Figure 4.2, all the s-urine solutions caused a significant increase in skin blood flow compared to baseline values ( $p<0.05$ ). In particular, and by looking at the percentage change table (Table 4.1), it is evident that 12 volunteers demonstrated an increase in skin blood flow following exposure to the different s-urine solutions, whereas the remaining three volunteers presented a decrease with at least one s-urine solution (different in each case). In addition, nine of the volunteers (4 males, 5 females) presented an increase in blood flow of at least 15% from baseline values with all the s-urine solutions. However, results from the Friedman test showed no significant differences in skin blood flow between the different s-urine solutions, including also the control ( $p>0.05$  in all cases). It is interesting to note, that there is also an increase in blood flow at the control site, compared to baseline ( $p>0.05$ ), suggesting that any changes in blood flow caused by s-urine treatments are not solely restricted to the area of exposure, but they might affect adjacent sites.



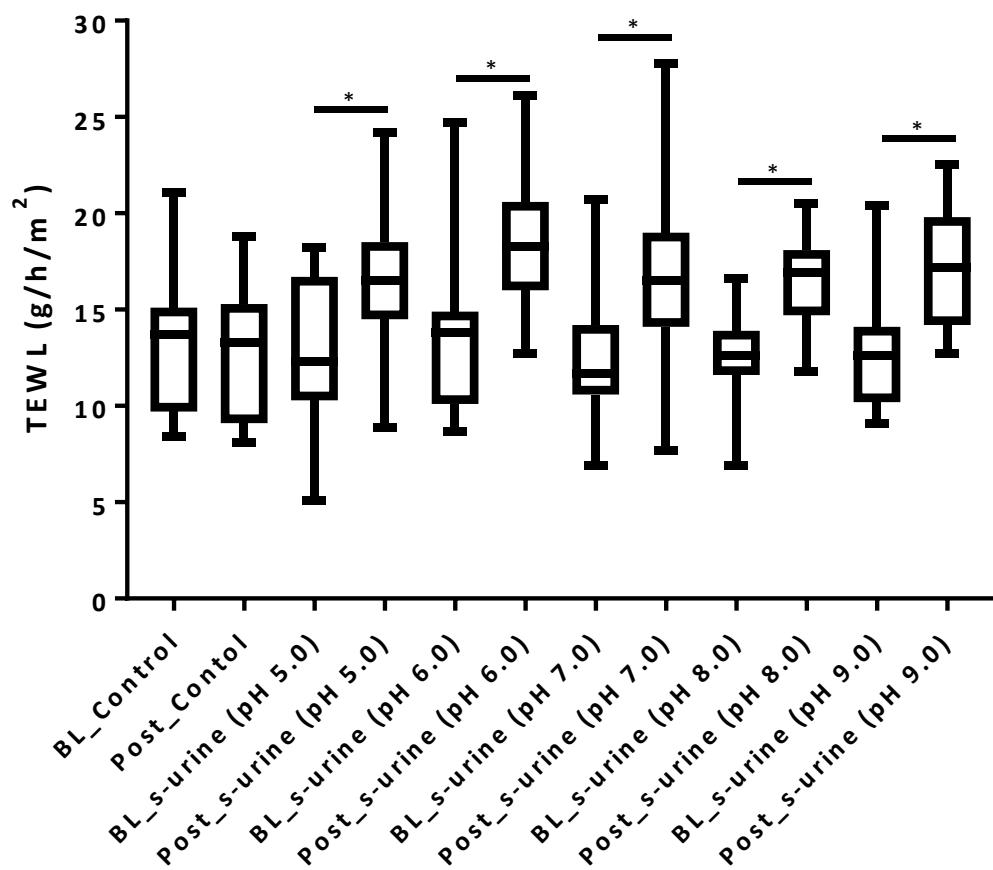
**Figure 4.2. Box and whiskers plot for skin blood flow at baseline and following exposure to s-urine solutions.** All s-urine solutions caused a significant increase to baseline ( $p<0.05$ ), however there were no significant differences between the s-urine solutions ( $p>0.05$ ). A non-significant increase ( $p>0.05$ ) in blood flow is also observed at the control site, and this is possibly due to changes in blood perfusion affecting adjacent sites. Significance is displayed with (\*).

**Table 4.1. Percentage change in blood flow following exposure to s-urine, compared to baseline values.**

<b>% change in blood flow Vs Baseline</b>						
<b>Participants</b>	<b>Synthetic-urine (<math>\pm 0.5</math>)</b>					
	<b>control</b>	<b>pH 5.0</b>	<b>pH 6.0</b>	<b>pH 7.0</b>	<b>pH 8.0</b>	<b>pH 9.0</b>
<b>P1</b>	-41%	13%	-2%	6%	53%	-14%
<b>P2</b>	7%	1%	29%	14%	7%	13%
<b>P3</b>	60%	65%	73%	38%	15%	60%
<b>P4</b>	-7%	16%	12%	-3%	50%	29%
<b>P5</b>	124%	132%	163%	131%	69%	53%
<b>P6</b>	56%	51%	25%	116%	49%	36%
<b>P7</b>	30%	38%	87%	79%	87%	76%
<b>P8</b>	28%	77%	88%	94%	46%	79%
<b>P9</b>	36%	69%	37%	52%	43%	19%
<b>P10</b>	9%	240%	104%	180%	6%	161%
<b>P11</b>	68%	52%	37%	72%	3%	74%
<b>P12</b>	41%	40%	62%	40%	67%	139%
<b>P13</b>	70%	-8%	31%	83%	-12%	137%
<b>P14</b>	34%	32%	39%	65%	57%	130%
<b>P16</b>	96%	72%	93%	133%	60%	85%
<b>mean</b>	<b>41%</b>	<b>59%</b>	<b>58%</b>	<b>73%</b>	<b>40%</b>	<b>72%</b>
<b>SD</b>	<b>41%</b>	<b>61%</b>	<b>43%</b>	<b>52%</b>	<b>29%</b>	<b>52%</b>
<b>p value</b>	<b>0.56</b>	<b>0.004</b>	<b>0.003</b>	<b>0.003</b>	<b>0.01</b>	<b>0.001</b>

## TEWL

With reference to TEWL measurement, and consequently skin barrier function, following exposure to s-urine there was a significant increase in this parameter compared to baseline values ( $p<0.05$ , Figure 4.3). In particular, all volunteers presented an increase in TEWL with all s-urine solutions, except P1 with the s-urine solution of pH 9.0. Additionally, for eight participants (2 males, 6 females) exposure to all the s-urine solutions caused a 10% increase from baseline values (Table 4.2), however, results from the Friedman test showed no significant differences between the various s-urine solutions ( $p>0.05$ ).



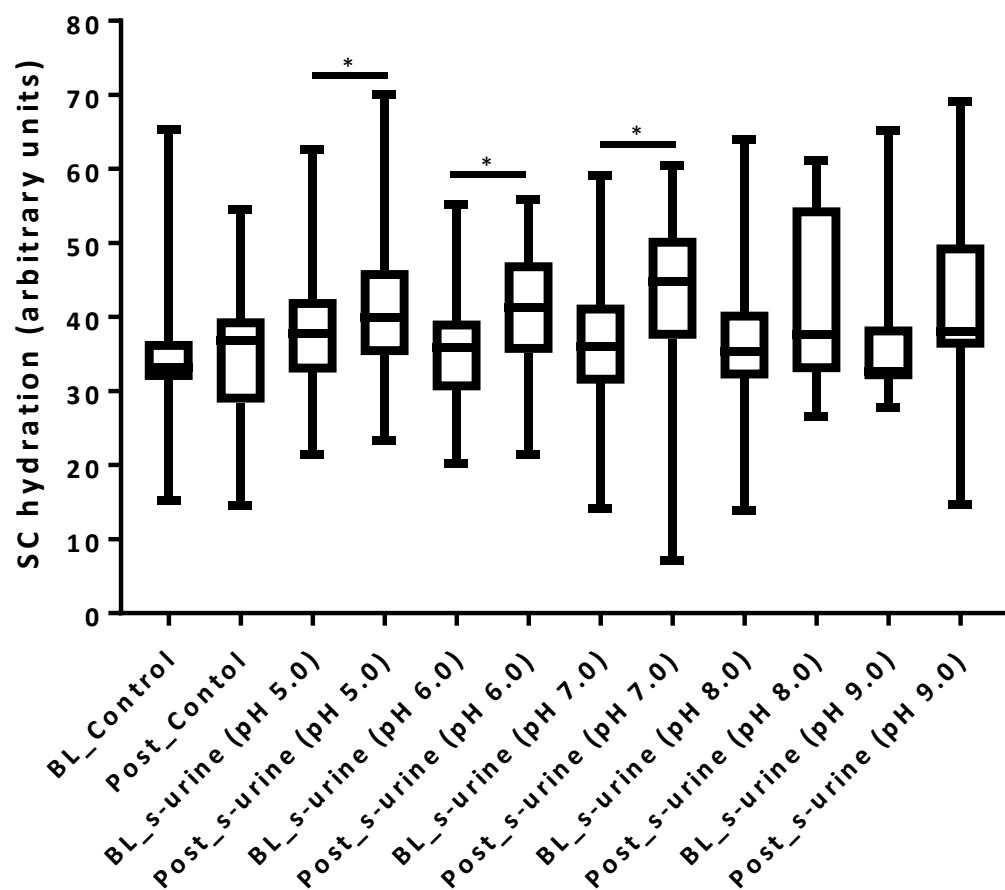
**Figure 4.3. Box and whiskers plot for TEWL measurement at baseline and following exposure to s-urine solutions.** All the s-urine solutions caused a significant rise in TEWL, and a consequent disruption of the skin barrier, compared to baseline values ( $p<0.05$ ). However, there were no significant differences between the s-urine solutions ( $p>0.05$ ). Significance is displayed with (\*).

**Table 4.2. Percentage change in TEWL following exposure to s-urine, compared to baseline values.**

<b>% change in TEWL Vs Baseline</b>						
<b>Participants</b>	<b>Synthetic-urine (<math>\pm 0.5</math>)</b>					
	<b>control</b>	<b>pH 5.0</b>	<b>pH 6.0</b>	<b>pH 7.0</b>	<b>pH 8.0</b>	<b>pH 9.0</b>
<b>P1</b>	12%	2%	65%	48%	25%	-5%
<b>P2</b>	32%	63%	20%	23%	15%	65%
<b>P3</b>	-3%	31%	91%	44%	14%	47%
<b>P4</b>	-12%	10%	6%	3%	48%	10%
<b>P5</b>	-24%	33%	6%	37%	31%	11%
<b>P6</b>	1%	149%	41%	12%	9%	18%
<b>P7</b>	-15%	60%	55%	33%	48%	40%
<b>P8</b>	20%	25%	58%	1%	19%	76%
<b>P9</b>	-20%	9%	38%	2%	26%	19%
<b>P10</b>	2%	11%	45%	27%	42%	40%
<b>P11</b>	-9%	37%	27%	51%	66%	52%
<b>P12</b>	-24%	15%	11%	79%	13%	73%
<b>P13</b>	-1%	22%	32%	34%	71%	39%
<b>P14</b>	-6%	4%	32%	51%	9%	26%
<b>P16</b>	2%	57%	54%	38%	40%	40%
<b>mean</b>	<b>-3%</b>	<b>35%</b>	<b>39%</b>	<b>32%</b>	<b>32%</b>	<b>37%</b>
<b>SD</b>	<b>16%</b>	<b>37%</b>	<b>24%</b>	<b>22%</b>	<b>20%</b>	<b>24%</b>
<b>p value</b>	<b>0.20</b>	<b>0.001</b>	<b>0.001</b>	<b>0.001</b>	<b>0.001</b>	<b>0.001</b>

## SC Hydration

As illustrated in Figure 4.4, exposure to s-urine resulted in an increase in the hydration state of the SC (Figure 4.4), a difference that was statistically significant compared to basal values ( $p<0.05$ ), with the exception of the s-urine solutions with a pH of 8.0 and 9.0 (Table 4.3). However, in one female participant (P10), treatment with the different s-urine solutions, except the one with a pH of 7.0, produced a decreased SC hydration. The corresponding percentage changes compared to baseline values are summarized in Table 4.3, showing that only three volunteers (1 male, 2 females) presented a consistent increase of more than 15% with all treatments compared to baseline. Again, there were no significant differences between the s-urine solutions ( $p>0.05$ ).



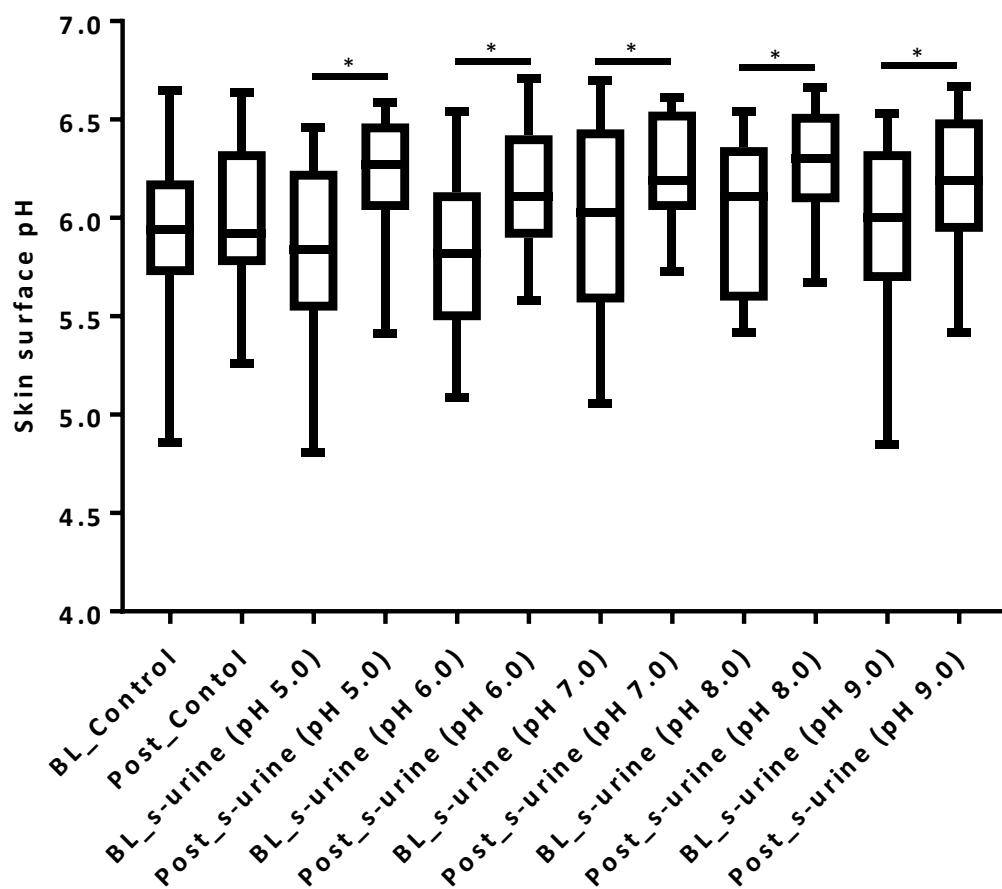
**Figure 4.4. Box and whiskers plot for SC hydration at baseline and following exposure to s-urine solutions.** Generally, there is an increase in the hydration status of the SC compared to baseline following exposure to varying pH s-urine solutions. There were no significant differences between the s-urine solutions ( $p>0.05$ ). Significance is displayed with (\*).

**Table 4.3. Percentage change in SC hydration following exposure to s-urine, compared to baseline values.**

<b>% change in SC hydration Vs Baseline</b>						
<b>Participants</b>	<b>Synthetic-urine (<math>\pm 0.5</math>)</b>					
	<b>control</b>	<b>pH 5.0</b>	<b>pH 6.0</b>	<b>pH 7.0</b>	<b>pH 8.0</b>	<b>pH 9.0</b>
<b>P1</b>	0%	21%	2%	28%	28%	16%
<b>P2</b>	-17%	12%	1%	2%	-5%	6%
<b>P3</b>	-11%	6%	5%	29%	3%	21%
<b>P4</b>	6%	24%	20%	27%	39%	17%
<b>P5</b>	5%	6%	19%	17%	7%	10%
<b>P6</b>	25%	46%	66%	64%	61%	52%
<b>P7</b>	24%	16%	32%	42%	43%	39%
<b>P8</b>	-13%	2%	-8%	16%	1%	-47%
<b>P9</b>	-4%	9%	6%	-50%	105%	-29%
<b>P10</b>	-5%	-4%	-5%	4%	-5%	-9%
<b>P11</b>	2%	-4%	3%	13%	-5%	9%
<b>P12</b>	12%	11%	29%	14%	27%	5%
<b>P13</b>	-11%	1%	10%	16%	-7%	21%
<b>P14</b>	5%	19%	6%	45%	23%	18%
<b>P16</b>	6%	2%	17%	12%	-6%	13%
<b>mean</b>	<b>2%</b>	<b>11%</b>	<b>14%</b>	<b>19%</b>	<b>21%</b>	<b>9%</b>
<b>SD</b>	<b>12%</b>	<b>13%</b>	<b>19%</b>	<b>25%</b>	<b>32%</b>	<b>24%</b>
<b>p value</b>	<b>0.90</b>	<b>0.002</b>	<b>0.01</b>	<b>0.001</b>	<b>0.10</b>	<b>0.07</b>

### Skin pH

Results regarding the skin surface pH vary due to inter-individual differences, although there was a tendency for pH to increase following exposure to s-urine. As shown in Figure 4.5, compared to baseline, all the s-urine solutions caused a significant increase in pH ( $p<0.05$ ). Indeed, in some volunteers ( $n=7$ ) the pH value increased following exposure to the different s-urine treatments, but in others there was a minimal or no change in pH levels. In particular, and as it can be seen from the percentage change table (Table 4.4), only 3 male participants presented an increase of 5% with all the s-urine solutions from baseline values. Again, statistical analysis showed no significant differences between the s-urine solutions ( $p>0.05$ ).



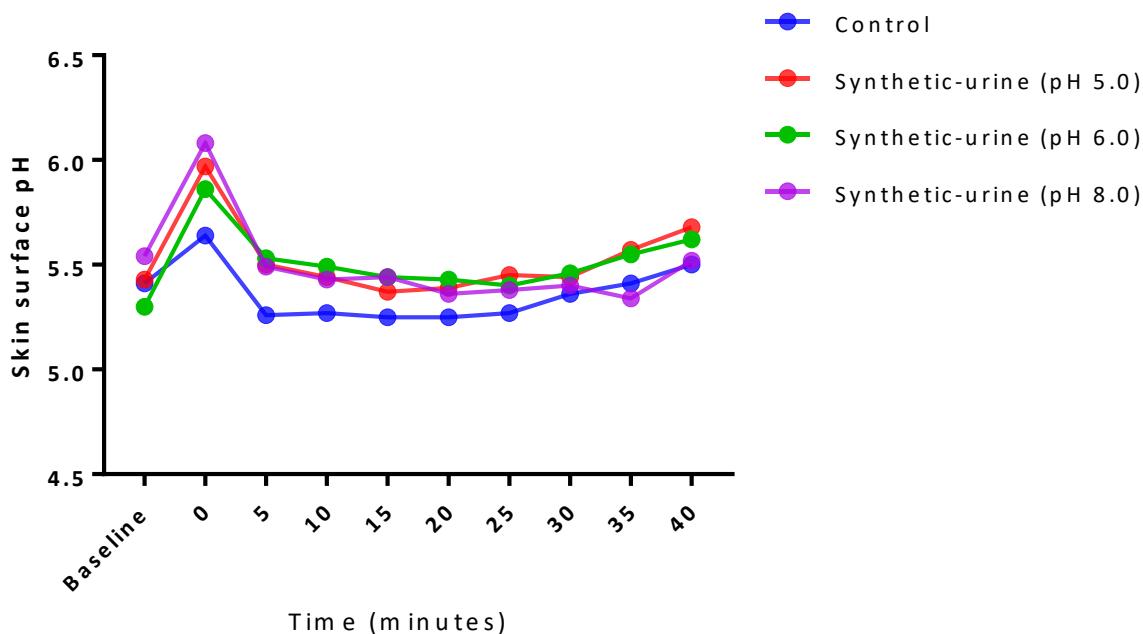
**Figure 4.5. Box and whiskers plot for skin surface pH at baseline and following exposure to s-urine solutions.** In some of the volunteers there was an increase in pH compared to baseline values following exposure to s-urine. No significant effect was found.

**Table 4.4. Percentage change in skin pH following exposure to s-urine, compared to baseline values.**

% change in skin pH Vs Baseline						
Participants	Synthetic-urine ( $\pm 0.5$ )					
	control	pH 5.0	pH 6.0	pH 7.0	pH 8.0	pH 9.0
<b>P1</b>	8%	-2%	2%	2%	1%	12%
<b>P2</b>	2%	9%	10%	10%	9%	14%
<b>P3</b>	1%	0%	-3%	3%	2%	-1%
<b>P4</b>	0%	1%	9%	8%	-1%	8%
<b>P5</b>	10%	30%	13%	22%	23%	12%
<b>P6</b>	2%	3%	6%	0%	3%	3%
<b>P7</b>	-5%	10%	8%	9%	8%	4%
<b>P8</b>	3%	10%	12%	12%	13%	9%
<b>P9</b>	-2%	12%	6%	1%	11%	1%
<b>P10</b>	2%	3%	3%	0%	-1%	4%
<b>P11</b>	7%	7%	7%	-2%	5%	6%
<b>P12</b>	-1%	3%	0%	-1%	4%	-4%
<b>P13</b>	6%	5%	5%	3%	2%	2%
<b>P14</b>	0%	1%	1%	0%	0%	0%
<b>P16</b>	3%	4%	4%	2%	5%	4%
<b>mean</b>	<b>2%</b>	<b>6%</b>	<b>5%</b>	<b>5%</b>	<b>6%</b>	<b>5%</b>
<b>SD</b>	<b>4%</b>	<b>8%</b>	<b>4%</b>	<b>6%</b>	<b>6%</b>	<b>5%</b>
<b>p value</b>	<b>0.90</b>	<b>0.004</b>	<b>0.004</b>	<b>0.01</b>	<b>0.005</b>	<b>0.01</b>

#### 4.8.2 The buffering capacity of the skin

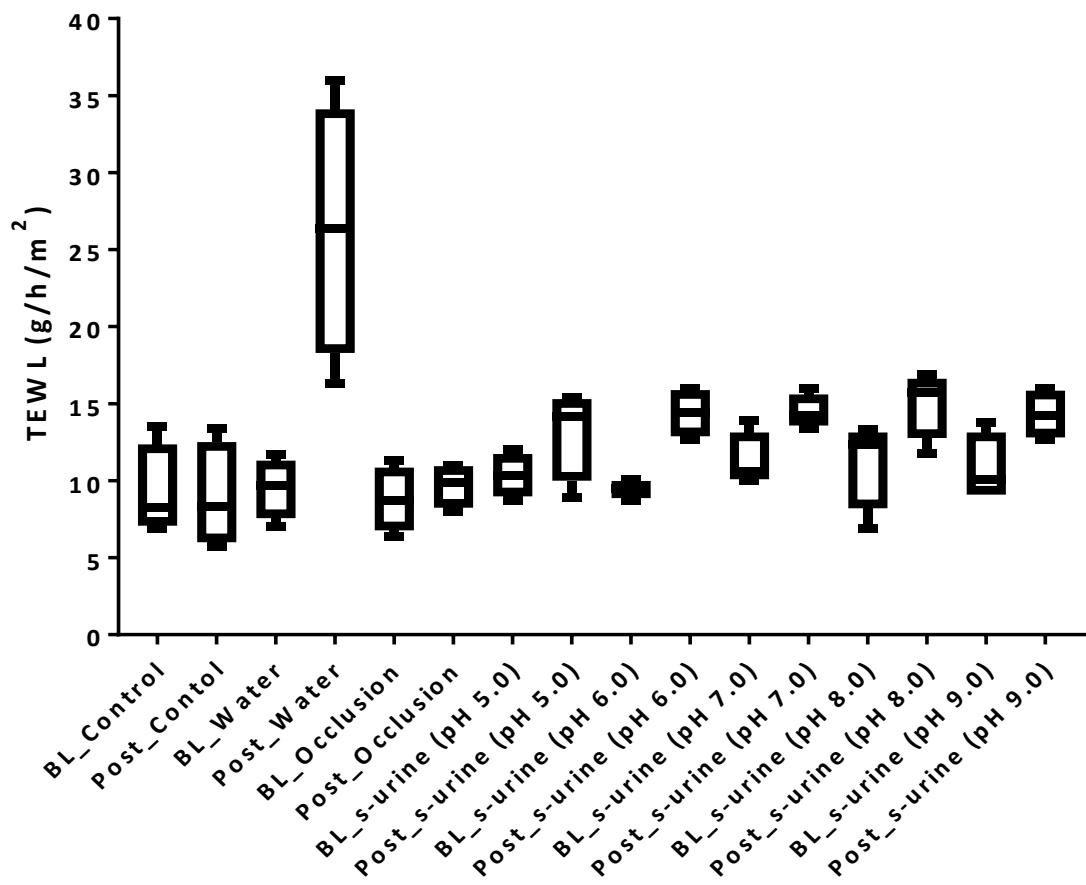
The second study examined the temporal changes in skin pH when exposed to urine solutions of three different pH values. The results, as shown in Figure 4.6, indicate a temporary increase in skin pH when compared to baseline control. However, the skin pH is restored back to basal values within five minutes after removal of the s-urine challenges.



**Figure 4.6. Measurement of pH over time.** The skin's buffering capacity was investigated after exposure to different s-urine solutions (pH 5.0, 6.0 and  $8.0 \pm 0.7$ ). After an initial increase in pH in all skin sites, skin pH returns to baseline values within 5 minutes post-application.

#### 4.8.3 The effects of water exposure and occlusion on the skin barrier function

In the final study, the effects of water exposure and occlusion per se were investigated, and results were pooled together with the results from the first study. Taken together, these findings clearly show that water exposure caused an increased TEWL in all volunteers, ranging between 108-256% compared to baseline values (Table 4.5). On the contrary, occlusion only caused a minimal increase in TEWL compared to baseline values. With respect to the different s-urine treatments, an increased TEWL was also observed, however this increase was much lower compared to water exposure, but higher than occlusion. Only one participant (P2) presented a decrease compared to baseline values following exposure to two s-urine solutions (pH 5.0 and pH 9.0), which was also lower when compared to the occluded site.



**Figure 4.7.** Box and whiskers plot for TEWL at baseline and following exposure to water, occlusion and s-urine, varying pH.

**Table 4.5.** Percentage change in TEWL following exposure to water, occlusion and s-urine, varying pH, compared to baseline values.

% change in TEWL Vs Baseline								
Participants	Synthetic-urine							
	control	Water	Occlusion	pH 5.0	pH 6.0	pH 7.0	pH 8.0	pH 9.0
P1	-1%	108%	-4%	25%	58%	1%	19%	76%
P2	-25%	256%	14%	2%	65%	48%	25%	-5%
P3	10%	205%	16%	22%	32%	34%	71%	39%
P5	0%	133%	25%	57%	54%	38%	40%	40%
mean	-1%	238%	16%	22%	42%	33%	30%	25%
SD	15%	152%	13%	22%	27%	19%	27%	39%
p value	0.60	0.07	0.14	0.70	0.70	0.70	0.70	0.14

## 4.9 Discussion

Incontinence represents the main risk factor for the development of IAD, and it has been proposed that prolonged exposure to urine disrupts the skin barrier function, reflected by increased TEWL and pH (Beeckman *et al.*, 2009). However, there is no empirical evidence to support this and hence the exact mechanism by which urine and its inherent pH lead to IAD development has not been clarified yet. Therefore, the main aim of the current investigation was to examine the effects of s-urine at varying pH (from pH 5.0-9.0), of physiological relevance, on the functional characteristics of healthy skin.

Exposure of healthy skin to various s-urine solutions led to an increase in skin microcirculatory blood perfusion (Figure 4.2). However, there was no significant difference between the s-urine solutions ( $p>0.05$ ). This is in contrast with what has been reported in the literature, that exposure to s-urine leads to a decrease in skin erythema (Mayrovitz and Sims, 2001). An increase in blood flow was also observed in the untreated control site, and a possible explanation for this is that any changes in blood flow might affect adjacent sites. With regards to TEWL (Figure 4.3) and SC hydration (Figure 4.4), s-urine led to an increase in both these parameters, but again without any significant differences ( $p>0.05$ ) between the various urine solutions. These results suggest that the effects of s-urine are not pH-dependent, and are in contrast with the results obtained in Larner *et al.*'s study that reported that an increase in alkaline pH was associated with more severe disruption of barrier function. With respect to skin surface pH (Figure 4.5), although results indicated a significant change ( $p<0.05$ ) following exposure to s-urine solutions, the size of this change was small (5-6%). This can be explained by the fact that this study was performed on healthy volunteers with an inherent physiological buffering capacity, such that any pH changes are rapidly diminished (Agache, 2004; Levin and Maibach, 2008). This was demonstrated in the second part of this investigation, in which the temporal profile of skin pH following exposure to acidic and alkaline urine solutions was examined. Results showed that all solutions (including control) caused a transient increase in skin pH (Figure 4.6), returning to baseline levels within 5 minutes. Skin pH was also increased with acidic solutions, an unexpected observation as the opposite has been reported in cadaver skin (Zhai *et al.*, 2009). Nevertheless, a direct comparison with that study should be avoided, as the skin function post-mortem is debatable and cell viability was not determined (Zhai *et al.*, 2009; Zheng *et al.*, 2012).

## Chapter 4

In the last stage of this investigation, the effects of water and occlusion on the skin barrier function were determined, and compared against the findings from the initial stage, in an attempt to further elucidate the effect of s-urine (water content and/or chemical constituents). Following removal of the challenges and prior to skin barrier assessment, the skin was dried with filter papers. By doing that it was ensured that what was actually measured was TEWL and not wet skin. Pooled results showed that both the various s-urine solutions and the water solution led to an increase in TEWL, and indeed water exposure was found to cause the highest increase in TEWL (Figure 4.7). By contrast, occlusion only caused a minimal increase in TEWL. This latter finding supports a previous study in which occlusion was reported not to produce any profound effects on the barrier function of intact skin (Jungersted *et al.*, 2010). When comparing the effects of s-urine to water and occlusion, it is evident that the former is less damaging to the skin barrier function than water exposure, but causes a higher increase in TEWL compared to occlusion on its own. These findings suggest that the chemical constituents of urine may alleviate the symptoms of water exposure. A possible explanation for this, is the presence of urea in urine that is a known humectant, that attracts water and keeps the SC in an hydrated state (Voegeli, 2012c).

To sum up, the present study was designed to examine the hypothesis that an increase in urinary pH disrupts SC function, leading to an increased TEWL and pH, compromising thus the integrity of the skin. The data presented here clearly inform the current theoretical framework of IAD development, as it was shown that while urine compromises the functional characteristics of the skin, its effects are not pH-dependent. This proposes that urinary pH is not damaging on healthy skin, suggesting that urine per se is not sufficient for IAD development. It should be acknowledged that the current investigation was performed on the volar forearm, as it is an easily accessible site, and is frequently used in clinical research. However, IAD occurs in the perineum and in areas which are in contact with urine/faeces, and consequently the differences in biophysical measurements, and in particular TEWL, between anatomical sites, should be taken into consideration (Pinnagoda *et al.*, 1990; Fader *et al.*, 2011).

### 4.10 Chapter summary

The studies in this chapter examined the hypothesis that an increase in urinary pH disrupts SC function, leading to an increased TEWL and pH, thus compromising the integrity of the skin. Findings showed that s-urine disrupts the functional characteristics of the skin, however its effects are not pH-dependent, which is in contrast with previous findings, which reported that an increase in alkaline pH was associated with an enhanced disruption of the barrier function (Lerner *et al.*, 2015). This can be explained by the differences in study designs with respect to exposure time and the degree of alkalinity of s-urine.

There was also a small change in skin surface pH following exposure to s-urine due to the inherent buffering capacity of able-bodied volunteers. Further investigation also showed that s-urine caused a higher degree of damage compared to occlusion, although the damage was less than the effects of wetness (water). Findings presented in this chapter provided additional motivation for the next study discussed in Chapter 5, namely to examine the effects of urine on damaged skin with a compromised buffering capacity.



# Chapter 5: The susceptibility and permeability of intact and compromised skin to synthetic-urine

## 5.1 Introduction

Incontinent patients require a structured skin care regimen to prevent skin breakdown and promote skin health. This is accomplished by removing the irritant moisture source from the skin by cleansing and applying a skin protectant (Gray *et al.*, 2002; Beeckman *et al.*, 2015). The most common method to cleanse the skin involves the use of soap, water and a regular towel or washcloth to dry the skin, as this represents an economic and convenient option for nursing care (Voegeli, 2008; Beeckman *et al.*, 2015). Soaps or surfactants are usually involved in cleansing routines as some of the impurities present on the skin surface are not water-soluble (Voegeli, 2008; Mukhopadhyay, 2011). Additional surfactants may also be added to soap preparations to decrease the surface tension of water. However, many of these can be irritating to the skin (Voegeli, 2008). For instance, a common surfactant found in soaps is SLS, is in fact a strong irritant, as several experimental studies have shown that exposure to SLS compromises skin integrity, characterized by increased TEWL and a visible erythema (Kligman, 1966; Tupker *et al.*, 1997; Kirsner and Froelich, 1998; Held and Agner, 1999; Held *et al.*, 2001; de Jongh *et al.*, 2006; De Jongh *et al.*, 2007b). Besides this, surfactants can cause: 1) after wash tightness, 2) skin dryness, and 3) itch (Imokawa *et al.*, 1989; Wilhelm *et al.*, 1994a). Their alkaline nature (pH 9.0-10.0) has also been considered as a contributing factor to skin irritation, as it can disrupt the protective acid mantle of the skin surface, promoting the growth of pathogens (Korting *et al.*, 1987; Korting and Braun-Falco, 1996). In the SC, surfactants cause disorganization of the lipid bilayers and eventually lead to a reduction of its natural moisturizing factor (Prottey and Ferguson, 1975; Bikowski, 2001; Draelos, 2008). At the cellular level, surfactants destroy the cell membrane of keratinocytes, as they bind to keratin causing protein denaturation (Bikowski, 2001). Commonly, following cleansing, the skin is dried using a washcloth or a towel through a mechanical action. Nonetheless, this process has also been reported to disrupt skin barrier function, by increasing TEWL, and stimulate the release of pro-inflammatory mediators, such as TNF- $\alpha$ , IL-8 and IL-10 (Nickoloff and Naidu, 1994). This may be further aggravated by the frictional damage caused by the texture of regular washcloths (Beeckman *et al.*, 2011).

With reference to the theoretical framework of IAD development, Beeckman and colleagues (2009) stated that physical and chemical irritation, exacerbated by frequent cleansing routines, increase skin permeability and therefore increase the risk of IAD (Beeckman *et al.*, 2009).

## Chapter 5

Indeed previous work within the local research group has shown that frequent cleansing compromises skin integrity, however, the permeability of the skin following cleansing activities has not been investigated fully (Voegeli, 2008). Therefore, this study was designed to examine the hypothesis that frequent skin cleansing activities increase the permeability of the skin and consequently its susceptibility to urine.

### 5.2 Aim of the study

- Determine the susceptibility and permeability of intact and compromised skin to s-urine

### 5.3 Objectives

- Determine the permeability of intact and damaged skin to s-urine
- Examine the effects of tape stripping and SLS treatment (surrogates for frequent cleansing techniques) on the functional characteristics of healthy skin
- Investigate the effects of s-urine on the functional characteristics of healthy and damaged skin
- Monitor the temporal effects of tape stripping and SLS exposure on blood perfusion

### 5.4 Participants

The study was completed in two stages, and ethical approval was gained from the local university ethics committee (FHS no: 9349). Volunteer recruitment was based on inclusion and exclusion criteria, detailed in Table 3.11. Although a previous power calculation (section 4.4) indicated that 15 participants were required to detect a change of 25% in the measures for skin barrier assessment, only 10 volunteers were recruited in the current study.

- In the initial stage, ten healthy volunteers were recruited (mean age  $\pm$  SD:  $29.40 \pm 10.52$ , 7 males, 3 females), and the effects of urine on intact and compromised skin were investigated.
- In the subsequent stage, five healthy volunteers (mean age  $\pm$  SD:  $47.20 \pm 14.65$ , 1 male, 4 females) were recruited, two of whom had participated in the first study. This study examined the temporal effects of tape stripping and SLS on skin blood flow.

Written informed consent was obtained from all participants prior to any testing. Volunteers were also requested not to apply any cosmetic products on their skin, such as moisturizers, as these have been reported to influence the biophysical measurements (Held *et al.*, 1999; Lodén, 2012).

The study was conducted in the bioengineering laboratory, within the Faculty of Health Sciences at University Hospital Southampton, at an ambient temperature ( $22^{\circ}\text{C} \pm 3^{\circ}\text{C}$ ) and humidity (40±5%). All participants were allowed to rest for at least 30 minutes before any testing commenced.

## 5.5 Methods

The methods used in this study are detailed in Chapter 3. Briefly, two distinct irritant models were used as surrogates for frequent cleansing techniques, namely, tape-stripping and SLS treatment. Tape stripping is a well-established model that removes the superficial layers of the SC, and therefore simulating the damage caused from physical irritation (such as repeated towel drying), whilst SLS is found in soap preparations and has been widely used in dermatological research as a model of chemical irritation. Tape stripping (n=20) was performed using equal sized adhesive tapes (Sellotape™, UK) and constant application pressure was ensured by using a roller, whilst 500 $\mu\text{l}$  SLS (0.5% w/v) were impregnated into HillTop chambers (25mm, HillTop Labs, Miamiville, USA) and applied on the skin for 24 hours, which is adequate to provoke an erythematous response (Tupker *et al.*, 1997). Intact skin was also represented by an untreated control area. S-urine (pH=7.9) was also applied on intact and damaged skin for two hours (Mayrovitz and Sims, 2001), as described previously (section 4.5). To evaluate the effects of s-urine on intact and compromised skin, non-invasive biophysical techniques were employed. Measurements of blood perfusion in the cutaneous microcirculation were obtained using a laser Doppler imaging system (LDI, Moor Instruments Ltd, Axminster, UK) that allows continuous measurement of the velocity and concentration of red blood cells over a large skin area. TEWL measurement (Tewameter® TM 300 wireless probe, MP WL, Courage & Khazaka) was used to assess the efficiency of the skin barrier function, and it represents the gold-standard method (Fluhr *et al.*, 2006b). SC hydration was quantified by measuring the electrical capacitance of the skin, using a standard method, corneometry (Corneometer® CM 825, wireless probe, MP WL, Courage & Khazaka), and skin pH was measured using a surface pH probe (Skin-pH-Meter® 905, wireless probe, MP WL, Courage & Khazaka). Measurements were obtained at baseline, following tape stripping/SLS challenges and subsequent to s-urine exposure. Upon removal of occluded conditions, there is an accumulation of excess water (TEWL) in the SC, which gradually returns to basal levels. This is known as skin surface water loss (SSWL), and may be captured by plotting desorption curves of TEWL. Accordingly, following removal of the s-urine containing patches, TEWL was taken first and recorded continuously for 10 minutes, in order to measure desorption curves of TEWL. Skin permeability, and in particular the amount of s-urine penetrating the skin, was then determined by calculating SSWL from the area under the resulting desorption curves, a method reported by Fader and colleagues (2011).

## 5.6 Experimental protocol

In the initial stage, volunteers attended the laboratory for three consecutive days. On Day 1, baseline measurements of LDI, TEWL, SC hydration and skin pH were obtained at three skin sites (20mmx20mm) on the left volar forearm, which was the non-dominant arm for all volunteers. A ruler was used to ensure correct positioning, as previously described (Chapter 4). To avoid any selection bias and to minimize variability, treatments were randomized using an online software package (Randomization., 2007). Following randomization, an SLS-containing HillTop chamber was applied to one site and left *in situ* for 24 hours. On another site, tape stripping (n=20) was performed, and TEWL measurements were taken after every five strips to evaluate the severity of barrier disruption. The remaining site served as the untreated control. Following tape stripping, the biophysical measurements were repeated, and s-urine was applied on both the tape-stripped and untreated sites for two hours, separated by a 10-minute interval. After exposure time, the chambers were removed, the skin was gently pat dried using filter papers to absorb excess moisture, and TEWL was recorded continuously for 10 minutes, followed by the other measurements. On Day 2, and after 24 hours of SLS exposure, the SLS-containing chambers were removed, and biophysical measurements repeated. Then, s-urine was applied for two hours, after which the skin was gently dried, as previously, and TEWL was recorded for 10 minutes, and the other biophysical measurements were repeated. On Day 3, and specifically 24 hours after removal of the SLS treatment, measurement of SC hydration was obtained on SLS-compromised skin, in order to examine the temporal effects of SLS on skin hydration levels. However, due to volunteer availability, this was only done with four volunteers. An overall diagram describing the initial study is presented in Figure 5.1.

In the second stage, volunteers attended the laboratory on two occasions. On Day 1, baseline measurements of LDI were obtained at three skin sites on the left volar forearm. Then, following randomization, SLS was applied on one site for 24 hours, on another site tape stripping was performed (n=20), and the remaining site represented an untreated control. Following tape stripping, LDI was measured immediately on the tape-stripped and untreated sites, and then every 30 minutes for a total period of 2 ½ hours. On Day 2, and after 24 hours of SLS exposure, the SLS-containing chambers were removed, and LDI measurements were repeated as previously.

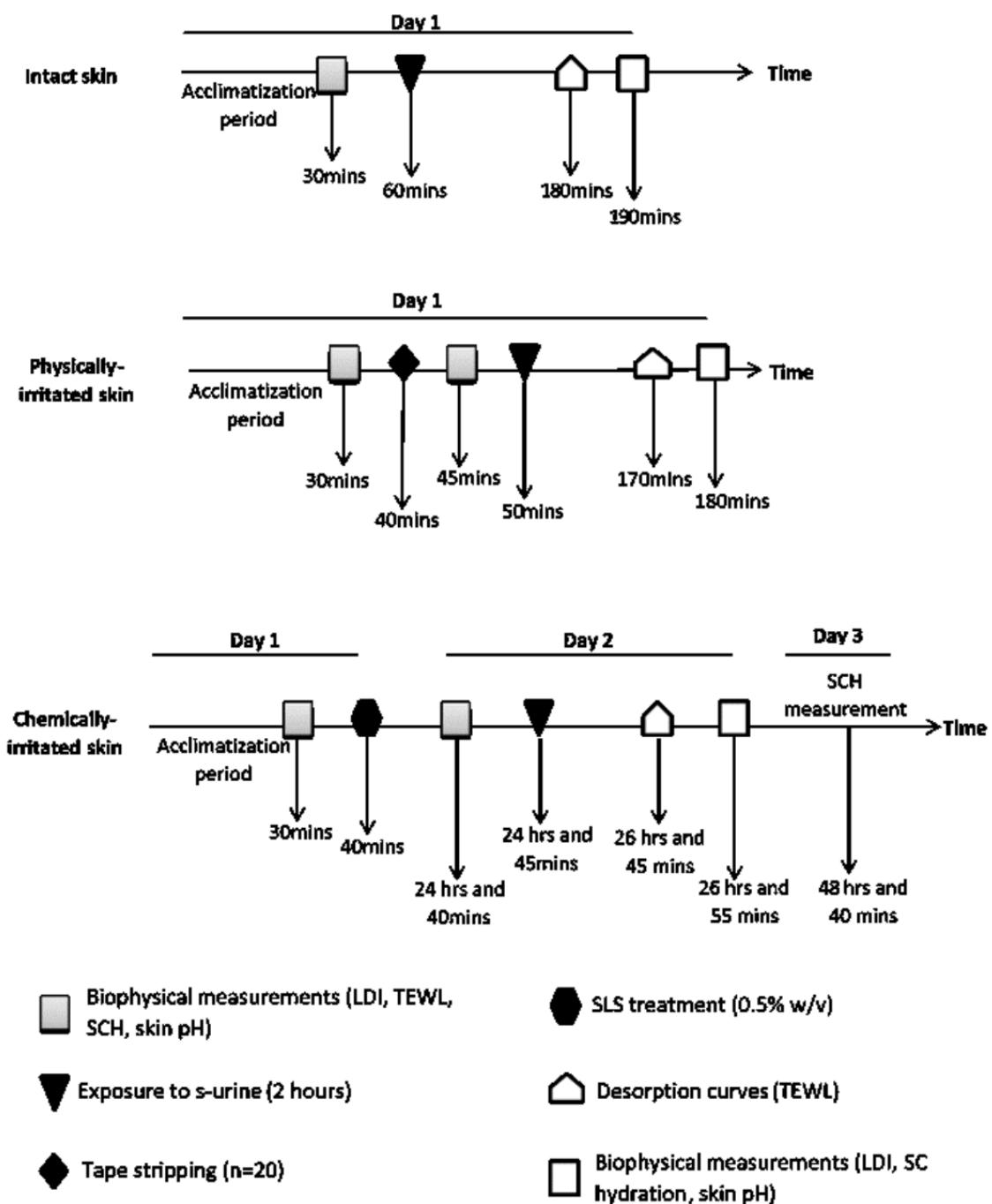


Figure 5.1. Flow diagram of the initial study of the current investigation.

## 5.7 Data Analysis

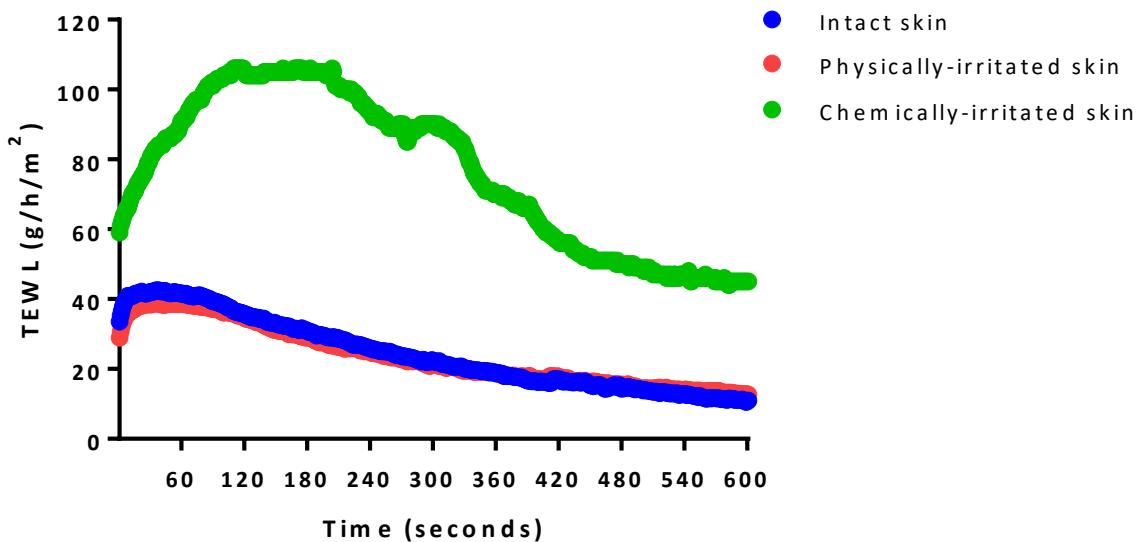
Data are presented in box plots, where boxes represent 25th to 75th percentile range; horizontal line represent median value and whisker represent min and max values, in all figures unless otherwise stated. Percentage change compared to baseline values following each challenge and the subsequent exposure to s-urine are also shown for each parameter. Analysis of perfusion images from the LDI was performed using the manufacturer's software (mLDI Main version 53, Moor Instruments Ltd, Axminster, UK), as described previously in section 4.7.

Statistical analysis was conducted using IBM SPSS version 21.0 (SPSS Inc., Chicago, IL, USA). Non-parametric tests were performed because due to small sample size the data was assumed to be non-normal in distribution. To determine the effects of skin condition on permeability and the subsequent exposure to s-urine on each of the biophysical measurements, the Friedman test was used and pairwise comparisons were performed using the Wilcoxon signed-rank test. Correlation analysis between the number of tape strips and TEWL was also examined using the Spearman's rank-order correlation. Statistical significance was set at  $p<0.05$ .

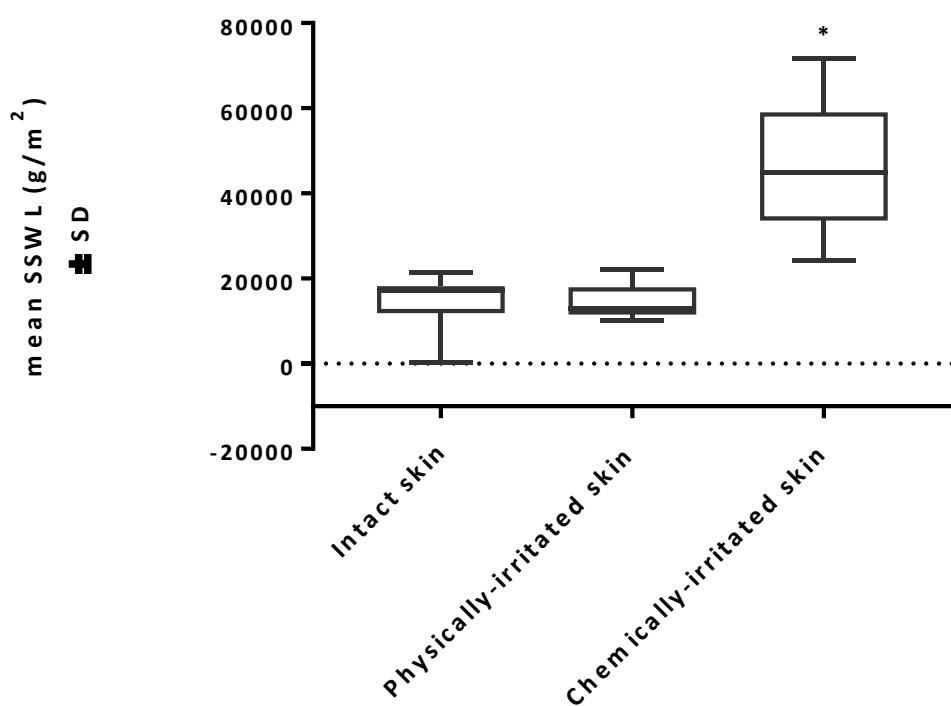
## 5.8 Results

### 5.8.1 The permeability of intact and compromised skin to s-urine

Following exposure to s-urine, desorption curves for intact and compromised skin were constructed by subtracting mean baseline TEWL value from post-challenge TEWL for each time point, as illustrated in Figure 5.2. For two volunteers, the desorption curves of tape-stripped skin presented an anomalous shape compared to the curves of the other volunteers, possibly due to movement artefacts and therefore were removed. Subsequently, the area under the curve was calculated for each volunteer using a trapezoidal integration approach, and the mean SSWL was then determined for each condition. Results, as illustrated in Figure 5.3, show that SLS-compromised skin presented a significantly ( $p<0.05$ ) higher SSWL compared to both intact and tape-stripped skin. By contrast, there was no significant difference ( $p>0.05$ ) between intact and tape-stripped skin.



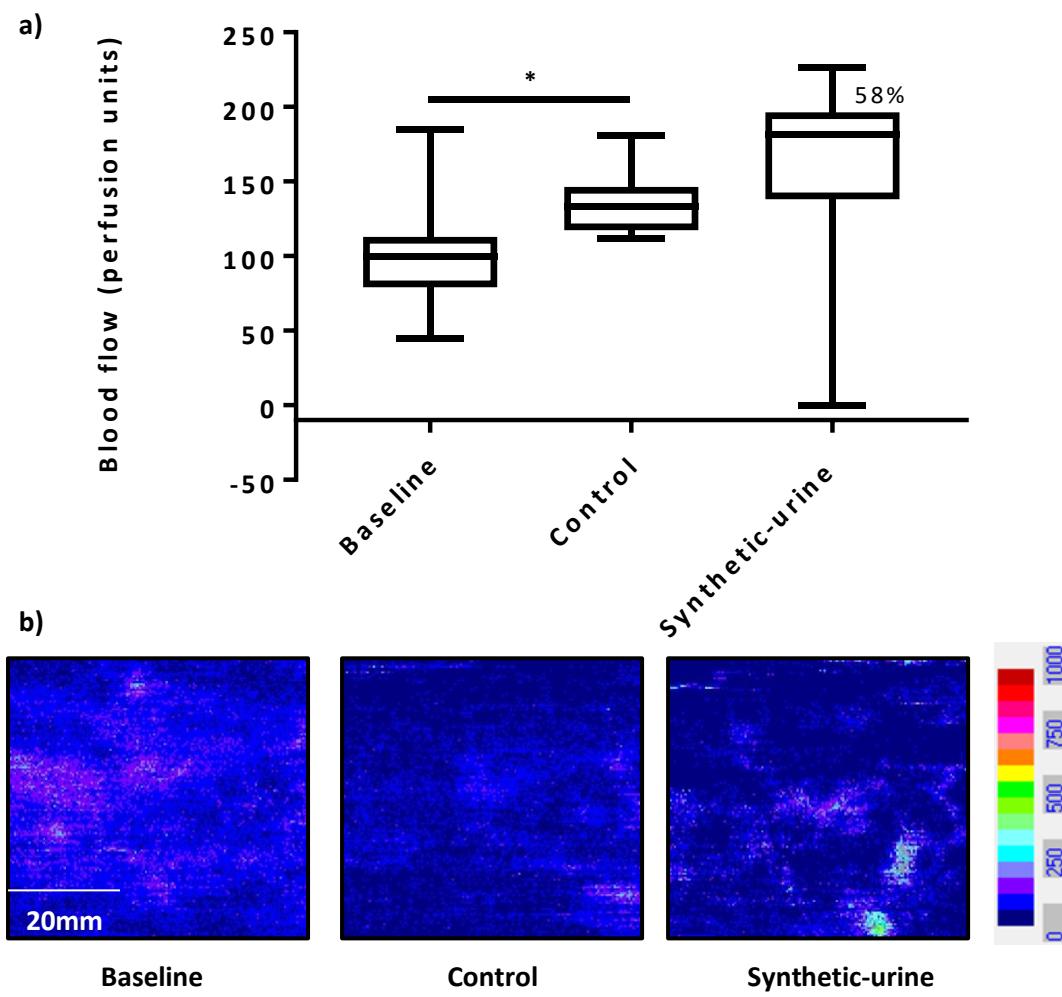
**Figure 5.2. Desorption curves of TEWL of intact and compromised skin exposed to s-urine.** Mean baseline TEWL was subtracted from post-challenge TEWL, and plotted against time. There is an initial increase in TEWL following removal of urine patches; however the TEWL value decreases with time, as skin surface water is evaporating. It is evident from the graph that chemically-irritated skin presents an increased susceptibility to s-urine penetration.



**Figure 5.3. Box and whiskers plot showing the amount of skin surface water loss for intact and compromised skin following exposure to urine.** The amount of SSWL for chemically-irritated skin is significantly higher compared to both intact and mechanically-irritated skin ( $p<0.05$ ). There is no significant difference between intact and mechanically-irritated skin ( $p>0.05$ ). Significance is displayed by (\*).

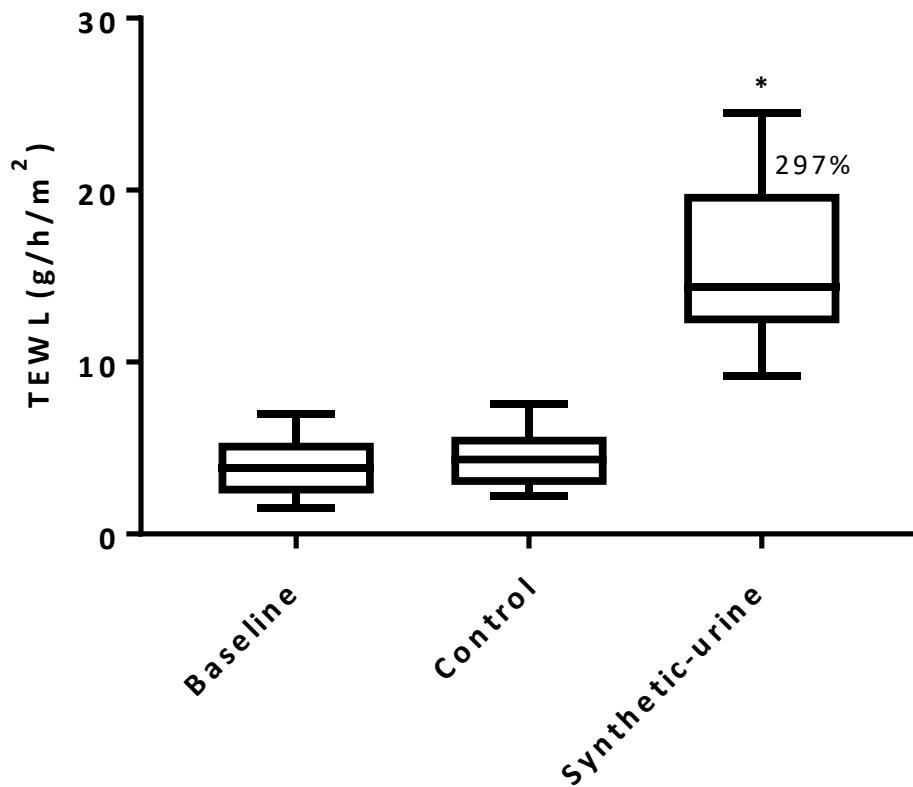
### 5.8.2 Susceptibility of intact skin to s-urine

After s-urine exposure of intact skin for two hours, changes in blood flow were investigated using the LDI, showing that s-urine leads to an increase in blood flow (mean blood flux  $\pm$  SD=  $157.64 \pm 63.61$ ) compared to both baseline (mean blood flux  $\pm$  SD=  $99.78 \pm 38.84$ ) and control (mean blood flux  $\pm$  SD=  $135.72 \pm 21.75$ ) values, as shown in Figure 5.4a. However, no significant difference was found ( $p>0.05$ ). There is also an increase in blood flow at the control site when compared to baseline ( $p<0.05$ ); however, this may be attributed to the influence of adjacent-exposed sites. Typical perfusion images from the LDI for one individual are shown in Figure 5.4b.

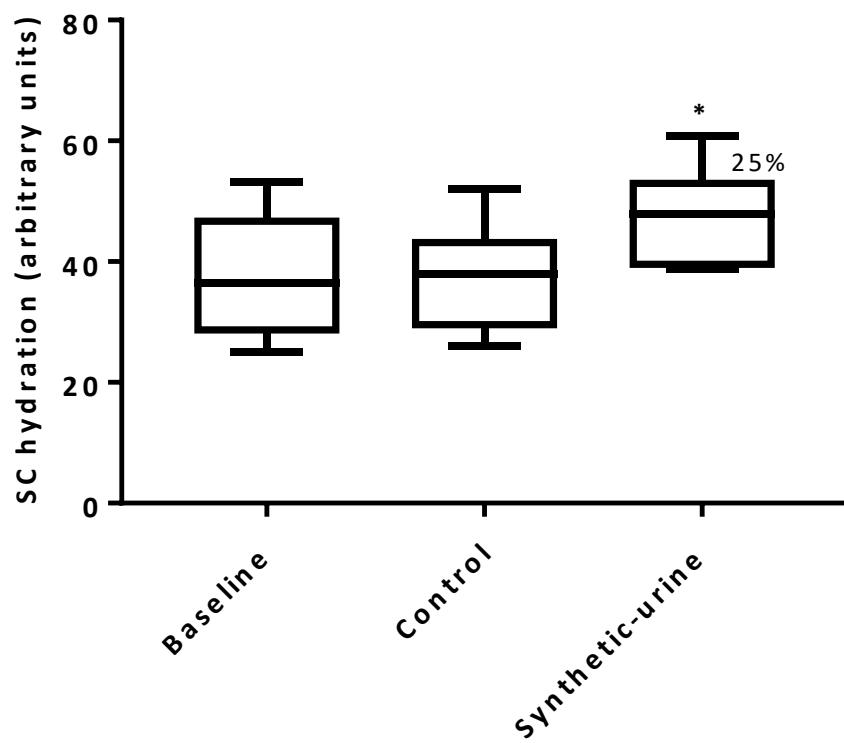


**Figure 5.4. The effect of s-urine on blood perfusion of intact skin. a)** Box and whiskers plot of skin blood flow of intact skin. Exposure to s-urine leads to increased blood perfusion, compared to both baseline and control values ( $p>0.05$ ). Percentage change compared to baseline is also shown, **b)** Generated perfusion images of an individual at baseline, control and following exposure to synthetic-urine (baseline subtracted).

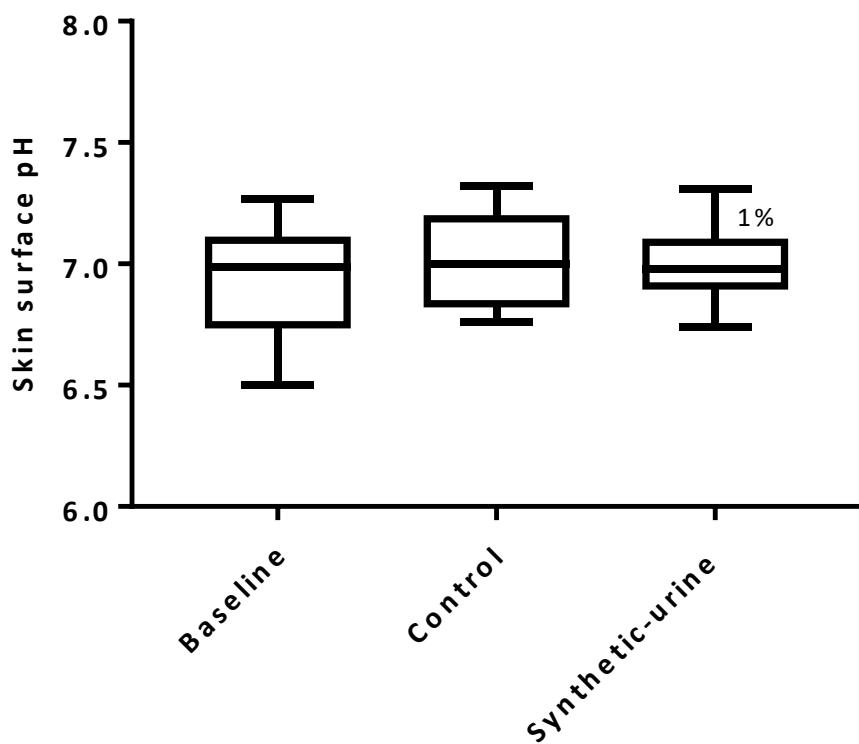
The results for both skin barrier function and SC hydration, as shown in Figures 5.5 and 5.6 respectively, indicate that exposure to s-urine caused a significant increase in both parameters when compared to both baseline and control values ( $p<0.05$ ). By contrast, s-urine exposure did not cause any significant change ( $p>0.05$ ) in skin pH, as shown below in Figure 5.7.



**Figure 5.5. Box and whisker plot showing the effect of s-urine on skin barrier function of intact skin.** Exposure to s-urine caused a significant increase in TEWL compared to both baseline and control values ( $p>0.05$ ). Percentage change compared to baseline is also shown. Significance is displayed with (\*)



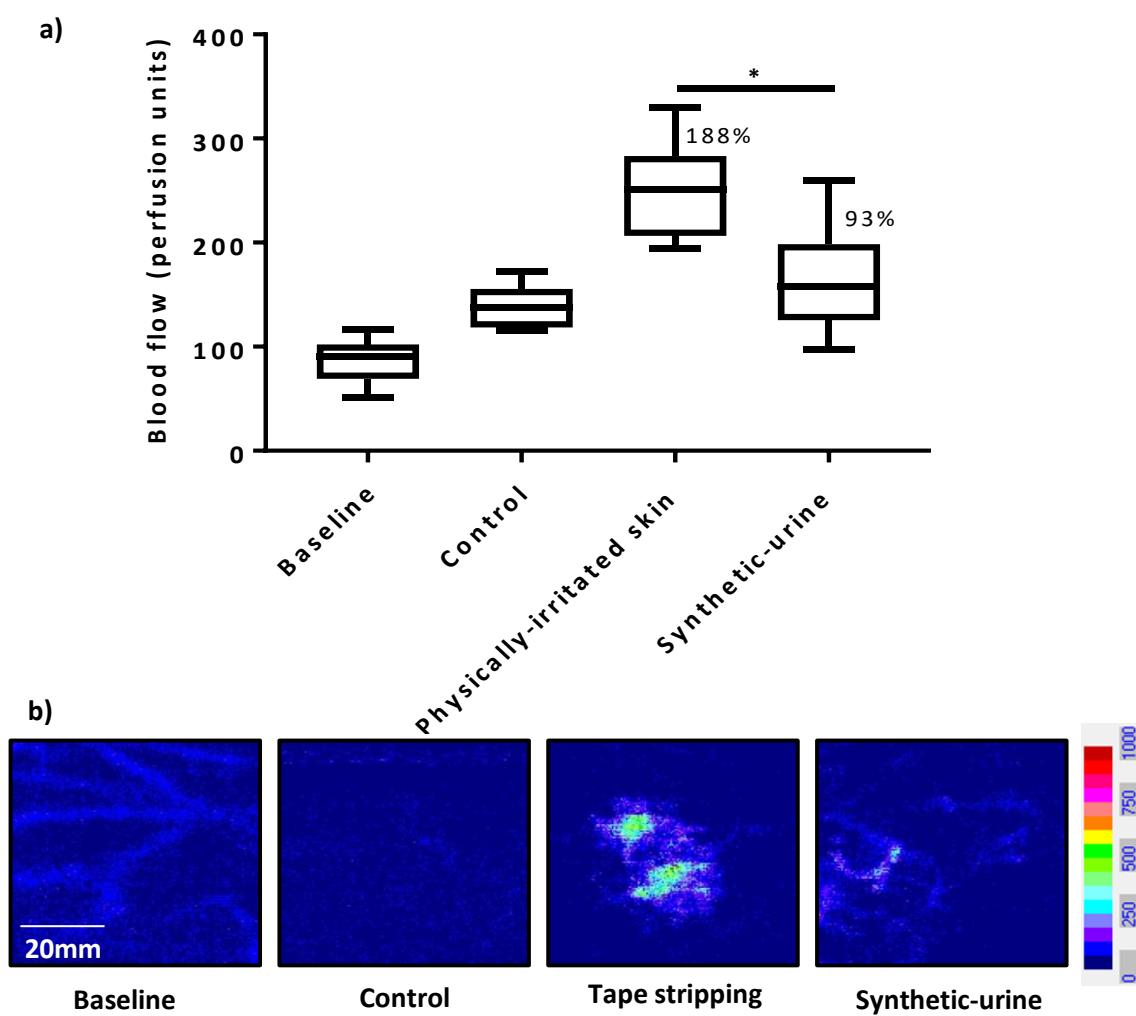
**Figure 5.6.** Box and whisker plot showing the effect of s-urine on SC hydration of intact skin. S-urine causes a significant ( $p<0.05$ ) increase in skin hydration, compared to both baseline and control values, as indicated (\*). Percentage change compared to baseline is also shown.



**Figure 5.7.** Box and whisker plot showing the effect of s-urine on the skin acid mantle of intact skin. Exposure of the skin to urine led to a small increase (1%) in skin pH from baseline, but this was not significant ( $p>0.05$ ).

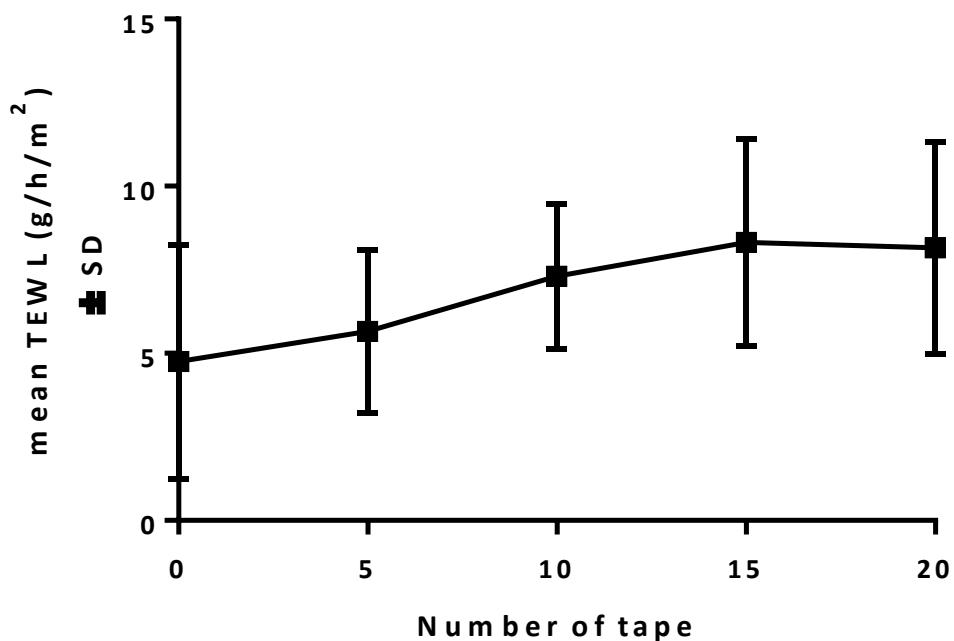
### 5.8.3 Susceptibility of physically-irritated skin to s-urine

Tape stripping the skin produced an erythematous response, also reflected by the significant ( $p<0.05$ ) increase in blood perfusion compared to baseline and control values (Figure 5.8). However, following exposure to synthetic urine there was a significant decrease in blood flow compared to tape stripping ( $p<0.05$ ), as shown in Figure 5.8a. Nevertheless, this was still significantly higher compared to baseline values ( $p<0.05$ ). As reported previously (section 5.8.2), at the control site there is an increase in blood flow compared to baseline values ( $p<0.05$ ), a finding probably attributable to the influence of adjacent-exposed sites. A typical set of perfusion images from an individual participant is shown in Figure 5.8b.

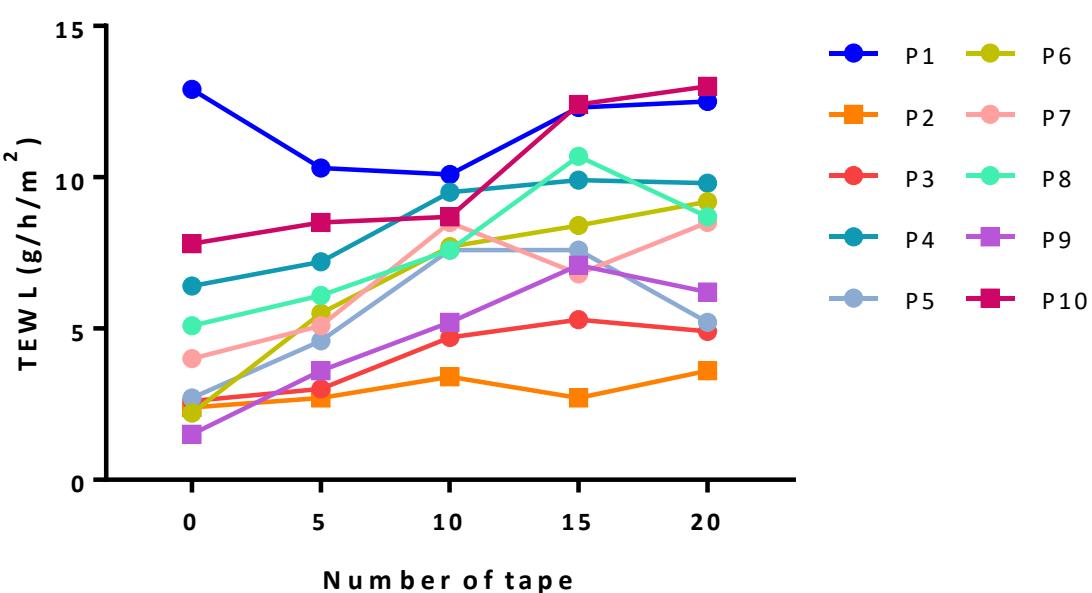


**Figure 5.8. The effect of s-urine on blood perfusion of physically-irritated skin. a)** A box and whisker plot of skin blood flow of physically-irritated skin. Following tape stripping, there was a significant increase in blood flow, compared to both baseline and control values ( $p<0.05$ ). However, exposure to s-urine led to a significant decrease in blood flow compared to tape stripping ( $p<0.05$ ), but blood perfusion was still higher than basal values ( $p<0.05$ ). There was also a significant rise in blood flow at the control site ( $p<0.05$ ). Significance is displayed with (\*). Changes compared to baseline values are also expressed as percentages. **b)** Images from the LDI measurement, showing blood perfusion at baseline, the control site, and following tape stripping and urine challenges. Baseline image has been subtracted from both tape stripping and s-urine images.

Additionally, tape stripping caused an increase in TEWL and consequently disruption of the skin barrier function, and this increase was positively correlated with the number of tapes ( $R=0.90$ ,  $p<0.05$ ), as shown in Figure 5.9. Following the 20<sup>th</sup> tape strip, however, the TEWL value is slightly decreased compared to the 15<sup>th</sup> tape. The variability between individuals in response to tape stripping is also shown in Figure 5.10, with a CV ranging between 30-75%.

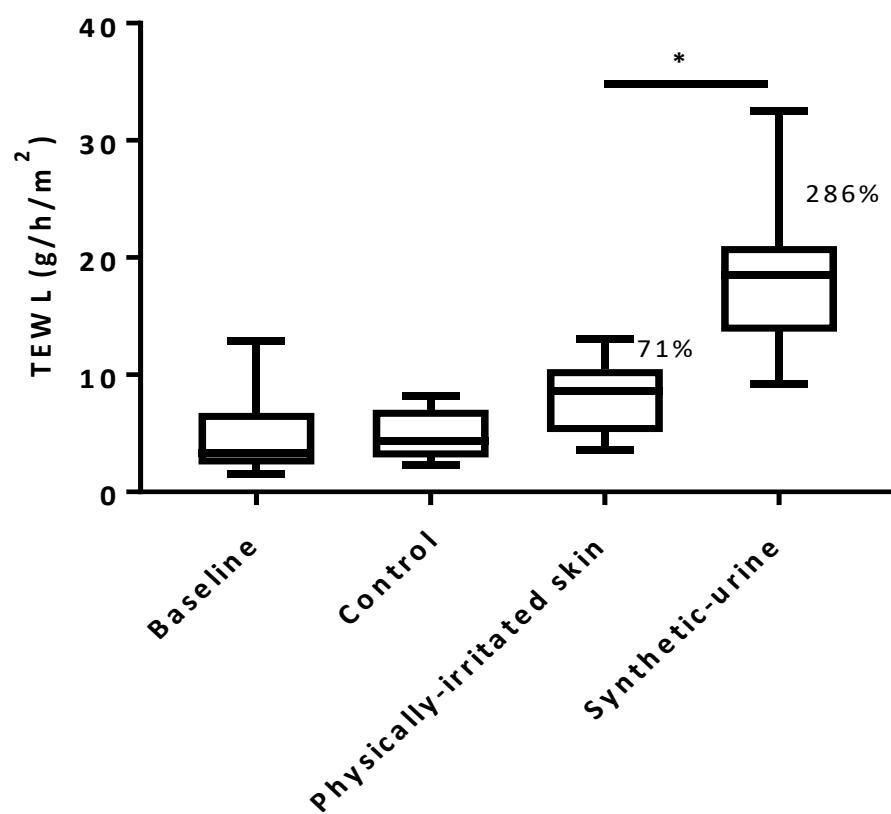


**Figure 5.9. The effect of tape stripping on the skin barrier function.** Tape stripping causes a disruption of the skin barrier function, evidenced by the increase in TEWL compared to baseline values (tape 0).

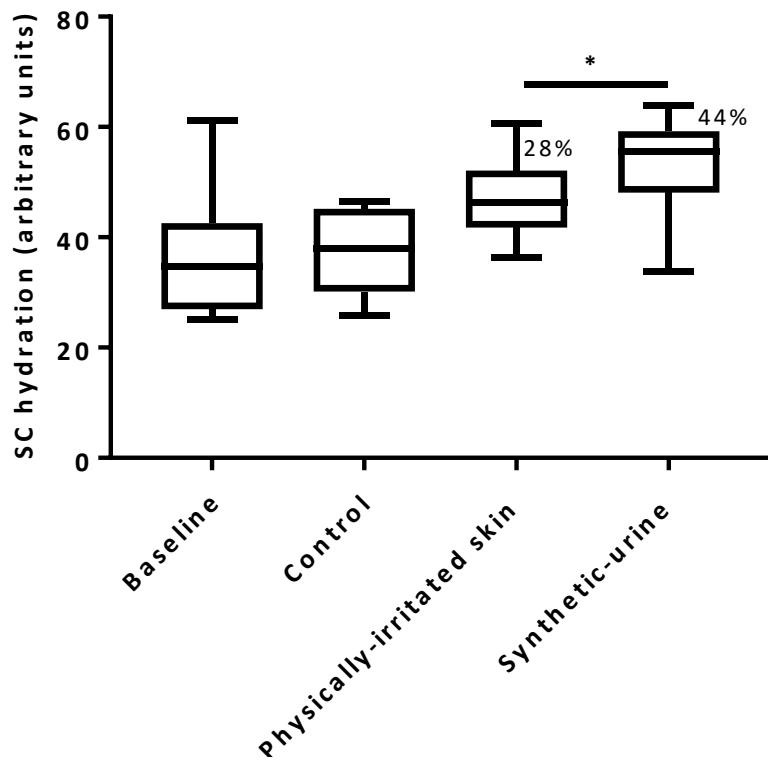


**Figure 5.10. Inter-individual variability in tape stripping the skin.** The inter-individual variation ranges between 30-75%.  $\circ$ =males,  $\square$ =females

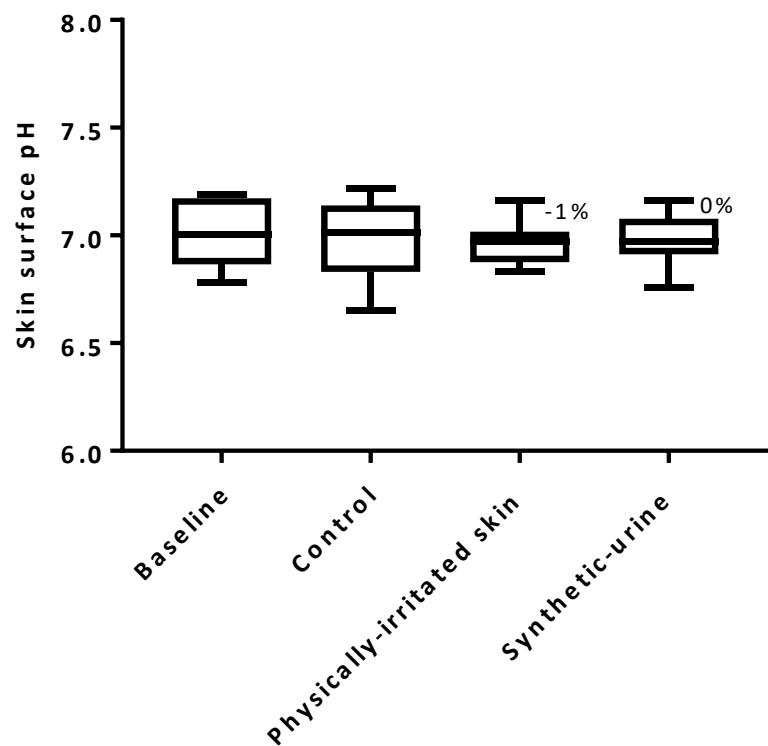
With reference to TEWL measurement and SC hydration, results showed that both tape stripping and s-urine caused a significant increase ( $p<0.05$ ) in both these parameters compared to baseline and control values, as illustrated respectively in Figures 5.11 and 5.12. In particular, s-urine further increased both TEWL and the hydration levels of the SC to a significant degree ( $p<0.05$  in both cases). In spite of the disruptive effects of tape stripping, it only caused a small decrease in skin pH (mean skin pH  $\pm$  SD=  $6.97\pm0.10$ ) (Figure 5.13). In a similar manner, following exposure to s-urine the pH value (mean skin pH  $\pm$  SD=  $6.99\pm0.12$ ) did not alter significantly ( $p>0.05$ ), compared to baseline.



**Figure 5.11. Box and whisker plot showing the effect of s-urine on skin barrier function of physically-irritated skin.** Tape stripping and the following exposure to s-urine caused a significant increase in TEWL compared to both baseline and control values ( $p>0.05$ ). Changes compared to baseline values are also expressed as percentages. Synthetic urine also caused a significant increase ( $p<0.05$ ) in TEWL compared to tape stripping, marked with (\*).



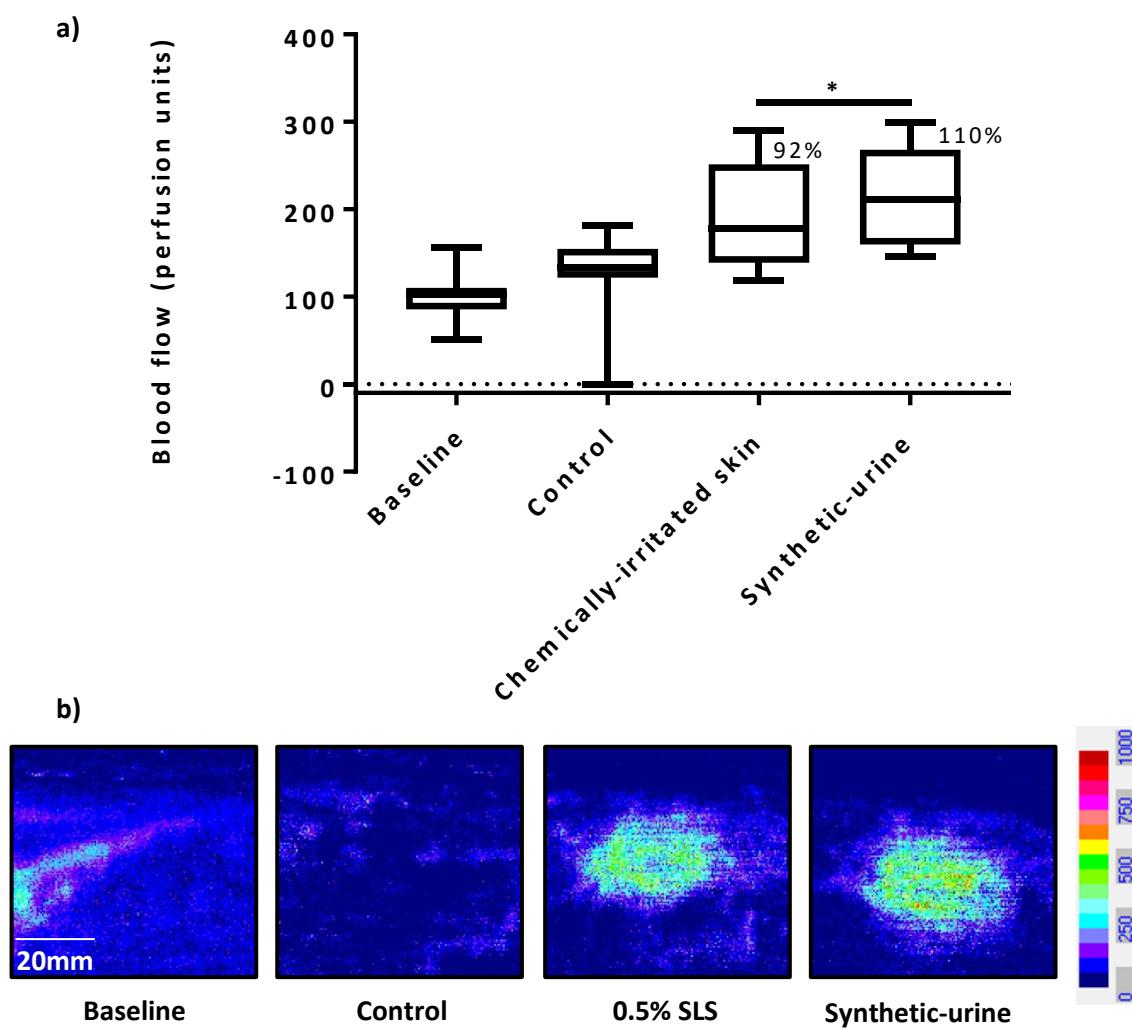
**Figure 5.12. Box and whisker plot showing the effect of s-urine on SC hydration of physically-irritated skin.** SC hydration increases with both tape stripping and exposure to synthetic-urine, and this is significant compared to both baseline and control values ( $p<0.05$ ). % change compared to baseline values is also shown. Significance is indicated with (\*).



**Figure 5.13. Box and whisker plot showing the effect of s-urine on the acid mantle of physically-irritated skin.** Both tape stripping and s-urine did not cause any significant change ( $p>0.05$ ) in skin surface pH. Changes compared to baseline are also expressed as %.

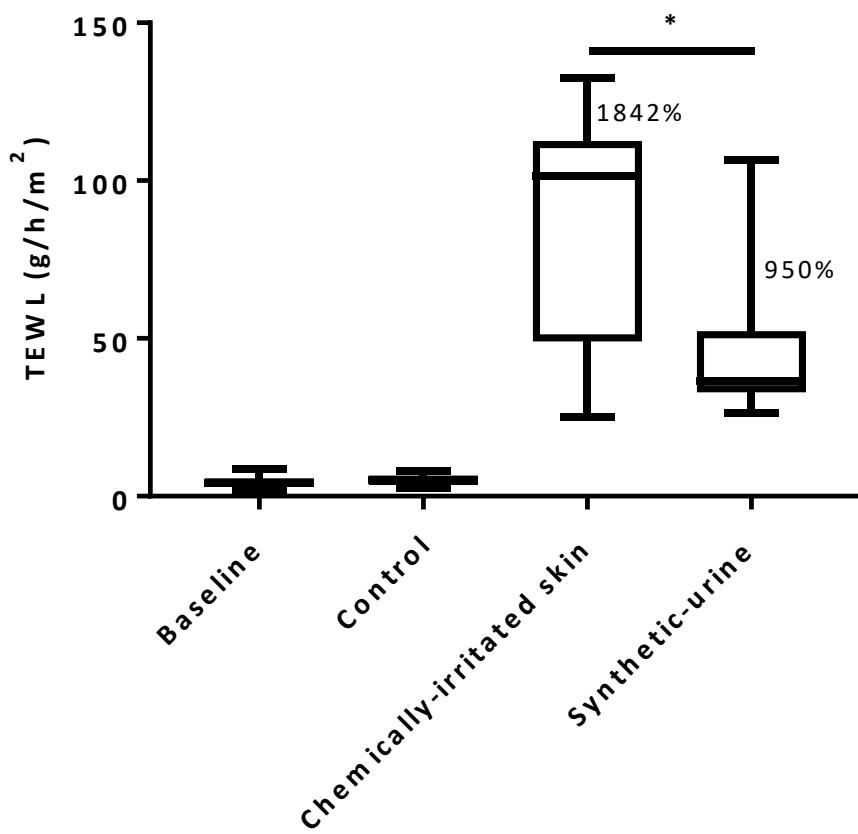
### 5.8.4 Susceptibility of chemically-irritated skin to urine

In this study, exposure to 0.5% SLS (w/v) for 24 hours caused a significant increase in blood perfusion (Figure 5.14a), and an erythematous response. In addition, the subsequent exposure to s-urine also resulted in an increased blood perfusion compared to both baseline and control values, but also to a significant further increase when compared to the values associated with SLS ( $p<0.05$ ). As reported previously (section 5.8.2), at the control site there is an increase in blood flow compared to baseline values and this is because of the influence of adjacent-exposed sites. Representative perfusion images from an individual are shown in Figure 5.14b.

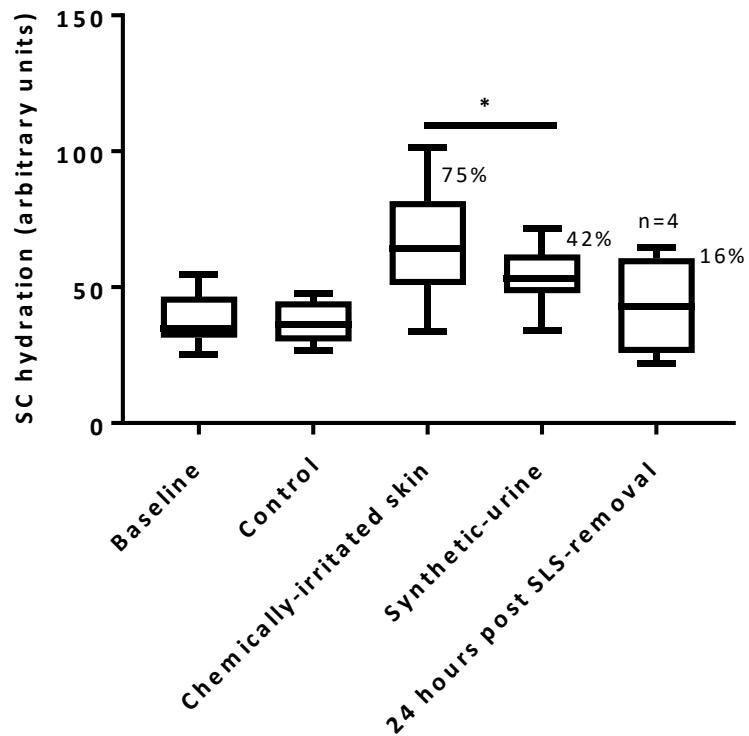


**Figure 5.14. The effect of s-urine on blood perfusion of chemically-irritated skin. a)** Box and whisker plot of skin blood flow of chemically-irritated skin. Both SLS and s-urine exposure caused a significant increase ( $p<0.05$ ) in skin blood flow compared to baseline, 92% and 110% respectively, and control values. S-urine exposure also caused a further increase in blood flux compared to SLS ( $p<0.05$ ). **b)** Images from LDI measurement. Baseline image has been subtracted from both SLS and s-urine post-exposure images. Significance is displayed by (\*).

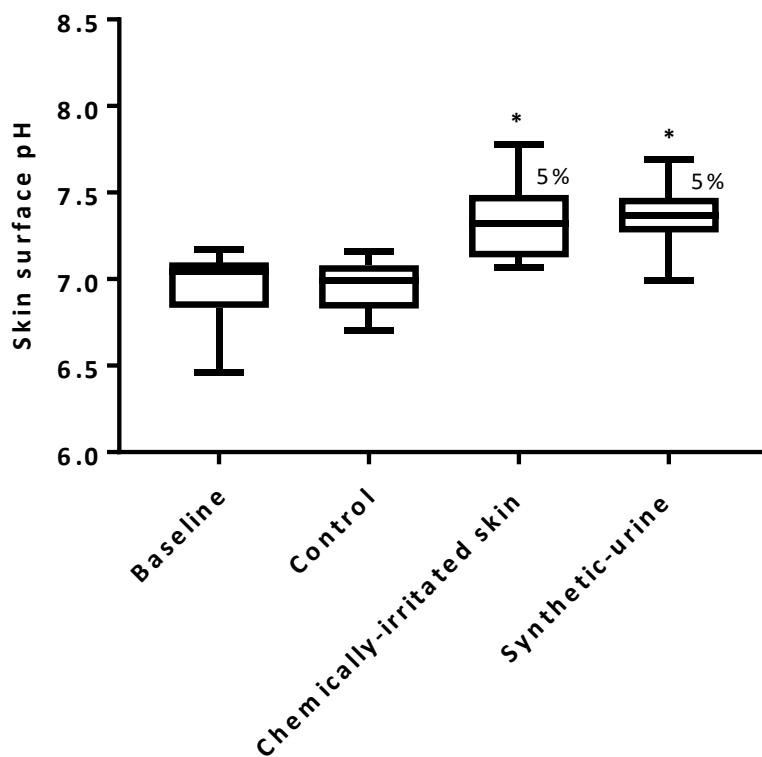
With regards to TEWL measurement and SC hydration, both the SLS and subsequent urine treatments caused a significant increase in both skin parameters compared to baseline and control values ( $p<0.05$ ), as illustrated in Figures 5.15 and 5.16 respectively. It is interesting to note that the SLS treatment evoked a significantly higher increase in both biophysical parameters when compared to corresponding values evoked by s-urine ( $p>0.05$ ). It is also evident that there is a decrease in the levels of SC hydration 24 hours post SLS treatment (Figure 5.16). With respect to skin pH, both the SLS and the subsequent exposure to urine resulted in a significant increase in skin pH compared to baseline and control values ( $p<0.05$ ), as shown in Figure 5.17. There was no significant difference ( $p<0.05$ ) between the treatments.



**Figure 5.15. Box and whisker plot showing the effect of s-urine on skin barrier function of chemically-irritated skin.** Exposure to SLS led to a disrupted skin barrier, reflected by the significant increase in TEWL compared to both baseline and control values. However, following exposure to urine, TEWL was still significantly higher than baseline, but significantly lower compared to SLS ( $p<0.05$ ). % change compared to baseline values is also shown. Significance is marked with (\*).



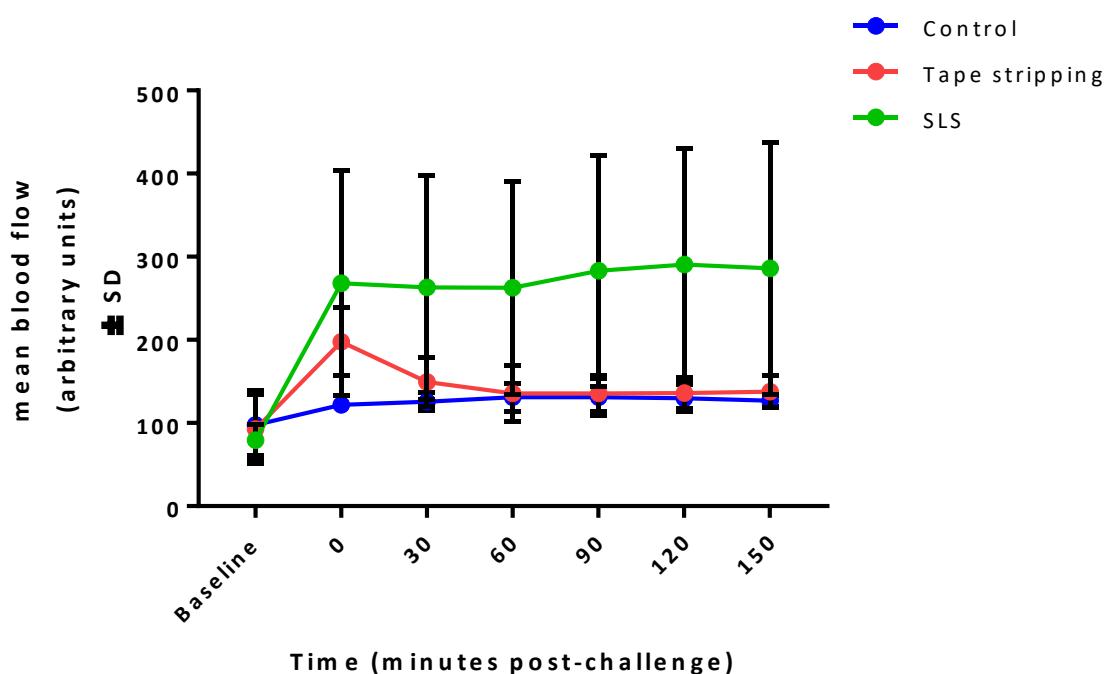
**Figure 5.16. Box and whisker plot showing the effect of s-urine on SC hydration of chemically-irritated skin.** Both the SLS and urine caused a significant increase in skin hydration compared to baseline and control values ( $p<0.05$ ), and indeed exposure to s-urine led to a significant decrease ( $p<0.05$ ) in hydration levels. Interestingly, 24 hours following SLS treatment, SC hydration further decreased. Change (%) compared to baseline is also shown. Significance is marked with (\*).



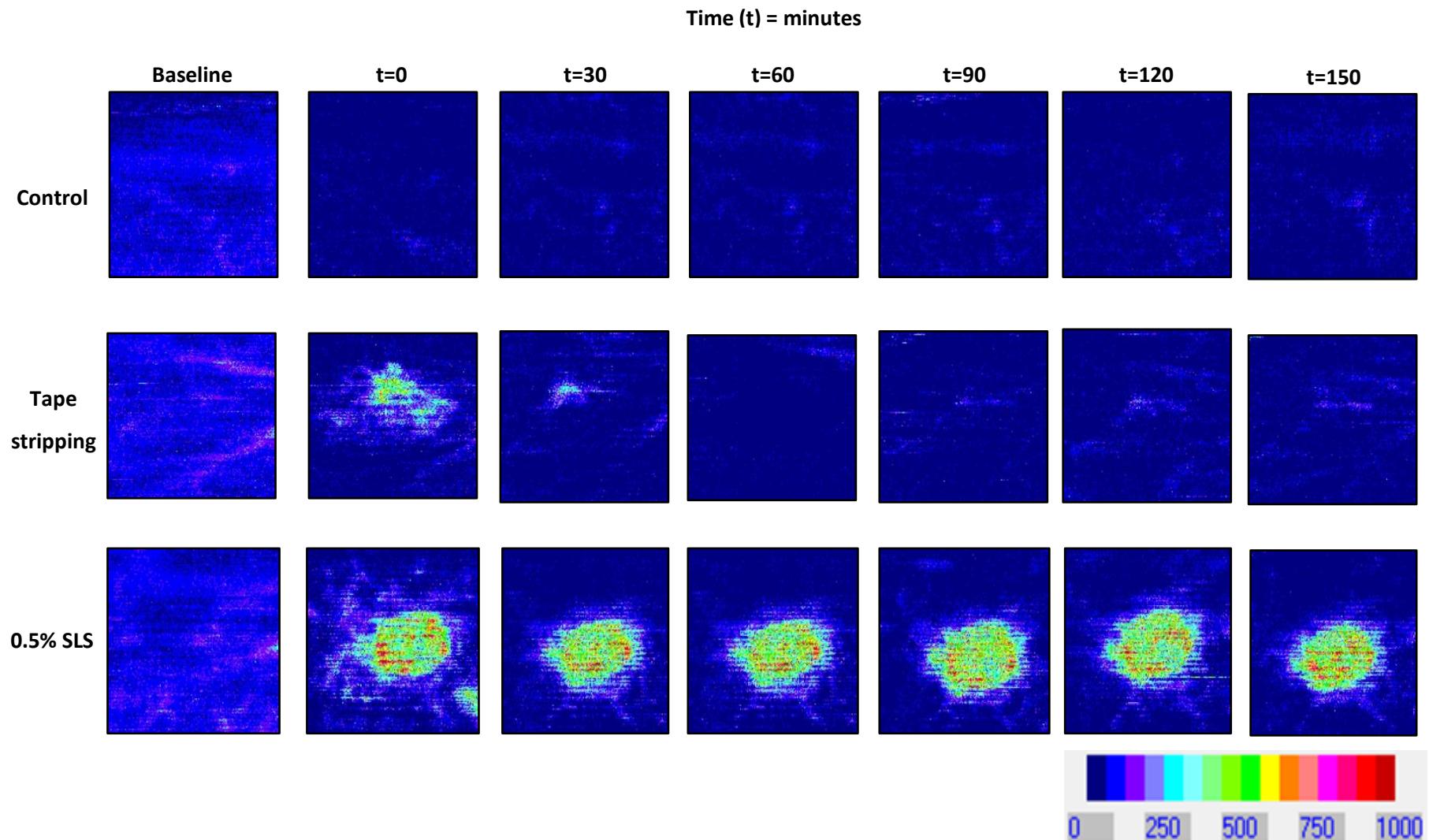
**Figure 5.17. The effects of s-urine on the acid mantle of chemically-irritated skin.** Both the SLS and urine led to a significant increase ( $p<0.05$ ) in skin pH compared to both baseline (+5%) and control values, however there was no significant difference between these two ( $p>0.05$ ). Significance compared to baseline and control values is indicated with (\*).

### 5.8.5 The temporal effects of tape stripping and SLS on blood perfusion

Subsequent to the initial study, it was important to investigate the temporal profiles of changes following both the mechanical- and chemical- based insults. This was examined with respect to blood perfusion. Typical examples of the two insults compared to intact skin, in the form of perfusion maps, are shown in Figure 5.19. By pooling the data, Figure 5.18 reveals differences in the temporal profiles. In particular, immediately following tape stripping there is a sharp increase in mean blood flux which subsequently is restored to baseline values. By contrast, following SLS treatment the increase in blood flux is maintained up to 150 minutes post insult.



**Figure 5.18. The temporal effects of tape stripping and SLS on blood perfusion.** Tape stripping causes a transient increase in blood perfusion compared to baseline values; however this returns back to basal levels and control values, within 30 minutes post-challenge. SLS causes a prolonged increase compared to baseline values, evident after 2 ½ hours post-challenge.



**Figure 5.19. Perfusion images of tape stripping and SLS over time.** It is clear that SLS causes a prolonged increase in blood perfusion. On the contrary, tape stripping initially causes an increase in blood perfusion, but following 30 minutes post-challenge it returns back to baseline and control levels. Baseline images have been subtracted from all post-challenge images.

## 5.9 Discussion

Maintaining skin integrity and promoting skin health is an important goal for clinical practice, especially for patients prone to incontinence. To achieve this, skin cleansing regimens are important, and commonly involve the use of soap and water, followed by drying the skin through a mechanical action using a towel (Voegeli, 2008). With respect to IAD development, it has been proposed that frequent cleansing leads to increased permeability and susceptibility of the skin to irritants (Beeckman *et al.*, 2009). Indeed one study which examined the effects of washing and drying techniques on skin biophysical properties, reported an increase in both TEWL and pH, thus compromising the integrity of the skin (Voegeli, 2008). However, the permeability of the skin to urine following cleansing activities has never been investigated, and this prompted the current investigation. Additional motivation was also provided by the findings of the previous study within this research thesis (Chapter 4). Therefore, the current study examined the hypothesis that an increase in urinary pH disrupts stratum corneum function leading to an increase in TEWL and skin surface pH, thereby compromising the integrity of the skin. The data presented here inform existing knowledge on IAD pathophysiology and enhance our understanding on current prevention strategies.

In the initial stage of the present investigation, ten healthy volunteers were recruited, and tape stripping and SLS were used as surrogates for frequent cleansing techniques, to compromise skin integrity (Pinkus, 1951; Schnetz *et al.*, 2000). S-urine was applied on both intact skin and on skin that had been compromised by both tape stripping and SLS. The original formulation of pH=7.9 was used (Mayrovitz and Sims, 2001), as a previous study within this thesis has shown that the effects of s-urine on the skin are not pH-dependent (chapter 4). When the skin is exposed to wetness for a fixed period, the resulting increase in TEWL subsequently returns back to baseline values, referred to as SSWL, equivalent to the amount of water that has penetrated the skin and is now effectively evaporating from the skin surface (Fader *et al.*, 2011). To quantify SSWL, desorption curves (Figure 5.2) were measured following s-urine exposure, by recording TEWL for 10 minutes and the area under the curve was calculated, corresponding to SSWL (Figure 5.3). To the best of author's knowledge, this is the first time that desorption curves of TEWL following exposure to s-urine have been estimated. As shown in Figure 5.3, SLS-compromised skin demonstrated a significantly increased permeability ( $p<0.05$ ) to s-urine, reflected by an increased SSWL, compared to the other two skin conditions. By contrast, no significant difference was found between intact and tape-stripped skin.

This finding may be due to the increased cohesiveness of deeper SC layers (Alikhan and Maibach, 2010), as tape stripping only removed superficial layers of SC and it is well-established that skin permeability depends on the full thickness of the SC (Fluhr *et al.*, 2002). In addition, increased permeability of SLS-damaged skin can be attributed to its mechanism of causing more severe and deeper damage, effectively penetrating the dermal-epidermal junction (Scheynius *et al.*, 1984; Ferguson *et al.*, 1985). SLS has been further proposed to disrupt the lipid composition of the SC, which is also linked with increased permeability (Wilhelm *et al.*, 1994b). These findings suggest that permeability to s-urine is dependent on skin integrity. With reference to IAD and the clinical relevance of findings, these propose that frequent cleansing routines might increase the risk of IAD, and specifically washing with soap which will lead to chemical irritation of the skin. Therefore, the frequency of skin cleansing regimens needs to be re-evaluated.

With regards to the effects of s-urine on the functional characteristics of intact skin, results are in agreement with a previous study (chapter 4), showing that exposure to s-urine led to an increased blood flow (Figure 5.4,  $p>0.05$ ), TEWL (Figure 5.5,  $p<0.05$ ) and SC hydration (Figure 5.6,  $p<0.05$ ). There was also no significant effect on skin pH (Figure 5.7). Physical irritation caused by towel drying and/or friction was simulated by tape stripping, which led to an increased blood perfusion (Figure 5.8) and a visible erythema. Additionally, it caused an increase in TEWL, and a positive correlation was found between the number of tapes and TEWL, up to a threshold value of 15 tape strips (Figure 5.9). The subsequent exposure to s-urine caused a significant reduction ( $p<0.05$ ) in blood flow (Figure 5.8), which was further examined in the second study to elucidate the role of s-urine. Results demonstrated that tape stripping causes a transient increase in the microcirculation which is restored back to baseline and control levels approximately 30 minutes post-challenge (Figure 5.18 and 5.19). This clearly suggests opposite effects of s-urine, that indeed it causes an increase in blood perfusion. This highlights the importance of investigating the temporal profile associated with tape stripping when examining the effect of s-urine treatment after 2 hours, in order to determine whether the results are caused by s-urine *per se* or not. By contrast, exposure of s-urine further increased both TEWL (Figure 5.11) and SC hydration (Figure 5.12), suggesting that its constituents exacerbate the effects of mechanical irritation with a subsequent delay of its barrier function recovery. This hypothesis is in agreement with a previous study which reported the effects of pressure loading on wet skin, exposed to s-urine (Mayrovitz and Sims, 2001). Other studies also reported a slowed but progressive barrier recovery followed disruption by tape stripping, based on TEWL measurements (Tanaka *et al.*, 1997; Fluhr *et al.*, 2002; Koopman *et al.*, 2004). In contrast, results for skin pH showed that both tape stripping and exposure to s-urine did not cause any significant changes in skin pH (Figure 5.13), and actually, tape stripping caused an initial decrease in skin pH which has been previously reported (Fluhr *et al.*, 2002).

## Chapter 5

The inability of tape stripping to disrupt the skin's acid mantle, and consequently its buffering capacity is possibly due to the limited number of tapes used or the tape brand. This contrasts with one study which reported that urine significantly increased pH on tape-stripped skin, at a level higher than that associated with an SLS-positive control (Farage *et al.*, 2014). A possible explanation for this is the discrepancy in the exposure period, which was three days in the previous study. Other limitations with this latter study include: a) the source of urine, as infant urine was applied on adult skin, and, b) the use of different anatomical sites, which are not comparable due to variation in TEWL. In view of these, the findings from that study are of limited physiological and clinical relevance.

To simulate chemical irritation from frequent cleansing with soap, the skin was challenged with 0.5% SLS for 24 hours. This resulted in an increased blood flow (Figure 5.14), visible as erythema, and a disrupted skin barrier function, evident by increased TEWL (Figure 5.15), due to hyperhydration of the SC and disorganization of the lipid bilayers (Wilhelm *et al.*, 1994b). The subsequent exposure to s-urine led to a further increase in blood perfusion (Figure 5.14), however, this might not be attributed to s-urine per se, as SLS treatment causes a prolonged response in blood perfusion, up to at least 150 minutes post challenge (Figures 5.18 and 5.19). This supports previous work reporting that following SLS exposure erythema is evident after 18 days (Wilhelm *et al.*, 1994b). This implies that s-urine may not have any significant effects on blood perfusion of severely damaged skin. Indeed, s-urine led to a decrease in TEWL (Figure 5.15), showing a slowed recovery of the barrier function. This is supported by the literature, as it has been reported that following SLS treatment, TEWL continues to increase, up to 14 days (Bruynzeel *et al.*, 1982; Tupker *et al.*, 1990; Wilhelm *et al.*, 1994b). The observed increase in the hydration status of the skin following SLS exposure can be explained by its continuous disruptive action on keratin proteins exposing new water-binding sites (Wilhelm *et al.*, 1994b). The subsequent s-urine treatment led to a decrease in skin hydration (Figure 5.16), but without any significant difference between the two challenges. Again this finding should be interpreted with caution, as SC hydration was also examined on SLS-compromised skin 24 hours following patch removal, showing decreased hydration levels (Figure 5.16). Therefore, it remains unclear whether decreased SC hydration is part of the response to SLS or due to s-urine per se. In contrast with tape stripping, SLS disrupted the buffering capacity of the skin as it increased skin pH significantly compared to basal and control values (Figure 5.17). However, the subsequent s-urine treatment produced little further change. Noteworthy, apart from TEWL measurement, there was no significant effect of urine on SLS-damaged skin. The increased permeability of chemically-irritated skin and the associated increase in skin pH, highlights the need for use of pH-balanced cleansers in clinical practice, to maintain the skin acid mantle and promote skin health.

It is clear that urine can be disruptive through different mechanisms, and indeed the severity of damage depends on the state of the skin. This contrasts the findings from a previous study which reported that the susceptibility to urine is not dependent on skin condition. Nevertheless, as mentioned above, that study is characterized by limited physiological relevance as infant urine was applied on adult skin (Farage *et al.*, 2014). Findings from the current study clearly add to the theoretical framework of IAD pathophysiology (Beeckman *et al.*, 2009), suggesting that urine itself can damage the integrity of the skin, however in combination with other factors, such as frequent cleansing activities, the risk of IAD is increased.

Further research is required to elucidate the role of skin care regimens in the development of IAD, and future studies should focus on characterizing the inflammatory response triggered following cleansing activities as literature is still limited on this topic. Importantly, the current investigation forms the basis for further studies to evaluate alternative skin care routines, including continence cloths and liquid cleansers. This will enhance our understanding on IAD development in clinical settings and will be a step forward towards more targeted preventive strategies. In the present investigation, tape stripping the skin with 20 strips was inadequate to compromise the buffering capacity of the skin, and accordingly in future studies an increased number of tapes should be used.

To summarize, informative findings have emerged from this investigation, as this is the first study to report the permeability and susceptibility of the skin to urine following irritation, such as from frequent cleansing routines. The data presented propose that when the skin is chemically-irritated it is associated with an increased susceptibility to urine, and consequently the frequency of skin cleansing with soap-based products should be determined.

## 5.10 Chapter summary

In Chapter 4, the effects of s-urine were investigated on intact skin and shown that its effects on skin barrier function are not pH-dependent. This provided motivation to examine the effects of s-urine on damaged skin. Accordingly, the present studies described in chapter 5 not only investigated the hypothesis that chemical- and physical- irritation from frequent cleansing activities increase the permeability of the skin but also its susceptibility to urine. Results showed that the permeability to urine was elevated on chemically-irritated skin with an elevated skin pH. This suggests that pH-balanced cleansers should be used in clinical practice to maintain the acid mantle of the skin surface.

## Chapter 5

Additionally, it was shown that urine can be disruptive through different mechanisms depending on the state of the skin, with urine having minimal effects on severely damaged skin. After determining the effects of urine on intact and compromised skin, Chapter 6 investigated the inflammatory response evoked by the skin following exposure to both urine and a proteolytic solution representative of faecal-incontinence.

# **Chapter 6: The inflammatory response triggered in the skin upon exposure to synthetic-urine, and a proteolytic-model of faecal-incontinence**

## **6.1 Introduction**

As discussed in section 2.4.5, pro-inflammatory cytokine release is considered to be critical for the development of IAD and the pathophysiological features of this condition. However, research to date has generally focused on prevention and management strategies for IAD, and the underlying inflammatory mechanisms have never been fully investigated, thus our knowledge remains incomplete. This research gap was addressed in the current mechanistic study, designed to provide an in depth insight into the inflammatory events triggered in IAD by investigating the hypothesis that exposure of the skin to urine and faeces leads to a release of inflammatory mediators .

## **6.2 Aim of the study**

- To investigate the release of pro-inflammatory cytokines following exposure of the skin to s-urine and to a proteolytic model of faecal-incontinence.

## **6.3 Objectives**

- Compare the effects of s-urine and a proteolytic-solution, against known irritant models on the functional characteristics of healthy skin
- Identify possible responsive markers of skin damage from incontinence
- Monitor the temporal effects of tape stripping and SLS exposure on cytokine release
- Compare the recovery of inflammatory mediators from the interstitium with microdialysis and from the skin surface with Sebutape

## 6.4 Participants

This study was completed in two stages and ethical approval was gained from the local university ethics committee (FHS no: 9349). Volunteer recruitment was based on previous inclusion and exclusion criteria (Table 3.11). In contrast to a previous power calculation (section 4.4) due to challenges in recruitment, mainly due to the invasive nature of the microdialysis technique and time pressures, only 10 volunteers were recruited.

- In the first stage, ten healthy volunteers (mean age  $\pm$  SD:  $33.80 \pm 9.07$ , 3 males, 7 females) were recruited to investigate the inflammatory response triggered in the skin, and was completed in two sessions. In the first session, s-urine, SLS and tape stripping were investigated, and in the second session the proteolytic solution and an untreated control site were included.
- In the second stage, four volunteers were involved (mean age  $\pm$  SD:  $28.75 \pm 5.38$ , 2 males, 2 females), three of which had participated in the initial stage. Volunteers had their volar forearm exposed to SLS for 24 hours and Sebutape samples from the skin surface were collected at baseline and post-exposure. This enabled the comparison of cytokine recovery from the SLS treated site following 2 and 24 hours of exposure.

Volunteers' informed consent was obtained before all investigations. As in previous studies, the use of any cosmetic products was restricted as they influence biophysical measurements (Held *et al.*, 1999; Lodén, 2012). The study was conducted in the bioengineering laboratory, within the Faculty of Health Sciences at University Hospital Southampton, under controlled temperature ( $22^\circ\text{C} \pm 3^\circ\text{C}$ ) and humidity (40-45%) conditions. Upon arrival to the laboratory, participants were left to adjust to the ambient conditions for at least 30 minutes.

## 6.5 Methods

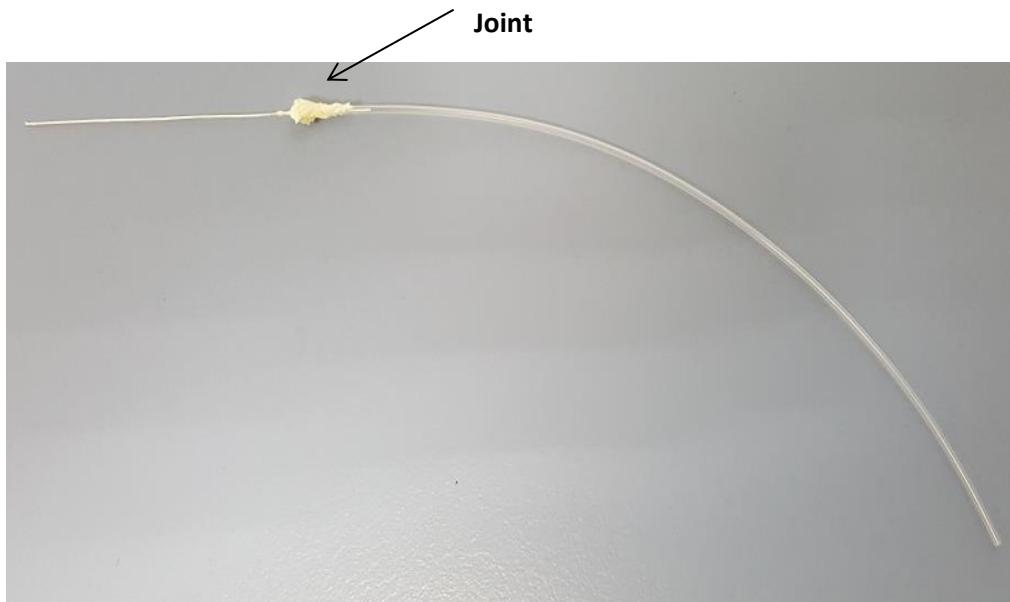
A combination of biophysical and sampling methods was employed to assess the functional characteristics of the skin and the cutaneous inflammatory response, respectively. These have been described in detail in Chapter 3. To simulate skin exposure to urine and faeces, s-urine ( $\text{pH}=7.9$ , Table 3.6), firstly described by Mayrovitz and Sims (2001), and a proteolytic solution (Andersen *et al.*, 1994; Mugita *et al.*, 2015) containing proteolytic enzymes and bile salts ( $\text{pH}=7.9$ , Table 3.7) were used, respectively. Two well-established skin challenges were also included and served as positive controls, namely, tape stripping and SLS (0.5% w/v, prepared in distilled water), as previously described (Chapter 5). SLS, s-urine and the proteolytic solution were applied to the skin for two hours using HillTop chambers (25mm, HillTop Labs, Miamiville, USA), soaked with 500 $\mu\text{l}$  solution, and a medical tape (3M, Transpore tape, UK) was used to keep them in place.

Tape stripping was performed 20 times using a commercially available tape (Sellotape™), and a pressure roller was used for constant application (Löffler *et al.*, 2004).

Changes in blood flow in the microcirculation were measured by the laser Doppler imaging system (LDI, Moor Instruments Ltd, Axminster, UK), which is able to provide continuous recordings. The Tewameter® TM 300 (MP WL, Courage & Khazaka, Germany) was used to measure TEWL, and represents the gold-standard method to assess skin barrier function, SC hydration was measured by electrical capacitance using a standard method, corneometry (Corneometer® CM 825, MP WL, Courage & Khazaka, Germany), and the Skin-pH-Meter® 905 (MP WL, Courage & Khazaka, Germany) was used to measure skin surface pH. Biophysical measurements were obtained at each skin site at baseline and following each challenge. To investigate the release of inflammatory mediators, two distinct sampling techniques were utilized, namely, the Sebutape™ (Cuderm Corporation, Dallas, TX, USA) absorption method and the minimally-invasive microdialysis that collect sample from the skin surface and the interstitium, respectively. These have been extensively used in several studies within the local research group (Voegeli *et al.*, 1999; Worsley *et al.*, 2016).

### 6.5.1 Construction of microdialysis fibres

Two different probes are mainly used in microdialysis studies, namely the linear and the concentric. These are available in different membrane types (i.e. hollow fibre) and pore sizes, ranging from 6 to 3000 kilo Daltons (kDa) (Schnetz *et al.*, 1999b; Schnetz and Fartasch, 2001). Due to the financial cost of commercial microdialysis fibres, these were manufactured inhouse (Figure 6.1). Briefly, haemophan fibres obtained from a haemofiltration cartridge (molecular weight cut off of 1000kDa) were cut into 50mm lengths and then a straightened medical grade stainless steel wire (diameter of 0.1mm ± 10%, Goodfellow, Cambridge Ltd, UK) was inserted using a magnifier, to ease implantation into the skin by making fibre manipulation easier. Each fibre was attached to a fine bore polyethylene tubing (100mm, inner diameter= 0.58mm, outer diameter= 0.96mm, Portex, Kent), and the joint was secured using a smooth silicone sealant (Unibond, UK). This was allowed to air-dry for 24 hours, and then the fibres were sealed into autoclave envelopes in groups of five and sterilised (Meridian Medical Ltd, UK). The main limitation with these home-made fibres is that they are characterized by increased fragility compared to the commercial counterparts, requiring careful handling. Frequently, the joint is the part that is damaged easily due to movement of the test area, and in case of a broken fibre *in situ* repair was conducted with a UV sensitive instant adhesive (Loctite 3301, Dusseldorf, Germany). Non-repairable fibres were discarded, and sample was obtained on the functional sites only.



**Figure 6.1. Construction of microdialysis fibres.** A stainless steel wire was inserted into the fibres and then attached to the polyethene tubing. The joint was sealed with a smooth sealant. In case of a damaged joint, Loctite instant adhesive was used for repair.

### 6.5.2 Efficiency of microdialysis

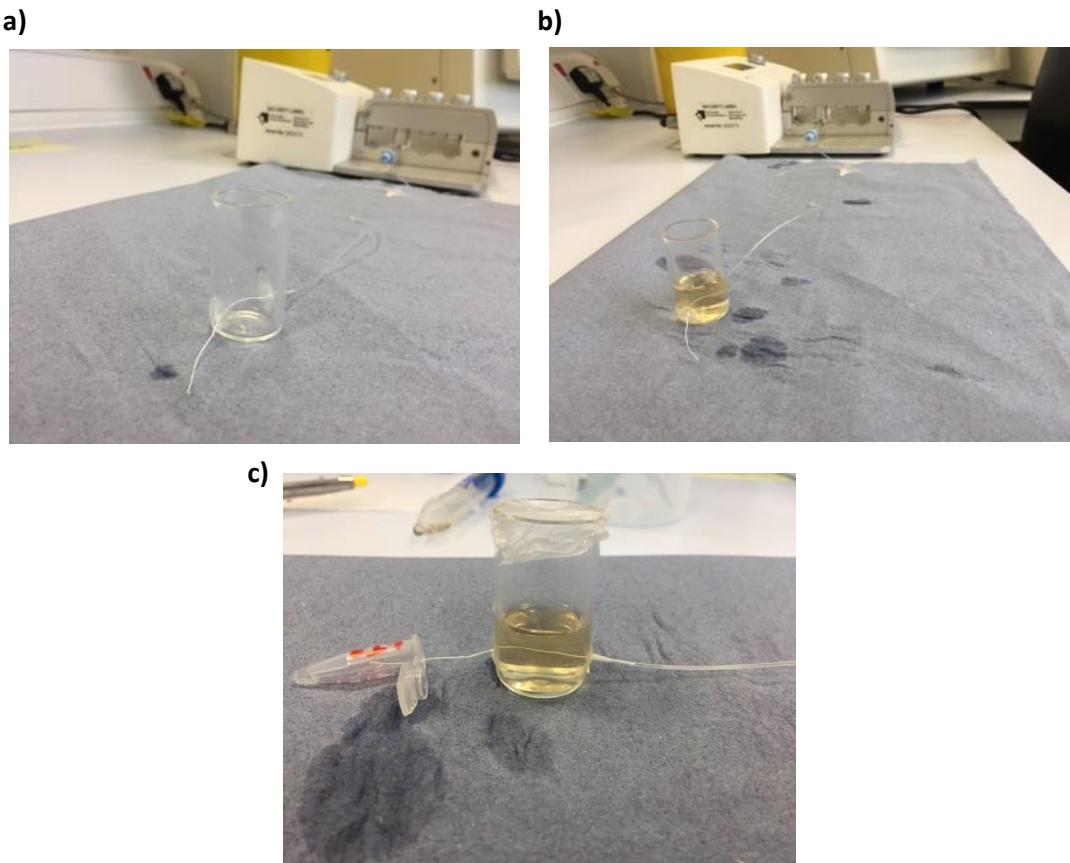
Microdialysis is considered to be a semiquantitative method, as the concentration of solutes collected in the dialysate does not correspond to the actual concentration in the extracellular fluid (Petersen, 1998). This is because the equilibrium between the extracellular fluid and the perfusate is incomplete, which facilitates diffusion across the membrane (Brunner *et al.*, 2000). Therefore, several methods have been developed to examine the efficiency of microdialysis, and in particular to establish the correlation between dialysate concentrations and *in vivo* concentrations. Examples of these methods include: 1) the zero and ultraslow flow rate method, 2) the dynamic no-net-flux method, 3) the no-net-flux or difference method, and 4) retrodialysis (Jacobson *et al.*, 1985; Lonnroth *et al.*, 1987; Wang *et al.*, 1991; Menacherry *et al.*, 1992; Olson and Justice, 1993; Wang *et al.*, 1993). In the current investigation, and prior to the study, it was important to determine the efficiency of microdialysis to recover the analytes of interest (IL-1 $\beta$ , IL-6, IL-8, TNF- $\alpha$ ). This was performed using the no-net-flux method, in which a microdialysis fibre was perfused with varying concentrations of the analyte of interest, in steady-state conditions. This experiment was performed at ambient temperature in the lab, and the following procedure was followed, based on previous work (Voegeli, 2001):

1. A single microdialysis fibre was passed through holes in both walls of a glass beaker and sealed with a PVA adhesive (Langlow, UK) (Figure 6.2a).

2. The bath was then filled with 5ml of the diluent provided in the assay kit containing all analytes (IL-1 $\beta$ , IL-6, IL-8, TNF- $\alpha$ ) at a known concentration (IL-1 $\beta$ =275.04pg/ml, IL-6=356.64pg/ml, IL-8=276.96pg/ml, TNF- $\alpha$ =176.64pg/ml), consisting of reconstituted Pro-inflammatory Panel 1 Human Calibrator Blend from the multiplex assay (K15053D-1, MesoScale Discovery, Rockville, Maryland, USA), and covered with wrapping film (Parafilm M<sup>TM</sup>, Fischer Scientific) to prevent changes in bath concentration due to evaporation. The fibre was immersed in the bath for a length of 20 mm (Figure 6.2b).
3. The fibre was then perfused at a flow rate of 5 $\mu$ l/min with two solutions with low concentrations of the analytes of interest and with one solution with higher concentrations to that of the bath, as summarized in Table 6.1. A 30 minutes equilibration period was allowed following the commencement of perfusion, and with each change in perfusate concentration, before samples were collected. Dialysates were then collected in vials at the exit of the fibres in 30 minutes intervals, for a total period of 90mins (Figure 6.2c).
4. As this method is based on steady-state conditions, it was assumed that the concentration of analytes in the bath would remain constant. Therefore, samples from the bath were also taken by pipette at the beginning and at the end of each perfusion.
5. Samples were analysed in duplicates using the MSD multiplex electrochemiluminescence assay, described at a later section.
6. For each analyte, the mean concentration perfused against mean concentration in dialysate minus concentration perfused was plotted, and microdialysis efficiency was determined from the slope of the regression line.

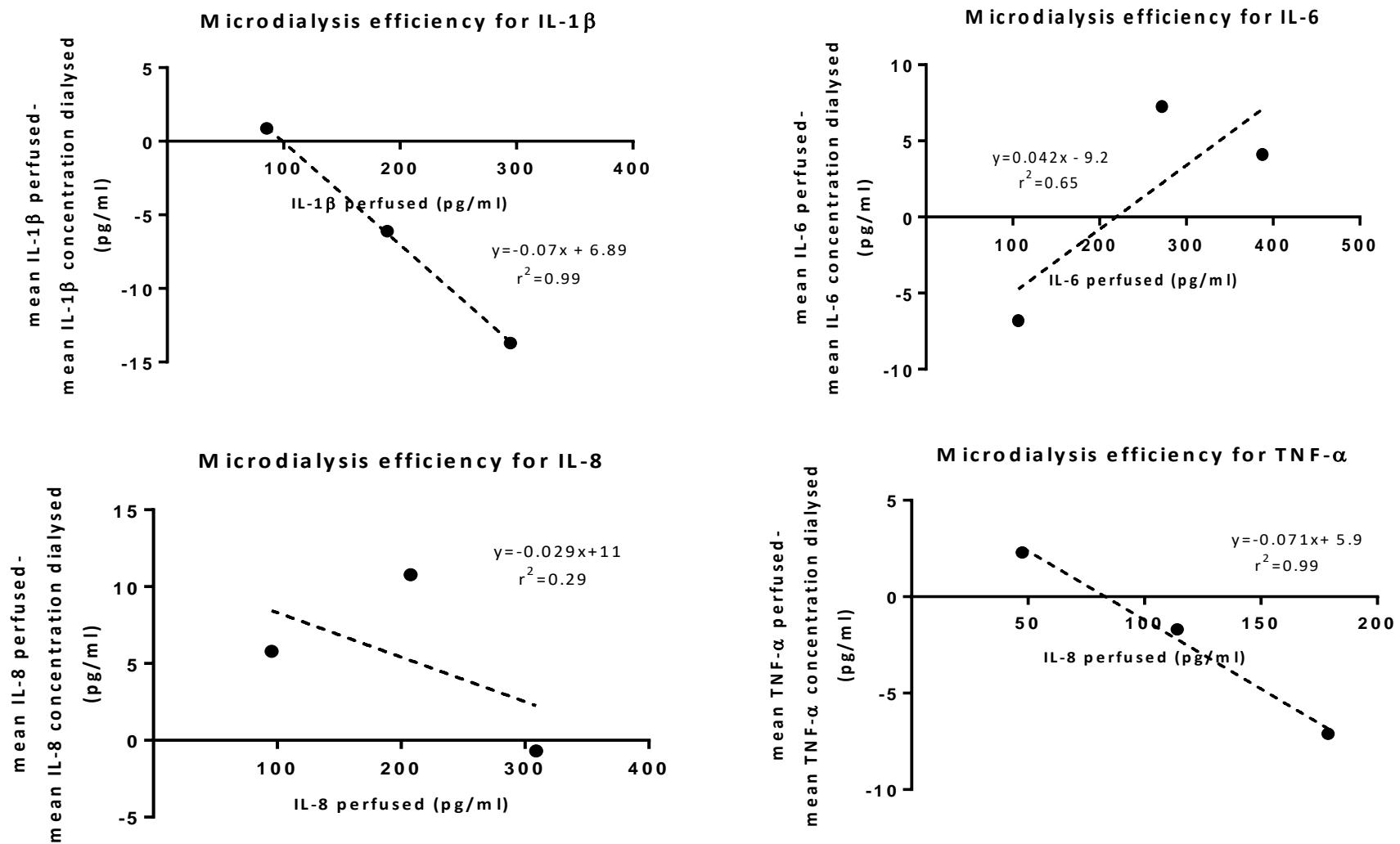
**Table 6.1. Investigation of efficiency of microdialysis for the recovery of analytes of interest.**

Analytes of interest	Lowest concentrations	Lower concentrations	Bath Solution concentrations	Higher concentrations
IL-1 $\beta$	85.95 pg/ml	189.09 pg/ml	275.04 pg/ml	280.77 pg/ml
IL-6	111.45 pg/ml	245.19 pg/ml	356.64 pg/ml	364.07 pg/ml
IL-8	86.55 pg/ml	190.41 pg/ml	276.96 pg/ml	282.73 pg/ml
TNF- $\alpha$	55.20 pg/ml	121.44 pg/ml	176.64 pg/ml	180.32 pg/ml



**Figure 6.2. No-net-flux method for the determination of microdialysis efficiency.** a) A microdialysis fibre was passed through the holes of a glass beaker and sealed. b) the bath was then filled with 5ml diluent containing known concentration of the analytes, and c) the fibre was perfused with different concentrations of analytes of interest (Table 6.1) at a flow rate of 5 $\mu$ l/min for 90 minutes. Dialysates were collected in vials in 30 minutes intervals and subsequently analysed using the electrochemiluminescence assay.

Results (Figure 6.3) showed that the efficiency of recovery was relatively low for all the analytes, ranging between 3-7%. In particular, the microdialysis efficiency was higher for IL-1 $\beta$  ( $7\% \pm 0.12\%$ ) and TNF- $\alpha$  ( $7\% \pm 1\%$ ). IL-6 presented an efficiency of  $4\% \pm 3\%$ , while IL-8 had the lowest efficiency ( $3\% \pm 5\%$ ). Nevertheless, these efficiency rates are higher compared to a previous study within the host group, which reported a low efficiency of microdialysis for these analytes of interest, ranging between 1-2.50% (Gray, 2017). The results obtained in the current study can be explained by the fact that microdialysis is affected by several parameters, including: 1) perfusate flow rate, 2) membrane length and pore size, 3) temperature, 4) perfusate composition and 5) the properties of analyte of interest (Hsiao *et al.*, 1990; Fartasch *et al.*, 1997; de Lange, 2013). Accordingly, these findings suggest that future studies involving microdialysis sampling should aim at increasing the efficiency of recovery by improving the design and manufacture of the fibres and/or by using a low perfusate flow rate.



**Figure 6.3. Efficiency of microdialysis for IL-1 $\beta$ , IL-6, IL-8 and TNF- $\alpha$ .** For each of the analyte, mean data were plotted and the efficiency was determined by the slope of the regression line. Microdialysis performed relatively poor, and the efficiency was between 3-7%.



## 6.6 Experimental protocol

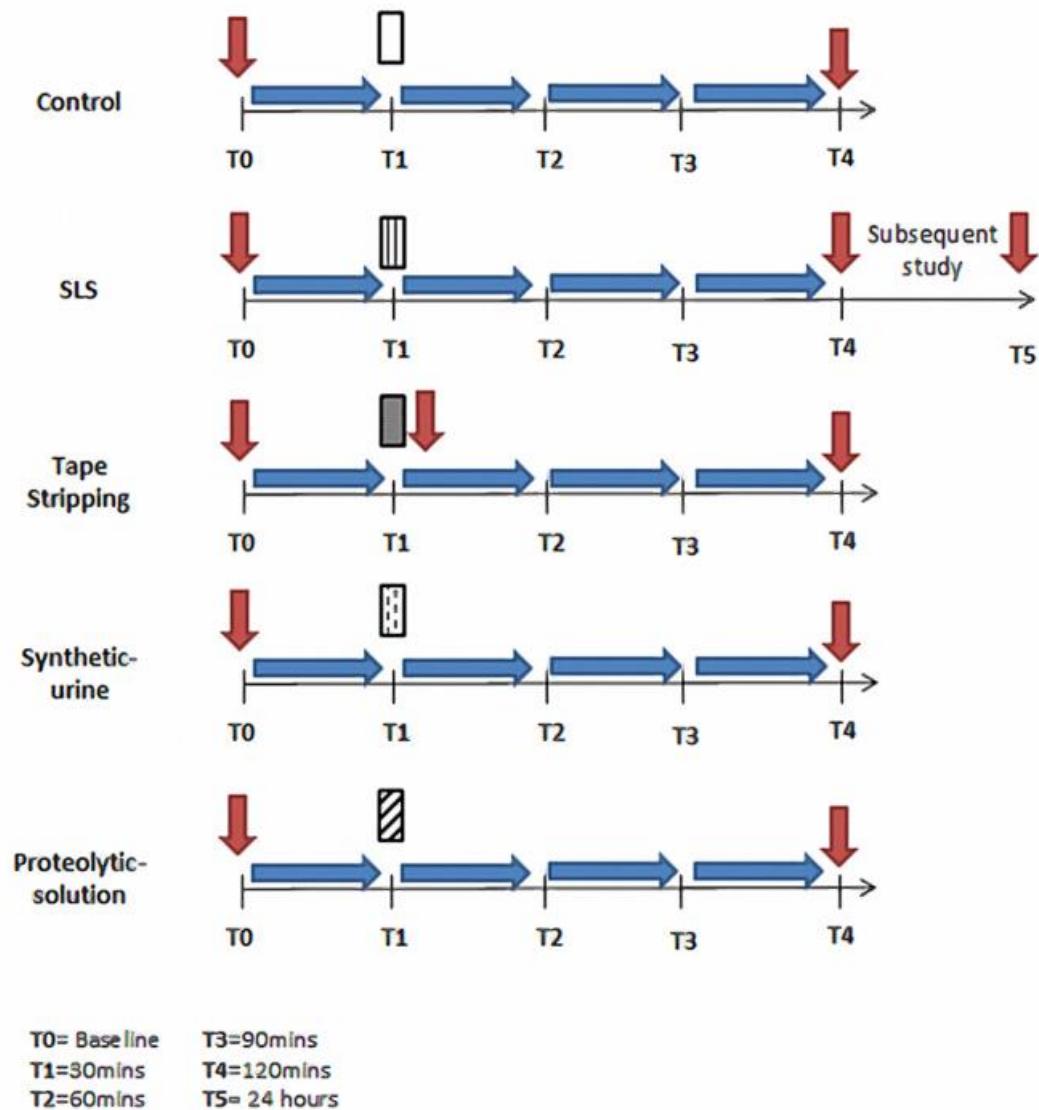
In the initial stage of the current study, volunteers attended the laboratory on two sessions, separated by two weeks, to ensure that any effects from the first visit had diminished. Prior to commencement, the integrity of microdialysis fibres was tested by perfusing sterile phosphate-buffered saline (PBS, Tayside Pharmaceuticals, Ninewells Hospital, Dundee) to ensure no leaks (Figure 6.4a). In the first session, volunteers applied a topical lidocaine/prilocaine anaesthetic cream (EMLA, Astra Zeneca) under an occlusive dressing (Tegaderm, 3M) for at least 90 minutes, on the three injection sites on the non-dominant arm, the left volar forearm, before fibre insertion (Figure 6.4b). Following arrival to the laboratory, the EMLA was removed, and the areas where the cream was applied were confirmed as numb by pinprick. Then, three skin sites were marked for needle insertion (Figure 6.4c) and three green hypodermic needles (21 gauge, Becton Dickinson, Microlance, Ireland) were inserted superficially at a length of 20mm and a depth of approximately 0.5-0.8mm (Figure 6.4d). The microdialysis fibres were then inserted through the needles and the needles removed, leaving just the fibres underneath the skin (Figure 6.4e). Entry sites were protected using a medical tape (Micropore, 3M), the fibres were covered with a moist dressing (Melolin, Smith and Nephew, UK) using 0.9% sodium chloride and bandaged, and the skin was allowed to rest for 2 hours, to recover from needle insertion and from any trauma caused (Groth and Serup, 1998). Subsequently, the bandage and dressing were removed, and the microdialysis fibres connected to a microinfusion pump (CMA 400 syringe pump, CMA, Sweden). The fibres were then perfused with sterile PBS (Tayside Pharmaceuticals, Ninewells Hospital, Dundee) at a constant flow rate of 5  $\mu$ l/ min. This rate was chosen to allow for sufficient sample volume for subsequent immunoassays. Baseline dialysate was collected for 30 minutes (Figure 6.4f). During baseline dialysate collection, biophysical measurements of LDI, TEWL, SC hydration and pH were taken at all skin sites. Baseline Sebutape samples (T0) were also collected (Figure 6.4g) from each site by applying the tape on the skin surface for 2 minutes using gloves, to avoid cross-contamination of proteins, and sterilized blunt forceps. A pressure roller was again used to ensure optimum contact with the skin. Upon removal, tapes were stored in vials on ice with their adhesive side facing upwards, as described by Perkins and colleagues (2001). Then, challenges were randomized to avoid selection bias and minimize variability using an online programme (Randomization., 2007). Tape stripping was performed on one site, with 0.5% SLS and s-urine applied on the remaining sites (Figure 6.4h). Sebutapes were also collected at the end of tape stripping to compare the recovery of cytokines immediately after disruption (T1) and at the end of the protocol (T4).

During each skin challenge, dialysate was collected for a total period of two hours, with collection vials changed at intervals of 30 minutes (T1-4). At the end of the collection time, all skin challenges were removed and the skin was gently pat dried with filter papers, and biophysical measurements and Sebutapes repeated at all sites. Samples obtained from microdialysis and Sebutapes were stored at -80°C until subsequent batch analysis. In the second session, an identical protocol was followed, but this time only two skin sites were used on the left forearm, with one being treated with a proteolytic-model of faecal incontinence, and the remaining site serving as an untreated control. Samples were again stored at -80°C until analysis. Microdialysis samples were collected throughout the course of the study, whilst Sebutape samples were collected at baseline (T0) and at specific time points at each site, as illustrated in Figure 6.5.

In the second stage, and to compare cytokine release from sites exposed to SLS for different times, SLS was applied on one site located on the left-forearm, and left *in situ* for 24 hours. Sebutape samples were taken at baseline and following removal of treatment (T5, Figure 6.5). Sebutapes were again stored at -80°C until analysis.



**Figure 6.4. Protocol to investigate the inflammatory response in the skin.** **a)** Integrity of the fibres was checked, **b)** EMLA was applied for 90 mins, **c)** three sites were marked, **d)** needles were inserted superficially at a length of 20mm, **e)** fibres were inserted through the needles and the needles removed, **f)** baseline dialysate was collected for 30 mins, **g)** baseline Sebutapes were collected, and **h)** challenges were applied and dialysate was collected for 2 hours



**Figure 6.5. Sample collection timings.** Microdialysis samples were collected throughout the study, as indicated by the blue arrows, whilst the red arrows correspond to the collection points of Sebutapes.

### 6.6.1 Sebutape™ sample preparation

Absorbed cytokines collected by the Sebutapes were extracted based on the protocol described by Perkins and colleagues (2001). However, a modification was made to the protocol, as previous work within the local research group revealed that the addition of 0.05% Tween™ 20 (a surfactant) to the PBS increased the recovery of the total protein from the Sebutapes and hence the quantification of cytokines (Gray, 2017). Accordingly, the tapes were thawed to the ambient temperature of the laboratory, and then 1.70 ml of PBS (Sigma) containing 0.05% Tween™ 20 (Fischer Scientific) was added to each tube, and ensured that the tape was fully submerged.

After immersion for 1 hour, the tapes were sonicated for 10 minutes to release proteins from the tapes into solution, vortexed vigorously for 1 minute and additionally mixed with a pipette tip. Then tape extracts were refrozen again at -80°C until cytokine analysis and total protein assays were performed.

### **6.6.2 Normalizing for total protein**

Due to the variation in cytokine levels recovered by each Sebutape, it was important to normalize the amount of cytokines extracted to the total protein concentration recovered from the Sebutapes. This was determined by the Bicinchoninic acid (BCA) protein assay kit (#23235, Pierce, Rockford, IL, USA), and was performed following manufacturer's protocol. The main principle underlying BCA protein assay is the detection of cuprous cation ( $Cu^{+1}$ ), which is formed by the reduction of  $Cu^{+2}$  by protein in an alkaline environment using BCA-contained reagent. It is a colorimetric technique based on a purple-coloured reaction product that is proportional to protein concentration. To review the protocol briefly, an 8-point standard curve (two-fold dilutions from 2000 to 0 mg/mL) from the supplied bovine serum albumin (BSA), and 150 $\mu$ l of standards and samples were pipetted in duplicates into a 96-well plate. Then 150 $\mu$ l working reagent was added to each well, and the plate was sealed and incubated at 37°C for 2 hours. Subsequently, the plate was cooled to the ambient temperature in the lab and absorbance was measured at 562nm using a VersaMax™ Microplate Reader (Molecular Devices, Sunnyvale, California, USA). The raw absorbance data obtained was corrected for background signal, by subtracting the average absorbance of the blank standards from the measurements of standards and samples. Unknown values were extrapolated from the standard curve, using a 2nd-order polynomial curve fitting equation, calculated using the inbuilt plate reader software (SoftMax Pro, Molecular Devices, Sunnyvale, CA). For every experiment, the goodness of fit for each standard curve was assessed using the  $r^2$  value, also known as the coefficient of determination, and was  $r^2 > 0.98$ , indicating a nearly perfect correlation.

### **6.6.3 Multiplex electrochemiluminescence immunoassays**

The inflammatory cytokines recovered by both the Sebutapes and microdialysis were quantified using commercially available electrochemiluminescence immunoassays (MesoScale Diagnostics, Rockville, Maryland, USA). For Sebutape samples, analysis of inflammatory markers was performed following the sample preparation protocol, whilst no preparation was required for microdialysis samples. For the analytes of interest, both singleplex and multiplex assays were purchased. In particular, IL-1 $\alpha$  and IL-1RA were investigated in singleplex assays, whilst IL-1 $\beta$ , IL-6, IL-8 and TNF- $\alpha$  were investigated in multiplex assays.

It should be noted here that as each dialysate corresponded to 150µl and each assay was performed in duplicates, requiring 50µl/well, samples from microdialysis were only analysed for IL-1 $\beta$ , IL-6, IL-8 and TNF- $\alpha$ . The median lower limit of detection (LLOD) and dynamic range of each of the cytokines examined are shown in Table 6.2, as reported by the manufacturer. For every assay the LLOD varied as it is calculated by the MSD software following analysis of the data. According to manufacturer's instructions the LLOD for each assay is set as the mean of the zero standard + 2.5 standard deviations.

**Table 6.2. Detection limit and dynamic range for each of the cytokines investigated.**

Cytokine	Median LLOD (pg/ml)	Dynamic range (pg/ml)
<b>IL-1<math>\alpha</math></b>	0.09	0.09-278
<b>IL-1RA</b>	1.12	1.12-650
<b>IL-1<math>\beta</math></b>	0.04	0.04-375
<b>IL-6</b>	0.06	0.06-488
<b>IL-8</b>	0.04	0.04-375
<b>TNF-<math>\alpha</math></b>	0.04	0.04-248

Each assay was performed according to the manufacturer's supplied protocol. To review briefly, all reagents were brought to room temperature prior to use. Then, a multianalyte lyophilized standard was reconstituted in 1ml of diluent (calibrator 1), depending on the assay, and then 7 more standards were prepared by sequential 4-fold dilutions of Calibrator 1 with the specified diluent. This resulted in an 8-point standard curve. At first, 50µl of standard or sample were added in duplicate to wells and the plate was sealed and shaken at 700rpm for 2 hours at the ambient temperature in the lab. Subsequently, the plate was incubated overnight at 4°C to increase the sensitivity of the assay and the binding of mediators to the capture antibodies. This is particularly important for low expressed mediators. The next day, the detection antibodies were prepared. As these were supplied in 50X stock solutions, they were diluted as required to the working concentrations (1X), using diluent 3, up to a total volume of 3ml. For example, for the multiplex assays, 60µl of each antibody were combined and 2760µl of Diluent 3 were added. Then, following three washes with 150µl of wash buffer (PBS+0.05% Tween™ 20), 25µl of detection antibody solution was added to each well, and the plate was shaken again, to accelerate capture of the mediator to the antibody attached to the well, for 2 hours.

During this period, the supplied read buffer (4X) was brought to room temperature and diluted with distilled water to a working concentration of 2X. After incubation time, the plate was washed again three times, and 150µl of read buffer was added per well and the plate was read immediately by the plate analyzer (MESO QuickPlex SQ 120, MSD, UK). Analysis was performed by the inbuilt software which provided the mean intensity signal and the concentration of cytokine in each well; in picograms (pg) per ml. Typical standard curves for each of the analytes investigated are shown in the Appendix C.

## 6.7 Data Analysis

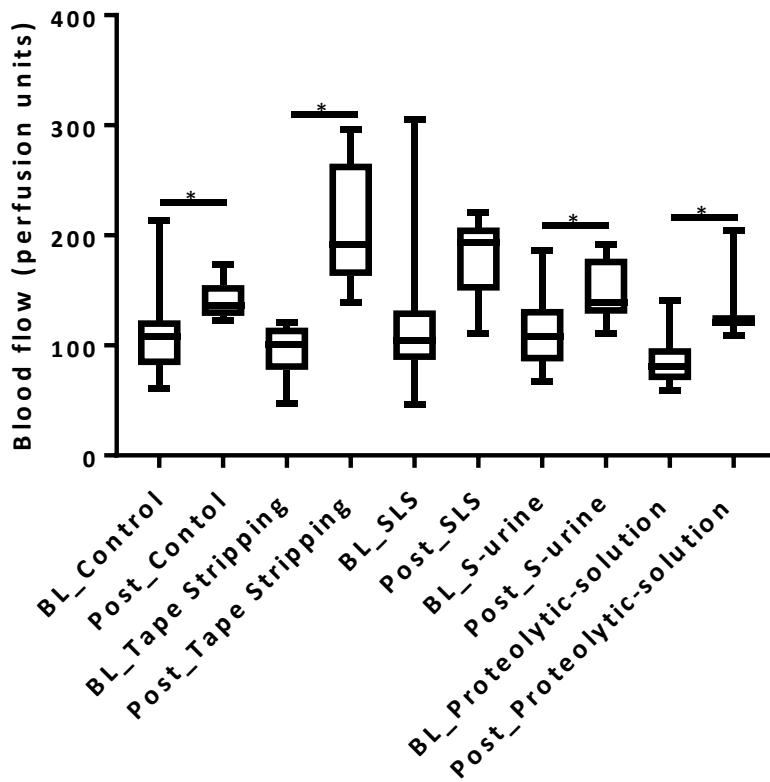
Results from the biophysical measurements were plotted as box plots with min to max whiskers at baseline (BL) and after each challenge. For each measurement, the percentage change to baseline is also presented in separate tables. Perfusion images from the LDI were analysed using the manufacturer's software (mLDI Main version 5.3, Moor Instruments Ltd, Axminster, UK), as described in section 4.7. With regards to cytokine analysis, for Sebutape samples, normalization for total protein seemed to disguise the findings as the resulting increase in cytokines for each challenge was not apparent. This has also been reported previously (Gray, 2017). Therefore, and by also considering the inter-individual variation in cytokine response profile, data are presented as ratio values post- to baseline for all treatments and for each cytokine. Data are also summarized in a separate table (Table 6.7) using non-parametric descriptors, namely median and interquartile ranges (IQR). Cytokine concentrations from microdialysis are presented as ratios to baseline (T0) for each time point (T1-4) for each challenge in separate tables. The raw data from Sebutape and microdialysis are also presented in Appendix D.

Statistical analysis was conducted using IBM SPSS version 21.0 (SPSS Inc., Chicago, IL, USA). Non-normal distribution of the data was assumed, due to small sample size, and non-parametric tests were performed. For each parameter post-exposure values were compared to baseline values, using the Wilcoxon signed-rank test. Subsequently, to evaluate the effect of treatment on each biophysical measurement and on cytokine release, data were analysed using the Friedman test and pairwise comparisons were performed using the Wilcoxon signed-rank test. It should be mentioned that statistical analysis was not performed on cytokine data from microdialysis on the control (n=2) and tape-stripped (n=1) sites due to the limited number of samples. Correlation analysis was also conducted to examine any relationship between cytokine groups, namely the IL-1 family and IL-1 $\alpha$ /IL-1RA. This was examined using the Spearman's rank-order correlation. Statistical significance was set at p<0.05.

## 6.8 Results

### 6.8.1 Skin Blood Flow

Results, as illustrated in Figure 6.6, showed that all challenges caused an increase in blood flow in the majority of the volunteers. In addition, by looking at Table 6.3, it is evident that tape stripping caused the highest average increase to baseline compared to the other challenges. Only 3 volunteers (P6, P8, and P10) showed a higher increase following SLS treatment. Exposure to s-urine and to the proteolytic solution also caused an increase in blood flow, and indeed in the majority of the volunteers the increase caused from the proteolytic solution was higher than s-urine. Additionally, two volunteers presented the highest increase in blood perfusion following treatment with the proteolytic solution. Percentage changes from baseline values (Table 6.3) were analysed using the Friedman test, and results showed significant differences ( $p<0.05$ ) between treatments. Further pairwise comparisons revealed that both tape stripping and SLS caused a significantly higher increase in blood flow compared to s-urine alone ( $p<0.05$ ). Although the proteolytic solution caused a higher increase in blood flow than s-urine, the difference was not significant ( $p>0.05$ ). In addition, no significant difference was found between tape stripping and SLS treatment ( $p>0.05$ ). However, when pre-post changes were examined, a significant change ( $p<0.05$ ) in skin blood flow was found at the tape-stripped site and on sites exposed to s-urine and the proteolytic solution. A significant increase ( $p<0.05$ ) was also observed at the control site compared to baseline, and this could be attributed to the effect of adjacent exposed sites.



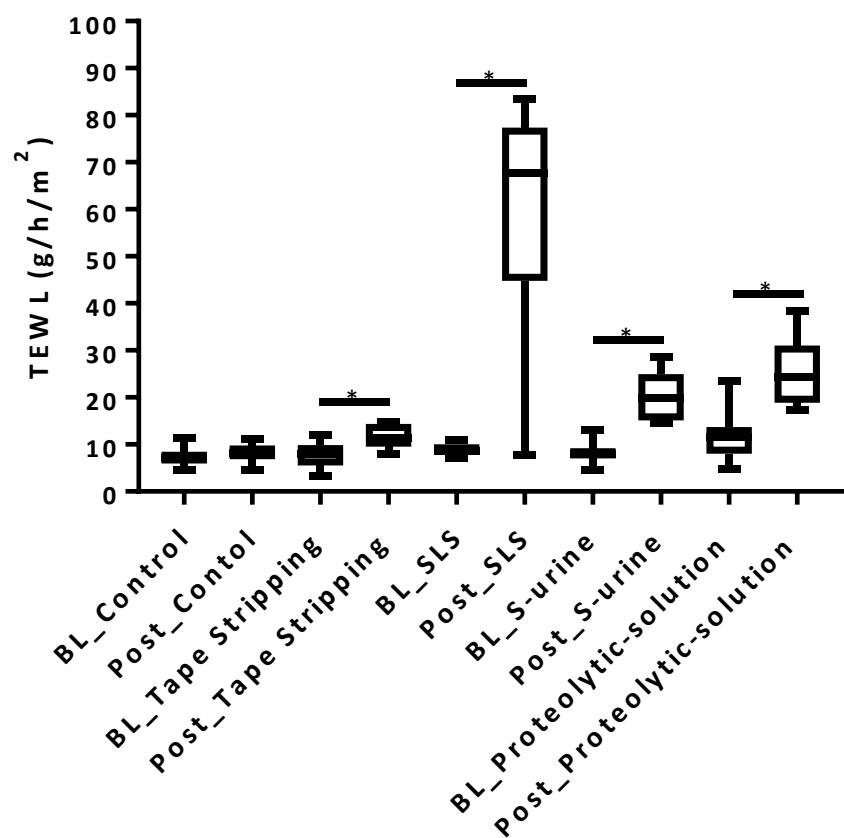
**Figure 6.6. Box-and-whisker plots for skin blood flow at baseline and after each challenge.** Tape stripping caused the highest average increase in blood perfusion. Both s-urine and the proteolytic solution led to increased blood perfusion, but the highest increase was observed with the latter. No significant differences were found between challenges on blood perfusion ( $p>0.05$ ). However, when assessing pre- and post- values, a significant increase was detected following tape stripping and exposure to s-urine and the proteolytic solution ( $p<0.05$ ). A significant increase was also observed at the control site compared to baseline values ( $p<0.05$ ). Significance is displayed with (\*).

**Table 6.3. Percentage change in blood flow from baseline at the control site and following exposure to s-urine, tape stripping, SLS and a proteolytic solution.** The highest mean increase in blood perfusion was observed with tape stripping. Exposure to S-urine and the proteolytic solution caused a milder increase in perfusion compared to both tape stripping and SLS.

Participants	% change in blood flow Vs Baseline				
	Control	Tape stripping	0.5% SLS	S-urine	Proteolytic-solution
P1	-19%	68%	-37%	-3%	58%
P2	11%	138%	82%	40%	39%
P3	19%	95%	31%	11%	103%
P4	117%	529%	139%	63%	13%
P5	31%	52%	-10%	9%	172%
P6	45%	69%	81%	97%	-16%
P7	69%	273%	177%	69%	19%
P8	15%	39%	109%	18%	27%
P9	50%	117%	76%	31%	88%
P10	38%	79%	131%	50%	110%
mean	38%	146%	78%	39%	61%
SD	37%	150%	67%	31%	57%
p value	0.03	0.01	0.06	0.01	0.01

### 6.8.2 TEWL

All challenges led to an increased TEWL value and to a consequent disruption of skin integrity (Figure 6.7). In particular, SLS treatment caused the highest increase in TEWL, and this was statistically significant compared to all the other treatments ( $p<0.05$ ). With respect to s-urine and the proteolytic solution, by looking at Table 6.4, describing the mean percentage change in TEWL compared to baseline values, it is clear that a similar response was caused, with the mean percentage change to baseline being 160% and 163%, respectively. However, s-urine was found to cause a higher increase in TEWL in seven of the volunteers. Statistical analysis showed no significant difference in TEWL between s-urine and the proteolytic solution ( $p>0.05$ ). Additionally, pre- and post- data analysis for each site revealed that all challenges caused a significant increase compared to baseline values ( $p<0.05$ ).



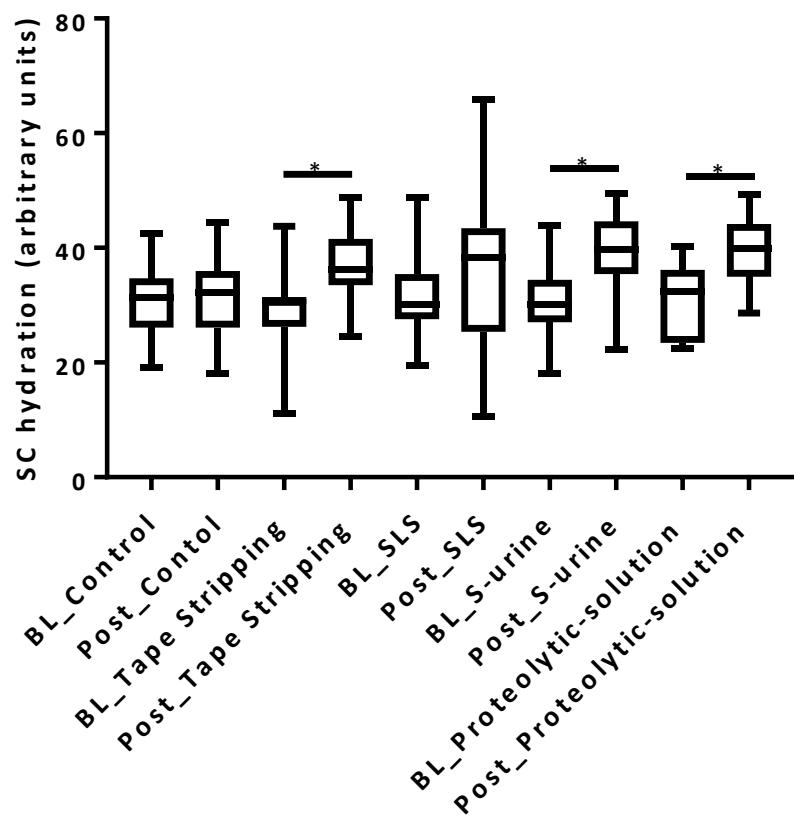
**Figure 6.7. Box-and-whisker plots for TEWL at baseline and after each challenge.** SLS caused the highest increase in TEWL and a severe disruption of the skin barrier function. Both s-urine and the proteolytic solution caused a significant increase in TEWL compared to baseline values, however no significant difference was found between them ( $p>0.05$ ). Data also revealed a significant increase in TEWL following each challenge compared to baseline values ( $p<0.05$ ). Statistical significance is displayed with (\*).

**Table 6.4. Percentage change in TEWL from baseline at the control site and following exposure to s-urine, tape stripping, SLS and a proteolytic solution.** It is evident that SLS caused a significantly higher TEWL compared to the other treatments ( $p<0.05$ ).

% change in TEWL Vs Baseline					
Participants	Control	Tape stripping	0.5% SLS	S-urine	Proteolytic-solution
P1	-18%	68%	393%	133%	103%
P2	-2%	46%	1%	121%	682%
P3	15%	39%	822%	263%	127%
P4	5%	24%	874%	246%	82%
P5	-2%	142%	453%	122%	80%
P6	30%	94%	693%	227%	147%
P7	-8%	17%	361%	88%	138%
P8	59%	21%	656%	95%	188%
P9	26%	81%	894%	238%	29%
P10	18%	57%	480%	69%	50%
mean	12%	59%	563%	160%	163%
SD	22%	39%	280%	74%	188%
p value	0.18	0.001	0.001	0.001	0.001

### 6.8.3 SC Hydration

An increase in the hydration levels of the SC was caused by all challenges, with tape stripping and the SLS treatment producing the highest and the smallest increase compared to baseline, respectively (Figure 6.8). In a similar manner to TEWL, there was no significant difference in SC hydration values following treatment with s-urine and the proteolytic solution (Table 6.5). By looking at the variation between individuals, it is clear that P2 presented low hydration values compared to the others. Analysis revealed no significant differences between challenges on SC hydration ( $p>0.05$ ). In a similar manner to blood perfusion, analysis of pre- and post-data revealed that tape stripping, s-urine and the proteolytic solution caused a significant increase in SC hydration compared to baseline values ( $p<0.05$ ).



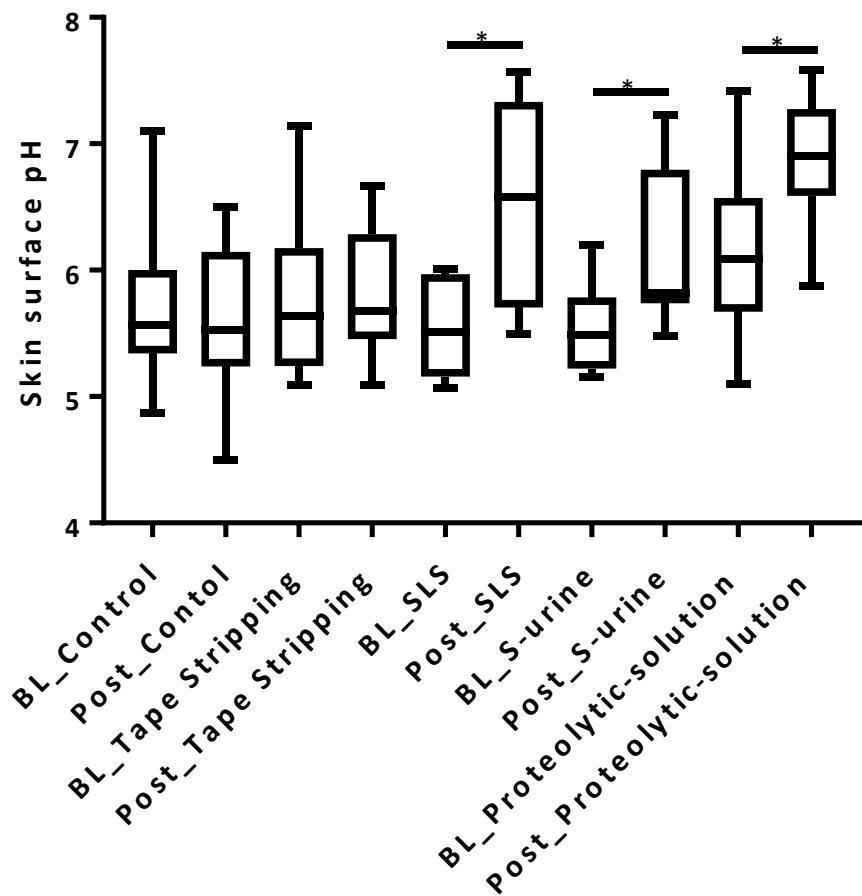
**Figure 6.8. Box-and-whisker plots for SC hydration at baseline and after each challenge.** All challenges caused an increase in SC hydration compared to baseline, but no significant differences were found between challenges ( $p>0.05$ ). Pre- and post-data revealed a significant increase in SC hydration following tape stripping and treatment with s-urine and the proteolytic solution ( $p<0.05$ ). Significance is shown with (\*).

**Table 6.5. Percentage change in SC hydration from baseline at the control site and following exposure to s-urine, tape stripping, SLS and a proteolytic solution.** All challenges increased the hydration status of the skin barrier, with s-urine and the proteolytic solution having a similar effect. No significant differences were found ( $p>0.05$ ).

% change in SC hydration Vs Baseline					
Participants	Control	Tape stripping	0.5% SLS	S-urine	Proteolytic-solution
<b>P1</b>	1%	13%	-11%	16%	22%
<b>P2</b>	-5%	121%	-46%	22%	42%
<b>P3</b>	2%	35%	37%	34%	26%
<b>P4</b>	10%	6%	-2%	39%	17%
<b>P5</b>	8%	26%	9%	58%	1%
<b>P6</b>	-1%	23%	59%	27%	55%
<b>P7</b>	-3%	22%	-25%	65%	21%
<b>P8</b>	4%	48%	11%	19%	58%
<b>P9</b>	5%	12%	35%	1%	38%
<b>P10</b>	5%	34%	59%	20%	22%
<b>mean</b>	<b>2%</b>	<b>34%</b>	<b>13%</b>	<b>30%</b>	<b>30%</b>
<b>SD</b>	<b>5%</b>	<b>33%</b>	<b>35%</b>	<b>20%</b>	<b>18%</b>
<b>p value</b>	<b>0.07</b>	<b>0.01</b>	<b>0.18</b>	<b>0.01</b>	<b>0.01</b>

#### 6.8.4 Skin pH

Exposure of the skin to s-urine and the proteolytic solution led to an increase in skin pH from baseline values, as shown in Figure 6.9. Only one participant failed to produce any change in pH following exposure to the proteolytic solution. However, both challenges revealed a similar response, with no statistically significant difference ( $p>0.05$ ), as the mean percentage change to baseline was 11% and 13% for s-urine and the proteolytic solution, respectively. The highest increase was caused by the SLS treatment (Table 6.6). However, and in spite of the fact that SLS, s-urine and the proteolytic solution caused a significant increase in skin pH compared to basal values ( $p<0.05$ ), no significant difference was found between them ( $p>0.05$ ). By contrast, tape stripping did not result in any significant changes in pH compared to basal values ( $p>0.05$ ).



**Figure 6.9. Box-and-whisker plots for skin surface pH at baseline and after each challenge.** SLS, s-urine and proteolytic solution caused a significant increase ( $p<0.05$ ) in skin pH from baseline, however no differences were found between challenges ( $p>0.05$ ). There was no significant effect of tape stripping on skin pH ( $p>0.05$ ). Significance is shown with (\*).

**Table 6.6. Percentage change on skin surface pH from baseline at the control site and following exposure to s-urine, tape stripping, SLS and a proteolytic solution.** All the challenges, except tape stripping, caused an increase in skin pH, with the highest increase caused by the SLS treatment. Mean percentage change for s-urine and proteolytic solutions was very similar, 11% and 13% respectively.

% change in skin surface pH Vs Baseline					
Participants	Control	Tape stripping	0.5% SLS	S-urine	Proteolytic-solution
P1	5%	-1%	8%	6%	19%
P2	-2%	-3%	3%	5%	-2%
P3	-3%	10%	26%	12%	13%
P4	3%	0%	9%	8%	4%
P5	-12%	4%	3%	7%	35%
P6	-5%	-2%	20%	8%	2%
P7	5%	-2%	28%	4%	13%
P8	3%	9%	44%	21%	13%
P9	-9%	-8%	23%	22%	15%
P10	2%	1%	26%	18%	15%
mean	-1%	1%	19%	11%	13%
SD	6%	5%	13%	7%	10%
p value	0.76	0.10	0.01	0.01	0.01

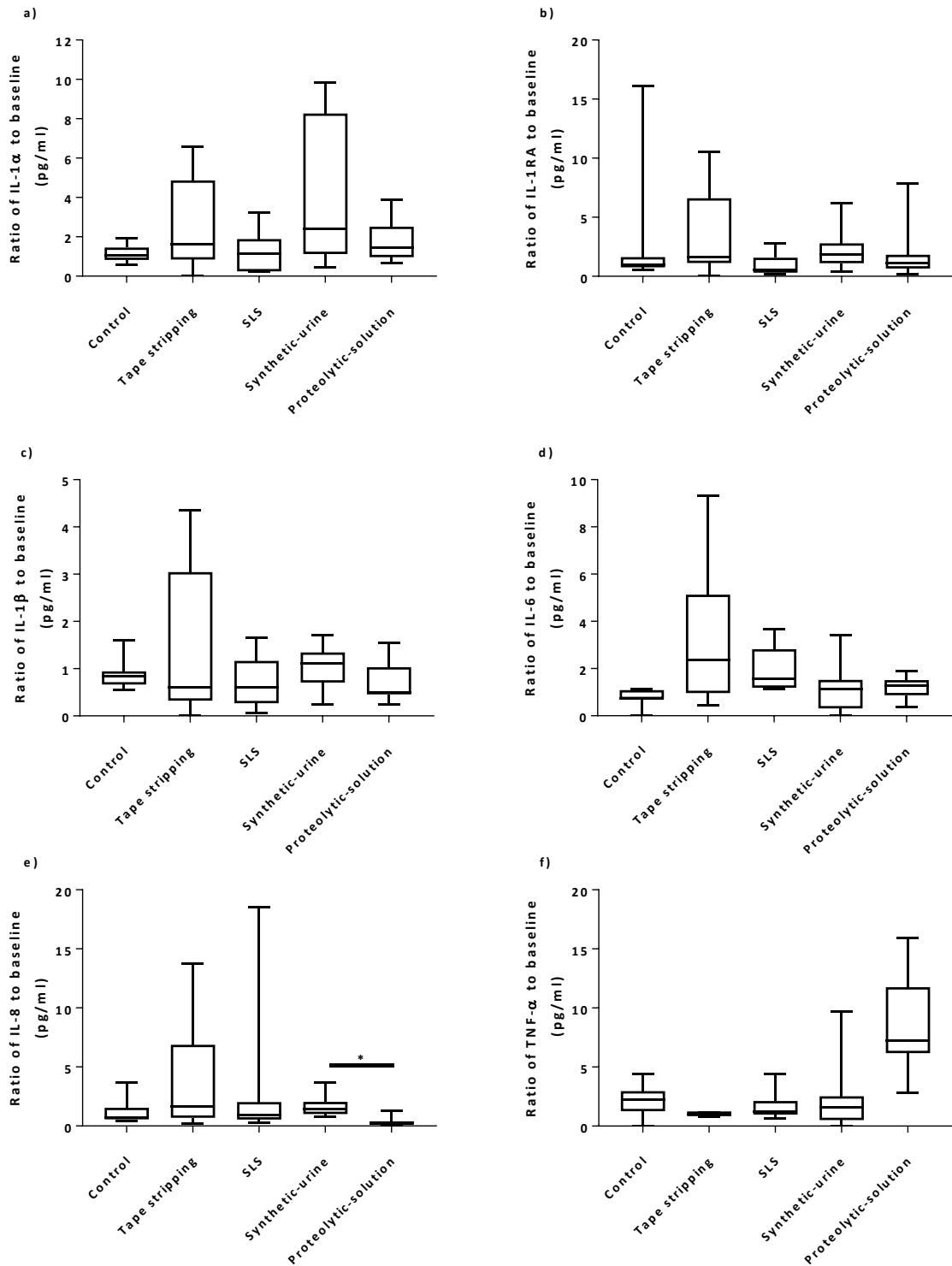
### 6.8.5 Release of inflammatory mediators

#### Cytokine analysis- Sebutapes

The pro-inflammatory cytokines IL-1 $\alpha$ , IL-1 $\beta$ , IL-8 and the anti-inflammatory IL-1RA were successfully quantified from Sebutape samples of all challenges. For IL-1 $\alpha$  (Figure 6.10a) and IL-1RA (Figure 6.10b), results showed that all challenges led to an increase in their levels compared to basal levels, except the SLS treatment, in which the majority of the volunteers showed either no change or a minimal decrease. Additionally, exposure to s-urine resulted in the highest increase in both IL-1 $\alpha$  and IL-1RA with a median ratio to baseline of 2.43 and 1.83 (Table 6.7), respectively, however the differences were not statistically significant ( $p>0.05$ ). Indeed, as can be seen from the raw data in Appendix D, P8 presented an extreme increase in IL-1 $\alpha$  levels following s-urine exposure (851 pg/ml). For IL-1 $\beta$  (Figure 6.10c), results showed that none of the challenges caused any considerable change in its levels, and indeed there was a tendency for this cytokine to decrease in the majority of the volunteers. For IL-8, all challenges, apart from the proteolytic-solution, resulted in an upregulation of IL-8 production (Figure 6.10d), and indeed there was a significant difference between s-urine and the proteolytic solution ( $p<0.05$ ). Additionally, tape stripping caused the highest median ratio change when compared to baseline (1.67). For IL-6 (Figure 6.10e) more than half of the samples were below the detection limit of the assay. In a similar manner, a number of samples were below the limit of detection for TNF- $\alpha$  (Figure 6.10f). Accordingly, for analysis and to calculate ratio changes to baseline, values below the LLOD were substituted with one half of the detection limit, as reported in previous studies (Djuardi *et al.*, 2010; Djuardi *et al.*, 2013). However, TNF- $\alpha$  was quantified in all samples collected from the site treated with the proteolytic solution. Indeed, there was a high increase from baseline, with a median ratio of 7.25 (Figure 6.10f), although the differences were not significant ( $p>0.05$ ). A small increase in cytokine levels was also observed at the untreated control site and particularly for IL-1RA and TNF- $\alpha$ . Considering the results from skin blood flow measurement (section 6.7.1), these findings also suggest that adjacent compromised sites influence cytokine release at the untreated site.

**Table 6.7. Ratio to baseline values of cytokines following each challenge.** Data is expressed as median (IQR).

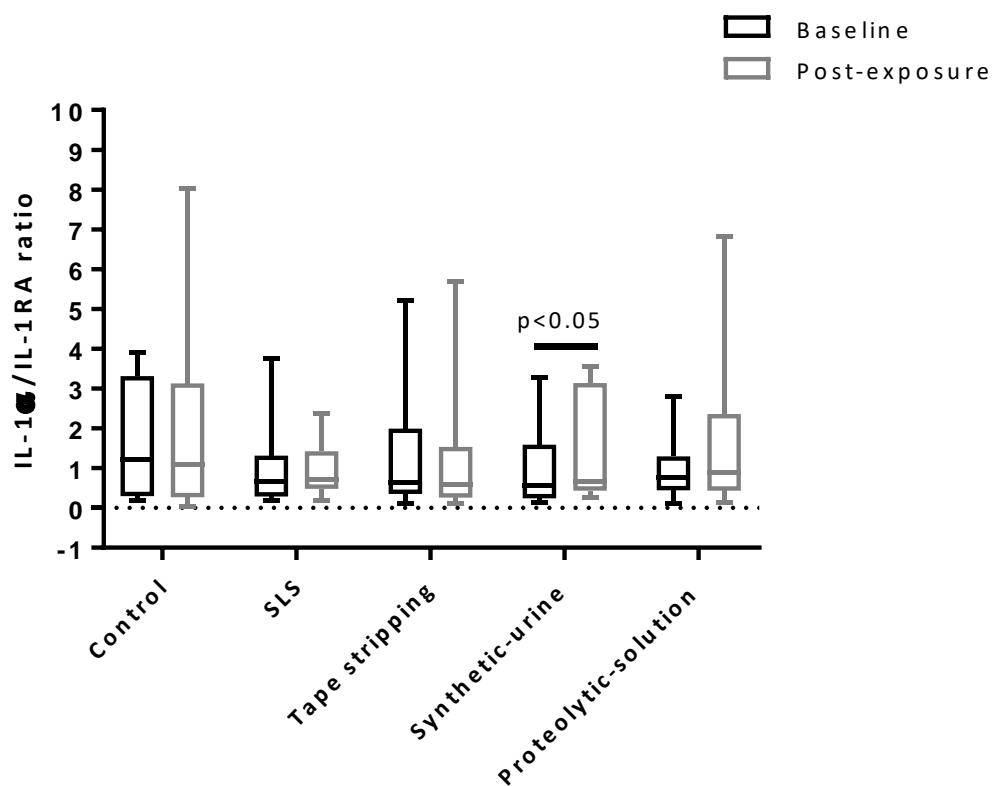
Cytokines, median (IQR)	Ratio change to baseline				
	Control	0.5% SLS	Tape stripping	S-urine	Proteolytic- solution
IL-1 $\alpha$	1.04 (0.88- 1.32))	1.16 (0.27- 1.74)	1.72 (1.35- 4.52)	2.43 (1.44- 6.77)	1.43 (1.01-2.25)
IL-1RA	0.98 (0.79- 1.48)	0.57 (0.35- 1.47)	1.69 (1.44- 6.38)	1.83 (1.42- 2.41)	1.13 (0.77-1.44)
IL-1 $\beta$	0.84 (0.68- 0.87)	0.60 (0.27- 1.01)	0.76 (0.39- 2.82)	1.11 (0.74- 1.27)	0.50 (0.48-0.88)
IL-6	0.86 (0.57- 1.00)	1.00 (1.00- 3.27)	1.38 (1.00- 2.81)	1.00 (1.00- 1.00)	1.05 (0.68-2.25)
IL-8	0.67 (0.58- 0.95)	0.90 (0.58- 1.88)	1.67 (0.73- 6.52)	1.41 (1.09- 1.70)	0.31 (0.14-0.36)
TNF- $\alpha$	2.52 (1.49- 3.50)	1.00 (0.93- 1.08)	1.23 (0.99- 1.97)	1.20 (1.00- 1.63)	7.25 (6.64-10.34)



**Figure 6.10. Box and whiskers plots of cytokine ratio changes to baseline (n=10, except n=9 for tape stripping).** a) S-urine caused the highest median increase in IL-1 $\alpha$ , b) Highest increase in IL-1RA was also observed with s-urine, c) IL-1 $\beta$  results varied and there was a tendency to decrease, d) most of the data for IL-6 were below the detection limit of the assay, and no meaningful data could be extracted, e) All challenges caused a an increase in IL-8, except the proteolytic solution. S-urine caused a significant increase in IL-8 levels compared to the proteolytic solution and f) the proteolytic solution caused a high increase in TNF- $\alpha$ ., with a median ratio change to baseline of 7.25. Significance is marked with (\*).

### Ratio of IL-1 $\alpha$ and IL-1RA

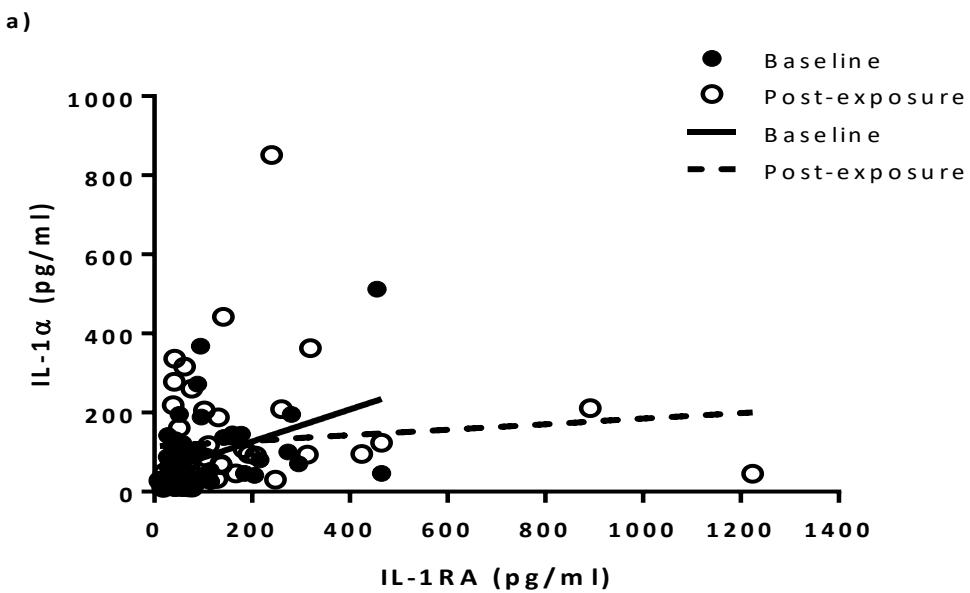
Tight regulation of IL-1 $\alpha$  activity is essential in the skin, and this is achieved by the binding of the anti-inflammatory IL-1RA to IL-1RI. Therefore, further analysis examined the changes in the ratio of IL-1 $\alpha$ /IL-1RA, as this is critical for the cutaneous inflammatory response (Robinson *et al.*, 2003; De Jongh *et al.*, 2006). Results, as presented in Figure 6.11, showed that there is an increase in this ratio with all challenges, except tape stripping, and indeed this was significant at the s-urine treated site ( $p<0.05$ ). Additionally, this was observed in the majority of the volunteers ( $n=9$ ). A small decrease was also observed at the control site post-challenge.



**Figure 6.11.** Box-whisker plot of IL-1 $\alpha$ /IL-1RA ratio at baseline and following each challenge. All challenges caused an increase in the ratio of IL-1 $\alpha$ /IL-1RA, except tape stripping. However, this was only significant on the s-urine treated site ( $p<0.05$ ).

### Relationship between cytokine groups

Results from all challenges were pooled together and secondary analysis was performed to explore a possible relationship between IL-1 $\alpha$  and IL-1RA, and between members of the IL-1 family, at baseline and post-challenge. Results showed a significant correlation between IL-1 $\alpha$  and IL-1RA at baseline ( $R=0.43$ ,  $p<0.05$ ) and following the challenge ( $R=0.10$ ,  $p<0.05$ ), as illustrated in Figure 6.12a. Similarly, a significant correlation was also found between IL-1 $\alpha$  and IL-1 $\beta$ , at baseline ( $R=0.30$ ,  $p<0.05$ ) and following the challenge ( $R=0.37$ ,  $p<0.05$ ), as illustrated in Figure 6.12b.



Baseline

$$y = 0.41x + 46$$

$$R^2 = 0.19$$

$$r = 0.43$$

$$p < 0.05$$

Post-exposure

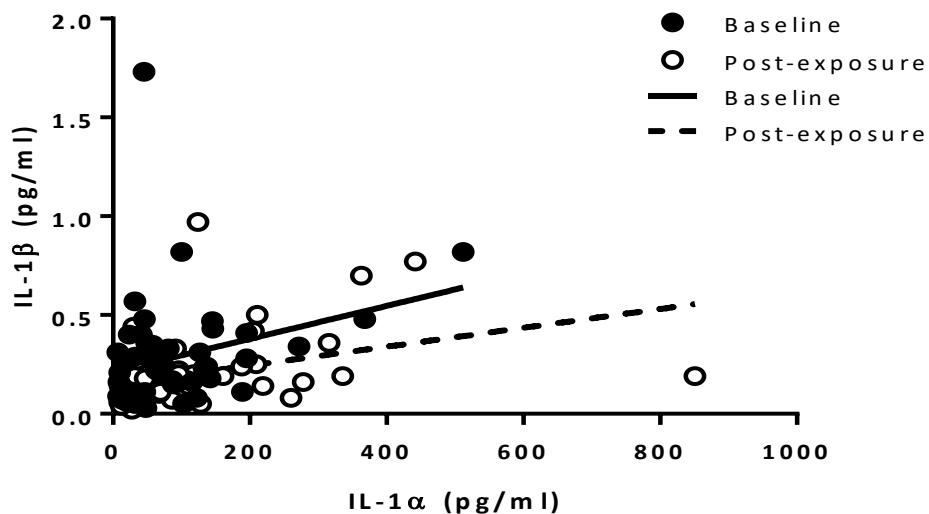
$$y = 0.07x + 115$$

$$R^2 = 0.01$$

$$r = 0.10$$

$$p < 0.05$$

b)



Baseline

$$y = 0.00083x + 0.21$$

$$R^2 = 0.09$$

$$r = 0.30$$

$$p < 0.05$$

Post-exposure

$$y = 0.0005x + 0.15$$

$$R^2 = 0.14$$

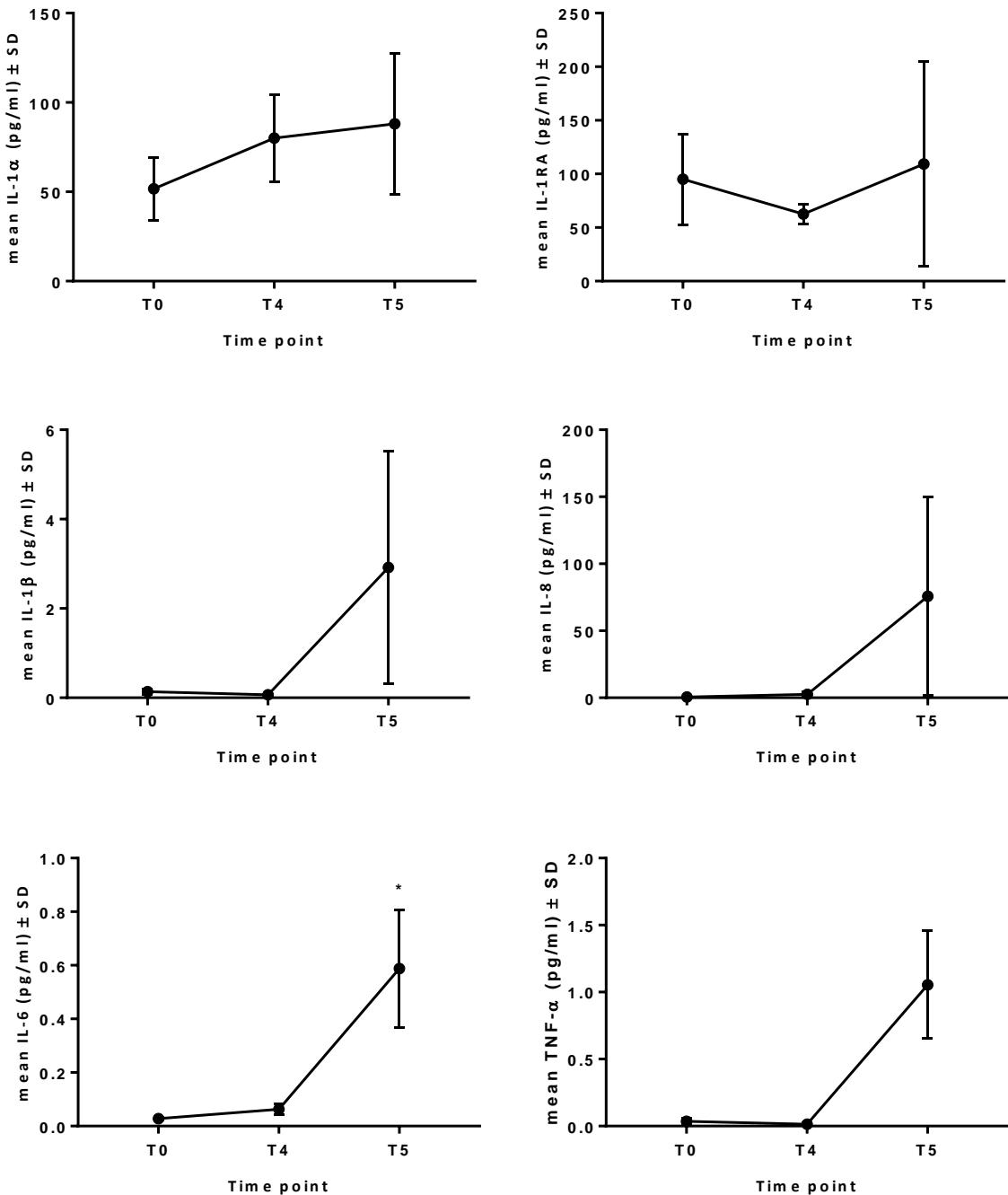
$$r = 0.37$$

$$p < 0.05$$

**Figure 6.12. Relationship between cytokine groups.** a) a significant correlation was found between IL-1 $\alpha$  and IL-1RA, b) a significant correlation was found between IL-1 $\alpha$  and IL-1 $\beta$

### Comparison of cytokine levels at the SLS-treated site at T0, T4 and T5

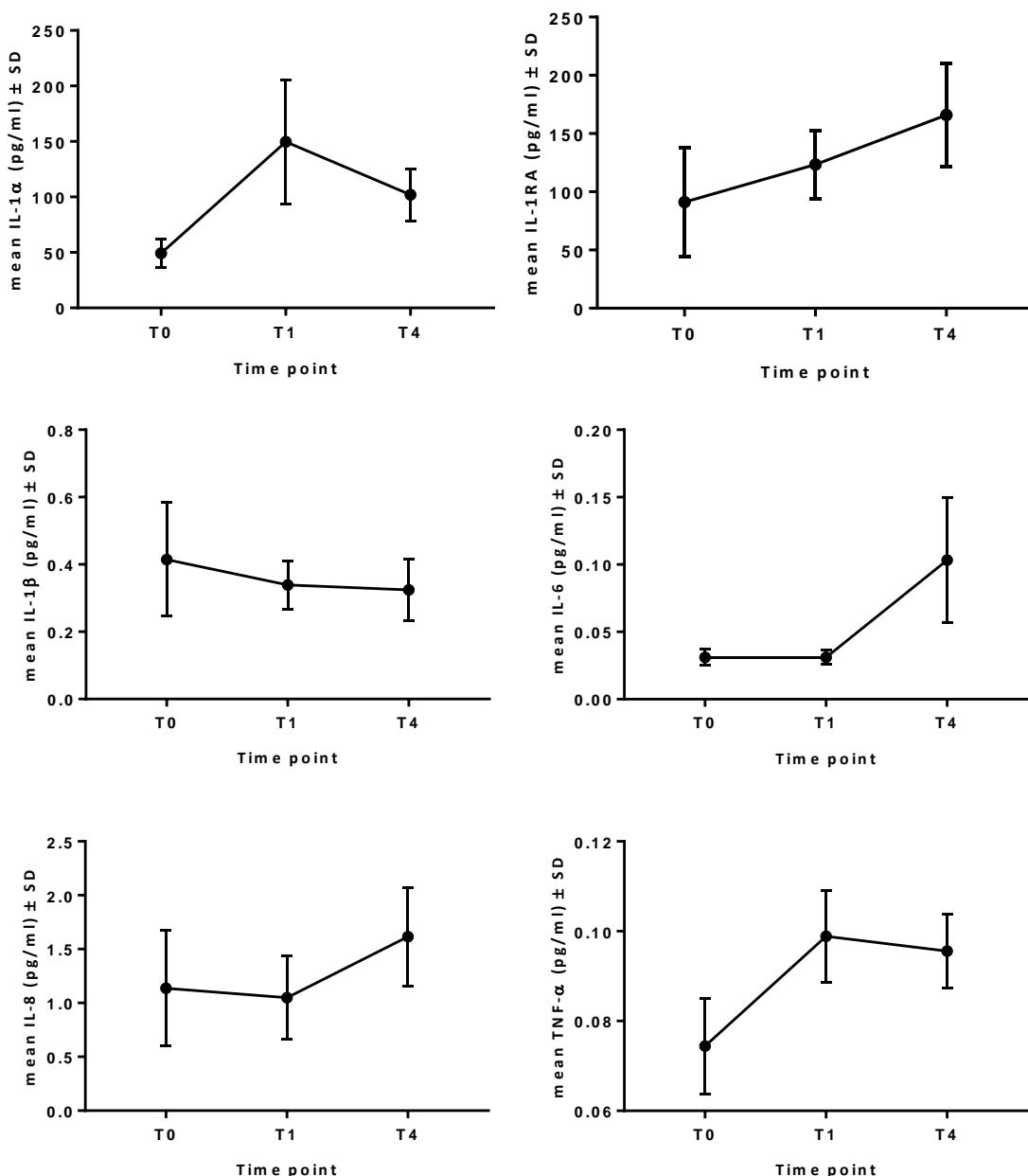
Cytokine release was also compared following exposure to SLS for two (T4) and 24 (T5) hours and results showed increased levels of all cytokines at T5 compared to T4 (Figure 6.13), however no significant differences were found between the two time points ( $p>0.05$ ). Only IL-6 presented significantly increased levels at T5 compared to T0 ( $p<0.05$ ).



**Figure 6.13. Cytokine levels at T0, T4 and T5 following exposure to SLS.** There is a general increase in cytokines at T5 compared to T4, however this was not significant ( $p>0.05$ ). A significant increase is only observed for IL-6 at T5 compared to baseline (T0) levels ( $p<0.05$ ). Significance is marked with (\*).

### Comparison of cytokine levels at the tape-stripped site at T0, T1 and T4

Sebutape samples from the tape-stripped site were obtained at three different time points, allowing thus the comparison of the levels of cytokines recovered at T1 and T4 in relation to T0 (baseline). Results, as presented in Figure 6.14, showed a small increase in the concentrations of IL-1RA, IL-6 and IL-8 at T4. On the contrary, a slight decrease was evident in the levels of IL-1 $\alpha$  and TNF- $\alpha$ , compared to T1. Nevertheless, no significant differences were found ( $p>0.05$ ). Compared to baseline, increased levels of IL-1 $\alpha$ , IL-1RA and TNF- $\alpha$  were detected at both T1 and T4, while for IL-6 and IL-8 increased levels were observed at T4 only. IL-1 $\beta$  also didn't show any major changes at T1 and T4, compared to T0.



**Figure 6.14. Cytokine levels at T0, T1 and T4 at the tape-stripped site.** A number of cytokines (IL-1RA, IL-6, IL-8) were increased at T4 compared to T1, whilst others (IL-1 $\alpha$ , TNF- $\alpha$ ) presented a decrease at T4, two hours after the challenge. However, no significant differences were found ( $p>0.05$ ).

### Cytokine analysis- Microdialysis

In contrast with the Sebutape samples, all the pro-inflammatory cytokines were successfully detected in microdialysis samples, and only a small number of samples were below the detection limit of the TNF- $\alpha$  assay.

#### Control site

Consistent with the results from Sebutapes, a small increase in cytokine levels (IL-1 $\beta$ , IL-8 and TNF- $\alpha$ ) compared to baseline was also observed at the control site (Table 6.8). Samples from one volunteer were below the detection limit of TNF- $\alpha$  assay and hence were assigned values half the detection limit (LLOD= 0.09pg/ml) of the assay.

**Table 6.8. Ratio changes to baseline of cytokine concentrations at the control site (n=2).**

Cytokine, median (IQR)	Control			
	T1	T2	T3	T4
IL-1 $\beta$	0.77 (0.70-0.84)	1.18 (1.17-1.20)	2.98 (2.79-3.17)	3.14 (3.08-3.21)
IL-6	0.91 (0.87-0.96)	1.48 (1.25-1.72)	0.99 (0.85-1.13)	0.65 (0.53-0.77)
IL-8	0.67 (0.45-0.88)	2.17 (1.37-2.96)	2.95 (1.99-3.92)	3.22 (1.82-4.62)
TNF- $\alpha$	0.69 (0.37-1.02)	2.57 (1.31-3.84)	2.66 (1.35-3.96)	5.15 (2.60-7.70)

#### SLS treatment

Varied results were obtained from cytokine analysis of the SLS-treated site (Table 6.9), requiring caution interpretation. In particular, for IL-1 $\beta$ , only one volunteer presented a consistent increase at T1-4, while P4 and P8 produced increased levels at T3-4. On the contrary, three volunteers (P3, P6, and P7) did not show any increase in the levels of IL-1 $\beta$ . For IL-6, only one volunteer presented a consistent increase compared to baseline. Additionally, for IL-8 and TNF- $\alpha$  there was a decrease in these cytokines in the majority of the volunteers, and only P2 and P3 showed an increase in IL-8 and TNF- $\alpha$  levels, respectively. Statistical analysis revealed no statistically significant differences for cytokine levels at T1-4 ( $p>0.05$ ).

**Table 6.9. Ratio changes to baseline of cytokine concentrations at the SLS-treated site (n=8).**

Cytokine, median (IQR)	SLS			
	T1	T2	T3	T4
IL-1 $\beta$	0.95 (0.77-1.24)	0.60 (0.41-0.95)	0.99 (0.79-1.65)	1.12 (0.60-1.38)
IL-6	0.94 (0.81-2.37)	1.39 (0.43-1.55)	1.67 (0.86-3.58)	0.94 (0.50-1.75)
IL-8	1.03 (0.64-1.81)	0.79 (0.38-1.33)	1.31 (0.56-2.70)	0.72 (0.39-0.94)
TNF- $\alpha$	0.84 (0.39-1.16)	0.37 (0.00-0.95)	1.28 (0.67-1.55)	0.80 (0.67-0.97)

**Tape stripping**

On the tape stripped site, dialysates were collected from one volunteer, and as shown in Figure 6.17, the levels of IL-1 $\beta$  were decreased, while the other cytokines were considerably increased at T3-4, suggesting a time-dependent relationship. For TNF- $\alpha$  most of the samples were below the detection limit of the assay, and were assigned a value equal to half of the LLOD of the assay (LLOD= 0.05pg/ml).

**Table 6.10. Ratio changes to baseline of cytokine concentrations at the tape-stripped site (n=1).**

Cytokine, median (IQR)	Tape stripping			
	T1	T2	T3	T4
IL-1 $\beta$	0.49 (0.49-0.49)	0.51 (0.51-0.51)	2.04 (2.04-2.04)	0.69 (0.69-0.69)
IL-6	0.78 (0.78-0.78)	0.59 (0.59-0.59)	7.63 (7.63-7.63)	0.84 (0.84-0.84)
IL-8	1.48 (1.48-1.48)	0.31 (0.31-0.31)	5.45 (5.45-5.45)	0.80 (0.80-0.80)
TNF- $\alpha$	1.00 (1.00-1.00)	1.00 (1.00-1.00)	1.00 (1.00-1.00)	4.61 (4.61-4.61)

**Synthetic-urine**

Commonly there was an increase in the levels of all pro-inflammatory cytokines investigated following exposure to s-urine. Indeed, cytokine concentration increased with time, and this was more evident with IL-1 $\beta$  (Table 6.11). For the other cytokines, a time-dependent increase was observed, which tended to plateau at T3-4. In particular, for IL-1 $\beta$ , the majority of the volunteers presented an increase. Specifically, three volunteers (P2, P4, and P6) showed a consistent increase at T1-4, whereas in two volunteers (P5, P9) IL-1 $\beta$  was increased at T3-4 only. On the contrary, the remaining volunteers displayed a decrease in the levels of this mediator. The levels of IL-6 were increased in all volunteers, except P3 and P7 who showed the opposite. Likewise, IL-8 and TNF- $\alpha$  also presented an increase during s-urine exposure, evident in all volunteers.

In addition, P4 showed consistent increase in all cytokines, and particularly for IL-8 the levels detected were extremely higher compared to baseline (112-177 pg/ml). Table 6.11 also shows the ratio changes to baseline for each cytokine. No significant differences were found between cytokine levels at T1-4 ( $p>0.05$ ).

**Table 6.11. Ratio changes to baseline of cytokine concentrations at the s-urine treated site (n=9).**

Cytokine, median (IQR)	Synthetic-urine			
	T1	T2	T3	T4
IL-1 $\beta$	0.88 (0.58-1.34)	0.95 (0.77-1.53)	1.17 (0.61-1.97)	1.43 (0.49-1.83)
IL-6	0.74 (0.00-1.06)	0.91 (0.65-2.35)	1.17 (0.00-2.52)	0.86 (0.68-0.95)
IL-8	1.37 (0.80-1.62)	2.10 (0.82-5.19)	1.47 (1.04-2.83)	1.38 (0.93-5.85)
TNF- $\alpha$	0.77 (0.49-0.90)	0.78 (0.00-1.62)	1.40 (0.95-2.44)	1.24 (1.20-1.52)

### Proteolytic-solution

Results from cytokine analysis of the site exposed to the proteolytic-solution are summarized in Table 6.12. Only two volunteers (P1, P4) presented a consistent increase between T1-4. The other volunteers showed varied results which are dependent on the time point, and indeed three volunteers (P3, P7, and P8) presented a decrease in IL-1 $\beta$ . Noteworthy, P3 was characterized by high levels of IL-1 $\beta$  at baseline (2.19 pg/ml) but following exposure to the proteolytic-solution these were decreased. With regards to the other cytokines, results clearly showed a time-dependent increase in cytokine levels, evident in all volunteers. Again, no significant differences were found ( $p>0.05$ ).

**Table 6.12. Ratio changes to baseline of cytokine concentrations at the proteolytic-solution treated site (n=8).**

Cytokine, median (IQR)	Proteolytic-solution			
	T1	T2	T3	T4
IL-1 $\beta$	0.79 (0.57-1.19)	0.78 (0.49-1.06)	1.58 (0.82-2.35)	1.23 (0.90-2.00)
IL-6	2.04 (1.67-2.32)	2.11 (1.51-3.06)	3.21 (2.58-6.80)	3.09 (1.55-13.53)
IL-8	1.21 (1.02-2.47)	1.46 (0.85-2.52)	2.19 (1.10-4.33)	2.33 (0.55-8.72)
TNF- $\alpha$	1.02 (0.00-1.25)	0.90 (0.00-1.18)	1.42 (0.00-1.55)	1.20 (0.00-1.55)

## 6.9 Discussion

The inflammatory nature of IAD is well-known. However, the mechanisms triggered in the skin following exposure to incontinence have never been investigated. In this respect, the aim of the current mechanistic study was to investigate the release of pro-inflammatory cytokines following exposure of the skin to two experimental models of urinary- and faecal-incontinence. Their effects on the functional characteristics of the skin were also examined. Well-established skin irritation models such as SLS and tape stripping were also used for comparison.

Results from the biophysical measurements, showed that all challenges led to an increase in all parameters, with s-urine and the proteolytic solution presenting a similar response to TEWL and SC hydration. In addition, for skin blood flow (Figure 6.6) the increase caused by the proteolytic solution was slightly higher than s-urine. However, no visible erythema was observed from either s-urine or the proteolytic solution, and this is partly in agreement with previous studies that reported the appearance of erythema after prolonged exposure to urine and faecal material for 48 hours (Berg *et al.*, 1986; Farage *et al.*, 2014). By contrast, SLS and tape stripping were found to cause a higher response in skin blood flow (Figure 6.6), TEWL (Figure 6.7) and SC hydration (Figure 6.8). With regards to skin pH (Figure 6.9), SLS caused the higher increase while tape stripping failed to produce any changes in pH. This is consistent with the results from a previous study, described in Chapter 5, confirming again the inability of tape stripping to compromise the acid mantle of the skin. Both s-urine and the proteolytic-solution led to an increase in pH, with the latter treatment producing a higher increase in pH, which can be explained by the presence of proteolytic enzymes, digesting the SC and possibly disrupting the mechanisms responsible for acidification of the skin surface. In regards to s-urine exposure, this is in contrast with the findings from a previous study (Chapter 5) in which it was reported that s-urine only causes a minimal change in pH (1%). A possible explanation for this is the good buffering capacity of the skin, as it was demonstrated previously that s-urine challenges only cause a temporary increase in skin pH which returns back to basal values within five minutes post-removal of the challenges (section 4.8.2). Accordingly, as skin pH measurement was taken following the desorption curves of TEWL (10 minutes) any changes in pH would have diminished.

With reference to the inflammatory response and the mediators released, this was investigated by employing two distinct techniques, which sample the skin at different depths. Microdialysis was used to analyse the biomarkers released in the interstitial tissue (depth of approximately 0.5-0.8mm), while Sebutape™ is a lipophilic tape that collects sebum at the skin surface. This enabled a comparison between these two techniques, which is presented in Table 6.13.

**Table 6.13. A comparative critique of dermal microdialysis and Sebutape™ absorption method**

Characteristics	Microdialysis	Sebutape™
<b>Analytes</b>	<ul style="list-style-type: none"> <li>• IL-1<math>\beta</math>, IL-8 successfully quantified</li> <li>• Superior for IL-6 and TNF-<math>\alpha</math></li> <li>• Low recovery of analytes, which can be improved by low perfusate flow rate and fibre manufacture</li> </ul>	<ul style="list-style-type: none"> <li>• Suitable for IL-1<math>\alpha</math>, IL-1RA, IL-8 and IL-1<math>\beta</math> detection</li> <li>• Not appropriate for IL-6 and TNF-<math>\alpha</math></li> </ul>
<b>Experimental procedure</b>	<ul style="list-style-type: none"> <li>• Minimally-invasiveness</li> <li>• Tolerated by the volunteers</li> <li>• In situ repair is required for any non-functional fibres</li> </ul>	<ul style="list-style-type: none"> <li>• Non-invasive</li> <li>• Well-tolerated by the volunteers</li> </ul>
<b>Clinical adaptation</b>	<ul style="list-style-type: none"> <li>• Clinically adapted technique</li> </ul>	<ul style="list-style-type: none"> <li>• Can be easily used in clinical practice</li> </ul>
<b>Other measure outcomes</b>	<ul style="list-style-type: none"> <li>• Can be combined with biophysical measurements</li> </ul>	<ul style="list-style-type: none"> <li>• Can be combined with biophysical measurements</li> </ul>
<b>Protocol</b>	<ul style="list-style-type: none"> <li>• Integrity of fibres is checked prior to the study</li> <li>• Long protocols: a 90-minute period is required for local anaesthetic and after fibre implantation an equilibrium period of 2 hours is required</li> <li>• Requires surgical skills for fibre implantation</li> </ul>	<ul style="list-style-type: none"> <li>• Minimal preparation</li> <li>• Easy to perform, quick</li> </ul>
<b>Analysis of samples</b>	<ul style="list-style-type: none"> <li>• Limited number of dialysate, multiplex assays are preferred</li> </ul>	<ul style="list-style-type: none"> <li>• Extraction protocol to be improved- obtain a more concentrated solution of cytokines</li> </ul>

Cytokine analysis revealed that IL-1 $\alpha$  (Figure 6.10a), IL-1RA (Figure 6.10b) and IL-1 $\beta$  (Figure 6.10c), and IL-8 (Figure 6.10e) can be successfully quantified with the Sebutapes, while the majority of the samples were below the LLOD for IL-6 (Figure 6.10d) and TNF- $\alpha$  (Figure 6.10f) assays. On the contrary, IL-1 $\beta$ , IL-6, IL-8 and TNF- $\alpha$  were successfully recovered by the microdialysis technique, highlighting that this technique is superior to Sebutape for the recovery of IL-6 and TNF- $\alpha$ . Due to limited volume in dialysates, analysis for IL-1 $\alpha$  and IL-1RA was not performed. Informative results were obtained from cytokine analysis of both Sebutape and microdialysis samples. Indeed, both methods revealed that all challenges stimulated the upregulation of cytokines.

## Chapter 6

With respect to Sebutape samples, it was revealed that s-urine and the proteolytic solution caused an increase in IL-1 $\alpha$  (Figure 6.10a) and IL-1RA (Figure 6.10b) concentrations, with s-urine causing the highest median ratio change to baseline (Table 6.7). In addition, the ratio of IL-1 $\alpha$ /IL-1RA (Figure 6.11) was increased by both s-urine and the proteolytic-solution, however this was only significant with s-urine ( $p<0.05$ ). Pooled data also showed a positive correlation between IL-1 $\alpha$  and IL-1RA at baseline and post-exposure ( $p<0.05$ , Figure 6.12a). With regards to IL-8 (Figure 6.10e) and TNF- $\alpha$  (Figure 6.10f) results showed that the production of IL-8 is increased following exposure to all challenges, except the proteolytic-solution which showed the opposite effect ( $p>0.05$ ). For TNF- $\alpha$ , exposure to the proteolytic solution presented markedly increased TNF- $\alpha$  production with a median ratio change to baseline of 7.25. With regards to IL-1 $\beta$  (Figure 6.10c), this was shown to decrease with all challenges. When pooled together, the data also revealed a significant positive correlation between IL-1 $\alpha$  and IL-1 $\beta$  at baseline and post-exposure ( $p<0.05$ , Figure 6.12b). Noteworthy, Sebutape results of SLS-treated site varied for all cytokines, and except from IL-6 and IL-8 it didn't show any considerable increase in cytokine levels. A possible explanation is the effect of the time of exposure to SLS. This was demonstrated in a further study, as it shown that cytokine release is increased following exposure to SLS for 24 hours (Figure 6.13). In this study, the skin was exposed to SLS for 2 hours due to practical considerations, such as volunteer convenience and for the whole duration of the protocol. In a similar manner, analysis of samples from the tape-stripped site at T1 and T4 revealed that some of the cytokines are increased at T4, however elevated levels of IL-1 $\alpha$  and TNF- $\alpha$ , which drive the inflammatory response, are evident at T1 (Figure 6.14). These confirm the results of a previous study (Chapter 5), which reported a transient effect of tape stripping. Therefore, in future studies, involving these two irritant models, the time of exposure to SLS and sample collection after tape stripping are critical for cytokine recovery, and should be carefully considered. An increase in cytokine levels was also detected at the control site, suggesting that this may result from adjacent compromised sites.

With regards to the results from microdialysis (Tables 6.8-12), these strengthen the results from Sebutapes, as additional information on the temporal profile of cytokines following each challenge was provided. Results showed a trend to a time-dependent increase in cytokine release. Indeed, both the s-urine (Table 6.11) and the proteolytic-solution (Table 6.12) showed a time-dependent increase in cytokine concentrations, which is of particular clinical relevance as it highlights the importance of exposure time in the development of the inflammatory response in IAD. Regarding the SLS (Table 6.9) and tape stripping (Table 6.10) insults, the former produced varied results without a clear underlying pattern. On the contrary, tape stripping caused an elevation in IL-6, IL-8 and TNF- $\alpha$  levels at T3-4, also supported by the Sebutape results.

Also, in contrast with the results from Sebutapes, an increase in IL-1 $\beta$  levels was also observed following exposure to both s-urine and the proteolytic-solution.

With reference to IAD development, the results presented here add to the current state of knowledge, as until now, no studies have examined the release of inflammatory mediators following exposure to urine and faeces. The current findings strongly show that exposure to s-urine and the proteolytic solution lead to an increase in pro-inflammatory cytokine release. Additionally, as the ratio between IL-1 $\alpha$  and IL-1RA is critical in the development of cutaneous inflammation (Terui *et al.*, 1998) this was also assessed in the current study, and evidence was provided for the first time that increased IL-1 $\alpha$  levels and the consequent increase in the ratio of IL-1 $\alpha$ /IL-1RA is involved in the inflammatory milieu of IAD. Additionally, even though both challenges caused an increase in TNF- $\alpha$  production, this was more evident with the proteolytic-solution. This suggests that induced inflammation in IAD may be triggered by different mechanisms, depending on the moisture source of incontinence, urinary or faecal. This could possibly explain why the damage caused by double incontinence is more severe than urine or faeces *per se*. This suggests that IL-1 $\alpha$  and TNF- $\alpha$  might be useful markers of skin damage caused by incontinence in future studies or in the development of point of care diagnostics.

In conclusion, the clinical relevance of these findings is prominent as it was shown that upon exposure to s-urine and the proteolytic-solution there is a time-dependent increase in pro-inflammatory cytokines, highlighting the need to re-evaluate the frequency at which incontinent patients have their skin cleansed in clinical practice to remove the source of the irritants.

## 6.10 Chapter summary

The studies described in this chapter examined the hypothesis that following exposure to urine/faeces there is an increase in inflammatory cytokines. This study represents a significant advance to the body of knowledge on IAD, representing the first to report the release of inflammatory cytokines in an in vivo model of IAD. In particular, results demonstrated that s-urine resulted in an increase in IL-1 $\alpha$ , whereas the proteolytic solution caused a marked increase in TNF- $\alpha$ . These data propose that the mechanisms of inflammation in IAD depend on the moisture source, and that these two cytokines have the potential to serve as responsive biomarkers of skin damage from incontinence. An increase in the IL-1 $\alpha$ /IL-1RA ratio was also observed, although this was only significant at the site treated with s-urine. Microdialysis collected data also showed that there is a time-dependent increase in cytokine release following exposure to s-urine and the proteolytic-solution, highlighting that the duration of exposure is critical in the inflammatory response in IAD.

## Chapter 6

With respect to the functional characteristics of the skin, a similar response was observed from both the s-urine and the proteolytic solution, with an increase in TEWL and SC hydration. However, the proteolytic-solution elicited a higher increase in both blood perfusion and skin pH, due to its composition of proteolytic enzymes that can digest the SC and disrupt the mechanism responsible for the acidification of the skin surface. Chapter 7 introduces an in vitro approach to examine some of the findings from the previous in vivo studies.

## Chapter 7: *In vitro* investigations

### 7.1 Introduction

Keratinocytes are the building blocks of the SC, and are critical for skin homeostasis and the response to inflammation. Therefore, primary (obtained directly from living tissues) and immortalized keratinocyte cell lines are widely used in research to investigate the irritating effects of chemicals (Coquette *et al.*, 2000; Netzlaff *et al.*, 2005). Although the use of primary keratinocytes is limited, as these cells: 1) have a finite lifespan, 2) are difficult to grow as they require additional growth factors, 3) do not survive after induced-differentiation and 4) are characterized by increased variation depending on the donor (Deyrieux and Wilson, 2007). As a consequence, immortalized cell lines are preferred, which are inexpensive and easy to use, have limited complexity, and give informative and reproducible results (Van de Sandt *et al.*, 1999). Indeed, cultured monolayer keratinocytes represent a robust model in dermatological research and are frequently used in mechanistic studies, to provide the basis of experimental human studies (Boukamp *et al.*, 1988; Schoop *et al.*, 1999; Deyrieux and Wilson, 2007). An example is the HaCaT cell line, which has all the differentiation characteristics of normal keratinocytes. Various endpoint measurements are used in *in vitro* investigations to determine the effects of a substance, including assessment of cell viability and cytokine release. Cell viability is defined as the number of healthy cells in a sample, and can be determined by using commercially available assays, including the MTT assay, trypan blue exclusion assay, lactate dehydrogenase and ATP assays (Cook and Mitchell, 1989; Stoddart, 2011). Commonly, determining just the cell viability is not adequate to explore the effects of an irritant and further information on its action is required. Therefore, in mechanistic studies the release of inflammatory mediators is also considered as an important endpoint measurement, and can be simply assessed using a range of analytical methods, described in detail in section 3.1.3 (Fentem *et al.*, 2001).

In the theoretical framework of IAD development, by Gray and colleagues (2007) proposed that increased pH is associated with over-production of keratinocytes and cytokine release. However, there are no scientific studies to support this hypothesis. Therefore, the aim of this *in vitro* study was to investigate this hypothesis, by examining the effects of moisture from incontinence on epidermal keratinocytes and on the release of inflammatory mediators. Findings from the current investigation will further support the work with human volunteers, and provide insights of the damaging mechanisms at the cellular level.

## 7.2 Aim

- To investigate the hypothesis that increased pH is associated with an increased proliferation of keratinocytes and the release of inflammatory cytokines

## 7.3 Objectives

To determine:

- The optimum cell density of HaCaT cell line for assessing viability
- The effects of a known inflammatory stimulus on HaCaT cells
- The sensitivity of epidermal keratinocytes to pH changes
- The time- and dose- response effects of s-urine and the proteolytic-solution on cell viability
- The effects of incubation time and urinary pH on cell viability and on the release of inflammatory mediators, compared to a known inflammatory stimulus

## 7.4 Methods

### 7.4.1 Cell culture

An established HaCaT cell line was used in this study, and was a kind gift from Dr Veronika Jenei (Faculty of Medicine, University of Southampton).

#### Maintenance of HaCaT cells

Cells were maintained in Nunc™ culture flasks (culture area= 175cm<sup>2</sup>, Thermo Scientific), and were used at passages 13 to 23 as responder cells to the various stimuli. Keratinocytes were maintained in Minimum Essential Medium (MEM) - α (Thermo Scientific) supplemented with 10% Foetal Bovine Serum (FBS), 0.1% human Epidermal Growth Factor (EGF, Sigma), 0.4% hydrocortisone (Sigma), 0.05% insulin from bovine pancreas (Sigma) and 1% adenine (Sigma). 1% L-glutamine-, penicillin- and streptomycin- containing solution (Sigma) was also added to the growth medium to prevent bacterial growth. This will be referred to as keratinocyte growth medium (KGM). All cultures were maintained at 37°C in an incubator with 5% CO<sub>2</sub>.

#### Cell Passage (subculture)

Cells were passaged by trypsinization when they reached 80-90% confluence. Briefly, the medium was removed from the flask, washed twice with 10ml PBS (Sigma), and treated with 5ml of EDTA trypsin (Sigma), to detach adherent cells, for 5-10 minutes at 37°C and 5% CO<sub>2</sub>.

Subsequently, and after a gentle tapping of culture flasks, cell detachment was examined under an inverted light microscope set at x10 magnification. Then, 1ml of KGM was added to the flask to neutralize the enzymatic activity of trypsin, due to the presence of FBS which contains protease inhibitors, and cell suspension was transferred to a 50ml sterile falcon tube (Fisher Scientific), and the volume adjusted to 50ml with PBS. Cells were centrifuged to remove debris at 1500rpm for 5 minutes, and then the supernatant was gently removed, leaving the cell pellet intact at the bottom of the tube, which was suspended in 1ml of KGM. This was spread to new culture flasks (1:3 dilution); fresh KGM was added, and placed back in the incubator.

#### **Trypan Blue exclusion assay (cell counting method)**

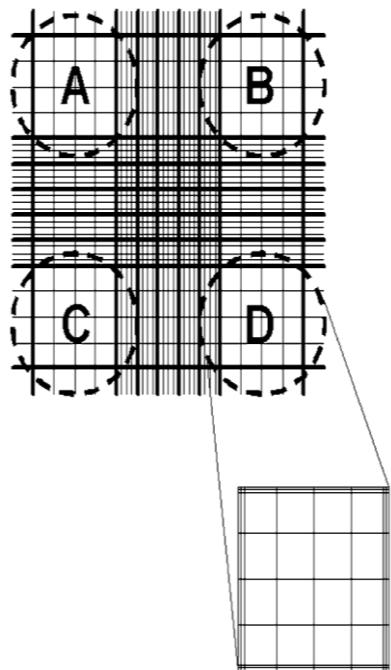
Cell counting is important to determine growth rates and to set up reproducible experiments. In the current investigation, the Trypan Blue exclusion assay was used as it represents the most direct way in quantifying cell numbers (Wiegand and Hipler, 2008). This was performed after trypsinization of cells. In this method, an equal amount (100 $\mu$ l) of 0.4% trypan blue (Thermo Scientific) and cell suspension were mixed, to stain non-viable cells blue. Then 20 $\mu$ l were transferred to a Fuchs-Rosenthal haemocytometer, a thick glass microscope slide with an indented chamber, and both the viable and non-viable cells were counted under a light microscope (Figure 7.1). Cells were counted at the four corners of the haemocytometer, and the average was calculated (Louis and Siegel, 2011; Stoddart, 2011).

The percentage of viable cells was determined by equation (1):

$$\% \text{ Viable cells} = \frac{\text{Number of viable cells}}{\text{Total number of cells}} \quad (1)$$

The number of viable cells per ml was calculated using equation (2), in which dilution factor was 2, as 100 $\mu$ l of cell suspension were diluted in a total volume of 200 $\mu$ l.

$$\text{Cell density} = \text{Number of viable cells} \times 10^4 \times \text{dilution factor} \quad (2)$$



**Figure 7.1. Trypan Blue exclusion assay.** Cells are counted at the four corner boxes and the average is calculated. Then cell density and the % viability are calculated. Figure adapted from Louis and Siegel, 2011

#### 7.4.2 Cell stimuli

Cells were seeded at the optimal density into 96-well plates (NUNC, UK) and incubated for 24 hours at 37°C and 5% CO<sub>2</sub> to allow the cells to attach to the bottom of the plate. Following that, the media was removed and each well was washed twice with 100µl PBS, to remove any residuals. Then, cells were challenged (100µl/well) with the following stimuli, in different experiments. All treatments were performed in six separate wells and KGM was used as negative control, unless otherwise stated. After each stimulus, cell supernatant was collected and stored at -80°C, and the viability of cells was assessed.

##### 1. Lipopolysaccharide

Lipopolysaccharide (LPS, Sigma, pH of 9.0 ± 0.5) is an endotoxin found in Gram negative bacteria, and is extensively used in experimental studies as a positive control, as it is known to induce an inflammatory response in keratinocytes, by stimulating the release of several cytokines, including IL-1 $\alpha$ , IL-1 $\beta$ , IL-6, IL-8 and TNF- $\alpha$ , (Kameda and Sato, 1994; Sweet and Hume, 1996). In the current investigation, cells were challenged with different concentrations of LPS, as indicated below:

**LPS concentrations (µg/ml):** 500, 250, 125, 62.5, 31.25, 16.5, 7.8, 3.9, 1.95 and 0 µg/ml (KGM)

## 2. Growth medium varying pH

To determine the effects of pH on keratinocytes viability, powdered MEM  $\alpha$  (Thermo Scientific) was obtained without sodium bicarbonate, which is responsible for its buffering capacity, and the pH level was adjusted to acidic or alkaline values, between pH 4.0-10.0 ( $\pm$  0.5), using hydrochloric acid (HCl) and ammonium hydroxide (NH<sub>4</sub>OH) respectively.

## 3. Synthetic-urine

S-urine, as described in section 3.1.2, was used as a model of urinary-incontinence to challenge the cells. In the first experiment, and to determine the time course and dose effects on keratinocytes, s-urine was used at various concentrations (v/v), between 5-100%, and prepared in KGM with a constant pH (pH=7.4), representing pH optimum for cell growth. Cells were incubated with these concentrations for 3, 6 and 24 hours.

Prior to investigating the effects of urinary pH on keratinocytes over time, an initial experiment was conducted to determine an optimum s-urine concentration to be used. Two concentrations (10 and 50%) of s-urine prepared in powdered MEM  $\alpha$ , without sodium bicarbonate, and the pH was adjusted as previously, with values ranging between 4.0 and 10.0 ( $\pm$  0.5). Cells were treated for 24 hours. In the second experiment, cells were treated with the optimum s-urine concentration at various pH levels for 3, 6, 24 and 72 hours.

## 4. Proteolytic-solution

To simulate faecal-incontinence, a proteolytic solution was used, containing enzymes and bile salts (section 3.1.2). To determine the effects of incubation time and dose on keratinocytes, different concentrations of the proteolytic-solution (v/v), were prepared, ranging between 5-100%. As the presence of FBS inactivates proteolytic enzymes, such as trypsin, these concentrations were prepared in serum-free media. Cells were treated with the different concentrations of the proteolytic-solution, and incubated for 3, 6 and 24 hours. In contrast with other experiments, serum free media KGM was used as negative control.

### 7.4.3 Measurement of cell viability

Cell viability is an important endpoint measurement in *in vitro* investigations. This can be assessed by using commercially available methods, including the MTT assay, trypan blue exclusion assay, lactate dehydrogenase and ATP assays (Cook and Mitchell, 1989), with the former being widely used in scientific research.

Indeed, the MTT assay has been used with several cell types, as it is inexpensive, easy and quick method, based on a standardized protocol, that gives reproducible results (Riss *et al.*, 2004; Kupcsik, 2011; Stoddart, 2011). Compared to other assays, the main advantage of the MTT is the application of microtiter plates, allowing thus the processing of many samples rapidly and simultaneously (Mosmann, 1983; Weyermann *et al.*, 2005). Therefore, in the current investigation cell viability was assessed using the MTT assay, which is a colorimetric assay, based on the metabolic activity of cells (Mosmann, 1983; Fotakis and Timbrell, 2006). The main principle underlying this method is the conversion of a yellow tetrazolium salt into a purple formazan product (crystals) by the living cells, which is measured by reading the absorbance at 570nm, with a reference wavelength of 630nm, using a spectrophotometer (Mosmann, 1983; Riss *et al.*, 2004).

### **MTT assay protocol**

The MTT assay protocol is based on the original publication by Mosmann (1983).

Preparation of stock solution and MTT reagent: MTT (Sigma) stock solution was prepared prior to experiments at a concentration of 5mg/ml in PBS, covered with foil as it is light-sensitive, and stored in the fridge at 4°C until subsequent use. On the day of the experiments, MTT reagent was prepared by diluting the stock solution in fresh KGM (1:4 dilution). Then the following protocol was performed:

- 1) 50µl was added to each well
- 2) Incubation (37°C, 5% CO<sub>2</sub>) of the plate for 3-4 hours. The plate was examined under the microscope every hour to check the formation of the crystals inside the cells.
- 3) After incubation time, media was discarded from the plate, 100µl/well dimethyl sulfoxide (DMSO, Sigma) was added to dissolve the insoluble formazan crystals, and plate was placed on a shaker for 5-10 minutes
- 4) Absorbance was then measured at 570nm and at 630nm (reference wavelength) using a VersaMax™ Microplate Reader (Molecular Devices, Sunnyvale, California, USA), and results exported to Microsoft Excel 2010 (Microsoft Corp., Redmond, WA, USA) for analysis.

### Analysis of results

To remove background signal, the absorbance readings obtained at 630nm were subtracted from the values at 570nm. The growth inhibition (% control) for each treatment was then calculated based on the following equation:

$$\% \text{ Growth inhibition} = \frac{\text{Absorbance}_{\text{control}} - \text{Absorbance}_{\text{treatment}}}{\text{Absorbance}_{\text{control}}} \times 100 \quad (3)$$

For each challenge, results are presented as mean growth inhibition (% control)  $\pm$  SD.

As the optimal cell concentration for the MTT assay is dependent on the cell line, an initial experiment was performed to determine this concentration for keratinocytes. Cells were seeded into a 96-well plate at a starting concentration of  $31.80 \times 10^5$  cells/ml. Then, 2-fold serial dilutions were performed down to a concentration of  $0.12 \times 10^5$  cells/ml. KGM represented a zero concentration. Cells were incubated for 24 hours at  $37^\circ\text{C}$  and 5%  $\text{CO}_2$ . The next day, the medium was removed and the MTT assay was performed.

#### 7.4.4 Quantification of the release of inflammatory cytokines

##### Multiplex electrochemiluminescence immunoassays

The release of inflammatory mediators is another important endpoint measurement in *in vitro* studies. In the present investigation commercial multiplex electrochemiluminescence immunoassays (MesoScale Diagnostics, Rockville, Maryland, USA) were used to measure cytokine release in cell supernatants. IL-1 $\alpha$  and IL-1RA were investigated in singleplex assays and IL-1 $\beta$ , IL-6, IL-8 and TNF- $\alpha$  were investigated in multiplex assays, as described in section 6.5. Samples were analysed in duplicates.

### 7.5 Data analysis

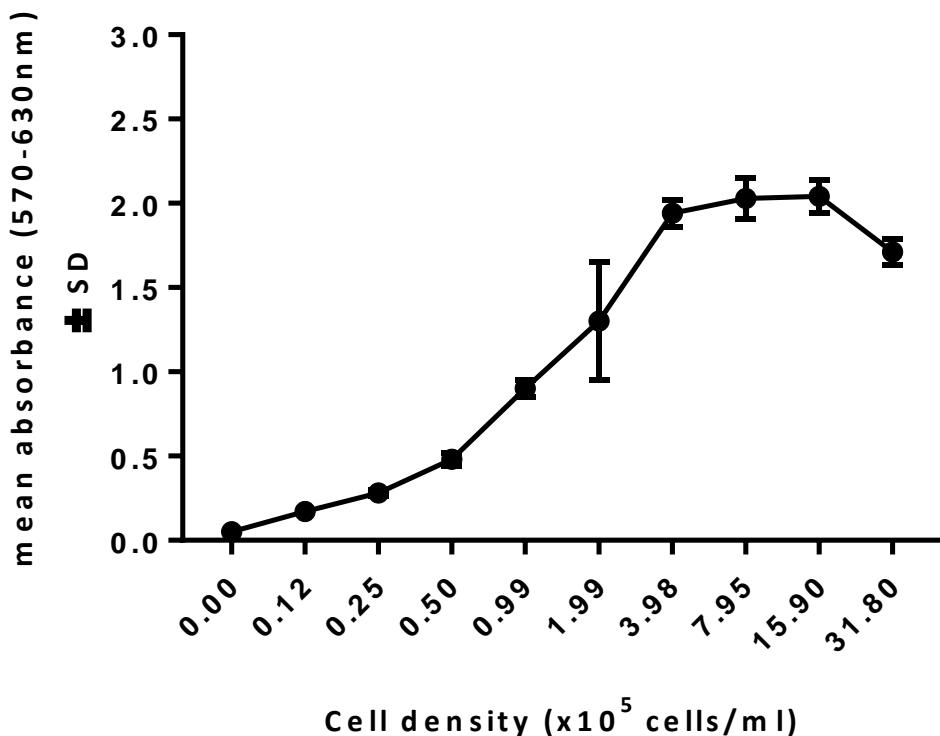
All data were expressed as mean  $\pm$  SD. Statistical analysis was performed using IBM SPSS version 21.0 (SPSS Inc., Chicago, IL, USA). The data was assumed to be non-normal in distribution and hence non-parametric tests were used. The statistical comparisons were performed using Mann-Whitney and Kruskal Wallis tests, for comparison between two or more groups, respectively. In particular, the Kruskal-Wallis test was performed to examine differences between treatments on growth inhibition, followed by pairwise Mann-Whitney tests.

Additionally, a two-way ANOVA was used to assess the effects of incubation times and pH on the growth inhibition of keratinocytes and on the release of cytokines. Although this is a parametric test, it is a robust test to violations of normality, providing valid results. Significance was set at  $p<0.05$ .

## 7.6 Results

### 7.6.1 The optimum concentration of HaCaT cells

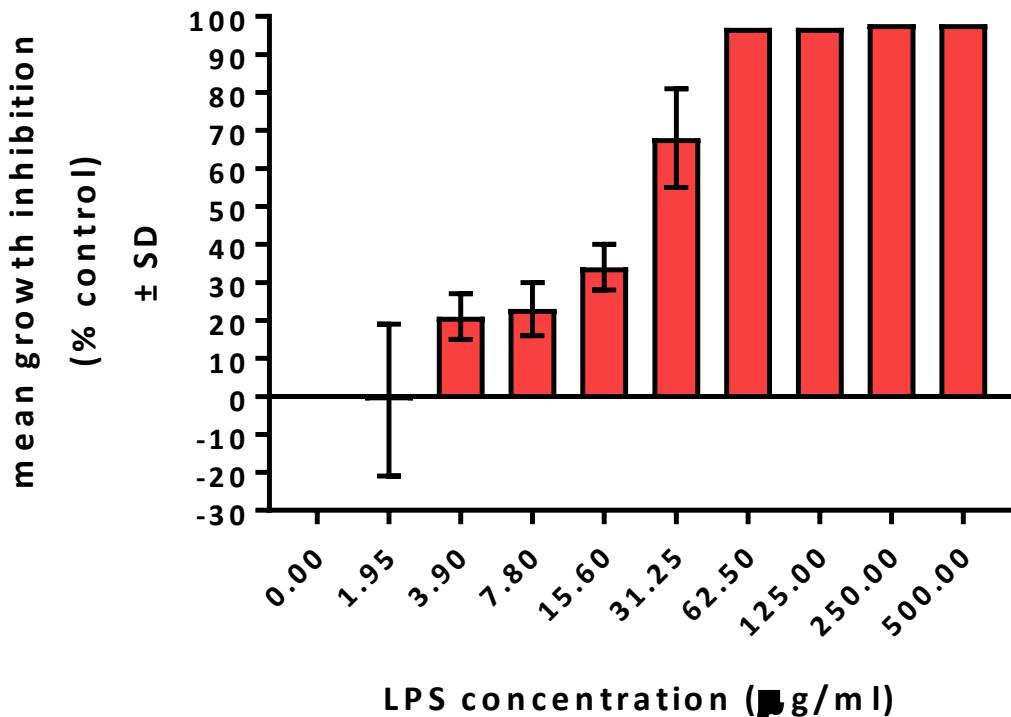
The results, as presented in Figure 7.2, demonstrated an S-shape response of cell density plotted against mean absorbance. To ensure a maximum sensitivity in response, a HaCaT cell concentration of  $0.99 \times 10^5$  cells/ml was selected as it corresponds to the central part of the linear region.



**Figure 7.2. The optimum cell density of HaCaT for cell viability assessment.** The optimum cell density of HaCaT corresponds to  $0.99 \times 10^5$  cells/ml. (n=6)

### 7.6.2 The effects of LPS on keratinocytes

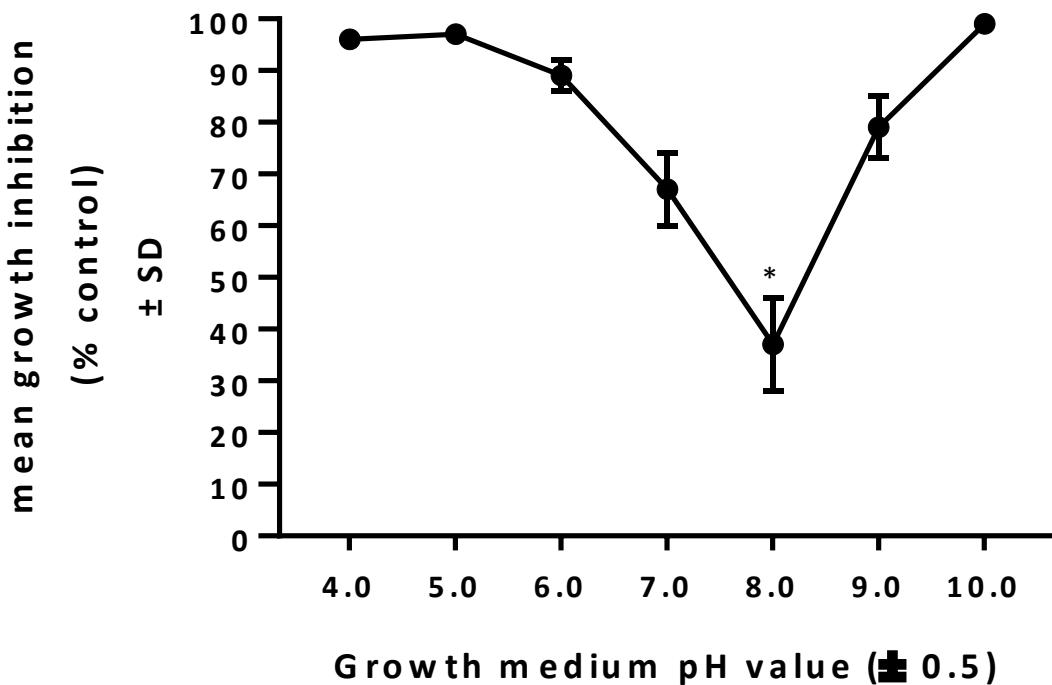
Results, as illustrated in Figure 7.3, showed that LPS had a significant effect on growth inhibition of keratinocytes ( $p<0.05$ ), and indeed at concentrations higher than 15.60  $\mu\text{g/ml}$  is toxic to the cells as growth is considerably inhibited by more than 50%.



**Figure 7.3. The effects of LPS on the growth inhibition of keratinocytes: a dose-response curve.** Growth in keratinocytes is inhibited over 50% when concentration of LPS is above 15.6  $\mu\text{g/ml}$ . ( $n=6$ )

### 7.6.3 The effects of pH on keratinocytes

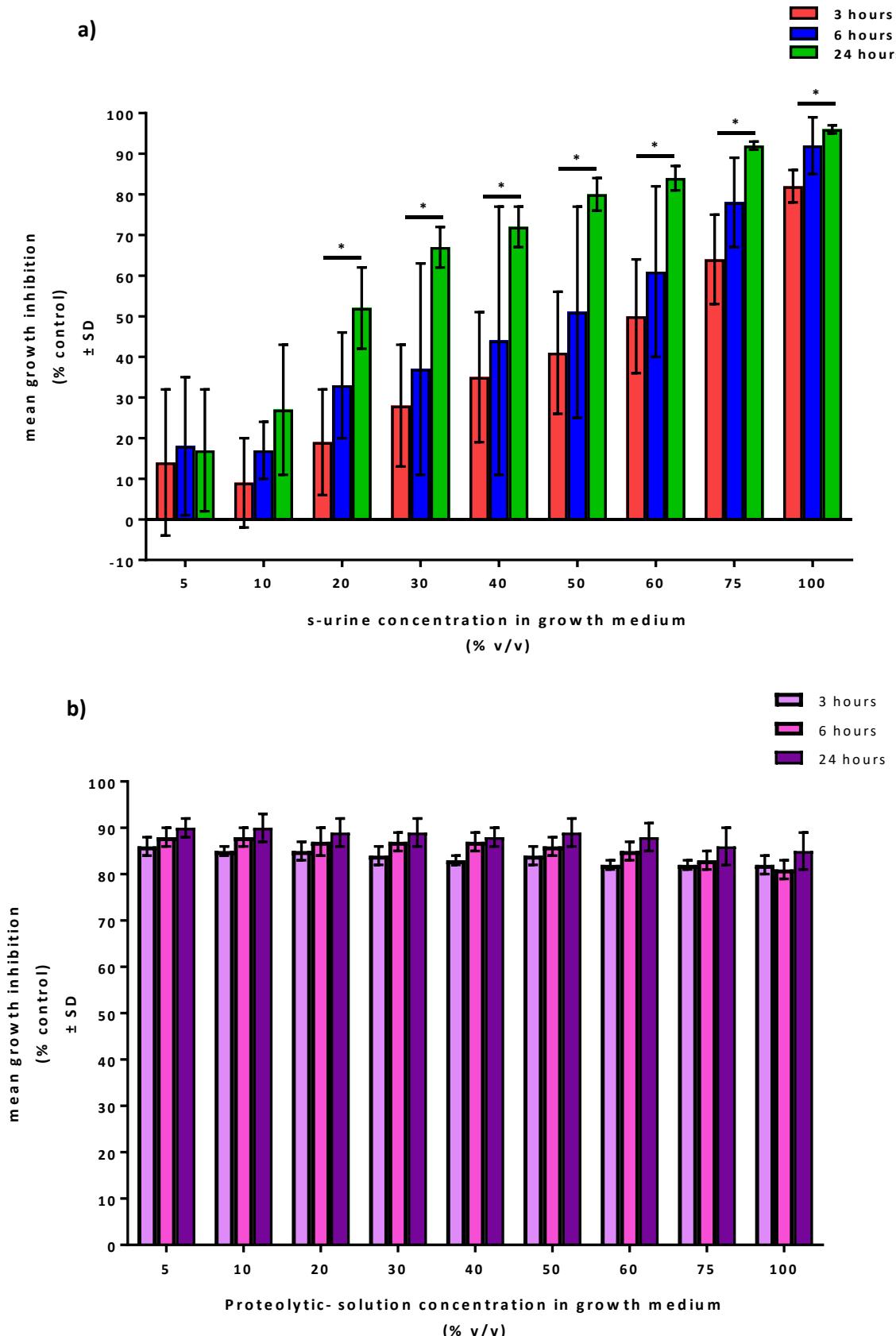
Prior to investigating the effects of s-urine and proteolytic-solution, the sensitivity of keratinocytes to pH changes was determined. The results, as illustrated in Figure 7.4, showed that there is a significant effect of pH on keratinocytes ( $p<0.05$ ), and indeed high levels of growth inhibition (>90%) were associated with extreme values of pH ( $\text{pH} \leq 5.0$  and  $\text{pH} 10.0$ ). By contrast, growth inhibition reduced significantly ( $p<0.05$ ) at pH values between 7.0 and 9.0, reaching the minimum mean value of 37% at pH 8.0. This data clearly reveals the sensitivity of growth inhibition on solution pH values.



**Figure 7.4. The effect of varying pH solutions on the growth inhibition of keratinocytes.** There was a significant effect ( $p<0.05$ ) of pH on keratinocytes' growth. In particular, at pH 8.0 there is a significant reduction ( $p<0.05$ ) in the growth inhibition of keratinocytes, compared to the other pH solutions. Significance is displayed with (\*). ( $n=6$ )

#### 7.6.4 The effects of various concentrations of s-urine and proteolytic-solution on keratinocytes over time

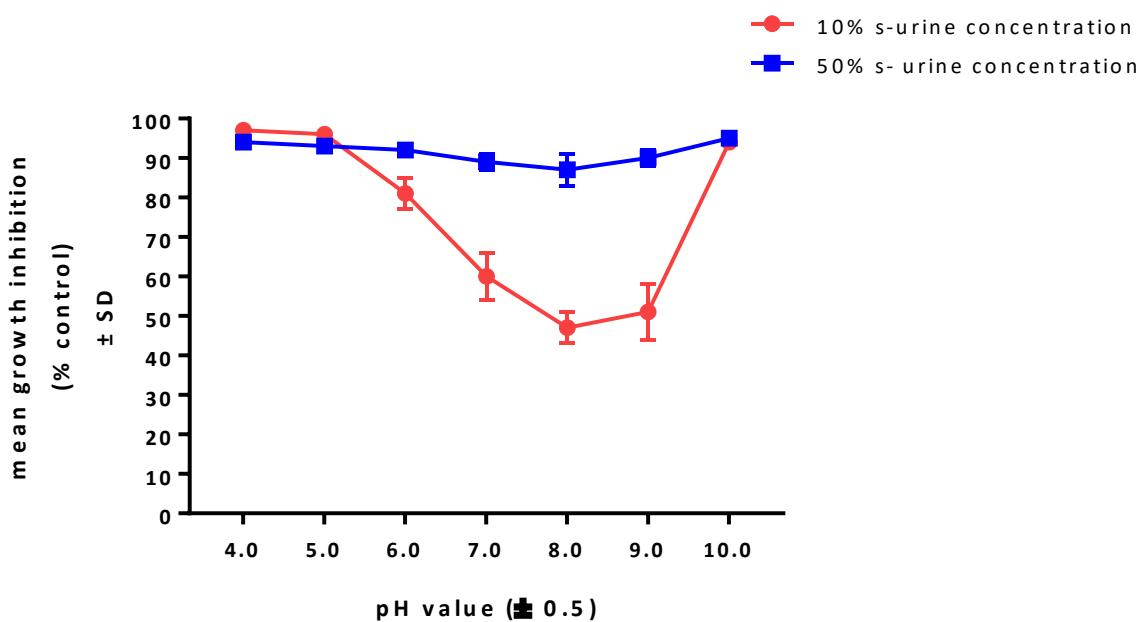
After showing the importance of pH on cell viability, the effects of different concentrations of s-urine and proteolytic-solution on keratinocytes were determined over time. Results, as shown in Figure 7.5, revealed that there is a gradual increase in the growth inhibition of keratinocytes with increasing s-urine and proteolytic-solution concentrations and exposure time. This is more evident with s-urine (Figure 7.5a), and indeed both the time of exposure and concentration had a statistically significant effect in growth inhibition ( $p<0.05$ ). In particular, for concentrations higher than 10%, there is a significant ( $p<0.05$ ) effect of exposure time on the growth inhibition of keratinocytes, with 24 hours resulting in the highest increase in the growth inhibition of keratinocytes when compared to the other exposure times ( $p<0.05$ ). Additionally, the growth inhibition of cells with the proteolytic solution (Figure 7.5b) is more than 80% at all incubation times, however no significant differences were found ( $p>0.05$ ).



**Figure 7.5. The effects of varying s-urine and proteolytic-solution concentrations on the growth inhibition of keratinocytes over time.** The effects of s-urine (a) and the proteolytic-solution (b) on keratinocytes are time- and dose- dependent. However, this is more evident with s-urine, which showed a significant increase in growth inhibition after 24 hours of exposure ( $p<0.05$ ). ( $n=6$ )

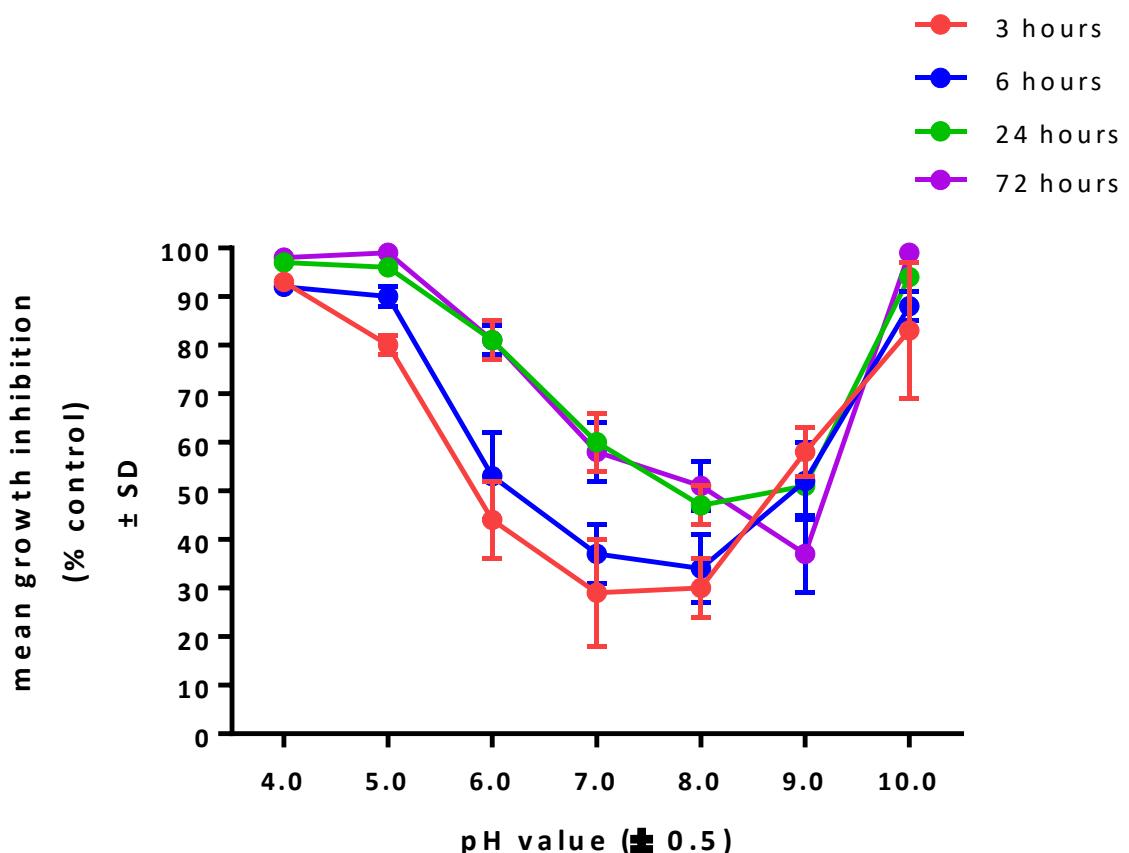
### 7.6.5 The effects of urinary pH on keratinocytes over time

After determining the effects of s-urine and the proteolytic-solution on the growth inhibition of keratinocytes, due to the detrimental effects of the proteolytic solution it was decided to proceed with s-urine only. Subsequently, one low and one high concentration of s-urine, 10% and 50% respectively, were chosen to investigate the effects of urinary pH on keratinocytes, at which the growth inhibition following 24 hours exposure is below 50% and above 80% (Figure 7.5a), respectively. Interestingly, there was clear difference between 10% and 50% s-urine concentrations, with the latter presenting an increased growth inhibition (Figure 7.6), although the differences were not statistically significant ( $p>0.05$ ). Additionally, there is a small decrease in growth inhibition between pH values of 7.0 and 9.0 with a 50% s-urine concentration, although the effects do not appear to be pH-dependent as the growth inhibition was relatively constant across all pH values. On the contrary, the effects of urinary pH were more evident with a low concentration of s-urine, at which it was shown that the growth inhibition decreases at pH values close to the physiological range for keratinocytes growth (pH 7.0-9.0) (Figure 7.4).



**Figure 7.6. The effects of a low and a high s-urine concentration, varying pH, on the growth inhibition of keratinocytes.** Keratinocytes were treated with varying pH s-urine solutions at two concentrations for 24 hours, and based on the growth inhibition of cells it was revealed that the effects of urinary pH are more evident with a low urine concentration (10%). However, no significant differences were found ( $p>0.05$ ). (n=6)

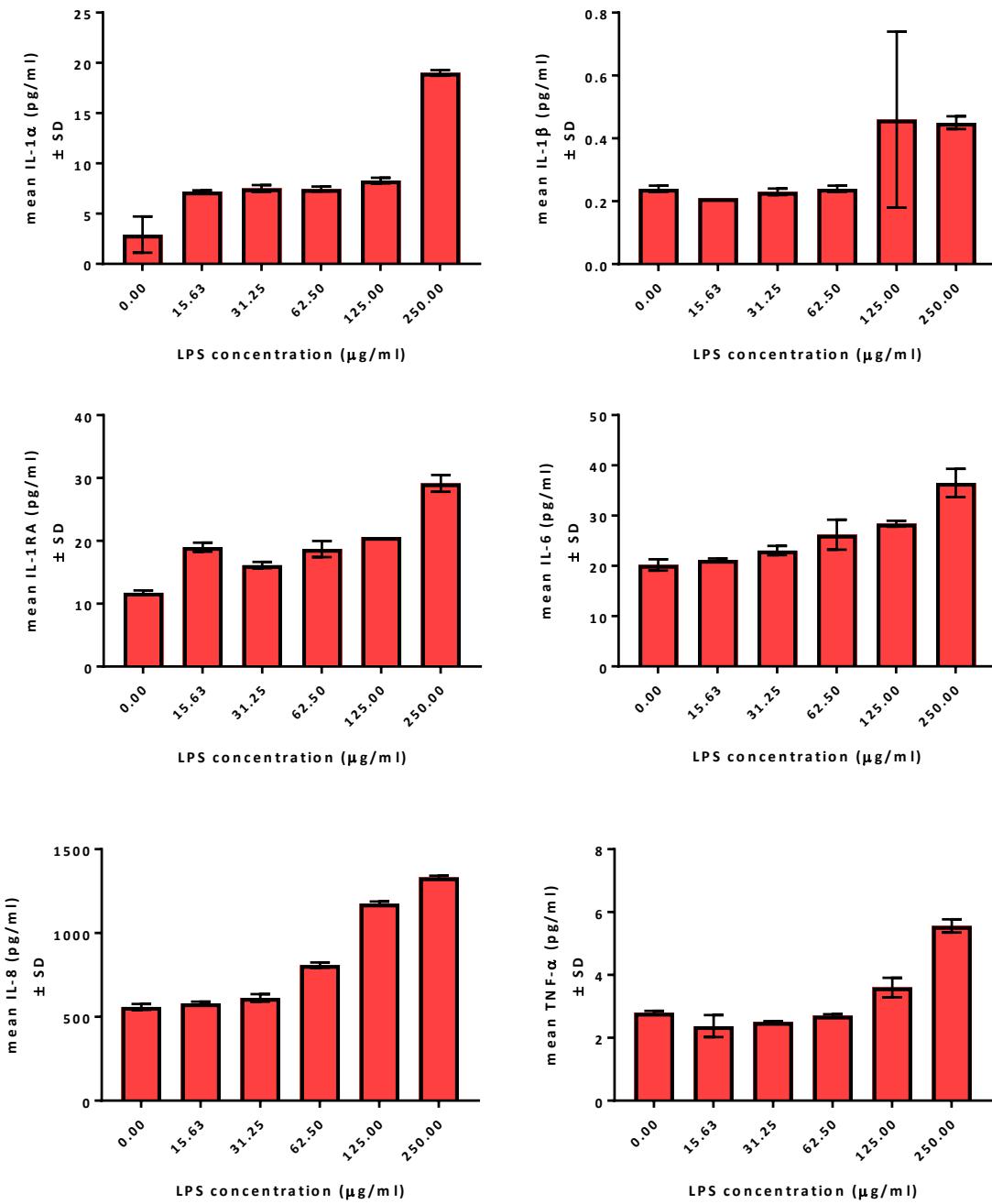
In view of this, the time course effects of urinary pH on keratinocytes were examined using a 10% s-urine concentration. As shown in Figure 7.7, there was a significant effect of exposure time, pH and the interaction between these two factors in the growth inhibition of keratinocytes ( $p<0.05$ ). Specifically, exposure to s-urine for 24 and 72 hours caused a significant increase in the growth inhibition of keratinocytes compared to the shorter exposure times of 3 and 6 hours ( $p<0.05$ ). However, no significant differences were found between 3 and 6 hours and between 24 and 72 hours of incubation ( $p>0.05$ ). Additionally, for all urinary pH values, except pH 9.0, the effects on keratinocyte behaviour are time-dependent. Indeed, a significantly increased growth inhibition was also observed at pH values of 4.0, 5.0 and 10.0 ( $p<0.05$ ). In agreement with previous results, between pH values of 7.0 and 9.0 the growth inhibition decreased.



**Figure 7.7. The effects of urinary pH on the growth inhibition of keratinocytes over time.** There is a time-dependent relationship between urinary pH and growth inhibition of keratinocytes. Exposure for 24 and 72 hours leads to a significant increase ( $p<0.05$ ) in growth inhibition compared to the other timings. (n=6)

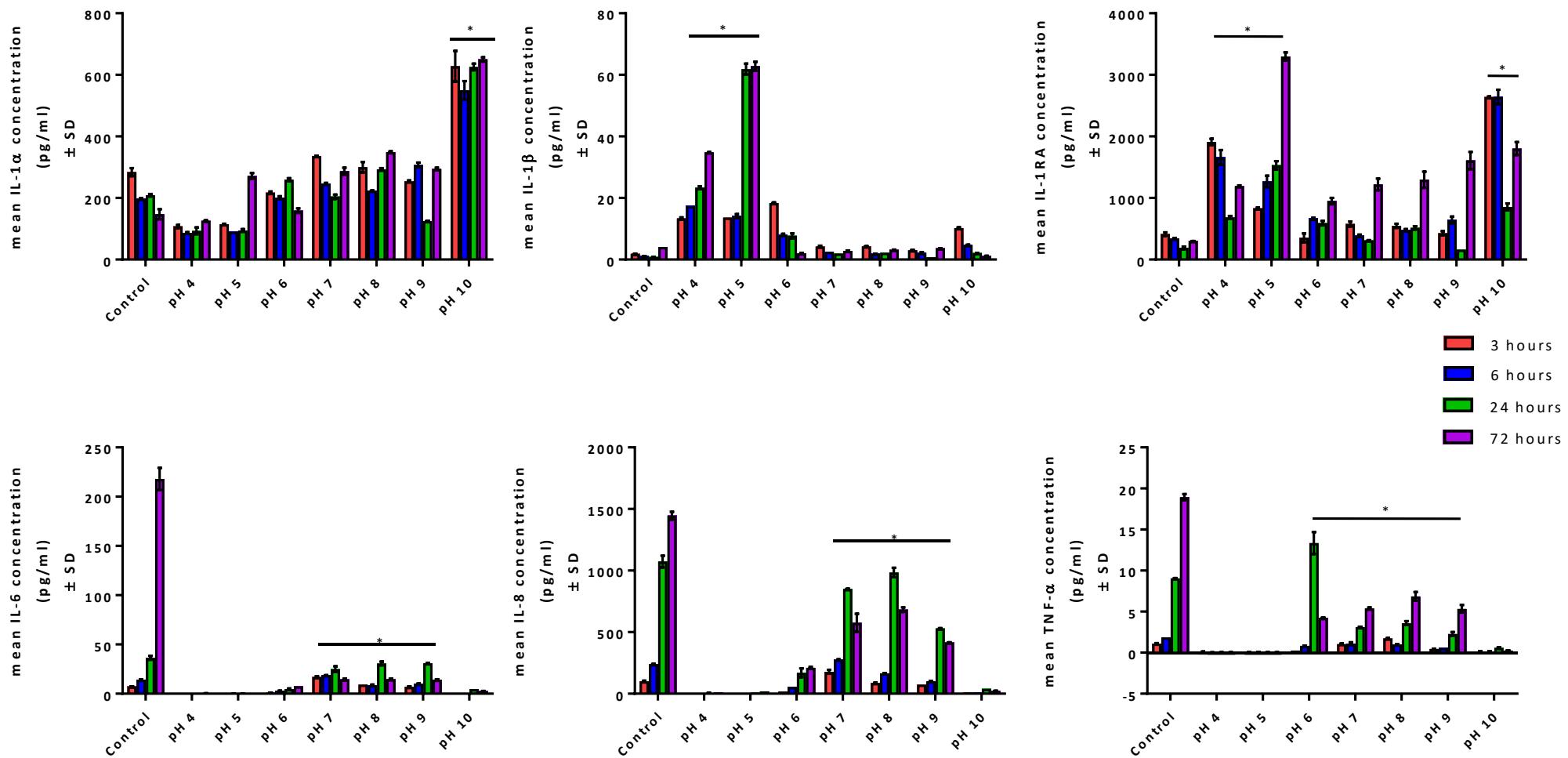
### 7.6.6 Cytokine release

All the inflammatory cytokines investigated were quantified in cell supernatants. Results from the LPS challenge, as shown in Figure 7.8, revealed a dose dependent increase in cytokine release, most evident with IL-6, IL-8 and TNF- $\alpha$ . No significant differences were found ( $p>0.05$ ).



**Figure 7.8. Cytokine release following stimulation with various LPS concentrations.** Results showed that cytokine production increases with increasing LPS concentration. No significant differences were found ( $p>0.05$ )

With reference to s-urine treatment, variable results were obtained specific to each cytokine (Figure 7.9). Statistical analysis revealed that there was a significant effect of the interaction between pH and exposure time on the release of pro-inflammatory cytokines ( $p>0.05$ ), however this was most notable with IL-1 $\alpha$ . Indeed, keratinocytes produced increased amounts of IL-1 $\alpha$ , with the highest increase caused by the most alkaline solution of pH 10.0 at all exposure timings ( $p<0.05$ ). In particular, the levels of IL-1 $\alpha$  increased with increasing alkalinity, decreased at optimum pH levels (pH 7.0-pH 9.0), and increased at a pH of 10.0. IL-1 $\alpha$  was also detected in the control group, although its concentration was lower compared to most s-urine solutions. In a similar manner, a significant rise in the levels of the anti-inflammatory cytokine, IL-1RA, was detected in supernatants of keratinocytes exposed to solutions of s-urine with pH values of 4.0, 5.0 and 10.0, compared to the other solutions at all timings ( $p<0.05$ ). For IL-1 $\beta$ , results showed an increase following exposure to acidic s-urine solutions (pH 4.0-5.0) only, and differences between these two solutions were evident with 24 and 72 hours of exposure. With regards to the other pro-inflammatory cytokines, results varied, as decreased levels of IL-6, IL-8 and TNF- $\alpha$  were detected at extreme acidic (pH 4.0-5.0) and alkaline (pH 10.0) solutions of s-urine (Figure 7.9). By contrast, IL-6, IL-8 and TNF- $\alpha$  presented increased levels in the control group following 72 hrs of incubation.



**Figure 7.9. Cytokine release following exposure to varying pH s-urine solutions at different incubation times.** There was a significant effect of exposure time and pH on the release of pro-inflammatory cytokines. Significant differences between the different pH solutions are marked with (\*).

## 7.7 Discussion

Keratinocytes are the most abundant cell type in the epidermis and are responsible for barrier homeostasis and the inflammatory response. Despite the fact that keratinocytes in the SC are dead, upon disruption of the skin barrier the penetration of harmful substances is increased and may affect the viability of living keratinocytes in the deeper layers of the epidermis. To the best of the author's knowledge, no previous studies have examined the underlying cellular mechanisms in IAD. This was investigated in the current study using a keratinocyte cell line that represents a robust model to characterize the mechanistic behaviour of epidermal keratinocytes *in vivo*.

After determining the optimal HaCaT concentration for viability assessment with the MTT assay (Figure 7.2) and prior investigating the effect of pH on keratinocytes, the effects of a known inflammatory stimulus (LPS) were examined. Results showed that the effects of LPS on the growth inhibition of keratinocytes are dose-dependent (Figure 7.3). Subsequently, when examining the effects of pH on keratinocytes it was revealed that keratinocytes are sensitive to changes in pH, and their optimum pH ranges between pH 7.0 and 9.0 (Figure 7.4). This was expected, as in physiological conditions the extracellular pH is  $7.4 \pm 0.1$  (Deutsch *et al.*, 1982), also supported by the results of a previous study that explored the migration of keratinocytes in wound healing conditions (Sharpe *et al.*, 2009). After highlighting the sensitivity of keratinocytes to pH, experiments were conducted with s-urine and a proteolytic-solution, to simulate the effects of urinary- and faecal- incontinence, respectively. At first, keratinocytes were treated with varying concentrations of s-urine and proteolytic-solution at different exposure times. Results showed that both insults were damaging to the cells, and indeed this was time- and dose- dependent (Figure 7.5). However, this was more profound with s-urine, as all the proteolytic-solutions, irrespective of incubation time and concentration, caused a high growth inhibition ( $>80\%$ ) for keratinocytes. This is attributed to the presence of trypsin and other proteolytic enzymes which damage keratinocytes, and especially with long incubation periods which can strip cell surface proteins and eventually lead to cell death. Taking this into consideration, further experiments were conducted with s-urine only.

With respect to IAD and its pathophysiology, these findings strongly support that prolonged exposure to s-urine and to a proteolytic-solution impairs keratinocytes viability and proliferation, and eventually the formation of an intact barrier. This is also of potential clinical relevance as adequate evidence was provided regarding the damaging effects of faecal incontinence which indeed has a direct killing effect on keratinocytes and this can explain why the severity of IAD in faecal incontinence is higher than urine per se.

To investigate the effects of urinary pH, two concentrations of s-urine were used, and it was revealed that only with a low concentration of s-urine (10%) the effects of pH are evident, and indeed any shifts in pH away from the optimum range lead to increased growth inhibition (Figure 7.6). This finding is partly in agreement with a previous study, involving a tissue-engineered epidermis, that also reported the damaging effects of alkaline urinary pH with time (Sonsma *et al.*, 2006). However, a major limitation of that study is that the skin model was grown in absence of CO<sub>2</sub>, and this per se decreases the viability of cells, as CO<sub>2</sub> mimics the physiological conditions in which cells grow *in vivo*. Additionally, there is no report of the concentration of urine used. Subsequently, results showed that the effects of urinary pH (pH 4.0-10.0) are time-dependent (Figure 7.7). In comparison with earlier findings (Figure 7.4), it is clear that even at the optimal pH levels (pH of 7.0-9.0) and exposure for 24 hours, s-urine caused a slightly higher increase (47%) in growth inhibition compared to the varying pH solutions of growth medium (37%), suggesting that the chemical composition of s-urine exacerbates the effects of pH. By looking at both graphs, it is clear that keratinocytes exhibit optimum growth at pH levels of 7.0 to 9.0, which represents their buffering capacity, and are only tolerant to small changes in pH and not in extreme acidic and alkaline pH environments.

In the second part of the analysis, the release of inflammatory mediators from keratinocytes treated with s-urine was investigated, and compared with LPS. Results showed that indeed LPS stimulation elicited an upregulation of cytokine production in a dose dependent manner (Figure 7.8). Informative findings were also obtained from s-urine treatment (Figure 7.9), as there was a significant effect of time and pH on cytokine release ( $p<0.05$ ). In particular, the release of IL-1 $\alpha$  was found to be pH-dependent, and indeed the highest increase was observed with the most alkaline solution (pH 10.0). This increase was significant compared to the other pH solutions at all exposure timings ( $p<0.05$ ). Additionally, the s-urine stimulus elevated the levels of the anti-inflammatory IL-1RA significantly ( $p<0.05$ ) and this was more profound at extreme acidic (pH 4.0-5.0) and alkaline conditions (pH 10.0). An explanation for this is that this cytokine is a natural inhibitor of IL-1 $\alpha$  activity and is increased in damaging and pathological conditions to suppress inflammation. Compared to the control group the levels of IL-1RA are higher in optimum pH conditions, suggesting that the chemical composition of s-urine may be responsible for this. The levels of IL-1 $\beta$  were only increased in acidic conditions, and were higher compared to the control group. On the contrary, low levels of IL-6, IL-8 and TNF- $\alpha$  were detected at extreme acidic and alkaline environments, suggesting that this may be due to increased growth inhibition associated with these solutions (>80%).

With regards to the pathophysiology of IAD, it has been hypothesized that an increase in pH is associated with increased proliferation of keratinocytes and cytokine release (Gray *et al.*, 2007). Certainly the data presented here partly contradict this hypothesis, as it was shown that pH is an important regulator of keratinocytes function, and any shifts in pH outside their optimum growth conditions (pH 7.0-9.0) drastically influence the viability and the proliferation of keratinocytes, and consequently skin barrier function. However, evidence is provided to support the hypothesis that increased pH modulates the release of inflammatory mediators, as indeed it was shown that in extreme alkaline conditions an increased production of the pro-inflammatory IL-1 $\alpha$  is evident. These findings are of clinical relevance as they highlight that the alkaline conditions developed following exposure to incontinence disrupt keratinocytes function and lead to increased production of pro-inflammatory cytokines. This is more prominent with faecal incontinence, in which proteolytic enzymes digest the SC and are highly active in an alkaline environment (Beeckman *et al.*, 2015). This latter finding further supports the results from a previous study with human volunteers (Chapter 6) which also reported that exposure to s-urine leads to an increase in IL-1 $\alpha$ . Together these data highlight the potential of this mediator to serve as a responsive marker in future studies of skin damage from incontinence. The present findings have also implications for clinical practice as they strongly suggest that internal pH should be tightly regulated. As such, any barrier creams, ointments or lotions used in incontinence care should be aiming at retaining this internal pH to facilitate normal barrier function. By contrast, in a previous study within this research thesis (Chapter 5), it was highlighted that s-urine is more damaging on compromised skin, characterized by increased pH, and therefore skin pH-balance cleansers should be used to maintain the acid mantle (pH 4.2-6.1). Apparently, the findings from both studies contradict each other, and propose that two distinct mechanisms, an internal and an external, which are pH-dependent, are involved in the maintenance of skin integrity. Accordingly, skin homeostasis depends on the synergistic effect of these mechanisms.

To sum up, results from *in vitro* investigations clearly showed that keratinocytes do not tolerate changes in pH and that exposure to s-urine for prolonged periods exacerbate these effects. Additionally, any changes in pH influence cytokine release and consequently the inflammatory response. Therefore, it is important to maintain this optimum pH for keratinocytes to facilitate normal barrier function and to prevent skin breakdown.

## 7.8 Chapter summary

The in vitro investigations discussed in this chapter examined the hypothesis that increased pH results in an increased proliferation of keratinocytes and the associated secretion of inflammatory cytokines. Results showed that epidermal keratinocytes are sensitive to pH changes and their optimum growth ranges between pH 7.0-9.0. Keratinocytes were subsequently treated with s-urine and a proteolytic solution, at different concentrations and exposure times. Results showed that the damage caused by both challenges is time- and dose- dependent, although these effects were more profound with s-urine. Subsequently, the effects of urinary pH were further explored, and it was revealed that there is an increase in growth inhibition at any pH values away from the optimum range for keratinocytes, in a time-dependent manner. This contrasts with the hypothesis from Gray et al., 2007, who proposed that an increase in pH stimulates the proliferation of keratinocytes. However, the present findings revealed that increased pH leads to a release of inflammatory cytokines, particularly evident with IL-1 $\alpha$ . These data propose that an internal pH mechanism is also implicated in the pathophysiology of IAD, which needs to be further elucidated.

# Chapter 8: General Discussion

## 8.1 Addressing the research aim and hypotheses

This thesis was designed to investigate the underlying mechanisms involved in IAD development through specific hypotheses that were generated from existing knowledge on IAD, based on the theoretical frameworks of IAD pathophysiology developed by Gray and colleagues (2007) (Figure 2.4) and by Beeckman and colleagues (2009) (Figure 2.5). Accordingly, this study examined the following hypotheses:

- 1) An increase in urinary pH disrupts stratum corneum function leading to an increase in TEWL and skin surface pH, thereby compromising the integrity of the skin
- 2) Frequent skin cleansing activities increase the permeability of the skin and consequently its susceptibility to urine
- 3) Exposure of the skin to urine and faeces leads to a release of inflammatory mediators
- 4) At the cellular level, increased pH is associated with an increased proliferation of keratinocytes and the release of inflammatory cytokines

These were successfully investigated using a combination of *in vivo* studies with healthy volunteers and *in vitro* studies using a keratinocyte cell model (HaCaT). An input-output model was adopted, as schematically shown in Figure 8.1, with the methods used to compromise the integrity of the skin and challenge the cells serving as the input, and the different biophysical, biochemical and bioengineering methods to assess skin barrier function, cell viability and the release of biomarkers serving as the output.

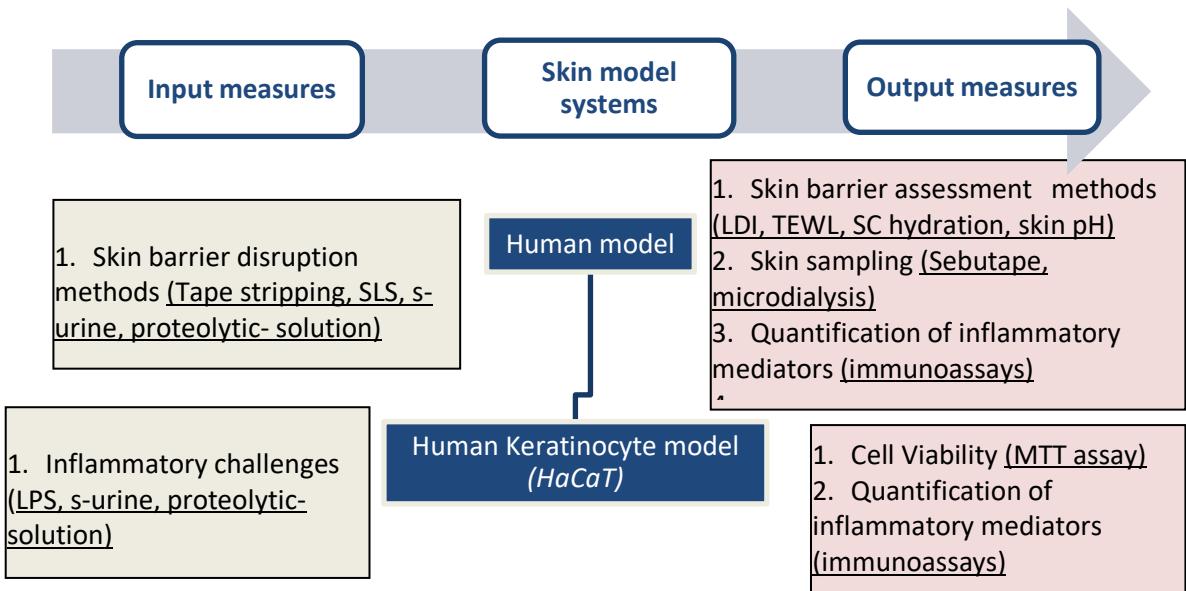


Figure 8.1. The study design to address the research hypotheses.

## Chapter 8

The first study, described in Chapter 4, examined the hypothesis that an increase in urinary pH disrupts stratum corneum function leading to an increase in TEWL and skin surface pH, thereby compromising the integrity of the skin. The findings from this study elucidated the role of urine and its inherent pH in the development of IAD. In particular, it demonstrated that urine disrupts the functional characteristics of the skin (Figures 4.2-4.5), however its effects are not pH-dependent, due to the inherent buffering capacity of able-bodied volunteers. This is in contrast with a previous study which reported that alkaline s-urine solutions are associated with severe barrier disruption (Larner *et al.*, 2015). However, the conditions imposed in the two studies were different. For example, Larner and colleagues (2015) employed alkaline urinary values (pH 7.9-10.7), which are beyond those of human urine (pH 4.8-8.0), and hence the physiological relevance of their study must be questioned. By contrast, the present study investigated a range of urinary values from pH 5.0-9.0 which resemble the pH value of physiological urine. Additionally the exposure times were different as in the current study the skin was exposed to s-urine solutions for two hours compared to six hours in Larner's study. This 2-hour exposure period was selected as it was considered to reflect clinical practice, corresponding to the frequency at which incontinent patients are checked for wetness (Voegeli, 2008). However, it should be acknowledged that with the increasing use of absorbent pads to contain incontinence, reflecting the primary preventive strategy for IAD (Beeckman *et al.*, 2015), changing intervals can vary and therefore urine may remain in contact with the skin for extended periods. These findings are also in contrast with another study, which reported that when intact skin is exposed to urine for three days, there is a significant elevation in the surface pH (Farage *et al.*, 2014). However, the results of that study should be interpreted with caution, due to the limited physiological and clinical relevance as infant urine was applied on adult skin. By contrast, the findings from the present investigation are of direct clinical and physiological relevance to IAD development, and propose that although urine compromises the integrity of the skin, on its own it is not sufficient to cause a shift in pH, away from the protective acid mantle, due to the buffering capacity of the skin. Furthermore, the current study suggests that urinary pH is not damaging to intact skin, which clearly contradicts the hypothesis that an increase in urinary pH disrupts the integrity of the skin, as reflected by increased TEWL and pH. This apparent contradiction provided additional motivation to the next study, namely to explore the effects of s-urine on damaged skin with a compromised buffering capacity.

The study described in Chapter 5 examined the hypothesis that frequent skin cleansing activities increase the permeability of the skin and consequently its susceptibility to urine. Results showed that permeability to urine depends on skin condition, with chemically-irritated skin presenting an increased permeability, as reflected by the significant increase ( $p<0.05$ ) in SSWL (Figure 5.3).

Furthermore, chemical irritation of the skin by SLS, a common surfactant found in soap preparations, provoked an increase in skin pH (Figure 5.17), suggesting that disruption of the acid mantle of the skin is associated with increased permeability to irritants. This finding has important clinical relevance. Existing studies in the literature have highlighted the importance of maintaining the acid mantle of healthy skin to prevent skin colonization and invasion by pathogens (Voegeli, 2008; Beeckman *et al.*, 2009), however this is the first study to report that a shift in skin pH away from the protective acid mantle is also associated with increased skin permeability. By contrast, intact and physically-irritated skin presented a lower permeability to urine, as reflected in the differences between the two conditions which were not statistically significant ( $p>0.05$ ). This result may be explained by the fact that there were no changes in skin surface pH in intact and physically-irritated skin, due to the inherent buffering capacity of intact skin and the inability of the tape stripping to compromise this buffering capacity. With regards to the susceptibility of the skin to urine, results again demonstrated that the severity of damage depends on skin condition. In particular, exposure of intact and physically-irritated skin to s-urine resulted in an increase in all skin properties i.e. blood flow, TEWL and SC hydration, which are in agreement with earlier findings (chapter 4). These findings also suggest that s-urine constituents aggravate the effects of physical irritation. By contrast, in intact and physically-irritated skin s-urine resulted in minimal changes in pH. With chemically-irritated skin, there were also minimal effects of s-urine exposure with only a decrease in TEWL values (Figure 5.15), suggesting that the chemical constituents of s-urine may alleviate the symptoms of chemical irritation, favouring gradual recovery of the barrier function. These findings contrast with those from Farage and colleagues (2014), which suggested that intact and compromised skin present the same susceptibility to urine, nevertheless it should be noted that tape stripping was employed to disrupt skin integrity and this could possibly explain why no differences were found with intact skin. This earlier study also treated adult skin with infant urine, thereby limiting direct comparisons with the present findings. Taken together, these findings suggest that urine can be disruptive through different mechanisms, as its irritation potentially depends on the state of the skin. Importantly, this study provided empirical evidence to support the hypothesis that frequent cleansing activities contribute to the development of IAD and further elucidated the role of urine in IAD development.

Another mechanism that has been proposed to be involved in IAD development is the release of cytokines, which are responsible for initiating the inflammatory response and modulating keratinocyte function. In this respect, the study described in Chapter 6, examined the hypothesis that exposure of the skin to urine and faeces results in a release of inflammatory mediators and present findings provided an insight into the inflammatory mechanisms associated with IAD.

In particular, results from the Sebutape samples showed that exposure to s-urine and the proteolytic-solution resulted in increased cytokine concentrations (Figure 6.10). In addition, s-urine caused a higher increase from baseline for IL-1 $\alpha$  and IL-1RA (Table 6.7), whereas the proteolytic-solution caused a higher increase in the levels of TNF- $\alpha$ . This proposes that induced-inflammation in IAD depends on the moisture source and that a combination of pro-inflammatory cytokines are implicated. Indeed an elevation in the ratio of IL-1 $\alpha$ /IL-1RA was also noted, which is critical in the balance of the inflammatory response (Terui *et al.*, 1998; Robinson *et al.*, 2003; De Jongh *et al.*, 2006), although the change was only significant ( $p<0.05$ ) at the site treated with s-urine (Figure 6.11). Furthermore, the results obtained from microdialysis provided additional information, namely, the temporal profile of cytokines' release. Of interest, a time-dependent increase in cytokine concentrations was evident following exposure to s-urine and the proteolytic solution (Tables 6.11-12). This is of particular clinical importance and supports that the increase in severity of damage is proportional to the time of exposure (Gray *et al.*, 2007). Most importantly, the current study is the first to report the successful recovery of cytokines in an in vivo model of IAD. Indeed, empirical evidence was provided to support the hypothesis that inflammatory cytokines are released following exposure to urine/faeces.

In addition to investigating the inflammatory response, the effects of s-urine and the proteolytic solution following a two-hour exposure period on selected biophysical parameters of the skin were examined. Findings revealed that both challenges produced similar responses with respect to TEWL (Figure 6.7) and SC hydration (Figure 6.8), while the proteolytic solution alone caused an increase in skin blood perfusion (Figure 6.6). However, no visible erythema was observed due to the relatively short exposure time, supporting studies which suggest that erythema only occurs after prolonged exposure for about 48 hours (Berg *et al.*, 1986; Farage *et al.*, 2014). While both challenges resulted in an increase in pH (Figure 6.9), the effect was more marked with the proteolytic-solution where the inherent proteolytic enzymes can degrade the SC and hence compromise the mechanisms responsible for the acidification of the skin surface. The increase in pH following exposure to s-urine differs to previous findings with a percentage increase from baseline of 11% compared to 1% observed in the previous study (Chapter 5). This difference could be attributed to the fact that in the earlier study, the desorption curves of TEWL were measured first for 10 minutes and then SC hydration and skin pH measurements were recorded. Therefore, any changes in surface pH would have diminished by the time of measurement due to the well-documented highly efficient buffering capacity of the skin. Indeed, as has been demonstrated, any changes in skin pH are only temporary in nature and are restored back to basal values within five minutes after removal of a challenge (Figure 4.6).

With reference to the underlying mechanisms of IAD at the cellular level, it has been hypothesized that increased pH is associated with an increased proliferation of keratinocytes to restore the skin barrier function and up-regulate cytokine release (Gray *et al.*, 2007). This notion was examined in the in vitro investigations described in Chapter 7. Findings from the in vitro investigations revealed that epidermal keratinocytes are not tolerant to changes in pH, and indeed any shifts away from their optimum pH range (pH 7.0-9.0) are associated with increased growth inhibition (Figure 7.4). This finding supports previous research on wound healing mechanisms (Sharpe *et al.*, 2009). Treatment with both s-urine and the proteolytic solution resulted in an inhibition of cell growth, which progressively increased with exposure time and concentration of challenge (Figure 7.5). The proteolytic solution caused more severe damage as it contained proteolytic enzymes, such as trypsin, which can degrade protein from the cell surface and result in cell death. In addition, it was demonstrated that for any urinary pH values outside the optimum range for keratinocytes the growth inhibition increased in a time-dependent manner (Figure 7.7). This has also been reported previously in a tissue-engineered model of human epidermis, however, these results are based in an environment in the absence of CO<sub>2</sub>, which is clearly required if the experiments are to simulate the physiological conditions *in vivo* (Sonsma *et al.*, 2006). The effects of various factors on the release of inflammatory cytokines revealed a significant impact with both incubation time and pH of s-urine solutions ( $p<0.05$ ). For example, it was evident that an increase in pH was associated with an elevation in IL-1 $\alpha$  levels, with the highest up-regulation corresponding to the most alkaline s-urine solution of pH 10.0 (Figure 7.9). Consequently, these in vitro-derived data confirmed *in vivo* findings (Chapter 6) and clearly showed that exposure to urine modulates cytokine release, suggesting that IL-1 $\alpha$  can be a robust responsive marker of skin damage in future studies. A significant increase ( $p<0.05$ ) in IL-1RA concentration was also evident at extreme acidic (pH 4.0-5.0) and alkaline conditions (pH 10.0), and this might have been predicted as this anti-inflammatory cytokine is released by keratinocytes in response to damaging conditions to block the activity of IL-1 $\alpha$  (Corsini and Galli, 2000). In addition, the levels of IL-1RA were higher when compared to the control and pH values of 7.0-9.0, suggesting that this may be attributed to the chemical composition of s-urine. Interestingly, results for the other pro-inflammatory cytokines varied, as IL-1 $\beta$  was only increased in acidic conditions (pH 4.0-5.0) compared to IL-6, IL-8 and TNF- $\alpha$  which were decreased considerably at extreme acidic (pH 4.0-5.0) and alkaline (pH 10.0) pH values. This latter finding is in contrast to previous *in vivo* data (Chapter 6) and may be due to increased growth inhibition associated with these solutions ( $>80\%$ ). Findings from the in vitro investigations highlighted the importance of pH in regulating keratinocytes function and consequently the maintenance of the skin barrier function. Accordingly, increased pH results in decreased cell viability and consequently proliferation of keratinocytes, which contradicts the proposed hypothesis of Gray and colleagues (2007).

However, evidence was provided that increased pH is associated with the release of inflammatory mediators, more pronounced with IL-1 $\alpha$ .

## 8.2 Critical Analysis of Techniques and Results

### 8.2.1 *In vivo* studies

#### Experimental protocols

*In vivo* studies described in this thesis were conducted on a relatively young cohort of able-bodied volunteers (mean age of  $33.7 \pm 10.9$  y.), and although age is not considered a risk factor for the development of IAD, it is associated with a high prevalence of incontinence (Kottner *et al.*, 2014). As a consequence, findings cannot be extrapolated to the sub-population suffering from incontinence and who are at increased risk of IAD. Additionally, the small sample size in the studies precludes robust conclusions about the mechanisms involved in the development of IAD, and hence findings must be viewed with caution. With regards to testing, the volar aspect of the forearm was used in the studies as it represents an accessible site, frequently used in clinical research. Nevertheless, it should be acknowledged that due to differences in barrier characteristics and skin reactivity between anatomical sites, results may not be applicable to other anatomical sites (Pinnagoda *et al.*, 1990), such as the perineum, which is commonly affected in IAD. In addition, exposure time to s-urine/proteolytic-solution was consistently prescribed at two hours, which is clinically relevant since it represents the frequency at which incontinent patients are checked for wetness in clinical practice (Voegeli, 2008). However, as reported in the literature and based on clinical experience, duration of exposure is critical in the development of IAD. Therefore, the 2-hour exposure time effectively represents an acute irritant reaction. In spite of these inherent limitations, the *in vivo* protocols successfully established that investigation of IAD is feasible in a lab-based setting.

With reference to the two irritant models, both the SLS and tape stripping disrupted the skin barrier function, as reflected by an increased TEWL, however, the latter treatment failed to compromise the acid mantle of the skin. This can be explained by the increased cohesiveness of deeper layers of the SC, as tape stripping (n=20) only removed the superficial layers of SC and it is well-established that skin permeability depends on the full thickness of the SC (Fluhr *et al.*, 2002).

### **Microdialysis and Sebutape™ absorption method**

In the current investigation, two distinct skin sampling techniques were employed to investigate cytokine release, namely microdialysis and the Sebutape™ absorption method. Microdialysis was well-tolerated by the volunteers and the cytokines of interest were successfully quantified in all collected dialysates. Due to the restriction of limited volume (150 $\mu$ l), samples were not quantified for IL-1 $\alpha$  and IL-1RA. To increase sample volume a longer protocol is required or an increased perfusate flow rate (>5 $\mu$ l/min), however these would have impacted the convenience of the volunteers and the recovery of cytokines, respectively. As microdialysis is a semi-quantitative method, an *in vitro* approach was adopted to examine its efficiency in cytokine recovery. This demonstrated a low recovery of cytokines, with values ranging between 3 and 7% (Figure 6.3). However, this recovery is higher to that previously reported (1-2.50%) within the host group (Gray, 2017). Moreover, the recovery rates of IL-1 $\beta$ , IL-8 and TNF- $\alpha$  were higher in the present study compared to a previous study that reported efficiencies of 0% for IL-1 $\beta$  and IL-8, and 4% for TNF- $\alpha$ . In fact, the authors reported that an increased recovery of 75% and 35% for IL-1 $\beta$  is only achieved with very low perfusate rates of 0.3 $\mu$ l/min and 1 $\mu$ l/min, respectively. By contrast, in that study the recovery for IL-6 was higher (11%) (Wælgaaard *et al.*, 2006). To increase the recovery of microdialysis, a lower perfusate flow rate can be used in future studies (Shippenberg and Thompson, 2001) than the one adopted in the present study i.e. 5 $\mu$ l/min, chosen on practical grounds to ensure sufficient volume over the collection period for quantitative cytokine analysis. If a lower perfusate flow rate had been used then a longer collection period may have affected the comfort of volunteers who rested in a supine position throughout the study. In addition, the recovery of cytokines by microdialysis can be increased by the inclusion of a trapping agent in the perfusate, including albumin, lipids and cyclodextrins (Sun and Stenken, 2003; Trickler and Miller, 2003; Ernberg and Alstergren, 2004). In spite the low recovery of cytokines by microdialysis in the current study, trends in cytokine levels following each challenge were evident. In respect to IAD, this was the first time that this was reported and indeed responsive markers of skin damage by incontinence were determined. Furthermore, microdialysis fibres were implanted superficially in the skin at a shallow depth of approximately 0.5-0.8mm. Although this depth was not characterized, it was assumed that a consistent shallow depth was achieved in all volunteers according to the protocol. In spite of this, it has been reported in the literature that the implantation depth of the fibres does not affect the recovery of cytokines (Hegemann *et al.*, 1995; Voegeli, 2001). Additionally, home-made microdialysis fibres constructed in the lab were used, as opposed, to commercial fibres with their inherent higher costs. Occasionally, the joint between the fibres and the tubing was damaged due to movement of the test area (Figure 6.1), but *in situ* repair was conducted immediately with a UV sensitive instant adhesive.

## Chapter 8

It should be noted that in some cases repair was not successful and hence, sample collection from all skin sites was not plausible. Nevertheless, pro-inflammatory cytokines were successfully quantified in all dialysates collected; highlighting that *in vivo* microdialysis sampling provides a powerful technique for real-time monitoring of the inflammatory response in the skin.

It is interesting to compare the differences between the cytokine levels measured from Sebutape samples, collected for 2 minute periods according to a previous protocol (Perkins *et al.*, 2001), and microdialysis samples collected over a 30 minute period. Results from microdialysis samples revealed that this technique was able to recover all the cytokines of interest, and indeed was found to be more sensitive to the measurements of both IL-6 and TNF- $\alpha$  concentration (Table 6.13). These differences can be explained by the fact that the application time of Sebutapes on the skin surface was not sufficient to saturate the tapes, and hence longer application periods are required. Another explanation for this might be the fact that these two methods sample the skin at different depths, as the Sebutape collects sebum from the skin surface whilst microdialysis collects sample from the interstitium. This suggests that inflammatory mediators released from epidermal keratinocytes and cells in the dermis are faster accumulated in the interstitial space rather than sebum. In addition to this, these differences may also attributed to the different cytokine profiles sampled by each technique. In particular, Sebutape collects sebum from the skin surface and studies in the literature propose that sebocytes are also involved in the inflammatory response in the skin by releasing cytokines in sebum, whereas microdialysis samples the interstitium and cytokine concentrations correspond to those released by epidermal keratinocytes and have crossed the DEJ. The inherent limitations associated with the Sebutape sample preparation protocol is also a possible factor of why some of the cytokines were not quantified, as absorbed cytokines on Sebutape samples were diluted in 1.70ml PBS + 0.05% Tween<sup>TM</sup> 20, and such large sample volumes may have resulted in the levels of the extracted cytokines being close to, or below, the LLOD. This proposes that in future studies the current protocol should be revisited and improved so that more concentrated samples are prepared, with a lesser dilution factor, and this in conjunction with extended collection periods will potentially increase the recovery of cytokines from Sebutapes. Noteworthy, although cytokine concentrations from Sebutape samples were normalized for total protein, this masked the actual changes in cytokine levels. This is consistent with previous results within the host group (Gray, 2017). Therefore, considering that Sebutape<sup>TM</sup> is a lipophilic tape collecting sebum from the skin surface, and that is important to correct cytokine concentrations for each challenge, then perhaps normalizing for total lipid content, measured by commercially available assays, might prove a more appropriate option.

### 8.2.2 *In vitro* investigations

Keratinocytes represent a robust *in vitro* skin model, frequently used in experiments to provide a basis for experimental human studies. However, it should be acknowledged that the threshold concentration required to provoke a defined response will be much lower than that encountered in *in vivo* conditions (Welss *et al.*, 2004). In addition there are differences in response between cell lines. As an example, it has been reported that inflammatory mechanisms triggered in HaCaT cells are different to those in living epidermal keratinocytes (Muller *et al.*, 2003). As a consequence, results obtained from *in vitro* studies cannot be directly correlated to the *in vivo* situation. To bridge the gap between epidermal keratinocytes and HaCaT cells, more complex reconstructed human epidermis models have been developed (Cannon *et al.*, 1994; Roguet *et al.*, 1994; Ponec *et al.*, 2002). Additional work using a full-thickness skin model was initially planned to be included in this research, however due to time and funding restrictions this was not completed.

## 8.3 Future Directions

### 8.3.1 Clinical Validation

The novel findings presented in this thesis shed light into the inflammatory mechanisms implicated in IAD, demonstrating that both IL-1 $\alpha$  and TNF- $\alpha$  can serve as responsive markers of skin damage from incontinence. Nevertheless, the use of experimental models of urinary- and faecal- incontinence highlights the necessity to confirm these findings in a clinical setting, involving patients suffering from IAD. Both sampling techniques have the potential for clinical adaptation, and with respect to microdialysis, preliminary work has been performed in developing a wearable microfluidics device for *in situ* measurement of inflammatory cytokines and other analytes of interest (Gowers *et al.*, 2015). In a similar manner, Sebutape<sup>TM</sup> can be easily adapted to a clinical setting as it is non-invasive and easy to perform. Nonetheless, inherent limitations with the protocol need to be addressed so that direct sample preparation and analysis is feasible. Such validation will not only strengthen the value of current approaches but will also facilitate future studies intended to detect early damage requiring prevention and to aid treatment.

### 8.3.2 Further characterization of the damage at the cellular level

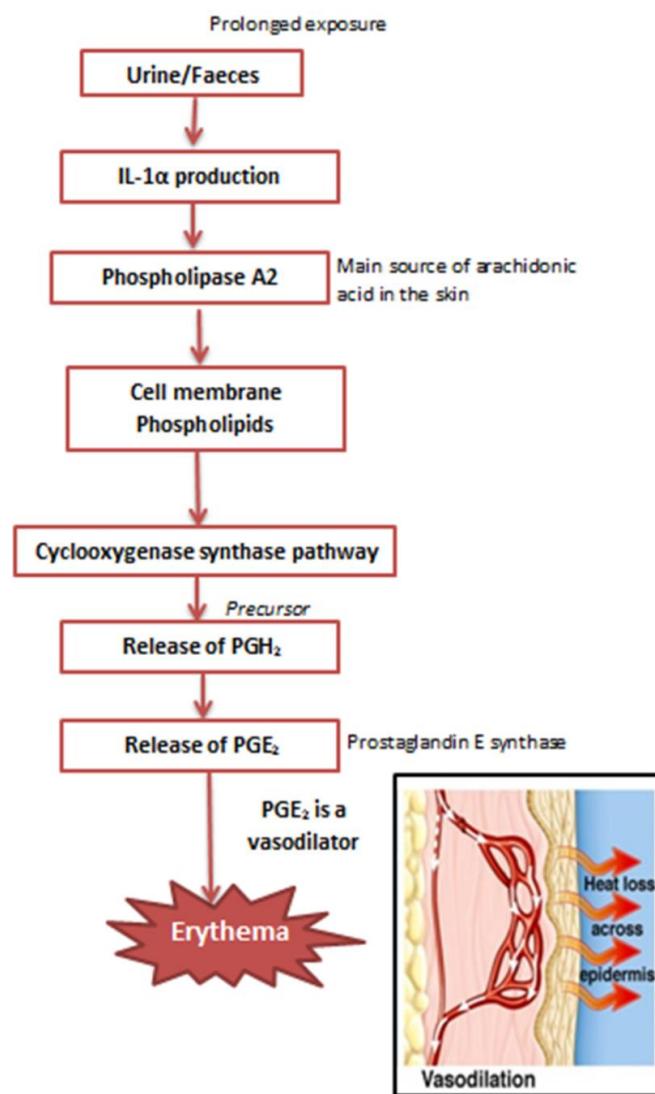
Findings from *in vitro* experiments with the HaCaT cell line demonstrated that s-urine and the proteolytic solution have detrimental effects on the viability of cells, which increase with time of exposure. This also led to the modulation of inflammatory mediators, with a more profound response seen with IL-1 $\alpha$ .

However, given that isolated HaCaT cells do not fully represent *in vivo* situations, it is recommended that further research is undertaken with a more relevant physiological model of skin barrier function, such as a full-thickness skin model. Such models closely resemble human skin as it is consisted of human-derived keratinocytes and fibroblasts, which are cultured and formed a multi-layered, highly differentiated human dermis and epidermis, and consequently a functional SC. This will allow detailed characterization of damage at the cellular level, as well as histopathological changes in tissue structure following exposure to urine and faeces could be determined. Additionally, the presence of fibroblasts will also strengthen the relevance to the *in vivo* situation, as these cells are critical for the formation of the skin barrier function and consequently the maintenance of cutaneous homeostasis. Indeed the modulation of cytokine release by both keratinocytes and fibroblasts and their interactions, which are critical in cellular communication (Varkey *et al.*, 2014), could also be investigated.

### **8.3.3 Other inflammatory mediators involved in the inflammatory response following exposure to urine and faeces**

Although the mechanisms underlying the pathophysiology of IAD are unknown, this study began to explore these mechanisms using innovative *in vitro* and *in vivo* skin models and has significantly enhanced our understanding on the inflammatory response associated with skin exposure to urine and faeces. The novel findings presented in this thesis demonstrated the release of pro-inflammatory mediators following exposure to simulated urine and faeces. However, further work is required to fully elucidate the underlying mechanisms in IAD. To achieve this, it is necessary to explore the possible pathophysiological mechanisms and the release of other biomarkers whereby the clinical symptoms of IAD, such as erythema and itching, are experienced. With regards to erythema, this represents the immediate local response of the skin to irritants. Studies in the literature suggest that the release of prostaglandin E<sub>2</sub> (PGE<sub>2</sub>), stimulated by IL-1 $\alpha$ , is one of the primary compounds responsible for the production of erythematous responses in the skin (Figure 8.2) (Hagermark and Strandberg, 1977; Terry *et al.*, 1999). Additionally, PGE<sub>2</sub> is abundantly produced by epidermal keratinocytes in healthy and diseased conditions, including psoriasis and eczema (Reilly *et al.*, 2000; Nicolaou *et al.*, 2011; Nicolaou, 2013). Therefore, this mediator may indeed be implicated in the development of IAD and future studies should investigate the release of PGE<sub>2</sub> following exposure to excessive moisture from incontinence. Both microdialysis and Sebutape methods used in the current thesis could be adopted and the levels of PGE<sub>2</sub> could be quantified in skin samples using commercially available immunoassays. Accordingly, in the case of increased levels of PGE<sub>2</sub> in the skin following exposure to urine/faeces, ibuprofen may be used to inhibit the production of PGE<sub>2</sub>, as reported in the literature (Shetty *et al.*, 2013), offering thus a possible treatment for the erythematous response in IAD.

The release of histamine has also been proposed to be involved in IAD development (Gray *et al.*, 2007) as it is an important mediator in inflammation, responsible for the itch sensation in skin (Shim and Oh, 2008). A series of studies have reported elevated levels of histamine in several skin inflammatory conditions, including AD (Ruzicka and Glück, 1983; Damsgaard *et al.*, 1997; Gruber *et al.*, 2011). Pivotal studies investigated the role of histamine receptors on the surface of keratinocytes, (Greaves and Davies, 1982), particularly their role on skin barrier recovery following tape stripping has been examined in mice suggesting that histamine delays barrier recovery (Ashida *et al.*, 2001). Therefore, in order to elucidate the exact mechanisms and factors associated with IAD, the role of histamine should be further investigated.



**Figure 8.2. Proposed mechanism of PGE<sub>2</sub> and the production of erythema in IAD.** Based on (Welss *et al.*, 2004; Nicolaou, 2013)

### **8.3.4 The effect of urine and faeces on SC serine proteases**

Serine proteases are expressed in the SC and have an essential role in epidermal proliferation, differentiation, and in lipid barrier homeostasis (Voegeli *et al.*, 2008). These enzymes, including the stratum corneum tryptic (KLK5) and chymotryptic (KLK7) are also critical for the physiological regulation of desquamation (Rawlings and Matts, 2005). Both enzymes have been found to be involved in inflammatory conditions in the skin and, in particular, evidence was provided in a clinical study involving patients suffering from AD and psoriasis (Komatsu *et al.*, 2005; Komatsu *et al.*, 2007). It has also been proposed that increased expression of these enzymes is associated with skin barrier disruption and a consequent degradation of lipid processing enzymes. Interestingly, an increase in skin pH has been associated with increased activity of these enzymes (Hachem *et al.*, 2005), and this is a key issue with respect to a possible implication in IAD development. Therefore, future studies should also aim to elucidate the effects of exposure to urine and faeces on these serine proteases. An identical protocol to that described in Chapter 6 can be adopted and the release of these enzymes can be quantified using commercial ELISA kits.

### **8.3.5 The role of occlusive conditions caused by incontinence products in the development of IAD**

Incontinence products are important in clinical practice for the prevention of IAD as they limit the exposure of the skin to urine and/or faeces and avoid over-hydration of the skin. However, the inevitable occlusive conditions created by these products represent a separate proposed mechanism involved in the development of IAD (Beeckman *et al.*, 2009). While the effects of occlusion were determined in the current investigation and distinguished from the effects of s-urine (Chapter 4, section 4.8.3), these were not explored with commercial incontinence products used in clinical settings. Therefore, to elucidate the role of occlusive conditions in the development of IAD, further work is required to explore the complex interaction between incontinence pads and the functional characteristics of the skin, including TEWL, SC hydration and skin surface pH. This can be examined with the non-invasive biophysical methods used within this thesis. In addition to this, changes in skin microclimate conditions (temperature and humidity) caused by incontinence products should also be investigated to further characterize the interface with the skin.

### **8.3.6 The effect of skin care regimens on the release of inflammatory mediators**

Novel findings in this thesis suggested that the traditional skin cleansing activities with soap, water, and drying using a towel may increase the risk of IAD. This has implications for future work, as the inflammatory response triggered following these regimens should be examined to further elucidate their role in the development of IAD.

Importantly, the findings from the current study provide a basis for future studies to evaluate alternative skin care routines and products, including continence cloths and liquid cleansers, in an attempt to optimize care for incontinent patients. It should be highlighted that these were not investigated as they were beyond the scope and research aim of the current research thesis.

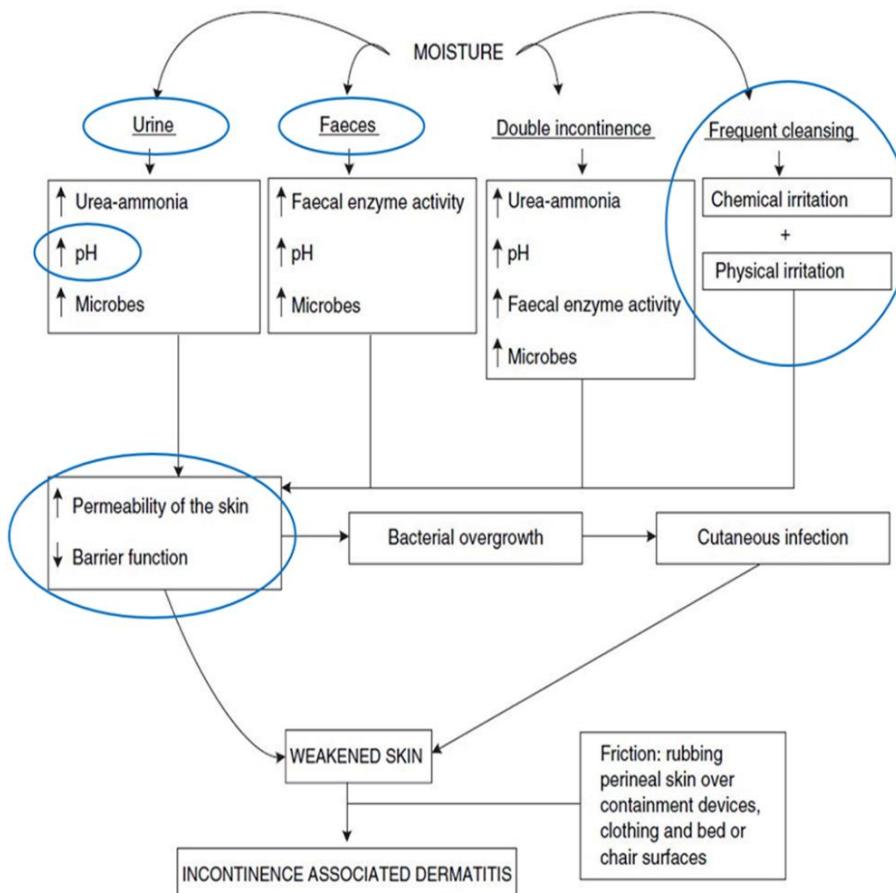
#### **8.4 Implications for IAD research/clinical practice**

Although there are some limitations associated with small sample sizes in each of the studies, the implications of the overall research for IAD research and clinical practice are undeniable. The most important implication is the successful development of an *in vivo* model of IAD, which can serve as indicator to future studies. The results from the study investigating the release of inflammatory mediators have also implications for future research, as it was shown that IL-1 $\alpha$  and TNF- $\alpha$  can serve as responsive markers of early skin damage caused by incontinence. In addition, it has been demonstrated that when tape stripping and SLS are included in such models, the time of both skin sampling after tape stripping and exposure to SLS are critical for cytokine recovery, and hence should be carefully considered.

With respect to clinical practice, an immediate implication is that the frequency of skin cleansing regimens with soap-based products should be re-evaluated as these may increase the risk of IAD. Results also showed that increased pH is associated with increased skin permeability, suggesting that the choice of skin care products in clinical settings should be carefully considered, favoring the use of pH-balanced cleansers to protect the skin acid mantle and promote skin health. Furthermore, cytokine analysis showed that the inflammatory response in IAD develops in a time-dependent manner, and this emphasizes the need to re-evaluate the frequency at which incontinent patients have their skin cleansed. It was also highlighted the need for incontinence care products to maintain the optimum internal pH to facilitate keratinocytes viability and consequently normal barrier function.

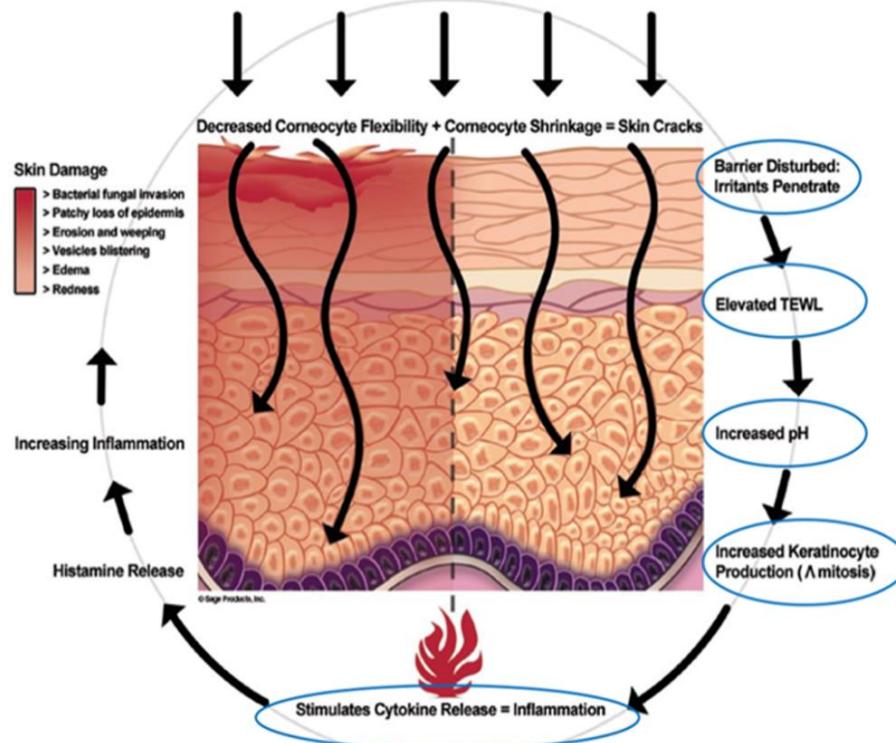
#### **8.5 Contribution of the thesis to the current state of knowledge**

Findings from the present investigation extend the state of current knowledge on IAD, as previously the mechanisms responsible for IAD development were restricted to clinical observations and were lacking in robust empirical evidence. Thus, the major contribution of this thesis is that empirical evidence is provided with respect to the proposed underlying mechanisms of IAD, which can inform future research directions (Figure 8.3).



**SKIN DAMAGE DEPENDENT UPON:**

1. Irritant: Moisture < Urine < Formed Stool < Urine and Stool < Liquid Stool
2. Duration of Exposure
3. Frequency of Exposure



Urinary pH is not  
damaging on  
healthy skin

The irritation potential  
of urine depends on the  
integrity of the skin

Frequent Cleansing  
activities may increase  
the risk of IAD

Exposure to urine/faeces  
modulates the release of  
inflammatory markers

Increased pH  
influences cell viability  
and cytokine release

**Figure 8.3. Contribution of this thesis to current knowledge.** Based on existing theoretical frameworks on IAD pathophysiology, this thesis investigated the proposed mechanisms in IAD development (displayed in blue circles). Empirical evidence provided by this research is marked in red line dotted boxes.

In this research, these mechanisms were examined in an experimental setting to simulate clinical and physiological conditions. Although it is not possible to draw definitive conclusions, findings of this research allow us to propose that:

- urinary pH is not damaging on intact skin
- the irritation potential of urine depends on the state of the skin
- frequent cleansing activities can potentially increase the risk of IAD
- the release of inflammatory mediators is implicated in the development of IAD
- an increase in the internal pH influences keratinocytes' viability and cytokine release

These mechanisms seem to be involved in the development of IAD and should be further explored in a large sample in future research. With respect to the role of pH in the development of IAD, these data suggest that while it is important to protect the acid mantle and consequently maintain the integrity of the skin barrier, it is also critical to maintain an optimum pH for keratinocytes function. This suggests the co-existence of both an internal and an external mechanism in the maintenance of skin integrity. Importantly, this study represents a novel approach to quantify inflammatory mediators in an in vivo model of IAD, using techniques and protocols that could be adapted for routine clinical usage. Findings inform future studies as it was shown that IL-1 $\alpha$  and TNF- $\alpha$  could serve as responsive markers to skin damage caused by factors associated with incontinence. In addition, evidence has also been provided for the first time suggesting that there are differences in the inflammatory mechanisms of IAD, depending on the moisture source, and this can explain the enhanced effects observed with doubly incontinent patients, and offers a novel line of investigation for other forms of moisture-associated skin damage. Overall, the studies described in this thesis established the methods to investigate IAD in an experimental setting, which can be translated to clinical studies.



## Appendix A     Ethics Application

### A-1: Reviewers' Comments and requested revisions

#### **ERGO Revisions Requested Form (Reviewers' form for new RGO Ethics form)**

Ethics No	
Applicant	Sofoklis Koudounas
Short Title of Study (6 words)	Inflammatory mechanisms and skin damage

Please make the requested revisions using track changes to highlight the revisions you have made. This will make it easier and faster for the reviewers to assess these revisions. Please submit your revised application via ERGO.

<b>Section</b>	<b>Revisions requested</b>
1. Applicant details	
<b>2. Study Details</b>	
2.1 Title of study	
2.2 Type of study	
2.3 Proposed start date	
2.3 Proposed end date	
2.4 What are the aims and objectives of this study?	Make sure you define all acronyms at first time of use eg. SLS application.
2.5 Background to the study	
2.6 Research question	
2.7 Study design	<p>'Small cohort studies' are the people recruited onto phase 1 the same people who be recruited onto phase 2, or are you hoping to recruit a separate group of 20 participants, i.e. are you recruiting 20 people for the studies detailed in this application or 40 people (20 for phase 1, 20 for phase 2)? The PIS suggests the former. This aspect of the study design needs clarification.</p> <p>Need to mention that the study design involves repeated visits to the laboratory.</p>
<b>3. sample and Setting</b>	
3.1 How are the participants to be approached?	More details of the recruitment are needed.
3.2 Who are the proposed sample and where are they from?	
3.3 Describe the relationship between researcher and sample	
3.4 Describe how you will ensure that fully informed consent is being given	<p>The minimum time participants will have to read to the PIS needs to be longer than 24hrs, particularly as one of the methodological stipulations is that participants should not use skin care products 24hrs prior to testing.</p> <p>Please define what you mean by skin care products,</p>

## Appendix A

	does this include soap, body wash, etc?
<b>4. Research procedures, interventions and measurements</b>	
4.1 Give account of the procedure as experienced by the participants	<p>Acronyms SLS and TEWL need explanation.</p> <p>How is TEWL measured?</p> <p>If the same people are taking part in both phases, is there any carry-over effect from the procedures undertaking in phase 1? Does there need to be a minimum rest period between phase 1 and phase 2?</p> <p>You stated that participants will need to attend the laboratory for a day, please be specific and state how long each data collection session will take.</p> <p>What will the participants be able to do whilst they are waiting for 2hrs in phase 1.1, are they able to read, watch videos, use the toilet, etc? What provisions will you have available for the participants?</p> <p>The section describing the application of the filter paper discs for 24hrs onwards is rather confusing and implies that the tape stripping protocol will be performed for 24hrs. The section needs rewriting for clarification making it clear what will happen to the participant.</p> <p>Please explain tape stripping.</p> <p>Will the application of the discs for 24hrs prevent the participant from performing every day activities, e.g. washing and self care?</p> <p>Please state who, with reference to their relevant qualifications, will be inserting the needles in phase 2.</p> <p>How are Dialysates collected?</p>
<b>5. Study management</b>	
5.1 state any potential for psychological or physical discomfort and/or distress?	The applicant needs to list potential sources of discomfort, not just stating that adverse effects will unlikely occur. The skin irritation protocols and inserting needles, for example, will cause some discomfort. Please expand on this section and consider your protocol from the perspective of the participant who will likely be naïve to your procedures.
5.2 Explain how you intend to alleviate any psychological or physical discomfort and/or distress that may arise? (if applicable)	Please state how you minimise the discomfort considering the above comment.
5.3 Explain how you will care for any participants in 'special groups'	
5.4 Please give details of any payments or incentives being used to recruit participants (if applicable)?	The protocol is time intensive for the participant. It would be appropriate to offer refreshment if they are with you for a day.
5.5 How will participant	

confidentially be maintained (if applicable)?	
5.6 How will personal data and study results be stored securely during and after the study?	
5.7 Who will have access to these data?	This section implies that images will be taken of the participants skin in response to the procedures, if this is the case, please state this in the 'what will happen to participants section'.

**PIS:**

1. The PIS needs to be completely re-written using lay language. Avoid overly scientific terms (e.g. "These will be perfused with a sterile saline solution to enable us to collect and measure the inflammatory mediators released by your skin in response to SLS, tape stripping and synthetic urine"), don't assume prior knowledge of the subject area (e.g. a person might not understand the consequences of differing pH levels in synthetic urine), explain procedures in simple terms (e.g. what does tape stripping involve, will it be painful?).
2. Within the 'Visit 1' section, please explain to the reader, in lay terms, what synthetic urine is.
3. Please state what the participants will be allowed to do whilst during the two hours.
4. In the ethics form it states that the local anaesthetic cream will be applied prior to visiting the lab, however, this is not mentioned here. Will you be providing the cream? If so, please state.
5. The application of the discs for 24hrs needs to be clarified, i.e. the discs will be applied then after the other measurements are taken they will leave the laboratory and go about their normal routine until they visit the lab the following day to have the discs removed. Please state whether any activities of daily living will be prevented through wearing of the discs.
6. Please state under the risks section whether discomfort will occur through the application of the needles and/or the tape stripping procedure.
7. Rephrase sentence 'Up to four small microdialysis fibres...' as it is not the EMLA that places the fibres under the skin.
8. Can you state how long the participant will be with you on visit 2 and 4?

**Poster**

1. Language on poster needs to be more lay.

**Risk assessment form**

1. On risk assessment for the age range is 18 - 70 years on the ethics application it is 18 - 65 years. Please adjust.
2. Risk assessment student signature needs dating.

**A-2: Response to reviewer's comments****Response to Reviewer's comments on RGO Ethics form**

Ethics No	
Applicant	Sofoklis Koudounas
Short Title of Study (6 words)	Inflammatory mechanisms and skin damage

Section	Revisions requested	Changes made
<b>2. Study details</b>		
2.4 What are the aims and objectives of this study?	Make sure you define all acronyms at first time of use e.g. SLS application	<p><b>Abbreviations added:</b> moisture- associated skin damage (MASD). synthetic-urine (s-urine)</p> <p><b>Acronyms defined:</b> sodium lauryl sulfate (SLS) application</p>
2.7 Study design		
	'Small cohort studies' are the people recruited onto phase 1 the same people who be recruited onto phase 2, or are you hoping to recruit a separate group of 20 participants, i.e. are you recruiting 20 people for the studies detailed in this application or 40 people (20 for phase 1, 20 for phase 2)? The PIS suggests the former. This aspect of the study design needs clarification. Need to mention that the study design involves repeated visits to the laboratory.	This section has been rewritten to make it clear that a total group of up to 20 volunteers will be recruited, and that these same volunteers will be asked to take part in each phase of the study. The PIS has been rewritten to make the time commitment required clearer.
<b>3. Sample and Setting</b>		
3.1 How are the participants to be approached?	More details of the recruitment are needed.	The usual practice of this research group is to recruit volunteers by poster advertisement. Posters are displayed on internal noticeboards within the faculty, following favourable ethics review. This section has been expanded slightly, although it is not totally clear what further details of recruitment are required or could be given?
3.4 Describe how you will	The minimum time participants will have to read to the PIS needs	A Patient Information Sheet (PIS) will be given to the participants a

ensure that fully informed consent is being given	to be longer than 24hrs, particularly as one of the methodological stipulations is that participants should not use skin care products 24hrs prior to testing.	minimum of 48 hours and maximum of 1 week prior to the study. This will be further discussed and any questions answered, at least 24 hours prior to visit 1. If the volunteers are then happy to proceed, they will be required to sign an informed consent form at visit 1, and be given a copy of this.
4. Research procedures, interventions and measurements		
4.1 Give account of the procedure as experienced by the participants	<ol style="list-style-type: none"> <li>1. Please define what you mean by skin care products, does this include soap, body wash, etc?</li> <li>2. Acronyms SLS and TEWL need explanation.</li> <li>3. How is TEWL measured?</li> <li>4. If the same people are taking part in both phases, is there any carry-over effect from the procedures undertaking in phase 1? Does there need to be a minimum rest period between phase 1 and phase 2?</li> <li>5. You stated that participants will need to attend the laboratory for a day, please be specific and state how long each data collection session will take.</li> <li>6. What will the participants be able to do whilst they are waiting to for 2hrs in phase 1.1, are they able to read, watch videos, use the toilet, etc? What provisions will you have available for the participants?</li> <li>7. The section describing the application of the filter paper discs for 24hrs onwards is rather confusing and implies that the tape stripping protocol will be performed for 24hrs. The section needs rewriting for clarification making it clear what will happen to the participant. Please explain tape stripping.</li> <li>8. Will the application of the discs for 24hrs prevent the participant from performing every day activities, e.g. washing and self-care?</li> <li>9. Please state who, with reference to their relevant qualifications, will be inserting the needles in phase 2.</li> </ol>	<ol style="list-style-type: none"> <li>1. Skin care products defined as moisturizers and any other skin care creams, but not excluding them from following their normal washing routine.</li> <li>2. Acronyms are explained in previous sections. SLS is defined in section 2.4 and TEWL in section 2.7</li> <li>3. Explained in methods section 2.7.</li> </ol> <p>Transepidermal Water Loss (TEWL) (TM300, MPA9, Courage &amp; Khazaka, Germany) to assess the status of the skin barrier (Fluhr et al., 2006; Gao et al., 2013). This will be measured using an open-chamber system that consists of two sensors (temperature and relative humidity) that measure the amount of water evaporating from the skin (Worsley and Voegeli, 2013)</p> <ol style="list-style-type: none"> <li>4. The same volunteers will take part in Phases 1 and 2 and for this reason a refractory period of two weeks will be adopted between the two phases to minimize any carry-over effects from Phase 1.</li> <li>5. This visit will last approximately 3 ½ hours, as detailed in the revised protocol, ERGO form and the participant information sheet.</li> <li>6. S-urine will be applied for 2 hours. During this time, participants will be able to leave the laboratory if they wish. If they choose to stay in the laboratory they will be able to read, listen to music or watch videos, and offered</li> </ol>

	<p>10. How are Dialysates collected?</p>	<p>refreshments as appropriate.</p> <p>7. This section was re-written accordingly to clarify the procedure to be followed. Please see below:</p> <p>Phase 1.2: Response to chemical and mechanical-induced damage, using SLS and the tape stripping method, and s-urine</p> <p>Same volunteers, as in Phase 1.1, will be asked to attend the laboratory for two days, each visit lasting between 1 and 3½ hours. On day 1, after a period of acclimatisation (20 minutes) at an ambient temperature of 22°C, baseline biophysical measurements of skin blood flow (SLDi, 50x50 mm scan lasting 2 minutes), TEWL, skin hydration and skin pH will be performed on four areas (20x20mm) of both volar forearms (2 sites on each arm). A ruler will be used to ensure correct positioning. At one site, a filter paper disc impregnated with 0.5% w/v SLS solution will be applied for 24 hours. Two sites will also be allocated for tape stripping and s-urine challenges that will be performed the following day. The remaining site will serve as the untreated control. On day 2, and after 24 hours of SLS application, volunteers will return to the laboratory, SLS-containing disc will be removed, followed by biophysical measurements on both the untreated and SLS-treated sites, to determine the effects of chemical irritation on the skin barrier. Then, tape stripping will be performed on the allocated site, using a commercially available adhesive tape. Tapes with a defined size (20mmx20mm) matching the test area will be used. A pressure roller will also be used to apply the tapes on the skin, to ensure a constant application pressure, as this has been shown to be critical for the removal of the stratum corneum (SC) (Ghadially <i>et al.</i>, 1995). A pressure roller will be used to press each tape 10 times. The tape stripping procedure will last no longer than 10 minutes. Tape stripping will be performed until there is no more increase in the</p>
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		<p>TEWL value or until the skin glistens (Morgan et al., 2003), and TEWL measurements will be recorded every 5 tape strips. After tape stripping, s-urine will also be applied for 2 hours on a third allocated site, at the optimum pH obtained from Phase 1.1. Biophysical measurements to determine the effects of each skin challenge on the skin barrier compared to the untreated site will be taken after every challenge.</p> <ol style="list-style-type: none"> <li>8. The application of SLS-containing discs will not affect daily activities. The volunteers will just be advised to be careful not to remove them.</li> <li>9. All work performed in the laboratory within the Clinical Academic Facility will be supervised by clinically qualified staff (DV). Insertion of the needles for microdialysis in Phase 2 will be performed by a registered nurse (DV)</li> <li>10. Dialysates will be collected in standard laboratory microtubes every 30 minutes for a maximum period of 3 hours. After collection, tubes will be stored in -80°C until subsequent analysis.</li> </ol>
<b>5. Study management</b>		
5.1 state any potential for psychological or physical discomfort and/or distress?	The applicant needs to list potential sources of discomfort, not just stating that adverse effects will unlikely occur. The skin irritation protocols and inserting needles, for example, will cause some discomfort. Please expand on this section and consider your protocol from the perspective of the participant who will likely be naïve to your procedures.	This section has been revised to reflect the minor discomfort that may occur following skin challenge and microdialysis fibre insertion.

## Appendix A

5.4 Please give details of any payments or incentives being used to recruit participants (if applicable)?	The protocol is time intensive for the participant. It would be appropriate to offer refreshment if they are with you for a day.	There will be no payments or incentives offered to participants. However, light refreshments, such as tea or coffee will be offered to the volunteers as appropriate.
5.7 Who will have access to these data?	This section implies that images will be taken of the participants skin in response to the procedures, if this is the case, please state this in the 'what will happen to participants section'.	The only images that will be taken from the volunteers are computer generated blood flow images from the SLDI and these will be anonymized. This section has been revised to aid clarity.

### PIS:

1. The PIS needs to be completely re-written using lay language. Avoid overly scientific terms (e.g. "These will be perfused with a sterile saline solution to enable us to collect and measure the inflammatory mediators released by your skin in response to SLS, tape stripping and synthetic urine"), don't assume prior knowledge of the subject area (e.g. a person might not understand the consequences of differing pH levels in synthetic urine), explain procedures in simple terms (e.g. what does tape stripping involve, will it be painful?).

Correction: PIS has been re-written using simple language, easier to understand

2. Within the 'Visit 1' section, please explain to the reader, in lay terms, what synthetic urine is.

Insertion: In this visit, synthetic urine (s-urine) will be used to challenge the skin barrier. S-urine is a water solution with the same composition as human urine.

3. Please state what the participants will be allowed to do whilst during the two hours.

Insertion: During this time, participants will be able to leave the laboratory as it is not required for the volunteers to be bedbound in this phase. If chosen to stay in the laboratory they will be able to read, listen to music or watch videos, and if chosen to leave the laboratory they will be offered tea or coffee.

4. In the ethics form it states that the local anaesthetic cream will be applied prior to visiting the lab, however, this is not mentioned here. Will you be providing the cream? If so, please state.

Correction- Visit 4: This visit will occur a minimum of two weeks after visit 3. This visit will not last longer than 5 hours. Prior commencing of the study, you will be provided with an anaesthetic cream (EMLA) which you will be asked to apply to your forearm at least 1½ hours, before arriving to the lab. You will be asked to attend the laboratory for no longer than 3 ½ hours. After arriving to the lab, EMLA will be removed and up to four microdialysis fibres will be inserted superficially under the skin. These will be perfused with a sterile saline solution to enable us to collect and measure the chemicals released by your skin in response to SLS, tape stripping and s-urine. Another method to measure these chemicals will also be used at the same time, so that we can compare both methods. This involves the use of a special 'sticky' tape called Sebutape, which absorbs the chemicals released by your skin.

5. The application of the discs for 24hrs needs to be clarified, i.e. the discs will be applied then after the other measurements are taken they will leave the laboratory and go about their normal routine until they visit the lab the following day to have the discs removed. Please state whether any activities of daily living will be prevented through wearing of the discs.

Correction: Visits 2 & 3: These visits will occur two-weeks after visit 1 to ensure any effects of the synthetic urine have resolved. Each visit will last a minimum of 1 and a maximum of 3½ hours. At visit 2, you will be required to attend the laboratory for approximately 1 hour. After arrival, baseline measurements of skin barrier function and skin blood flow will be recorded at four sites on your forearm. On one site, a filter paper disc filled with 0.5%w/v sodium lauryl sulfate (SLS) solution will be applied and left in place for 24 hours (being removed the next day at visit 3). Two sites will be used for the other two skin challenges (tape stripping and s-urine) and the remaining site will be left untreated (control). After this you will be free to leave the laboratory and continue your normal routine, without restriction, until you return to the laboratory the next day. The application of the SLS will not affect any daily activities but you are kindly requested to be careful not to remove it.

6. Please state under the risks section whether discomfort will occur through the application of the needles and/or the tape stripping procedure.

Insertion: It is most unlikely that you will suffer any discomfort or adverse effects from this study, although some may experience a mild, painless, short-lasting skin irritation following the application of SLS or tape stripping. The use of the EMLA cream means the needles and microdialysis fibres can be inserted without pain. Very occasionally some minor bruising may result from the microdialysis, but this normally resolves within 3 days.

7. Rephrase sentence 'Up to four small microdialysis fibres...' as it is not the EMLA that places the fibres under the skin. DONE

8. Can you state how long the participant will be with you on visit 2 and 4?

Visit 2: At visit 2, you will be required to attend the laboratory for approximately 3 ½ hours.

Visit 4: This visit will occur a minimum of two weeks after visit 3. This visit will not last longer than 5 hours.

#### Poster

1. Language on poster needs to be more lay. Poster has been re-written.

#### Risk assessment form

1. On risk assessment for the age range is 18 – 70 years on the ethics application it is 18 – 65 years. Please adjust. Adjusted
2. Risk assessment student signature needs dating. Dates will be updated on the date of resubmission.

## Appendix A

## Appendix B     Approved documents

### B-1: ERGO application form



#### ERGO application form – Ethics form

All mandatory fields are marked (M\*). Applications without mandatory fields completed are likely to be rejected by reviewers. Other fields are marked "if applicable". Help text is provided, where appropriate, in italics after each question.

#### 1. APPLICANT DETAILS

1.1 (M*) Applicant name:	Sofoklis Koudounas
1.2 Supervisor (if applicable):	Dr David Voegeli (DV) Prof Dan L Bader (DLB)
1.3 Other researchers/collaborators (if applicable): <i>Name, address, email, telephone</i>	

#### 2. STUDY DETAILS

2.1 (M*) Title of study:	Investigation of the inflammatory mechanisms leading to moisture-associated skin damage
2.2 (M*) Type of study (e.g. Undergraduate, Doctorate, Masters, Staff):	Doctorate
2.3 i) (M*) Proposed start date:	01/05/2015
2.3 ii) (M*) Proposed end date:	01/05/2016

2.4 (M*) What are the aims and objectives of this study?
Aims and Objectives of in vivo work
The overall aim of this programme of work is to investigate the inflammatory mechanisms leading to disruption of the skin barrier as evident in moisture-associated skin damage (MASD).
Specific Objectives:
1. Investigation of the effects of different skin challenges on the skin barrier function.
1.1. Determination of an optimum pH value of synthetic-urine (s-urine) at which a skin response is triggered
1.2. Response to chemical- and mechanical- challenge, using sodium lauryl sulfate (SLS) application and tape stripping respectively, and s-urine
2. Investigation of the release profile of inflammatory mediators following each of the mechanical and chemical challenges.

**2.5 (M\*) Background to study (a brief rationale for conducting the study):**

The effects of moisture on skin function and barrier properties have been well-documented, however, the exact structural mechanisms leading to skin breakdown have not been clearly revealed (Gray et al., 2011). Prolonged exposure of the skin to moisture, such as urine, stool, perspiration, skin tears and wound exudate can cause chronic inflammation and erosion that ultimately leads to skin breakdown (Gray et al., 2011).

A general term, moisture-associated skin damage (MASD) is used to cover a range of distinct categories, depending on the source of damage.

Incontinence is believed to be one of the major risk factors for skin breakdown and the development of incontinence-associated dermatitis (IAD) (Copson, 2006).

IAD is a multifactorial condition whose cause is the prolonged exposure to urine/faeces (Gray et al., 2007; Voegeli, 2010b). It is associated with inflammation of the skin and is the most widely studied type of MASD (Voegeli, 2010b; Beeckman et al., 2011; Gray et al., 2012). The precise mechanisms of IAD are not well understood (Voegeli, 2010b), however, several assumptions have been made with respect to its inflammatory component. Thus it has been proposed that faeces and liquid stool overhydrate the epidermis and upregulate their constituent proteolytic enzymes leading to an increase in skin pH to alkali levels, thus disrupting the acid mantle. It has also been suggested that the presence of urine per se does not lead to IAD (Gray et al., 2007; Voegeli, 2010).

Skin damage from prolonged exposure to moisture (urine/faeces) represents a major health issue for patients in both hospital and community settings. Minimizing the prolonged exposure of skin to moisture is clearly critical in preventing MASD. For this reason, it has been suggested that four main strategies should be adopted, namely:

- i. the use of a standardized skin care regimen
- ii. the use of products that absorb moisture from the skin,
- iii. the removal of the source of excessive moisture and
- iv. the treatment of any secondary infections (Gray et al., 2011)

Skin care regimens have been developed and adopted to protect the skin from the damaging effects of moisture. Skin regimens usually include the use of soap and water to cleanse the area at risk and the use of a moisturizer and/or a skin protectant (Gray et al., 2007). Skin care regimens are important, but further research is required in order to establish a more structural approach to prevent the development of IAD (Gray et al., 2007).

Indeed, by defining the inflammatory mechanisms involved in the pathogenesis of IAD, future research will enable more targeted preventive and treatment strategies.

**2.6 (M\*) Key research question (Specify hypothesis if applicable):**

This study will investigate the inflammatory mechanisms that lead to the development of incontinence-associated dermatitis (IAD). Skin challenges simulating the effects of prolonged exposure to urine/faeces and mechanical damage will be used in conjunction with a series of well established physical and biochemical analysis techniques.

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## 2.7 (M\*) Study design (Give a brief outline of basic study design)

*Outline what approach is being used, why certain methods have been chosen.*

A small cohort of up to 20 volunteers will be recruited to address the objectives of this research. Volunteers will participate in both phases 1 and 2, and will be required to attend the laboratory on four separate occasions spread over a 5 week period. Each visit will last a minimum of 3 ½ hours and a maximum of 5 hours.

Skin Barrier assessment and induced-damage will be performed using well-established quantitative skin biophysical methods that are available in the Clinical Academic Faculty and regularly employed in dermatological research. These include:

### Skin Barrier Disruption methods:

- 1) Chemically-induced irritation will be performed using a well-established sodium lauryl sulphate (SLS) model validated by Held and Agner (1999).
- 2) Mechanically-induced irritation will be performed using the tape stripping method as described by Gao et al., (2013).
- 3) Synthetic urine (s-urine) will also be used as an irritant source, experienced by patients with IAD, previously used by Mayrovitz and Sims (2001).

### Skin Barrier Assessment methods:

- 1) Scanning Laser Doppler Imaging (SLDi) to examine skin blood flow in response to damage. This will involve the use of a non-invasive laser Doppler imaging system (Moor Instruments Ltd, Axminster, UK).
- 2) Transepidermal Water Loss (TEWL) (TM300, MPA9, Courage & Khazaka, Germany) to assess the status of the skin barrier (Fluhr et al., 2006; Gao et al., 2013). This will be measured using an open-chamber system that consists of two sensors (temperature and relative humidity) that measure the amount of water evaporating from the skin (Worsley and Voegeli, 2013).
- 3) Skin Hydration assessed using a corneometer (Corneometer MPA9, Courage & Khazaka, Germany), which represents the gold-standard method for measuring skin hydration in dermatological research (Berardesca et al., 1990).
- 4) Skin surface pH assessed utilising a commercial pH probe (MPA9, Courage & Khazaka, Germany).
- 5) Analysis of inflammatory markers will be performed utilizing a non-invasive tape absorption method (Sebutape), previously described by Perkins et al. (2001).
- 6) Microdialysis will be used as the gold standard method to assess the inflammatory response caused by the different skin challenges and as a reference for the Sebutape /tape stripping method, a protocol previously outlined by Schnetz and Fartasch (2001).

All work performed on volunteers in the laboratory will be supervised by clinically qualified staff (DV) and all researchers have received NIHR GCP training.

### 3. SAMPLE AND SETTING

**3.1 (M\*) How are participants to be approached?** Give details of what you will do if recruitment is insufficient. If participants will be accessed through a third party (e.g. children accessed via a school) state if you have permission to contact them and upload any letters of agreement to your submission in ERGO.

Participants will be recruited by poster advertisement designed to attract the staff and student populations of the University of Southampton, as is usual practice within this research group and no recruitment problems are anticipated.

**3.2 (M\*) Who are the proposed sample and where are they from (e.g. fellow students, club members)?** List inclusion/exclusion criteria if applicable. NB The University does not condone the use of 'blanket emails' for contacting potential participants (i.e. fellow staff and/or students).

*It is usually advised to ensure groups of students/staff have given prior permission to be contacted in this way, or to use of a third party to pass on these requests. This is because there is a potential to take advantage of the access to 'group emails' and the relationship with colleagues and subordinates; we therefore generally do not support this method of approach.*

*If this is the only way to access a chosen cohort, a reasonable compromise is to obtain explicit approval from the Faculty Ethics Committee (FEC) and also from a senior member of the Faculty in case of complaint.*

Up to 20 healthy participants will be recruited from the staff and student population of the University of Southampton through advertisement.

Inclusion criteria:

- Aged 18-65 years
- No history of skin diseases

Exclusion criteria:

- Pregnancy
- pre-existing medical condition that is known to affect the dermal vasculature (e.g. diabetes mellitus, peripheral vascular disease, Raynaud's phenomenon; pregnancy)
- treatment with any vasoactive medication (e.g. beta-blockers, nitrates, calcium channel blockers, angiotensin-converting-enzyme (ACE) inhibitors, antihistamines; nonsteroidal anti-inflammatory drugs (NSAIDS); steroids)
- pre-existing dermatological condition
- inability to give informed written consent;

**3.3 (M\*) Describe the relationship between researcher and sample** (Describe any relationship e.g. teacher, friend, boss, clinician, etc.)

Participants may be colleagues or students.

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**3.4 (M\*) Describe how you will ensure that fully informed consent is being given: (include how long participants have to decide whether to take part)**

A Participant Information Sheet (PIS) will be given to volunteers a minimum of 48 hours and maximum of 1 week prior to the study. This will be further discussed and any remaining questions answered, at least 24 hours prior to visit 1. If the volunteers are then happy to proceed, they will be required to sign an informed consent form at visit 1, and be given a copy of this.

## 4. RESEARCH PROCEDURES, INTERVENTIONS AND MEASUREMENTS

**4.1 (M\*) Give a brief account of the procedure as experienced by the participant (Make clear who does what, how many times and in what order. Make clear the role of all assistants and collaborators. Make clear total demands made on participants, including time and travel). Upload any copies of questionnaires and interview schedules to your submission in ERGO.**

Participants will be instructed not to use any skin care product, such as moisturizers and any other skin care creams, but not excluding them from following their normal washing routine, on the day prior to each study, and the day of each visit, as it has been shown that the application of moisturizers and other cosmetic products influence TEWL and consequently the susceptibility to skin irritation (Lodén, 1996; Held et al., 1999; Buraczewska et al., 2007; Lodén, 2012).

Objective 1: Investigation of the effect of different skin challenges on the skin barrier function

To address the needs of this objective, volunteers (up to 20) will be recruited and the skin barrier will be challenged using SLS, tape stripping, and s-urine. Skin biophysical measurements will be used to determine the effect of each challenge on the barrier function. It is also anticipated that these studies will provide informative results relating to the next objective, which will examine the inflammatory mechanisms in response to skin challenge. This objective will be divided in two phases depending on the skin challenge. A two-week period will be prescribed between the two phases, in order for the skin barrier to fully recover from any irritating effects occurring from the exposure to the different s-urine formulations in Phase 1.1.

Phase 1.1: Investigation of the effects of different s-urine formulations on the skin barrier

Volunteers (up to 20) will be asked to attend the laboratory. This visit will last approximately 3½ hours, as detailed in the protocol and the participant information sheet. After a 20 minute-period of acclimatisation, at an ambient temperature of 22°C, baseline biophysical measurements of skin blood flow (SLDi 50x50 mm scan lasting 2 min), TEWL, skin hydration and skin pH will be performed on six areas

(20x20mm) on the volar aspects of both forearms.

A ruler will be used to ensure correct positioning, with different s-urine solutions, of varying pH, applied on five sites. S-urine preparation will be based on the formulation used by Mayrovitz and Sims (2001), and application via filter paper discs. Ammonium hydroxide will be added to the formulations, to adjust the pH of each solution (pH 5.0, 6.0, 7.0, 8.0, 9.0). The remaining site will serve as the untreated control. S-urine will be applied for 2 hours. During this time, participants will be free to leave the laboratory if they wish. If they choose to stay in the laboratory they will be able to read, listen to music or watch videos, and will be offered refreshments. After the 2 hour exposure time, the s-urine discs will be removed and the biophysical measurements will be repeated at all six sites (5 experimental, 1 control).

**Phase 1.2: Response to chemical and mechanical-induced damage, using SLS and the tape stripping method, and s-urine**

Following a minimum period of two weeks to ensure there is no residual effect from phase 1.1, the same volunteers, will be asked to attend the laboratory for two days, each visit lasting between 1 and 3½ hours. On day 1, after a period of acclimatization (20 minutes) at an ambient temperature of 22°C, baseline biophysical measurements of skin blood flow (SLDi, 50X50mm scan lasting 2 minutes), TEWL, skin hydration and skin pH will be performed on four areas (20x20mm) of both volar forearms (2 sites on each arm). A ruler will be used to ensure positioning. At one site, a filter paper disc impregnated with 0.5% w/v SLS solution will be applied and left in place for 24 hours. Two sites will also be allocated for the tape stripping and s-urine challenges that will be performed the following day. The remaining site will serve as the untreated control. The application of SLS-containing discs will not affect daily activities; volunteers will just be advised to be careful not to remove them. The next day, and after 24 hours of SLS application, volunteers will return to the laboratory, the SLS- containing disc will be removed, followed by biophysical measurements on both the untreated and SLS-treated sites, to determine the effects of chemical irritation on the skin barrier. Then, tape stripping will be performed on the allocated site, using a commercially available tape (e.g. Sellotape™). Tapes with a defined size (20mmx20mm) matching the test site will be used. A pressure roller will be used to apply the tapes on the skin, to ensure a constant application pressure, as this has been shown to be critical for the removal of the stratum corneum (SC) (Ghadially *et al.*, 1995). A pressure roller will be used to press each tape 10 times. The tape stripping procedure will last no longer than 10 minutes. The tape stripping procedure will last no longer than 10 minutes. Sequential tape strips will be performed until there is no more increase in the TEWL value observed or until the skin glistens (Morgan *et al.*, 2003) and TEWL measurements will be recorded every 5 tape strips. After tape stripping, s-urine will also be applied for 2 hours on a third allocated site, at the optimum pH determined in Phase 1.1. Biophysical measurements to determine the effects of each skin challenge on the skin barrier compared to the untreated site will be taken after every challenge.

**Objective 2: Investigation of the profile of inflammatory mediators released following skin challenge**

To address this objective, the same volunteers will be invited to return to the laboratory a minimum of two weeks after phase 1.2. This visit will last 5 hours and dermal microdialysis and Sebutape will be performed to investigate the release of inflammatory markers upon chemical- and mechanical- challenge, using SLS and tape stripping respectively, and exposure to s-urine. Both analysis techniques will be compared in an attempt to establish the sensitivity and validity of each method in quantifying the release of inflammatory mediators. The same volunteers will take part in Phases 1 and 2 and for this reason a refractory period of two weeks will be adopted between the two phases in an attempt to minimize any carry-over effects from Phase 1.

**Protocol:**

Prior to returning to the laboratory for this visit, volunteers will be provided with a local anaesthetic cream (Eutectic Mixture of Local Anaesthetic, EMLA) and instructed how to apply it to four areas of the dominant volar forearm (at a distance of 20mm from each other) where the microdialysis fibres will be placed at least 1.5 hours beforehand. After arrival to the lab, the EMLA will be removed followed by 20 minutes of acclimatization at an ambient temperature of 22°C. Then, four hypodermic needles (21g) will be inserted superficially, at a depth of 0.5-0.8mm under the skin for a distance of 20mm. Insertion of the needles at this stage, will be performed by a registered nurse (DV). The microdialysis fibres will then be inserted through the needles and the needles removed, leaving just the fibres underneath the skin. All fibres will be perfused with sterile phosphate buffered saline (PBS) at a rate of 3-5 µl/ min and dialysate collected 30 minutes prior to skin challenge (baseline). Sebutape will also be attached on each site at baseline. Then, after randomization, 0.5% SLS will be applied on one site using filter paper discs (20 mm diameter), tape stripping will be performed on another site, and s-urine will be applied on a third site. The pH of the s-urine solution will depend on findings from Objective 1, phase 1.1. The remaining site will serve as the untreated control. Dialysates will then be collected in standard laboratory microtubes every 30 minutes for a maximum period of 3 hours. Sebutape will also be performed in all sites at the end. Samples will then be stored at -80°C until subsequent batch analysis for inflammatory mediators using a Meso Scale Discovery (MSD) electrochemiluminescence multiplex assay.

A schematic detailing each of the test protocols is provided in the Research Protocol document.

## 5. STUDY MANAGEMENT

5.1 (M*) State any potential for psychological or physical discomfort and/or distress?
<p>Minor discomfort may be experienced during SLS application and microdialysis. Skin irritation and itching after SLS application is very mild and normally resolves after 24 hours.</p> <p>If adverse effects are observed by the experimenters (immediate damage to the skin surface) or by the study participants (extended periods of erythema, skin dryness) the study will be terminated and protocol reviewed. The methods for inducing skin damage are non-invasive and safe, and are not associated with a risk of developing an occupational skin disease.</p> <p>Microdialysis is a minimally-invasive and painless technique due to the use of EMLA. Some volunteers might experience some minor bruising following microdialysis fibre insertion, however this normally resolves after 3 days.</p> <p>The laser in the scanning Laser Doppler Imager (SLDi) manufactured by Moor Instruments Ltd, Axminster, UK is a single wavelength, red 633 nm, 1.5 mW Class 3a laser. As such it is certified safe for use in humans.</p> <p>Synthetic urine (pH 7.8) will be based on a formulation described by Mayrovitz and Sims (2001), including: 25g/l urea, 9g/l sodium chloride, 2.5g/l disodium hydrogen orthophosphate, 3g/l ammonium chloride, 2g/l creatine, 3g/l sodium sulfite (unhydrated).</p>

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**5.2 (M\*) Explain how you intend to alleviate any psychological or physical discomfort and/or distress that may arise? (if applicable)**

A minor degree of irritation and mild bruising might occur from the different skin challenges and from microdialysis, and will likely fade away within 2-3 days but if not and the participants are concerned, they will be asked to return to the laboratory for further examination. Also, to minimize any discomfort during needle insertion for microdialysis, an anaesthetic cream (EMLA) will be used. All operators placing the microdialysis have considerable experience in using this technique.

**5.3 Explain how you will care for any participants in 'special groups' (i.e. those in a dependent relationship, vulnerable or lacking in mental capacity) (if applicable)?**

N/A

**5.4 Please give details of any payments or incentives being used to recruit participants (if applicable)?**

There will be no payments or incentives offered to participants. However, light refreshments, such as tea or coffee, will be offered to the volunteers as appropriate.

**5.5 i) How will participant anonymity and/or data anonymity be maintained (if applicable)?**

*Two definitions of anonymity exist:*

*i) Unlinked anonymity - Complete anonymity can only be promised if questionnaires or other requests for information are not targeted to, or received from, individuals using their name or address or any other identifiable characteristics. For example if questionnaires are sent out with no possible identifiers when returned, or if they are picked up by respondents in a public place, then anonymity can be claimed. Research methods using interviews cannot usually claim anonymity - unless using telephone interviews when participants dial in.*

*ii) Linked anonymity - Using this method, complete anonymity cannot be promised because participants can be identified; their data may be coded so that participants are not identified by researchers, but the information provided to participants should indicate that they could be linked to their data.*

Linked anonymity will be maintained in this series of studies. All samples will be anonymised using an identification code that is allocated upon recruitment. No volunteer personal details will be stored. Data (raw or processed) will be stored on the computers of the academic group generating the data, cited in the Clinical Academic Faculty.

The results of all the analyses, along with images on skin responses, will also be stored in an anonymised electronic format in a centralised repository together with a backup copy of all raw data. Access to the data will be limited to those parties taking part in this work. Anonymised images and data will be stored on encrypted memory sticks for transfer from the laboratory for analysis. All study data will be archived in accordance with the University policy for research data management (<http://www.southampton.ac.uk/library/research/researchdata/>).

**5.5 ii) How will participant confidentiality be maintained (if applicable)?**

*Confidentiality is defined as the non-disclosure of research information except to another authorised person. Confidential information can be shared with those who are already party to it, and may also be disclosed where the person providing the information provides explicit consent.*

Access to the data will be limited to those parties taking part in this work.

**5.6 (M\*) How will personal data and study results be stored securely during and after the study?** *Researchers should be aware of, and compliant with, the Data Protection policy of the University. You must be able to demonstrate this in respect of handling, storage and retention of data.*

At the end of the study, data collected will be securely stored for a minimum of ten years in accordance with the University policy for research data management (<http://www.southampton.ac.uk/library/research/researchdata/>).

**5.7 (M\*) Who will have access to these data?**

Data (raw or processed) will be stored on the computers of the academic groups generating the data. The results of all the analyses will also be stored in an anonymised electronic format in a centralised repository together with a backup copy of all raw data. Access to the data will be limited to those parties taking part in this work. Anonymised images (computer generated blood flow images from the SLDi) and data will be stored on encrypted memory sticks for transfer from the laboratory for analysis.

**N.B. – Before you upload this document to your ERGO submission remember to:**

1. Complete ALL mandatory sections in this form
2. Upload any letters of agreement referred to in question 3.1 to your ERGO submission
3. Upload any interview schedules and copies of questionnaires referred to in question 4.1

**B-2: Recruitment Poster**



## Research Study

### Investigation of the inflammatory mechanisms leading to moisture-associated skin damage

**Are you generally healthy and aged between 18-65?  
We need you!**

We are investigating the inflammatory mechanisms activated in the skin upon persistent exposure to moisture.

#### What the study involves:

The study will take place in the Faculty of Health Sciences in the South Academic Block at Southampton General Hospital. You will be asked to attend on 4 separate occasions, spread over a 5 week period. Each visit will last a minimum of 1 hours and maximum of 5 hours. Non-invasive, painless skin measurements will be recorded before and after different skin challenges on your forearm skin to mimic the skin damage experienced by patients with urinary incontinence. In the final visit we will measure the chemicals released in the skin in response to these challenges using a technique called microdialysis. Up to four small microdialysis fibres will be placed just under the skin surface in one of your forearms following the application of a local anaesthetic cream (EMLA). The fibres will be left to settle for 1 ½ hours and then perfused with a sterile saline solution to collect the chemicals released in the skin. Microdialysis samples will be collected for a period of up to three hours. After this the fibres will be removed, your skin cleaned and you will be free to leave. This would complete the study.

**FOHS Ethics number: 9349**

**RGO number:**

If you are interested in taking part in this study or would like further details please contact:

Sofoklis Koudounas MASD study sk5e13@soton.ac.uk								
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## B-3: Participant Information Sheet

Health  
Sciences

UNIVERSITY OF  
Southampton

### Participant Information Sheet

**Study Title:** Investigation of the inflammatory mechanisms leading to moisture-associated skin damage

**Researcher:** Sofoklis Koudounas

**Supervisors:** Dr David Voegeli, Prof Dan Bader

**Faculty of Health Sciences Ethics number:** 9349

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it would involve. Please take time to read the following information carefully and discuss it with friends and relatives. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you would like to take part.

Thank you for reading this.

#### **What is the purpose of the study?**

This study aims to explore the skin irritation caused by prolonged contact with moisture, known as moisture associated skin damage (MASD). If the skin is exposed to excessive moisture for any length of time it can become inflamed (as often seen in individuals who suffer from urinary incontinence). Although several explanations for why this occurs exist, the exact mechanisms involved in the inflammation have not been fully studied. As well as moisture / wetness, other factors such as friction, the pH of the urine and the chemicals within urine, are thought to contribute to the inflammation. We now wish to explore how the skin reacts to these factors in order to better understand this common problem. We will do this using non- invasive techniques to measure how intact the outer layer of the skin is (barrier properties of the skin), and skin redness (skin blood flow imaging), before and after the application of different skin challenges to mimic the effects of friction, and moisture. In a separate part of this study the challenges will be repeated and the chemicals released by the skin cells will be collected using two techniques - Sebutape and microdialysis.

#### **Why have I been chosen?**

You have been chosen because you may be eligible to be involved in this study and have responded to our advertisement.

#### **Do I have to take part?**

It is up to you whether or not to take part. If you decide to take part you will be given this information sheet to keep and be asked to sign a consent form. You are free to withdraw from the study at any time and without giving a reason.

## **What will happen to me if I take part?**

You will be asked to answer a few questions about yourself. If you meet the criteria of the study and agree to take part you will be enrolled to the study.

You will be asked to attend the clinical laboratory in the Faculty of Health Sciences at University Hospital Southampton on 4 separate occasions, spread over a 5 week period. Each visit will last a minimum of 1 hour and a maximum of 5 hours.

**Visit 1:** This visit will last approximately 3½ hours. At this visit, synthetic urine (s-urine) will be used to challenge the skin barrier. S-urine is a water-based solution with the same composition as human urine. Baseline measurements of skin barrier function will be recorded at five areas on your forearm, and then filter paper discs containing small volumes of different synthetic urine formulations will be applied to each area for 2 hours. During this period, you will be free to leave the laboratory if you wish. After two hours the will be removed and the skin barrier measurements repeated. This will allow us to determine the ideal formulation of synthetic urine for use in other parts of this study.

**Visit 2 &3:** These visits will occur two-weeks after visit 1 to ensure any residual effects of the s-urine have resolved. At visit 2, you will be required to attend the laboratory for approximately 1 hour. After arrival, baseline measurements of skin barrier function and skin blood flow will be recorded at four sites on your forearm. On one site, a filter paper disc filled with 0.5%w/v sodium lauryl sulfate (SLS) solution will be applied and left in place for 24 hours (being removed the next day at visit 3). Two sites will be used for the other two skin challenges (tape stripping and s-urine) and the remaining site will be left untreated (control). After this you will be free to leave the laboratory and continue your normal routine, without restriction, until you return to the laboratory the next day. The application of the SLS will not affect any daily activities but you are kindly requested to be careful not to remove it.

The following day (visit 3), you will be asked to return to the laboratory for a period of 3½ hours. The SLS disc will be removed, and the skin barrier and skin blood flow measurements repeated. Tape stripping and s-urine application will also be performed at the other sites on your arm and the skin barrier measurements repeated at these sites. Tape stripping (to remove the uppermost layer of skin) involves the repeated application and removal of up to 20 2cm x 2cm strips of sticky tape to a small area of skin, and is a painless procedure. This mimics the skin damage caused by friction (e.g. rubbing of clothes or bed linen on the skin). At another site, synthetic urine will be applied for two hours, as in visit 1. Following each of these skin challenges, measures of skin barrier function and skin blood flow will be repeated, and then you will be free to leave the laboratory.

**Visit 4:** This visit will occur a minimum of two weeks after visit 3 and will not last longer than 5 hours. Prior to this visit you will be provided with an anaesthetic cream (Eutectic Mixture of Local Anaesthetic, EMLA ) and shown how to apply it. You will be asked to apply the EMLA to your forearm

at least 1½ hours, before arriving at the lab. After arriving at the laboratory the EMLA will be removed and up to four microdialysis fibres will be inserted just under the skin using hypodermic needles. These will be perfused with a sterile saline solution to enable us to collect and measure the chemicals released by your skin in response to the different challenges previously outlined. Another method to measure these chemicals will also be used at the same time, so that we can compare both methods. This involves the use of a special adhesive tape called Sebutape, which absorbs the chemicals released by your skin on contact.

The measures of skin barrier function and skin blood flow that will be used in this study are:

*Skin blood flow:* will be measured using Scanning Laser Doppler Imaging (SLDI). This uses a harmless laser beam to scan the skin surface, and pick up the movement of red blood cells to create an image of blood flow in the skin.

*Skin barrier function:* will be assessed using transepidermal water loss (TEWL). This uses a small probe that sits on top of your skin and measures the amount of water evaporating from the skin surface. We will also measure how hydrated your skin is, and any changes, using a small probe called a corneometer, that is pressed gently on your skin. Skin surface pH will be assessed using a skin pH-meter.

*Microdialysis:* this is a minimally invasive, and painless, procedure used to measure substances produced within the skin. Hollow microfibres no wider than a needle are placed just under the skin surface following the application of a local anaesthetic cream (Eutectic Mixture of Local Anaesthetic, EMLA). These fibres are then slowly perfused with a sterile saline solution to enable the chemicals being released in the skin to be collected. At the end of the study the fibres are removed, leaving little visible evidence that they were there.

### **Are there any risks involved?**

It is most unlikely that you will suffer any discomfort or adverse effects from this study, although some may experience a mild, painless, short-lasting skin irritation following the application of SLS or tape stripping. The use of the EMLA cream means the needles and microdialysis fibres can be inserted without pain. Very occasionally some minor bruising may result from the microdialysis, but this normally resolves within 3 days. If this does occur, and you are concerned, you will be asked to return to the laboratory for a subsequent visit to monitor the skin irritation. All the methods for assessing skin barrier function are non-invasive.

### **Are there any benefits in my taking part?**

You would not receive any benefits directly. However, this study will increase our understanding of the damaging effects of moisture on the skin, and could possibly lead to improved methods of skin care in the future.

### **What happens if something goes wrong?**

If you have any complaints or concerns during this study you should immediately inform the investigator. In the unlikely event that something goes wrong during the study indemnity insurance has been provided.

If you have a concern or a complaint about this study you should contact Trudi Bartlett, Research Governance Officer, at the University of Southampton (Address: Research Integrity & Governance Office, Building 37, Room 4079, University of Southampton, Highfield, Southampton SO17 1BJ. Email: [rgoinfo@soton.ac.uk](mailto:rgoinfo@soton.ac.uk); Tel: 023 8059 5058). If you remain unhappy and wish to complain formally the Research Integrity & Governance Office can provide you with details of the University of Southampton Complaints Procedure.

**Would my taking part in this study be kept confidential?**

All data will be treated in compliance with the Data Protection Act and the University of Southampton policy for the storage of data. Your details will be coded and no identifiable personal information will be stored on computer.

**What will happen to the results of the research?**

It is hoped that the results from this study will be published in suitable professional and scientific journals. It will not be possible to identify any individuals from any of the data presented. You will be asked whether you wish to be personally informed of the results of this study at the end.

**What happens if I change my mind?**

You have the right to withdraw from this study at any time, without giving a reason.

**Who has reviewed the study?**

This study has been reviewed by the Faculty of Health Sciences research ethics committee.

**Contacts for further information:**

Sofoklis Koudounas – email: [sk5e13@soton.ac.uk](mailto:sk5e13@soton.ac.uk)

Dr David Voegeli- email: [D.Voegeli@soton.ac.uk](mailto:D.Voegeli@soton.ac.uk) Tel: 023 8077 7222  
Extension: 3162

**You will be given a copy of this information sheet and a signed consent form to keep.**

**Thank you for taking the time to read this information.**

## Appendix C Typical standard curves for cytokines

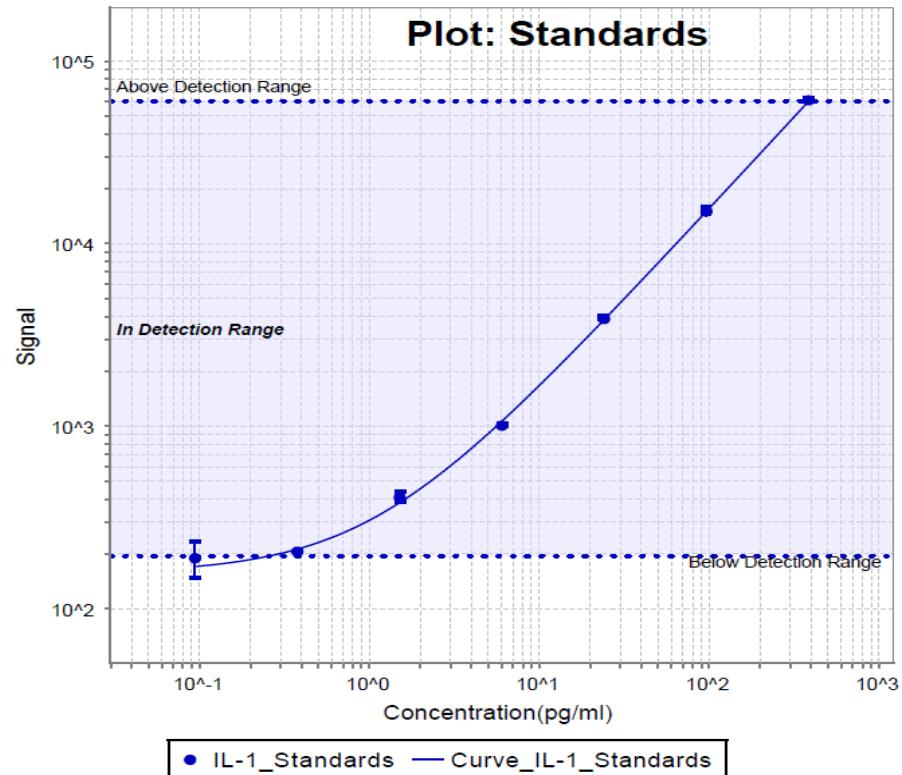


Figure C 1: Standard curve of IL-1 $\alpha$

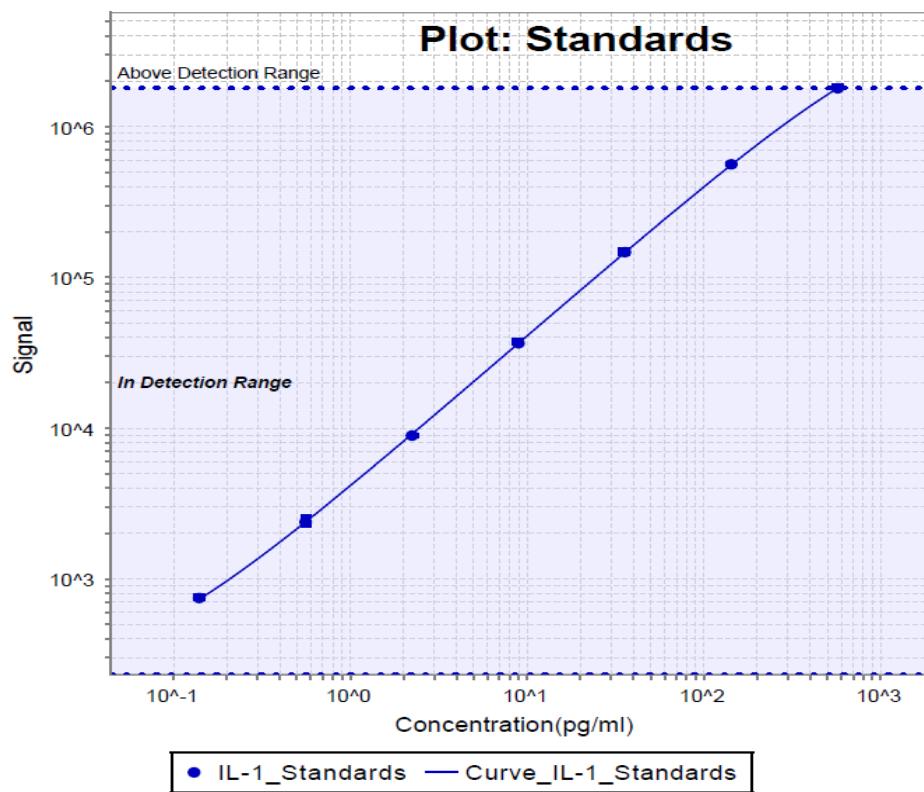


Figure C 2: Standard curve of IL-1 $\beta$

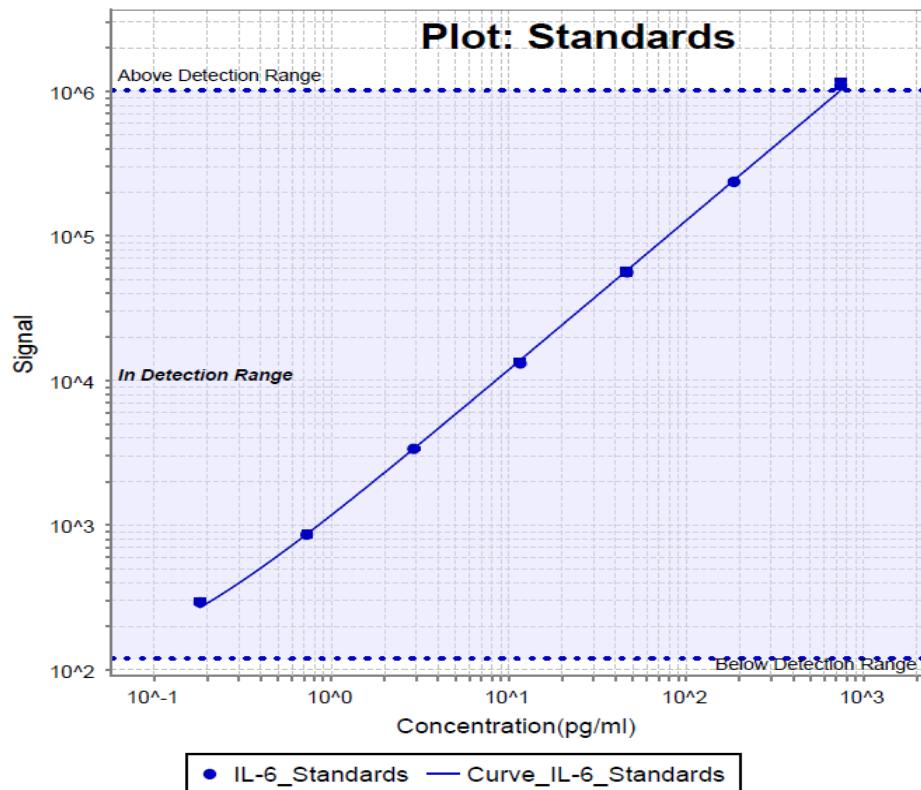


Figure C 3: Standard curve of IL-6

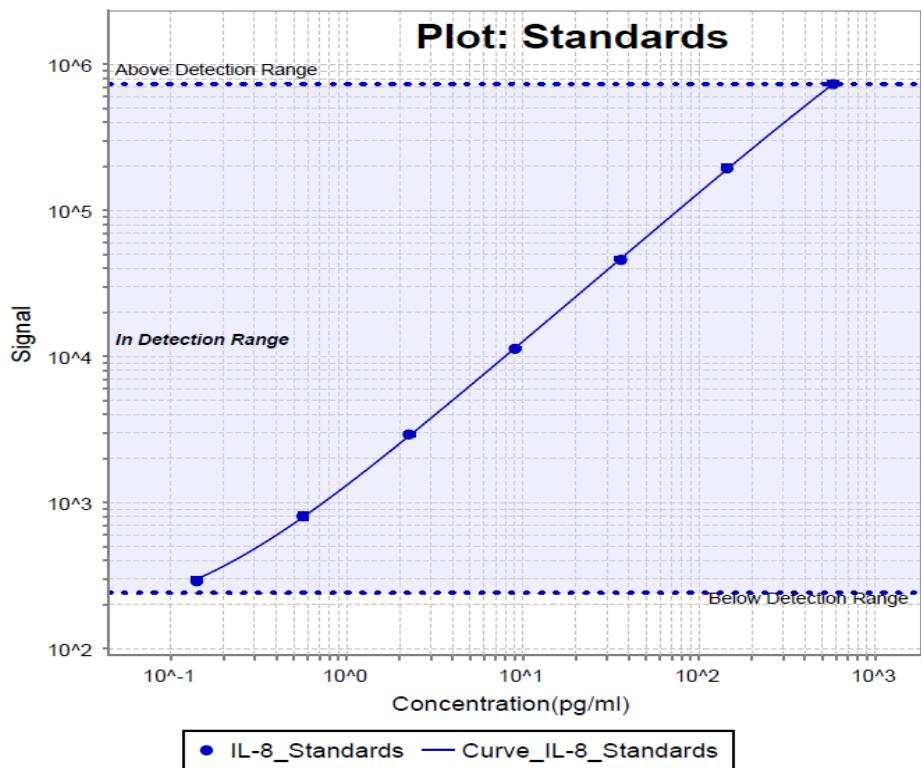


Figure C 4: Standard curve of IL-8

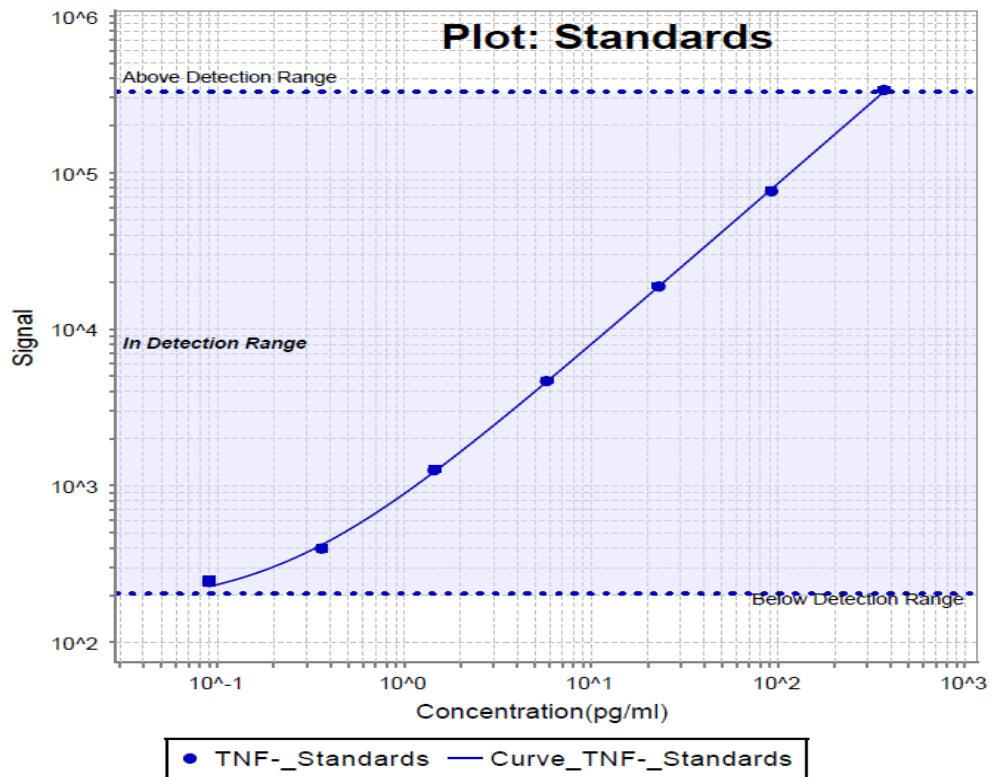
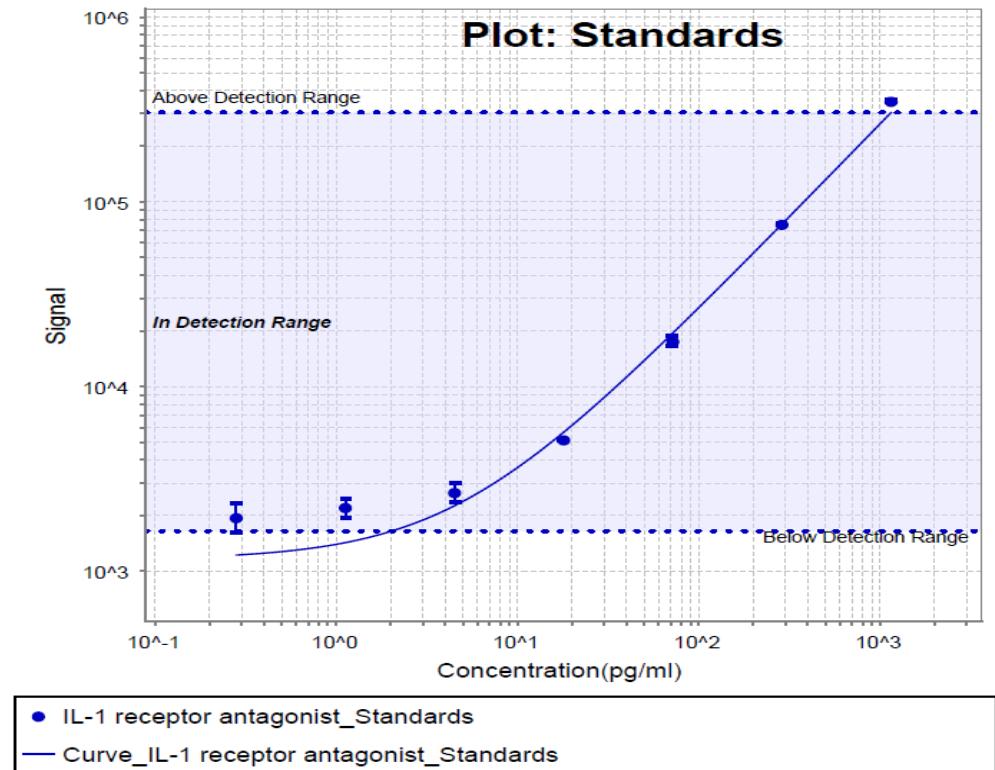
Figure C 5: Standard curve of TNF- $\alpha$ 

Figure C 6. Standard curve of IL-1RA

## Appendix C

## Appendix D Cytokine Concentrations in Sebutape and microdialysis samples

### D-1: Sebutape data

#### IL-1 $\alpha$

	CONTROL		TAPE STRIPPING		SLS		S-URINE		PROTEOLYTIC SOLUTION	
	BASELINE (T0)	POST (T4)	BASELINE (T0)	POST (T4)	BASELINE (T0)	POST (T4)	BASELINE (T0)	POST (T4)	BASELINE (T0)	POST (T4)
P1	23.50	45.10	-	-	80.60	27.10	103.00	128.00	6.83	10.40
P2	195.00	336.00	45.90	31.90	8.00	12.30	25.80	12.00	145.00	209.00
P3	48.10	44.30	31.90	56.30	20.90	37.90	31.60	86.40	31.90	36.90
P4	115.00	111.00	22.30	30.10	26.30	31.60	46.40	94.00	127.00	316.00
P5	41.60	46.10	15.50	91.60	13.40	14.90	9.88	33.40	54.10	211.00
P6	368.00	442.00	58.10	51.90	10.00	21.00	8.44	66.70	195.00	278.00
P7	70.20	95.70	18.90	124.00	24.30	78.50	7.57	70.70	100.00	95.80
P8	272.00	161.00	142.00	219.00	189.00	47.90	86.30	851.00	72.40	188.00
P9	137.00	118.00	45.40	205.00	133.00	27.60	87.90	69.40	49.10	46.90
P10	512.00	363.00	62.90	108.00	46.00	9.45	122.00	260.00	146.00	94.40

#### IL-1 $\beta$

	CONTROL		TAPE STRIPPING		SLS		S-URINE		PROTEOLYTIC SOLUTION	
	BASELINE (T0)	POST (T4)	BASELINE (T0)	POST (T4)	BASELINE (T0)	POST (T4)	BASELINE (T0)	POST (T4)	BASELINE (T0)	POST (T4)
P1	0.40	0.27	-	-	0.33	0.09	0.05	0.05	0.31	0.15
P2	0.28	0.19	0.48	0.19	0.09	0.08	0.06	0.04	0.47	0.25
P3	0.29	0.25	0.29	0.10	0.06	0.07	0.05	0.07	0.57	0.27
P4	0.16	0.14	0.10	0.44	0.07	0.03	0.11	0.16	0.31	0.36
P5	0.40	0.24	0.12	0.33	0.07	0.06	0.21	0.05	0.32	0.50
P6	0.48	0.77	0.35	0.16	0.14	0.04	0.16	0.10	0.41	0.16
P7	0.20	0.22	0.26	0.97	0.09	0.14	0.08	0.13	0.82	0.20
P8	0.34	0.19	0.18	0.14	0.11	0.03	0.17	0.19	0.25	0.24
P9	0.24	0.20	1.73	0.42	0.23	0.02	0.15	0.11	0.36	0.18
P10	0.82	0.70	0.22	0.17	0.04	0.06	0.08	0.08	0.43	0.21

**IL-1RA**

	CONTROL		TAPE STRIPPING		SLS		S-URINE		PROTEOLYTIC SOLUTION	
	BASELINE (T0)	POST (T4)	BASELINE (T0)	POST (T4)	BASELINE (T0)	POST (T4)	BASELINE (T0)	POST (T4)	BASELINE (T0)	POST (T4)
<b>P1</b>	76.00	1224.00	-	-	216.00	39.30	89.10	41.90	70.10	77.70
<b>P2</b>	51.30	41.90	465.00	128.00	41.50	61.10	115.00	46.70	178.00	260.00
<b>P3</b>	37.10	76.50	50.00	72.10	30.80	85.80	43.30	105.00	53.70	51.00
<b>P4</b>	36.50	42.00	83.90	248.00	91.80	63.90	185.00	313.00	45.30	61.70
<b>P5</b>	205.00	167.00	28.30	207.00	45.50	20.50	35.50	69.80	114.00	892.00
<b>P6</b>	94.40	141.00	32.30	48.90	13.30	19.30	22.00	137.00	280.00	40.80
<b>P7</b>	295.00	424.00	44.30	465.00	37.60	71.00	57.70	136.00	273.00	193.00
<b>P8</b>	88.60	51.30	27.30	38.50	95.80	20.20	61.00	240.00	44.40	131.00
<b>P9</b>	142.00	111.00	60.40	102.00	35.60	11.60	26.80	45.30	41.60	48.20
<b>P10</b>	455.00	319.00	28.70	183.00	42.10	18.00	57.70	76.40	160.00	81.30

**IL-6**

	CONTROL		TAPE STRIPPING		SLS		S-URINE		PROTEOLYTIC SOLUTION	
	BASELINE (T0)	POST (T4)	BASELINE (T0)	POST (T4)	BASELINE (T0)	POST (T4)	BASELINE (T0)	POST (T4)	BASELINE (T0)	POST (T4)
<b>P1</b>	0.05	0.04	-	-	0.03	0.09	0.03	0.03	0.02	0.05
<b>P2</b>	0.02	0.02	0.07	0.03	0.03	0.03	0.03	0.03	0.02	0.02
<b>P3</b>	0.04	0.02	0.02	0.02	0.03	0.03	0.03	0.06	0.04	0.02
<b>P4</b>	0.02	0.04	0.04	0.27	0.03	0.10	0.03	0.05	0.02	0.02
<b>P5</b>	0.04	0.02	0.02	0.05	0.03	0.07	0.03	0.03	0.04	0.05
<b>P6</b>	0.02	0.02	0.03	0.04	0.03	0.03	0.03	0.03	0.04	0.02
<b>P7</b>	0.04	0.05	0.04	0.41	0.03	0.03	0.03	0.03	0.02	0.05
<b>P8</b>	0.06	0.05	0.03	0.02	0.03	0.12	0.03	0.03	0.06	0.02
<b>P9</b>	0.02	0.02	0.02	0.02	0.03	0.03	0.03	0.03	0.02	0.05
<b>P10</b>	0.05	0.02	0.03	0.07	0.03	0.03	0.03	0.03	0.02	0.04

\* Values below the detection limit of the assay are shown in red font

## IL-8

	CONTROL		TAPE STRIPPING		SLS		S-URINE		PROTEOLYTIC SOLUTION	
	BASELINE (T0)	POST (T4)	BASELINE (T0)	POST (T4)	BASELINE (T0)	POST (T4)	BASELINE (T0)	POST (T4)	BASELINE (T0)	POST (T4)
P1	0.40	1.48	-	-	0.73	1.76	0.34	0.45	7.33	0.18
P2	0.44	0.22	4.90	1.05	0.79	0.80	0.19	0.56	0.80	0.24
P3	0.25	0.20	0.36	0.60	0.41	7.68	0.43	1.59	0.57	0.18
P4	0.34	0.21	0.32	4.45	0.60	0.27	0.66	1.14	0.59	0.19
P5	1.04	0.70	0.47	3.07	0.40	0.76	0.54	0.53	0.86	1.13
P6	0.32	0.21	0.31	1.59	0.46	0.37	1.68	1.71	7.41	0.06
P7	0.37	1.18	2.68	1.95	1.04	0.63	0.50	0.38	0.63	0.29
P8	0.36	0.21	0.46	0.36	4.10	0.99	0.86	1.15	0.45	0.06
P9	0.85	0.36	0.58	0.37	0.60	0.34	0.52	0.84	0.59	0.12
P10	0.29	0.29	0.15	1.10	0.23	0.43	0.31	0.47	0.30	0.11

TNF- $\alpha$ 

	CONTROL		TAPE STRIPPING		SLS		S-URINE		PROTEOLYTIC SOLUTION	
	BASELINE (T0)	POST (T4)	BASELINE (T0)	POST (T4)	BASELINE (T0)	POST (T4)	BASELINE (T0)	POST (T4)	BASELINE (T0)	POST (T4)
P1	0.03	0.09	-	-	0.04	0.04	0.04	0.04	0.10	0.96
P2	0.03	0.10	0.12	0.07	0.05	0.04	0.07	0.11	0.06	0.82
P3	0.03	0.13	0.02	0.07	0.04	0.04	0.08	0.04	0.08	0.58
P4	0.09	0.05	0.10	0.13	0.09	0.04	0.12	0.12	0.08	0.57
P5	0.07	0.32	0.02	0.10	0.06	0.07	0.04	0.04	0.07	0.19
P6	0.07	0.10	0.07	0.07	0.04	0.04	0.04	0.11	0.10	1.10
P7	0.03	0.11	0.08	0.08	0.04	0.04	0.04	0.06	0.06	0.90
P8	0.10	0.15	0.07	0.13	0.14	0.14	0.09	0.15	0.10	0.47
P9	0.03	0.06	0.09	0.10	0.04	0.04	0.04	0.11	0.11	0.72
P10	0.07	0.14	0.09	0.11	0.04	0.04	0.15	0.18	0.14	0.97

\* Values below the detection limit of the assay are shown in red font

## D-2: Microdialysis data

IL-1 $\beta$ 

	CONTROL					TAPE STRIPPING				
	T0	T1	T2	T3	T4	T0	T1	T2	T3	T4
<b>P1</b>	0.17	0.15	0.21	0.57	0.56	1.13	0.55	0.28	0.57	0.39
<b>P2</b>	0.29	0.18	0.33	0.75	0.86	-	-	-	-	-
<b>P3</b>	-	-	-	-	-	-	-	-	-	-
<b>P4</b>	-	-	-	-	-	-	-	-	-	-
<b>P5</b>	-	-	-	-	-	-	-	-	-	-
<b>P6</b>	-	-	-	-	-	-	-	-	-	-
<b>P7</b>	-	-	-	-	-	-	-	-	-	-
<b>P8</b>	-	-	-	-	-	-	-	-	-	-
<b>P9</b>	-	-	-	-	-	-	-	-	-	-
<b>P10</b>	-	-	-	-	-	-	-	-	-	-
	SLS					S-URINE				
	T0	T1	T2	T3	T4	T0	T1	T2	T3	T4
<b>P1</b>	0.83	1.11	0.44	1.05	0.81	0.15	0.03	0.10	0.00	0.00
<b>P2</b>	0.18	0.22	0.17	0.15	0.33	0.21	0.26	0.20	0.26	0.41
<b>P3</b>	0.80	0.46	0.29	0.51	0.50	0.24	0.14	0.06	0.07	0.09
<b>P4</b>	0.29	0.24	0.27	0.32	0.40	0.85	1.14	1.00	1.00	1.22
<b>P5</b>	0.29	0.54	0.79	0.80	0.36	0.31	0.28	0.24	0.62	0.57
<b>P6</b>	1.31	0.19	0.05	0.17	0.09	0.03	0.27	0.10	0.32	0.31
<b>P7</b>	0.53	0.51	0.23	0.45	0.29	0.07	0.10	0.07	0.05	0.04
<b>P8</b>	0.28	0.26	0.19	0.81	0.38	0.41	0.23	0.82	0.33	0.44
<b>P9</b>	-	-	-	-	-	0.24	0.17	0.36	0.94	0.39
<b>P10</b>	-	-	-	-	-	-	-	-	-	-

	PROTEOLYTIC SOLUTION				
	T0	T1	T2	T3	T4
<b>P1</b>	0.08	0.20	0.27	0.37	0.53
<b>P2</b>	0.46	0.08	0.07	0.43	0.54
<b>P3</b>	2.19	0.31	0.31	0.55	1.52
<b>P4</b>	0.11	0.16	0.20	0.25	0.21
<b>P5</b>	0.16	0.13	0.15	0.33	0.47
<b>P6</b>	0.08	0.10	0.07	0.22	0.11
<b>P7</b>	0.18	0.12	0.11	0.18	0.17
<b>P8</b>	0.09	0.07	0.07	0.04	0.01
<b>P9</b>	-	-	-	-	-
<b>P10</b>	-	-	-	-	-

## II-6

	CONTROL					TAPE STRIPPING				
	T0	T1	T2	T3	T4	T0	T1	T2	T3	T4
<b>P1</b>	2.23	1.84	4.36	2.84	0.93	2.22	1.72	1.01	7.71	6.47
<b>P2</b>	12.22	12.30	12.37	8.73	10.83	-	-	-	-	-
<b>P3</b>	-	-	-	-	-	-	-	-	-	-
<b>P4</b>	-	-	-	-	-	-	-	-	-	-
<b>P5</b>	-	-	-	-	-	-	-	-	-	-
<b>P6</b>	-	-	-	-	-	-	-	-	-	-
<b>P7</b>	-	-	-	-	-	-	-	-	-	-
<b>P8</b>	-	-	-	-	-	-	-	-	-	-
<b>P9</b>	-	-	-	-	-	-	-	-	-	-
<b>P10</b>	-	-	-	-	-	-	-	-	-	-
	SLS					S-URINE				
	T0	T1	T2	T3	T4	T0	T1	T2	T3	T4
<b>P1</b>	1.71	1.70	0.50	1.92	0.93	0.00	0.11	0.94	0.00	0.03
<b>P2</b>	0.76	4.12	1.35	2.34	5.47	0.00	0.00	0.08	0.35	1.42
<b>P3</b>	2.74	8.52	3.76	2.58	0.98	0.08	0.07	0.06	0.04	0.05
<b>P4</b>	1.58	1.30	2.85	3.50	2.32	2.69	8.43	10.23	6.76	6.58
<b>P5</b>	4.03	8.54	5.93	2.53	3.05	6.25	4.65	5.66	8.68	5.38
<b>P6</b>	1.19	0.92	0.57	0.30	0.05	0.53	0.56	1.84	1.70	0.50
<b>P7</b>	1.23	1.09	1.72	8.76	3.20	0.04	0.00	0.03	0.00	0.03
<b>P8</b>	0.18	0.06	0.00	0.91	0.20	3.92	7.68	9.21	14.23	15.85
<b>P9</b>	-	-	-	-	-	0.12	0.07	0.11	0.15	0.11
<b>P10</b>	-	-	-	-	-	-	-	-	-	-

	PROTEOLYTIC SOLUTION				
	T0	T1	T2	T3	T4
<b>P1</b>	1.12	2.60	3.41	3.88	4.04
<b>P2</b>	1.29	1.74	2.81	4.25	2.25
<b>P3</b>	4.72	8.38	9.72	14.82	14.69
<b>P4</b>	0.42	1.13	2.19	2.86	5.70
<b>P5</b>	1.59	2.01	1.88	4.30	4.87
<b>P6</b>	0.84	1.67	1.36	1.83	0.68
<b>P7</b>	0.03	0.14	0.25	1.11	3.58
<b>P8</b>	0.08	0.16	0.08	0.06	0.07
<b>P9</b>	-	-	-	-	-
<b>P10</b>	-	-	-	-	-

## IL-8

	CONTROL					TAPE STRIPPING				
	T0	T1	T2	T3	T4	T0	T1	T2	T3	T4
<b>P1</b>	1.16	0.27	0.67	1.18	0.48	8.75	12.90	3.95	21.53	17.32
<b>P2</b>	13.96	15.36	52.37	68.25	84.01	-	-	-	-	-
<b>P3</b>	-	-	-	-	-	-	-	-	-	-
<b>P4</b>	-	-	-	-	-	-	-	-	-	-
<b>P5</b>	-	-	-	-	-	-	-	-	-	-
<b>P6</b>	-	-	-	-	-	-	-	-	-	-
<b>P7</b>	-	-	-	-	-	-	-	-	-	-
<b>P8</b>	-	-	-	-	-	-	-	-	-	-
<b>P9</b>	-	-	-	-	-	-	-	-	-	-
<b>P10</b>	-	-	-	-	-	-	-	-	-	-
	SLS					S-URINE				
	T0	T1	T2	T3	T4	T0	T1	T2	T3	T4
<b>P1</b>	37.44	49.49	16.22	53.21	24.10	0.20	0.33	2.92	0.05	0.06
<b>P2</b>	0.74	3.17	1.23	2.13	7.93	0.05	0.06	0.25	0.85	7.90
<b>P3</b>	17.20	39.21	34.95	45.32	15.17	0.34	0.24	0.26	0.06	0.31
<b>P4</b>	11.58	6.34	11.96	13.81	9.16	81.22	159.05	177.69	119.32	112.12
<b>P5</b>	36.19	59.82	44.05	24.53	17.82	28.47	22.70	21.91	58.69	32.80
<b>P6</b>	2.10	1.39	0.43	0.22	0.09	0.13	0.31	0.88	9.18	0.74
<b>P7</b>	18.69	13.81	10.14	71.90	21.10	0.04	0.03	0.03	0.04	0.04
<b>P8</b>	8.48	0.64	0.17	1.64	0.79	15.59	24.96	32.73	44.12	52.67
<b>P9</b>	-	-	-	-	-	0.32	0.32	0.38	0.38	4.16
<b>P10</b>	-	-	-	-	-	-	-	-	-	-

	<b>PROTEOLYTIC SOLUTION</b>				
	<b>T0</b>	<b>T1</b>	<b>T2</b>	<b>T3</b>	<b>T4</b>
<b>P1</b>	13.05	32.30	32.90	36.86	32.49
<b>P2</b>	4.73	0.97	1.64	1.70	1.08
<b>P3</b>	48.12	65.81	67.16	62.43	104.94
<b>P4</b>	3.14	8.10	10.14	16.13	27.38
<b>P5</b>	20.48	21.44	31.11	88.73	106.44
<b>P6</b>	10.51	10.90	9.53	16.45	6.95
<b>P7</b>	1.24	3.47	7.20	22.20	62.19
<b>P8</b>	0.40	0.39	0.27	0.20	0.08
<b>P9</b>	-	-	-	-	-
<b>P10</b>	-	-	-	-	-

TNF- $\alpha$ 

	CONTROL					TAPE STRIPPING				
	T0	T1	T2	T3	T4	T0	T1	T2	T3	T4
<b>P1</b>	0.05	0.05	0.05	0.05	0.05	0.02	0.02	0.02	0.02	0.11
<b>P2</b>	0.13	0.17	0.67	0.69	1.34	-	-	-	-	-
<b>P3</b>	-	-	-	-	-	-	-	-	-	-
<b>P4</b>	-	-	-	-	-	-	-	-	-	-
<b>P5</b>	-	-	-	-	-	-	-	-	-	-
<b>P6</b>	-	-	-	-	-	-	-	-	-	-
<b>P7</b>	-	-	-	-	-	-	-	-	-	-
<b>P8</b>	-	-	-	-	-	-	-	-	-	-
<b>P9</b>	-	-	-	-	-	-	-	-	-	-
<b>P10</b>	-	-	-	-	-	-	-	-	-	-
	SLS					S-URINE				
	T0	T1	T2	T3	T4	T0	T1	T2	T3	T4
<b>P1</b>	0.22	0.21	0.16	0.22	0.16	0.06	0.06	0.18	0.16	0.18
<b>P2</b>	0.08	0.05	0.00	0.12	0.07	0.06	0.04	0.00	0.00	0.09
<b>P3</b>	0.07	0.13	0.13	0.18	0.10	0.08	0.12	0.06	0.11	0.09
<b>P4</b>	0.10	0.05	0.09	0.15	0.11	0.25	0.47	0.57	0.40	0.38
<b>P5</b>	0.32	0.56	0.33	0.23	0.14	0.19	0.09	0.16	0.27	0.18
<b>P6</b>	0.11	0.00	0.00	0.06	0.08	0.07	0.06	0.00	0.17	0.09
<b>P7</b>	0.13	0.13	0.00	0.20	0.07	0.09	0.07	0.07	0.09	0.11
<b>P8</b>	0.07	0.00	0.00	0.02	0.06	0.12	0.05	0.20	0.34	0.37
<b>P9</b>	-	-	-	-	-	0.00	0.05	0.00	0.03	0.06
<b>P10</b>	-	-	-	-	-	-	-	-	-	-

	PROTEOLYTIC SOLUTION				
	T0	T1	T2	T3	T4
<b>P1</b>	0.12	0.17	0.18	0.17	0.17
<b>P2</b>	0.00	0.05	0.05	0.07	0.07
<b>P3</b>	0.36	0.36	0.31	0.52	0.56
<b>P4</b>	0.00	0.10	0.15	0.17	0.24
<b>P5</b>	0.16	0.17	0.19	0.49	0.57
<b>P6</b>	0.10	0.22	0.21	0.19	0.10
<b>P7</b>	0.00	0.00	0.07	0.15	0.48
<b>P8</b>	0.05	0.07	0.05	0.08	0.10
<b>P9</b>	-	-	-	-	-
<b>P10</b>	-	-	-	-	-



## References

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