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CORRELATES OF DERMIS THICKNESS IN MOUSE MODELS WITH A RANGE OF OBESITY, INSULIN RESISTANCE AND DIABETIC STATES

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ABSTRACT
Diabetic complications of the skin regarding skin structure have been well documented and researched, notably in relation to wound healing. Recently it has also been discovered that dermis thickness may also be reduced in type 2 diabetic patients. Peripheral damage in diabetes has been attributed to inflammation, as well as hyperglycaemia resulting from insulin resistance. However, this has not been investigated specifically in relation to dermis thickness. This study used mouse models with a range of obesity, insulin resistance and diabetic states to investigate the extent of reduction in dermis thickness that results from these conditions and to elucidate the correlation of dermis thickness with both biomarkers of insulin resistance and whole-body and local proinflammatory cytokine levels, which can both directly damage tissues and be the causative factor of the insulin resistance. The results suggest that the reduced dermis thickness observed in type 2 diabetes is likely a result of hyperglycaemia resulting from insulin resistance rather than the increased proinflammatory milieu resulting from insulin resistance and obesity.

Keywords: dermis depth, obesity, diabetes, hyperglycemia, inflammation

IINTRODUCTION
Most individuals diagnosed with type 2 diabetes mellitus experience skin complications. These range from relatively benign epidermal manifestations, such as *acanthosis nigricans*, through to potentially catastrophic delays in wound healing (Bermudez *et al*., 2011; Romano *et al*., 1998; Hu *et al*., 2019; Sani *et al*., 2020). Peripheral damage in diabetes has been attributed to elevated levels of reactive glucose molecules, chronic hyperinsulinaema and inflammation (Singh *et al*., 2001; Aronson, 2008, Hijazi *et al*., 2020; Hosseini *et al*., 2021). Recent reports have suggested that dermal thickness is reduced in type 2 diabetic patients (Niu *et al*., 2012; Su *et al*. 2018; Rodriguez *et al*., 2022). Isolating these processes in humans is difficult as skin presentation is complex and heterologous. Murine models of type 2 diabetes are, therefore, useful in the study of skin lesions in increasingly hyperglycaemic and hyperinsulinaemic states.

Wild-type animals exposed to calorie-rich diets, or genetic mutants that lack either the satiety regulator leptin (C57BL/6 Lep^{ob}/Lep^{ob} mice) or its receptor (C57BLKS/J $Lepr^{db}/Lepr^{db}$ mice), are widely used in the study of type 2 diabetes. These models are associated with obesity, insulin-resistance and hyperglycaemia. In the case of C57BLKS/J $Lepr^{db}/Lepr^{db}$ mice, there is deterioration in pancreatic β-cell function resulting in insufficient insulin secretion to overcome the insulin resistance and, consequently, overt hyperglycaemia and diabetes (Hummel *et al*., 1966; Surwit *et al*., 1988). These animals also display the compromised re-epithelialisation and chronic inflammation characteristic of non-healing diabetic wounds in humans; thus, they are widely used in wound healing studies (Frank *et al*., 2000; Stachura *et al*., 2022). There are, however, few reports that investigate other aspects of skin pathology in type 2 diabetes, most notably the specific impact on the dermis.

Underlying the dermis is a layer of adipose tissue, the subcutis, which acts both as an energy store and a component of innate immunity that could contribute to inflammatory disease, and the increase in fat deposition found in obesity is associated with increased proinflammatory cytokine release (Chen *et al*., 2019). Many of these cytokines modulate wound healing, notably tumour necrosis factor [TNF]-α and interleukin-1 [IL-1]-β) (Trayhurn & Wood, 2004; Trayhurn, 2013; Mu *et al*., 2022). Thus, prolonged exposure to cytokines released from an expanded, subcutis could impair adjacent skin function and impact the integrity of the dermis.

Insulin resistance and the direct action of cytokines on the cells of the integument are known to be important in the pathophysiology skin diseases in obesity-induced metabolic syndrome and type 2 diabetes (Hu *et al*., 2019). Keratinocytes and the stromal cells of the skin are insulin sensitive, with insulin promoting glucose uptake, proliferation and differentiation (Spravchikov *et al*., 2001; Schüppel *et al*., 2008) and so defects in insulin signalling could also provoke cutaneous complications (Caro *et al*., 1986; Olefsky, 1976; Chen *et al*., 2019). Pro-inflammatory cytokines also stimulate keratinocyte proliferation and stimulate antimicrobial peptide production. However, high levels of pro-inflammatory cytokines or chronic exposure is inhibitory (Xiao *et al*, 2020). This phenomenon has not, however, been investigated in relation to the integrity of the dermis. This study is designed to investigate the extent to which obesity and insulin resistance affects dermis depth in a range of mouse models of obesity and diabetes. We also investigate a possible correlation between dermal thickness and both proinflammatory cytokines and markers of insulin resistance.

MATERIALS AND METHODS

Animals

All procedures were conducted in accordance with the UK Government Animals (Scientific Procedures) Act 1986 and approved by the University of Buckingham Ethical Review Board (Bu13/012). Male mice (C57BL/6J, C57BL/6-*Lep^{ob}/Lep^{ob}* (ob/ob), C57BLKS/J (ksj) and C57BLKS/J-*Lepr^{db}/Lepr^{db}* (db/db) were obtained from Charles River (Marston, Kent, UK) at 6 weeks of age. Animals were maintained on either standard laboratory chow (Beekay Number 1, B&K Universal Ltd., Hull, UK) or a high fat diet (HFD; 60% of calories by fat, Research Diets Inc., New Brunswick, New Jersey, USA, cat D12492) *ad libitum* for 12 or 46 weeks. Mice were housed in pairs at 21 – 23 °C with lights on at 08.00h, lights off at 20.00h.

Body composition

Mice were weighed at 18 or 52 weeks of age. Body fat was measured using a Minispec LF90II Nuclear Magnetic Resonance (Bruker Corporation, Germany).

Dermal depth

Animals were terminated by cervical dislocation followed by exsanguination. Dorsal skin was shaved and fixed in 10% neutral buffered formalin (BDH, VWR International Ltd, Lutterworth, UK) for 6 hours at 40°C prior to paraffin wax embedding and preparation of 4µM sections. Care was taken in ensuring that sections were cut perpendicular to the horizontal plane by using a fixed cutting angle, and by careful orientation of skin during the embedding process. Tissues were dewaxed and rehydrated as standard before staining. Staining was differentiated in 95% ethanol containing a drop of eosin Y prior to clearing and mounting. Hematoxylin and eosin (H&E) staining was performed as standard (Bancroft & Gamble, 2008). Our histology methods are described in more depth in a report by Al-Habian et al (Al-Habian *et al*., 2014). Dermal depth was measured using ImageJ (imagej.nih.gov).

Oral glucose tolerance test

After fasting for five hours, mice were dosed with glucose (3 g.kg^{-1}, body weight PO by gavage). Blood samples were collected from the tail at -30, 0, +30, +60 and +120 minutes, relative to glucose dosing. Blood glucose was measured using a glucose oxidase reagent kit (Gluc-PAP, GL2623; Randox, Crumlin, UK). Plasma insulin was measured at t = -30 (Ultra-sensitive Mouse Insulin ELISA kit, Cat #: 90080; Crystal Chem, Downers Grove, IL, USA), leptin (Mouse Leptin ELISA kit, Cat #: 90030; Chrystal Chem).

Termination: Plasma and skin cytokines

Animals were terminated by cervical dislocation followed by exsanguination. Blood collected in EDTA-coated microvettes (Cat #: 16.440.100; Sarstedt, Nümbrecht, Germany). Plasma measurement of cytokine levels was quantified using the Meso Scale™ Sector Imager 2400 ultrasensitive mouse pro-inflammatory multiplex assay (Meso Scale Discovery, Rockville, Maryland, USA), according to the manufacturer's recommendations. Back skin was shaved and aseptically cleaned. A skin area of approximately 4cm^2 was washed in sterile phosphate buffered saline (PBS) and placed epidermis down to allow complete separation of the hypodermis by scraping with a scalpel. The remaining tissue was then washed twice in PBS to remove any remaining fat, prior to transferring the tissue from both compartments (fat and outer skin) to pre-warmed Krebs-Ringer-HEPES buffer pH7.4 containing 1% endotoxin free bovine serum albumin (BSA). Samples were immediately placed into DMEM/F-12 Ham's (1:1) (supplemented with 5mM L-glutamine, 15mM HEPES and 1% BSA) and minced prior to incubation at 37°C for 90 minutes with shaking at 150 oscillations per minute (IKA Vibrax VXR B82, Staufen, Germany). The culture medium was collected and frozen at -80ºC prior to analysis of cytokines using a multiplex cytokine assay as previously described (Meso Scale Discovery, USA) (Kępczyńska *et al*., 2013). Results were determined using the Meso Scale Sector Imager 2400 according to the manufacturer's recommendations.

Statistics

A priori power analysis was conducted using G*Power3 (Faul *et al*., 2007). All data was analysed using Prism ™ software version 9.02 (GraphPad, La Jolla, California, USA). Statistical analyses for figures 1 - 4 were performed by one-way ANOVA followed by Šidák's post-test. The strength of correlations of all other data to dermis depth was performed by Spearman's Rank Correlation Rho value grouping individual mice from all groups together (Ramsey, 1989; Fowler *et al*., 2009). Results are presented as means ± SEM. Statistical significance for effects in the tables and figures is given as **P*<0.05; ***P*<0.01; ****P*<0.001; *****P*<0.0001.

RESULTS

Animal models of obesity and diabetes had both increased bodyweight and increased body fat percentage (table 1). High-fat diets (HFD) did not affect dermis depth in C57Bl6 mice; however, dermis depth was reduced by 43% in chow-fed *ob/ob* (baseline 544 µm in chow-fed C57Bl6 mice and endpoint 315 µm in chow-fed *ob/ob* mice) and by 78% in chow-fed *db/db* mice (baseline 597 µm in chow-fed KSJ mice and endpoint 129 µm in chow-fed *db/db* mice) (Fig. 1A). The subcutaneous fat layer was thicker in all the animal models of obesity and diabetes: C57Bl6 HFD 12 wks by 443% (baseline 182 µm and endpoint 808 µm); C57Bl6 HFD 46 wks by 480% (baseline 201 µm and endpoint 964 µm); *ob/ob* chow by 887% (baseline 182 µm and endpoint 1614 µm); *ob/ob* HFD by 1485% (baseline 182 µm and endpoint 1902 µm); *db/db* by 452% (baseline 203 µm and endpoint 918 µm); Fig. 1B).

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Table 1. Body composition (N = 12 for all groups)

Group	Strain/mutation	Diet (weeks on diet)	Bodyweight (g)	% Body fat
A	C57Bl6/J	Chow (12 weeks)	25.4 ± 0.6	11.9 ± 2.6
B	KSJ	Chow (12 weeks)	26.0 ± 2.1	19.1 ± 2.4
C	C57Bl6/J	Chow (46 weeks)	39.4 ± 1.0 ****	12.3 ± 2.6 ****
D	C57Bl6/J	HFD (12 weeks)	46.2 ± 4.5 ****	50.8 ± 3.8 ****
E	C57Bl6/J	HFD (46 weeks)	55.3 ± 4.2 ****	59.4 ± 4.3 ****
F	ob/ob	Chow (12 weeks)	45.5 ± 4.0 ****	49.1 ± 3.9 ****
G	ob/ob	HFD (12 weeks)	44.3 ± 4.2 ****	46.5 ± 1.3 ****
H	db/db	Chow (12 weeks)	40.9 ± 2.5 ****	45.1 ± 1.3 ****

Table 1. Body composition. Body weight and % body fat of mouse strains after 12 or 46 weeks on chow or HFD. Data is presented as mean ± S.E.M. (N = 12 for all groups). ★★★★ P < 0.0001.

HFD-feeding increased blood glucose concentration by 140% when fed for 46 weeks (baseline 5.2 mmol.l^{-1} in chow-fed C57Bl6 mice and endpoint 7.3 mmol.l^{-1} in HFD-fed C57Bl6 mice). Similarly, blood glucose levels were raised by 152% in the *ob/ob* mice (baseline 4.8 mmol.l^{-1} in chow-fed C57Bl6 mice and endpoint 7.3 mmol.l^{-1} in chow-fed *ob/ob* mice) and HFD-feeding further elevated blood glucose levels by 197% (baseline 7.3 mmol.l^{-1} in chow-fed *ob/ob* mice and endpoint 14.4 mmol.l^{-1} in HFD-fed *ob/ob* mice) and this concentration matched the blood glucose levels in *db/db* mice. Similarly, HFD-feeding increased plasma insulin levels by 624%

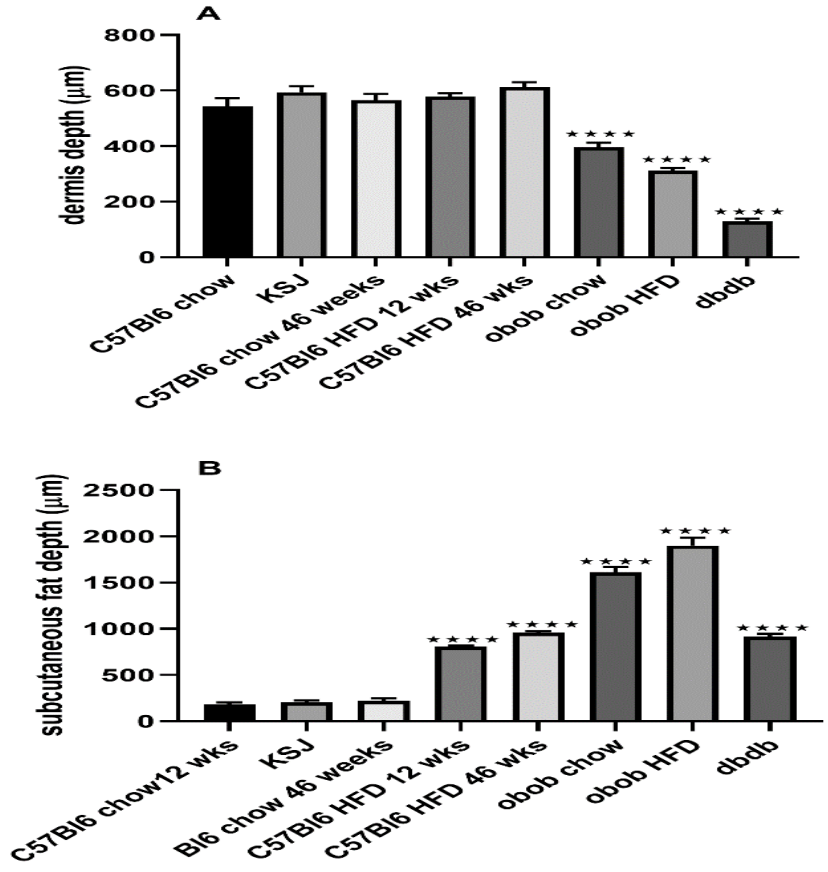

Figure 1. Skin layer thickness. Histologically measured dermis (A) and subcutaneous fat layer (B) thickness in mouse models of insulin resistance after 12 or 46 weeks on chow or HFD. Data is presented as mean ± S.E.M. (N = 12 for all groups). ★★★★ P < 0.0001.

(baseline 97 pmol.l^{-1} in chow-fed C57Bl6 mice and endpoint 605 pmol.l^{-1} in HFD -fed C57Bl6 mice) and glucose intolerance by 153% when fed for 12 weeks (baseline 1335 mmol.min.l^{-1} in chow-fed C57Bl6 mice and endpoint 2038 mmol.min.l^{-1} in HFD-fed C57Bl6 mice). Extending the duration of HFD-feeding to 46 weeks increased plasma insulin by 3981% (baseline 106 pmol.l^{-1} in chow-fed C57Bl6 mice and endpoint 4220 pmol.l^{-1} in HFD-fed C57Bl6 mice) and glucose intolerance by 233% (baseline 1385 mmol.min.l^{-1} in chow-fed C57Bl6 mice and endpoint 3222 mmol.min.l^{-1} in HFD-fed C57Bl6 mice). Genetically diabetic (*db/db)* mice had statistically similar plasma insulin levels and glucose intolerance to the ob/ob mice fed a HFD. Plasma HbA1c levels were increased in the mouse models by the same extent as blood glucose concentrations (Fig. 2).

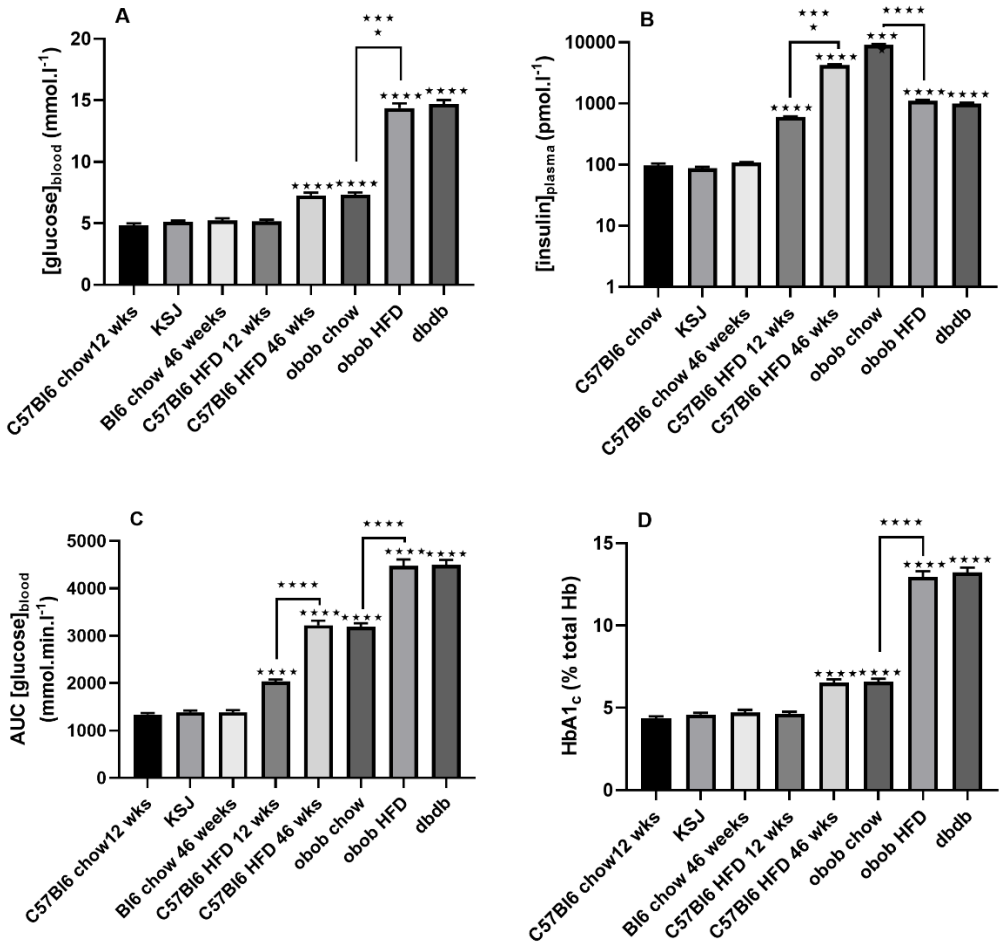

Figure 2. Markers of insulin resistance. Fasting blood glucose concentrations (A), fasting plasma insulin concentrations (B), Area Under Glucose concentration curve in oral glucose tolerance test (C) and plasma HbA1c (D) in mouse models of insulin resistance after 12 or 46 weeks on chow or HFD. Data is presented as mean ± S.E.M. (N = 12 for all groups). ★★★★ P < 0.0001.

Plasma concentrations of the proinflammatory cytokines IL-1β, IL-6, IL-12, KC, TNF-α, MCP1 and RANTES levels were increased in all mouse models of insulin resistance. However, plasma concentrations of IFN-γ and GM-CSF were only elevated in the obese, insulin resistant, but non-diabetic groups (Fig. 3). Similarly, skin secretion of IL-1β, IL-6, TNF-α and MCP1 were significantly elevated in the obese mouse models regardless of diabetic state. In contrast, IFN-γ secretion was increased only in the C57Bl/6J mice fed HFD for 46 weeks and the *ob/ob* mice fed chow diet, but not in the diabetic groups. Skin secretion of TNF-α was increased by age (Fig. 4).

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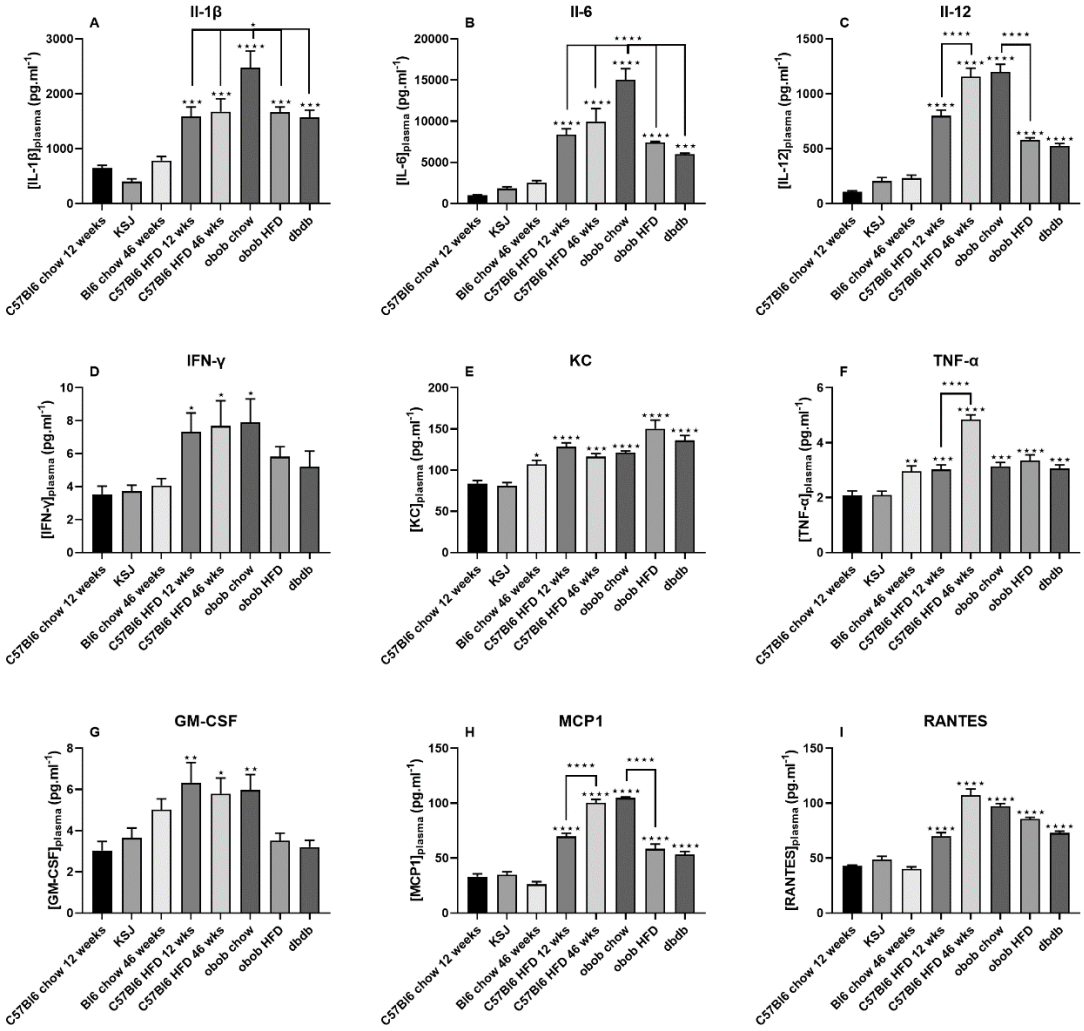

Figure 3. Plasma proinflammatory cytokine concentrations. Terminal plasma concentrations of IL-1β (A), IL-6 (B), IL-12 (C), IFN-γ (D), KC (E), TNF-α (F), GM-CSF (G), MCP1 (H) and RANTES (I) in mouse models of insulin resistance after 12 or 46 weeks on chow or HFD. Data is presented as mean ± S.E.M. (N = 12 for all groups). ★ P < 0.05, ★★ P < 0.01, ★★★ P < 0.001, ★★★★ P < 0.0001.

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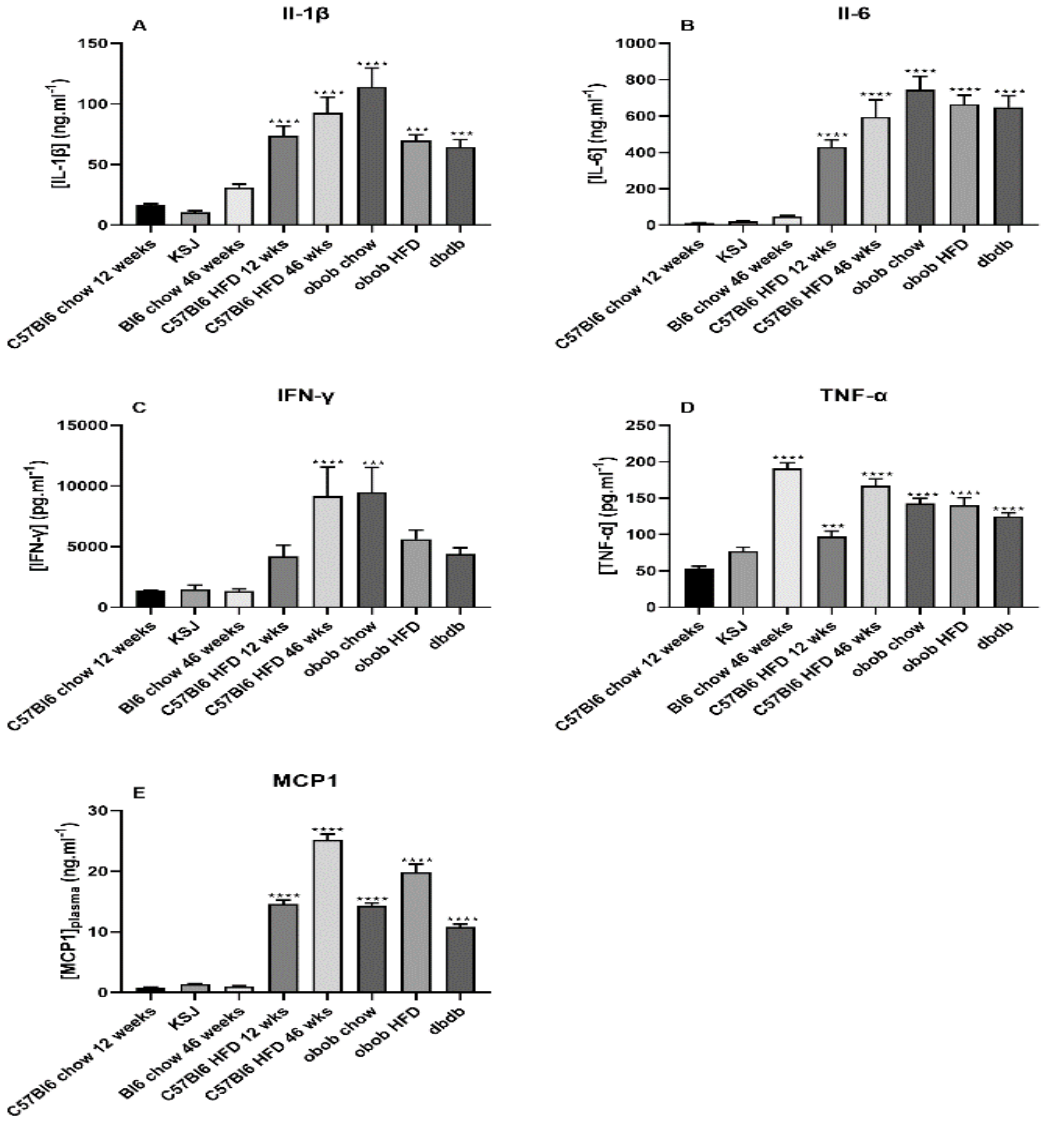

Figure 4. Skin proinflammatory cytokine secretion. Ex vivo incubations of skin sections concentrations of IL-1β (A), IL-6 (B), IFN-γ (C), TNF-α (D) and MCP1 (E) in mouse models of insulin resistance after 12 or 46 weeks on chow or HFD. Data is presented as mean ± S.E.M. (N = 12 for all groups). ★★★ P < 0.001, ★★★★ P < 0.0001.

Plasma HbA1c (rho = -0.8791), fasting blood glucose (rho = -0.8092), plasma insulin (rho = -0.7549) and OGTT AUC (rho = -0.7461) negatively correlated strongly with dermis thickness (table 2). Only one proinflammatory cytokine, plasma GM-CSF, did not correlate significantly with dermis thickness. Plasma IFN-γ and both plasma and skin-secreted TNF-α showed a weak, but significant, negative correlation with dermis thickness. All the other proinflammatory cytokines measured in the plasma or secreted from skin showed a moderate negative correlation with dermis thickness.

Table 2. Correlations of dermis thickness with biomarkers of hyperglycaemia, insulin resistance and proinflammatory cytokines (N=96)

Inverse correlation strength	Biomarker	Rho (P value)
Strong (Spearman's rho = -0.7 to -0.9)	Plasma $HbA1_c$	-0.8791 (P<0.0001)
	Fasting $[glucose]_{blood}$	-0.8092 (P<0.0001)
	Fasting $[insulin]_{plasma}$	-0.7549 (P<0.0001)
	OGTT	-0.7461 (P<0.0001)
Moderate (Spearman's rho = -0.4 to -0.7	*Ex vivo* skin Il-6 secretion	-0.6481 (P<0.0001)
	$[RANTES]_{plasma}$	-0.6472 (P<0.0001)
	Ex vivo skin MCP-1 secretion	-0.5962 (P<0.0001)
	Subcutaneous fat layer depth	-0.5807 (P<0.0001)
	Ex vivo skin IFN-γ secretion	-0.5679 (P<0.0001)
	$[MCP\text{-}1]_{plasma}$	-0.5077 (P<0.0001)
	$[Il\text{-}1\beta]_{plasma}$	-0.5071 (P<0.0001)
	$[Il\text{-}12]_{plasma}$	-0.5022 (P<0.0001)
	Ex vivo skin Il-1β secretion	-0.485 (P<0.0001)
	$[Il\text{-}6]_{plasma}$	-0.4738 (P<0.0001)
	$[KC]_{plasma}$	-0.4655 (P<0.0001)
	% body fat	-0.4354 (P<0.0001)
	Body weight	-0.4274 (P<0.0001)
Weak (Spearman's rho = -0.2 to -0.)	$[TNF\text{-}\alpha]_{plasma}$	-0.363 (P<0.001)
	Ex vivo skin TNF-α secretion	-0.3558 (P<0.001)
	$[IFN\gamma]_{plasma}$	-0.3029 (P<0.01)
Very weak (Spearman's rho = -0 to -0.2)	$[GM\text{-}CSF]_{plasma}$	-0.03723 (*ns*)

Table 2. Correlations of dermis thickness with biomarkers of hyperglycaemia, insulin resistance and proinflammatory cytokines. Spearman's rank correlations of dermis thickness (figure 1A data) with markers of hyperglycaemia, insulin resistance (figure 2 data), plasma proinflammatory cytokines (figure 3 data) and skin secretion proinflammatory cytokines (figure 4 data) in mouse models of insulin resistance after 12 or 46 weeks on chow or HFD. Correlations are presented in order of strength of the inverse correlation (N = 96).

DISCUSSION

This study was designed to investigate the strength of correlation between dermis thickness with proinflammatory cytokines and insulin resistance. All the proinflammatory cytokines measured in the plasma and secreted in *ex vivo* skin incubations were increased in all murine models of obesity and diabetes, except for systemic GM-CSF and IFN-γ (systemically and locally), which were increased in the obese models but not in the diabetic models. Previous studies have shown IFN-γ to be a strong inducer of insulin resistance, type 2 diabetes and its complications (Hoggard *et al*., 1997; McLaughlin *et al*., 2017) and despite only a modest increase in these models there was still a significant correlation with dermis depth. TNF-α is also strongly associated with causing insulin resistance (Goren *et al*., 2006; Ali *et al*., 2022) although in this study, whilst the correlation with dermis depth was statistically significant, the correlation strength may have been artificially weakened by the strong ageing effect on TNF-α secretion which has been previously documented to have complex interaction with obesity and insulin resistance (Lerman *et al*., 2003; Guarner & Rubio-Ruiz, 2015). The dermis layer was reduced in the ob/ob and db/db mouse groups, but not in the HFD C57Bl/6J mice.

These observations raise the possibility that a lack of leptin or leptin signalling promotes cutaneous degradation. Leptin has the capacity to act as a local cytokine in tissues including the skin (Herbelin *et al*., 1990, Ikeda *et al*., 2022) and leptin-deficient ob/ob and db/db mice show impaired wound repair (Herbelin *et al*., 1990; Frank *et al*. 2000; Vlahos *et al*., 2006; Stachura *et al*., 2022). However, this has been demonstrated to be due to the systemic imbalance of glucose homeostasis, rather than a direct action of leptin (Mahlangu *et al*., 2020). Moreover, impaired dermal fibroblast growth and increased senescence in db/db mice only occurred following the onset of insulin resistance and hyperglycaemia (Hotamisligil & Spiegelman, 1994). However, a disadvantage of this study is that a direct leptin effect was not studied, and therefore cannot be ruled out as a possibility for the reduced dermis depth. The present study also does not distinguish between the different cytokine-secreting cells in the skin, including inflammatory cells (Hänel *et al*., 2013), and the effect that each may have may be masked by correlating dermis depth with whole skin incubations. Further studies would be required to investigate this.

The present study found the greatest correlation with dermis thickness to be plasma HbA1c levels, then fasting blood glucose, plasma insulin levels and the degree of glucose intolerance, indicating that dermis depth is highly integrated with hyperglycaemia, and particularly with glycated hemoglogin. $HbA1_c$ is a precursor for hemoglobin advanced glycation end product (Hb-AGE) (Turk *et al*., 1998). This is important because advanced glycation end products (AGEs) have been strongly associated with both the progression of diabetic complications (Vistoli *et al*., 2013, Mengstie *et al*. 2022) and reduced dermis thickness (Niu *et al*., 2012). Dermis thickness also correlates with microvascular damage (Sulli *et al*., 2014) and oxidative stress (Niu *et al*., 2012). Both of these have been reported to be caused by AGEs (Niu *et al*., 2012; Yamagishi *et al*., 2007). AGEs may, therefore, be important factors linking the correlations observed.

In conclusion, skin complications of type 2 diabetes may arise from both the proinflammatory cytokines and insulin resistance (Hu *et al*., 2019). Dermis depth is significantly associated with both of these factors, although the main cause appears to be hyperglycemia.

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