IMPAIRED WOUND HEALING AND INFLAMMATION: THE ROLE OF THE DERMAL FIBROBLASTS

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Impaired Wound Healing and Inflammation: The Role of the Dermal Fibroblast

Phenotypic Changes in the Human Dermal Fibroblast with Inflammation; Potential Impact on Wound Healing

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Abstract

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Keywords: wound healing, fibroblasts, inflammation, skin, diabetes

Dermal fibroblasts positively contribute throughout the wounding response by secreting a profile of pro- and anti-inflammatory cytokines in the wound milieu. However, a chronically inflamed environment will, cause detrimental effects on the functional, secretory, and molecular properties of these cells. This study aims to understand how the effect of the pro-inflammatory cytokine TNF-α modulates both healthy and diabetic dermal fibroblast phenotype.

To mimic a chronic inflammatory environment and assess whether fibroblasts respond similarly in different anatomical sites, donor-matched fibroblasts from face and scalp were pre-incubated for 3 days with different concentrations (2.5, 25 or 250 ng/ml) of TNF-α. All concentrations significantly impaired proliferation by day 14 in cells from both sites and stimulated (papillary) metabolic activity at day 14.

However, this did not correlate with an increase in papillary cell senescence since this did not appear until passage 17, and then only at a supra-pathophysiological concentration.

Migration of dermal fibroblasts, assessed by the scratch assay. TNF-α significantly inhibited the cells migration, particularly in diabetic fibroblasts, suggesting they are more sensitive to TNF-α. Since TNF-α may stimulate the secretion of soluble paracrine factors by dermal fibroblasts, conditioned medium was collected to assess its effect on other dermal fibroblasts, however, this had no significant effect on migration.

However, using gelatin zymography, it was found that TNF-α did stimulate the secretion of soluble paracrine factors that induce MMP activity in non-diabetic fibroblasts, mirroring previous observations that a pro-inflammatory environment can increase proteolytic activity, and indicating that diabetic fibroblasts were again more sensitive than healthy. No difference was observed with MMP-9 activity and nor did the results with dermal fibroblasts reach statistical significance, perhaps because of a relatively low n-number.

The ability of TNF-α to modulate the expression of genes associated with the ECM (MMP-1, -2, -9, TIMP-1, and -2) and senescence (Sirt1 and 6) was investigated. There was no change in Sirt1 and Sirt6 expression and no evidence of paracrine effects (conditioned medium) on any of the genes. TNF-α significantly induced mRNA expression of MMP-1 in healthy non-scratched and scratched diabetic fibroblasts, and TIMP-1 in healthy non-scratched cells. There was also considerable donor variability that prevented statistical significance being achieved under the other conditions.

The secretion of various cytokines associated with inflammation was compared in healthy and diabetic fibroblasts in the presence and absence of TNF-α. Seven cytokines were secreted, by healthy and diabetic male and female fibroblasts, although diabetic female fibroblasts did not secrete two of them. TNF-α stimulated secretion of cytokines in healthy and diabetic, male and female cells but the profiles of those released were different between the different groups. There was no TNF-α induced paracrine effect on cytokine secretion by healthy dermal fibroblasts.

In conclusion, changes in the microenvironment and the influx of pro-inflammatory cytokines may significantly alter the dermal fibroblast phenotype. Understanding these functional and molecular changes in response to inflammatory cytokines will give a better understanding of the differences between fibroblast activity in normal physiological wound healing and chronic or diabetic non-healing wounds.

I dedicate this work to my mother and my only son... and to all faithful friends who helped and supported me.

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Abbreviations:

AceCS2: Acetyl-CoA synthetase 2

AGEs: Advanced glycation end products

AP-1: Activating protein 1

APS: Ammonium persulphate

ASK1: Apoptosis signal regulating kinase1

ATF2: Activating transcription factor 2

Bax: Bcl2 associated X protein

bFGF: Basic fibroblast growth factor

Blimp1: B lymphocyte induced maturation protein

CCL1/I-309: Chemokine C-C motif ligand1/ Human Cytokine I-309

CCL2/MCP-1: Chemokine C-C motif ligand 2/ monocyte chemoattractant protein 1

CCL5/RANTES: Chemokine C-C motif ligand 5/ RANTES

CD40Ligand/TNFSF5: Member of TNF-α superfamily/ CD154

CD90: Cluster Differentiation 90

c-Jun: c-Jun N-terminal kinase

CM: Conditioned media

Complement component C5/C5a: Complement component 5 (complement system)

CPS1: Carbamoyl phosphate synthetase 1

CtIP: C-terminal binding protein interacting protein

CtIP: C-terminal binding protein interacting protein

CVLUs: Chronic venous leg ulcer

CXCL1/GROα: Chemokine C-X-C motif ligand 1/GROα

CXCL10/IP-10: Chemokine C-X-C motif ligand 10/ Interferon gamma-induced protein 10 (IP-10)

CXCL11/I-TAC: Chemokine C-X-C motif ligand 11/ Interferon-inducible T-cell alpha chemoattractant

CXCL12/SDF-1: Chemokine C-X-C motif ligand 12/ stromal cell-derived factor 1 (SDF-1)

DD: Death domain

DEJ: Dermal-Epidermal junction

DF: Dermal fibroblast

Dlk1: Delta-like homologue 1

DMEM: Dulbecco's Modified Eagle Medium

DMF: Dimethylformamide

DNA: Deoxyribose nucleic acid

DTT: Dithiothreitol

ECM: Extracellular matrix

EGF: Epidermal growth factor

En1: Engrailed-1

ERK: Extracellular signal- regulated kinase

FACS: Fluorescence Assisted Cell Sorting

FADD: FAs associated death domain

FAP: Fibroblast activation protein

FBS: Foetal bovine serum

FGF a and b: Fibroblasts growth factors acidic and basic

FOXO: Forkhead box O

FXR: Farnesoid X receptor

GAGs: Glycosaminoglycans

GCK: Germinal center kinase

G-CSF: Granulocyte-colony stimulating factor

GDH: Glutamate dehydrogenase

GLβ1 or (SA-β-Gal) or (β-Gal): beta-galactosidase

GM-CSF: Granulocyte-macrophage colony-stimulating factor

H3K56: Histone 3 acetyl-lysine 56

H3K9: Histone 3 acetyl-lysine 9

H4: Histone 4

HA: Hyaluronic acid or hyaluronan

HAS2: Hyaluronic acid synthase 2

HAT: Histone acetyltransferase

HDAC: Histone deacetylase

HF: Hairy (Scalp skin) fibroblasts

HGF: Hepatocyte growth factor

Hif: Hypoxia-inducible factor

HMG-CoA synthase 2: Hydroxy-3-methylglutaryl CoA synthase 2

HSF1: Heat shock factor 1

I L-1, 4, 6 and 8: Interleukin-1, 4, 6 and 8

ICAM-1/CD54: Intercellular Adhesion Molecule/CD54

IDH2: Isocitrate dehydrogenase 2

IFN-γ: Interferon gamma;

IGF-1: Insulin-like growth factor

IGFBP6: Insulin-like growth factor binding protein-6

IKK: I kappa B kinase

IL-10: Interleukin-10

IL-12 p70: Interleukin-12/ heterodimer p70

IL-13: Interleukin-13

IL-16: Interleukin-16

IL-17A or E: Interleukin-17A, or Interleukin-17E

IL-18/IL-1F4: Interleukin-18/ IL-1F4 Protein

IL-1ra/IL1-F3: Interleukin-1 receptor antagonist (IL-1RA)

IL-1α/IL-1F1: Interleukin-1 alpha/ IL-1F1 Protein

IL-1β/IL-1F2: Interleukin-1 beta/ IL-1F2 Protein

IL-1β: Interleukin-1beta

IL-2: Interleukin-2

IL-21: Interleukin-21

IL-27: Interleukin-27

IL-32α: Interleukin-32α

IL-5: Interleukin-5

INF-γ: Interferon gamma

JNK: Jun N-terminal kinase

KGF: Keratinocyte growth factor

LCAD: Long chain acyl CoA dehydrogenase

Lrig1: leucine-rich repeats and immunoglobulin-like domains protein 1

LXR: Liver X receptor

MAPK: Mitogen activated protein kinase or MAP kinase

MCP-1: Monocyte chemoattractant protein

MEKK1: Mitogen-activated protein kinase kinase kinase 1

MHC: Major histocompatibility complex

MIF: Macrophage migration inhibitory factor

MIP-1: Macrophage inflammatory protein

MIP-1α/MIP-1β: Macrophage Inflammatory Proteins (α and β)

miRNAs: MicroRNAs

MKKs: MAPK kinases

MLK2 and 3: Mixed lineage kinases 2 and 3

MMPs: Matrix metalloproteinases

MnSOD: Mn-superoxide dismutase

MT-MMP: Membrane type matrix metalloproteinase

NCTC 2544: Human keratinocyte cell line

NF-$_k$B: Nuclear factor-kappa B

NHF: Non-hairy (Facial skin) fibroblasts

P38: P38 mitogen-activated protein kinase

P53: Tumour suppressor protein 53

PBS: Phosphate buffered saline

PDGF: Platelet derived growth factor

PDGFRα: pan-fibroblast marker platelet derived growth factor receptor alpha

PER2: Period circadian protein homolog 2

PGC-1α: Peroxisome proliferator-activated receptor gamma coactivator 1α

PIC: Protease inhibitor cocktail

PMA: Phorbol 12-myristate 13-acetate

Rac: Superfamily of GTP hydrolases

RIP: Receptor interacting protein

RIPA buffer: Radio immunoprecipitation assay buffer

ROS: Reactive oxygen species

Sac1: Stem cell antigen 1

SASP: Senescence associated secretory phenotype

SDS: Sodium dodecyl sulphate

Serpin E1/PAI-1: Serpin E1/Plasminogen activator inhibitor-1

SF medium: Serum free medium

SOD2: Superoxide dismutase 2

SODD: Silencer of death domains

SREBP: Sterol regulatory element binding protein

STAT3: Signal transducer and activator of transcription 3

TAK 1: TGF-β-activated kinase 1

TEMED: N, N, N,'N'- Tetramethylethylene diamine

TGF-β: Transforming growth factor- beta

TIMPs: Tissue inhibitors of metalloproteinases

TNFR1and 2: TNF-α receptors 1 and 2

TNF-α: Tumour necrosis factor alpha

TORC2/CRTC2: Transcriptional coactivator for the transcription factor CREB

TRADD: TNFR associated death domain protein

TRAF2: TNF-α receptor associated factor

TREM-1: Triggering receptor expressed on myeloid cells-1

UVR: Ultraviolet radiation

VEGF: Vascular endothelial growth factor

α-SMA: Alpha- smooth muscle actin

1 Introduction

1-1: Skin function

Human skin is considered to be the largest organ, because it covers the whole body and is continuous at entry and exits points of the body. It contains all four types of primary tissues epithelial, connective, muscle and nerve. Also, the blood vessels present in the dermis supply nutrients to the skin (Pullar *et al.,* 2017). Thus, skin functions are related to its structure (Mitchall and Peel, 2009; Chen *et al.,* 2014; Young *et al.,* 2014). One of the most important roles of skin is to act as a barrier; it provides protection against microbes, protection against chemical and physical damage, protects from damage by ultraviolet radiation (UVR) by increasing pigmentation, prevents excess water loss and regulates the body temperature via the sweat glands, while specialised Merkel cells are responsible for sensing the environment. In addition, the skin is the main site for the production of vitamin D (Mitchall and Peel, 2009; Chen *et al.,* 2014; Young *et al.,* 2014).

1-2: Skin structure

The skin consists of three major layers; the outer layer is called the epidermis (the epithelial tissue), and beneath that is the dermis that is mainly structured from connective tissue. The dermis is superficial to the hypodermis the deeper layer, which is also composed of connective tissue (Fig 1-1) and may be referred to, as the superficial fascia, or subcutaneous tissue (Mitchall and Peel, 2009; Bettes *et al.,* 2013; Young *et al.,* 2014).

1-2-1: The epidermis

The epidermis is derived from the ectodermal layer during development and is composed of a stratified squamous keratinized epithelium. The most abundant cell is the keratinocyte, although other cells such as Merkel cells, Langerhans cells and melanocytes are also present (Richmond and Harris, 2014). An important function of the keratinocytes is to provide a waterproof barrier and protect the underlying tissues. The epidermis usually consists of four layers; although five layers are found only in thick skin (Fig 1-2), such as the soles of the feet and palms of the hands (reviewed by Rinnerthaler *et al.,* 2015).

The layers from the innermost are; the stratum germinativum or basale which is the deepest layer of the epidermis and contains highly proliferating epithelial cells as stem cells for the epidermis which maintains the homeostasis of the skin (Mitchall and Peel, 2009). The migration of cells from the bottom layer, the stratum germinativum to the stratum corneum in human skin takes approximately 4 weeks depending on the location and the thickness of the skin (Candi *et al.,* 2005). The stratum spinosum is the thickest layer, which is comprised of the prickle cells, this layer is rich with desmosomes which are important in holding the cells together, resisting shearing forces and are responsible for layer's spiny appearance (William, 2017). The stratum granulosum, is formed of keratinocytes, which are characterized by the presence of cytoplasmic granules of keratohyalin; some granules consist of lipids that increase the waterproofing of epidermal superficial layers when released, also cells of this layer form the outmost epidermal layer, the stratum corneum (Piotrowska *et al.,* 2016). The stratum lucidum is a thin layer of keratinocytes only found in thick skin, it acts as a barrier to water, and

decreases the friction in thick skin (Betts *et al.,* 2013). The stratum corneum is the outmost layer of the epidermis and consists of corneocytes, non-living cells that are constantly shed and sealed together with lipid-rich domains, forming a water-impermeable barrier (Pullar *et al.,* 2017). This epidermis is separated from the underlying dermis by a basement membrane (Martini and Ober, 2006).

1-2-2: The dermis

The dermis is derived from the embryonic mesoderm and is classified as a connective tissue, made up of fibres including collagen, reticulin and elastin, and dermal fibroblasts. Dermal fibroblasts are responsible for the production of the extracellular matrix (ECM).

The dermis also contains nerves, lymphatic and blood vessels, sweat glands, sebaceous glands, hair follicles and nail roots (Mitchall and Peel, 2009; Young *et al.,* 2014). The dermis can be divided into two layers with no distinct boundary (Fig 1-3), the papillary and reticular layers (Martini and Ober, 2006). The papillary layer is the upper layer of the dermis and is composed of loose areolar connective tissue, with capillary loops that provide the skin with blood and sensory nerve fibres. Collagen fibres are produced as thin bundles 10μm in diameter. In general, the fibre bundles are thin and randomly arranged (Sanders *et al.,* 1999). The principal type of collagen in this layer is type III (Sorrell and Caplan, 2004; Bettes *et al.,* 2013). Also, the human papillary dermis contains rete ridges, which project into the epidermis (stratum basale) to form

finger-like dermal papillae that help to increase the interactions between epidermis and dermis (reviewed by Sriram *et al.,* 2015).

The reticular layer sits under the papillary layer and connects the skin to the hypodermis. It is composed of dense irregular connective tissue, with predominant collagen and elastin fibres, but is less cellular than the papillary layer (Carroll, 2007). The collagen fibres in this layer are produced as parallel dense collagen bundles of more than 50µm in diameter (Sanders *et al.,* 1999; Marcos-Garces *et al.,* 2014). The principal type of collagen in this layer is type I (Sorrell and Caplan, 2004). In addition, the hair follicle bulb, the functional parts of the sebaceous glands and sweat glands are also located in the reticular dermis (Alberts *et al.,* 2008).

Molecular studies in cultured human primary dermal fibroblasts from different anatomical regions/sites and different aged donors (foetal and adult), have shown differences in location (topography), especially for the genes that are expressed for biological processes such as; growth, differentiation, ECM production, migration and metabolism (Chang *et al.,* 2002; Rinn *et al.,* 2006*;* Bryant and Nix, 2012).

Korosec and her colleagues (2018), have found that fibroblasts can change the expression of a large number of their genes *in vitro* immediately, therefore it is difficult to use their subset-specific markers to isolate them from the cultures. Interestingly, they identified two cell surface markers, FAP and CD90 (proliferation markers), which enable the isolation of the papillary from reticular fibroblasts in human skin via the fluorescence activated cell sorting (FACS) technique. Also, in humans, there were many differences between papillary and reticular dermis, in addition to their structure, both *in vivo* and *in vitro* include;

size and morphology, the collagen type III to type I ratio, differences in ECM protein composition, protein secretion/expression, and organization. All these differences have a significant effect on fibroblast behaviour, role, response in wound healing and scar formation, as well as at the cellular level of dermis (Sorrell and Caplan, 2004; reviewed by Sriram *et al.,* 2015).

In mice, it has been shown that different lineages of dermal fibroblasts have different gene expressions, the gene expression of different lineages of murine dermal fibroblasts are listed in (Table 1-1) (reviewed by Sriram *et al.,* 2015).

References	Gene Expression (in mice)
Driskell *et al.,* 2013	PDGFRα, Lrig 1 and Blimp1 (Papillary DFs) PDGFRα, Dlk1 and Sca1 (Reticular & Hypodermal DFs)
Rinkevich *et al.,* 2015	En1 (Embryonic DFs); responsible for deposition of dermal connective tissue in future lineage.

Table 1-1: Some genes that are expressed in different lineages of murine dermal fibroblasts (reviewed by Sriram *et al.,* 2015).

1-2-3: The hypodermis

The hypodermis is composed of a thick layer of adipocytes encasing the bulbs of terminal anagen hair follicles. This layer has no distinct boundaries to the dermis and it connects the upper skin layers (dermis and epidermis) to underlying tissues such as bones and muscles (Betts *et al.,* 2013). This layer of adipocytes can also referred to as subcutaneous adipose, hypodermis, and subcutis. It has multiple functions including; fat storing, maintaining the body

heat via insulation, and as a protectial cushion. In addition, the development of dermal adipocytes (hypodermis) is structurally independent from that of subcutaneous adipose tissue (reviewed by Gonzaga da Cunha *et al.,* 2014). Some studies have identified a common developmental precursor for dermal fibroblasts and intradermal adipocytes (Joe *et al.,* 2010; Uezumi *et al.,* 2010; Schmidt and Horsley, 2013).

Furthermore, the role of the adipocytes has been shown to be of significance for epidermal homeostasis during hair follicle regeneration and wound healing in mice (Schmidt and Horsley, 2013; Driskell *et al.,* 2014). In human skin, adipocytes can secrete pro-inflammatory cytokines, because they can store the inflammatory fatty acids in the form of lipids (Richmond and Harris, 2014).

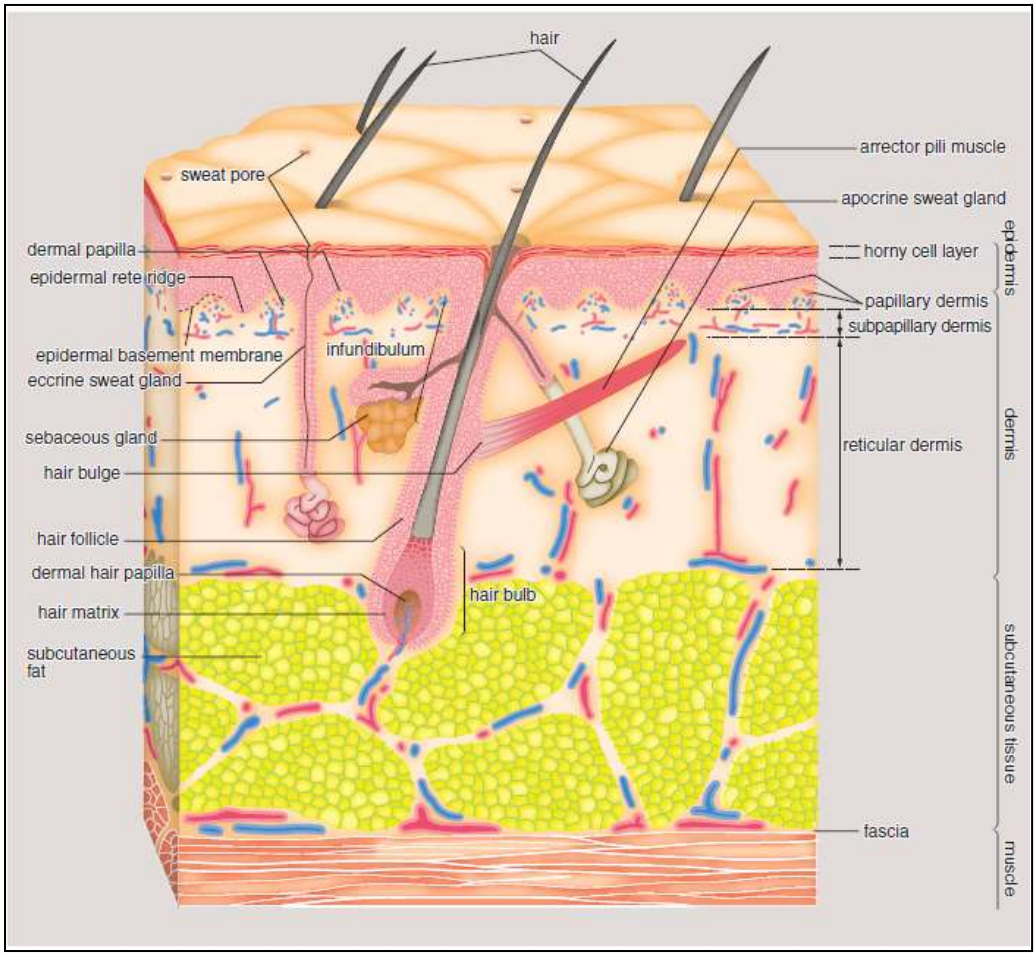

Fig 1-1: Human skin structure.

The human skin consists of three main layers; the epidermis, made of keratinocytes, the dermis, made of connective tissue, fibres and dermal fibroblasts. Beneath the dermis, the hypodermis, composed mainly of loose connective and fatty tissues. Also, skin appendages e.g. hair follicles and sweat glands (Nakayama, 2001).

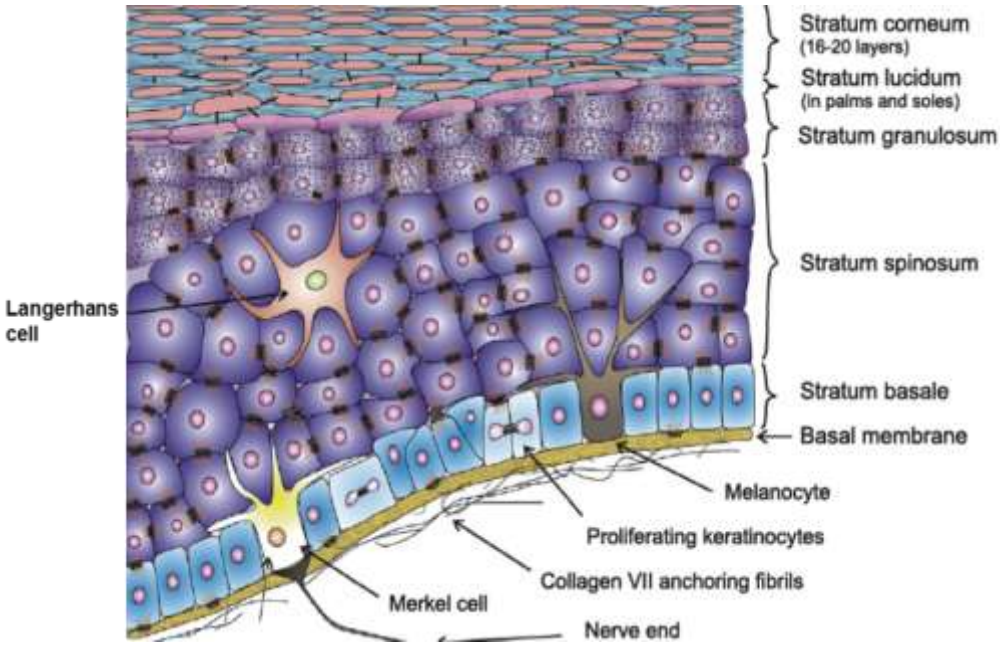

Fig 1-2: The different layers of human epidermis.

The five layers of the epidermis in glabrous skin. The layers from top to bottom; the stratum corneum, the stratum lucidum, the stratum granulosum, the stratum spinosum and the stratum basale (germinativum). The stratum basale is separated from the underlying papillary dermis by a basement membrane (Yagi and Yonei, 2018).

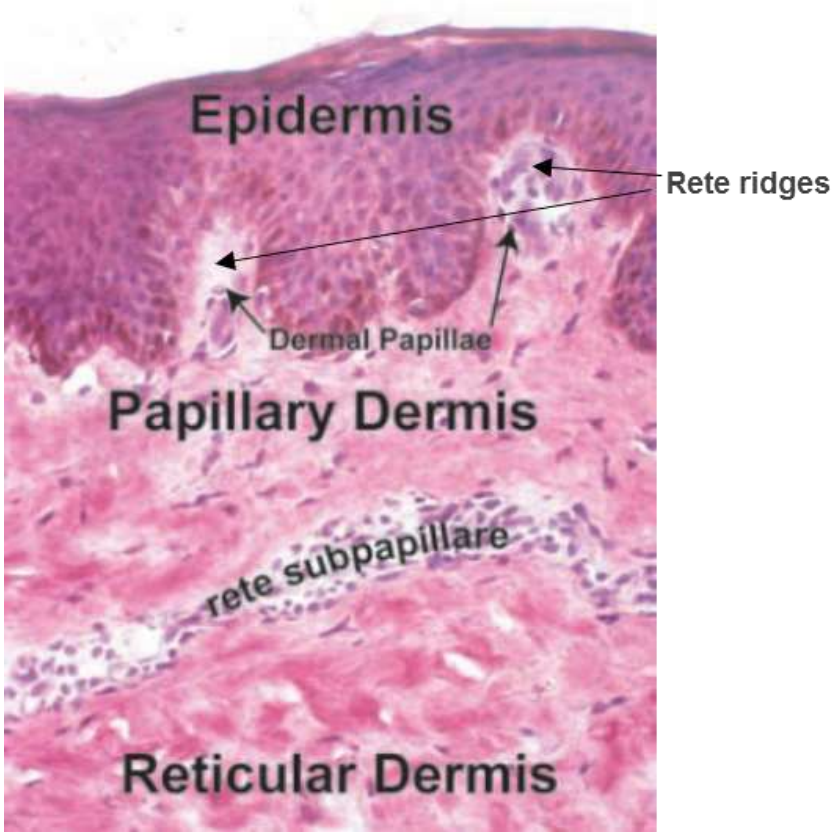

Fig 1-3: The papillary and reticular dermis.

The two major layers of human dermis; the papillary dermis and reticular dermis are separated by the rete subpapillare. The papillary dermis project into the epidermis by rete bridges or dermal papillae (Sorrell and Caplan, 2004).

1-3: Skin appendages

The skin appendages include, sweat and sebaceous glands, hair follicles and the nail. Sweat glands are present as two secretory types; the eccrine and apocrine sweat glands. Sweat glands are characterized by their existence all over the human body, the secretory cells are located deep in the dermis and even in the hypodermis, and the duct opens on the surface of the skin. In contrast, the apocrine sweat gland is present only in certain anatomical regions,

such as the axilla, and around the nipples, and forms part of the pilosebaceous unit. Unlike the eccrine glands, they secrete directly into the hair follicle (Mitchall and Peel, 2009), in a similar way to the sebaceous glands. In contrast, the sebaceous glands secrete sebum, a lipid substance produced by holocrine secretion, which helps to lubricate the hair fibre, acts as anti-microbial and waterproofs the skin and hair. Sebaceous glands are larger on the face and scalp, but absent on the palms and soles of the hands and feet, that have no pilosebaceous units (Young *et al.,* 2014).

Human hair follicles exist as three types, which can be described as terminal or vellus in adult human skin, and the third type called lanugo is found in the skin of the foetus (Buffoli *et al.,* 2013). There is also sometimes said to be a type of terminal hair called intermediate hair. These are smaller, and less pigmented than terminal hairs, and they do not penetrate as deep into the dermis as terminal hair follicles (Miranda *et al.,* 2010).

Lanugo hair is very soft and unpigmented and is shed before birth, when it is replaced by terminal or vellus hair follicles depending on the site in the human body. Vellus hair follicles are small, fine and unpigmented, whereas terminal hair follicles produce a large amount of pigment, such as those found on the scalp, eyebrows and eye lashes (Lai-Cheong and McGrath, 2013).

Interestingly, these appendices such as the hair follicles and sweat glands contain epithelial cells and a reservoir of stem cells, which are important in the regeneration of the epidermis (re-epithelialisation) in wound healing and thus, wounds heal faster in haired skin (Millar, 2002; Martin and Nunan, 2015).

The hair follicle structure is divided into two parts, the upper permanent part, which does not take part in the hair cycle, and the lower transient part, which is remodelled with each hair cycle. The hair follicle is composed of eight epithelial layers; these are the cuticle, cortex and medulla (hair shaft), the inner root sheath composed of Henle's, Huxley's and the inner root sheath cuticle, the companion layer and outer root sheath (Fig1-4) (Buffoli *et al.,* 2013). The bulge contains the stem cells and is part of the outer root sheath, and is located at the level of the insertion of the arrector pili muscle (Fig1-5). The bulb consists of the mesenchyme-derived dermal papilla, which is continuous with the dermal sheath; a connective tissue which surround the whole hair follicle (reviewed by Westgate, 1997; Ross and Pawlina, 2011). Finally, the dendritic melanocytes derived from the neural crest are also located in the bulb and produce the hair pigment (Cotsarelis, 2006 b; Rittie and Fisher, 2015).

Importantly, hair growth is cyclical, which means new hairs are produced throughout life. The hair cycle involves four main phases which are anagen, a phase with active growth; catagen, a regressing phase; telogen when the hair follicle enters a resting stage; and exogen, when the hair is shed (reviewed by Langan *et al.,* 2015) (Fig 1-5).

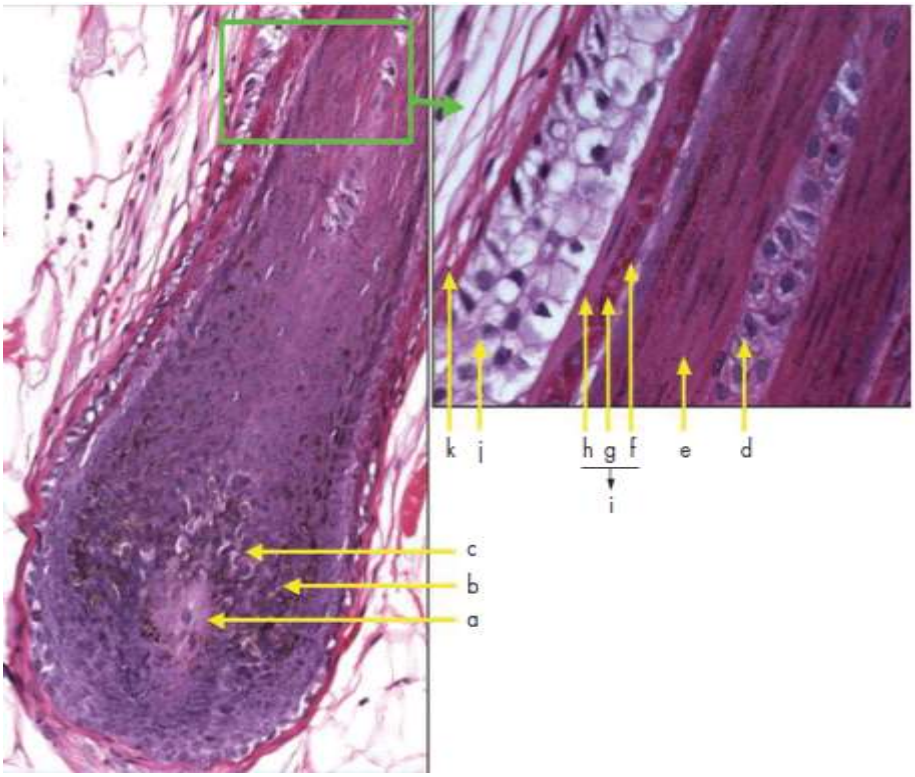

Fig 1- 4: The structure of the human hair follicle.

A histological section displays the hair bulb layers (a) mesenchymal dermal papilla (b) epithelial matrix (c) melanocytes (d) medulla (e) hair cortex (f) hair cuticle (g) Huxley's layer (h) Henle's layer (i) the inner root sheath (j) the outer root sheath, and (k) connective tissue sheath (Alsaad *et al.,* 2007).

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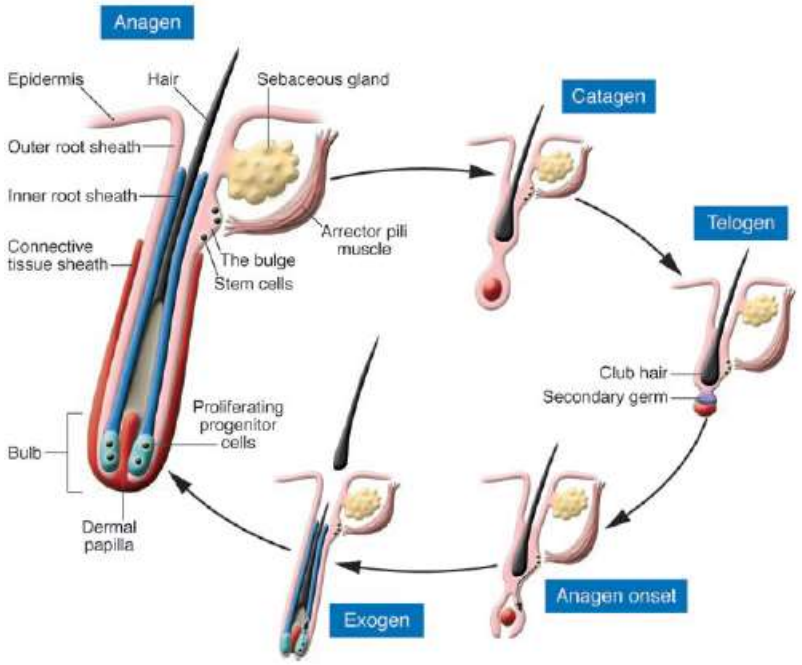


Fig 1-5: The hair follicle cycle.

The human hair follicle stages; anagen, forming a new hair. Upon cessation of anagen, the follicle regresses in catagen, returning to telogen. The old hair is shed in exogen when the new hair grows (Cotsarelis, 2006 a).

1-4: The dermal fibroblast

Dermal fibroblasts are the predominant mesenchymal cell type in the skin dermis, with the central role of synthesizing the extracellular matrix (ECM) (Alberts, 2008). The ECM consists of proteins such as proteoglycans and glycosaminoglycans with crucial functions, such as regulation of tissue structure, and maintenance of the cellular microenvironment by the secretion of growth factors and cytokines, and the production of matrix metalloproteinases (MMPs). Fibroblasts are responsible for the composition of fibres especially

collagen fibres in addition to elastic and reticular fibres (Mine *et al.,* 2008). Because dermal fibroblasts are changing with age, the fibres will change with age too. Therefore, these cells became the researchers' main focus for their crucial role in aging (Tigges *et al.,* 2014; Korosec *et al.,* 2018).

One important property of dermal fibroblasts is their heterogeneity, Harper and Grove (1979) reported differences in function and structural features between the papillary and reticular dermis, and they suggested that, the dermal layers contain two different fibroblast lineages with distinct morphology and functions, in humans. Subsequently, in mice it has been confirmed that dermal fibroblasts arise from two distinct lineages (Driskell *et al.,* 2013; reviewed by Thangapazhum *et al.,* 2014; Rinkevich *et al.,* 2015). First from the upper "papillary dermis" and the second from the deeper "reticular dermis", called papillary fibroblasts and reticular fibroblasts respectively (Driskell *et al.,* 2013; Mastrogiannaki *et al.,* 2016). More reports have recognized that fibroblasts from different micro-anatomic regions have specific properties depending on their particular niche, and they can be further divided into fibroblasts that are associated with the hair follicle, including the dermal sheath, which acts as a reservoir for new dermal papilla cells (Jahoda, 2003) and dermal papilla cells, which have a role in hair follicle regeneration (Rompolas *et al.,* 2012) (reviewed by Thangapazhum *et al.,* 2014).

Representation of the heterogeneous populations of dermal fibroblasts with a variety of features should be considered in any study (Mine *et al.,* 2008; Driskell *et al.,* 2013; reviewed by Thangapazhum *et al.,* 2014; Rinkevich *et al.,* 2015). In human mammary skin, it has been observed that papillary dermal fibroblasts and reticular dermal fibroblasts have different functional and genetic markers

demonstrating the regional importance of fibroblast sub populations (Pageon *et al.,* 2012; Rinkevich *et al.,* 2015) (Table 1-2). Papillary dermal fibroblasts were characterized by a higher proportion of smaller sized cells with low granularity and a higher growth/clonogenic potential than reticular dermal fibroblast populations (Mine *et al.,* 2008).

Papillary markers	Reticular markers
Podoplanin (PDPN)	**Calponin1 (CNN1)**
Netrin 1 (NTN1)	**Transglutaminase 2 (TGM2)**
C-C Chemokine receptor type 11(CCRL1)	**Cadherin 2 (CDH2)**

Table 1-2: Markers of the papillary and reticular dermal fibroblasts
Some markers that distinguish human papillary fibroblasts from reticular dermal fibroblasts (Janson *et al.,* 2013).

The other difference is that papillary dermal fibroblasts divide faster than reticular dermal fibroblasts in culture (Sorrell and Caplan, 2004). During aging, a reduction in the growth capacity of papillary dermal fibroblasts may result from a decline in their response to growth/mitogenic factors, leading to a decrease in their number (Reenstra *et al.,* 1993; Pageon *et al.,* 2012). Interestingly, after long series of passages (P16- P22) in monolayer culture, papillary dermal fibroblasts transform into reticular fibroblasts (Janson *et al.,* 2013).

Generally, dermal fibroblasts play a key role the regulation of epidermal morphogenesis, especially cells which have been shown to affect the formation of the basement membrane and can directly communicate with keratinocytes (Sorrell and Caplan, 2004; Lee and Cho, 2005; Mine *et al.,* 2008). Also, the modification of papillary dermal fibroblast secretions, such as the secretion of

cytokines, growth factors and MMPs; these proteinases degrade the ECM and are regulated by the tissue inhibitors of metalloproteinases (TIMPs), and together can have a significant impact on the production and remodelling of the ECM components (Werner *et al.,* 1994 ; Odorisio *et al.,* 2002).

1-5: Wound Healing

A wound can be defined as a break in the barrier of the skin extending to the dermis, muscle and even bone. It may be delineated in many ways; by its aetiology, anatomical location, by the method of closure, by symptoms or by the appearance of the tissue types in the wound bed, and whether it is an acute or chronic wound (Enoch and Price, 2004).

1-5-1: Phases of wound healing

Wounds are classified into two types, acute and chronic wounds, depending on the healing process. In general, normal wound healing is a complex process involving a series of overlapping events, which leads to the repair of the injured skin/tissue. The phases of normal wound healing consist of several interacting overlapping events, which are in the sequence of haemostasis, inflammation, proliferation and remodelling (Singer and Clark, 1999; Greaves *et al.,* 2013; reviewed by Thangapazhum *et al.,* 2014) (Fig 1-6) (Fig 1-7).

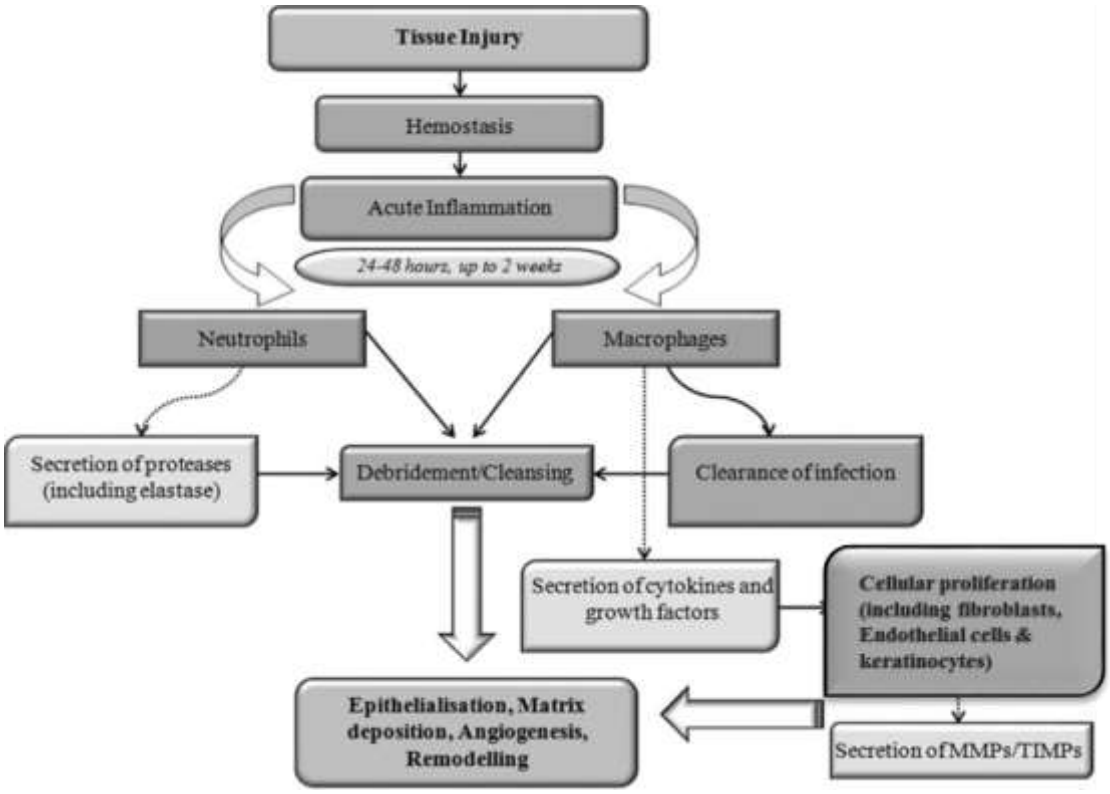


Fig 1-6: Mechanism of normal human wound healing.

Tissue injury starts with haemostasis (clot formation), and inflammation, which involves the recruitment of macrophages and neutrophils that secrete cytokines and growth factors to promote cellular migration and proliferation of keratinocytes, fibroblasts and endothelial cells. Proteases (MMPs) also are secreted by skin cells that aid in the remodelling phase (McCarty and Percival, 2013).

1. Haemostasis

Haemostasis or coagulation occurs immediately after injury, when platelets begin to adhere and clump at sites of exposed collagen in the wound. The activated platelets, following activation by thrombin, will undergo a change in their morphology (platelet aggregation), which is more suitable for clotting in order to stop the bleeding. The clot consists of fibrin, fibronectin and vitronectin (Darby *et al.,* 2014; Olczyk *et al.,* 2014). Then platelets release multiple growth factors (Weyrich and Zimmerman, 2004), including platelet derived growth factor (PDGF), insulin-like growth factor-1 (IGF-1), epidermal growth factor (EGF), and transforming growth factor beta (TGF-β) (Singer and Clark, 1999). All these growth factors initiate the wound healing cascade which attract and activate fibroblasts, endothelial cells, neutrophils and macrophages (Demidova-Rice *et al.,* 2013; Darby *et al.,* 2014).

2. Inflammation

During this phase the release of growth factors and pro-inflammatory cytokines from endothelial cells, keratinocytes and fibroblasts induce the chemotaxis, attraction and proliferation of neutrophils and macrophages, the inflammatory cells (Singer and Clark, 1999). They cooperate to remove dead cells, bacteria/pathogens and debris from the wounds by phagocytosis (Wilgus, 2008; Martin and Nunan, 2015), and release reactive oxygen species (ROS) that can act as an antimicrobial agent (Demidova-Rice *et al.,* 2013). In addition to the release of inflammation stimulating molecules, the damage-associated molecular patterns (DAMPs) that are secreted by wound scratched or dead cells, and pathogen-associated molecular patterns (PAMPs) that are secreted

by pathogens of infection, will initiate the inflammatory responses (Qian *et al.,* 2016; Roh and Sohn, 2018). Macrophages are key regulators of wound healing as they release further cytokines and growth factors into the wound, to recruit fibroblasts, keratinocytes and endothelial cells, to repair the wound (Darby *et al.,* 2014). The removal of inflammatory cells by apoptosis, a programmed cell death, indicates the resolution of this phase (Eming *et al.,* 2007).

3. Proliferation

Proliferation or growth of new tissue is characterized by the replacement of the provisional fibrin/fibronectin matrix with new granulation tissue. In this phase, fibroblasts proliferate, migrate, synthesise new collagen, and form new granulation tissue to allow re-epithelialisation to occur (Robson, 2003; Darby *et al.,* 2014; Olczyk *et al.,* 2014). Fibroblasts differentiate into myofibroblasts in response to TGF-β1 signalling via Smad2/3 phosphorylation (Li and Wang, 2011) and together migrate into the wound site and produce the matrix proteins including fibronectin, hyaluronic acid (HA), collagens and proteoglycans, to form a new provisional ECM that gives more support for further ingrowth of cells (Singer and Clark, 1999; Midwood *et al.,* 2004; Demidova-Rice *et al.,* 2013). Collagen synthesis is essential since collagens provide strength and integrity. These collagens are essential components of all phases of wound healing, because they will become the foundation of the ECM; fibroblasts synthesise and secrete type III collagen to form a new matrix during wound healing (Zhu *et al.,* 2016). Vascular endothelial cells form new blood vessels (angiogenesis), to replace damaged capillaries (Montesinos *et al.,* 2002; Chin *et al*., 2005; Mohd *et al,* 2012). Granulation tissue formation is made up of capillaries and tissue

macrophages in a matrix of collagen (Nowak and Olejek, 2004), and glycosaminoglycans (GAGs) including HA (Kozma *et al.,* 1998; Tomasek *et al.,* 2002).

Finally re-epithelialisation occurs where a single layer of epithelial cells migrate from the wound edges, and skin appendices (Martin and Nunan, 2015), such as the hair follicles and sweat glands. Further proliferation is stimulated by EGF- is released from injured epidermis and KGF- is released from mesenchymal cells, which are potent stimulators of epithelial mitogenesis and chemotaxis to provide cover for the new tissue (Demidova-Rice *et al.,* 2013; Darby *et al.,* 2014).

4. Remodelling

The remodelling or contraction phase, is where matrix synthesis, remodelling and wound contraction occur simultaneously. In this phase, the granulation tissue matures to form a scar. Fibronectin and HA are broken down, and collagen bundles increase in diameter. Type I collagen is replaced by collagen type III (Kuwaba *et al.,* 2001; Reinke and Sorg, 2012; Olczyk *et al.,* 2014). A balance between MMPs and their inhibitors TIMPs is important in remodelling wounds. As TIMP activity increases, it thereby down regulates levels of pro-inflammatory cytokines that stimulate MMP production. Thus, MMP activity is decreased (Trengove *et al.,* 1999; Zhao *et al.,* 2013).

Wound remodelling occurs when the underlying contractile connective tissue brings the wound margins closer together by increasing tissue tension. Contraction is an interaction between myofibroblasts (dermal fibroblasts which differentiate into myofibroblasts) and the ECM, which is influenced by factors

such as TGF-β, PDGF and fibroblast growth factor (FGF) (Hinz, 2007; Demidova-Rice *et al.,* 2013).

These myofibroblasts express alpha smooth muscle actin (α-SMA), microfilaments that exhibit contractile properties and can be used as a myofibroblast defining marker (Hinz *et al.,* 2007; Desmouliere *et al.,* 2005; Olczyk *et al.,* 2014). Cells that are no longer needed such as macrophages and myofibroblasts are removed by apoptosis, and are replaced by dermal fibroblasts, which produce the collagen type I, and maintain the homeostasis of the ECM (Sorrell and Caplan, 2004; Darby *et al.,* 2014).

1-5-2: The acute wound

An acute wound is one which heals normally and within an expected time frame (Fig 1-7).

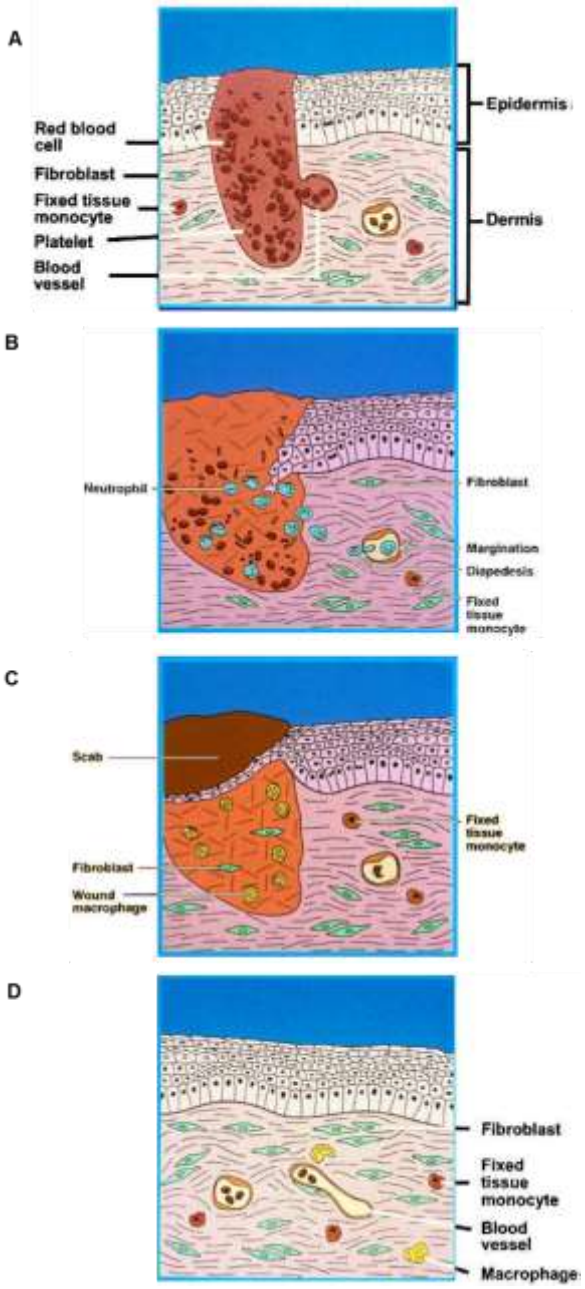


Fig 1-7: Acute human wound healing stages.

(A) The haemostasis phase (the platelets aggregate and release growth factors); **(B)** the inflammation phase (neutrophils migrate and accumulate); **(C)** the proliferation phase (keratinocyte migration and proliferation, recruitment of fibroblasts and synthesis of collagen, neoangiogenesis); **(D)** the remodelling phase (balance between synthesised and degraded ECM) (Diegelmann and Evans, 2004).

1-5-3: Dermal fibroblasts in wound healing

Fibroblasts are pivotal during the wound healing process, after wounding they are attracted from the wound edges, or from the mesenchymal stem cells and start to proliferate and migrate into the wound bed to produce ECM proteins (reviewed by Thangapazhum *et al.,* 2014; Shi *et al.,* 2015). ECM proteins act as a scaffold for inflammatory cell migration and granulation tissue generation (Kendall and Feghali-Bostwick, 2014), and during the inflammatory phase dermal fibroblasts produce a spectrum of cytokines (Werner *et al.,* 2007) (Table 1-3). The main roles of the dermal fibroblasts in the wound healing process has been described in section 1-5-1.

In addition to the production of ECM, fibroblasts secrete a variety of growth factors and MMPs that promote or regulate wound healing (Greaves *et al.,* 2013) (Table 1-3). Basic fibroblast growth factor (bFGF) is a potent mitogen that stimulates the migration, proliferation and differentiation of cells including fibroblasts, keratinocytes, endothelial cells and melanocytes (Shi *et al.,* 2015), and regulates ECM production (Shi *et al.,* 2013).Vascular endothelial growth factor (VEGF) promotes angiogenesis through its receptors on endothelial cells (Kendall and Feghali-Bostwick, 2014), and hepatocyte growth factor (HGF) all promote wound healing. (Ko *et al.,* 2011; Loyd *et al.,* 2012). HGF has a spectrum of effects on wound healing including stimulation of angiogenesis, regulation of matrix deposition and degradation, and promoting re-epithelialisation of keratinocytes by stimulating keratinocyte migration and proliferation (Conway *et al.,* 2006). There are many proteins expressed by fibroblast specific proteases MMPs such as, MMP-2, which are required during the remodelling phase (Trengove *et al.,* 1999; Gill and Parks, 2008; Olaso *et al.,*

2011). In mice, the expression of MMP-9 and MMP-13 during the late remodelling phase is believed to promote scar-free healing (Gawronska-kozak, 2011; reviewed by Thangapazhum *et al.,* 2014).

Interestingly, Driskell *et al*, (2013) explained that wound repair in mice is mediated by reticular dermal fibroblasts, which are responsible for producing the ECM that initiates the wound healing process. These fibroblasts synthesis a matrix rich tissue that lacks hair follicles, while the papillary dermal fibroblasts are responsible for hair follicle formation, in mice.

Name	Abbreviation	Class	Produced by	Function
Interleukin 1,6 ,8	IL1, IL6, IL8	Cytokine	Macrophages, Keratinocytes	Pro-inflammatory; recruit fibroblasts and keratinocytes
Interleukin 2	IL2	Cytokine	T-lymphocytes	Recruits fibroblasts
Interleukin 4	IL4	Cytokine	T-lymphocytes	Inhibits TNF, IL1, IL6, inhibits fibroblast proliferation
Tumour Necrosis factor alpha	TNF-α	Cytokine	Macrophages	Pro-inflammatory: promotes collagen synthesis by DFs
Epidermal growth factor	EGF	Growth Factor	Platelets, macrophages, keratinocytes	Promotes keratinocyte and fibroblast proliferation, Keratinocyte migration, and granulation tissue formation
Fibroblast growth factor acidic and basic	FGF-a and –b	Growth factor	Endothelial cells, fibroblasts, macrophages, T-lymphocytes	Cause angiogenesis, fibroblast chemotaxis and proliferation
Keratinocyte growth factor 1 and 2	KGF	Growth factors	Fibroblasts	Stimulate keratinocyte division and proliferation
Platelet derived growth factor (PDGF exists in several forms: AA, BB, AB, others)	PDGF	Growth factor	Platelets, macrophages, also fibroblasts, endothelial cells	Cause neutrophil and fibroblast chemotaxis, fibroblast proliferation, and synthesis of matrix proteins, MMPs, and stimulates angiogenesis
Transforming growth factor (alpha and beta)	TGF- α,β	Growth factor	Platelets, macrophages, fibroblasts, keratinocytes, T-lymphocytes	Cause fibroblast and keratinocyte chemotaxis, angiogenesis, upregulates TIMP, inhibits production of MMPs and keratinocyte proliferation and induces TGF-β production
Vascular endothelial growth factor (a family of peptides)	VEGF	Growth factor	Endothelial cells, keratinocytes, platelets, macrophages, fibroblasts	Cause angiogenesis (mitogenic for endothelial cells). Expression increased in the presence of hypoxia
Tissue inhibitor of metalloproteinase	TIMP	Enzyme	Most mesenchymal cells, fibroblasts	Inhibits MMPs
Matrix metalloproteinase	MMP	Enzyme	Monocytes, macrophages, endothelial cells, fibroblasts	Degrades the extracellular matrix

Table 1-3: The main cytokines, growth factors and proteases that participate in wound healing (Mohd *et al,* 2012).

1-6: The role of the dermal fibroblast in inflammation

Dermal fibroblasts have a significant role in switching from acute inflammation to chronic persistent inflammation, and they can modulate the quality, quantity and duration of inflammation by a number of pathways (Flavell *et al.,* 2008; Kumar *et al.,* 2013).

The interaction between circulating leukocytes from the blood stream and endothelial cells helps the emigration of the leukocytes through the vascular endothelial barrier, to reach the underlying tissue (Zittermann and Issekutz, 2006; Kumar *et al.,* 2013). After activation of endothelial cells, leukocytes extravasate and induce an inflammatory response in the microenvironment, via the release of pro-inflammatory stimuli, such as tumour necrosis factor (TNF-α) and interferon gamma (INF-γ). Chemotactic factors, including interleukin 8 (IL-8) and monocyte chemoattractant protein (MCP-1), act to recruit monocytes, neutrophils, and lymphocytes, as well as regulating migration and infiltration of monocytes, and are involved in attracting the leukocytes to the site of inflammation (Johnston and Butcher, 2002; Kumar *et al.,* 2013; Linthout *et al.,* 2014).

Interestingly, fibroblasts accelerate the homing of the leukocytes and promote activation of endothelial cells at the same time (Nash *et al.,* 2004), since they produce chemokines including MCP-1, at high levels compared with macrophage inflammatory protein (MIP-1) and express chemokine receptors (Hogaboam *et al.,* 1998). MCP-1 also stimulates collagen expression and endogenous up-regulation of TGF-β expression in fibroblasts, leading to stimulation of collagen synthesis (Gharaee-Kermani *et al.,* 1996). The induction

of chemokines following activation of fibroblasts with inflammatory stimuli such as TNF-α is regulated by the pro-inflammatory nuclear transcription factor NF-κB (Enzerink, and Vaheri, 2011; Linthout et *al.,* 2014).

In mice, the lack of RelB expression (a member of the NF-κB family of transcription factors, which activates and regulates the KB-regulated genes expressed in lymphoid tissue) in fibroblasts leads to an increase in pro-inflammatory chemokine production via fibroblasts and increased inflammatory cell accumulation (Smith *et al.,* 1997), indicating that NF-κB activity may cause persisting inflammation (Newton *et al.,* 1997; Linthout *et al.,* 2014). Also fibroblasts can promote or inhibit the recruitment of leukocytes on endothelial cells via the induction, or inhibition, of cytokine-induced expression of adhesion molecules on endothelial cells (McGettrick *et al.,* 2009). Another important function of fibroblasts in inflammation is that they regulate the behaviour of the immune cells and their retention and survival in damaged tissue, via crosstalk between the fibroblasts and immune cells (Smith *et al.,* 1997).

1-7: Chronic wounds

A chronic wound fails to heal in the standard time frame, resulting in a non-healing wound (Fig1-8). It is associated with an impairment of the normal healing process, which is interrupted at one or more phases, usually this results in a halting of the process in the inflammatory or proliferative phases (Singer and Clark, 1999; Al-Mulla *et al.,* 2011). Chronic wounds are characterized by an increased incidence of bacterial biofilms, leading to persistent inflammation, and an excessive proteolytic activity that destroys the ECM (Chin *et al.,* 2005), due

to high levels of MMPs with lower expression of TIMPs. Also non-healing wound fluid does not have growth factors, while the pro-inflammatory cytokines such as IL-1, IL-6 and TNF-α are elevated (Trengove *et al.,* 2000). Fibroblasts within these wounds are unable to proliferate and/or migrate, which means their responses are poor (Agren *et al.,* 2000; Mohd *et al.,* 2012), since they have lost their functional receptors that respond to cytokines and growth factors (Mohd *et al.,* 2012; Demidova-Rice *et al.,* 2013).

Furthermore, there are many causes and types of chronic wounds, for instance chronic venous leg ulcers (CVLUs), pressure ulcers, also arterial ulcers and diabetic disease (Enoch and Price, 2004; Demidova-Rice *et al.,* 2013). There are similarities among these pathophysiologic phenomena and/or diseases, which include a prolonged inflammatory phase, persistent infection, formation of drug- resistance microbial biofilms, and the inability of dermal and/or epidermal cells to respond to reparative stimuli, which collectively results in a failure of these wounds to heal (Edwards and Harding, 2004; Demidova-Rice *et al.,* 2013). A complication of aging and diabetes can lead to, and exacerbate vascular pathologies, including both arterial and venous insufficiencies, and worsen pressure ulcers. Another complication that leads to the development of chronic wounds in diabetic patients is neuropathy, often linked to vascular impairment, deficiencies in muscle metabolism, and a number of microvascular pathologies. The wounds are recognized by cellular phenotypic abnormalities, including low mitogenic potential and the inability to respond to environmental signals (Demidova-Rice *et al.,* 2013).

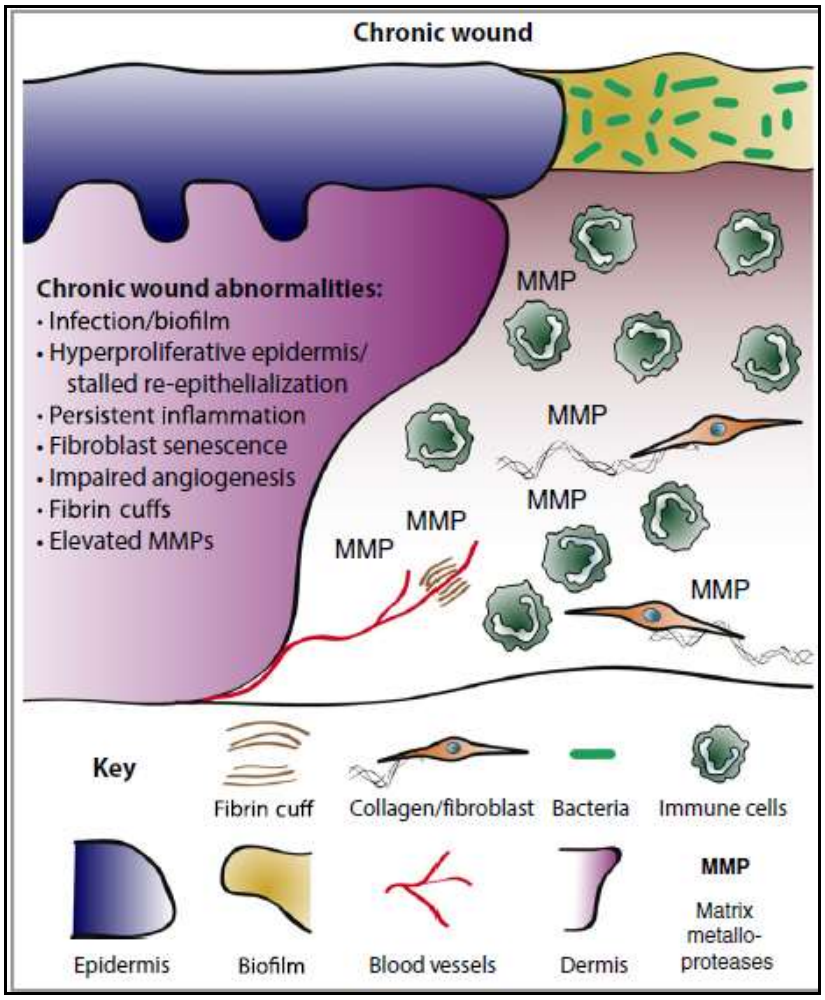


Fig 1-8: Chronic wound biology.

The diagram illustrates the chronic wound which can be infected with biofilm microorganisms, and exhibit lengthened and excessive inflammation. Re-epithelialisation stalls while hyperproliferation of keratinocytes occurs. High expression of MMPs, leads to defective granulation tissue, and poor fibroblast infiltration. This is accompanied by impaired angiogenesis, elevated levels of oxygen free radicals, abundance of collagen type III and an increase in inflammatory cell numbers (Martin and Nunan, 2015).

1-8: The inflammatory phase in chronic wounds

Inflammation is an important stage in wound healing that is disrupted and deregulated in chronic wounds (Satish, 2015). The inflammatory phase takes place early in the sequence lasting only a few days, followed by progression to tissue regeneration including re-epithelialization, granulation tissue formation, angiogenesis and tissue remodelling (Zhao *et al,* 2013).

Non-healing wounds are characterized by a prolonged inflammatory phase, which alters the progression of wound healing and can be accompanied with microbial infection that contributes to the prolongation of this inflammation (Zhao *et al,* 2013). Moreover, this prolongation may lead to chronic inflammation by altered production of pro- and anti-inflammatory cytokines, causing an impaired angiogenesis process, and decreasing granulation tissue formation by reducing migration and proliferation of keratinocytes and fibroblasts (Goova *et al.,* 2001; Blakytny and Jude, 2008). The extended inflammatory phase may relate to an imbalance between pro and anti-inflammatory cytokines in chronic wounds, particularly the high levels of interleukins IL-1, IL-6 and Il-8 and TNF-α (Fivenson *et al.,* 1997; Satish, 2015). The continuous exposure to pro-inflammatory cytokines may stimulate the production of MMPs, while inhibiting the synthesis of TIMPs (Trengove *et al.,* 1999; McCarty and Percival, 2013), whereas IL-1 and TNF-α enhance collagenase secretion (Barchowsky *et al.,* 2000). Another explanation is that the inflammatory phase disrupts the normal balance between deposition and degradation of ECM components. The degradation and remodelling of the ECM by MMPs, which is a major factor in wound repair, and the failure in the control of its expression results in excessive ECM degradation with a delay in healing.

Also it is important in the recruitment of leukocytes, regulation of angiogenesis and re-epithelialisation (Diegelmann and Evans 2004; Schultz and Wysocki, 2009; McCarty and Percival, 2013).

1-9: Tumour necrosis factor-alpha

Tumour necrosis factor- alpha (TNF-α) or cachexin is a pleiotropic and pro-inflammatory cytokine, which is considered the most important inflammation marker for it is present in both acute and chronic inflammation processes (Wang and Lin, 2008; Agren *et al.,* 2015). TNF-α expression levels are responsible for controlling the inflammation process switching from acute to chronic, which exhibit the key role of TNF-α. Furthermore, the excessive expression of TNF-α can lead to chronic, inflamed, non-healing wounds and/or inflammatory diseases (Han *et al.,* 2001a; Intiso *et al.,* 2003; Arican *et al.,* 2005; Gohel *et al.,* 2008; Montgomery and Bowers, 2012).

Although the inflammatory cells such as the macrophages are the main producer of this cytokine, it can be secreted by other immune cells that are involved in inflammation responses, and even by cells such as fibroblasts and neurons (Han *et al.,* 2001a; Intiso *et al.,* 2003; reviewed by Xu *et al.,* 2013).

TNF-α exists in two forms; the trans- membrane (membrane-bound) and the secreted form, and both are biologically functional and active (Varfolomeev and Vucic, 2018). The central function of TNF-α is to regulate the immune response by regulating and controlling the immune cells and it aids in maintaining homeostasis of the immune system (reviewed by Xu *et al.,* 2013; Varfolomeev and Vucic, 2018). In addition, TNF-α can participate in many biological functions

such as cell activation, proliferation and apoptosis, depending on its concentration and the target cell type (Ruckert *et al.,* 2000; Petrache *et al.,* 2000) (Fig 1-9). The TNF-α cytokine has two main receptors, which are TNFR1 (receptor type one; p55) and TNFR2 (receptor type two; p75) (Theiss *et al.,* 2005; reviewed by Xu *et al.,* 2013). Receptor TNFR1 is 55kDa and receptor TNFR2 is 75kDa, TNFR1 can be found in most cell types and can be activated by both forms of TNF-α (membrane- bound and soluble). Also it is believed that most TNF-α signalling pathways are performed by binding to this receptor, whereas TNFR2 can be found in immune cells and endothelial cells, and binds with high affinity to the membrane- bound form (Montgomery and Bowers 2012; Poggi *et al.,* 2013).

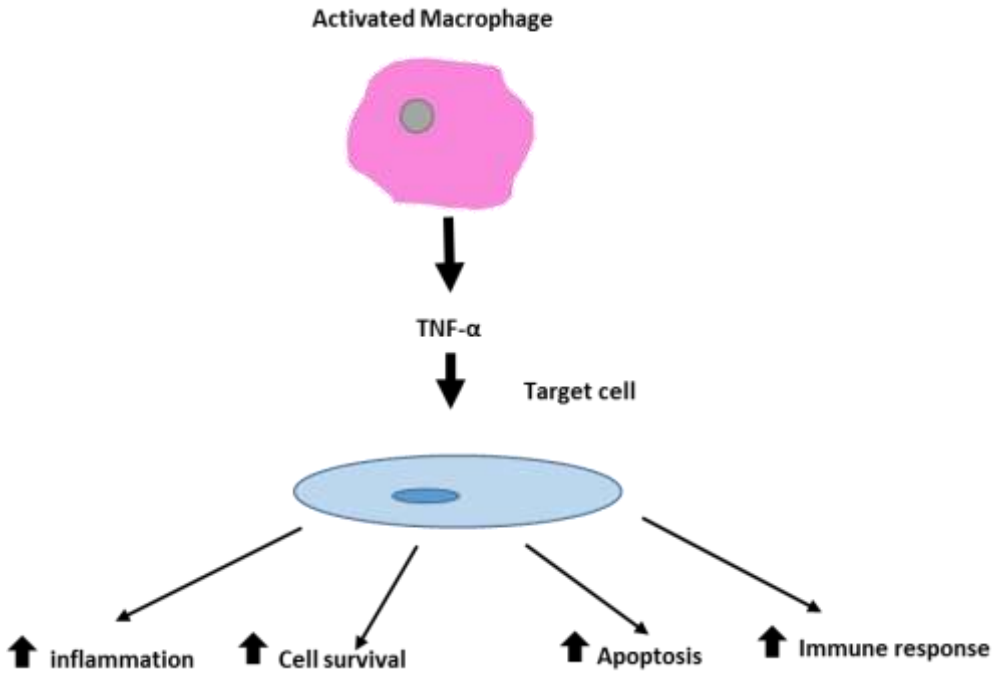


Fig 1-9: Effects of TNF-α on target cells.

The various effects of TNF-α at the cellular level lead to inflammation, cell survival (proliferation), cell apoptosis and immune response.

1-9-1: TNF- α signalling pathways

TNF-α effects are mediated by binding to the two types of cell membrane receptors TNFR1 and TNFR2 (Poggi *et al.,* 2013; Moelants *et al.,* 2013), or binding to receptor type one (TNFR1) (Bose, 2015). One of three main TNF-α pathways, the inflammation or NF-$_k$B activation pathway (Fig 1-10); starts by the binding of TNF-α to the extracellular domain of its receptor TNFR1, causing conformational changes in the receptor itself, which leads to dissociation of the inhibitory protein Silencer of death domains (SODD) from the death domain (DD). This allows the adapter protein molecule interacts with TNF-R1-associates death domain protein and form (TRADD), which attaches to the intracellular side of the receptor and provide a scaffold for the sequenced binding events (reviewed by Turner *et al.,* 2014). TRADD recruits two molecules, (TNF-α receptor associated factor 2) TRAF2 and RIP1 (receptor interacting protein), then TRAF2 recruits I_k-B kinase, or IKK complex- an enzyme transfer phosphate group from high energy compound. The IKK complex phosphorylates I_k-Bα resulting in subsequent events through ubiquitin-mediated proteasomal degradation. Targeted degradation of I_k-Bα leads to release of NF-$_k$B (free NF-$_k$B dimers are mainly p50/p65 or p50/c-Rel), which is a transcription factor that translocates to the nucleus. NF-$_k$B mediates the transcription of vast numbers of pro-inflammatory cytokines and inflammatory responses (Kawahara *et al.,* 2011; reviewed by Xu *et al.,* 2013; Bose, 2015).

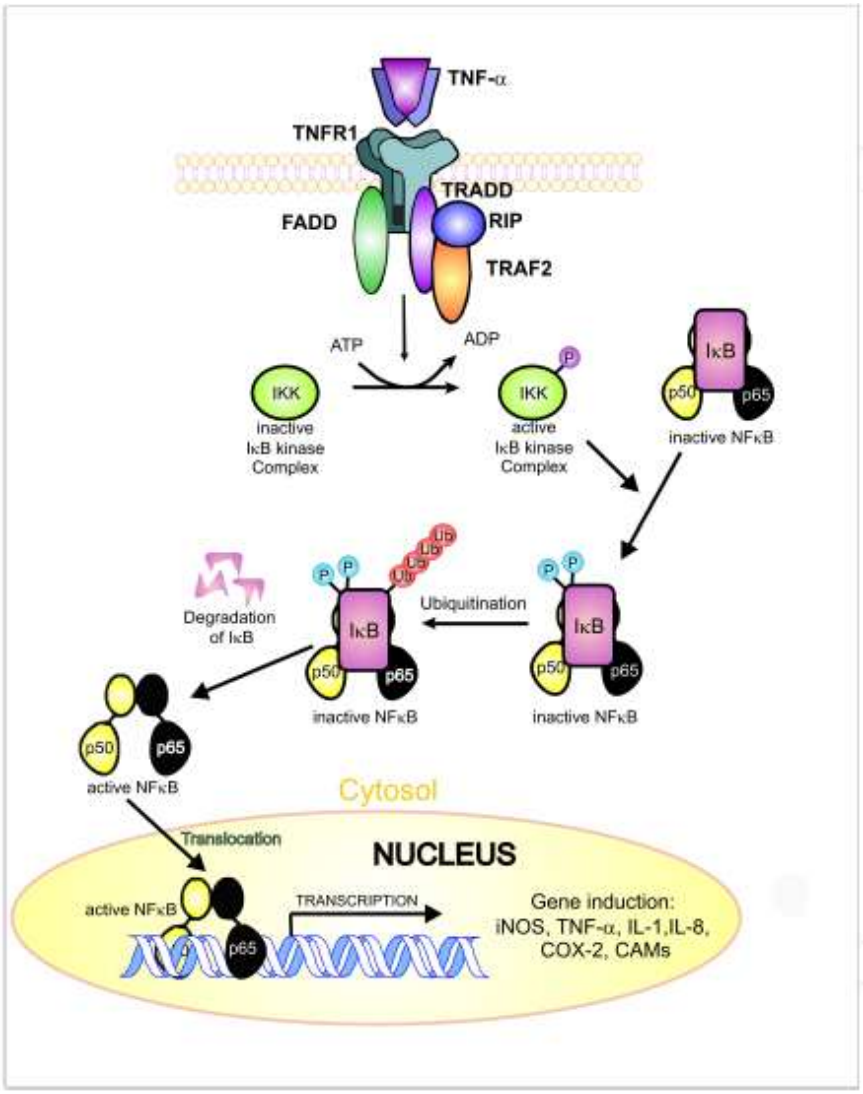


Fig 1-10: Triggering of the NF-$_k$B pro-inflammatory signalling pathway by TNF-α.

NF-$_k$B signalling pathway starts by binding the trimeric cytokine to it is receptor TNFR1, which attracts TRADD, followed by TRAF2. RIP1binding to TRADD, leads to phosphorylation of IKK enzyme, phosphorylation of Ik-Bα by IKK, resulting in Ik-Bα promotes its polyubiquitination and degradation, and thus releasing of NF-$_k$B dimers to the nucleus causes transcription of pro-inflammatory genes. (Ingaramo *et al.,* 2013).

1-9-2: TNF-α and wound healing

The pro-inflammatory cytokine TNF-α has a significant role in the inflammatory phase in the process of wound healing, but excessive levels of this cytokine may cause chronic, inflamed non-healing wounds by lengthening this phase and increasing the inflammatory cell numbers such as neutrophils and leukocytes (Tellechea *et al.,* 2010; reviewed by Xu *et al.,* 2013). Some researchers reported that elevated TNF-α levels lead to a significant increase in chronic wounds, such as chronic venous leg ulcers, compared to acute or healing wounds (Gohel *et al.,* 2008; Tellechea *et al.,* 2010; Khanna *et al.,* 2010). TNF-α has various effects on the dermal fibroblast. The cytokine inhibits both the differentiation of dermal fibroblasts to myofibroblasts, which is important to contract the wound. TGF-β1 via the Smad signalling pathway both *in vitro* and *in vivo* induces the expression of the α-SMA gene (Goldberg *et al.,* 2007), and the production of ECM proteins such as fibronectin and type I collagen. In addition, TNF-α stimulates MMP (MMP-1,-2,-3,-9 and -13) expression, all these effects may lead to non-healed and chronic wounds (Arancibia *et al.,* 2013; Zhu *et al.,* 2016). The *in vitro* effects of TNF-α are time, and dose-dependent; low concentrations of TNF-α promote remodelling/healing of injured and senescent tissue indirectly by stimulating inflammation and increasing the number of macrophages, which release growth factors that stimulate fibroblast proliferation (Tracey and Cerami, 1990; Barrientos *et al.,* 2008). However, high concentrations of TNF-α (for a long time) can induce apoptosis of dermal fibroblasts and have a deleterious effect on healing (Petrache *et al.,* 2000; Ruckert *et al.,* 2000).

1-10: Cytokines

Cytokines are small molecular weight proteins of 5-20 kDa, which are also called soluble factors (Gulati *et al.,* 2016). Cytokines have a crucial regulatory role in mediating the inflammatory and immunity responses (Cameron and Kelvin, 2003; Dinarello, 2007; Holdsworth and Gan, 2015), as well as mediating the reactions of a number of biological processes such as: immune cell proliferation and differentiation, haematopoiesis, inflammation, angiogenesis, tumorigenesis, infections, and induction of wound healing, through specific receptors (Behm *et al.,* 2011; Gulati *et al.,* 2016; Mclnnes, 2017). Thus, they can be used as diagnostic markers for health or disease, and as therapeutic agents (Dinarello, 2007; Holdsworth and Gan, 2015). Cytokines are secreted from a range of different cells, in particular immune cells, but also other cells such as fibroblasts, endothelial cells and stromal cells (Chung, 2009, Turner *et al.,* 2014). They include many families; the chemokine family, the interferons, the interleukins, the lymphokins, lymphocyte growth factors, the tumour necrosis factor family and adipokines (Zhang and An, 2007; Bishara, 2012; Feng *et al.,* 2016). Cytokines can act in a number of ways; on the cells that secreted them (autocrine), or modulate the neighbouring cells (paracrine), and also action on long distance cells (endocrine) (Mirza and Koh, 2015; Feng *et al.,* 2016). They can be classified depending on their function, or the secreted cells, or the target of action and/or biological chemical structure (Anaya *et al.,* 2013; Gulati *et al.,* 2016).

As mentioned before, wound healing is a complex process, which involves the coordination of different types of immune and skin cells. Their migration, infiltration, recruitment, proliferation and differentiation at the site of the wound

are controlled and regulated by a complex signalling/interaction network, which involves a large number of intracellular messengers including several cytokines, chemokines and growth factors, in overlapping wound healing process phases, as described in section 1-5-2. Dysregulation in the expression or function of these molecules will alter or delay the normal healing process (Behm *et al.,* 2011).

The inflammatory phase is mediated by multiple growth factors and cytokines and/or chemokines. The role of the chemokines are to recruit leukocytes, macrophages and inflammatory cells via migration and proliferation to the wound site as sources for growth factors, and to remove the debris, as well as to provide host defence, for example CX3CL1, which induces macrophages recruitment (Ishida *et al.,* 2006; Ishida *et al.,* 2008; Behm *et al.,* 2011). Also, in this stage, there is an increase in the expression of pro-inflammatory genes such as IL-1α, IL-1β, IL-6, and TNF-α, which induce inflammation (Mohd *et al.,* 2012; Satish, 2015; Ligi *et al.,* 2016a). High levels of IL-1 or IL-6 were detected in chronic wounds, which are considered as markers for these wounds, and their prolonged inflammatory phase (Chin *et al.,* 2005; Satish, 2015). In the proliferative phase, there is a switch to collagen synthesis and granulation tissue formation, via releasing IL-4, which acts as anti-inflammatory cytokine and triggers the production of precursor collagen proteins. IL-4 also induces the expression of IL-27, which inhibits the production of pro-inflammatory cytokines. In addition, the IL-6 mediates the infiltration of immune cells indirectly, collagen deposition, angiogenesis, and keratinocyte proliferation via inducing growth factors. In the remodelling phase. Some interleukins are considered to act as regulators of MMPs, e.g. MMP-3 induction via IL-22 would have a positive

impact on epidermal skin cell division (Yates *et al.,* 2007; McCarty and Percival, 2013), similarly, IL-1 inhibits TIMP-1 and induces MMP-1,-3 and -9 in fibroblasts. And for chemokines, both CXCL10 and CXCL11 mediate pathways that initiate the remodelling phase after mediating the end of the proliferative phase, via CXCR3 receptor (Satish, 2015).

1-11: Matrix Metalloproteinases MMPs

Matrix metalloproteinases MMPs are a family of zinc (Zn^{2+}) -dependent endopeptidases, 23 genes have been identified for the members of this family in humans, these members can be found as soluble or membrane-type (Itoh, 2015). MMPs have a specific degrading ability for extracellular matrix ECM proteins, the major component of the dermis. They are involved in many biological functions such as; development, morphogenesis, regeneration and wound healing, facilitate cell migration or invasion, and skin aging (Gohel *et al.,* 2008; Gawronska-kozak, 2011; Frankowski *et al*., 2013; Chen *et al.,* 2013). These proteolytic enzymes are also considered as inflammation markers in inflammatory diseases and inflamed chronic non-healing wounds (Weckroth *et al.,* 1996, Han *et al.,* 2001a, b). Due to their proteinase actions they are presented in all wound healing phases; i.e. influx of immune cells, keratinocyte and dermal fibroblast migration, angiogenesis and ECM remodelling (Gill and Park, 2008; Gawronska-kozak, 2011). Importantly, the expression of MMPs in the remodelling phase without balancing by their inhibitors (TIMPs) that neutralise their impact will lead to non-healing wounds.

Thus, an imbalance between MMPs and TIMPs leads to impaired wound healing and chronicity (Diegelmann and Evans, 2004; Gawronska-kozak, 2011; reviewed by Xu *et al.,* 2013).

The MMP family include the collagenases (MMP-1, -8, -13), which cleave unique sites of collagen types I, II, III and V; the gelatinases (MMP-2, -9) which degrade gelatin; the stromelysins (MMP-3, -10 and -11) that degrade a range of ECM molecules, macrophage metalloelastases (MMP-12), and membrane-type MT-MMPs e.g. (MMP-14) (Gawronska-kozak, 2011; Frankowski *et al*., 2013). In addition, MMPs mediate the release of growth factors and pro-inflammatory molecules such as FGFs, IGF, TGF-β and TNF-α. MMPs are structured from a single peptide, a pro-peptide, a catalytic domain (has a zinc binding site), and hemopexin-like C-terminal domain (Chen *et al.,* 2013) (Fig 1-11).

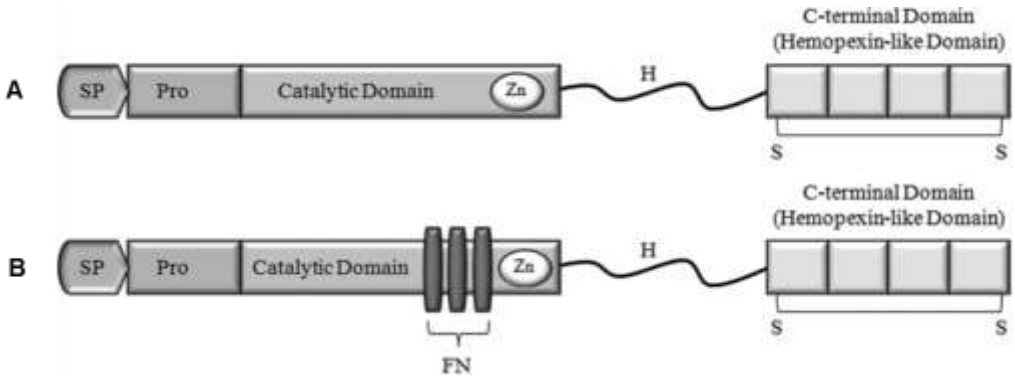


Fig 1-11: MMPs simple structure.

(A) MMPs (collagenases and stromelysins) contain SP, Pro, catalytic and hemopexin domains, (B) Gelatinases have a similar structure with additional FN domains (SP: single peptide, Pro: propeptide, catalytic domain has zinc binding site hemopexin-like c-terminal domain (made up 4 repeated units binding by s-s bridge and inserted FN: 3 fibronectin type II repeats. The hinge region H links hemopexin-like C-terminal domain and catalytic domain), (McCarty and Percival, 2013).

The gelatinolytic enzymes (MMP-2, -9) or gelatinase A and gelatinase B are two important members which are comprised of fibronectin-like domains for collagen binding, and both can cleave type IV and/or types I, V, VII, XI collagen and laminin as well as gelatin (Han *et al.,* 2001a; Frankowski *et al*., 2013). Both have a functional role in the wound healing process because of their ability in remodelling the basement membrane, and this feature controls cell migration and proliferation during both metastasis in cancer and in wound healing (Han *et al.,* 2001a; Gohel *et al.,* 2008). MMP-2 and MMP-9 are secreted into the tissues in the form of pro-MMP-2 and pro-MMP-9 (inactivated forms) and their conversion to the active form is controlled by removing the N-terminal prodomain by autocatalytic cleavage (Han *et al.,* 2001a; Frankowski *et al*., 2013). Elevated levels of MMP-3, MMP-1 and MMP-7 activate pro-MMP-9, while elevated levels of MMP-14 and MMP-16 activate pro-MMP-2 (Murphy *et al*., 1999; Morrison *et al*., 2001).

1-12: TIMPs- tissue inhibitors of matrix metalloproteinase

MMP activity can be regulated/inhibited by four inhibitors, named tissue inhibitors of metalloproteinases (TIMPs), 21–34 kDa proteins in size, which are endogenously expressed (Lambert *et al.,* 2004; Brew and Nagase, 2010; Moore and Crocker, 2012). They are secreted by variety of cells including fibroblasts, epithelial and endothelial cells, smooth muscle cells and many tumour cells (Shapiro, 2009; Ulrich *et al.,* 2010), and in human skin by dermal fibroblasts and keratinocytes (Lopez-Lopez *et al.,* 2014). TIMPs can inhibit MMPs via tight, non-covalent binding with 1:1 stoichiometry (Ulrich *et al.,* 2010; Giannandrea

and Parks, 2014). TIMP-1, TIMP-2, and TIMP-4 are secreted proteins (non-bound), whereas TIMP-3 is bound to the extracellular matrix to promote MMP inhibition (Arpino *et al.,* 2015). Interestingly, TIMP-1 can strongly inhibit a range of MMPs, but has a low affinity for the membrane-type MMPs (MT) - MMPs, which is different to others (Brew and Nagase, 2010; Löffek *et al.,* 2011).

TIMPs are considered to play a key role in ECM homeostasis, and in the progression of wound healing. The imbalance between MMP and TIMP activity would cause a chronic wound, and excessive MMP activity leads to enhanced degradation of ECM, essential growth factors and cytokines causing persistent inflammation in chronic wounds (Moore and Crocker, 2012; Lopez- Lopez *et al.,* 2014; Arpino *et al.,* 2015). Furthermore, in fibroblasts, TIMP-2 has to form a complex relationship with MMP-2 to be secreted. As TIMP-2 can inhibit MMP-2, it also can dock proMMP-2 to the cell membrane, where the enzyme is activated by (MT) - MMPs (Shapiro, 2009). In diabetic wound studies, a decrease in TIMP-2 expression, while an increase in MMPs expression resulted in the failure of diabetic wounds to heal (Lobmann et *al.,* 2002; Lopez- Lopez *et al.,* 2014).

TIMP-3 controls the ECM remodelling during wound healing. In rodent skin studies, *Timp-3* knockout mice displayed abnormal collagen and fibronectin remodelling (Caley *et al.,* 2015). TIMP-4 has an important role in inflammatory diseases such as psoriasis. Psoriatic studies in human, showed that TIMP-4 is the most important inhibitor of psoriatic skin degeneration, which is increased by dermal MMP-2 inhibition (Sidhom *et al.,* 2015).

1-13: Skin ageing

Skin ageing is a chronological process that leads to structural and functional changes, and can be induced by both intrinsic (chronological) and extrinsic (environmental) factors. Aging skin is characterized by a reduction in thickness, alterations in biochemical properties and reduced collagen production, with increased fragmentation of dermal collagen fibrils that is a result of elevated MMP-1 activity, all of which slows wound healing (Percival, 2009; Fisher *et al.,* 2009; Quan*. et al.,* 2012). This reduction is also associated with disorganization of type I collagen, a decline in collagen I production resulting in impaired function, an increase in type III collagen, an increase in skin laxity, a decrease in HA content, and reduction in elastin, which leads to prominent wrinkles (Montagna and Carlisle, 1979; Percival, 2009; Baumann *et al.,* 2009; Reviewed by Kammeyer and Luiten, 2015). These aging signs arise from the changes in ECM properties that leads to a defective remodelling phase, impairing or delaying wound closure (Coppe *et al.,* 2010). In addition, the proliferative ability of keratinocytes and fibroblasts become decreased. A decline in the rate of keratinocyte proliferation leads to epidermal atrophy (Gilchrest, 1983), while fibroblasts appear senescent and the proportion of senescent fibroblasts increases with age (Frants *et al.,* 2010). Senescent fibroblasts have a reduced migratory capacity and are unresponsive to stimulatory growth factor signals, so they are unable to replicate. They also have increased MMP expression, while TIMP expression is reduced (reviewed by Kammeyer and Luiten, 2015). In addition, senescent fibroblasts result in high levels of fibronectin in the ECM due to an increase in the rate of synthesis (Kondo and Yonezawa, 1992; Coppe *et al.,* 2010), and an alteration in the physical nature of the fibronectin causes a

decrease in cell adhesive properties, so generally the ability of fibroblasts to produce ECM proteins is impaired in older people (Ashcroft *et al.,* 1995).

Studies on aging associated with murine wound healing have shown that after wounding there is a delay in the formation of granulation tissue in older mice due to the decrease in fibroblast numbers and collagen density (Kligman and Lavker, 1998). There is also an association between skin aging and the delay in macrophage infiltration, angiogenesis and re-epithelialisation.

In humans, TGF-β1 plays a key role in wound healing *in vivo* (Al-Mulla *et al.,* 2011). TGF-β1 expression is decreased in the wounds of elderly females compared with younger females (Ashcroft *et al.,* 1997). Interestingly, TGF-β is a major growth factor involved in collagen synthesis by activation of fibroblasts to produce collagen (Quan *et al.,* 2012).

1-13-1: Senescent cells

The first description for cellular senescence was in 1965 by Hayflick, as a genetic program that limits the proliferation of normal human cells (Campisi and d'Adda di, 2007; Daniel *et al.,* 2015). Cellular senescence is now linked to tumour suppression and aging (cellular senescence refers to irreversible growth arrest which means the loss of proliferative ability) (Hayflick and Moorhead, 1961; Campisi and d'Adda di, 2007; Rodier and Campisi, 2011). Cellular senescence contributes to aging since senescence associated phenotypes can participate in both tumour progression and inhibit normal tissue repair (Rodier and Campisi, 2011). *In vitro*, cultured dermal fibroblasts can be induced into

senescent cells by replicative senescence (long-term passaging), or stress induced premature senescence, or by causing nuclear DNA damage using ionizing radiation (Coppe *et al.,* 2008; Coppe *et al.,* 2011; Daniel *et al.,* 2015).

Senescent cells can be recognized by their enlargement and flattened shape and expression of senescence- associated β-galactosidase (SA-β-Gal) activity (Dimri *et al.,* 1995; Quan *et al.,* 2012; Rock *et al.,* 2015). Other properties of senescent cells include irreversible growth arrest; enhanced heterochromatinization (Kreiling *et al.,* 2011); they have an altered secretory phenotype, with robust secretion of numerous growth factors such as insulin-like growth factor binding protein-6 (IGFBP6), interleukins (IL- 4, IL-8 and IL-1β), cytokines (TNF-α), and proteases (MMP-1, MMP-3 and MMP-10). In addition to other senescence–associated secretory phenotype (SASP). Furthermore, SASP proteins such as these described above in some studies may facilitate wound healing, participate in biological processes and contribute to aging (Rodier and Campisi, 2011; Daniel *et al.,* 2015; Ohgo *et al.,* 2015). Interestingly, senescent cells remain viable and metabolically active, along with an increased resistance for apoptotic death (Dimri *et al.,* 1995).

A study by Röck *et al* (2015) found some microRNAs (miRNAs) which are noncoding RNAs, have a functional role in regulating skin cell senescence *in vitro* and dermal aging via binding to HAS2 (membrane enzyme hyaluronic acid synthase 2, which mediates HA synthesis). This results in reduced HAS2, which leads to induction of senescence markers and inhibition of proliferation (Mancini *et al.,* 2012).

1-14: Skin alterations with aging

Scientific studies of skin aging have focused on the dermis as the most affected part of the skin by aging alterations (Quan *et al.,* 2015). The dermis becomes damaged, thin and the collagen fibrils are thinning, reduced in their organization and increased fragmentation (Quan *et al.,* 2012). Moreover, alterations in collagen fibrils will impair the structural integrity of the skin and its functional properties, resulting in increased skin fragility (Quan *et al.,* 2012). Furthermore, age- dependent alterations in the dermal ECM create a microenvironment that facilitates the formation and growth of epithelial cancer (Kudravi and Reed, 2000; Bissell *et al.,* 2005).

Both impaired function and migration of dermal fibroblasts can have a significant influence on skin aging (Quan *et al.,* 2010; Quan *et al.,* 2015).

Fibroblasts exhibit reduced spreading and contact with collagen fibrils, causing the loss of their typical morphology and have a collapsed appearance (Varani *et al.,* 2004; Fisher *et al.,* 2008; Qin *et al.,* 2014). The reduction of fibroblast spreading has two effects; it up-regulates MMP-1 expression through the activation of the transcription factor (AP-1) which controls MMP-1 expression and causes collagen fragmentation, and secondly causes a down-regulation of collagen production through TGF-β inhibition, which is the major factor in collagen synthesis and other ECM protein production (Varga *et al.,* 1987; Quan *et al.,* 2010). Furthermore, these alterations expand to the aged epidermis at the intersection of the epidermis and dermis, which is named the dermal-epidermal junction (DEJ), which becomes more flattened with age (Percival, 2009; Baumann *et al.,* 2009).

1-15: Sirtuins

Silent regulation information 2 (SIR 2) was the first sirtuin discovered to be responsible for lifespan extension in yeast (reviewed by Li and Kazgan, 2011; Hubbard and Sinclair, 2013; Ma *et al.,* 2014). Sirtuin enzymes function by oxidizing their NAD^+ (reviewed by Kelly, 2010a), they can regulate chromatin or genes; respond to epigenetic changes, which are heritable changes in gene expression without any modulations in the DNA sequence, and these changes have pivotal roles in skin growth and homeostasis, including wound healing (Perdigoto et al., 2012; Plikus et al., 2015; Zhang and Duan, 2015; reviewed by Saldanha et al., 2016).

Also, sirtuins are effective in an organism's response to stress and toxicity. Therefore, they contribute in lifespan/longevity, obesity/cellular metabolism, inflammation, heart disease, cancer, neurological activity and aging (reviewed by Kelly, 2010b; Hubbard and Sinclair, 2013). Sirtuin substrates and functions are summarized in Table 1-4.

Histone acetylation can be explained as adding the acetyl group from acetyl Co A onto the lysine residues in the histone tail. Two basic enzymes that control the acetylation are histone acetyltransferase (HAT) and histone deacetylases (HDACs), (Kiefer, 2007; Ruthenburg *et al.,* 2007; Arrowsmith *et al.,* 2012). Moreover, acetylation is considered as a dynamic balance between the two enzymes (Kiefer, 2007). In mammals, HATs can be found in 5 families, while HDACs can be divided into 4 basic classes, the class III HDACs include the nicotinamide adenine dinucleotide+-dependent (NAD+-dependent) sirtuins (Ma *et al.,* 2014). In mammals, there are seven sirtuin genes which express the Sirt1-7 enzymes. Sirt 1, 2, 6 and 7 are expressed primarily in the nucleus, Sirt 2

is localised in the cytoplasm, while the last group Sirt 3, 4 and 5 are located in the mitochondria (reviewed by Kelly, 2010a; reviewed by Li and Kazgan, 2011).

In humans, there is a strong relationship between sirtuins and wound healing. The pro-inflammatory cytokine IL-22 modulates human keratinocyte proliferation and differentiation, its effect can be controlled by Sirt1 through acetylation of the STAT3-dependent pathway, which inhibits the IL-22 inflammatory effects and regulates keratinocyte proliferation (Dal Farra and Domloge, 2006; Blander *et al.,* 2009; Sestito *et al.,* 2011). In mice, the lack of Sirt 6 in diabetic wounds is characterized by activation of the NF-$_k$B pathway activation, resulting in inflammation and impaired angiogenesis, caused by the decrease in VEGF expression and increase in oxidative stress (Thandavarayan *et al.,* 2015).

Sirtuin	Activity	Substrates	Functions
SIRT1	Deacetylase	P53, Foxo1, Foxo3, Bax, Hif-1α, Hif-2a,HSF1,Ku70,b-catenin,E2F1, Myc, STAT3,PGC-1a, NF-κB, TORC2,LXR,FXR,SREBP, PER2,CLOCK	Energy metabolism, stress response
SIRT2	Deacetylase	Tubulin,H4,Foxo3a	Cell cycle, tumour suppressor
SIRT3	Deacetylase	Oxidative phosphorylation complex I, AceCS2, LCAD, HMG-CoAsynthase2,IDH2, MnSOD,SOD2	ATP production, anti-oxidative stress, thermogenesis
SIRT4	ADP-ribosyl-transferase	GDH	Insulin secretion, fatty acid oxidation
SIRT5	Deacetylase	CPS1	Urea cycle
SIRT6	Deacetylase, ADP-ribosyl-transferase	H3K9, H3K56, CtIP, SIRT6	DNA repair, metabolism, inflammation
SIRT7	Deacetylase	P53	rDNA transcription

Table 1-4: Sirtuins of mammals
The mammalian sirtuins 1-7, activity, their substrates and sirtuin functions (reviewed by Li and Kazgan, 2011). p53: tumour suppressor protein 53; Foxo: forkhead box O; Bax: Bcl2 associated X protein; Hif: hypoxia-inducible factor; HSF1: heat shock factor 1; STAT3: signal transducer and activator of transcription 3; PGC-1α: peroxisome proliferator-activated receptor gamma coactivator 1α: TORC2/CRTC2: a transcriptional coactivator for the transcription factor CREB; LXR: liver X receptor; FXR: farnesoid X receptor; SREBP: sterol regulatory element binding protein; PER2: period circadian protein homolog 2; H4: histone 4; AceCS2: acetyl-CoA synthetase 2; LCAD: long-chain acyl CoA dehydrogenase; HMG-CoA synthase 2: 3-hydroxy-3-methylglutaryl CoA synthase 2; IDH2: isocitrate dehydrogenase 2; MnSOD: Mn-superoxide dismutase; SOD2: superoxide dismutase 2; GDH: glutamate dehydrogenase; CPS1: carbamoyl phosphate synthetase 1; CtIP: C-terminal binding protein interacting protein; H3K9: histone 3 acetyl-lysine 9; H3K56: histone 3 acetyl-lysine 56; CtIP, C-terminal binding protein interacting protein.

1-16: Type 2 diabetes mellitus (T2DM)

Type 2 diabetes is a metabolic disorder, which is characterized by elevated glucose levels in the blood, and it is the most common type of diabetes (Leon and Maddox, 2015; reviewed by Tadic and Cuspidi, 2015). Type 2 diabetes can cause serious long-term health problems which include; eyes (vision loss and blindness), heart (cardiovascular diseases and stroke), chronic wounds, which fail to close, along with aging, (reviewed by Baltzis *et al.,* 2014; Mirza and Koh, 2015), and kidney failure (Amutha and Mohan, 2016). Moreover, neuropathy, microvascular disorders and angiopathy are all complications of diabetes and contribute to a 12-25% lifetime risk of developing diabetic ulcers, which are the main cause of limb amputations (Das *et al.,* 2016). Type 2 diabetes can be caused by a defect in insulin hormone secretion, which is normally produced by the beta cells in the pancreas, and is responsible for controlling and regulating the amount of glucose in the blood. It can be associated with obesity, or due to having a family history (can be inherited) of type 2 diabetes (Kaku, 2010; reviewed by Wu *et al.,* 2014).

The main difference between diabetes type 1 and 2 is that, type one is insulin-dependent diabetes mellitus, while type 2 is influenced by the environment, or due to insulin deficiency (low production levels), or insulin resistance (Kaku, 2010; De Lade *et al.,* 2016), e.g. the target tissue of multiple organs is resistant to the action of insulin (Alves *et al.,* 2008). Insulin resistance is accompanied by persistent hyperglycemia- very high levels of circulating glucose, with pancreatic β-cell dysfunction. The glucose is not used as fuel for energy and remains in the circulation, causing an attenuation in protein, lipid and carbohydrate metabolism, which is a classical feature of type 2 diabetes (Kaku, 2010; Saini,

2010). The molecular explanation for insulin resistance phenomena is that an attenuation in the normal insulin activation signalling pathway IRS-1/PI3K/Akt, which is responsible for the regulation of glucose transport in response to insulin, leads to insulin resistance (Choi and Kim, 2010).

Patients with type 2 diabetes suffer from impaired wound healing and persistent inflammation, micro- and macro-circulatory dysfunction, hypoxia, and impaired neuropeptide signalling. Diabetic chronic wounds manifested with impaired angiogenesis, increased MMP levels, increased levels of pro-inflammatory cytokines, hyperglycemia, which leads to glycation of collagen, plasma, and other proteins. Protein glycation forms the advanced glycation end products (AGEs). All of which reduce the solubility of the ECM and most inflammatory alterations, resulting in impaired wound closure (Argyropoulos *et al.,* 2016; Feng *et al.,* 2016). Interestingly, in human, AGEs participate in the pathogenesis of diabetic complications and other diseases such as; rheumatoid arthritis, osteoporosis and even aging (Singh *et al.,* 2014). Some studies have shown that, collagen glycation can increase the formation and migration of myofibroblasts, resulting in the development of fibrosis in diabetes (Yuen *et al.,* 2010; Singh *et al.,* 2014).

Aims:

While the role of dermal fibroblasts in the production of the ECM has long been recognised, additional roles of the dermal fibroblasts in inflammation, particularly during cutaneous wound healing, are only now starting to emerge.

The hypothesis of this study is that the dermal fibroblast acts as a gatekeeper of the inflammatory response in human skin during wound healing with a central role in regulating acute and chronic inflammation, fibrosis, and the resolution of inflammation.

Since chronic inflammation leads to phenotypic changes detrimental to wound repair, primary human dermal fibroblasts will be cultured in the presence of the pro-inflammatory cytokine TNF-α to mimic a pro-inflammatory chronic wound environment.

The first aim of this study is to determine functional changes in dermal fibroblasts, e.g. proliferation, metabolic activity and migration in the presence of different concentrations of TNF-α, and for different lengths of exposure.

Human dermal fibroblasts are an extremely heterogeneous population arising from two different lineages with different phenotypes depending upon anatomical location, their position in the dermis, whether they are from hair-bearing skin, and the age and sex of the donor. Therefore, primary dermal fibroblasts will be cultured from the skin of women of a similar age, from a similar anatomical site to reduce heterogeneity.

The next aim of this study is to identify the secretory phenotype of dermal fibroblasts in response to inflammatory cytokines, such as TNF-α. The senescent phenotype induces an irreversible arrest of proliferation, but the fibroblast is still metabolically active, with an altered senescent associated secretory phenotype (SASP). An increase in the percentage of senescent fibroblasts in response to TNF-α will be measured by the expression of β-galactosidase, and the culture media will be collected for further analysis.

In order to analyse changes in the secretory phenotype of the dermal fibroblasts, this study will measure the changes in the secretion of the gelatinases, MMP-2 and MMP-9 by zymography for healthy donors and diabetic patients, to determine whether they are induced by the pro-inflammatory cytokine TNF-α.

In order to gain a better understanding of the molecular differences that may discriminate fibroblasts in chronically, inflamed wounds from these in acute wounds, dermal fibroblasts from healthy donors and diabetic patients will be cultured in presence of different concentrations of TNF-α and changes in the expression of extracellular matrix and senescence genes, such as (MMP-1, MMP-2, MMP-9, TIMP-1, TIMP-2, Sirt1 and Sirt6), will be quantitated by real-time PCR.

The final aim of this study is to establish whether dermal fibroblasts exposed to a chronic inflammatory environment will demonstrate changes in their secretion profile of cytokines that may help to identify markers for new therapeutic targets to improve wound healing, especially in diabetic patients.

2 Materials and Methods

2-1: Cell culture

Primary human dermal fibroblasts were obtained from previously established cells frozen in liquid nitrogen. The original skin samples were obtained from Ethical Tissue Bank from female cosmetic surgery (facelift) skin, which were established as described in (Kamala, 2014). Cells used in all experiments are listed in Table 2-1.

Donor	Age	Gender	Passage No	Scalp skin	Facial skin	Techniques
OK15	36	F	P3		✓	Migration, Gelatin zymography, RT-PCR
AH557	45	F	P5, P8		✓	Alamar blue, CyQUANT
OK13	52	F	P6, P7	✓	✓	DF proliferation, Migration assay recipient, Migration assay CM donor
C513	55	F	P10, P11 P13,P17		✓	Gelatin zymography, Senescence β-gal assay
OK18	56	F	P3, P4,P5	✓	✓	Migration, Gelatin zymography RT-PCR, Human cytokine array, DF proliferation
AH 554	57	F	P3, P4,P7,P8		✓	Migration, Gelatin zymography RT-PCR Alamar blue, CyQUANT
JT555	60	F	P3, P5,P8		✓	Migration, Gelatin zymography RT-PCR Alamar blue, CyQUANT
RB532	60	F	P8		✓	Migration assay CM donor
OK21	64	F	P3 P8	✓	✓	DF proliferation, Migration assay CM donor
AH558	67	F	P3, P4,P7		✓	Migration, Gelatin zymography RT-PCR Alamar blue, CyQUANT
C495	77	F	P6		✓	Comparison of 2% and 10% growth media

Table 2-1: Healthy dermal fibroblasts donor details. Age, gender, passage, skin source and assays used for all healthy dermal fibroblast donors. Note that phenotype varies with passage number so different techniques are used for their characterisation.

2-2: Diabetic cell culture

Diabetic primary human dermal fibroblasts were obtained from diabetic patient's leg skin following amputation. The consent from diabetic patients and ethical approval were obtained. Cells used in all experiments are listed in Table 2-2.

Donor	Age	Gender	Passage No	(Leg skin -type2 diabetes)	Techniques
10541	52	F	P3	✓	Migration assay, Gelatin zymography, RT-PCR
10209	65	M	P3	✓	Migration assay, Gelatin zymography, RT-PCR, Human cytokine array,
10539	65	F	P3	✓	Migration assay, Gelatin zymography, RT-PCR, Human cytokine array,
10206	66	M	P3	✓	Migration assay, Gelatin zymography, RT-PCR

Table 2-2: Type 2 diabetic mellitus (T2DM) dermal fibroblasts patient details. Age, gender, passage, skin source and assays used for all diabetic dermal fibroblast patients. All lines were used for other diabetic experiments, for individual details, see relevant chapters.

2-2-1: Isolation of primary diabetic dermal fibroblasts

After obtaining the skin samples from diabetic patients (type 2 diabetes mellitus), the fat layer was removed and discarded using a small forceps and scissor till reaching the dermis layer which appeared white. Then the skin sample was cut to small pieces, 1cm^2 for each using forceps and scalpel. After that, the pieces were washed and sterilised twice in phosphate buffer saline (PBS) containing 1% penicillin/streptomycin (100U/ml and 100µg/ml) (Gibco) and 1% of amphotericin B (250µl/ml) (Gibco) in Falcon tubes (50ml). These pieces were transferred into 50ml Falcon tubes or Petri dishes which contain dispase II (protease) solution (Sigma-Aldrich, UK) (100mg of dispase II in 100ml PBS) in fridge for one overnight at 4˚C- to facilitate separating the epidermis from dermis. Next day, by using two forceps and 100 mm Petri dishes, the epidermis were pulled out from the dermis and the pieces were washed in tubes containing normal growth media for 2-3 times to remove dispase II traces. Quickly, the pieces were moved to T75 culture flasks contain 12ml of growth media and incubated at 37˚C in 5% CO_2, for 4 weeks with changing the media each 1-2 days till the dermal fibroblasts started emerging from the dermis, attaching to the flask and proliferating. After, the attached dermal fibroblasts would be ready for passaging and experiments purposes (Fig 2-1).

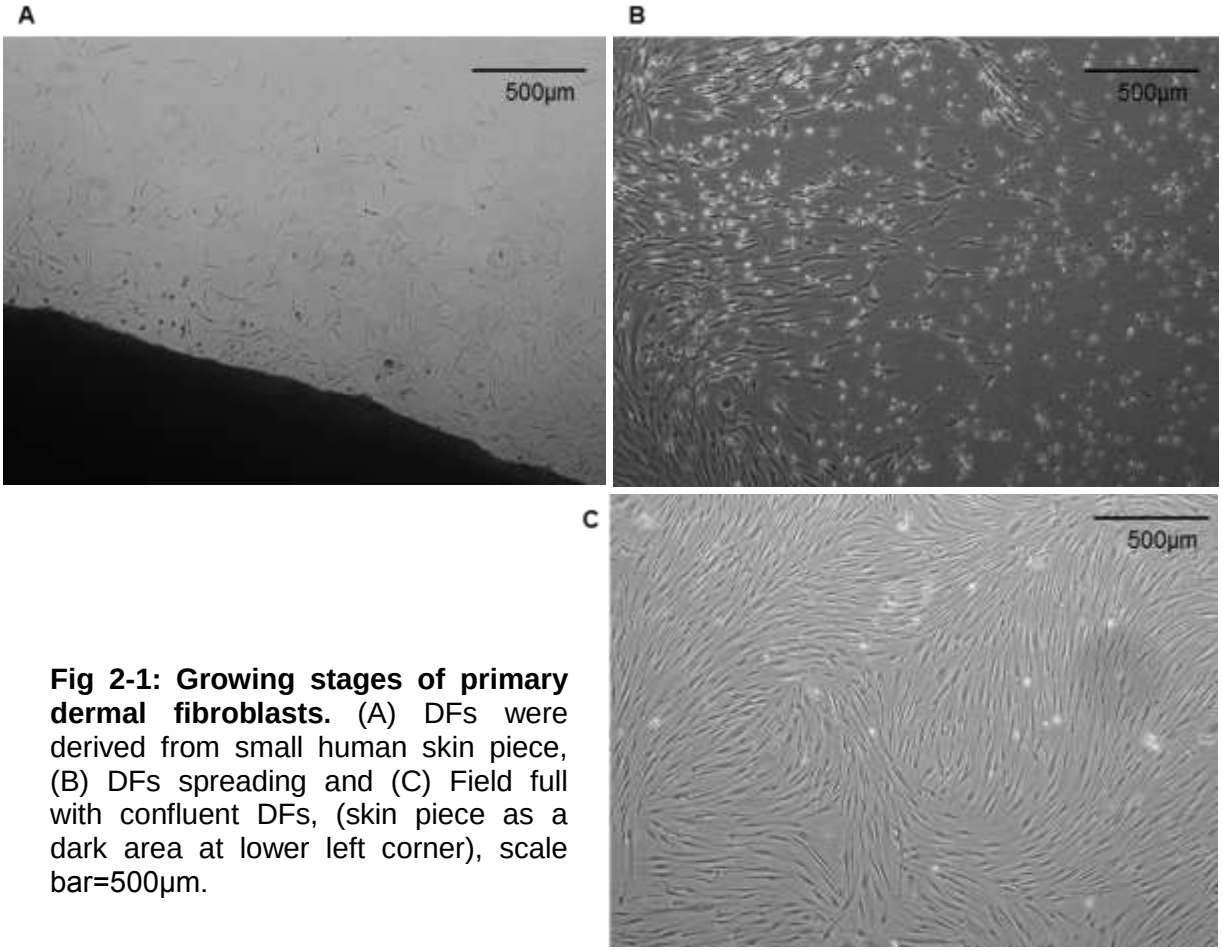


Fig 2-1: Growing stages of primary dermal fibroblasts. (A) DFs were derived from small human skin piece, (B) DFs spreading and (C) Field full with confluent DFs, (skin piece as a dark area at lower left corner), scale bar=500µm.

2-3: Tumour necrosis factor 2.5, 25 and 250 (ng/ml) concentrations

The physiological levels of TNF-α in the human differ with the different tissues that in which TNF-α presents, such as blood serum or plasma, human tissue samples such as skin, wounds and wound fluids, and also differ with different health conditions, such as chronic inflammation, sepsis, and diabetes. The normal circulating levels of TNF-α is (11.2 ± 7.3 pq/ml) (Arican *et al.,* 2005), while in human normal skin, TNF-α levels are (1470.86 ± 3186.6 pg/g) (Grellner *et al.,* 2000). Endo (1993) has shown, that TNF-α plasma levels range from 109 - 358 pq/ml in burns patients, which results from bacterial infection/septicaemia. Interestingly, the experimental concentration of TNF-α that induces the maximal response is (10 ng/ml) greatly exceeding levels in normal human plasma and acute-sepsis patient's serum, indeed a serum concentration of (5 ng/ml) was not found in patients with acute sepsis (Turner *et al.,* 2010). In heart inflammation, the concentration of (15-30 ng/ml) induced maximal inflammatory response (Lin *et al.,* 2015). In diabetes, the serum TNF-α level was (94.28 ± 5.01 pg/mL), and urinary TNF-α level was (7.12 ± 8.09 pg/mg) (Lampropoulou *et al.,* 2014), with a correlation between TNF-α serum levels and diabetes duration (Danielson *et al.,* 2016).

Present study concentrations, 2.5 and/or 25 ng/ml are higher than the normal conditions of TNF-α and supra-physiological concentrations were chosen to simulate the inflammatory environment and to investigate any effects on dermal fibroblasts function and secretory phenotype, while 250 ng/ml is a supra-pathophysiology concentration and was used to investigate whether TNF-α has a concentration-dependent effects, and as a maximal stimulus.

2-4: Normal growth media

Dermal fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, UK) supplemented with 10% foetal bovine serum FBS, L-glutamine (10mM), penicillin/streptomycin (100U/ml and 100µg/ml) and amphotericin B (250µl/ml), cells were incubated at 37˚C in 5% CO_2 incubator (Thermo Scientific Heracell, UK) in T75 culture flasks (Sarstedt, UK), and the media was changed every 2-3 days.

2-5: Serum free assay media (serum free DMEM media)

In some experiments, cells were incubated in serum-free growth medium DMEM that contains all the necessary supplements as described in 2-4, but lacking the serum, at 37˚C in 5% CO_2 overnight. This medium was used to remove any traces of serum from previous treatment media.

2-6: Passaging of cells

Once cells had reached 80-90% confluence, they were passaged and transferred to a new T75 cm^2 culture flask or seeded into 6-well plates (Clear Sterile, UK). First, the growth medium was discarded and the cell monolayers were washed 2-3 times with 10ml PBS. After the PBS was discarded, the cells were trypsinized using 0.05% trypsin/EDTA (Gibco) in amounts of 3ml for T75 and 1.5 ml for T25 flasks, and incubated for 5-10 minutes at 37˚C in 5% CO_2. The cells were examined under the phase contrast microscope (x4 and x10) to

determine whether they were detaching. Then the trypsin was neutralized by adding growth medium (3.5-7ml) dependent on flask size, containing 10% FBS to inhibit the trypsin effects. The cells were centrifuged for 5 minutes at 1000 rpm, the supernatant was discarded and the cell pellet was re-suspended in 5-10 ml growth medium dependent on flask size containing 10% FBS. The cells were re-seeded into new culture flasks (Corning, UK), with a split ratio of 1:3. The cell suspension was transferred into a T75 flask and topped up with 10 ml growth medium; for T25 flasks the cell suspension was topped up with 5ml growth medium.

2-7: Freezing and thawing

Cells that were not required for immediate use were stored in liquid nitrogen. Cells were trypsinized, neutralized with 7ml 10% FBS growth medium and transferred into a 15ml Falcon tube, and centrifuged to form a pellet. The supernatant was discarded and the cell pellet re-suspended in 1ml freezing medium Cryo-SFM (PromoCell) and transferred into Cryo Pure tubes (Sarstedt, UK). The tubes were placed in liquid nitrogen vapour for 24 hours before transferring into liquid nitrogen until required.

When needed, the cells were thawed quickly after moving from liquid nitrogen by immersion in warm water, transferred immediately into a T75 flask to which 10 ml 10% FBS growth medium had been added. Cells were incubated at 37˚C in 5% CO_2 and after 24 hours, they were checked to confirm they had attached to the flask and the medium was changed.

2-8: Cell counting

The cells were trypsinized and neutralized with 10% FBS growth medium. The suspended cells were counted using a Neubauer haemocytometer (Hawksley, UK) slide. An aliquot (100µl) was mixed with 100µl trypan blue exclusion dye (Sigma-Aldrich, UK). Then, 10µl of this was added to each chamber of the haemocytometer-slide. The total number of cells in 1ml was calculated by counting the number of the cells in each of the four corner squares of the grid and calculating the mean. The final value was calculated by multiplying by 10^4 and the dilution factor (the cell suspension was mixed in 1:1 ratio with trypan blue dye, therefore the dilution factor was 2) to give cell/ml.

2-9: A comparison of dermal fibroblast proliferation in medium supplemented with 2% and 10% FBS

Dermal fibroblasts were seeded into 6-well plates, at a cell density of 20,000 cells per well. Triplicate wells were incubated in either 2ml of medium containing 2% FBS, or with 2ml of medium supplemented with 10% FBS (i.e. normal growth medium). The cells were incubated at 37˚C in 5% CO_2, and the media was replaced every two days. The number of cells per well was counted in duplicate for each well, as described in section 2-8, triplicate wells were counted every two days for a total of 12 days.

2-10: The effect of TNF-α on dermal fibroblast proliferation

2-10-1: Manual cell counting- trypan blue

Matched dermal fibroblasts derived from scalp and facial skin of the same donor were seeded at a density of 10,000 cells per well into 12-well plates (Corning Incorporated Costar, 3512, UK). Triplicate wells were incubated with either 1ml 10% FBS growth medium, or 1ml 10% FBS growth media containing either 2.5, 25 or 250 ng/ml of TNF-α (Sigma-Aldrich, UK) at 37˚C in 5% CO_2 for 72h. After 3 days, the media was replaced without TNF-α. The number of the cells in triplicate wells were counted every on days 3, 5, 7, 10, 12 and 14, and each well was counted in duplicate using the trypan blue exclusion dye, as described in section 2-8. Media on the remaining cells was also replaced at these time points.

2-10-2: CyQUANT assay (direct cell proliferation)

CyQUANT is a fluorescence-based proliferation microplate assay for determining the number of proliferating cells (Invitrogen, 35011, USA). The principle of the assay is a cell-permeant DNA-binding dye (component A) in combination with a background suppression reagent (component B). The nucleic acid stain is a masking dye that blocks the staining of dead cells, and also cells with compromised cell membranes, resulting only in the staining of healthy cells, by which the assay can measure cell proliferation accurately.

Component A is a green fluorescent nucleic acid stain with a feature of a live cell-permeable reagent that mainly concentrates in the nucleus of mammalian

cells, while component B is a background suppression dye and is impermeable in living cells and will suppress the green fluorescence, so both dyes together provide an assay based on both DNA content and membrane integrity.

To generate a standard curve (standard wells), fully confluent cells were trypsinized, neutralized with 10% FBS growth medium, and re-suspended in a medium. Using a Neubauer haemocytometer, one millilitre of cell suspension should contain 10^5-10^6 cells per ml (as recommended). Five serial dilutions were generated in the wells of microtiter plate from the one millilitre of cell suspension and growth media with cell number ranging from 1000-5000 in 100µl volume. A standard curve is in Appendix 8-2.

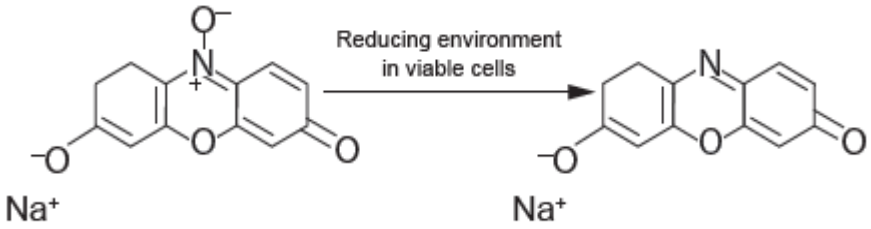
In the sample wells, cells were seeded in 96 well microtiter plates (Corning Incorporated Costar, UK) at cell density of 2,000 cells per well. Four replicate wells were used for standards, samples, and controls. Three microtiter plates were set up at the same time for analysis after day 3, 7 and 14. Prior to the assay, cells were incubated in 100µl of 10%FBS growth media with or without TNF-α at three different concentrations (2.5, 25 and 250 ng/ml) and incubated at 37˚C in 5% CO_2 for 72h. At day 3 the media was replaced with 100µl of 10% FBS growth media, and replenished with 10%FBS every three days.

According to the manufacturer's instructions 2X detection reagent was prepared by mixing 11.7ml of growth media, 48µl of component A and 240 µl of component B, then 100µl of 2X detection reagent was added into cell culture media for each well, and the cells were incubated for 60 minutes at 37˚C in 5% CO_2. Changes in fluorescence were measured using Infinite 200 PRO microplate reader (Tecan, Switzerland), with excitation at 508 nm and emission at 527 nm. A standard curve was performed and ranged from (1000-5000) cells,

to calculate the cells numbers (for converting fluorescence values into cell numbers) for days 3, 7 and 14.

2-11: TNF-α induces the metabolic activity of dermal fibroblasts at day 14

Cell metabolism was measured using the Alamar blue assay (colorimetric assay) (Invitrogen, USA), a reagent used to quantify cellular metabolic activity. This reagent contains the non-fluorescent blue coloured molecule resazurin (oxidised form), and its reduction by metabolically active cells will convert resazurin into resorufin (reduced form), which is a highly fluorescent pink coloured molecule.



Resazurin weakly fluorescent **Resorufin highly fluorescent**

Fig 2-2: Chemical structures of resazurin and resorufin. Resazurin is reduced to fluorescent resorufin by metabolically active cells.

Cells were seeded in 96-well microtiter plates at a cell density of 2000 cells per well. Under all conditions, the samples, controls and blanks were performed in four replicate wells. Three microtiter plates were set up at same time for measuring metabolic activity after day 3, 7 and 14. Prior to performing the Alamar blue assay, cells were incubated in 200µl of 10% FBS growth medium

with, or without, TNF-α at three different concentrations (2.5, 25 and 250 ng/ml) at 37˚C in 5% CO_2 for 72h. After 3 days the media was replaced with 100µl of 10% FBS growth media, which was replenished every 3 days. Ten microliters of the Alamar blue reagent was added to all the wells (100µl capacity) directly into the cell growth media, then the plates were wrapped in foil and cells were incubated at 37˚C in 5% CO_2 for 4 hours when the colour changed into pink. Directly, the absorbance was measured at 570nm using Infinite 200 PRO microplate reader (Tecan, Switzerland), and plots were drawn, the absorbance versus treatment concentrations.

2-12: Induction of senescence in cultured dermal fibroblasts

The senescence-associated β-galactosidase assay is a cytochemical assay that detects the expression of senescence-β-Galactosidase (β-Gal) at pH 6, as a marker of senescent cells; *in vitro* (Dimri *et al.,* 1995).

Dermal fibroblasts were seeded at a low density of 7000-7500 cells per well in 6-well plates and cultured in 10% FBS growth medium for 1-2 days. Cells were washed twice with 1ml PBS and incubated with 2.5, 25 or 250 ng/ml of TNF-α in 10% FBS growth medium, or 10% FBS growth medium, or serum free assay medium for 48h. Cells were washed once with 2ml PBS before fixing with 1x fixative solution (Cell Signaling Technology, USA).

According to the manufacturer's instructions, the 1x fixative solution was prepared by diluting 10x fixative solution with distilled water, and then 1ml was added to each well and left for 10-15 minutes at room temperature. The cells were washed twice with 2ml PBS to remove the fixative solution and incubated

with 1ml of the 1x β-galactosidase staining solution at pH 6 (Cell Signaling Technology, USA). The 1x β-galactosidase staining solution was prepared by diluting the 10x staining solution with distilled water and, one millilitre of 1x β-galactosidase staining solution contained; 930µl 1x staining solution, 50µl 20mg/ml X-gal stock solution in dimethylformamide (DMF) (Sigma-Aldrich, UK), 10µl solution A and 10µl solution B. The pH was adjusted to 6 with HCl. Immediately, the plate was sealed with parafilm to prevent the solution from evaporating and placed in CO_2-free incubator at 37 ˚C (Genlab, UK) for 24h. The cells were photographed under the light microscope for the development of a blue colour indicating senescent cells. For long- term storage, the staining solution was removed from each well and replaced with 1 ml 70% glycerol in H_2O (Sigma-Aldrich, UK), before the cells were stored at 4˚C. At least five images were taken radially for each well using an Olympus Nikon TS100 digital camera connected to the phase contrast microscope (Ernst Leitz Wetzlar GMBH, Germany), at x40 (eyepiece x10 and objective x4) magnification. The level of cellular senescence after 24h was quantitated by counting the number of blue cells and dividing by the total number of the cells and then multiplying by 100%.

2-13: Preparation of conditioned media to determine changes in MMP-2 and -9 secretion

Dermal fibroblasts were seeded into either 6-well plates, or 12.5 cm^2 flasks at a cell density of 50,000 cells per well. Cells were incubated with 1ml 10% FBS growth medium at 37˚C in 5% CO_2 and grown to confluence (90-100%). The cells were washed 2-3 times with 2ml PBS and were growth arrested by

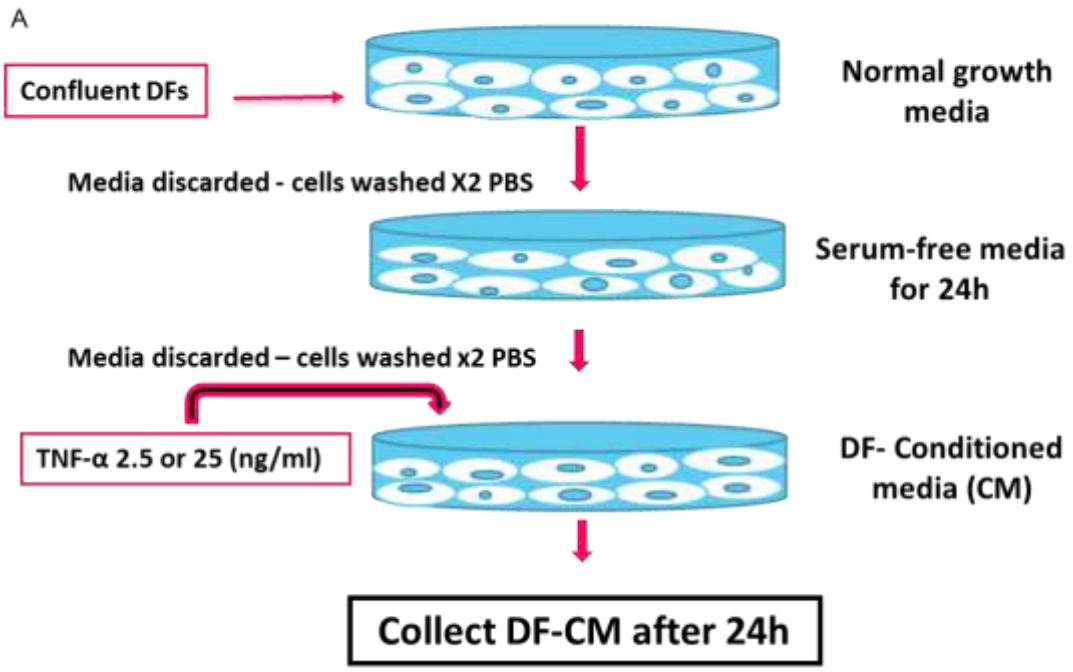
incubating in 2ml serum free media for 24h. Cells were washed twice with 2ml PBS and incubated with either 1ml 10% FBS growth medium containing 2.5 or 25 or 250 ng/ml of TNF-α, or with 1ml 100 nM phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich, UK) in 1% FBS medium, or with 1ml 10% FBS growth medium, or 1ml 1% FBS medium for 48h. The cell media was collected and centrifuged at 900 rpm for 6 minutes to remove the cell debris, then frozen at -80 ˚C until used in the zymography assays.

2-14: Conditioned media CM

Cells were cultured in 6-well plate at cell density of 50,000 cells per well, and incubated with 2ml 10% FBS growth medium at 37˚C in 5% CO_2 for 72h, till they were confluent (90-100%). The cells were washed twice with PBS, and incubated with serum free DMEM media for 24h. After that, cells were washed two times with PBS and treated/incubated with 1ml of TNF-α in two different concentrations 2.5 or 25 (ng/ml), or without TNF-α as control, the conditioned media was collected after 24h (Fig 2-3 A). The conditioned media CM was centrifuged at 1200 rpm for 5 minutes and stored at -80 ˚C until used for:

- Migration
- Zymography
- Cytokine profile array.

In some experiment conditions the conditioned media was added to another dermal fibroblasts (Fig 2-3 B).



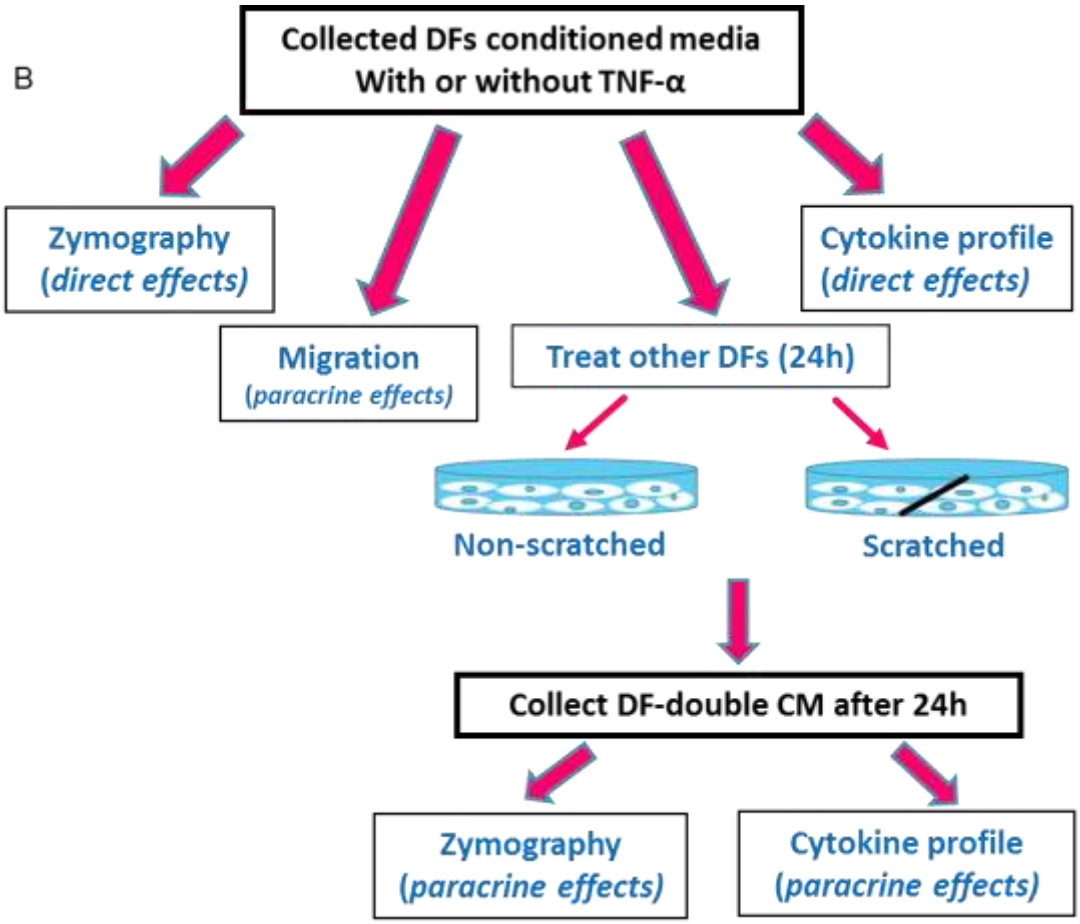


Fig 2-3: Preparation of conditioned media. (A) Confluent cultured DFs were washed with PBS and incubated with serum-free DMEM media for 24h, then cells were washed again, and incubated with or without TNF-α 2.5 or 25 ng/ml for 24h. After, DFs conditioned media were collected, and frozen (B) The collected CM was tested in the zymography, and human cytokine profile assays to measure the TNF-α effect and/or added to another recipient cells (scratched or non-scratched), collected again after 24h, and tested in migration, and the same assays above to measure the paracrine effect.

2-15: Scratched wound migration assay

Cells were seeded into 6-well plates, at cell density of 50,000 cells per well, and incubated in 2ml of 10% FBS growth medium for 72h till cells were fully confluent. At the same time parallel 6-well plates were set up that were treated identically, but not scratched. Prior to making the scratch, both scratched and non-scratched cells were serum-starved for 24h using serum free DMEM medium. For scratched cells, a linear scratch was made across the diameter of each well using the scratch wound device designed by Dr. Kamala (Kamala, 2014), which removes a fixed width (0.8 mm) of cells.

Both scratched and non-scratched monolayers were washed with 2ml PBS twice to remove cell debris. The effect of TNF-α was tested by adding two millilitre of 10% FBS of growth medium (control), or 2ml of 2.5 or 25 (ng/ml) of TNF-α prepared in 10% FBS growth medium, to triplicate wells. This effect was assessed for healthy and diabetic scratched and non-scratched dermal fibroblasts. For scratched (migrated) cells, the migration of the cells between the wound edges was determined at after 24h. The measurement of the cell migration (distance) between the wound edges was made from the images of the scratch wound taken using a digital camera (Olympus Nikon TS100, Japan) connected to the phase contrast microscope (Ernst Leitz Wetzlar GMBH, Germany) at the same six points in triplicate wells and the average was taken. All images were taken at x40 total magnification (eyepiece x10 and objective x4). The migration of the cells between the wound edges was measured at the same six points in each of triplicate wells for each treatment.

The average migratory distances at different time points were calculated, and the data statistically analysed using GraphPad Prism7. For non-scratched (non-migrated cells), they were incubated without imaging for 24h. After, the conditioned media (direct TNF-α conditioned media) were collected from both scratched and non-scratched cells and kept at -80˚C until requiring for zymography assay and the remaining cells were used for performing RT-PCR assay.

2-16: Soluble factors and indirect effect of TNF-α on the migration of dermal fibroblasts

The soluble factors and the indirect effect of TNF-α (conditioned TNF-α) were performed by adding 2ml of 10% FBS conditioned growth medium (control conditioned), or 2ml 10% FBS conditioned media prepared in the presence of 2.5 or 25 (ng/ml) TNF-α, or 2ml of conditioned growth medium with 2.5 or 25 (ng/ml) of TNF-α in 10% FBS growth medium (direct/fresh TNF-α), to triplicate wells for scratched and non-scratched healthy dermal fibroblasts. The doubled-conditioned media were collected from both scratched and non-scratched cells and kept at -80˚C until requiring for zymography assay and the remaining cells were used for performing RT-PCR assay.

2-17: Gelatin zymography

Gelatin zymography was used to quantitate the gelatinolytic MMP-2 (Gelatinase A) and MMP-9 (Gelatinase B) activity of the conditioned media from dermal fibroblasts, as described in section 2-13 and 2-14.

The conditioned media was diluted as appropriate (1:40) with 2x non-reducing sample buffer (Appendix 8-1). The electrophoresis gels were prepared with acrylamide gels containing a final concentration of 1.5 mg/ml gelatin (Sigma-Aldrich, UK). The separating gel contained 3.75ml of separating gel buffer (composed of 1.5M Tris base pH 8.8), 150µl 10% Sodium dodecyl sulphate (SDS), 3.7ml 30% acrylamide /0.8% bis-acrylamide mix, 75µl 10% ammonium persulfate (APS) (Sigma-Aldrich, UK) and 1.325ml distilled water, while the stacking gel contained 1.875ml stacking gel buffer (0.5M Tris base pH 6.8), 75µl 10% SDS, 0.975µl 30% acrylamide /0.8% bis-acrylamide mix, 37.5µl 10% APS and 4.58ml distilled water (see Appendix 8-1 for all buffer compositions). Tetramethylethylene diamine (TEMED) (Sigma- Aldrich, UK) was added to polymerize the gels; 30µl for the separating gel and 15µl for the stacking gel.

Twenty microliters of diluted conditioned medium was loaded into the gels and the electrophoresis was performed for 110 minutes at 120V in 350ml running buffer (Appendix 8-1). Then the gels were washed three times (each time 20 minutes in 80ml 5x washing buffer for two gels) with gentle agitation, using a shaker (Grant-Bio, UK) at room temperature. The gels were incubated with 1x incubation buffer (Appendix 8-1) to reactivate the gelatinases activity (100ml for each gel), for 24h in incubator at 37 ˚C -without CO_2 supplier (Genlab, UK). Finally, to visualize the enzymes as clear bands of lysis on a blue background, the gels were stained in Coomassie brilliant blue staining solution (Appendix 8-

1) (100ml to stain two gels) for 20 minutes with shaking at room temperature until the bands appeared. Images were taken using a UV/white light conversion screen-visualization system (Bio-Rad, UK) and analysed using Image J to calculate the percentage of MMP-2 and MMP-9 compared to controls.

2-17-1: Analysis of gelatin zymography

Stained gels images were taken using a UV/white light conversion screen-visualization system (Bio-Rad, UK). The images were analysed using the Image J programme. This programme converts the images to densitometry values (data) which were then converted to percentages. Bands were quantified using densitometry, which was representative of MMP activity and expressed relative to the control on each gel (Fig 2-4).

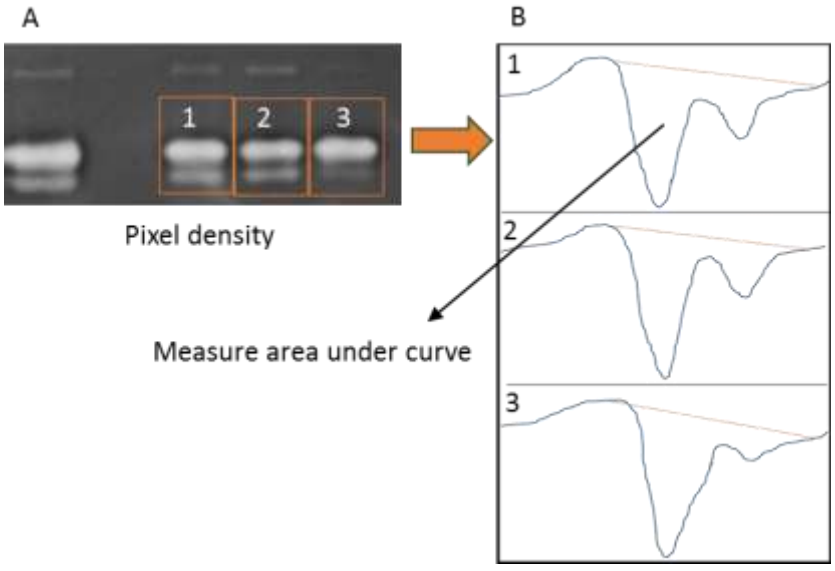


Fig 2-4: ImageJ analysis of zymograms. (A) Images of zymograms were opened in Image J, and identical lanes identified. (B) Densitometric analysis of identified bands.

2-18: Quantitative real time PCR (RT-qPCR)

RT-qPCR involves; total RNA extraction, cDNA synthesis and finally the real time PCR which amplifies and measures the amount of cDNA containing the gene of interest.

2-18-1: Total RNA extraction

Cells were seeded into 6-well plates at cell density of 50,000 cells per well and incubated in 10%FBS growth media at 37˚C of 5% CO_2 until fully confluent. Then cells were serum-starved for 24h using serum free DMEM medium. Some seeded cells were scratched linearly and some seeded cells were not (both cells were taken from migration assay section 2-15). Both scratched and non-scratched cells were washed with 2ml PBS twice to remove debris or scratched cells.

The total RNA was extracted from these treated Scratch and non-scratched cells using RNeasy mini kit (Qiagen, 74104, UK) silica column method. Cells were washed with PBS twice to remove treatment traces. According to manufacturer's instructions, cells were lysed by adding 250µl/well lysis buffer RLT and collected in micro-centrifuge tubes (on ice), then the tubes were vortexed for homogenization. A volume of 350µl of 70% ethanol was added for binding conditions (precipitates nucleic acids), and the solution was mixed by pipetting. The entire 700µl volume of the homogenised solution was transferred to spin RNeasy columns and centrifuged for 15 seconds at 12,000 rpm (this allow the RNA to bind to the column), the flow-through was discarded, and the

column was washed with 350µl RW1 washing buffer for 15 seconds at 12,000 rpm to remove any salts and proteins and the flow-through was discarded. A mixture of 10µl of DNase I in 70µl of RDD was added to the column and incubated at room temperature for 15 minutes to allow the degradation of any DNA that may have bound to the column. Again, the column was washed with 350µl RW1 washing buffer and centrifuged for 15 seconds at 12,000 rpm, and the flow-through was discarded.

Two washing steps were made, Buffer RPE of 500µl volume was added to the centre of the column and was centrifuged for 15 seconds at 12,000 rpm, the flow-through was discarded, and this step was repeated with extending the centrifuging time for two minutes, the flow-through was discarded. The RNeasy columns were transferred to a new collection tubes 2ml and were centrifuged to full speed of 12,000 rpm for one minute, then the RNeasy column was placed in a new collection tubes 1.5ml and 45µl RNase free water was added directly to the column. These tubes were centrifuged for 1minute at 12,000 rpm to elute the RNA. To get a high concentration of RNA, the last step was repeated (by re-using the eluted water back on the same column), then the RNA was stored at -80 ˚C in the same collection tube.

2-18-1-1: Quantification of total RNA extraction

The concentration of total RNA was quantified using NanoPhotometer P-330 (IMPLEN) in ng/µl. The Nanophotometer can measure the concentration and quality of RNA by reading the absorbance at 230, 260 and 280nm, so photometer was blanked by using RNase DNase free water (solvent) and the

RNA purity/quality was measured. Optical density ratios O.D. 260/ O. D. 280 ≥ 1.8 and O. D. 260/O.D. 230 >_2 were considered as pure. If the ratio is lower than 1.8 or 2, it gives an indication of contamination existence such as proteins or phenol red that can be absorbed at 280nm.

2-18-2: cDNA synthesis through reverse transcription

Reverse transcription of total RNA was performed to synthesis the cDNA from extracted RNA using the High-Capacity cDNA reverse transcription kit (AB Applied Biosystems, UK) according to the manufacturer's instructions (manual protocol). The 2x master mix components was prepared before preparing cDNA reaction tubes (Table 2-3). Fifteen microliters of master mix (RT) was added to 5µl of purified RNA sample per tube, giving a final volume of 20µl.

Component	Volume
10X Reverse Transcription Buffer	**2 µl**
25X dNTPs Mix (100mM)	**0.8 µl**
10X RT Random primers	**2 µl**
MultiScribe™ Reverse Transcriptase (RT), 50 U/µL	**1 µl**
RNase Inhibitor	**1 µl**
Nuclease-free H_2O	**8.2 µl**
Total amount	**15 µl**

Table 2-3: The 2X RT master mix components.

The thermocycler (Techne, USA) was programmed to heat the RNA to 25˚C for 10 minutes for annealing conditions; then to increase in temperature to 37 ˚C for 120 minutes to allow the reverse transcriptase enzyme to catalyse second strand synthesis, after the reaction temperature increased to 85˚C for 5 minutes for inactivate the reaction and then dropped to 4˚C to final hold. The yield cDNA was stored at (-20 to - 25˚C) until use.

2-18-3: Real time PCR

RT-qPCR is a method to amplify the target gene from cDNA strand. Taqman gene expression assay is based on 5' nuclease chemistry, which uses a fluorogenic probe to enable the detection of a specific PCR product as it accumulates during PCR. RT-qPCR reaction was performed in duplicate in 96-well plate (Bio-Rad, USA), and each reaction contains as shown in table 2-4.

Component	**Volume**
Primer (pair of specific unlabelled primers)	**1.25 µl**
Taqman Universal PCR Master Mix-FAM™	**12.5 µl**
cDNA	**2.5 µl**
Nuclease-free H_2O	**8.75 µl**
Final volume	**25µl**

Table 2-4: The RT-PCR reaction mixture.

RT-qPCR was carried out on Bio-Rad CFX Connect™ Real-Time PCR Detection System, USA, and CFX Manager Software, (USA).

The PCR plate was ran according to the protocol of the TaqMan® gene expression assay. The plate was heated to 95˚C for 10 minutes to activate the DNA polymerase/Taq activation, after a standard PCR reaction was undertaken (denaturation of the DNA template at 95˚C for 15 s, annealing of the primer and probe to their specific target sequences and complementary second strand synthesis at 60˚C for 1 minutes/Extension), 50 cycles.

During the Taqman gene expression assay, cDNA is denatured, primers and probe annealed, and during polymerization the gene-specific probe is cleaved and fluorescence emitted. With each cycle the intensity of the fluorescence is proportional to the amount of synthesized products (which is proportional to original mRNA in RNA samples), which can be detected (Fig 2-5).

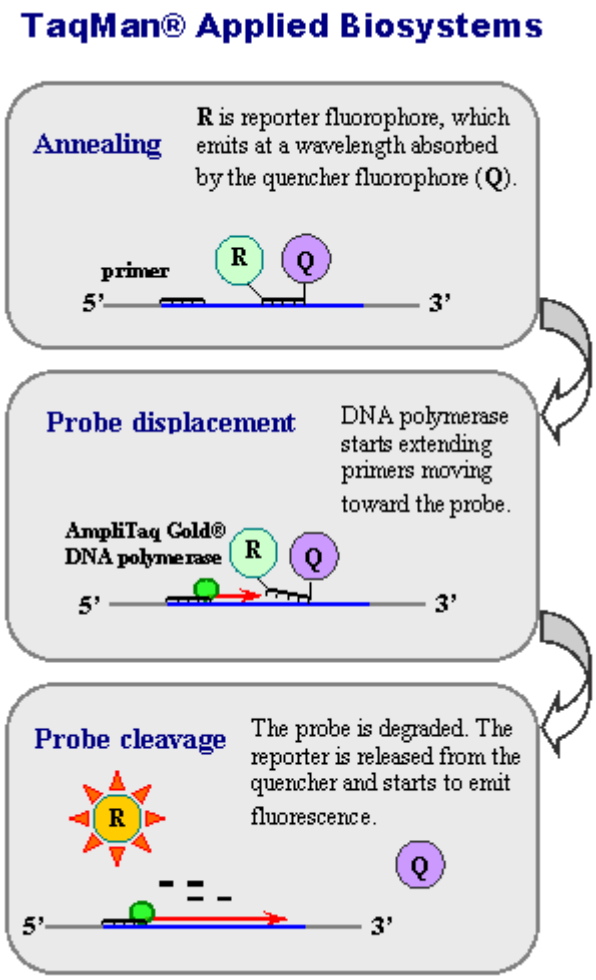


Fig 2-5: Taqman assay principles. The figure adapted from the web site of (https://www.ncbi.nlm.nih.gov/core/assets/probe/images/qpcr_principle1.gif)

2-18-3-1: Analysis of RT-PCR data

The CFX Manager Software was used for analysing the data, which generates the C_T values for all target genes (MMP-1, MMP-2, MMP-9, TIMP1, TIMP2, Sirt1, and Sirt6) and endogenous control (housekeeping gene) GAPDH (Table 2-5). ΔC_T was calculated from C_T for the target gene (sample) and the reference GAPDH gene, using the equation:

$$\Delta C_T = C_T \text{ (target gene)} - C_T \text{ (reference gene)}$$

This normalizes to the reference gene (GAPDH). A further calculations, $2^{-\Delta CT}$ was generated to convert the negative number into positive (in considering the relationship between C_T and expression are inverse). Then the $2^{-\Delta CT}$ value for the target gene was multiplied with 100 to find out the % GAPDH, which was plotted and analysed by GraphPad Prism7.

Catalogue N.	Primer
4331182	- Hs02786624_g1 for human GAPDH - Hs00899658_m1 for human MMP1 - Hs01548727_m1 for human MMP2 - Hs00957562_m1 for human MMP9 - Hs01092512_g1 for human TIMP1 - Hs00234278_m1 for human TIMP2 - Hs01009006_m1 for human SIRT1 Hs00966002_m1 for human SIRT6

Table2-5: All primes used Taqman gene expression assay.

2-19: Human cytokine array assay (antibody array)

Relative levels of human cytokines and chemokines were identified by the antibody array method, which acts as a tool to simultaneously detect 36 cytokines differences between samples. The principle of the assay is, that capture antibodies have been spotted in duplicate on nitrocellulose membranes, and the cell conditioned media (samples) contain the proteins (antigens), then immune complexes were formed on the correspondence spots on the membrane with different intensity that can be visualized by streptavidin-HRP (streptavidin conjugated to horseradish-peroxidase) and chemiluminescent detection reagents- Chemi reagents (1 and 2).

The spots are arranged in each membrane as; positive controls 3 pairs of spots, negative controls 1 pair of spot and 36 pairs of spots for determining cytokines difference levels in different samples, (Appendix 8-4).

One millilitre from each conditioned medium, that collected from the migration assay (sections 2-15 and 2-16, the direct and paracrine effect of TNF-α) from healthy and diabetic donor cells of; 10%FBS growth conditioned media (direct control), or 10%FBS double growth conditioned media (paracrine control), or 2.5 (ng/ml) of TNF-α conditioned media (direct TNF-α), and 2.5 (ng/ml) of TNF-α double conditioned media (paracrine TNF-α) were assayed to determine the relative expression levels of 36 human cytokines.

The assay was carried out according to the manufacturer's instructions (R & D systems bio-techne, ARY005B, USA). The nitrocellulose membranes were blocked by adding 2ml of array buffer 4 (block buffer) into each well of the 4-Well Multi-dish, on a shaker for one hour with array number should be facing

upward. In meanwhile the samples (centrifuged conditioned media) were prepared in separated tubes, by mixing 1ml from each conditioned media with 500µl of array buffer 4 with a final volume of 1.5ml, then 15 µl of human cytokine array detection antibody cocktail (prepared according to the manufacturer's instructions) was added for each sample, these mixtures of sample/antibody were mixed and incubated at room temperature for one hour. After, the array buffer 4 was removed from the wells of the 4-Well Multi-dish and replaced with the sample/antibody mixtures for each sample, covered with the lid and incubated for overnight at 2-8 ˚C on a shaker.

Next, the membranes were washed in individual square Petri dishes with 20ml of 1x washing buffer (was prepared by diluting 40ml of 25x of washing buffer in 960ml of distilled water) for three times in total (each time for 10 minutes on a shaker at room temperature). The streptavidin-HRP was diluted in array buffer 5 using the dilution factor (1µl HRP: 2000µl array buffer 5) and 2ml of diluted streptavidin-HRP was taken into each well of the 4-Well Multi-dish. After the membranes were transferred into the 4-Well Multi-dish containing the diluted streptavidin-HRP, the plate was covered with the lid and incubated for 30minutes at room temperature on a shaker. The membranes were washed again for three times as described above. The membranes were dried and placed on the bottom of the plastic sheet protector (kit supplier), and the chemi reagent 1 and 2 mixture (chemiluminescent detection reagents) was prepared instantly. Finally, to visualize the intensity of spots, 1ml of chemi reagent 1 and 2 mixture was added onto each membrane, then covered with the top plastic sheet protector and were incubated for 1 minute at room temperature (wrapped in foil). Images were taken using a UV/white light conversion screen-

visualization system (Bio-Rad, UK) and Image J software was used to analyse the images.

2-19-1: Analysis of human cytokine array

The images were analysed using Image J programme, which measures the densitometry of the spots and converts pixels into values, the average signal (pixel density) was calculated for each pair of duplicated spots (each pair representing a cytokine depending on transparency- coordinate reference) (kit supplier), a background subtractions were carried out for all spots. The negative values were dismissed, while the positive values were considered and these values compared to identify any differences between signals on control or TNF-α treated samples.

2-20: Statistical analysis

Data is displayed in as mean ± SEM. The tests that used were ANOVA (one-way and two-way ANOVA). An ANOVA test is used to find out the differences between three or more groups (to determine significance of mean group differences). One-way ANOVA is used to compare one categorical variable or single factor with three or more groups, while two-way ANOVA is used to compare two or more categorical variable or two factors with multiple groups. The p-values; *$p<0.05$, **$p<0.01$ and ***$p<0.001$ were considered as significant.

3 Results

3-1: Comparison of the rate proliferation of dermal fibroblasts in media supplemented with 2% or 10% FBS

It is well established that FBS stimulates proliferation of human dermal fibroblasts. However, the presence of serum may mask the effects of growth factors and hormones added to the culture media. Therefore to determine the effect of reducing the concentration of FBS on the growth of papillary dermal fibroblasts in culture, two different concentrations of FBS were compared in the same cell type.

To determine the differences in dermal fibroblast proliferation in response to growth factors, hormones and attachment factors in serum that stimulate cell growth as well as the role of serum as a spreading factor, cells were incubated in either 2% or 10% FBS supplemented media for up to 12 days. (Figure 3-1).

There was a significant difference in the proliferation rate of dermal fibroblasts incubated in two different concentrations of 2% and 10% FBS over a 12 day period. From day 6 the rate of proliferation of fibroblasts incubated in 10% FBS was significantly faster ($p<0.001$) than corresponding cells cultured in 2% FBS for up to 12 days.

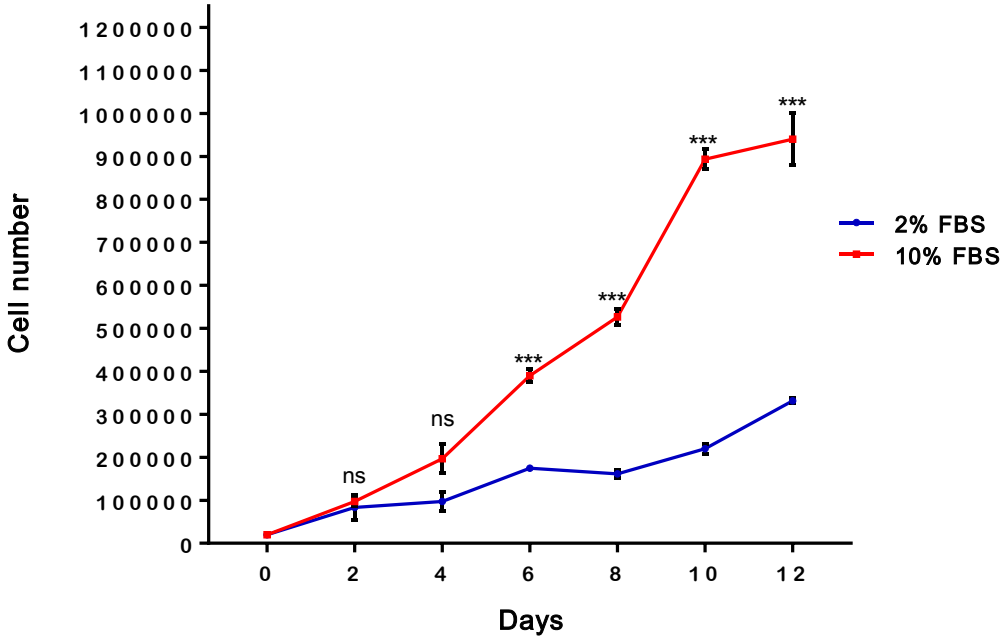


Figure 3-1: Proliferation of dermal fibroblasts cultured in different FBS concentrations of 2% and 10%. DFs were cultured in either 2% or 10% FBS and counted every 2 days for 12 days. Each point represents the mean of triplicate wells +/- SEM from one donor. (Female, facial skin, age 77, P6); *** denoted $p < 0.001$ using two-way ANOVA.

3-2: TNF-α inhibits dermal fibroblast proliferation

3-2-1: Manual cell counting

A comparison in growth rate between two different populations of dermal fibroblasts was undertaken. This was comparable to the design of previous experiments for papillary dermal fibroblasts (Kamala, 2014).

To determine whether dermal fibroblasts derived from scalp (haired skin) and facial skin (non-haired) showed differences in proliferation, three donor-matched cultures were grown in 10% FBS supplemented media for up to 14 days. There was no significant difference in the basal rate of proliferation over this time period (Fig 3-2).

When dermal fibroblasts were pre-incubated with TNF-α (2.5, 25 and 250 ng/ml) for 3 days, cell proliferation was significantly reduced by day 14 at all concentrations of TNF-α in dermal fibroblasts derived from female scalp (Fig 3-3 A). This was mirrored in donor-matched dermal fibroblasts derived from facial skin (Fig 3-3 B).

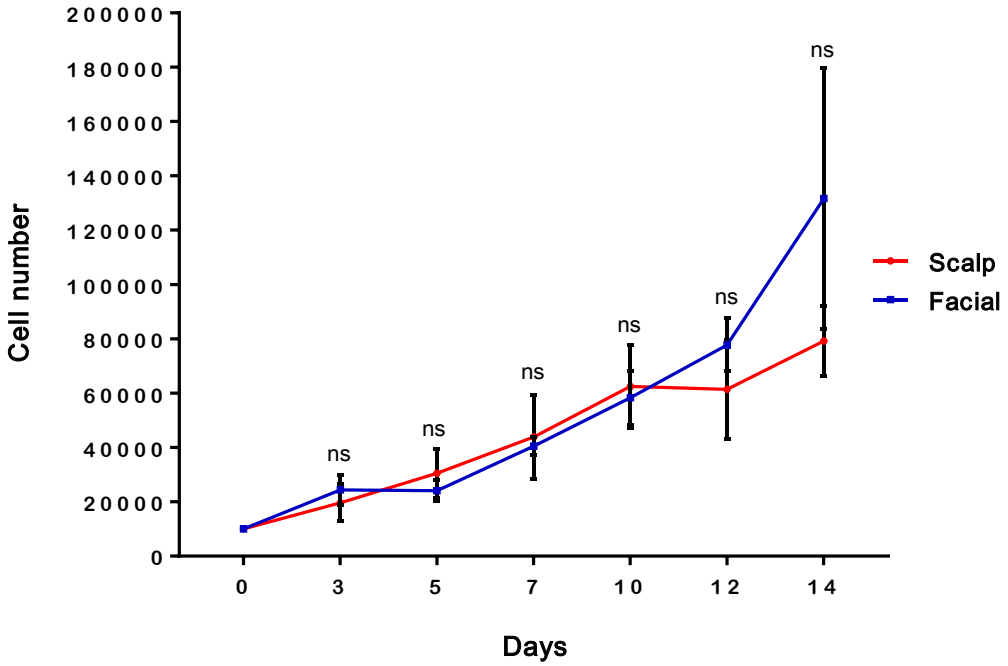


Figure 3-2: Comparison of the rate proliferation of donor-matched cultures of dermal fibroblasts derived from facial and scalp skin. Primary cultures of DFs from three matched females donors scalp (n=3) and facial (n=3) skin, (P4-6), were cultured for up to 14 days and counted every 2-3 days. Each point represents the mean of 3 donors +/- SEM, and proliferation was assessed in three triplicate wells for each donor. Age range (52-64 years); ns= non-significant, two-way ANOVA.

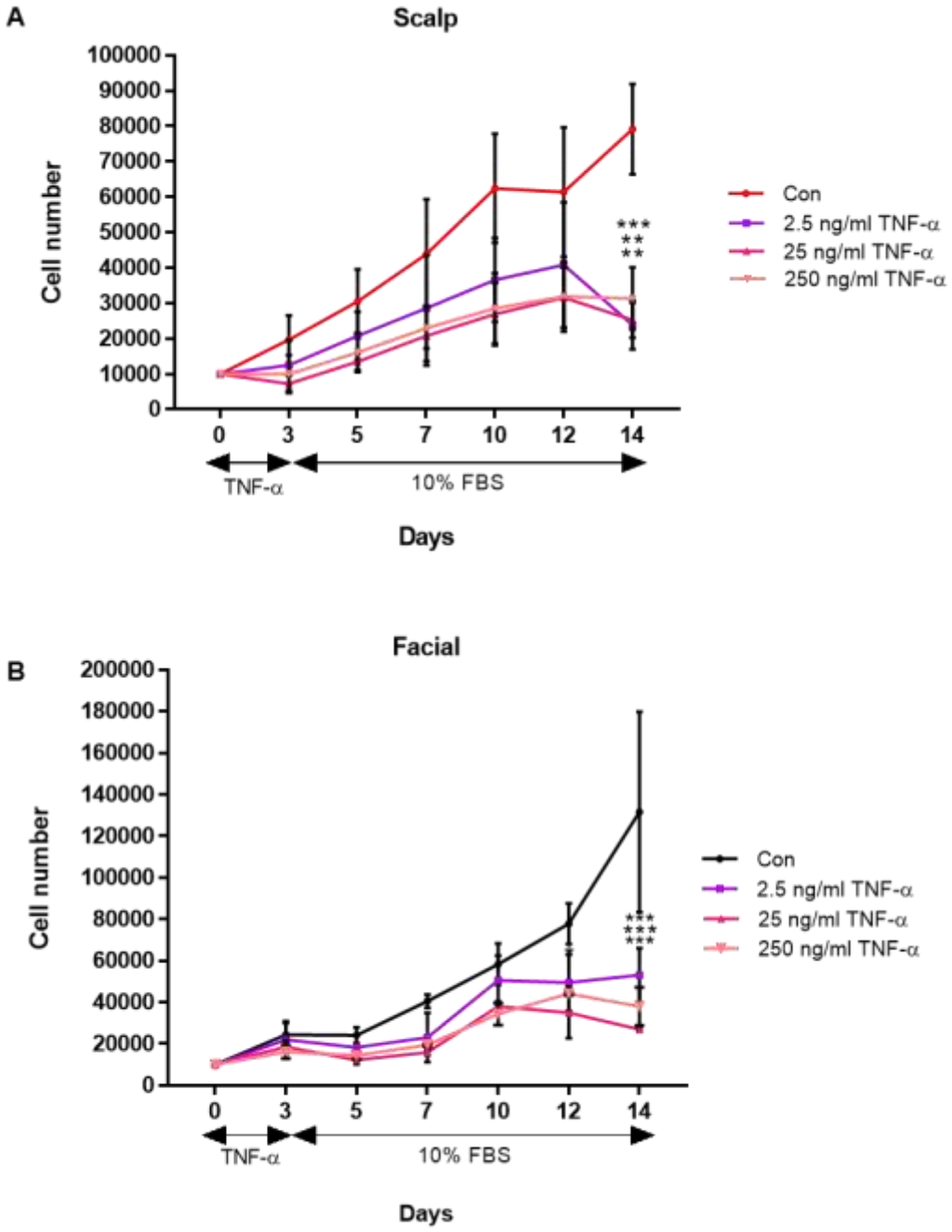
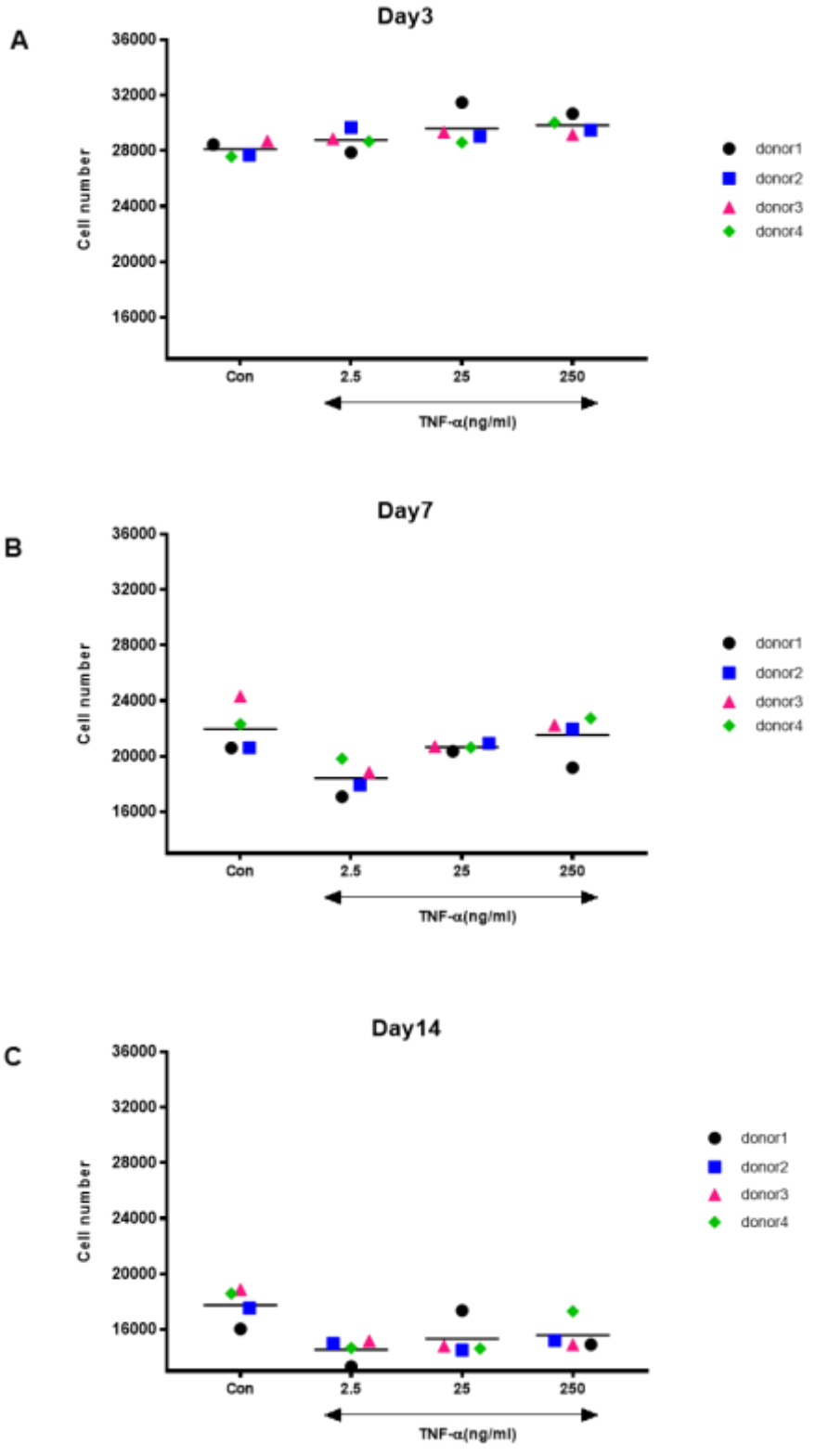


Figure 3-3: Pre-incubation with TNF-α inhibits the proliferation of donor-matched cultures of dermal fibroblasts derived from facial and scalp skin. Primary cultures of DFs were incubated with different concentrations of TNF-α for 3 days and then replenished with 10% FBS growth medium for a further 11 days. Triplicate wells were counted in duplicate every 2-3 days. Cells were established from two different sites of the same donors (A) DFs derived from female (haired) scalp and (B) corresponding facial (non-haired) skin (n= 3 donors). Each point represents the mean of 3 donors +/- SEM. Female donors, (P4-6), age range (52-64 years); * $p<0.05$, ** $p<0.01$ and *** $p< 0.001$, two-way ANOVA.

3-2-2: Direct cell proliferation- CyQUANT

To confirm the effect of TNF-α on dermal fibroblast proliferation quantitated by manual cell counting, another direct cell proliferation CyQUANT assay was used. Dermal fibroblasts from four healthy donors were pre-incubated with three different concentrations of TNF-α (2.5, 25 and 250 ng/ml) for 3 days as in the previous experiment (section 3-2-1) and then for 11 days in growth medium containing 10% FBS without TNF-α (Fig 3-4). Significant differences were observed at day 7 and 14 in the rate of cell proliferation. At day 7, the proliferation rate was reduced significantly following pre-incubation with 2.5 ng/ml of TNF-α, and a significant decrease in cell proliferation was observed at day 14 following pre-incubation with the lowest concentrations of TNF-α (2.5 and 25 ng/ml) (Fig 3-4 B and C).


A
Day3
36000
32000
28000
24000
20000
16000
Cell number
Con
2.5
25
250
TNF-α(ng/ml)
donor1
donor2
donor3
donor4
B
Day7
36000
32000
28000
24000
20000
16000
Cell number
Con
2.5
25
250
TNF-α(ng/ml)
donor1
donor2
donor3
donor4
C
Day14
36000
32000
28000
24000
20000
16000
Cell number
Con
2.5
25
250
TNF-α(ng/ml)
donor1
donor2
donor3
donor4

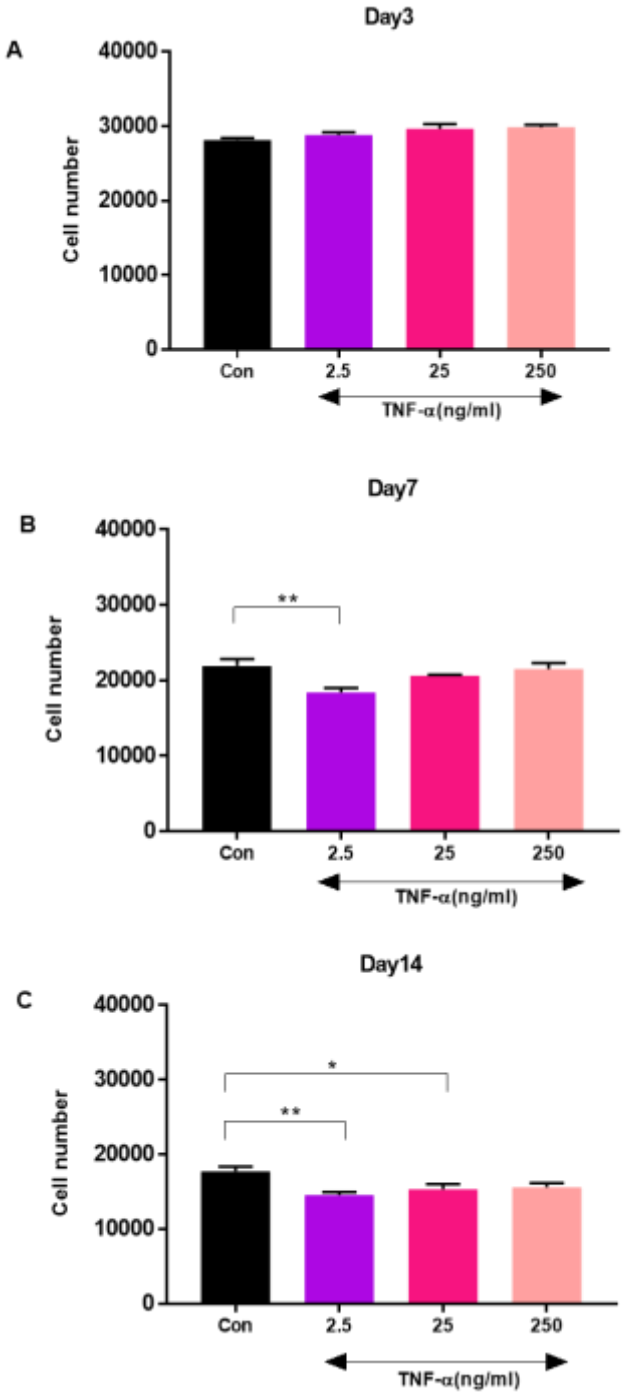
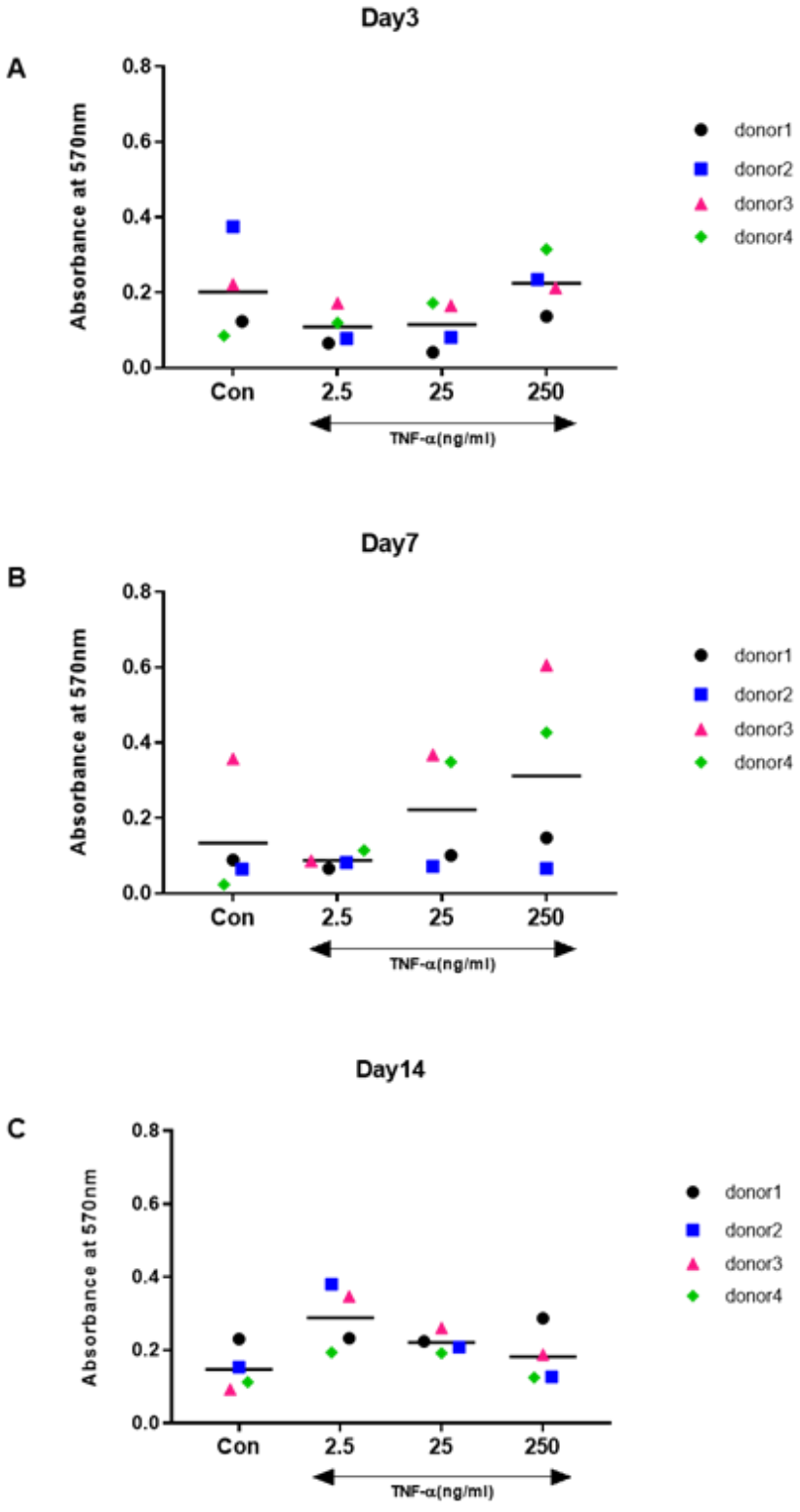


Figure 3-4: CyQUANT direct proliferation assay. DFs derived from female facial skin were pre-treated with three different concentrations of TNF-α (2.5, 25 and 250 ng/ml) for 3 days. Media was replenished with 10% FBS growth media every 3 days for 14 days (A) The proliferation rate at day 3, (B) at day 7 and (C) at day 14. Each point represents the mean of four replicates wells +/- SEM of four donors (n=4), (P7-8), age range (45-67y). The data are presented twice: Scatter Plots A-C show the means for each individual by group; Histograms A-C show the means per group. Proliferation was significantly reduced by the lowest concentrations (2.5 and 25 ng/ml) of TNF-α at day 14;* $P<0.05$ and ** $p<0.01$, one-way ANOVA. The fluorescent measurements were taken using plate reader with excitation at 508nm and emission at 527nm.

3-3: Pre-incubation with TNF-α for 3 days leads to an increase in the metabolic activity of dermal fibroblasts at day 14

Because TNF-α inhibits the proliferation of dermal fibroblasts by day 14, there is the possibility that TNF-α is reducing metabolic activity, so the metabolic activity of the cells was assayed till day 14.

Dermal fibroblasts from facial skin of four healthy donors were assayed for changes in their metabolic activity after pre-treatment with TNF-α at three different concentrations of (2.5, 25 and 250 ng/ml) for 3 days followed by incubation in growth media containing 10% FBS without TNF-α for a further 11 days. There was no significant change in metabolic activity until day 14 (Fig 3-5), when the metabolic activity was increased significantly by pre-treatment with the lower concentration of 2.5 ng/ml TNF-α when compared to the control (Fig 3-5 C).


Day3
A
Absorbance at 570nm
0.8
0.6
0.4
0.2
0.0
Con
2.5
25
250
TNF-α(ng/ml)
donor1
donor2
donor3
donor4
Day7
B
Absorbance at 570nm
0.8
0.6
0.4
0.2
0.0
Con
2.5
25
250
TNF-α(ng/ml)
donor1
donor2
donor3
donor4
Day14
C
Absorbance at 570nm
0.8
0.6
0.4
0.2
0.0
Con
2.5
25
250
TNF-α(ng/ml)
donor1
donor2
donor3
donor4

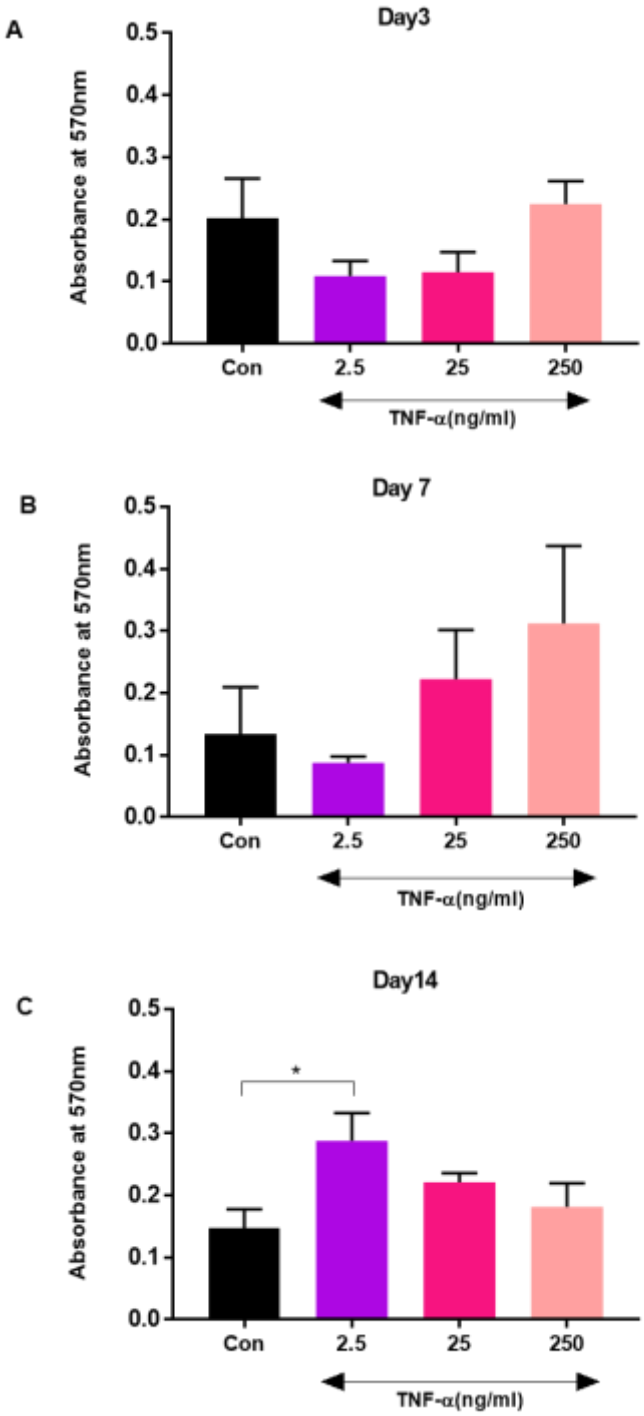


Figure 3-5: Pre-incubation with TNF-α stimulates metabolic activity. Alamar blue metabolic activity assay. DFs derived from female facial skin were incubated with increasing concentrations of TNF-α (2.5, 25 and 250 ng/ml) for 3 days. Then the medium was replenished without TNF-α every 3 days for up to 14 days. Each point represents the mean of four replicates wells +/- SEM of four donors (n=4), (P4-5) age range (45-67y). The data are presented twice: Scatter Plots A-C show the means for each individual by group; Histograms A-C show the means per group. Changes in metabolic activity are shown at day 3 (A) day 7 (B) and day 14 (C). Metabolic activity was significantly higher following pre-incubation with 2.5 ng/ml TNF-α at day 14;* $P<0.05$, one-way ANOVA. Absorbance was measured using plate reader at 570nm.

3-4: Induction of senescence in cultured dermal fibroblasts by TNF-α

As TNF-α reduced the proliferation of dermal fibroblasts and seemed to increase metabolic activity, which suggests the cells may become senescent cells (cells are not dividing, but are metabolically active). Its ability to modulate senescence was therefore measured using beta-galactosidase assay. When cultured dermal fibroblasts were incubated with a concentration of 250 ng/ml of TNF-α for 48h, representative images shown in (Fig 3-6), there was a significant increase in the percentage of dermal fibroblasts expressing beta-galactosidase compared to the controls ($p=0.001$), (Fig 3-7), while the lower concentrations (2.5 and 25 ng/ml) of TNF-α had no effect. This was only observable in cells which had proliferated in culture up to passage 17, and was not detected in cells at lower passages.

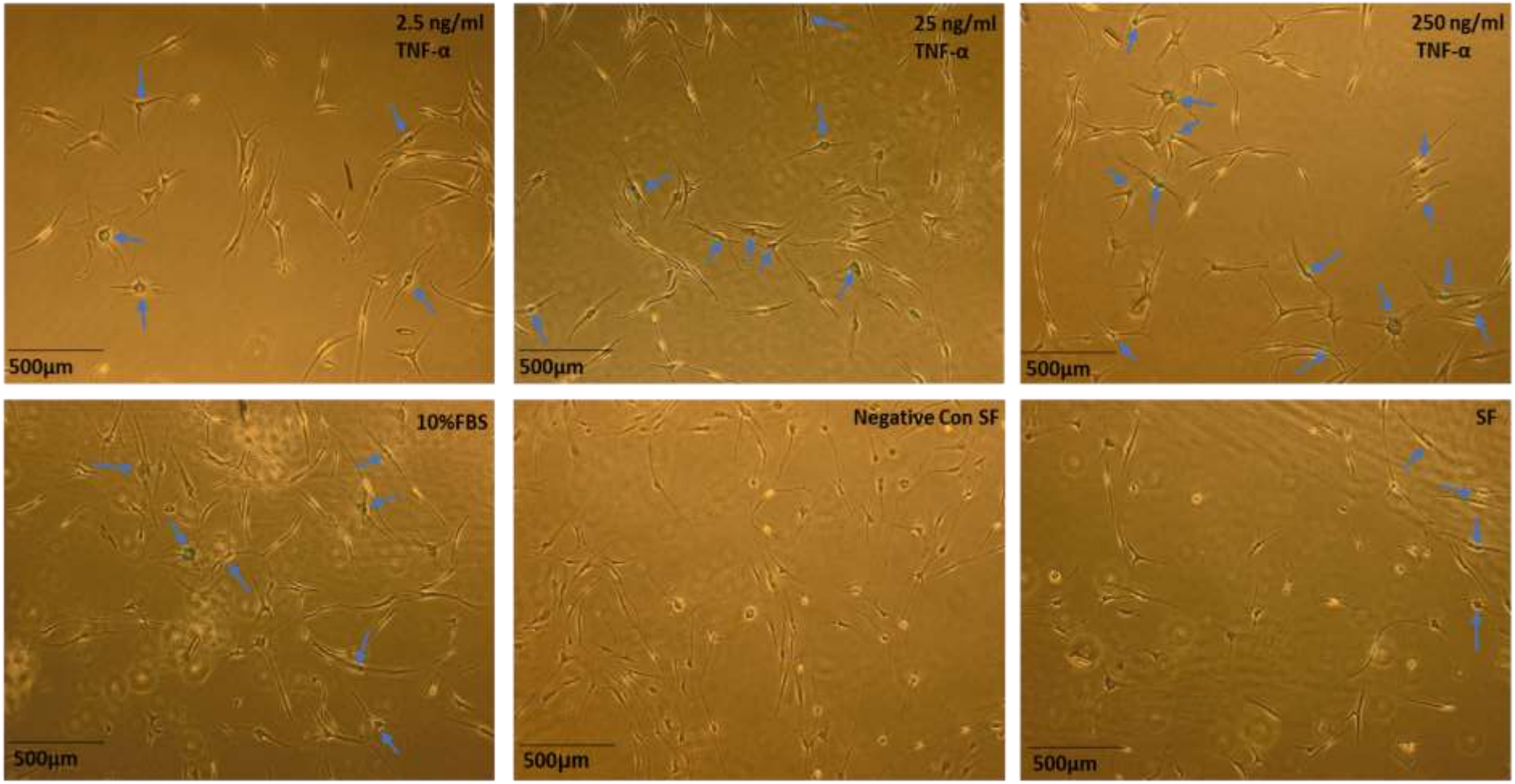


Figure 3-6: Beta-galactosidase expressed in cultured human dermal fibroblasts. Cells were incubated either in upper row; different concentrations of TNF-α 2.5 or 25 or 250 ng/ml, or in lower row; 10% FBS or negative Con or SF for 48h. Senescent cells were detected using β-galactosidase staining at pH 6.0 after 24h and counted in 5 radially selected images. DFs were derived from female, facial skin, age 55y, at passage P17. Image magnification x40, scale bar=500 µm, arrows indicate to senescent cells.

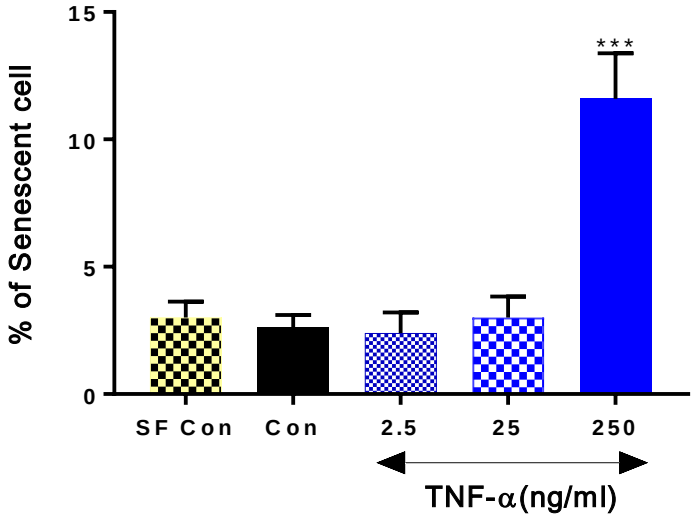


Figure 3-7: TNF-α induces senescence. DFs were incubated with three concentrations of TNF-α (2.5 or 25 or 250 ng/ml) for 48h in 10% FBS before staining for beta-galactosidase at pH 6.0 for 24h. DFs were derived from female, facial skin, age 55y, Passage 17. Each point represents the mean of five technical replicates (five images for each well) +/- SEM; *** p< 0.001, one-way ANOVA.

3-5: TNF-α inhibits migration of dermal fibroblasts derived from healthy donors and diabetic patients

From the previous experiment (senescence assay), it seemed the cells are not senescent because if the wound healing environment was inflamed or chronic (cells incubated in an inflammatory environment) the migration response of the cells would be reduced/decreased, but as senescent cells, they could not migrate.

In order to measure the impact of TNF-α on dermal fibroblast migration, TNF-α was added in two different concentrations 2.5 or 25 ng/ml to scratched cells. In healthy dermal fibroblasts, TNF-α significantly impaired migration after 24h in the concentration of 25 ng/ml (Fig 3-8) and (Fig 3-10 A). However, TNF-α significantly inhibited diabetic dermal fibroblast migration after 24h at both concentrations of 2.5 and 25 ng/ml (Fig 3-9) and (Fig 3-10 B).

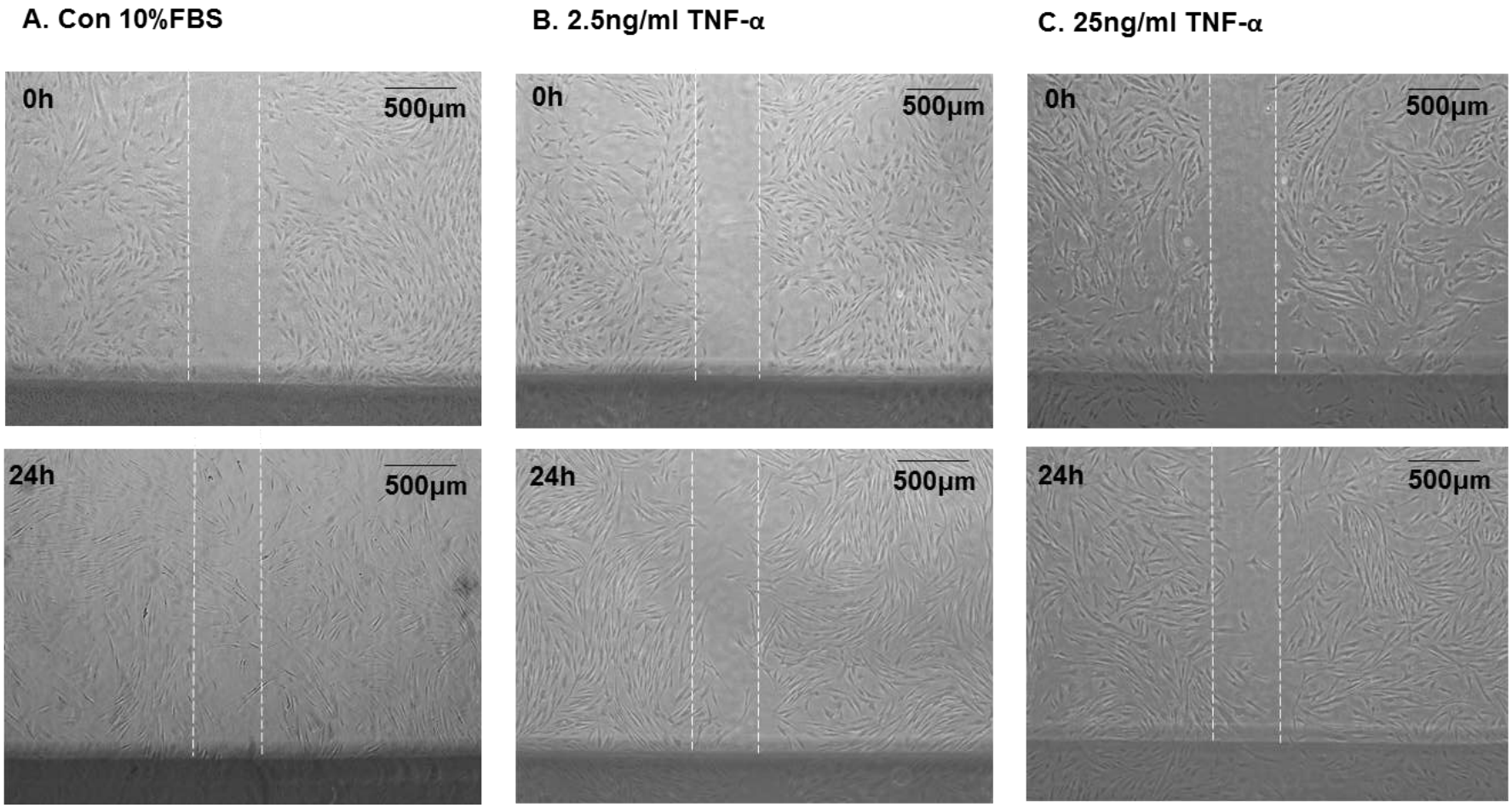


Figure 3-8: Representative images of migration assay for normal (healthy) dermal fibroblasts. Cultured DFs from female, facial skin, Passage 3, age 57y. DFs were scratched to produce a wound in the centre of the well and incubated for 24h with (A) Control 10% FBS (B) 2.5 ng/ml TNF-α (C) 25 ng/ml TNF-α. Image magnification x40, scale bar=500 µm, with hatched lines indicate initial wound boundaries.

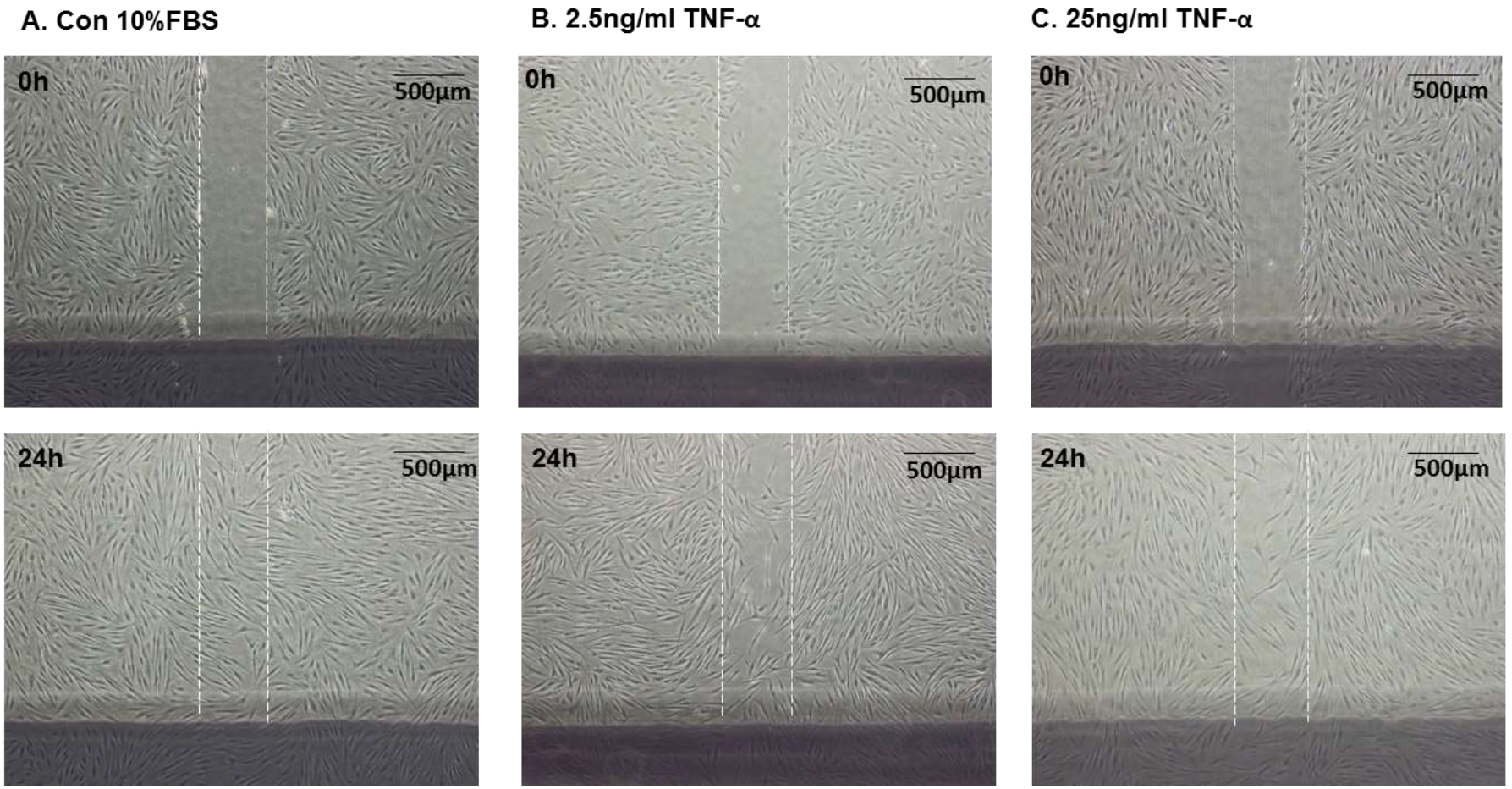


Figure 3-9: Representative images of migration assay for diabetic dermal fibroblasts. Cultured DFs from female diabetic patient, lower leg, Passage 3, age 52y. DFs were scratched to produce a wound in the centre of the well and incubated for 24h with (A) Control 10% FBS (B) 2.5 ng/ml TNF-α (C) 25 ng/ml TNF-α. Image magnification x40, scale bar=500 µm, with hatched lines

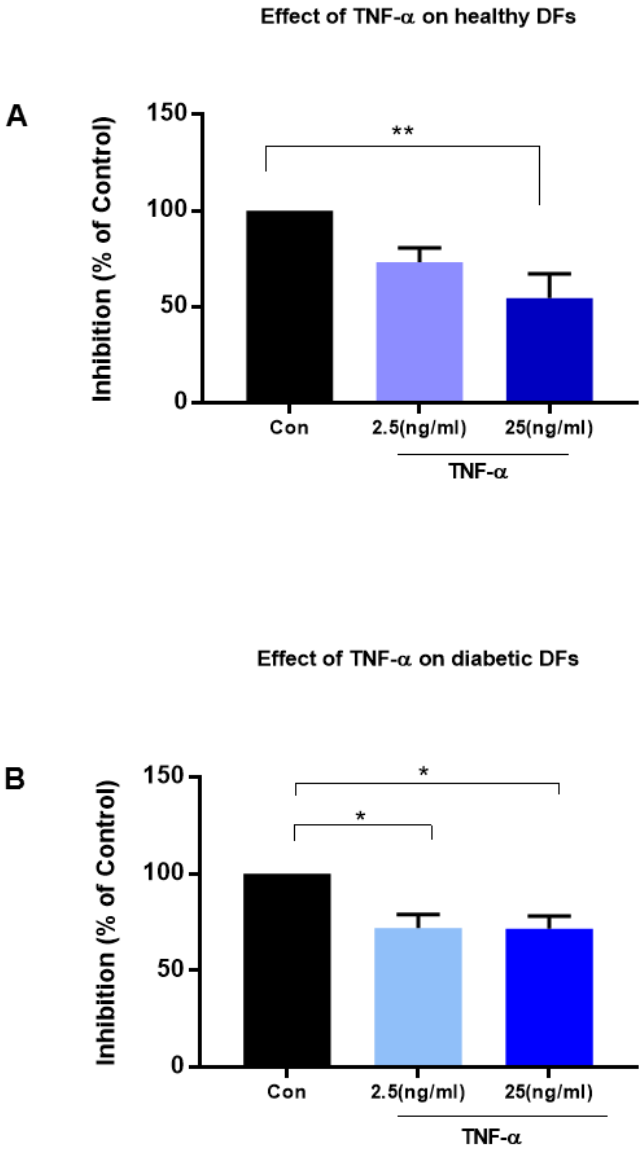


Figure 3-10: TNF-α inhibits migration of dermal fibroblasts from healthy donors and diabetic patients after 24h. (A) Scratched DFs from female donors (n=6), facial skin, P3, age range (36-67y) were incubated in the presence or absence of TNF-α (2.5 or 25 ng/ml). Each point represents the mean of triplicate wells +/- SEM of 6 donors. ** p<0.01, one-way ANOVA. (B) Diabetic DFs from (2males/2females) (n=4), lower leg, P3, age range (52-66y). Migrated diabetic DFs were incubated in the same conditions (2.5 or 25 ng/ml) of TNF-α. * P<0.05, one-way ANOVA.

3-6: Effect of paracrine factors from TNF-α conditioned media on healthy dermal fibroblast migration

As TNF-α can cause the up-regulation of secreted factors by treated cells, conditioned media was collected and added to healthy dermal fibroblasts to determine whether any paracrine effects modulated migration of other fibroblasts, while there was an inhibitory trend for the TNF-α paracrine effect to inhibit healthy dermal fibroblast migration after 24h, this did not reach statistical significance (Fig 3-11) and (Fig 3-13), although it appeared to mirror the direct effect of TNF-α (Fig 3-10). This appeared to be a direct effect of TNF-α rather than a result of any soluble factors increased, since the combination of conditioned control media with fresh TNF-α (2.5 or 25 ng/ml) added to it , had a similar effect on healthy dermal fibroblast migration after 24h (Fig 3-12) and (Fig 3-13).

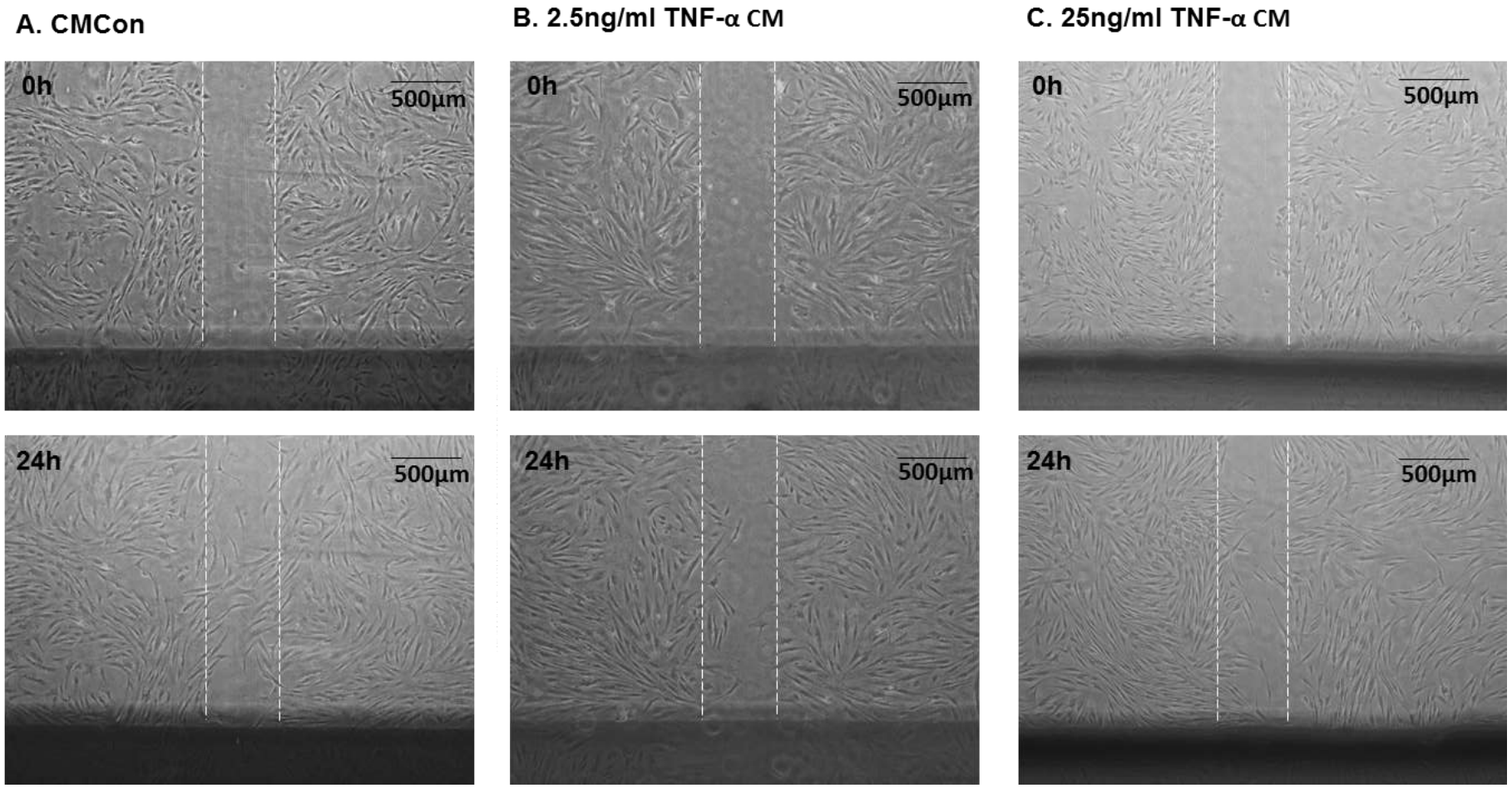


Figure 3-11: Representative images of migration assay for normal (healthy) dermal fibroblasts. Cultured DFs from female, facial skin, Passage 3, age 67y. DFs were scratched to produce a wound in the centre of the well and incubated for 24h with (A) Conditioned media Control (B) 2.5 ng/ml TNF-α CM (C) 25 ng/ml TNF-α CM. Image magnification x40, scale bar=500 µm, with hatched lines indicate initial wound boundaries.

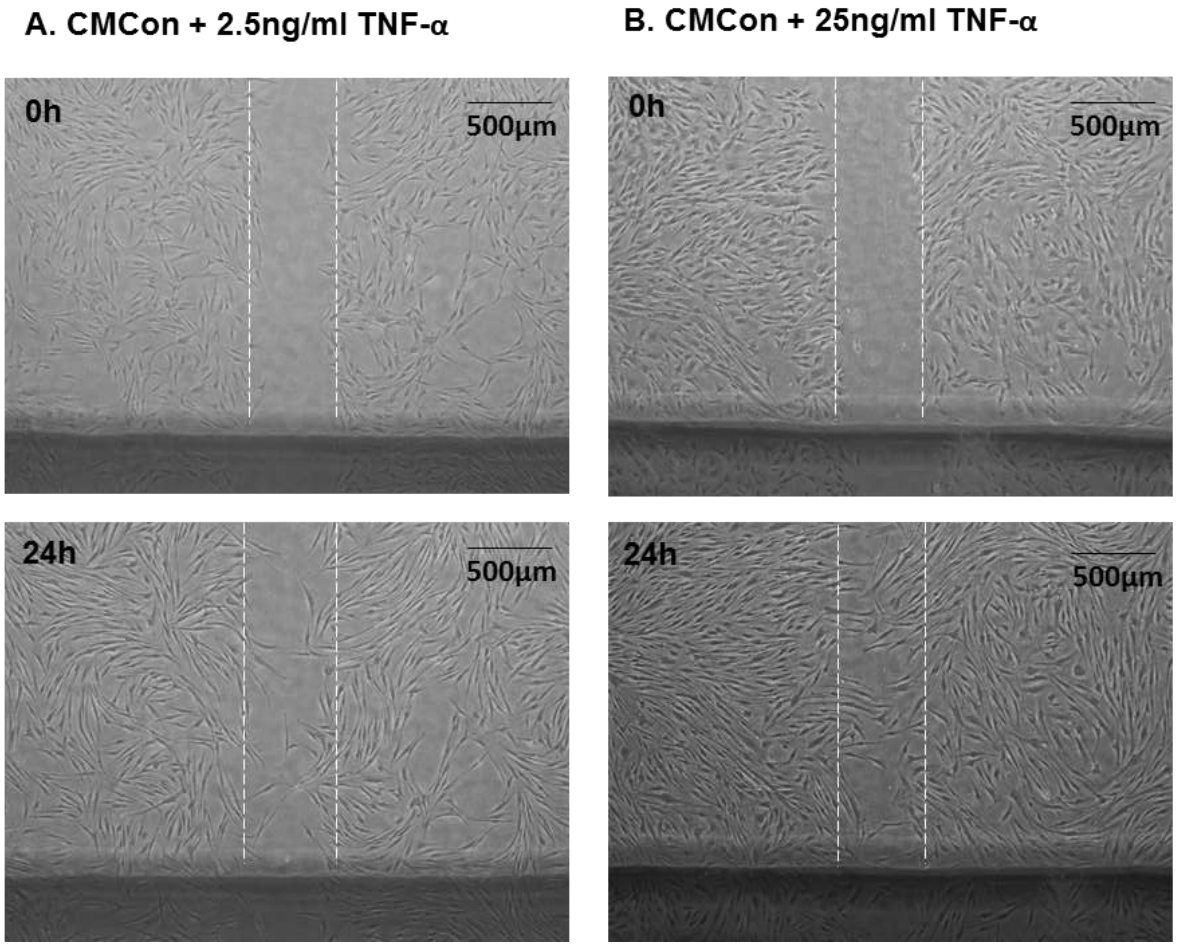


Figure 3-12: Representative images of migration assay for normal (healthy) dermal fibroblasts. Cultured DFs from female, facial skin, Passage 3, age 67y. DFs were scratched to produce a wound in the centre of the well and incubated for 24h with (A) Conditioned media Control + 2.5 ng/ml TNF-α (B) Conditioned media Control + 25 ng/ml TNF-α . Image magnification x40, scale bar=500 µm, with hatched lines indicate initial wound boundaries.

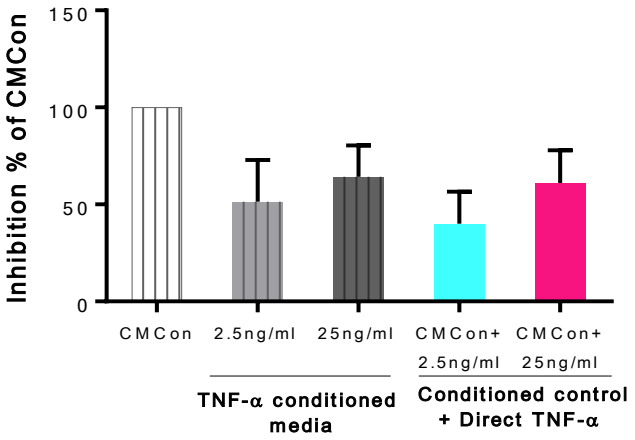


Figure 3-13: Paracrine effect of TNF-α on healthy dermal fibroblasts migration after 24h. Conditioned media was collected from two healthy female donors (n=2), facial skin, P7-8, age range (52-60y), which had been incubated in the presence or absence of TNF-α, 2.5 or 25 ng/ml and was added to healthy recipient DFs. The addition of TNF-α, 2.5 or 25 ng/ml TNF-α CM to the healthy recipient dermal fibroblasts from five female donors (n=5), facial skin, P3, age range (36-67y); non-significant, one-way ANOVA. The application of TNF-α (2.5 or 25 ng/ml) combined with conditioned control media (CMCon-without TNF-α) was added to same recipient DFs; no significant differences after 24h, one-way ANOVA.

3-7: Optimisation of the zymography technique for quantifying MMP-2 and MMP-9

Levels of the inflammatory markers MMP-2 and MMP-9 were determined to confirm whether the wounded and non-wounded cells reflected those in an inflammatory environment and whether diabetic wounded and non-wounded cells are more sensitive to an inflammatory environment as shown by increased MMPs secretion. High MMPs secretion would cause a breakdown of wound healing and induced the production of pro-inflammatory cytokines.

Gelatin zymography evaluates the degradation of gelatin by MMP-2 and -9 in conditioned media. If the conditioned medium is too concentrated, degradation can be over saturated, which makes quantitation inaccurate. Therefore, it is important to perform a series of dilutions to achieve a defined tight band of lysis (Fig 3-14). All conditioned media for optimisation of the assay were collected from dermal fibroblasts established from female facial skin (donor age 55y) and assayed at passage 10. The phorbol ester (PMA) was included as a positive control as it a known inducer of MMP-2 and -9 activity, *in vitro* (Agren *et al.,* 2015). From this, a dilution of (1:40) was chosen for all future assays.

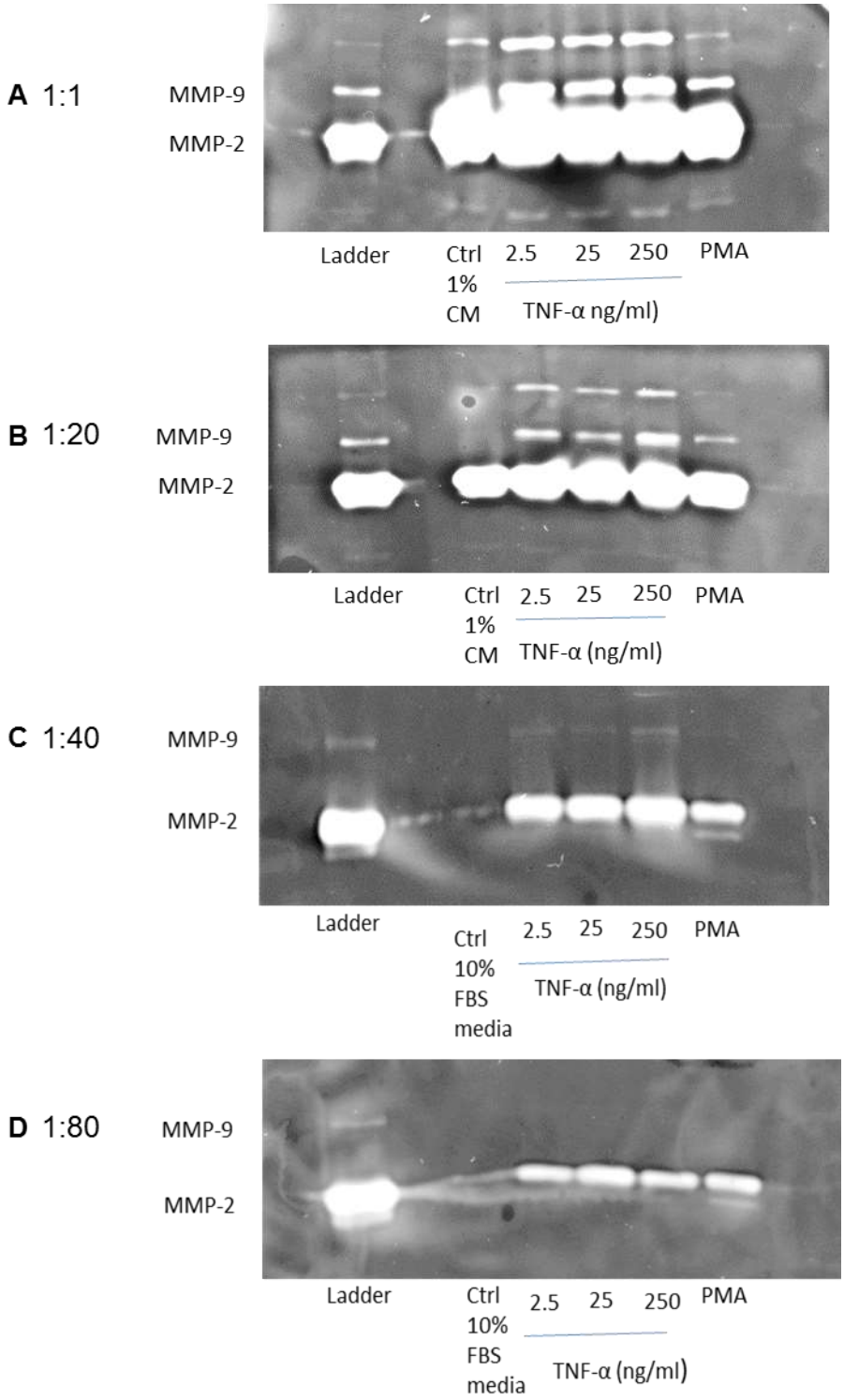


Figure 3-14: Optimisation of zymography loading. DFs were derived from female, facial skin, age 55y, Passage 10. DFs were cultured with TNF-α (2.5 or 25 or 250 ng/ml) in 10% FBS, or 100 nM PMA (Phorbol 12-myristate 13-acetate) for 48h and conditioned media were collected. This conditioned media was diluted (A) 1:1, (B) 1:20, (C) 1:40 or (D) 1:80 with loading buffer and the resulting digested bands imaged. The MMP-2 and MMP-9 bands appear as a clear band of lysis on a dark background of intact gelatin substrate.

3-8: MMP-2 and MMP-9 activity in conditioned media of scratched and non-scratched healthy and diabetic dermal fibroblasts

3-8-1: Secretion of MMP-2 in conditioned media from scratched dermal fibroblasts

Conditioned media was collected from dermal fibroblasts that had been scratched and cultured in the presence of 2.5 or 25 ng/ml TNF-α for 24h (migration assay 2-15) and MMP-2 activity assayed by zymography. Data was normalized to growth medium (10% FBS) taken from the bottle. For healthy dermal fibroblasts, TNF-α had no effect on MMP-2 secretion (Fig 3-15 A). However, in diabetic dermal fibroblasts, 25 ng/ml TNF-α increased MMP-2 expression by 261%, although this was highly variable and not statistically significant (Fig 3-15 B).

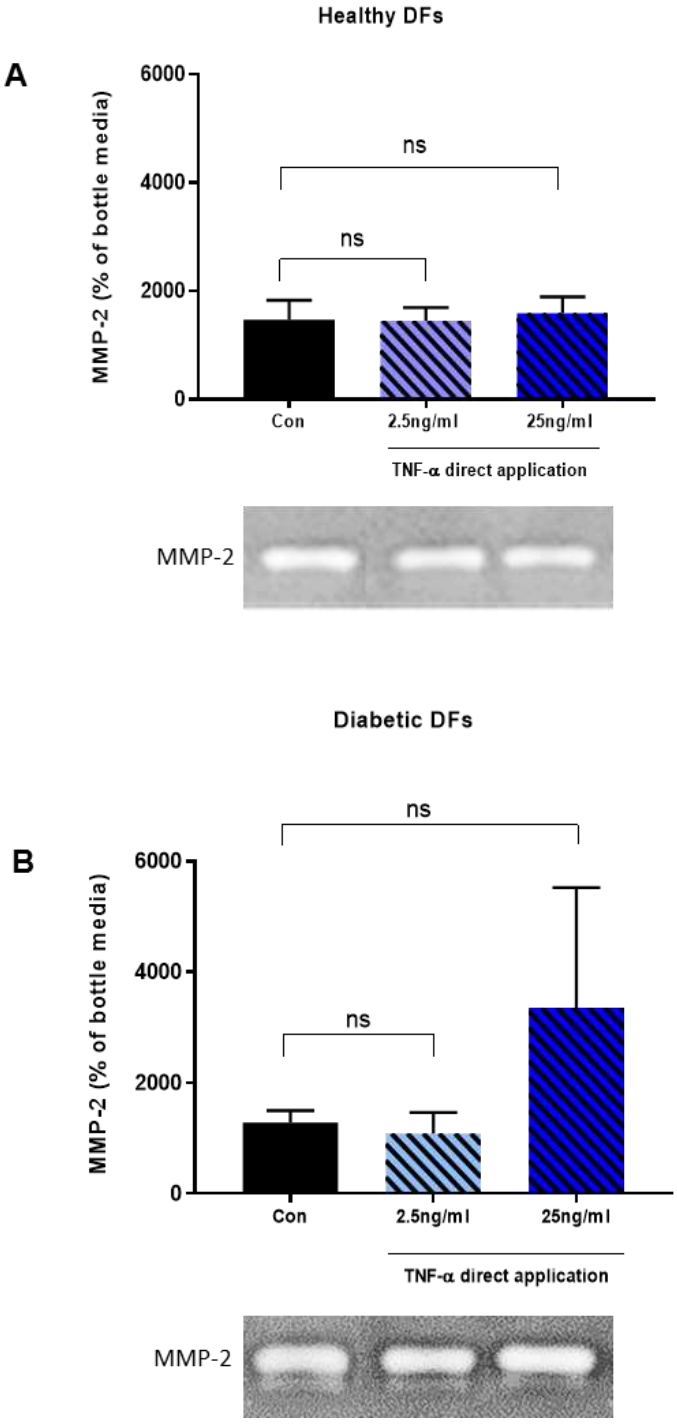


Figure 3-15: Effect of TNF-α on MMP-2 secretion in healthy and diabetic scratched dermal fibroblasts. Conditioned media was collected from scratched cells cultured with TNF-α after 24h from (section 3-5). (A) Healthy DFs from female donors (n=6), facial skin, P3 and age range (36-67y). (B) Diabetic DFs from 2males/2females (n=4), lower leg, P3 and age range (52-66y), ns= non-significant, one-way ANOVA.

3-8-2: Secretion of MMP-9 in conditioned media from scratched dermal fibroblasts

TNF-α had a variable effect on MMP-9 secretion which was consistently lower than MMP-2 secretion. In healthy dermal fibroblasts, both concentrations tended to increase MMP-9 secretion, but this was not statistically significant or dose-dependent (Fig 3-16 A). In diabetic dermal fibroblasts, TNF-α appeared to dose-dependently increase MMP-9 secretion, but again this was not significant due to high variability (Fig 3-16 B).

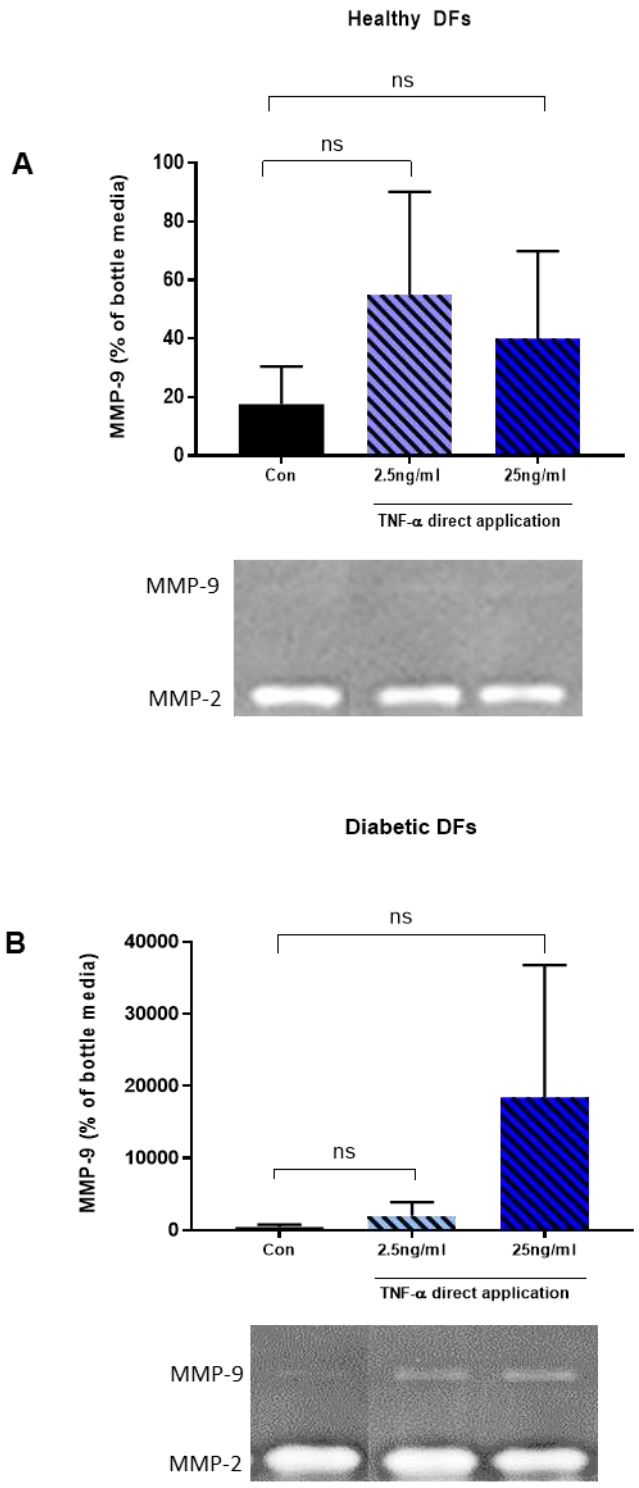


Figure 3-16: Effect of TNF-α on MMP-9 secretion in healthy and diabetic scratched dermal fibroblasts. Conditioned media was collected from scratched cells cultured with TNF-α after 24h from (section 3-5). (A) Healthy DFs from female donors (n=6), facial skin, P3 and age range (36-67y). (B) Diabetic DFs from 2males/2females (n=4), lower leg, P3 and age range (52-66y), ns= non-significant, one-way ANOVA.

3-8-3: Secretion of MMP-2 in conditioned media from non-scratched dermal fibroblasts

MMP-2 secretion was also measured in parallel dermal fibroblasts that had been treated with TNF-α, but had not been scratched. In both healthy and diabetic dermal fibroblasts, there was no significant, or dose-dependent (Fig 3-17 A and B) effect on MMP-2 secretion.

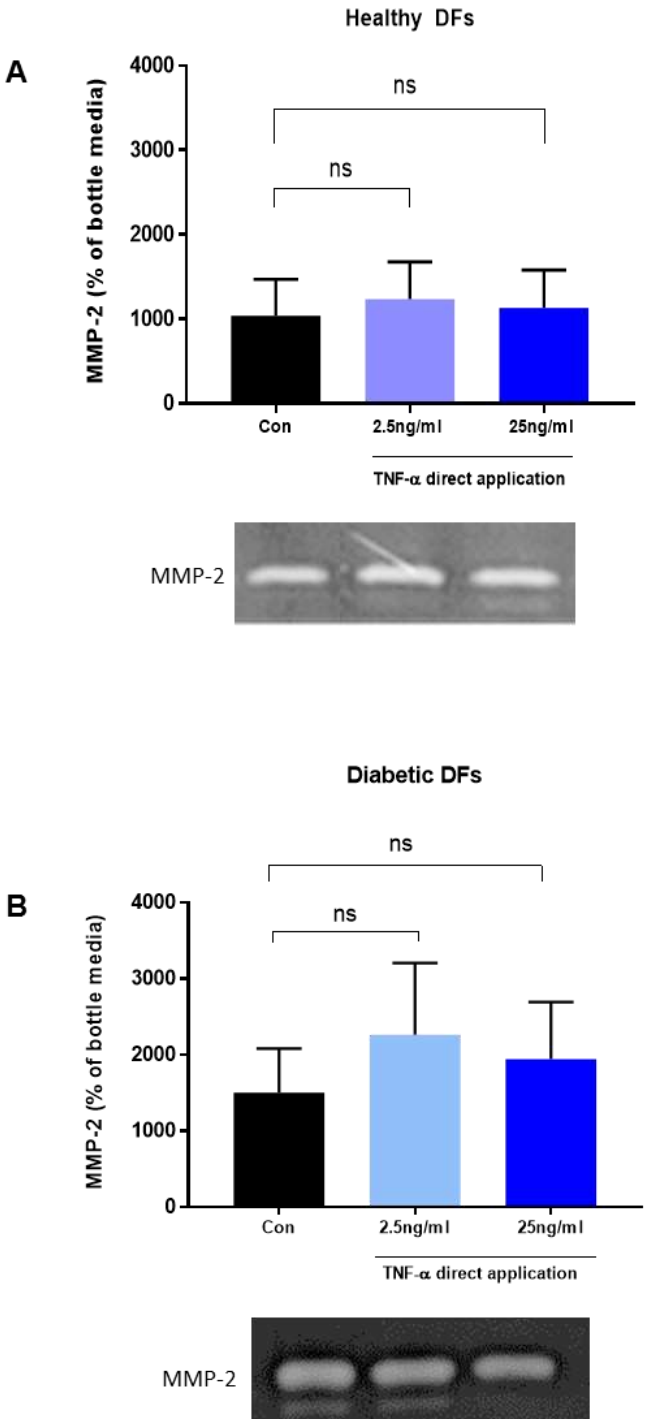


Figure 3-17: Effect of TNF-α on MMP-2 secretion in healthy and diabetic non-scratched dermal fibroblasts. Conditioned media was collected from cells cultured with TNF-α after 24h from (section 3-5). (A) Healthy DFs from female donors (n=6), facial skin, P3 and age range (36-67y). (B) Diabetic DFs from 2males/2females (n=4), lower leg, P3 and age range (52-66y), ns= non-significant, one-way ANOVA.

3-8-4: Secretion of MMP-9 in conditioned media from non-scratched dermal fibroblasts

In non-scratched dermal fibroblasts, MMP-9 secretion was again lower than MMP-2. Levels of MMP-9 secretion in both healthy and diabetic dermal fibroblasts under TNF-α stimulation were higher than the controls in a dose-dependent manner, but this was not statistically significant (Fig 3-18 A and B).

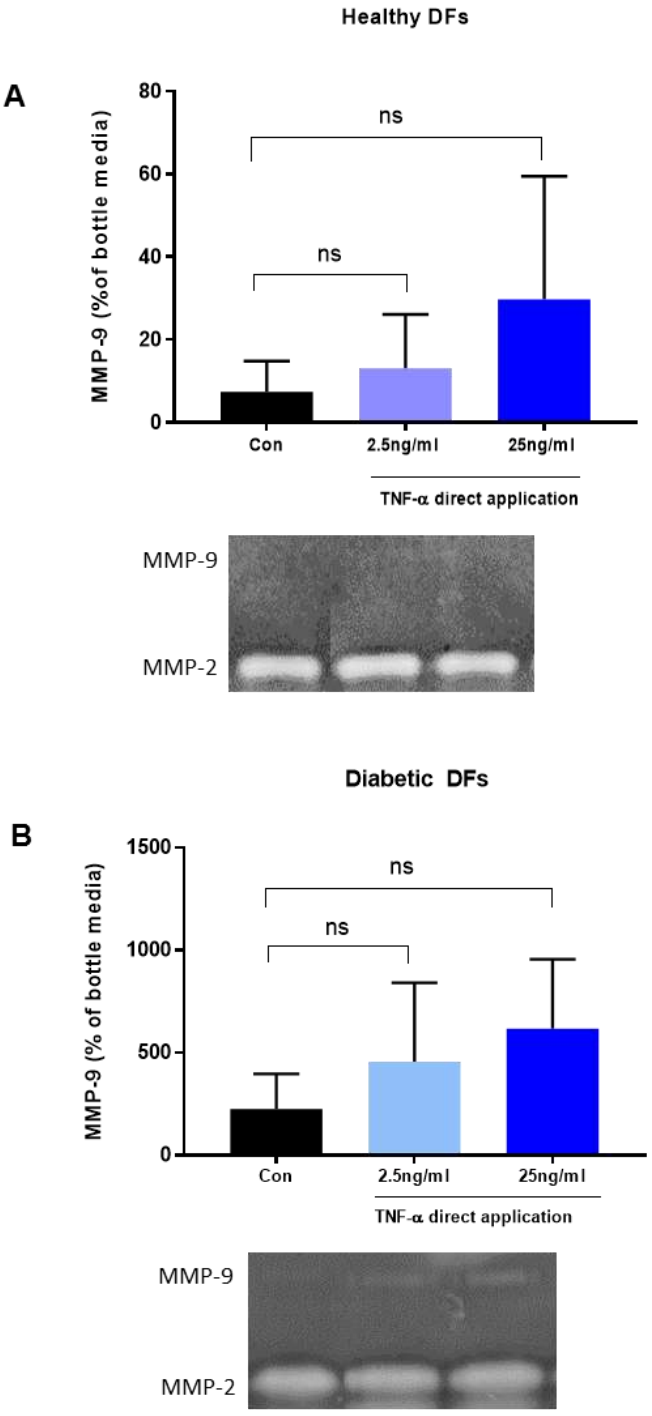


Figure 3-18: Effect of TNF-α on MMP-9 secretion in healthy and diabetic non-scratched dermal fibroblasts. Conditioned media was collected from cells cultured with TNF-α after 24h from (section 3-5). (A) Healthy DFs from female donors (n=6), facial skin, P3 and age range (36-67y). (B) Diabetic DFs from 2males/2females (n=4), lower leg, P3 and age range (52-66y), ns= non-significant, one-way ANOVA.

3-9: Effect of TNF-α conditioned media from healthy dermal fibroblasts on MMP-2 and -9 secretion

To determine the influence of paracrine factors and to investigate whether TNF-α has an indirect paracrine effect on MMP-2 and -9 secretion, conditioned media was collected from cells that had been cultured in the presence and absence of the TNF-α in the scratch assays of section 2-16, and from parallel non-scratched cells that were cultured under the same conditions.

The stimulation of paracrine factors by TNF-α conditioned medium significantly increased MMP-2 secretion by healthy scratched dermal fibroblasts at both concentrations of 2.5 and 25 ng/ml TNF-α in the conditioned media, but adding TNF-α directly to previously conditioned media control had no effect (Fig 3-19 A). MMP-2 induction in non-scratched dermal fibroblasts was also higher than the controls at 2.5 and 25 ng/ml TNF-α, but this was highly variable and not significant (Fig 3-19 B).

The paracrine effect on MMP-9 secretion by healthy scratched dermal fibroblasts was variable, but not significant and this effect was similar to direct application of TNF-α combined with the conditioned control, which had no significant effect (Fig 3-20 A). In non-scratched dermal fibroblasts, there was also no statistically significant difference, and for the conditioned media control with TNF-α added to it, the effect was variable and not significant (Fig 3-20 B).

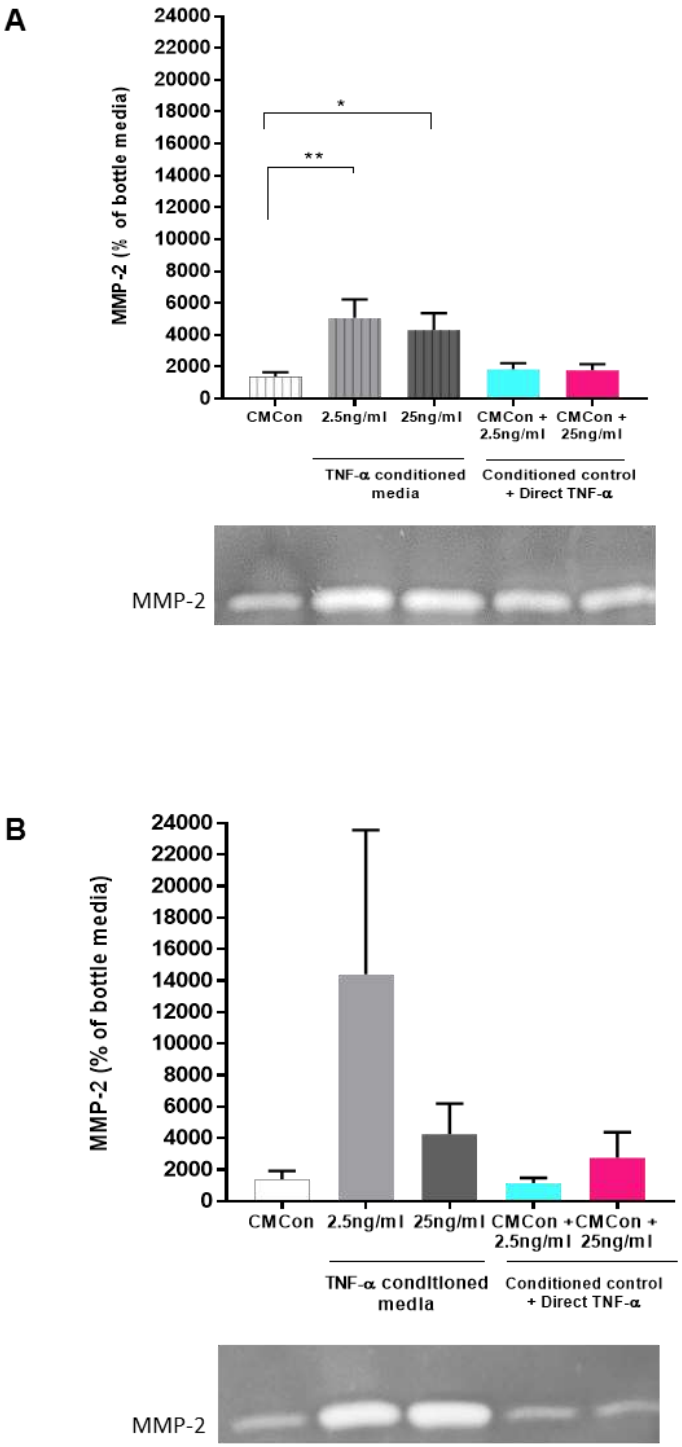


Figure 3-19: Paracrine effect of TNF-α on MMP-2 secretion in healthy scratched and non-scratched dermal fibroblasts. Conditioned media was collected from scratched and non-scratched cells that had been cultured in the presence or absence of 2.5 or 25 ng/ml TNF-α for 24h. DFs, from female donors (n=6), facial skin, P3 and age range (36-67y). (A) Scratched DFs, both 2.5 and 25 ng/ml CM induced MMP-2 significantly; *p<0.05 and **p<0.01, one-way ANOVA, and (B) Non-scratched DFs no significant differences, one-way ANOVA.

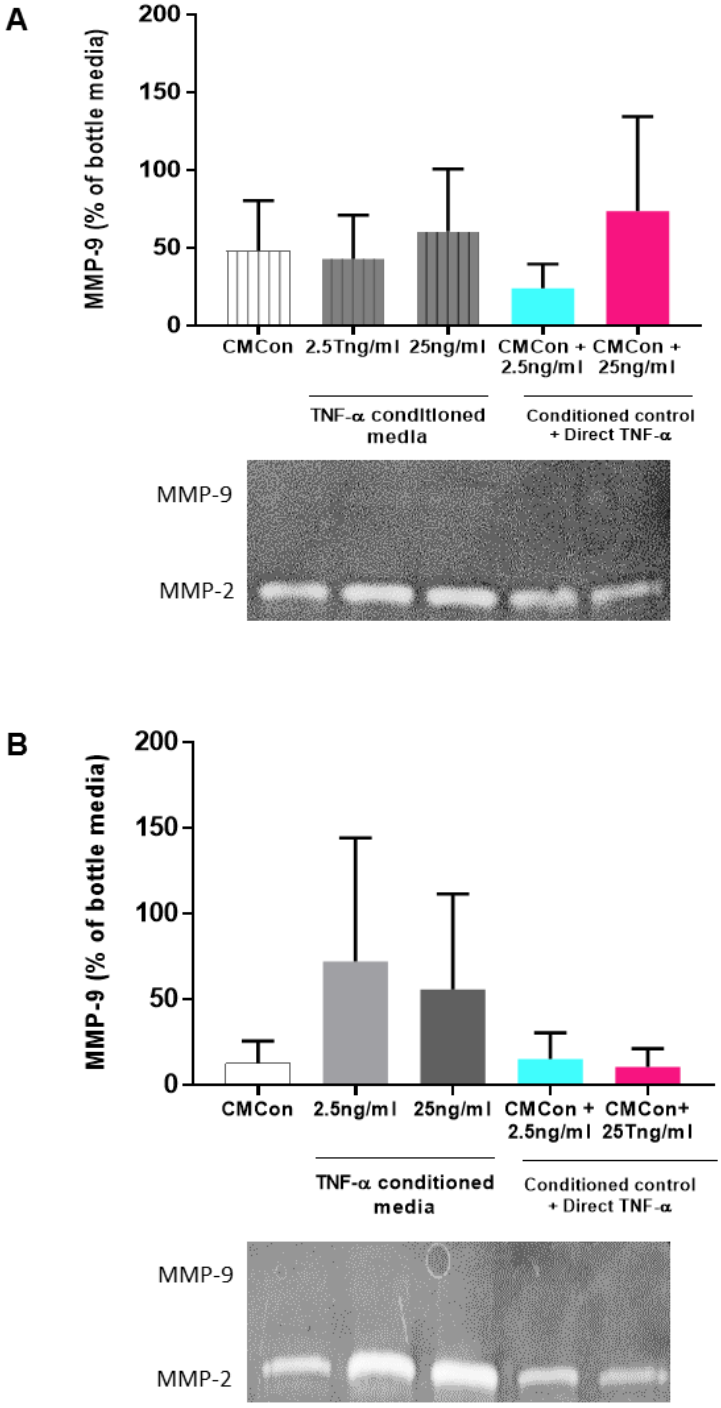


Figure 3-20: paracrine effect of TNF-α on MMP-9 secretion in healthy scratched and non-scratched dermal fibroblasts. Conditioned media was collected from scratched and non-scratched cells that had been cultured in the presence or absence of 2.5 or 25 ng/ml TNF-α for 24h. DFs, from female donors (n=6), facial skin, P3 and age range (36-67y). (A) Scratched DFs, TNF-α paracrine had no significant differences, one-way ANOVA, and (B) Non-scratched DFs, TNF-α paracrine had no significant differences, one-way ANOVA.

3-10: Changes in gene expression in response to TNF-α

3-10-1: The effect of TNF-α on MMP-1 mRNA expression in healthy and diabetic dermal fibroblasts

As TNF-α had functional effects on dermal fibroblasts (section 3-5 and 3-6) and induced the secretion of MMP (section 3-7 to 3-9), its ability to modulate the expression of a range of extracellular matrix and senescence genes was investigated. All RNA samples were of high concentration (12-34 ng/µl) and purity (260/280 ≥ 1.8, 260/230 ≥ 2).

In healthy non-scratched dermal fibroblasts, TNF-α significantly increased MMP-1 gene expression at both concentrations of TNF-α (2.5 and 25 ng/ml) compared to control. This increase in MMP-1 mRNA expression was also mirrored in scratched cells, but was not significant (Fig 3-21 A).

The effect of TNF-α on MMP-1 expression in diabetic dermal fibroblasts cultured under the same conditions was also investigated. Interestingly, diabetic control (basal) gene expression of MMP-1 was considerably higher than that seen in healthy cells; 1637% vs 235% (scratched cells) and 1669% vs 478 % (non-scratched cells) (Fig 3-21 A and B).

In diabetic dermal fibroblasts, under both conditions, MMP-1 expression with TNF-α stimulation was higher in a dose-dependent manner but this was only significant at the highest concentration of TNF-α in diabetic scratched cells (Fig 3-21 B).

In order to determine whether gender plays a role in MMP-1 gene expression in response to TNF-α in diabetic patients, the effect of TNF-α on dermal fibroblasts derived from female and male patients was compared (Fig 3-21 C and D). In diabetic male dermal fibroblasts (n=2) MMP-1 gene expression was higher than the controls at the highest concentration of TNF-α for both non-scratched and scratched cells but not significantly so. In diabetic female dermal fibroblasts (n=2), the effect of TNF-α on MMP-1 expression under both culture conditions was more variable and no significant differences were seen.

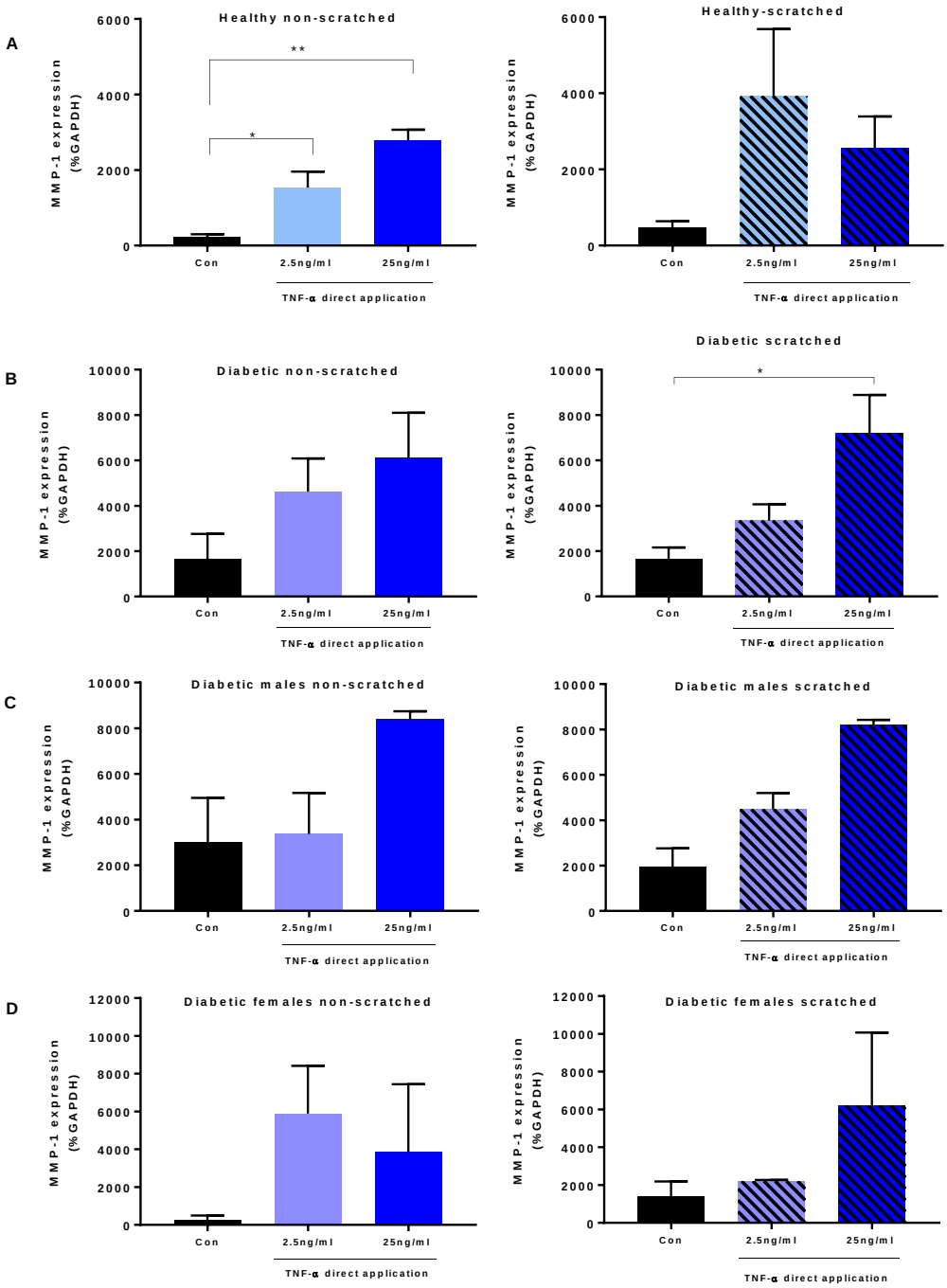


Figure 3-21: Effect of scratching and TNF-α incubation on MMP-1 mRNA expression. DFs were incubated in the presence and absence of TNF-α for 24h at two different concentrations 2.5 or 25 ng/ml. DFs were either scratched or parallel DFs were left unscratched (intact) for healthy donors and diabetic patients before RNA was extracted. (A) Expression levels of MMP-1 in healthy cells comparing scratched and non-scratched, in the presence of TNF-α (2.5 or 25 ng/ml), n=5 female donors, facial skin, P3, (36-67y). (B) Diabetic DFs from 2males/2females (n=4), P3, lower leg, (52-66y), and (C) The effect of TNF-α on MMP-1 gene expression in DFs from male diabetic patients (n=2), and (D) in DFs from female diabetic patients (n=2). *p<0.05 and **p<0.01, one-way ANOVA.

3-10-2: The effect of TNF-α on MMP-2 mRNA expression in healthy and diabetic dermal fibroblasts

MMP-2 gene expression was analysed as TNF-α had previously been shown to modulate protein expression by zymography (section 3-8). In healthy dermal fibroblasts, TNF-α had no significant effect on MMP-2 expression under both cell conditions at either concentration of TNF-α (Fig 3-22 A).

In diabetic dermal fibroblasts, TNF-α caused no statistically significant difference in MMP-2 expression in both non-scratched and scratched cells (Fig 3-22 B). In non-scratched cells, (basal) MMP-2 gene expression was higher in diabetic cells compared to healthy cells (1936% vs. 650%, respectively). Noticeably, MMP-2 expression in both healthy basal was (2103% control scratched vs. Less than 1000%, control non-scratched) (Fig 3-22 A and B).

No significant differences have observed on MMP-2 mRNA expression in both non-scratched and scratched cells of both genders (Fig 3-22 C and D).

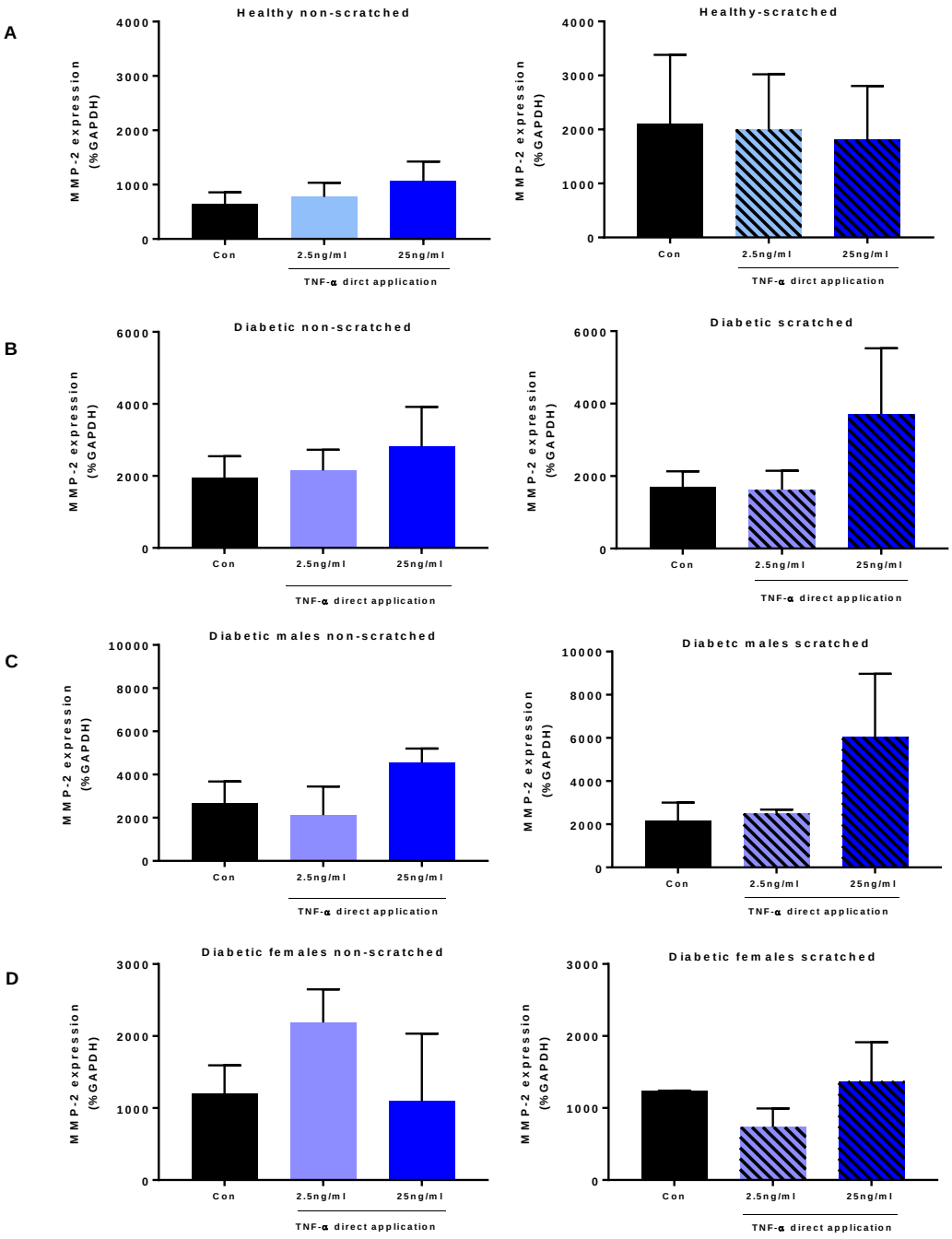


Figure 3-22: Effect of scratching and TNF-α incubation on MMP-2 mRNA expression. DFs were incubated in the presence and absence of TNF-α for 24h at two different concentrations 2.5 or 25 ng/ml. DFs were either scratched or parallel DFs were left unscratched (intact) for healthy donors and diabetic patients. (A) Expression levels of MMP-2 in healthy cells comparing scratched and non-scratched, in the presence of TNF-α (2.5 or 25 ng/ml), n=5 female donors, facial skin, P3, (36-67y). (B) Diabetic DFs from 2males/2females (n=4), P3, lower leg, (52-66y), and (C) The effect of TNF-α on MMP-2 gene expression in DFs from male diabetic patients (n=2), and (D) in DFs from female diabetic patients (n=2). All non-significant, one-way ANOVA.

3-10-3: The effect of TNF-α on MMP-9 mRNA expression in healthy and diabetic dermal fibroblasts

In healthy dermal fibroblasts, there was no statistically significant upregulation in MMP-9 mRNA expression TNF-α in non-scratched and scratched cells (Fig 3-23 A).

In diabetic dermal fibroblasts, MMP-9 mRNA expression was higher than in the controls, in both scratched and non-scratched cells, but did not reach statistical significance (Fig 3-23 B). There was no difference in basal MMP-9 mRNA expression between diabetic and healthy controls under either condition (Fig 3-23 A and B).

TNF-α had no effect on MMP-9 expression in male and female diabetic cells, under both conditions (Fig 3-23 C and D).

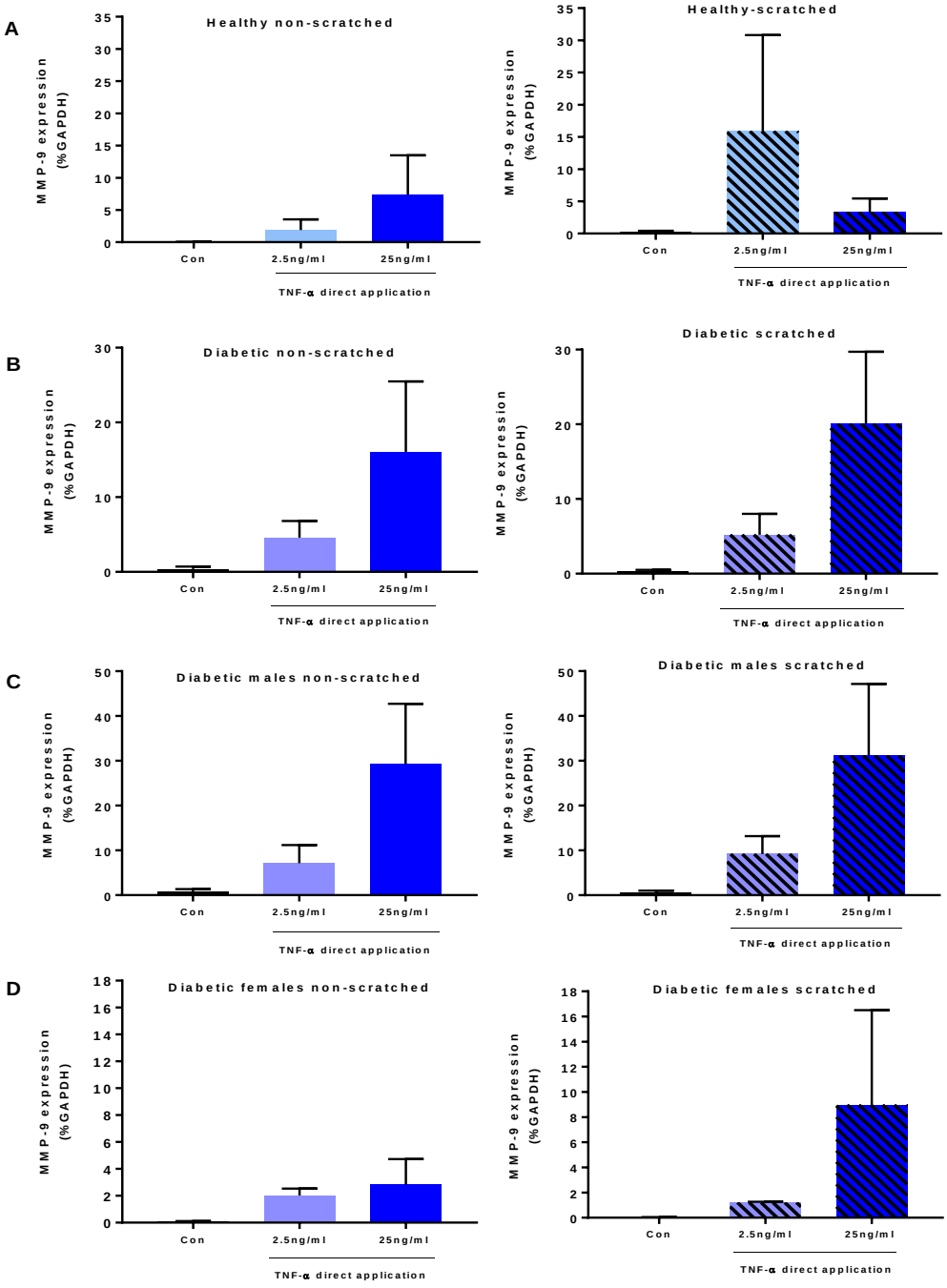


Figure 3-23: Effect of scratching and TNF-α incubation on MMP-9 mRNA expression. DFs were incubated in the presence and absence of TNF-α for 24h at two different concentrations 2.5 or 25 ng/ml. DFs were either scratched or parallel DFs were left unscratched (intact) for healthy donors and diabetic patients. (A) Expression levels of MMP-9 in healthy cells comparing scratched and non-scratched, in the presence of TNF-α (2.5 or 25 ng/ml), n=5 female donors, facial skin, P3, (36-67y). (B) Diabetic DFs from 2males/2females (n=4), P3, lower leg, (52-66y), and (C) The effect of TNF-α on MMP-9 gene expression in DFs from male diabetic patients (n=2), and (D) in DFs from female diabetic patients (n=2). All non-significant, one-way ANOVA.

3-10-4: The effect of TNF-α on TIMP-1 mRNA expression in healthy and diabetic dermal fibroblasts

In healthy dermal fibroblasts, TNF-α significantly increased TIMP-1 mRNA expression by (234%) at a concentration of 25 ng/ml in non-scratched cells. Although a similar effect of TNF-α (225%) was observed at a 2.5 ng/ml concentration in scratched cells, it was not significant (Fig 3-24 A).

In diabetic dermal fibroblasts, TNF-α did not significantly alter TIMP-1 expression in non-scratched cells (Fig 3-24 B). Control (basal) expression was higher in diabetic fibroblasts compared to healthy control fibroblasts under non-scratched conditions (1028% vs. 307%). Nevertheless, this difference was not apparent in scratched fibroblasts since TIMP-1 mRNA expression in diabetic fibroblasts was similar to that in non-diabetic cells (Fig 3-24 A and B).

When the diabetic fibroblasts were split by gender, no significant differences were found but the sample size was only n = 2 (Fig 3-24 C and D).

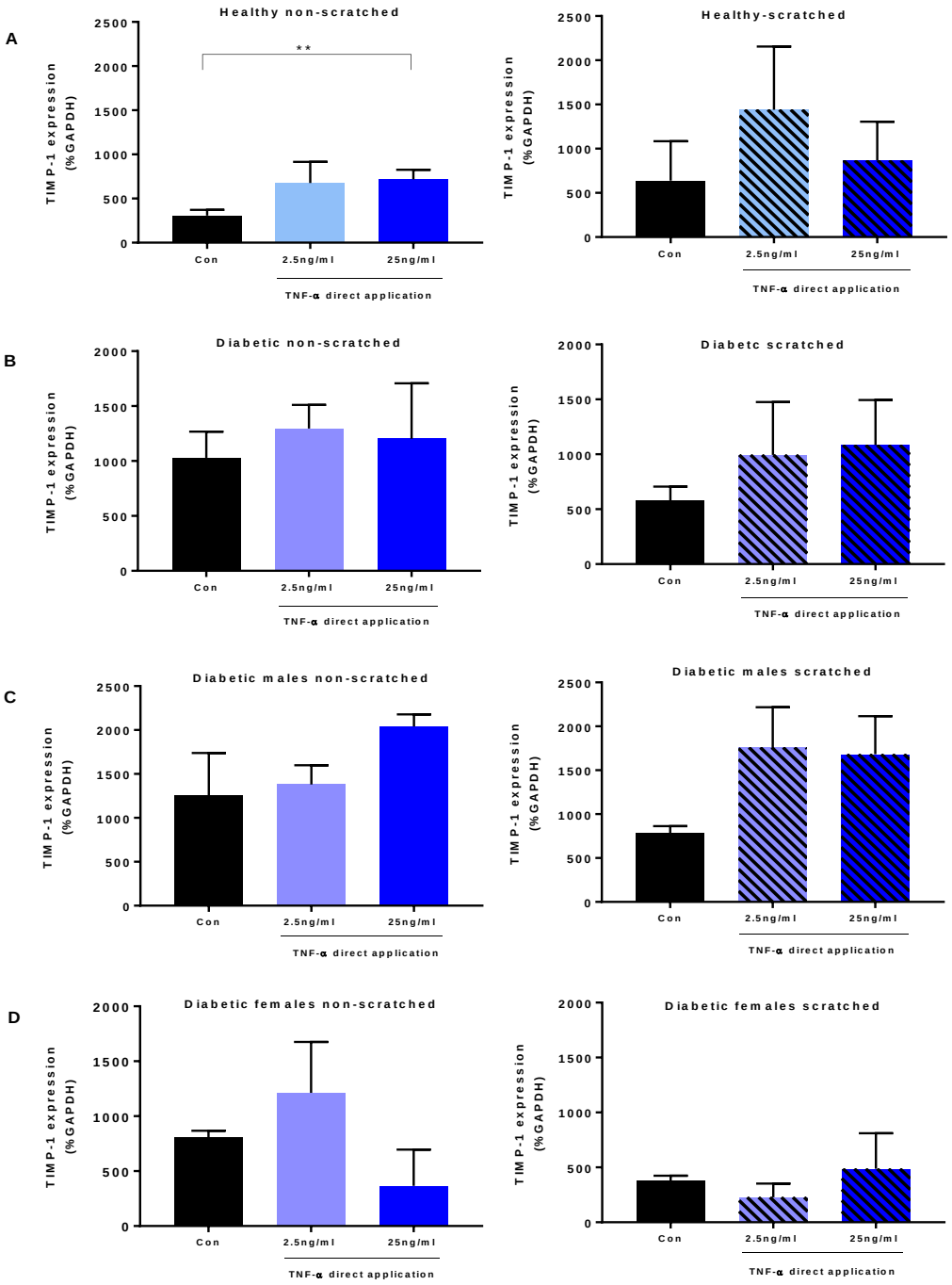


Figure 3-24: Effect of scratching and TNF-α incubation on TIMP-1 mRNA expression. DFs were incubated in the presence and absence of TNF-α for 24h at two different concentrations 2.5 or 25 ng/ml. DFs were either scratched or parallel DFs were left unscratched (intact) for healthy donors and diabetic patients. (A) Expression levels of TIMP-1 in healthy cells comparing scratched and non-scratched, in the presence of TNF-α (2.5 or 25 ng/ml), n=5 female donors, facial skin, P3, (36-67y). (B) Diabetic DFs from 2males/2females (n=4), P3, lower leg, (52-66y), and (C) The effect of TNF-α on TIMP-1 gene expression in DFs from male diabetic patients (n=2), and (D) in DFs from female diabetic patients (n=2). **p<0.01. All rest non-significant, one-way ANOVA.

3-10-5: The effect of TNF-α on TIMP-2 mRNA expression in healthy and diabetic dermal fibroblasts

In healthy dermal fibroblasts, whether scratched or non-scratched conditions, TNF-α had no statistically significant effect on TIMP-2 expression (Fig 3-25 A).

Similarly, in diabetic dermal fibroblasts, under non-scratched and scratched conditions (Fig 3-25 B), there was no significant effect of TNF-α. The expression of TIMP-2 in the diabetic control dermal fibroblasts was higher than the healthy dermal fibroblasts under both conditions (686% vs. 509%) in non-scratched, and in scratched cells (641% vs. 340%) (Fig 3-25 A and B).

As with TIMP-1 expression, no gender differences could be demonstrated but the sample size was only n = 2 (Fig 3-25 C and D).

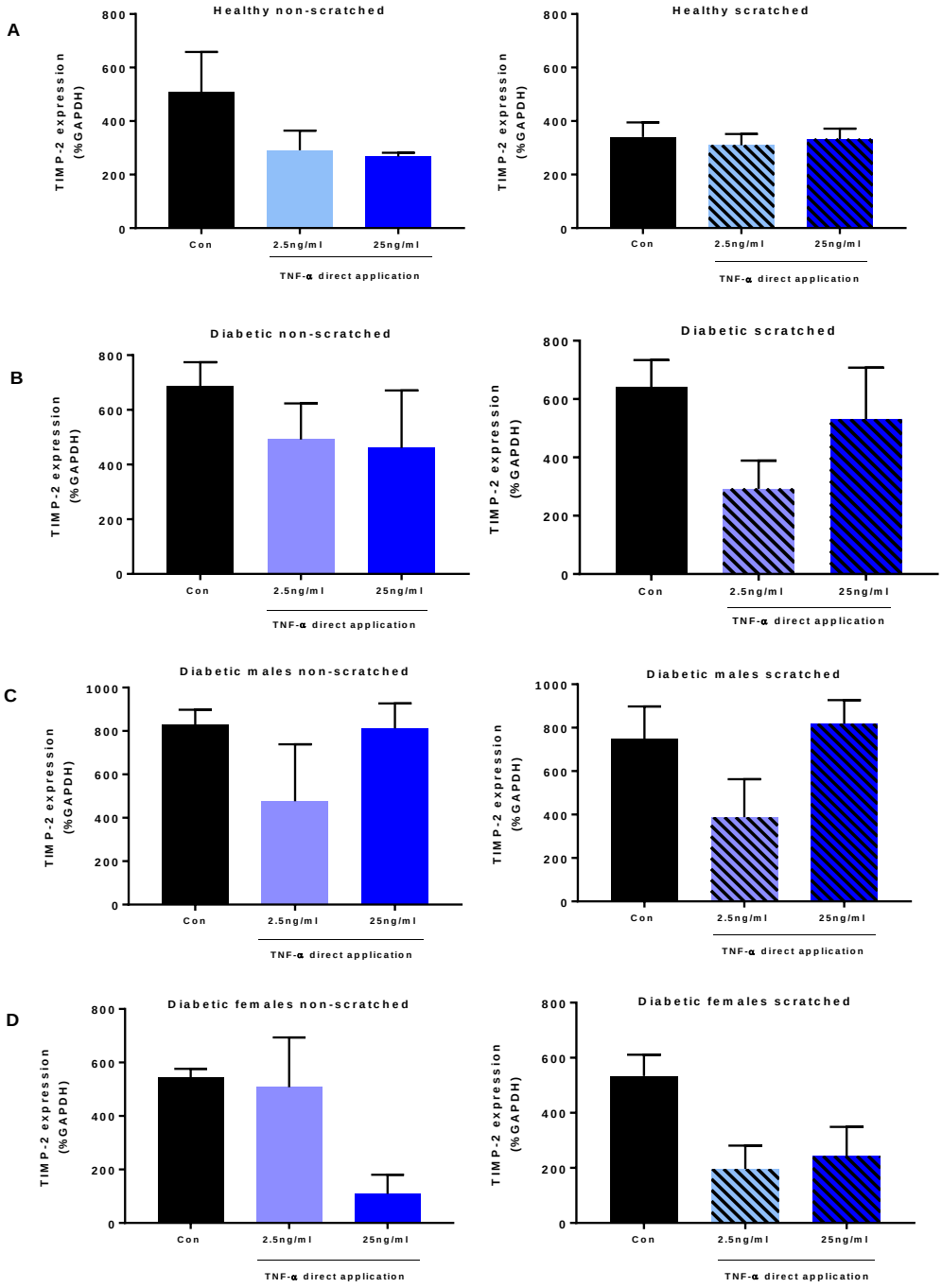


Figure 3-25: Effect of scratching and TNF-α incubation on TIMP-2 mRNA expression. DFs were incubated in the presence and absence of TNF-α for 24h at two different concentrations 2.5 or 25 ng/ml. DFs were either scratched or parallel DFs were left unscratched (intact) for healthy donors and diabetic patients. (A) Expression levels of TIMP-2 in healthy cells comparing scratched and non-scratched, in the presence of TNF-α (2.5 or 25 ng/ml), n=5 female donors, facial skin, P3, (36-67y). (B) Diabetic DFs from 2males/2females (n=4), P3, lower leg, (52-66y), and (C) The effect of TNF-α on TIMP-2 gene expression in DFs from male diabetic patients (n=2), and (D) in DFs from female diabetic patients (n=2). All non-significant, one-way ANOVA.

3-10-6: The effect of TNF-α on Sirt1 mRNA expression in healthy and diabetic dermal fibroblasts

In healthy dermal fibroblasts, TNF-α had no effect on Sirt1 mRNA expression in either scratched or non-scratched dermal fibroblasts (Fig 3-26 A).

In diabetic dermal fibroblasts, TNF-α also had no significant effect on Sirt1 expression under both conditions (Fig 3-26 B). The diabetic control cells expressed at least double the levels of Sirt1 mRNA compared to healthy control dermal fibroblasts under both conditions (7% vs. 3.6%) in non-scratched cells, and (6% vs. 2%) in scratched cells (Fig 3-26 A and B).

No significant observations in diabetic male and female dermal fibroblasts in Sirt1 expression were made. The pattern of Sirt1 mRNA in response to TNF-α under both conditions was similar, but had a sample size of n=2 (Fig 3-26 C and D).

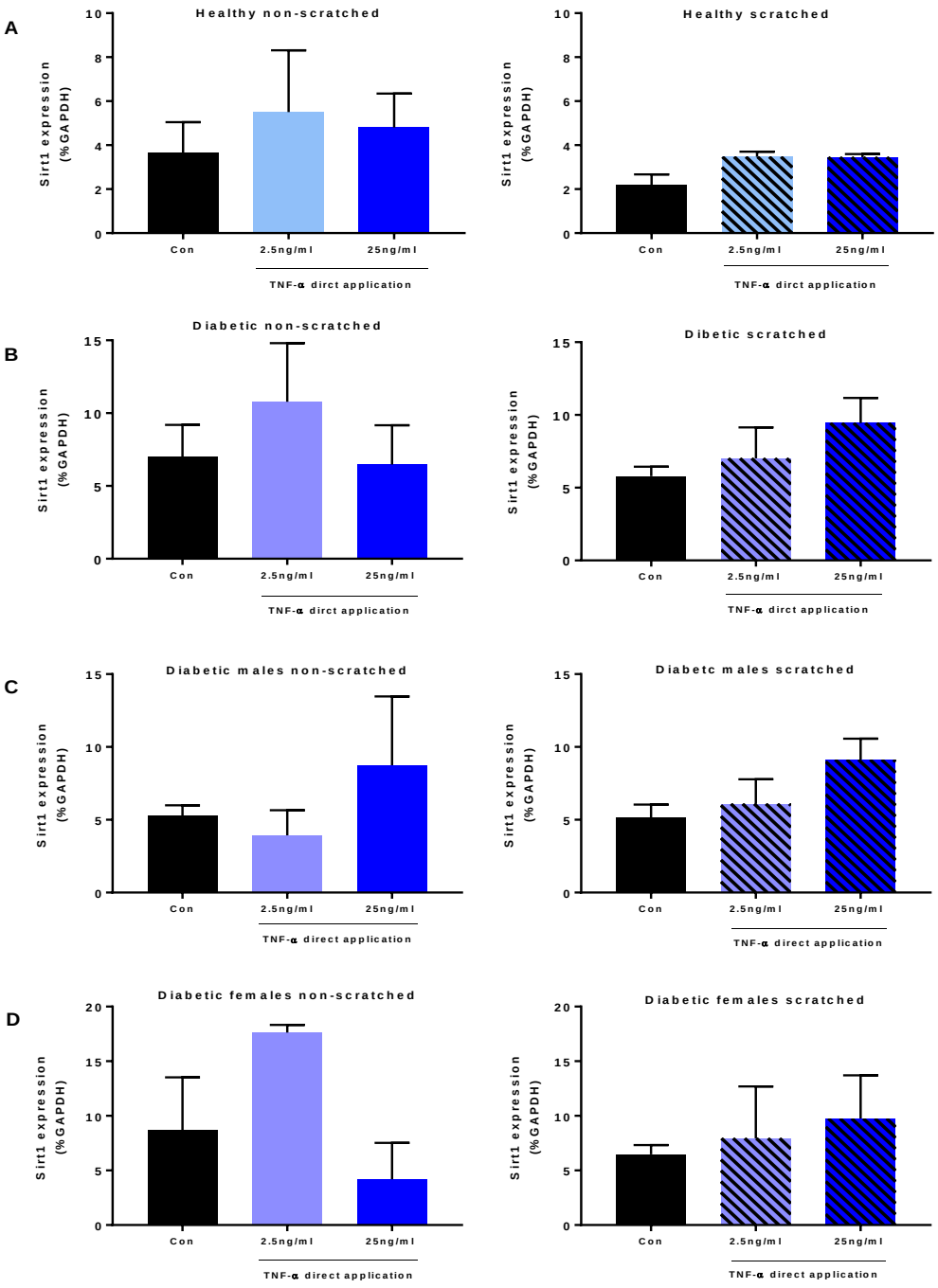


Figure 3-26: Effect of scratching and TNF-α incubation on Sirt1 mRNA expression. DFs were incubated in the presence and absence of TNF-α for 24h at two different concentrations 2.5 or 25 ng/ml. DFs were either scratched or parallel DFs were left unscratched (intact) for healthy donors and diabetic patients. (A) Expression levels of Sirt1 in healthy cells comparing scratched and non-scratched, in the presence of TNF-α (2.5 or 25 ng/ml), n=5 female donors, facial skin, P3, (36-67y). (B) Diabetic DFs from 2males/2females (n=4), P3, lower leg, (52-66y), and (C) The effect of TNF-α on Sirt1 gene expression in DFs from male diabetic patients (n=2), and (D) in DFs from female diabetic patients (n=2). All non-significant, one-way ANOVA.

3-10-7: The effect of TNF-α on Sirt6 mRNA expression in healthy and diabetic dermal fibroblasts

In healthy dermal fibroblasts, TNF-α had no effect on Sirt6 mRNA expression under non-scratched and scratched conditions (Fig 3-27 A).

Similarly, in diabetic dermal fibroblasts, TNF-α had no effect on Sirt6 mRNA expression under both conditions (Fig 3-27 B). As for Sirt1 expression, the control (basal) expression in diabetic cells was higher than in healthy dermal fibroblast under both conditions (5% vs 2%) in non-scratched cells, and (4% vs 1.3%) in scratched cells (Fig 3-27 A and B).

In both gender cells no significant effect was observed on Sirt6 expression by TNF-α under both conditions (Fig 3-27 C and D).

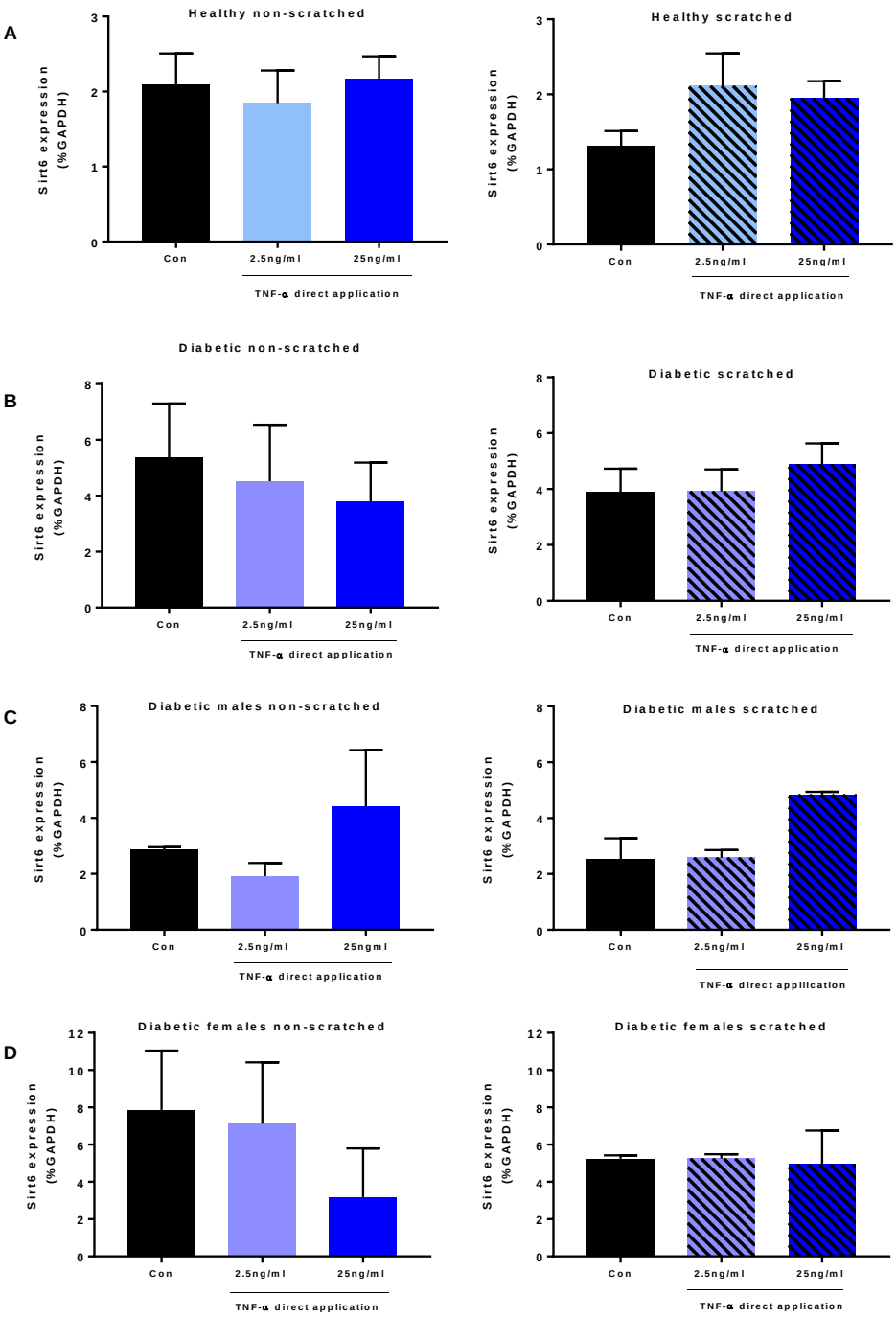


Figure 3-27: Effect of scratching and TNF-α incubation on Sirt6 mRNA expression. DFs were incubated in the presence and absence of TNF-α for 24h at two different concentrations 2.5 or 25 ng/ml. DFs were either scratched or parallel DFs were left unscratched (intact) for healthy donors and diabetic patients. (A) Expression levels of Sirt6 in healthy cells comparing scratched and non-scratched, in the presence of TNF-α (2.5 or 25 ng/ml), n=5 female donors, facial skin, P3, (36-67y). (B) Diabetic DFs from 2males/2females (n=4), P3, lower leg, (52-66y), and (C) The effect of TNF-α on Sirt6 gene expression in DFs from male diabetic patients (n=2), and (D) in DFs from female diabetic patients (n=2). All rest non-significant, one-way ANOVA.

3-11: Paracrine effect of TNF-α on MMP-1, MMP-2 and MMP-9 mRNA expression by healthy dermal fibroblasts

To determine whether TNF-α had an indirect effect on MMPs expression by stimulating soluble paracrine factors, the effect of media conditioned by dermal fibroblasts in the presence of TNF-α was investigated, in healthy dermal fibroblasts under both conditions; either with scratching and without scratching.

In non-scratched dermal fibroblasts, the expression of MMP-1 mRNA was several-fold higher with TNF-α conditioned media (25ng/ml) (Fig 3-28 A) but this was not statistically significant. Similar results were obtained when dermal fibroblasts were incubated directly with TNF-α (Fig 3-21 A). Adding TNF-α directly to the conditioned media induced a similar upregulating of MMP-1, indicating that TNF-α in the medium rather than secreted paracrine factors stimulated this response. A similar response observed in scratched dermal fibroblasts, but was significant (Fig 3-28 B).

There was no difference in MMP-2 mRNA expression in response to TNF-α conditioned media under either cell condition (Fig 3-29 A and B), which was similar to what was observed when dermal fibroblasts were incubated directly with TNF-α (Fig 3-22 A).

TNF-α conditioned media had no effect on MMP-9 mRNA expression, in either non-scratched or scratched dermal fibroblasts (Fig 3-30 A and B). Interestingly, there appeared to be an additive effect of TNF-α supplemented conditioned medium in a dose-dependent manner, but this was highly variable and did not achieve statistical significance (Fig 3-30 A and B).

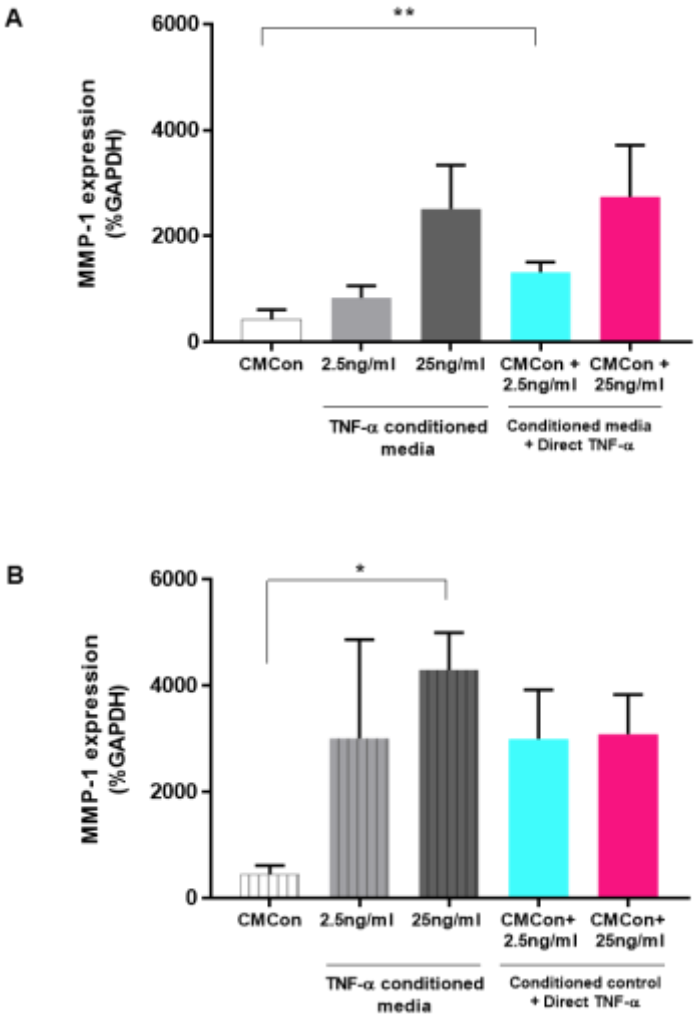


Figure 3-28: Effect of scratching and TNF-α paracrine incubation on MMP-1 mRNA expression. DFs were incubated in the presence or absence of TNF-α for 24h at two different concentrations 2.5 or 25 ng/ml. DFs were scratched and a parallel DFs were left unscratched (intact), (n=5) female donors, facial skin, P3, age range (36-67y). (A) Expression levels of MMP-1 in healthy non-scratched cells, and (B) in healthy scratched cells. *p<0.05 and **p<0.01, one-way ANOVA.

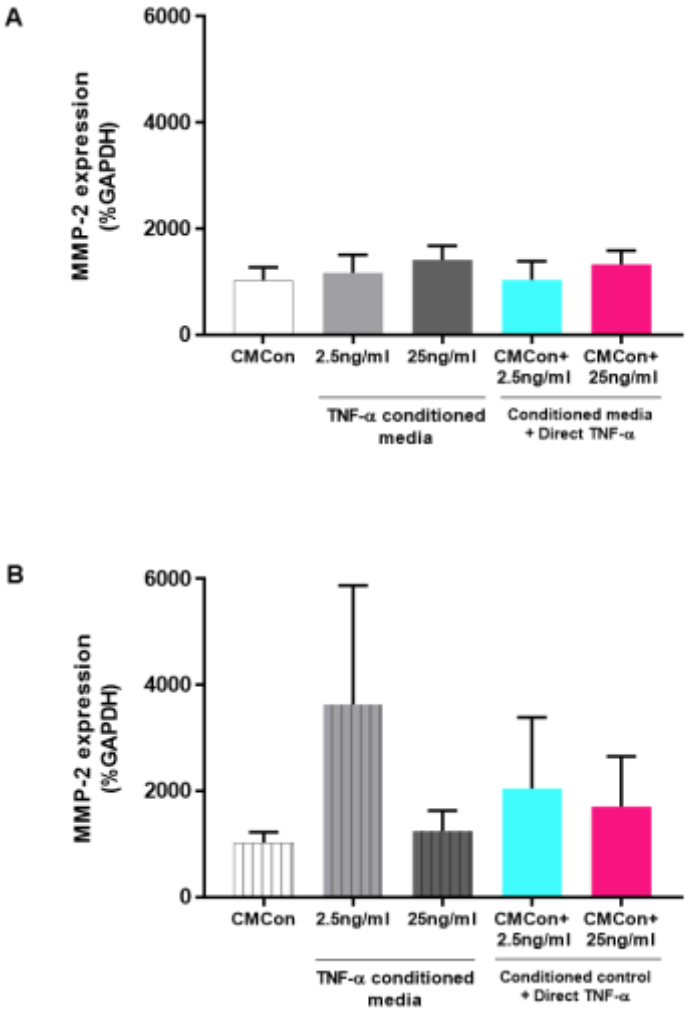


Figure 3-29: Effect of scratching and TNF-α paracrine incubation on MMP-2 mRNA expression. DFs were incubated in the presence or absence of TNF-α for 24h at two different concentrations 2.5 or 25 ng/ml. DFs were scratched and a parallel DFs were left unscratched (intact), (n=5) female donors, facial skin, P3, age range (36-67y). (A) Expression levels of MMP-2 in healthy non-scratched cells, and (B) in healthy scratched cells. All non-significant, one-way ANOVA.

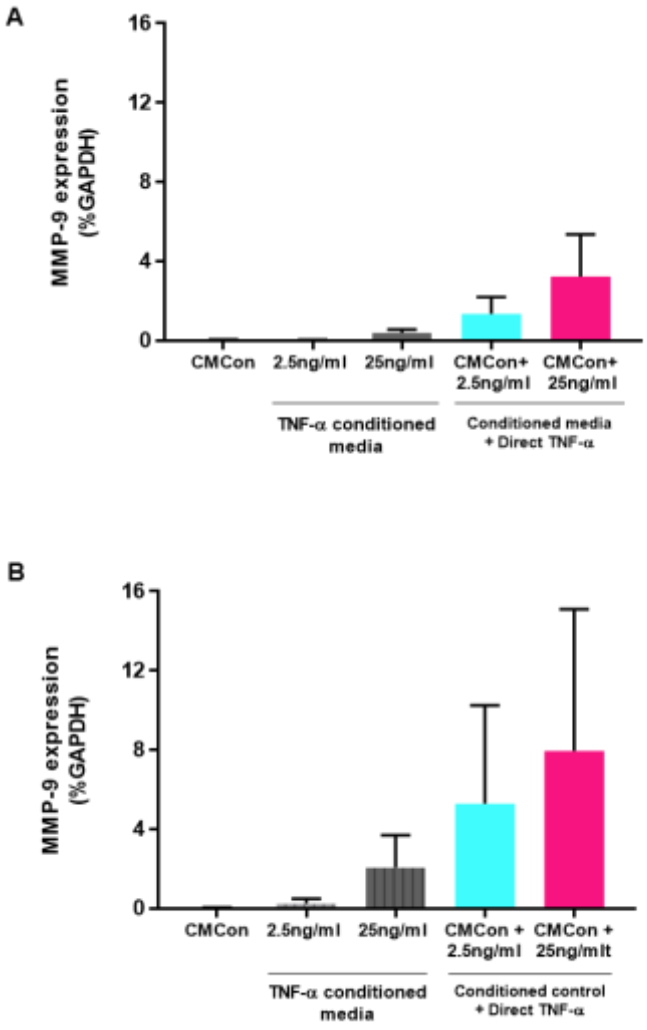


Figure 3-30: Effect of scratching and TNF-α paracrine incubation on MMP-9 mRNA expression. DFs were incubated in the presence or absence of TNF-α for 24h at two different concentrations 2.5 or 25 ng/ml. DFs were scratched and a parallel DFs were left unscratched (intact), (n=5) female donors, facial skin, P3, age range (36-67y). (A) Expression levels of MMP-9 in healthy non-scratched cells, and (B) in healthy scratched cells. All non-significant, one-way ANOVA.

3-12: Paracrine effect of TNF-α on TIMP-1 and TIMP-2 mRNA expression by healthy dermal fibroblasts

The pattern of expression of TIMP-1 mRNA in response to TNF-α conditioned media (Fig 3-31 A) was similar to that seen when TNF-α was added directly (Fig 3-24 A). Supplementation of control conditioned media with TNF-α similarly had no effect. In scratched cells, TNF-α conditioned media also showed no significant effect on TIMP-1 mRNA expression (Fig 3-31 B).

TNF-α conditioned media did not significantly affect TIMP-2 expression at both concentrations in non-scratched cells. Supplementation of control conditioned media with fresh TNF-α had a similar effect on TIMP-2 expression (Fig 3-32 A). This pattern was similar to that observed when TNF-α was added directly to dermal fibroblasts (Fig 3-25 A). There was no change in TIMP-2 expression in response to TNF-α in scratched cells (Fig 3-32 B), which again was similar to the response of scratched dermal fibroblasts when TNF-α was added directly (Fig 3-25 A).

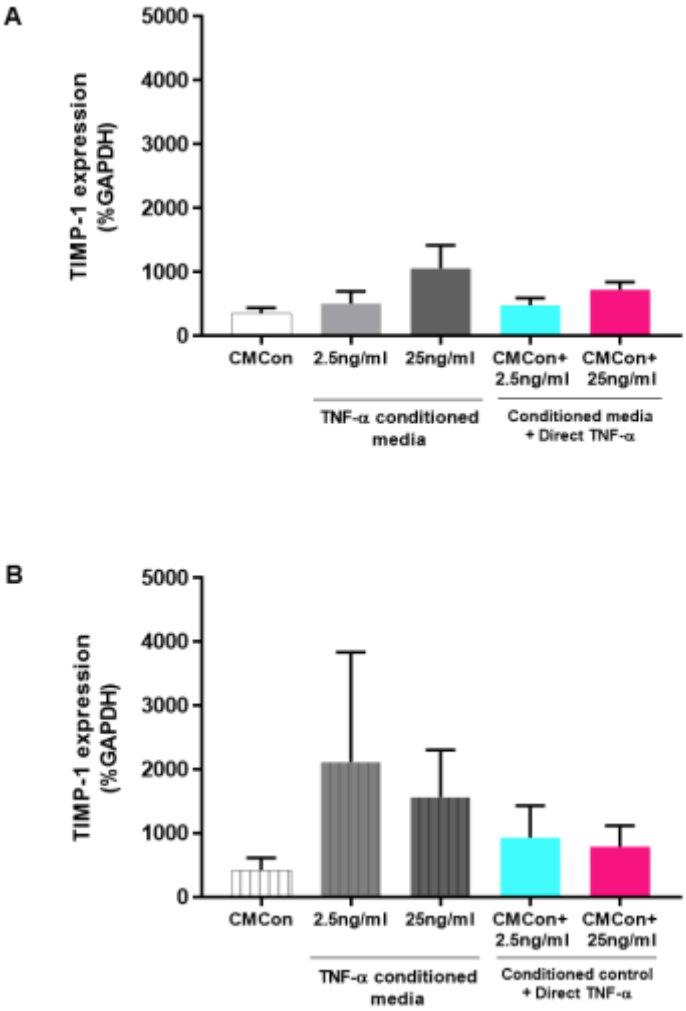


Figure 3-31: Effect of scratching and TNF-α paracrine incubation on TIMP-1 mRNA expression. DFs were incubated in the presence or absence of TNF-α for 24h at two different concentrations 2.5 or 25 ng/ml. DFs were scratched and a parallel DFs were left unscratched (intact), (n=5) female donors, facial skin, P3, age range (36-67y). (A) Expression levels of TIMP-1 in healthy non-scratched cells, and (B) in healthy scratched cells. All non-significant, one-way ANOVA.

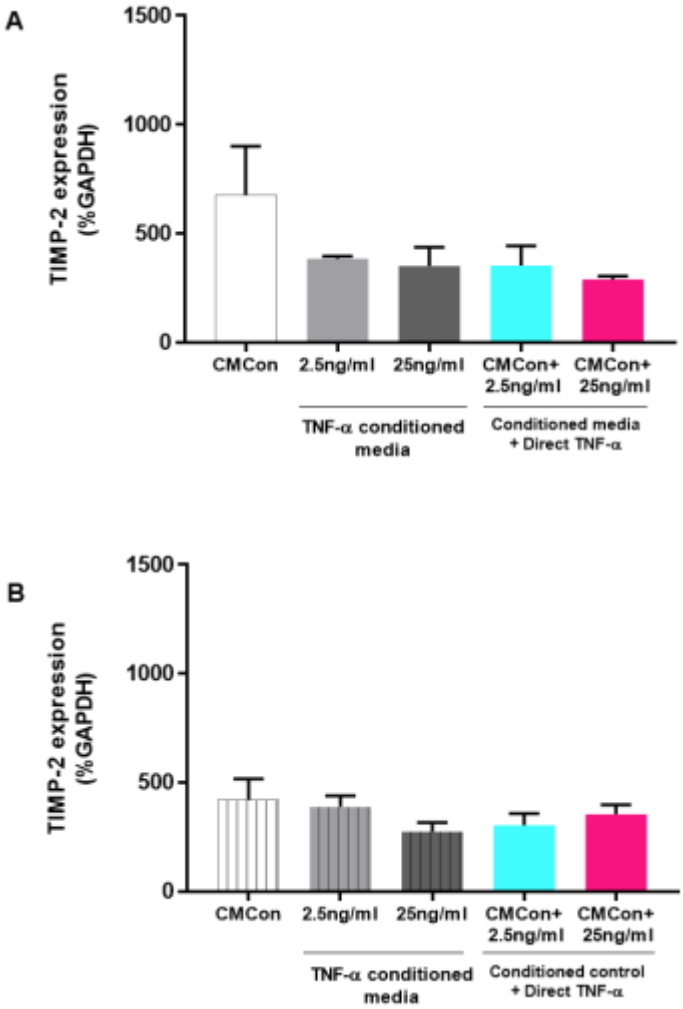


Figure 3-32: Effect of scratching and TNF-α paracrine incubation on TIMP-2 mRNA expression. DFs were incubated in the presence or absence of TNF-α for 24h at two different concentrations 2.5 or 25 ng/ml. DFs were scratched and a parallel DFs were left unscratched (intact), (n=5) female donors, facial skin, P3, age range (36-67y). (A) Expression levels of TIMP-2 in healthy non-scratched cells, and (B) in healthy scratched cells. All non-significant, one-way ANOVA.

3-13: Paracrine effect of TNF-α on Sirt1 and Sirt6 mRNA expression by healthy dermal fibroblasts

TNF-α conditioned media had no significant effect on Sirt1 gene expression at both concentrations, in non-scratched cells. The non-significance is due to high donor variability so either increasing sample size or repeating expression assay may confirm any biological effect of TNF-α conditioned media on Sirt1 expression). Supplementation of control conditioned media with TNF-α also had no statistically significant effect (Fig 3-33 A). In scratched cells, there was no change in Sirt1 expression under any conditions (Fig 3-33 B), which was similar to the results seen when TNF-α was added directly (Fig 3-26 A).

There was no paracrine effect of TNF-α on Sirt6 expression either in non-scratched cells or scratched cells (Fig 3-34 A and B). This was similar to the results in dermal fibroblasts when TNF-α was added directly (Fig 3-27 A).

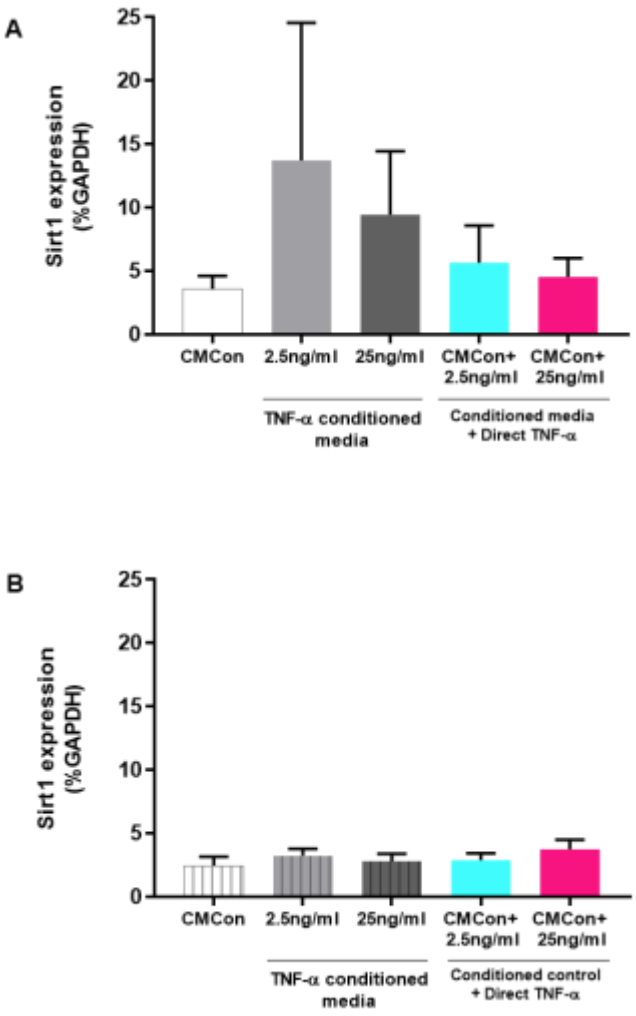


Figure 3-33: Effect of scratching and TNF-α paracrine incubation on Sirt1 mRNA expression. DFs were incubated in the presence or absence of TNF-α for 24h at two different concentrations 2.5 or 25 ng/ml. DFs were scratched and a parallel DFs were left unscratched (intact), (n=5) female donors, facial skin, P3, age range (36-67y). (A) Expression levels of Sirt1 in healthy non-scratched cells, and (B) in healthy scratched cells. All non-significant, one-way ANOVA.

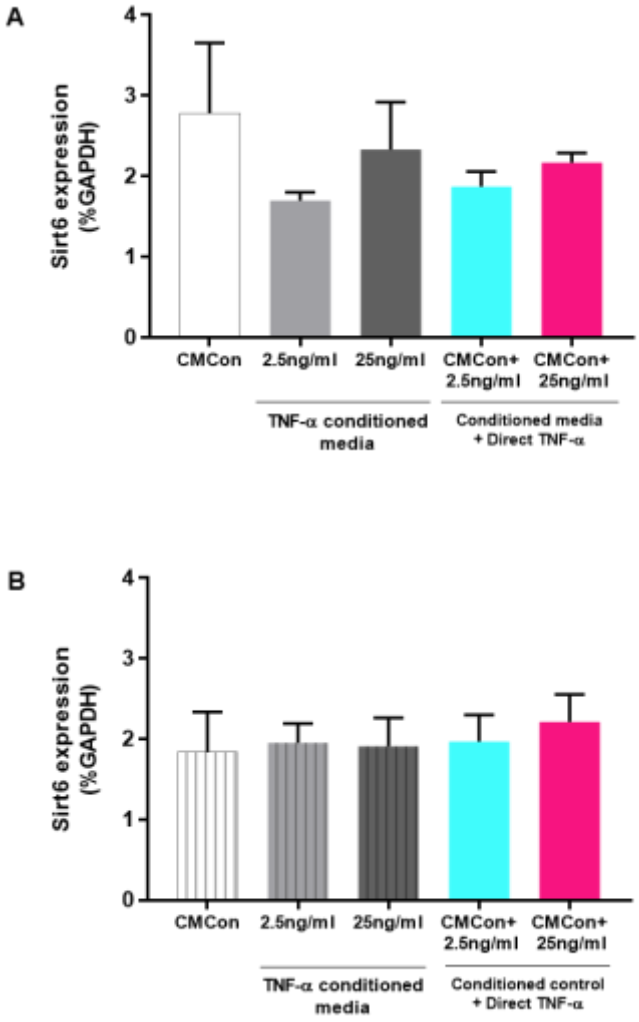


Figure 3-34: Effect of scratching and TNF-α paracrine incubation on Sirt6 mRNA expression. DFs were incubated in the presence or absence of TNF-α for 24h at two different concentrations 2.5 or 25 ng/ml. DFs were scratched and a parallel DFs were left unscratched (intact), (n=5) female donors, facial skin, P3, age range (36-67y). (A) Expression levels of Sirt6 in healthy non-scratched cells, and (B) in healthy scratched cells. All non-significant, one-way ANOVA.

3-14: The effect of TNF-α, or TNF-α induced paracrine factors on cytokine release by healthy and diabetic dermal fibroblasts

The release of cytokines, growth factors, and chemokines by cultured dermal fibroblasts in response to TNF-α was quantitated 24h after scratching. The Proteome Profiler Array measures 36 different cytokines as described in Table 3-1. The description of these cytokines and representative images of their expression are in (Appendix 8-4). In healthy dermal fibroblasts, under normal conditions, the secretion of 7/36 cytokines (CCL2/MCP-1, CXCL1/GROα, CXCL12/SDF-1, IL-6, IL-8, MIF and Serpin E1/PAI-1) were detected. The same seven cytokines were detected in diabetic male dermal fibroblasts under basal conditions, while in diabetic female dermal fibroblasts, the cytokines (CCL2/MCP-1 and IL-6) were not detected (see section 4-13).

In healthy dermal fibroblasts, the presence of 2.5 ng/ml TNF-α stimulated the secretion of an additional five cytokines (MIP-1α/ MIP-1β, G-CSF, GM-CSF, IL-32α and TNF-α), which were not detected under basal conditions, Table 3-1 and (Fig 3-35 A). In addition, TNF-α appeared to stimulate an increase in the secretion of CXCL1 (87.45%) and IL-6 (207.13%), in contrast, it seemed to decrease MIF secretion by 68.51% (Fig 3-35 A) (see section 4-13).

In female diabetic dermal fibroblasts, TNF-α induced the secretion of a further 6 cytokines that were not detected under its basal condition. These were (CCL2/MCP-1, CCL5/RANTES, ICAM-1/CD54, GM-CSF, IL-6, and TNF-α), Table 3-1 and (Fig 3-35 B). In addition, TNF-α further increased secretion of CXCL1/GROα by 52.01%, but decreased secretion of MIF and Serpin E1/PAI-1 by 83.64% and 49.1% respectively (Fig 3-35 B).

In male diabetic dermal fibroblasts, TNF-α also induced the secretion of ICAM-1/CD54 and TNF-α, which were not detected under basal conditions (Table 3-1). However, in contrast to female diabetic dermal fibroblasts, TNF-α did not induce secretion of GM-CSF or CCL5/RANTES, (Table 3-1) and (Fig 3-35 C).

In a similar manner to female diabetic dermal fibroblasts, TNF-α further increased the secretion of CXCL1/GROα by 90.34%, and decreased secretion of MIF and Serpin E1/PAI-1 by 76.93% and 52.3% respectively in male diabetic dermal fibroblasts (Fig 3-35 C). However in contrast to female diabetic dermal fibroblasts, in male diabetic dermal fibroblasts, TNF-α induced CXCL12/SDF-1 secretion by 98.29% and stimulated secretion of IL-6 by 13.23% (Fig 3-35 C).

To investigate whether dermal fibroblasts paracrine factors modulate cytokine secretion, healthy dermal fibroblasts were incubated with dermal fibroblast media that had been conditioned in the presence or absence of TNF-α. There was no difference in cytokine secretion between control conditioned (CMCon) or TNF-α 2.5 ng/ml conditioned media. The recipient dermal fibroblasts secreted the same 7 cytokines (CCL2/MCP-1, CXCL1/GROα, CXCL12/SDF-1, IL-6, IL-8, MIF and Serpin E1/PAI-1) in the presence and absence of TNF-α, and there was no change in the level of secretion in the presence of TNF-α CM (Fig 3-36). In contrast to the direct effect of TNF-α on dermal fibroblasts, the TNF-α conditioned media did not stimulate the secretion of MIP-1α/ MIP-1β, G-CSF, GM-CSF, IL-32α or TNF-α (Table 3-1).

Cytokine, Chemokine and growth factor	Healthy donor ♀				Diabetic patient ♀		Diabetic patient ♂	
	Con 10% FBS	2.5ng/ml TNF-α	CMCon	2.5ng/ml TNF-α CM	Con 10% FBS	2.5ng/ml TNF-α	Con 10% FBS	2.5ng/ml TNF-α
CCL1/I-309	–	–	–	–	–	–	–	–
CCL2/MCP-1	✓	✓	✓	✓	–	✓	✓	✓
MIP-1α/MIP-1β	–	✓	–	–	–	–	–	–
CCL5/RANTES	–	–	–	–	–	✓	–	–
CD40Ligand/TNFSF5	–	–	–	–	–	–	–	–
Complement component C5/C5a	–	–	–	–	–	–	–	–
CXCL1/GROα	✓	✓	✓	✓	✓	✓	✓	✓
CXCL10/IP-10	–	–	–	–	–	–	–	–
CXCL11/I-TAC	–	–	–	–	–	–	–	–
CXCL12/SDF-1	✓	✓	✓	✓	✓	✓	✓	✓
G-CSF	–	✓	–	–	–	–	–	–
GM-CSF	–	✓	–	–	–	✓	–	–
ICAM-1/CD54	–	–	–	–	–	✓	–	✓
IFN-γ	–	–	–	–	–	–	–	–
IL-1α/IL-1F1	–	–	–	–	–	–	–	–
IL-1β/IL-1F2	–	–	–	–	–	–	–	–
IL-1ra/IL1-F3	–	–	–	–	–	–	–	–
IL-2	–	–	–	–	–	–	–	–
IL-4	–	–	–	–	–	–	–	–
IL-5	–	–	–	–	–	–	–	–
IL-6	✓	✓	✓	✓	–	✓	✓	✓
IL-8	✓	✓	✓	✓	✓	✓	✓	✓
IL-10	–	–	–	–	–	–	–	–

IL-12 p70	–	–	–	–	–	–	–	–
IL-13	–	–	–	–	–	–	–	–
IL-16	–	–	–	–	–	–	–	–
IL-17A	–	–	–	–	–	–	–	–
IL-17E	–	–	–	–	–	–	–	–
IL-18/IL-1F4	–	–	–	–	–	–	–	–
IL-21	–	–	–	–	–	–	–	–
IL-27	–	–	–	–	–	–	–	–
IL-32α	–	✓	–	–	–	–	–	–
MIF	✓	✓	✓	✓	✓	✓	✓	✓
Serpin E1/PAI-1	✓	✓	✓	✓	✓	✓	✓	✓
TNF-α	–	✓	–	–	–	✓	–	✓
TREM-1	–	–	–	–	–	–	–	–

Table 3-1: Cytokine, Chemokine, and growth factors were up-regulated and/or downregulated by control or 2.5 ng/ml or CMCon or 2.5 ng/ml CM by the healthy donor and diabetic patients DFs conditioned media

Human 36 Cytokine, Chemokine and growth factors, detected and non-detected. CCL1/I-309: Chemokine, (C-C motif) ligand1/ Human Cytokine I-309; CCL2/MCP-1: Chemokine, (C-C motif) ligand 2/ monocyte chemoattractant protein 1; MIP-1α/MIP-1β: Macrophage Inflammatory Proteins (α and β); CCL5/RANTES: Chemokine, C-C motif ligand 5/ RANTES; CD40Ligand/TNFSF5: Member of TNF-α superfamily/ CD154; Complement component C5/C5a: Complement component 5 (complement system); CXCL1/GROα: Chemokine, C-X-C motif ligand 1/GROα; CXCL10/IP-10: Chemokine, C-X-C motif ligand 10/ Interferon gamma-induced protein 10 (IP-10); CXCL11/I-TAC: Chemokine, C-X-C motif ligand 11/ Interferon-inducible T-cell alpha chemoattractant; CXCL12/SDF-1: Chemokine, C-X-C motif ligand 12/ stromal cell-derived factor 1 (SDF-1); G-CSF: Granulocyte-colony stimulating factor; GM-CSF: Granulocyte-macrophage colony-stimulating factor; ICAM-1/CD54: Intercellular Adhesion Molecule/CD54; IFN-γ: Interferon gamma; IL-1α/IL-1F1: Interleukin-1 alpha/ IL-1F1 Protein; IL-1β/IL-1F2: Interleukin-1 beta/ IL-1F2 Protein; IL-1ra/IL1-F3: Interleukin-1 receptor antagonist (IL-1RA); IL-2: Interleukin-2; IL-4: Interleukin-4; IL-5: Interleukin-5; IL-6: Interleukin-6; IL-8: Interleukin-8; IL-10: Interleukin-10; IL-12 p70: Interleukin-12/ heterodimer p70); IL-13: Interleukin-13; IL-16: Interleukin-16; IL-17A: Interleukin-17A; IL-17E: Interleukin-17E; IL-18/IL-1F4: Interleukin-18/ IL-1F4 Protein; IL-21: Interleukin-21; IL-27: Interleukin-27; IL-32α: Interleukin-32α; MIF: Macrophage migration inhibitory factor; Serpin E1/PAI-1: Serpin E1/Plasminogen activator inhibitor-1; TNF-α: Tumour necrosis factor-α; TREM-1: Triggering receptor expressed on myeloid cells 1. **The symbols ✓: detected, - : not detected.**

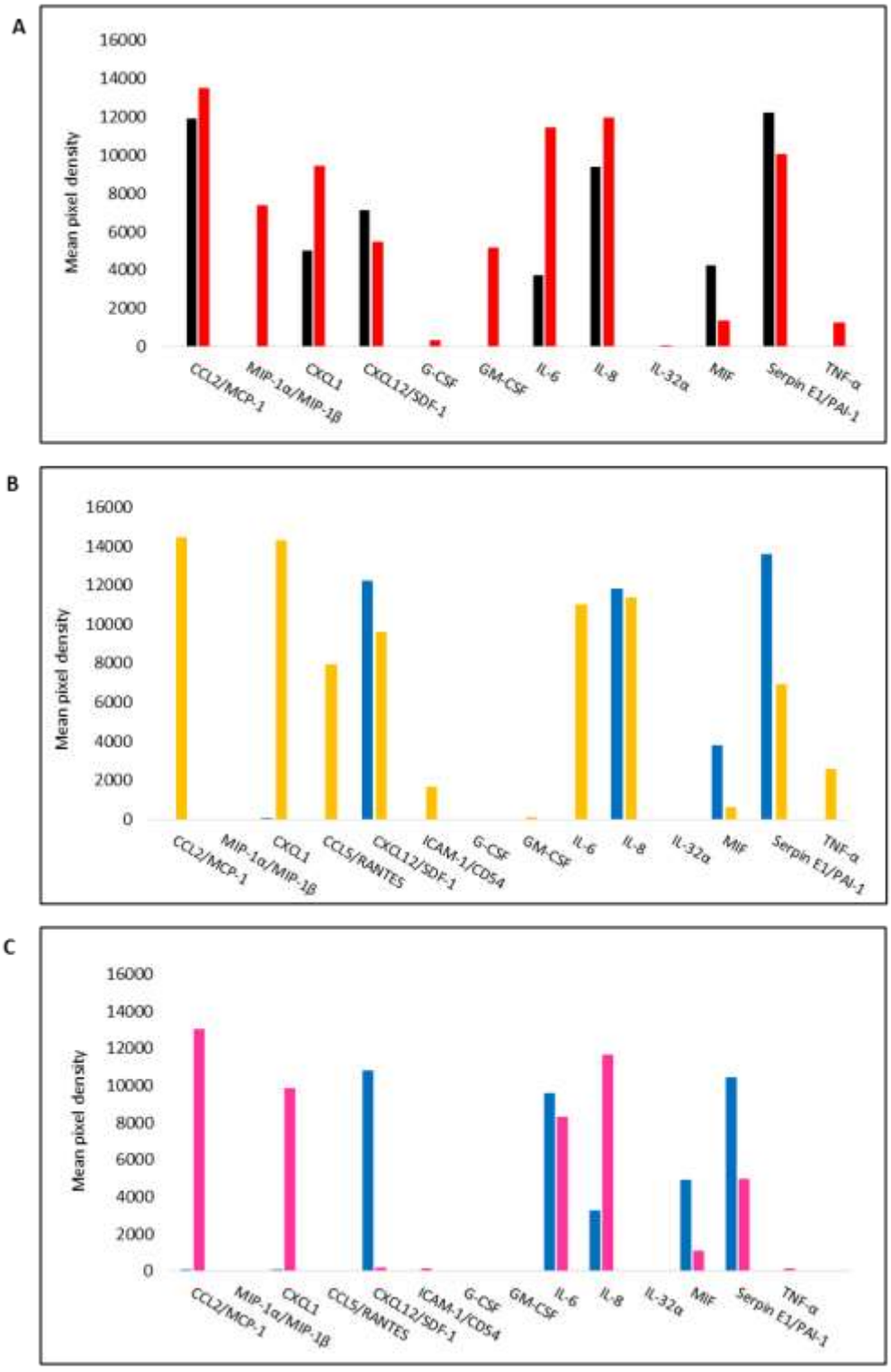


Figure 3-35: The effect of 2.5 ng/ml of TNF-α on the secretion of cytokines, chemokines, growth factors and soluble proteins by secreted dermal fibroblasts after 24h. Conditioned media collected from (migration assay 3-5), (A) Healthy female DFs, facial skin, P3 and age 56y. (B) Diabetic female DFs, and (C) Diabetic male DFs, lower leg, P3 and ages 65y. Displayed data represents the mean of duplicate spots pixel density. Dark and blue columns: control, other coloured columns: 2.5 TNF-α.

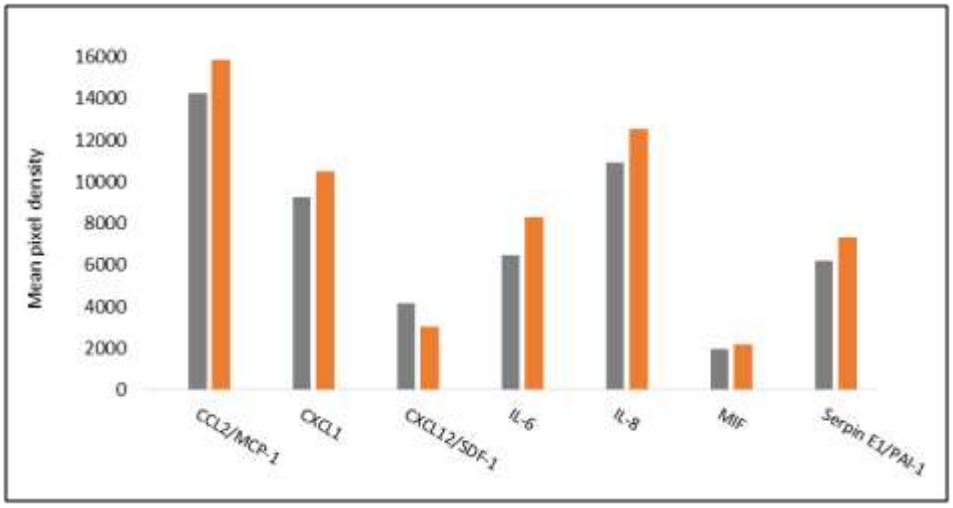


Figure 3-36: The paracrine effect of 2.5 ng/ml of TNF-α on the production of cytokines, chemokines, growth factors and soluble proteins in dermal fibroblasts conditioned media after 24h. Double conditioned media (conditioned media from healthy females DFs (n=2), facial skin, P7-8, age range (52-60y), was added to scratched DFs of the recipient). Double conditioned media was collected from (3-6 migration assay), female healthy recipient DFs, facial skin, P3 and age 56y and pooled. DFs were incubated in the presence and absence of TNF-α of 2.5 ng/ml CM for 24h. Displayed data represented the mean of duplicate spots pixel density. Grey columns: control, apricot columns: 2.5 TNF-α.

4 Discussion

4-1: Comparison of the rate proliferation of dermal fibroblasts in media supplemented with 2% or 10% FBS

In vivo, human dermal fibroblasts are not rapidly dividing cells; their primary function is to maintain the homeostasis of the dermis by collagen synthesis and modelling of the ECM (Ejiri *et al.,* 2014). However, during wound healing proliferation of dermal fibroblasts is increased and it is also well established that dermal fibroblasts can be stimulated to proliferate *in vitro* (Darby *et al.,* 2014; Chen *et al.,* 2014; Shi *et al.,* 2015). Fig 3-1, illustrates the proliferative response of dermal fibroblasts to serum, even in fibroblasts cultured from an older donor (age 77yrs). A concentration of 10% FBS stimulated proliferation significantly faster compared to 2% FBS after 6 days culture and continued to do so over a 12-day period.

The difference in cell proliferation in presence of 2% or 10% serum reflects the differences in the relative amounts of growth factors, carriers of water-insoluble nutrients, hormones and protease inhibitors, as well as the binding and neutralization of toxic moieties in the different concentrations of FBS.

Human dermal fibroblasts are not a homogenous population and they display significant variations when isolated from different anatomical regions, even within the dermis (reviewed Sriram *et al.,* 2015). It is clear that dermal fibroblast sub-populations display differences in morphology, proliferation rates, and biochemical activity. The upper papillary layer of dermis contains the papillary dermal fibroblasts and the lower reticular layer of dermis contains the reticular

dermal fibroblasts (Ohyama *et al.,* 2010; Honardoust *et al.,* 2012). A number of differences have been characterised that allow these two lineages of dermal fibroblasts to be distinguished from one another including differences in ECM composition and organisation, which has an effect on controlling the fibroblast behaviour and their response in wound healing.

In vitro, papillary dermal fibroblasts differ from reticular dermal fibroblasts, with a higher growth potential and lower contractile property, and when they become senescent their expression of collagen XVI and integrin-α5 decline, which leads to an impairment of their adhesion properties (Mancini *et al.,* 2012). Moreover, senescent papillary dermal fibroblasts (*in vitro* and even in organotypic cultures) release high levels of KGF, VEGF, and MMP-1, 2 and 3, TIMP-1 and 2 (Mine *et al.,* 2008; Pageon *et al.,* 2012). Papillary dermal fibroblast exhibit more heterogeneity in size, with less potential growth in culture, and they are more susceptible to changes caused by aging, which drives them to differentiate into fibroblasts with a reticular phenotype (Janson *et al.,* 2013; reviewed by Sriram *et al.,* 2015).

A study has reported that human dermal fibroblasts from terminal hair-bearing skin e.g. scalp and donor-matched vellus skin e.g. female face, display differences *in vitro* (Kamala, 2014). Fluorescence-activated cell sorting (FACs) analysis demonstrated that papillary fibroblasts from female facial skin were smaller and more granular than donor-matched fibroblasts from scalp skin, and at early passages (P3) proliferated faster in medium containing 5% stripped serum (Kamala, 2014). In addition, the expression of inhibitors of apoptosis proteins (IAPs) was higher in facial dermal fibroblasts compared to

corresponding donor-matched scalp fibroblasts (Kamala, 2014). Another study by Chipev and Simon (2002), also supports these findings; they reported that dermal fibroblasts from glabrous skin are smaller, more proliferative, their production of TGF-β1 and expression of TGF-β1-receptors II, α-SMA and fibronectin are less than the dermal fibroblasts from non-glabrous skin (reviewed by Sriram *et al.,* 2015).

A comparison of the proliferation rates between donor-matched papillary dermal fibroblasts from these two sites (facial and scalp) for up to 14 days, in 10% FBS showed no significant difference (Fig 3-2). This is a comparison whether the growth rates of papillary dermal fibroblasts from different sites differ, and the results of the present study are comparable to previous reports for papillary dermal fibroblasts Kamala (2014). The proliferation rate of scalp papillary fibroblasts in the Kamala (2014) study was significantly lower than the proliferation rate of facial donor-matched fibroblast. However, in the present study, the fibroblasts were grown in 10% FBS which provides optimal growth, while in the Kamala study, fibroblasts were grown in 5% stripped serum which slows the growth in a similar manner to 2% FBS.

One important factor in the comparison of dermal fibroblasts is passage number. It has been reported that high numbers of passaging in culture result in a lower replicative rate and may lead to aging of the papillary dermal fibroblasts (Gunin *et al.,* 2011). In addition, a study by Janson et al., (2013) reported that the papillary dermal fibroblasts take on a reticular dermal fibroblast phenotype after long term passaging *in vitro*, so they may become more like reticular dermal fibroblasts leading to a slower proliferation rate.

4-2: TNF-α inhibits both donor-matched facial and scalp dermal fibroblast proliferation

TNF-α is a pro-inflammatory cytokine, with normal circulating levels of (11.2 ± 7.31 pg/ml) (Arican *et al.,* 2005). In human skin, TNF-α reduces collagens by increasing MMPs production by human dermal fibroblasts (Brembilla *et al.,* 2015). However, TNF-α in human diabetic foot ulcers increases dermal fibroblast apoptosis, collagen degradation and decreases angiogenesis. (Kasiewicz and Whitehead, 2016). Since *in vitro* effects of TNF-α are time and dose-dependent (Barrientos *et al.,* 2008), in this study, three concentrations, supra-physiological (2.5, 25 ng/ml) and supra-pathophysiological (250 ng/ml) of TNF-α were chosen to simulate the inflammatory environment and to investigate changes in the cultured dermal fibroblast phenotype. When fibroblasts were pre-incubated with TNF-α at all 3 concentrations for 3 days, cell proliferation was inhibited by day 14, even though the medium was replenished with 10% normal growth media (Fig 3-3 A and B) for the following 11 days. TNF-α had the same inhibitory effect on papillary dermal fibroblast proliferation regardless of whether they were derived from scalp or facial skin of the same donors. This inhibition may be due to a cytotoxic effect of TNF-α in a rat hepatocytes cell line at high concentration (10 ng/ml) (Jones *et al.,* 2000). A study by Wang et al., (2014) reported that the proliferation of human fibroblasts was inhibited due to overexpression of TNF-α, but this was in diabetic wounds. Another explanation is that the cells may have been stimulated to secrete their own TNF-α, or other pro-inflammatory cytokines, and did not recover (permanent effect), even when the endogenous TNF-α was removed after 3 days. Another explanation for reduced dermal fibroblast

number, maybe due to their differentiation into myofibroblasts induced by exposure to TNF-α (reviewed by Distler *et al.,* 2008). To confirm this the expression of α-SMA (protein) in dermal fibroblasts exposed to TNF-α could be quantitated.

TNF-α can also be a potent pro-apoptotic stimulus, a recent study displayed that (20 ng/ml) TNF-α induces fibroblast (dermal fibroblast and vascular adventitial fibroblast) apoptosis by increasing the expression of cleaved caspase -8/3, which downregulates Bcl-2/Bax and upregulates FOXO1, an apoptosis transcription factor (Wang *et al.,* 2014). Pre-incubation with TNF-α for 3 days may induce irreversible changes, one of which may be due to increased numbers of senescent cells. However, manual cell counts using a haemocytometer-slide and trypan blue exclusion dye is not the most sensitive method to qualify cell viability. Cells may have detached and floated off into the media prior to counting. Since this was not collected, this may not be a true representation of the percentage viability.

Therefore these results were also confirmed using a more sensitive and reliable direct cell proliferation assay; CyQUANT direct cell, to confirm this observed effect of TNF-α on dermal fibroblast proliferation (Fig 3-4). The experimental design was identical to that used for manual cell counting, with the trypan blue exclusion dye. The CyQUANT direct cell proliferation assay also confirmed a significant reduction in the rate of dermal fibroblast proliferation after a 3 day incubation with TNF-α at the lowest concentrations 2.5 and 25 ng/ml at day 14. The inhibitory effect of TNF-α at day 14 was similar to that seen using the manual cell counting method. Although the inhibitory effect of TNF-α at the

highest concentration of 250 ng/ml did not reach statistical significance, it was close (Fig 3-4 C). Another observation using the CyQUANT assay was that the inhibitory effect was seen earlier at day 7 (Fig 3-4 B), although it was seen at day 12 in the facial using the manual method, at the lower concentrations of 2.5 and 25 ng/ml respectively. These similarities using two different methods confirm that dermal fibroblasts proliferation inhibited by TNF-α.

4-3: pre-incubation with TNF-α leads to an increase in the metabolic activity of dermal fibroblasts at day 14

Human dermal fibroblasts that are repeatedly treated with (10 ng/ml) of TNF-α every 2 days for at least 20 treatments become senescent. Thus, long-term exposure to TNF-α induces senescence of human dermal fibroblasts, decreases the proliferation rate and increases β-galactosidase expression (Mavrogonatou *et al.,* 2018).

Pre-incubation of dermal fibroblasts with TNF-α at doses between (2.5- 250) ng/ml for 3 days, followed by incubation with normal growth media (10% FBS) for up to 14 days, demonstrated a significant increase in their metabolism at the lowest concentration of 2.5 ng/ml by day 14 (Fig 3-5).

One possible explanation for the decrease in dermal fibroblast proliferation in response to TNF-α may be a result of increased differentiation into a myofibroblast phenotype, which is highly metabolically active fibroblasts, and the TNF-α ability to induce dermal fibroblasts differentiation into myofibroblasts may be in direct, or indirect way.

4-4: Induction of senescence in cultured dermal fibroblasts by TNF-α

One possible explanation for the inhibition of dermal fibroblast proliferation (Fig 3-3 A and B) and upregulation metabolic activity is cell senescence. Senescent cells are also characterized by altered and enhanced MMP activity (reviewed by Borg *et al.,* 2013). An increase in MMP expression leads to degradation of growth factor receptors, and dermal fibroblasts are unable to recognise their growth factors, resulting in a low proliferative ability, with a high proportion of senescent dermal fibroblasts (Chen *et al.,* 2013). Telomere shortening can be induced through repeated replications (long term passaging) and can result in cellular senescence induced by cell stress via inflammatory factors and inflammatory diseases, resulting in increased ROS (Lopez-Otin *et al.,* 2013; Lagouge and Larrson, 2013). Therefore the ability of TNF-α to induce senescence in this study was analysed using β-galactosidase as a marker.

TNF-α is believed to have two different roles in preventing tumorigenesis and stimulating tumorigenesis, so the excessive proliferation of cells can be limited by arresting the cell cycle, resulting in cellular senescence (Wang and Lin, 2008). Senescence as a cellular stress response, leads to the blockade of cell proliferation that is characterized by morphological changes and the expression of the senescence marker β-galactosidase (Campisi and d'Adda di, 2007; Hirano *et al.,* 2015).

A recent study in mice, reported that TNF-α at low concentrations (0.5 ng/ml) induces p21 (a trigger for cellular senescence) expression in mesangial cells (kidney), while higher concentrations of TNF-α (2.5 ng/ml) did not induce p21

expression, yet expression was blocked at a concentration of 50 ng/ml of TNF-α, demonstrating a dose-dependent effect (Hirano *et al.,* 2015).

TNF-α induces permanent senescence by increasing β-galactosidase expression with persistent ROS production and oxidative stress. In senescent human dermal fibroblasts, TNF-α leads to an elevation of ROS levels and continual, rapid, transient, p38 MAPK activation, even after long term exposure to TNF-α (Kandhaya-Pillai *et al.,* 2017).

In the present study, incubation with TNF-α induced cell senescence at (250 ng/ml), the highest level of senescence was seen in cells exposed to a concentration of 250 ng/ml TNF-α (Fig 3-6 and 7). The passage number is an important factor that contributes to induce β-galactosidase expression. In the present study, β-galactosidase was only expressed in cultured dermal fibroblasts when they reached passage number 17 (Fig 3-6). However, the same cells at earlier passage numbers did not express a significant increase in β-galactosidase in response to TNF-α, demonstrating that TNF-α induced senescence depends on aging *in vitro*. It has also been reported that β-galactosidase induction differs among different cell types. An *in vitro* study by Müller et al 2008, found lung fibroblast cultures from emphysema patients exhibited a high expression of β-galactosidase when exposed to smoke extract as a stress, while dermal fibroblasts from the skin of the same patients did not express β-galactosidase, which demonstrate the importance of exposure to levels of chronic irritation, as well as tissue specificity in driving cellular senescence.

4-5: Effect of TNF-α on dermal fibroblast migration

Although the release of pro-inflammatory cytokines is important for wound healing, but an excessive and prolonged production may result in a chronic non-healing wound.

In order to measure the impact of TNF-α on the migration of dermal fibroblasts, cells were incubated with TNF-α at two concentrations 2.5 or 25 ng/ml. Migration was assessed in both healthy and diabetic dermal fibroblasts. In healthy female facial dermal fibroblasts 25 ng/ml of TNF-α significantly inhibited cell migration ($P<0.01$) after 24h (Fig 3-8 and 3-10 A).

In diabetic dermal fibroblasts, both concentrations of TNF-α 2.5 and 25 ng/ml inhibited cell migration significantly ($P< 0.05$) after 24h (Fig 3-9 and 3-10 B), However, the percentage inhibition compared to the control was similar in both concentrations 2.5 and 25 ng/ml (28% and 28.2%, respectively).

A previous study by AL-Mulla et al., (2011) reported that 1ng/ml of TNF-α impaired the migration of human dermal fibroblasts by blocking Smad2/3 and inducing Smad7 (Smad7- is required for differentiation into myofibroblasts and migration of dermal fibroblasts).

Interestingly, the TNF-α impacts on cellular migration appear to depend on the cell type/tissue specific. For example, a concentration of 1ng/ml TNF-α induced endothelial migration, while 100 ng/ml TNF-α inhibited endothelial cell migration. Conversely, low concentration of 1ng/ml TNF-α inhibited the migration of epidermal dendritic cells and human dermal fibroblasts (Takemura *et al.,* 2006). In the present study, only a higher concentration 25 ng/ml of TNF-α inhibited the

migration of healthy dermal fibroblasts, while dermal fibroblasts derived from diabetic skin were also inhibited by the low concentration 2.5 ng/ml of TNF-α (Fig 3-10).

Since MMPs are strongly upregulated in inflammatory diseases, excessive expression of MMPs induced via pro-inflammatory cytokines such as TNF-α leads to excessive degradation of cell-cell and cell-ECM receptors (Ligi *et al.,* 2016a). This can result in the inhibition of cell migration, while under normal conditions the release of MMPs facilitates cell migration to the wound site by ECM degradation (Löffek *et al.,* 2011; Chen *et al.,* 2013; Brembilla *et al.,* 2015).

TNF-α also stimulates the release of MMPs, including the collagenases (MMP-1,-3 and -13), and the gelatinases (MMP-2 and -9), but not the TIMPs. Increased activity of all these MMPs has been shown to impair dermal fibroblast migration in an inflamed environment, in human (Barrientos *et al.,* 2008; reviewed by Xu e*t al*., 2013; Agren *et al.,* 2015).

There is increasing evidence that diabetes has an effect on essential aspects of fibroblast biology such as migration. Diabetic mice/human dermal fibroblasts have displayed a reduction in proliferation and migration, but an elevation in apoptosis compared to non-diabetic fibroblasts (Desta *et al.,* 2010; reviewed by Baltzis *et al.,* 2014; Shi *et al.,* 2015).

Diabetic, murine dermal fibroblasts exhibited an impaired migratory ability, they migrate 75% less than the normal dermal fibroblasts (Lamers *et al.,* 2011). However, the present study showed that human diabetic dermal fibroblasts migrate faster than the non-diabetic dermal fibroblasts by 25%, but under TNF-α stimulation, they migrate similarly to non-diabetic at 2.5 ng/ml concentration of

TNF-α, and these diabetic cells migrated faster (17%) than non-diabetic cells only at the concentration of 25 ng/ml of TNF-α (Fig 3-10), which shows the different impact of different concentrations of TNF-α (concentration-dependent).

Lamers and colleagues also have shown that high glucose levels (hyperglycemia) mediate oxidative stress, which causes the loss of cell polarity and impaired cell migration. Moreover, wound healing can be blocked by impairing fibroblast functions; migration and proliferation via AGEs (proteins or lipids become glycated by high glucose) accumulation products, which mediates the activation of ROS (Loughlin and Artlett, 2010).

Several studies have demonstrated the imbalance between MMPs and TIMPs in the chronic diabetic wound environment, with an increase in MMP-1, -2, -8 and -9, and a decrease in TIMP-2, which may explained the impaired cell migration (reviewed by Xu *et al.,* 2013; Ligi *et al.,* 2016b).

In human skin, Shi (2015), has reported that diabetic wounds are recovery poorly due to chronic hyperglycemia (high glucose levels), and the migration of human diabetic dermal fibroblasts is impaired under these conditions.

4-6: Does TNF-α stimulate secretion of paracrine factors that can modulate neighbouring dermal fibroblast migration?

Paracrine soluble factors including cytokines, chemokines, growth factors and proteases (MMPs) (Davalos *et al.,* 2010) can affect neighbouring cells by changing the microenvironment through activating various cell surface receptors and corresponding signal transduction pathways. To determine whether TNF-α

induces a change in the secretory phenotype of dermal fibroblasts, the conditioned media in the presence and absence of TNF-α was collected and other dermal fibroblasts were assessed for their ability to migrate in its presence.

Dermal fibroblasts from healthy donors were incubated with, or without, TNF-α in concentrations of 2.5 and 25 ng/ml in normal growth media for 3 days to produce conditioned media. Other dermal fibroblasts from different healthy donors were incubated with this conditioned media (Fig 3-11). The conditioned media, in the presence of TNF-α showed a lower migration of healthy dermal fibroblasts by 48.6% and 35.8%, similar to what was seen by adding TNF-α directly (Fig 3-10), although this did not achieve statistical significance (Fig 3-13). A similar effect was observed when control conditioned media had TNF-α added after conditioning) (Fig 3-12) and (Fig 3-13). These results need confirming but may imply that TNF-α has a direct effect on dermal fibroblasts by inhibiting their migration, but it does not stimulate paracrine factors that have any significant effect on modulating fibroblast migration.

4-7: Secretion of active MMP-2 and -9 by dermal fibroblasts in response to TNF-α

In order to determine whether the TNF-α conditioned media of cultured dermal fibroblasts represents an inflammatory *in vitro* environment, the activity of the MMP-2 and -9 profile as a potential marker for chronic inflammation and their action as gelatinolytic enzymes was assessed by Zymography. In the

optimisation of this study. The diluted conditioned media at all dilutions (1:1, 1:20, 1:40 and 1:80), exhibited MMP-2 and MMP-9 activity after incubation for 48h with TNF-α (Fig 3-14 A, B, C, and D). The bands were the clearest, with a dilution of 1:40 which was identified as the optimum dilution, where the bands were not saturated and clearly showed the gelatin lysis activity that supports the activity of high levels of MMPs seen in chronic inflammatory states. The results here can be explained that increased activity of these MMP-2 and -9 are inflammatory markers, and can be induced by inflammatory cytokines such as TNF-α.

Both MMPs are active as they can carry out their degradational function for gelatin as identified by specific bands (Parks *et al.,* 2004). *In vivo,* both gelatinases can degrade the basement membrane which contains collagen type IV and laminin as substrates for them (Löffek *et al.,* 2011). A functional basement membrane is essential for keratinocyte migration, proliferation and re-epithelialization to restore the barrier membrane (Barrientos *et al.,* 2008).

A recent study examined increased gelatinase activity as a possible cause for impairing dermal fibroblast migration during wound healing. A study in mice by Gao et al., (2015), showed that increased MMP-9 is involved in delayed/impaired diabetic wound healing, while MMP-9 deficiency leads to impaired wound healing. This is probably due to the fact that MMP-9 also contributes to keratinocyte migration during the re-epithelialization phase (Caley *et al.,* 2015). The expression of MMP-2 in acute wounds is associated with increased keratinocyte migration and laminin expression (Caley *et al.,* 2015).

The present findings indicate that MMP-2 and MMP-9 secretion was higher than the controls under TNF-α stimulation in both healthy and diabetic dermal fibroblasts under both scratched and non-scratched conditions but there were no statistically significantly differences (Fig 3-15, 16, 17 and 18).

There was no difference in MMP-2 secretion in scratched or non-scratched healthy dermal fibroblasts incubated with either concentration of TNF-α (2.5 or 25 ng/ml) (Fig 3-15 A and17 A). Furthermore, due to donor variability, diabetic dermal fibroblasts demonstrated no significant differences in MMP-2 secretion at either concentration of TNF-α (Fig 3-15 B and 17 B).

Similarly there was higher MMP-9 secretion under scratched and non-scratched conditions in both healthy dermal fibroblasts and diabetic dermal fibroblasts, but due to donor variability, a significant difference at either concentration of TNF-α (2.5 or 25 ng/ml) was not demonstrated (Fig 3-16 and 18).

The study by Han et al., (2001a), reported that concentrations of (5, 10 and 50 ng/ml) of TNF-α all induced MMP-2 secretion by human dermal fibroblasts in monolayer cultures. They confirmed that exposing dermal fibroblasts to TNF-α leads to activation of the NF-$_k$B pathway, which stimulates the degradation of I-$_k$B by a cascade of events that lead to the translocation of NF-$_k$B to the nucleus and thereby the transcription of target genes. Thus, NF-$_k$B induces MT1-MMP activation, and the MT1-MMP, in turn, activates MMP-2.

In humans, MMP-9 is upregulated by inflammatory signals such as TNF-α, and as MMP genes are controlled by the transcription factors AP-1 and NF-$_K$B, TNF-

α will increase the binding of these two transcription factors to the MMP-9 DNA sequence, which leads to upregulation of MMP-9.

The present study showed a much higher secretion of MMP-9 in diabetic both scratched (1983% vs 55%) and (18,432% vs 40%) and non-scratched (456% vs 13%) and (617% vs 30%) dermal fibroblasts compared to healthy scratched and non-scratched cells (MMP-9 was below the level of detection in dermal fibroblasts cultured from most of the healthy donors) (Fig 3-16 B) and (Fig 3-18 B). Furthermore, the secretion of MMP-9 was increased following scratching in the presence of TNF-α in a dose-dependent fashion, particularly in the diabetic fibroblasts. A recent study has shown that MMP-9 is upregulated only in diabetic wounds (Gao *et al.,* 2015) suggesting a possible detrimental role.

Most published studies indicate that normal human dermal fibroblasts do not express MMP-9, but Gawronska-kozak et al., (2011), reported that both (1ng/ml) of TGF-β1 and (10 ng/ml) of TNF-α stimulate and regulate MMP-9 expression in human dermal fibroblasts from explants of skin (reconstructive surgery), by zymography and western blot.

Pathologically elevated levels of MMP-2 and -9 are indicated in impaired wound healing. A study in mice by Lerman et al., (2003), suggests that MMP-9 is elevated in chronic wounds, due to high glucose levels depending on the cell type. Previous studies have reported elevated MMP-9 levels are the cause of prolonged inflammation in mice, or maybe due to microbial infection *in vivo* (Lerman *et al.,* 2003).

Furthermore, diabetic chronic wounds display an imbalance between MMPs and TIMPs with increased expression levels of MMP-1, -2 and -9, and reduced

TIMP-1 and TIMP-2 expression (Menghini *et al.,* 2013; reviewed by Baltzis *et al.,* 2014). Also, the prolonged inflammation in diabetic chronic wounds directs the inflammatory cells to produce TNF-α, which then acts synergistically with IL-1β to increase the MMPs levels and decrease the TIMPs (McCarty and Percival, 2013).

From recent results, it seems that incubation cell monolayers with TNF-α does not modulate the secretion of MMP-2 and -9, in either healthy or diabetic dermal fibroblasts under either scratched and non-scratched conditions. A recent study by Argyropoulos et al., (2016), reported that MMP-1 and MMP-2 mRNA expression increased significantly in diabetic skin compared with normal skin. Patel et al., (2016), suggested in terms of MMPs to TIMPs ratio, the mean MMP-9/TIMP-1 ratio in fluids decreased significantly with increased ulcer healing chances. Thus, the lower the mean MMP-9/TIMP-1 ratio collected from wound fluids, the higher ultimately healed wounds compared with non-healed wounds, which showed the harmful effect of MMP-9 in chronic wounds. This supports our findings especially MMP-9 was overexpressed in diabetic wounds and led to inhibit wound healing in migration assay, although of the inhibitory effect of TNF-α.

4-8: TNF-α paracrine effect on the secretion of active MMP-2 and -9 by healthy dermal fibroblasts

To assess whether TNF-α has a paracrine effect on MMP-2 and -9 secretion, the conditioned media from healthy dermal fibroblasts was assessed under

scratched and non-scratched conditions, (Fig 3-19 and 20). In scratched dermal fibroblasts, doubled conditioned media at both concentrations (2.5 and 25 ng/ml) of TNF-α induced MMP-2 secretion significantly, while in non-scratched cells, due to donor variability, there was no significant difference in MMP-2 secretion at either concentration of TNF-α (Fig 3-19). This was not due to the presence of TNF-α since when TNF-α was added to the control media this stimulation in MMP-2 secretion was not seen.

With MMP-9 secretion, there was no significant difference at either concentration of TNF-α, by scratched or non-scratched dermal fibroblasts (Fig 3-20), suggesting TNF-α does not stimulate the secretion of paracrine factors that modulate the secretion of MMP-9.

Many possible explanations arise here, MMP-2 secretion by scratched cells (Fig 3-19 A), is stimulated directly by low levels of TNF-α which are still present in the double conditioned media and not by soluble factors, or there could be an alternate explanation. As TNF-α conditioned media was pooled from two donors, TNF-α can induce the secretion of other cytokines, which modulate the production of MMPs, and then interfere with ECM protein secretion by fibroblasts via autocrine and paracrine pathways (Wong *et al.,* 2001). Furthermore, soluble factors may involve pro-inflammatory cytokines such as IL-1 and IL-6, which can normally induce MMP-2 secretion directly, or these pro-inflammatory cytokines induced by TNF-α and they, in turn, stimulate the MMP-2 secretion indirectly. Scratching may also induce the MMP-2 secretion.

4-9: Changes in gene expression in response to TNF-α

4-9-1: Effect of TNF-α on MMP-1 mRNA expression in healthy and diabetic dermal fibroblasts

Dermal fibroblasts secrete MMP-1 a protease, which degrades collagen particularly type I and III in connective tissue and ECM in response to pro-inflammatory cytokines such as TNF-α during the wound healing process (Brown Lobbins *et al.,* 2017).

The results of this study of the effect of TNF-α on MMP-1 mRNA expression in healthy dermal fibroblasts have shown that, in healthy non-scratched dermal fibroblasts TNF-α induces a significant increase in the MMP-1 expression at both concentrations of TNF-α (2.5 and 25 ng/ml).

In scratched fibroblasts, there were no significant differences, due to donor variability (Fig 3-21 A). A study by Lindner et al., (2012), where the human dermal fibroblasts were incubated with (10 ng/ml) TNF-α for 24h, reported that MMP-1 mRNA expression was also significantly increased, while for wounded dermal fibroblasts, an older study by Dayer (1985) had reported that TNF-α induces the MMP-1 mRNA and protein expression in dermal fibroblasts, concluding that the MMPs are upregulated in response to exogenous cytokine signals temporarily in skin which resulting in alterations in cell-to-cell contacts.

The results of diabetic dermal fibroblasts, showed TNF-α produced a significant increase in the expression of MMP-1 only at the highest concentration of TNF-α at 25 ng/ml in scratched cells (Fig 3-21 B). It is well known that TNF-α can also induce other pro-inflammatory cytokines such as IL-1 and IL-6 in diabetic

conditions, which together can induce MMP-1 mRNA expression (Bauer *et al.,* 2009).

Interestingly, basal levels of MMP-1 mRNA were higher in diabetic dermal fibroblasts (1637% vs 235%) compared to healthy control fibroblasts (1669% vs 478%) under control conditions (Fig 3-21 A and B). Hence, since the levels of MMP-1 expression in response to TNF-α were similar in both while the level of expression in the presence of TNF-α in diabetic fibroblasts was approximately double that seen in normal fibroblasts. However, these suggestions require confirmation with larger sample sizes as they did not reach statistical significance. Other recent studies have reported similar findings, for example, a study by Argyropoulos (2016), reported that MMP-1 and MMP-2 expression was significantly (3.2 fold) and (4.4 fold) higher in diabetic skin compared with non-diabetic skin. Further quantification of the protein levels by western blot confirmed that MMP-1 was elevated in diabetic skin. For diabetic wounds, a study by Lobmann (2002), demonstrated that MMP-1 expression was elevated by 65 fold in biopsies taken from diabetic foot ulcers (DFU) compared to those from acute wounds. This study was further confirmed by the Lopez- Lopez (2014) study, when they found the same upregulation in MMP-1 expression in DFU compared to the control.

The effect of TNF-α on MMP-1 mRNA expression in both gender cells (two females) and (two males) was also compared (Fig 3-21 C and D). No significant observations have seen due to sample small size. MMP-1 expression was higher at the highest concentration of TNF-α in both scratched and non-scratched cells. In female cells and under both conditions MMP-1 expression

was more variable. These results may due to how long they have had diabetes or how long they had a chronic wound before amputation.

4-9-2: Effect of TNF-α on MMP-2 mRNA expression in healthy and diabetic dermal fibroblasts

MMP-2, or (gelatinase A) is a major protease which degrades denatured collagens and gelatins. The results of healthy dermal fibroblasts showed, a non-significant increase in MMP-2 mRNA expression TNF-α in non-scratched cells, while the results from the scratched cells showed no effect of TNF-α on MMP-2 mRNA expression at either concentration (Fig 3-22 A). However, scratching the cells induced expression of MMP-2 by 3-fold, even in the absence of TNF-α (2104% vs 650%). Interestingly, zymography results in healthy dermal fibroblasts are mirrored to MMP-2 mRNA expression results, TNF-α had no effect on MMP-2 secretion in scratched cells (Fig 3-15 A), and a dose-dependent effect on MMP-2 secretion in non-scratched cells (Fig 3-17 A). Also scratching increased secretion of MMP-2 (about 1000 in non-scratched – just under 2000 in scratched) (1230% vs 1450%) and (1130% vs 1587%, respectively) (Fig 3-15 and 17 A). In contrast to the present results, Lindner et al., (2012), found that 10 ng/ml of TNF-α increased the MMP-2 mRNA expression significantly in dermal fibroblasts after 24h incubation.

In non-scratched diabetic dermal fibroblasts, MMP-2 mRNA gene expression was more than 2 fold higher than in healthy fibroblasts at basal levels (Fig 3-22 A and B). This result mirrors the results of TNF-α on MMP-2 secretion in non-

scratched diabetic dermal fibroblasts at basal levels in zymography (Fig 3-17 A and B). In contrast to healthy fibroblasts, scratching diabetic fibroblast monolayers did not alter MMP-2 expression (Fig 3-22 B). Conversely, scratched diabetic dermal fibroblasts did alter MMP-2 secretion in zymography (Fig 3-15 B).

In contrast, to healthy scratched fibroblasts, MMP-2 mRNA expression following incubation with TNF-α was double that of the controls although due to donor variability this did not achieve statistical significance (Fig 3-22 B). This result mirrors the results obtained for TNF-α on MMP-2 secretion in scratched diabetic dermal fibroblasts and was non-significant (Fig 3-15 B). A report by Lauhio et al., (2008) displayed that MMP-2 levels increased in the serum of diabetic patients, while another study reported a high decrease in MMP-2 mRNA expression in type 2 diabetic patients (Xu *et al.,* 2014).

When the diabetic fibroblasts were split by gender, TNF-α had no significant effect on MMP-2 expression in both genders under both conditions due to the small sample size (Fig 3-22 C and D). The variant in data of genders may also be due to differences in diabetes duration, sex hormones and in response to diabetes.

This increased expression of MMP-1 and MMP-2 in the phenotypic profile of diabetic dermal fibroblasts indicates a combined action of elevated levels of these MMPs in the dermis of diabetic skin may be a significant factor that leads to chronic non-healing wounds in diabetic skin.

4-9-3: Effect of TNF-α on MMP-9 mRNA expression in healthy and diabetic dermal fibroblasts

MMP-9 or gelatinase B is a protease which degrades denatured collagens and gelatins. The results of this study showed that MMP-9 was only expressed when non-scratched healthy dermal fibroblasts were incubated in the presence of TNF-α (Fig 3-23 A). MMP-9 secretion in zymography is similar to MMP-9 mRNA expression in non-scratched cells with too low MMP-9 secretion at the control (Fig 3-18 A). There was no statistically significant effect of MMP-9 mRNA expression in response to TNF-α in non-scratched cells. This is similar to MMP-9 secretion in zymography in non-scratched cells (Fig 3-18 A).

In scratched cells MMP-9 mRNA expression was highly variable (Fig 3-23 A), and similar to MMP-9 secretion in zymography in scratched cells. There was no differences in basal MMP-9 mRNA expression between healthy and diabetic controls under scratched and non-scratched conditions (Fig 3-23 A and B).

In diabetic dermal fibroblasts the presence of TNF-α also had no statistically significant effect on MMP-9 mRNA but was several-fold higher than the controls in a concentration-dependent manner under both scratched and non-scratched conditions (Fig 3-23 B) and was approximately twice that seen in the healthy fibroblasts (16% vs 7%) in non-scratched cells and (20% vs 3%) in scratched cells. This suggests they may be more sensitive to TNF-α but requires confirming with larger sample sizes. This is similar to that seen in MMP-9 secretion (zymography), (Fig 3-16 and 18).

Lobmann et al., (2002), have found MMP-9 expression in diabetic wounds was 14 fold compared to acute wounds. Also, a study by Zhou et al., 2000 in human found, an over expression levels of MMP-9 were evident in non-healed chronic

wounds, and very low levels in acute, healed-wounds. In addition, these authors reported that in dermal fibroblasts isolated from healed and non-healed wounds incubated with 10 ng/ml of TNF-α for 16h, that TNF-α induced MMP-9 mRNA expression via activation of the PAK1 pathway, which in turn activates the JNK pathway resulting in transcription of MMP-9.

This concurs with a study showing MMP-9 expression levels are higher in DFU biopsies and patient serum, and associated with poor healing especially when compared with healed DFU (Liu *et al.,* 2009).

When the diabetic fibroblasts were split by gender, the responses to TNF-α were similar, particularly in the scratched cells (Fig 3-23 C and D). The actual increase in MMP-9 mRNA expression in male fibroblasts in response to TNF-α was approximately 3 times that seen in female fibroblast (7% vs 2%) and (29% vs 3%) in non-scratched cells, while it was higher (9% vs 1%) and (31% vs 9%) in scratched cells. Gender is more likely to explain these differences, or gender differences inflammatory responses but the group sizes were extremely small so any repeat should be with larger groups sizes.

4-9-4: Effect of TNF-α on TIMP-1 mRNA expression in healthy and diabetic dermal fibroblasts

Dermal fibroblasts also secrete the MMP inhibitors such as TIMP-1, which is a specific inhibitor of MMP-9 (gelatinase B), but also has overlapping inhibitory activities on other MMPs (reviewed by Ayuk *et al.,* 2016).

The data of healthy dermal fibroblasts showed that, TNF-α significantly increased TIMP-1 mRNA expression at the highest concentration in non-scratched cells, while the TNF-α effect was not significant in scratched cells (Fig 3-24 A). A previous study using neonatal dermal fibroblasts from foreskin reported that different concentrations of TNF-α (0.1, 10 or 100 ng/ml) did not modulate TIMP-1 expression (Wong *et al.,* 2001), while another study by Dasu et al., (2003) agree with the current data, since when they incubated human dermal fibroblasts with 10 ng/ml of TNF-α for 6 h, the TIMP-1 protein levels and mRNA expression levels were increased significantly.

In diabetic dermal fibroblasts, TNF-α had no effect on TIMP-1 mRNA expression in non-scratched and scratched cells (Fig 3-24 B). Previous studies (Rysz *et al.,* 2007; Xu *et al.,* 2014) have found circulating TIMP-1 levels in patient with diabetic nephropathy to be decreased, while others have reported an increase in TIMP-1 in diabetic patients sera (Lauhio *et al.,* 2008; Xu *et al.,* 2014). Furthermore, TIMP-1 mRNA expression was found to be significantly higher in DFU compared to control biopsies (Lopez- Lopez *et al.,* 2014). It is suggested that the high ratio of MMP-1/TIMP-1 is associated with impaired wound healing in DFUs, due to possibility that high levels of TIMP-1 may delay wound healing in DFU biopsies (Muller *et al.,* 2008).

The basal expression of TIMP-1 mRNA was higher in diabetic cells than in healthy cells under non-scratched conditions. There was no significant effect of scratching increased TIMP-1 expression in healthy or diabetic cells (Fig 3-24 A and B). According to the prediction of Muller et al., basal diabetic MMP-1 and TIMP-1 mRNA expression was higher than healthy non-scratched cells (Fig 3-21 B) and (Fig 3-24 B), while this prediction is changed in cells under scratched

condition. However, the sample sizes in the present studies were very small so the results need confirmation. Similarly, gender results showed no effect on TIMP-1 expression, again due to the small size of the sample (Fig 3-24 C and D).

4-9-5: Effect of TNF-α on TIMP-2 mRNA expression in healthy and diabetic dermal fibroblasts

TIMP-2 is a specific inhibitor of MMP-2 (gelatinase A) (reviewed by Ayuk *et al.,* 2016). The results of healthy dermal fibroblasts showed that, TNF-α had no effect on TIMP-2 expression under both cell conditions (Fig 3-25 A). A study by Wong et al., (2001) reported that TIMP-2 expression remained unchanged when neonatal dermal fibroblasts were incubated with TNF-α for 24h.

Basal levels of TIMP-2 mRNA expression in diabetic fibroblasts was higher than healthy controls under both conditions (Fig 3-25 A and B). This is in contrast to an *in vivo* study by Argyropoulos (2016), which reported there was no difference between diabetic skin and healthy skin in relation to the mRNA levels of TIMP-1 and TIMP-2 and a study by Lobmann, *et al.,* (2002), who reported that in diabetic wounds TIMP-2 expression was 2-fold lower than that seen in non-diabetic wounds. Furthermore, a study by Lopez- Lopez (2014) found no difference in TIMP-2 mRNA expression between healthy skin (control) and DFU biopsies.

In diabetic dermal fibroblasts, under both cell conditions, TNF-α had no significant effect on TIMP-2 expression (Fig 3-25 B). Both gender cells had no

significant effect on TIMP-2 expression because of n=2 for each males and females (Fig 3-25 C and D).

4-9-6: Effect of TNF-α on Sirt1 mRNA expression in healthy and diabetic dermal fibroblasts

Sirt1 is a member of the family of mammalian sirtuins, which can stimulate dermal fibroblast migration, keratinocyte proliferation and differentiation, and has an important role in aging and inflammation (Kown *et al.,* 2016; Qiang *et al.,* 2017). The results of RT-qPCR showed there was no effect of TNF-α on Sirt1 mRNA expression in either non-scratched or scratched in healthy dermal fibroblasts (Fig 3-26 A). Present results in contrast with Saini et al., (2012) who reported that Sirt1 mRNA expression was elevated in mouse skeletal myoblasts when incubated with (10 ng/ml) TNF-α for 48h, which means that Sirt1 down-regulates the TNF-α effect, via its elevation trying to keep the balance against the pro-inflammatory cytokine, other explanations, this elevation in Sirt1 expression is due to TNF-α dose-dependent, or Sirt1 effects are tissue dependent.

In addition, Sirt1 functions in wound healing are modulated depending on cell-type or wound healing stage-specific. In mice, overexpression of Sirt1 impaired the corneal epithelial wound healing (diabetic keratopathy) *in vitro* (Wang *et al.,* 2013).

In diabetic dermal fibroblasts, TNF-α also had no effect on the expression of Sirt1 in non-scratched and scratched cells (Fig 3-26 B). High glucose levels in diabetes (hyperglycemia) have been reported to induce the downregulation of

Sirt1 expression in human myocardium tissue (Sulaiman *et al.,* 2010; Frati *et al.,* 2017). Basal levels of Sirt1 mRNA were higher in diabetic fibroblasts compared to healthy fibroblasts under both conditions (Fig 3-26 A and B). Aioi, (2017) reviewed that Sirt1 and Sirt6 inhibit MMP-9 expression, and this may explain the upregulation in Sirt1 mRNA expression by diabetic basal cells led to inhibit the MMP-9 mRNA expression levels in diabetic basal cells (Fig 3-23 B), although the same effect was seen in healthy basal cells (Fig 3-23 A). Both gender cells showed no significant observations on Sirt1 expression but the sample size was 2 (Fig 3-26 C and D).

4-9-7: Effect of TNF-α on Sirt6 mRNA expression in healthy and diabetic dermal fibroblasts

Sirt6 is also a member of the family of mammalian sirtuins, which is involved in cell metabolism, metabolic diseases such as diabetes, aging, and inflammation (reviewed by Bae, 2017). Similar to Sirt1, healthy dermal fibroblasts showed that, TNF-α had no effect on Sirt6 mRNA expression in non-scratched and scratched cells (Fig 3-27 A). Present results in contrast to He et al., (2017) study in rodent vascular adventitial fibroblasts treated with TNF-α in concentrations (5, 10, 20 and 40 ng/ml) for 8h, showed a decrease in Sirt1 and Sirt6 expression concentration-dependently.

In diabetic dermal fibroblasts, TNF-α had no effect on Sirt6 expression under both cell conditions (Fig 3-27 B). Present results also not agree with a study in mice, where found high levels of Sirt6 expression in diabetic and an old mice (hepatocytes) compared to control mice (Ghiraldini *et al.,* 2013). Diabetic

wounds of mice that treated with Sirt6 siRNA showed a significant delay in healing in full-thickness skin wounds compared to control wounds (non-siRNA treated wounds), which means Sirt6-knockdown delays wound healing in diabetic mice (Thandavarayan *et al.,* 2015).

Basal levels of Sirt6 mRNA were higher in diabetic fibroblasts compared to healthy fibroblasts under both conditions (Fig 3-27 A and B). It has been reported that Sirt6 expression is significantly higher in human psoriatic skin biopsy than in control skin biopsy, this can be explained that Sirt6 upregulates TNF-α synthesis, which induces chronic inflammation, and detrimental effect on psoriasis (Rasheed *et al.,* 2016). Both diabetic genders did not show significant observations on Sirt6 expression because n=2 (Fig 3-27 C and D).

4-10: Paracrine effect of TNF-α on MMP-1, MMP-2 and MMP-9 mRNA expression by healthy dermal fibroblasts

Since it is possible that as well as having a direct effect on MMP secretion, TNF-α may also stimulate the secretion of other pro-inflammatory factors that may modulate MMPs (McCarty and Percival, 2013). The results of incubating normal dermal fibroblasts with conditioned medium taken from other dermal fibroblasts that had been incubated in the presence or absence of TNF-α are shown in (Fig 3-28 A). These data showed that the expression of MMP-1 mRNA with 25ng/ml TNF-α conditioned medium was several-fold higher than the controls, similar to what had been previously seen when fibroblasts were incubated directly with TNF-α (Fig 3-21 A). Although the results were not

statistically significant, adding TNF-α to the control conditioned media induced the same response so if confirmed, this possible increase in MMP-1 mRNA expression would be likely to be directly stimulated by TNF-α.

In scratched cells, the pattern was the same as seen with the direct application of TNF-α with the TNF-α conditioned media significantly increasing MMP-1 mRNA at the highest concentration of TNF-α (Fig 3-28 B), again suggesting that is due directly to the presence of TNF-α rather than any additional induced soluble factors.

In contrast, TNF-α conditioned medium had no effect on MMP-2 expression in either non-scratched or scratched fibroblasts (Fig 3-29 A and B), which concurs with the results seen when fibroblasts were incubated directly with TNF-α (Fig 3-22 A). The results with TNF-α conditioned medium were similar for MMP-9 mRNA expression in both non-scratched and scratched cells (Fig 3-30 A and B) as seen with the direct effect; when TNF-α was added to the control conditioned medium, the result was even higher, although this also did not reach statistical significance and was very variable in the scratched cells. However, this may indicate an additive effect of TNF-α with a soluble paracrine factor in the conditioned medium, which warrants further investigation. Data of MMP-1,-2 and -9 expressions show that there is no paracrine effect on their expression.

4-11: Paracrine effect of TNF-α on TIMP-1 and TIMP-2 mRNA expression by healthy dermal fibroblasts

TNF-α conditioned media yielded a similar result to the direct application of TNF-α on the expression of TIMP-1 mRNA in non-scratched cells, which did not reach statistical significance (Fig 3-31 A). In scratched cells the results were also non-significant (Fig 3-31 B). A previous study by Wong et al., (2001) found that TNF-α had no modulating effect on TIMP-1 and TIMP-2 expression in the supernatant of dermal fibroblasts using enzyme-linked immunosorbent assays ELISA. TNF-α conditioned media decreased TIMP-2 mRNA expression at both concentrations in non-scratched cells (Fig 3-32 A), in a similar way to what was seen in with the direct application of TNF-α. It had no effect on the expression of TIMP-2 mRNA in scratched cells (Fig 3-32 B), again concurring with what was observed with the direct effect of TNF-α. TIMP-1 and TIMP-2 expression results show that there is no paracrine effect.

4-12: Paracrine effect of TNF-α on Sirt1 and Sirt6 mRNA expression by healthy dermal fibroblasts

TNF-α conditioned media had no effect on Sirt1 mRNA in either non-scratched cells (Fig 3-33 A), or scratched cells (Fig 3-33 B), which mirrored the results when TNF-α was added directly to the culture medium (Fig 3-26 A).

Similarly, there was no effect of TNF-α conditioned media on the expression of Sirt6 mRNA in either non-scratched or scratched cells (Fig 3-34 A and B), which

again was similar to the results obtained with the direct addition of TNF-α to the culture medium (Fig 3-27 A). The results indicate that TNF-α has no effect on Sirt1 or Sirt6 expression either directly or via induction of soluble paracrine factors.

4-13: TNF-α induces the secretion of different cytokines secretion in dermal fibroblast from normal and diabetic skin

The human dermal fibroblast secreted protein profile can be determined by antibody arrays. This study sought to clarify which cytokines associated with inflammation are modulated by TNF-α and secreted into the media by dermal fibroblasts as potential candidates responsible for influencing proliferation, cell migration and/or the release of wound healing mediators (Appendix 8-4).

The findings from the human cytokine array analysis have shown that under basal conditions, healthy female dermal fibroblasts, secrete of 7/36 factors associated with inflammation. There are (Chemokine C-C motif ligand 2/ monocyte chemoattractant protein-1 CCL2/MCP-1, Chemokine C-X-C motif ligand 1/Growth-Regulated Protein alpha CXCL1/GROα, Chemokine C-X-C motif ligand 12/ stromal cell-derived factor 1 CXCL12/SDF-1, Interleukin-6 IL-6, Interleukin-8 IL-8, Macrophage migration inhibitory factor MIF and Serpin E1/Plasminogen activator inhibitor-1 SerpinE1/PAI-1). CCL2/MCP-1 has a role in recruiting monocytes, neutrophils and lymphocytes, thus it facilitates their migration and infiltration, and also recruits the leukocytes to the site of inflammation (Linthout *et al.,* 2014). Interestingly, dermal fibroblasts secrete

CCL2/MCP-1 to accelerate leukocytes homing and promote endothelial activation in inflammation (Nash *et al.,* 2004). CXCL1/GROα is a pro-inflammatory cytokine, which promotes neutrophil infiltration. Full-skin substitutes and autograft studies have shown high levels of IL-6, CCL2/MCP-1, CXCL1/GROα, and CXCL8/IL-8 were secreted by keratinocytes and dermal fibroblasts, and these mediators are important for wound healing (Spiekstra *et al.,* 2007).

In normal and psoriatic human skin, CXCL12/SDF-1 is the chemokine that is produced by human dermal fibroblasts (particularly by papillary dermal fibroblasts), and stimulates keratinocyte proliferation (Quan *et al.,* 2015).

Kim *et al.,* 2008, suggested that MIF is a pro-inflammatory cytokine that inhibits random migration of macrophages, and induces other cytokines such as; TNF-α, INF-γ, IL-1, IL-2, IL-6, and IL-8. In the wound healing process, MIF secreted by endothelial cells, so it is enhanced after wounding, and in dermal fibroblasts from wound sites compared to non-wounded sites (reviewed by Shimizu *et al.,* 2005; Simons *et al.,* 2011). In mice, inhibition of SerpinE1/PAI-1 in wounds, prevents wound closure, impairs keratinocyte re-epithelialisation and dermal fibroblast proliferation, recruitment and differentiation into myofibroblasts via antagonist PAI-1, *in vitro* (Simone *et al.,* 2015).

The same seven cytokines were also secreted by male diabetic dermal fibroblasts under basal conditions, however, in diabetic female dermal fibroblasts under basal conditions, secretion of the cytokines CCL2/MCP-1 and IL-6 were not observed, Table 3-1 and (Fig 3-35 B and C). In diabetic wounds, and particularly at the early inflammatory stage, the macrophages respond

poorly due to insufficient CCL2/MCP-1 (Satish, 2015). These results concur with these studies, since CCL2/MCP-1 was not detected at basal levels in diabetic female fibroblasts, and although it was secreted by diabetic male fibroblasts, levels were very low and almost below the level of detection.

Differences between the male and female secretion of IL-6 may be explained by the fact that IL-6 is increased significantly in chronic wounds due to wound bacterial infection (Satish, 2015). While we do not have this data on the donors, future studies may need to take this into account.

The much higher level of CCL2/MCP-1 secretion by healthy dermal fibroblasts compared to male diabetic dermal fibroblasts, under basal conditions (Fig 3-35), agree with an *in vivo* study in mice that showed the lack in this chemokine delayed wound healing by impairing re-epithelialisation, angiogenesis and tissue remodelling (reviewed by Ridiandries *et al.,* 2018). In contrast, the elevated secretion of IL-6 by diabetic male fibroblasts which is important (during the inflammatory phase) of wound healing, is highly expressed in chronic wounds in diabetic mice, which results in impaired wound healing caused by an infiltration of leukocytes (reviewed by Behm *et al.,* 2011; Buskermolen *et al.,* 2017).

When healthy dermal fibroblasts were incubated in the presence of 2.5 ng/ml TNF-α (Fig 3-35 A), the secretion of 5 additional cytokines was induced (Macrophage Inflammatory Proteins α and β MIP-1α/β, Granulocyte-colony stimulating factor G-CSF, Granulocyte-macrophage colony-stimulating factor GM-CSF, Interleukin-32α IL-32α and Tumour necrosis factor-alpha TNF-α), which were not observed under basal conditions. The MIP-1α/β chemokines are

important in attracting and recruiting the monocytes and T cells to inflammatory sites. In humans, MIP-1 secretion is restricted to hematopoietic cells and fibroblasts and is induced by inflammatory cytokines such as IL-1 and TNF-α (Coondoo, 2012).

G-CSF and GM-CSF can be induced by TNF-α, which leads to an increase in white blood cell number (haematopoiesis) (Gulati *et al.,* 2016). The G-CSF cytokine has an important role in wound healing, via inducing the neutrophil differentiation, maturation and mobilization into the bloodstream (Shiomi and Usui, 2015). GM-CSF stimulates the mobilization of hematopoietic and non-hematopoietic cells, and thus it increases the number of neutrophils at the wound site and induces keratinocyte proliferation (Wong and Crawford, 2013). Moreover, IL-32α is a pro-inflammatory cytokine that is expressed in response to TNF-α (Alsaleh *et al.,* 2010; Kandhaya-Pillai *et al.,* 2017) and also it secreted by fibroblast-like synoviocytes of rheumatoid arthritis patients.

For TNF-α observation, the most likely explanation is that TNF-α is still in the conditioned media (TNF-α direct addition to dermal fibroblasts).

The production of IL-6 can be induced by TNF- α in human dermal fibroblasts (Khan, 2009). Finally, the increase in CXCL1/ GRO-α chemokine that is important in promoting angiogenesis may be explained by the fact that dermal fibroblasts secrete CXCL1 in response to TNF-α (Kolar *et al.,* 2012), since *in vitro* human endothelial cells also secrete CXCL1 when induced by TNF-α (LO *et al.,* 2014).

Female diabetic dermal fibroblasts, the presence of TNF-α induced the additional cytokines (CCL2/MCP-1, Chemokine C-C motif ligand 5/RANTES

CCL5/RANTES, Intercellular Adhesion Molecule/CD54 ICAM-1/CD54, GM-CSF, IL-6 and TNF-α), Table 3-1 and (Fig 3-35 B). A study by Galindo et al., (2001) reported that incubation of dermal fibroblasts with TNF-α at 10, 25 and 50 U/ml (Units per millilitre, 1U/ml=1.66054e-15 ng/ml), stimulated a dose-dependent increase of CCL5/RANTS, CCL2/MCP-1, CXCL10/IP-10, and IL-8. A study has shown elevated levels of CCL5/RANTES in diabetic type 2 patients (Pais *et al.,* 2014), Nakajima and colleagues (2003), suggest that an increase in the secretion of pro-inflammatory cytokines such as TNF-α or IL-1 β in diabetic type 2 (nephropathy) resulting from high glucose levels, which in turn induce the CCL5/RANTES secretion in mesangial cells in the kidney.

For ICAM/CD54, TNF-α upregulates high expression of the ICAM adhesion molecule on dermal fibroblasts indirectly in presence of INF-γ, which leads to the recruitment of a high number of inflammatory cells during chronic inflammation (Coondoo, 2012). Also, ICAM has been reported to be a biomarker of diabetes/diabetic neuropathy due to its high levels in patients, while high gene expression in the kidney of a diabetic animal model has also been reported (Gu *et al.,* 2013). Fang and others (2010), showed that GM-CSF decreased significantly by 50% in murine diabetic wounds compared to control, and it can enhance wound healing via stimulating pro-inflammatory cytokines such as IL-6 and MCP-1. In patients with diabetic foot ulcers, there is a significant elevation of IL-6 level compared to control (Weigelt *et al.,* 2009).

Again TNF-α was detected in the supernatants which may be the residual TNF-α, although, in diabetic wounds there is also an increase in the level of pro-inflammatory cytokines including TNF-α (Mohd *et al.,* 2012). DeClue and

Shornick (2015), have reported that the pro-inflammatory cytokines IL-1, IL-6, and TNF-α are significantly elevated in diabetic wounds, while TGF-β and IL-10 are not.

In the male diabetic dermal fibroblasts, the presence of TNF-α also induced ICAM-1/CD54 and TNF-α similar to what was seen in diabetic female dermal fibroblasts, although in contrast there was no secretion of GM-CSF or CCL5/RANTS in response to TNF-α Table 3-1 and (Fig 3-35 C). The absence of both GM-CSF and CCL5/RANTS may due to how long the male had diabetes before amputation.

The diabetic fibroblasts show similarities in cytokine secretion in both genders induced by TNF-α, e. g. an increase in CXCL1/GROα, with a decrease in MIF and Serpin E1/PAI-1, Table 3-1 and (Fig 3-35 B and C). Interestingly, MIF has shown to have an immunological role induced by pro-inflammatory cytokines such as TNF-α in autoimmune diseases (Bautista-Herrera *et al.,* 2017). In contrast, Abe and colleagues (2000) have shown an increase in MIF levels would improve wound healing in rats. However, in human chronic venous ulcers, MIF levels are significantly increased (reviewed by Shimizu, 2005).

High levels of Serpin E1/PAI-1 are considered to be biomarkers for diabetes type2 (Yasar Yildiz *et al.,* 2014). Miyashita *et al.,* 2012, also reported that Serpin E1/PAI-1 is associated with obesity, diabetes type2 obesity, and insulin resistance, but an older study by Chandler, (1991) displayed that Serpin E1/PAI-1 levels showed variations even in normal populations which may explain these results.

To explain these variations, it is well known that diabetic patients differ in their duration of diabetes, which in turn may affect the secreted cytokine profile, in

addition to any differences between genders including biological, psychosocial factors, sex hormones, obesity, and inflammatory response.

4-14: There was no TNF-α induced a paracrine effect on cytokine secretion by healthy dermal fibroblast

To determine whether dermal fibroblasts secrete soluble factors that may alter the secretory profile of neighbouring dermal fibroblasts in a paracrine manner, medium was also collected from fibroblasts that had been treated with conditioned medium from other cells that had been conditioned both in the presence and absence of 2.5 ng/ml TNF-α. This secretory profile was identical, showing similar amounts of the same 7 cytokines were secreted, table 3-1 and (Fig 3-36). This suggests that secreted cytokines derived from aged dermal fibroblasts (passage 7-8), are similar to soluble factors seen in healthy dermal fibroblasts conditioned media.

5 Conclusion

Data from this study has shown a significant difference in the proliferation rate between dermal fibroblasts cultured in different percentages of FBS (2% and 10%) from the same donor, with cells proliferating faster in 10% than corresponding cells in 2% FBS. However, no differences between the rates of proliferation of dermal fibroblasts cultured from facial and scalp (haired) skin from the same donor in normal growth media (10% FBS) was observed, suggesting that there are no significant anatomical differences in the response to TNF-α in terms of proliferation.

Interestingly, pre-treatment (3 days) of both scalp and facial dermal fibroblasts with TNF-α significantly decreased proliferation by day 14. The inhibitory effect of TNF-α was only seen after this protracted time period, and this may suggest that *in vitro* studies should be carried out over longer periods.

Additional studies using the same experimental conditions, demonstrated that dermal fibroblasts pre-incubated with TNF-α for 3 days also increased their metabolic activity significantly by day 14.

One explanation for decreased proliferation and increased metabolic activity is that TNF-α is inducing cellular senescence. However, since β-galactosidase was not detected in cultured dermal fibroblasts until they reached passage 17, and was not observed at the earlier passage numbers of P4-8 when the proliferation and metabolic assays were performed, this does not seem to be the obvious explanation. Notwithstanding, TNF-α induced senescence in high passage dermal fibroblasts, which confirms that they become more susceptible to senescence induced by inflammation with aging.

In a scratch wound assay, TNF-α inhibited migration of both healthy and diabetic dermal fibroblasts. However, the higher concentration of TNF-α (25 ng/ml) was required to inhibit the migration of healthy dermal fibroblasts, while in diabetic dermal fibroblasts the lower concentration of TNF-α (2.5 ng/ml) was also able to inhibit cell migration after 24h, suggesting that fibroblasts derived from diabetic donors are more susceptible to inhibition by inflammatory cytokines during wound healing.

To determine whether TNF-α induced dermal fibroblasts to secrete paracrine factors that may influence neighbouring cells, conditioned media was collected from pre-treated dermal fibroblasts with TNF-α (soluble factors), but this had no effect on migration of other dermal fibroblasts after 24h. However, whether a longer exposure is required warrants further investigation, as does effects on other parameters of fibroblast biology during wound healing such as ECM synthesis.

To investigate the proteolytic activity, zymography of media collected from both healthy and diabetic dermal fibroblasts demonstrated that TNF-α induced an inflammatory environment which upregulates gelatinases (MMP-2 and -9) activity, which are potential candidates for the inhibitory factors that impair the migration of dermal fibroblasts.

Interestingly, there was an independent paracrine effect on MMP-2 secretion by healthy scratched dermal fibroblasts, suggesting TNF-α induced soluble factors on MMP-2 activity, which was not due to the direct effect of TNF-α.

Exposure of healthy and diabetic dermal fibroblasts to TNF-α at concentrations of 2.5 and 25 ng/ml under both scratched and non-scratched conditions allowed changes in expression of genes associated with the ECM (MMP-1, -2, -9, TIMP-

1, and -2) and senescence (Sirt1 and 6) to be quantitated by RT-qPCR. TNF-α at both concentrations upregulated mRNA expression of MMP-1 significantly in healthy non-scratched cells, providing further support that TNF-α increases MMP expression in dermal fibroblasts which may significantly impact on ECM turnover. In scratched diabetic dermal fibroblasts the highest concentration of TNF-α was required to increase MMP-1 expression. While a significant increase in TIMP-1 mRNA expression in the presence of 25 ng/ml TNF-α was observed in healthy non-scratched cells, this was not mirrored in diabetic fibroblasts, suggesting the regulation of MMPs by TIMPs may be impaired. While TNF-α also modulated MMP-2, MMP-9 and TIMP-2 mRNA expression in some donors, there was also significant donor variability. TNF-α plays an important role in tissue inflammatory, repair and remodelling processes or modulating the synthesis of ECM proteins. No changes were seen in Sirt1 and Sirt6 mRNA expression in the presence of TNF-α and there was no evidence of any paracrine effect on any of the genes studied, at least in the healthy cells.

The cytokine arrays provide the profile of the changes in the inflammatory secretome of the healthy and diabetic dermal fibroblasts under scratched conditions in the presence of TNF-α.

In healthy female and diabetic male dermal fibroblasts, under basal conditions, the same seven cytokines were secreted (CCL2/MCP-1, CXCL1/GROα, CXCL12/SDF-1, IL-6, IL-8, MIF and Serpin E1/PAI-1). These cytokines are involved in the biological phases/events of wound healing process such as coagulation, inflammation, and angiogenesis. Also, their target cells are immune cells.

In healthy cells, TNF-α stimulated the secretion of a further five cytokines (MIP-1α/ MIP-1β, G-CSF, GM-CSF, IL-32α and TNF-α). Therefore, TNF-α can induce other pro-inflammatory cytokines that attract, stimulate and recruit the neutrophils to the wound site mimic to *in vivo* inflammatory stage.

In female diabetic cells, TNF-α stimulated the secretion of a further six cytokines, which were not secreted under basal conditions (CCL2/MCP-1, CCL5/RANTES, ICAM-1/CD54, GM-CSF, IL-6, and TNF-α). Therefore, adhesion molecules involved in the inflammation process can be induced by TNF-α, as well as chemotactic cytokine for leukocytes for prolonged inflammatory process at the wound site.

In male diabetic cells, TNF-α induced the secretion of the same cytokines as seen in female diabetic cells, with the exception of GM-CSF and CCL5/RANTES. This variation in secreted cytokines may be due to gender and/or how long the donor has had type 2 diabetes.

To investigate the paracrine effect of TNF-α in healthy dermal fibroblasts the conditioned media was collected from dermal fibroblasts that had been treated with conditioned medium with or without TNF-α (soluble factors), but there was no differences in cytokine secretion between the control conditioned medium or TNF-α conditioned media.

In summary, TNF-α impairs dermal fibroblast proliferation, without reducing cell metabolic activity, but can increase senescence. It also inhibits the migration of healthy and diabetic dermal fibroblasts. TNF-α had no significant effect on MMP-2 and MMP-9 secretion by healthy and diabetic fibroblasts under scratched and non-scratched conditions. However, TNF-α upregulates the paracrine secretion of factors which induce MMP-2 activity in healthy scratched

fibroblasts. TNF-α increased the expression of MMP-1 mRNA expression in healthy and diabetic cells, and also increased TIMP-1 mRNA expression in healthy cells, but had no effect on Sirt1 and Sirt6 mRNA expression.

TNF-α induces secretion of wound healing cytokines in both healthy and diabetic fibroblasts. While there may be possible gender differences in response to TNF-α in diabetic fibroblasts, this was not confirmed in healthy donors, since they were all derived from female donors TNF-α did not induce any paracrine effects on cytokine secretion by healthy dermal fibroblasts, at least in the time period observed.

6 Future studies

One possible explanation for a decrease in dermal fibroblast proliferation may be the result of an increase in the differentiation of dermal fibroblasts to myofibroblasts. In future studies, the ability of TNF-α to directly or indirectly induce dermal fibroblast differentiation can be investigated by quantifying changes in α-SMA expression using qRT-PCR and immunocytochemistry.

TNF-α induced senescence in high-passage dermal fibroblasts, which confirms that they become more susceptible to senescence induced by inflammation with aging. In future studies, it is possible to compare the senescence expression of dermal fibroblasts derived from older donors e.g. 60 years and more, and from diabetic patients, to find out whether diabetic cells show a more aged phenotype and become more susceptible, which could help explain why these patients experience more chronic wounds.

In future studies, to determine whether TNF-α induced dermal fibroblasts to secrete paracrine factors that may influence neighbouring cells, the scratch wound assay can be used to assay the paracrine effect of TNF-α on other cells such as keratinocytes (affect re-epithelialization), endothelial cells (affect angiogenesis), and immune cells whether they attract inflammatory cells e.g. neutrophils, monocytes, macrophages, mast cells etc to wound site and cause a chronic wound.

Since changes in ECM proteins are also significantly altered in chronic inflammation that can lead to impaired wound healing, changes in the synthesis of specific ECM proteins such as laminin, fibronectin, collagen in wound healing assays can be assessed in the presence of TNF-α by western blot.

The cytokine arrays provide the profile of the changes in the inflammatory secretome of the healthy and diabetic dermal fibroblasts under scratched conditions in the presence of TNF-α. In future studies, the cytokines profile measurement can be done using ELISA and Western blot. In addition, the most important secreted cytokines that induced by TNF-α by scratched healthy and diabetic dermal fibroblasts can be validated by qPCR, such as (CCL2/MCP-1, GM-CSF, CXCL1/GROα, lL-6, Serpin E1/PAI-1and MIF).

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8 Appendix

8-1: Solutions Preparation

8-1-1: Gelatine zymography Reagents and solutions

8-1-2: Separating Gel Buffer (1.5M Tris base)

1. 90.86g Tris-base, final concentration 1.5M
2. 275 ml D.W
The Tris -base was dissolved in 275 ml D.W and made up to 500ml D.W, the pH was adjusted to 8.8, and stored at 4˚C.

8-1-3: Stacking Gel Buffer (0.5M Tris base)

1. 6g Tris base, final concentration 0.5M
2. 60ml D.W
The Tris -base was dissolved in 60 ml D.W and made up to 100ml, the pH was adjusted to 6.8 and stored at 4˚C.

8-1-4: Running Buffer

1. 15g Tris base, final concentration 25 mM
2. 72g Glycine, final concentration 200 mM
3. 5g SDS, final concentration 0.5 % w/v
The Trise-base, glycine and SDS were dissolved in 1L using D.W and was stored at 4˚C .The prepared solution was diluted as (280ml D.W plus 70ml Running Buffer) when was used.

8-1-5: 2× Non-reducing Sample Buffer

1. 3.125ml **(**0.5 M Tris base, pH 6.8) Stacking gel buffer, final concentration 62.5 mM
2. 10ml 10% SDS, final concentration 40 % v/v
3. 10ml glycerol, final concentration 40 % v/v
4. 1.87 ml D.W
5. 125µl Bromophenol Blue
All components were mixed gently and the final solution was stored at RT.

8-1-6: 5x Washing Buffer

1. 125 ml Triton X-100.
2. 875 ml D.W
The125 ml Triton X-100 was added as 5 parts until full dissolved and stored at RT. The solution was diluted as (200ml D.W plus 50ml washing buffer) when was used.

8-1-7: 10x Incubation Buffer

1. 78.8g Tris base, final concentration 50 mM
2. 14.7g $CaCl_2$. $2H_2O$, final concentration 100 mM
3. 29.2g NaCl, final concentration 50 mM
4. 5 ml Brij-35, final concentration 0.5 % v/v
All components were dissolved in 1L D.W, the pH was adjusted at 7.6, and stored at 4˚C. The solution was diluted as (180ml D.W plus 20ml Incubation Buffer) when was used.

8-1-8: Coomassie Blue Staining Solution

1. 0.2g Coomassie brilliant blue R-250, final concentration 0.02 % v/v
2. 100 ml glacial acetic acid, final concentration 10% v/v
3. 250 ml Methanol, final concentration 25 % v/v
All components were dissolved in 1L D.W with magnetic stirring overnight, and the solution was stored at RT.

8-1-9: Destain Solution

1. 50 ml Methanol, final concentration 5% v/v
2. 50 ml Glacial acetic acid, final concentration 5% v/v
The Methanol and Glacial acetic acid were dissolved in 1L D.W, and was stored at RT.

8-1-10: 10% SDS Solution

1. 10g SDS.
2. 80 ml D.W
The SDS was dissolved in 80ml DW and made up to 100ml D.W, and when fully dissolved was stored at RT.

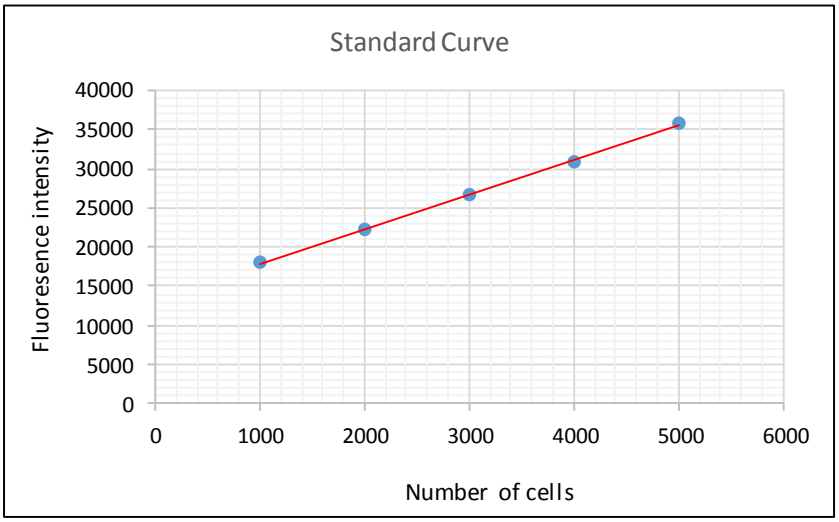
8-1-11: 10% APS Solution

1. 0.12g ammonium persulfate (APS).

2. 1.2 ml D.W

The APS dissolved in 1.2 D.W, and was stored -20˚C, until used.

8-2: The standard curve of CyQUANT- direct proliferation assay



Representative standard curve for the CyQUANT- (direct cell proliferation). The fluorescence intensity measured at peak excitation wavelength of 508nm and at peak emission wavelength of 527nm.

8-3: ORAL AND POSTER PRESENTATIONS

1. ORAL PRESENTATIONS

a) **A Al-Rikabi**, K Riches, DJ Tobin, MJ Thornton. *Changes in the secretory phenotype of human dermal fibroblasts by a pro-inflammatory environment: implications for wound healing.* British Inflammation Research Association Meeting **2018,** Manchester, Alderley Edge, UK

b) **A Al-Rikabi**, K Riches, DJ Tobin, MJ Thornton. *A pro-inflammatory environment modulates the human dermal fibroblast phenotype: implications for chronic wounds.* 13th World Congress on Inflammation Conference **2017**, London, UK (published in the conference proceeding)

2. POSTER PRESENTATIONS

a) **A Al-Rikabi**, K Riches, DJ Tobin, MJ Thornton. *A pro-inflammatory environment induces changes in the phenotype of human dermal fibroblasts derived from diabetic and non-diabetic donors: implications for wound healing.* British Society of Investigative Dermatology Annual Meeting **2019***,* Bradford, UK

b) **A Al-Rikabi**, K Riches, DJ Tobin, MJ Thornton. *A pro-inflammatory environment modulates the human dermal fibroblast secretory phenotype: implications for chronic wounds.* International Investigative Dermatology Conference **2018**, Orlando, Florida, USA (published as a late breaking abstract in the JID)

c) **A Al-Rikabi**, K Riches, DJ Tobin, MJ Thornton. *A pro-inflammatory environment modulates the human dermal fibroblast secretory phenotype: implications for chronic wounds. British J. Dermatol. Mar (2018),**178:*** P48

d) **A Al-Rikabi**, K Riches, DJ Tobin, MJ Thornton. *Tumour necrosis factor-α modulates the human dermal fibroblast phenotype: implications for inflammation, impaired wound healing and ageing. British J. Dermatol. Apr 2017; **176***:e58

e) **A Al-Rikabi**, K Riches, DJ Tobin, MJ Thornton. *TNF-α and dermal fibroblasts: Implications for inflammation, impaired wound healing and ageing.* North of England Cell Biology Meeting **2016**, Bradford, UK

8-4: Human cytokine array

8-4-1: List of the human cytokines array coordinator-key table (2015, R&D Systems, ARY005B)

Coordinate	Target/Control	Gene/ function and location
A1, A2	**Reference spots**	**Nil**
A3,A4	**CCL1/I-309**	**Chemokine, (C-C motif) ligand1/ Human Cytokine I-309, produced by activated Tells, monocytes chemoattractant and other immune cells, inflammatory cytokine**
A5,A6	**CCL2/MCP-1**	**Chemokine, (C-C motif) ligand 2/ monocyte chemoattractant protein 1, produced by macrophage, monocytes and dendritic cells, chemotactic for immune cells**
A7,A8	**MIP-1α/MIP-1β**	**Chemokine, Macrophage Inflammatory Proteins (α and β/ CCL3 and CCL4), produced by macrophages, dendritic cells and lymphocytes, chemotactic, activates granulocytes and caused (neutrophilic inflammation), stimulates pro-inflammatory cytokines from fibroblasts**
A9,A10	**CCL5/RANTES**	**Chemokine, C-C motif ligand 5/ RANTES, produced by activated T cells, chemotactic for immune cells especially for recruiting leukocytes into inflammatory sites**
A11,A12	**CD40Ligand/TNFSF5**	**Protein, (member of TNF-α superfamily)/ CD154 expressed on T cells, produced by CD4 cells, and other immune cells, it binds to (APCs)**
A13,A14	**Complement component C5/C5a**	**Protein ,Complement component 5 (complement system) can be cleaved into C5a and C5b, coded by C5 gene, a role in chemotaxis and attacks immune complexes**
A15,A16	**CXCL1/GROα**	**Chemokine, C-X-C motif ligand 1/GROα, produced by macrophages, neutrophils and epithelial cells, a chemoattractant to neutrophils, a role in inflammation, wound healing and angiogenesis**
A17, A18	**CXCL10/IP-10**	**Chemokine, C-X-C motif ligand 10/ Interferon gamma-induced protein 10 (IP-10), produced by monocytes, endothelial cells and fibroblasts in response to IFN-γ, a chemoattractant to immune cells**
A19,A20	**Reference spots**	**Nil**

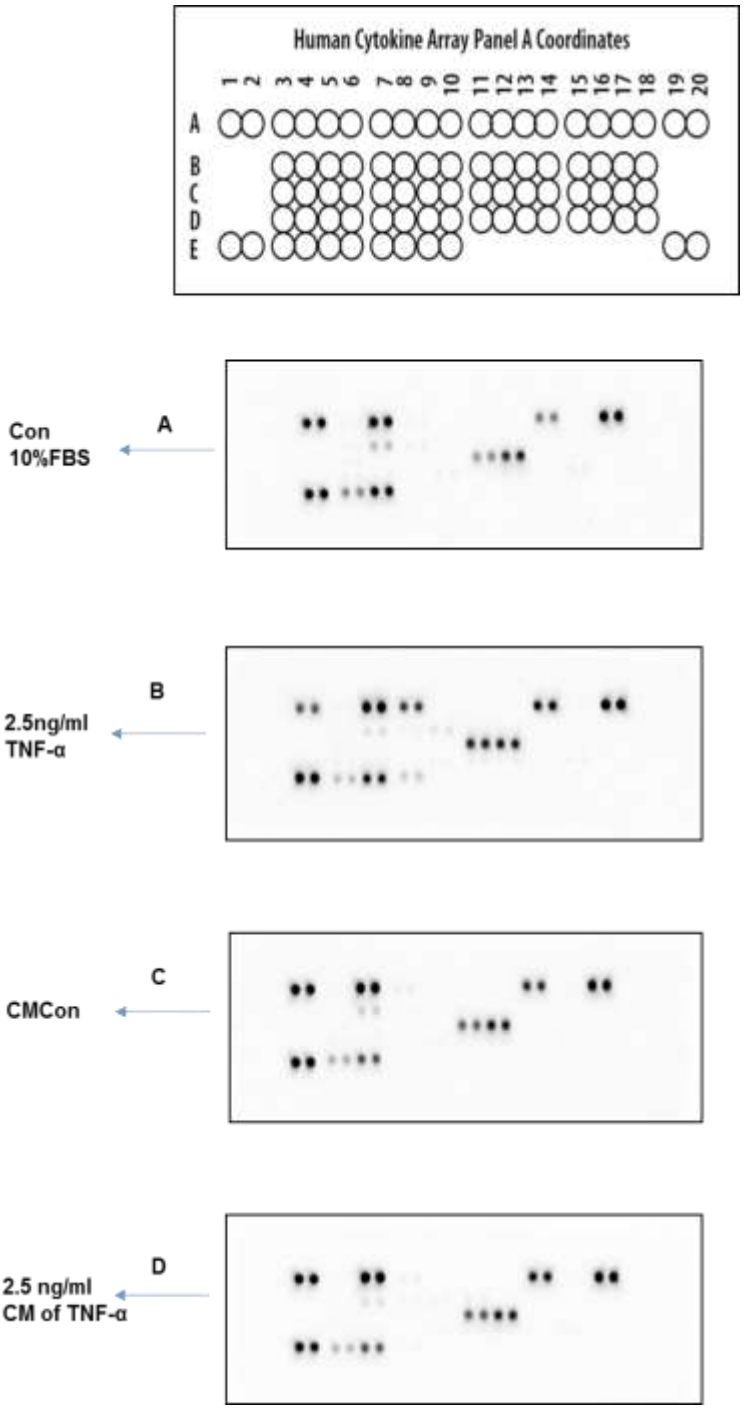
B3,B4	**CXCL11/I-TAC**	**Chemokine, C-X-C motif ligand 11/ Interferon-inducible T-cell alpha chemoattractant, produced by blood cells/leukocytes pancreas and liver, a chemotactic**
B5,B6	**CXCL12/SDF-1**	**Chemokine, C-X-C motif ligand 12/ stromal cell-derived factor 1 (SDF1), produced by many tissues e.g. brain, thymus...etc., lymphocytes chemotactic, induced by TNF-α**
B7,B8	**G-CSF**	**Granulocyte-colony stimulating factor, Glycoprotein, as cytokine, produced by tissues, an inducer for granulocytes and stem cells in bone morrow to release into blood stream, a neutrophils stimulator**
B9,B10	**GM-CSF**	**Granulocyte-macrophage colony-stimulating factor, Glycoprotein, produced by macrophages, immune cells fibroblasts and endothelial cells as stimulator cytokine**
B11,B12	**ICAM-1/CD54**	**Intercellular Adhesion Molecule/CD54, expressed by endothelial and immune cells, induced by TNF-α, a molecule for stabilizing cell-cell signalling and facilitating leukocyte endothelial transmigration**
B13,B14	**IFN-γ**	**Cytokine, Interferon gamma, produced by activated T cells and macrophages, defends against virus**
B15,B16	**IL-1α/IL-1F1**	**Interleukin-1 alpha/ IL-1F1 Protein, produced by activated macrophages, neutrophils, endothelial and epithelial cells, promotes release of multiple inflammation mediators**
B17,B18	**IL-1β/IL-1F2**	**Interleukin-1 beta/ IL-1F2 Protein, produced by activated macrophages, monocytes and other immune cells, promotes inflammation response, fever and sepsis**
C3,C4	**IL-1ra/IL1-F3**	**Interleukin-1 receptor antagonist (IL-1RA), produced by immune cells, epithelial cells and adipocytes, binds to IL-1 receptor, a natural inhibitor of IL-1 α and β**
C5,C6	**IL-2**	**Interleukin-2, produced by activated CD4+ T cells, immunity-immune tolerance, influences all lymphocytes differentiation and regulation, can recognize self and non-self antigens**
C7,C8	**IL-4**	**Interleukin-4, produced by T helper type 2 CD4 T cells, basophils, and mast cells. stimulates the differentiation of Th0cells into Th2 cells and B-cells into plasma cells**
C9,C10	**IL-5**	**Interleukin-5, produced by T helper type 2 and mast cells, stimulates B cells growth, increases IgG and IgA secretion as mediator of eosinophil activation,**

C11,C12	**IL-6**	**Interleukin-6, produced by osteoblasts, smooth muscle cells of blood vessels, its role a pro-inflammatory and an anti-inflammatory cytokine (inhibitor effect to TNF-α), mediates fever and acute phase response**
C13,C14	**IL-8**	**Interleukin-8, produced by macrophages, epithelial cells, airway smooth muscle cells and endothelial cells, it's a neutrophil chemotactic factor, stimulates phagocytosis and promotes angiogenesis**
C15,C16	**IL-10**	**Interleukin-10, produced by Th2 cells, monocytes and other immune cells, anti –inflammatory cytokine**
C17,C18	**IL-12 p70**	**Interleukin-12/ (active heterodimer is p70), produced by APCs, acts as T cells stimulating factor, T cell differentiation into Th1 cells, role in NK cells activation**
D3,D4	**IL-13**	**Interleukin-13, produced by T helper type 2 CD4 t cells, NK cells, mast cell and granulated white cells, it mediates allergic, inflammation e.g. Asthma**
D5,D6	**IL-16**	**Interleukin-16, produced by lymphocytes, CD4 T cells and epithelial cells, a chemoattractant for CD4 T cells**
D7,D8	**IL-17A**	**Interleukin-17A, produced by activated T cells (T helper 17 cells), a pro-inflammatory cytokine (allergic responses),**
D9,D10	**IL-17E**	**Interleukin-17E or IL-25, produced by mast cells and T helper type 2, epithelial cells and granulated white cells, induces NF-$_k$B activation, stimulates for IL-8**
D11,D12	**IL-18/IL-1F4**	**Interleukin-18 belongs to IL-1 family , produced by macrophages and other immune cells, pro-inflammatory cytokine**
D13,D14	**IL-21**	**Interleukin-21, produced by activated CD4 T cells, it reregulates the immune cells functions e.g. NK cells, cytotoxic t cells**
D15,D16	**IL-27**	**Interleukin-27, a member of IL-12 family, expressed by APCs, stimulates T cells differentiation, upregulates IL-10 secretion**
D17,D18	**IL-32α**	**Interleukin-32α, produced by NK cells, macrophages and other immune cells, fibroblasts, epithelial and endothelial cells, pro-inflammatory cytokine**
E1,E2	**Reference spots**	**Nil**
E3,E4	**MIF**	**Macrophage migration inhibitory factor, produced by , regulates the innate immunity, inflammatory**

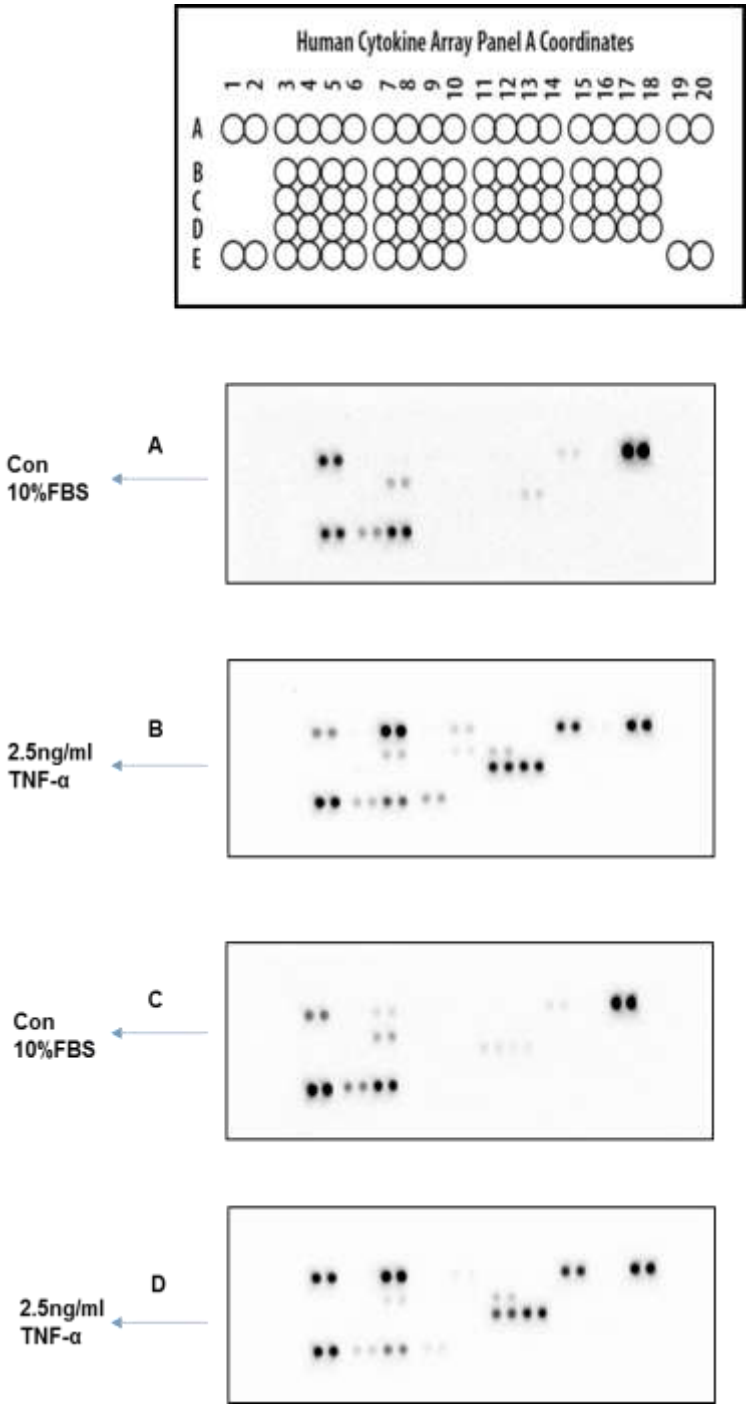
		cytokine
E5,E6	**Serpin E1/PAI-1**	**Serpin E1/Plasminogen activator inhibitor-1, an enzyme-a specific inhibitor of tissue plasminogen activator (tPA), produced by endothelial cells and adipose tissue, controls biological processes e.g. coagulation, inflammation, its high levels are indicator of thrombosis and atherosclerosis**
E7,E8	**TNF-α**	**Tumour necrosis factor-α, produced by macrophages and other immune cells, a pro-inflammatory cytokine**
E9, E10	**TREM-1**	**Receptor, Triggering receptor expressed on myeloid cells 1, primes myeloid cells to respond to other stimuli**
E19, E20	**Negative control**	**Nil**

(2015, R&D Systems)

8-4-2: Coordinate reference and representative images



1. Representative images of human cytokine Array. Conditioned media's (supernatant's), antigens binding to capture antibodies on membranes. (A) In 1ml of control (B) In 1ml of 2.5ng/ml TNF-α (C) In 1ml of conditioned control, and (D) In 1ml of 2.5ng/ml TNF-α conditioned media. All samples from female healthy donor.



2. Representative images of human cytokine Array. Conditioned media's (supernatant's), antigens binding to capture antibodies on membranes. (A) In 1ml of control, and (B) In 1ml of 2.5ng/ml TNF-α, both from female diabetic patient. (C) In 1ml of control, and (D) In 1ml of 2.5ng/ml TNF-α, both from male diabetic patient.

8-5: Quantitate the TNF-α levels (ELISA)

To quantitate the relative levels of human TNF-α cytokine in the diabetic and non-diabetic dermal fibroblasts supernates (conditioned media), 10% FBS control conditioned and 2.5 ng/ml of TNF-α conditioned media were assessed to measure the TNF-α concentration), which were collected from migration assay (section 2-14). The ELISA kit (Abcam TNF-α SimpleStep ELISA, ab18142, UK) was used to carry out these measurements with a limitation of (15.6- 2000 pg/ml). The principle of the assay is, that labelled capture antibodies and detector antibodies which immunocaptured the sample analyte (protein) in solution, forming complexes of (capture antibody- analyte- detector antibody) are fixed in the wells. Reagents were prepared according to manufacturer's instructions, 1X wash buffer PT (was prepared by diluting 5ml of 10X wash buffer PT with 45 ml distilled water with a final volume of 50 ml), and also 3 ml of antibody cocktail mixture was prepared (by mixing 300µl of capture antibody and detector antibody with 2.4 mL of antibody diluent 5BI). All standards, samples, and controls wells were performed in duplicate of 50 µl volume of each in one plate. The standard curve was generated, by adding 500 µl of sample diluent NS to lyophilized TNF-α recombinant protein, mixing, and incubating at room temperature (RT) for 10 minutes to get stock standard solution with a concentration of 10000 pg/ml. Eight standards tubes were prepared (7 standard tubes + 1 blank tube -no protein), after 360 µl of sample diluent NS was added to standard micro tube1 and 150 µl were added for the 7 left standards microtubes. To prepare the standard tube1 (40 µl of stock standard solution was added and mixed), then 150 µl was taken from standard

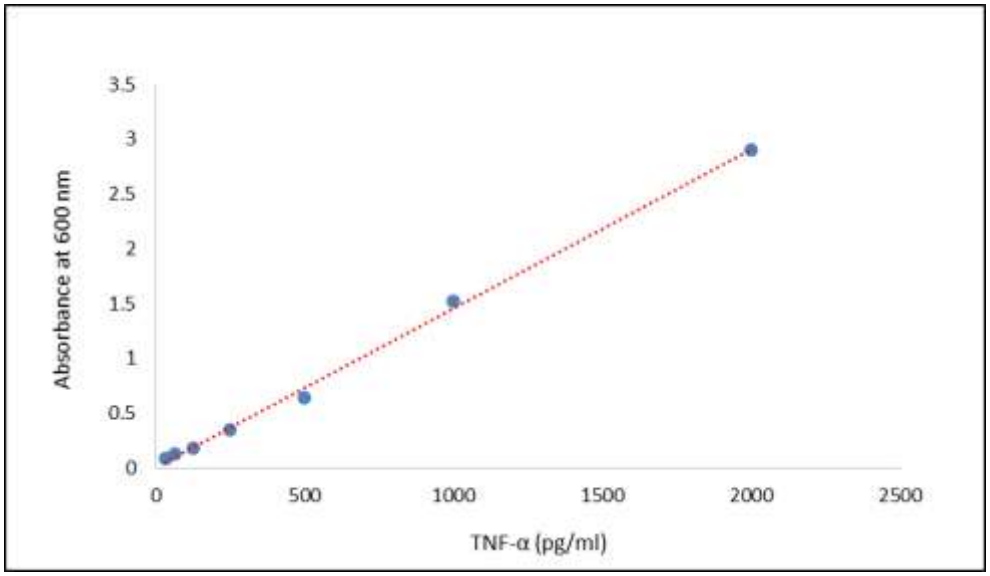
tube1 and added to standard tube 2 (by this way the standard tube 2 is prepared), this way was repeated to prepare the other 3-7 standard tubes, while standard tube 8 is blank (zero TNF-α protein).

Fifty microliter from each standards, conditioned media and controls were added into appropriate wells of 96 microtiter well plate. Then, 50 µl of antibody cocktail mixture was added to each well, the plate was wrapped in foil (kit supplier) to avoid the light and incubated for an hour at RT on a shaker at 400 rpm. After the wells were washed for three times with 350 µl of 1X wash buffer PT and the plate was dried carefully by inverting the plate on a paper towel after the final wash. TMB development solution was added in the amount of 100 µl to each well and the wrapped plate incubated for 10 minutes on a shaker (at 400 rpm) at RT. Immediately, when the blue colour was developed the absorbance readings were taken at 600 nm using Infinite 200 PRO microplate reader (Tecan, Switzerland), and the standard curve was performed, the absorbance versus treatment concentrations.

8-5-1: Analysing of TNF-α levels (ELISA)

The average of duplicate absorbance readings was calculated for each well and the average zero (standard blank) was subtracted from all absorbance readings. A standard curve was performed by excel, which used to find out the concentration of TNF-α for the unknown samples. GraphPad prism7 is used to draw the plot, the TNF-α concentration in (pg/ml) against conditioned media treatments.

8-5-2: The standard curve and graphs of TNF-α (ELISA)



Representative standard curve of TNF-α (ELISA). The absorbance measured at 600 nm.

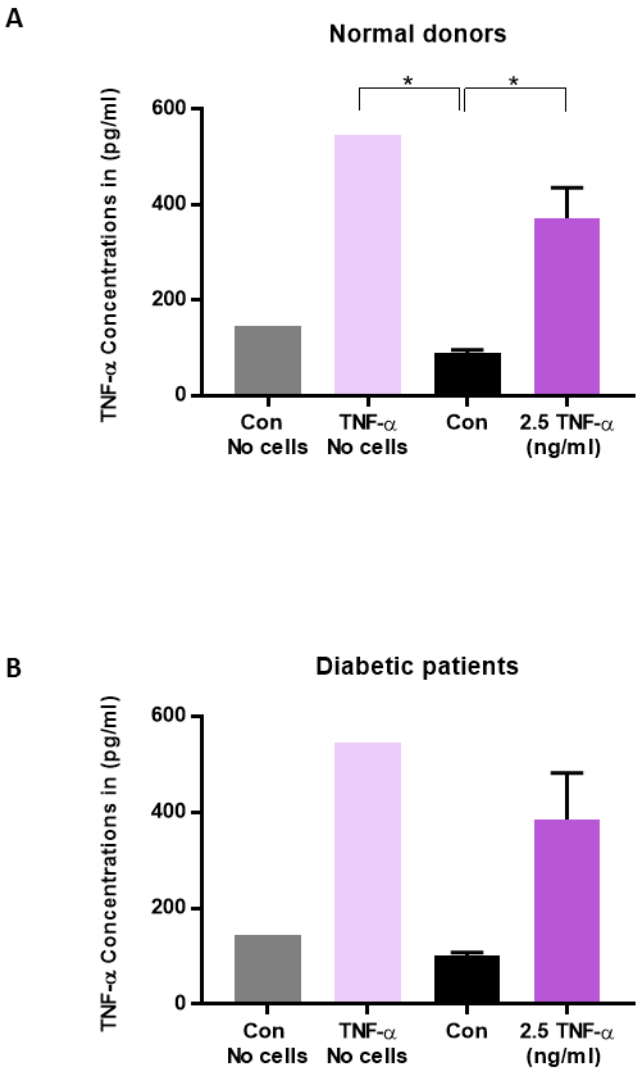


Fig1: The TNF-α levels in conditioned media of diabetic and non-diabetic DFs in (pg/ml). Dermal fibroblasts were scratched and incubated with or without 2.5 ng/ml of TNF-α for 24h, and the conditioned media were collected. (A) Healthy DFs from female donors (n=4), facial skin, P3, and age range (45-67y). (B) Diabetic DFs from 2females/2males (n=4), lower leg, P3, and age range (55-67y). *P<0.05, one-way ANOVA.

8-5-3: The quantitative determination of TNF-α for diabetic and non-diabetic dermal fibroblasts

The conditioned media (biomarkers) were used to assess and compare the TNF-α level measurements of scratched diabetic and non-diabetic dermal fibroblasts, to find out whether these levels are pathological that lead to inflammation or progress diabetes and its complications or as a result of ageing.

In cutaneous of old people, TNF-α was found as a key responsible of collagen degradation via upregulating the expression of MMP9 in dermal fibroblasts (all of donors /patients are aged (45-67 years) (Borg *et al.,* 2013), as well as the inflammation, is associated with diabetes and its complications, such as micro- and macro- vascular especially in type 2. More, diabetes is characterised by chronic hyperglycemia which promotes the increase of pro-inflammatory cytokines as TNF-α which in turn leads to apoptosis of pancreatic β-cells resulting in diabetes progression (Ping *et al.,* 2017). Results have shown, a similarity of both healthy donors and diabetic patient's plots, but there was a significant increase ($P< 0.05$) in the concentration of TNF-α in healthy cells that incubated with (2.5 ng/ml) compare to control (Fig 1). The concentration of TNF-α in control of diabetic cells was (92-119 pg/ml), while for healthy cells was (77-107 pg/ml), while the concentration of TNF-α of diabetic cells that incubated in (2.5 ng/ml) was (146 – 471 pg/ml), while for healthy cells was (212- 521 pg/ml), from present results it seems that TNF-α has induced TNF-α/inflammation in healthy cells that incubated in (2.5 ng/ml) more than diabetic. This may be due to that diabetic patients may have taken medicine or the stage of diabetes.