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Assessing mitochondrial function, oxidative stress, and vitamin D status in dark-skinned patients with keloid disease

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Declaration of Originality

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Assessing mitochondrial function, oxidative stress, and vitamin D status in dark-skinned patients with keloid disease

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Executive Summary

Keloid disease is a challenging condition that occurs worldwide and is mainly experienced by dark-skinned population groups. Keloids are characterised by excessive fibrous tissue formation. Unlike normal scars, keloids extend beyond the boundaries of the original wound and continue to grow for months or years. They are firm, shiny, and irregular in shape. Symptoms, such as itching and pain, are associated with keloid disease. Keloids tend to appear in areas such as the chest, shoulders, earlobes, and upper back and have a high recurrence rate after treatment. Recent evidence has demonstrated that mitochondria play a critical role in meeting the substantial energy requirements of wound repair and facilitate healthy wound healing through cytoskeletal remodelling, mediated by mitochondrial morphological alterations and reactive oxygen species (ROS) signalling pathways. Although the exact pathophysiology remains unclear, emerging evidence suggests that mitochondrial dysfunction and oxidative stress play critical roles. Vitamin D (Vit D), known for its antioxidant properties and its influence on mitochondrial health, may also be implicated, especially in populations prone to Vit D deficiency.

This study aimed to assess mitochondrial function, oxidative stress, and Vit D status in dark-skinned patients with keloid disease. Specifically, we explored the complex correlation between mitochondrial dysfunction and oxidative stress, as indicated by malondialdehyde (MDA) levels over a range of Vit D concentration levels.

Keloid tissue and blood samples were collected from a cohort of patients with dark-skinned keloids. A total of 44 patients were included in the study, of which 27 were male and 17 were female and were between the ages of 18 – 40 years old. Vitamin D status was assessed by measuring plasma Vit D concentrations, and lipid peroxidation was assessed by measuring MDA levels in the blood plasma, which is a biomarker for oxidative stress in cells. Mitochondrial morphology was examined using Transmission Electron Microscopy (TEM), focusing on the mitochondrial size, membrane integrity, and abundance. Correlation analysis was conducted to evaluate the complex relationship between mitochondrial integrity, MDA levels, and Vit D status.

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The results revealed a significant negative correlation (r = -0.52) between viable mitochondrial DNA (mtDNA) and MDA levels, indicating that higher levels of oxidative stress are associated with greater mitochondrial dysfunction. Patients that had lower concentration levels of Vit D showed elevated MDA levels within their blood plasma and more severe mitochondrial damage, such as no clear double membrane, improper folding of the cisternae, and small mitochondria compared to those with higher concentration levels of Vit D. In contrast, 32 dark-skinned patients had adequate Vit D levels. The TEM analysis highlighted significant mitochondrial abnormalities in keloid cells, including reduced size, damaged membranes, and decreased mitochondrial quantity. These defects were more pronounced in patients with Vit D deficiency.

In conclusion, the findings of this study suggest that mitochondrial integrity and oxidative stress are key components of keloid pathogenesis, particularly in dark-skinned patients with low concentration levels of Vit D. The significant association between reduced mitochondrial function and increased oxidative stress highlights the potential role of Vit D in modulating these processes. This study emphasises the need for further research on Vit D supplementation along with calcium as a therapeutic intervention for keloid disease, particularly in dark-skinned individuals prone to low concentration levels of Vit D and keloid formation.

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List of Abbreviations

ATP	Adenosine triphosphate
ADP	Adenine diphosphate
BHT	Butylated hydroxytoluene
Ca^{2+}	Calcium
CAT	Catalase
CHBAH	Chris Hani Baragwanath Academic Hospital
CI	Confidence interval
DNA	Deoxyribonucleic acid
S-1	Dimethylaminoethanol
DMSO	Dimethyl sulfoxide
dH_2O	Distilled water
DDSA	Dodecenyl succinic anhydride
ER	Endoplasmic reticulum
ETC	Electron transport chain
ECM	Extracellular matrix
FBS	Fetal bovine serum
$FADH_2$	Flavin adenine dinucleotide
gDNA	Genomic deoxyribonucleic acid
GSH	Glutathione
GA/FA	Glutaraldehyde-formaldehyde
GPx	Glutathione peroxidase
HIV	Human immunodeficiency virus

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HLA	Human leukocyte antigen
HCL	Hydrochloric acid
H_2O_2	Hydrogen peroxide
IMM	Inner mitochondrial membrane
IL-1	Interleukin-1
IL-6	Interleukin-6
IMS	Intermembrane space
KEAP1	Kelch-like ECH-associated protein 1
KFs	Keloid fibroblasts
Li-hep	Lithium heparin
MDA	Malondialdehyde
MMP's	Matrix metalloproteinases
MNA	Methyl nadic anhydride
mtDNA	Mitochondrial DNA
mVSS	Modified Vancouver Scar Scale
NADH	Nicotinamide adenine dinucleotide
Nrf2	Nuclear factor erythroid 2-related factor
PBMC's	Peripheral blood mononuclear cells
PGC-1α	Peroxisome proliferator-activated receptor-γ coactivator-1-alpha
PBS	Phosphate buffer saline
H_3PO_4	Phosphoric acid
qPCR	Quantitative polymerase chain reaction
ROS	Reactive oxygen species
RT	Room temperature

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SIRT3	Sirtuin 3
SDS	Sodium dodecyl sulphate
NaOH	Sodium hydroxide
SA	South Africa
SEM	Standard error of the mean
SBAH	Steve Biko Academic Hospital
SOD2	Superoxide dismutase
SMAD	Suppressor of mothers against decapentaplegic
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reactive substances
TIMPs	Tissue inhibitors of metalloproteinases
TGF-β	Transforming growth factor-beta
TEM	Transmission electron microscopy
TNF-α	Tumour necrosis factor-alpha
Coenzyme Q	Ubiquinone
NEDD4	E3 ubiquitin-protein ligase
UVB	Ultraviolet B
Vit D	Vitamin D
Vit D3	Vitamin D3
VDR	Vitamin D receptor

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List of Symbols and Units

cm	Centimetre
mm^3	Cubic Millimetre
°C	Degree Celsius
g	Gram
kV	Kilovolts
µL	Microlitre
µm	Micrometre
µM	Micromole
mL	Millilitre
mM	Millimole
M	Mole
ng	Nanogram
nm	Nanometre
nM	Nanomole

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Chapter 1: Background and Literature Review

1.1 Background

Keloid disease is a chronic, fibrotic skin disorder characterised by excessive formation of scar tissue at sites of skin injury.(1) Unlike normal scars, keloids grow beyond the boundaries of the original wound, creating raised, firm, and often painful or itchy lesions.(1) While keloids are benign, they can cause significant functional and psychological distress owing to their unsightly appearance and persistent nature.(1, 2) The exact etiology of keloid formation is complex and remains poorly understood. However, it is believed to involve an aberrant wound healing response driven by dysregulated cellular processes such as fibroblast hyperproliferation, excessive collagen deposition, and inflammation.(3)

Emerging research suggests that investigating mitochondrial function and oxidative stress could be key to understanding the pathophysiology of keloid disease.(4) Mitochondria play a critical role in cellular energy production, and dysfunction of these organelles can lead to excessive ROS production, contributing to oxidative stress.(5) Elevated oxidative stress has been implicated in various pathological processes, including abnormal wound healing, which may exacerbate inflammation and fibroblast activity, thereby promoting the overgrowth of scar tissue in keloids.(6)

Additionally, Vit D has been recognised for its immunomodulatory and antifibrotic properties, which make it a potentially important factor in keloid formation.(7) Deficiency in Vit D may impair normal wound healing processes, leading to an increased susceptibility to excessive scar formation. Investigating the relationship between Vit D status, mitochondrial dysfunction, and oxidative stress in keloid patients could provide new insights into the mechanisms underlying keloid development and pave the way for novel therapeutic interventions.

1.2 Keloid pathophysiology

A keloid is characterised as a dermal tumour, wherein excessive collagen deposition occurs in response to inflammation or trauma to the skin of the individual.(8) Unlike normal scars, keloids grow beyond the original margins of the scar, but show no signs of regression.(1) Keloids usually start to develop after three months, when there has

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been any sort of injury or trauma to the skin, but can also take several years to form.(1, 2) The exact pathophysiology of keloids is not fully understood, but several factors contribute to their formation.

1.2.1 Fibroblast dysfunction in keloid pathogenesis

Fibroblasts are cells found in connective tissue that play a crucial role in the maintenance, repair, and structural integrity of tissues in the body. Fibroblasts are responsible for producing and organising the extracellular matrix (ECM), a network of proteins such as collagen and elastin that provides structural support to tissues.(9) In keloids, fibroblast activity is dysregulated in the following ways:

Hyperproliferation of fibroblasts

Hyperproliferation refers to an abnormal increase in the number of fibroblasts, a condition that occurs when these cells undergo excessive or uncontrolled division. In keloids, fibroblasts multiply more rapidly than they would during normal tissue repair, and as a result, cause an excessive build-up of fibroblasts at the site of tissue injury or within the tissue matrix, leading to abnormal tissue growth.(10)

Excessive collagen production

Keloid fibroblasts (KFs) produce abundant amounts of collagen, especially type I and type III collagen.(11) The collagen bundles in keloids are thick, disorganised, and arranged arbitrarily, which contributes to the raised and firm nature of keloid scars.(12) In normal wound healing, collagen production slows down during the remodelling phase; however, in keloids, fibroblasts produce collagen long after the wound has healed.(12)

Aberrant cell signalling pathway

Hyperproliferation of fibroblasts is often driven by the overactivation of certain growth factors and cytokines, particularly transforming growth factor-beta (TGF-β).(13) The role of TGF-β is to not only boost collagen production but also reduce the levels of matrix metalloproteinases (MMPs), resulting in the build-up of ECM.(13) Furthermore, TGF-β promotes the recruitment and transformation of fibroblasts into myofibroblasts, which have stronger contractile abilities and contribute to the raised, firm structure of keloid scars.(14)

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Reduced apoptosis

Under normal circumstances, once wound healing is complete, fibroblasts undergo apoptosis (programmed cell death) to decrease in number and inhibit collagen production.(15) However, under hyperproliferative conditions, fibroblasts resist apoptosis, remain active, and continue to produce collagen and other ECM proteins.(15) The inability to reduce fibroblast activity leads to excessive tissue growth, as observed in keloids.

1.2.2 Clinical and histological features of keloids

Clinical features

A keloid is a firm, raised scar with a flat and shiny surface that exhibits viscoelastic properties. Unlike normal scars, they extend beyond the original wound area.(16) Keloids vary in colour as they are often hyperpigmented (darker) or erythematous (reddish) depending on the individual's skin tone and the age of the keloid.(16) Over time, they may fade to a more skin-toned colour but often retain a darker or redder tone. Keloids have irregular shapes and are significantly larger than the initial wound.(17) They tend to grow gradually over time, often taking months or years to achieve their maximum size.(17) They are present in various sizes ranging from a few millimetres to several centimetres in diameter.(17) The most common locations for keloids to form include the upper arms, chest, shoulders, overlying skin joints, and head-neck regions. However, they can develop anywhere on the skin that has been injured.(18) Many keloids are asymptomatic, but some may cause symptoms such as pain, pruritus, burning, and movement limitations, particularly when they are actively growing.(16) Keloids usually start to develop after three months of injury but can also take several years to form.(17) Even after surgical excision, keloids have a high recurrence rate between 45% and 100%, often growing back larger than before.(19)

Histological features

Keloids are marked by the overproduction of collagen, particularly types I and III. The collagen fibres in keloids are thick, disorganised, and arranged irregularly, unlike normal scars, where the fibres are aligned parallel to the skin surface.(16) Under a microscope, the collagen in keloids often presents as dense, glassy (hyalinised) bundles, which are distinctive to keloids and aid in differentiating them from hypertrophic scars.(20) Keloids have an abnormally high number of active fibroblasts,

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which are responsible for producing collagen and other ECM components.(16) These fibroblasts are more proliferative and continue to produce collagen long after normal healing has stopped.(16) During normal wound healing, enzymes known as MMPs play a role in the breakdown of excess collagen. In keloids, MMPs activity is diminished, while tissue inhibitors of metalloproteinases (TIMPs) are elevated, resulting in reduced collagen degradation.(13) Keloids generally have enhanced vascularity with numerous blood vessels, which contributes to their thicker and more vascular appearance, especially at the periphery of the lesion.(21, 22) In some keloids, there is an accumulation of mucin, a gel-like substance rich in glycoproteins, within the ECM of keloid tissue.(23) Mucin can contribute to the thickened and firm texture characteristics of keloids. As mucin is a hydrophilic substance, it allows water absorption, which may contribute to the swelling and persistence of keloids.(24) The presence of mucin, when found in a keloid, adds another level of complexity to the lesion structure. Lastly, keloids contain inflammatory cells, such as lymphocytes and macrophages, indicating that chronic inflammation contributes to the persistence of abnormal scar growth.(25)

1.2.3 Difference between keloids and hypertrophic scars

Keloids and hypertrophic scars are both forms of abnormal scarring, yet they differ significantly in appearance, growth behaviours, and underlying biological processes.

Keloids extend beyond the original wound site, invading the surrounding healthy skin, whereas hypertrophic scars remain confined within the boundaries of the wound.(10, 26) Keloids may take months to form and continue to grow for years, whereas hypertrophic scars typically develop soon after the injury and stabilise within a few months.(10, 26) In terms of collagen structure, hypertrophic scars have collagen organised more parallel to the skin surface, while keloids exhibit thick, dense, and haphazardly arranged collagen bundles.(10) Additionally, keloids have a high rate of recurrence even after treatment, while hypertrophic scars generally improve over time and are less likely to recur after excision or therapy.(19)

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1.2.4 Clinical assessment of keloids

The evaluation of keloid severity involves both clinical examination and, in some cases, quantitative methods. According to the latest procedures, keloid severity is assessed through a combination of clinical assessments and scoring systems.

The clinical assessment method entails visual and physical examination of the keloid by a healthcare professional. Key aspects that are evaluated during the clinical assessment include the following: shape (the dimensions of the keloid, often measured with a ruler or calliper, and the keloid's contours and whether it is flat or raised), colour (the pigmentation of the keloid in comparison to the surrounding skin), texture (the texture of the keloid, which may be smooth, rough, or nodular), whether the keloid is suppurative, and symptoms (such as itching, pain, or tenderness). Along with clinical assessment, the health professional will use one of several standardised scoring systems to evaluate the severity of the patient's keloid.

The modified Vancouver Scar Scale (mVSS) is the most used scar scoring system. The mVSS is widely used to assess scar severity, particularly hypertrophic scars, and keloids. The mVSS evaluates four key parameters: pliability, height, vascularity, and pigmentation. Each parameter is scored, and the total score provides an overall assessment of keloid severity and acts as a guide for treatment decisions.(27)

1.2.5 Prevalence of keloids

According to research, keloids are most prevalent in individuals aged between 11 and 30 years, with an equal preference in both sexes.(28-31) The prevalence of keloid disease differs between all geographical areas around the world, but is correlated with skin pigmentation, as dark-skinned population groups are more at risk.(32) The available data are limited and contradictory. Table 1.1 indicates the various prevalence percentages of keloid disease in different countries. Ethnic groups that are dark-skinned have an estimated incidence of between 5 – 10%.(33) In Africa, there have been a few research studies conducted on keloids, specifically in Cameroon(34), Ghana(35), Democratic Republic of Congo(36), Zambia(37), and Kenya(36) however, there is currently no research data available on the prevalence of keloid disease in South Africa (SA), but in the clinical setting, it is observed frequently. Keloid disease is considered to have a genetic predisposition as it is usually inherited in a familial pattern, characterised by an autosomal dominant inheritance pattern with variable

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expressivity and incomplete dominance.(38) Several genes have been implicated in keloid formation, though no single causative gene has been universally identified. Genetic studies suggest that dysregulation in the TGF-β signalling pathway plays a central role, with variations in genes such as TGF-β1, TGF-β2, TGF-β3, and Suppressor of Mothers against Decapentaplegic (SMAD), (SMAD2, SMAD3, SMAD7), influencing fibroblast proliferation and extracellular matrix deposition. Other candidate genes, including E3 ubiquitin-protein ligase (NEDD4) and Human Leukocyte Antigen (HLA) variants (HLA-DRB1 and HLA-DQA1), have also been linked to keloid susceptibility in certain populations.(39)

Genetic associations with keloids appear to differ across ethnic groups. Studies indicate that individuals of African and African-American descent have a higher genetic susceptibility, potentially due to variations in TGF-β-related genes. In contrast, East Asian populations have shown associations with HLA gene variants, while keloids are less common in European populations, with limited genetic studies available for this group.

Table 1.1 Prevalence of keloid disease in various countries

Country	Keloid incidence (%)	References
Great Britain	0.09%	(33, 36)
Japan	0.1%	(33)
Democratic Republic of Congo	16.0%	(36)
Zambia	9.0%	(37)
Kenya	8.5%	(36)

Although a significant amount of progress has been made to understand keloid pathophysiology, the primary cause and treatment are still unknown.(40). When surgical excision is the only method used, there is a recurrence rate of 45 – 100% for the keloid to start forming again.(19) There are many treatment possibilities available that have been supported including corticosteroids, 5-fluorouracil, bleomycin, interferon, cryotherapy and verapamil that have not yet produced optimal treatment results.(41) Keloids have a high recurrence rate and, therefore, add strain on the overworked South African healthcare system. Understanding the pathophysiology of keloids provides a crucial foundation for exploring the role of mitochondrial function in

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keloid disease, as mitochondrial integrity is recognised as a key factor influencing keloid development and persistence.

1.3 Mitochondrial function in keloid disease

Mitochondria are vital organelles responsible for producing cellular energy in the form of adenosine triphosphate (ATP) through oxidative phosphorylation, which supports various cellular functions including tissue repair and regeneration.(42) During normal wound healing, mitochondrial activity is essential for regulating processes, such as cellular proliferation, collagen synthesis, and programmed cell death.(43) In keloid disease, these functions are disrupted.(44) Given the role of mitochondria in energy production and cellular repair, understanding how these organelles function in keloid pathology is essential. This section examines the disruption of mitochondrial energy production in KFs and its contribution to the abnormal wound healing response characteristic of keloids in contrast to normal scar formation.

1.3.1 Oxidative phosphorylation

Normal oxidation phosphorylation

Oxidative phosphorylation, illustrated in Figure 1.1, starts with electron transfer from high-energy molecules such as nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide ($FADH_2$) into the electron transport chain (ETC), which consists of four protein complexes found within the inner mitochondrial membrane.(45) Electrons get transferred from NADH to Complex I, which utilises the released energy to pump protons (H^+) from the mitochondrial matrix into the intermembrane space, thereby creating a proton gradient.(45) The electrons are then passed to ubiquinone (coenzyme Q), which transports them to Complex III.(46) Flavin adenine dinucleotide donates electrons directly to Complex II, which passes electrons to coenzyme Q and transfers them to Complex III.(45) Coenzyme Q delivers electrons to Complex III and pumps more protons into the intermembrane space, thereby further increasing the proton gradient.(46) Electrons are then passed to cytochrome c, a mobile electron carrier that transports electrons to Complex IV.(45) Electrons are then transferred from cytochrome c to Complex IV, which pumps additional protons and transfers electrons to oxygen.(45) Oxygen is the final electron acceptor and combines with protons to form

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water (H_2O), thus completing the electron transfer process.(46) Lastly, the proton gradient created by the ETC pushes protons back into the mitochondrial matrix through ATP synthase. The flow of protons energizes ATP synthase, which catalyses the conversion of adenine diphosphate (ADP) to ATP.(45) This process is known as chemiosmosis, and is how the cell efficiently produces ATP.(47)

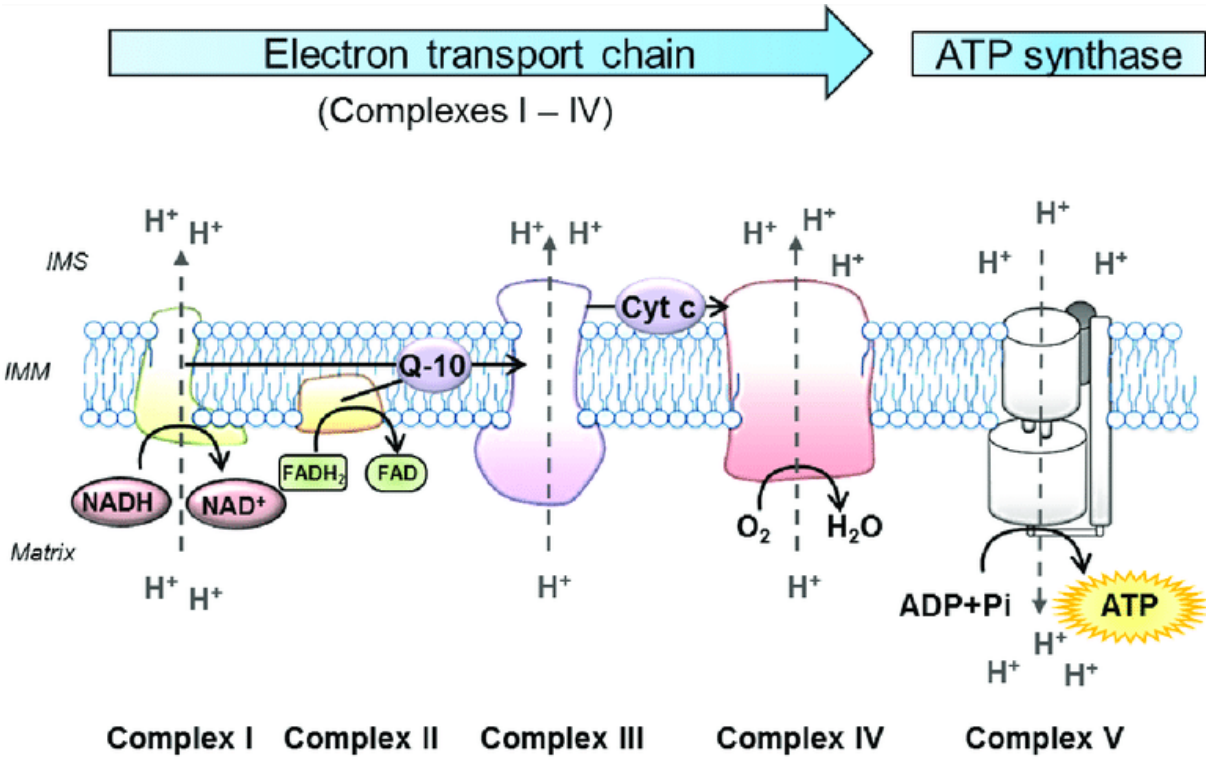

Figure 1.1 Overview of the key steps in oxidative phosphorylation. This figure illustrates the normal process of oxidative phosphorylation, highlighting the electron transport chain and ATP synthesis.(48) Inner mitochondrial membrane (IMM), intermembrane space (IMS).

Altered oxidative phosphorylation in keloid disease

In keloid disease, oxidative phosphorylation is altered, which contributes to abnormal wound healing and excessive scar formation. Due to the extremely high levels of ROS found within KFs, Complexes I – III become inactivated, therefore decreasing its ability to effectively transfer electrons from NADH to coenzyme Q.(45, 49) As a result, this can lead to reduced proton pumping efficiency, which would cause a decrease ATP production.(43) In keloid disease, there is often a further increase in ROS production due to inefficient electron transfer at Complexes I and III.(43) The increased ROS levels not only damage mitochondrial components but also enhance oxidative stress,

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which further impairs ETC function and causes damage to lipids, proteins, and deoxyribonucleic acid (DNA).(50) Inefficient electron transfer through ETC can decrease the proton gradient across the inner mitochondrial membrane.(45) As a result of fewer protons pumped into the intermembrane space, the electrochemical potential driving ATP synthesis weakens, which can lead to reduced production of ATP.(45) In KFs, the reduced proton gradient also affects ATP synthase, which relies on the flow of protons back into the mitochondrial matrix to generate ATP. A decreased proton gradient results in lower levels of ATP synthesis, leading to an energy deficit in cells. Complex IV in keloid cells is also altered, as inefficient electron transfer to oxygen can slow down the entire ETC, thereby decreasing the production of water and ATP.(45) These disruptions in mitochondrial function may contribute to the excessive fibroblast activity observed in keloid formation, a relationship that this study aims to investigate further.

1.3.2 Differences in wound healing in keloid disease

Wound healing is significantly altered in keloid disease, leading to excessive and abnormal scar formation.(51) The normal wound-healing process involves a well-coordinated sequence of phases designed to restore tissue integrity, including haemostasis, inflammation, proliferation, and remodelling. However, several key aspects of this process are dysregulated in keloid disease, resulting in uncontrolled scar tissue growth.(51) The following sections explore how keloid formation alters these phases of wound healing.

Haemostasis phase

The haemostasis phase begins immediately after injury and can last for several hours.(52) In keloid formation, this phase is only minimally affected because it primarily focuses on stopping bleeding and initiating the inflammatory process. However, keloids may indirectly influence haemostasis by affecting blood vessels and surrounding tissue.(22)

Keloids can affect the structure and function of blood vessels at the wound site and exhibit abnormal angiogenesis and the formation of new blood vessels.(22) Although this process is typically more relevant to the later stages of wound healing, it might

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indirectly influence the initial haemostatic response by altering blood flow patterns and the integrity of blood vessels.(22) Keloids create a persistent inflammatory environment characterized by the prolonged presence of inflammatory molecules such as cytokines and chemokines.(53) While these molecules primarily affect the later stages of wound healing, they may subtly influence the haemostasis phase by modulating the activity of platelets and other cells involved in clot formation and vascular repair. Altered tissue architecture and composition of keloids may affect the mechanical stability of the wound site.(54) While haemostasis primarily involves physical sealing of blood vessels to prevent excessive bleeding, the presence of keloid tissue may impact the formation and stability of the initial blood clot.

Inflammation phase

The inflammatory phase begins within minutes of injury, peaks within two to three days, and can persist for one to two weeks.(55) Keloids sustain a chronic inflammatory environment characterised by the persistent presence of inflammatory mediators such as cytokines, chemokines, and growth factors.(53) This prolonged inflammatory response extends beyond the normal duration of the inflammatory phase in wound healing, leading to continuous tissue damage and delayed healing.

Keloids allow increased infiltration of immune cells, such as macrophages, lymphocytes, and mast cells, into the wound site.(25) These immune cells release pro-inflammatory cytokines and growth factors, thereby preserving the inflammatory cascade and contributing to keloid pathogenesis.(25)

Keloid tissue shows decreased expression of cytokines and chemokines, with increased levels of pro-inflammatory cytokines, such as tumour necrosis factor-alpha (TNF-α), interleukin-1 (IL-1), and interleukin-6 (IL-6).(56) These cytokines promote fibroblast activation, collagen synthesis, ECM deposition, sustaining the inflammatory environment, and keloid formation. Inflammation in keloids contributes to sustained fibroblast activation, with fibroblasts exhibiting increased proliferation, migration, and collagen synthesis.(25, 56) Pro-inflammatory cytokines and growth factors released during the inflammation phase stimulate fibroblast activity, leading to excessive collagen deposition and scar formation characteristic of keloids.(25)

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Proliferation phase

The proliferative phase, also known as the tissue generation phase, typically begins three days post-injury and lasts for two to three weeks.(55) Fibroblasts are crucial in the proliferation phase of wound healing, where they migrate to the wound site and proliferate to produce ECM components including collagen.(57) In keloids, there is excessive and sustained fibroblast proliferation, leading to the accumulation of fibroblasts within the scar tissue.(25)

KFs exhibit enhanced collagen synthesis, resulting in the accumulation of excessive collagen fibres within the scar tissue, as shown in Figure 1.2.(58) This leads to the characteristic raised and firm texture of keloid scars, which extend beyond the boundaries of the original wound.(1, 59)

The ECM in keloids undergoes abnormal remodelling, characterised by increased collagen deposition, and altered collagen fibre organisation. Collagen fibres in keloids are often thick, irregularly arranged, and densely packed, contributing to the rigid and fibrous nature of keloid scars.(60, 61) There is simultaneous continuation of inflammation during the proliferation phase of keloid formation, with the ongoing release of pro-inflammatory cytokines and growth factors that stimulate fibroblast proliferation and collagen synthesis.(25) This sustained inflammatory environment contributes to the preservation of abnormal cellular activity and ECM deposition in the keloids.

Remodelling phase

The remodelling phase begins two to three weeks after injury and can last for a year or longer depending on the size of the scar.(55) In normal wound healing, collagen fibres undergo remodelling, becoming more organized and cross-linked to improve the strength and integrity of the scar tissue.(60, 61)

In keloids, collagen remodelling is dysregulated, resulting in the persistence of thick, disorganized collagen fibres within scar tissue, as illustrated in Figure 1.2.(62) Keloids exhibit changes in ECM composition, with increased deposition of collagen and altered

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expression of other ECM components such as proteoglycans and glycoproteins.(60) These alterations contribute to the stiff, fibrous texture of keloid scars and impair the normal maturation of scar tissue.

Inflammation endures throughout the remodelling phase of keloid formation, with the ongoing release of proinflammatory cytokines and growth factors that contribute to the dysregulated ECM remodelling process.(53) The prolonged inflammatory conditions sustain the activation of fibroblasts and synthesis of collagen, thereby contributing to the persistence of abnormal scar tissue.(25, 55)

The prolonged inflammation and excessive fibroblast activity observed in keloid wound healing create an environment full of cellular stress, which is further intensified by the addition of oxidative stress, a key factor contributing to the progression and persistence of keloid formation.

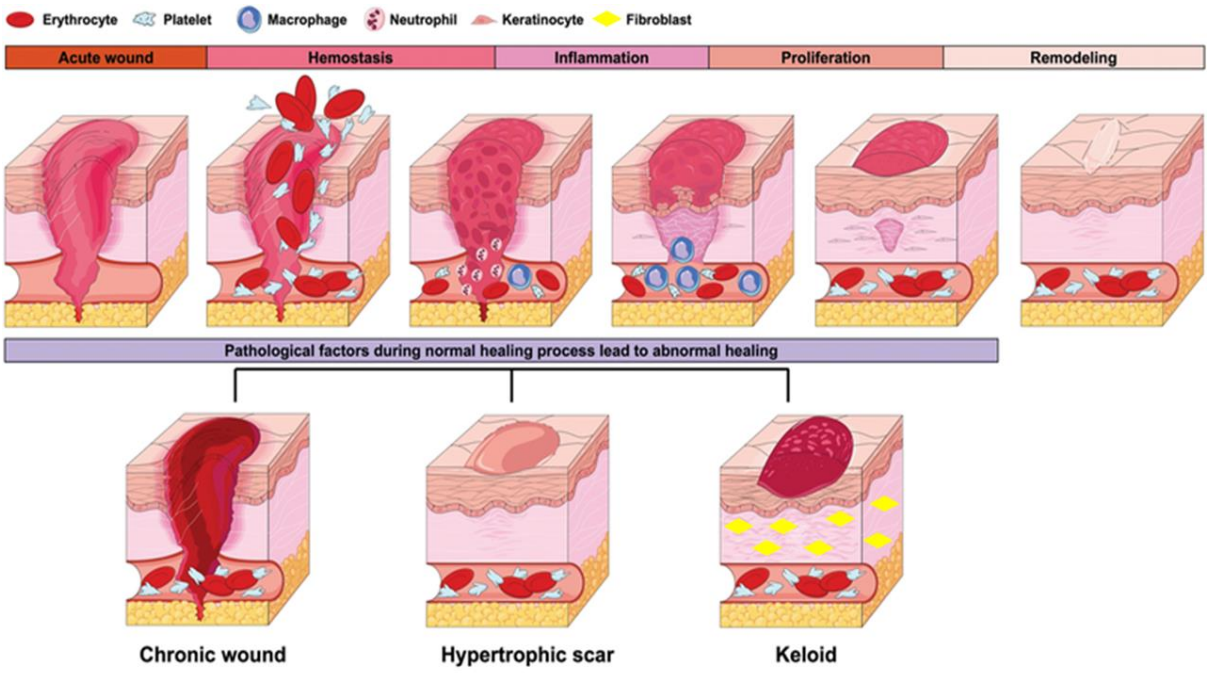

Figure 1.2 Comparative analysis of normal and keloid scars during the wound healing phases.(63) This figure illustrates the key differences in collagen organisation and remodelling between normal scars and keloid scars at various stages of wound healing. It highlights the presence of numerous fibroblasts in keloids, that produce thick collagen fibrils which contribute to their characteristic of raised and fibrous texture compared to normal scar tissue.

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The altered progression of wound healing phases in keloid-prone individuals not only disrupts normal repair processes but also creates an environment where cellular stress responses, such as oxidative stress, may be heightened, potentially accelerating fibroblast proliferation and collagen deposition, which are key factors in keloid formation.

1.3.3 Oxidative stress and keloid formation

In normal healthy wound healing, during the proliferation phase, mitochondria synthesise ATP for cellular activity, producing by-products known as ROS or free radicals. A free radical is an unstable molecule containing oxygen that can freely react with other molecules in the cell.(5) The most commonly produced ROS is superoxide (O_2^-). In the human body, these free radicals often contribute to aging, cancer, and redox signalling. When there is an abundance of free radicals within the cell, they can cause substantial damage; therefore, they are removed via antioxidant defence mechanisms.(64)

Oxidative stress occurs when antioxidant defence mechanisms do not remove free radicals effectively and cause an imbalance between ROS and antioxidants, as shown in Figure 1.3.(65) This imbalance of free radicals within the cell causes oxidative damage towards lipids, proteins, and mitochondrial DNA (mtDNA).(65)

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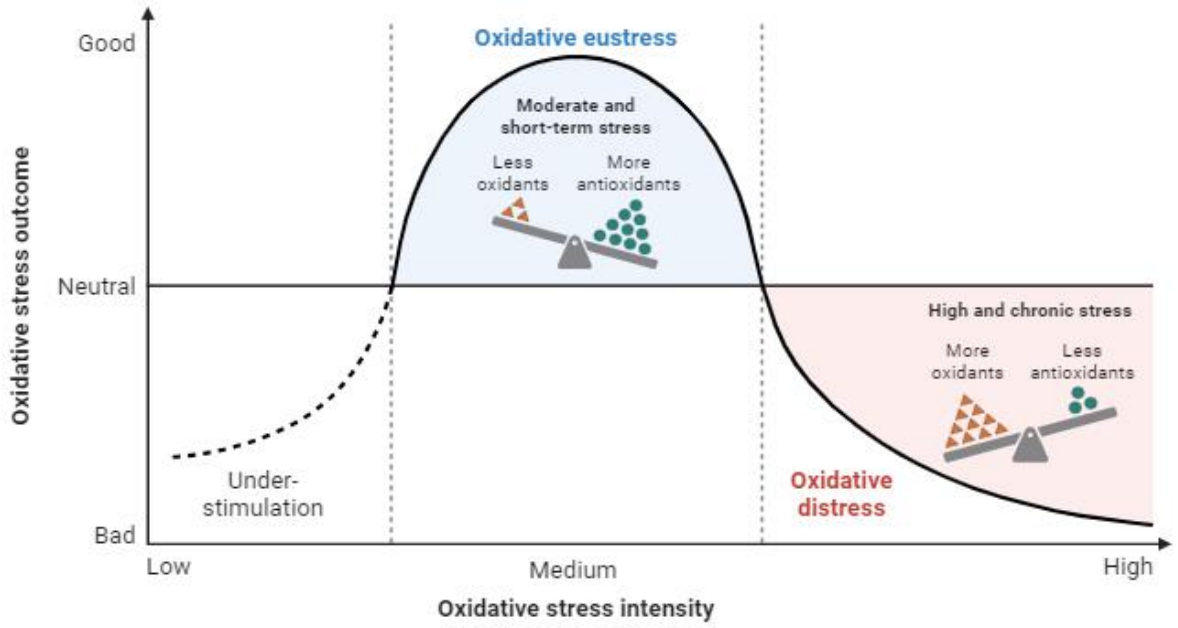

Figure 1.3 Relationship curve between oxidative stress and eustress as modulated by antioxidant levels. This figure illustrates that an excess of antioxidants relative to oxidants results in oxidative eustress, which can be characterised as beneficial stress. When the concentration of oxidants exceeds that of antioxidants, it results in oxidative stress, which is considered detrimental as it induces severe and prolonged. Available at https://app.biorender.com/biorender-templates/figures/all/t-6470c7367e20baa208370e7d-impacts-of-oxidant-and-antioxidant-imbalance-on-oxidative-st.

Mitochondria then undergo structural and functional changes, ultimately altering their integrity.(64) The altering of mtDNA is said to be a crucial cause of metabolic reprogramming in KFs.(4) During wound healing, mitochondria are involved in various cell signalling pathways, and when they become dysfunctional, these pathways can then be disrupted, potentially leading to abnormal fibroblast production and collagen deposition, as seen in keloids.(66) ROS can activate signalling pathways within fibroblasts, including mitogen-activated protein kinase and TGF-β.(67, 68) These pathways collectively contribute to the excessive fibroblast activity observed in keloids. Oxidative stress stimulates fibroblasts to increase collagen synthesis. In keloids, there is an imbalance between collagen synthesis and breakdown, causing excessive build-up of collagen type I in the ECM.(69) The activity of MMP's, enzymes involved in the degradation of ECM components, can also be affected by oxidative stress. In keloids, MMP activity decreases, which further contributes to the accumulation of collagen.(70)

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The role of oxidative stress in keloid formation highlights the need for effective antioxidant defence mechanisms that can help neutralise excess ROS and potentially lower the damaging effects that contribute to keloid scar development.

1.3.4 Modifications to antioxidant systems in keloid disease

The majority of cellular ROS are primarily generated within the mitochondria during normal oxidative phosphorylation. This occurs when electrons escape from the ETC.(71) Antioxidant systems are crucial for protecting cells from oxidative stress by neutralising ROS and maintaining cellular redox balance. Within the antioxidant system, there are a few crucial compounds, such as glutathione (GSH), glutathione peroxidase (GPx), superoxide dismutase (SOD2), and catalase (CAT).(71, 72)

The enzyme GPx reduces hydrogen peroxide (H_2O_2) and lipid hydroperoxides to water and lipid alcohols, respectively, using GSH as the substrate.(73) This prevents the build-up of harmful peroxides and protects the cells from oxidative damage.(73) However, in KFs, GPx activity can be impaired in KFs, leading to the accumulation of hydrogen peroxide and other ROS.(74) This promotes oxidative stress, which contributes to prolonged inflammation and abnormal fibroblast activity, including excessive collagen production.(74) GSH is a small antioxidant molecule that plays a key role in detoxifying ROS and maintaining cellular redox balance. It is a cofactor for several antioxidant enzymes including GPx. GSH also directly neutralises free radicals and regenerates other antioxidants, such as vitamins C and E, to their active forms.(75) Reduced levels of GSH are observed in KFs and impair the ability of cells to neutralise ROS and regenerate antioxidants. This deficiency contributes to oxidative stress, which stimulates fibroblast proliferation, inhibits apoptosis, and leads to enhanced deposition of collagen and ECM.(76) The enzyme SOD2 is located within the mitochondria and converts superoxide (O_2^-), a highly reactive ROS produced during cellular respiration, into H_2O_2. By detoxifying superoxide, SOD2 prevents oxidative damage to the mitochondrial proteins, lipids, and DNA.(77) In KFs, decreased SOD2 activity is observed, leading to the accumulation of superoxide radicals, causing mitochondrial dysfunction and increased ROS production.(74) This promotes oxidative stress and contributes to the abnormal wound healing process, including persistent fibroblast activity and increased scar tissue formation.(74) CAT is an enzyme that converts hydrogen peroxide into water and oxygen, thereby preventing

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the harmful effects of hydrogen peroxide on build-up. Catalase works together with other antioxidants, such as GPx and SOD, to maintain the cellular redox balance.(78) In keloid disease, decreased CAT activity leads to elevated hydrogen peroxide levels, and increasing oxidative stress.(74) This heightened oxidative stress contributes to abnormal wound healing in keloids by amplifying inflammation, encouraging fibroblast proliferation, and triggering excessive collagen production.(79)

Redox-signalling pathways

Maintaining an appropriate balance of oxidation in aerobic organisms is crucial because impaired antioxidant function can result in mutagenicity, cytotoxicity, and carcinogenicity.(80) The imbalance in oxidative and antioxidant systems in keloid disease affects redox-sensitive signalling pathways.(81) These pathways regulate processes, such as cell proliferation, apoptosis, and collagen synthesis. Disrupted redox signalling can lead to prolonged fibroblast survival, continuous collagen deposition, and persistent inflammation, all of which are hallmarks of keloid formation.(81)

Under normal conditions, nuclear factor erythroid 2-related factor (Nrf2) binds to a protein called Kelch-like ECH-associated protein 1 (keap1) in the cytoplasm.(82) Keap1 promotes the ubiquitination and degradation of Nrf2, thereby maintaining its low levels. When oxidative stress levels start to rise, the cysteine residues in Keap1 are modified, which disrupts the Keap1-Nrf2 interaction.(82) This disruption impairs Nrf2 activation, resulting in the expression of many antioxidant genes, including those encoding enzymes such as GSH, GPx, CAT, and SOD2.(82)

In KFs, because of the high levels of ROS(83), peroxisome proliferator-activated receptor-γ coactivator-1-alpha (PGC-1α) expression appears to be downregulated compared to that in normal skin fibroblasts because of the high levels of ROS.(82, 84) Since PGC-1α is crucial for maintaining mitochondrial function, its downregulation leads to mitochondrial dysfunction, causing reduced ATP production and an imbalance in ROS management.(84) The decrease in PGC-1α weakens the cell's ability to control oxidative stress, contributing to oxidative damage and persistent inflammation that characterise keloid formation.(83, 84)

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Sirtuin 3 (SIRT3), a mitochondrial enzyme involved in regulating oxidative stress and maintaining mitochondrial function, is downregulated by excessive ROS production in keloid disease.(85, 86) Reduced levels of SIRT3 in KFs, because of increased ROS levels, inhibits the cell's function to regulate ROS, leading to a further increase in oxidative stress levels.(87) This dysregulation contributes to mitochondrial dysfunction, enhanced fibroblast activity, and excessive collagen production, which are the key features of keloid formation.(87) The decrease in SIRT3 also disrupts energy metabolism, further promoting the abnormal wound healing and persistent inflammation seen in keloid scars.(88)

These pathways, which are sensitive to oxidative changes, are integral to the regulation of cellular responses to stress. In keloid disease, dysregulation due to excessive ROS contributes to persistent fibroblast activation, chronic inflammation, and excessive scar tissue formation, typical of keloid scars. Understanding the imbalance in antioxidant systems and redox signalling pathways in keloid formation provides a foundation for exploring the potential role of Vit D, a known regulator of the immune response and oxidative stress, in influencing keloid development and progression.

1.4 Vitamin D and keloid disease

1.4.1 Vitamin D in dark-skinned populations

The Fitzpatrick scale is a numerical classification schema for human skin colour. There are six different types that start from (I) pale white colour (meaning they burn more easily and never tan when in the sun) all the way, until (VI) dark brown to black skin (meaning they always tan and never burn when in the sun). Keloids are usually seen in populations with Fitzpatrick skin types (IV-VI).(89)

Vitamin D deficiency is more prevalent in dark-skinned populations because of the higher melanin content in the skin(90), which reduces the ability of the skin to produce Vit D when exposed to sunlight. A meta-analysis published in 2020 found that specific ethnic groups, especially those with dark skin, have high rates of Vit D deficiency.(91)

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The primary way the human body produces Vit D is through skin exposure to ultraviolet B (UVB) radiation from sunlight. When UVB rays penetrate the skin, they convert 7-dehydrocholesterol, a cholesterol precursor in the epidermis, into preVit D3. This undergoes thermal isomerization to form cholecalciferol (Vit D3), which then enters the bloodstream. In the liver, Vit D3 is hydroxylated to 25-hydroxyvitamin D [25(OH)D], the major circulating form of vitamin D. It is further hydroxylated in the kidneys to form 1,25-dihydroxyvitamin D [1,25$(OH)_2$D], the biologically active form that regulates calcium and phosphate homeostasis, bone mineralization, and immune function..(92) Dark skin pigmentation inhibits UVB light penetration, causing decreased vitamin D3 production in the body.(93) Vitamin D is a steroidal hormone that regulates the uptake of calcium (Ca^{2+}) and phosphate and plays a primary role in bone growth and wound healing.(94) Dark-skinned individuals are thought to have more active fibroblasts, the cells that produce collagen, and when combined with Vit D deficiency, impairs the regulation of collagen production, which leads to excessive collagen deposition in response to injury.(95) A deficiency in Vit D within dark-skinned populations may cause an overactive inflammatory response to skin injury, which can delay the wound healing process, making it more difficult for the body to resolve inflammation and promote proper tissue remodelling.(96) Vitamin D deficiency disrupts the balance of collagen production, increasing the likelihood of excessive collagen accumulation in scar tissue.(97) In dark-skinned individuals, who may already have a genetic predisposition for higher collagen activity, Vit D deficiency can further enhance this risk.(16) The role of Vit D in regulating gene expression related to cell growth and apoptosis can affect how the body responds to skin injury(98), but without adequate Vit D, the normal control of cell proliferation and collagen regulation may fail, impairing the body's ability to regulate inflammation, collagen production, apoptosis, and wound healing, thereby increasing the likelihood of keloid formation and persistence in these populations.

Although Vit D deficiency is a significant factor in keloid formation, the role of the Vit D receptor (VDR) further highlights the complexity of this relationship, as it influences cellular processes such as fibroblast activity and collagen regulation, which are crucial in keloid development.

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1.4.2 Vitamin D receptor and keloid disease

VDR plays a critical role in the regulation of skin health and wound healing(99), and its involvement in keloid formation has been an area of increasing research. Vitamin D receptor is a nuclear receptor that, upon binding with active Vit D [1,25$(OH)_2$D], regulates the expression of various genes involved in cellular growth, differentiation, inflammation, and collagen production.(100) In individuals with Vit D deficiency, VDR may not function optimally, which can exacerbate keloid formation in several ways.

Vitamin D activates VDR, which regulates the genes involved in inflammation, collagen production, and cell proliferation. In dark-skinned individuals with low Vit D levels, VDR activation is impaired(90), limiting its ability to decrease excessive fibroblast proliferation and collagen synthesis.(101) This results in uncontrolled collagen build-up. Without sufficient Vit D, VDR cannot effectively control the inflammatory response, resulting in prolonged inflammatory signals that promote abnormal scarring.(53, 102) The VDR normally helps maintain a balance between collagen types (I and III) in wound healing.(103) During a Vit D-deficient state, the ability of the VDR to maintain this balance is compromised, leading to excessive collagen deposition. The VDR plays a key role in the regulation of the expression of genes related to cell growth and apoptosis.(104) When an individual is Vit D-deficient, the activity of VDR is decreased, which can lead to impaired control over cell proliferation and reduced apoptosis of excessive fibroblasts, contributing to the abnormal growth of scar tissue seen in keloids.(105)

Vitamin D deficiency weakens the ability of VDR to control critical processes, such as inflammation, collagen synthesis, and fibroblast activity, thereby increasing the risk of keloid formation**.** Figure 1.4 illustrates the mechanism by which decreased levels of vit D and VDR contribute to keloid development. Figure 1.4 shows an overview of the proposed involvement of Vit D signalling through the VDR in keloid development and suggests that genetic risk factors, dark skin pigmentation, and Vit D deficiency act as predisposing factors that enhance inflammation levels, potentially helping the formation of keloids. This process is partly mediated by lower circulating levels of Vit D and reduced VDR expression, which promote pro-inflammatory and pro-fibrotic

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effects in various cell types. This interaction leads to a sustained inflammatory state that contributes to keloid formation.(101)

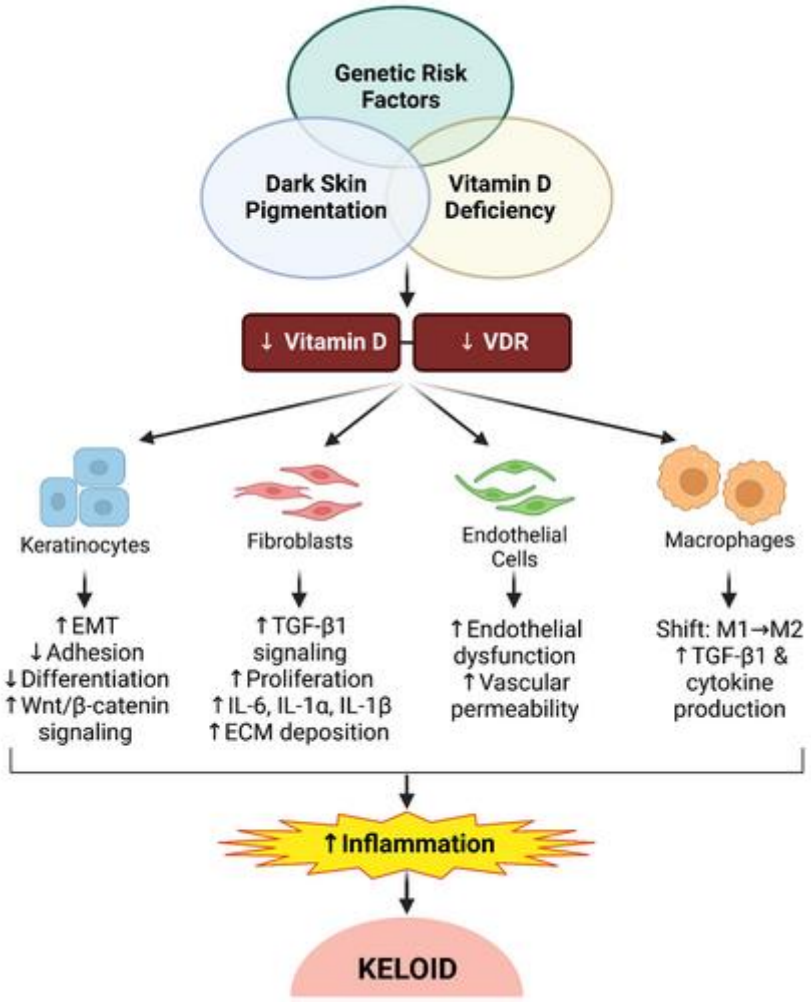

Figure 1.4 Mechanistic pathways linking genetic and environmental factors to keloid formation via Vit D deficiency and inflammatory responses.(101) This figure illustrates the interplay between genetic risk factors, dark skin pigmentation, and Vit D deficiency in contributing to keloid formation through a reduction in Vit D levels and VDR expression. These deficiencies affect various cell types such as keratinocytes, fibroblasts, endothelial cells, and macrophages.

Understanding the role of VDR in keloid formation not only highlights how Vit D deficiency enhances collagen production, inflammation, and fibroblast activity but also underscores the receptor's critical involvement in maintaining mitochondrial integrity, further influencing the cellular environment conducive to keloid development.

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1.4.3 Vitamin D receptor and mitochondrial integrity

In individuals with Vit D deficiency, VDR function is impaired(90), which can affect mitochondrial integrity through several mechanisms.(106) Mitochondria are essential for energy production, cellular metabolism, and the regulation of cell survival.(107)

The VDR plays an important role in maintaining mitochondrial function by influencing the expression of genes related to mitochondrial energy production and biogenesis.(106) In a Vit D-deficient state, VDR activation is diminished(108), decreasing its ability to support the maintenance and proper functioning of the mitochondria.(31) This could result in decreased ATP production.(107) The VDR is also involved in protecting cells from oxidative stress and without adequate Vit D, VDR's role of VDR in regulating antioxidant defences is weakened, thereby increasing ROS levels that cause mitochondrial damage.(98) Both Vit D and VDR activation promote mitochondrial biogenesis by regulating pathways such as PGC-1α, a key regulator of mitochondrial production, but in a Vit D-deficient environment, this pathway may be impaired, reducing the number of healthy mitochondria and negatively affecting cellular energy balance.(109) Pathways that control apoptosis are also influenced by VDR activity; however, during Vit D deficiency, VDR activity is reduced, which can lead to mitochondrial dysfunction, which is closely tied to the release of pro-apoptotic factors such as cytochrome c from damaged mitochondria.(104, 110) This could result in increased cell death and impaired tissue regeneration, contributing to overall tissue health decline. Calcium homeostasis is regulated by Vit D and VDR and is vital for mitochondrial function.(111) Mitochondria help buffer cellular Ca^{2+} levels, and any imbalance in Ca^{2+} regulation can impair mitochondrial processes.(112) In a Vit D-deficient state, Ca^{2+} maintenance is disrupted, which can lead to mitochondrial dysfunction and trigger cell damage or apoptosis.(111, 112)

In individuals with Vit D deficiency, impaired VDR function can adversely affect mitochondrial integrity by disrupting mitochondrial biogenesis, reducing antioxidant defences, altering cellular metabolism, promoting inflammation, and affecting Ca^{2+} homeostasis. Collectively, these factors can lead to mitochondrial dysfunction, which contributes to the development of keloids.

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1.6 Problem statement

Keloid disease is a chronic condition characterised by abnormal wound healing and excessive collagen build-up, predominantly affecting individuals with darker skin tone. Despite considerable research, the precise molecular mechanisms that drive keloid formation remain poorly understood. Key processes, such as fibroblast behaviour, mitochondrial dysfunction, oxidative stress, and inflammation, have not been fully elucidated. Additionally, the role of Vit D deficiency, a common condition in dark-skinned populations, on keloid formation, warrants further investigation.

Current treatments for keloid, including surgical interventions and pharmacological approaches, frequently result in high recurrence rates, underscoring the need for a more targeted therapeutic strategy. Recent studies suggest that mitochondrial dysfunction and oxidative stress play critical roles in keloid development, which is potentially exacerbated by Vit D deficiency. However, comprehensive investigations into the interplay between these factors in keloid pathogenesis are lacking. Furthermore, while racial and ethnic disparities in keloid prevalence are well-documented, there is insufficient understanding of how these differences contribute to disease progression.

This study aimed to address these research gaps by examining the interplay among mitochondrial function, oxidative stress, and Vit D levels in dark-skinned patients with keloid disease. The goal was to provide insights into the molecular underpinnings of keloid formation, which could contribute to the development of more effective and tailored treatment strategies.

1.6.1 Hypothesis

This study hypothesised that mitochondrial dysfunction and increased oxidative stress are key contributors to the pathophysiology of keloid disease in dark-skinned patients, and that Vit D deficiency exacerbates these processes, leading to more severe keloid formation. Specifically, the following hypothesis is proposed:

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1. Keloid tissues exhibit significant mitochondrial dysfunction and abnormal morphology including altered mtDNA viability in the blood plasma.
2. Elevated levels of oxidative stress, as indicated by increased lipid peroxidation, are present in blood plasma of patients diagnosed with keloid disease.
3. There is a negative correlation between Vit D levels and mitochondrial dysfunction as well as oxidative stress in dark-skinned patients with keloid disease.

1.6.3 Aim and objectives

Aim

This study aimed to determine the relationship between mitochondrial function, oxidative stress, and Vit D levels in dark-skinned patients with keloid disease.

Objectives

1. **To quantify mtDNA viability in blood plasma of dark-skinned patients with keloid diseaseusing quantitative polymerase chain reaction (qPCR).**

2. **To evaluate oxidative stress levels in blood plasma by measuring lipid peroxidation using the thiobarbituric acid reactive substance (TBARS) assay.**

3. **To analyze mitochondrial morphology and quantity in keloid tissue using TEM.**

4. **To correlate Vit D levels with mitochondrial function and oxidative stress in keloid disease.**

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Chapter 2: Materials and Methods

2.1 Study design

This was a cross-sectional analytical study. Blood and keloid tissue samples were collected. Sample analysis for the MSc project was conducted on keloid tissue and blood plasma samples collected from dark-skinned patients of various ethnic groups who had been diagnosed with keloid disease. The study design is shown in Figure 2.1.

2.2 Study setting

Keloid tissue and blood samples were obtained from patients attending consultations at the Plastic Surgery outpatient clinic on the 6th floor of Steve Biko Academic Hospital (SBAH), Kalafong Hospital, and Chris Hani Baragwanath Academic Hospital (CHBAH). These patients were seen by Dr. Jaco Kotze, Dr. Siganga, and other attending surgeons. Keloid tissue samples were surgically removed as part of routine clinical management. Histological specimens were stored at -80°C in Room 9-30 of the Basic Medical Sciences building, Prinshof Campus, University of Pretoria.

2.3 Study population and sampling

2.3.1 Vitamin D screening

A Vit D blood test was performed on all the patients to obtain their Vit D status. A clinic nurse drew the patient's blood for the Vit D test. The patient's blood was labelled with an ID code, collection date, and time. The blood samples were sent to Ampath Laboratories, an accredited pathology laboratory for Vit D screening. All records were kept in the laboratory book, which included patient number, date, and time of Vit D screening.

The Vit D screening test was done by Ampath by using gas chromatography-mass spectrometry, which measured 25-hydroxyvitamin D [25(OH)D], the major circulating form of Vit D and the best biomarker for assessing Vit D status. The patients' blood

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samples were grouped according to Vit D levels from Ampath. The three groups consisted of patients with insufficient Vit D levels (21 – 30 ng/mL), deficient Vit D levels (< 20 ng/mL), and sufficient Vit D levels (> 31 ng/mL).(113)

2.3.2 Inclusion and exclusion criteria

Insufficient vitamin D level group

Inclusion criteria:

Patients could be of any biological sex but had to be dark-skinned with Fitzpatrick skin types between IV and VI and between 18–40 years of age. They must have been clinically diagnosed with keloid disease and had vitamin D levels in the physiological range, between 21 and 30 ng/mL.(113)

Exclusion criteria:

Patients who were light skinned with Fitzpatrick skin type below IV and Vit D levels beyond the range of 21 – 30 ng/mL were excluded

Deficient vitamin D level group

Inclusion criteria:

Patients of any biological sex and were dark-skinned with Fitzpatrick skin type between IV and VI and between 18 – 40 years of age were included. Patients must have been clinically diagnosed with keloid disease and have a Vit D level below the normal physiological range of 20 ng/mL.(113)

Exclusion criteria:

Patients who were light skinned with Fitzpatrick skin type below IV and Vit D levels above 20 ng/mL were excluded.

Sufficient vitamin D level group

Inclusion criteria:

Patients of any biological sex that were dark-skinned with Fitzpatrick skin type between IV and VI and between 18 – 40 years of age were included. The patient was clinically diagnosed with keloid disease and had Vit D levels at the normal physiological range of 31 ng/mL and above.(113)

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Exclusion criteria:

Patients who were light skinned with Fitzpatrick skin type below IV and Vit D levels below 31 ng/mL were excluded.

Exclusion criteria for all three groups

Patients with chronic renal disease or renal failure, chronic liver disease or liver failure, chronic medication, smokers, human immunodeficiency virus (HIV) positive, on vitamin supplements, or with any known immune-compromised conditions. Excluding these individuals helps ensure that the study results focus on the primary factors being investigated, without interference from external or unrelated health conditions that could complicate the findings.

2.3.3 Recruitment process

Participants were recruited from the dermatology clinic in SBAH and hospitals, including Kalafong Hospital and CHBAH, with a focus on dark-skinned individuals who were clinically diagnosed with keloid disease. Potential participants were identified based on their medical records. Eligible participants who met the inclusion criteria were asked whether they wanted to participate in the study.

Upon initial screening, participants were informed about the study's purpose, procedures, risks, and benefits. Informed consent was obtained prior to sample collection (blood or keloid tissue), ensuring that the participants fully understood their involvement and the confidentiality of their data.

2.3.4 Ethical considerations

This study was approved by the Faculty of Health Sciences Research Ethics Committee on the 25th of October 2022, renewed on the 8th of November 2023, and again on the 16th of October 2024 (Ethics Reference No. 532/2022). The certificates can be seen in Appendices A, B and C respectively. The participants were thoroughly informed of the purpose, procedures, risks, and benefits of the study before written

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consent was obtained. The participants' personal information was kept confidential. Keloid tissue and blood samples, along with any related personal data, were anonymised by assigning unique identifiers to ensure that individual participants remained unidentifiable.

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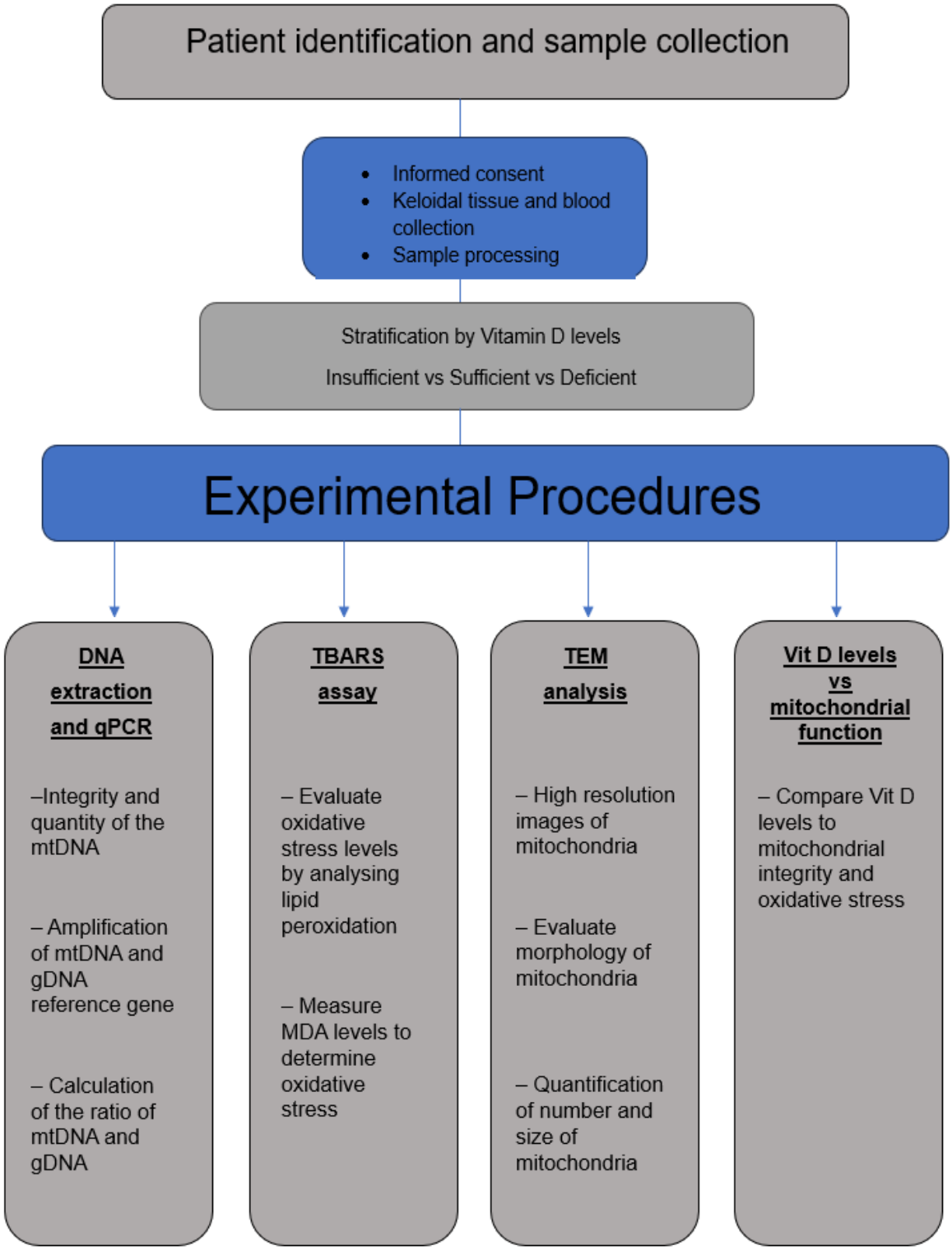

Figure 2.1 Overview of the study design

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2.4 Sample collection and processing

Tissue samples were collected during routine keloid excision procedures by a qualified medical professional to ensure minimal risk to the patient. Keloid tissues harvested under sterile conditions were immediately stored in the appropriate preservation media for TEM viewing.

Four tubes of blood (4 mL each), two red top vacutainer tubes, and two Lithium-heparin (Li-hep) green top vacutainer tubes were collected from each patient. Blood tubes were kept in a blood box at room temperature (RT) and processed within four hours of the first blood draw to prevent agglutination.

Media stock preparation was performed at least one or two days before the planned blood collection. The storage media was prepared by thawing 45 mL of foetal bovine serum (FBS) from the -20°C freezer and then added 5 mL of dimethyl sulphoxide (DMSO), and then stored in the fridge at 4°C. Thereafter, 50 mL of 0.1 M phosphate buffered saline (PBS) was then aliquoted into an empty 50 mL tube, and was stored in the fridge at 4°C. Lastly, 3 mL of Histopaque 1077® was aliquoted into eight 15 mL conical tubes which were also stored in the fridge at 4°C.

In the laboratory, the red top blood tubes were placed in a centrifuge at 1000 x g for 10 minutes. The top layer of the centrifuged red top blood tubes was serum. In a biological safety cabinet, 500 µL of serum was aliquoted into 3 x 1.5 mL tubes, and the remainder was aliquoted into a 4th 1.5 mL tube. The tubes were then stored at -80°C until further analysis.

The following was prepared in a biological safety cabinet: For each sample, the whole blood in Li-hep tubes was layered onto the 3 mL Histopaque 1077®. The 15 mL conical tubes with layered blood were then spun in a centrifuge at 400 x g for 30 minutes at RT. The top layer of the spun 15 mL conical tubes was plasma, and 500 µL was aliquoted into 3 x 1.5 mL tubes and the remainder, if any into a 4th 1.5 mL tube. The tubes were stored at -80°C until further analysis. The samples were labelled with unique identifiers to ensure participant anonymity.

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2.4.1 Sample types and experimental procedures

This study utilised both whole blood and keloid tissue samples, depending on specific experimental requirements. Keloid tissue samples were used for TEM to analyse mitochondrial morphology. Whole blood was processed into serum and plasma samples. Whole blood was used for the extraction of DNA and quantification of mtDNA whereas blood plasma was used for oxidative stress markers, including the TBARS assay and Vit D levels. The use of blood plasma was necessary due to the limited availability of tissue samples and the challenges in homogenising tissue for certain biochemical procedures. This approach ensured that the best possible data were obtained from the available samples and tailored to the needs of each experimental protocol.

2.5 Reagents and consumables

2.5.1 Reagents

All the reagents used in the study and their respective sources are listed in Table 2.1.

Table 2.1 All Reagents and their source

Reagent	Source
Agar100	Advanced Laboratory Solutions, SA
Butylated hydroxytoluene (BHT)	Sigma-Aldrich, SA
Dimethylaminoethanol (S-1)	Advanced Laboratory Solutions, SA
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich, SA
DNA elution buffer	Quick-DNA™ MiniPrep Kit
Dodecenyl succinic anhydride (DDSA)	Advanced Laboratory Solutions, SA
Absolute Ethanol	Sigma-Aldrich, SA
Fetal bovine serum (FBS)	Sigma-Aldrich, SA
Formaldehyde	Sigma-Aldrich, SA
gDNA wash buffer	Quick-DNA™ MiniPrep Kit
Genomic lysis buffer	Quick-DNA™ MiniPrep Kit
Glutaraldehyde	Sigma-Aldrich, SA
Histopague	Sigma-Aldrich, SA
Hydrochloric acid (HCL)	Sigma-Aldrich, SA

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Lead citrate	Merck (Pty) Ltd, SA
Methyl nadic anhydride (MNA)	Advanced Laboratory Solutions, SA
Osmium tetraoxide	Sigma-Aldrich, SA
Phosphate buffer saline (PBS)	Sigma-Aldrich, SA
Phosphoric acid (H_3PO_4)	Sigma-Aldrich, SA
Prewash buffer	Quick-DNA™ MiniPrep Kit
Sodium dodecyl sulfate (SDS)	Sigma-Aldrich, SA
Sodium Hydroxide (NaOH)	Sigma-Aldrich, SA
Thiobarbituric acid (TBA)	Sigma-Aldrich, SA
Uranyl acetate	Merck (Pty) Ltd, SA

2.5.2 Consumables

Consumables that were critical to the study are listed in Table 2.2.

Table 2.2 Vital consumables used in the study

Consumable	**Used in**
Lithium Heparin (Li-hep) vacutainer tube (green top)	Blood collection
Red top vacutainer tube	Blood collection
Zymo-spin™ IICR column	DNA extraction
Zymo-spin™ collection tube	DNA extraction
Cool Cell	Freezing of PBMCs

2.6 Experimental procedures

2.6.1 Extraction and quantification of mtDNA using qPCR

This section details the procedure for extracting DNA from the blood plasma and performing qPCR to assess mtDNA viability. This method directly supported Objective 1, which focused on assessing the integrity and quantity of mtDNA in the blood plasma of patients diagnosed with keloid disease, a key factor in understanding the mitochondrial function in keloid pathology. Assessment of mtDNA viability was critical for understanding mitochondrial dysfunction, which may play a key role in the pathophysiology of keloid formation.

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The qPCR technique was selected for its high sensitivity and accuracy in quantifying DNA concentration, making it well suited for detecting small variations in mtDNA copy number. By comparing mtDNA levels to a gDNA reference (β-globin), this method provides valuable insights into mitochondrial health in patients with keloid disease.

Overview: DNA was extracted from blood plasma using the Quick-DNA™ MiniPrep kit, following the protocol for blood plasma. Blood plasma samples were mixed with genomic lysis buffer at a 1:4 ratio to lyse the cells, followed by purification through multiple wash and centrifugation steps. Quantification of mtDNA was performed using qPCR with SYBR Green Supermix and specific primers for mtDNA and the β-globin reference gene.

DNA extraction

Blood plasma was used for DNA extraction. The blood plasma was mixed in a (1:4) ratio with the genomic lysis buffer, and 100 µL of blood plasma and 400 µL of genomic lysis buffer were used. This was mixed by vortexing for 4 – 6 seconds, and then letting it stand for 10 minutes at RT. The mixture was then transferred to a Zymo-Spin™ IICR Column in a collection tube and centrifuged at 10 000 x g for 1 minute at 4°C. The collection tube was then discarded with flow-through. The Zymo-Spin™ IICR Column was then transferred to a new collection tube, and 200 µL of DNA pre-wash buffer was added to the spin column and centrifuged at 10 000 x g for 1 minute at 4°C. Next, 500 µL of gDNA wash buffer was added to the spin column and centrifuged at 10 000 x g for 1 minute at 4°C. The spin column was then transferred to a clean microcentrifuge tube and 50 µL of DNA elution buffer was added to the spin column. This was then incubated for 5 minutes at RT and centrifuged at the top speed (18 407 x g) for 30 seconds at 4°C to elute the DNA. The isolated DNA was quantified using a Nanodrop® 2000 spectrophotometer. The spectrophotometer was always calibrated on each use, with the blank by using nuclease-free water. The eluted DNA was stored in a freezer box at -20°C until further analysis.

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Reaction optimisation

To ensure the accuracy and reproducibility of the qPCR results, several optimisation steps were performed. These steps included adjusting the primer concentrations, DNA template concentrations, and cycle conditions to maximise amplification efficiency and minimise variability.

Firstly, the stock solutions of both forward and reverse primers for mtDNA and β-globin were diluted to a working concentration of 100 µM from an initial concentration of 20 µM. The working stock (20 µM) was subsequently diluted to prepare four concentrations: 500, 250, 100, and 50 nM. The mtDNA primer concentrations were analysed in triplicate, whereas β-globin primer concentrations were analysed as single samples. These prepared concentrations were utilised in the initial optimisation run, which also included Bio-Rad SYBR® Green Supermix and DNA samples standardised to a concentration of 10 ng/µL. The initial optimisation cycle was conducted over 28 cycles of denaturation. For each primer set (mtDNA and β-globin), a no-template control (using RNase-free water instead of the DNA template) was included in every run to verify the absence of contamination.

Secondly, it was observed that utilising a 20 µM diluted stock solution posed significant challenges in micro-pipetting because of the resultant small volumes. Consequently, the original 20 µM working stock solution was re-diluted to establish a new working concentration of 2 µM. This modification enhanced the volumes required for micropipetting, thereby facilitating more efficient handling. During the subsequent optimisation process, four distinct concentrations were systematically evaluated (500 nM, 250 nM, 100 nM, and 50 nM) of mtDNA and β-globin in conjunction with two different SYBR Green formulations: BioRad SYBR® Green Supermix and PowerUp™ SYBR™ Green Master Mix (Thermo Fisher Scientific, Vilnius, Lithuania). Two different DNA template concentrations (10 ng/µL and 20 ng/µL) were employed, and the number of denaturation cycles was increased to 38°C.

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Finally, a third optimisation was conducted to further investigate the mtDNA concentrations that yielded the most favourable results, with the aim of determining the optimal concentration for each SYBR Green formulation and DNA template.

qPCR reaction setup

The isolated DNA was quantified using a Nanodrop® 2000 spectrophotometer and standardised to 10 ng/µL. A Roche LightCycler 480 (Roche Diagnostics) was used to perform qPCR. A reaction volume of 12.5 µL consisting of 6.25 µL SYBR Green Supermix (BioRad, Hercules, CA, USA), 500 nM of forward (5'-TGAGGCCAAATATCATTCTGAGGGC-3') and reverse primer (5'-TGCACCTGCTCTCTGTGATTATGACTATCCCACAGTC-3'), and 10 ng DNA template was made up in nuclease free water. Changes in mtDNA amplification were calculated based on the $2^{-\text{delta delta cycle threshold}}$ ($2^{-\Delta\Delta Ct}$) method described by Livak and Schmittgen(114) and reported as percentage mtDNA amplification relative to untreated control (100 %). The housekeeping gene β-globin (sense: 5'-ACATGATTAGCAAAGGGCCTAGCTTGACTCAGA-3'; antisense: 5'-TGCACCTGCTCTGTGATTATGATATCCCACAGTC-3') was amplified simultaneously. Cycling conditions included an initial denaturation step (94°C for 3 minutes), followed by 38 cycles of denaturation (94°C for 20 seconds), annealing (58°C for 10 minutes), extension (72°C for 10 minutes), and a plate read. This was followed by a melting curve and a final hold (25°C for 5 minutes). The composition of the reaction mixture is shown in Table 2.3.

Table 2.3 Reaction mix composition

Reagent	Volume
BioRad SYBR® Green Supermix	6.25 µL
Forward + Reverse primer (500 nM/strand)	5.0 µL
DNA template (10 ng/µL)	1.25µL
Final volume per well	**12.5 µL**

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Data normalisation and analysis

Mitochondrial DNA was quantified relative to β-globin using the ΔΔCt method following the protocol described by Livak and Schmittgen.(114) The relative mtDNA copy number was calculated and all samples were run in triplicate. Negative controls were included to ensure no contamination. The results are reported as the percentage of mtDNA amplification relative to untreated controls (100%).

2.6.2 Measurement of oxidative stress using TBARS assay

The TBARS assay was used to evaluate lipid peroxidation, a marker of oxidative stress, in blood plasma samples of dark-skinned patients with keloid disease. This method directly addressed Objective 2, which aimed to quantify oxidative stress within blood plasma of patients with keloid disease by analysing lipid peroxidation, an indicator of cellular damage caused by ROS.

Sample preparation

The reagents had to be prepared before the reaction setup could start. All the reagents were prepared in a beaker or conical flask under a fume hood. The reagents were appropriately labelled to prevent confusion. Table 2.4 demonstrates the preparation method for each reagent.

Table 2.4 Reagent preparation

Reagent	Preparation steps followed
2% H_3PO_4	1177 µL of H_3PO_4 in 48.823 mL of distilled water (dH_2O)
7% H_3PO_4	4.1 mL of H_3PO_4 in 45.9 mL of dH_2O
1 M HCL	Add 50 mL of dH_2O then add 4.92 mL of 32% HCL to water
1 M NaOH	Add 2 g of NaOH to 30 mL of dH_2O, once dissolved top up with dH_2O up until 50 mL
3 mM HCL	30 µL from1 M HCL stock into 9.97 mL dH_2O
20 mM BHT stock	440.8 mg of 20 mM BHT stock in 100 mL ethanol
TBA/BHT solution	Add 0.1 g of NaOH, 0.5 g of TBA, and 250 µL BHT (from 20 mM stock). Then dissolve in 40 mL of dH_2O, once dissolved top up dH_2O until 50 mL

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Reaction setup

Plasma samples were removed from the -80°C freezer and left at RT to thaw. Once the samples were thawed, 250 µL of the plasma sample was added to a glass tube and labelled. Next, 100 µL of 2% H_3PO_4 was added the glass tube and vortexed for 3 seconds. Subsequently, 200 µL of 7% H_3PO_4 was added to the glass tube and vortexed again for 3 seconds. Thereafter, 200 µL TBA/BHT was added to the glass tube.

Controls

A positive control was prepared as follows in a glass tube: 100 µL of 2% H_3PO_4, 200 µL of 7% H_3PO_4, 200 µL of TBA/BHT, and (0.5 µL of MDA in 249.5 µL ionised water).

A negative control was prepared as follows in a glass tube: 100 µL of 2% H_3PO_4, 200 µL of 7% H_3PO_4, 200 µL of TBA/BHT, and 250 µL of 3 mM HCL.

When the glass tubes were boiled in a beaker, there was at least one positive and one negative control.

Processing method

A 1 L beaker was filled to 400 mL with distilled water, into which the glass tubes were submerged. The assembly was heated on a hot plate at 100°C for 15 minutes. The positive control was extracted after 5 minutes, or once it exhibited a pink hue. Subsequently, all glass tubes were transferred to a tube rack for equilibration at RT. Subsequently this, 1 mL of 100% butanol was introduced into each glass tube containing the samples and subjected to being vortexed for 10 seconds, facilitating the separation of the samples into two distinct phases. The butanol phase was then carefully aspirated and 200 µL was aliquoted into a 96-well microtiter plate in triplicate.

Measurement and quantification

The absorbance of the MDA-TBA complex was measured using a spectrophotometer at 532 nm and a reference wavelength of 600 nm, and MDA levels were calculated in nM/mL. All samples were analysed in triplicate to ensure accuracy and reproducibility.

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2.6.3 Transmission electron microscopy

This section detailed the analysis of mitochondrial morphology and quantity in keloid tissue samples by TEM. This study specifically focused on mitochondrial dimensions, membrane integrity, and mitochondrial abundance, addressing Objective 3: to analyse mitochondrial morphology and quantity in keloid tissues. This objective focused on observing and quantifying structural changes in the mitochondria, providing insight into mitochondrial dysfunction in keloid tissue.

Sample preparation

The keloid tissue samples were sectioned into approximately 1 mm^3 fragments and placed in a 2.5% glutaraldehyde/formaldehyde (GA/FA) fixative solution (composed of 1 mL glutaraldehyde, 1 mL formaldehyde, 3 mL distilled water, and 5 mL PBS) for 1 hour at room temperature. Following fixation, the tissues were rinsed three times with PBS, and each wash lasted 15 minutes. Post-fixation was performed in a fume hood using 1% osmium tetroxide for 1 hour at room temperature, followed by an additional series of PBS rinses.

Dehydration and embedding

The tissue samples were dehydrated using a graded ethanol series (30%, 50%, 70%, 90%, and three changes of 100%) for 15 minutes at each concentration. The tissue samples were then left in fresh 100% ethanol overnight. Following dehydration, the samples were infiltrated with a 50:50 mixture of 100% ethanol and resin mixture (which is illustrated in Table 2.5) for 30 minutes. The amount of resin made up was depended on the number of samples that need to be embedded. The first three reagents (Agar 100, MNA, DDSA) were mixed and then there after the S-1 reagent was added and mixed again.

Table 2.5 Resin mixture preparation

Reagents	5 g	10 g	15 g	20 g	50 g
Agar 100	2.617 g	5.233 g	7.85 g	10.467 g	26.167 g
MNA	1.767 g	3.533 g	5.3 g	7.067 g	17.667 g
DDSA	0.683 g	1.367 g	2.05 g	2.733 g	6.833 g
S1	0.05 g	0.10 g	0.152 g	0.203 g	0.507 g

*MNA - Methyl nadic anhydride, DDSA - Dodecenyl succinic anhydride, S1 - Dimethylaminoethanol

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The prepared resin mixture was then placed in a 1.5 mL test tube with the sample and an identifying label together with 100% ethanol to form a (50:50) mixture and mixed well. The test tube was slowly rotated at 30rpm for 30 minutes. The sections were placed in a 100% resin mixture and rotated again at 30rpm for a further 4 hours. The resin mixture and allocated sample numbers were transferred to moulds (Advanced Laboratory Solutions, SA). The moulds were placed in an oven at 60ºC for 38 hours for polymerisation.

Ultramicrotome sectioning and staining

The samples were removed from the moulds and trimmed using a single-edge razor knife. After the optimal cutting area had been obtained the sample was cut with a ultramicrotome (Leica, HistoCore BIOCUT) glass knife until the sample surface area was smooth and the sample was reached. The sample was then further cut with a DiATOME diamond knife with a thickness of 50 – 90 nm (until a gold-like colour started to form), picked up with a copper grid (Advanced Laboratory Solutions, SA), and placed in a grid holder (Advanced Laboratory Solutions, SA). The sample was then placed in an enclosed container to prevent photo-oxidation and contrasted with uranyl acetate for 5 minutes at RT with the container closed, followed by 2 minutes in lead citrate at RT with the container closed.

Imaging and mitochondrial analysis

TEM was performed using a JEOL JEM 2100F microscope operating at 120 kV. Random micrographs were captured at 5,000x, 10,000x, and 20,000x machine magnifications to analyse mitochondrial properties. The following parameters were assessed.

1. **Mitochondrial enlargement**: The size of individual mitochondria was measured using the ImageJ(115) software to assess potential enlargement, a sign of mitochondrial stress in keloid tissues. The average mitochondrial size was calculated across a minimum of 5 micrographs.
2. **Membrane integrity**: The structural integrity of mitochondrial membranes was evaluated by focusing on any visible membrane damage or disintegration. Mitochondrial membrane damage is often associated with oxidative stress in keloid pathology.

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3. **Mitochondrial count**: The number of mitochondria per field of view was recorded to determine mitochondrial density within the keloid tissue. A lower count indicates mitochondrial depletion or dysfunction.

Quantification

Mitochondrial size, membrane integrity, and cell counts were analysed using ImageJ software. Manual thresholding was applied to accurately segment the mitochondria, and all measurements were averaged across five random micrographs per sample for statistical analysis.

2.6.4 Correlation between vitamin D levels and mitochondrial function/oxidative stress

This section outlines the methodology employed to assess the correlation among Vit D levels, mitochondrial function, and oxidative stress in patients with keloid disease. This method directly addressed Objective 4, which aimed to investigate the relationship between plasma Vit D concentration and indicators of mitochondrial health and oxidative stress, specifically mtDNA viability, lipid peroxidation, and mitochondrial morphology.

Vitamin D measurement

Vitamin D status was assessed by measuring 25-hydroxyvitamin D levels using gas chromatography-mass spectrometry. Plasma was obtained from the two Li-hep blood tube as described previously and were aliquoted into 1.5 mL plasma tubes. Vit D levels were reported in ng/mL, and patients were categorised into three groups based on their levels: deficient (< 20 ng/mL), insufficient (21 – 30 ng/mL), and sufficient (> 30 ng/mL).

2.6.5 Data integration and statistical analysis

The correlation between Vit D levels and mitochondrial function (mtDNA viability, mitochondrial morphology via TEM) and oxidative stress (lipid peroxidation via TBARS assay) was assessed using Pearson's correlation coefficient. Each patient's mtDNA copy number (from qPCR results), TBARS-derived MDA levels, and TEM-determined

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mitochondrial morphology were compared to their corresponding Vit D levels to identify potential trends or correlations. Statistical significance was set at ($P < 0.05$).

Method

Statistical analyses were performed using GraphPad Prism 9.0, (San Diego, CA, USA). Data were summarised as means, standard deviations, medians, ranges, and 95% confidence intervals (CI) for continuous variables and as frequency, percentage, and 95% CI for categorical (binary/discrete) data.

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Chapter 3: Results

All data were tested for normality of distribution. Only the Vit D levels passed the normality test; therefore, parametric tests were performed on the data. Nonparametric tests were applied to all other variables. In total there were 44 patients included in the study, of which 27 were male and 17 were female and all patients were between the ages of 18 – 40 years of age. Patients were then split into their respective Vit D groups, 32 patients in the Vit D sufficient group, 11 patients in the Vit D insufficient group and 1 patient in the Vit D deficient group.

3.1 Testing significance between vitamin D levels

The analysis was conducted using Welch's t-test, which allowed for an accurate comparison of mean Vit D levels across the three groups: sufficient, insufficient, and deficient. The results of the Vit D levels are shown in Figure 3.1.

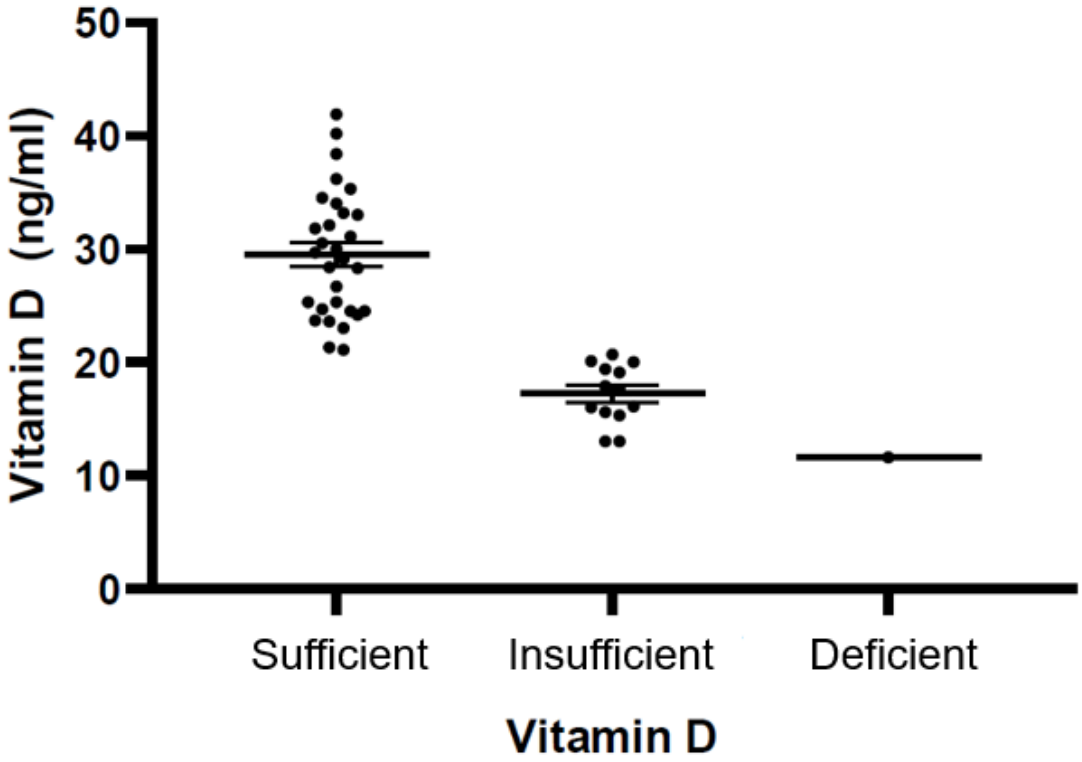

Figure 3.1 Comparison of Vit D levels across the sufficient, insufficient, and deficient groups. This figure indicates a mean difference of -12.30 and standard error of the mean (SEM) ±1.25, with a statistical significance of ($p < 0.05$) between the insufficient and sufficient Vit D groups. Note: the deficient group had limited data points.

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The means of the sufficient group (29.52 ng/mL) and the insufficient group (17.22 ng/mL) indicate notable variation in Vit D levels across the study population of dark-skinned individuals. A mean difference of -12.30 with a relatively small SEM of ±1.25 suggests that the difference between the two groups is both statistically significant ($p < 0.05$) and precisely estimated. The negative mean difference indicates that, on average, the insufficient group has a substantially lower value than the sufficient group by approximately 12 ng/mL, and this difference is consistent with the low variability around this estimate. The 95% CI indicated that the true mean difference lies between -14.75 ng/mL and -9.85 ng/mL.

3.2 Measuring viable mitochondrial DNA levels

3.2.1 Evaluating viable mitochondrial DNA levels according to vitamin D levels

This section explores the relationship between viable mtDNA and Vit D levels, as a follow-up analysis to the prior categorical approach. Due to the limited number of data points in the Vit D-deficient group, statistical group-based comparisons were less robust for this category, prompting the use of a continuous variable analysis to provide a broader view of the relationship. The approach of analysing viable mitochondrial DNA (mtDNA) levels across the range of Vit D concentrations allows leveraging the full dataset to detect trends, even if the sample size of the deficient group is small. Spearman's rank-order correlation test was applied to assess whether there was a significant association between viable mtDNA and Vit D levels. This analysis aimed to determine whether changes in Vit D levels were linked to variations in viable mtDNA levels. The results of the analysis are illustrated in Figure 3.2, which depicts the relationship between mtDNA and Vit D levels.

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3.3 Measuring malondialdehyde levels

3.3.1 Evaluating malondialdehyde levels according to vitamin D groups

This section outlines the findings from the analysis of MDA levels categorised by Vit D groups. As a complementary analysis to the previous categorical approach, the limited number of data points in the Vit D-deficient group made group-based statistical comparisons less reliable. This limitation led to the adoption of a continuous variable analysis, offering a more comprehensive perspective on the relationship. By examining MDA levels across the full spectrum of Vit D concentrations, this approach utilises the entire dataset to identify trends, despite the small sample size in the Vit D deficient group. Malondialdehyde was assessed to determine how variations in Vit D status across the three different groups correlated with levels of oxidative stress, as shown in Figure 3.4.

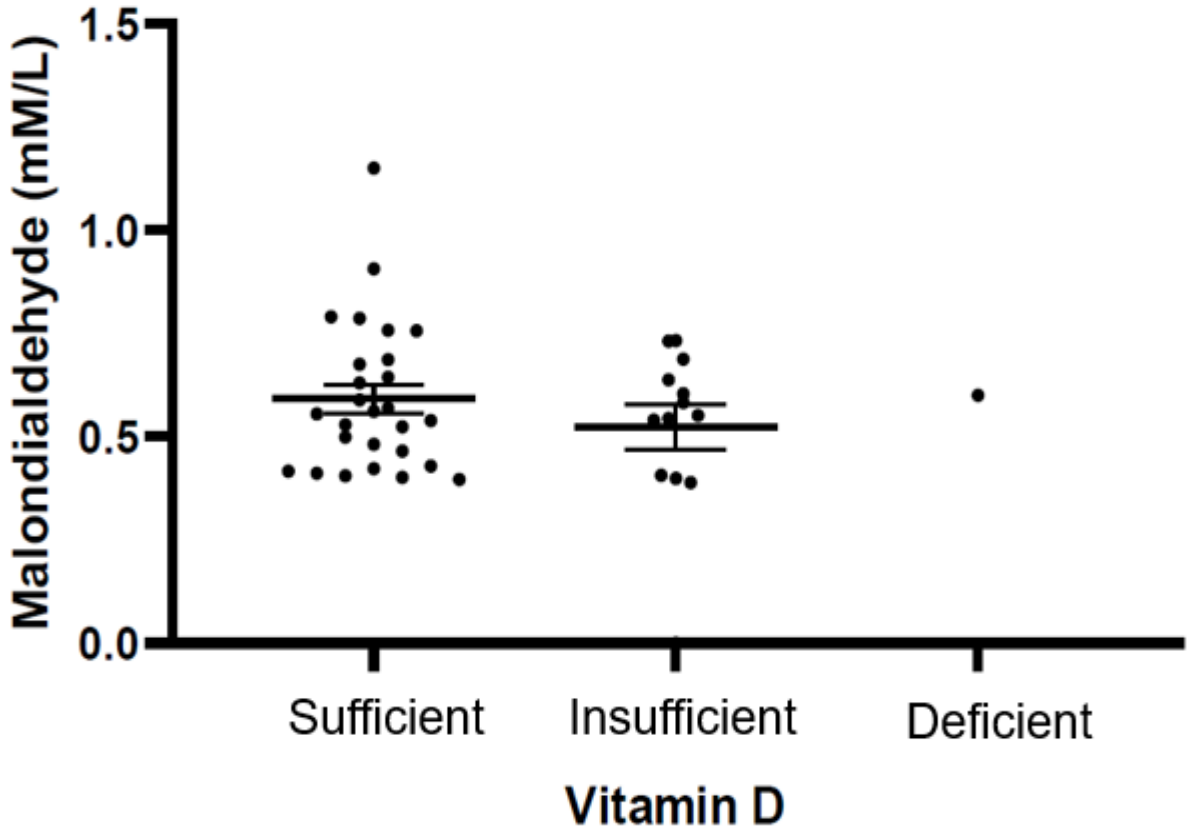

Figure 3.4 Association between MDA levels across different Vit D groups (sufficient, insufficient, and deficient). This figure indicates a U statistic of 157 and ($p > 0.05$) showing that no significant differences in MDA levels were observed among the groups, suggesting that oxidative stress, as measured by MDA, is not significantly influenced by Vit D status in this cohort. Note: the deficient group had limited data points.

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The analysis yielded a U-statistic of 157, indicating the aggregated ranks of the sufficient group relative to the insufficient group. The corresponding p-value of 0.61 exceeded ($p > 0.05$), suggesting that the observed differences in the distributions of the two groups were not statistically significant. This lack of significance implies that there is insufficient evidence to conclude that groups differ in their central tendencies or distributions regarding the measured outcome. Consequently, these findings suggest that any observed variations between the groups may be attributed to random chance rather than to a systematic effect between MDA levels and Vit D groups.

3.3.2 Evaluating malondialdehyde levels according to vitamin D levels

This analysis examined the association between MDA levels, a marker of oxidative stress, and Vit D levels in the study population. Spearman's correlation test was applied to evaluate the strength and direction of the relationship between these two variables, as the data did not meet parametric assumptions. The results are presented in Figure 3.5, which provides a detailed overview of the relationship between MDA and Vit D levels.

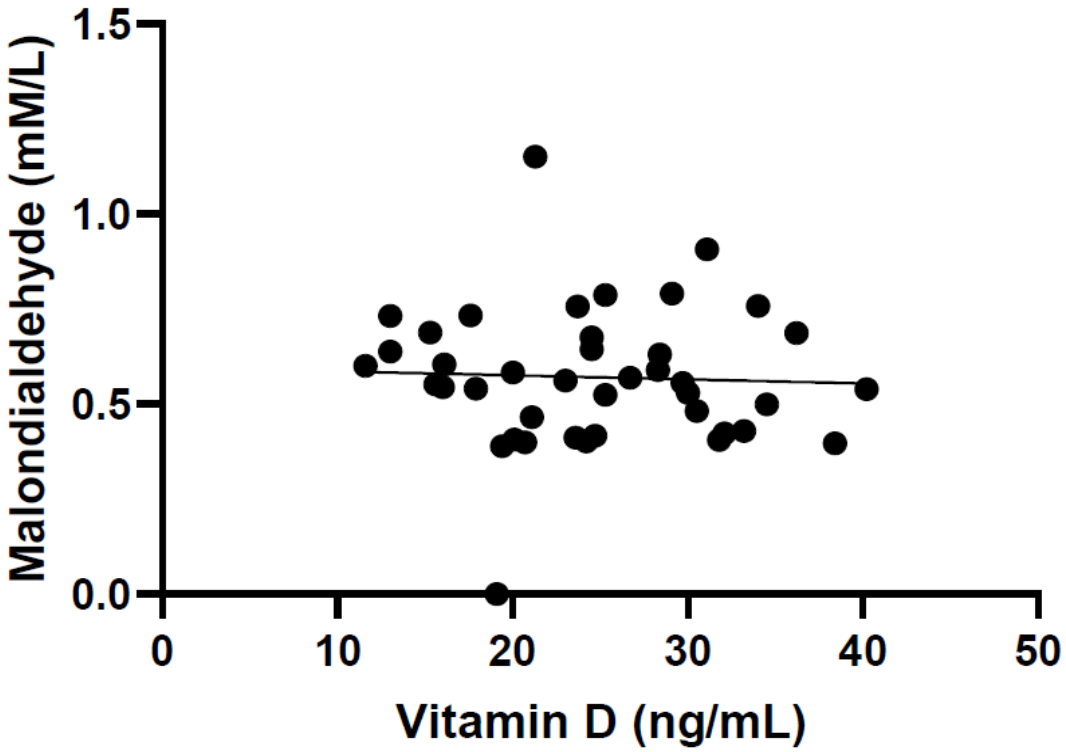

Figure 3.5 Correlation between MDA levels and Vit D levels. The analysis yielded a very weak negative correlation ($r = -0.09$), with no significant ($p > 0.05$) association between Vit D levels and oxidative among dark-skinned individuals with keloid disease.

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As shown in Figure 3.5, the test produced a very weak negative correlation (r = -0.09) between MDA and Vit D levels. The 95% CI for the correlation coefficient ranged from -0.40 to 0.23, indicating no statistical significance (p > 0.05). This CI falls within the range that indicates a negligible effect size, which means that there is no meaningful relationship between the two variables, suggesting that changes in Vit D levels are unlikely to be associated with changes in MDA levels.

3.4 Comparing the relationship of viable mitochondrial DNA and malondialdehyde levels

A Spearman's correlation test was applied to evaluate the strength and direction of the relationship between viable mtDNA and MDA levels, as the data did not meet parametric assumptions. The results are presented in Figure 3.6, which provides a detailed overview of the relationship between the viable mtDNA and MDA levels.

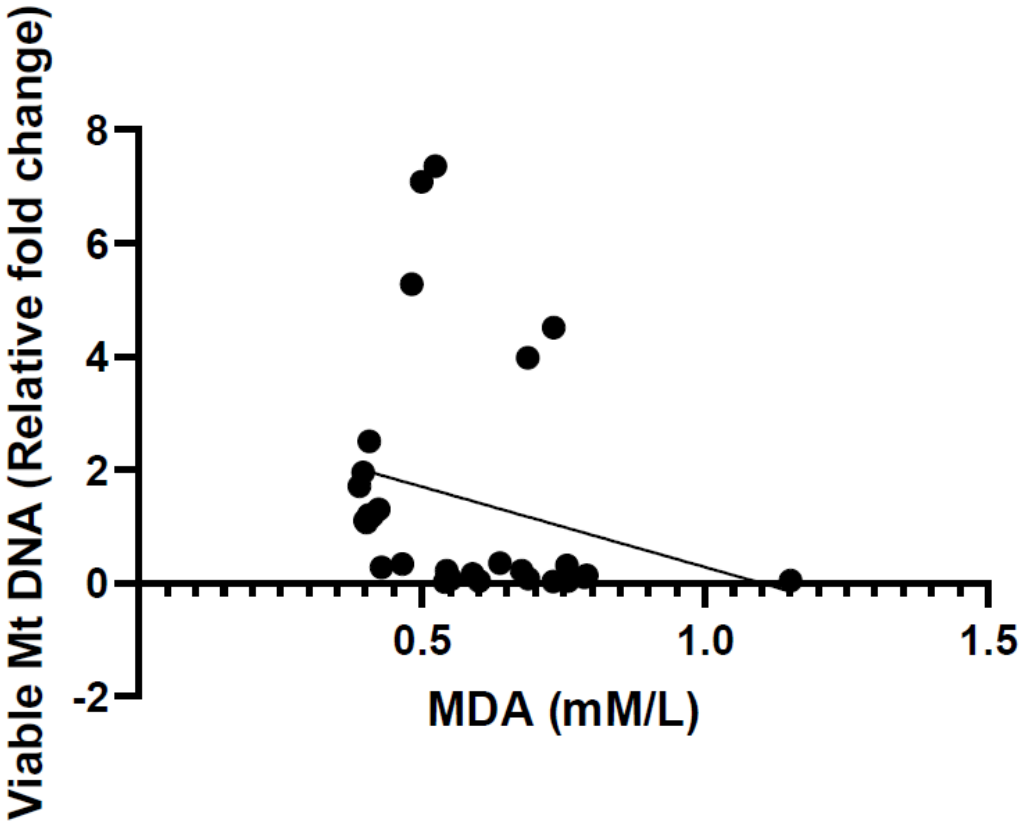

Figure 3.6 Correlation between viable mtDNA and MDA levels among dark-skinned individuals with keloid disease. This figure demonstrates a moderate negative correlation (r = -0.52), with a (p < 0.05) indicating a significant difference, suggesting an inverse relationship between oxidative stress and viable mtDNA in this cohort.

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The analysis revealed a correlation coefficient (r = -0.52), indicating a moderate negative relationship between MDA levels and viable mtDNA. This finding suggests that as MDA levels, a marker of oxidative stress, decrease, viable mtDNA levels also increase. The calculated p-value of 0.01 was found to be statistically significant ($p < 0.05$), indicating that the observed relationship is unlikely to be attributable to chance. Additionally, the 95% confidence interval for the correlation coefficient ranged from -0.75 to -0.18, reinforcing the significance of the association between MDA and viable mtDNA.

3.5 Analysing mitochondria organelle under TEM

The ultrastructure of the keloid tissue, collected and examined under TEM, was analysed with particular emphasis on the mitochondria. The keloid tissues collected were separated into three different groups according to the patient's Vit D level from which the keloid was collected. No normal skin samples were collected as the focus was to see how the keloid tissue differs between keloid samples with different Vit D levels categorised into three groups. These three groups were used to study the morphological differences between the groups to determine whether Vit D levels contributed to morphological mitochondrial changes. An image of a normal healthy mitochondrion in a eukaryotic cell was used for illustration. The normal mitochondria found in skin cells are shown in Figure 3.7. Figures 3.8 to 3.10 show TEM analysis of mitochondria found within the three Vit D groups (deficient, insufficient, and sufficient) in keloid tissue respectively.

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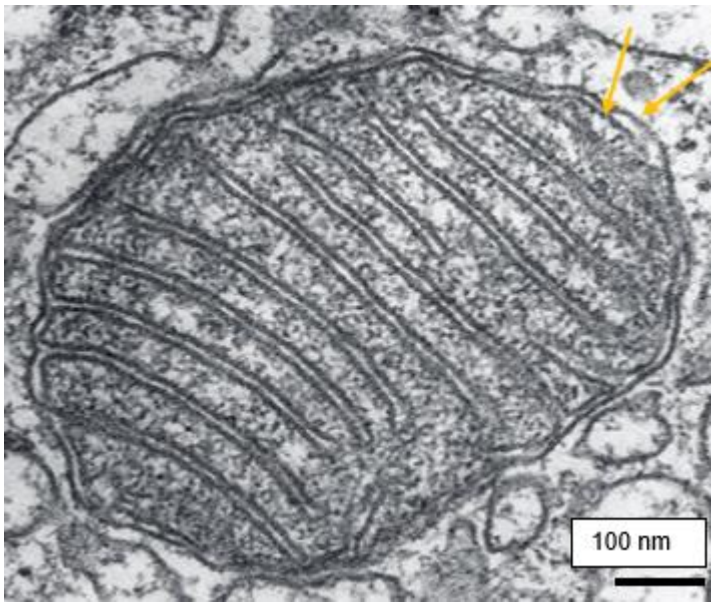

Figure 3.7 TEM image of a normal mitochondrion found in a eukaryotic cell. The mitochondrion features a double membrane architecture, comprising of an outer and an inner membrane, as indicated by the yellow arrows. The inner membrane is characterised by numerous invaginations known as cristae, which project into the mitochondrial matrix, facilitating increased surface area for metabolic processes.(116)

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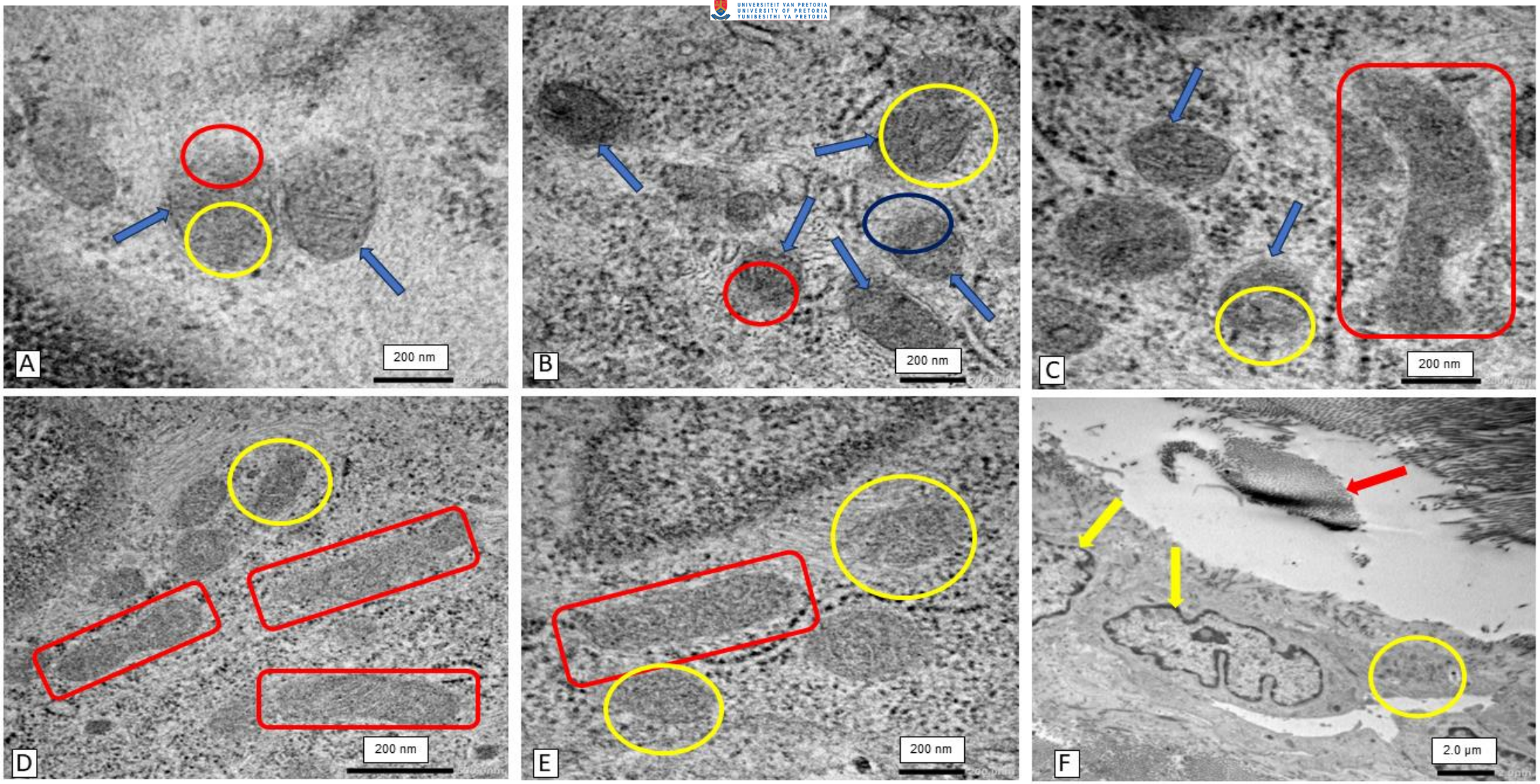

Figure 3.8 TEM analysis of mitochondria from Vit D-deficient keloid tissue. Image (A) indicates two mitochondria organelles Image (A) demonstrates damage towards the ultrastructure of the mitochondria (blue arrows) clearly. Image (B) and (C) indicates multiple mitochondria organelles (blue arrows) that show damage towards their integrity. Image (D) highlights multiple mitochondria that are elongated. Image (E) also shows numerous mitochondria that are diminutive and elongated, images (A - E) was taken at 20 000x. Image (F) illustrates the limited number of mitochondria (yellow circle) that are found in deficient Vit D keloid cells, taken at 2 500x.

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In Figure 3.8, all keloid tissue samples were analised and findings were consistent across all specimens. The mitochondria in images (A – F) do not exhibit distinct double membranes (red, blue, and yellow circles), potentially due to the low Vit D levels and high ROS levels observed in these patients. Image (A) depicts two damaged mitochondrial organelles (blue arrows) that show a missing double membrane (red circle) and improper cristae folding (yellow circle). Image (B) clearly demonstrates the absence of a discernible double membrane (blue and red circles), suggesting potential damage to the mitochondrial membrane, and the mitochondrial cristae appear improperly folded (yellow circle). In images (C, D, and E), several mitochondria displayed abnormal morphology, characterised by severe elongation, and stretching. In image (C), mitochondria can be seen as elongated organelles (red block) and shows signs of improper cristae folding (yellow circle). Image (D) shows more abnormal mitochondria that are elongated and stretched out (red blocks), with more improper cristae folding (yellow circle). Image (E) shows another mitochondrion that is elongated (red block), with more mitochondria displaying improper cristae folding (yellow circles). Image (F) illustrates an overview of the keloid cell that shows very few mitochondrial bundles present (yellow circle), two fibroblasts can be seen (yellow arrows) along with some collagen that was present (red arrow). Mitochondria are typically large, oval-shaped organelles, ranging from 0.5 to 10 µm in length. However, in images (A – E), mitochondrial sizes were observed to range between 150 and 600 nm in length, which were significantly smaller than those in normal healthy cells.

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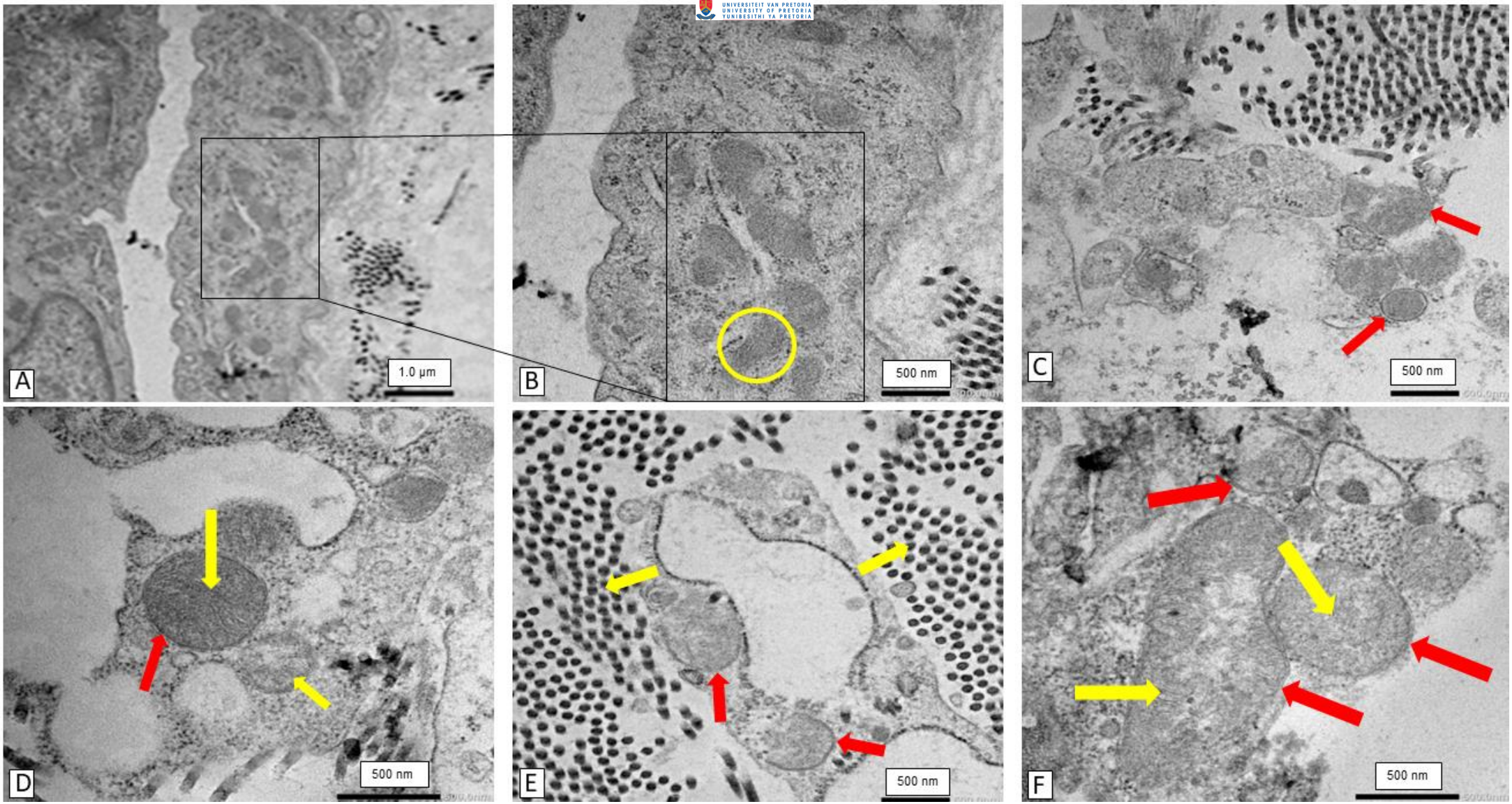

Figure 3.9 TEM analysis of mitochondria from Vit D-insufficient keloid tissue. Image (A) illustrates an overview of the cell with a small cluster of mitochondria present, taken at 5 000x. Image (B) demonstrates a magnified image of image (A) showing a close-up of the mitochondria morphology. Image (C) shows a bundle of mitochondria grouped together. Image (D) depicts a large mitochondrion. Image (E) shows two mitochondria organelles next to a large vacuole alongside collagen fibrils (yellow arrows). Image (F) displays mitochondria that exhibit normal morphology. Images (B – F) was taken at 10 000x. Red arrows represent double membrane mitochondria.

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In Figure 3.9 in image (A) the overview of the cell is shown that highlights a small bundle of mitochondria organelles grouped together, throughout the cell the mitochondria were not over abundantly present, this was one area where there were more mitochondria than normal. Image (B) serves as a close-up of image (A), where there were no clear double membranes visible around the mitochondria, but there were mitochondria that had improper folding of their cristae (yellow circle). Image (C) depicts two mitochondria with clear double membranes (red arrows). Image (D) illustrates a large mitochondrion with a double membrane (red arrow) and evident cristae that folded properly (yellow arrows). Image (E) shows two mitochondria with double membranes (red arrows) alongside collagen fibrils (yellow arrows). Image (F) shows two large and one small mitochondrion that present double membranes (red arrows), and the two large mitochondria also show signs of proper cristae folding (yellow arrows). Mitochondrial morphology appears to be normal, as evidenced by the increasingly round or oval shape observed in the images (A – F). The size of the mitochondria also increased, compared to the mitochondria observed in the Vit D deficient keloids. These mitochondria can be observed in images (A – F), with lengths ranging from 500 nm – 1.5 μm.

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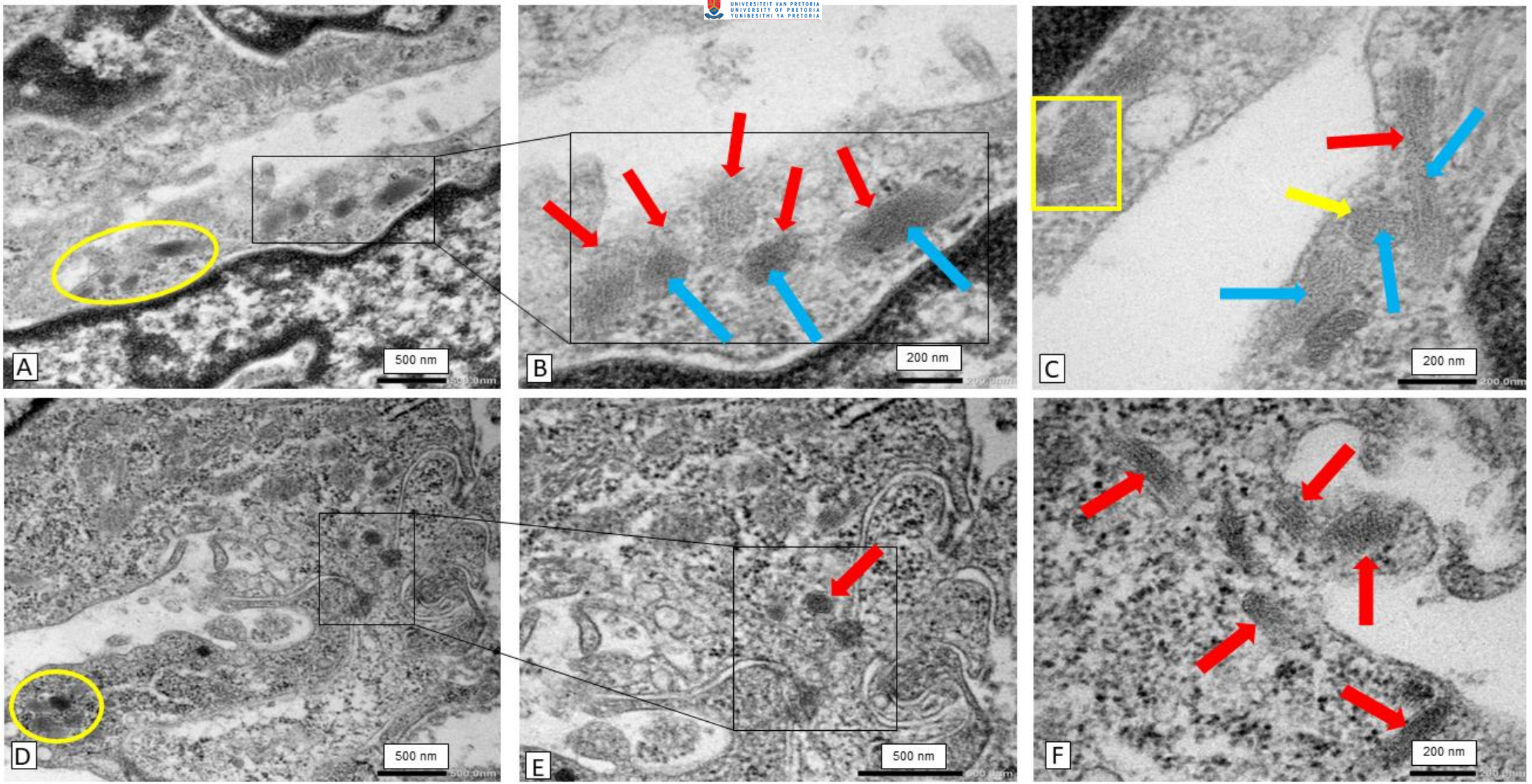

Figure 3.10 TEM analysis of mitochondria from Vit D-sufficient keloid tissue. Image (A) illustrates a cluster of mitochondria adjacent to a fibroblast. Image (B) shows a magnified view of image (A) to see the morphology of mitochondria more clearly. More bundles of mitochondria are seen in image (C). Image (E) provides a close-up of image (D). Image (E) further details the abundance of mitochondrial clusters in these cells. Images (A, D and E) were taken at 10 000x and images (B, C and F) were taken at 20 000x. Red arrows point towards the membrane of the mitochondria, whereas the yellow arrow highlights a double membrane layer and the blue arrows indicate the cristae of the mitochondria.

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In Figure 3.10 image (A), an overview of the cell is presented and depicts clusters of mitochondria that were present mostly throughout the cell. In image (B), mitochondria lack a clearly defined double membrane structure (red arrows) and do not show any signs of proper cristae folding (blue arrows). In image (C), there is one mitochondrion displayed with a clear double-membrane structure (yellow arrow) and another elongated mitochondrion (red arrow). The mitochondria present in this image exhibit cristae folding within the inner membrane, although the cristae are not prominently defined (blue arrows). The mitochondrion represented in the (yellow block) display a bilobed structure, which is rarely seen in this population group. Image (D) illustrates two bundles of mitochondria (yellow circle and black block). Image (E) Magnified image of (D) displaying a mitochondrion with a double membrane (red arrow). Image (F) shows another bundle of mitochondria with no clear double membranes (red arrows). The mitochondrial morphology predominantly appears round or oval in images (B, C, and D); however, certain mitochondria present a bilobed structure in the image (C, yellow block) or an elongated, stretched morphology in the image (C, red arrow). Mitochondria are also of diminutive size, ranging from 180 – 1.5 µm in length.

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3.7 Analysing rough endoplasmic reticulum under TEM

The objective of this chapter was to investigate the mitochondrion organelle; however, during the examination of keloid tissue, notable changes were observed in the endoplasmic reticulum (ER), warranting attention. The rough endoplasmic reticulum (RER) plays a critical role in protein synthesis and folding, which are essential processes in collagen production and secretion. Since excessive collagen deposition is a hallmark of keloid disease, examining the RER could provide insights into the dysregulation of these processes in keloid pathophysiology. Figure 3.11 provides an illustrative depiction of the normal morphology of the ER found in healthy eukaryotic cells, serving as a baseline reference. In Figures 3.12 to 3.14 observations were subsequently compared across the different Vit D groups (deficient, insufficient, and sufficient) respectively, within the keloid tissue samples.

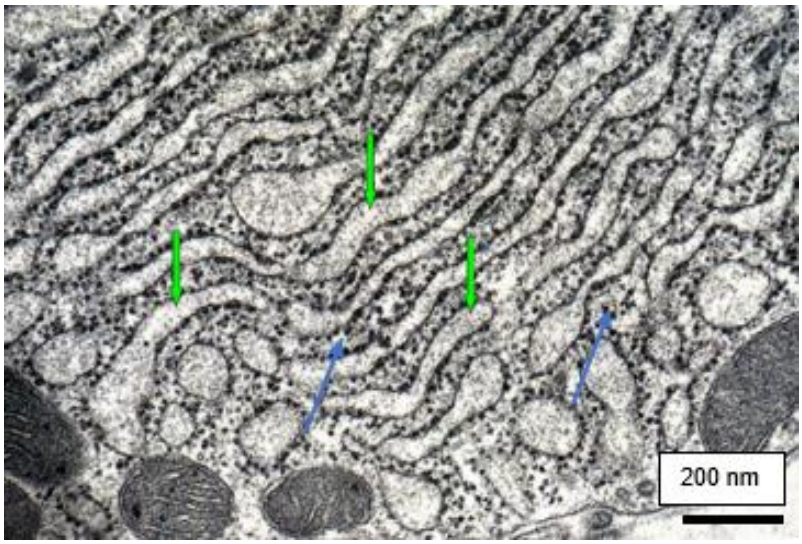

Figure 3.11 TEM image of a normal mitochondrion found in a eukaryotic cell. The cisternae of the RER are commonly arranged in parallel stacks or layers, creating a distinct, highly organised structure (green arrows). The intracisternal spacing of the RER is typically between 20 and 30 nm. The surface is densely populated with ribosomes, imparting the characteristic "rough" appearance. These ribosomes are attached to the cytoplasmic side of the RER membrane and appear as small, dark, spherical particles under TEM (blue arrows). Due to their electron-dense nature, ribosomes on the RER membrane exhibit a darker contrast compared to other cellular structures, facilitating clear identification of the RER in TEM images.(117)

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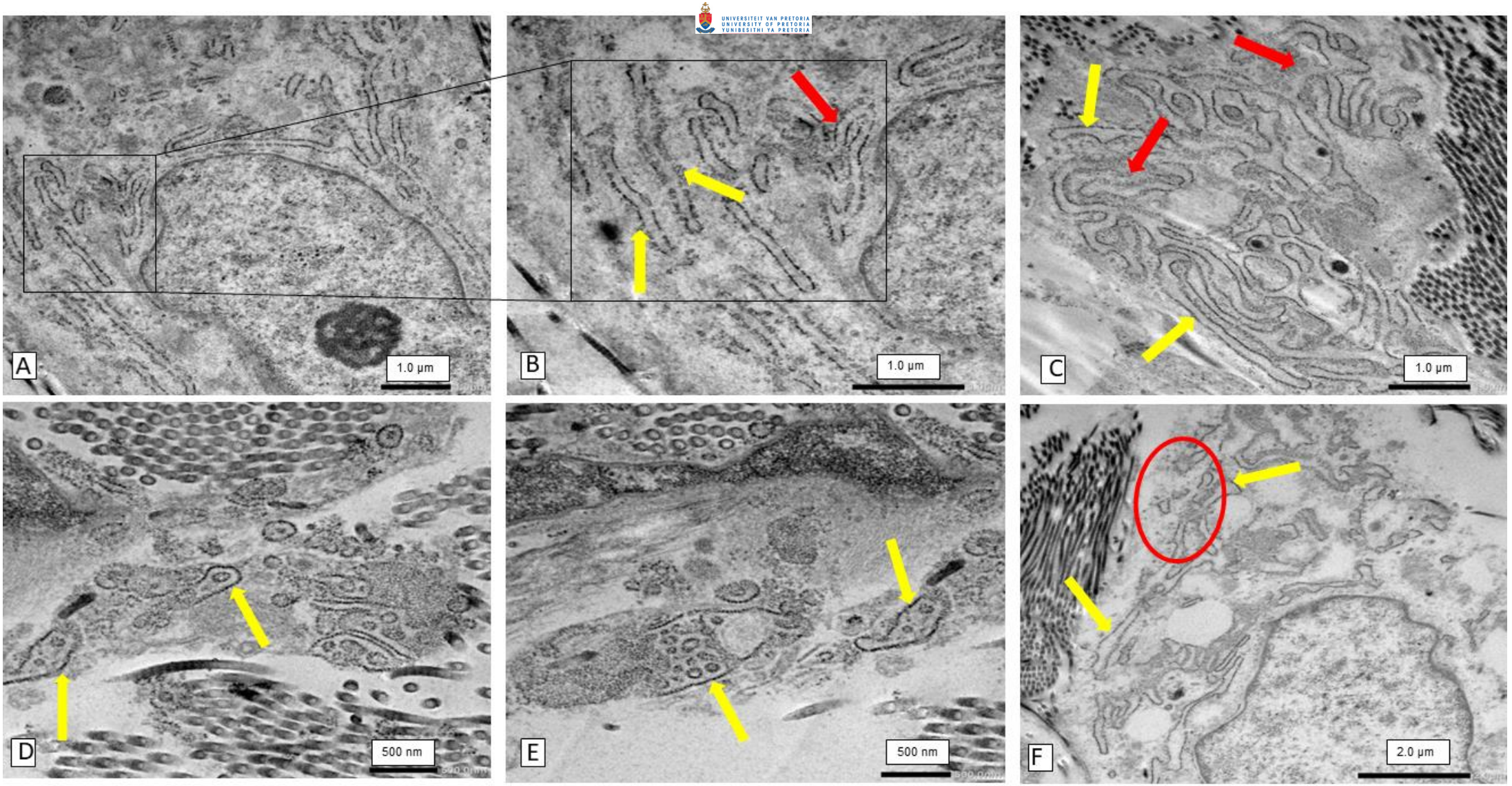

Figure 3.12 TEM analysis of RER from Vit D-deficient keloid tissue. Image (A) revealed how sparsely the RER was scattered around the fibroblast cell. Image (B) magnified image (A) to observe the morphology of the RER more closely. Image (C) highlights no form of parallel lines between RER, images (A – C) were taken at 5 000x. Image (D) depicted minute RER with abnormal structure. In image (E) the RER exhibited no distinct parallel lines and were less abundant in the cell. Images (D and E) were taken at 10 000x. Image (F) displayed an overview of the cell with diminished RER, taken at 2 500x. Yellow arrows point towards ribosomes and red arrows towards parallel alignment.

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In Figure 3.12 in image (A) displays an overview of the cell showing how limited the RER is in the tissue. Image (B) serves as a magnified image of image (A), illustrating how light in colour the ribosomes appear (yellow arrows) and how the RER is disorganised and lacks a parallel arrangement (red arrow). Image (C) shows plentiful RER that also lacks parallel formation (red arrows) but has more electron-dense ribosomes (yellow arrows). Images (D and E) show an abnormal RER parallel formation (yellow arrows). Image (F) illustrates an overview of the cell showing how sparsely the RER is available within the cell, displaying disorganisation of the parallel formation (red circle). Intracisternal spacing notably expanded in (images A, B, and F), indicating atypical morphology.

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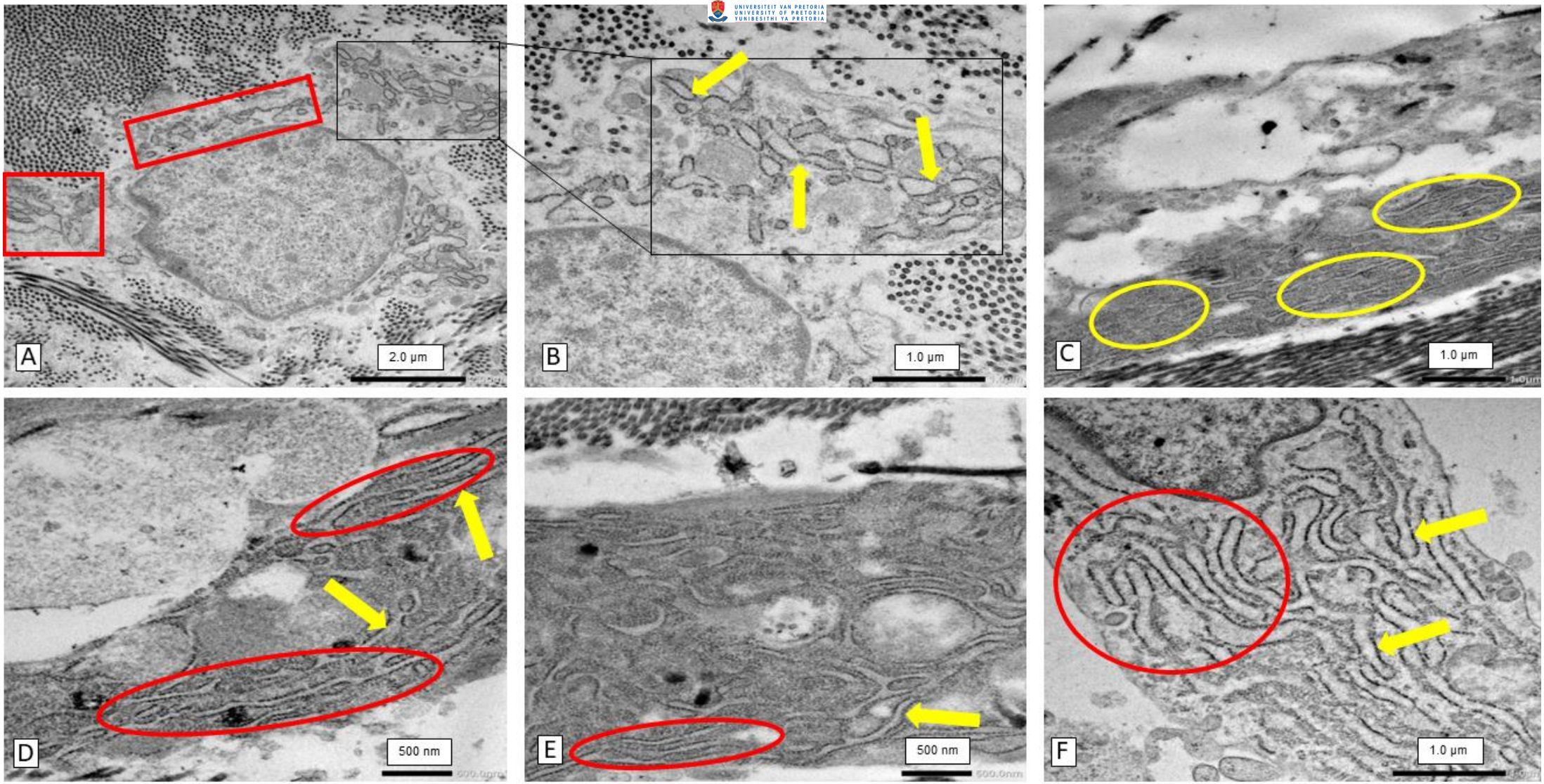

Figure 3.13 TEM analysis of RER from Vit D-insufficient keloid tissue. Image (A) depicts numerous clusters of RER in the cell, taken at 2 500x. Image (B) is a magnification of image (A) highlighting the morphology of the RER. The RER starts to form parallel lines in image (C). Both images (B and C) were taken at 5 000x. The intracisternal space is appearing to be normal and more RER in parallel lines are displayed in image (D), taken at 10 000x. The RER appear in abundance in the cell as illustrated in image (E), taken at 5 000x. Image (F) exhibits RER with an enlarged intracisternal space with RER forming more parallel lines, taken at 5 000x. Yellow arrows point towards ribosomes.

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In Figure 3.13 image (A) displays an overview of the cell, highlighting the over-abundance of RER within the cell. Image (B) serves as a magnified image of image (A) showing ribosomes that are more electron-dense (yellow arrows) with large intracisternal spacing between RER. Image (C) represents the abundance of RER within the cell that exhibits proper organisation of parallel lines (yellow circles). Image (D) shows more RER that are in parallel line formation, which means they are organised (red circles), and contain ribosomes that are more electron-dense (yellow arrows). Image (E) shows some RER that show some form of proper organisation (red circle), with electron-dense ribosomes (yellow arrow). Images (C – E) show narrow intracisternal spacing between RER. Image (F) shows more electron-dense ribosomes (yellow arrows) and displays RER in parallel formation (red circle). However, the intracisternal space was slightly enlarged in the image (F).

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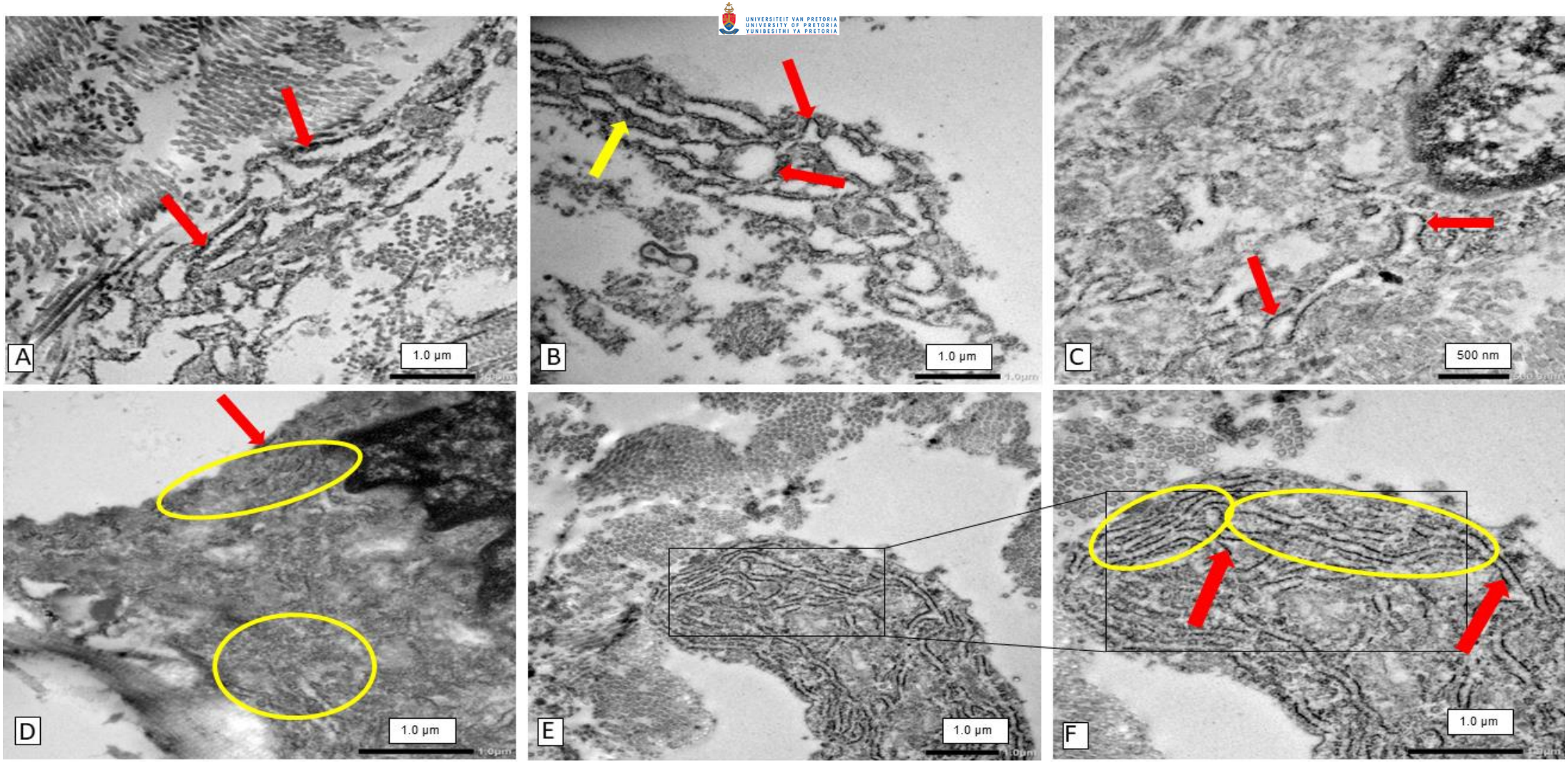

Figure 3.14 TEM analysis of RER from Vit D-sufficient keloid tissue. Image (A) reveals a high electron density of ribosomes attached to the RER, although the intracisternal space appears enlarged. In image (B), the RER structures are sparse within the tissue, displaying abnormally widened intracisternal spaces. Image (C) shows fragmented RER structures with a reduced ribosomal presence and a lack of parallel cisternal layering, captured at a 10,000x. Image (D) illustrates severely damaged RER, devoid of typical structural characteristics. In contrast, image (E) provides a cellular overview where RER exhibits normal intracisternal spacing and some formation of parallel layers. Image (F), a magnification of image (E), highlights the abundance of ribosomes attached to the RER. Despite the sufficient Vit D levels in these individuals, the RER structures remain abnormal. Images (A, B, D–F) were taken at 5,000x. Red arrows point towards ribosomes and yellow arrow shows the intracisternal space.

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In Figure 3.14 illustrates the variability in RER abundance and organisation across cellular structures. Images (A – D, red arrows, and yellow circles) show scarce RER compared with the prominent bundles in the images (E – F, yellow circles). RER cisternae appear disorganised with minimal parallel layering in images (B – D), although some parallel alignment was observed in the image (F, yellow circles). The RER appeared to be sparsely spaced in images (A – D) but appeared more abundant in images (E and F). Intracisternal spacing is abnormally large in images (A – C) but tightly packed in image (F). Ribosomes appeared to be darker in colour and electron-dense in this population group.

3.8 Analysing collagen under TEM

The objective of this chapter was to investigate the mitochondrion organelle; however, collagen abnormalities are known to be apparent in keloids; therefore, the collagen fibrils were analysed as well. Figure 3.15 presents an illustrative depiction of the normal structure of collagen in healthy eukaryotic cells, serving as a baseline reference. Comparative assessments were subsequently conducted across the different Vit D groups (deficient, insufficient, and sufficient) within the keloid tissue samples, as illustrated in Figures 3.16 to 3.18 respectively.

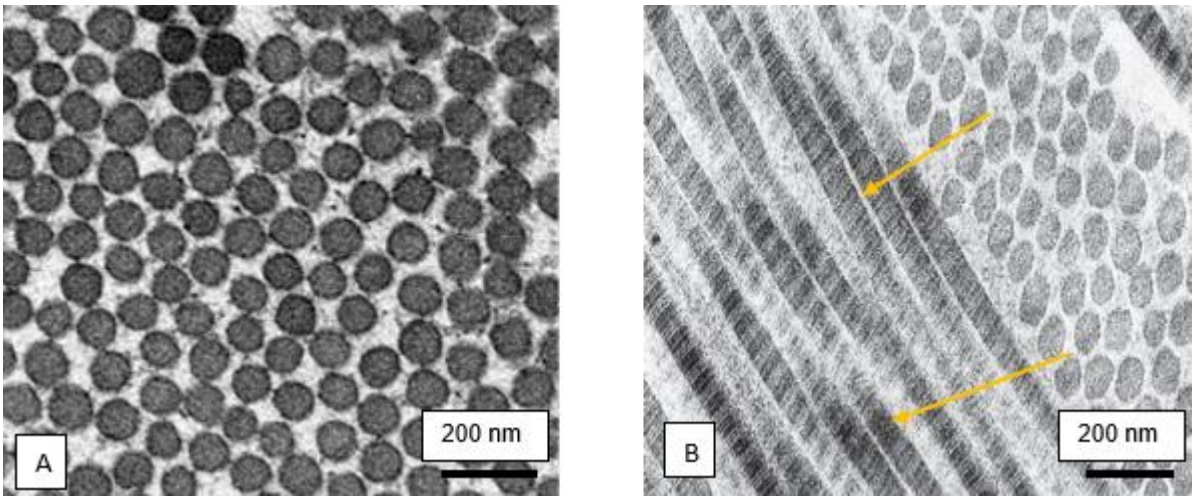

Figure 3.15 TEM image of normal collagen found in a eukaryotic cell.

In Figure 3.15, image (A) represents transverse collagen in a cross-sectional orientation. The fibrils often appear as circular or oval structures. Image (B) illustrates

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longitudinal collagen fibrils, which are mostly elongated, cylindrical, and unbranched structures that typically align parallel to each other. The alternating dark and light bands result from variations in electron density, where the dark bands (regions of high electron density) correspond to areas of dense collagen molecular packing and the light bands (regions of low electron density) represent gaps between collagen molecules. This pattern, known as D-periodicity, is a distinctive feature of collagen fibrils, with a typical periodic length of approximately 67 nm arising from the staggered alignment of collagen molecules. In image (A), fibril diameter varies based on collagen type and tissue origin, generally ranging from 20 to 500 nm(118), with Type I collagen fibrils frequently observed within a range of 50 to 100 nm(119) in diameter.(120)

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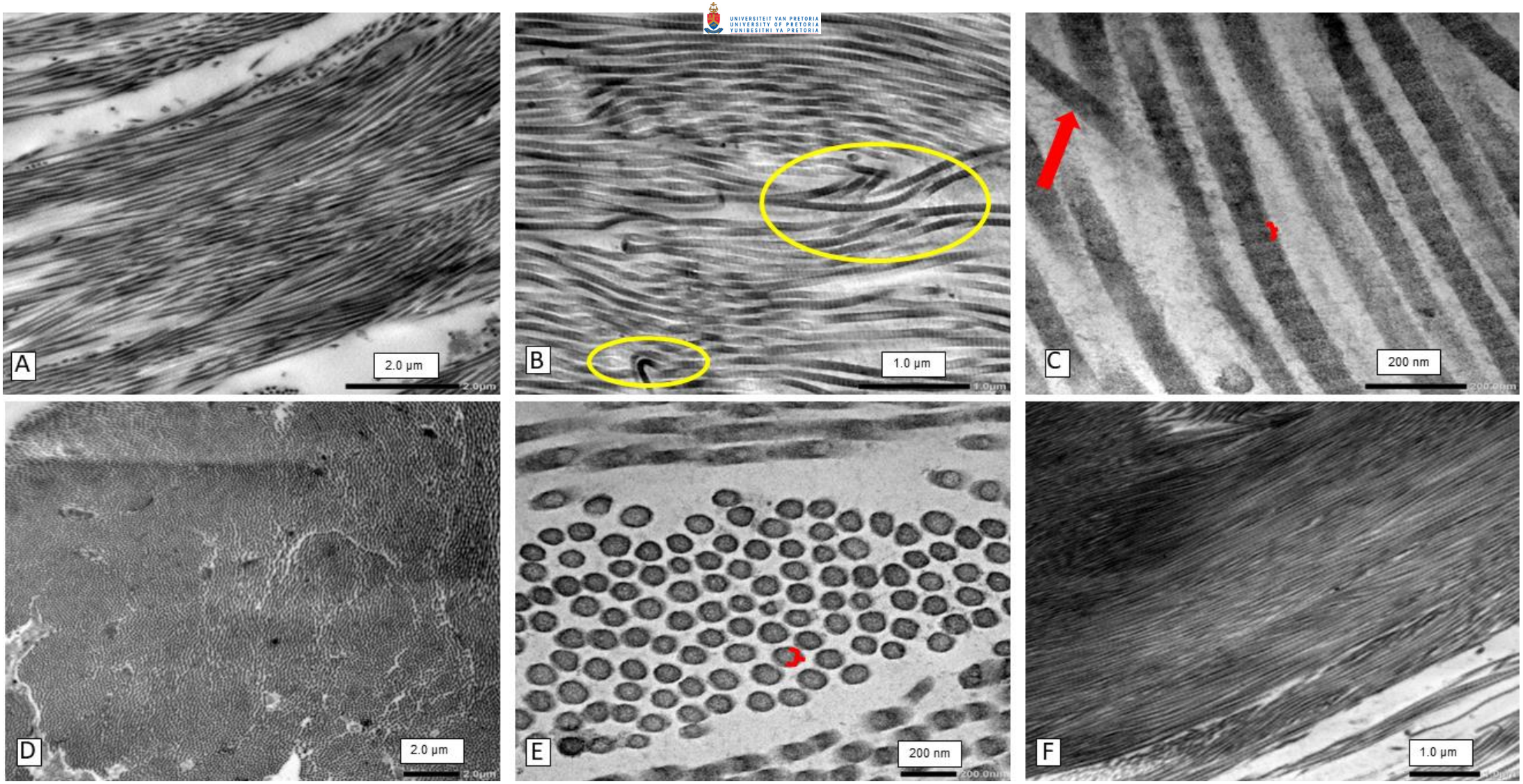

Figure 3.16 TEM analysis of collagen from Vit D-deficient keloid tissue. This figure illustrates TEM micrographs (A – C, and F) of longitudinal collagen fibrils and (D and E) of transverse collagen fibrils. Image (A) shows numerous collagen fibrils with slight deviations from strict parallel alignment, captured at 2 500x. In image (B), the collagen fibrils are oriented in multiple directions, lacking parallel organisation, taken at 5 000x. Image (C) provides a high-magnification view of a single collagen fibril, highlighting the periodicity characteristic observed in collagen, taken at 20 000x. An excess of collagen fibrils is observed in keloid tissue, as shown in image (D) at 2 500x. Image (E) depicts transverse collagen fibrils and their diameters, taken at 20 000x. Image (F) displays additional transverse fibrils, emphasising their morphology, taken at 10 000x. Red arrow shows nonparallel formation.

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In Figure 3.16, images (A and D) illustrate an overabundance of collagen fibrils in keloid cells. Some longitudinal fibrils exhibited a non-parallel alignment and deviated from a linear configuration in the images (B and C, yellow circles, red arrow). The observed D-band periodicity is approximately 40 nm, which is smaller than the typical 67 nm, with the image (C, red bracket) showing a D-periodicity measurement of 3 µm. Transverse collagen fibrils in images (E and F) display notable interfibrillar spacing, with fibrils not in direct contact with the membrane. Furthermore, these transverse fibrils exhibit poorly defined and blurred outer membranes. The diameter of the transverse collagen fibrils in the image (E, red bracket), approximately 80 nm, falls within the normal range, with a fibril diameter measurement of 4 µm.

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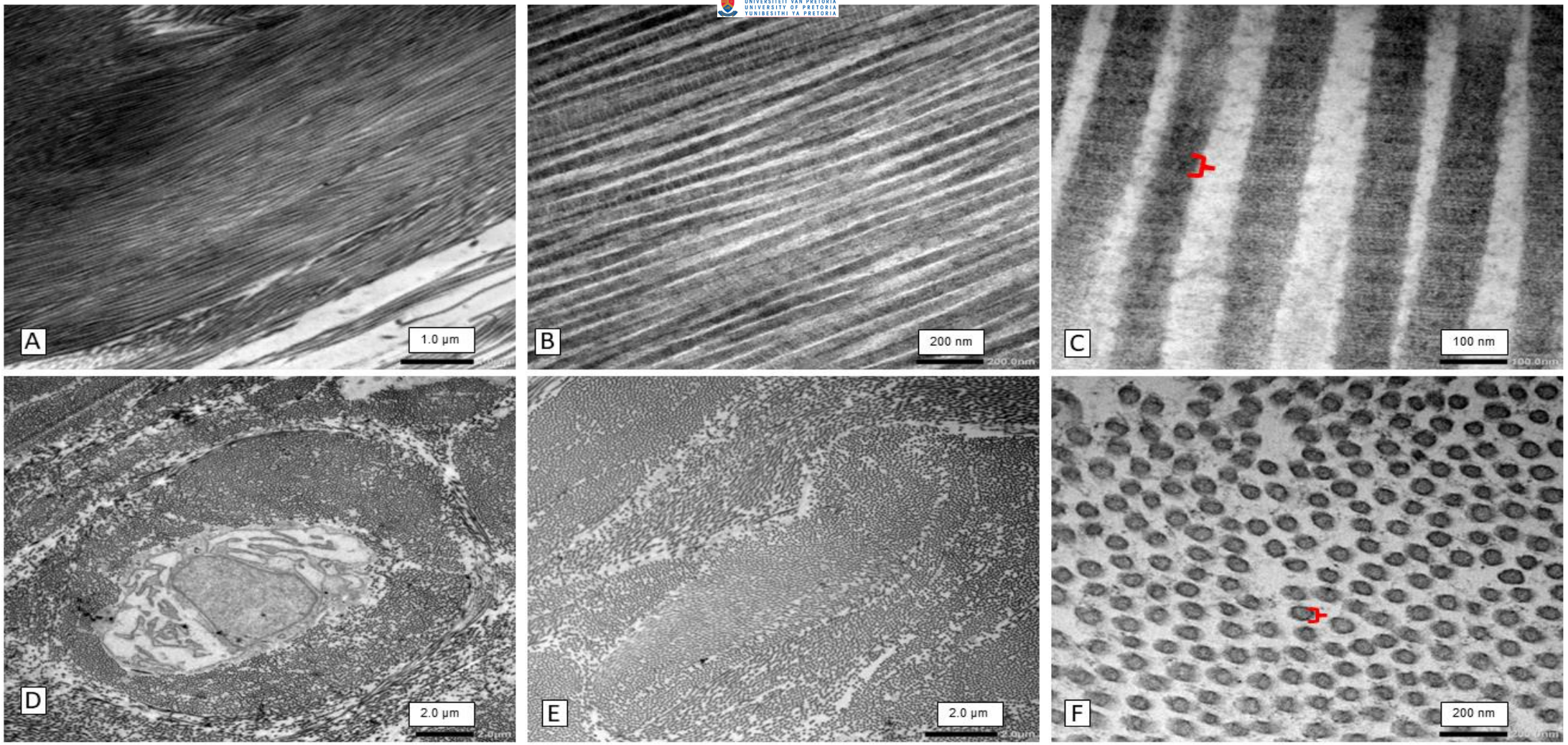

Figure 3.17 TEM analysis of collagen from Vit D-insufficient keloid tissue. This figure illustrates TEM micrographs (A – C) of longitudinal collagen fibrils and (D – F) of transverse collagen fibrils. Image (A) reveals a dense arrangement of collagen fibrils aligned in parallel layers, captured at 5 000x. Image (B) further emphasises the excessive accumulation of collagen fibrils characteristic of keloid tissue, shown at 20 000x. Image (C) provides a high-magnification view of individual collagen fibrils, illustrating the characteristic D-band periodicity, captured at 30 000x. Image (D) demonstrates the substantial collagen fibril density within keloid tissue. Image (E) highlights the densely packed nature of collagen within this tissue type; both images (D and E) were taken at 2 500x. Image (F) offers a close-up view of transverse collagen fibrils to assess detailed morphological features, taken at 20 000x.

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Figure 3.17 demonstrates the overabundance of collagen in keloid cells images (A, B, D, and E). Longitudinal collagen fibrils (images A – C) exhibited straight, parallel alignment, indicative of an organised structure. The D-band periodicity is approximately 55 nm, slightly smaller than the typical 67 nm image (C, red bracket) with a 3 µm measurement. Transverse collagen fibrils (image F) show significant interfibrillar spacing with no membrane contact, and their outer membranes appear poorly defined. The diameter of these fibrils, approximately 60 nm, is within the normal range as seen in image (F, red bracket) with a diameter of 3 µm.

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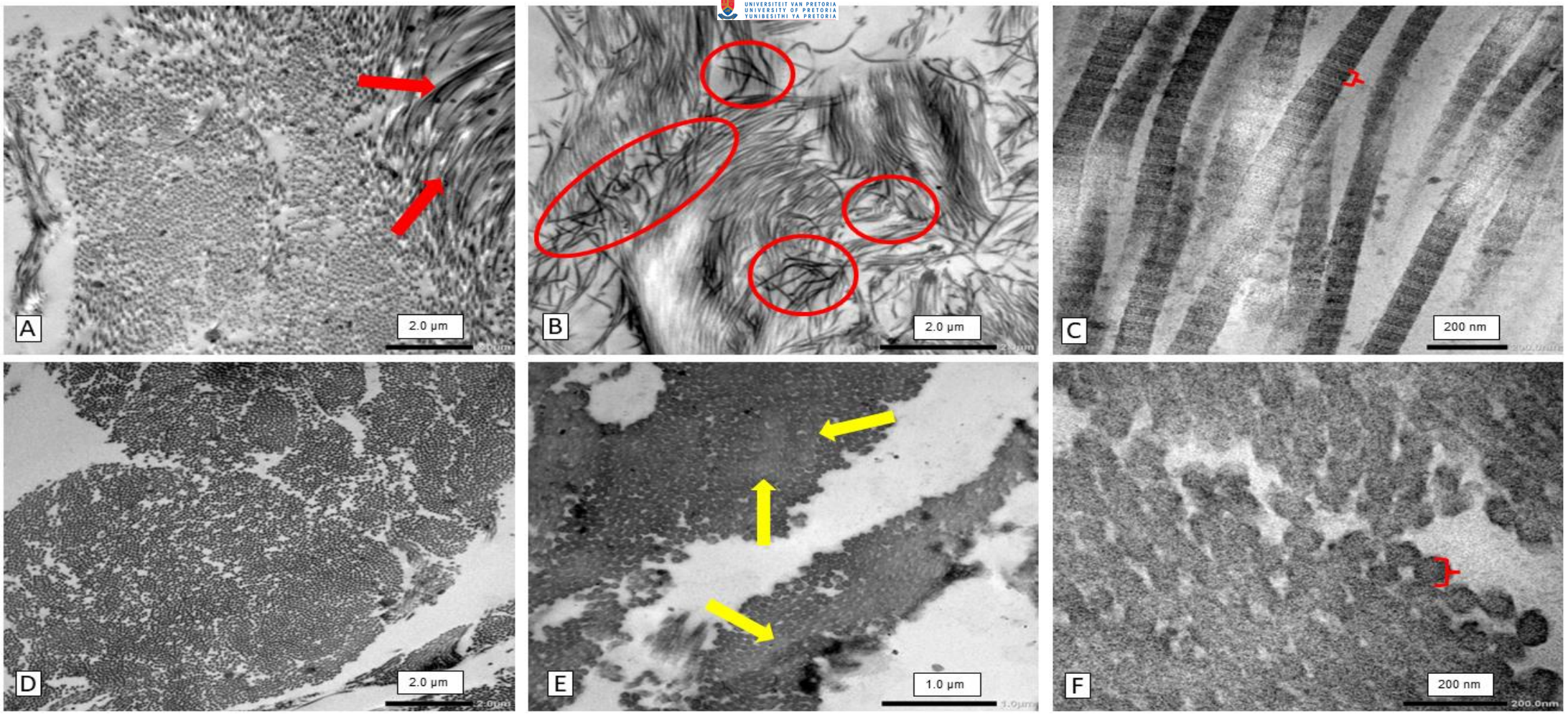

Figure 3.18 TEM analysis of collagen from Vit D-sufficient keloid tissue. This figure illustrates TEM micrographs (A – C) of longitudinal collagen fibrils and (D – G) of transverse collagen fibrils. Image (A) reveals collagen fibrils exhibiting disorganisation, lacking the formation of parallel layers. Image (B) shows a random arrangement of collagen fibrils oriented in multiple directions, with no evidence of parallel alignment. Both images (A and B) were captured at 2 500x. Image (C) highlights individual collagen fibrils, emphasising their characteristic D-band periodicity, taken at 20 000x. Image (D) provides an overview of keloid cells, illustrating the abundant presence of collagen fibrils within the cellular environment, taken at 2 500x. Image (E) depicts closely spaced collagen fibrils that are in contact with each other, taken at 5 000x. Finally, image (F) presents a close-up view of the collagen fibrils, reinforcing the observation of their close packing and membrane contact, taken at 20 000x. Red arrows show nonparallel formation and yellow arrows show collagen fibrils with their membranes in contact with each other.

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In Figure 3.18, keloid cells are characterised by an abundance of collagen fibrils, as illustrated in images (A, B, D, and E). Disorganised longitudinal collagen is observed in the images (A and B, red arrows, and red circles), where the fibrils fail to exhibit a straight parallel alignment. The D-band periodicity measures approximately 72 nm, which is slightly above the normal range of the 67 nm image (C, red bracket) with a measurement of 4 mm. Transverse collagen fibrils depicted in images (E and F) exhibit minimal interstitial spacing, with some fibril membranes in direct contact (yellow arrows). Additionally, the outer membranes of the fibrils appeared distinct and well defined, as shown in image (F). The diameter of the transverse collagen fibrils was approximately 71 nm, which falls within the normal range image (F, red bracket) with a measurement of 5 mm.

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Chapter 4: Discussion

The objective of the present study was to evaluate mitochondrial function, oxidative stress, and Vit D status in dark-skinned patients with keloid disease. This investigation aimed to elucidate the complex interplay between these factors in the pathophysiology of keloid formation. The findings obtained in this study indicate potential associations between mitochondrial dysfunction, elevated oxidative stress, and Vit D status in the dark-skinned population group. These results were analysed in the context of existing literature, addressing how they differ from and align with previous studies, while also considering the potential therapeutic mechanisms, clinical implications, and limitations of the study.

4.1 Vitamin D status in keloid disease

4.1.1 Difference between sufficient and insufficient vitamin D groups

The results obtained from the patients' Vit D status revealed a significant difference ($p < 0.05$) in the mean levels between the Vit D sufficient group (29.52 ng/mL) and the Vit D insufficient group (17.22 ng/mL), with the sufficient group exhibiting a substantially higher mean with a mean difference of (-12.30 ng/mL) with a relatively small SEM of (±1.25). In this study it was noted that 73% of the overall population had sufficient Vit D levels, 25% of the overall population had insufficient Vit D levels, and 2% of the overall population had deficient Vit D levels. A recent study done in 2021 which looked at the Vit D levels of individuals without keloid disease from African ancestry in the Cape Town region also showed high population values within the Vit D range of 22 – 24 ng/mL(121) which correlates to the 25% of the population found in this study having sufficient Vit D levels. Additionally, according to data from the National Health and Nutrition Examination Survey 2001 – 2010, the prevalence of Vit D [25(OH)D] deficiency (< 20 ng/mL) was 75% for individuals that are non-Hispanic blacks(121), which contradicts our findings, as our population only represents 27% of individuals that have Vit D [25(OH)D] levels below 20 ng/mL. This significant difference aligns, to some extent, with the existing literature (as the Vit D insufficient group showed Insufficient levels) suggesting that lower Vit D levels may be associated with dark-skinned individuals diagnosed with keloid disease.(121) However, despite this

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significant difference, the findings within the Vit D sufficient group showed substantially high Vit D levels, which does not align with the existing literature, which suggests that Vit D levels are typically lower in individuals with keloid disease.(122)

In this study Vit D [25(OH)D] was measured and was found to be high in most of the population group but this does not necessarily mean that the active form of Vit D [1,25$(OH)_2$D] is also high. Kidney dysfunction, low parathyroid hormone, chronic inflammation, and genetic mutations can affect the way in which Vit D is metabolised causing this imbalance between the two different Vit D states. The high levels of Vit D [25(OH)D] in these dark-skinned patients could be due to many factors, such as environmental factors, dietary intake, genetic variability, and the inflammatory state. Firstly, as Vit D synthesis relies on sunlight exposure, it is plausible that some patients may have resided in regions with higher solar radiation or may have experienced increased direct sun exposure. Secondly, these patients could consume a Vit D-rich diet that includes (fatty fish such as salmon, trout, and tuna, canned fish such as herring and sardines, egg yolks, and beef or fish liver). Thirdly, genetic variations in polymorphisms in Vit D-binding proteins and receptors (115) could play a significant role (by promoting Vit D production) in these patients, potentially inherited from their parents, suggesting that keloids are inherited in an autosomal dominant pattern (116), or that these patients may metabolise and store Vit D differently. Lastly, it is crucial to consider the inflammatory response associated with keloid disease, as this could elevate Vit D levels despite an underlying Vit D deficiency. Further research is necessary to assess the role of these factors in Vit D production.

According to previous studies, the expected relationship between a dark-skinned patient with a keloid and their Vit D status showed a decrease in the Vit D status.(121) Previous studies have indicated that individuals with darker skin pigmentation tend to have lower levels of Vit D, attributed to the higher melanin content in their skin, which reduces the skin's capacity to synthesise Vit D.(123, 124) There have been recent studies that have linked the potential Vit D deficiency in these individuals to keloid disease(122), as Vit D plays a vital role in skin healing and inflammation. These studies directly support the results, as it was found that dark-skinned patients with keloid disease generally had lower levels of Vit D.

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According to literature, skin conditions, such as psoriasis or eczema, which share some pathological features similar to those of keloids, can affect Vit D levels.(125, 126) These studies also support the findings as eczema and psoriasis are known to have low Vit D levels.(125, 127) Keratinocyte activity is altered in skin diseases; therefore, causing inflammation which can affect the way in which Vit D is metabolised.(127, 128) The role between keratinocytes and keloids is not fully understood, but can be a potential avenue for exploration.

The data obtained predominantly challenges the common generalisation in the literature that darker skin impairs Vit D synthesis.(91, 121, 123, 124), which suggests that individuals with darker skin exhibit lower Vit D levels. The findings in this study potentially indicates that the South African dark-skinned population groups with keloid disease generally possess significantly higher Vit D levels than the dark-skinned population groups with no keloid disease examined in previous research. It highlights the need for more region-specific research.

Furthermore, it is noteworthy that despite these individuals possessing sufficient Vit D levels, they still developed keloids, which contradicts existing literature, as research has demonstrated that elevated Vit D levels are associated with the treatment of keloids by reducing their thickness.(129) It also suggests that Vit D's protective role in keloid prevention may not apply universally or may interact differently in the South African context due to genetic, metabolic, or environmental factors.

In addition, this study highlights a potential geographic and contextual difference in Vit D levels among dark-skinned populations. While previous research has primarily associated darker skin with lower Vit D levels, the findings in this cohort suggest that South African dark-skinned individuals may maintain higher Vit D levels, likely due to greater sunlight exposure. This divergence underscores the importance of considering regional and lifestyle factors in interpreting Vit D-related health outcomes.

Interestingly, despite sufficient Vit D levels, keloid formation persisted in this cohort. This observation suggests that while Vit D may play a role in wound healing, other factors such as Vit D receptor activity, keratinocyte function, and broader metabolic pathways may influence keloid development in dark-skinned populations. Further

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research is required to explore these mechanisms, particularly in the South African context.

The study's findings challenge existing literature by demonstrating sufficient Vit D levels in some dark-skinned patients with keloids. This could reflect unique environmental, genetic, or dietary factors in the South African context. This aspect deserves further investigation to refine thresholds and treatment protocols for this population.

4.2 Mitochondrial function and vitamin D in keloid disease

Mitochondrial function and Vit D play key roles in the normal wound healing process.(43, 130, 131) In keloid disease, abnormalities in mitochondrial function may contribute to the excessive fibrotic response seen in keloids(132), while Vit D is thought to benefit the mitochondrial function and wound healing process.(131) When mitochondrial activity is disturbed it can lead to dysfunction.(66) Dysfunctional mitochondria can lead to increased oxidative stress levels, which then interfere with normal cell communication(133) and can trigger excessive collagen production in keloids. Understanding mitochondrial dysfunction observed in keloid disease provides valuable insights into the mechanisms underlying keloid formation and may facilitate the identification of novel therapeutic approaches for managing keloid disease.

4.2.1 Viable mitochondrial DNA levels in relation to vitamin D levels

The analysis conducted in this study revealed a moderate positive correlation between viable mtDNA and Vit D levels: the moderate positive correlation ($r = 0.40$, $p < 0.05$) supports a role for Vit D in maintaining mitochondrial function by reducing oxidative stress and promoting mitochondrial biogenesis. While this does not establish causation, it aligns with literature suggesting a protective effect of Vit D on mitochondrial integrity. This study revealed a statistically significant positive correlation, supporting the hypothesis that Vit D plays a role in mitochondrial health. This association implies a potential link between Vit D status and mitochondrial health; however, the moderate strength of the correlation suggests that other factors such as age, genetic background, inflammatory status, and lifestyle variables (e.g., nutrition,

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smoking, and alcohol consumption) may also exert a significant influence on mtDNA viability. Further studies incorporating these additional variables are warranted to clarify the relative contribution of Vit D to the mitochondrial function in this population.

Firstly, Vit D can attenuate oxidative stress levels, potentially safeguarding viable mtDNA from disruption. A prominent characteristic of keloids is the elevated oxidative stress relative to that observed in normal tissue cells. Elevated oxidative stress can inflict damage to both the nucleus and mtDNA. Consequently, increased levels of Vit D may mitigate oxidative stress to a degree that facilitates the preservation of mtDNA. Secondly, Vit D, in conjunction with PGC-1α, may further enhance mitochondrial biogenesis through replication and maintenance of healthy mitochondria. Therefore, higher concentrations of Vit D could correlate with an increased presence of viable mtDNA, thereby ensuring optimal energy production and cellular functionality within the keloid tissues. The moderate positive correlation observed between viable mtDNA and Vit D in this study suggests that other factors such as genetic predisposition, inflammation, and oxidative stress may exert a more substantial influence on viable mtDNA than Vit D alone.

Certain individuals may be genetically predisposed to factors that impair normal mitochondrial function and compromise the mitochondrial integrity. Subjects carrying these inherited mutations would exhibit dysfunctional mitochondria, resulting in low viable mtDNA despite elevated Vit D levels. Additionally, keloids exist in a chronic inflammatory state characterised by the sustained release of pro-inflammatory cytokines such as IL-6 and TNF-α, which contribute to elevated oxidative stress levels. Excessive oxidative stress may overwhelm the protective effects of Vit D, resulting in mitochondrial damage and subsequent decrease in viable mtDNA. Furthermore, inflammation disrupts mitochondrial dynamics, including fusion and fission, thereby impairing the capacity of organelles to generate adequate ATP. Insufficient ATP production can compromise the integrity of viable mtDNA and negatively affect overall mitochondrial health. Viable mtDNA levels in keloid patients are regulated by a complex interplay of multiple factors that are interconnected and extend beyond Vit D levels alone. Consequently, it is essential to adopt a multifactorial research approach to enhance the understanding of keloid pathophysiology.

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Recent studies have evaluated viable mtDNA in relation to Vit D levels, but none have correlated it with dark-skinned individuals with keloid disease. Recent studies have elucidated the significant relationship between Vit D and VDR in the mitochondria. According to Genzen-Ak, D (2023), their "results showed for the first time that VDR was able to bind and regulate mtDNA transcription and interact with mitochondrial transcription factor A, even in the human brain."(106) This finding demonstrates the critical nature of the interaction between Vit D and VDR in the maintenance of mitochondrial health. Furthermore, a study conducted by Salles, J (2022) reported that long-term Vit D deficiency tended to decrease mtDNA(134), which directly correlates with the present study, as Vit D deficiency reduces viable mtDNA levels. Considering the results of this study and previous research, it can be postulated that Vit D plays a minimal role in protecting mitochondrial organelles in dark-skinned patients with keloid disease.

Given the moderate positive correlation between viable mtDNA and Vit D levels observed in this study, along with findings from previous studies indicating that VDR plays a critical role in the maintenance of mitochondrial health, it is recommended that dark-skinned individuals classified as Vit D insufficient (12 – 20 ng/mL) or Vit D deficient (< 20 ng/mL) strive to maintain sufficient levels of Vit D (> 20 ng/mL). Achieving this threshold might enhance the protective effects of Vit D on mitochondrial integrity and contribute to the preservation of viable mtDNA.

4.3 Mitochondrial malondialdehyde levels and vitamin D in keloid disease

Keloid disease is associated with mitochondrial dysfunction and elevated oxidative stress levels. In this study, lipid peroxidation was assessed by measuring MDA levels within the blood plasma, which is a by-product of lipid peroxidation and serves as a biomarker for oxidative stress and mitochondrial damage. It is important to take note that in this study the high levels of ROS observed in the findings refers to ROS levels found within the blood plasma in circulation and therefore is an indirect measure of what might be the contributing factor within keloids.

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4.3.1 Malondialdehyde levels in relation with vitamin D levels

This study revealed no significant ($p > 0.05$) differences between the Vit D groups, as there were very limited data points for the Vit D-deficient group; therefore, continuous data analysis was used to address this limitation. Additionally, a weak negative correlation ($r = -0.09$) between Vit D and MDA levels is consistent with the narrative that other factors (e.g., chronic inflammation, genetic predisposition) might overshadow Vit D's protective effects in this population. Chronic inflammation in keloids likely elevates oxidative stress to levels that surpass the protective capacity of Vit D. This is supported by the weak correlation between Vit D and MDA, despite Vit D's known antioxidant effects. Consequently, these findings do not support the conclusive effect of Vit D on reducing oxidative stress, as measured by MDA levels, in dark-skinned patients with keloid disease. The lack of a meaningful correlation may be attributed to limitations such as the small sample size ($n = 44$), which likely restricted the statistical power. Interestingly, patients with elevated Vit D levels in this study also exhibited increased oxidative stress markers, a finding that contrasts with the established literature suggesting that optimal Vit D levels are typically associated with enhanced antioxidant capacity and reduced oxidative stress. Conversely, the observation that low Vit D levels corresponded to heightened oxidative stress is consistent with previous findings. This unexpected association between high Vit D [25(OH)D] levels and oxidative stress may suggest complex regulatory mechanisms of oxidative balance mediated by Vit D [25(OH)D], potentially implicating alternative, or compensatory pathways in the different antioxidant systems. The antioxidant systems could be affected in three different ways, namely the endogenous, exogenous, and mitochondrial specific antioxidants. With endogenous antioxidants the metabolites GSH and coenzyme Q are affected, additionally in exogenous antioxidants the metabolites vitamin C and E and GSH peroxidase are affected, furthermore in mitochondrial specific antioxidants the metabolites affected include NADH, SOD, and CAT.

Prior research has demonstrated that Vit D supplementation significantly elevates the total antioxidant capacity and GSH levels ($p < 0.001$) and markedly reduces MDA concentrations ($p < 0.001$).(135) Antioxidants primarily neutralise ROS, inhibit oxidative chain reactions, and thereby protect cellular lipids from peroxidative damage

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to cell membranes. Limiting lipid degradation is essential for mitigating oxidative damage and subsequently reducing MDA formation, an indicator of lipid peroxidation. Glutathione similarly neutralises ROS, with an additional role in regenerating other antioxidants, such as vitamins E and C, thereby amplifying cellular antioxidant capacity. Together, antioxidants and GSH play pivotal roles in reducing MDA levels by counteracting ROS and minimising lipid peroxidation.

In contrast, keloid pathology is characterised by elevated oxidative stress levels, which may indicate a deficiency in endogenous antioxidants and GSH, resulting in higher MDA levels.

Complementary studies have examined the impact of Vit D supplementation in combination with calcium in diabetic rat models, revealing significant increases in antioxidant enzyme activities, including SOD2, GPx, and CAT, compared with untreated groups.(136) Notably, Vit D and calcium treatments also resulted in a significant reduction in MDA levels.(136) As established, these enzymes play crucial roles in reducing oxidative stress by directly neutralising ROS, underscoring the potential of Vit D as a modulator of oxidative defence mechanisms.

4.4 Mitochondrial function and oxidative stress in keloids

4.4.1 Viable mitochondrial DNA in relation to malondialdehyde levels

The relationship between viable mtDNA and MDA provides critical insight into mitochondrial function and its role in oxidative damage. Mitochondria play a central role in energy production and are vulnerable to oxidative stress, which leads to the release of viable mtDNA. Investigating the correlation between viable mtDNA and MDA levels could help clarify the extent to which mitochondrial dysfunction contributes to oxidative stress. In this study, it is essential to recognise that the elevated ROS levels observed pertain to those present in circulating blood plasma, serving as an indirect indicator of potential factors contributing to keloids. This study revealed a statistically significant moderate negative correlation ($r = -0.52$, $p < 0.05$) between mtDNA and MDA levels and aligns with known mechanisms where oxidative stress impairs

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mitochondrial viability. This finding supports the hypothesis that mitochondrial dysfunction is central to keloid pathophysiology.

Reduced mtDNA viability, in conjunction with elevated MDA levels, suggests that oxidative stress likely compromises mitochondrial integrity, leading to abnormal cellular activity. This disrupted cellular homeostasis could potentially affect fibroblast function and proliferation, promoting excess collagen deposition and altering the extracellular matrix, which is a characteristic of keloid formation.

Furthermore, the moderate correlation between Vit D and viable mtDNA highlights Vit D's protective role, but the stronger inverse relationship between viable mtDNA and MDA suggests oxidative stress plays a more dominant role. The data indicate a multifactorial interplay involving genetic predisposition, chronic inflammation, and oxidative stress that extends beyond Vit D levels alone.

4.5 Evaluating mitochondrial morphology and vitamin D levels by using TEM

4.5.1 Mitochondria morphology in relation to deficient vitamin D levels

Evaluating TEM images revealed significant mitochondrial damage (e.g., reduced size, membrane disruption) in dark-skinned patients with Vit D deficiency and keloid disease compared to Vit D sufficient keloid patients. The reduced size and damaged membrane integrity could be due to the high oxidative stress and low Vit D levels experienced in this population group, suggesting a compromised ATP production ability. Additionally, with constant chronic inflammation within the keloid cell, it maintains the level of oxidative stress at a high level, and the antioxidant levels are too low to decrease the ROS levels, causing mitochondria to become damaged and deformed in structure, and inhibiting the cristae from folding properly within the mitochondria. The high levels of ROS within this population group could be the reason for the decreased number of mitochondria observed within keloid cells.

This study also highlighted a substantial reduction in mitochondrial abundance within affected cells, reinforcing the hypothesis of mitochondrial damage. Given these structural abnormalities, it can be inferred that the mitochondria in keloid cells may be functionally impaired, likely resulting in lower ATP yields due to damage caused by

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elevated oxidative stress. Previous studies have established that Vit D plays a crucial role in mitochondrial protection, and that a deficiency in Vit D is associated with increased oxidative stress, which can compromise mitochondrial integrity.(137) Findings from this study contribute to the growing body of evidence linking Vit D deficiency to mitochondrial dysfunction, suggesting that it may exacerbate oxidative damage in keloid-prone tissues, thereby playing a role in the progression of keloid pathology in dark-skinned individuals.

In addition, TEM findings and the relationship between mtDNA and MDA suggest that Vit D deficiency contributes to mitochondrial damage but is not the sole driver. This complexity underscores the need for a multifactorial approach to understanding and managing keloid pathology.

4.5.2 Mitochondria morphology in relation to insufficient vitamin D levels

Examination of the TEM images revealed that mitochondria in cells with insufficient Vit D levels displayed a distinct double membrane and more round or oval morphology, indicative of structurally intact and functionally healthy mitochondria, mainly due to more balanced oxidative stress and antioxidant levels. A notable difference in mitochondrial size was observed, with mitochondria in Vit D-deficient cells measuring between 150 – 600 nm in length, whereas those in Vit D insufficient cells ranged from 500 nm to 1.5 µm, suggesting improved mitochondrial stability and growth with adequate Vit D levels. Furthermore, internal mitochondrial organisation was enhanced, with well-defined cristae folding observed in many mitochondria, a feature associated with improved mitochondrial function and reduced structural damage. As the mitochondrial organelles appear to be more normal in structure, this could suggest that ATP production should return to normal levels and oxidative stress levels should be reduced. Mitochondrial abundance within cells was also higher in Vit D-insufficient samples, in contrast to the reduced mitochondrial count observed in Vit D-deficient patients. These findings align with those of Quigley et al.(2022), who proposed that Vit D facilitates mitochondrial homeostasis by modulating the redox balance and regulating key metabolic pathways.(138) Thus, the current study provides additional support for the role of insufficient Vit D levels in preserving the mitochondrial integrity and functionality. These data suggest that Vit D and VDR may be important factors for cellular resilience against oxidative stress and potential therapeutic targets for keloid

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disease, highlighting the importance of Vit D in maintaining mitochondrial health in this context.

4.5.3 Mitochondria morphology in relation to sufficient vitamin D levels

The findings of this study provide partial support for previous research, indicating that Vit D levels above the normal range (> 30 ng/mL) may help preserve mitochondrial integrity. Observations included mitochondria with intact double membranes, typical round or oval morphology, average lengths of 500 nm to 1.5 µm, and properly folded cristae, all characteristics consistent with healthy functional mitochondria. These observations suggest that elevated Vit D levels can mitigate some aspects of mitochondrial damage, potentially enhancing structural stability and supporting normal mitochondrial function.

However, several findings diverge from the established literature, revealing structural abnormalities even in the presence of sufficient Vit D levels. Some mitochondria exhibited an absent or incomplete double membrane, atypical morphologies, such as mitochondria with two lobes, reduced sizes averaging between 180 – 500 nm, and incomplete cristae folding. These structural anomalies suggest that elevated Vit D levels alone may not be sufficient to prevent mitochondrial damage in keloids. The persistence of mitochondrial abnormalities despite sufficient Vit D suggests that additional factors, including genetic predisposition, diet, heightened oxidative stress, and inflammation may have a more substantial impact on mitochondrial health.

4.6 Evaluating rough endoplasmic reticulum and vitamin D levels by using TEM

4.6.1 Rough endoplasmic reticulum in relation to deficient vitamin D levels

The TEM images in this study revealed that RER in Vit D-deficient keloid cells appeared diminished in both quantity and organisation, with cisternae that do not form the typical parallel arrays observed in Vit D-insufficient and sufficient groups. Additionally, the intracisternal spacing was slightly larger, measuring approximately 20 – 30 nm, while the ribosomes on the RER membrane appeared fewer and less electron-dense than those in typical RER structures. These observations suggest that Vit D deficiency and high levels of oxidative stress may interfere with the integrity and function of RER. This disruption in RER organisation raises the possibility that low Vit

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D levels with high ROS levels could impair protein synthesis and processing within the RER, which may have downstream effects on cellular health and resilience. Disruptions in protein synthesis may also impair fibroblast function, which could produce more collagen than normal levels. The connection between mitochondria and RER, as reported in previous studies, suggests that RER disruption could affect mitochondrial function and cellular metabolic balance, given that these organelles closely collaborate to regulate cell survival and apoptosis pathways.(139)

4.6.2 Rough endoplasmic reticulum in relation to insufficient vitamin D levels

The TEM images illustrated in this study revealed that RER structures in cells from participants with insufficient Vit D levels and lower ROS levels than in the deficient group resembled those of typical characteristics of healthy RER, suggesting preserved functionality. These characteristics include an abundant presence of RER, organised cisternae in parallel arrays, and densely populated, electron-dense ribosomes. Additionally, the intracisternal spacing was smaller than that of the deficient group and was maintained within the normal range (20 – 30 nm), further indicating structural integrity.

These findings suggest that higher Vit D and lower oxidative stress levels may support optimal RER function, which could have downstream effects on cellular health by supporting efficient protein synthesis and cristae folding. Fibroblast functionality could be minimally impacted by protein synthesis. This could also allow for the formation of a reduced amount of collagen. The observation of healthier and more structurally intact mitochondria in this group aligns with the literature describing the close functional relationship between RER and mitochondria. Given that these organelles collaborate in calcium signalling and the cellular stress response, the findings imply that Vit D may contribute indirectly to mitochondrial stability by preserving RER integrity.

These insights underscore the potential role of RER in supporting mitochondrial health, particularly in the context of keloid pathology.

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4.6.3 Rough endoplasmic reticulum in relation to sufficient vitamin D levels

The analysis of TEM images from this population cohort revealed that the interaction between Vit D and oxidative stress levels with RER is more intricate than initially hypothesised. The data exhibited considerable variability, which prevented the establishment of a clear relationship. Specifically, some cells exhibited a markedly reduced abundance of RER, whereas others displayed profusion of this organelle. Notably, morphological assessments revealed a lack of organised parallel cisternae in some cells, in contrast to others that demonstrated a degree of parallelism. Additionally, ribosomal density and electron density varied significantly among cells, with certain specimens showing a greater abundance of darker ribosomes, indicative of active protein synthesis, whereas others appeared less robust. Furthermore, the intracisternal space between RER structures was found to be significantly enlarged in many cells compared to the inefficient Vit D group, which is atypical and suggests a potential dysfunction. Despite adequate Vit D levels, RER structural abnormalities persisted, suggesting that Vit D sufficiency alone does not fully restore RER integrity or fibroblast functionality. These findings underscore the complex and heterogeneous relationship between RER morphology, Vit D, and oxidative stress levels, mirroring similar observations in mitochondrial morphology among cells with elevated Vit D levels. This variability suggests that, while Vit D may influence RER integrity and function, other factors likely contribute to the observed heterogeneity.

4.7 Evaluating collagen and vitamin D levels by using TEM

4.7.1 Collagen in relation to deficient vitamin D levels

Previous studies have demonstrated that Vit D reduces collagen expression, thereby decreasing the amount of collagen observed in cells.(97) The findings of the present study corroborate this assertion, as an excess of collagen fibrils was observed in patients with Vit D deficiency who developed keloids. Disturbances indicating pathological alterations, such as longitudinal collagen fibrils, exhibit a lack of organisation, with many failing to align in parallel or form straight lines, as typically observed in healthy collagen. The measurement of the D-band periodicity indicated an average value of approximately 40 nm, significantly below the normal range of 64.7 to 65.3 nm (140), suggesting a potential impairment in collagen assembly and structural integrity. Additionally, the transverse collagen fibrils presented indistinct

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membranes, exhibiting considerable spacing between fibrils, which may compromise the tensile strength and overall function of the collagen matrix. Although the diameter of the transverse Type I collagen fibrils was 80 nm, which falls within the normative range of 50 to 100 nm, the observed irregularities in fibril organisation and membrane clarity are concerning. These structural alterations suggest collagen abnormalities characterised by excessive deposition and disordered fibril architecture, which contribute to the formation of dense fibrotic scar tissue observed in keloid pathology. These findings imply that Vit D plays a regulatory role in collagen homeostasis; however, the deficient Vit D levels documented in this population group likely result in dysregulation of collagen synthesis and organisation. This dysregulation may contribute to the persistence and exacerbation of keloid diseases.

4.7.2 Collagen in relation to insufficient vitamin D levels

Analysis of collagen morphology revealed a significant presence of collagen within the cells, demonstrating an emerging trend towards greater organisation, as evidenced by the increased formation of parallel fibrillar structures. The measurement of the D-band periodicity yielded a value of 55 nm, which represents an improvement from the values observed in the Vit D-deficient group; however, it remained slightly below the normative range of 64.7 to 65.3 nm.(140) This enhancement suggests a partial restoration of the collagen structure, yet underscores the ongoing dysregulation associated with suboptimal Vit D levels. Despite this increase in organisation, the transverse collagen fibrils exhibited similar characteristics to those in the Vit D-deficient group, including poorly defined membranes and a lack of contact between fibrils. The measured diameter of the transverse collagen fibrils was approximately 60 nm, which was smaller than that in the Vit D-deficient cohort and remained within the established normal range of 50 to 100 nm. While some collagen features are typical, the observed structural abnormalities suggest excessive deposition, which is a hallmark of keloid pathology. These observations indicated that the relationship between Vit D and collagen synthesis is complex and multifaceted. The partial improvements in collagen organisation and periodicity may suggest that Vit D plays a modulatory role in collagen homeostasis, yet persistent abnormalities indicate that there are mechanisms that play a crucial role in the regulation of collagen synthesis.

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4.7.3 Collagen in relation to sufficient vitamin D levels

The TEM images indicated a notable abundance of collagen fibrils within the cells, despite the presence of sufficient Vit D levels. Most of the longitudinal collagen fibrils exhibited a parallel alignment, yet some fibrils displayed disorganised structures, indicating potential dysregulation of the collagen architecture. Measurement of the D-band periodicity revealed a value of approximately 72 nm, which exceeds the established normal range of 64.7 to 65.3 nm.(140) This suggests a deviation from the typical collagen fibril organisation and may reflect alterations in collagen synthesis or post-translational modifications. In contrast, the transverse collagen fibril membranes demonstrated contact with one another, exhibiting minimal interfibrillar spacing, which suggests a degree of structural integrity and cohesiveness not seen in previous Vit D-deficient or insufficient groups. The outer membranes of these fibrils appear well-defined and distinct, indicating a level of normalcy in their structural morphology. Furthermore, the diameter of the transverse collagen fibrils was measured at 71 nm, positioned between the diameters observed in the Vit D-deficient (80 nm) and Vit D-insufficient (60 nm) groups, but remained within the normative range of 50 to 100 nm. These findings underscore the intricate and multifactorial relationship between Vit D and collagen regulation in keloid disease. Although adequate Vit D levels appear to contribute positively to collagen organisation and integrity, they do not fully account for the observed abnormalities. This suggests that Vit D is not the sole regulator of collagen synthesis and organisation. Other factors, particularly oxidative stress, may exert a more pronounced influence on collagen homeostasis.

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Chapter 5: Conclusion

The objective of this study was to evaluate mitochondrial function, oxidative stress, and Vit D status in dark-skinned individuals with keloid disease. The correlation between mitochondrial integrity, as indicated by membrane damage and morphological alterations, and oxidative stress, as quantified by MDA levels, was examined. Additionally, the potential influence of Vit D status on these parameters was investigated.

5.1 Summary of key findings

The findings of this study revealed several key insights into the pathology of keloid disease in the context of mitochondrial dysfunction and varying levels of vitamin D concentrations. Firstly, the study identified a very weak negative correlation ($r = -0.09$) between Vit D and MDA levels, suggesting that no significant relationship exists between Vit D and the reduction of oxidative stress in dark-skinned patients with keloids. This finding highlights the multifactorial nature of keloid pathology, where oxidative stress is likely driven by mechanisms other than low concentrations of Vit D, such as mitochondrial dysfunction or chronic inflammation. This underscores the need to explore other contributors to oxidative stress and suggests that addressing a low concentration level of Vit D alone may be insufficient to mitigate keloid formation. Secondly, a moderate negative correlation ($r = -0.52$) was observed between viable mtDNA and MDA levels, indicating that higher oxidative stress is associated with decreased mtDNA viability. These findings may contribute to keloid pathology by impairing energy production, increasing ROS levels, and promoting chronic inflammation. While Vit D exhibits a protective role by maintaining mitochondrial viability and mitigating oxidative stress, its effects appear to be overshadowed by the chronic inflammatory state characteristic of keloids, which could drive fibroblast hyperactivity, excessive collagen deposition, and abnormal tissue remodelling, which are the hallmark features of keloids. The association between viable mtDNA and MDA levels highlights mitochondrial dysfunction as a key driver of oxidative stress and fibroproliferative activity during keloid formation. This finding emphasises the role of dysfunctional mitochondria and oxidative stress in keloid pathophysiology. Thirdly,

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TEM images revealed mitochondrial damage in Vit D-deficient keloid patients, including disrupted membranes and abnormal morphology, suggesting that Vit D deficiency exacerbates mitochondrial dysfunction. Damaged mitochondria can increase ROS production and impair cellular energy metabolism, driving chronic inflammation, fibroblast hyperactivity, and excessive collagen deposition, which are key processes in keloid pathology. Conversely, healthier mitochondrial structures in Vit D-sufficient patients highlight the potential role of Vit D in preserving mitochondrial integrity and mitigating some aspects of keloid development. Fourthly, the analysis of collagen fibrils showed that Vit D levels partially influence collagen organisation, with improved alignment and diameter in the Vit D-sufficient groups. However, persistent deviations in D-band periodicity and disorganised collagen highlight the complex regulation of the ECM in keloid pathology. This suggests that while Vit D may help modulate collagen structure, other factors such as oxidative stress likely play a significant role in driving the excessive and disorganised collagen deposition characteristic of keloid scars. Finally, the complex relationship between Vit D and RER function, with variability in RER abundance and organisation, suggests that disruptions in RER activity may influence protein synthesis and secretion, including excessive collagen production in keloid pathology. The potential link between RER function and mitochondrial integrity highlights a feedback loop in which mitochondrial dysfunction and RER stress amplify each other, driving fibroblast hyperactivity and abnormal ECM deposition. This underscores the need to further investigate the role of Vit D in modulating the RER activity and its contribution to keloid formation.

Ultimately, these results underscore the multifactorial nature of keloid disease, highlighting the importance of both Vit D and additional biological factors, such as oxidative stress, in regulating mitochondrial function and collagen homeostasis. These insights may inform future therapeutic strategies for the management of keloid diseases.

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5.2 Study limitations

While this study produced valuable findings, there are certain considerations that could enhance future research. Firstly, due to the limited number of data points in the Vit D-deficient group, categorical pairwise analysis with this group was not possible. However, this prompted the incorporation of a continuous variable analysis across the range of Vit D concentrations, providing a broader view of the relationship between Vit D levels and correlated variables. Therefore, this approach leveraged the full data set to detect trends in correlation patterns while ensuring statistically valid comparisons between variables. Secondly, although TEM analysis offered important insights into mitochondrial morphology, incorporating functional assays in future work could further elucidate the bioenergetic capacity of the mitochondria, complementing morphological observations. Finally, this study specifically examined dark-skinned patients with keloid. Expanding future research to include patients without keloids and individuals from different skin groups will provide a more comprehensive understanding of Vit D thresholds in various populations and help clarify the distinct roles of mitochondrial dysfunction and Vit D deficiency in keloid pathogenesis.

5.3 Future research directions

Conducting longitudinal studies to monitor changes in Vit D levels, oxidative stress markers, and mitochondrial function over time could provide deeper insight into the interrelationships and temporal dynamics of keloid disease progression. Additionally, expanding the sample size and including diverse populations may enhance the statistical power of our findings and allow for more robust conclusions regarding the effects of Vit D on oxidative stress and mitochondrial integrity. Furthermore, investigating the specific biochemical pathways through which Vit D influences mitochondrial function and collagen synthesis could help elucidate the mechanisms underlying keloid disease and identify potential therapeutic targets. Future research should aim to design interventional trials to assess the effects of Vit D supplementation on oxidative stress, mitochondrial function, and collagen characteristics in keloid patients, and provide evidence for clinical applications and treatment strategies. Finally, future studies should also consider the importance of re-evaluating Vit D thresholds and environmental influences (e.g., sunlight exposure) specific to dark-

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skinned South African populations. Furthermore, roles of other factors, such as genetic predisposition, inflammatory mediators, and lifestyle variables that may influence mitochondrial health and collagen regulation in keloid disease. These findings highlight the need for a multifactorial research approach and tailored clinical strategies to address the unique aspects of keloid disease in this demographic.

5.4 Conclusion

In conclusion, this study provides pivotal insights into the complex interplay among mitochondrial dysfunction, oxidative stress, and Vit D deficiency in dark-skinned patients with keloid disease. These findings underscore the critical role of mitochondrial health in keloid pathogenesis and suggest that mitochondrial dysfunction may be a central driver of keloid formation. Although Vit D appears to exert a minor protective effect, its influence is nuanced and warrants further exploration. These results not only expand the understanding of the underlying mechanisms of keloid disease, but also open new avenues for innovative therapeutic strategies targeting mitochondrial function. Addressing these key factors could lead to more effective management and treatment approaches for keloid, contributing to advancements in clinical practice and patient outcomes.

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Appendix A – Ethics approval letter

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences

Institution: The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.

- FWA 00002567, Approved dd 18 March 2022 and Expires 18 March 2027.
- IORG #: IORG0001762 OMB No. 0990-0278 Approved for use through August 31, 2023.

Faculty of Health Sciences **Research Ethics Committee**

27 October 2022

Approval Certificate
New Application

Dear Mr G Hattingh

Ethics Reference No.: 532/2022
Title: Assessing mitochondrial function, oxidative stress, and vitamin D status in dark-skinned patients with keloid disease

The New Application as supported by documents received between 2022-09-27 and 2022-10-25 for your research, was approved by the Faculty of Health Sciences Research Ethics Committee on 2022-10-25 as resolved by its quorate meeting.

Please note the following about your ethics approval:

Ethics Approval is valid for 1 year and needs to be renewed annually by 2023-10-27.

Please remember to use your protocol number (532/2022) on any documents or correspondence with the Research Ethics Committee regarding your research.

Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.

Ethics approval is subject to the following:

The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.

Yours sincerely

On behalf of the FHS REC, Dr R Sommers
MBChB, MMed (Int), MPharmMed, PhD
Deputy Chairperson of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria

The Faculty of Health Sciences Research Ethics Committee complies with the SA National Act 61 of 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 and 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes, Second Edition 2015 (Department of Health)

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Fakulteit Gesondheidswetenskappe
Lefapha la Disaense tša Maphelo

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Appendix B – First ethics renewal letter

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences

Institution: The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.
- FWA 00002567, Approved dd 18 March 2022 and Expires 18 March 2027.
- IORG #: IORG0001762 OMB No. 0990-0279 Approved for use through June 30, 2025 and Expires 07/28/2026.

Faculty of Health Sciences **Research Ethics Committee**

9 November 2023

Approval Certificate
Annual Renewal

Dear Mr G Hattingh,

Ethics Reference No.: 532/2022 – Line 2
Title: Assessing mitochondrial function, oxidative stress, and vitamin D status in dark-skinned patients with keloid disease

The **Annual Renewal** as supported by documents received between 2023-10-12 and 2023-11-08 for your research, was approved by the Faculty of Health Sciences Research Ethics Committee on 2023-11-08 as resolved by its quorate meeting.

Please note the following about your ethics approval:

- Renewal of ethics approval is valid for 1 year, subsequent annual renewal will become due on 2024-11-09.
- The Research Ethics Committee (REC) must monitor your research continuously. To this end, you must submit as may be applicable for your kind of research:
  a) annual reports;
  b) reports requested *ad hoc* by the REC;
  c) all visitation and audit reports by a regulatory body (e.g. the HPCSA, FDA, SAHPRA) within 10 days of receiving one;
  d) all routine monitoring reports compiled by the Clinical Research Associate or Site Manager within 10 days of receiving one.
- The REC may select your research study for an audit or a site visitation by the REC.
- The REC may require that you make amendments and take corrective actions.
- The REC may suspend or withdraw approval.
- Please remember to use your protocol number (532/2022) on any documents or correspondence with the Research Ethics Committee regarding your research.

Ethics approval is subject to the following:

- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.

Yours sincerely

On behalf of the FHS REC, Dr R Sommers
MBChB, MMed (Int), MPharmMed, PhD
Deputy Chairperson *of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria*

The Faculty of Health Sciences Research Ethics Committee complies with the SA National Act 61 of 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 and 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes, Second Edition 2015 (Department of Health).

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Appendix C – Second ethics renewal letter

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences

Institution: The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.
- FWA 00002567, Approved dd 18 March 2022 and Expires 18 March 2027.
- IORG #: IORG0001762 OMB No. 0990-0279 Approved for use through June 30, 2025 and Expires 07/28/2026.

Faculty of Health Sciences **Research Ethics Committee**

16 October 2024

Approval Certificate
Annual Renewal

Dear Mr G Hattingh,

Ethics Reference No.: 532/2022 – Line 3
Title: Assessing mitochondrial function, oxidative stress, and vitamin D status in dark-skinned patients with keloid disease

The **Annual Renewal** as supported by documents received between 2024-10-03 and 2024-10-16 for your research, was approved by the Faculty of Health Sciences Research Ethics Committee on 2024-10-16 as resolved by its quorate meeting.

Please note the following about your ethics approval:

- Renewal of ethics approval is valid for 1 year, subsequent annual renewal will become due on 2025-10-16.
- The Research Ethics Committee (REC) must monitor your research continuously. To this end, you must submit as may be applicable for your kind of research:
  a) annual reports;
  b) reports requested *ad hoc* by the REC;
  c) all visitation and audit reports by a regulatory body (e.g. the HPCSA, FDA, SAHPRA) within 10 days of receiving one;
  d) all routine monitoring reports compiled by the Clinical Research Associate or Site Manager within 10 days of receiving one.
- The REC may select your research study for an audit or a site visitation by the REC.
- The REC may require that you make amendments and take corrective actions.
- The REC may suspend or withdraw approval.
- Please remember to use your protocol number (532/2022) on any documents or correspondence with the Research Ethics Committee regarding your research.

Ethics approval is subject to the following:

- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.

Yours sincerely

On behalf of the FHS REC, Dr R Sommers
MBChB, MMed (Int), MPharmMed, PhD
Deputy Chairperson *of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria*

The Faculty of Health Sciences Research Ethics Committee complies with the SA National Act 61 of 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 and 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes, Second Edition 2015 (Department of Health).

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