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THE DEVELOPMENT OF A LIPOSOMAL FORM SECUKINUMAB AN IL-17 PATHWAY INHIBITOR IN THE TREATMENT OF PSORIASIS

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PHD

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THE DEVELOPMENT OF A LIPOSOMAL FORM SECUKINUMAB AN IL-17 PATHWAY INHIBITOR IN THE TREATMENT OF PSORIASIS

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Abstract

Name: Gazala Ibrahim Abdulkarim Layas

Title: The development of a liposomal form Secukinumab – an IL 17 pathway inhibitor in the treatment of psoriasis.

Keywords: Secukinumab, liposomes, geno-protective, lymphocytes, psoriasis, comet, MN assay, oxidative stress, genotoxicity

Various approaches are currently used to treat and manage psoriasis, and biological treatments are often the latest approaches. All biological treatments have major side effects as they are given systemically via injections. One of the latest biological treatments for psoriasis, one which has shown great efficacy with fewer side effects, is Secukinumab. Secukinumab is an anti-IL17 antibody that works by stopping the action of IL17, a cytokine that is known to have a major role in the pathogenesis of psoriasis. This work is based on the development of a new way to commence drug therapy to reduce the side effects of the treatment.

Our work is based on the studies of the genotoxicity of the drug Secukinumab in its bulk and liposome form using comet and micronucleus assays on lymphocytes. The results from both assays have illustrated the safety of the drug and demonstrated the reduction of the DNA damage induced in both healthy individuals and patients with psoriasis. Secukinumab significantly decreases-H_2O_2 induced damage and efficiently attenuates its adverse effects both in the comet ($p<0.0001$) and micronucleus assays ($p<0.01$). The two concentrations of Secukinumab used (2.1 and 2.8μg/ml) efficiently decreased H_2O_2-induced DNA damage in both groups to nearly the level of the negative control. Overall, Secukinumab reveals protective and anti-genotoxic effects by demonstrating its

potential in reducing DNA damage caused by oxidative stress and by not inducing any further damage in the lymphocytes of either healthy individuals or patients. Liposomes are highly versatile which have been proven efficient for therapy and research applications. The discovery of new therapies in the treatment of psoriasis is a considerable challenge and is now a necessity. Our study was the first one to determine the genotoxicity of various concentrations of the drug in the lymphocytes of psoriasis patients compared to healthy individuals. In the MTT assay, the data showed a decrease in % cell survival rates after exposure to different concentrations of Secukinumab. Also, the results demonstrated no statistically significant differences on confounding factors such as ethnicity, smoking, drinking habits, gender and age among psoriasis patient and healthy controls. The regulation of gene expression levels of IL-17, IL-22 and RORC were assessed after treatment with Secukinumab in the bulk and liposome form via RT-PCR analysis. Secukinumab bulk (2.1µg/ml) treatment significantly down-regulated gene expression of IL-17, IL22 and RORC to 0.46-fold, 0.47-fold and 0.5-fold, respectively. However, Secukinumab liposome (2.1µg/ml) only decreased the expression of IL-17 and IL-22 significantly, by 0.46-fold and 0.53-fold, respectively. On the other hand, studying the expression of P53 and P21 using qPCR revealed that Secukinumab bulk and liposome has no effect on the expression of these genes in lymphocytes from healthy individuals and psoriasis patients.

Western blotting was used to investigate the effect of Secukinumab in both forms on protein expression levels IL-17, IL-22 and RORC. Analysis of the results showed that Secukinumab bulk and liposome had no significant effect on expression levels of any of these proteins in lymphocytes derived from healthy individuals. However, there was a statistically significant down-regulation observed in the protein expression levels of IL-17, IL-22 and RORC in lymphocytes obtained from the psoriasis patients, confirming the sensitivity of the compromised lymphocytes from patient

group to Secukinumab treatment. With Secukinumab (bulk form) administration, a 0.5-fold decrease was observed in IL-17, 0.59-fold decrease in IL-22, and a 0.6-fold decrease in RORC expression. However, liposome form reduced their levels to 0.47–fold, 0.5-fold and 0.47–fold, respectively, when compared to the control group. While it had no significant effect on expression of P53 and P21 proteins in lymphocytes from healthy individuals and psoriasis patients and there was no difference observed in their regulation. In conclusion, the use of Secukinumab liposome as topical drug delivery system may be suitable replacement for improving the drug bioavailability and its side effects.

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Finally, a debt of gratitude to my family as, without their support, this work would have been impossible. My parents, Ibrahim and Fazia Layas, who have given me all the support I have ever needed throughout my life are forever in my thoughts. To my husband, Amjad Bader, a special thanks for his encouragement and confidence in me. Gratitude also to my brothers Abdulkarim, Saad, and Mohamed and sisters Kauther, Soad and Basma for always finding them beside me when I needed help.

I also give thanks to my four wonderful children Aya, Amane, Ahmed, and Esra for their inspiration every day.

Dedication

This work is devoted to my God for keeping me focused, and strong. I also dedicate this work to my father Ibrahim Layas and my mother Mrs Faiza Layas for believing in me and for raising me to be strong. Gratitude for all their love, prayers and emotional support. I could not have imagined coming this far without her enduring support. Most importantly, this thesis is dedicated to my loving husband Amjad Bader without whose love and unconditional support nothing would have been possible. I feel blessed to have my siblings always there for me.

Declaration

I solemnly declare that this work is my own work and, where the work of others is used, this has been correctly acknowledged. The content of my thesis has not been submitted for the award of a PhD elsewhere.

Abbreviations

ADAs:	Anti-drug antibodies
AMPs	Antimicrobial peptides
CBMN:	Cytokinesis-Blocked Micronucleus
CMHP:	Committee for Medicinal Products for Human Use
DCs	Dendritic Cells
DLS	Dynamic Light Scattering
DMSO:	Dimethyl sulphoxide
DNA:	Deoxyribonucleic acid
DSB:	Double-strand break of DNA
EB	Ethidium bromide
IGA	Investigator’s Global Assessment
Il17:	Interleukin 17
KC	Keratinocytes
LMP:	Low melting point agarose
LC	Langerhans cell
mAb:	Monoclonal antibody
MAPPs:	Major histocompatibility complex-associated peptide proteomics

MNi:	Micronucleus
NaCl:	Sodium chloride
NaOH:	Sodium hydroxide
NBUDs:	Nuclear buds
NC:	Negative control
NDI:	The nuclear division index
NMP:	Normal melting point agarose
NPs:	nanoparticles
NPB:	Nucleoplasmic bridges
OTM	Olive Tail Moment
PASI:	Psoriasis Area and Severity Index of the diseases
PBLs	peripheral blood lymphocytes
PBS	Phosphate buffered salin
PC	positive control
PHA:	Phytohaemagglutinin
ROR	related orphan receptors
ROS:	Reactive oxygen species
RT	room temperature

SEM	Scanning Electron Microscope
SSB:	Single strand break of DNA
STAT:	signal transduction and activators of transcription 3
TCR	T- cell receptor
TEM	Transmission Electron Microscope
Th:	T-helper
TNFα:	Tumour necrosis factor-alpha

Chapter 1.

Introduction

Psoriasis is a skin condition that causes red, flaky, crusty patches of skin to appear which are covered with silvery scales. These patches usually appear on the knees, elbows, scalp and lower back, but can occur in any part of the body. The vast majority of patients are only affected by small patches, although in others the condition can spread across the entire body. In some cases, these patches can be itchy or painful. Psoriasis affects around 2% of people in the UK and can start at any age, but onset is most frequently observed in adults under the age of 35 and the condition affects both men and women equally. The severity of psoriasis varies greatly from person to person. For some individuals, it is just a minor irritation, but for others, it can affect their quality of life significantly. Psoriasis is a chronic disease, one that usually involves periods in which the disease is stable, followed by periods where the symptoms are severe and affect the patient's quality of life. Psoriasis is an autoimmune disorder affecting the skin and may also manifest as a systemic inflammatory disorder affecting the joints leading to a severe impact on the patient's quality of life (Raychaudhuri, 2013)

1.1 Histopathology of psoriasis

To understand the pathogenesis of psoriasis, the understanding of skin pathology is essential. The skin is the largest organ of the body with an area of over 2 m^2 and a weight of around 5 kg. The thickness of the skin differs from less than a millimetre in the eyelids to 4 mm on the heels of the feet. The skin is the major barrier between the internal organs and the external environment, which is divided into three layers, namely the epidermis, an outer keratinised and stratified squamous epithelium. The term keratinised derives from keratin, a special durable fibrous type of protein. In this layer dead cells may be found which are reduced to flattened scales, or squames, and these are filled with densely packed keratin. The epidermis is vital for the protection of the skin. The basal layers of this epithelium are folded to form dermal papillae. Skin contains four to five layers, depending on the thickness. The first layer adjacent to the dermis is the basal cell layer (stratum basale), comprising a single layer of cells, in which the stem cells divide and migrate. Then comes the prickle cell layer (stratum spinosum) comprising around 8-10 layers of cells with many desmosomes (a type of bond that anchor the cells to one another and contain thick mesh of intermediate filaments). Above the stratum spinosum, there is a layer of 3-5 cell rows called the granule cell layer (stratum granulosum) that contains lipid-rich granules which serve as a sealant. As the cells migrate to the surface, they become keratinised as they lose their nuclei and granules. In thick skin, a fifth layer (stratum lucidum) may be present between the granular cell layer and the uppermost layer (Figure 1.1) (Gisondi *et al.*, 2020).

The dermis itself is a thick connective tissue layer that lies beneath the skin. It plays important roles in sensation, protection, and thermoregulation by virtue of the presence of nerves, blood vessels, fibroblasts, sweat glands and hair. The first layers of the dermis are folded to form dermal papillae, and these are particularly prominent in thick skin. Underneath the dermis is the hypodermis which contains both adipose tissue and sweat glands. The dermal adipose tissue has additional important metabolic functions in that it is responsible for the production of vitamin D and triglycerides (Rendon & Schäkel, 2019).

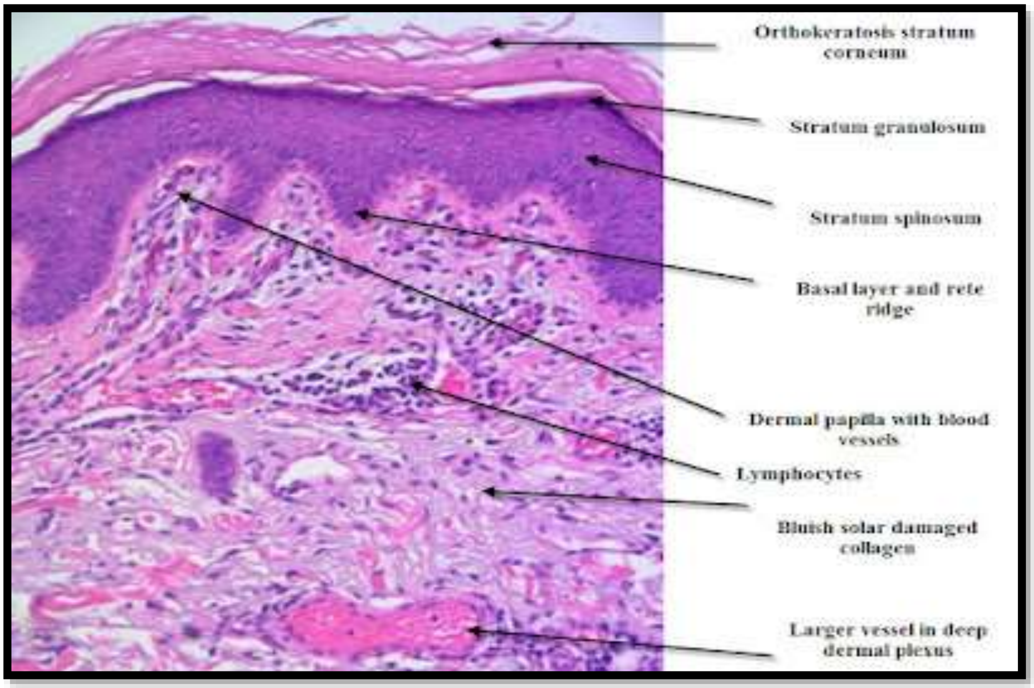

Figure 1-1. Skin histology showing different part of the skin. Illustrating the different layer of skin; Epidermis **-** most superficial, Dermis **-** deep layer, Hypodermis **-** deepest layer with loose connective and adipose tissue. http://dermpathmadesimple.blogspot.com/2010/09/introduction-to-skin-histopathology.html

The pathogenesis or manner in which psoriasis develops is not yet fully understood as there may be more than just one pathway involved in developing the disease. However, the immunopathogenesis of psoriasis seems to result from the complex interactions arising between genetic, immunological, and environmental factors. Initially considered a primary disease of the epidermis, it is now known that the cutaneous manifestations of psoriasis result from a dysregulation of the immune system with the involvement of T cells (Elain, Jeanneau, Rutkowska, Mir, & Dev, 2014). In the1960s, psoriasis was thought to be a primary disease of the epidermis caused only by the overproduction of keratinocytes. The immune system was first implicated in the 1990s, when lymphocyte-targeted therapies were demonstrated to be an effective way to treat the disease (Gottlieb *et al*., 1995). The pathogenesis of psoriasis was initially anticipated to depend on Th1 responses, based on the identification of an elevated expression of Th1 cytokines such as IFNγ, TNFα, and IL-12. As a consequence of research, a monoclonal antibody (Ustekinumab), designed to block the p40 subunit of IL-12, was synthesised (Brembilla, Senra, & Boehncke, 2018). One of the first theories argues that psoriasis patients present a phenomenon of T lymphocyte auto-immunoreactivity, which is triggered after contact with superantigens, some of which are bacterial proteins (e.g., streptococcal superantigens).

The autoreactive T lymphocytes may target different target organs or remain in circulation, depending on the presence or absence, respectively, of specific organ receptors (Janeway *et al.*, 2001). Among the activated T cells, those with receptors for the skin come into contact with several biomolecules, recognising some as autoantigens, thus initiating a cascade of cellular molecular events that result in the formation of psoriatic plaques (Felisbino, Santos-Filho, Piancini, Cestari, & Leme, 2018). However, it has been known that psoriasis is an extremely common disorder, one that is mediated by the human immune system (Griffiths & Barker, 2007). The hallmark of psoriasis is sustained inflammation that leads to uncontrolled keratinocyte proliferation and dysfunctional differentiation. The histology of the psoriatic plaque exhibits acanthosis (epidermal hyperplasia) (Figure 1.2) which overlies inflammatory infiltrates composed of dermal dendritic cells, macrophages, T cells and neutrophils, with neovascularisation a prominent feature (Rendon & Schäkel, 2019).

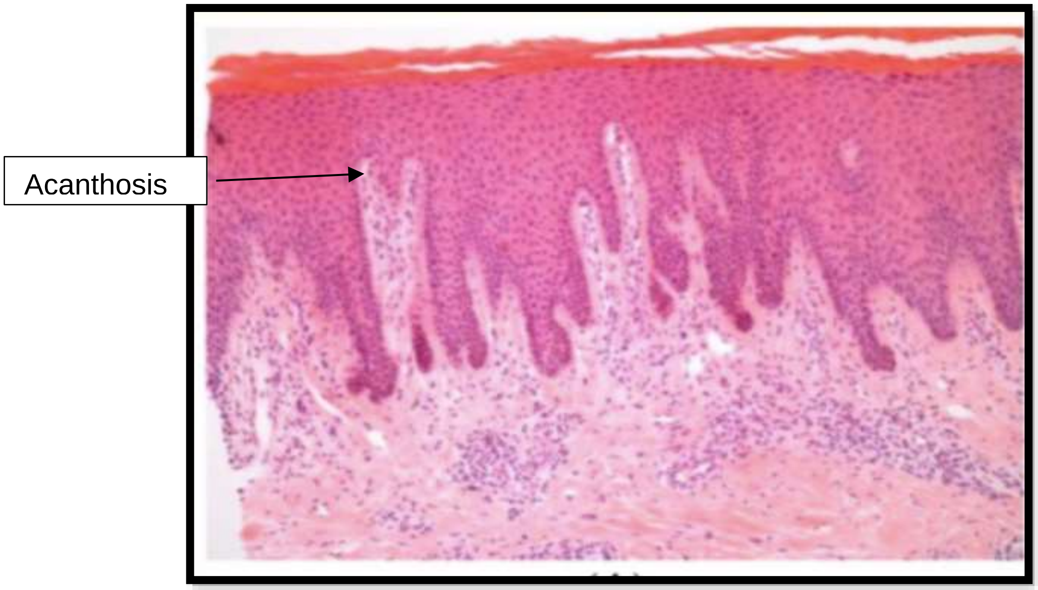

Figure 1-2. Histopathology of psoriasis (from Rendon & Schäkel, 2019)

Illustrating the increase thickening of epidermis (acanthosis)

The main clinical features of psoriasis are evident at the outermost layer of the skin, one which is made up of the proliferation of keratinocytes. However, the development of the psoriatic plaque is not itself restricted to inflammation within the epidermal layer, but rather is shaped by the interaction of keratinocytes with many different cell types (associated with the innate and adaptive

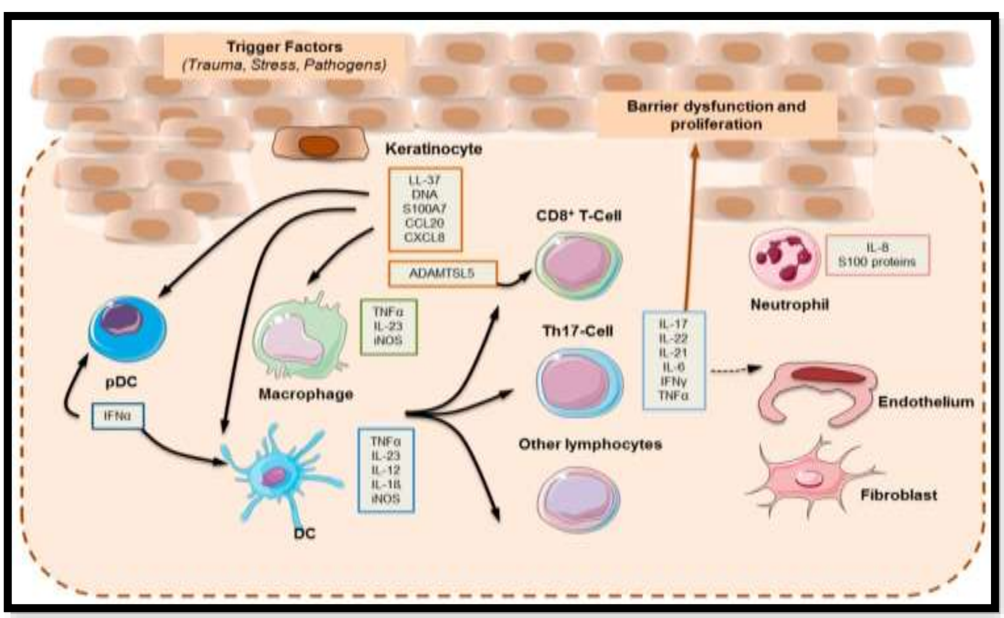

Figure 1-3. Pathogenesis of psoriasis. (Rendon & Schäkel, 2019): Illustrating the initiation phase and maintenance phase of skin pathogenesis of psoriasis

immune cells and vasculature) which span the dermal layer. The pathogenesis of psoriasis can be divided into an initiation phase, one that is possibly triggered by trauma (Koebner phenomenon), infection, or drugs, and a maintenance phase that is characterised by a chronic clinical progression (Figure 1.3) (Rendon & Schäkel, 2019).

1.2 Pathogenesis of psoriasis

Psoriatic inflammation is caused by the disturbances in the innate and adaptive cutaneous immune responses. Activation of the innate immune system driven by endogenous 'danger signals' and cytokines characteristically coexists with an autoinflammatory process in some patients or T cell-driven autoimmune reactions in others. Thus, psoriasis shows traits in common with an autoimmune disease on an (auto)inflammatory background, with both mechanisms overlapping and even potentiating one another (Liang, Sarkar, Tsoi, & Gudjonsson, 2017).[1]

The recognition of psoriasis as an immune-mediated disease has reclassified this pathology alongside other chronic inflammatory diseases such as rheumatoid arthritis and Crohn's disease, sharing their increased risk of associated systemic co-morbidities such as cardiovascular disease, metabolic syndrome and diabetes (Gisondi, Bellinato, Girolomoni, & Albanesi, 2020). This association results from the common genetic factors and shared identical inflammatory pathways between psoriasis and multiple co-morbidities. The primary cause of psoriasis is a dysregulation of immune responses, which manifests in those individuals carrying one or more psoriasis susceptibility genes, either those which are skin-specific or related to immune functions, and these are triggered after their exposure to certain environmental triggers (Gisondi *et al.*, 2020).

[1] Note: The innate immune system is composed of physical and chemical barriers, phagocytic leukocytes, dendritic cells, natural killer cells, and plasma proteins. The adaptive immune system is composed of B cells and T cells and is produced in response to exposure to a foreign substance.

Psoriasis immunopathogenesis is an inflammatory disease that is characterised by the expansion and activation of helper T cells (Th1, Th17, and Th22), and by the production of associated cytokines, among which interferon INF-γ, tumour necrosis factor-alpha (TNF-α), IL-17 and IL-22 are highlighted (Cai *et al.*, 2012). T lymphocytes constitute, in conjunction with B lymphocytes, the acquired immune system, and are responsible for specific immune responses and immune memory, respectively. T cells can have different characteristics that classify them into subclasses with different functions, such as CD4+ T-helper lymphocytes and CD8+ cytotoxic T lymphocytes. The first T-helper lymphocytes to be described included only two types, Th1 and Th2, and these are mediators of cellular and humoral immunity, respectively. Both are derived from the same common cell line, the T-helper naive lymphocytes, and are characterised by cytokine patterns that are specific to each of these subtypes. However, since the Th1/Th2 paradigm could not explain some observed phenomena, the investigation of the immune system led to the identification of the third lineage of T-helper lymphocytes, named Th17 due to their high production of IL-17 and their functional specificities (Harrington *et al.*, 2005). Interleukin is a human pro-inflammatory cytokine. This cytokine interleukin, also known as (IL)-17A, originates from the Rh17 CD4+ T-helper set of cells and other adaptive and innate immune cells (Kolbinger *et al.*, 2016). The induction of the Th17 phenotype from naive T-helper cells occurs by the action of multiple cytokines, including IL-23, TGF-β, IL-6, IL-21; various intracellular factors such as the transcription factors ROR and STAT3; Th1 and Th2 lymphocyte cell lines; and regulatory T cells themselves. Th17 lymphocytes play an important role in eliminating extracellular bacteria and fungi and in interacting with other immune cells and other cell types (Felisbino *et al.*, 2018). The production of IL-17A, IL-17F, and IL-22 by Th17 results in the recruitment and activation of neutrophils and macrophages and immune activation factors by keratinocytes /macrophage colony-stimulating factor (GM-CSF), IL-8 or TNF-α24 (Felisbino *et al.*, 2018).

Dendritic cells are dedicated antigen-presenting cells and play an important role in initiating the disease. However, the manner of their activation in psoriasis is not entirely clear. One of the proposed mechanisms involves their recognition of antimicrobial peptides (AMPs) which are secreted by keratinocytes in response to injury and are characteristically found to be overexpressed in psoriatic lesions. Studies have found that most psoriasis-associated AMPs are LL37, β-defensins, and S100 proteins (Morizane & Gallo, 2012). LL37, known as cathelicidin, is released by damaged keratinocytes and subsequently forms complexes with self-genetic material from other damaged cells. LL37, when bound to DNA, stimulates toll-like receptor (TLR) 9 in plasmacytoid dendritic cells (pDCs) (Morizane & Gallo, 2012). The activation of pDC is key in initiating the development of the psoriatic plaque and is characterised by the production of Type I IFN (IFN-α and IFN-β). Signals from Type I IFN stimulate the phenotypic maturation of myeloid dendritic cells (mDC), implicated in Th1 and Th17 differentiation and function, inducing IFN-γ and interleukin (IL)-17 production, respectively (Hansel *et al.*, 2011; Santini *et al.*, 2011) (figure 1.4). Defined T cells are responsible for the activation of the adaptive immune response which drives the maintenance phase of psoriatic inflammation, including the Th17 cytokines IL-17, IL-21, and IL-22 which activate keratinocyte proliferation in the epidermis (Figure 1-5) (Matsuzaki & Umemura, 2018; Nestle, Turka, & Nickoloff, 1994).

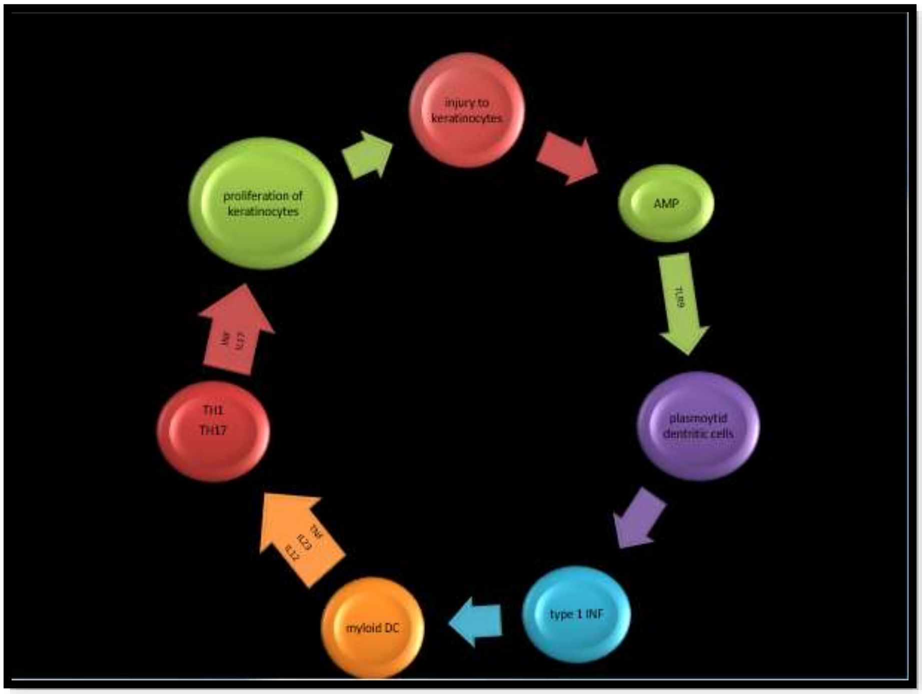

Figure 1-4: Illustration simplifying one of the pathways implicated in the pathogenesis of psoriasis

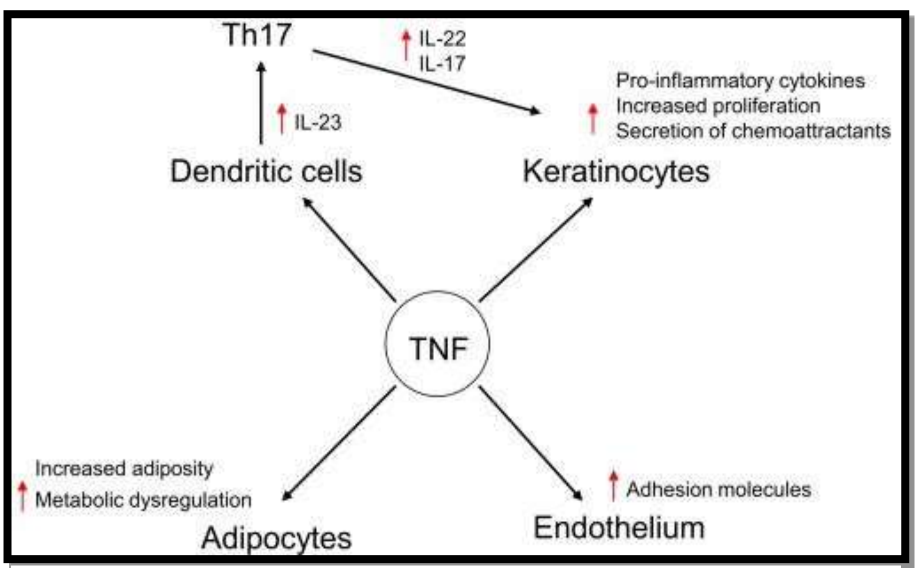

Figure 1-5. Pathogenesis of psoriasis (Yost & Gudjonsson, 2009)

showing the role of cytokines in psoriasis pathogenesis

Numerous cytokines are produced in psoriasis. Three factors, in particular, contribute to the pathogenesis of the disease, namely TNFα, dendritic cells, and T cells (Griffiths & Barker, 2007), and there is evidence that all of this is driven by T cells (Yost & Gudjonsson, 2009). Patients with the skin disease have been found to have raised TNFα levels in skin lesion and blood circulation (Zaba *et al*., 2007). Moreover, TNFα is secreted by both the antigen-presenting cells (APCs) and T cells in skin lesions and is thus a central mediator in the psoriasis disease process. The role of TNFα in disease pathogenesis is evident from the fact that it is a pro-inflammatory cytokine that increases inflammation via various specific pathways. This results in inflammatory cells entering skin lesions by inserting adhesion molecules onto endothelial vascular cells which, in turn, stimulates keratinocyte generation of more pro-inflammatory mediators. Lastly, keratinocytes trigger both dendritic cells and dermal macrophages to develop the disease further (Figure 1.5) (Yost & Gudjonsson, 2009).

The essential effector cytokines are IL-23 and IL-17, although TNF-α also plays a crucial role likely by contributing to the potentiation of the effects of these cytokines and/or the maintenance of the cells that produce these cytokines. In this study, the greatest focus will be on IL-17.

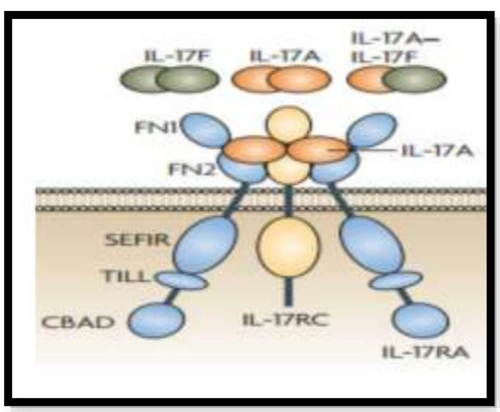

Figure 1-6. Illustration of IL17-receptor (adapted from Gaffen, 2009). Illustrating the parts of IL17 receptor head and tail and their attachments

1.2.1 Pathophysiology of IL-17 in psoriasis

The IL-17 cytokine family is comprised of six members of designated nomenclature IL-17A–F. They are produced by a range of different cell types, including Th17 cells, CD8+ T cells (Tc17), invariant NKT cells, TCR γδ T cells, ILC3 cells (which lack expression of T cell or B cell receptor) and neutrophils (Matsuzaki & Umemura, 2018).The cytokine IL-17A is produced by T-helper 17 (TH17) cells, a subset of CD4+ T cells. Although IL-17 is produced by different cell types, all IL-17 family cytokines promote inflammation, either in host defence or inflammatory pathology. Biological treatments targeting IL-17 have shown great promise in the treatment of psoriasis (Jin & Dong, 2013).

IL-17RA, a known target for IL-17A and IL-17F, was the first established member of the IL-17R receptor family. IL-17RA has the biggest cytoplasmic tail, providing docking sites for a number of different signalling pathways. Working through a SEF/IL-17R (SEFIR) domain, exclusive to the IL-17R family, IL-17RA plays an important role in inducing the activation of nuclear factor-κB (NF-κB), mitogen-activated protein kinase (MAP kinase) and CCAAT/enhancer-binding protein signalling pathways, all of which play a key role in the inflammation process (Figure 1.6)(Gaffen, 2009).

Many researchers have targeted IL-17 family cytokines, notably IL-17A and IL-17RA, for the prospective treatment of autoimmune diseases. It was always known that an understanding of the molecular features of this cytokine family would provide useful information for therapeutic advances, and now there is a treatment in the market that inhibits IL17 activity known as Secukinumab.

Traditional TH1 and TH2 signature cytokines signal through JAK-STAT-mediated pathways, with IFNγ activating a JAK1/2-STAT1-dependent pathway, and IL-4 activating a JAK1/3- STAT6-

dependent pathway. Activation of these pathways is critical, not only for the effector functions of these cells but also for their development. In contrast, IL-17A and IL-17F derived from TH17 cells promote ACT1/TRAF6/NF-κB-dependent signalling pathways, reminiscent of those receptors associated with innate immunity such as IL-1-family receptors and TLR ligands. Interestingly, another TH17 hallmark cytokine is IL-22, which not only activates a JAK-STAT3 signalling pathway but also shares downstream gene targets which are strikingly similar to those induced by IL-17. Thus, TH17 cells promote signals which are typical of early inflammatory events and, in this sense, serve to bridge innate and adaptive immune processes (Figure 1.7) (Gaffen, 2009).

IL-17 cytokines are secreted as dimers and bind to members of the IL-17 receptor family which include IL-17 R/IL-17RA, IL-17B R/IL-17RB, IL-17RC, IL-17RD/SEF, and IL-17RE. All of these receptors contain type I transmembrane protein subunits which hold two fibronectin type III-like domains in their extracellular domain and an inner cytoplasmic SEF/IL-17 R/TIR (SEFIR) domain (Pappu, Ramirez-Carrozzi, & Sambandam, 2011). Where IL-17RA diverges from other IL-17 family receptors is in the presence of an inner cytoplasmic Toll/IL-1 R-like loop (TILL) and a C/EBP beta activation domain (CBAD) (Figure 1-6) (Shen *et al.*, 2009). The receptors of IL-17 work together to form complex structures. For example, two IL-17RA subunits and one IL-17RC subunit can bind together to form a functional receptor (Pappu *et al.*, 2011).

One of the most important single factor in the aetiology of psoriasis is mediated mostly by IL-17A and IL-17F which converge upon the same receptor but have different actions, as IL-17A has a stronger effect than IL-17F. IL-17A binds to its trimeric receptor complex, composed of two IL-17RA subunits and one IL-17RC subunit, resulting in the recruitment of the ACT1 adaptor protein. The interaction between ACT1 and the IL-17 receptor complex leads to the activation of a series of intracellular kinases including extracellular signal-regulated kinase (ERK), p38 MAPK, TGFbeta-activated kinase 1 (TAK1), I-kappa B kinase (IKK), and glycogen synthase kinase 3 beta (GSK-3 beta) (Figure 1.7). These kinases enable the NFκB, AP-1, and C/EBP-directed transcription of pro-inflammatory cytokines, chemokines, and antimicrobial peptides (Harden, Krueger, & Bowcock, 2015). The role of the IL-17 family in autoimmunity is as potent inducers of inflammation, which act as promoters of cell infiltration and producers of an assortment of cytokines and pro-inflammatory chemokines. IL-17A was the first to be discovered, followed by the identification of several proteins possessing a high degree of homology (which may be as high as 50% between IL-17A and IL-17F).

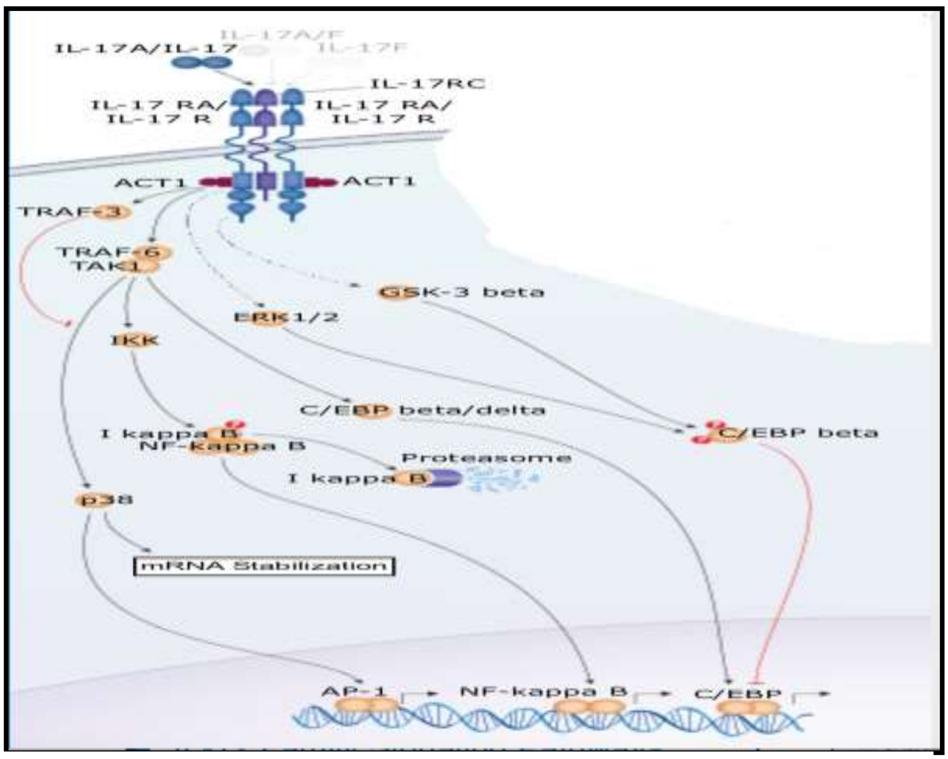

Figure 1-7, IL-17 family signalling pathway
(https://www.rndsystems.com/pathways/il17-family-signaling-pathways)

In the skin, IL-17A primarily acts on keratinocytes and exerts two major effects, specifically the induction of different inflammatory mediators and the facilitation of the abnormal proliferation of keratinocytes. Through the synergistic activation of TNF-α, IL-17A induces the transcription of genes of various inflammatory cytokines, including IL-1β, IL-6, IL-8, and TNF-α. IL-1β which, together with IL-23, augment IL-17A production in IL-17A-producing cells. L-23 promotes the survival and proliferation of T-helper (Th) 17 cells, whereas IL-12 promotes the development of Th1 cells (Figure 1.8).

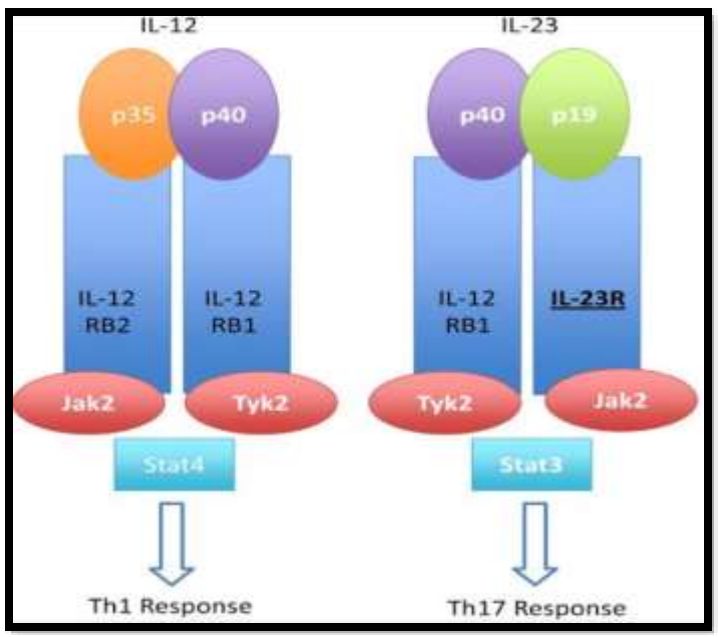

Figure 1-8. The IL-23 axis genes associated with psoriasis (Harden et al., 2015)

Many components of the IL-23 axis have genetic associations with psoriasis, including the IL-23 cytokine itself, the IL-23 receptor, and downstream signalling through the IL-23 receptor.

1.2.2 Role of ROR in the pathology of psoriasis

The retinoid-ROR (related orphan receptors) is the key transcription factor in the differentiation programme of Th17 (Ivanov *et al.*, 2007). All of the cytokine pathways involved in Th17 cell differentiation result in an up-regulation of RORγt expression. The IL-6/IL-21/IL-23 signalling pathways also activate STAT3 which binds directly to the IL-17 and IL-21 promoter regions. When cells were transduced with both RORγt and an active form of STAT3 (STAT3C), there were more IL-17+ cells and a higher level of IL-17 expression in each cell, consistent with a synergistic activation by RORγt and STAT3 at transcriptional target. (Ivanov *et al.*, 2007) (Figure 1.9).

IL-17 gene transcription is controlled by the RORγ and RORα transcription factors. Th17 cell differentiation is driven via RORs that are specific to them. Thus, ROR is essential for the differentiation of Th17 cells and the production of IL-17. Inhibition of ROR is considered a promising strategy in the treatment of Th17-mediated autoimmune diseases (Sun, Guo, & Wang, 2019). Human IL-17A producing cells are derived from a cluster of differentiation 161 (CD161) + CD4+ progenitor cells which express retinoic acid-related orphan receptor variant 2 (RORC2). The RORC2 gene encodes the ROR-related orphan receptor gamma (RORγ) which is a dominant regulator of human Th17 cells. Naive CD4+ T cells may differentiate into Th17 cells depending on the initial effects of such cytokines as transforming growth factor beta (TGFβ), IL-1β, IL-6, and IL-23, with the help of other regulatory factors including aryl hydrocarbon receptor (Ghoreschi, 2010) (Ramirez, 2010). In a study by Ivanov II et al. (2007), mice with RORγt-deficient T cells exhibited diminished autoimmune disease and the absence of tissue-infiltrating Th17 cells. This finding suggests that RORγt is a key regulator of immune homeostasis and highlights its potential role as a therapeutic target in inflammatory disease (Figure 1.9).

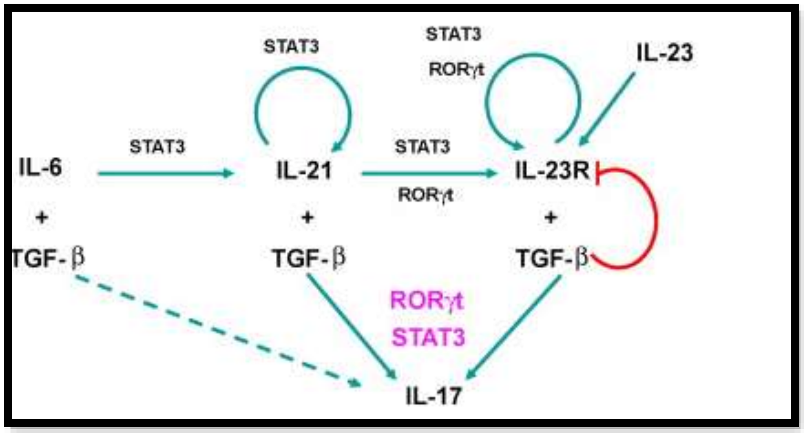

Figure 1-9 Sequential roles of cytokines in the differentiation of the Th17 cell lineage. IL-6 induces IL-21, which in turn induces its own transcription in a STAT3-dependent manner. Full differentiation of Th17 cells requires the cooperative action of RORγt and STAT3**(Ivanov *et al.*, 2007)**.

RORs are members of a superfamily of ligand-regulated transcription factors which bind to DNA response elements known as ROR response elements (ROREs), providing the consensus core motif AGGTCA that is preceded by a 5′ A/T-rich sequence located within the regulatory regions of the target genes. The ROR family comprises three members, namely RORα (NR1F1), RORβ (NR1F2), and RORγ (NR1F3) which are encoded by the *RORa*, *RORb*, and *RORc* genes, respectively. RORγt is expressed in thymocytes at the double-positive stage of T cell development, but is it is not found in mature thymocytes and in mature naive T cells in spleen and peripheral lymph nodes. In 2006, RORγt has been noticed in IL-17-producing T cells, and it has been shown to play a vital role in Th17 differentiation. The transcription of RORγt is introduced by activation of the promoter RORC2 into the *RORC* locus (Capon 2020).

1.2.3 The role of Il-22 in the pathology of psoriasis

Th17 cell-specific cytokines, notably IL-17A and IL-22, have been recorded at high levels in psoriasis, and Th17 cells have been identified by flow cytometry in psoriatic lesions (Harper *et al.*, 2009). Various environmental risk factors trigger the body's immune response, whereupon naive

T cells located within the epidermis, particularly Langerhans cells, are triggered by APCs which can release cytokines such as interleukin IL12 and IL23, resulting in the differentiation of naive T cells into Th1 and Th17 cells. IL23 and IL12 are released by inflammatory myeloid dendritic cells to activate IL17-producing T cells, Th1 cells and Th 22 cells to produce an abundance of the psoriatic cytokines IL17, IFNγ, TNF, and IL22 (Mohammed & Mutlag, 2020).

IL22 inhibits the terminal differentiation of keratinocytes and induces the production of antimicrobial peptides and some chemokines. This leads to epithelial remodelling and inflammation. Research has shown that the unaffected regions of the skin of psoriasis patients have a lower expression of IL-22 than the lesional and perilesional skins (Martin *et al.*, 2017). IL-17A and IL-22 induce STAT3 activation which leads to the proliferation of keratinocytes and an inflammatory response. IL-6, produced by keratinocytes, also induces STAT3 activation (Figure 1.10) (Furue, Furue, Tsuji, & Nakahara, 2020).

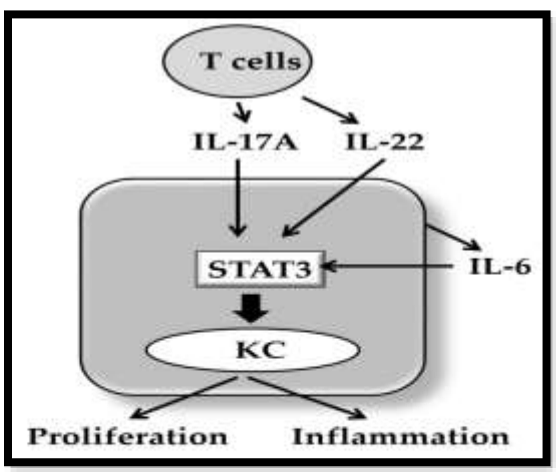

Figure 1-10. Pivotal role of signal transducer and activator of transcription 3 (STAT3) in psoriasis

The activation of STAT3 promotes keratinocyte (KC) proliferation and an inflammatory response. IL-17A and IL-22 induce STAT3 activation. IL-6 produced from KCs also induces STAT3 activation (Furue et al., 2020)

IL-17A, together with TNF and IL-22, have been reported to upregulate the expression of IL-1-like family member IL-36 which, in turn, augments the function of Th17 cytokines, thereby revealing the existence of a feedback loop between Th17 and IL-36 cytokines. IL-17A (and possibly IL-20 and IL-22) induce the production of IL-19 and IL-36 by keratinocytes (KCs) and are considered to be the very cytokines that cause the abnormal proliferation of KCs in psoriasis (Brembilla *et al.*, 2018). IL-17A and IL-22 are co-expressed by Th17 cells and play a significant role in forming psoriatic plaques (Jin & Dong, 2013). Interleukin-17A and IL-22 positive cells are highly represented in peripheral blood mononuclear cells (PBMCs) and also in the psoriatic lesions of the patients (Noack, 2016).

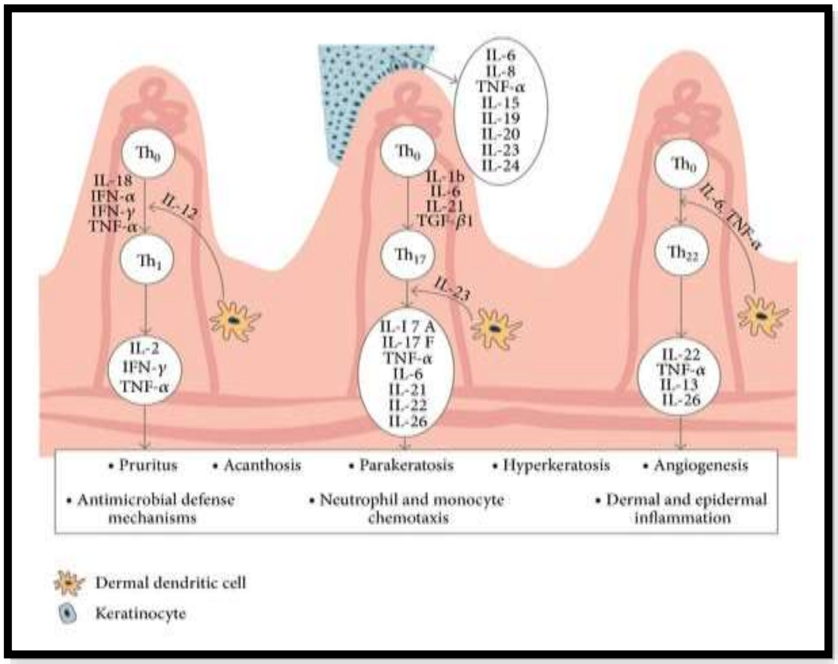

Figure 1-11. Th1, Th17, and Th22 cytokines in the pathogenesis of psoriasis (Michalak-Stoma et al., 2013). Showing how the pathology of psoriasis involves all 3 types of T Cell (Th1, Th17, and Th22)

Th17 cells produce IL-17, IL-6, IL-21, IL-22, and TNF. Native T cells become differentiated to Th 17 in response to TGF-β1, IL-6, IL-23, and IL-15. TGF-β1 is secreted by activated T cells and initiates T cell and fibroblast activation, angiogenesis and neovascularisation. IL-6, secreted by macrophages, endothelial cells, and epithelial cells, is responsible for the expansion of KC hyperplasia and an invasion of macrophages and T cells. IL-15, produced by monocytes, macrophages, DCs, and T cells, can appear to induce angiogenesis, immune cell recruitment, and the activation of KCs. Once the Th17 phenotype is achieved, IL-23 maintains a cellular Th17 polarisation. Cytokines produced by Th17 cells were found to initiate acanthosis, hyperkeratosis, and parakeratosis. Th17 cells have been demonstrated to have an involvement in neutrophil and monocyte chemotaxis, T cell migration and activation, and neovascularisation. IL-22 is responsible for the observed angiogenesis as shown in Figure 1.11 (Michalak-Stoma *et al.*, 2013).

Th22 cells have, more recently, been redefined as inflammatory CD4+ T cells which release such cytokines as IL-22, IL-26, and IL-13, of which IL-22 is likely the most important. Th22 cells do not express IL-17A or IFN-γ (Nograles, Davidovici, & Krueger, 2010). Recent studies have shown that IL-6 and TNF-α, with the aid of plasmacytoid dendritic cells, promote the Th22 phenotype (Zhang, Pan, & Ye, 2011). The variation of the three primary T cell subsets involved in the pathogenesis of psoriasis (as well as Th1, Th17, and Th22 cytokine production) are illustrated in Figure 1-11. In psoriasis, an increase in IL-17 has been detected in the skin of patients in addition to IL-22, a cytokine which is also produced by Th17 cells that apparently contributes to the induction of KC proliferation and the production of antimicrobial peptides and cytokines. IL-17 triggers changes in KCs which are characteristic of psoriasis through the synergy of this cytokine with other mediators such as γ-interferon, TNFα and IL-22 (Michalak-Stoma *et al.*, 2013**).**

IL-22 is primarily expressed by CD4 T cells, although a variety of other immune cell subsets have also been shown to express this cytokine. IL-22 acts on epithelial cells and fibroblasts and may mediate deleterious or protective pro-inflammatory effects. While IL-22 is an established critical pro-inflammatory mediator of psoriasis, it also plays a role in host defence against extracellular bacteria at the mucosal surfaces of the lung, gut and skin. IL-22 is specifically produced by Th17 cells and mediates the acanthosis induced by IL-23. Further, IL-22 mediates IL-23-induced acanthosis and dermal inflammation via the activation of Stat3 in vivo. Although IL-23 or IL-6 can directly stimulate the production of IL-22 from naive T cells, the production of IL-22 and IL-17 from Th17 cells is differentially regulated. TGFβ, although crucial for IL-17 production, actually inhibits IL-22 production. IL-22, as an effector cytokine produced by T cells, thereby serves as an intermediary between the immune system and epithelia (Zheng *et al*., 2007)(Furue et al., 2020). IL-22 has been shown to induce acanthosis and hypergranulosis in skin by downregulating those genes involved in its terminal differentiation.

IL-17A and TNF-α also induce the release of Chemokine C-C-Motif Ligand 20 (CCL-20), which plays a critical role in the recruitment of IL-17-producing cells such as Th17 cells and γδT cells (Harper et al., 2009). These infiltrating cells produce inflammatory mediators and further expand psoriatic inflammation which leads to cell proliferation. The abnormal hyperproliferation of KCs, which leads to epidermal hyperplasia – a hallmark of psoriasis – is also indirectly regulated by IL-17A.

Genome-wide association studies also affirm the role of the immune system in the pathogenesis of psoriasis, with the HLA-Cw6 allele being attributable to approximately 50% of the disease's heritability. The detection of dissimilarities in those loci containing genes involved in the IL-23/Th17 pathway implicates the specific involvement of IL-23R and IL-12B which lie upstream of the IL-17 expression, and also of STAT3 and ACT1 which lie downstream of the IL-17 receptor (Brembilla *et al*., 2018).

The effects of IL-17A are not restricted to KCs and include numerous other cells, including endothelial cells, chondrocytes, fibroblasts and synovial cells. IL-17A is obviously of major importance in the context of psoriasis-associated comorbidity in the form of psoriatic arthritis (Zheng *et al*., 2007).

Table 1-1. Cellular and molecular elements comprising the immunity of the skin's immune system (Bos *et al*., 2015).

Skin immune system: Elements of innate immunity	Expression in psoriasis
Cellular	
Keratinocytes: epithelial barrier	Hyperplastic
Phagocytes: neutrophilic granulocytes, monocytes/macrophages	Increased and activated
Dendritic cells (DCs)	Increased and activated, even in uninvolved skin
Natural killer (NK) cells and NK T cells	Increased and activated
Humoral	
TLRs, heat shock proteins (HSPs)	Up-regulated
Antimicrobial peptides: defensins, cathelicidins	Up-regulated
Complement system	Activated

Skin immune system: Elements of innate immunity	Expression in psoriasis
Cytokines of innate immunity: TNF-α, IFN-γ, IL-1, IL-6, IL-12, IL-15, IL-18	Up-regulated
Chemokines: CXC8 (IL-8)	Up-regulated

In addition to the central role of Th17 cells, the increase in cytokine levels within skin lesions was found to have impacted other cell types of the immune system. Studies have shown a predominance of T-helper lymphocytes in psoriatic lesions over cytotoxic T lymphocytes, with the Th1 pattern characterised by an increase in levels of IFN-γ, IL-1β, IL-12p40, and TNF-α. On the other hand, dysregulation of the innate immune system is also involved in the immunopathogenesis of psoriasis (Damsker, 2010). Dendritic cell numbers are increased in psoriatic plaques, as is their level of activation, and the levels of the main cytokine produced by this cell type, INF-α, are also increased. This cytokine participates in the induction of T cell proliferation, potentiating autoreactivity within the immune system, the production of TNF-α, and the expression of the inducible isoform of nitrogen monoxide synthesis with its marked pro-inflammatory effects. The KC also plays an essential role in the immune reactions in the skin by producing cytokines in response to stimulation by other inflammatory mediators such as IL-17. The pathophysiology of psoriasis encompasses multiple complex and interrelated immune phenomena involving different pathways of the innate and acquired immune systems (Table 1.1). Knowing the complex physio-pathogenesis of psoriasis has allowed the identification of new potentially effective therapeutic targets (Bos, De Rie, Teunissen, & Piskin, 2005).

CD4 T cells, when stimulated under pro-inflammatory conditions in the presence of cytokines IL-6, IL-23, IL-21, and TGFβ, will differentiate into the Th17 phenotype and produce IL-17 (Tesmer, Lundy, Sarkar, & Fox, 2008). In healthy individuals, IL-17 is undetectable in serum, although increased levels have been detected in several autoimmune diseases including rheumatoid arthritis, systemic lupus erythematosus, inflammatory bowel disease, spondylitis, as well as psoriasis, among others (Vincent, Northcott, Hoi, Mackay, & Morand, 2013). In systemic lupus erythematosus, some studies have shown that mice with IL-17 or IL-17R deficiency are protected from lupus nephritis, indicating a further important role for IL-17. Additionally, genetic factors known to predispose to systemic lupus erythematosus are implicated in signalling pathways relating to Th17 cells, highlighting the potential importance of IL-17 in the disease (Paquissi & Abensur, 2021).

In a study, the involvement of IL-17 in inflammatory bowel diseases has revealed that there seems to be a pattern of cytokine secretion in ulcerative colitis which strongly suggests a role for IL-17. The burgeoning scientific evidence for the role of IL-17 in the pathophysiology of systemic inflammatory reactions has motivated new therapeutic strategies targeting IL-17 and its IL-17R. The development of such therapeutic strategies aimed at IL-17/IL-17R and Th17 cells has thus become one of the main targets of research in the treatment of chronic inflammatory diseases, with encouraging clinical results to date (Műzes *et al*.,2012) (Miossec, 2017).

The most direct way to block IL-17-mediated functions is to inhibit either IL-17 or the IL-17R receptor itself. Two monoclonal antibodies directed against IL-17A are currently under development, namely Secukinumab (AIN457; an utterly human antibody developed by Novartis) which has completed phase III studies for psoriasis; phase II for rheumatoid arthritis; and is currently undergoing phase II studies for ankylosing spondylitis. The second is Ixekizumab

(LY2439821; a humanised antibody developed by Eli Lilly) which has completed a phase II study for psoriasis and has a phase IV study for psoriatic arthritis. Ixekizumab is a human monoclonal antibody that binds to interleukin-17A and inhibits the release of pro-inflammatory cytokines and chemokines (Di Lernia, 2020).

IL-17, along with T-helper (Th) 17 cells, plays a central part in the disease's pathogenesis. IL-17A, usually referred to simply as IL-17, is a fundamental component of the IL-17 group. Therefore, the human T cell family has several subsets, including Th17, which produces the cytokine IL-17. Psoriasis is thought to be driven by Th1; as research findings from knockout animals and experimental findings from humans suggest that Th17 and IL-17 do indeed play a significant part in the pathogenesis of this disease (Brembilla *et al*., 2018). Studies have identified Th17 cells in dermal extracts from patients' psoriatic lesions. Raychaudhuri (2013) and his research reviewers confirmed the finding that Th17 cells appear to be strengthened when present in the papillary dermis of patients' psoriatic plaques. They are also enriched in newly separated effector T lymphocytes isolated from the patient's synovial fluid. They determined that most cells were IL-17+, and CD4+ T cells 'are of memory phenotype CD4RO+CD45RA-CD11a+' (Raychaudhuri, 2013). In addition, their research has shown the existence of a functional IL-17 receptor in synovial fibroblast of psoriatic arthritis patients and the contribution of Th17 cytokines (IL-17 and IL-22) in the disease severity (Raychaudhuri, 2013).

S100A9, S100A7, and S100A8 are other effects of IL-17, which contribute to the strengthening of the mechanisms of the epidermal inflammatory response. Expression studies revealed that IL-17 controls those genes which encode for proteins associated with differentiation, antimicrobial defence, and immune cell recruitment and activation, including filaggrin, S100A7, DEFB4, CCL20, and IL-8 (CXCL8). Moreover, the expression of chemokines and cytokines, such as

CCL20 and IL-8, marshals the recruitment of immune cells, including neutrophils, which can further exacerbate the inflammatory progression of psoriatic lesions through crosstalk with KCs (Pfaff, Marquardt, Fietkau, Baron, & Lüscher, 2017).

On the other hand, IL-17 reduces the expression of filaggrin, and genes involved in the adhesion of KCs, seemingly contributing to the skin barrier's interference in inflammatory diseases mediated via the actions of IL-17. It also extends to skin cell types other than KCs, including myeloid dendritic cells, macrophages, fibroblasts and endothelial cells, thereby enhancing the inflammatory characteristics observed in psoriasis (Gutowska-Owsiak *et al.*, 2012). The selectivity of the treatments used in psoriasis has important implications in terms of efficacy and safety, as has been shown from experiences of the use of anti-TNF-α agents and their impact on innate and adaptive immunity, as such treatments led to an increased incidence of infections caused by intracellular bacterial and granulomatous infections. Although IL-17 contributes to the defence against some pathogenic microbial agents, this cytokine may provide a more specific target in the treatment of autoimmune diseases given that IL17 deficiency has not been linked with severe complications or the induction of autoimmune symptoms. So, the inhibition of this pathway may have a favourable benefit/risk ratio.

1.3 Oxidative stress and psoriasis

DNA can be defined as a molecule composed of two chains that combine to form a complex chemical structure carrying the genetic instructions used to direct the growth of both eukaryotic and prokaryotic cells (Davis, 2012). DNA changes or modifications are related to exposure to various environmental elements and, within a given biological system, many possibly result in mutations that cause the pathogenesis of some diseases (Clancy, 2008). The integrity and stability of DNA are required for normal physiological functioning. DNA damage has been linked to causative factors such as cigarette smoking leading to damage to the epithelial cells of the lung and cancer of this tissue. Environmental factors such as exposure to UV radiation can induce DNA damage skin cancer and other skin conditions (Lord & Ashworth, 2012). Free radicals, for example, hydrogen peroxide, have been shown to cause oxidative stress by increasing the level of reactive oxygen. Each damaged cell may be able to express up to one million DNA lesions daily. Consequently, the regulation of DNA repair is very important in resolving this magnitude of damage to preserve normal cell functions. Many proteins are involved in completing the DNA repair process (Abbotts, Thompson, & Madhusudan, 2014). Different repair pathways have been shown to be involved in repairing DNA damage. These DNA repair mechanisms can be classified into several types, including base excision repair (BER), double-strand break repair, mismatch repair (MMR), and nucleotide excision repair (NER) (Klapacz *et al*., 2016).

Cells need O_2 for survival and O_2 travels dissolved in blood plasma, although the solubility of O_2 in water at body temperature is limited. Therefore, O_2 is carried by haemoglobin, a protein comprised of 4 subunits, each of which carries a heme group bound to an iron ion in the Fe^{2+} state. The O_2 reversibly attaches to heme, binding at high O_2 concentrations as blood flows through the lungs and dissociating again at a lower O_2 level in tissues. Free radicals are defined as any species capable of independent existence that contains one or more unpaired electrons. Unpaired electrons occupy a solitary atomic or molecular orbital and are denoted by a superscript dot. This free electron makes the free radical more attractive to a magnetic field and highly reactive. They are formed by gaining or losing an electron or when a covalent bond is broken. When two free radicals meet, they join to form a covalent bond. However, most biological molecules are non-radicals and therefore a chain reaction of damage may occur (Halliwell & Gutteridge, 2015).

Oxidative stress has been linked to a number of pathologies through the elevation of intracellular levels of reactive oxygen species (ROS) that cause damage to lipids, proteins and DNA. However, elevated ROS are also signalling molecules (i.e., redox biology that maintains physiological functions). Oxidative stress has been shown to have a major role in different inflammatory conditions, including psoriasis (Wagener, Carels, & Lundvig, 2013). ROS are short-lived and are continuously generated at low levels during the course of normal metabolism. ROS includes single oxygen ($^1O_2$), superoxide anion (O_2^-), and H_2O_2, the hydroxyl radical ($OH^•$). $^1O_2$ is formed via the transfer of physical or chemical energy to molecular oxygen (O_2), which, at normal temperatures, acts as a triplet and is paramagnetic due to its unpaired electrons. $^1O_2$ has no unpaired electrons and is a very strong oxidant. The reduction of O_2 leads to the formation of O_2^-, H_2O_2, and $OH^•$. Free radical-driven reactions are commonly chain reactions. For instance, working as an electron donor, O_2^- can lead to the production of $OH^•$, by an O_2^--driven Fenton reaction, while interaction with nitrous oxide (NO), can produce highly reactive peroxynitrite ($ONOO^-$). Molecular oxygen interacts freely to produce other free radicals. An extra source of oxygen free radicals derives from infiltrating activated leukocytes that generate O_2^- and hypochlorite, vital sources of ROS in situ. The essential function for the release of such vast quantities of ROS during the inflammatory process is to kill or terminate invading microorganisms and/or to destroy damaged tissue structures. It is the non-specific targeting of ROS that can induce oxidative stress in nearby normal cells thereby triggering the inflammatory reaction (Bickers & Athar, 2006) (Hancock *et al*., 2001).

Fe^{2+} is a central reagent for producing free radical-driven ROS via the Fenton reaction. Cu^+ is nearly as active as Fe^{2+} as a reagent but is a more powerful mutagen due to its capacity to interact directly with DNA bases. Antioxidant defence systems have also evolved alongside aerobic metabolism to neutralise the damaging effects of ROS and reduce their capacity for tissue damage.

Cellular damage from ROS accumulates throughout life, despite antioxidant defence mechanisms (Hancock *et al*., 2001).

Higher levels of ROS may arise due to overproduction or inadequate sifting. These may engender oxidative stress, leading to harmful effects via the: (1) Oxidative modification and damage of biomolecules, altering the organisation and/or function of lipids, proteins and DNA; (2) irreversible oxidation of reactive protein thiol groups which is a classical feature of oxidative stress and; (3) dysregulation of cell signalling pathways, activating downstream signalling cascades leading to different patterns of cytokine release and an increase in inflammation. All this excess ROS leads to pathological changes in cells and tissues, as demonstrated by inflammatory skin conditions like psoriasis (Wagener *et al*., 2013).

There is a significant increase in oxidative stress markers in patients with psoriasis such as malondialdehyde, lipid hydroperoxide, etc. Concomitantly, there is a decrease in the level of antioxidants such as serum vitamin E (Pujari, Ireddy, Itagi, & Kumar, 2014). A positive correlation has been noted between oxidative stress markers and psoriasis area and severity index (PASI) values, while a negative correlation exists between antioxidant markers and PASI values in patients with psoriasis (Lin & Huang, 2016). For example, decreased antioxidant levels are low along with elevated levels of lipid peroxidation markers in the blood of psoriasis patients. Also, serum levels of catalase were high in psoriatic patients (catalase is an enzyme that catalyses the decomposition of hydrogen peroxide to water and oxygen and in this way protects the cells from the oxidative damage caused by ROS) (Winiarska-Mieczan, 2020). Such catalysis occurs through the Fenton reaction:

$$H_2O_2 + Fe\ (II) = Fe\ (III) + OH^{\bullet} + OH^{-}$$

In the absence of organic ligands (an ion or molecule attached to a metal atom by coordinate bonding), no OH• products can be calculated under conditions where H_2O_2 is the main oxidant of Fe (II). Therefore, it can be concluded that inorganic Fe (II) and H_2O_2 react to form an intermediate that is not OH•. Generally, the Fenton reaction will only result in the production of OH• if Fe (II) is in the presence of an organic ligand (Miller, Rose, & Waite, 2016). Oxidative stress is involved in the pathogenesis of psoriasis. Redox imbalance found in the blood and skin of the patients indicates that psoriasis is in a state of oxidative stress. Oxidative stress could improve the capacity of dendritic Cells (DCs) to promote T-cell activation, proliferation, and proinflammatory cytokine production, consequently driving the immunopathological process in psoriasis. TCR-mediated ROS production might function to regulate T-cell activation. Oxidative stress might also contribute to the deficient counter-regulatory capacity of Th2 on Th1 response in psoriasis. ROS might play a role involving activation of transcription factors in KCs, leading to KC hyperproliferation. Angiogenic and oxidative mechanisms both underlie the pathophysiology of psoriasis. ROS can induce angiogenic processes through both VEGF-dependent and VEGF-independent pathways. In addition, ROS act as secondary messengers that modulate transcription factors in a variety of inflammatory signalling pathways including MAPK, NF-κB, and JAK-STAT. All these play a vital role in the pathogenesis of psoriasis (Lin, 2016).

In addition, there is an increase in the levels of superoxide dismutase (SOD) activity, an enzyme that catalyses the dismutation (a redox reaction in which one compound of intermediate oxidation state is converted into two compounds, one of a higher and one of a lower oxidation state) of (O^{2-}) radicals into normal molecular (O_2) and (H_2O_2). SOD is an antioxidant enzyme that scavenges superoxide radicals and requires metal cofactors such as Cu, Zn, or Mn for full activity. It was found that SOD3, in particular, participates in the control of immune responses and signal transduction. In addition, SOD3 controls DC, Langerhans (LC), and T cell production and migration in skin inflammation. Furthermore, SOD3 controls TLR4 signalling by regulating TLR4 translocation to lipid rafts in macrophages which, in turn, controls NF-KB signalling-mediated inflammatory molecule production. Even DC maturation is controlled by SOD3. Considering that SOD3 expression is significantly down-regulated in psoriatic patients and that psoriasis occurs due to non-ideal T-helper cells responses, it is likely that SOD3 controls the T-cell-mediated adaptive immune response (Kwon, Kim, Lee, & Kim, 2012).

Besides the directly damaging effects of unregulated ROS production, dysregulation of several pro-inflammatory pathways, including MAPK, NF-κB, and JAK-STAT, have also been considered to play a role in the pathogenesis of psoriasis. The NF-κB pathway regulates pro-inflammatory cytokine production, leukocyte recruitment and cell survival, all of which are important contributors to the inflammatory response. Dysfunction in psoriasis is found at the level of KCs as well as in the innate and acquired immune responses, especially relating to T cell-related processes. NF-κB links altered keratinocyte and immune cell states through its effects on cellular proliferation, differentiation and apoptosis, as well as cytokine and chemokine production. Members of the innate immune system, including TLR2 and caspase-5, recognise when pathogen-associated molecular patterns (PAMPs) are up-regulated in psoriasis and rely on the downstream activity of NF-κB through the elaboration of pro-inflammatory cytokines. Apoptotic signals are dampened within epidermal keratinocytes, leading to altered rates of programmed cell death and an epidermal cell cycle almost 9-fold longer than that of normal skin (36 hrs versus 311 hrs). Altered NF-κB signalling changes the balance of apoptotic signals such as bcl-xL and cyclins. Levels of survivin, another anti-apoptotic factor increased in psoriasis, also relies on NF-κB signalling (Goldminz, Au, Kim, Gottlieb, & Lizzul, 2013).

Dysregulation of NF-κB-mediated signalling may cause further exacerbation of the disease, as the NF-κB-mediated up-regulation of pro-inflammatory cytokines initiates NF-κB via a positive feedback loop. Notably, inhibition of NF-κB nuclear translocation and DNA binding activity decreases the inflammation of psoriasis. Several of the cytokines implicated within the pathology of psoriasis induce a further proliferation of keratinocytes, and these signals are via ROS-mediated action transmitted to transcription factors and associated proliferation pathways. An imbalance between oxidant and antioxidant activity will lead to a sustained inflammation in psoriasis (Wagener *et al.*, 2013).

Therefore, it is widely accepted that keratinocytes play a main role in the exacerbation of psoriasis. Cytokine interactions in psoriasis result in the hyperproliferation of keratinocytes. The T cell type 1 pathway, involving IL-2, IL-12 or IFN-γ, has been shown to be a central inducer, via the activation of STAT1 and NF-κB. In addition, novel cytokines have recently been shown to be an integral part of these networks. This is thought to be important because of its ability to stimulate pro-inflammatory cytokines, such as IL-6 and IL-8, as well as intercellular adhesion molecule-1 (ICAM-1). Many cytokines that are involved in psoriasis induce KC proliferation. The signals are eventually transmitted to transcription factors, such as STAT or NF-κB, either directly or through ROS. ROS mainly play a role in the activation of transcription factors in keratinocytes rather than by inducing cytokine release by KCs and lymphocytes (Bito & Nishigori, 2012).

1.4 Management and treatment of Psoriasis

The psoriasis treatments available are allocated based on the clinical severity of the disease. This is ascertained via a range of indices, including the PASI (Psoriasis Area and Severity Index of the diseases), Physician Global Assessment (PGA), and Dermatology Life Quality Index (DLQI).

Another factor is the type of psoriasis. Plaque psoriasis is the most common form and usually appears on the elbows, knees, lower back and scalp. Guttate psoriasis is mostly triggered by a bacterial infection such as Streptococcus. It appears as small, drop-shaped, scaling lesions on the trunk, arms or legs. Inverse psoriasis arises in the form of smooth patches of red skin that mainly affect the skin folds of the groin, buttocks and breasts. Pustular psoriasis comprises pus-filled lesions and is not very common. Nail psoriasis affects the fingernails and toenails, causing pitting, abnormal nail growth and discolouration. Erythrodermic psoriasis is rare and could be life-threatening, as the body becomes red, and the patient is susceptible to dehydration, hypothermia and electrolyte imbalance. Psoriatic arthritis is characterised by swollen, painful joints and such symptoms are typical of arthritis.

Mild forms can be successfully treated with topical therapy, which is often recommended as the first line of treatment. However, moderate-to-severe cases need photo- or systemic therapeutic approaches or even a combination of the two.

Phototherapy is administered a couple of times per week and, if not effective and the patient life quality is affected by the disease, then systemic therapy is given. In relation to systemic therapy, two methods can be offered, namely non-biological (cyclosporine, acitretin and methotrexate, etc.) or biological (adalimumab, infliximab, Ustekinumab, etc.). Among biological treatments, Secukinumab is the drug ultimately used in the treatment of severe psoriasis (Vlahovic, 2019). As concerns the cost per sustained response analysis, Secukinumab showed the lowest value observed over the other biological treatments , because of its high response rate (86% vs. 60–80%), which was achieved early in time (Zagni *et al*, 2021).

1.5 Biological treatment

Biological treatments are a type of treatment that use substances derived from living organisms to treat disease. These substances may occur naturally in the living body or else are made in the laboratory. Biological treatment often refers to bioengineered monoclonal antibodies. These are prepared by inoculating laboratory animals against a target antigen. The antibodies selected are monoclonal in that they are clones of each other and attach to the same epitope on the target antigen. Biological treatments are used to treat severe and recalcitrant cases of autoimmune diseases. Although biological treatments are very effective, they are reserved for patients who have failed at least two other treatments because of their expense and side effects (Wang, Shammas, & Hung, 2010).

Biological treatment may be in the form of proteins that regulate the activity of other proteins and cellular processes, genes that control the production of essential proteins, modified human hormones, or cells which release materials that suppress or activate components of the immune system. Biological products may be referred to as biological response modifiers due to their ability to change the actions of naturally occurring intracellular and cellular processes (Levine& Kroemer, 2019). In our research, our focus was on the biological drug Secukinumab

Table 1-2 classification of biological treatments in psoriasis

Drug	FDA approved to treat psoriasis	Pathogenesis
Cimzia®(certolizumab pegol)	X	Binds TNF to neutralize its effects

Cosentyx® (Secukinumab)	X	IL 17 inhibitor
Enbrel® (etanercept)	X	Binds TNF to neutralize its effects
Humira® (adalimumab)	X	Binds TNF to neutralize its effects
Ilumya™ (tildrakizumab)	X	IL-23 inhibitor
Remicade® (infliximab)	X	Binds TNF to neutralize its effects
Siliq1™ (brodalumab)	X	IL 17 inhibitor
Simponi® (golimumab)		Binds TNF to neutralize its effects
Skyrizi™ (risankizumab)	X	IL-23 inhibitor
Stelara® (ustekinumab)	X	Blocks the actions of IL-12 and IL-23
Taltz® (ixekizumab)	X	IL-17 inhibitor
Bimekizumab	X	IL-17 inhibitor
Tremfya™ (guselkumab)	X	IL-23 inhibitor

1.6 Secukinumab

Secukinumab is a first-in-class recombinant high-affinity, fully human monoclonal anti-human antibody of the IgG1/kappa isotype that selectively targets Interleukin 17A (IL-17A) and neutralises the bioactivity of this cytokine. Secukinumab is a monoclonal antibody (mAb), also known as anti-IL-17A, which is used to treat many inflammatory diseases such as psoriasis and inflammatory bowol diseases. IL-17A is a naturally occurring cytokine responsible for regular immune and inflammatory responses. One of the conditions it is involved in is the pathogenesis of psoriasis. Secukinumab has IgG1/K-class properties and has been produced in Chinese Hamster Ovary (CHO) cells using recombinant DNA technology (Kolbinger *et al*., 2016). The name is devised according to the WHO system, where S stands for bone (target antigen), E for hamster and U for human (the species on which sequences of the antibody are based), K for interleukin, and MAB for monoclonal antibodies (Wang *et al*., 2010) (see Appendix 6).

Cosentyx (the trade name of Secukinumab) is a commercially available medicinal product that is available in three different pharmaceutical forms based on two formulations. These include (1) 150 mg powder for solution for injection in a vial; (2) a 150 mg/1mL solution for injection in a pre-filled syringe, and (3) a 150 mg/1mL pre-filled bulk syringe assembled within a pen/auto-injector (BNF). The syringe and the pen/auto-injector forms use the same bulk syringes and have the same formulation. In this research the pre-filled syringe formula was used.

Secukinumab is an anti-IL17A antibody also referred to as AIN457, which has been used as a treatment for both moderate and severe psoriasis (Elain *et al*., 2014), as well as for psoriatic arthritis (Spindeldreher *et al*., 2018) and other disorders such as multiple sclerosis (Elain *et al*., 2014) and ankylosing spondylitis (Kammuller *et al*., 2017).

Secukinumab is superior to other biological treatments having achieved a PASI rating of 75 (a score that is used to assess the severity of the disease) (Thaci *et al*., 2015) (Miossec, 2017). Also, it can sustain its efficacy over time as compared to other biological treatments since Secukinumab has been demonstrated to have low immunogenicity and prevalence of detected anti-drug antibodies (Jabbar-Lopez *et al*., 2017; Mrowietz *et al*., 2015).

1.6.1 Secukinumab 's mechanism of action as the treatment of psoriasis

Secukinumab works in the treatment of psoriasis by neutralising and binding to aspects of the cytokine interleukin-17A (IL-17A). The pharmaceutical corporation Novartis, in their presentation on the 12th of September 2014 in an Advisory Committee Meeting of the Ophthalmic and Dermatological Drugs briefing, stated exactly how Secukinumab can treat psoriasis as it is a 'fully human monoclonal antibody which selectively binds and neutralises the pro-inflammatory cytokine interleukin-17A (IL-17A)' (Novartis, 2014: 2). They have described the therapeutic effects of Secukinumab when used in cases of psoriasis. Secukinumab completely neutralises the activity of IL-17A without affecting IL-17F or Th17 cells. Additionally, it has no direct influence on the individual's Th1 pathway. The ultimate result of this action is that skin histology returns to normal, and most patients experience clear skin recovery.

Based on the International Council for Harmonisation (ICH) Guidelines for the Preclinical Safety Evaluation of Biotechnology-Derived Pharmaceuticals (ICH S6(R1) 2011), genotoxicity studies have not been conducted for Secukinumab, which is acceptable to Committee for Medicinal Products for Human Use (CHMP, 2014).

Treatment with Secukinumab induced statistically significant reductions in the following parameters in the psoriatic lesional skin: acanthosis (hyperplasia of the epidermis); epidermal thickness; Ki67 (proliferative marker); Munro's abscesses (accumulation of polymorphonuclear leukocytes in the keratinous layer and a histological hallmark of psoriasis); parakeratosis (retention of nuclei in the stratum corneum); and MPO positive cell counts (reflecting polymorphonuclear leucocyte count). Reductions in several histopathological features which are typical for the lesional skin of psoriatic patients were observed after Secukinumab treatment at doses of between 1x3mg/kg and 3x10mg/kg paralleled by clinical PASI improvement (CHMP ,2014).

In a study, Secukinumab was evaluated in 36 patients with moderate-to-severe plaque psoriasis, in a proof-of-placebo-controlled concept, showing a significant increase in mean PASI 75 at week 4 (58% vs. 4%; p = 0.0001), with benefits maintained until week 12. In this study, fewer than 10% of patients experienced a recurrence until 15 weeks after the last dose, and no immunogenicity phenomena or reactions at the site were observed during administration, although in some cases, mild-to-moderate neutropenia without associated clinical manifestations was observed (Hueber *et al.*, 2010).

The focus on blocking IL-17/IL-17R in the treatment of psoriasis is based on the central role of this cytokine in the pathophysiology of the disease and the potential advantage of such a high specificity of action. This may significantly reduce any adverse effects and, in particular, serious infections. The guidelines for the prescription of agents in the treatment of psoriasis recommend the initial use of anti-TNFα and anti-inflammatory agents. However, if it occurs shortly after the approval of these new biological agents with a different mechanism of action, they are likely to become an essential therapeutic alternative, whether in psoriasis or other inflammatory diseases. As the scientific evidence becomes more robust and confirms its superior efficacy with a better safety profile as to the risk of infection, and eventually, disease neoplasticity given the biological agents already available, this new group of drugs could yet become a first-line therapy in the treatment of moderate-to-severe psoriasis (K.-A. Cho, Suh, Lee, Kang, & Woo, 2012).

As with all biotherapies, monoclonal antibodies (mAbs) may cause immunogenic effects in some patients, resulting in the development of some ADAs which may, in turn, lead to a loss of response and a variety of adverse incidences such as hypersensitivity reactions (Spindeldreher *et al.*, 2018). Various studies, discussed below, have assessed the likelihood of the production of ADAs in patients with psoriasis.

A study by Spindeldreher and colleagues evaluated the efficacy of IL-17A antibodies Ixekizumab and Secukinumab, which have only recently been approved, and two older biological treatments Ustekinumab and Adalimumab, side-by-side in one group of healthy donors (Spindeldreher *et al.*, 2018). The in vitro study was specifically conducted to discover if these four antibodies produce anti-drug T cell reactions and to calculate if they can develop ADAs in actual patients. The researchers found that their donors had fewer pre-existing T cells and demonstrated fewer T cell responses to Secukinumab than to Adalimumab and Ixekizumab. Additionally, the researchers found lower responses to the antibody Ustekinumab although the observed difference was not statistically significant. The researchers firstly concluded that there is a significantly lower chance of immunogenicity. Secondly, the results explain the lower incidence of clinical immunogenicity induced by Secukinumab as compared to other approved alternative therapeutic antibodies for plaque psoriasis treatment (Spindeldreher *et al.*, 2018).

A body of research (Maródi & Casanova, 2010; Whibley *et al.*, 2016) has shown that biotherapeutics may lead to a higher level of opportunistic infections in patients, since they block various critical mediators of both adaptive and innate immunity. IL-17A plays a part in the immune defence within mucocutaneous barrier tissues (Weaver, Elson, Fouser, & Kolls, 2013). A previous study (van de Kerkhof *et al*., 2016) has demonstrated that psoriasis patients do benefit from a 52-week specific treatment programme using Secukinumab. They had a low level of temporary, mild or moderate cutaneous and mucosal candidiasis, which was dependent on the dose of the drug. Such incidences were controlled by regular therapy and there were no resulting discontinuations in either phase 2 or phase 3 studies involving patients with either severe or moderate plaque psoriasis. There is a suggestion of associations between Secukinumab and latent tuberculosis infection but less common that other systemic treatment (*Elewski et al, 2021*).

Kammuller investigated the association between Secukinumab and the reactivation of latent tuberculosis infection, although this was not significant (Kammuller *et al*., 2017). They focused specifically on patients who received Secukinumab after testing positive for latent tuberculosis following screening or those who had pulmonary tuberculosis historically. Additionally, the researchers investigated the effect of using Secukinumab on dormant M tuberculosis mycobacteria and compared it to the antibody treatment known as anti-TNFx, which had a higher rate of activation of tuberculosis (Kammuller *et al*., 2017).

Kolbinger (Kolbinger *et al*., 2016) discussed factors which affect biotherapeutic immunogenicity, including structural homology in terms of sequences of amino acids in humans and posttranslational modifications. The elimination of foreign sequences of amino acids from antibodies lowers the occurrence of ADAs and patient hypersensitivity reactions (Nelson, Dhimolea, & Reichert, 2010). It is thought that human antibodies have a reduced immunogenicity potential as compared to chimeric constructs (Jullien, Prinz, & Nestle, 2015). Nevertheless, human antibodies, such as Adalimumab, and various human proteins, including erythropoietin and interferons, show high immunogenicity rates. A variety of factors impact immunogenicity, such as the dose amount and frequency, formulation of the product, and any impurities or aggregates

(Jullien *et al*., 2015). Furthermore, treatment and patient elements such as concomitant drug usage, disease and genetic background of patients, also create difficulties when comparing patient immunogenicity rates across clinical studies and dissimilar biotherapeutics.

In their study, Karle and colleagues (Karle *et al*., 2016) focused on evaluating the possibility of immunogenicity in the biotherapeutics of in vitro assays. The researchers obtained biotherapeutics from a pharmacy that were comparable to the drugs prescribed to patients. The researchers applied two distinct in vitro assays. One contained T cell activation and the other involved MAPPs which are known to be involved in the pathogenesis of the disease. Indicating an approximate correlation between in vitro assay results and clinical immunogenicity incidence which both confirmed the low immunogenicity of Secukinumab. (Karle *et al*., 2016).

The uses of Secukinumab and the phase of trial are shown in the Table below (Tabarkiewicz, Pogoda, Karczmarczyk, Pozarowski, & Giannopoulos, 2015)

Table 0-2. Use of Secukinumab and the phase of the trial

I	Psoriasis	Leonardi et al. (2008)
II	Psoriasis	Kasper et al. (2006)
II	Psoriatic arthritis	Chioato et al. (2012)
II	Rheumatoid arthritis	Krueger et al. (2007)
II	Psoriasis/rheumatoid arthritis/uveitis	Leonardi et al. (2008)
II	Crohn’s disease	Hueber et al. (2012)
III	Noninfectious uveitis	Dick et al. (2013)
I	Vaccinated healthy adults	Chioato et al. (2012)

1.7 Nanoparticles

Nanotechnology aims to manipulate the form and application of structures in the nanometre range. Thus, nanotechnology is a broad term that defines a rapidly evolving interdisciplinary field of technology based on manipulating matter at a sub-micron scale and embraces objects, mechanisms, assemblies, and various drug delivery systems. The prefix "nano" derives from the Greek word "nanos" meaning dwarf. There has been significant development in the nanotechnology field across different science systems, including medical science, biomedical imaging, chemical engineering, computing, materials science, agriculture, military applications and communications. Its application in medicine includes drug delivery, both in vitro and in vivo, and diagnostics (C. R. Martin, 2006).

Nanotechnology is a technique of nanostructure development, one that is used widely across the pharmaceutical and cosmetic industries. This is a technique in which designed nanostructures are assembled. Nanomaterials have been successfully used for different processes, including protein characterisation, biosensing, drug delivery, enzymes, and biometric assemblies (Nalwa, 2014). Many drugs have large side effects because they are delivered to normal cells as well as diseased cells. This has influenced many industries to develop a target drug delivery system which provides a more selective distribution of medications (Alavi, Karimi, & Safaei, 2017).

The European Technology Platform on Nanomedicine describes nanomedicine as the ‘application of nanotechnology to health. It exploits improved and novel physical, chemical, and biological properties of materials at the nanometric scale’. Nanomedicine has a potential impact on the prevention, early and reliable diagnosis and treatment of disease and thus is the systematic study of a specific system at the nano level (1-100nm). (Initiative, 2000). The US National Nanotech

Initiative defines nanomedicines as those drugs with particle sizes between 1 and 100nm. However, most of the literature accepts that nanomedicines can range from 500nm to a micron (Martin, 2006).

Nanotechnology provides further opportunities for material science research, medicine, biology and several other disciplines through the manipulation of single atoms and molecules in a certain manner that befits a specific application (Nie *et al.*, 2007). This similarity between nanostructures and biological molecules is significant in designing and manufacturing modern nano-assemblies with crucial biotechnological and medical applications (Yang *et al.*, 2015).

1.7.1 Properties of nanoparticles (NP)

NPs are found in between the form of the bulk material and the atomic structure of a compound. Although bulk materials show persistent physiochemical characteristics with their substituent NPs, they also show entirely diverse properties (Hutter & Maysinger, 2011). NPs are agglomerated, as they are held together by different kinetic forces, which affects the solubility of their component particles. As the size decreases to the nanometre scale, so the surface area to volume ratio increases. This enhances diffusion capacity and reactivity, particularly at high temperatures. Further, any reduction in size lowers the melting temperature of the particles and changes various other catalytic properties (Allouni, Cimpan, Høl, Skodvin, & Gjerdet, 2009).

1.7.2 Applications of nanotechnology

The use of NPs for the delivery of drugs is valuable due to their high surface-to-volume ratios, modifiable platforms, and smaller size. Nanomedicine has profited deeply from the exclusive properties of NPs. The minuscule size of nanoparticles is essential and critical to their application in drug delivery processes, as the carrier size controls the perfusion of materials through the endothelium and the diffusion of materials through tissues (Athar & Das, 2014). Scientists are considering the development of a multifunctional nanoparticle that could directly target specific cells (Brown *et al*., 2010). Due to ineffective lymphatic drainage, NPs favourably accumulate at the target site because of their reduced size. This could be useful when considering NPs in future treatments targeting specific cells and replacing existing therapies (Leary, Liu, & Apuzzo, 2006).

Other possible future medical uses of NPs include photodynamic therapy (Yamakoshi, Sueyoshi, & Miyata, 1999), surgery (Matteini *et al*., 2010) and cellular repair, etc. (Moghimi, Hunter, & Murray, 2005). However, commercially NPs are used in consumer products such as sunscreens, cosmetics, foodstuffs, external furniture polishes, surface glaze, paint nanofiltration, optics and the textile industry (DelaiáSun, 2010). Liposomes have proven highly versatile in their analytical, therapeutic and research applications, as they are biocompatible, biodegradable, and non-immunogenic.

Delivering drugs through the skin has many advantages, for instance evading first-pass metabolism, lower fluctuations in plasma drug levels, targeting of the active substance for the best local effect and good patient compliance. However, the barrier nature of skin makes it difficult for most drugs to penetrate. Therefore, a means of infiltrating the skin was needed. The topical delivery of drugs by lipid vesicles was developed (Mezei & Gulasekharam, 1980), demonstrating the efficacy of liposomes in skin-mediated delivery. The first commercialised therapeutic liposome treatment using lipid vesicles on the skin was econazole (an anti-mycotic agent). However, more recently it was found that classic liposomes do not penetrate the skin and, therefore, transdermal drug delivery is of no value since the drug remains in the upper layers of the stratum corneum (Natsheh *et al*, 2020).

1.8 Liposomes

The name liposome is derived from two Greek words, ‘lipos’ meaning fat and ‘soma’ meaning body. Liposomes are tiny, microscopic vesicles comprised of one or more concentrated lipid bilayers, usually separated by an aqueous channel. As such they are made of the same material as the cell membrane. Liposomes can encapsulate hydrophilic substances present in the aqueous medium and entrap hydrophilic vesicles by insertion or adsorption onto the membrane. Liposomes are composed of phospholipids (synthetic or natural), antioxidants, and sterols. The most used fatty acids in synthesising peptide-loaded liposomes are phosphatidylcholines, and these are extensively used in liposome formulation studies due to their excellent stability in a saline medium or upon exposure to variations in pH. Peptide-loaded liposomes are characterised by a phase transition temperature (Tc) wherein the membrane transforms from the gel phase into a crystalline phase. In the gel phase, the lipid bilayer's hydrocarbon chain is structurally organised. In contrast, within the liquid-crystal phase, the molecules within the lipid bilayer move relatively freely, and the hydrophilic group radicals achieve complete hydration (Filipczak, Pan, Yalamarty, & Torchilin, 2020).

Liposomes and lipid nanoparticles (LNPs) are similar by design, but slightly different in composition and function. Both are lipid nano formulations and excellent drug delivery vehicles, transporting cargo of interest within a protective, outer layer of lipids. In application, however, LNPs can take a variety of forms. LNPs are liposome-like structures especially geared towards encapsulating a broad variety of nucleic acids (RNA and DNA); and as such, they are the most popular non-viral gene delivery system. Traditional liposomes include one or more rings of lipid bilayer surrounding an aqueous pocket, but not all LNPs have a contiguous bilayer that would qualify them as lipid vesicles or liposomes. Some LNPs assume a micelle-like structure, encapsulating drug molecules in a non-aqueous core (Zabielska-Koczywąs, & Lechowski, 2017).

The permeability provides the exact measurements needed to determine the flow rate at which the solute separates from the aqueous medium through the bilayer. The permeability of peptide-loaded liposomes depends solely upon membrane fluidity and the nature of the solute. Research has determined that the widespread application of nanocarriers for transdermal delivery of drugs/genes has been successful (Das Kurmi, Tekchandani, Paliwal, & Rai Paliwal, 2017).

Long-circulating liposomes or long-term liposomes are synthesised in vivo and are obtained by different methods, including surface coating liposomes with naturally hydrophilic components like monosialoganglioside GM1 and phosphatidylinositol, or synthetic hydrophilic polymers, specifically polyethylene glycols (PEGs) (Daraee, Etemadi, Kouhi, Alimirzalu, & Akbarzadeh, 2016). Phospholipids offer great biocompatibility and a special amphiphilicity (have both lipophilic and hydrophilic properties). When presented within an aqueous environment, phospholipids self-assemble, generating different supramolecular structures, including liposomes. Phospholipids have good emulsifying properties, which can stabilise the resulting emulsions. Moreover, phospholipids act as surface-active wetting agents which can coat the surface of crystals to enhance the hydrophility of hydrophobic drugs. These unique characteristics make phospholipids suitable pharmaceutical excipients, and they have a vast range of applications in drug delivery systems (Li *et al*., 2015).

The hydrophilic surface layer of these polymers increases the circulation time of the liposomes, thus preventing the recognition and consequent association with the plasma opsonin (proteins act as tags to label particles in the body that should be phagocytosed by phagocytes), thereby inhibiting molecular recognition and capture by cells of the mononuclear phagocytic system (mainly Kupffer cells in the liver) (Figure 1-12). In an attempt to increase the specificity of the interaction of liposomes with receptors and increase the number of drugs released in these cells, research in this field has focused on the development of targeted, or site-specific, liposomes. The latter use ligands coupled to its surface, which provide selectivity to distribute the drug encapsulated in the desired action site (Vibeke Strand et al., 2017). The liposomal membrane can be altered through different lipids that facilitate the binding of a variety of recognition elements. Reactive lipid derivatives include those with an amino group that consists of phosphatidylethanolamine (PE), and a group hydroxyl, using cholesterol or polyethene glycol. Some examples of recognition ligands are antibodies, glycopeptides, polysaccharides, viral proteins, and lectins (Elain *et al*., 2014).

Due to its limitations in the treatment of psoriasis, most available therapies rely mainly upon alleviating symptoms and containing their progression to improve the clinical conditions of psoriasis patients. The treatment selected by the clinician will depend on many elements (such as the type of lesions; their extent and location; the patient's tolerance; and possible past treatment

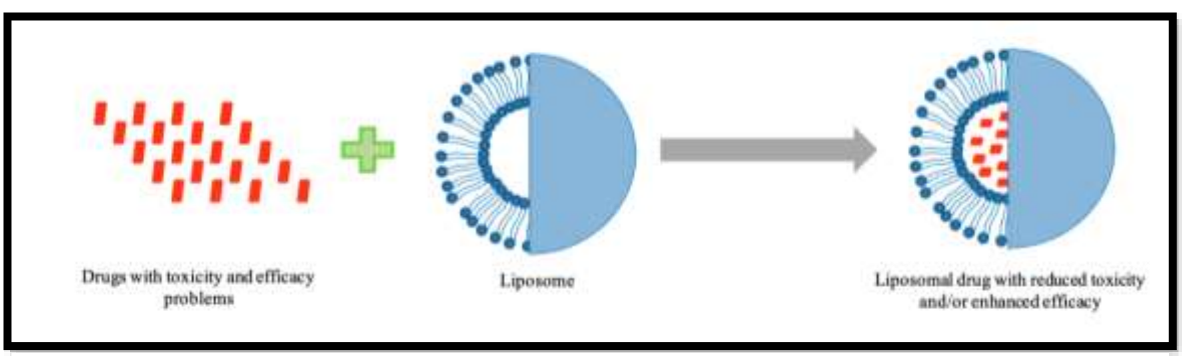

Figure 1-12. Schematic representation showing the advantages of formulating drugs in liposomes (Bulbake, Doppalapudi, Kommineni, & Khan, 2017)

failures). The treatments for psoriasis are often lifelong, owing to the chronicity of the condition, making the market highly lucrative for pharmaceutical companies and global healthcare organisations. Thus, discovering new therapies for the treatment of psoriasis presents a considerable and necessary challenge. The pharmaceutical industry is losing momentum in terms of the provision of novel agents, instead focusing on optimising pre-existing molecules by using innovative vector systems. One of the rapidly expanding areas in the pharmaceutical industry is the field of innovative drug vectors based on drug encapsulation. In recent years, relatively few new molecules have been discovered. Undeniably, consecutive failures have discouraged further investment. Currently, research initiatives are more oriented toward refining extant treatments, an approach that is cost-effective and safer for both the pharmaceutical companies and their patients.

Different advantages have been put forward, including the improvement of side effects by administering lower doses; improving the efficacy of novel drugs with reduced bioavailability or increased metabolism; and achieving better comfort for patients (repetitive doses, injections). Therefore, drug industries have provided a sustainable solution to many potential leading compounds to improve pathologies. However, they have also made it possible to rediscover therapeutic molecules with high potential, compounds which may have shown efficacy during preclinical trials but were prohibited due to their cytotoxicity during clinical trials. Table 1.3 shows different liposome-based products currently available on the market(Bulbake *et al.*, 2017).

Although liposomes are often referred to as nanoparticles, they differ from classical nanoparticles in both, their structure and in their stability . Liposomes are therefore not nanoparticles in a narrower sense. Nanoparticles are made of solid materials. Liposomes can be between a few nanometres to even 10 microns in size. They consist of certain lipids (phospholipids) together with other materials and form a hollow sphere consisting of one or more double membranes (bilayers).

They are filled with water and require a hydrophilic environment. Their bilayers are hydrophilic on the outside and also inside of the hollow sphere. They are usually less stable than nanoparticles.

1.8.1 Liposome Technologies for Delivery of Therapeutics

1.8.1.1 PEGylation Liposomes

In this technology, liposomes have been developed to make it difficult for the mononuclear phagocyte system to detect them by attaching them to PEG. This system provides less clearance and, therefore, remains in circulation longer (e.g. Doxil) (Veronese & Harris, 2002).

1.8.1.2 DepoFoamTM Liposome Technology

Drugs are encapsulated in a multivesicular liposomal platform without modification of their molecular structure, enabling them to be released over a prerequisite period. Mainly composed of synthetic analogues of naturally existing lipids (e.g., DOPC, DPPG, cholesterol). One example is Depocyt.

1.8.1.3 Lysolipid Thermally Sensitive Liposome (LTSL) Technology

Here lipids with a transition temperature of between 40-45°C are used to form the liposomes and, when applying heat, they transform from the gel to liquid phase, so allowing a 25-fold greater concentration of drug within the treatment area. An example of this technology is ThermoDox which is currently in phase 3 of its clinical trials (Slingerland, Guchelaar, & Gelderblom, 2012).

Table 1-3. Clinically used liposome-based products (Bulbake *et al.*, 2017).

SN	Clinical Products (Approval Year)	Administration	Active Agent	Lipid/Lipid:Drug Molar Ratio	Indication	Company
1.	Doxil® (1995)	i.v.	Doxorubicin	HSPC:Cholesterol:PEG 2000-DSPE (56:39.5 molar ratio)	Ovarian, breast cancer, Kaposi's sarcoma	Sequus Pharmaceuticals
2.	DaunoXome® (1996)	i.v.	Daunorubicin	DSPC and Cholesterol (2:1 molar ratio)	AIDS-related Kaposi's sarcoma	NeXstar Pharmaceuticals
3.	Depocyt® (1999)	Spinal	Cytarabine/Ara-C	DOPC, DPPG, Cholesterol and Triolein	Neoplastic meningitis	SkyPharma Inc.
4.	Myocet® (2000)	i.v.	Doxorubicin	EPC:Cholesterol (55:45 molar ratio)	Combination therapy with cyclophosphamide in metastatic breast cancer	Elan Pharmaceuticals
5.	Mepact® (2004)	i.v.	Mifamurtide	DOPS:POPC (3:7 molar ratio)	High-grade, resectable, non-metastatic osteosarcoma	Takeda Pharmaceutical Limited
6.	Marqibo® (2012)	i.v.	Vincristine	SM:Cholesterol (60:40 molar ratio)	Acute lymphoblastic leukaemia	Talon Therapeutics, Inc.
7.	Onivyde™ (2015)	i.v.	Irinotecan	DSPC:MPEG-2000:DSPE (3:2:0.015 molar ratio)	Combination therapy with fluorouracil and leucovorin in metastatic adenocarcinoma of the pancreas	Merrimack Pharmaceuticals Inc.
8.	Abelcet® (1995)	i.v.	Amphotericin B	DMPC:DMPG (7:3 molar ratio)	Invasive severe fungal infections	Sigma-Tau Pharmaceuticals
9.	Ambisome® (1997)	i.v.	Amphotericin B	HSPC:DSPG:Cholesterol:Amphotericin B (2:0.8:1:0.4 molar ratio)	Presumed fungal infections	Astellas Pharma
10.	Amphotec® (1996)	i.v.	Amphotericin B	Cholesteryl sulphate:Amphotericin B (1:1 molar ratio)	Severe fungal infections	Ben Venue Laboratories Inc.
11.	Visudyne® (2000)	i.v.	Verteporphin	Verteporphin:DMPC and EPG (1:8 molar ratio)	Choroidal neovascularisation	Novartis
12.	DepoDur™ (2004)	Epidural	Morphine sulfate	DOPC, DPPG, Cholesterol and Triolein	Pain management	SkyPharma Inc.
13.	Exparel® (2011)	i.v.	Bupivacaine	DEPC, DPPG, Cholesterol and Tricaprylin	Pain management	Pacira Pharmaceuticals, Inc.
14.	Epaxal® (1993)	i.m.	Inactivated hepatitis A virus (strain RGSB)	DOPC:DOPE (75:25 molar ratio)	Hepatitis A	Crucell, Berna Biotech
15.	Inflexal® V (1997)	i.m.	Inactivated hemaglutinine of Influenza virus strains A and B	DOPC:DOPE (75:25 molar ratio)	Influenza	Crucell, Berna Biotech

i.v. (intravenous); i.m. (intramuscular); HSPC (hydrogenated soy phosphatidylcholine); PEG (polyethylene glycol); DSPE (distearoyl-sn-glycero-phosphoethanolamine); DSPC (distearoylphosphatidylcholine); DOPC (dioleoylphosphatidylcholine); DPPG (dipalmitoylphosphatidylglycerol); EPC (egg phosphatidylcholine); DOPS (dioleoylphosphatidylserine); POPC (palmitoyloleoylphosphatidylcholine); SM (sphingomyelin); MPEG (methoxy polyethylene glycol); DMPC (dimyristoyl phosphatidylcholine); DMPG (dimyristoyl phosphatidylglycerol); DSPG (distearoylphosphatidylglycerol); DEPC (dierucoylphosphatidylcholine); DOPE (dioleoly-sn-glycero-phophoethanolamine).

1.9 Genotoxicity assay

Methods used in the assessment of genotoxicity (DNA damage), chromosomal instability, and gene and protein expressions in human whole blood and peripheral blood lymphocytes.

1.9.1 Genotoxicity assessment

As per our knowledge, genotoxicity studies were not conducted for Secukinumab. In addition, based on the ICH Guidelines for the Preclinical Safety Evaluation of Biotechnology-Derived Pharmaceuticals (ICH S6 (R1) 2011), genotoxicity studies have not yet been conducted for Secukinumab, which is acceptable to CHMP (2014).

Hence, our study will be the first to determine the genotoxicity of various concentrations of the drug in psoriasis patients as compared to healthy individuals.

1.9.2 Comet assay

The comet assay is a common test used to detect and quantify DNA damage and repair in individual eukaryotic cells (also known as single-cell gel electrophoresis, or SCGE). The name comes from the formation of a comet shape as a result of the negatively charged fragments of DNA that are pulled through an agarose gel in response to an electric field (Glei, Schneider, & Schlörmann, 2016). The comet assay technique was developed by Swedish scientists Ostling and Johanson (1984) to assess the effects of gamma-irradiation on mammalian DNA under neutral pH conditions. Later, Singh *et al.* (1988) discovered that the neutral pH condition limited the use of the assay to assess different types of DNA damage and, as such, mainly detected DNA double-strand breaks (DSB) (Andrew R Collins, 2004). In 1990, an alkaline assay was introduced for further modified and optimised by introducing electrophoresis under alkaline pH condition (pH >13) to detect both SSB and DSB (Olive et al. 1990; Tice et al. 2000). However, this was later contradicted by Andem et al. as they detected single-stranded breaks (SSB) under neutral pH conditions (Andem, Agbor, & Ekpo, 2013).

Since its modification, its use has dramatically increased and it has been successfully used to study DNA repair mechanisms, toxicology, environmental and human monitoring, etc. Genotoxicity studies using Comet assays have revealed several advantages for alkaline elution and DNA synthesis assays. These advantages include identifying minor DNA damage, its straightforward procedure, low cost, easy preparation of solutions, rapidity, and the relatively small sample required to conduct the test (Tice *et al.*, 2000).

Even though the Comet assay test is extremely sensitive in identifying single or DSBs and areas of alkaline lability, it is unable to identify crosslinks in DNA. Crucially DNA crosslinks can stabilise DNA and prevent its migration (Hartmann *et al.*, 2003). Tice *et al.* (2000) argued that

DNA crosslinks are an important element in mutagenesis and, consequently, additional work will need to be carried out in this field (Tice *et al*., 2000).

The comet assay technique is frequently utilised in molecular epidemiology to identify both carcinogenesis and oxidative stress in patients. However, lifestyle factors must be considered as confounding elements in research data, since both physical exercise and increased age increase DNA migration (Anderson *et al*., 2014); (A. R. Collins, 2014); (Hartmann *et al*., 2003).

The assay process commences with the application of 1% normal agarose to slides. Low melting point agarose and treated cells are mixed together and coated onto previous slides, and this is followed by an additional coating of agarose. Then the slides are laid out for five minutes on an ice-cold surface before entering a tray of lysis solution which consists of detergent material and salts. This is left overnight. The cellular compartments and membrane structure gradually dissolve. The DNA on the slides is unwind under alkaline conditions in an electrophoresis tank. When an electric field is applied, the DNA (with an overall negative charge) moves towards the positively charged anode. Undamaged DNA strands are too large and do not leave the cell’s centre unlike smaller damaged fragments, which migrate away from the nucleus.

A neutral buffer solution is applied to the slides three times to wash them. They are then stained with a dye such as ethidium bromide (EB). EB is an aromatic planer dye that readily intercalates between base pairs. Ten minutes later the slides are subsequently scored with the aid of a fluorescence microscope. The shape of the comet can then identify any DNA damage and migration occurring through the process of electrophoresis. The shape is determined by the speed at which DNA migrates from the DNA nucleus. The tail end of the shape contains DNA circles, and the thickness of the tail shows the extent of the DNA breaks, while the length of the tail indicates how long these DNA loops are (Collins, 2009). Both the OTM (Olive Tail Moment) and tail DNA are recorded as findings of the comet assay test. The OTM is defined as the product of the tail length and the fraction of the total DNA in the tail. The tail moment incorporates a measure of both the smallest detectable size of migrating DNA (reflected in the comet tail length) and the number of relaxed/broken pieces (as represented by the intensity of DNA in the tail) (Collins, 2009).

1.9.3 Genotoxicity assessment and the Comet assay

Genotoxicity is defined as a property of a chemical that brings harm to the DNA, abolishes its structure and function, and thus destroys its integrity as a store of genetic information. This damage causes mutations, leading to harmful effects, especially in the absence of an effective repair process, ultimately leading to carcinogenesis (Magdolenova, Lorenzo, Collins, & Dusinska, 2012). Eukaryotic cells have a very advanced and efficient internal system to counter any genotoxic substances and ROS. They are able to control their levels compatible with cellular integrity and maintain homeostasis using both enzymatic and non-enzymatic defence mechanisms (Schins, 2002). However, if genotoxicity is not prevented, either by repair systems or cellular death, the damage persists, continuing to mutagenicity and finally tumorigenesis. All genotoxic chemicals are not mutagenic, however, but all mutagenic substances are genotoxic and, therefore, mutagenicity is a worse property (Leitão, Al-Shaikh, Hassan, Hamadou, & Bach, 2017). Various genotoxic agents employ their own individual modes of action to cause mutagenicity, but the prime target of their mutagenicity is DNA. Mutations in DNA initiate cancer development and it is well known that compromised genetic material is associated with cancer progression. DNA damaging effects in populations are not the same as they are in individuals, since they have different capacities to detoxify genotoxic substances. Hence, cancer incidence within populations also differs depending on various factors, including differences in the efficiency of repair mechanisms and inheritance of specific genes (Felisbino *et al*., 2018).

The Comet assay is a sensitive and very suitable method to detect the genotoxicity of any substance and has been used for decades to assess and quantify DNA damage and repair (Gopalan *et al*., 2011; Najafzadeh, Reynolds, Baumgartner, & Anderson, 2009; Anderson *et al*., 2014).

1.10 Cytogenetic assay

1.10.1 Cytokinesis-Blocked Micronucleus (CBMN) Assay

The cytokinesis-blocked micronucleus (CBMN), also known as the micronucleus (MN) assay, is the most comprehensive technique used to detect and quantify the extent of DNA damage, cytotoxicity, and cytostatic events in cells (Fenech, 2007). The CBMN assay can detect and measure changes in both the structure and the number of chromosomes of dividing cells. For this reason, cells are prevented from dividing. Some of the whole chromosomes or chromosome fragments, therefore, lag at the anaphase stage or are simply unable to migrate effectively to the spindle during mitosis and subsequently become intertwined, leading to the formation of micronuclei (MNi).

1.10.2 The micronucleus assay - background and advantages

Micronuclei were consistently found once cells had been exposed to radiation and were assigned a host of different names and definitions. It was believed that micronuclei originate from acentric fragments which are extended from two daughter nuclei at a later stage of mitosis (Kirsch-Volders & Fenech, 2019). Micronuclei were discovered as a form of cytogenetic damage when the efficacy of gamma rays was being investigated versus neutrons in Vicia faba roots (Evans, Neary, & Williamson, 1959).

The development of the micronucleus assay to study cytogenetic damage was described decades ago by Countryman and Heddle in 1976. However, various flaws were observed by Michael Fenech in the assay due to some notable limitations. Fenech decided that this assay would only work in its optimal form when the identification of the divided cells was established. The short-lived binucleated stage of telophase is the only point where this can occur as only dividing cells can express micronuclei and so, only in dividing cells can chromosomal damage be detected. Therefore, there was a need to block cytokinesis to stop cells from completing their cellular division once completed in the nucleus. It was found that cytochalasin B, the most efficient type of cytochalasin, is able to stop cytokinesis in lymphocytes and other cells (Michael Fenech, 2009).

A micronucleus serves as a biomarker of DNA damage and occurs as a result of lesions at the chromosomal and protein levels, as its formation generally needs mitotic or meiotic division. There was limited information as to the understanding of MNi formation and the methods used to determine cell propagation and MNi generation. Hence, the validity of MNi as an in vitro genotoxicity assay was delayed until further developments were made to fully validate the assay. The Human MicroNucleus (HUMN) project aims to work towards its establishment and better understanding (Fenech, 2009).

The micronucleus assay is a highly reliable, simple, and appropriate procedure through which to investigate DNA damage at a chromosomal level, particularly when determining mutagenicity (Hovhannisyan, 2010). As such, it is presently the most frequent method used for the genotoxicity assessment of herbal extracts (Sponchiado *et al.*, 2016) and for scoring MNi frequency in lymphocytes. DNA damage or injury is scored specifically at the binucleated (BN) stage of cell division and comprises three components: Micronuclei (MNi) – biomarkers of chromosomal breakage and/or the loss of an entire chromosome; Nucleoplasmic bridges (NPBs) - biomarkers of DNA disrepair/telomere end-fusions; and Nuclear buds (NBUDs) – biomarkers of amplified DNA exclusion and/or DNA repair complexes. Cytotoxicity is measured through necrotic and/or apoptotic cell ratios while cytostatic events (inhibition of cell growth) are measured according to the proportions of mono-, bi- and multinucleated cells (Fenech, 2009).

1.11 Gene Expression assay

1.11.1 Polymerase Chain reaction PCR

PCR was discovered by the American biochemist Kary Mullis and gained the Nobel Prize in Chemistry in 1993. PCR is used to amplify small sections of DNA or a gene. This yields millions of copies which help in the study of DNA sequences or in detecting the presence or absence of a gene. PCR involves a process of heating and cooling DNA together with specific primers in a thermal cycler. There are three main stages. The first is denaturation: the DS template DNA is heated to 'melt' it into two single strands, then the temperature is lowered to allow the DNA primers to attach to the template DNA, a process called annealing. This is followed by an extension phase in which the temperature is elevated, and a new strand of DNA is made by the Taq (*Thermus aquaticus*) polymerase enzyme. These three stages are repeated up to 40 times, doubling the number of DNA copies during each cycle, giving rise to the term 'molecular photocopying'.

1.11.2 Quantitative reverse transcription PCR RT-qPCR

Quantitative reverse transcription PCR (RT-qPCR) is often used when the starting material is RNA. RNA is first transcribed into complementary DNA (cDNA) by reverse transcriptase from a sample of total RNA or messenger RNA (mRNA). The cDNA is then used as the template for the qPCR reaction as this cDNA is not subject to RNase degradation, making it more stable than RNA, and then the amplification of the PCR continues as described above. RT-qPCR is used for a range of purposes including gene expression analysis, RNAi validation, microarray validation, pathogen revealing, genetic testing, and other research (Ho-Pun-Cheung et al., 2009). RT-qPCR can be a single or two-step process as indicated in the following Table. For this research, the two-step process has been used (Table 1.4).

Table 1-4. Advantages and disadvantages of one-step and two-step reactions

	One-step	Two-step

advantages	• Both reactions take place in the same tube therefore less variation. • Less contamination due to less pipetting. • Easy processing of multiple samples for repetitive tests. • Faster	• A stable cDNA pool is made which can be stored for long periods and used for multiple reactions. • The target and reference genes can be copied from the same cDNA pool without multiplexing. • Flexible priming options
disadvantages	• Difficult to do two reactions together • Less sensitive • Detection of fewer targets per sample	• Higher risk of DNA contamination • Time-consuming • Needs more optimisation than one-step

1.12 Western blot

Western blotting (WB) is one of the most commonly used techniques for detecting specific proteins in a sample of tissue homogenate or extract. WB was initially described in 1979 by Towbin and colleagues (Towbin, Staehelin, & Gordon, 1992). However, the name was coined by Burnette (Burnette, 1981) two years later in 1981 as an acknowledgement of Southern blotting and because their lab was located on the west coast. Southern blotting is used to detect specific DNA molecules within an array of many other DNA molecules. WB is based on the separation of proteins via gel electrophoresis according to their molecular size, followed by protein transfer or blotting from gel to a suitable membrane (e.g. PVDF). This is then followed by probing with primary antibodies and detection with secondary antibodies, followed by visualisation with chemiluminescent substrates, after which the bands are quantified using appropriate software.

There are two methods, direct and indirect (Burnette, 2009). In this research, indirect WB was used as shown below.

Table 1-5. Two methods of Western blotting.

Direct WB	**Indirect WB**
Benefits • Only need one antibody • Avoids problems with secondary antibody cross-reactivity Drawbacks • Label may interfere with target binding • Possibility of false reading if antibody specificity for target is weak • Conjugated primary antibodies may be expensive • Limitation of options of conjugated primary antibodies	Benefits • Signal amplification by secondary antibody • Wide selection of conjugated secondary antibodies • Different primary antibodies can be used one secondary antibody • Use of secondary antibody does not reduce primary antibody target binding • Use of labelled secondary antibodies provides options for multiple detection methods Drawbacks • Non-specific staining may increase background • A longer procedure

1.13 Aims and Objectives of this research

- To determine the % cell survival after treatment with different doses of Secukinumab by using CCK8 and MTT.
- To assess the level of DNA damage on lymphocytes from psoriasis patients compared to healthy controls after treatment with Secukinumab bulk and liposome form at various doses by using Comet assay.
- To investigate the genotoxicologic impact of Secukinumab bulk compared to the liposome form on lymphocytes from psoriasis patients and healthy individuals
- To investigate the anti-oxidative effects of Secukinumab against H_2O_2-induced damage in lymphocytes from psoriasis patients compared to healthy individuals by using Comet assay.
- To determine the cytogenetic effects of Secukinumab bulk and liposome form lymphocytes from psoriasis patients and healthy individuals using the micronucleus assay.
- To determine the best concentration of Secukinumab to be used for preparing a liposomal form.
- To develop a new form of drug delivery system by transforming the bulk Secukinumab into the liposome form.
- To characterize liposome forms of Secukinumab to understand their physicochemical properties, such as the mean hydrodynamic diameter/ particle size, shape, agglomeration state, and surface charge in aqueous dispersion and in the dry state. We would achieve this objective using the Zetasizer Nano Instrument for Dynamic Light scattering (DLS) and

Zeta Potential (ZP), followed by an electron microscope using the Scanning Electron Microscope (SEM) and Transmission Electron Microscope (TEM), respectively.

- To evaluate the effects of Secukinumab bulk and liposomes on the gene expression of specific repair gene and their protein expression to GAPDH from psoriatic and the results compared with those from healthy individuals using the RT-qPCR and Western Blotting techniques, respectively.

Chapter 2. Materials and Methods

2.1 Materials

Table 2-1. List of materials and chemicals used for the current study

Chemicals and Reagents	**Company / Distributor**	**CAS Number**
Secukinumab	Novartis	_
Low melting point agarose	Invitrogen, UK	39346-81-1
Normal melting point (NMP)agarose Invitrogen, UK 9012-36-6	Invitrogen, UK	9012-36-6
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich, UK	67-68-5
EDTA (Na2EDTA.2H2O)	Sigma-Aldrich, UK	E5134
Ethidium bromide	Sigma-Aldrich, UK	E1510
Phosphate buffered saline (PBS)	Sigma-Aldrich, UK	N/a
Sodium chloride (NaCl)	Sigma-Aldrich, UK	7647-14-5
Sodium hydroxide (NaOH)	Sigma-Aldrich, UK	1310-73-2
Triton X-100	Sigma-Aldrich, UK	9002-93-1
Trizma base	Sigma-Aldrich, UK	77-86-1
RPMI-1640 medium	Sigma-Aldrich, UK	R8758
Distilled water(dH2O)	n/a	n/a
Eppendorf ® tubes (1ml)	Sigma-Aldrich, UK	n/a

Cover glass	VWR	631-0147
Superfrost microscope slides Slides	Thermo Scientific	10504182
FourErgoOne® single-channel pipettes	ErgoOne® Starter Pack 4	STARTERPACK42.5
Pipette tips	VWR	n/a
Black box	Sigma-Aldrich, UK	Z742411
Lithium heparin tube 9ml(LH)	Griener Bio-One, Austria	455084/A18033VS
Safety blood collection set Holder	Griener Bio-One, Austria	Lot:18C08
Fetal bovine serum	Sigma-Aldrich, UK	F7524
UltraPure™ Low Melting Point Agarose	Invitrogen (by Thermo Fisher ScientificTM), UK Fisher ScientificTM), UK	16520-050
2-Mercaptoethanol	Sigma-Aldrich, UK	60-24-2
Trypan blue	Sigma, UK	72-57-1
Potassium chloride	Sigma-Aldrich, UK	7447-40-7
penicillin-streptomycin solution	Sigma-Aldrich, UK	P4333
Glacial Acetic Acid	Fisher Scientific, UK	64-19-7
Mitomycin C	Sigma-Aldrich, UK	M0503
Phytohaemagglutinin	Sigma-Aldrich, UK	L1668
Sodium phosphate diabasic	VWR, UK	7558-79-4

Sodium phosphate monobasic	VWR, UK	7558-80-7
DPX Mountant	Sigma-Aldrich, UK	n/a
Ethanol	Sigma-Aldrich, UK	64-17-5
Giemsa stain	VWR	51811-82-6
Cell counting kit - 8 (CCK8)	Sigma-Aldrich, UK	96992F
96 well plates	VWR	10861-666
Falcon tubes	BD, Swindon, UK	n/a
SLS lab basics pipette filler	Scientific laboratory supplies (SLS)	B2B4830
Bradford Protein Assay Kit 1 Bio	Bio-Rad	5000201
Protease inhibitor cocktail	Sigma-Aldrich, UK	66701-25-5
Acrylamide 30%	Sigma-Aldrich, UK	79-06-1
Ammonium persulphate APS	Sigma-Aldrich, UK	7727-54-0
Recombinant Anti-Mutant p53 antibody	Abcam, UK	ab32049
ROR gamma (RORC) Human qPCR Primer Pair (NM_005060)	Origene ,UK	HP2308499 ROR gamma (RORC) Human qPCR Primer Pair (NM_005060)
Recombinant Anti-p21 antibody	Abcam, UK	ab109520
Donkey Anti-Rabbit IgG HandL	Abcam, UK	ab6802
Bovine serum albumin	Sigma-Aldrich, UK	9048-46-8
Bromophenol blue	Sigma-Aldrich, UK	115-39-9
Western ECL Substrate, 200 ml	Bio-Rad	1705060

FAST SYBR, GREEN	Fisher Scientific, UK	163795-75-3
iScript™ cDNA Synthesis Kit	Bio-Rad, UK	1708890
QIAamp® RNA Blood Mini kit	Qiagen	52304
Hs_ACTB_1_SG QuantiTect Primer(B-Actin) QuantiTect	Qiagen	QT00095431
P53 primer	Qiagen	QT00050785
Hs_BcL2_1_SG QuantiTect Primer assay	Qiagen	QT00025011
Hs_CDKN1A-va. 1-SG_1_SG(p21))000P))(((QuantiTect Primer assay (p21)	Qiagen	QT00062090
Mini Gel Tank	Thermo Fisher Scientific	A25977
Bolt™ 4-12% Bis-Tris Plus Gels	Thermo Fisher Scientific	NW04120BOX
20X Bolt™ MES SDS Running Buffer	Thermo Fisher Scientific	B0002
4X Bolt™ LDS Sample Buffer	Thermo Fisher Scientific	B0007
10X Bolt™ Sample Reducing Agent	Thermo Fisher Scientific	B0009

RIPA Lysis and Extraction Buffer	Thermo Fisher Scientific	89900
cytochalasin B	Sigma	14930

Table 2-2. Equipment used

Equipment and other Services	**Company / Distributor**
Amersham™ Hybond™ Polyvinylidene difluoride (PVDF) Blotting Membrane (0.45 µM x 150 mm)	GE Healthcare Life Sciences, Germany
BioDrop™ Touch Duo Spectrophotometer	BioDrop Ltd, Cambridge, UK
Bolt™ Welcome Pack + iBlot™ 2 System	Thermo Fisher Scientific
BRAND® Filter Flask with lateral socket / Vacuum glass bottle	Sigma-Aldrich, UK
BRAND® Staining Trough / incubation box with tray	Sigma-Aldrich, UK
Centrifuge Mistral 3000	MSE, Albertville, USA
Centrifuge (biofuge 28 RS)	Heraeus, Sepatech, Germany
CCD camera	Hitachi KPMI/EK Monochrome, Japan
Coplin jar	VWR, Lutterworth, UK
Corning® 15 mL centrifuge Tubes	Sigma-Aldrich, UK
Corning® cell culture flasks (25 cm2)	Sigma-Aldrich, UK
Culture flasks (25and75 Cm^3)	Corning Incorporated Costar®, NY, USA
Dry incubator (37º C) LKB BIOCHROM	Leec LTD, Nottingham, UK
Electrophoresis power supply	Consort (E861), Belgium

Electrophoresis tank (HU20)	Scie-Plas, Renfrewshire, UK
Falcon tubes	BD, Swindon, UK
Freezer -20º C	Sanyo, Ultra-low, Japan
Freezer -80º C	Sanyo, Ultra-low, Japan
Fluorescent microscope	Leica, Weztler, Germany
Fume cupboard	Milton, UK
Fume hood ray air	Maiche Aire, Bolton, UK
Ice maker (Scotsman AF 100)	Namur, Belgium
Incubator 37º C with 5% CO_2	Andor Technology Ltd, Belfast Heraeus, Germany
Light microscope	Nikon, Japan
Komet 6 software	Kinetic Imaging, Nottingham, UK
Microcentrifuge MSE	GMI, Alberville, USA
Microplate reader	Dynex technology, Sussex, UK
Microscope (ortholux)	Leitz, Sturttgart, Germany
Mini Gel Tank	Thermo Fisher Scientific, UK
Pipettes	Gilson, Middleton, WI, USA
pH meter	Dunmow, UK
Powerpack supply	Pharmacia LKB, Uppsala, Sweden
Water bath	Grant instruments, Cambridge, UK

2.2 General methods

2.2.1 Ethical Approval

Ethical approval has been granted for studying genetic and environmental effects on lymphocytes from different cancerous, precancerous and inflammatory conditions using various genetic endpoints (as reviewed by Leeds East Research Ethics Committee (REC) Ref. No. 12/yh/0464). All peripheral blood samples were collected after informed consent from healthy individuals and patient groups.

2.2.2 Recruitment of volunteers

Every participant, to each whom we are indebted for their cooperation, as this project would be impossible to accomplish without their help, was given an information sheet consisting of two paper parts (see Appendix 1 and 2**)** and these were explained along with any verification required:

Part 1: Informing the participant, the purpose of study and the reason why they had been invited, followed by an emphasis on the voluntary nature of participation.

Part 2: Providing more detailed information about what would happen to participants if they took part in the study, noting that study tests were non-predictive for individual participants; an explanation of which people could not take part in the study; and whom to contact for queries.

Healthy volunteers (males and females, aged between 18 and 70 years) from mixed ethnicities were recruited from staff and students at the University of Bradford with some residents of Bradford and Leeds taking informed consent. They were recruited between August 2018 and January 2021.

For healthy normal volunteers, the inclusion criteria were:

- A willingness to complete the informed consent form.
- Agreeing to have blood samples taken or stored at the University of Bradford.
- Data collected might be examined by individuals from the NHS Trust and the University of Bradford Sub-Committee for the healthy Individuals.
- Free from any medical disease such as cancer or any inflammatory condition.
- Not presently receiving any medications.

Patients with psoriasis were recruited from the clinic of Dr Nader Ghadari at St Luke's Hospital, Bradford, West Yorkshire, UK. Inclusion criteria were:

- A willingness to complete the informed consent form.
- Agreement to have blood samples taken or stored at the University of Bradford.
- Any data collection might be assessed by individuals from the NHS Trust or researchers at the University of Bradford.
- Have been clinically diagnosed as having psoriasis.
- Not presently on any biological treatment.

Confounding factors such as age, gender, ethnicity, and cigarette smoking habits were taken into consideration for both groups.

2.2.3 Blood Collection

Whole blood was collected from 20 healthy individuals and 20 patients by venipuncture after informed consent and stored in 9 mL heparinised tubes (Vacuette®, LH Lithium Heparin). Their basic information about health and lifestyle factors was taken using a questionnaire completed by the volunteers. Once collected, the whole blood was diluted 1:1 with RPMI 1640 media and supplemented with 10% dimethyl sulphoxide (DMSO). The resulting suspension was aliquoted into Eppendorf tubes and stored at -80°C for use in the Comet assay, whereas freshly collected whole blood was used for the micronucleus assay, PCR and western blot. Information about the blood samples derived from healthy individuals and patients with psoriasis may found in Tables 2.3 and 2.4, respectively.

Table 2-3. Brief information on healthy individuals

No	**Age**	**Ethnicity**	**Gender**	**Smoking history**
1	33	Western	F	Non-smoker
2	34	Asian	M	Past smoker
3	20	Asian	F	Non-smoker
4	42	Asian	F	Non-smoker
5	21	British	F	Non-smoker
6	20	Asian	M	Non-smoker
7	21	Asian	M	Non-smoker
8	22	Asian	F	Non-smoker
9	21	British	F	Non-smoker
10	22	Asian	F	Non-smoker
11	24	Asian	F	Non-smoker
12	35	Western	F	Non-smoker
13	37	Western	M	Non-smoker
14	40	Asian	M	Non-smoker

15	39	African	F	Non-smoker
16	33	Asian	M	Non-smoker
17	39	Caucasian	M	Non-smoker
18	28	Caucasian	M	Non-smoker
19	35	Arab	Male	Non-smoker
20	48	Arab	Male	Non-smoker

Table 2-4. Brief information of the patient group

No	**Age**	**Ethnicity**	**Gender**	**Smoking history**
1	31	Caucasian	M	Ex-smoker
2	41	Asian	M	Non-smoker
3	65	Caucasian	F	Ex-smoker
4	64	Asian	F	Non-smoker
5	23	Caucasian	F	Non-smoker
6	47	Caucasian	M	Ex-smoker
7	50	Caucasian	M	Non-smoker
8	35	Caucasian	F	Non-smoker
9	38	Caucasian	M	Smoker
10	80	Caucasian	F	Non-smoker
11	64	Caucasian	M	Non-smoker

12	62	Caucasian	F	Non-smoker
13	44	Caucasian	M	Non-smoker
14	80	Caucasian	F	Ex-smoker
15	53	Caucasian	F	Non-smoker
16	63	Caucasian	F	Ex-smoker
17	35	Caucasian	M	Smoker
18	55	Caucasian	F	Non-smoker
19	42	Caucasian	F	Non-smoker
20	35	Asian	M	Non-smoker

2.2.4 Preparation of the chemicals

Secukinumab solutions were prepared by dissolving the drug in distilled water (150mg/ml). The drug was provided by Novartis with the help of Professor Andrew Wright. A 75µg/ml stock solution was prepared.

2.2.5 Determining the concentration of chemicals

The optimal concentration of hydrogen peroxide was determined using a concentration-response curve. Different concentrations of hydrogen peroxide (25, 50, 75 and 100 µM) were used and 75 µM was found to be the concentration that caused the highest levels of DNA damage without inducing apoptosis. This was thus used as a positive control (PC) throughout the research study. The optimal concentration of Secukinumab (2.1µg/ml) was also determined using the dose-response curve, i.e., the one that caused the least DNA damage and produced maximum viability.

2.2.6 Preparation and characterisation of peptide-loaded Liposome

Liposomes were prepared using the thin film rehydration method. DPPG (1,2-Dipalmitoyl-sn-glycero-3-phosphoglycerol), DOPE (1,2-Dioleoyl-sn-glycero-3-phosphoethanolamine) and cholesterol were dissolved in DCM (Dichloromethane) and methanol (3:1 v/v) and the resulting solution was transferred to a rotary evaporator flask. The organic solvent was evaporated at 40°C under vacuum for 6 hrs to ensure that no traces of organic solvent remained. The thin film was then hydrated by adding 10 ml of distilled water mixed with 1ml of Secukinumab for 1 hr at 60°C using a bath sonicator (150 W). The sample was then subjected to 4 repeated freeze-thaw cycles (freezing at -20°C and thawing at 60°C in the bath sonicator). The final solution was diluted to 75μg/ml using distilled water. This was done with the help and instruction of Dr Mohammad Isreb.

The average size and poly-disparity index (PDI) of the liposome preparations were determined by dynamic light scattering (DLS) using a Zetasizer ZS (Malvern Instruments, UK). All measurements were performed in triplicate. Measurements were found to be <800 nm.

2.2.7 Lymphocyte isolation

Human PBLs (peripheral blood lymphocytes) from healthy individuals and patients were isolated from whole blood using the Lymphoprep TM density gradient centrifugation method with a slight modification (Boyum, 1986; STEMCELL Technologies 2017). Human whole blood was diluted 1:1 in NaCl (0.9%) and gently mixed (See Figure 2.1). 3 mL of LymphoprepTM was added to a 15 mL Falcon tube together with 6 mL of the diluted blood sample. The blood-NaCl mixture was carefully layered on top of the LymphoprepTM without disturbing the mixture. The Falcon tubes were centrifuged at 1,900 rpm (800 x g) for 20 min at room temperature (RT) leading to the formation of four different layers of blood components. The red blood cells (erythrocytes and granulocytes) descended to the bottom; then came the LymphoprepTM layer; followed by a layer of white blood cells (lymphocytes and monocytes) and platelets; and finally, a supernatant layer of plasma.

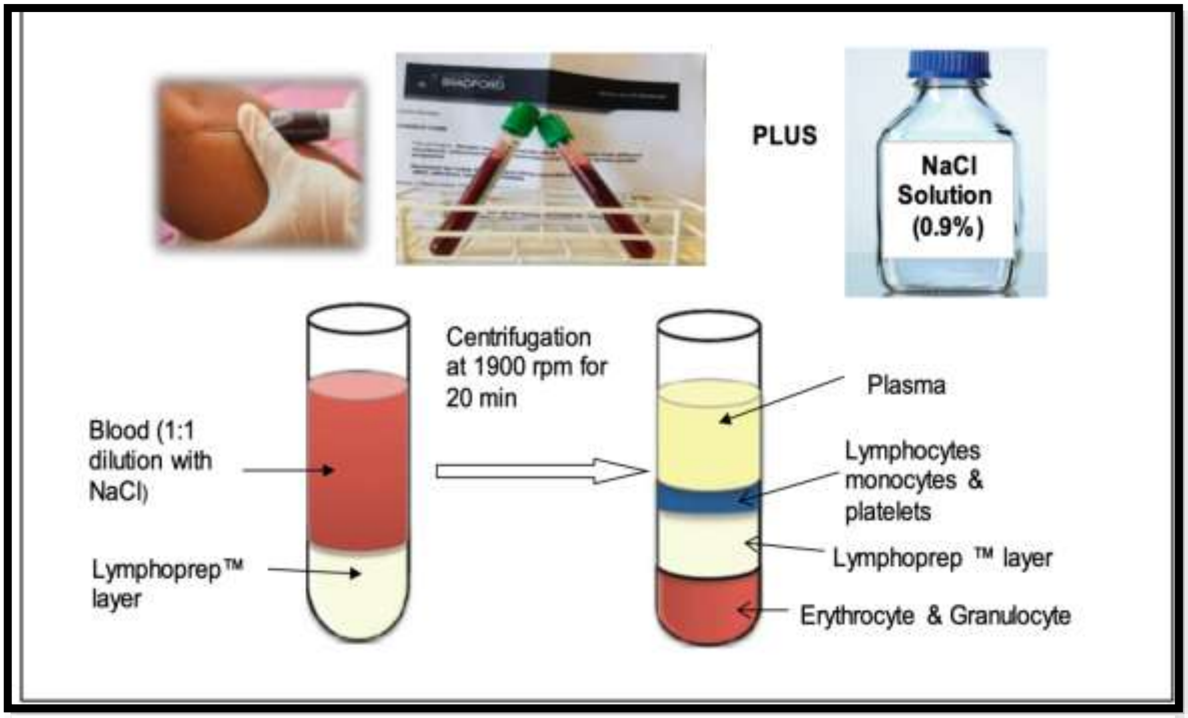

Figure 2-1. Schematic illustration of isolation of lymphocytes from human whole blood using density gradient LymphoprepTM (adapted Bharadwaj et al., 2012)

The cloudy, buffy coat layer containing the white blood cells (lymphocytes and monocytes) and platelets (immediately above the LymphoprepTM layer) was then carefully collected from the interface using a Pasteur pipette. The lymphocyte-containing layer was transferred into a 15 mL universal tube pre-filled with 10 mL of 0.9% NaCl. The universal tube containing the lymphocyte-NaCl mixture was then centrifuged at 1,500 rpm (630 x g) for 15 min at RT to concentrate the cells. The resulting supernatant was discarded, leaving the pellets on the base of the tube undisturbed which were collected in an Eppendorf.

2.2.8 Cell Counting with Haemocytometer

A 10 μL cell suspension was mixed with Trypan blue stain at a 1:1 ratio and then pipetted and transferred into a Neubauer haemocytometer. Cells were observed under an inverted microscope using 10x eyepiece lenses at a 100x magnification (objective lenses) and cells were counted from 4 big squares covering each of the 4 corners [1 square = 1 mm; = 16 smaller squares of 0.05 mm (= 2.5 x 10^{-3} mm^2)]. If the cell membrane is weak, then the Trypan blue penetrates the cell membrane and gives a blue colouration, indicating cell death (Chan, Rice, & Qiu, 2020). Therefore, the percentage of viable cells was counted using a Neubauer haemocytometer under the microscope. The concentrations with a cell viability of ≥75% were used in all experiments (Avelar-Freitas *et al.*, 2014).

2.2.9 Determination of cell concentration

An average of the four readings was taken and the result (**a**) was multiplied by 10^4 cells/μL to give the concentration of cells in the cell-RPMI mixture. That is, C1 = **a** x 10^4 cells/μL; where **a** = average cell counts, and 10^4 cells = the standard number of cells in a 1 square volume of the haemocytometer.

Overall, the following equation was used:

$$C1V1 = C2V2 \text{ or } V1 = {}^{\boldsymbol{C2V2}}/_{\boldsymbol{C1}}$$

Where: V1 = unknown volume of the lymphocyte-RMPMI-1640 mixture we require for incubation and treatment with chemicals.

C2 = 20 x104 cells/μL (a standard cell concentration).

V2 = 1 mL (1,000 μL - a standard final volume).

C1 = known concentration (a) of lymphocytes counted earlier.

2.2.10 Cell Viability determination

The cell counting kit 8 from VWR was used to measure the viability and cytotoxicity of the lymphocytes. It is an efficient one-step method for obtaining highly reliable and accurate results. Cell cultures were prepared by adding thawed blood samples in RPMI-1640 medium and the chemicals in Eppendorf tubes were as described later in the comet assay treatment step. The tubes were centrifuged for 5 mins at 3,000 rpm (705 g) and the resulting supernatant was discarded. The remaining cell pellet was resuspended by gently patting the tubes. The contents of the kit were directly added to the cell suspension and the procedure was followed according to the manufacturer's protocol (Appendix 7). The treatment groups which produced viability >80% were considered for use in the current research study. The first step was achieved by cell inoculation (100 μl/well) in a 96-well plate and the plate was pre-incubated in a humidified incubator (37 °C, 5% CO_2). Secondly, 10 μl of the CCK-8 solution was added to each well of the plate (avoiding bubbles as they interfere with the reading). The plate was incubated for 4 hrs. Finally, the absorbance was measured at 450 nm using a microplate reader (triplicate readings were used).

2.2.11 MTT Cell Proliferation /Cell survival Assay

The MTT assay, developed by Tim Mossman, is a colourimetric assay that measures the metabolic activities of mitochondria. The MTT assay was chosen because it is a specific and sensitive test used in drug screening due to its ability to give linearity of results over a wide range of cell densities and cytotoxic risks (Niles, Moravec, & Riss, 2009). As the cells undergo metabolic redox reaction, the mitochondrial reductase enzymes convert the soluble tetrazolium MTT (3- (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) dye from its yellow form into insoluble, purple formazan crystals. This procedure was performed according to the manufacturer's protocol from Abcam (Appendix 8).

Human lymphocyte-RPMI mixtures pipetted into four wells (2, 3, 4, and 5) were treated with 3 different concentrations of Secukinumab, while the 5th well, the negative control (NC), was treated with fresh RPMI-1640 (1X) medium supplemented with glutamine (GlutaMaxTM), plus 15% heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin. After that, they were incubated overnight for 24 hrs in an incubator (37°C, 5% CO_2) and the cells were allowed to adhere to the base of the plate.

After an overnight incubation, the cells were transferred into Eppendorf tubes and centrifuged at 1,000 rpm (≈ 418 x g) for 5 min to concentrate the cells and the resulting medium was discarded. The pellets were resuspended in 100 μL of fresh RPMI-1640 medium and then transferred in triplicate into a 96-well plate to commence treatment with the MTT dye. The first wells of the 96-well plate were loaded with 100μL RMPI-1640 medium to serve as the NC/ blank (with no cells), while the remainder of the wells each contained 100 μL of cells in triplicate. Ten microlitres (10 μL) of 12 mM MTT stock solution were added to each well to differentiate between genotoxicity and cytotoxicity in the pathogenesis of DNA DSB. Thereafter, they were incubated at 37°C for 4 hrs, followed by removal of all medium save for 25 μL per well. After that, the MTT dye absorbed by the cells in each well was solubilised by adding 50 μL of Dimethyl sulfoxide (DMSO) and thoroughly mixed by gentle pipetting or a vortex mixer. They were incubated at 37°C for 15 min and absorbance readings were taken immediately.

The amounts of MTT dye absorbed by cells are directly proportional to the proliferative activities of the cells. They were quantified by measuring the absorbance readings at 540 nm using the MultiscanTM FC Microplate reader. A calculation of the percentage of cell survival was performed (Figure 2.2). First, the corrected absorbance readings were obtained by subtracting all the background readings (i.e., absorbances of empty wells) from each of the absorbance readings from treated cells (NC, 2.1, 2.8, and 3.751 μg/mL, respectively), followed by an averaging of the triplicate readings (n=3). The control readings were obtained from the absorbance readings of the first wells containing only the RPMI medium (with no cells) and MTT stock solution. The corrected absorbance values are proportional to the number of proliferating cells. The % cell survival rates, a measurement of metabolic activity and cytotoxicity, were calculated using the following equation:

$$\%\ \text{Cell survival} = \frac{Absorbance\ (Treated\ Cells)}{Absorbance\ of\ Control\ (Medium\ only)} \times 100$$

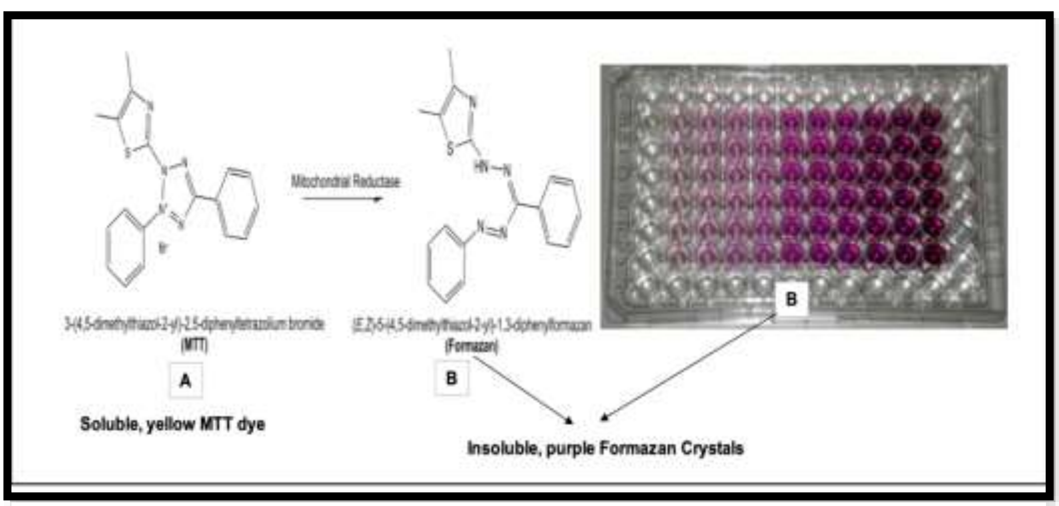

Figure 2-2. Principle of MTT assay Conversion of soluble, yellow tetrazolium MTT dye (A) to insoluble, purple Formazan crystals (B) by reductase enzymes inside the mitochondria of living cells (Shinryuu 2010)

2.2.12 Statistical Analysis

Percentage cell survival rates in the MTT Assay and CCK8 of treated lymphocytes (healthy, and psoriatic/ patients) were plotted against different concentrations of the drug, while the untreated lymphocytes (NC = 0 µg/ml) had a 100% absorbance. Graphs were plotted using GraphPad Prism® software version 9 (Fay Avenue, La Jolla, CA, USA), and statistical analysis was performed using the same software with a built-in One-Way ANOVA and Dunnett's post-hoc multiple comparison tests to determine any significant differences in the cytotoxicity of treated lymphocytes relative to controls.

2.2.13 The Comet assay

2.2.13.1 Cell Treatment

Blood samples collected from 20 healthy individuals and 20 patients were supplemented with RPMI medium and 10% DMSO and immediately stored at -80°C. To start the experiment. A total of 100µl of each thawed blood sample was added to an Eppendorf tube along with 890 RPMI-1640 media and 10µl of the respective treatment compounds. Different treatment groups were used to conduct the experiment, including a NC (untreated), which only contained the blood sample and RPMI 1640 medium. The first treatment was the PC containing media, blood, and a total of 10µl 75µM/ml H_2O_2, while the others were two treatment groups containing different concentrations of Secukinumab (2.8µg /ml, 2.1µg/ml) with or without H_2O_2. Once all contents had been gently mixed, the Eppendorf tubes were incubated at 37°C for thirty minutes. Following incubation, the tubes were centrifuged for three minutes at 3,000 rpm (705 g). A total of 960 µL supernatant was removed from each Eppendorf and 100µl of low melting agarose was added to the cell pellet and mixed thoroughly. 100µl of the cell suspension was dropped onto the precoated slides with 1% of

(NMP) low melting point agarose. Coverslips were applied and the slides were stored on an ice plate for approximately 5 minutes to settle the gel. Then this was repeated with bulk and liposome forms of the drug.

2.2.13.2 Lysis

Once the agarose was set, cell lysis was commenced by the removal of the coverslips and immersion of the slides in a black box that contained a freshly prepared lysis buffer (2 ml of %Triton X-100, 178 ml of lysis stock solution plus 20 ml of 10% DMSO) at pH. Slides were then stored at 4°C overnight to dissociate protein bonding. The lysis stock solution contained 100 mM EDTA, 2.5 mM NaCl, 10 mM Trizma base.

2.2.13.3 Electrophoresis

The next step of the process involved electrophoresis, whereupon slides were placed horizontally on the electrophoresis gel tank and incubated in freshly made, cold alkaline solution (60ml 10M NaOH enabling alkaline pH >13 and 10 ml 200mM EDTA) for 30 minutes at 4°C to unwind the DNA. A power supply of 300 A at 25 volts (0.78 V/cm for the electrophoresis tank) was then applied and the electrophoresis was run for another 30 minutes. The electrophoretic field draws the DNA fragments and single strands away from the nucleus and towards the anode (Liao, McNutt, & Zhu, 2009). Under alkaline conditions (pH>13), the double-stranded DNA is denatured and becomes single-stranded.

2.2.13.4 Neutralisation

The slides were removed from the electrophoresis buffer and washed three times for five minutes in neutralisation buffer (0.4M Tris, pH 7.5).

2.2.13.5 Staining the slides

A total volume of 60µl of the EB stain solution (20µg/ml) was added to the slides to stain and visualise the DNA in a dark room, and coverslips were placed over the slides.

2.2.13.6 Scoring of the slides

All microscope slides were correctly coded as per the treatment groups. 100 cells were counted from each slide using a fluorescence microscope equipped with a CCD camera and connected to a computer terminal. Data were produced by randomly scoring the cells at a magnification of 20X using the two established parameters of the comet assay, the OTM and % tail DNA on Komet 6 software (Kinetic Imaging).

2.2.13.7 Statistical analysis

The data were analysed via one-way ANOVA and t-tests to determine the significant values, wherein a P-value of <0.05 was considered statistically significant. Analysis was performed on Graph Pad Prism 8.

2.2.14 Cytokinesis block micronucleus assay (CBMN)

Fresh blood samples were obtained from healthy individuals and the patient group for the micronucleus assay.

2.2.14.1 Medium preparation

A fume hood was used for all the culture steps under sterile conditions. The culture medium was prepared by adding the RPMI 1640 (25mM HEPES and L-Glutamine) to 1% penicillin-streptomycin and 15% FBS.

2.2.14.2 Cell culture

The basic cell culture medium, 4.5ml, was added to 25cm^3 vented cap Corning flasks as per the treatment protocol and stored at -20°C for later use. When needed, flasks were equilibrated at 37°C in the presence of 5% CO_2 for 30 minutes.

2.2.14.3 Culturing blood and treatment

130µl of phytohaemagglutinin (PHA) and 350µl of fresh blood sample were added to the flasks containing the basic medium and were slowly shaken to mix the ingredients. The flasks were left to incubate for 24 hrs at 37°C in the presence of 5% CO_2.

After the incubation period, 50µl of the test chemicals (Secukinumab and H_2O_2) were added to each flask. A basic medium was used for the untreated NC, with Mitomycin C (0.4µM) and H_2O_2 (75µM) serving as the positive controls. The blood cultures were incubated under the same conditions for another 20 hrs.

After treatment, 30µl of cytochalasin B (cyt B at 1mg/ml) was added to each culture to block cytokinesis but not mitosis at 44 hrs. Flasks were incubated again for 24-28 hrs after the addition of cyt B. At 72 hrs, the cultures were removed from incubation and all contents were carefully transferred to 15ml Falcon tubes as sterile conditions were no longer required.

The tubes were taken to the centrifuge tank and centrifuged at 800 rpm for 8 mins. The supernatant was aspirated from the tubes using a vacuum pump until only 500µl remained. Then 5 ml of chilled KCl was added to each tube to cause hypotonic shock while being vortexed. The resulting lysate was incubated at 4°C for 15 mins. After incubation, the tubes were centrifuged, and the supernatant was aspirated.

2.2.14.4 Fixation

First, 5 ml of freshly prepared Carnoy's solution (1 part glacial acid and 3 parts methanol) was added to the tubes while vortexing. This was followed by the addition of 3 drops of 38% formaldehyde to each tube. The contents were mixed using a plastic Pasteur pipette. The tubes were then centrifuged, and the supernatant discarded. The remaining cell pellet was resuspended, and these steps were repeated twice without adding formaldehyde. The tubes were left overnight at 4°C.

2.2.14.5 Slide preparation

The following day, the Falcon tubes were centrifuged to remove the supernatant and the tubes were aspirated until only 100µl of the precipitate remained. Carnoy's solution (100 to 500µl) was mixed together with the cell suspension according to the cell density and the size of pellet. Then, the cell concentration was adjusted as desired and 2 x 20µl of the suspension was dropped on a plain, pre-labelled microscope slide. Two slides were prepared for each treatment group and left overnight at room temperature to dry.

2.2.14.6 Staining

The next day, the slides were stained with freshly prepared 5% Giemsa solution for 20 mins and then carefully washed with distilled water for a couple of minutes and left to air dry. To preserve the slides, three drops of DPX mounting solution were applied to each slide and coverslips added.

2.2.14.7 Micronuclei (MN) scoring

The frequency of MNi was determined by scoring 1,000 cells per treatment group under 40X magnification using a light microscope according to the criteria adapted from Fenech (2007). The

MNi frequency in binucleated and mononucleated cells, NDI, percentage of binucleated cells per 1,000 cells scored, and other parameters of the micronucleus assay were also determined.

2.2.14.8 Statistical analysis

The experiment was repeated three times for 5 different individuals selected at random from both the healthy and patient groups. Normality was performed to determine the differences arising between various treatment groups. All data were analysed on GraphPad Prism 8 using one-way ANOVA and t-tests.

Box 1. Scoring criteria

Criteria for scoring viable mono-, bi- and multinucleated cells

Mono-, bi and multinucleated cells are viable cells with an intact cytoplasm and normal nuclear morphology containing one, two, or three or more nuclei, respectively. They may or may not contain one or more MNi or NBUDs and, in the case of bi- and multinucleated cells, they may contain one or more NPBs.

Criteria for scoring apoptotic cells

Early apoptotic lymphocytes can be identified by the presence of condensed chromatin within the nucleus and intact cytoplasmic and nuclear membranes.

Criteria for scoring necrotic cells

Early necrotic cells can be identified by their pale cytoplasm, the presence of numerous vacuoles (mainly in the cytoplasm and sometimes in the nucleus), damaged cytoplasmic membrane and a fairly intact nucleus.

Criteria for selecting BN cells suitable for scoring MNi, NPBs and NBUDs

The cells should be binucleated. The two nuclei in a binucleated cell should have intact nuclear membranes and be situated within the same cytoplasmic boundary. The two nuclei in a binucleated cell should be approximately equal in size, staining pattern and staining intensity. The two nuclei within a BN cell may be attached by a nucleoplasmic bridge no wider than $1/4^{th}$ of the nuclear diameter. The two main nuclei in a BN cell may touch but ideally should not overlap each other. A cell with two overlapping nuclei can be scored only if the nuclear boundaries of each nucleus are distinguishable.

Criteria for scoring micronuclei

MNis are morphologically identical to, yet smaller than nuclei. The diameter of MNis in human lymphocytes usually varies between $1/16^{th}$ and $1/3^{rd}$ of the mean diameter of the main nuclei. MNis are non-retractile and they can therefore be readily distinguished from artefact. MNis are not connected to the main nuclei. MNis usually have the same staining intensity as the main nuclei but occasionally staining may be more intense.

Criteria for scoring nucleoplasmic bridges

An NPB is a continuous DNA-containing structure linking the nuclei in a binucleated cell. NPBs should also have the same staining characteristics as the main nuclei. On rare occasions, more than one NPB may be observed within one binucleated cell. A binucleated cell with an NPB may contain one or more MNis.

Criteria for scoring nuclear buds

NBUDs are similar to MNis in appearance, with the exception that they are connected with the nucleus via a bridge. NBUDs usually have the same staining intensity as MNis.

Calculation of the NDI

NDI is calculated using the formula NDI = (M1 + 2M2 + 3M3 + 4M4)/N, where M1–M4 represent the number of cells with 1-4 nuclei, and N is the total number of viable cells scored (excluding necrotic and apoptotic cells). The lowest NDI value possible is 1.0, which occurs when all of the viable cells have failed to divide during the cytokinesis block period and are therefore all mononucleated. If all viable cells completed one nuclear division and are therefore all binucleated, the NDI value is given as 2.0. An NDI value can only be greater than 2.0 if a substantial proportion of viable cells have completed more than one nuclear division during the cytokinesis block phase and are therefore binucleated.

Figure 2-3. Brief MN scoring criteria (after Fenech, 2007)

2.2.15 Real-time PCR

2.2.15.1 Extraction of Total RNA from human lymphocytes

Total RNA was isolated from peripheral blood lymphocytes from healthy individuals and patients using an RNeasy Mini Kit (Qiagen). First, lymphocytes were isolated then treatment with Secukinumab, bulk, liposome, IL17 or a PC (H_2O_2 and NC) in 6-well Petri dishes. The cells were incubated in a cell culture incubator (37°C, 5% CO_2) for 24 hrs. Thereafter, they were transferred to Eppendorf tubes in a sterile fume hood and centrifuged at 400g for 3 mins to precipitate the cells. The supernatant RPMI medium was completely aspirated, and the cells were placed on an ice tray under the sterile fume hood. 350 µL of RLT buffer (10µl B-mercaptoethanol per ml) was added and centrifuged at a maximum speed. The lysate was then put into a QIAshredder spin column in a 2ml collection tube and centrifuged at full speed for another 2 mins. 70% ethanol was added to the lysate and mixed carefully via a pipette. This sample, including any precipitate that may have formed, was transferred into a new QIAamp spin column, placed in a 2ml collection tube and centrifuged for 15s at 8,000g. The QIAamp spin column was transferred into a new 2 ml collection tube then washed with 700µl of RW1 Buffer and centrifuged for a further 15s at 8,000g. The QIAamp spin column was again placed in a new 2 ml collection tube and washed twice with 500µl RPE buffer before being centrifuged at 8000g for a further 3 mins to avoid carrying over any ethanol contamination. Finally, the QIAamp spin column was transferred to a new 1.5ml microcentrifuge tube and the RNA was eluted by pipetting 50µl RNase-free water directly onto the centre of a QIAamp membrane followed by centrifugation for 1 min at 8,000g. The purity and quality of RNA were measured using a NanoDrop™ 1000 spectrophotometer which measures the absorbance of UV light and calculates the 260/280nm ratio. The RNA samples used for cDNA synthesis were stored at -80°C prior to qPCR.

2.2.15.2 Complementary DNA synthesis

Synthesis of the cDNA from total RNA was conducted using an iScript cDNA synthesis kit (Bio-Rad) by mixing the following components in PCR tubes:

- 4µl of 5xiScript reaction mix.
- 1µl of iScript Reverse Transcriptase.

- 11 µl of RNase-free water.
- 4µl of extracted DNA.

PCR reactions were transferred to a Bio-Rad PTC-200 Peltier Thermal Cycler (Bio-Rad Laboratories Inc., Hercules, CA, USA), and incubated for 5 min at 250°C; followed by reverse transcription for 20 mins at 460°C; RT inactivation for 1 min at 950°C and then a step holding at 40°C for 10 mins. The cDNA samples were stored at -20°C until use for the next step.

2.2.15.3 The quantitative real-time PCR method

After synthesis of the cDNA, the real-time PCR reaction was set up in 96 PCR reaction plates and sealed using MicroAmp® Optical 8-Cap Strips.

The total volume of the RT-PCR reactions was 20µl as delineated in the following Table.

Table 2-5. Total volume of RT-PCT reactions

Primer (p53, p21, IL17, IL22, β-Actin)	2µl
Fast SYBRTM Master Mix	10µl
RNase/DNase-free water	4µl
diluted cDNA	4µl

β-Actin was used as a housekeeping gene for the normalisation of the reaction and the assays were performed in triplicate to reduce any variance in the measurement. The 96 well plates were placed on a plate spinner to remove any bubbles present and to settle the solution at the bottom of the plates. The plates were then placed into a StepOnePlus RT-PCR Detection System (Applied Biosystems, Warrington, UK), and the qRT-PCR thermal conditions were set as follows: denaturation at 95°C for 10 mins; followed by 40 cycles of denaturation at 95°C for 15s; annealing and extension at 60°C for 1 min. Then, finally, a melting curve was performed at 95°C for 15s, then 60°C for 15s and 95°C for 15s.

2.2.15.4 Data analysis

The data were analysed using CFX Manager™ Software to determine gene expression levels and normalised against the housekeeping reference gene β-Actin. 2-ΔΔCT (Livak and Schmittgen, 2008) was used to determine the fold-change differences in gene expression between untreated lymphocytes (control cells) derived from patients and healthy volunteers and those lymphocytes treated with 75 μM H_2O_2 and Secukinumab bulk and liposome.

2.2.15.5 Statistical analysis

Each experiment was repeated in triplicate for three different healthy individuals and three patients and the results were presented as means ± SD. The data analysis was performed using GraphPad Prism® software version 8.4.0 with built-in two-way ANOVA and Tukey's multiple comparison tests.

2.2.16 Western blotting

2.2.16.1 Cell culture

Fresh blood samples from psoriatic patients or healthy volunteers were used to isolate the lymphocytes. A concentration of $1x10^6$ lymphocytes was cultured in six-well plates containing RPMI 1640 medium supplemented with Glutamax-I, 15% FBS and 1% penicillin-streptomycin solution under sterile conditions with the addition of PHA 2.5% final concentration. This mixture was incubated at 37°C in the presence of 5% CO_2 in air for 72 hours. After 24 hrs treatments were added, specifically Secukinumab bulk and liposome and H_2O_2 (as a PC).

2.2.16.2 Protein extraction

The culture medium was scrapped from the plate and transferred into eppendorfs, then centrifuged at 400g for 3 mins. The resulting supernatant was discarded, and the lymphocyte pellets washed twice with cold PBS. The pellets were then resuspended in 100 µl RIPA buffer plus 2 µl of protease inhibitor cocktail before resting the cells for 30 mins on ice. Then the resulting suspension was sonicated for approximately 5s at 30 W followed by centrifugation at 14,243g for 30 mins and the resulting supernatant was collected and transferred to other labelled tubes. From each sample, 5 µl of the extracted protein was taken for protein quantification by Bradford assay and the rest of the cell lysates were stored at -80ºC.

2.2.16.3 Protein quantification

Total protein concentration was measured by using a Bio-Rad Bradford assay kit. The assay is based on an absorbance shift in the dye coomassie (red colouration) which changes and stabilises into coomassie blue by the binding of the protein. The characteristic blue colour has a maximum absorbance at 595 nm, which is proportional to the amount of bound dye, and thus to the concentration of protein present in the sample. In this assay, a range of different concentrations (0.125, 0.250, 0.5, 0.750, 1.00, 1.5 and 2 mg/mL) of bovine serum albumin was used to produce a standard curve. Five µl of each unknown sample and standard were pipetted into separate wells of 96 well plates in triplicate along with 250µl of diluted Bio-Rad dye reagent. The optical densities of BSA standards and unknown samples were read at 595 nm.

2.2.16.4 Sodium dodecyl sulphate-polyacrylamide gel (SDS- PAGE)

Prepared gels were used and set in the cassettes. The combs were removed and, in each well, a protein sample was loaded slowly. The protein samples were prepared by mixing each sample with the 2X sample buffer (a 1:1 ratio) and then the samples were heated in boiling water for 5 min. The Laemli buffer was used because it contains 2-mercaptoethanol which reduces the disulfide bonds. It also contains SDS detergent which denatures the proteins and gives each protein a negative charge. Accordingly, each protein then separates based on its size and not its negative charge. In addition, bromophenol blue was used because its colour makes it easier to locate the samples, whereas glycerol was used to increase sample viscosity and density. Samples were then centrifuged for 10s after boiling and then loaded into separate lanes, with the first (left-hand) lane preserved for the pre-stained ladder reference. Power PackTM Basic (Bio-Rad, UK) was used to run all the gels. The gel was initially run more slowly through the stacking gel at 50V to give clear and sharp bands and thereafter at a 100V constant voltage throughout the resolving gel. The pre-stained protein ladder and the dye front were monitored to determine the leading front of the electrophoresis separation process. The gels were immersed in electrophoresis buffer (25 mM Tris base, 192 mM glycine and 0.1% w/v SDS) within the running tank and 30 µg of protein was loaded per well. On each gel, one lane contained 10 µl of protein ladder (Precision Plus Protein™ Dual Colour Standards). The gel was run for approximately 2 hrs at a constant voltage of 100V. The SDS gel was used to separate proteins based on their respective sizes (Figure 2.4).

Figure 2-4. Steps involved in preparing a PAGE gel showing small steps done by our self to help explain the procedure.

2.2.16.5 Transfer to membrane

After the electrophoresis, proteins were transferred to a blotting nitrocellulose membrane using the iBlot® Gel Transfer Device (Invitrogen) for 7 min at a constant voltage of 25V (Figure 2.5).

Figure 2-5. iBlot® Gel Transfer Device

2.2.16.6 Blocking

After transfer, the nitrocellulose membranes were incubated with the blocking solution containing 5% (w/v) BSA in Tris-buffered saline containing Tween 20 (TBS-T; 150 mM NaCl, 20 mM Tris base and 0.1% (v/v) Tween 20, pH 7.4) for 1 hr at RT with gentle shaking. Membranes were washed 3 times for 10 mins with TBS-T (figure 2.6).

2.2.16.7 Primary antibody

The blotted membranes were incubated overnight at 4ºC in a shaking tray containing the primary antibody. GAPDH rabbit monoclonal primary antibody was used as a loading control. A monoclonal antibody was used because it gives a better signal and lower background as it has a higher specificity. The primary antibodies used included [GAPDH] (1:10,000 dilution); P53 (1:1,000 dilution); P21 (1:1,000 dilution); IL17 (1:1000 dilution), IL22 (1:1000 dilution). All dilutions were performed with TBS-T containing 5% (w/v) BSA. Membranes were then washed 3 times for 10 mins with TBS-T.

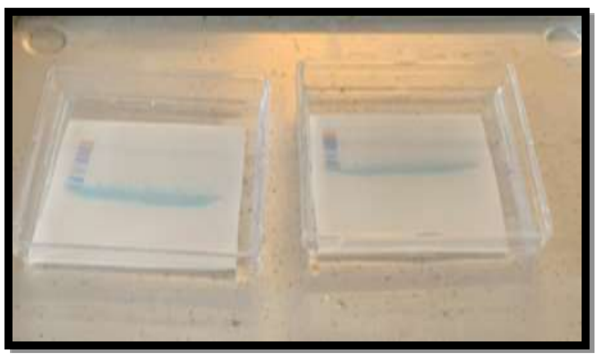

Figure 2-6. Blocking solution membrane putting the membranes in the blocking solution ready to go on the shaking machine

2.2.16.8 Secondary antibody

A secondary antibody (donkey anti-rabbit IgG HandL HRP) was added to uncover those locations where the primary antibody actively bound. The secondary antibody was diluted 1:3,000 with 5% (w/v) BSA in TBS-T solution. The membrane was incubated at RT with gentle agitation for 1 hr and the membrane was washed in TBS-T 4 times for 15 mins.

2.2.16.9 Detection

The membrane was stained with enhanced chemiluminescence (ECL) kit solutions by incubating the membrane with the same amount of ECL solutions 1 and 2 for 1 min at RT. Excess detection reagent was removed and the membrane was placed on a GB Box (Gene flow, UK). Images were captured and saved for later analysis (Figure 2.7).

Figure 2-7. GB Box

2.2.16.10 Data Analysis

Relative expression of the protein was determined using Image J software. GAPDH served as the protein loading control. The data were presented as means ± SE of three independent experiments in isolated lymphocytes derived from 3 healthy individuals and 3 patients. To analyse the significant difference between the different treatment concentrations the data were analysed using one-way ANOVA followed by Dunnett's post-hoc test for significant differences.

Chapter 3. Determination of the optimum dose of Secukinumab

3.1 Introduction

Secukinumab is expressed in CHO cells (MCB 060428 CHO-HPT1 cell line) using recombinant DNA technology. Culture media were prepared in T-flasks and roller bottles in the absence of serum and with low levels of protein. They contained no animal or human-derived raw materials and were harvested and then purified as a single active substance batch. The purification procedure comprised the following steps, including harvesting, chromatography and filtration, before final freezing and storage at ≤60°C.

Secukinumab, approved in January 2015 by the European Medicines Agency (EMA); is a monoclonal antibody. Human IgG1 explicitly binds to IL17A, thereby inhibiting its interaction with the IL-17 receptor, which is expressed in several cell types, including KCs. Consequently, Secukinumab inhibits the release of pro-inflammatory cytokines, chemokines, and mediators of tissue damage and thus reduces IL17A-mediated contributions to autoimmunity and inflammation. The recommended weekly dosage, in the induction phase, is 150-300mg for the first 4 weeks, followed by a dose of 150-300mg every 4 weeks in the maintenance phase, both of which are administered subcutaneously (Lebwohl *et al.*, 2017). The safety and efficacy of Secukinumab have been assessed in four independent phase III trials which are randomised, double-blind and placebo-controlled, conducted in patients with psoriasis in moderate-to-severe plaques [the four trials were referred to as ERASURE, FIXTURE, FEATURE, JUNCTURE]. The ERASURE trial (Efficacy of Response and Safety of Two Fixed Secukinumab Regimens in Psoriasis) evaluated 738 randomised patients at a 1: 1: 1 ratio, all of whom received doses of either 150 or 300 mg Secukinumab (or placebo) at weeks 0, 1, 2, 3, and 4 and monthly intervals thereafter. The

FIXTURE trial (Full Year Investigative Examination of Secukinumab vs. Etanercept Using Two Dosing Regimens to Determine Efficacy in Psoriasis) evaluated 1306 randomised patients at a 1: 1: 1: 1 ratio, all of whom received either doses of 150 or 300 mg Secukinumab at weeks 0, 1, 2, 3, and 4, and at monthly intervals thereafter, placebo or Etanercept (50 mg twice a week for 12 weeks, followed by 50 mg once a week). In both studies, patients were randomised to receive placebo. Those who were non-responders at week 12 were moved on to the treated group Secukinumab (150 mg or 300 mg) at weeks 12, 13, 14, and 15, followed by the same dose every month from week 16. All patients were monitored up to 52 weeks after the first administration of the study treatment. The co-primary objectives of controlled studies with active drug or placebo included the proportion of patients who achieved a PASI 75 response and a score 0,1 IGA (a measurement of disease severity).

In the ERASURE study, the results obtained for PASI 75 for the 12th week were 81.6%, 71.6%, and 4.5% for patients undergoing Secukinumab at 300mg, 150mg, and placebo, respectively. For IGA, the figures were 65.3%, 51.2%, and 2.4% of the patients, respectively. After 52 weeks, the percentage of patients reaching a PASI 75 score who had been treated with Secukinumab at dosages of 300mg or 150mg were 74.3% and 60.1%, respectively, with associated IGA 0,1 scores of 60.4% and 41.4%. In the FIXTURE study, the percentage of patients achieving a PASI 75 score by the 12th week was 77.1%, 67%, 44%, and 4.9%, respectively, in the groups treated with Secukinumab 300mg and 150mg, Etanercept and placebo, respectively (Langley et al, 2014). During the placebo-controlled clinical trials of plaque psoriasis (a total of 1,382 patients treated with Secukinumab and 694 patients treated with placebo for 12 weeks), infections were reported in 28.7% of patients treated with Secukinumab and 18.9% of those treated with placebo. Most of these consisted of non-severe and mild-to-moderate upper respiratory tract infections, including nasopharyngitis and rhinitis, but there were also cases of herpes and oral candidiasis reported with some frequency. The occurrence of serious infections was low, and these figures were similar between the two treatments (150 and 300 mg). Fewer than 1% of patients treated with Secukinumab developed drug antibodies. Contraindications to this agent included severe hypersensitivity reactions to the active substance and active infection, both of which were clinically relevant. The evolution of the knowledge of the immunopathogenesis of psoriasis has allowed and will facilitate the development of new therapies which act on immunoinflammatory processes that play a critical role in the maintenance and induction of psoriatic lesions, enabling even more effective, safer, and more comfortable dosages. Currently, based on the evidence that supports the importance of the complex inflammatory processes involving IL-23/Th17 and IL-17, more treatments will, in future, be approved for the treatment of psoriasis (Krueger *et al.*, 2001; Menter *et al.*, 2016). Another clinical trial (CLEAR) compared the efficacy of Secukinumab relative to Ustekinumab. This study lasted 52 weeks and involved 676 patients presenting with moderate-to-severe plaque psoriasis who had been randomised into two equal groups, receiving either 300 mg Secukinumab or Ustekinumab (Puig et al, 2018). Those receiving Secukinumab achieved both the study's primary and secondary goals, showing improved outcomes versus Ustekinumab in terms of their PASI 90 response at week 16 (primary objective), speed of onset of their PASI 75 response to week 4, and long-term PASI 90 responses to week 52.

serum level of IL-17 was 6.24±4.65 pg/mL and 2.0 ± 0.75 pg/mL in psoriatic patients and control group, respectively (P=0.001). The mean±SD of PASI severity scale of patients was 10.68 ± 6.65 and 6.16±4.2 before and after treatment, respectively (P > 0.001) .The mean±SD of serum level of IL-17 of patients before and one month after treatment were 6.24±4.65 and 3.34±1.77 pg/mL, respectively (Sobhan, et al, 2022).

Although the efficiency and safety of the drug have been proven clinically and the optimal dosage identified for clinical use, dose toxicity and the optimal dose for use in vitro have yet to be established, to the best of our knowledge. According to the ICH, genotoxicity studies have not yet been conducted for Secukinumab, a treatment that is already acceptable to the Committee for Medicinal Products for Human (CHMP). In this Chapter, the optimal dose for Secukinumab is identified via the viability, MTT, CCK8, and Comet assay.

Since the concentration of serum Secukinumab of 10 μg/mL was considered toxic by the manufactory. Thus, a series of doses below this range have been tried and tested. We started with the viability test because it is quick, easy and gives us a range at which the dose becomes measurably toxic. Among the different doses checked, 10 μg/mL was found to be toxic as all the cells treated died. Accordingly, for the purposes of this study, three doses have been selected, namely 2.1, 2.8 and 3.75 μg/mL. These doses were prepared randomly according to how convenient for us to diluted.

3.2 Material and Methods

This was described in detail in chapter 2.

3.3 Viability test

Cells that take up the dye are identified as non-viable, whereas those that do not are viable. High viability is regarded by many as a prerequisite for the comet assay. However, Trypan blue does not measure viability but rather simply indicates whether cell membranes are intact. Cells with damaged membranes are Trypan blue-positive but may recover and survive after that. Therefore, the viability test does not identify damaged DNA, even though 10 μg/mL was found to be toxic, causing damage to the cells.

3.4 Cell survival and cytotoxicity assessment using the MTT assay

Human lymphocytes from healthy individuals and patients were treated with different concentrations of Secukinumab for 24 hours followed by treatment with MTT dye (4 hrs) to determine the cells' rate of proliferation, metabolic activity and survival. In the MTT assay, the data showed a decrease in % survival rates after exposure to different concentrations of Secukinumab (2.1, 2.8 and 3.75 µg/mL). After treatment with 3.75 µg/mL, the % cell survival rate decreased sharply in a concentration-dependent manner and was statistically significant. A rate of cell survival of over 80% was considered a safe dose and, therefore, we considered only 3.75µg/mL

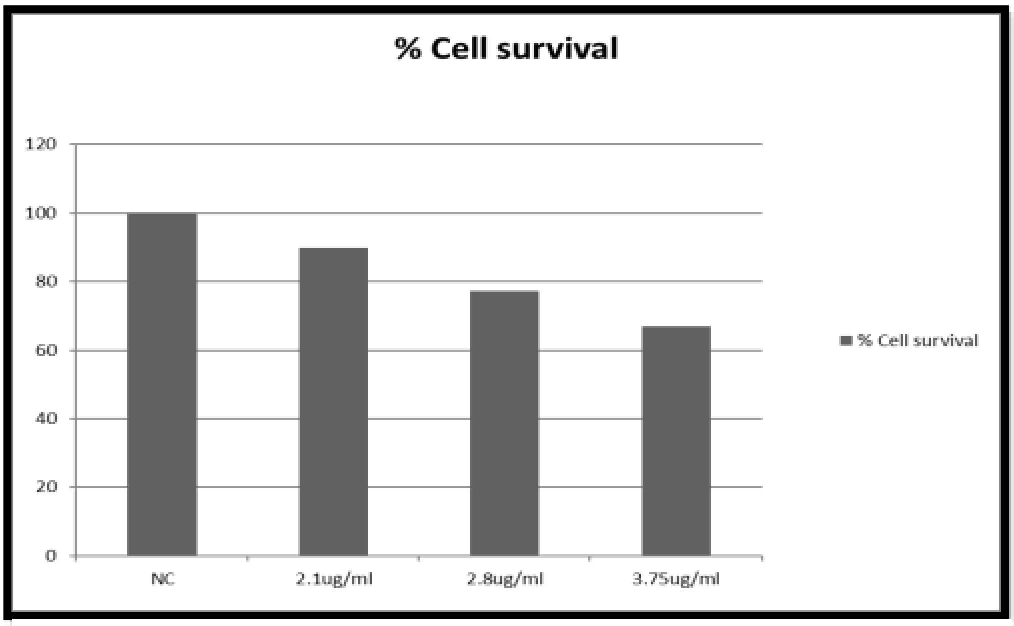

Figure 3-1. Cytotoxicity of Secukinumab in peripheral human lymphocytes after 24 hrs exposure to different concentrations (2.1, 2.8, and 3.751 µg/mL). Mitochondrial activities were assessed with MTT assays demonstrating the higher dose was toxic unlike 2.1 and 2.8 which were non-toxic (NC=negative control).

to be toxic as only a 66.9% survival rate was observed with this concentration (Figure 3.1). Theses doses was chosen randomly just for the simplicity of preparing the diluted from.

3.5 Cell viability using CCK8 kit

A cell counting kit was used to measure the cellular viability and integrity of lymphocytes. Cell viability is defined as the number of live or viable cells as a percentage of the total number counted. Different concentrations of Secukinumab were screened for their cytotoxicity in peripheral lymphocytes from healthy individuals. The results revealed concentration-dependent cytotoxicity of Secukinumab in lymphocytes. A higher concentration of Secukinumab (3.75µg/ml) was found to be cytotoxic, although the two lower concentrations of the drug tested, 2.1µg/ml and 2.8µg/ml, had cell survival rates of over 75%, confirming the results of the MTT assay (Figure 3.2). Therefore, these concentrations were considered non-toxic and were used throughout the research studies presented here.

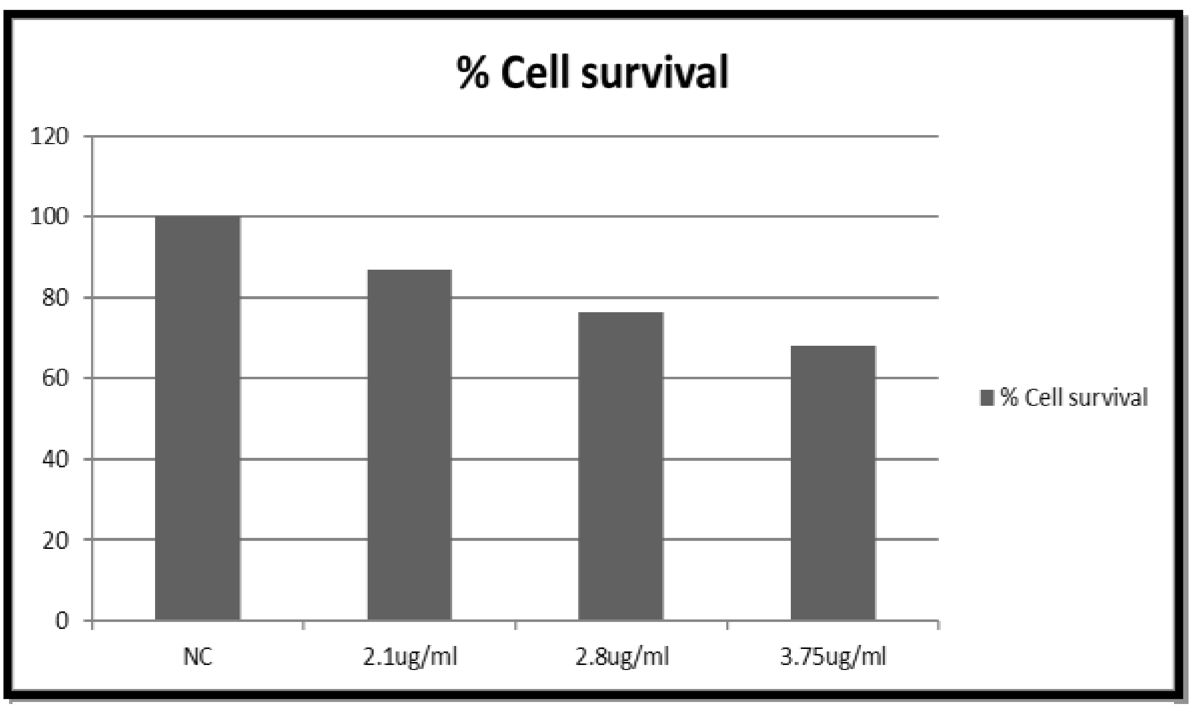

Figure 3-2. % Cell survival in response to Secukinumab in healthy lymphocytes using a cell counting kit. Results were evaluated for three concentrations of the drug as compared to the untreated group (NC), which showed the safety of the 2.11µg/ml and 2.81µg/ml dose and the toxicity of the 3.751µg/ml.

3.6 Identifying the genotoxicity of the treatment dose

In this chapter, identification of the best dose of the treatments (H_2O_2 and Secukinumab) to be used throughout the research.

3.6.1 Concentration-dependent responses to hydrogen peroxide (H_2O_2)

H_2O_2 is known to induce DNA damage in cells (Driessens et al., 2009). To determine whether the dose which is going to be used in this research is genotoxic, a comet assay was performed. First, we established the highest concentration of H_2O_2 (μM) (PC) that induced the most significant DNA damage after treating whole human blood from healthy individuals in the alkaline comet assay relative to untreated whole blood in the absence of cytotoxicity. This gave us a PC by which to compare treatment groups. Whole blood samples from healthy individuals were treated with four incremental concentrations of H_2O_2 (25, 50, 75, 100 and 125 μM). In the absence of any chemical treatment, untreated blood samples served as the negative control (NC). DNA damage was expressed as an OTM and percentage tail DNA (% tail DNA) (Olive and Durand, 2005).

The in vitro treatment of healthy lymphocytes with different concentrations of H_2O_2 in the comet assay reveals that 75μM H_2O_2 induces the most significant levels of DNA damage. Therefore, this optimal concentration was adopted as a PC throughout the research studies (Figure 3.3).

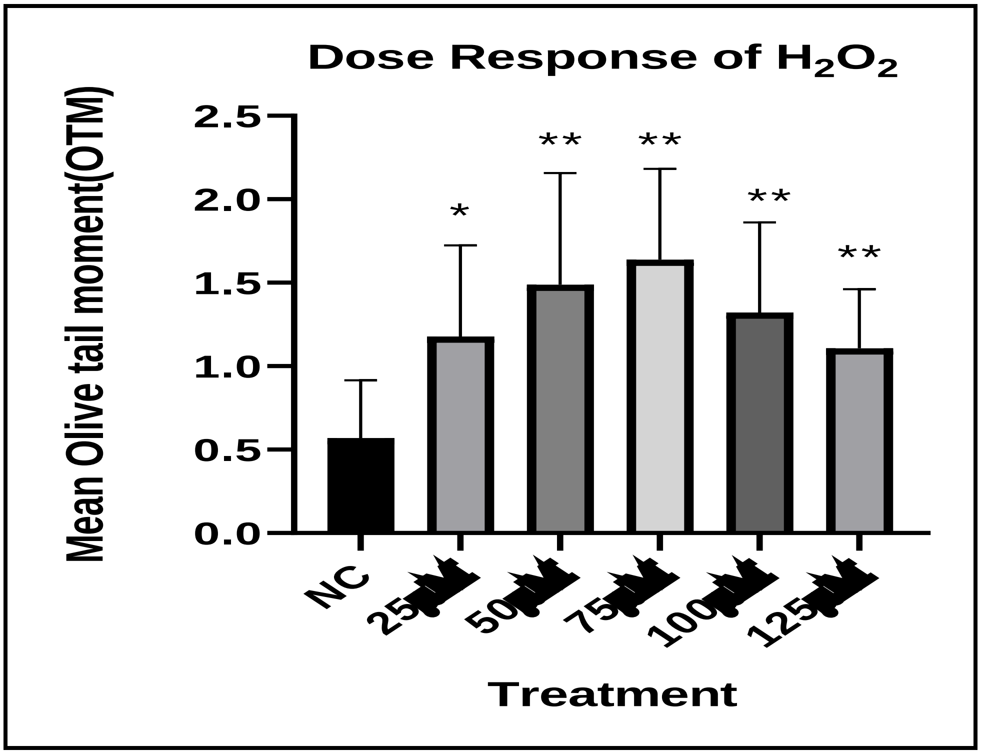

Figure 3-3. The dose-response to H2O2 in lymphocytes from healthy individuals by measuring OTM. Data were evaluated as means ± SE from 20 individual experiments, each assessing 100 cells. The various concentrations of H_2O_2 considered were 25, 50, 75, 100 and 125μM (p-values for all the treatment groups were 0.02, 0.004, 0.001, 0.009, and 0.001, respectively) NC=negative control.

3.6.2 The effect of Secukinumab at different concentrations before and after H_2O_2 treatment of lymphocytes from healthy individuals

After treatment with 2.1µg/ml Secukinumab, no significant difference was observed in terms of DNA damage in healthy lymphocytes relative to controls. However, H_2O_2 (PC) alone induced significant levels of DNA damage ($p<0.001$) in the treated cells. When 75µM H_2O_2 was added alongside 2.1 or 2.8µg/ml Secukinumab, DNA damage was significantly reduced to levels comparable to the NC according to the parameters of the comet assay, OTM and % tail DNA, as shown in Figure 3.4 and 3.5. This means that Secukinumab either has a protective or a repair mechanism.

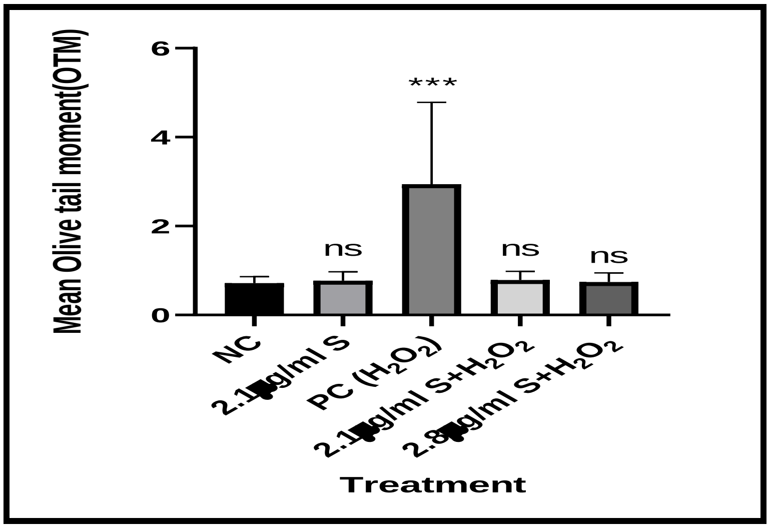

Figure 3-4. Effects of Secukinumab and H2O2 on lymphocytes from healthy individuals according to measurements of OTM. The results are presented as means of 20 experiments from 20 healthy volunteers using 5 different treatment groups: NC (untreated group), PC (positive control)(75µM H_2O_2), 2.1µg/ml Secukinumab, 2.1µg/ml Secukinumab + H_2O_2, and 2.8µg/ml Secukinumab + H_2O_2. A total of 100 cells were counted for each group. (ns indicates not significant, ***p<0.0001).

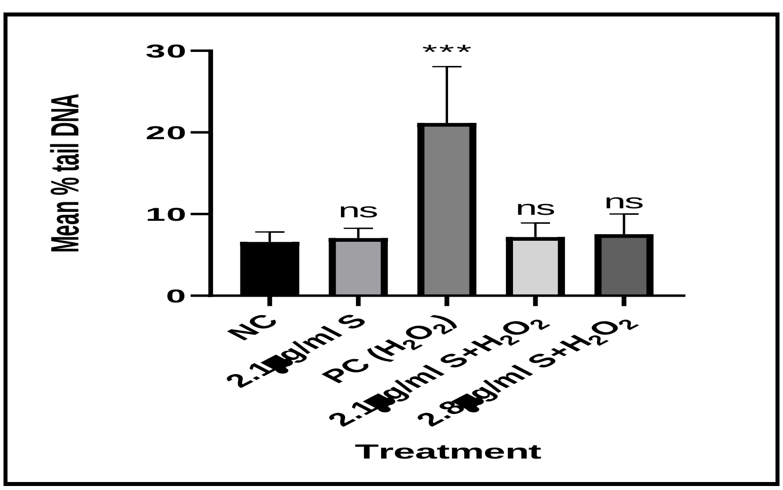

Figure 3.5 The effects of Secukinumab and H_2O_2 on the DNA from healthy lymphocyte cells using % tail DNA. The results demonstrate mean SE of 20 experiments done in 20 healthy volunteers using 5 different treatment

groups: NC (untreated group), PC(positive control) (75µM of H2O2), 2.1µg/ml of Secukinumab, 2.1µg/ml of Secukinumab (S) with H2O2 and 2.8µg/ml of Secukinumab with H2O2. A total of 100 cells were counted for each group. (ns=not significant, ***p<0.0001).

3.6.3 The effects of Secukinumab at basal levels plus on H_2O_2-induced DNA damage in lymphocytes from the patient group

In vitro treatment of lymphocytes from psoriasis patients with Secukinumab 2.1µg/ml showed no significance difference in the DNA damage when compared against the NC whereas H_2O_2 (75µM) has induced a high level of DNA damage as expected. By co-supplementing the H_2O_2 with different concentrations of Secukinumab, the DNA damage was reduced to a significant level, compared to the PC alone. Hence, Secukinumab could potentially extenuate the adverse effects of H_2O_2 and proficiently reduced the DNA damage caused by it. Results from OTM and % tail DNA are shown below in figure 3.6 and 3.7.

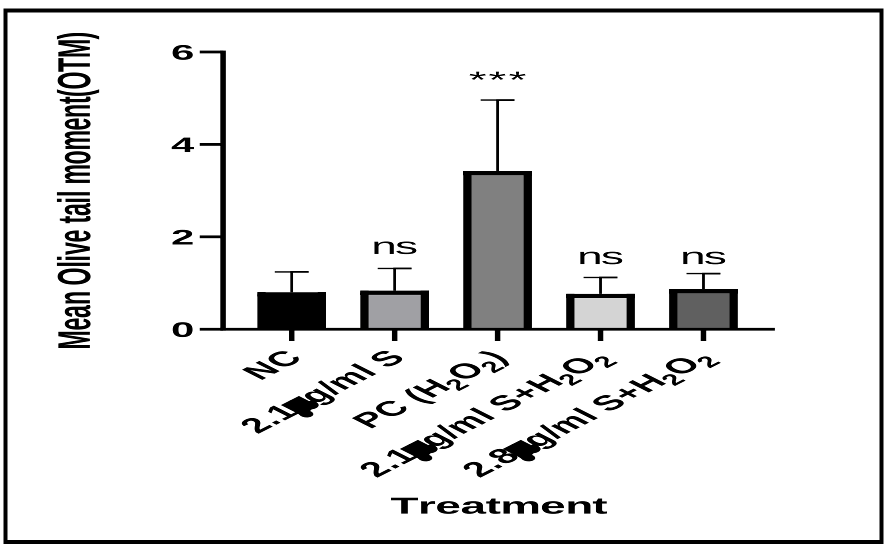

Figure 3.6. The effects of Secukinumab and H_2O_2 on the DNA damage in the lymphocytes from patient group using OTM. The results demonstrate mean SE of 20 individuals' experiments done in 20 psoriasis patients using 5 different treatments groups: NC (untreated group), PC(positive control) (75µM of H2O2), 2.1µg/ml of

Secukinumab, 2.1µg/ml of Secukinumab with H2O2 and 2.8µg/ml of Secukinumab with H2O2. A total of 100 cells were counted for each group. (ns=not significant, ***p<0.0001)

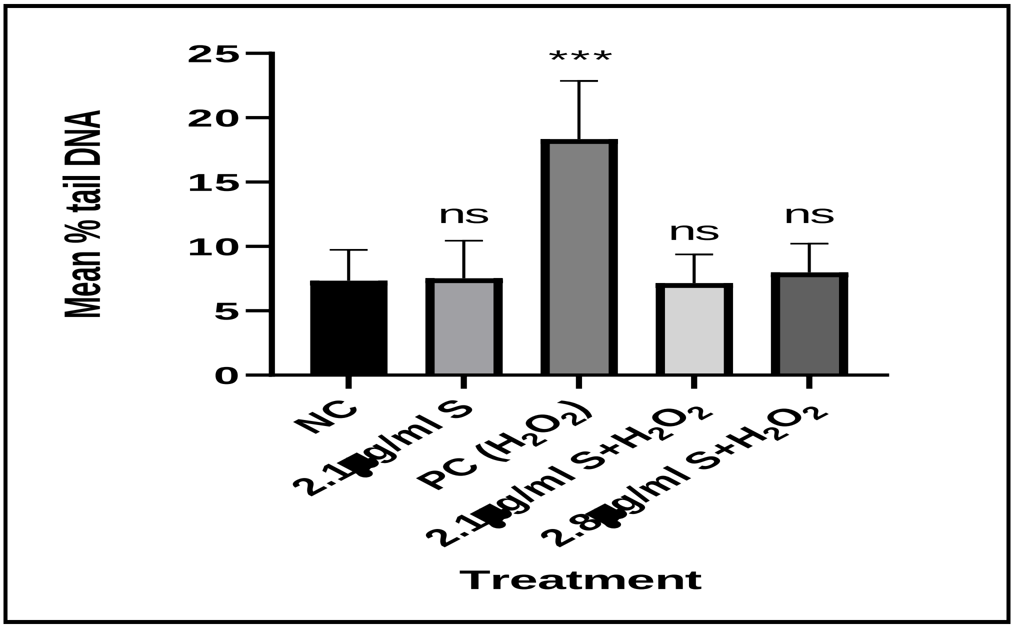

Figure 3.7 The effects of Secukinumab and H_2O_2 on the DNA damage in lymphocytes from psoriasis patients using % tail DNA. The results demonstrate mean SE of 20 individuals' experiments done in 20 patients using 5 different treatment groups; NC (untreated group), PC (positive control) (75µM of H2O2), 2.1µg/ml of Secukinumab, 2.1µg/ml of Secukinumab with H2O2 and 2.8µg/ml of Secukinumab with H2O2. A total of 100 cells were counted for each group. The mean NC and PC values were 7 and 18 respectively. (ns=not significant, ***p<0.0001)

3.6.4 Comparison between the effects of Secukinumab on lymphocytes from healthy individuals and psoriasis patients

Secukinumab exhibits similar effects in terms of DNA damage induced in lymphocytes caused by H_2O_2 in the patient group as compared to those derived from healthy individuals. Secukinumab (2.1µg/ml) induced no significant level of DNA damage in lymphocytes obtained from either group. However, the two concentrations of Secukinumab tested (2.1 and 2.8µg/ml) efficiently decreased H_2O_2-induced DNA damage in both groups. Similar results were observed for all treatments in both groups and a Secukinumab concentration of 2.1 µg/ml was used for all ensuing experiments (Figures 3.8).

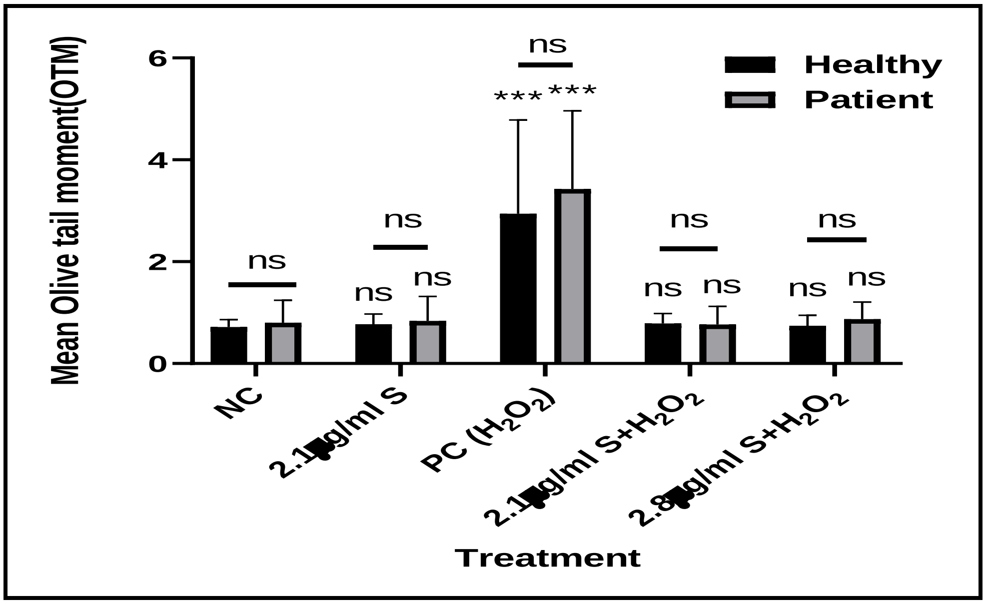

Figure 3-8. Concentration responses of Secukinumab and H2O2 on DNA damage induced in lymphocytes from healthy individuals versus psoriasis patients as measured via OTM

The results are presented as the means of 20 experiments performed on lymphocytes from 20 healthy volunteers and patients using 5 different treatment groups: specifically NC (untreated group), PC (positive control) (75µM H2O2), 2.1µg/ml Secukinumab, 2.1µg/ml Secukinumab + H2O2 and 2.8µg/ml of Secukinumab + H2O2. A total of 100 cells were counted for each group. The horizontal line on the graph and the statistics presented above indicate the statistical differences arising between groups (ns=not significant, ***p<0.0001).

3.6.5 The genotoxicity determination of IL-17 in lymphocytes

IL-17 plays a vital role in the pathogenesis of psoriasis and, since Secukinumab is an anti-IL17 antibody, we have evaluated the genotoxicity of IL17 in lymphocytes after identifying the best dose of IL-17 to use. The effects of IL-17 were measured alongside those of Secukinumab.

3.6.5.1 Optimum concentration of IL-17

The results reveal that differences in DNA damage at various doses of IL-17 were insubstantial, and so we elected to use a medium dose throughout our current research study. Gathered from different studies as discussed below the doses 2, 20, 200 μg/ mL were used, then the 20 μg/ mL was used throughout the research.

In a previous study by Noonan *et al* on PBLs, the optimum dose of IL-17 established was 10 μg/mL (Noonan *et al.*, 2010). In another prior study on the possible pathogenic role of Th17 cells in atopic dermatitis, three doses of IL-17 (2, 20, 200 μg/ mL) were used (Eyerich *et al.*, 2001). In research conducted in lymphocytes, 10 μg/mL IL-17 was used to study the support for tumour progression by tumour-infiltrating IL-17-producing γδ-T cells' via the promotion of angiogenesis (Yang *et al.* 2014). Our results from the comet assay revealed that the three different concentrations of IL-17

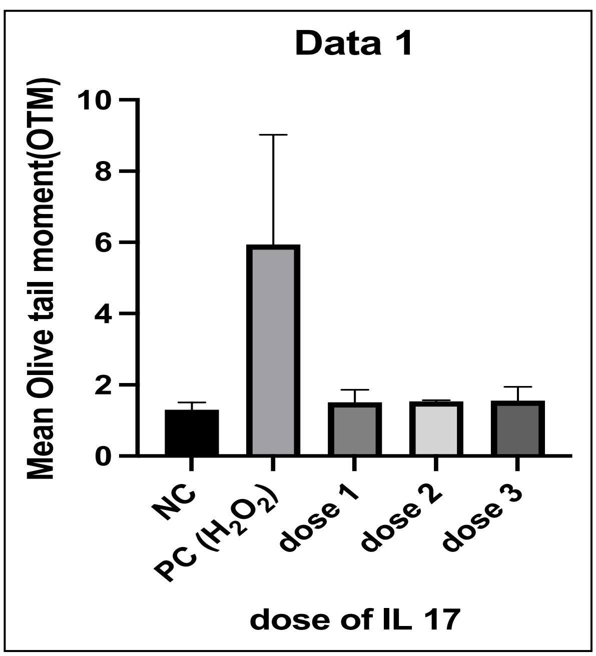

Figure 3-9. Dose-response relationship of IL-17 on mean OTM. Three different concentrations (2,20,200 μg/ mL) of IL-17 were used to assess genotoxicity while compared against the untreated cells (NC) and PC (positive control) 75 μM H_2O_2

tested had the same effects on human lymphocytes which were similar to those exerted on untreated lymphocytes.

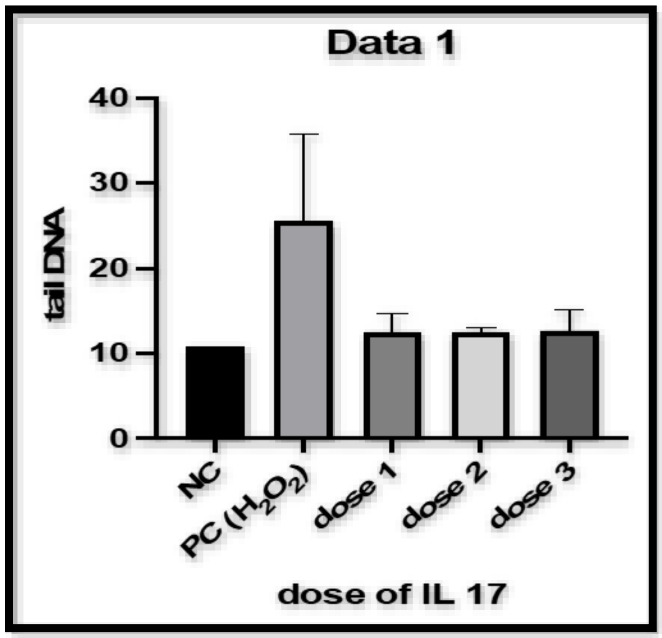

Figure 3-10. Dose-response relationship of IL-17 on % tail DNA. Three different concentrations (2, 20, 200 μg/mL) of IL-17 were used to assess genotoxicity as compared to the untreated NC and (positive control) 75 µM H_2O_2. No significant difference of the effect of the 3 doses on lymphocytes ($p<0.0001$)

No significant difference arose between the DNA damage caused by different concentrations of IL-17, revealing that IL-17, in of itself, does not induce any DNA damage and hence has only non-genotoxic effects on lymphocyte cells. Although IL-17 is critical to the progression and pathogenesis of psoriasis, in our study it has not caused any visible genotoxicity in lymphocytes which could be due to several reasons, including a lower sensitivity and enhanced integrity of lymphocytes towards IL-17. However, the various tissues and cells of our body can behave differentially on exposure to different drugs and agents (Figure 3.9 and 3.10)

3.7 Analysis of Confounding Factors

Confounding factors such as smoking, drinking habits and age etc. are considered potential contributors to DNA damage, causing mutagenicity and ultimately leading to more adverse effects, including cancer development. These lifestyle and environmental factors tend to increase one's susceptibility to cancer and cardiovascular disease (Fenech 2007). Therefore, an investigation was conducted to determine the effects of these confounding factors (age, ethnicity, gender and smoking) in both the healthy volunteers and patient groups using the comet values. Our results demonstrated that there was no significant relationship found among the variables in any of the treatment groups (Table 3.1).

3.7.1 Age

There was a significant difference in age between the healthy and patient groups. However, these were the only patient samples available at the time of this study and the age of healthy individuals was possibly best matched by considering the patients' age during the volunteer recruitment process. Table 3.1 below shows that the oldest patient groups (45-60 and >60) expressed higher levels of basal DNA damage than the younger age group or healthy individuals. However, the observed difference was not statistically significant when compared to the control group. This could be due to the disease condition and compromised biological mechanisms in the older patient group.

Secukinumab bulk treatment showed DNA damage levels similar to that of the untreated group for all age groups, whereas the liposomal form of Secukinumab exhibits a non-significant reduction in DNA damage, irrespective of the age or group type. H_2O_2 induced significant levels of DNA damage in all groups, as expected ($p<0.001$).

3.7.2 Ethnicity

The results demonstrated no statistically significant differences among different healthy and patient ethnic groups. However, the liposomal form of Secukinumab induced a non-significant decrease in DNA damage within all the groups.

3.7.3 Gender

No relationship was found between gender differences and comet parameters, nor was there any effect on DNA damage caused by the various treatment groups.

3.7.4 Smoking Habits

The results showed that former smokers and non-smoking patient groups demonstrated a higher level of basal damage as compared to the non-smoking control group, although this observed difference was again statistically insignificant. Otherwise, there was no significant effect of smoking status upon either comet parameters or OTM values.

Table 3-1. Analysis of confounding factors

Treatment Group and confounding factor		NC(Untreated)	PC(H_2O_2 75µM)	SB(2.1 µg/ml)	SL(2.1 µg/ml)
Age	Controls 20-35	0.6 ± 0.3	4.1 ± 2.1 ***	0.9 ± 0.4 ^{ns}	0.7 ± 0.3 ^{ns}

	Controls 35-50	0.8 ± 0.5 ^{ns}	5.2 ± 2.2***	1.3 ± 0.8 ^{ns}	0.9 ± 0.2 ^{ns}
	Patients 30-45	1.2 ± 0.7 ^{ns}	6.3 ± 0.9***	1.0 ± 0.7 ^{ns}	0.9 ± 0.5 ^{ns}
	Patients 45-60	2.1 ± 1.3^{ns}	6.6 ± 2.7***	1.9 ± 1.4 ^{ns}	1.9 ± 0.4^{ns}
	Patients >60	2.2 ± 0.8^{ns}	6.4 ± 1.5 ***	2.7 ± 1.5 ^{ns}	2.2 ± 1.3 ^{ns}
Smoking History	Non-smoking Controls	0.8 ± 0.1	6.3 ± 1.3 ***	0.9 ± 1.1 ^{ns}	0.7 ± 0.4 ^{ns}
	Ex-smoking Patients	2.4 ± 0.3 ^{ns}	5.3 ± 2.0 **	2.5 ± 0.2 ^{ns}	1.8 ± 0.1^{ns}
	Non-smoking patients	1.9 ± 1.0 ^{ns}	5.8 ± 1.0 ***	2.1 ± 1.1 ^{ns}	1.8 ± 0.2 ^{ns}
Ethnicity	Caucasian controls	0.8 ± 0.1	5.1 ± 1.5 ***	1.0 ± 0.2 ^{ns}	0.6 ± 0.1 ^{ns}
	Caucasian patients	2.0 ± 0.2^{ns}	6.5 ± 1.4***	2.2 ± 1.0^{ns}	1.7± 0.8 ^{ns}
	Asian controls	1.2 ± 0.4^{ns}	5.5 ± 1.9***	1.5 ± 0.5 ^{ns}	1.0 ± 0.3^{ns}
	Asian patients	2.2 ± 1.0^{ns}	5.7 ± 1.0***	2.0 ± 1.1 ^{ns}	1.9 ± 1.2 ^{ns}
Gender	Female ControlsMale Controls	1.0 ± 0.21.1 ± 0.4^{ns}	4.6 ± 1.4***5.0 ± 1.0 ***	1.1 ± 0.5 ^{ns} 0.9 ± 0.3^{ns}	1.0 ± 0.4 ^{ns} 0.9 ± 0.2 ^{ns}
	Female PatientsMale Patients	1.3 ± 0.9^{ns}1.1 ± 1.0^{ns}	6.0 ± 1.2***5.8 ± 1.1***	1.4 ± 1.0^{ns} 1.1 ± 1.0 ^{ns}	1.1 ± 0.4^{ns}0.9 ± 0.6^{ns}

3.8 Discussion

Psoriasis is a chronic inflammatory disease and one which may be associated with diabetes, heart complications, arthritis, social isolation, depression and anxiety. All these conditions can impact employment status and personal relationships and hence psoriasis can increase lifelong healthcare costs. The pathogenesis of the disease is related to the IL-23/Th-17 pathway, in which IL-17A is likely implicated in the systemic inflammation and skin plaques observed. Therapies include the inhibition of IL-17A, which was found to be very efficacious in the long term. Secukinumab targeted IL-17A signalling across many parts of the body, as evidenced by various clinical trials. This drug has also been approved by the FDA for the treatment of plaque psoriasis. In trials a dosage of 300 mg resulted in a 78-82% PASI efficacy as compared to 67-72% efficacy for 150 mg. Randomised, placebo-controlled trials also revealed better outcomes for Secukinumab as compared to Etanercept. In all trials, Secukinumab was generally reported as being well tolerated in patients in the management of psoriasis. The safety of Secukinumab was also evaluated in short-term studies which confirmed an acute clinical improvement in patients, especially in the more difficult-to-treat areas such as palmo-plantar, genital, head and scalp regions (Galluzzo *et al.*, 2020).

Various past clinical and laboratory trials have demonstrated the superiority of Secukinumab versus other biologics and available treatments in the treatment of psoriasis (Johar *et al.*, 2019). Secukinumab has been shown to efficiently clear the patient's skin (Blauvelt *et al.*, 2017), maintaining its efficacy over long periods as it exhibits low levels of immunogenicity and an absence of any identified ADAs (Hsu, Snodgrass, & Armstrong, 2014; Strand et al., 2017). Since, it is its immunogenic effect rather than its biologic effect that causes the induction of antibodies that enhance drug neutralisation via its binding ability and its clearance from the system (Hsu *et al.*, 2014). Additionally, the efficacy of Secukinumab was unaffected, even when the presence of ADAs was detected (Mrowietz *et al.*, 2015). Moreover, a recent study has demonstrated that psoriasis patients treated with Secukinumab did not relapse, even after two years of discontinuation (Lebwohl *et al.*, 2017). The psoriasis modifying potential of Secukinumab in new-onset, moderate-to-severe psoriasis is still under investigation in an ongoing trial. Owing to its various aforementioned advantages, dermatology experts recommend Secukinumab as a first treatment option for moderate-to-severe psoriasis (Johar *et al.*, 2019).

Our results from the Comet assay indicate that Secukinumab does not induce any significant DNA damage in lymphocytes from healthy individuals relative to the patient group or the control untreated group. The results were almost equivalent to those from the negative control (Figure 3.7). This suggests that the administration of 2.1 and 2.8µg/ml of Secukinumab is non-genotoxic, despite reports of its actions at higher doses than 150 to 300 mg (Galluzzo *et al.*, 2020).

Several groups have previously demonstrated that H_2O_2, an oxidative stress-inducing compound, causes significant levels of oxidative stress-induced DNA damage in peripheral lymphocytes according to the comet assay (Najafzadeh *et al.*, 2009; Anderson *et al.*, 2014; Stanic *et al.*, 2016). Our results are thus congruent with such studies in which H_2O_2 induces significant DNA damage in both healthy individuals and psoriasis patients. However, when these groups were treated with two different concentrations of Secukinumab, namely 2.1 and 2.8µg/ml, then the ROS-related DNA damage induced by H_2O_2 was significantly decreased as compared to the positive controls alone. In addition, the damage was reduced to a level similar to that of the untreated control population. The results were the same as those indicated by the two parameters of the comet assay, OTM and % tail DNA. Several molecular properties of Secukinumab could be involved in the reduction of such damage. Best dose of IL-17 to be used in vitro was assessed and a dose of 20 µg/ml was decided to be used as not much difference was observed.

In conclusion, the studies showed that DNA damage increased significantly with increased H_2O_2 concentration and 75 µg/ml had the highest cytotoxic effect, however, higher doses showed less cytotoxicity, and this could be explained by full cell apoptosis. Therefore, 75 µg/ml dose of H_2O_2 was used throughout the research. The two concentrations of Secukinumab used in this study have shown no significant effects in terms of DNA damage compared to the NC experiments of either healthy individuals or psoriasis patients. However, in those experiments where H_2O_2 was added, Secukinumab significantly decreased H_2O_2-induced damage and efficiently attenuated its adverse effects. The untreated patient group had a higher level of DNA damage as compared to the comparative healthy untreated group throughout this in vitro study. Overall, Secukinumab (2.1µg/ml) demonstrated more effective results against H_2O_2-mediated DNA damage. These results suggest that both concentrations of Secukinumab may not induce any significant levels of genotoxicity, instead producing protective effects against H_2O_2-induced oxidative stress and possibly even facilitating repair. However, other possible molecular mechanisms may be involved, which will need further work. In addition, best dose of IL-17 was identified in this chapter, and it was 20 µg/ml.

Chapter 4. Preparation of Liposomes

Since liposomes have a large surface area, which makes them more active and diffuse easily through the membranes of different cells, this may rise the possibility of being more toxic at the same dose. (Bharali and Mousa, 2010). NPs can enter our tissues through different routes i.e., breathing (Oberdorster, 2001), the digestive system (Jung et al., 2000) and perhaps through the skin (Kreilgaard, 2002). The use of engineered liposomes in research and various fields requires emphasis on toxicological matters related to liposomes exposure. liposomes have the potential to cause DNA damage, as they are considered as forging bodies. Different mechanisms of liposomes causing DNA damage could be due to the production of oxidative stress, direct interaction with DNA by disturbing spindle fibre plus other components or by inducing genome instability through epigenetic mechanisms. Some damage inducing properties of liposomes include their size, agglomeration state, release of toxic metal ions and their capability to produce ROS. Hence detection of genotoxicity, induced by liposomes is very important and crucial for reducing their effects. (Karlsson et al., 2015). For these reasons, the effect of the liposome form was compared to the bulk form, after preparing the liposome with less toxicity possible (No metals included).

4.1 Material and methods

This was described in detail in chapter 2.

4.2 Results

4.2.1 Agglomeration state/particle size distribution and zeta potential (surface charge) analysis

After preparation of the liposomes, determination of the size of liposomes was deemed to be essential as well as the stability of liposomes. Dynamic Light Scattering (DLS) and zeta potential (ZP) was used in evaluating the agglomeration state/particle size distribution and surface charge in aqueous solution and in the liposome form which the difference in the size is clear. For the size distribution, we observed that Secukinumab liposomes (SL) were well dispersed after gentle shaking before the experiment. The average hydrodynamic size (Z-Average; d.nm) ranged from 693.8 to 806. The stability of the liposomes was confirmed by measuring their size over a month.

Although, the particle size determined for the liposome form of Secukinumab was >100nm, we still refer to them as liposomes. Nano particles of the drug suspended in excipient mixture were compared to the solution prepared from its bulk form in the same excipient mixture. Results show the average particle of the bulk and nanoparticles as 1983 and 760nm, respectively (table 4.1). Nanoparticles of Secukinumab were much smaller compared to the bulk particles. Nanoparticles have been shown to penetrate cell membranes via phagocytosis and endocytosis and thus provide a higher effective dose within the cells (Gradinaru et al., 2010). It is reported that particles in the size range of 250 nm to 3µm are mainly internalised by phagocytosis whereas those below 200nm are mainly up taken by endocytosis (Amit et al., 2014). Nano medicine has been shown to exhibit different biological activity compared to solutions of the same drug.

Table 4-1. Average particle size of Secukinumab bulk and liposome

Suspension name	Zeta average particle size	Quality
Secukinumab Liposome	760 nm	good
Secukinumab bulk	1983 nm	good

4.2.2 Stability of Secukinumab liposome form

The stability of the particles for both forms of Secukinumab was assessed by checking their particle size and the difference was less than 1% over 1 month observation. Hence, these were considered stable to be used for the study. The suspensions were also sonicated for 10 mins before each use to avoid sedimentation and control aggregation.

4.2.3 TEM Analysis

TEM (Transmission Electron Microscope) micrographic images of the aqueous dispersion of Secukinumab at 2.1 concentration were measured at 20,000X magnification as shown in (Figure 4.1a).Then the measurement was taken for the liposome form (Figure 4.1b).

Characterisation of the agglomerated state of nanoparticles is commonly performed via DLS using optical techniques to analyse their dynamic properties and size distribution. The Zetasizer works by observing the Brownian motion of particles after passing a monochromatic laser light through a solution. The intensities of the scattered light waves spread out in all directions and are translated into particulate size (Mountain, 1992). Since these particles move according to the laws of Brownian motion, particle sizes may fluctuate (Wills et al., 2017).

To address some of the limitations of the DLS technique, alternative methods such as SEM (Scanning Electron Microscope) and TEM were used. TEM offers very high resolution because it uses an electron beam with shorter wavelengths. Regardless of any advantages offered by TEM, a number of limitations were noted in this technique (Winey, Meehl, O'Toole, & Giddings, 2014).TEM can only analyse one sample at a time – termed drop-cast TEM – where samples dispersed in a medium are dropped and allowed to air dry on a TEM grid before imaging.

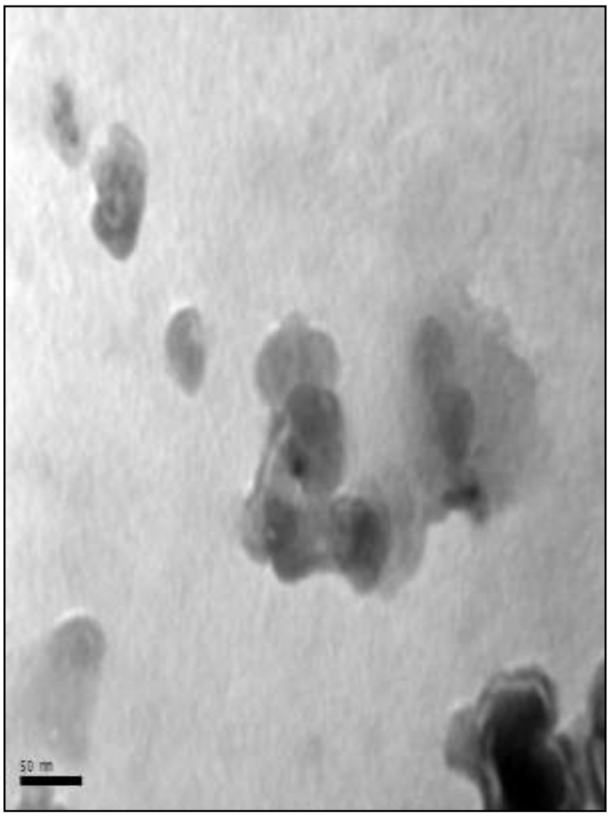

Figure 4-1a. TEM analysis of the aqueous dispersion of Secukinumab

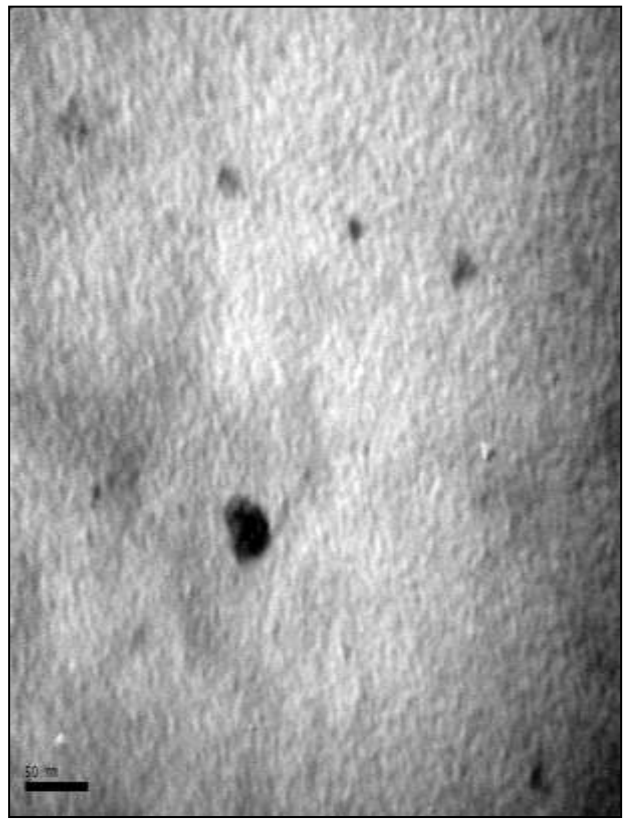

Figure 4-1b. TEM imaging showing liposomal form of Secukinumab

Although the particle sizes, shapes and sample compositions can be analysed via TEM imaging, this technique may not be a reliable method to measure particle agglomeration since the particles tend to agglomerate as the liquid components evaporate (Hondow et al., 2012; Wills et al., 2017).

Another limitation is that the intensity of the ionising radiation can damage the samples during TEM analysis and may not be suitable for thermolabile samples. A further critical limitation of characterisation via TEM is that the micrographs produced are cross-sectional 2D images of agglomerated particles, with several images observed at various points during the analysis, usually 3D images.

The scale bar at the bottom left of each image below gives the approximate length of respective particle shown in the image (Figure 4.1). To reduce and avoid the possibility of dust particles' clinging to the surface of charged TEM grids, sample preparation was carried out in a clean fume hood cabinet using covered petri dishes and other equipment. Hence, chances of dust particles overlapping with the particles were made limited, enhancing the effectiveness of TEM visualization of Secukinumab bulk and liposomes and best pictures was used in this thesis.

Chapter 5. Cytotoxicity of Secukinumab bulk and liposome forms in human lymphocytes from healthy individuals and patients

Although psoriasis is normally a disease that manifests locally on the skin, Secukinumab is usually administered subcutaneously by injection. This is the only route that is currently commercially available. Therefore, this project seeks to be the first step in the clinical process of offering a modified form that can be applied topically as a cream or via micro-dermabrasion, thereby avoiding any unnecessarily systemic side effects when treating this skin disease. Therefore, a liposome form was prepared and discussed before and the need to study it toxicity is mandatory with comparing it to the bulk form.

This Chapter summarises the genotoxic effect of Secukinumab in human lymphocytes and discusses its effect on their metabolic activities using the comet assay. The use of human peripheral blood lymphocytes (PBLs) in the assessment of cytotoxicity can be traced back over half a century. Human PBLS exhibit a remarkable sensitivity, both in vitro and in vivo, leading to their inclusion in the WHO guidelines on the human biomonitoring of genotoxicity, describing PBLs as surrogate cells and genotoxic endpoints (Albertini *et al.*, 2000).

In this Chapter, we evaluate the impacts of Secukinumab in both its bulk and liposomal forms on human whole blood via the alkaline comet assay and CBMN. Human whole blood was selected in preference to isolated lymphocytes as it is simpler and faster to detect and monitor changes in blood samples during patients' early medical treatment than relying upon late detection when much more damage may have been inflicted, thereby leading to poor prognosis. In this Chapter, we are mindful of the fact that genotoxicity can occur in the absence of cytotoxicity (Henderson, Wolfreys, Fedyk, Bourner, & Windebank, 1998).

5.1 Materials and Methods

The methods were as described in Chapter 2.

5.1.1 Cytotoxicity of Secukinumab in human lymphocytes from healthy individuals and patients

Please refer to Chapter 3.

5.1.2 H_2O_2-induced DNA Damage

As described in Chapter 3.

5.1.3 Genotoxic effects of bulk and liposome forms of Secukinumab in vitro in human whole blood from healthy individuals and psoriatic patients

In the previous Chapter, we established that using the alkaline comet assay to measure human whole blood derived from both patients and healthy individuals treated with different concentrations of Secukinumab has shown no significant effects in terms of DNA damage, indicating that Secukinumab is not genotoxic in these cells. In this Chapter, we will discuss the differences arising between the genotoxic effects of the bulk and liposomal forms of Secukinumab using the comet assay which affords microscopic detection of DNA damage. The damaged DNA is separated from the intact DNA, thereby generating a ‘comet tail’. Measurement of the damage arising is conducted either through a visual scoring of cells or else using software that automatically recognises the extent of damage.

The main limitation of the comet assay is that it does not directly measure the number of specific DNA lesions, but rather the migration of DNA in agarose gels as a consequence of the strand breaks induced under alkaline environments. There are substantial variations in comet assay procedures and primary descriptors (such as the % DNA in the tail) between laboratories. These variations might confound inter-laboratory data comparisons and interpretation, as well as any attempts to standardise the method and promote the use of reference standards. For this reason, the Minimum Information for Reporting Comet Assay (MIRCA) recommendations were taken into consideration when conducting this assay (Møller *et al.*, 2020). However, this technique also confers various advantages in that the comet assay also identifies DNA damage at the level of an individual cell, allowing for more robust statistical analysis. This reduced the number of cells needed for a sample, augmenting the sensitivity and enabling the use of any eukaryotic single-cell population (either in vitro or in vivo) for eco-genotoxicological studies (Kumaravel, Vilhar, Faux, & Jha, 2009).

The comet images (Figure 5.1) derived from patients reveal various levels of DNA damage induced by different treatments. Since 75 μM H_2O_2 induces the highest levels of DNA damage in healthy individuals as compared to controls, it was used as a PC. When DNA damage is induced by an exogenous agent or any treatment, it moves towards the anode during electrophoresis revealing the extent of the DNA damage. The greater the DNA damage, the longer the comet tail, whereas non-damaged DNA stays intact in the head and is much shorter when comets are analysed. Two parameters of the comet assay, namely the OTM and % tail DNA are used to measure the intensity and extent of DNA damage caused by any treatment.

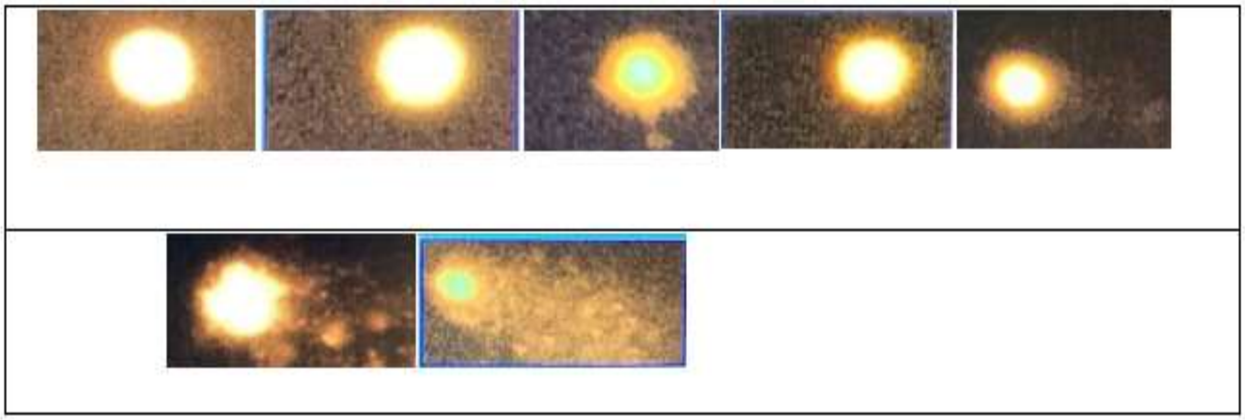

Figure 5-1. Comet images derived from lymphocytes treated with different concentrations of various agents. The first line shows a well-defined head with little damage, whereas the 2nd line shows damage to the DNA indicated by the presence of a tail giving the image its characteristic comet shape.

DNA damage, expressed as OTMs and % tail DNA assessed at basal levels was slightly higher in psoriatic patients as compared to healthy individuals. This could be due to the disease state in the patient group. However, DNA damage levels were almost the same in both investigative groups when comparing the effect of bulk and liposomal forms of Secukinumab.

The results from the comet assay showed that H_2O_2 had induced significant levels of DNA damage in both groups although, Secukinumab administered in both forms (liposomal and bulk) suppressed the effect of H_2O_2 and significantly attenuated the adverse effects caused by this oxidising agent as shown in Figures 5.2and 5.3. Again, there was not much difference in DNA damage between healthy and patient groups after treatment with both forms of Secukinumab when supplemented with H_2O_2, although H_2O_2 induced more damage in healthy individuals than in patients at basal levels. Our results indicate that Secukinumab had no adverse effect on lymphocyte DNA when administered at 2.1µg/ml in both liposomal and bulk forms together with 75µM H_2O_2 as measured via % DNA tail and OTM analysis. However, it suppressed the damaged caused by H_2O_2 even when adding IL17.

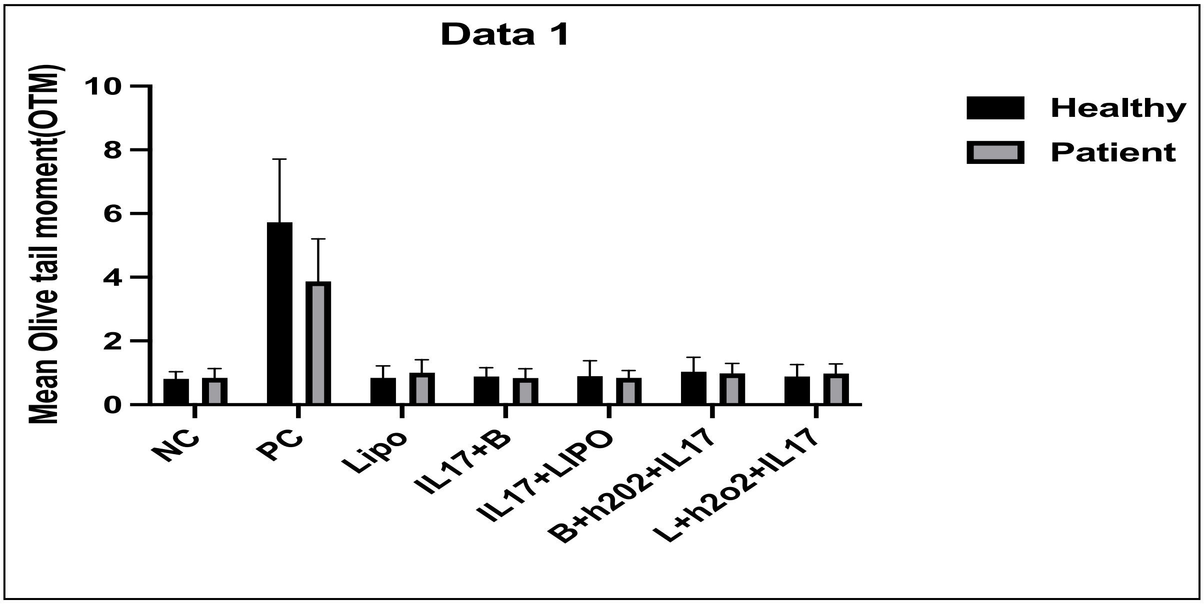

Figure 5-2. Olive tail moments (OTMs) in lymphocytes derived from healthy individuals as compared to those from the patient group

Different treatment groups included untreated negative control (NC); 75 µM H_2O_2 as positive control (PC); Secukinumab in liposomal form; IL-17 + Secukinumab in bulk form; IL-17 + Secukinumab in liposomal form; IL-17 + Secukinumab in bulk form + H_2O_2; and IL-17 + Secukinumab in liposomal form + H_2O_2. Data are presented as means ±SD from 20 individual experiments. All the groups were compared against the PC and NC as well as with each other

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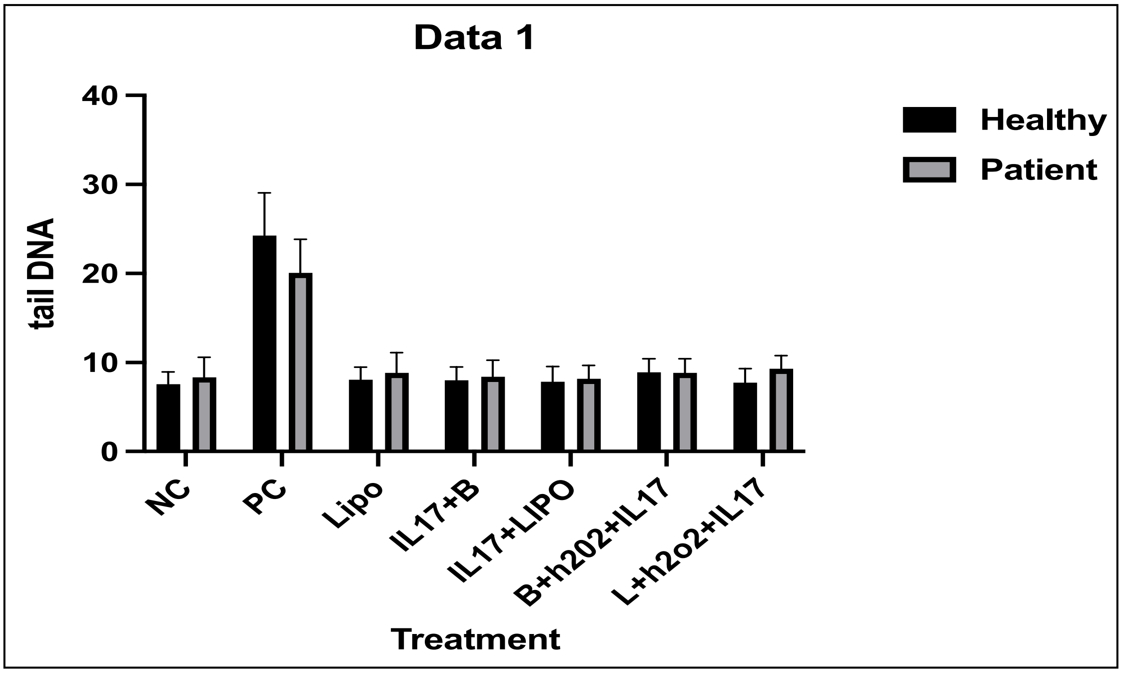


Figure 5-3. Bar graph showing % tail DNA in lymphocytes derived from healthy individuals in comparison to those from patient group

Different treatment groups included: untreated negative control (NC); 75 µM H_2O_2 as positive control (PC); Secukinumab in liposomal form; IL-17 + Secukinumab in bulk form; IL-17 + Secukinumab in liposomal form; IL-17 + Secukinumab in bulk form + H_2O_2; and IL-17 + Secukinumab in liposomal form + H_2O_2. Data are presented as means ± SD. All the groups were compared against the PC and NC as well as with each other

5.1.4 Analysis of the effects of Secukinumab on genetic instability endpoints (Chromosome aberration and Micronuclei (MNi)) in healthy individuals and psoriatic patients

5.1.4.1 Cytokinesis-Blocked Micronucleus (CBMN) Assay

Previously, we established from the alkaline comet assay that human whole blood derived from both patients and healthy individuals treated with Secukinumab for 30 min were non-genotoxic.

We also observed that DNA damage broadly equivalent in both patients and heathy individuals. When we compared the levels of DNA damage in the treated samples and NC in both healthy and patients, no significant difference was observed, although cells treated with PC had the highest level of DNA damage and treatment with Secukinumab markedly reduced this damage. Having established the validity of the comet assay as a measure of DNA damage, this Chapter goes on to assess chromosomal damage parameters using the CBMN assay. In this Chapter, we also investigated the cytogenetic effects in terms of chromosomal breakage through disturbance of the chromosomal formation. During a normal human cell cycle, a cell undergoes series of events. It grows in size, then replicates its DNA, an event that is followed by cellular division. Cell division is comprised of two phases, namely mitosis (nuclear division) and cytokinesis (cytoplasmic division), leading to the production of two identical daughter cells. These newly formed daughter cells then grow and repeat the whole process when proliferating. After each phase of the cell cycle has been successfully completed, the cells move on to the next phase. However, sometimes cells fail to progress and are unable to complete the process, instead entering a resting phase (G0-phase).

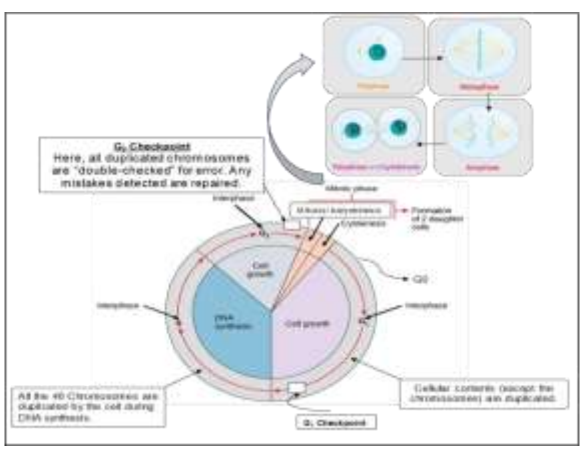


Figure 5-4. Schematic illustration of the cell cycle comprising interphase (G0, G1, S and G2) and the mitotic phase (mitosis and cytokinesis). From Lumen Learning, 2017; CNX 2019).

This is also known as quiescence (Daignan-Fornier & Sagot, 2011). Once cells enter this state, they do not normally divide again and remain in this state permanently.

During interphase, cell growth occurs during G1 until the first cell cycle checkpoint is reached. These so-called checkpoints of the cell cycle involved crucial cellular machinery that safeguards the healthy working and progression of the cell cycle. Cells are assessed at the G1 checkpoint for any potential growth errors or defects. Once the cells have successfully passed this checkpoint, they progress onto S phase where DNA synthesis occurs, and all chromosomes divide into two. Thereafter, cells enter G2 phase. The G2 checkpoint is a critical point in the cell cycle as, at this stage, the newly duplicated cells are "double-checked" for any defects before progressing to the next phase of nuclear division (mitosis/karyokinesis).

The mitotic phase of cell division is comprised of four successive steps, namely prophase, metaphase, anaphase and telophase. All these steps have to be completed in a coordinated and sequential manner so that the duplicated chromosomes can be segregated and apportioned into daughter nuclei. Mitosis is immediately followed by cytokinesis whereby cytoplasmic division takes place to form two new daughter cells, each with its own nucleus. The two daughter cells can either start a new cycle or enter the quiescent G0 state (Figure 5.4). The cell cycle is thus an organised sequence of events that are timed in a precise manner (Malumbres & Barbacid, 2009).

In the G1 phase, the cells actively grow and accumulate building blocks, including chromosomes and proteins essential for DNA synthesis, and the necessary components for DNA replication and synthesis are checked at the **G1 checkpoint** in readiness for the next phase of the cell cycle. In **S phase**, all 46 chromosomes are duplicated to yield two identical DNA sister-chromatids which then progress into G2 phase. Here, the cells continue to grow, producing yet more proteins and cellular components, including the various organelles. At the G2 checkpoint, chromosomal arrangements are actively checked for any defects and any identified abnormalities are repaired in preparation for the mitotic or M phase.

In M phase, the nuclear DNAs are condensed into chromosomes and the cells undergo nuclear divisions to form two nuclei through 4 sub-phases as previously described and illustrated in Figure 4.7. In the final phase of the cell cycle (cytokinesis), the cytoplasm divides to form two new daughter cells (Rodrigues et al., 2018). The duration of the cell cycle varies, depending on the cell type. In a rapidly dividing human cell, the cell cycle could be completed in 24 hrs with 11 hrs spent in G1 phase; 8 hrs in S phase; 4 hrs in G2 phase and 1 hr in M phase (Malumbres & Barbacid, 2009).

The basis of the CBMN assay is its sensitivity in detecting chromosomal aberrations (e.g., BiNC, MNi, etc.) after exposure to mutagenic agents in actively dividing cells. Cytokinesis commences immediately after mitosis (nuclear division) is complete. Cyto-B is a cell-permeable mycotoxin that blocks the cytokinesis stage of the cell cycle and disrupts actin formation – a contractile microfilament ring assembly that is vital for the cell morphology (shape), cytoskeletal framework and structure of cells (Heng & Koh, 2010). In addition to cytokinesis inhibition, Cyto-B has an intrinsic DNA damaging property, leading to the accumulation of damaged chromosomes (clastogenicity) as well as loss of chromosomes (aneugenicity) (Schwarzbacherova, Šiviková, Holečková, & Dianovský, 2016). A CBMN assay was used to assess these chromosomal instability parameters in cells derived from human whole blood after exposure to treatment and Mitomycin C (MMC) or H_2O_2 as PC. Mitomycin C (MMC) is a widely recognised anti-tumour antibiotic and acts as a clastogenic and genotoxic compound.

The CBMN assay has long been an important test in the detection of the genotoxicity of different compounds (Cho *et al.*, 2020). By measuring micronuclei and other chromosomal abnormalities, such as NPBs (a biomarker of dicentric chromosomes) and NBUDs (biomarkers of gene amplification) we investigated the effects of Secukinumab at a chromosomal level. MNis are cytoplasmic bodies that are produced as a result of the incapacity of centric fragments or whole chromosomes to traverse to opposite poles during anaphase, during which time the nuclear envelope forms around the lagging chromosomes and fragments which gradually take on the morphology of an interphase nucleus which is smaller than the main nuclei in the cell (Fenech et al., 2011; Luzhna, Kathiria, & Kovalchuk, 2013). To arrest cell division Cyto-B was used (Doherty, Bryce, & Bemis, 2016) (Figure 5.5).

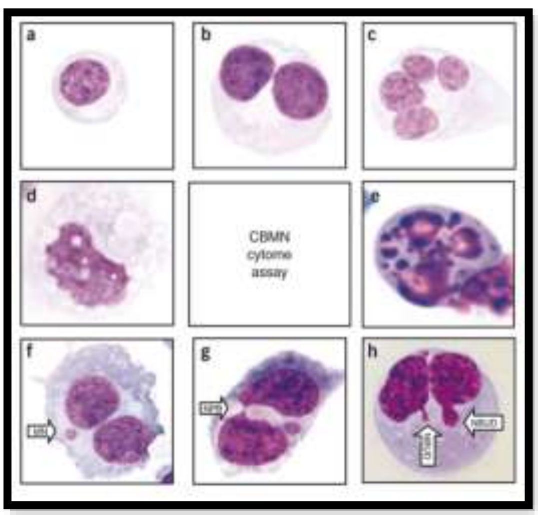


Figure 5-5. Photomicrographs of different types of cells scored in the CBMN assay. a) Mononucleated cell; (b) BN cell; (c) multinucleated cell; (d) necrotic cell; (e) late apoptotic cell; (f) BN cell containing one or more MNi; (g) BN containing an NPB (and a MN); (h) BN cell containing NBUDs (Benassi-Evans et al. 2007; Fenech 2007).

Here we have tested the genotoxicity of Secukinumab and its ability to assist in the repair of DNA damage that has occurred due to H_2O_2 oxidation. It was found that Secukinumab is genoprotective and has the ability to reverse the oxidative damage produced by H_2O_2, confirming the results from the comet assay.

5.1.4.2 The micronucleus (MN) results

Lymphocytes from five healthy individuals and five psoriasis patients were treated variously with 0.4µM MMC, 75µM H_2O_2, 2.1µg/ml of Secukinumab, 2.1µg/ml Secukinumab plus H_2O_2, and 2.8µg/ml Secukinumab plus H_2O_2. The concentrations of the chemicals used in this assay are the optimal doses determined via dose-response curves.

5.1.4.2.1 MNi in binucleated cells (BiNC)

In vitro treatment of lymphocytes from healthy individuals and psoriasis patients with MMC and H_2O_2 (PCs) significantly induced the appearance of MNis in BiNC as compared to the respective control untreated group. Secukinumab (at 2.1µg/ml) on its own caused no significant detectable levels of genotoxicity. However, when different concentrations of Secukinumab were co-administered with H_2O_2, MNi frequency in BiNC decreases as compared to H_2O_2 alone. All treatment groups showed similar induction of MNis in BiNC in both investigated groups, although the differences between them were not statistically significant (Figure 5.6).

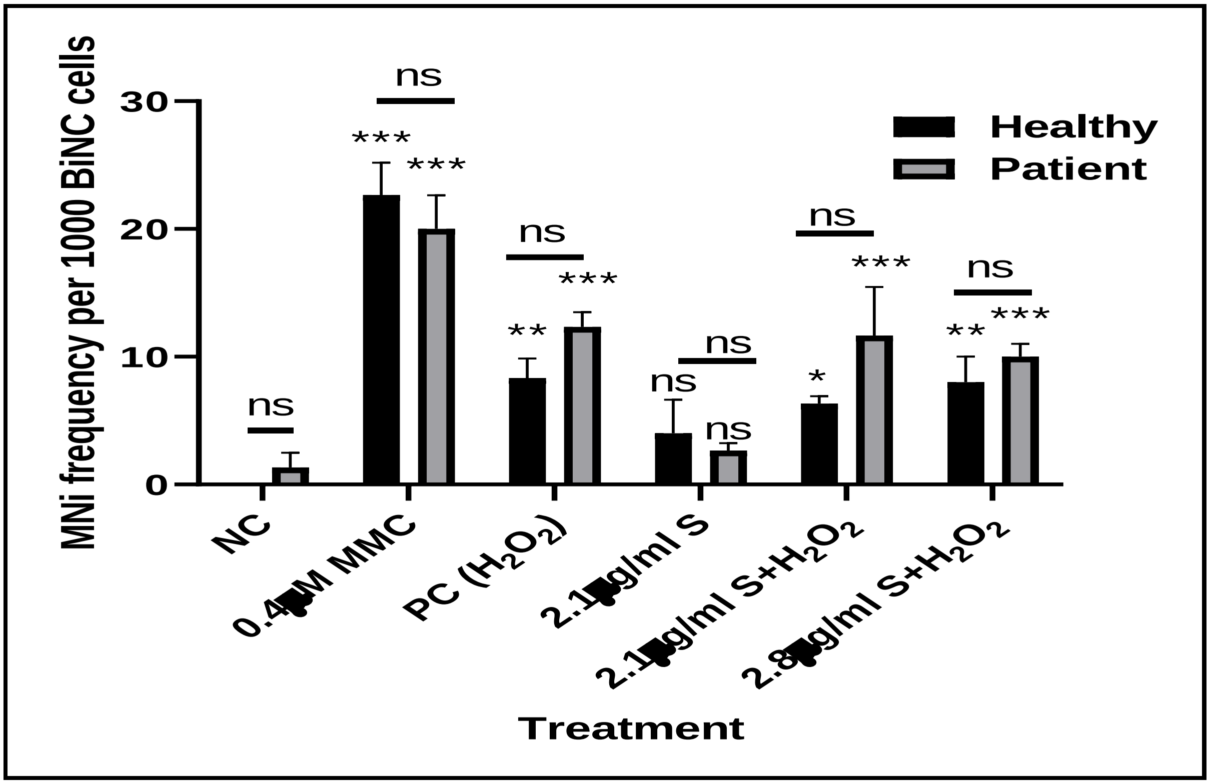


Figure 5-6. The average frequency of MNis in BiNC scored per 1,000 cells from 5 independent experiments on cells from healthy individuals and psoriasis patients (n=1,000). Data are expressed as means ± SE

The six treatment groups included the NC(negative control), two PC (positive control) groups, 0.4μM MMC, 75μM H_2O_2, 2.1μg/ml Secukinumab, 2.1μg/ml Secukinumab + H_2O_2, and 2.8μg/ml of Secukinumab + H_2O_2, where *** represents a P<0.0001, **p<0.001, *p<0.02, *p<0.018, and ns indicates that the findings were not significant (horizontal lines and the statistics above them show the differences between the groups).

5.1.4.2.2 MNis in mononucleated cells

After treating lymphocytes from both groups with the PCs (0.4μM MMC, 75μM of H_2O_2), there was a significant increase in MNi frequency observed in mononucleated cells (MonoNCs). Secukinumab treatment (2.1μg/ml) showed a slight reduction in MNi induction in the patient group, while inducing MNi in the healthy control group, although the levels were not statistically significant when compared to the respective NC. A significantly higher level of MNi in MonoNC was calculated in the patient group without treatment (NC). Addition of Secukinumab at 2.1µg/ml or 2.8µg/ml with H_2O_2 reveals some attenuation of the effects caused by H_2O_2 (Figure 5.7).

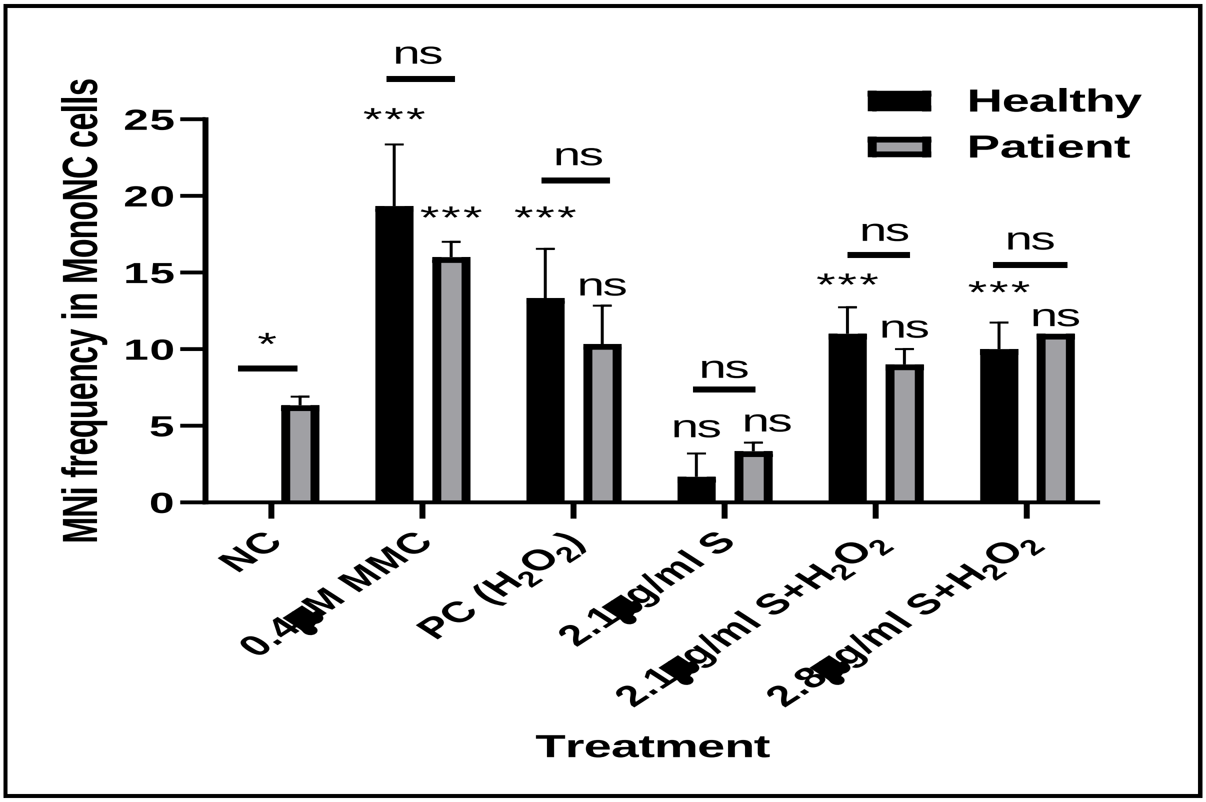


Figure 5-7. The average MNi frequency in MonoNCs from 5 independent experiments performed on cells derived from healthy individuals and psoriasis patients. The six treatment groups included the negative control (NC), two positive control groups (MMC 0.4µM and 75µM H_2O_2), 2.1µg/ml Secukinumab, and 2.1µg/ml of Secukinumab with H_2O_2 and 2.8µg/ml of Secukinumab with H_2O_2 (*** represents P< 0.001, **p<0.001, *p<0.01, and ns indicates no statistical significance. The horizontal lines and the statistics above them show the differences arising between the groups).

5.1.4.2.3 Other biomarkers of MN assay

Table 4.1 shows the other important determinants of DNA damage in the MN assay. No significant difference arises between the NDI of different treatment groups. The untreated patient group (NC) reveals a greater number of MNis in MoNCs without treatment as compared to the group treated with 2.1μ/ml Secukinumab. Secukinumab at 2.1μ/ml induces no significant difference in the number of MNis in either group. Most of the % of BiNC observed within different groups arises within the normal range.

Table 5-1. The average of various parameters measuring chromosomal damage in the micronucleus assay after treating lymphocytes from healthy and patient groups with different chemicals

Subject	**Treatment Group**	**NDI**	**% BiNC**	**Per 1000 BiNC CELLS**			**% MNi in MoNC**
				BiMNi	BiNPB	BiBuds	
Healthy individuals	NC	1.9	60	0	0	0	0
	0.4μM MMC	1.9	59	22	1	0	19
	75μM H_2O_2	2	60	8	0	0	13
	2.1μg/ml S	1.9	60	4	0	0	2
	2.1μg/ml S+75μM H_2O_2	1.9	61	6	0	0	11
	2.8μg/ml S+75μM H_2O_2	1.9	60	8	0	0	10
Psoriasis patients	NC	1.9	56	1	0	1	6
	0.4μM MMC	1.8	60	20	0	2	16
	75μM H_2O_2	1.9	60	12	1	1	10
	2.1μg/ml S	1.9	60	2	0	0	3
	2.1μg/ml S+75μM H_2O_2	2	54	11	0	0	9
	2.8μg/ml S+75μM H_2O_2	2	55	10	0	0	11

Nuclear division indices per treatment on lymphocytes from healthy individuals and psoriasis patients are presented. Key: NDI – nuclear division index; BiNC- binucleated cells; MoNC – mononucleated cells; Multi-NC – multinucleated cells; BiMNi – binucleated cells with micronuclei; BiNPB – binucleated cells with nucleoplasmic bridges; BiBuds – binucleated cells with nuclear.

We then studied the effect of the liposomes and compared these with the data from the bulk form of Secukinumab in healthy and patient groups. There was no significant difference arising between bulk and liposomal forms of Secukinumab treatment, and the treatment had no observable genotoxic effect on either investigative group. Further, we checked the cytotoxicity of IL17 in healthy individual and patient groups and found no evidence of any DNA damage, thereby confirming the results from the comet assay.

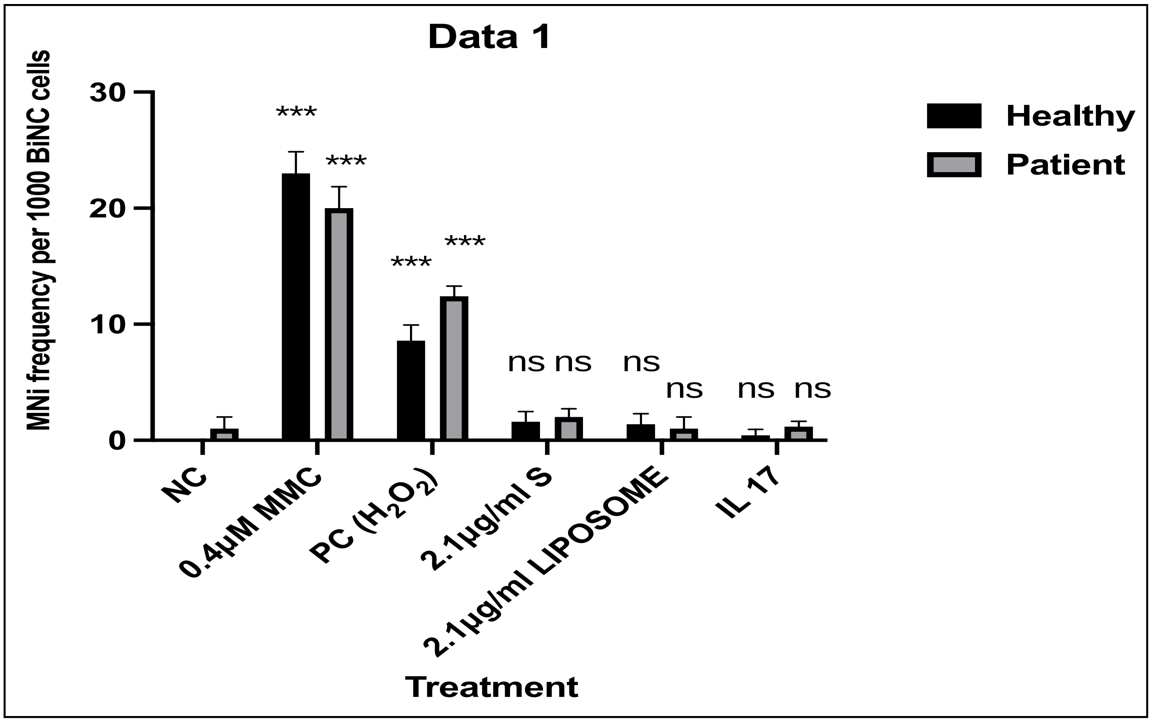


Figure 5-8. The average of MNis in BiNC scored per 1,000 cells from 5 independent experiments derived from healthy individuals and psoriasis patients (n=1,000). Data are expressed as means ±SE. The six treatment groups included the negative control (NC), two PC (positive control) **groups 0.4µM MMC and 75µM of H_2O_2, 2.1µg/ml Secukinumab, 2.1µg/ml Secukinumab in liposomal form and the effect of IL17. (ns = not significant,***P<0.001)**.

Then the effect of the liposome was studied. The lymphocytes from healthy individuals treated with Secukinumab in both forms had no effect on MNi frequency when compared with untreated cells. The MMC and 75 µM H_2O_2 both induced a significant increase in the Mni frequency in lymphocytes (***p<0.001), yet Secukinumab (bulk or liposome co-supplemented with H_2O_2) showed significant decreases of MNi in lymphocytes (*p<0.05). It was evident that liposomal Secukinumab caused a significant reduction in the number of MNis in patients (***p<0.001 as compared to untreated cells). Furthermore, the addition of MMC and 75 µM H_2O_2 showed a significant increase (***p<0.001) in the MNi number in lymphocytes as compared to the untreated cells (Figure 5.8).

Table 5-2. The average of various parameters of chromosomal damage in the micronucleus assay after treating lymphocytes from healthy and patient groups with different chemicals

Subject	Treatment Group	NDI	% BiNC	Per 1000 BiNC CELLS			% MNi in MoNC
				BiMNi	BiNPB	BiBuds	
Healthy individuals	NC	1.9	60	0	0	0	0
	0.4µM MMC	1.9	59	23	1	1	18
	75µM H_2O_2	2	60	8	1	0	14
	2.1µg/ml S	1.9	60	4	0	0	2
	2.1µg/ml liposome	1.9	61	6	0	0	11
	IL17	1.9	60	0	0	0	1
Psoriasis patients	NC	1.9	56	1	0	1	7
	0.4µM MMC	1.8	60	20	0	2	17
	75µM H_2O_2	1.9	60	12	1	1	10
	2.1µg/ml S	1.9	60	2	0	0	3
	2.1µg/ml S Liposome	2	54	11	0	0	9
	Il17	1.9	55	1	0	0	7

Presenting NDI per treatments on lymphocytes from healthy individuals and psoriasis patients. Key: NDI – nuclear division index, BiNC – binucleated cells, MoNC – mononucleated cells, Multi-NC – multinucleated cells, BiMNi – binucleated cells with micronuclei, BiNPB – binucleated cells with nucleoplasmic bridges, BiBuds.

Cytological scoring parameters are numerous and include various biomarkers of mitotic division in mononucleated cells (MoNCs), BiNCs, and multinucleated cells (MultiNCs). From the values of these parameters, the NDI was calculated for both healthy individuals and patients. The mean values for the NDI for all treatment groups were within the normal range, as shown in Table 4.2.

Table 4.2 also displays parameters of chromosomal instability in the form of the mean of NPBs, NBUDs per 1000 BiNC, and MNi frequency in MoNCs. The Table clearly shows that the frequency of MNis in the BiNC of untreated cells is higher than in lymphocytes treated with Secukinumab in psoriatic patients. The NDI values were calculated using the data from the proportions of MonoNCs, BiNCs, and MultiNCs per 1,000 cells scored. A review of the NDI data reveals a peculiar pattern. Their NDI was with in normal range. In healthy and patients, the various treatments did not cause a significant induction or reduction of NDI.

5.2 Discussion

The current in vitro study examined the effects of Secukinumab on human peripheral lymphocytes from twenty psoriasis patients and twenty healthy individuals using the comet assay. A further from five volunteers from each group were screened using the cytokinesis block micronucleus assay. In addition, the potential protective effects of Secukinumab on H_2O_2-induced DNA damage were determined for both study groups. Although, the total DNA damage of Secukinumab as the NC was negligible; in the presence of H_2O_2 Secukinumab protected cells from DNA damage. Secukinumab significantly decreased the percentage of DNA damage in lymphocytes using the comet assay and these results suggest that Secukinumab has the capacity to arrest and reduce DNA damage induced by H_2O_2 in healthy individuals and psoriasis patients. This could be due to the fact that oxidative stress results in the production of many pro-inflammatory cytokines and, since

Secukinumab is an anti-IL-17, it will logically reverse many of the effects of oxidation (Woods & Davies, 2003).

Although, the CBMN assay is claimed to be an affordable and swift assay, one which is widely used in genetic toxicology studies (İpek *et al.*, 2017), which in this study encountered various limitations during the course of our experiments. First, it identified dead cells which had accumulated after cytokinesis had been blocked making the method time-consuming and laborious as it took up to 8 days before the initial results could be obtained, including 72 hrs of cell treatments under sterile conditions, and 1-2 hrs of cell fixation, slide preparation, and Giemsa staining. According to our experience, each duplicate slide took over 30 min to 1 hr to score, making the micronucleus assay unsuitable for field studies where rapid results are required. Furthermore, the CBMN involves the tedious process of manually scoring 1,000 cells (i.e., 500 cells per concentration using hand-held tally counters). This counting process could lead to human error and inter-laboratory discrepancies among various researchers (Fenech, 2009).

In this study, Secukinumab shown DNA repairing influence against DNA damage in human lymphocytes obtained from patients and healthy. Furthermore, The Data illustrated that all samples with 75Mm H_2O_2 had high levels of DNA damage in both OTM and % tail DNA and a significant reduction in DNA repair capacity. The combination of the Secukinumab with H_2O_2 reduced the frequency of MNi compared to H_2O_2 in patients' lymphocytes.

Our results from the Comet and micronucleus assays showed that Secukinumab bulk and liposome did not induce significant DNA damage in the lymphocytes from healthy individuals and patients. Secukinumab has shown genoprotective effects in lymphocytes from patients by significantly inhibiting MNi induction in MoNC. This could be possible due the repair mechanism of the drug. These findings suggest that Secukinumab in both forms has anti-genotoxic properties depending on the concentration. However, at non genotoxic concentration Secukinumab could protect the lymphocytes of healthy individuals and patients against DNA damage and possibly facilitate the repair, however, this will need further studies.

Chapter 6. Gene and protein expression levels of various cytokines in lymphocytes of healthy individuals and psoriasis patients

6.1 Real-time Polymerase Chain Reaction (RT-PCR)

Based on our previous results from the comet and micronucleus assays we established that Secukinumab has anti-genotoxic effects on lymphocytes derived from healthy individuals and psoriatic patients. Secukinumab exhibited geno-protective effects in lymphocytes from psoriatic patients by significantly reducing the MNi induction in MoNCs. To identify the molecular mechanisms involved in this effect, we studied the influences of Secukinumab on the gene expression levels of p53, P21, RORC, IL-22, IL-17 in patients versus healthy lymphocytes and ATM kinase mRNA expression in patient lymphocytes. RORC, IL-17 and IL-22 genes were chosen due to their significant roles in the pathogenesis of psoriasis and their effects on IL-17 and, unlike other cytokines, little research has been done on them. P53 and P21 was chosen for their known involvement in the DNA damage cascade in human lymphocytes. P53 is a suppressor gene, encodes the p53 protein, a vital component in human cells, and provides a key role in stress control factor in human cells (Horn and Vousden, 2007). The first p53 target gene to be identified was (p21), a gene which encodes the cyclin-dependent kinase inhibitor which plays a main role in the cell cycle regulation (Georgakilas et al. 2017).

6.1.1 Effect of Secukinumab bulk and liposome forms on gene expression levels of cytokines

Our results from the comet and micronucleus assays showed that Secukinumab, in both bulk and liposomal forms (2.1µg/ml), caused anti-genotoxic effects at basal levels and exhibited antioxidant potential by attenuating H_2O_2-induced DNA damage in lymphocytes from healthy individuals and psoriasis patients. In order to understand the molecular mechanisms involved in the geno-protective effects of Secukinumab in psoriasis patients, we studied the gene expression levels of some important cytokines which are known to play a vital role in the pathogenesis of the disease. The regulation of gene expression levels of IL-17, IL-22 and RORC were assessed after treatment with Secukinumab bulk (2.1µg/ml) and Secukinumab liposome (2.1µg/ml) treatment forms via RT-PCR analysis. Total RNA was isolated from blood samples of healthy volunteers and psoriasis patients pre-treated with both forms of Secukinumab and exposed to quantitative RT- PCR.

Treatment groups included an untreated negative control (NC), two PC groups (H_2O_2 75µM and IL-17), Secukinumab bulk (SB) 2.1µg/ml, and Secukinumab liposome (SL) 2.1µg/ml. Results from healthy individuals demonstrated that the oxidative stress inducer H_2O_2 (at 75µM) significantly up-regulates gene expression of all three cytokines (IL-17, IL-22 and RORC)

. However, IL-17 (PC) only up-regulated gene expression levels of IL-17a and had no effect on IL-22 or RORC. Secukinumab bulk (SB) 2.1µg/ml and Secukinumab (SL) 2.1µg/ml also had no significant effect on the regulation of any of these genes under investigation as compared to the NC group (Figure 6.1).

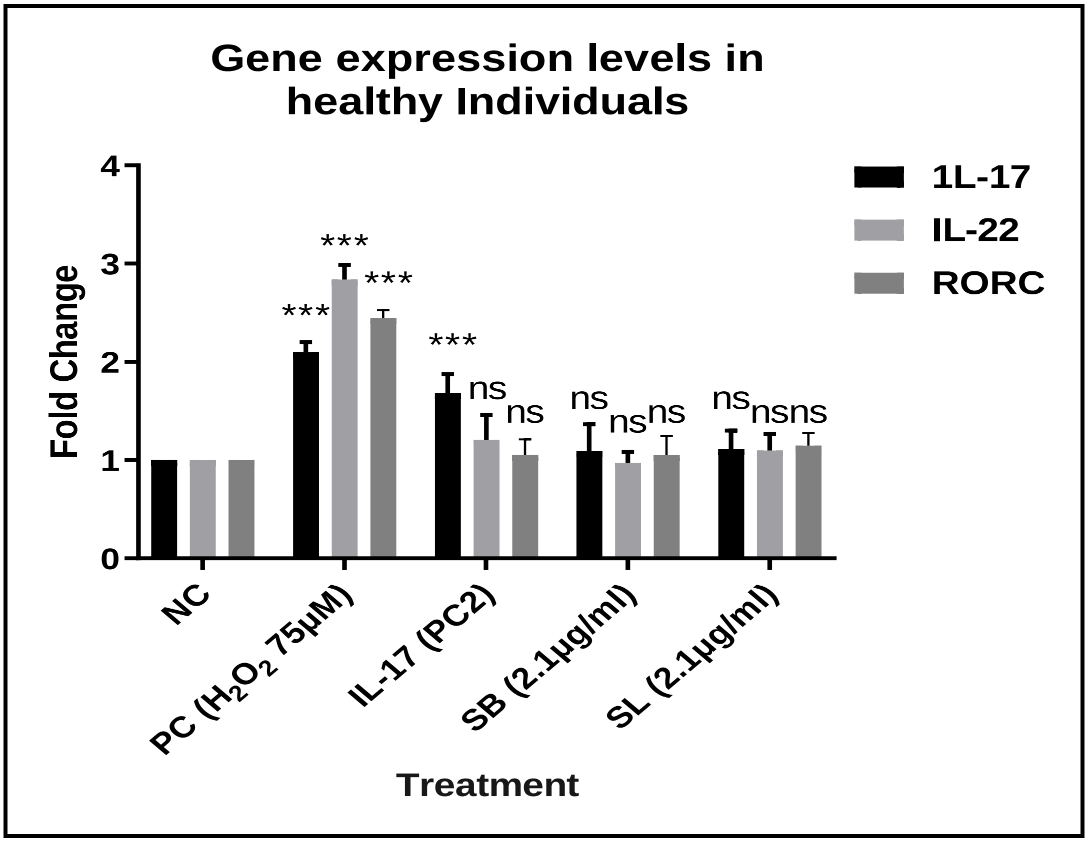


Figure **6**-1. The effects of Secukinumab bulk and liposome treatments on the gene expression levels of IL-17, IL-22 and RORC in healthy individuals. All data from the treatment groups were compared against the control group (C) and normalised against the internal housekeeping gene β-actin. Experiments were repeated three times for three different individuals. The treatment groups included an untreated negative control (NC), H_2O_2 75µM as a positive control (PC) group, IL-17 as a PC2, Secukinumab bulk (SB) 2.1µg/ml and Secukinumab liposome (SL) 2.1µg/ml. Secukinumab in both forms did not show any significant effect on levels of IL-17, IL-22 and RORC expression (ns = not significant, $^{***}P<0.001$**).**

When same treatments were applied on psoriasis patient group, H_2O_2 induced a significant up-regulation ($p<0.001$) of IL-17, IL-22 and RORC levels, as observed in healthy individuals. The PC, IL-17, also up-regulated ($p<0.001$) the expression levels of all three cytokines, a result that diverged from its behaviour in the healthy group where it only affected IL-17. Secukinumab bulk

(SB) 2.1µg/ml treatment significantly down-regulated gene expression of IL-17, IL22 and RORC to 0.46-fold, 0.47-fold and 0.5-fold, respectively. However, Secukinumab liposome (SL) 2.1µg/ml only decreased the expression of IL-17 and IL-22 significantly, by 0.46-fold and 0.53-fold, respectively (Figure 6.2).

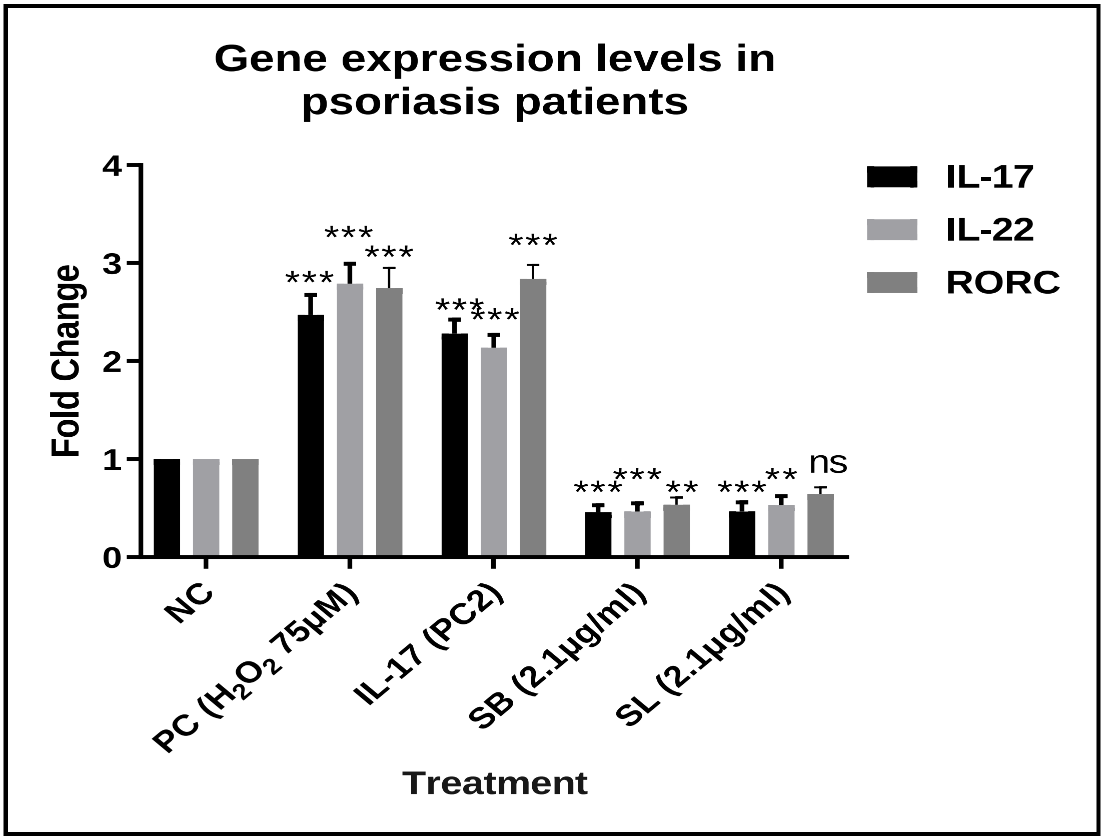


Figure 6-2. Effect of Secukinumab in bulk and liposomal form on the gene expression levels of IL-17, IL-22 and RORC in psoriasis patients. All data from the treatment groups were compared against the control group (C) and normalised against the internal housekeeping gene, β-actin. Experiments were repeated three times in three different individuals. The treatment groups included an untreated negative control (NC), H_2O_2 at 75µM as a positive control (PC), IL-17 as a PC2, Secukinumab bulk (SB) 2.1µg/ml and Secukinumab liposome (SL) 2.1µg/ml. Secukinumab in both forms significantly down-regulated the expression levels of IL-17, IL-22 and RORC expression (ns = not significant, **P<0.01,***P<0.001).

This indicates that the protective effects caused by Secukinumab bulk and liposomal forms in psoriasis patients might be caused by suppressing the activity of IL-17, IL-22 and RORC. Time PCR was conducted to analyse (P53 and P21), however, there was no significant effect on the P53 and P21. The results showed that treatment do not significantly regulate the expression of these genes at in lymphocytes from healthy individuals and patients (Figure 6.3 and 6.4 respectively). H2O2 showed up regulation of P53 and P21 in healthy, however, this was not significant. This was significant in patient with P53. P21 (p <0.001, 0<0.1 respectively)

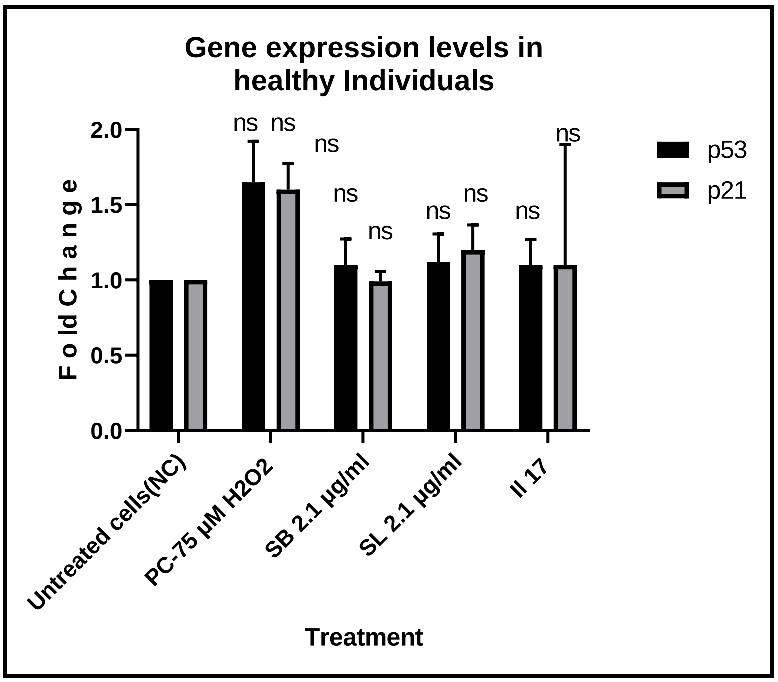


Figure **6**-3. The effects of Secukinumab bulk and liposome treatments on the gene expression levels of P5**3** and P21 in healthy individuals. All data from the treatment groups were compared against the control group (C) and normalised against the internal housekeeping gene β-actin. Experiments were repeated three times for three different individuals. The treatment groups included an untreated negative control (NC), H_2O_2 75µM as a positive control (PC) group, P53 and P21, Secukinumab bulk (SB) 2.1µg/ml and Secukinumab liposome (SL) 2.1µg/ml. Secukinumab in both forms did not show any significant effect on levels of IL-17, IL-22 and RORC expression. (ns=not significant)

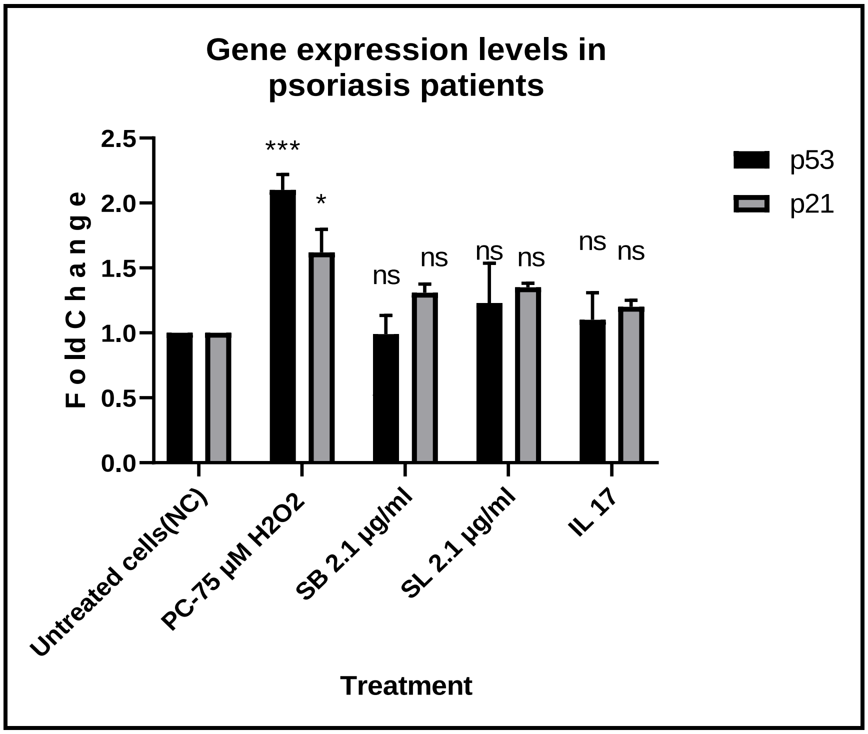


Figure 6-4. Effect of Secukinumab in bulk and liposomal form on the gene expression levels of P53 and P21 in psoriasis patients. All data from the treatment groups were compared against the control group (C) and normalised against the internal housekeeping gene, β-actin. Experiments were repeated three times in three different individuals. The treatment groups included an untreated negative control (NC), H_2O_2 at 75µM as a positive control (PC), IL-17 as a PC2, Secukinumab bulk (SB) 2.1µg/ml and Secukinumab liposome (SL) 2.1µg/ml. Secukinumab in both forms did not show any significant effect on P53 and P21. (***p <0.001, *0<0.1, ns = not significant)

6.2 Western blotting

6.2.1 The effect of Secukinumab bulk and liposome forms on IL-17, IL-22 P53, P21 and RORC protein expression levels in isolated lymphocytes

We conducted several experiments using the Western blotting technique to determine the protein expression levels of IL-17, IL-22, P53, P21 and RORC and validate the results from RT-PCR. Western blotting was used to investigate the effect of Secukinumab bulk (SB, 2.1 mg/ml) and

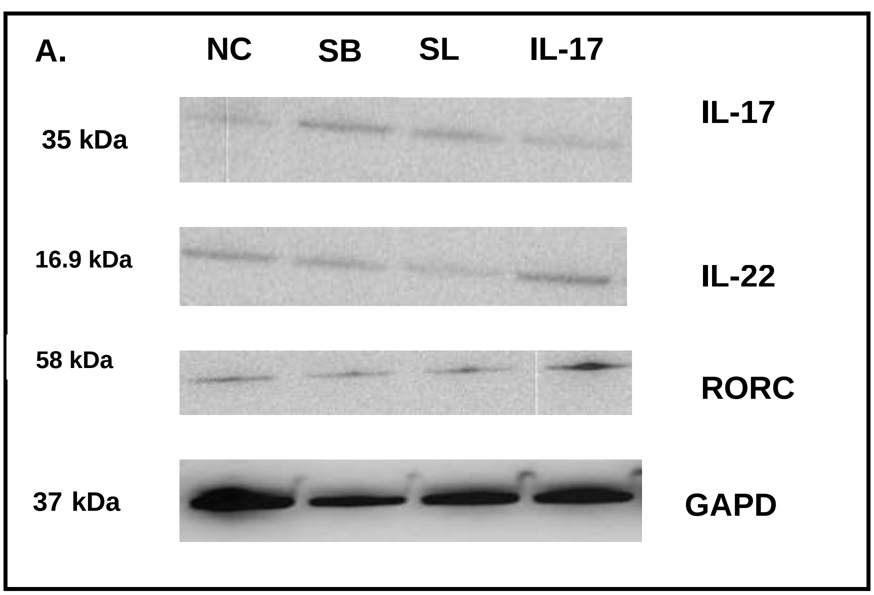
Secukinumab liposome (SL, 2.1µg/ml) on protein expression levels of major genes associated with the pathogenesis of psoriasis, namely P53, P21, IL-17, IL-22 and RORC as expressed in lymphocytes from healthy individuals and psoriasis patients.

Analysis of the results showed that Secukinumab bulk 2.1 mg/ml and Secukinumab liposome 2.1µg/ml had no significant effect on expression levels of any of these proteins in lymphocytes derived from healthy individuals. Moreover, no significant difference was observed in their regulation. However, there was a statistically significant down-regulation observed in the protein expression levels of IL-17, IL-22 and RORC in lymphocytes obtained from the psoriasis patients, confirming the sensitivity of the compromised lymphocytes from patient group to Secukinumab treatment. With SB (2.1 mg/ml) administration, a 0.5-fold decrease was observed in IL-17, 0.59-fold decrease in IL-22, and a 0.6-fold decrease in RORC expression. However, SL (2.1 mg/ml) reduced their levels to 0.47–fold, 0.5-fold and 0.47–fold, respectively, when compared to the control group. These results indicated that Secukinumab (at 2.1 mg/ml) in both forms has potential to suppress the adverse effects of psoriasis in lymphocytes from patients by decreasing the expression levels of IL-17, IL-22 and RORC, ultimately generating a protective effect. Further, the liposomal form has proven to be more effective than its bulk counterpart. Hence, the liposomal form of the drug could contribute towards the better management and treatment of the disease. Furthermore, our results from the Western blot analysis were in accordance with our results from the RT-PCR technique (Figure 6.5 and 6.6).

All data from the treatment groups in healthy individuals were compared against control group and normalised against the internal housekeeping gene GAPDH. Secukinumab, in both forms, showed no significant effect on IL-17, IL-22 and RORC protein expression levels (ns = not significant).

All data from the treatment groups of patients were compared to the control group and normalised against the internal housekeeping gene, GAPDH. Secukinumab, in both forms, caused a significant down-regulation of IL-17, IL-22 and RORC protein expression (***P<0.001) in patients. On the other side, had no significant effect on expression of P53 and P21 proteins in lymphocytes from healthy individuals and patients. Analysis of the results showed that Secukinumab had no significant effect on expression of any of these proteins in lymphocytes from healthy individuals and patients and there was no difference observed in their regulation (Figure 6.7 and 6.8).

When studying the effect of IL-17, there was no significant effect on expression of P53, P21, IL-22 and RORC proteins in lymphocytes from healthy individuals. However, it showed non-significant rise in the expression of IL-17. On the other hand, there was a up regulation of IL-17, IL-22 and RORC in the lymphocytes from patients (P<0.1). This confirms the results from the RT-PCR.



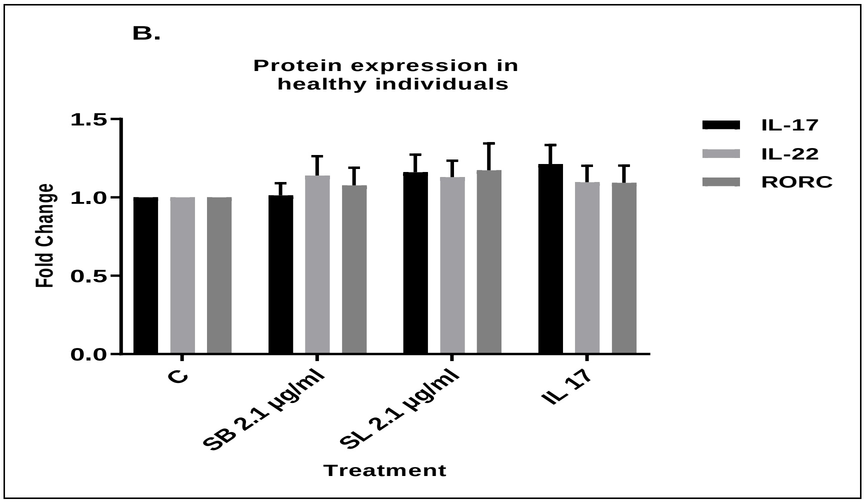
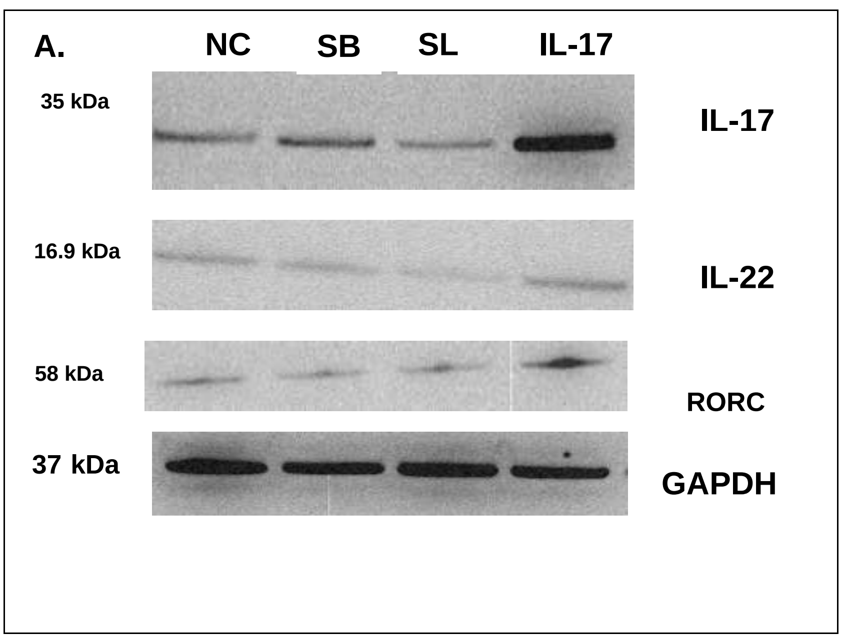


Figure 6-5. The effect of Secukinumab bulk and liposome forms 2.1 mg/ml on the protein expression levels of IL-17, IL-22 and RORC in lymphocytes from healthy individuals determined using Western blotting. (A) Immunoblot analysis of the IL-17, IL-22 and RORC proteins in lymphocytes from healthy individuals treated with SB, SL and IL-17. **(B)** Bar graphs exhibiting fold changes in protein expression levels. Data are represented as the mean ± SD of three experiments conducted in three different individuals. All data from the treatment groups were compared against control group and normalized against the internal home gene, GAPDH. Secukinumab in both forms did not show any significant effect on IL-17, IL-22 and RORC protein expression. (ns=not significant)



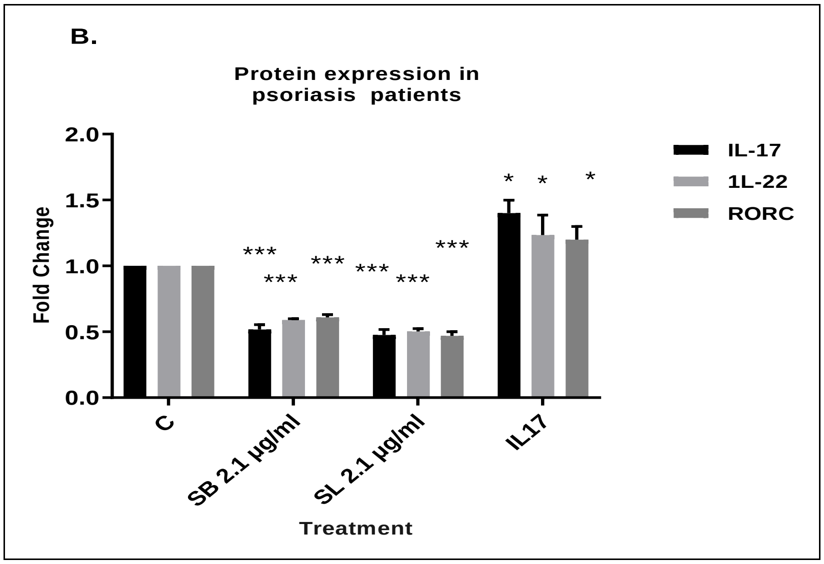


Figure 6-6 The effect of Secukinumab bulk and liposome forms 2.1 mg/ml on the protein expression levels of IL-17, IL-22 and RORC in lymphocytes from psoriasis determined using Western blotting. (A) Immunoblot analysis of the IL-17, IL-22 and RORC proteins in lymphocytes from healthy individuals treated with SB, SL and IL-17 **(B)** Bar graphs exhibiting fold changes in protein expression levels. Data are represented as the mean ± SD of three experiments conducted in three different patients (***P<0.001, *P<0.1)

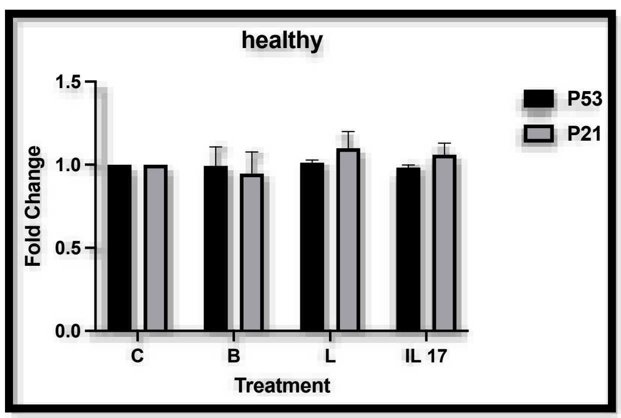


Figure 6-7. The effects of Secukinumab bulk and liposome forms and IL-17 on protein expression levels of P53 and P21 in lymphocytes obtained from healthy individuals as determined via Western blotting. Bar graphs exhibiting fold changes in protein expression levels of P53 and P21 which did not show any significant effect with all treatments on lymphocytes of healthy individuals. Data are represented

as the means ±SD of three experiments conducted on lymphocytes derived from three different patients. This diagram illustrates the there is no significant effect on these proteins.

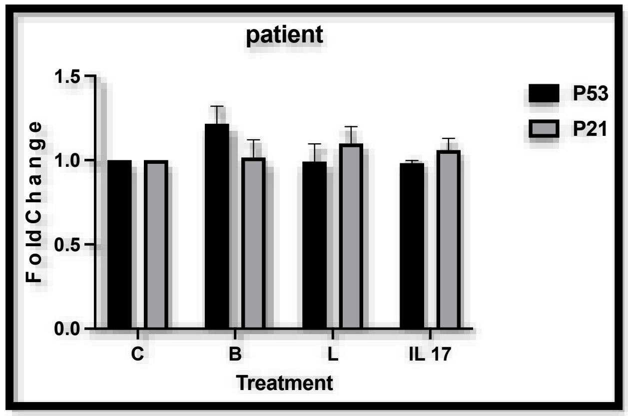


Figure 6-8. **The effects of Secukinumab bulk and liposomal forms and IL-17 on protein expression levels of P53 and P21 in lymphocytes derived from psoriasis patients as measured using Western blotting**. **Bar graphs exhibiting fold changes in protein expression levels of P53 and P21 which showed no significant effect on patient lymphocytes with all treatments. Data are represented as the means ±SD of three experiments conducted on lymphocytes derived from three different patients.** This diagram illustrates the there is no significant effect on these proteins.

Chapter 7. Discussion

Psoriasis is an inflammatory condition with small itchy or painful patches on the skin. Psoriasis is associated with many interleukins which play pivotal role in inflammation (Bodoor et al., 2020). IL17 is one such cytokine and is involved in the adaptive and innate immune mechanisms of psoriasis. During these events, the activation of other cytokines occurs, including IL17A, IL17F and IL22 in keratinocytes, as induced by neutrophils and macrophages during inflammation (Tokuyama & Mabuchi, 2020). IL17RC is a co-receptor of IL17RA that triggers IL17A- and IL17F-mediated signalling in the pathogenesis of the disease (Deng et al., 2021). Efforts to target IL17 and IL17RA are underway for treatment of autoimmune diseases such as psoriasis. Now, there is treatment available on the market that works by blocking IL17 activity in the form of Secukinumab.

Psoriasis is a skin inflammatory disorder characterised by the production of inflammatory cytokines and an associated activation of the immune system (Ortega *et al.*, 2009). Th17 has been identified within a subset of lymphocytes which are referred to as Th17/IL-17-producing cells. These play a vital role in the development of autoimmune disorders (Zhang, Jin, Peng, Ramgolam, & Markovic-Plese, 2008). IL-17-producing lymphocytes (Th17) are thus considered key intermediaries in the pathogenesis of psoriasis (Ortega *et al.*, 2009). In the case of inflammation, the up-regulation of certain markers, including IL-17, IL-22 and RORC, was observed. The up-regulation of IL-22 induces inflammation in skin disorders such as acanthosis and psoriasis. CD8 IL-17-producing cells produce various inflammatory mediators including TNF-α, IFN-γ, IL-17, IL-21 and IL-22, as well as a significant up-regulation of RORC transcription factor (Ortega *et al.*, 2009). In current study, a down-regulation of IL-17, IL-22 and RORC by Secukinumab was demonstrated which may explains the associated with a decreased level of inflammation when using the drug.

Molecular studies also revealed an increased expression of p53 in psoriatic patients in half of the patients, while the other half showed decreased expression (Jasim, Lioe, McKenna, Robson, & Ouhtit, 2006). In another study, an accumulation of p53 protein was observed in cutaneous carcinoma, but not in psoriasis. However, in psoriasis, p21 may be overexpressed via a p53-independent pathway (Chang, Sung, Moon, Koh & Choi, 1999), which was not the case in this research. No parallel increase in p53 expression was observed in normal human KCs. An up-regulation of p21WAF1 was also observed in response to treatment in other studies (Todd & Reynolds, 1998). However, this research could not prove that p53 and p21 had specific involvement in psoriasis.

ROR is essential for differentiation of Th17 cells and the production of IL17. Inhibition of ROR is considered a promising strategy in the treatment of Th17-mediated autoimmune diseases (Tang et al., 2018). Human IL17A producing cells derive from cluster of differentiation 161 $^{+}$CD4^{+} progenitor cells, which express ROR variant 2 (RORC2) (Baricza, 2018). The RORC2 gene is a dominant regulator of human Th17 cells. Psoriasis vulgaris is mediated by T cells and dendritic cells. IL23 and IL12 are released by inflammatory myeloid dendritic cells to activate IL17-producing T cells, Th1 cells, and Th22 cells to produce abundant psoriatic cytokines IL17, IFNγ, TNF, and IL22. IL22 inhibits the terminal differentiation of keratinocytes and induces the production of antimicrobial peptides and some chemokines. This, in turn, leads to epithelial remodelling and inflammation (Lopez & Kongsbak-Wismann, 2022). Research has shown that non-affected patches of skin in psoriasis patients demonstrate lower expression levels of IL22 than does the skin of healthy controls (Ghafouri-Fard, Eghtedarian, Taheri, & Rakhshan, 2020). IL17 reduces the expression of filaggrin and other genes involved in the adhesion of keratinocytes, seemingly contributing to the skin barrier's interference in inflammatory diseases to the effects of IL17 (Bozó et al., 2021).

Among biologics, Secukinumab is the ultimate drug used in the treatment of severe psoriasis (Blair, 2021). Secukinumab is a first-in-class, recombinant, high-affinity monoclonal antibody (mAb), also known as anti-IL17A, and is used in the treatment of many inflammatory diseases, including psoriasis. Secukinumab completely neutralises the activity of IL17A without affecting IL17F and Th17 cells. Serum levels of total IL17A (free and Secukinumab-bound IL17A) are initially increased in patients receiving Secukinumab. A study of researchers evaluated two existing anti-IL17A antibodies, Ixekizumab and Secukinumab. The researchers found that their donors had fewer pre-existing T cells and demonstrated fewer T cell responses to Secukinumab than others (Morita et al., 2020).

Liposomes are highly versatile vehicles for analytical, therapeutic, and research applications as they are biocompatible, biodegradable, and non-immunogenic. Topical delivery systems for drugs via lipid vesicles were developed and the first report to show the effectiveness of liposomes for skin delivery was by More, Nandgude, & Poddar (2016).

Available treatments for psoriasis mainly rely upon alleviating symptoms and containing their progression to improve clinical outcomes. Thus, the development of new therapeutic strategies in the treatment of psoriasis is a considerable challenge and a necessity. Our study is the first to determine the genotoxicity of various concentrations of the drug in psoriasis patients. Although the efficiency and safety of the drug has been proven clinically and the best dose had previously been identified for clinical use, the dose toxicity and optimal dose for use in vitro has not previously been identified. Based on the ICH guidance, genotoxicity studies had not yet been conducted for Secukinumab to the satisfaction of CHMP. The three doses selected for assays were 2.1, 2.8 and 3.75 μg/mL as 10 μg/mL was found to be toxic and to cause damage to cells. In the MTT assay, the data showed decreases in % cell survival rates after exposure to different concentrations of Secukinumab (at 2.1, 2.8 and 3.75 μg/mL). A survival rate of over 80% was considered a safe dose and, therefore, 2.1 and 2.8 μg/mL were deemed safe dosages, while 3.75 μg/mL was considered toxic as there was only a 66.9% survival rate. Results from the cell viability assay revealed concentration-dependent cytotoxicity of the drug in lymphocytes. However, the two lower concentrations of the drug were considered non-toxic and carried forward throughout the research studies presented in this thesis, confirming the results of MTT. In vitro treatment of healthy lymphocytes with different concentrations of H_2O_2 in the comet assay revealed that 75μM H_2O_2 produced the most significant levels of DNA damage when compared to the negative control (NC) group. Secukinumab has shown similar effects on the DNA damage in the lymphocytes caused by H_2O_2 in the patient group as compared to healthy individuals. Secukinumab 2.1μg/ml induced no significant levels of DNA damage in lymphocytes from either group. Thus 2.1 μg/ml became a standardised dosage for this thesis.

Confounding factors such as smoking, drinking habits and age were considered as potential contributors to DNA damage owing to their potential mutagenicity and, ultimately, potential role in cancer development. These lifestyle and environmental factors tend to increase one's susceptibility to cancer, and diseases. Therefore, an investigation was carried out to determine the effects of the confounding factors of age, ethnicity, gender and smoking amongst both the healthy volunteers and patient group using the comet values. The results demonstrate that there was no significant relationship found among any of these variables in any of the treatment groups or between healthy and patient ethnic groups. However, Secukinumab liposomal treatment did elicit a statistically insignificant decrease in DNA damage in all groups. In the current study, the down-regulation of IL17, IL-22 and RORC by Secukinumab was observed and thus there was a decreased level of inflammation after treatment with Secukinumab as compared to disease group, while the levels of these markers after treatment were almost equivalent to those of the healthy group.

There could be several molecular properties of Secukinumab involved in the reduction of this damage, including antioxidant defence. The two concentrations of Secukinumab used in this study exhibited no significant effects on DNA damage in the NC experiments of healthy individuals and psoriasis patients. However, in those experiments where H_2O_2 was added, Secukinumab significantly decreased H_2O_2-induced damage and efficiently attenuated its adverse effects, as measured in the comet and micronucleus assays. The patient untreated group had a higher level of DNA damage as compared to the comparative healthy untreated group throughout the in vitro study. Secukinumab (2.1µg/ml) demonstrated more effective results against H_2O_2-mediated DNA damage, overall. These results suggest that Secukinumab not only induced no significant signs of genotoxicity, but also produced protective effects against H_2O_2-induced oxidative stress, possibly even facilitating repair at the two lower concentrations tested. However, other possible molecular mechanisms may be involved which may form the focus of future work.

In this research, also observed that DNA damage was equivalent in patients and healthy individuals. When the DNA damage levels were compared in the treated samples and the NC in both healthy individuals and patient groups, there was no statistically significant difference. It was observed that cells treated with PC had the highest level of DNA damage, while treatment with Secukinumab reduced this damage. Moreover, it was studied the effect of the liposomal form of the drug and compared it with bulk treatment in both healthy and patient groups. This showed that there was no significant difference in the outcome between bulk and liposomal forms and that neither treatment was likely to be genotoxic. Further, when assessing the cytotoxicity of IL17 on cells from healthy individuals and patients, which did not show DNA damage to the cells. Secukinumab did not cause any overt DNA damage, validated through the results of the comet assay, CBMN, PCR and WB. The current in vitro study examined the effects of Secukinumab on human peripheral lymphocytes from twenty psoriasis patients and twenty healthy individuals using the comet assay in addition to a further five volunteers from each group using the cytokinesis block micronucleus assay. In addition, the potential protective effects of Secukinumab on H_2O_2-induced DNA damage were determined for both study groups. Although the total DNA damage of Secukinumab as a negative control proved ineffective, in the presence of H_2O_2, the effects of Secukinumab on the extent of DNA damage became evident, as Secukinumab significantly decreased the degree of DNA damage induced in lymphocytes according to the comet assay. These results suggested the ability of Secukinumab to arrest and reduce the DNA damage caused by H_2O_2 in healthy individuals and psoriasis patients. This could be due to the fact that oxidative stress results in the production of many pro-inflammatory cytokines and, since Secukinumab is an anti-IL17, it reverses the action of the oxidation. This indicates that the protective effects caused by Secukinumab, in both bulk and liposomal forms in psoriasis patients, might be caused by suppressing the activity of IL17, IL22 and RORC. Analysis of the results showed that bulk and liposomal treatments with Secukinumab had no significant effect on the expression levels of any of these proteins in lymphocytes derived from healthy individuals, and no significant difference was observed in their regulation. However, there was a statistically significant down-regulation observed in protein expression levels of IL17, IL22 and RORC in lymphocytes obtained from psoriasis patients, confirming the sensitivity of the compromised lymphocytes from the patient group to Secukinumab treatment.

In conclusion, Psoriasis is a chronic disease which cannot be controlled in some patients and the need for a safer treatment is mandatory. As the innovation of new medication takes a long journey, the development of an already existing treatment is less time-consuming and more affordable. Secukinumab was chosen for its known effect on the treatment of psoriasis with fewer side effects. The best dose of Secukinumab in the bulk and liposome form to be used in the lab was identified. The cytotoxicity of the drug was assessed and compared to positive control and showed that the drug is safe at 2.1 µg/ml. It also reversed the oxidative effect caused by H_2O_2. Although IL-17 is known to have a role in the pathogenesis of psoriasis, its addition of it did not cause an increase in cell damage. The results from real-time PCR show that there is no significant effect seen on the regulation of ATM kinase in lymphocytes from psoriasis patients and healthy individuals when checking the P53 and P21expression. Results in healthy individuals and psoriatic patients demonstrated that the stress inducer H_2O_2 (75µM) has significantly up-regulated the gene expression of IL17, IL-22 and RORC. However, IL-17 only up-regulated the gene expression levels of IL-17 cytokine and had no effect on IL-22 and RORC in heathy individuals, unlike in patients where IL-17 up-regulated ($p<0.001$) the expression levels of all three cytokines. Secukinumab bulk and liposome had no significant effect on the regulation of any of these investigative genes compared to the NC group in healthy individuals. On the other hand, the Secukinumab bulk and liposome significantly down-regulated gene expression of IL-17, IL22 and RORC in patients, which shows a protective or a repair mechanism of the drug through these genes. This was emphasized by the results of the western blot. Therefore, liposome version could be an efficient substitute with less DNA damage and better repair and suitable for the topical drug delivery system.

Further work

Although there are many data obtained from this research, there is still the need of further studies.

Identification of the Mechanism of the repair of Secukinumab to the oxidative damage caused by

H_2O_2 is mandatory. More research needs to be put in to study the stability of the liposomes. All the experiments done in this thesis should be repeated on skin cultures to study how much of the drug is absorbed into the cells and its stability in it. More research on the bioavailability of the liposomes in the cells, also, on the effect of the liposomes on the 3D model of psoriasis, keratinocytes, fibroblasts and vascular system from psoriasis patients.

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Appendices

Appendix 1. Data Collection Form

UNIVERSITY of BRADFORD
Faculty of Life Sciences

School of Life Sciences

DATA COLLECTION FORM (To be completed by the Doctor)

STUDY TITLE: Genetic and environmental effects in lymphocytes from different cancerous, precancerous and inflammatory conditions using various genetic endpoints

REVIEWED BY LEEDS East RESEARCH ETHICS COMMITTEE (REC)

(REC REFERENCE NUMBER: 12/YH/0464)

PATIENT NUMBER			DATE OF SAMPLE	
AGE				
SEX (PLEASE TICK)	M	F	CONSENT	Y / N
ETHNIC GROUP			INFORMATION SHEET	Y / N

CURRENT SMOKER	Y/N	PAST SMOKER	Y/N	HOW MANY/MUCH PER DAY?
CIGARETTES		CIGARS		PIPE
ALCOHOL	Y/N	UNITS PER WEEK		

DIET	WESTERN	ASIAN	OMNIVORE	VEGETARIAN	VEGAN

Any systemic treatment for psoriasis	
Any biological treatment	
Any UVB / PUVA	
Date of receiving biological treatment	
Recurrent or flare up	
Resistant to biological treatment	

Current diagnosis and PASI score

List of medications

Psoriasis

EXTENT		SITE		HISTOLOGY	

OTHER MEDICAL CONDITIONS
(PLEASE LIST)

Family history

Appendix 2. Consent Form

School of Life Sciences

Patient Number:

CONSENT FORM

Title of Project: **Genetic and environmental effects in lymphocytes from different cancerous, precancerous and inflammatory conditions using various genetic endpoints**

Reviewed by Leeds East Research Ethics Committee (REC)

(REC reference number: 12/YH/0464)

Names of Researchers: Prof. D Anderson, Dr. Mojgan Najafzadeh, Prof Andrew Wright, Dr Gazala Layas **Initials**

1. I confirm that I have read and understand the information sheet for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected. ☐

3. I understand that relevant sections of my medical notes and data collected during the study may be looked at by individuals from the NHS Trust or the University of Bradford, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records. ☐

4. I agree that the sample I have given and the information gathered about me can be stored at the University of Bradford, as described in the attached information sheet. ☐

5. I agree to take part in the above study. ☐

Patient number Date Signature

Patient name in capital

Name of Person taking consent | Date | Signature

When completed, 1 for patient; 1 for researcher site file; 1 (original) to be kept in medical notes

(Version 1 April 2019)

School of Life Sciences

Participant Information Sheet for patients (Version 4, 28/01/2013)

Study title: Genetic and environmental effects in lymphocytes from different cancerous, precancerous and inflammatory conditions using various genetic endpoints

Reviewed by Leeds East Research Ethics Committee (REC)

(REC reference number: 12/YH/0464)

Invitation to the research study

We should like to invite you to take part in a research study. Before you decide you need to understand why the research is being done and what it would involve for you. Please take time to read the following information carefully. Talk to others about the study if you wish and you will be allowed around 24 hours to consider this.

(Part 1 tells you the purpose of this study and what will happen to you if you take part).

Part 2 gives you more detailed information about the conduct of the study).

Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you want to take part.

Part 1

What is the purpose of the study?

In this study blood cells will be treated in a test tube with very small chemical particles or UVA (Ultra Violet A light) to determine if patients with different diseases are more at risk after exposure compared to healthy individuals. For example, chemicals and UV can break and damage the DNA of white blood cells. In this specific study we want to see the effect of Secukinumab and UVB on healthy individuals and patients with psoriasis.

Further examination of this resulting damage may improve our knowledge of psoriasis and other inflammatory diseases. The tests are not predictive for any kind of diseases and the test results will not impact on you or the healthy volunteers with whom you are compared. Blood samples for the study will be taken during the clinic visit in which you are recruited, if possible at the same time as the samples taken for your routine care. You will not be asked to return back for any other procedures or questions regarding the study.

A blood sample of around 1-2 teaspoons (5-10 ml) will be taken. Samples will be stored only until the end of the study (after 8 years) and used for studies of similar nature or to check original responses. The research is also used for some PhD programmes.

Why have I been invited?

Because you are a patient at the Clinic and we should like to determine if these small chemical particles or UVA could be more harmful to you than to people without the disease.

Do I have to take part?

No, it is up to you to decide. We shall outline the study and go through this information sheet, which we shall then give to you. We shall ask you to sign a consent form to show you have agreed to take part. You are free to withdraw at any time, without giving a reason. This would not affect the standard of care you receive.

Part 2

What will happen to me if I take part?

Only a single blood sample will be taken for this research study. A brief questionnaire will need to be completed by the researchers.

Each individual will be given a coded study number so that your clinical data will be linked in an anonymous way with the research results.

<u>The study tests are not predictive for you.</u>

The data obtained will only be available to the research team and will **not** be returned to you. Responses will be compared only on group basis i.e. collective responses from patients with that individual disease compared to collective responses from people without that disease. Results could be published in the form of scientific papers. The work may benefit the medical and scientific community at large, but will not be of direct benefit to you as an individual. If, however, you would like more information, the appropriate consultant will be prepared to talk to you individually about study results.

The data will be stored until the study is completed at the end of 8 years.

People who cannot take part in the study

People who are not well enough to take part will be excluded e.g. those with anaemia.

If you have any further questions, you could contact the research team:

Prof Andrew Wright, St Luke’s Hospital, Bradford Teaching Hospitals NHS Foundation Trust, BD5 0NA. Telephone: 01274 542200

Professor Diana Anderson, Established Chair in Biomedical Sciences, BSc, MSc, PhD, DipEd, FIBiol, FATS, FRCPath, FIFST, FBTS, FHEA, University of Bradford, Richmond Road, Bradford, BD7 1DP and Honorary Research Consultant to Bradford NHS Trust.

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Dr Gazala Layas MBBS, MSc, PhD researcher at University of Bradford Richmond Road, Bradford, BD7 1DP

Appendix 3. Product datasheet 1

OriGene Technologies, Inc.
9620 Medical Center Drive, Ste 200
Rockville, MD 20850, US
Phone: +1-888-267-4436
https://www.origene.com
techsupport@origene.com
EU: info-de@origene.com
CN: techsupport@origene.cn

Product datasheet for HP230849

ROR gamma (RORC) Human qPCR Primer Pair (NM_005060)

Product data:

Product Type:	qPCR Primer Pairs
Gene ID:	6097
Forward Sequence:	GAGGAAGTGACTGGCTACCAGA
Reverse Sequence:	GCACAATCTGGTCATTCTGGCAG
ACCN:	BC031554, NM_001001523, NM_005060, NM_005060.1, NM_005060.2, NM_005060.3, NM_001001523.1, BC031554.1, BC110571, BI520481, NM_001001523.2, NM_005060.4
UniProt ID:	P51449
Synonyms:	IMD42; NR1F3; RORG; RZR-GAMMA; RZRG; TOR
Components:	1 vial of lyophilized qSTAR qPCR primer mix (1 nmol each primer, sufficient for 200 reactions)
Quality Control:	The primer mix has been tested to generate satisfactory qPCR data on ABI 7900HT by using the following PCR program: Stage 1: Activation: 50 °C for 2 min; Stage 2: pre-soak:95 °C for 10 min; Stage 3: Denaturation: 95 °C for 15 sec, Annealing: 60°C for 1 min; Stage 4: Melting curve: 95°C for 15 sec, 60°C for 15 sec, 95°C for 15 sec.
Storage:	The primer mix is stable for one year from date of shipping. Store at -20°C.

Appendix 4. Product datasheet 2

OriGene Technologies, Inc.
9620 Medical Center Drive, Ste 200
Rockville, MD 20850, US
Phone: +1-888-267-4436
https://www.origene.com
techsupport@origene.com
EU: info-de@origene.com
CN: techsupport@origene.cn

Product datasheet for HP205926

IL17 (IL17A) Human qPCR Primer Pair (NM_002190)

Product data:

Product Type:	qPCR Primer Pairs
Gene ID:	3605
Forward Sequence:	CGGACTGTGATGGTCAACCTGA
Reverse Sequence:	GCACTTTGCCTCCCAGATCACA
ACCN:	NM_002190, NM_002190.1, NM_002190.2, BC066251, BC066252, BC066253, BC067503, BC067504, BC067505, NM_002190.3
UniProt ID:	Q16552
Synonyms:	CTLA-8; CTLA8; IL-17; IL-17A; IL17; ILA17
Components:	1 vial of lyophilized qSTAR qPCR primer mix (1 nmol each primer, sufficient for 200 reactions)
Quality Control:	The primer mix has been tested to generate satisfactory qPCR data on ABI 7900HT by using the following PCR program: Stage 1: Activation: 50 °C for 2 min; Stage 2: pre-soak:95 °C for 10 min; Stage 3: Denaturation: 95 °C for 15 sec, Annealing: 60°C for 1 min; Stage 4: Melting curve: 95°C for 15 sec, 60°C for 15 sec, 95°C for 15 sec.
Storage:	The primer mix is stable for one year from date of shipping. Store at -20°C.

Appendix 5. Product datasheet 3

OriGene Technologies, Inc.
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Product datasheet for HP213710

IL22 Human qPCR Primer Pair (NM_020525)

Product data:

Product Type:	qPCR Primer Pairs
Gene ID:	50616
Forward Sequence:	GTTCCAGCCTTATATGCAGGAGG
Reverse Sequence:	GCACATTCCTCTGGATATGCAGG
ACCN:	NM_020525, NM_020525.1, NM_020525.2, NM_020525.3, NM_020525.4, BC070261, BC070261.1, BC066263, BC066264, BC066265, BC066266, BC067509, BC067510, BC069112, BC069308, NM_020525.5
UniProt ID:	Q9GZX6
Synonyms:	IL-21; IL-22; IL-D110; IL-TIF; ILTIF; TIFa; TIFIL-23; zcyto18
Components:	1 vial of lyophilized qSTAR qPCR primer mix (1 nmol each primer, sufficient for 200 reactions)
Quality Control:	The primer mix has been tested to generate satisfactory qPCR data on ABI 7900HT by using the following PCR program: Stage 1: Activation: 50 °C for 2 min; Stage 2: pre-soak:95 °C for 10 min; Stage 3: Denaturation: 95 °C for 15 sec, Annealing: 60°C for 1 min; Stage 4: Melting curve: 95°C for 15 sec, 60°C for 15 sec, 95°C for 15 sec.
Storage:	The primer mix is stable for one year from date of shipping. Store at -20°C.

Appendix 6. How biological treatments are named

The World Health Organisation (WHO) dictates the names of generic drugs.

International Nonproprietary Names (INNs) for monoclonal antibodies shall be composed of a prefix that can be anything, a target (substem A), a species of origin (substem B), and a suffix (-mab) [2].

The prefix

The prefix can be random; the only requirement is to contribute to a pleasant-sounding and distinctive name.

Substem A

'Substem A' refers to the first of two internal stems that identify the type of drug. Substem A indicates the type of target antigen — a molecule, a cell, or an organ. A single letter is used when possible. If a vowel is also needed to aid pronunciation, use the vowel indicated in parentheses in Table 1.

Table 1: Naming a biologic: the WHO Substem A target

Target antigen type	Substem A
Bacterial	- b (a) -
Cardiovascular	- c (i) -
Fungal	- f (u) -
Interleukin	- k (i) -

Immunomodulating	- l (i) -
Neural	- n (e) -
Bone	- s (o) -
Toxin	- tox (a) -
Tumour	- t (u) -
Viral	- v (i) -

Substem B

With a single letter, Substem B describes the animal or human species or species (a chimera) on which the sequence of the monoclonal antibody is based.

Table 2. Naming a biologic: the WHO Substem B species

The species	**Substem B**
Rat	- a -
Rat + mouse	- axo -
Hamster	- e -
Primate	- i -
Mouse	- o -
Human (fully human)	- u -
Chimeric	- xi -
Chimeric + humanised	- xizu -

Humanised	- zu -

Chimeric antibody

A chimeric antibody is constructed from two sources: a foreign variable domain (ie, the V-D-J region) is synthesised or originates from a non-human species linked to a constant (Fc) region of human origin.

Humanised

A humanised antibody is a chimeric antibody constructed using antibody engineering. It consists of synthetic or non-human complementarity-determining regions of the variable domains and the remaining chain is of human origin.

The suffix

All monoclonal antibodies have the same suffix: -mab. This -mab suffix is used for all products containing an immunoglobulin variable domain that binds to a defined target.

Appendix 7

Cell Counting Kit-8

Cell Proliferation Assay and Cytotoxicity Assay

Technical Manual

for 100, 500, 1000, 3000, and 10000 tests

General Information

Cell Counting Kit-8 (CCK-8) allows very convenient assays by utilizing Dojindo's highly water-soluble tetrazolium salt. WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-*2H*-tetrazolium, monosodium salt] produces a water-soluble formazan dye upon reduction in the presence of an electron mediator, as shown in Figure 1.

CCK-8 is a one-bottle solution; no premixing of components is required. CCK-8, being nonradioactive, allows sensitive colorimetric assays for the determination of the number of viable cells in cell proliferation and cytotoxicity assays. WST-8 is reduced by dehydrogenases in cells to give an orange colored product (formazan), which is soluble in the tissue culture medium (Figure 2). The amount of the formazan dye generated by dehydrogenases in cells is directly proportional to the number of living cells.

Figure 3 shows that the cell proliferation assay using CCK-8 correlates well with the [^{3}H]-thymidine incorporation assay. Thus, the CCK-8 assay can also be substituted for the [^{3}H]-thymidine incorporation assay. As shown in Figure 4, the detection sensitivity using CCK-8 is higher than assays using other tetrazolium salts such as MTT, XTT, MTS or WST-1.

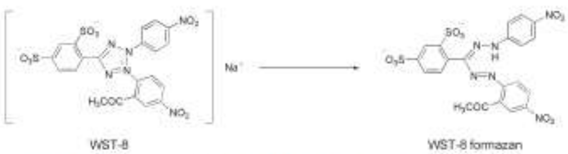

Figure 1. Structures of WST-8 and WST-8 formazan

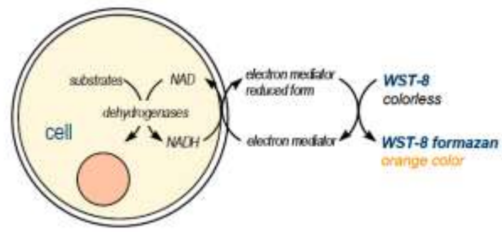

Figure 2. Principle of the cell viability detection with Cell Counting Kit-8.

Kit Contents

-	100 tests :	1 ml	x	1
-	500 tests :	5 ml	x	1
-	1000 tests :	5 ml	x	2
-	3000 tests :	5 ml	x	6
-	10000 tests :	100 ml	x	1

Storage

Store at 0-5℃.

CCK-8 is stable over one year at 0-5°C with protection from light. Store it at -20°C for longer storage. Repeated thawing and freezing causes an increase in the background, which interferes with the assay. Please store the kit at 0-5°C for frequent use.

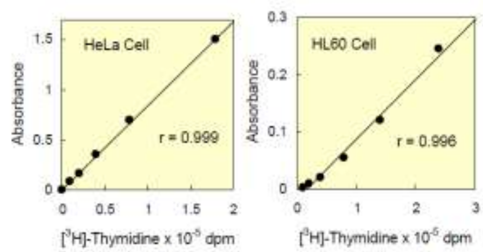

Figure 3. Correlation between CCK-8 assay and [^{3}H]-thymidine incorporation assay.

- plate reader (450 nm filter)
- 96-well plate
- CO_2 incubator
- 10 µl and 100 - 200 µl multi-channel pipettes

HL60.......RPMI1640, 10% FBS
Reagent : [^{3}H]-Thymidine.........37 KBq/well
CCK-8......................10 µl/well
Incubation : [^{3}H]-Thymidine assay..... 4 hours
CCK-8...........................3 hours

Cell Number Determination

1. Inoculate cell suspension (100 µl/well) in a 96-well plate. Pre-incubate the plate in a humidified incubator (e.g., at 37°C, 5% CO_2).
2. Add 10 µl of the CCK-8 solution to each well of the plate.
   Be careful not to introduce bubbles to the wells, since they interfere with the O.D. reading.
3. Incubate the plate for 1 - 4 hours in the incubator.
4. Measure the absorbance at 450 nm using a microplate reader.
   To measure the absorbance later, add 10 µl of 1% w/v SDS or 0.1 M HCl to each well, cover the plate and store it with protection from light at room temperature. No absorbance change should be observed for 24 hours.

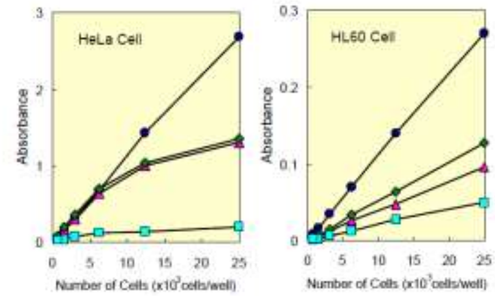

Figure 4. Cell number determination using CCK-8 and other reagents.

Medium : HeLa....... MEM, 10% FBS
HL60........ RPMI1640, 10% FBS
Incubation : HeLa........ 37°C, 5 % CO_2, 2 hours
HL60........ 37°C, 5 % CO_2, 3 hours
Detection : CCK-8 (●.... 450 nm, XTT (◆).... 450 nm
MTS (▲)....... 490 nm, MTT (■).... 570 nm

CK04 : Cell Counting Kit-8
Revised February 17, 2016

Cell Proliferation and Cytotoxicity Assay

1. Dispense 100 µl of cell suspension (5000 cells/well) in a 96-well plate. Pre-incubate the plate for 24 hours in a humidified incubator (e.g., at 37°C, 5% CO_2).
2. Add 10 µl of various concentrations of substances to be tested to the plate.
3. Incubate the plate for an appropriate length of time (e.g., 6, 12, 24 or 48 hours) in the incubator.
4. Add 10 µl of CCK-8 solution to each well of the plate.
   Be careful not to introduce bubbles to the wells, since they interfere with the O.D. reading.
5. Incubate the plate for 1 - 4 hours in the incubator.
6. Measure the absorbance at 450 nm using a microplate reader.
   To measure the absorbance later, add 10 µl of 1% w/v SDS or 0.1 M HCl to each well, cover the plate and store it with protection from light at room temperature. No absorbance change should be observed for 24 hours.

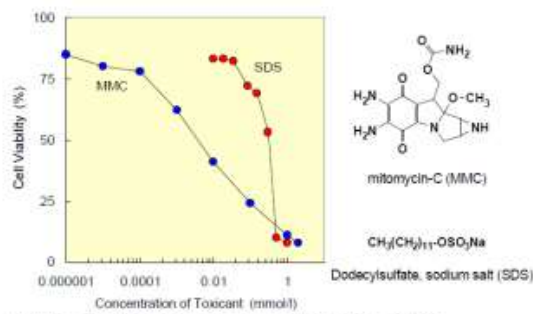

Figure 5. Toxicological test of chemicals using CCK-8.

Cell line : HeLa
Culture medium : MEM, 10% FBS
Chemicals : Mitomycin-C (MMC),
Dodecylsulfate, sodium salt (SDS)
Incubation : 37°C, 5% CO_2, 2 hours
Detection : 450 nm, Reference : 650 nm

1. Since the CCK-8 assay is based on the dehydrogenase activity detection in viable cells, conditions or chemicals that affect dehydrogenase activity in viable cells may cause discrepancy between the actual viable cell number and the cell number determined using the CCK-8 assay.
2. WST-8 may react with reducing agents to generate WST-8 formazan. Please check the background O.D. if reducing agents are used in cytotoxicity assays or cell proliferation assays.
3. Be careful not to introduce bubbles to the wells, since they interfere with the O.D. reading.
4. If the sterilization of the CCK-8 solution is necessary, please filter the solution with a 0.2 µm membrane.
5. The incubation time varies by the type and number of cells in a well. Generally, leukocytes give weak coloration, thus a long incubation time (up to 4 hours) or a large number of cells (~10^5 cells/well) may be necessary.
6. Measure and subtract the O.D. at 600 nm or higher from that of sample if there is a high turbidity in the cell suspension.

Appendix 8

Ab211091 – MTT Cell Proliferation Assay Kit

For the measurement of cell proliferation in cultured cells.

For overview, typical data and additional information please visit: www.abcam.com/ab211091 (use www.abcam.cn/ab211091 for China, or www.abcam.co.jp/ab211091 for Japan)

This product is for research use only and is not intended for diagnostic use

Precautions

- All kit components have been formulated and quality control tested to function successfully as a kit.
- We understand that, occasionally, experimental protocols might need to be modified to meet unique experimental circumstances. However, we cannot guarantee the performance of the product outside the conditions detailed in this protocol booklet.
- Reagents should be treated as possible mutagens and should be handled with care and disposed of properly. Please review the Safety Datasheet (SDS) provided with the product for information on the specific components.
- Observe good laboratory practices. Gloves, lab coat, and protective eyewear should always be worn. Never pipette by mouth. Do not eat, drink or smoke in the laboratory areas.
- All biological materials should be treated as potentially hazardous and handled as such. They should be disposed of in accordance with established safety procedures.

Storage and Stability: Store kit at -20°C in the dark immediately on receipt and check below for storage for individual components. Kit can be stored for 1 year from receipt.

- Refer to list of materials supplied for storage conditions of individual components.
- Observe the storage conditions for individual prepared components in the Materials Supplied section.
- Aliquot components in working volumes before storing at the recommended temperature.

Materials Supplied:

Item	Quantity	Storage temperature (before prep)	Storage temperature (after prep)
MTT Reagent	50 mL	-20°C	-20°C
MTT Solvent	150 mL	-20°C	-20°C

Materials Required, Not Supplied

These materials are not included, but will be required to successfully perform this assay:

- Microplate reader capable of measuring absorbance at OD 590 nm
- Pipettes and pipette tips, including multi-channel pipette
- Tubes for the preparation of reagents and buffer solutions
- 96 well plate with clear flat bottom
- General cell culture supplies
- Materials and instrumentation necessary to work under sterile conditions
- Foil

Version 2a, Last updated Friday, 23 July 2021

Technical Hints

- **This kit is sold based on number of tests. A "test" simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions.**
- Selected components in this kit are supplied in surplus amount to account for additional dilutions, evaporation, or instrumentation settings where higher volumes are required. They should be disposed of in accordance with established safety procedures.
- Avoid foaming or bubbles when mixing or reconstituting components.
- Avoid cross contamination of samples or reagents by changing tips between sample reagent additions.
- Ensure plates are properly sealed or covered during incubation steps.
- Ensure all reagents and solutions are at the appropriate temperature before starting the assay.
  Make sure all necessary equipment is switched on and set at the appropriate temperature.

Reagent Preparation: Briefly centrifuge small vials at low speed prior to opening.

1. **MTT Reagent (50 mL):**
   Ready to use as supplied.
   Equilibrate to room temperature before use and open vial under sterile conditions.
   Aliquot reagent so that you have enough volume to perform the desired no. of assay.
   Store at -20°C, protected from light.
2. **MTT Solvent (150 mL):**
   Ready to use as supplied.
   Equilibrate to room temperature before use.
   Store at -20°C. Once opened, use within 2 months.
   Tips for dissolving MTT crystals - add warm solvent (37°C max). Do not add cold MTT solvent.

Assay Procedure

- Equilibrate all materials and prepared reagents to RT just prior to use and gently agitate.
- Assay all standards, controls and samples in duplicate.
- conditions such as age of the culture, number of passages, and growth media can affect the result and must be taken into consideration when analyzing the data.

ΔNOTE: Serum or phenol red present in the culture medium can generate background. If your sample contains serum or phenol red, set up Sample Background Controls

1. Grow cells at varying densities (1-5 x106 cells per mL) in a clear plate according to the desired protocol

NOTE: Cells seeded at densities between 5,000-10,000 cells/well should reach optimal population densities within 48-72 hours. We recommend using appropriate incubation time depending on the individual cell type and cell concentration used.

2. If treating cells, dissolve compounds of interest in an appropriate solvent and treat cells with compound for desired time period.

NOTE: Prepare parallel well(s) as solvent control and use same volume of solvent as for the treated cells.

3. Discard treatment media:
4. For adherent cells, carefully aspirate the media.
5. For suspension cells, spin the 96 well plate at 1,000 X g, 4°C for 5 minutes in a microplate-compatible centrifuge and carefully aspirate the media.
6. Add 50 µL of serum-free media and 50 µL of MTT Reagent into each well.

NOTE: Background control wells: 50 µL MTT Reagent + 50 µL cell culture media (no cells)

7. Incubate the plate at 37°C for 3 hours.
8. After incubation, add 150 µL of MTT Solvent into each well.
9. Wrap the plate in foil and shake on an orbital shaker for 15 minutes.
10. Read absorbance at OD = 590 nm.

Data analysis

Cell Proliferation Assays

- Average the duplicate reading for each sample.
- Subtract the culture medium background from your assay reading. This is the corrected absorbance.
- Amount of absorbance is proportional to cell number.
  ANOTE: for cell counting, a standard curve can be established with known cell number and fixed incubation times with the assay reagent.

Cell Cytotoxicity Assays

- Average the duplicate reading for each sample.
- Subtract the culture medium background from your assay readings. This is the corrected absorbance.
- Calculate percentage cytotoxicity with the following equation, using corrected absorbance:

$$\%\,Cytotoxicity = \frac{(100 \times (Control - Sample))}{Control}$$

Troubleshooting

Problem	Reason	Solution
Assay not working	Use of ice-cold reagent	Warm reagent to assay temperature
	Plate read at incorrect wavelength	Check equipment and filter settings of instrument
Unanticipated results	Measured at incorrect wavelength	Check equipment and filter settings of instrument

Version 2a, Last updated Friday, 23 July 2021

Interferences

These chemical or biological materials will cause interferences in this assay causing compromised results or complete failure: Serum or phenol red present in the cell culture media.

FAQs

Should the media in background control well be serum-free media? Also, after 3-hour incubation with MTT reagent, should I remove MTT reagent or directly add MTT solvent without removing MTT reagent?
Use serum-free media for the background control wells too. Whether to remove the MTT reagent or not depends on the type of cells you have. For adherent cells, you can remove the MTT reagent gently and then add the MTT solvent. For suspension cells, one may lose the cells while removing the MTT reagent. Thus, we do not recommend removing the MTT reagent. The MTT solvent can be added directly and the total volume will be around 250 µl in the wells.

What are the known intereferences with this assay?
A variety of chemical compounds are known to interfere with the MTT assay. These are mostly reducing compounds that lead to non-enzymatic reduction of the MTT to formazan. Examples include ascorbic acid, vitamin A, sulfhydryl-containing compounds including reduced glutathione, coenzyme A, and dithiothreitol. Certain plant extracts and polyphenolic compounds can also interfere with the MTT assay.

Can you use media containing phenol red with this assay?
It's preferred not to use phenol red.

Why do I see high absorbance from media only?
The media contaminated with cells/bacteria or it might contain ascorbic acid. Always use sterile technique to work with the reagents. Try to work with sterile pipette tips and inside a biological hood.

Why is the MTT reagent blue/green in colour?
The reagent is either contaminated by a reducing agent or cell/bacterial contamination or it received excessive exposure to light. The reagent should be discarded. New reagent should be processed under sterile conditions and kept in the dark at 4°C.

Does MTT-medium need to be removed before addition of solvent?

Whether to remove the MTT reagent or not depends on the type of cells you have. For adherent cells, you can remove the MTT reagent gently and then add the MTT solvent. For suspension cells, for the fear that you may lose cells, please do not remove MTT reagent and you can add the MTT solvent directly and the total volume will be around 250 µl in the well.

Technical Support

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For all technical or commercial enquiries please go to:
www.abcam.com/contactus
www.abcam.cn/contactus (China)
www.abcam.co.jp/contactus (Japan)