Characterisation of the Relationship between Fatigue, Vitamin D level, Disease Activity and Interferon Signature Gene Expression in Systemic Lupus Erythematosus: a Population Based Study

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ABSTRACT

Vitamin D deficiency and fatigue are highly prevalent in Systemic Lupus Erythematosus (SLE). The main aim of this research was to analyse the relationship between vitamin D, fatigue, disease activity and interferon (IFN) signature gene expression in SLE.

92 patients with SLE were interviewed, had blood and urine tests, and filled questionnaires. Subsequently, 13 patients with vitamin D deficiency and 20 with vitamin D insufficiency were supplemented with vitamin D3. They were re-evaluated after 6 and 12 months. The expression of 12 IFN signature genes in blood at baseline and at 6 months was measured. In the final part of the research, the in vitro effect of calcitriol on the IFN signature gene expression in macrophages and dendritic cells was analysed.

56.5% of participants in the cross-sectional cohort had a high degree of fatigue (fatigue severity scale (FSS) >3.7). The strongest predictors of fatigue were depression ($p<0.001$) and pain ($p<0.001$) and no correlation with serum 25-hydroxyvitamin D and IFN signature gene expression was found. Higher disease activity measured by SLE disease activity index-2K (SLEDAI-2K) was noted in patients who were homozygous for the minor allele for the vitamin D receptor (VDR) polymorphism BsmI ($p=0.046$). In the prospective study, an improvement in SLEDAI-2K ($p=0.028$) and FSS ($p=0.011$) at 12 months were noted. The mean IFN signature gene expression score decreased from baseline to 6 months (from 2.69 to 2.10, $p=0.083$), and the IFN signature gene expression decreased for all 12 genes especially OAS1 ($p=0.032$) and SOCS1 ($p=0.005$). In the in vitro experiment, a significant reduction in IFN score was noted in dendritic

cell and macrophage samples with calcitriol (p=0.014, p=0.012). This was also the case for Interferon Regulatory Factor (IRF) 8 expression in the macrophage culture (p<0.001).

Vitamin D supplementation in SLE resulted in improved disease activity and fatigue. This could be explained by the suppression of the IFN signature gene expression, as confirmed in the cell culture experiment.

Keywords: Vitamin D, fatigue, interferon, Systemic Lupus Erythematosus

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ABBREVIATIONS

ACR	American College of Rheumatology
ANA	Anti-nuclear antibodies
Anti-dsDNA	Anti-double-stranded deoxyribonucleic acid
Anti-RNP	Anti-ribonucleoprotein
Anti-Sm	Anti-Smith
Anti-SSA	anti-Sjogren's syndrome related antigen A
Anti-SSB	anti-Sjogren's syndrome related antigen B
BILAG	British Isles Lupus Activity Group
BMI	Body Mass Index
C3	Complement 3
C4	Complement 4
CBC	Complete blood count
CD	Cluster of differentiation
CI	Confidence interval
CKD	Chronic kidney disease
CRP	C-reactive protein
DCs	Dendritic cells
DMARDs	Disease modifying anti-rheumatic drugs
DMEM	Dulbecco's Modified Eagle's Media
ECLAM	European Consensus Lupus Activity Measurement
EDTA	Ethylenediaminetetraacetic acid
eGFR	Estimated glomerular filtration rate
ENA	Extractable nuclear antigens

ESR	Erythrocyte sedimentation rate
EULAR	European League against Rheumatism
FBS	Fetal Bovine Serum
FITC	Fluorescein isothiocyanate
FSS	Fatigue Severity Scale
HADS	Hospital Anxiety and Depression Scale
HAQ	Health Assessment Questionnaire
IFN	Interferon
IgG	Immunoglobulin G
IL	Interleukin
IQR	Interquartile range
IRF	Interferon regulatory factor
ISRE	Interferon stimulated response element
K-S test	Kolmogorov-Smirnov test
LPS	Lipopolysaccharides
MFI	Median fluorescence intensity
mHAQ	Modified Health Assessment Questionnaire
PBMC	Peripheral blood mononuclear cell
PCR	Protein creatinine ratio
PE	Phycoerythrin
PSQI	Pittsburgh Sleep Quality Index
RCT	Randomized controlled trial
RNA	Ribonucleic acid
RNP	Anti-ribonucleoprotein
RPMI	Roswell Park Memorial Institute
S.D.	Standard Deviation

SDI	Systemic Lupus International Collaborating Clinics/American College of Rheumatology damage index
SELENA-SLEDAI	Safety of Estrogen in Lupus National Assessment - Systemic Lupus Erythematosus Disease Activity Index
SLAM	Systemic Lupus Erythematosus Activity Measure
SLE	Systemic Lupus Erythematosus
SLEDAI	Systemic Lupus Erythematosus Disease Activity Index
SLE-DAS	Systemic Lupus Erythematosus Disease Activity Score
SLICC	Systemic Lupus International Collaborating Clinics
TFT	Thyroid function test
UV	Ultraviolet
VAS	Visual analogue scale
VDR	Vitamin D receptor
VDRE	Vitamin D responsive elements

CHAPTER 1 – INTRODUCTION AND LITERATURE REVIEW

1.1 INTRODUCTION

This introduction provides a background on systemic lupus erythematosus (SLE) and its pathophysiology, including the role of interferon (IFN) alpha. It also provides an insight on vitamin D and its physiological roles.

1.1.1 SYSTEMIC LUPUS ERYTHEMATOSUS

SLE is a chronic autoimmune disorder with a wide range of clinical features involving multiple systems. The term “lupus” (Latin for “wolf”) started to be used in the middle ages to describe the erosive skin lesions similar to a “wolf’s bite”. The butterfly description of the malar rash was introduced in 1846 by Ferdinand von Hebra (1816-1880), a Viennese physician. Moriz Kaposi (1837-1902) recognised lupus as a systemic disease. This was further established by William Osler (1849-1919) and Josef Jadassohn (1863-1936). Other milestones included the description of endocarditis lesions by Emanuel Libman (1872-1946) and Benjamin Sacks (1896-1971), as well as the wire-loop lesions in glomerulonephritis by George Baehr (1887-1978). The discovery of the “LE” cell by Hargraves et al. in 1948 heralded the modern era (Norman, 2016).

The reported prevalence of SLE in Europe is 20 to 50 per 100,000 people, and the incidence rates in Europe and in North and South America are estimated from 1 to 23 per 100,000 per year (Bertsias et al., 2013; Pons-Estel et al., 2010). The condition affects females more frequently than males, at a ratio of 9:1. The onset of SLE occurs between the ages of 16 and 55 years in 65 per cent of cases. The aetiology includes a combination of environmental (including ultraviolet (UV) light, infections and drugs), genetic and epigenetic factors (including DNA methylation and histone modifications) that result in an irreversible break in immunological tolerance (Farivar et al., 2018). Genome-wide association studies have shown that a number of genes are associated with SLE. These include HLA-DR, PTPN22, STAT4 and IRF5 (Moser et al., 2009).

SLE starts with a pre-clinical phase during which there is the development of autoantibodies, predominantly anti-double-stranded deoxyribonucleic acid (anti-dsDNA) antibodies and antibodies targeting extractable nuclear antigens (ENA). This progresses to the clinical phase that is characterised by heterogeneous clinical manifestations that involve multiple systems including the mucocutaneous, musculoskeletal, renal, haematological, neurological, respiratory and cardiovascular systems (Tsokos, 2011). The clinical course waxes and wanes, leading to the accumulation of disease and therapy-related damage, predominantly infections, atherosclerosis and malignancies. This can be recorded by the Systemic Lupus International Collaborating Clinics/American College of Rheumatology damage index (SDI), which is a validated tool that measures damage (Gladman et al., 2000). Disease activity can be evaluated by a number of validated tools including Systemic Lupus Erythematosus Disease Activity Index (SLEDAI), British Isles Lupus Activity Group (BILAG), European Consensus Lupus Activity Measurement (ECLAM) and Systemic

Lupus Erythematosus Activity Measure (SLAM). (Griffiths et al., 2005). These tools are very useful in clinical practice since they allow adequate monitoring, enabling appropriate treatment changes. Active disease in SLE has a negative effect on patients' quality of life (Chaigne et al., 2017; Pereira et al., 2020).

SLE Classification criteria have been developed for research purposes by the American College of Rheumatology (ACR) in 1971 and have subsequently been revised in 1982 and in 1997. This was further revised by the Systemic Lupus International Collaborating Clinics (SLICC) group in 2012 (Petri et al.). This resulted in an improved sensitivity than the ACR criteria. The classification criteria for SLE have been further updated by the European League against Rheumatism (EULAR) and ACR in 2019 (Aringer et al.). These criteria use anti-nuclear antibodies (ANA) as an entry criterion. All criteria have individual weights (ranging from 2 to 10), and they are counted only if there is no alternative explanation. A classification of SLE is made if the total score is 10 or more. These criteria have an improved specificity compared to the SLICC criteria.

1.1.2 PATHOPHYSIOLOGY OF SLE AND INTERFERON SIGNATURE

The underlying pathogenesis in SLE is due to increased apoptosis of lymphocytes, excessive neutrophil extracellular trap formation and defective phagocytosis by macrophages. This results in the production of auto-antigen, which stimulates the plasmacytoid dendritic cells (DCs) to produce type 1 IFN cytokines which have a central role in SLE development (Crow, 2014, Kim et al., 2015; Huang et al., 2015; Rönnblom and Leonard 2019). Type 1 IFNs promote the myeloid DCs to produce various stimulators including a proliferation-inducing ligand and B lymphocyte stimulator.

These cytokines play a role in the survival of autoreactive B cells, which produce autoantibodies, resulting in the formation of immune complexes and tissue injury. There is an association between certain autoantibodies and clinical manifestations in SLE, for example anti-ribosomal P antibodies and neuropsychiatric SLE, and anti-dsDNA antibodies and lupus nephritis (Mok et al., 2003). The immune complexes stimulate the plasmacytoid dendritic cells via the endosomal toll-like receptors to release further type 1 IFNs. This is mediated by interferon regulatory factors (IRFs), particularly IRF5, IRF7 and IRF8 (Salloum et al., 2011).

The immune complexes result in complement activation which leads to tissue damage. Low levels of complement 3 and 4 (C3, C4) are used as diagnostic markers for SLE and to monitor disease activity (Weinstein et al., 2021). Inherited complement deficiencies (particularly C2, C4 and C1q) result in increased risk for SLE development (Mok et al., 2003). Defective clearance of immune complexes by phagocytic cells occurs due to decreased number of complement receptor 1, as well as polymorphisms of immunoglobulin G (IgG) receptors (FcγRIIA and FcγRIIIA) that have decreased affinity for Fc portions of IgG2 and IgG3 (Mok et al., 2003; Junker et al., 2020).

Type 1 IFNs result in decreased phagocytosis by the macrophages and decreased clearance of apoptotic cells (Ma et al., 2019). Moreover, type 1 IFNs stimulate the release of interleukin(IL)-6 and IL-23. These enhance T helper 17 cell responses that lead to IL-17 production that stimulates B cell hyper-reactivity, and tissue inflammation by recruiting lymphocytes, macrophages and neutrophils (Figure 1.1).

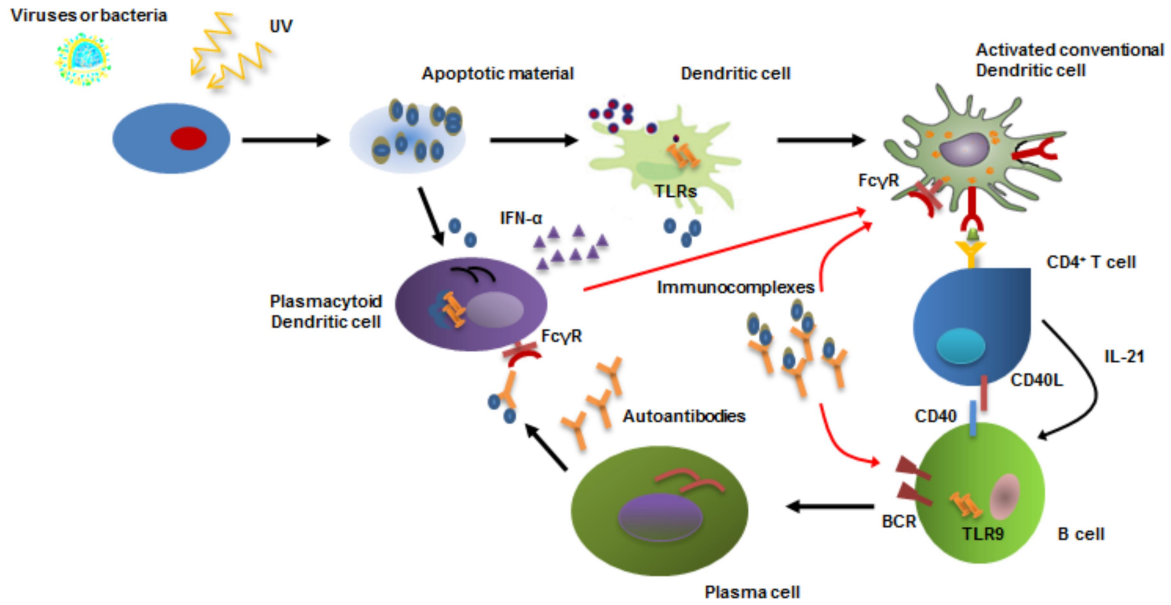

Figure 1.1. The various SLE disease activation pathways lead to the production of IFN alpha (Bertsias et al., 2012). (Figure used with permission.)

BCR, B cell receptor; CD, cluster of differentiation; IFN-α, interferon alpha; IL, interleukin; FcγR, Fcγ receptor; TLR, toll-like receptor; UV, ultraviolet.

Gene expression profiling studies have shown that type 1 IFN-regulated genes are overexpressed in the peripheral blood of SLE patients (known as the IFN signature). This increased expression is positively correlated with disease activity (Rai et al., 2016). Twelve such genes that are either IFN regulated or induced have been identified by meta-analysis (*CCL2, CXCL1, IFITM1, IFI35, IFIT3, IFIT1, MX1, SOCS1,OAS1, SOCS3, STAT1, STAT2*) (Arasappan et al., 2011). The overexpression of these genes differentiates SLE from other autoimmune conditions including rheumatoid arthritis and antiphospholipid syndrome. The IFN signature creates targets for the development of new medications for the treatment of SLE.

Type 1 interferons include all subtypes of IFN-α and IFN-β, which are produced in response to viral infections (Sim et al., 2022). They have anti-proliferative and

immunostimulatory roles and are used to treat viral infections, such as hepatits C, and certain haematological malignancies. Therapeutic IFN-α is associated with fatigue, sleep disturbances and depression (Raison et al., 2010). In a prospective cohort study including 30 participants who were started on IFN-α therapy for hepatitis C, IFN-α significantly increased symptoms of depression and enhanced right amygdala reactivity to negative versus neutral expressions (Davies et al., 2021). The latter was found to predict the development of symptoms of depression occurring 4 weeks after starting IFN-α. This creates interest as to whether the central role of type 1 interferons in the underlying pathogenesis of SLE, contributes to neuropsychiatric symptoms including fatigue.

1.1.3 FATIGUE

The literature does not provide a consensus definition for fatigue, but it is often described as whole-body tiredness or exhaustion, lack of energy, the overwhelming sensation of weakness, that is chronic, typically poorly relieved by rest and unrelated to over-exertion. Fatigue is highly common in SLE; present in up to 90% of SLE patients (Zonana-Nacach A et al., 2000). Around half of patients with SLE consider fatigue as the most disabling symptom (Krupp et al., 1989), and it has a negative effect on quality of life (Margiotta et al., 2019; Pereira et al., 2020).

The cause of fatigue in SLE is multi-factorial and includes behavioural components such as lack of physical activity (Daltroy et al., 1995; Tench et al., 2003) and co-morbid conditions (including sleep disorder (Du et al., 2018), fibromyalgia (Jump et al., 2005), pain (Azizoddin et al., 2019; Pinto et al., 2021); depression (Tench et al., 2000; Wang

et al., 1998; Omdal et al., 2003) and anxiety (Arnaud et al., 2019). Fatigue in neuropsychiatric SLE has been strongly associated with anxiety and depression, but no association was found with disease activity in a cohort study that included 348 patients with neuropsychiatric SLE (Monahan et al., 2021). Fatigue in SLE has also been associated with the presence of anti-N-methyl-D-aspartate receptor antibodies, which are associated with neuropsychiatric manifestations (Tay et al., 2017; Schwarting et al., 2019). Fatigue has been associated with a younger age at SLE diagnosis, disease duration and a worse quality of life (Elera-Fitzcarrald et al., 2020; Pinto et al., 2021).

Several questionnaires have been developed to measure fatigue (Neuberger 2003; Barbacki et al., 2019). Fifteen such instruments that have been used in SLE have been reviewed by the Ad Hoc Committee on SLE Response Criteria for Fatigue (2007). The Fatigue Severity Scale (FSS) was recommended as the instrument of choice (Krupp et al., 1989). The FSS was initially developed as a tool to be used in SLE and multiple sclerosis, but has since then also been used in other conditions including stroke and cirrhosis (Ozyemisci-Taskiran et al., 2019; Rossi et al., 2017). It is an easy to use, self-administered questionnaire that comprises nine descriptions associated with fatigue; each one is rated on a seven point Likert scale. The total score is calculated by finding the mean, and is positively correlated with the degree of fatigue.

A systematic review that looked into the management of fatigue in SLE, established that belimumab and aerobic exercise have the strongest evidence with regards to management of fatigue in SLE. N-acetylcysteine and UVA-1 phototherapy have low-to-moderate level of evidence; acupuncture, dietary manipulation, vitamin D supplementation and psychosocial interventions have weak evidence of efficacy (Yuen

and Cunningham, 2014). Two meta-analysis have concluded that exercise training results in an improvement in the level of fatigue in SLE (O'Dwyer et al., 2017; Wu et al., 2017). A systematic review that included six randomized controlled trials (RCTs) of non-pharmacologic therapies on fatigue in SLE, concluded that exercise and psychological intervention may help improve fatigue (Fangtham et al., 2019).

1.1.4 VITAMIN D

Vitamin D is a fat-soluble vitamin present in only few foods, chiefly in oily fish (such as salmon) and egg yolk. Its predominant source (80-90%) is its production in the skin from 7-dehydrocholesterol upon absorption of UV-B radiation. Hydroxylation to 25-hydroxyvitamin D occurs in the liver; and is then hydroxylated further to 1,25-dihydroxyvitamin D. The latter occurs not only in the kidneys, but also in a number of cells belonging to the immune system such as macrophages, dendritic cells and lymphocytes (Baeke et al., 2010). 1,25-dihydroxyvitamin D is the active form that enters the cytoplasm and binds to vitamin D receptor (VDR) that is found in the majority of cells. The compound then attaches to retinoic acid x-receptor and serves as a nuclear transcription factor by binding to vitamin D responsive elements (VDRE), also known as VDR binding sites, in genomic DNA (Carlberg and Seuter, 2009). As a result, vitamin D can control the transcription of more than 200 target genes, which include genes that regulate cell growth, apoptosis and proliferation.

Vitamin D is important for calcium absorption in the gastrointestinal tract, homeostasis of serum calcium and phosphate, and bone growth and remodelling (Wintermeyer et al., 2016). It also has other multiple roles, including inflammation reduction, immune

functions, cell growth modulation and neuromuscular functions (Iruretagoyena et al., 2015). Vitamin D deficiency is defined as serum 25-hydroxyvitamin D concentration of less than 20ng/mL. It is highly prevalent and it is estimated that 34% to 67% of adults in Europe have vitamin D deficiency (Spiro and Buttriss, 2014). Vitamin D deficiency has been associated with autoimmune diseases including multiple sclerosis, rheumatoid arthritis and autoimmune thyroid disease (Murdaca et al., 2019).

Vitamin D supplementation has been associated with an improvement in the level of fatigue in autoimmune diseases, such as multiple sclerosis (Glabska et al., 2021; López-Muñoz et., 2023). In rheumatoid arthritis, this relationship has not been observed, but vitamin D deficiency has been associated with higher disease activity and lower quality of life (Raczkiewicz et al., 2015; Soubrier et al., 2018; Jelsness-Jørgensen et al., 2020). In some conditions characterised by the presence of fatigue, a negative impact of vitamin D deficiency has been noted. In long COVID syndrome, vitamin D deficiency has been associated with a longer time to recovery (Chen et al., 2023). Lower vitamin D level has been associated with a higher disease impact in fibromyalgia, but it has not been found to contribute to the level of fatigue in chronic fatigue syndrome (Beserra et al., 2020; Earl et al., 2017).

An improvement in the level of fatigue has been noted with vitamin D supplementation in a prospective interventional study that included patients complaining of fatigue who had stable comorbid medical conditions (if at all), and low serum 25-hydroxyvitamin D levels (Roy et al., 2014). Several mechanisms are likely to be involved in manifesting the effect of vitamin D deficiency on fatigue, including type II muscle fibre atrophy, resulting in muscle weakness (Ceglia, 2009).

Vitamin D deficiency, and consequently higher levels of parathyroid hormone are associated with obesity (Guasch et al., 2012). Vitamin D is a fat soluble vitamin; the increased body fat acts as a storage reservoir for vitamin D decreasing its bioavailability (Lagunova et al., 2009). The therapeutic effects of vitamin D are influenced by body mass index (BMI). In a randomised controlled study on the effect of vitamin D supplementation on cancer incidence and major cardiovascular events, the VITAL trial, found a significant reduction in cancer risk in individuals with a normal BMI; however, this was not the case in overweight and obese individuals (Manson et al., 2020).

Vitamin D level is also influenced by seasonal variation as a result of the decreased UV-B availability in the Winter months. Cohort studies carried out in Ankara, Turkey (39^{o} N) and Nicosia, Cyprus (35^{o} N), having a similar latitude to Malta (35^{o} N), showed that vitamin D levels were significantly lower in Winter compared to Summer (Bozkurt et al., 2014; Beyitler et al., 2018). The prevalence of vitamin D deficiency varies across Europe due to decreased UV-B availability with increasing latitude. Even though there is a lower UV-B availability in Northern Europe (>60^{o} N) compared to mid-Europe (45-60^{o} N), vitamin D levels are higher in Northern Europe due to higher vitamin D intake (O'Neill et al., 2016).

1.1.5 VITAMIN D IN SLE

Vitamin D deficiency is more common in SLE patients (Kamen and Aranow, 2008). Four meta-analysis that included 18, 24, 19 and 34 studies respectively showed that Vitamin D was significantly lower in SLE patients compared to controls (Bae and Lee, 2018;

Wang et al., 2018; Guan et al., 2019; Islam et al., 2019). On stratification of the included studies according to ethnicity, the result was consistent in African, Asian and Caucasian patients. Vitamin D levels are significantly lower in African American SLE patients compared to Caucasian patients due to the reduced ability of UVB to convert 7-dehydrocholesterol to vitamin D in patients with darker skin pigmentation (Kamen et al., 2006). The low vitamin D in SLE could be a risk factor for SLE or its consequence (such as due to sun avoidance and use of drugs that influence vitamin D metabolism, including glucocorticoids and hydroxychloroquine). Vitamin D is thought to have a significant role in the pathogenesis of SLE in view of the expression of VDR by multiple immune cells (including DCs, B cells, T cells and macrophages) (Sakthiswary and Raymond, 2013).

Interferon regulatory factor 8 (*IRF8*) is a transcription factor belonging to the IRF family and is known to have a VDR binding site (Ramagopalan SV et al., 2010). IRF8 protein binds to the IFN-stimulated response element (ISRE) and controls expression of genes that are stimulated by IFN-alpha (including IFN signature genes). *IRF8* variants have been associated with an increased risk for development of SLE (Lessard CJ et al., 2012).

Several *VDR* gene polymorphisms have been reported, namely BsmI (rs1544410), ApaI(rs7975232), TaqI(rs731236) and FokI(rs2228570). A meta-analysis, that included 13 studies, published by Zhou et al. (2015) showed that the BsmI B allele, BB genotype and bb genotype, FokI f allele and ff genotype were associated with increased risk of SLE in Asians and Africans. In the latter an increased risk was also found with ApaI A allele, AA genotype and aa genotype. However, these associations were not found in Caucasians. Another meta-analysis, that included 11 case-control studies, also

concluded that BsmI and FokI polymorphisms are associated with a higher risk for SLE, especially in Asians (Xiong et al. 2014). FokI F allele was found to be associated with increased risk for SLE in the Arab populations in a meta-analysis by Bae et al. (2018).

VDR gene polymorphisms have been associated with certain SLE organ manifestations. A study by Mostokwska et al. demonstrated that SLE patients with FF and Ff genotypes of FokI *VDR* gene polymorphism, had an increased risk of developing lupus nephritis (Mostowska et al., 2013). In another study, BsmI BB, ApaI AA and FokI FF genotypes were associated with lupus nephritis and more severe disease activity (Emerah and El-Shal, 2013). Carvalho et al. (2015) concluded that SLE patients with CT genotype of FokI and TT genotype of TaqI had a higher SLICC damage score indicating more severe SLE.

1.1.6 Effect of Vitamin D on Cells

Penna et al. (2000) demonstrated in cell culture experiments that 1,25-dihydroxyvitamin D inhibited the differentiation of peripheral blood mononuclear cells (PBMCs) into immature DCs. In addition, 1,25-dihydroxyvitamin D prevented the maturation of immature DCs, and promoted DC apoptosis. In another experiment monocytes derived from buffy coat samples were differentiated to DCs, which were in turn cultured for 24 hours with or without the addition of calcipotriol (a vitamin D3 analogue) (Parnell et al. 2019). *IRF8* expression was found to be decreased with vitamin D, in tolerogenic DCs. The effect of vitamin D on DCs derived from patients with SLE has not been studied.

1.1.7 SLE IN MALTA

Previous research on SLE patients in Malta includes a study whereby a comparative analysis of clinical and immunological features of 55 female and 7 male SLE patients was made (Camilleri and Mallia, 1999). Another study by Camilleri and Mallia (1999) compared the clinical and laboratory features of SLE patients who were positive for anti-ribonucleoprotein (RNP) antibody with those who were negative. An audit carried out in 2015 by Cefai et al. on 50 SLE patients identified a number of aspects in monitoring of SLE patients that required improvement, including documentation of smoking status and assessment of urine for microalbuminuria.

A study carried out by J Grech Meli (2020) described the VDR polymorphisms in 59 SLE patients living in Malta, and compared them to 93 control samples. The study showed a significant decrease in SLE prevalence when ApaI polymorphism was present as a homozygote for the minor allele (OR=0.39, CI 0.17-0.87, p=0.02). Moreover, an increased prevalence of SLE by around 2 fold was found in the haplotype with the minor allele for FokI and all other wild-type alleles (OR= 1.95, CI 1.12-3.38, p=0.01) and in the haplotype with all wild-type alleles for the *VDR* gene (OR= 2.36, CI 1.13-4.91, p=0.02).

In Malta, SLE patients are followed up at Mater Dei Hospital by Consultant Rheumatologists. Prior to this research, there was no available up-to-date register of local patients with SLE, and as a result the incidence and prevalence of SLE in Malta was not known.

1.2 LITERATURE REVIEW

Vitamin D deficiency and fatigue are highly prevalent in SLE patients. This creates interest as to whether a relationship exists between these two factors, as well as whether they have any relationship with disease activity and IFN signature gene expression. The objective of this literature review is to outline the evidence on the relation between fatigue, vitamin D, IFN signature gene expression and disease activity in SLE. MEDLINE/PubMed was searched for publications in the English language up to July 2022, using the MeSH terms “systemic lupus erythematosus”, “interferon”, “gene expression”, “fatigue” and “vitamin D”. The references included in these articles were used to select additional publications. The abstracts were analysed, and the full text was considered for inclusion when relevant.

1.2.1 VITAMIN D AND INTERFERON SIGNATURE GENE EXPRESSION

A cross-sectional case control study, including 32 patients with SLE, found a higher mean serum IFN-α activity in SLE patients with vitamin D deficiency than those with normal levels (Ritterhouse et al., 2011). This was also the case in two other cross-sectional studies that demonstrated that serum 25-hydroxyvitamin D correlated negatively with plasma IFN-α and with IFN-α gene expression in SLE patients (Mandal et al., 2014; Abdel Galil et al., 2018). In a RCT, there was no significant change in IFN signature gene expression after 12 weeks when SLE patients with vitamin D deficiency were supplemented with vitamin D3 2000IU or 4000IU daily (Aranow et al., 2015). The reason for this, however, could have been that 48.5% of treated patients did not

obtain levels of 25-hydroxyvitamin D above 30ng/ml because a loading dose of vitamin D3 was not used. The authors of this trial stated that higher levels of 25-hydroxyvitamin D, maintained for a longer time, might be necessary to decrease IFN signature gene expression (Table 1.1).

Table 1.1. Studies that have evaluated the relation between serum 25-hydroxyvitamin D level and IFN signature gene expression in SLE.

Study (year)	**Country**	**Design**	**Study Population**	**Genes studied**	**Relation between IFN signature gene expression and vitamin D**
Ritterhouse LL et al. (2011)	United States	Cross-sectional case control	32 SLE 32 control	*MX1, PKR, IFIT*	*p*=0.02*
Mandal M et al. (2014)	India	Cross-sectional case control	129 SLE 100 control	*IFN-α* gene	*p*=0.0009* *r*=-0.45
Aranow C et al. (2015)	United States	Randomised, placebo controlled trial	54 SLE	*MX1, IFIT1, IFI44*	*p*=0.77
Abdel Galil SM et al. (2018)	Egypt	Cross-sectional case control	123 SLE 100 control	*IFN-α* gene	*p*<0.001* *r*=-0.454

*statistically significant results

IFN, interferon; SLE, systemic lupus erythematosus.

1.2.2 VITAMIN D AND DISEASE ACTIVITY

There are conflicting results on the relationship between disease activity and vitamin D. The majority of studies looking into this relationship are cross-sectional studies; few are prospective studies and RCTs. A significant inverse relationship between disease activity and vitamin D level was reported in thirty six studies but twenty six studies did not demonstrate this association (Table 1.2). Possible reasons for these conflicting results include different populations studied, with varying study designs and inclusion criteria. In addition, different validated scoring systems to determine disease activity in SLE have been used, including SLEDAI, BILAG, ECLAM, SLAM and SLE disease activity score (SLE-DAS). The reason for the inability of some prospective interventional studies to demonstrate a significant relationship between vitamin D level and disease activity could have been the short duration of the study (12 week duration in the study by Aranow et al.; 6 months duration in the studies by Terrier B et al., Karimzadeh et al. and Al-Kushi et al.) and the low percentage of patients achieving serum 25-hydroxyvitamin D >30ng/ml following supplementation. In the study by Ruiz-Irastorza et al. this was achieved in 29% and in the study by Andreoli et al. this was achieved in 75% and 28% in the intensive and standard regimen respectively. In the study by Al-Kushi et al. the mean serum 25-hydroxyvitamin D was 26.5ng/ml following supplementation, meaning that a significant proportion had not achieved the desired vitamin D level.

Table 1.2. Studies that have evaluated the relation between serum 25-hydroxyvitamin D level and disease activity in SLE.

Study (year)	Country	Design	Study Population	Disease activity measure	Significant association between vitamin D and disease activity
Becker A et al. (2001)	Germany	Cross-sectional cohort	57 SLE	SLAM	Yes r=-0.3, p=0.02
Chen S et al. (2007)	China	Cross-sectional case-control	112 SLE	SLEDAI	No *
Orbach H et al. (2007)	Israel	Cross-sectional cohort	138 SLE	ECLAM	No*
Ruiz-Irastorza G et al. (2008)	Spain	Cross-sectional cohort	92 SLE	SLEDAI	No p=0.94
Wu PW et al. (2009)	United States	Cross-sectional cohort	181 SLE	SLEDAI	Yes p=0.018
Borba VZ et al. (2009)	Brazil	Cross-sectional case-control	36 SLE 26 controls	SLEDAI	Yes r=-0.65, p<0.001
Wright TB et al. (2009)	United States	Cross-sectional case-control	38 paediatric SLE 207 controls	SLEDAI	Yes p=0.01
Amital H et al. (2010)	Israel	Cross-sectional cohort	378 SLE	SLEDAI-2K ECLAM	Yes r=-0.12, p=0.01
Ruiz-Irastorza G et al. (2010)	Spain	Prospective cohort	80 SLE	SLEDAI	No p=0.87
Toloza SM et al. (2010)	Canada	Prospective cohort	124 SLE	SLEDAI-2K	No association on multi- and univariate analysis
Ben-Zvi I et al. (2010)	United States	Cross-sectional cohort	198 SLE	SLEDAI	Yes r=-0.2, p=0.002
Hamza RT et al. (2011)	Egypt	Cross-sectional case control	60 SLE 60 controls	SLEDAI	Yes r=-0.9, p<0.01
Bonakdar ZS et al. (2011)	Iran	Cross-sectional cohort	40 SLE	BILAG	Yes r=-0.46, p=0.001
Szodoray P et al. (2011)	Hungary	Cross-sectional cohort	177 SLE	SLEDAI	Yes p=0.03
Kim HA et al.	Korea	Cross-sectional	104 SLE	SLEDAI	No

(2011)		case control	49 controls		p=0.7
Souto M et al. (2011)	Brazil	Cross-sectional cohort	159 SLE	SLEDAI-2K	No p=0.46
Lopez-Robles C et al. (2011)	Spain	Cross-sectional cohort	55 SLE	SLEDAI	No*
Ezzat Y et al. (2011)	Egypt	Cross-sectional case control	50 SLE 30 controls	SLEDAI	Yes p=0.006
Mok CC et al. (2012)	Hong Kong	Cross-sectional cohort	290 SLE	SLEDAI	Yes r=-0.19, p<0.003
Yeap SS et al. (2012)	Malaysia	Prospective cohort	38 SLE	SLEDAI-2K	Yes p=0.033
Reynolds JA el al. (2012)	United Kingdom	Cross-sectional cohort	75 SLE	SLEDAI-2K	Yes p=0.03
Munoz-Ortego J et al. (2012)	Spain	Cross-sectional cohort	73 SLE	SLEDAI	No p=0.31
Fragoso TS et al. (2012)	Brazil	Cross-sectional case control	78 SLE 64 controls	SLEDAI	No p=0.9
Sumethkul K et al. (2012)	Thailand	Cross-sectional cohort	108 SLE	SLEDAI	Yes r=-0.22, p=0.03
Terrier B et al. (2012)	France	Prospective cohort	20 SLE	SELENA-SLEDAI	No p=0.16
Bogaczewicz J et al. (2012)	Poland	Cross-sectional cohort	49 SLE	SLAM	No*
Monticielo OA et al. (2012)	Brazil	Cross-sectional case control	195 SLE 201 controls	SLEDAI	No p=0.19
Robinson AB et al. (2012)	United States	Cross-sectional cohort	37 paediatric SLE	SLEDAI	No*
Casella CB et al. (2012)	Brazil	Cross-sectional case control	57 juvenile SLE 37 controls	SLEDAI	Yes p=0.01
Abou-Raya A et al. (2013)	Egypt	Randomised placebo controlled trial	267 SLE	SLEDAI	Yes r=-0.5, p<0.05
Petri M et al. (2013)	United States	Prospective cohort	1006 SLE	SELENA-SLEDAI	Yes p=0.032
Chaiamnuay S et al. (2013)	Thailand	Cross-sectional cohort	101 SLE	SLEDAI	No r=-0.06, p=0.5
Attar SM et al.	Saudi Arabia	Retrospective	95 SLE	SLEDAI-2K	No*

(2013)		cohort			
Sahebari M et al. (2014)	Iran	Cross-sectional case control	82 SLE 49 controls	SLEDAI-2K	No r=0.0003, p=0.68
Mandal M et al. (2014)	India	Cross-sectional case control	129 SLE 100 control	SLEDAI	Yes r=-0.42, p<0.0001
Lertratanakul A et al. (2014)	International cohort – Europe, North America, Asia	Cross-sectional cohort	875 SLE	SLEDAI-2K	Yes*
Andreoli L et al. (2015)	Italy	Prospective cohort	34 SLE	SLEDAI-2K	No*
Aranow C et al. (2015)	United States	Randomised, placebo controlled trial	54 SLE	SELENA-SLEDAI	No*
Miskovic R et al. (2015)	Serbia	Cross-sectional cohort	46 SLE	SLEDAI	No r=0.194, p=0.195
Abaza NM et al. (2016)	Egypt	Cross-sectional case control	60 SLE 30 control	SLEDAI	Yes r=-0.495, p<0.001
Gao CC et al. (2016)	China	Cross-sectional case control	121 SLE 150 control	SLEDAI-2K	Yes r=-0.413, p<0.001
Salman-Monte TC et al. (2016)	Spain	Cross-sectional cohort	102 SLE	SLEDAI	No p=0.807
Lima GL et al. (2016)	Brazil	Randomised, placebo controlled trial	40 juvenile SLE	SLEDAI ECLAM	Yes p=0.011, p=0.006
Rifa'i A et al. (2016)	Indonesia	Open label clinical trial	39 SLE	SLEDAI	Yes p=0.000
Eloi M et al. (2017)	Brazil	Cross-sectional case control	199 SLE 150 control	SLEDAI	Yes p=0.042
Garcia-Carrasco M et al. (2017)	Mexico	Cross-sectional cohort	137 SLE	MEX-SLEDAI	No p=0.21
Karimzadeh H et al. (2017)	Iran	Randomised, placebo controlled trial	90 SLE	SLEDAI	No p=0.39
Singgih Wahono CS et al. (2017)	Indonesia	Prospective study	39 SLE	SLEDAI	Yes p=0.001

Lin TC et al. (2018)	Taiwan	Cross-sectional cohort	35 juvenile SLE	SLEDAI-2K	Yes r=-0.335, p=0.003 when SLEDAI $\leq$ 4; r=-0.373, p=0.016 when SLEDAI $\geq$5.
Abdel Galil SM et al. (2018)	Egypt	Cross-sectional case control	123 SLE 100 control	SLEDAI-2K	Yes r=-0.591, p<0.001
Al-Kushi AG et al. (2018)	Saudi Arabia	Prospective cohort	81 SLE	SLEDAI	No p=0.103
Mok CC et al. (2018)	China	Prospective cohort	276 SLE	SLEDAI	Yes p=0.02
Dutta C et al. (2019)	India	Cross-sectional case control	109 SLE 109 control	SLEDAI	Yes p<0.01
Ospina-Caicedo AI et al. (2019)	Colombia	Cross-sectional cohort	69 SLE	SLEDAI BILAG	Yes p=0.0001, p=0.039
Cardona-Cardona AF et al. (2020)	Colombia	Cross-sectional cohort	51 SLE	SLEDAI-2K	Yes r=-0.578, p<0.001
Tabra SAA et al. (2020)	Egypt	Cross-sectional case control	100 juvenile SLE 100 control	SLEDAI	Yes r=-0.545, p=0.001
Pakchotanon R et al. (2020)	Thailand	Randomised, placebo controlled trial	104 SLE	SLEDAI-2K	No p=0.101
Correa-Rodriguez M et al. (2021)	Spain	Cross-sectional cohort	264 SLE	SLEDAI-2K	Yes P=0.001
Abo-Shanab AM et al. (2021)	Egypt	Cross-sectional case control	50 juvenile SLE 25 control	SLEDAI-2K	Yes r=-0.431, p=0.022
Arshad A et al. (2021)	Pakistan	Retrospective	98 SLE	SLE-DAS	Yes p=0.001
Athanassiou L et al. (2022)	Greece	Cross-sectional case control	45 SLE	SLEDAI-2K	Yes r=-0.572, p<0.001
Hayashi K et al. (2022)	Japan	Cross-sectional cohort	870 SLE	SLEDAI	No p=0.40

*p value was not mentioned in the related article.

BILAG, British Isles Lupus Activity Group; ECLAM, European Consensus Lupus Activity Measurement; SELENA-SLEDAI, Safety of Estrogen in Lupus National Assessment - Systemic Lupus Erythematosus Disease Activity Index; SLAM, Systemic Lupus

Erythematosus Activity Measure; SLE, Systemic Lupus Erythematosus; SLEDAI, Systemic Lupus Erythematosus Disease Activity Index; SLE-DAS, Systemic Lupus Erythematosus Disease Activity Score.

A systematic review published in 2013 established that vitamin D had a significant inverse relationship with disease activity (Sakthiswary and Raymond, 2013). In this review there was not a significant association between vitamin D level and organ damage. In other studies, a low vitamin D level has been associated with renal disease (Kamen et al., 2008; Bogaczewicz et al., 2012) and cardiovascular disease in SLE (Reynolds et al. 2012; Ravenell et al. 2012).

The inverse relationship between vitamin D and disease activity in SLE was confirmed in a meta-analysis (Sahebari et al., 2014). This meta-analysis included 11 articles that analysed this relationship using Pearson's correlation coefficient (Borba et al., 2009; Amital et al., 2010; Ben-Zvi et al., 2010; Hamza et al., 2011; Mok et al., 2012; Sumethkul et al., 2012; Abou-Raya et al., 2013; Chaiamnuay et al., 2013; Sahebari et., 2014). The pooled Pearson correlation was calculated and found to be -0.365 (95% confidence interval (CI): -0.536 to -0.165). However, this meta-analyses did not include other publications that did not state the Pearson's correlation coefficient, including those that did not find a significant relationship. A more recent meta-analysis, that included 6 studies, also showed an inverse correlation between serum 25-hydroxyvitamin D and disease activity (pooled correlation coefficient= -0.50, 95% CI: -0.828 to -0.169) (Guan et al., 2019).

A meta-analysis was performed on three randomised placebo controlled trials conducted by Abou-Raya et al. (2013), Aranow et al. (2015) and Lima et al. (2016) in

which vitamin D was supplemented in SLE patients (Franco et al., 2017). In keeping with the above studies, this meta-analysis found a significant decrease in anti-dsDNA titre (p=0.005). However, the effect on SLEDAI was not analysed in this meta-analysis. Another meta-analysis by Zheng et al. (2019) analysed SLEDAI scores in four RCTs (Lima et al., 2016; Aranow et al., 2015; Rifa'i A et al., 2016; Karimzadeh et al., 2017). This meta-analysis showed an improvement in SLEDAI scores by vitamin D supplementation, however this lacked statistical significance when compared to placebo (p=0.070).

A more recent meta-analysis that included five clinical trials that assessed the effect of vitamin D supplementation on SLEDAI, showed a significant decrease (p<0.001) (Irfan et al., 2022). It included two studies in its analysis of the effect of vitamin D on anti-dsDNA titre, which showed a non-significant decrease (p=0.42). Of note, all the included clinical trials had a low number of participants that were supplemented with vitamin D (range 20-52).

1.2.3 VITAMIN D AND FATIGUE

Five cross-sectional studies, one prospective study and one open label RCT have studied the association between fatigue and vitamin D in adult SLE patients (Ruiz-Irastorza et al., 2008, 2010; Fragoso et al., 2012; Stockton et al., 2012; Salman-Monte et al., 2016; Abaza et al., 2016; Rifa'i et al., 2016) (Table 1.3). A significant inverse association between fatigue and serum 25-hydroxyvitamin D was found in the studies by Ruiz-Irastorza et al. (2010), Salman-Monte et al., Abaza et al. and Rifa'i et al. In the open label trial by Rifa'i et al., 20 vitamin D deficient SLE patients were supplemented

with 1200IU vitamin D3 for 3 months and 19 patients received placebo. In the vitamin D supplementation group, the FSS had a mean decrease of 2.25 (p=0.000) while there was no significant improvement in the placebo group (p=0.971). The studies by Stockton et al. and Rifa'i et al. were the only ones that measured fatigue with the FSS. In the other five studies fatigue was measured by the visual analogue scale (VAS). The cross-sectional cohort studies by Ruiz-Irastorza et al. (2008), Fragoso et al. (2012) and Stockton et al. (2012) failed to demonstrate a significant association between fatigue and vitamin D level, likely due to the lower level of evidence provided by such study design and the possible effect of confounding factors. In addition the study by Stockton et al. had a small sample size and included only 24 SLE patients.

The effect of vitamin D supplementation on fatigue, measured by Kids Fatigue Severity Scale (K-FSS), in juvenile-onset SLE was assessed in a RCT (Lima et al., 2016). This trial included 40 SLE patients who were up to the age of 25 years, and who had disease onset before 16 years of age. The patients received 50,000IU vitamin D3 weekly or placebo for 24 weeks. A statistically significant decrease in fatigue interfering in social life (a K-FSS constituent) was noted in the treatment group in comparison to the placebo group. However there was no significant improvement in the total K-FSS score. The characteristics of the patients included in this study were different to those in the other prospective studies by Ruiz-Irastorza et al. (2010) and Rifa'i et al. (2016), since it included younger patients with juvenile-onset SLE. In addition, it could have failed to demonstrate a significant improvement in K-FSS due to its small sample size.

A meta-analysis analysed the FSS scores reported in the two RCTs by Lima et al. and Rifa'i et al. (Zheng et al., 2019). The meta-analysis concluded that when pooling the

data from the two trials the improvement in FSS scores by vitamin D supplementation was statistically significant when compared to placebo (p=0.001). This analysis was also carried out in the meta-analysis by Irfan et al. (2022) that reached the same conclusion (p<0.001).

Table 1.3. Studies that have evaluated the relation between serum 25-hydroxyvitamin D level and fatigue in SLE.

Study (year)	Country	Design	Study Population	Fatigue measure	Relation between fatigue and vitamin D
Ruiz-Irastorza G et al. (2008)	Spain	Cross-sectional cohort	92 SLE	VAS	*p*=0.08
Ruiz-Irastorza G et al. (2010)	Spain	Prospective cohort	80 SLE	VAS	*p*= 0.015*
Fragoso TS et al. (2012)	Brazil	Cross-sectional case control	78 SLE 64 controls	VAS	*p*=0.808
Stockton KA et al. (2012)	Australia	Cross-sectional case control	24 SLE 21 controls	FSS	*r* = -0.12
Salman-Monte TC et al. (2016)	Spain	Cross-sectional cohort	102 SLE	VAS	*p*=0.009*
Abaza NM et al. (2016)	Egypt	Cross-sectional case control	60 SLE 30 control	VAS	*r*=-0.436, *p*<0.05*
Rifa'i A et al. (2016)	Indonesia	Open label clinical trial	39 SLE	FSS	*p*=0.000*
Lima GL et al. (2016)	Brazil	Randomised, placebo controlled trial	40 juvenile SLE	K-FSS	*p* = 0.008* (in social life component)

*statistically significant results

FSS, Fatigue Severity Scale; K-FSS, Kids Fatigue Severity Scale; SLE, Systemic Lupus Erythematosus; VAS, Visual Analogue scale.

1.2.4 VITAMIN D AND SLEEP QUALITY

A cohort study on 63 SLE patients found that serum vitamin D had a positive correlation with physical activity (r=0.310, p=0.015) and a negative correlation with poor sleep quality measured with Pittsburgh Sleep Quality Index (PSQI) (r=-0.262, p=0.043) and anxiety measured with Hospital Anxiety and Depression Scale (HADS) (r=-0.298, p=0.021) (Gholamrezaei et al., 2014). Vitamin D level was independently associated with sleep quality in a linear regression model (p=0.042). This highlighted a potential role of vitamin D supplementation in ameliorating the sleep quality of patients with SLE.

1.2.5 OTHER RELATIONSHIPS

IFN signature gene expression correlates positively with disease activity (Mandal et al., 2014; Rai et al., 2016). The data on the relationship between disease activity and fatigue is conflicting; in some studies there was a strong association (Wysenbeek et al., 1993; Tench et al., 2000; Tayer et al., 2001; Omdal et al., 2003; Rifa'i et al., 2016; Pinto et al., 2021), in others there was a moderate association (Arnaud et al., 2019), and in others a weak or no association was found (Wang et al., 1998; Bruce et al., 1999; Abaza et al. 2016; Yilmaz-Oner et al., 2016; Du et al., 2018; Azizoddin et al., 2019; Zonana-Nacach et al., 2000; Monahan et al., 2021). In a 10 year prospective study on 280 SLE patients, four latent dual trajectory patterns for fatigue and disease activity were identified (Moazzami et al., 2021). It was concluded that fatigue trajectories cannot be fully explained solely by disease activity. The relationship between the IFN

signature gene expression and fatigue in SLE has been studied in one published paper (Kellner et al., 2010). This cross-sectional case-control study on 58 SLE patients did not find a correlation between fatigue measured by Multidimensional Fatigue Inventory and the expression of three IFN signature genes (*MX1, Ly6E, IFI44*).

1.2.6 LITERATURE REVIEW CONCLUSIONS

This review includes several studies that have a different study design and include patients with diverse characteristics. Even though there are some conflicting results from multiple studies, the strongest form of evidence consisting of a meta-analysis including 11 cohort studies, indicated that a low level of vitamin D is correlated with a higher SLE disease activity (Sahebari et al., 2014). This meta-analysis was limited since it only included studies that reported the Pearson's correlation coefficient. Another meta-analysis, that included five clinical trials, concluded that vitamin D supplementation resulted in a significant improvement in SLEDAI (Irfan et al., 2022). Vitamin D deficiency could result in increased SLE disease activity due to the effect of vitamin D, via VDR, on gene expression resulting in the suppression of auto-immunity. In addition, active disease in particular lupus nephritis may result in vitamin D deficiency. This occurs as renal impairment results in decreased hydroxylation of 25-hydroxyvitamin D to 1,25-dihydroxyvitamin D, the latter being the active form.

Fatigue in SLE is associated with multiple factors such as sleep disorder, depression and decreased physical activity. The evidence on the associations between fatigue and (i) disease activity, and (ii) vitamin D is conflicting. Most studies addressing the latter

did not measure fatigue with the recommended instrument for SLE, the FSS. The strongest form of evidence looking into the relationship between fatigue and vitamin D in adult SLE patients is provided by an open label clinical trial that included only 20 patients treated with vitamin D3 (Rifa'i et al., 2016). This trial showed a significant amelioration in FSS with vitamin D3 supplementation but could have introduced an element of bias due to lack of blinding. The other RCT by Lima et al., (2016) included patients with juvenile SLE and found a significant improvement in the social life component of K-FSS, but there was no significant improvement in the total K-FSS score. The literature search identified one cross-sectional cohort study that studied the relationship between fatigue and the expression of three IFN signature genes; this was not found to be significant. This study used Multidimensional Fatigue Inventory to measure fatigue.

Studies looking into the association between vitamin D and IFN signature gene expression are also very limited and have measured the expression of up to three IFN signature genes. Even though the three cross-sectional cohort studies described showed an inverse relation between serum vitamin D and IFN signature gene expression, the RCT described did not, possibly due to inadequate vitamin D supplementation and the short duration of the study (Ritterhouse et al., 2011; Mandal et al., 2014; Abdel Galil et al., 2018; Aranow et al., 2015).

These conclusions are summarised in figure 1.2.

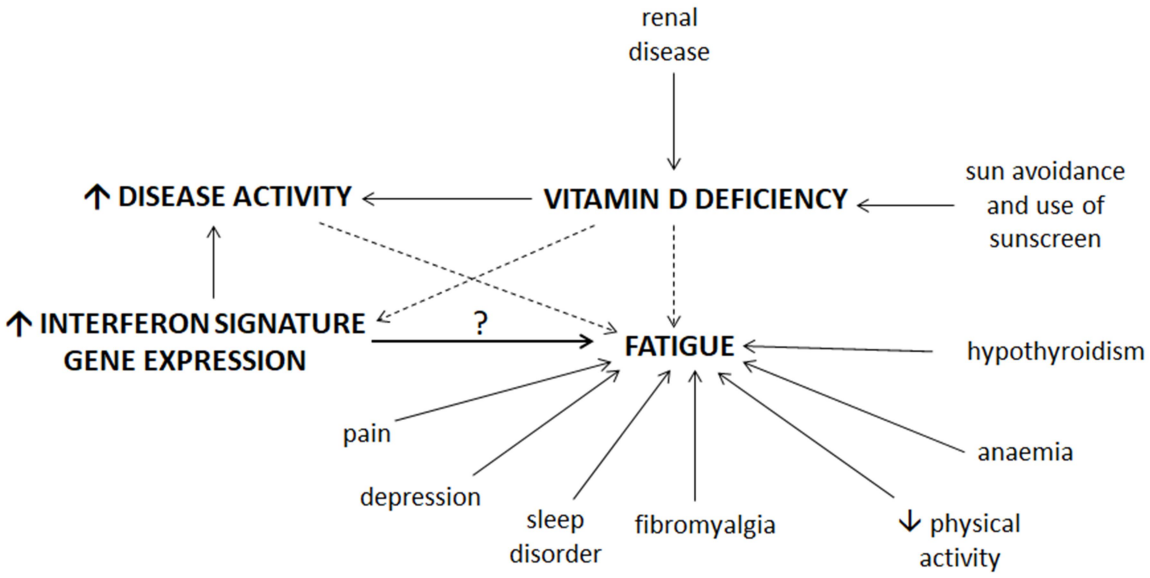

Figure 1.2. The relationship between serum vitamin D level, disease activity, fatigue and IFN signature gene expression in SLE. The continuous lines denote known associations; the dotted lines denote conflicting evidence; and the bold arrow denotes a relationship with minimal available information in the literature.

1.3 Development of the Research Questions

The literature review has shown that further research looking into the relationship between vitamin D, fatigue, disease activity and IFN signature gene expression in SLE is required. Such research needs to be done using the recommended FSS to measure fatigue. In the case of prospective interventional studies, adequate vitamin D supplementation for an adequate duration should be ensured. More specifically the clinical effect on fatigue and disease activity when treating vitamin D deficiency needs to be confirmed. If these parameters are found to improve with supplementation, the importance of screening for vitamin D deficiency in patients with SLE would be highlighted. Further research on the relationship between vitamin D and sleep quality

in SLE is required, since only one cohort study looking into this could be identified in the literature. In addition to this, the relationship between fatigue and disease activity needs to be studied in more detail as it could provide clinical guidance as to whether fatigue in SLE could be improved by a tighter control of disease activity.

The increased expression of the IFN signature genes in SLE is positively correlated with disease activity. Further research on the effect of vitamin D supplementation on the expression of the 12 IFN signature genes that are overexpressed in SLE, is necessary to establish whether vitamin D results in decreased expression of the IFN signature, possibly through the role of VDR in cells belonging to the immune system. This could provide an underlying mechanism of how vitamin D reduces disease activity. The relationship between the IFN signature gene expression and fatigue also needs to be addressed since the IFN pathway may offer new mechanisms in the treatment of fatigue.

1.4 HYPOTHESIS

The literature review has demonstrated that vitamin D deficiency is associated with a higher SLE disease activity. It is thus hypothesized that vitamin D supplementation in SLE results in improved disease activity, particularly when vitamin D is deficient. Since IFN alpha has a pivotal role in the underlying pathogenesis of SLE, it is postulated that the effect of vitamin D on disease activity is mediated by the influence of VDR on the IFN signature gene expression in dendritic cells and other cells belonging to the immune system. This could also provide an explanation for any negative correlation

between vitamin D level and fatigue, particularly since fatigue is a common side effect of IFN alpha when used as treatment for infectious diseases and malignancy (Felger et al. 2012).

1.5 AIMS AND OBJECTIVES

A population based prospective cohort study on patients with SLE who live in Malta will be carried out. The primary aim is to characterise the relationship between vitamin D, disease activity, fatigue and IFN signature gene expression in systemic lupus erythematosus.

The following are the secondary aims and objectives of the study:

1. Identify the effect of vitamin D supplementation in SLE patients with vitamin D deficiency/insufficiency, on fatigue, sleep quality, disease activity, functional disability, steroid use and IFN signature gene expression.
2. Establish the prevalence of vitamin D insufficiency and deficiency, and fatigue in the Maltese population of SLE patients.
3. Explore other factors related to vitamin D deficiency (including damage (measured by SDI), co-morbidities and sleep quality) and to fatigue (including depression, sleep quality, pain and functional disability).
4. Describe the disease characteristics of the SLE patients living in Malta, including organ involvement, disease activity and age at disease diagnosis.
5. Assess the relationship between IFN signature gene expression and other factors including damage, depression, sleep quality and function.

6. Compare the SLE disease characteristics (including disease activity, damage (measured by SDI), IFN signature gene expression, co-morbidities, autoantibody profile and organ manifestations) between the genotypes for the four VDR polymorphisms.
7. Establish the effect of calcitriol (1,25-dihydroxycholecalciferol) supplementation in vitro to cultured dendritic cells and macrophages obtained from a patient with SLE on the expression of the IFN signature genes and *IRF8*.
8. Translate, validate and perform cross-cultural adaptation of FSS, PSQI and the modified Health Assessment Questionnaire (mHAQ) into the Maltese language.

CHAPTER 2 – METHOD

2.1 METHODOLOGY

The first part of the research consisted of the translation and validation of the FSS, PSQI and mHAQ into the Maltese language so that these questionnaires could be used during the research. After the translation of the questionnaires according to the relevant guidelines, psychometric testing of the pre-final version of the Maltese translations was carried out by means of a cohort study including 20 bilingual SLE patients (Beaton et al., 2000; Sousa and Rojjanasrirat, 2011).

After the Maltese translation of the questionnaires was finalised, a population based cross-sectional cohort study including 92 SLE patients was carried out. A cross-sectional study was deemed appropriate to study the correlations between the variables of interest. By inviting all known SLE patients residing in Malta who fulfilled the inclusion criteria of the study, a population based cohort study could be carried out. This enabled the characterisation of SLE patients in Malta. The SLE patients with vitamin D deficiency or insufficiency identified in the cross-sectional cohort study were then invited to participate in an open label prospective interventional study, in which the participants were treated with vitamin D3. This study included 33 patients who were followed up after 6 and 12 months. This enabled the comparison of variables at baseline and after vitamin D supplementation.

A further in vitro experiment was carried out to study the effect of vitamin D on the expression of IFN signature genes and *IRF8* gene in dendritic cells and macrophages. This experiment was carried out in the form of a case study in which samples from one SLE patient and one control were used. The gene expression in cell cultures treated with calcitriol (1,25-dihydroxyvitamin D) was compared to untreated cell cultures.

2.2 TRANSLATION, VALIDATION AND CROSS-CULTURAL ADAPTATION OF THE QUESTIONNAIRES

Although Maltese and English are both official languages in Malta, a significant proportion of the Maltese population are not able to read and speak in English. In fact, a study showed that 26% of the Maltese population were not able to read an English newspaper article and 11% were unable to speak English (European Commission, 2012). Out of a random cohort of 65 SLE patients living in Malta, 36.9% stated that they preferred filling in a questionnaire in Maltese. This necessitated the need to translate, validate and perform cross-cultural adaptation of the FSS, PSQI and mHAQ into the Maltese language for use during the research. Guidelines on the translation and adaptation of self-report measures were followed (Beaton et al., 2000; Sousa and Rojjanasrirat, 2011).

2.2.1 Initial Translation into Maltese

Two translators, whose native language was Maltese, translated the original instruments into the Maltese language. One of the translators had knowledge on what was being assessed in the questionnaires and had a medical qualification. The other translator was not familiar with the issues being assessed as he did not have a medical background.

2.2.2 Formation of the Preliminary Initial Maltese Translation

A comparison of the two questionnaire translations was made. The two translators discussed the discrepancies between the two versions (Tables 2.1-2.3). A consensus was reached on the most suitable Maltese translation that truly expressed the content of the English version.

Table 2.1. Discrepancies noted between the two forward translations of the FSS. The last column shows the agreed version.

English version	**Translation 1**	**Translation 2**	**Preliminary Translation**
Strongly disagree	Ma naqbilx bil-qawwa	Ma naqbilx ħafna	Ma naqbilx ħafna
Strongly agree	Naqbel bil-qawwa	Naqbel ħafna	Naqbel ħafna
Exercise brings on my fatigue.	L-eżerċizzju jġib fuqi għajja kbira.	L-eżerċizzju jgħajjini.	L-eżerċizzju jġib fuqi għajja kbira.
Fatigue interferes with my physical functioning.	L-għajja taffettwa l-mod kif ġismi jista jaħdem fiżikament.	L-għajja ttellifni milli nagħmel moviment fiżiku.	L-għajja ttelifni milli nagħmel l-attivitajiet fiżiċi li nixtieq.

Table 2.2. Discrepancies noted between the two forward translations of the PSQI. The last column shows the agreed version.

English version	**Translation 1**	**Translation 2**	**Preliminary Translation**
How long (in minutes) has it taken you to fall asleep each night?	Kemm iddum (f'minuti) biex jirnexxielek torqod kull lejl?	Kemm tieħu minuti sakemm torqod?	Kemm domt (f'minuti) biex marret għajnejk bik kull lejl?
During the past month, how often have you had trouble staying awake while driving, eating meals, or engaging in social activity?	F'dan l-aħħar xahar, kemm-il darba kellek problema biex tibqa' mqajjem/mqajma waqt li kont qed issuq, tiekol jew f'xi attivita' soċjali?	Matul dan l-aħħar xahar, kemm-il darba batejt biex tibqa' mqajjem waqt li tkun qed issuq, tiekol jew tissoċjalizza?	Matul dan l-aħħar xahar, kemm-il darba batejt biex tibqa' mqajjem/mqajma waqt li kont qed issuq, tiekol jew tissoċjalizza ma' ħaddieħor?
During the past month, how much of a problem has it been for you to keep up enthusiasm to get things done?	F'dan l-aħħar xahar, kemm kienet problema għalik biex tkun entużjast(a) biex tlesti dak li għandek tagħmel?	Matul dan l-aħħar xahar, kemm-il darba kellek diffikultajiet biex izzomm l-entużjażmu sabiex tagħmel xi xogħol?	Matul dan l-aħħar xahar, kemm-il darba kellek diffikulta' biex tkun entużjast(a) biex tagħmel dak li għandek tagħmel?
Fairly good	Pjuttost tajjeb	Mhux ħazin	Pjuttost tajjeb
Fairly bad	Pjuttost ħazin	ħazin	Pjuttost ħazin

Table 2.3. Discrepancies noted between the two forward translations of the mHAQ. The last column shows the agreed version.

English version	**Translation 1**	**Translation 2**	**Preliminary Translation**
Without ANY difficulty	Bla EBDA diffikulta’	Mingħajr diffikulta’	Mingħajr EBDA diffikulta’
With SOME difficulty	DAQSXEJN diffiċli	B’xi ftit diffikulta’	B’xi FTIT diffikulta’
With MUCH difficulty	Diffiċli ĦAFNA	B’ħafna diffikulta’	B’ĦAFNA diffikulta’
UNABLE to do	MA NISTAX nagħmilha	Ma nistax nagħmel	MA NISTAX nagħmilha

The preliminary initial Maltese translation of the FSS, PSQI and mHAQ was formed as follows.

FATIGUE SEVERITY SCALE (FSS)

Jekk jogħġbok immarka b'ċirku n-numru bejn 1 u 7 li jaqbel l-iktar ma' dawn is-sentenzi. Dawn jirreferu għall-ħajja normali tiegħek f'din l-aħħar ġimgħa. 1 jindika "ma naqbilx ħafna" u 7 jindika "naqbel ħafna".

Aqra u mmarka numru b'ċirku.	Ma naqbilx ħafna			⇨			Naqbel ħafna
1. Il-motivazzjoni tiegħi hija iktar baxxa meta nkun għajjien(a) ħafna.	1	2	3	4	5	6	7
2. L-eżerċizzju jġib fuqi għajja kbira.	1	2	3	4	5	6	7
3. Ngħajja malajr.	1	2	3	4	5	6	7
4. L-għajja ttelifni milli nagħmel l-attivitajiet fiżiċi li nixtieq.	1	2	3	4	5	6	7
5. L-għajja spiss toħloqli problemi.	1	2	3	4	5	6	7
6. L-għajja ma tħallinix nagħmel xogħol fiżiku fit-tul.	1	2	3	4	5	6	7
7. L-għajja ttellifni milli naqdi wħud mid-dmirijiet u r-responsabbiltajiet tiegħi.	1	2	3	4	5	6	7
8. L-għajja hija fost l-iktar sintomi li jtellfuni f'ħajti.	1	2	3	4	5	6	7
9. L-għajja ttellifni f'xogħoli, mal-familja, jew fil-ħajja soċjali tiegħi.	1	2	3	4	5	6	7

PITTSBURGH SLEEP QUALITY INDEX (PSQI)

Il-mistoqsijiet li ġejjin huma dwar id-drawwiet tal-irqad tiegħek matul l-aħħar xahar biss. It-tweġibiet tiegħek għandhom jindikaw ir-risposta l-aktar eżatta għall-maġġoranza tal-jiem u l-iljieli f'dan l-aħħar xahar. Jekk jogħġbok wieġeb il-mistoqsijiet kollha.

Fl-aħħar xahar,

1. Fi x'ħin normalment dħalt fis-sodda? _____
2. Kemm domt (f'minuti) biex marret għajnejk bik kull lejl? _____
3. Fi x'ħin normalment qomt filgħodu?_____
4. Kemm-il siegħa torqod bil-lejl? (It-tweġiba tista tkun differenti minn kemm tqatta siegħat fis-sodda.) _____

Jekk jogħġbok immarka l-aħjar risposta:

	Qatt f'dan l-aħħar xahar	Inqas minn darba fil-ġimgħa	Darba jew darbtejn fil-ġimgħa	Tlett darbiet jew iktar fil-ġimgħa
5. Matul dan l-aħħar xahar, kemm-il darba kellek diffikulta' biex torqod minħabba li				
A. Ma stajtx torqod fl-ewwel nofs siegħa				
B. Tqum f'nofs ta' lejl jew filgħodu kmieni				
C. Ikollok tqum biex tuża l-kamra tal-banju				
D. Ma jirnexxielekx tieħu nifs komdu				
E. Tisgħol jew tonħor jgħajjat				
F. Tħoss ħafna bard				
G. Tħoss ħafna sħana				
H. Toħlom ikrah				

I. Tkun muġugħ(a)				
J. Għal xi raġuni(jiet) oħra. Jekk jogħġbok semmihom u inkludi kemm-il darba kellek diffikulta' biex torqod minħabba f'dawn ir-raġunijiet.				
6. Matul dan l-aħħar xahar, kollox ma' kollox, kif tikkunsidra li rqadt?	Tajjeb ħafna	Pjuttost tajjeb	Pjuttost ħażin	Ħażin ħafna
7. Matul dan l-aħħar xahar, kemm-il darba ħadt mediċina (bir-riċetta jew mingħajr riċetta tat-tabib) biex tgħinek torqod?				
8. Matul dan l-aħħar xahar, kemm-il darba batejt biex tibqa' mqajjem/mqajma waqt li kont qed issuq, tiekol jew tissoċjalizza ma' ħaddieħor?				
9. Matul dan l-aħħar xahar, kemm-il darba kellek diffikulta' biex tkun entużjast(a) biex tagħmel dak li għandek tagħmel?	Ma kienetx problema	Problema zgħira ħafna	Pjuttost problema	Problema kbira ħafna

MODIFIED HEALTH ASSESSMENT QUESTIONNAIRE (mHAQ)

Jekk jogħġbok immarka l-aħjar tweġiba skond l-abbiltajiet tiegħek.

Bħalissa, tista:	**Mingħajr EBDA diffikulta'**	**B'xi FTIT diffikulta'**	**B'ĦAFNA diffikulta'**	**MA NISTAX nagħmilha**
Tilbes waħdek, inkluż taqfel iż-żarbun u l-buttuni?				
Tidħol u tqum mis-sodda?				
Terfa' kikkra jew tazza biex tixrob?				
Timxi barra fil-wita'?				
Tinħasel u tixxotta ġismek kollu?				
Titbaxxa biex tiġbor il-ħwejjeg mill-art?				
Tiftaħ u tagħlaq vit?				
Tidħol u toħrog minn karozza?				

2.2.3 BACK TRANSLATION

Another two translators, whose native language was English, carried out back translation of the preliminary Maltese translations into English. They were completely unaware of the original questionnaire and they did not have a medical background. This was done to assure that the Maltese translations reflected the same meaning as the original English questionnaires and thus check their validity.

2.2.4 SYNTHESIS OF THE PRE-FINAL MALTESE TRANSLATION

A comparision of the back translated English questionnaires to the original English versions was made and any differences were identified. These variations in the Maltese versions were analysed by the principal researcher and the four translators, and adjustments were made to mirror the original English versions as precisely as possible (tables 2.4-2.6). The prefinal Maltese translation was thus produced as included in appendix 4.

Table 2.4. Changes made to the Maltese translation of the FSS following discrepancies noted between the original version and the back translations.

Original English version	**Preliminary Translation**	**Prefinal Translation**
Please circle the number between 1 and 7 which you feel best fits the following statements.	Jekk jogħġbok immarka b'ċirku n-numru bejn 1 u 7 li jaqbel l-iktar ma' dawn is-sentenzi.	Jekk jogħġbok indika kemm taqbel ma' kull sentenza billi timmarka b'ċirku t-tweġiba bejn 1 u 7.
Strongly disagree	Ma naqbilx ħafna	Ma naqbel xejn
Fatigue interferes with my physical functioning.	L-għajja ittelifni milli nagħmel l-attivitajiet fiżiċi li nixtieq.	L-għajja ittelifni milli nagħmel xogħol fiżiku.
Fatigue causes frequent problems for me.	L-għajja spiss toħloqli problemi.	L-għajja ta' spiss toħloqli problemi.

Table 2.5. Changes made to the Maltese translation of the PSQI following discrepancies noted between the original version and the back translations.

Original English version	**Preliminary Translation**	**Prefinal Translation**
When have you usually gone to bed?	Fi x'ħin normalment dħalt fis-sodda?	Fi x'ħin normalment dħalt torqod?
How many hours of actual sleep do you get at night?	Kemm-il siegħa torqod bil-lejl?	Kemm-il siegħa jirnexxielek torqod bil-lejl?
Cannot get to sleep within 30 minutes	Ma stajtx torqod fl-ewwel nofs siegħa	Ma tistax torqod fl-ewwel nofs siegħa
During the past month, how often have you had trouble staying awake while driving, eating meals, or engaging in social activity?	Matul dan l-aħħar xahar, kemm-il darba batejt biex tibqa' mqajjem/mqajma waqt li kont qed issuq, tiekol jew tissoċjalizza ma' haddieħor?	Matul dan l-aħħar xahar, kemm-il darba batejt biex tibqa' mqajjem/mqajma waqt li kont qed issuq, tiekol ikla jew tissoċjalizza ma' haddieħor?
During the past month, how much of a problem has it been for you to keep up enthusiasm to get things done?	Matul dan l-ahħħar xahar, kemm-il darba kellek diffikulta' biex tkun entużjast(a) biex tagħmel dak li għandek tagħmel?	Matul dan l-aħħar xahar, kemm kienet diffiċli żżomm l-entużjażmu biex tagħmel dak li għandek tagħmel?

Table 2.6. Changes made to the Maltese translation of the mHAQ following discrepancies noted between the original version and the back translations.

Original English version	**Preliminary Translation**	**Prefinal Translation**
Lift a full cup or glass to your mouth?	Terfa' kikkra jew tazza biex tixrob?	Terfa' kikkra jew tazza mimlija biex tixrob?

2.2.5 PILOT TESTING OF THE PRE-FINAL MALTESE TRANSLATIONS

The pre-final FSS, PSQI and mHAQ Maltese translations were filled in by twenty bilingual SLE patients, after giving informed consent to take part in the study. They also filled in a Maltese version of VAS fatigue. After completing the questionnaires, the participants were interviewed to evaluate the clarity of the questionnaires.

2.2.6 PSYCHOMETRIC TESTING OF THE PRE-FINAL MALTESE TRANSLATIONS IN BILINGUAL SLE PATIENTS

The original English FSS, PSQI and mHAQ questionnaires were filled in by the twenty participants, 4 to 7 days following the completion of the Maltese versions. The reliability, internal consistency and validity of the translations were assessed by means of psychometric testing. Kendall's tau test and Pearson's correlation test were used to test the reliability of the translation for statements with an ordinal scale and for variables with a metric scale respectively. The null hypothesis, accepted if the *p* value exceeded 0.05, specified a weak reliability. The alternative hypothesis, accepted if the *p* value was less than 0.05, specified a satisfactory reliability. Cronbach's alpha was used to assess the FSS, PSQI and mHAQ internal consistency. A value of 0.7 or higher was considered as being an acceptable level of internal consistency (Field, 2005). The validity of the FSS Maltese translation was tested by conducting Pearson's correlation test to assess the correlation between FSS and VAS fatigue. These statistical tests were carried out using IBM SPSS statistics 24.

2.3 CROSS-SECTIONAL COHORT STUDY OF SLE PATIENTS IN MALTA

A population based cross-sectional cohort study of SLE patients living in Malta was carried out.

2.3.1 IDENTIFICATION OF SLE CASES

SLE patients living in Malta were identified through various sources since a local SLE register was not available. These comprised:

(i) referral from all Rheumatologists and specialist trainees within the Department of Rheumatology at Mater Dei Hospital,

(ii) invitation of members of Arthritis and Rheumatism Association Malta (ARAM) and SLE Maltese support groups,

(iii) invitation of patients who receive treatment for Lupus Erythematosus on the national formulary.

Identified SLE cases who were over 18 years of age and who fulfilled the SLICC classification criteria for SLE (Petri et al., 2012), were invited to take part in the study. Patients with inability to give informed consent, such as due to mental illness, were excluded.

One hundred twenty nine patients were referred, out of which 22 were excluded as they did not fulfill the SLICC classification criteria. Fifteen patients refused to participate (Figure 2.1).

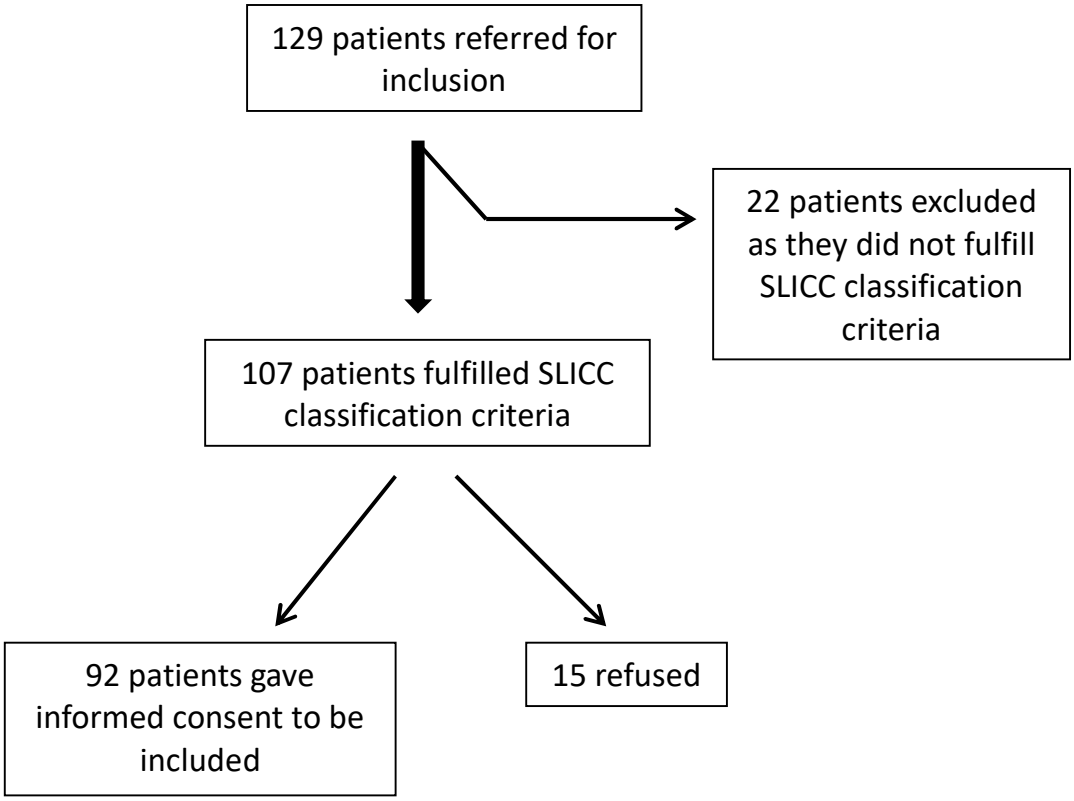

Figure 2.1. Flow chart depicting the number of patients referred for the study and the number of patients that were included.

SLICC, Systemic Lupus International Collaborating Clinics.

2.3.2 PATIENT INTERVIEW

The patients who were interested in taking part in the study were provided with all the details on the study by means of a patient information sheet that was available in both English and Maltese (appendix 1). Written informed consent to take part in the study was given by 92 patients. The consent form is included in appendix 2. A face-to-face interview was conducted with all participants by the researcher. The data gathered included: age, ethnicity, sex, disease duration, autoantibody status, co-morbidities, drug history, physical activity, BMI, smoking status, sunscreen use, SLEDAI-2K score and SDI. Information on the past history of SLE including past organ involvement was

noted from the medical file and by obtaining a history from the patients. The pro forma used to collect the data is included in appendix 3.

The patient interviews were performed from October 2016 to June 2017. The summer months were avoided to: (i) decrease any confounding effect of the higher ambient temperature on fatigue and (ii) minimise the seasonal effect on vitamin D levels due to higher sun exposure in the summer months.

2.3.3 QUESTIONNAIRES

Fatigue, sleep quality, depression, anxiety, pain and functional disability were assessed by means of the following questionnaires filled on the day of the interview: FSS, VAS fatigue, PSQI, HADS, VAS pain and mHAQ (Krupp e al., 1987; Buysse et al., 1989; Zigmond et al., 1983; Pincus et al., 1983). These questionnaires have been deemed most suitable for the scope of the research (Ad Hoc Committee on Systemic Lupus Erythematosus Response Criteria for Fatigue, 2007; Smarr et al., 2011; Omachi et al., 2011; Maska et al., 2011; de Almeida Macêdo et al., 2017). The patients were able to choose whether to complete the English or Maltese version of the questionnaires (appendix 4). The HADS questionnaire has been translated and validated into the Maltese Language by Baldacchino et al. (2002). The FSS, PSQI and mHAQ have been translated and validated as part of this research.

2.3.4 INVESTIGATIONS

The following blood tests were carried out for each participant: serum 25-hydroxyvitamin, complete blood count (CBC), complement 3 (C3), complement 4 (C4), anti-dsDNA titre, erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), thyroid function test (TFT), blood glucose, renal function, calcium and albumin. Estimated glomerular filtration rate (eGFR) was calculated using the Modified Diet in Renal Disease (MDRD) formula (National Kidney Foundation Calculator for Healthcare Professionals). Urinalysis and microscopy, and urine protein creatinine ratio (PCR) were carried out.

CBC was measured using Sysmex XN haematology analyser. C3, C4 and CRP were measured by immunochemistry (Siemens) and anti-dsDNA titre was measured by an enzyme-linked immunosorbent assay (Euroimmun). Serum 25-hydroxyvitamin D and TFT were measured by chemiluminescent immunoassay using the equipment Siemens Centaur. Blood glucose, renal function, calcium, albumin and urine PCR have been measured using the equipment Roche COBAS C501, and urinalysis was carried out using Roche Urisys 1800. Urine microscopy was performed manually using a wet film preparation and bright field microscopy.

2.3.5 STATISTICAL ANALYSIS

The statistical analysis was carried out using IBM SPSS statistics 24. The Kolmogorov–Smirnov test was used to establish whether continuous variables had a normal distribution. In this case, the null hypothesis stated that the variable was normally

distributed and was accepted if the *p* value obtained was larger than 0.05. On the other hand, the alternative hypothesis stated that the data was not normally distributed. It was accepted if the *p* value obtained was smaller than 0.05 (Pallant, 2010). The mean and standard deviation were used to describe normally distributed variables and the median and interquartile range were used for non-normally distributed variables.

Pearson's coefficient was used for correlation between two continuous variables. Spearman's correlation test was used as the non-parametric alternative. The independent samples t-test was used for comparisons of continuous normal variables between two groups; the Mann-Whitney U-test was the non-parametric alternative. Categorical variables between two groups were compared using the Chi-Squared test. For all tests the null hypothesis specified that there was no relationship between the two variables tested and was accepted if the *p* value was higher than the 0.05 level of significance. The alternative hypothesis for these tests stated that there was a significant relationship between the two variables tested and was accepted if the *p* value was below 0.05. ANCOVA regression model was used to explore the relationship of multiple continuous and categorical variables to the dependent variables, namely serum 25-hydroxyvitamin D, FSS and VAS fatigue. The generalised linear model for gamma distributed dependent variables was used for PSQI, mHAQ and SDI since they had a right skewed distribution.

2.4 Prospective Cohort Study of SLE patients with Vitamin D Deficiency or Insufficiency

The prospective part of the study included SLE patients who were found to have vitamin D insufficiency (21-29ng/ml) or vitamin D deficiency (<20ng/ml) in the cross-sectional cohort study. These patients were treated and followed up for one year. This open label prospective interventional study was registered with the ISRCTN registry (Trial ID: ISRCTN59058825). Registration can be viewed on https://www.isrctn.com/ISRCTN59058825.

2.4.1 Treatment of patients with vitamin D Insufficiency and Deficiency

Fourteen and 25 patients were diagnosed with vitamin D deficiency and insufficiency respectively in the cross-sectional cohort study. These patients were invited to take part in the prospective interventional part of the research, with the exception of two patients with stage 4 and 5 chronic kidney disease. Thirty three patients, 13 with vitamin D deficiency and 20 with vitamin D insufficiency agreed to be followed up for one year after receiving treatment with vitamin D3.

Patients with vitamin D insufficiency and deficiency were supplemented with vitamin D3 8000IU daily for 4 and 8 weeks respectively, followed by 2000IU daily maintenance as per guideline recommendations (Benhamou et al., 2011; Holick et al., 2011). Vitamin D supplementation according to this regime was sponsored for one year by

Quest Nutra Pharma, and the patients were able to pick up the treatment from Tony's Pharmacy in Gżira upon presentation of a letter.

2.4.2 PATIENT FOLLOW-UP

Serum 25-hydroxyvitamin D level and corrected calcium were measured after 3 months of vitamin D3 supplementation to make sure that target serum 25-hydroxyvitamin D levels had been reached and to check for any hypercalcaemia as a consequence of treatment. If the target serum 25-hydroxyvitamin D level (≥30ng/mL) was not achieved, the patients were contacted to ensure adherence and adjust vitamin D3 dosage if required.

The patients were then invited for another face-to-face interview after 6 months from the initial assessment. The following variables were recorded: drug history, current use of sunscreen, physical activity, disease activity (SLEDAI-2K) and damage (SDI). The patients once again, filled the following questionnaires on the day of the interview: FSS, VAS fatigue, HADS, VAS pain, PSQI and mHAQ (Krupp et al., 1987; Zigmond et al., 1983; Buysse et al., 1989; Pincus et al., 1983) (appendix 4).

Urinalysis, urine PCR and the following blood tests were taken on the same day: CBC, renal function, calcium, albumin, TFT, 25-hydroxyvitamin D, ESR, CRP, C3, C4 and anti-dsDNA titre. The interview, questionnaires, blood and urine tests were repeated at 1 year following the initial assessment. Figure 2.2 summarises the method used in the cross-sectional cohort and prospective studies.

At the end of the one year period, any changes in the dosages of prednisolone or disease modifying anti-rheumatic drugs (DMARDs) over the one year period were noted. The results of the patients who were initiated on DMARDs or who had an increase in the DMARD dose during the one year period (two patients) were excluded to ensure that any observed effects were not due to the DMARDs.

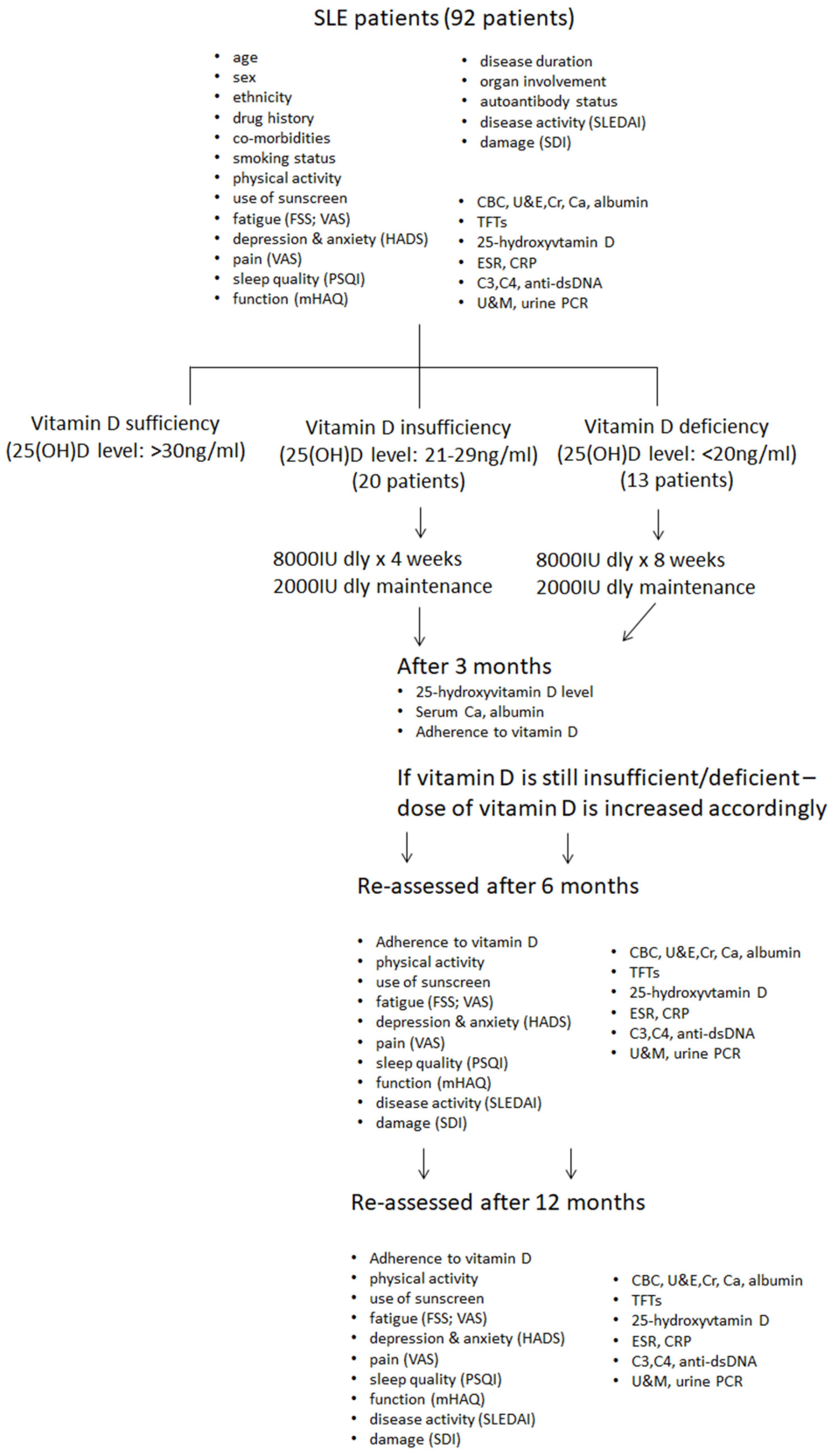

Figure 2.2. Summary of cross-sectional cohort and prospective studies. Anti-dsDNA, Anti-double-stranded deoxyribonucleic acid; C3, Complement 3; C4, Complement 4;

Ca, Calcium; CBC, Complete blood count; Cr, Creatinine; CRP, C-reactive protein; ESR, Erythrocyte sedimentation rate; FSS, Fatigue Severity Scale; HADS, Hospital Anxiety and Depression Scale; mHAQ, Modified Health Assessment Questionnaire; PCR, protein creatinine ratio; PSQI, Pittsburgh Sleep Quality Index; SDI, Systemic Lupus International Collaborating Clinics/American College of Rheumatology damage index; SLE, Systemic lupus erythematosus; SLEDAI, Systemic Lupus Erythematosus Disease Activity Index; TFTs, Thyroid function tests; U& E, Urea and electrolytes; U&M, Urinalysis and microscopy; VAS, Visual analogue scale.

2.4.3 STATISTICAL ANALYSIS

The statistical analysis was carried out using IBM SPSS statistics 24. The Kolmogorov–Smirnov test was used to assess the distribution of continuous variables. Comparison of continuous variables at baseline and after 6 and 12 months of vitamin D3 treatment was done using the paired samples t-test for normally distributed variables and Wilcoxon signed ranks test for variables that were not normally distributed. The Chi-Squared test was used to assess change from baseline to 6 and 12 months of vitamin D treatment for categorical variables. For these tests the null hypothesis specified that there was no change after vitamin D supplementation and was accepted if the *p* value was higher than the 0.05 level of significance. The alternative hypothesis for these tests specified that there was a significant change after vitamin D supplementation and was accepted if the *p* value was less than 0.05.

2.5 INTERFERON SIGNATURE GENE EXPRESSION

Blood samples drawn from patients participating in the cross-sectional cohort study and following 6 months of vitamin D supplementation in the prospective study were analysed in order to measure ribonucleic acid (RNA) expression of 12 IFN signature genes (*CCL2, CXCL1, IFI35, IFITM1, IFIT1, IFIT3, MX1, SOCS1, SOCS3, STAT1, OAS1, STAT2*) in white blood cells. These IFN-regulated genes have been identified in a meta-analysis as being overexpressed in the peripheral blood of SLE patients (Arasappan et al., 2011). This analysis was performed by the researcher at the University of Malta laboratories.

2.5.1 WHITE BLOOD CELL COLLECTION

Two 3ml blood samples were collected in ethylenediaminetetraacetic acid (EDTA) from each patient participating in the cross-sectional cohort study. Bloodletting was performed by the researcher during the interview with the patients. Two further 3ml blood samples collected in EDTA were obtained from patients found to have vitamin D insufficiency/deficiency, 6 months after vitamin D3 supplementation during the patient interview in the prospective part of the research. This time frame was chosen as in a RCT, supplementation of vitamin D to SLE patients with vitamin D deficiency, did not show an effect on the expression of IFN signature genes at 12 weeks (Aranow et al., 2015).

The blood samples were analysed in the laboratory within minutes of being drawn in order to avoid degradation of the RNA. They were stored at a temperature of 4^{o}C until the procedure could be started. The blood samples were centrifuged at 2000 x g for 15 minutes at room temperature. The plasma (top layer) was aspirated with a transfer pipette. The white blood cell layer (around 0.7ml) was then aspirated with a transfer pipette and transferred to a 15ml collection tube.

2.5.2 RNA EXTRACTION

QIAamp® RNA Blood Mini kit was used to extract RNA from white blood cells. The manufacturer's instructions were followed (Qiagen® 2016).

The concentration and purity of the extracted RNA was measured using the Thermo Scientific NanoDrop^{TM} spectrophotometer. The RNA was then stored at -80^{o}C at the Malta BioBank, University of Malta.

2.5.3 MEASUREMENT OF IFN SIGNATURE GENE EXPRESSION

QuantiGene® Plex Assay kit was then used to measure RNA expression for 12 IFN signature genes (*CCL2, CXCL1, IFI35, IFIT3, IFIT1, IFITM1, SOCS1, MX1, SOCS3, STAT1, OAS1, STAT2*) in the extracted RNA. Moreover RNA expression of 3 housekeeping genes (*RPL13A, HPRT1, TBP*) was also measured (Zhang et al., 2005; Ledderose et al., 2011; de Lima Rebouças et al., 2013). This technique enables the simultaneous detection and quantification of multiple RNA targets (Scerri et al., 2019). The

QuantiGene Plex Assay utilises capture beads to capture specific RNA molecules. The Luminex® flow cytometer is utilised to measure the consequent fluorescence signal linked with the capture beads, and is recorded as median fluorescence intensity (MFI). The latter is proportionate to the amount of target RNA molecules present.

The instructions provided in the QuantiGene Plex Assay kit user manual were followed (ThermoFisher Scientific 2017). The reported MFI results were normalised in accordance with the housekeeping genes. This was done by subtracting the mean background signal for the genes; and then dividing by the geometric mean of the result for the three reference genes. RNA expression was measured in 123 RNA samples; 91 samples from whole blood at baseline and 32 samples collected at 6 months in the prospective part of the study.

The IFN signature gene expression score for each sample was computed. This was done by first normalising the gene expression values for each gene by subtracting the gene expression by the minimum expression for that gene and then dividing by the range of expression. The maximum for each gene expression was thus 1. The IFN signature gene expression score was than calculated by summing up the normalised gene expressions for each of the 12 IFN signature genes.

2.5.4 STATISTICAL ANALYSIS

The statistical analysis was carried out by the statistical software IBM SPSS statistics 24. The Kolmogorov–Smirnov test was used to establish the normality of continuous variables. The relationship between normalised MFI signals and other continuous

variables was analysed using Pearson's or Spearman's coefficients depending on whether the variables were normally distributed or not, respectively. Continuous variables between two groups were compared using the independent samples t-test if the variables were normally distributed or the Mann-Whitney U-test if they were not. The normalised MFI signals at baseline and after 6 and 12 months of vitamin D3 supplementation were compared by using the paired samples t-test for normally distributed variables and the Wilcoxon signed ranks test for those that were not normally distributed. Multiple gene testing was compensated for by means of the Bonferroni correction. The characteristics of patients who had a decrease in the IFN signature gene expression score with vitamin D3 supplementation, were compared with those who had an increase, by using the independent samples t-test for normally distributed variables or the Mann-Whitney U-test for those that were not normally distributed.

2.6 VDR POLYMORPHISMS

A study carried out by J Grech Meli (2020) described the genotypes of the VDR polymorphisms (BsmI, FokI, ApaI, TaqI) in 59 SLE patients that have been included in the cross-sectional cohort study described in section 2.3. In this study DNA extraction was carried out from whole blood. The regions containing VDR polymorphisms were amplified by polymerase chain reaction and the genotypes were established by restriction fragment length polymorphism.

Statistical analysis was carried out to assess the relationship between the genotypes of the VDR polymorphisms and the variables measured in the cross-sectional cohort study. For continuous variables that were normally distributed one-way ANOVA test was used. Kruskal Wallis Test was used as the non-parametric alternative. To analyse the relationship of VDR polymorphisms with categorical variables, Chi-Squared test and Fisher's exact test were used. Odds ratios were also used for further analysis.

Statistical analysis was also carried out to compare the genotype of the four VDR polymorphisms between the patients who had a decrease in IFN signature gene expression score with vitamin D supplementation (11 patients) with those patients who did not (7 patients). The Freeman-Halton extension of Fisher's exact test was used for this analysis.

2.7 IN VITRO EFFECT OF CALCITRIOL SUPPLEMENTATION ON EXPRESSION OF IFN SIGNATURE GENES, *IRF8* AND *IRF7* IN PRIMARY CELL CULTURE

An in vitro cell culture experiment was carried out on blood collected from one SLE patient and one control. The SLE patient was a 41 year old Caucasian female who fulfilled the SLICC classification criteria for SLE. The patient had a SLEDAI-2K of 2, SDI was 0 and serum 25-hydroxyvitamin D was 25ng/ml at the time of the experiment. Her BMI was 22.7kg/m^2 and she had been diagnosed with SLE at the age of 23 years. She had a history of arthritis and mucocutaneous and haematological manifestations, with

positive anti-dsDNA and anti-Ro antibodies. She did not have any co-morbidities and was on regular hydroxychloroquine. The control was a 36 year old healthy Caucasian female who did not have any history of autoimmune disease or co-morbidities. The serum 25-hydroxyvitamin D was 25ng/ml and her BMI was 22.1kg/m^2.

In this experiment, monocytes from the SLE patient and from the control were differentiated to dendritic cells and macrophages. The cell cultures were treated with calcitriol and the expression of the 12 IFN signature genes (*IFITM1, IFI35, MX1, IFIT1, STAT1, IFIT3, OAS1, STAT2, CXCL1, SOCS1, CCL2, SOCS3*), *IRF8* and *IRF7* was measured at 24 and 48 hours using QuantiGene Plex Assay. This was compared to untreated cell cultures.

During this experiment strict precautions to avoid contamination were taken. This included wiping all items to be used with alcohol and working continuously under laminar flow. The incubator and 6 well plates were also wiped with alcohol each time they were removed from and placed back into the incubator.

2.7.1 ISOLATION OF PERIPHERAL BLOOD MONONUCLEAR CELLS

Thirty millilitres of blood was collected in EDTA from the control and the SLE patient respectively. The blood was centrifuged at 2500rpm for 25 minutes at room temperature. The white blood cell layer was then aspirated with a transfer pipette and transferred to a 50ml Falcon tube. 5ml of histopaque®-1077 were placed in an empty 15ml Falcon tube. The white cell layer was added slowly to the wall of the Falcon tube containing the histopaque®-1077 (figure 2.3 A). This was centrifuged at 2500rpm for

25 minutes (figure 2.3 B). After centrifugation the layer containing PBMCs was aspirated and placed in a 15ml Falcon tube. 0.9% saline was added to fill up the 15ml Falcon tube. This was then centrifuged at 1500rpm for 5 minutes at room temperature. The supernatant obtained was discarded. 3ml 0.9% saline was added to the pellet and mixed by pipetting multiple times.

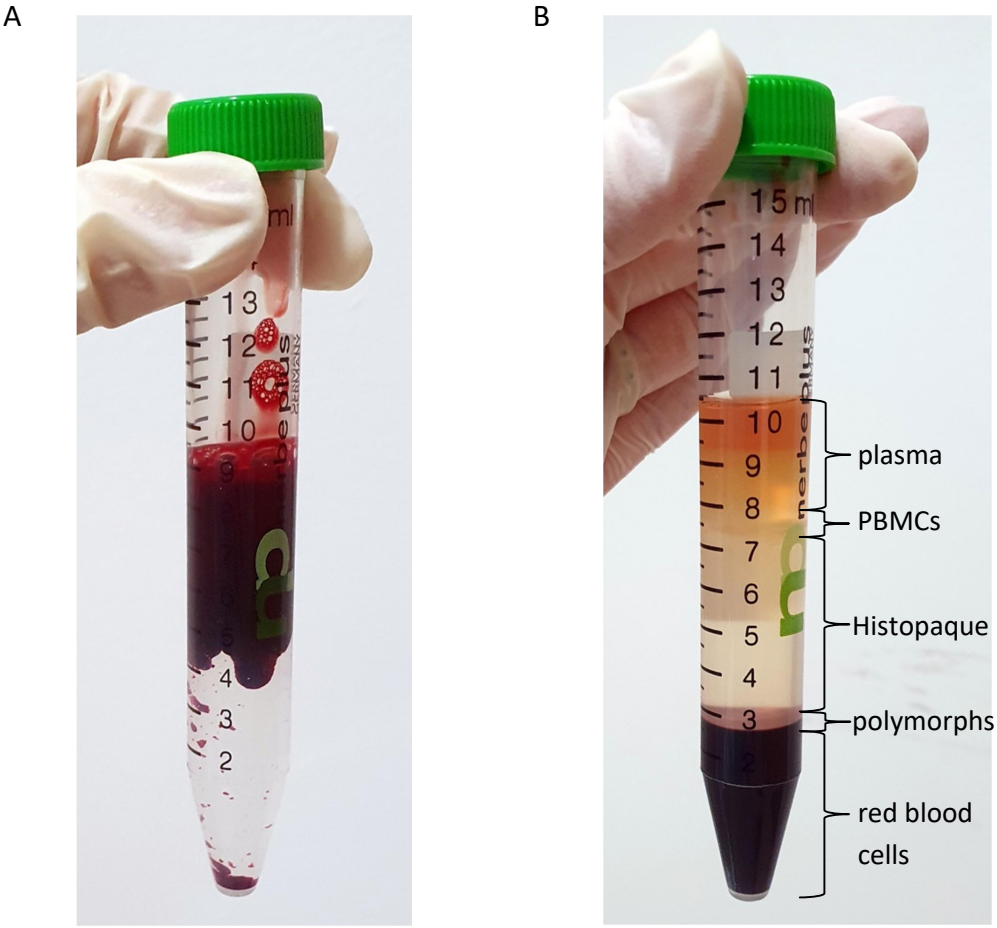

Figure 2.3. Figure showing Falcon tube containing white cell layer added to histopaque®-1077 before (A) and after (B) centrifugation.
PBMCs, peripheral blood mononuclear cells.

Thirty microlitres of the cell suspension was placed in a 1.5ml eppendorf tube and 3µl of trypan blue stain (0.4%) was added to it. 10µl of the mixture was placed on either side of the Countess™ cell counting chamber slide. The Countess™ automated cell counter was used to count the number of PBMCs and perform cell viability assay.

One hundred microlitres of the cell suspension obtained was added to: (i) 3µl of fluorescein isothiocyanate (FITC) anti-human cluster of differentiation (CD) 14 and 3µl of phycoerythrin (PE) anti-human CD83, (ii) 3µl FITC Mouse IgG1, κ isotype control and 3µl PE Mouse IgG1, κ isotype control. In another test tube 100µl of cell suspension was left unstained. These were mixed well by repetitive pipetting. The samples were left in the dark for 10 minutes and then mixed by vortexing. Flow cytometry was carried out using FACSCalibur.

The remaining cell suspension was divided in two and centrifuged at 1500rpm for 5 minutes at room temperature. The supernatant containing saline was discarded.

2.7.2 DIFFERENTIATION OF MONOCYTES TO DENDRITIC CELLS

Differentiation to DCs was carried out by following the ImmunoCult™ DC culture kit protocol (Stemcell™ Technologies 2020). Fifty millilitres of ImmunoCult™ DC differentiation medium was prepared by adding 500µl of ImmunoCult™-ACF dendritic cell differentiation supplement to 49.5ml of ImmunoCult™-ACF dendritic cell medium. 1ml of penicillin streptomycin (10,000IU/ml) was added. Twenty five millilitres of ImmunoCult™ DC differentiation medium was added to the pellet obtained from the SLE patient's cell suspension and mixed well by pipetting. 2.5ml of medium containing

suspended cells was placed in each well in two 6 well plates (10 wells). The same was done for the control. The 6 well plates were checked under the microscope and placed in an incubator at 37^{o}C.

On day 3, 50ml of ImmunoCult^{TM} DC differentiation medium was once again prepared. The 6 well plates were checked under the microscope. The medium containing non-adherent cells was pipetted from the wells and collected in Falcon tubes which were centrifuged at 1200rpm for 10 minutes. 2.3ml of ImmunoCult^{TM} DC differentiation medium was added to each well. The supernatant obtained on centrifugation was discarded. 2ml of ImmunoCult^{TM} DC differentiation medium was added to each pellet (from SLE patient and control) and mixed by pipetting. 200µl of medium containing suspended cells was added to each well. The 6 well plates were placed in an incubator at 37^{o}C.

On day 5, the wells were once again checked under the microscope. Twenty five microliters of ImmunoCult^{TM} Dendritic Cell Maturation supplement was added to each well. The 6 well plates were swirled gently to mix and placed back in the incubator.

2.7.3 DIFFERENTIATION OF MONOCYTES TO MACROPHAGES

The culture medium for macrophage differentiation was prepared by adding 20ml Roswell Park Memorial Institute (RPMI) 1640 Medium, 10ml Dulbecco's Modified Eagle's Media (DMEM), 10ml Nutrient Mixture F-12 Ham, 10ml Fetal Bovine Serum (FBS), 1ml penicillin streptomycin (10,000IU/ml) and 500µl Lipopolysaccharides (LPS) from Escherichia coli O55:B5. A protocol using LPS was used in order to induce M1

macrophages known to impact inflammation in SLE (ThermoFisher Scientific 2020; Niu et al., 2019). Twenty millilitres of the medium was added to the pellet obtained from the SLE patient's cell suspension and mixed well. Two millilitres of medium containing suspended cells was placed in each well in two 6 well plates (10 wells). The same was done for the control. The 6 well plates were checked under the microscope and placed in an incubator at 37^{o}C.

On day 3, 50ml of fresh culture medium was prepared as above. The 6 well plates were checked under the microscope. The medium containing non-adherent cells was pipetted from the wells and collected in Falcon tubes which were centrifuged at 1200rpm for 10 minutes. 1.3ml of fresh culture medium was added to each well. The supernatant obtained on centrifugation was discarded. Seven millilitres of culture medium was added to each pellet (from SLE patient and control) and mixed well by repetitive pipetting. Seven hundred microlitres of medium containing suspended cells was added to each well. The 6 well plates were placed in an incubator at 37^{o}C.

On day 5, the wells were once again checked under the microscope. Five hundred microlitres of fresh culture medium was added to each well. The 6 well plates were placed back in the incubator.

2.7.4 CHARACTERISATION OF DIFFERENTIATED CELL CULTURES AND RNA EXTRACTION

On day 7, the 6 well plates containing differentiated DCs and macrophages were checked under the microscope. The medium containing non-adherent cells was collected in separate Falcon tubes from (i) 4 wells from SLE patient cultures containing DCs, (ii) 4 wells from SLE patient cultures containing macrophages, (iii) 4 wells from control cultures containing DCs, (iv) 4 wells from control cultures containing macrophages. The Falcon tubes were centrifuged at 1500rpm for 5 minutes. The supernatant containing conditioned medium was collected in a Falcon tube. One hundred microlitres of 0.9% saline was added to each pellet.

One and a half millilitres of trypsin was added to each well and placed in an incubator at 37^{o}C for 5 minutes. The wall of the wells was washed with trypsin by using a pipette, and the trypsin containing suspended adherent cells was then collected in separate 15ml Falcon tubes. The supernatant obtained above was added to the trypsin and suspended cells to inactivate the trypsin. This was then centrifuged at 1500rpm for 5 minutes. The supernatant was discarded. One hundred microlitres of 0.9% saline was added to each pellet, mixed well and added to the pellet in saline obtained above.

Twenty microlitres of the cell suspension from (i) SLE patient macrophage, (ii) SLE patient DC, (iii) control macrophage, and (iv) control DC cultures were placed in separate 1.5ml eppendorf tubes and 2µl of trypan blue stain (0.4%) was added to each tube. Ten microlitres of the mixture was placed on either side of the Countess^{TM} cell

counting chamber slide. The Countess™ automated cell counter was used to obtain a cell count and perform a cell viability assay. This was repeated twice.

Fifty microlitres of the cell suspension obtained from two wells of (i) SLE patient macrophage, (ii) SLE patient DC, (iii) control macrophage, and (iv) control DC cultures were added to (a) 3µl of FITC anti-human CD14 and 3µl of PE anti-human CD83, (b) 3µl FITC Mouse IgG1, κ isotype control and 3µl PE Mouse IgG1, κ isotype control. These were mixed well by pipetting. The samples were left in the dark for 10 minutes and then mixed by vortexing. Flow cytometry was carried out using FACSCalibur.

The cell suspension obtained from two wells of (i) SLE patient macrophage, (ii) SLE patient DC, (iii) control macrophage, and (iv) control DC cultures was used for RNA extraction using PureLink™ RNA Mini kit as per manufacturer's instructions (ThermoFisher Scientific 2020). A Thermo Scientific NanoDrop™ spectrophotometer was used to measure the purity and concentration of the extracted RNA. This was stored at -80°C at the Malta BioBank, University of Malta.

2.7.5 TREATMENT OF PRIMARY CELLS WITH CALCITRIOL

One milligram of calcitriol was suspended in 2.40ml of absolute ethanol. The culture medium containing suspended non-adherent cells in 4 wells of (i) SLE patient macrophage, (ii) SLE patient DC, (iii) control macrophage, and (iv) control DC cultures was collected in separate Falcon tubes. Fresh culture medium was added to increase the volume to 10ml. 0.48µl of the calcitriol stock solution was added to the 10ml

culture medium and mixed well. 2.5ml of medium was placed in each well. Thus calcitriol at a concentration of 20nM was added to 4 of the wells.

2.7.6 RNA EXTRACTION AT 24 AND 48 HOURS FOLLOWING ADDITION OF CALCITRIOL

After 24 hours following the addition of calcitriol, the medium containing non-adherent cells was collected in separate Falcon tubes from 2 wells of calcitriol treated (i) SLE patient DC, (ii) SLE patient macrophage, (iii) control DC, and (iv) control macrophage cultures. It was also collected from 1 well of untreated (i) SLE patient DC, (ii) SLE patient macrophage, (iii) control DC, and (iv) control macrophage cultures. The Falcon tubes were centrifuged at 1500rpm for 5 minutes. The supernatant containing conditioned medium was collected in a Falcon tube. One hundred microlitres of 0.9% saline was added to the pellet.

One and a half millilitres of trypsin was added to each well and placed in an incubator at 37^{o}C for 5 minutes. The wall of the wells was washed with trypsin by using a pipette and the trypsin containing suspended adherent cells was collected in separate 15ml Falcon tubes. The supernatant obtained above was added to the trypsin and suspended cells to inactivate the trypsin. This was then centrifuged at 1500rpm for 5 minutes. The supernatant was discarded. 100µl of 0.9% saline was added to the pellet, mixed well and added to the pellet in saline obtained above.

The 200µl cell suspension obtained from each cell culture well was used to carry out RNA extraction using the PureLink^{TM} RNA Mini kit (ThermoFisher Scientific 2020). Once

again, the Thermo Scientific NanoDrop™ spectrophotometer was used to measure the purity and concentration of the extracted RNA. The latter was stored at -80°C at the Malta BioBank, University of Malta.

The above steps were repeated at 48 hours following treatment with calcitriol.

2.7.7 MEASUREMENT OF EXPRESSION OF IFN SIGNATURE GENES, *IRF8* AND *IRF7*

QuantiGene® Plex Assay kit was then used to quantify RNA expression for 12 IFN signature genes (*IFITM1, MX1, IFI35, IFIT1, STAT1, IFIT3, OAS1, STAT2, CCL2, SOCS1, CXCL1, SOCS3*), *IRF8* and *IRF7* in the extracted RNA. Moreover RNA expression of 4 housekeeping genes (*TBP, RPL13A, HPRT1, PPIB*) was also measured. This was carried out in triplicate for each RNA sample available. The instructions provided in the QuantiGene Plex Assay kit user manual were followed (ThermoFisher Scientific 2017). The MFI results obtained were then normalised. This was done by first subtracting the average background signal for each gene; and subsequently dividing by the geometric mean of the signal obtained for the four reference genes. The IFN signature gene expression score was calculated as detailed in section 2.5.3.

2.7.8 STATISTICAL ANALYSIS

The presence of biological duplicates and technical triplicates enabled statistical analysis. This was performed using the statistical software IBM SPSS statistics 24. The

Kolmogorov–Smirnov test was used to establish the normality of continuous variables. The two-tailed independent samples t-test and Mann-Whitney U-test were used to compare continuous variables between two groups in normally and non-normally distributed variables respectively.

2.8 ETHICS AND OTHER APPROVALS

Ethics approval was granted by the University Research Ethics Committee (ref no 54/2016).

Approval was also obtained from the:

(i) Chairman of the Department of Medicine at Mater Dei Hospital, Prof S Fava;

(ii) Rheumatology Consultants at Mater Dei Hospital, Prof A Borg, Dr F Camilleri, Dr B Coleiro, Dr PJ Cassar and Dr C Mercieca;

(iii) Chief Executive Officer at Mater Dei Hospital, Mr Ivan Falzon;

(iv) Data Protection Officer, Ms Sharon Young.

Dr L Kruppe, Prof DJ Buysse, Prof T Pincus were emailed in order to seek their approval to use the FSS, PSQI and mHAQ questionnaires respectively. Permission to use the HADS questionnaire was obtained from GL Assessment Ltd (appendix 5).

2.9 FUNDING

Funding to carry out this research has been obtained from the Faculty of Medicine and Surgery, University of Malta (appendix 6). The vitamin D3 treatment for patients with vitamin D deficiency or insufficiency has been sponsored by Quest Nutra Pharma.

CHAPTER 3 – RESULTS

3.1 TRANSLATION, VALIDATION AND CROSS-CULTURAL ADAPTATION OF THE QUESTIONNAIRES

Statistical testing was performed to determine the reliability, internal consistency and validity of the Maltese translation of the three questionnaires: FSS, PSQI and mHAQ.

3.1.1 PATIENT DEMOGRAPHICS

Twenty bilingual Maltese SLE patients filled in the Maltese and English versions of the questionnaire four to seven days apart. The patients were all Caucasian with a mean age of 37.7 years (S.D. 10.9 years). Table 3.1 shows the patient characteristics. The Kolmogorov-Smirnov test has been used to assess the distribution of the continuous variables described.

Table 3.1. Characteristics of the twenty SLE patients who filled in the Maltese and English translations of the questionnaires.

Characteristics	Values	Kolmogorov-Smirnov test statistic	Kolmogorov-Smirnov p value
Age, mean (S.D.) years	37.7 (10.9)	0.131	0.200
Female sex (n/N) %	19/20 (95)		
Maltese nationality, n/N (%)	20/20 (100)		
Caucasian race, n/N (%)	20/20 (100)		
Age at SLE diagnosis, mean (S.D.) years	29.7 (9.4)	0.170	0.134
Disease duration, median (IQR) years	7 (9)	0.204	0.029
Secondary level of education, n/N (%)	8/20 (40)		
Tertiary level of education, n/N (%)	12/20 (60)		
VAS Fatigue , median (IQR)	6 (5)	0.198	0.039
FSS, mean (S.D.)	4.82 (0.99)	0.127	0.200
PSQI, mean (S.D.)	7.25 (4.63)	0.156	0.200
mHAQ, median (IQR)	0.125 (0.469)	0.275	<0.001

The mean is used to describe normally distributed variables (having a Kolmogorov-Smirnov p value >0.05) and the median is used for those variables that are not normally distributed (having a Kolmogorov-Smirnov p value <0.05).

FSS, Fatigue Severity Scale; IQR, interquartile range; mHAQ, modified Health Assessment Questionnaire; PSQI, Pittsburgh Sleep Quality Index; S.D., standard deviation; VAS, Visual Analogue Scale.

3.1.2 RELIABILITY

The Kendall's Tau test was used to check the reliability of the translated statements that had an ordinal scale. Tables 3.2, 3.3 and 3.4 show results obtained for FSS, PSQI and mHAQ respectively. The reliability of the translated statements in PSQI having a

metric scale, was tested using Pearson's correlation test. Table 3.5 shows these results. Table 3.6 shows Kendall's Tau values and *p* values for VAS fatigue and pain. Since all the *p* values obtained were below 0.05, the alternative hypothesis was accepted. The translated questionnaires into the Maltese language had a satisfactory reliability.

Table 3.2. Kendall's Tau values and *p* values obtained for the FSS statements.

Statement	**Kendall's Tau value**	***p* value**
1. My motivation is lower when I am fatigued.	0.507	0.006
2. Exercise brings on my fatigue.	0.697	<0.001
3. I am easily fatigued.	0.309	0.047
4. Fatigue interferes with my physical functioning.	0.391	0.024
5. Fatigue causes frequent problems for me.	0.630	<0.001
6. My fatigue prevents sustained physical functioning.	0.408	0.041
7. Fatigue interferes with carrying out certain duties and responsibilities.	0.502	<0.001
8. Fatigue is among my most disabling symptoms.	0.400	0.029
9. Fatigue interferes with my work, family, or social life.	0.669	<0.001

FSS, Fatigue Severity Scale.

Table 3.3. Kendall's Tau values and *p* values obtained for the questions having an ordinal scale in PSQI.

Statement	**Kendall's Tau value**	***p* value**
5. During the past month, how often have you had trouble sleeping because you		
A. Cannot get to sleep within 30 minutes	0.711	<0.001
B. Wake up in the middle of the night or early morning	0.886	<0.001
C. Have to get up to use the bathroom	0.855	<0.001
D. Cannot breathe comfortably	0.769	0.003
E. Cough or snore loudly	0.840	<0.001
F. Feel too cold	0.551	0.001
G. Feel too hot	0.408	0.026
H. Have bad dreams	0.827	<0.001
I. Have pain	0.570	0.001
J. Other reason (s), please describe, including how often you have had trouble sleeping because of this reason(s)	0.557	0.027
6. During the past month, how would you rate your sleep quality overall	0.905	<0.001
7. During the past month, how often have you taken medicine (prescribed or "over the counter") to help you sleep?	0.993	0.007
8. During the past month, how often have you had trouble staying awake while driving, eating meals, or engaging in social activity?	0.400	0.035
9. During the past month, how much of a problem has it been for you to keep up enthusiasm to get things done	0.412	0.036

PSQI, Pittsburgh Sleep Quality Index.

Table 3.4. Kendall's Tau values and *p* values obtained for mHAQ.

Statement	**Kendall's Tau value**	***p* value**
At this moment, are you able to:		
1. Dress yourself, including tying shoelaces and doing buttons?	0.993	0.007
2. Get in and out of bed?	0.899	<0.001
3. Lift a full cup or glass to your mouth?	1.000	<0.001
4. Walk outdoors on flat ground?	0.858	0.003
5. Wash and dry your entire body?	1.000	<0.001
6. Bend down to pick up clothing from the floor?	0.859	<0.001
7. Turn faucets/taps on and off?	0.750	0.008
8. Get in and out of a car?	0.822	<0.001

mHAQ, modified Health Assessment Questionnaire.

Table 3.5. Pearson's R values and *p* values for questions having a metric scale in PSQI.

Statement	**Pearson's R value**	***p* value**
1. When have you usually gone to bed?	0.972	<0.001
2. How long (in minutes) has it taken you to fall asleep each night?	0.953	<0.001
3. When have you usually gotten up in the morning?	0.977	<0.001
4. How many hours of actual sleep do you get at night? (This may be different than the number of hours you spend in bed.)	0.915	<0.001

PSQI, Pittsburgh Sleep Quality Index.

Table 3.6. Kendall's Tau values and *p* values obtained for VAS fatigue and pain.

Statement	Kendall's Tau value	*p* value
VAS Fatigue	0.709	<0.001
VAS Pain	0.807	<0.001

VAS, Visual Analogue Scale.

3.1.3 INTERNAL CONSISTENCY

The internal consistency for the Maltese translation of the questionnaires was analysed by calculating Cronbach's alpha. Table 3.7 shows the Cronbach's alpha values obtained. The results show good internal consistency since the values obtained are greater than 0.8.

Table 3.7. Cronbach's alpha values for the Maltese translations.

	Cronbach's Alpha	Number of Items
FSS	0.877	9
PSQI - individual items	0.859	14
PSQI - 7 components	0.827	7
mHAQ	0.897	8

FSS, Fatigue Severity Scale; mHAQ, modified Health Assessment Questionnaire; PSQI, Pittsburgh Sleep Quality Index.

3.1.4 VALIDITY

The validity of the Maltese translation of the FSS was checked by using Pearson's correlation test to analyse the correlation between the Maltese translation of FSS and VAS fatigue. Pearson's R value obtained was 0.809 (p<0.001), hence a significant positive correlation was present (figure 3.1). This is in agreement with the original publication on FSS by Krupp et al. (1989) (r=0.68, p<0.001).

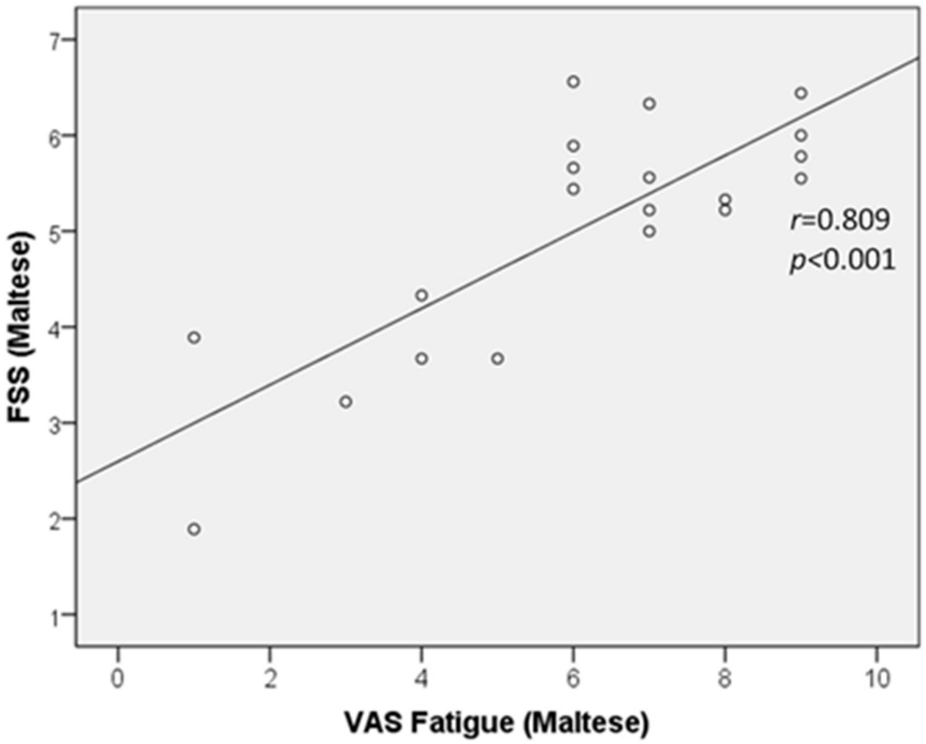

Figure 3.1. Correlation between the Maltese translation of FSS and VAS fatigue.
FSS, Fatigue Severity Scale; VAS, Visual Analogue Scale.

3.2 CROSS-SECTIONAL COHORT STUDY OF SLE PATIENTS IN MALTA

For this study, an attempt was made to identify all SLE patients in Malta who satisfy the SLICC classification criteria for SLE; hence the results reflect the characteristics of SLE patients in Malta. 107 such individuals were identified, of whom 92 consented to take part in the cross-sectional cohort study of SLE patients in Malta.

3.2.1 PATIENT DEMOGRAPHICS OF ENTIRE COHORT

93.5% of patients identified with SLE were female. Out of the entire cohort of 107 patients, 104 and 3 patients were of Caucasian and Asian ethnicity respectively. The mean age was 46.2 years (S.D. 13.9 years) and the median disease duration was 12 years (IQR 14 years). The mean age at disease diagnosis in this cohort was 33.1 years (S.D. 13.3 years) (figure 3.2). Co-morbidities were highly prevalent with 79.4% having at least one co-morbidity. 30.8% had osteopaenia/osteoporosis, 25.2% were known cases of hypertension, 11.2% were on lipid lowering drugs and 8.4% were diabetic. Rheumatologic co-morbidities were also highly prevalent with fibromyalgia occurring in 9.3% and antiphospholipid syndrome in 10.3%. Overlap syndrome with Sjogren's syndrome and rheumatoid arthritis was present in 3.7% and 2.8% respectively. 18.7% were current smokers and 22.4% were ex-smokers. Figures 3.3 and 3.4 show the frequency of organ manifestations in SLE and autoantibody profile respectively.

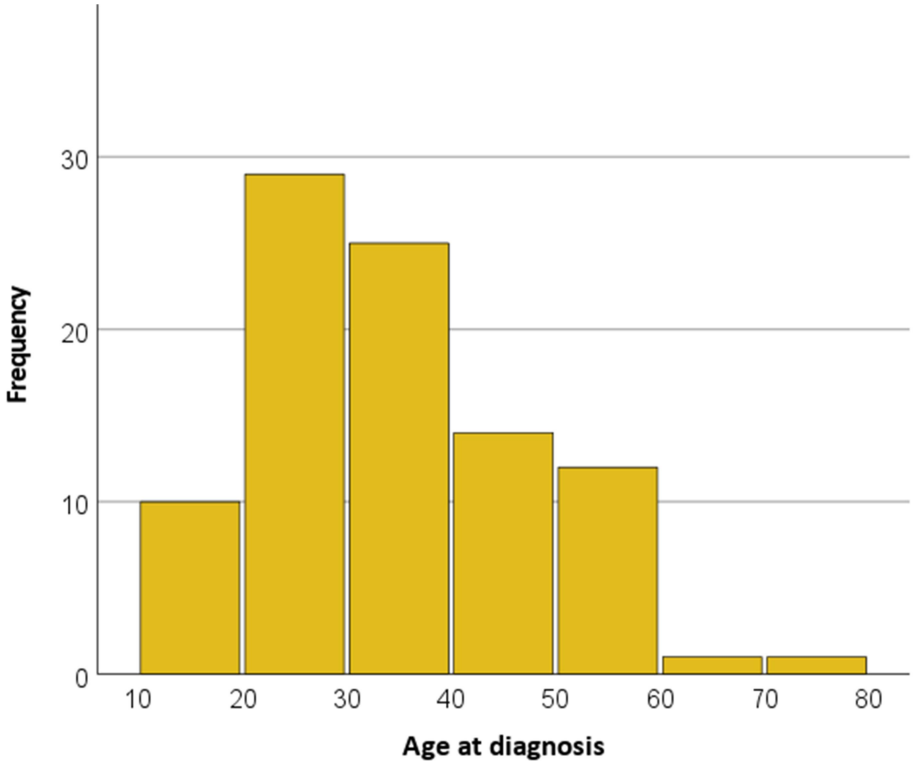

Figure 3.2. Histogram showing frequency of age at SLE diagnosis in the cohort studied.

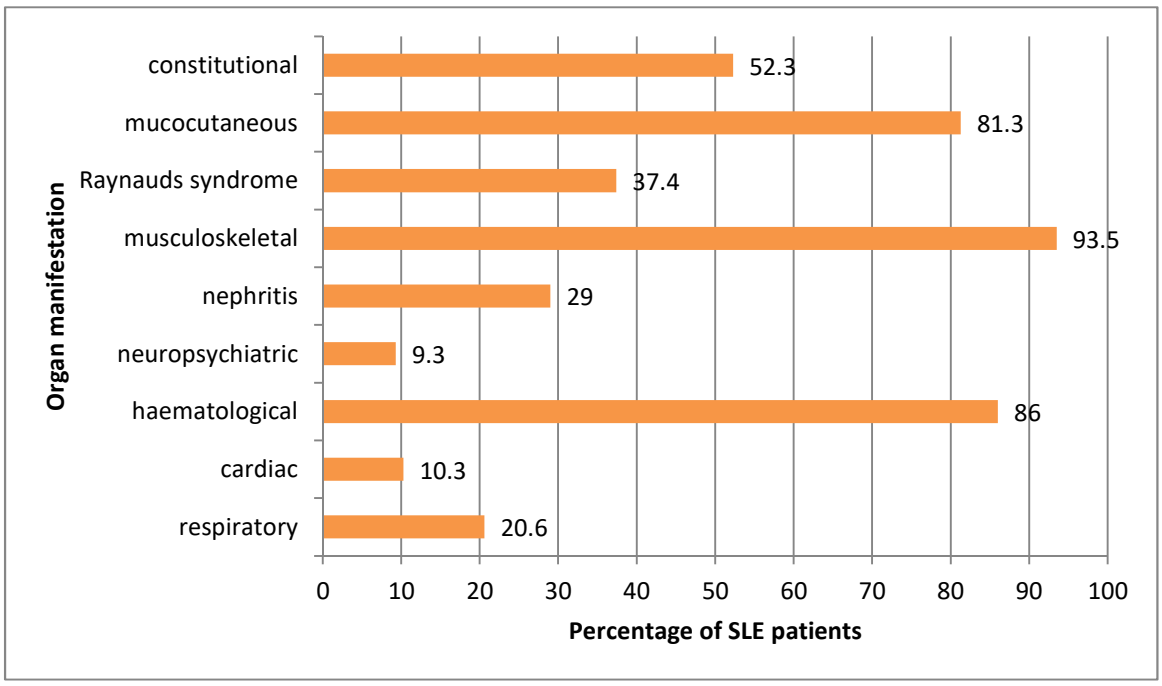

Figure 3.3. Frequency of organ manifestations in SLE at any time during the disease course.

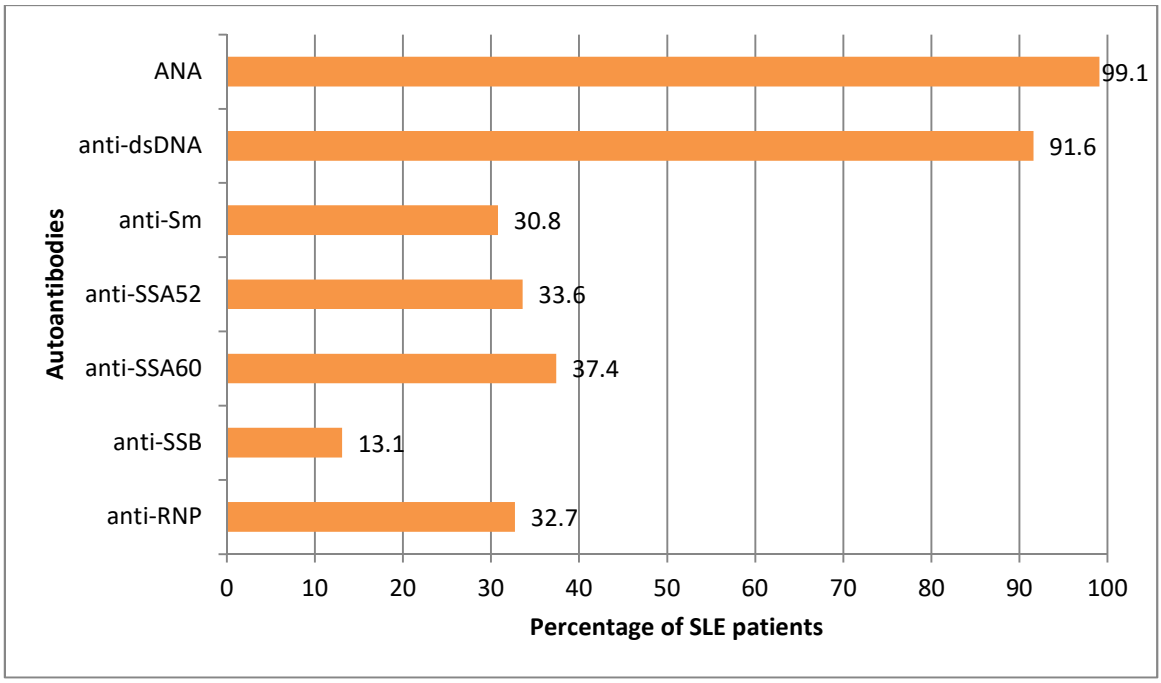

Figure 3.4. Frequency of positive autoantibodies in the entire SLE cohort of 107 patients.

ANA, anti-nuclear antibodies; anti-dsDNA, Anti-double-stranded deoxyribonucleic acid; anti-RNP, anti-ribonucleoprotein; anti-Sm, anti-Smith; anti-SSA, anti-Sjogren's syndrome related antigen A; anti-SSB, anti-Sjogren's syndrome related antigen B; SLE, Systemic Lupus Erythematosus.

Prednisolone use at a dose varying from 1.25mg to 15mg daily, was prevalent in 45.8% of SLE patients (figure 3.5). A high proportion of patients were on calcium and vitamin D supplementation (52.3% and 57.0% respectively). Other drugs included hydroxychloroquine in 60.7%, azathioprine in 20.6%, methotrexate in 9.3% and mycophenolate mofetil in 8.4%. Rituximab was being taken by one patient. 52.4% (22 patients) of the 42 patients not receiving hydroxychloroquine, were previously receiving the drug but had stopped it for a number of reasons, including side effects (table 3.8). These 22 patients had previously received hydroxychloroquine for a mean

of 1.6 years (range few days to 16 years). Documentation with regards to discussion on starting hydroxychloroquine, but being refused by the patients was found in 4 of the remaining 20 patients. No such documentation was found in the remaining 16 patients. Table 3.9 summarises the clinical characteristics of the cohort.

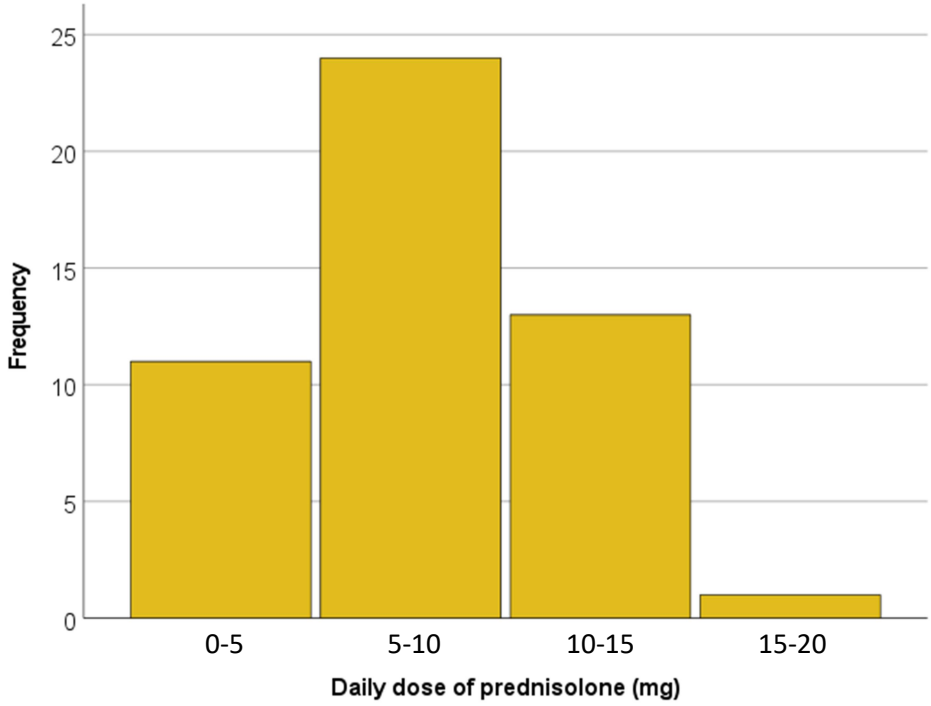

Figure 3.5. Histogram showing frequency of prednisolone daily dose taken by SLE patients on oral prednisolone.

Table 3.8. Table showing reasons hydroxychloroquine had been discontinued in 22 SLE patients.

Reasons for discontinuing hydroxychloroquine	Number of patients
Rash	6
Gastrointestinal side effects	1
Ophthalmic complications	6
Alopecia	1
Pregnancy, followed by disease remission	1
Disease remission	4
Persistent disease activity	1
Reason not documented	2

Table 3.9. Clinical characteristics of the entire cohort of 107 SLE patients who fulfilled the SLICC classification criteria.

Characteristics	**Values**
Age, mean (S.D.) years	46.2 (13.9)
Female sex , n/N (%)	100/107 (93.5)
Caucasian race, n/N (%)	104/107 (97.2)
Disease duration, median (IQR) years	12 (14)
Age at SLE diagnosis, mean (S.D.) years	33.1 (13.3)
Current smoker, n/N (%)	20/107 (18.7)
Ex-smoker, n/N (%)	24/107 (22.4)
Any co-morbidity, n/N (%)	85/107 (79.4)
Osteopaenia/osteoporosis, n/N (%)	33/107 (30.8)
Hypertension, n/N (%)	27/107 (25.2)
Hyperlipidaemia on treatment, n/N (%)	12/107 (11.2)
Diabetes mellitus, n/N (%)	9/107 (8.4)
Fibromyalgia, n/N (%)	10/107 (9.3)
Anti-phospholipid syndrome, n/N (%)	11/107 (10.3)
Sjogren's syndrome, n/N (%)	4/107 (3.7)
Rheumatoid arthritis, n/N (%)	3/107 (2.8)
Current prednisolone, n/N (%)	49/107 (45.8)
Prednisolone dose, median (range) mg/day	5.00 (1.25-15.00)
Current hydroxychloroquine, n/N (%)	65/107 (60.7)
Current azathioprine, n/N (%)	22/107 (20.6)
Current methotrexate, n/N (%)	10/107 (9.3)
Current mycophenolate, n/N (%)	9/107 (8.4)
Current rituximab, n/N (%)	1/107 (0.9)
Current calcium supplementation, n/N (%)	56/107 (52.3)
Current vitamin D supplementation, n/N (%)	61/107 (57.0)

Mean and standard deviation (S.D.) are given for normally distributed variables. Median and interquartile range (IQR) are given for variables that are not normally distributed.

3.2.2 PATIENT DEMOGRAPHICS OF PATIENTS STUDIED FURTHER

92 SLE patients consented to take part in the cross-sectional cohort study and were studied further. 92.4% of these patients were female and the mean age was 46.9 years (S.D. 13.9 years). Out of the 92 patients, 90 had a Caucasian ethnicity (of which 82 were Maltese) and 2 patients were of Asian origin. The median duration of SLE was 13 years (IQR 15 years) and the mean age at diagnosis of SLE in the cohort studied was 33.8 years (S.D. 12.8 years). Figures 3.6 and 3.7 show the frequency of organ manifestations in SLE and the autoantibody profile of the 92 patients studied further. The clinical characteristics of this cohort are summarised in table 3.10.

A family history of SLE in a first degree or second degree relative was present in 3.3% and 5.4% respectively. The median BMI was noted to be 26.5kg/m^2 (IQR 8.46kg/m^2); with 1.1%, 31.5% and 29.3% noted to be underweight (BMI < 18.5kg/m^2), overweight (BMI 25-30kg/m^2) and obese (BMI >30kg/m^2) respectively. There was a significant positive correlation between BMI and prednisolone daily dose (R=0.177, p=0.046). Sunblock was used regularly in 50% of SLE patients, with a frequency varying from once a week to more than once a day. Exercise was carried out regularly in 41.3%.

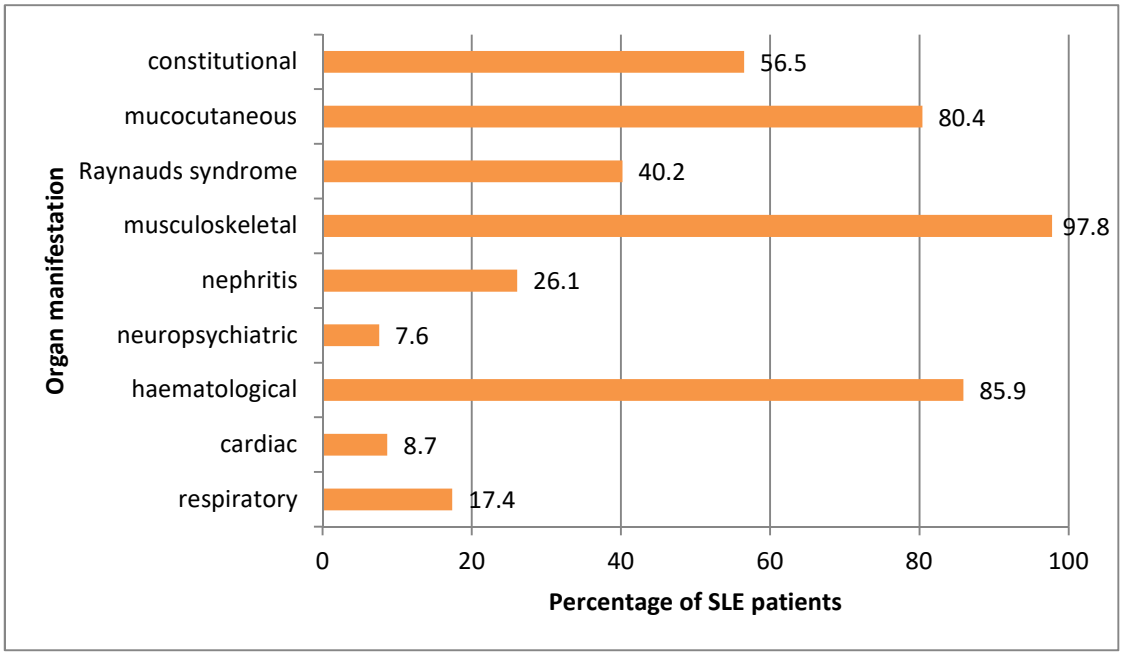

Figure 3.6. Frequency of organ manifestation in the 92 SLE patients that were studied further, at any time during the disease course.

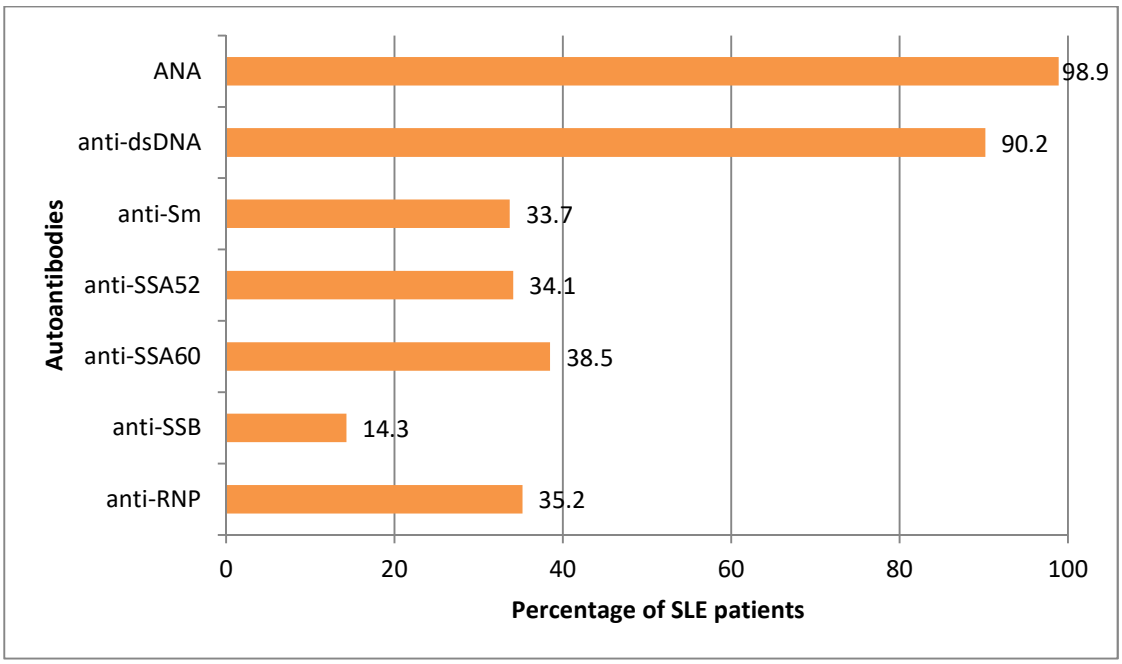

Figure 3.7. Frequency of positive autoantibodies in 92 SLE patients studied further. ANA, anti-nuclear antibodies; anti-dsDNA, Anti-double-stranded deoxyribonucleic acid; anti-RNP, anti-ribonucleoprotein; anti-Sm, anti-Smith; anti-SSA, anti-Sjogren's syndrome related antigen A; anti-SSB, anti-Sjogren's syndrome related antigen B; SLE, Systemic Lupus Erythematosus.

Table 3.10. Clinical characteristics of the cohort of 92 SLE patients studied further.

Characteristics	**Values**
Age, mean (S.D.) years	46.9 (13.9)
Female sex , n/N (%)	85/92 (92.4)
Caucasian race, n/N (%)	90/92 (97.8)
Disease duration, median (IQR) years	13 (15)
Age at SLE diagnosis, mean (S.D.) years	33.8 (12.8)
BMI, median (IQR) kg/ m^2	26.5 (8.46)
Current smoker, n/N (%)	14/92 (15.2)
Ex-smoker, n/N (%)	24/92 (26.1)
Current use of sunscreen, n/N (%)	46/92 (50)
Regular exercise, n/N (%)	38/92 (41.3)
Family history of SLE in first degree relative, n/N (%)	3/92 (3.3)
Any co-morbidity, n/N (%)	71/92 (77.2)
Osteopaenia/osteoporosis, n/N (%)	30/92 (32.6)
Hypertension, n/N (%)	22/92 (23.9)
Hyperlipidaemia on treatment, n/N (%)	11/92 (12)
Diabetes mellitus, n/N (%)	7/92 (7.6)
Fibromyalgia, n/N (%)	9/92 (9.8)
Anti-phospholipid syndrome, n/N (%)	7/92 (7.6)
Sjogren's syndrome, n/N (%)	4/92 (4.3)
Rheumatoid arthritis, n/N (%)	3/92 (3.3)
Current prednisolone, n/N (%)	41/92 (44.6)
Prednisolone dose, median (IQR) mg/day	5 (6.43)
Current hydroxychloroquine, n/N (%)	55/92 (59.8)
Current azathioprine, n/N (%)	20/92 (21.7)
Current methotrexate, n/N (%)	10/92 (10.9)
Current mycophenolate, n/N (%)	6/92 (6.5)
Current calcium supplementation, n/N (%)	48/92 (52.1)
Current vitamin D supplementation, n/N (%)	54/92 (58.7)
SLEDAI 2K, median (IQR)	4 (4)
SDI, median (IQR)	1 (1)

Mean and standard deviation (S.D.) are given for normally distributed variables. Median and interquartile range (IQR) are given for variables that are not normally distributed. SDI,

Systemic Lupus International Collaborating Clinics/American College of Rheumatology damage index; SLEDAI 2K, Systemic Lupus Erythematosus Disease Activity Index 2000.

Remission and low disease activity, with a SLEDAI-2K of 0 and 1-5 respectively, were noted in 23.9% and 52.2% of SLE patients. 20.7% had a moderate disease activity (SLEDAI-2K 6-10) and 3.3% had severely active disease (SLEDAI-2K 11-19). The median SDI was 1 (IQR 1) (figure 3.8).

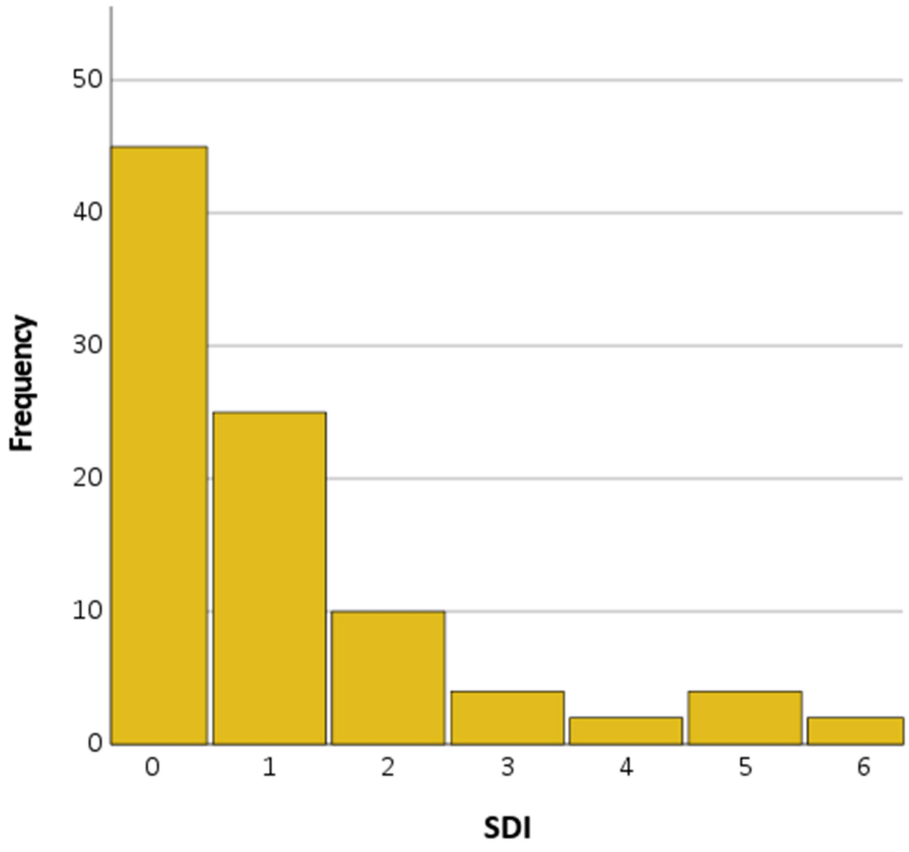

Figure 3.8. Bar chart showing frequency of SDI.

SDI, Systemic Lupus International Collaborating Clinics/American College of Rheumatology damage index.

Results obtained for the patient questionnaires are shown in table 3.11. A high level of fatigue (FSS >3.7) was present in 56.5% (Krupp et al., 1989). Depression was present in 6.5% (HADS D 11-21), with 18.5% noted to be borderline (HADS D 8-10). Anxiety was more common as it was present in 35.9% (HADS A 11-21), and 21.7% were considered to be borderline for anxiety (HADS A 8-10) (Smarr et al., 2011). 55.4% had poor sleep quality (PSQI >5) (Omachi et al., 2011), and the level of function was considered to be abnormally low (mHAQ >0.3) in 26.1% (Maska et al., 2011). The blood and urine test results are shown in table 3.12. Vitamin D deficiency (25-hydroxyvitamin D <20ng/ml) and insufficiency (25-hydroxyvitamin D 21-29ng/ml) were present in 15.2% and 27.2% respectively. 75.5% of the patients with a normal vitamin D level were already receiving vitamin D supplementation. Only 14.1% of the whole cohort of patients had a serum 25-hydroxyvitamin D level of $\geq$30ng/ml and were not being supplemented with vitamin D3. Figure 3.9 depicts the number of patients with normal vitamin D level, vitamin D insufficiency and deficiency. They have been subdivided according to whether they were receiving vitamin D supplementation or not. Chronic kidney disease (CKD) stage 4 (eGFR 15-29) was present in two patients, and a further two patients had CKD stage 5 (eGFR <15).

Table 3.11. Results obtained for the questionnaires filled in by SLE patients included in the study.

Questionnaires	**Values**
FSS, median (IQR)	4.17 (2.44)
VAS Fatigue, median (IQR)	5.00 (4)
VAS Pain, median (IQR)	4.00 (4)
HADS depression, median (IQR)	5.00 (6)
HADS anxiety, mean (S.D.)	8.48 (4.32)
PSQI, median (IQR)	6 (7)
mHAQ, median (IQR)	0.125 (0.375)

FSS, Fatigue Severity Scale; HADS, Hospital Anxiety and Depression Scale; IQR, interquartile range; mHAQ, modified Health Assessment Questionnaire; PSQI, Pittsburgh Sleep Quality Index; S.D.; standard deviation; VAS, Visual Analogue Scale.

Table 3.12. Table showing results for blood and urine investigations.

Investigations	**Values**
Haemoglobin, mean (S.D.) *[12.0-15.5 g/dL]*	13.07 (1.60)
Estimated glomerular filtration rate (eGFR), median (IQR) *[>60 mls/min/1.73m^2]*	97.0 (32)
Urine protein creatinine ratio, median (IQR) *[0-150 mg/g]*	96.9 (112.44)
25-hydroxyvitamin D, mean (S.D.) *[30-100 ng/mL]*	30.75 (9.53)
Corrected calcium, median (IQR) *[2.05-2.60 mmol/l]*	2.30 (0.11)
CRP, median (IQR) *[0-5 mg/L]*	1.65 (3.4)
ESR, median (IQR) *[18-22 mm 1st hr]*	21 (23)
C3, mean (S.D.) *[900-1800 mg/l]*	1010.5 (244.6)
C4, mean (S.D.) *[100-400 mg/l]*	232.07 (108.8)
Anti-dsDNA titre, median (IQR) *[0.0-100 IU/mL]*	92.9 (369.2)

Normal values are shown in square brackets.

Anti-dsDNA, anti-double-stranded deoxyribonucleic acid; C3, complement 3; C4, complement 4; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; IQR, interquartile range; S.D., standard deviation.

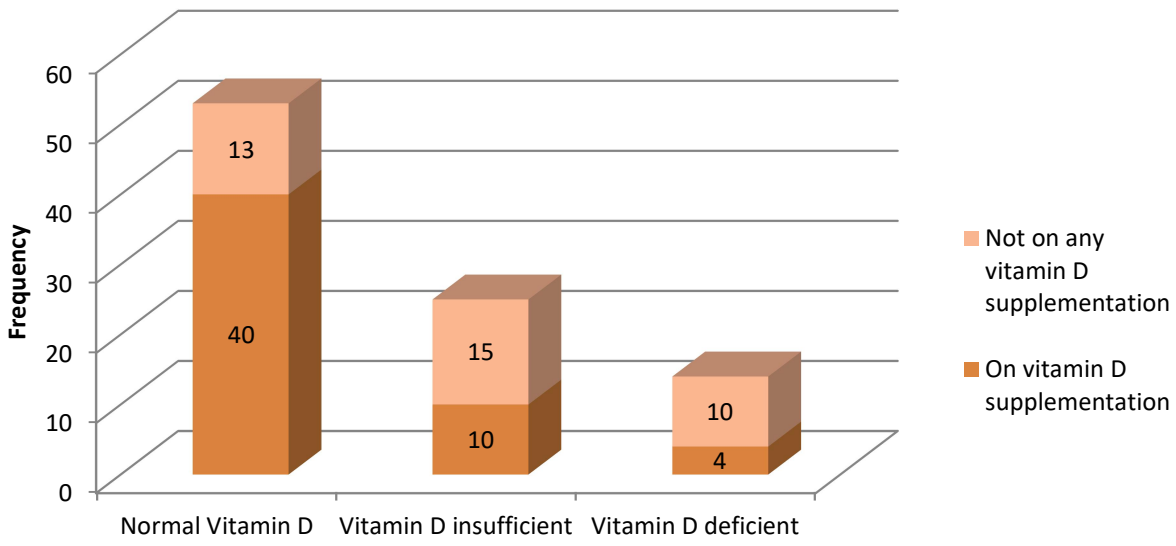

Figure 3.9. Bar chart showing number of patients with normal vitamin D level, vitamin D insufficiency and deficiency. The patients have been subdivided according to whether they were receiving vitamin D supplementation or not.

The demographic and clinical characteristics of the 92 patients who agreed to participate in the study, were compared with the clinical characteristics of the 15 patients who refused. The independent samples t-test and the Mann-Whitney U-test were used to compare continuous variables that were normally and non-normally distributed respectively. No significant difference was noted in age (p=0.736), age at SLE diagnosis (p=0.174), ethnicity (p=0.328), disease duration (p=0.628) and gender (p=0.269). This also applied to the autoantibody profile and co-morbidities documented, except in the case of anti-phospholipid syndrome (p=0.024). 7.6% of patients who consented had anti-phospholipid syndrome, whereas this was the case in 26.7% of those who were not willing to participate.

3.2.3 TESTING FOR NORMALITY OF DATA

In order to analyse the relationship between the variables noted for the 92 participants in the cross-sectional cohort study, statistical tests were carried out. The first step involved testing for normality of continuous variables since it indicated whether parametric or non-parametric tests were to be utilized during data analysis. The Kolmogorov-Smirnov test (K-S test) was carried out to test for normality of the distribution of continuous variables. Table 3.13 shows the K-S test results.

Table 3.13. Results for Kolmogorov-Smirnov test for the cross-sectional cohort study.

Variable	Test Statistic	p value
Age	0.066	0.200
BMI	0.118	0.003
SLE duration	0.147	<0.001
Age at disease diagnosis	0.088	0.073
Hydroxychloroquine daily dose	0.266	<0.001
Prednisolone daily dose	0.319	<0.001
Calcium daily dose	0.300	<0.001
Vitamin D daily dose	0.307	<0.001
SLEDAI-2K	0.195	<0.001
SDI	0.275	<0.001
FSS	0.097	0.032
VAS fatigue	0.133	<0.001
VAS pain	0.169	<0.001
HADS-D	0.108	0.010
HADS-A	0.083	0.145
PSQI	0.122	0.002
mHAQ	0.270	<0.001
Haemoglobin	0.078	0.200
eGFR	0.111	0.007
Urine PCR	0.290	<0.001
Serum calcium (corrected)	0.120	0.002
Serum 25-hydroxyvitamin D	0.081	0.180
CRP	0.308	<0.001
ESR	0.150	<0.001
C3	0.089	0.071
C4	0.066	0.200
Anti-dsDNA titre	0.229	<0.001

Anti-dsDNA, anti-double-stranded deoxyribonucleic acid; BMI, body mass index; C3, complement 3; C4, complement 4; CRP, C-reactive protein; eGFR, estimated

glomerular filtration rate; ESR, erythrocyte sedimentation rate; FSS, Fatigue Severity Scale; HADS-A, Hospital Anxiety and Depression Scale – anxiety subscale; HADS-D, Hospital Anxiety and Depression Scale – depression subscale; mHAQ, modified Health Assessment Questionnaire; PSQI, Pittsburgh Sleep Quality Index; SDI, Systemic Lupus International Collaborating Clinics/American College of Rheumatology damage index; SLEDAI, Systemic Lupus Erythematosus Disease Activity Index; VAS, Visual Analogue Scale.

The p value obtained by the K-S test for age, age at disease diagnosis, HADS-A, haemoglobin, 25-hydroxyvitamin D, C3 and C4 was higher than 0.05. The null hypothesis was accepted, meaning that they were normally distributed. The other continuous variables, whose p value obtained was less than 0.05, were not normally distributed.

3.2.4 ASSOCIATION BETWEEN SERUM 25-HYDROXYVITAMIN D LEVEL AND OTHER VARIABLES

The relationship between serum 25-hydroxyvitamin D and the other variables studied was assessed. With regards to continuous variables, correlation analysis was used to describe the relationship between two variables (Pallant, 2010). For those relationships in which both continuous variables were normally distributed, Pearson's correlation was used. For those relationships in which one of the variables was not normally distributed, Spearman's correlation test was used (table 3.14). In order to assess the relationship between 25-hydroxyvitamin D and categorical variables, the

independent samples t-test was used (table 3.15). This parametric test was used since 25-hydroxyvitamin D was found to be normally distributed.

Table 3.14. Correlation of continuous variables with serum 25-hydroxyvitamin D level.

Variable	R value	p value (2-tailed)
Age	-0.033	0.756
Disease duration	0.004*	0.968
Age at disease diagnosis	-0.001	0.991
BMI	-0.190*	0.070
Current prednisolone dose	0.058*	0.581
Current hydroxychloroquine dose	0.031*	0.766
Current vitamin D dose	0.471*	**<0.001**
Current calcium dose	0.285*	**0.006**
eGFR	-0.152*	0.148
Urine PCR	0.048*	0.652
SLEDAI-2K	-0.085*	0.423
SDI	0.096*	0.360
FSS	-0.014*	0.898
VAS Fatigue	0.055*	0.601
VAS Pain	0.108*	0.306
HADS-D	0.038*	0.719
HADS-A	0.054	0.610
PSQI	-0.064*	0.544
mHAQ	-0.039*	0.710
Haemoglobin	-0.002	0.983
Calcium (corrected)	0.111*	0.292
CRP	-0.071*	0.499
ESR	0.066*	0.532
Anti-dsDNA titre	0.065*	0.535

Pearson's correlation test was used for normally distributed variables. Spearman's correlation test was used when at least one of the variables was not normally distributed (R value marked with *). Anti-dsDNA, anti-double-stranded deoxyribonucleic acid; BMI, body mass index; CRP, C-reactive protein; eGFR, estimated

glomerular filtration rate; ESR, erythrocyte sedimentation rate; FSS, Fatigue Severity Scale; HADS-A, Hospital Anxiety and Depression Scale – anxiety subscale; HADS-D, Hospital Anxiety and Depression Scale – depression subscale; mHAQ, modified Health Assessment Questionnaire; PSQI, Pittsburgh Sleep Quality Index; SDI, Systemic Lupus International Collaborating Clinics/American College of Rheumatology damage index; SLEDAI-2K, Systemic Lupus Erythematosus Disease Activity Index 2000; VAS, Visual Analogue Scale.

Table 3.15. Table showing categorical variables, mean serum 25-hydroxyvitamin D level and standard deviation (S.D.) when the variable was present and when absent.

Variable		Mean 25-hydroxyvitamin D level (ng/mL)	S.D.	p value (2-tailed)
Gender	Female	30.84	9.666	0.767
	Male	29.71	8.180	
Current sunscreen use	Present	30.07	9.169	0.247
	Absent	31.43	9.923	
Smoking	Smoker	32.14	12.727	0.555
	Non-smoker	30.50	8.915	
Regular exercise	Present	30.71	9.174	0.974
	Absent	30.78	9.851	
Current prednisolone	Present	32.15	9.512	0.209
	Absent	29.63	9.480	
Current hydroxychloroquine	Present	31.53	9.483	0.343
	Absent	29.59	9.602	
Current azathioprine	Present	32.95	8.852	0.245
	Absent	30.14	9.674	
Current methotrexate	Present	27.70	9.878	0.286
	Absent	31.12	9.477	
Current mycophenolate	Present	33.50	7.259	0.468
	Absent	30.56	9.668	
Current calcium supplementation	Present	33.10	8.652	**0.012**
	Absent	28.18	9.862	
Current vitamin D suppplementation	Present	33.98	8.831	**<0.001**
	Absent	26.16	8.635	
Raynauds syndrome	Present	31.57	8.965	0.503
	Absent	30.20	9.928	
Osteopaenia/ Osteoporosis	Present	31.03	8.896	0.844
	Absent	30.61	9.884	
Hypertension	Present	30.14	10.375	0.731
	Absent	30.94	9.314	
Hyperlipidaemia	Present	35.09	12.045	0.108
	Absent	30.16	9.063	
Diabetes Mellitus	Present	25.86	11.202	0.159
	Absent	31.15	9.337	
Fibromyalgia	Present	34.44	14.143	0.418
	Absent	30.35	8.918	
Anti-phospholipid syndrome	Present	28.14	12.642	0.454
	Absent	30.96	9.288	
Sjogren's syndrome	Present	27.00	12.111	0.424
	Absent	30.92	9.443	
Rheumatoid Arthritis	Present	24.67	10.214	0.263
	Absent	30.96	9.495	

The p values shown were obtained by using the independent samples t-test.

Serum 25-hydroxyvitamin D level had a significant positive correlation with vitamin D supplementation (R=0.356, p<0.001) and calcium supplementation (R=0.226, p=0.030).

3.2.5 ASSOCIATION BETWEEN FATIGUE AND OTHER VARIABLES

Correlation analysis was used to describe the relationship between FSS and other continuous variables using Spearman's correlation test as FSS was not normally distributed (supplementary table S1). The above was repeated by using VAS fatigue as a measure for fatigue (supplementary table S2). The results show that fatigue measured by FSS was significantly positively correlated with VAS pain (p<0.001), HADS-D (p<0.001), HADS-A (p<0.001), PSQI (p<0.001), mHAQ (p<0.001) and current hydroxychloroquine dose (p=0.040) (figures 3.10-3.14). These relationships were confirmed when using VAS fatigue as the measure for fatigue. In addition to the above, a positive significant relationship was noted between SLEDAI-2K and VAS fatigue (p=0.018) (figure 3.15).

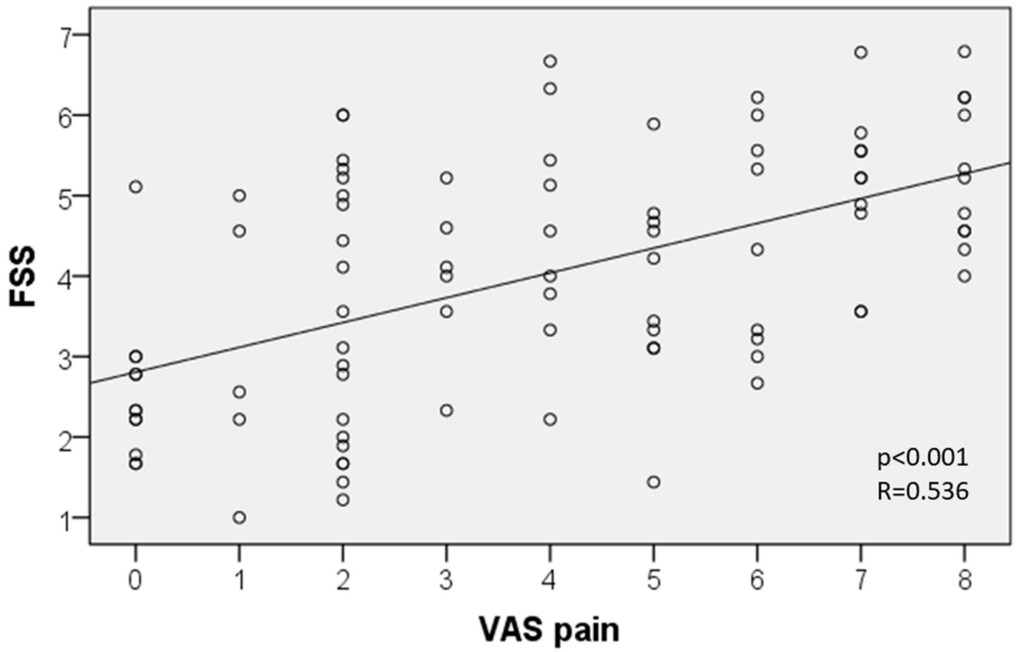

Figure 3.10. Scatter plot showing correlation between FSS and VAS pain. FSS, Fatigue Severity Scale; VAS, Visual Analogue Scale.

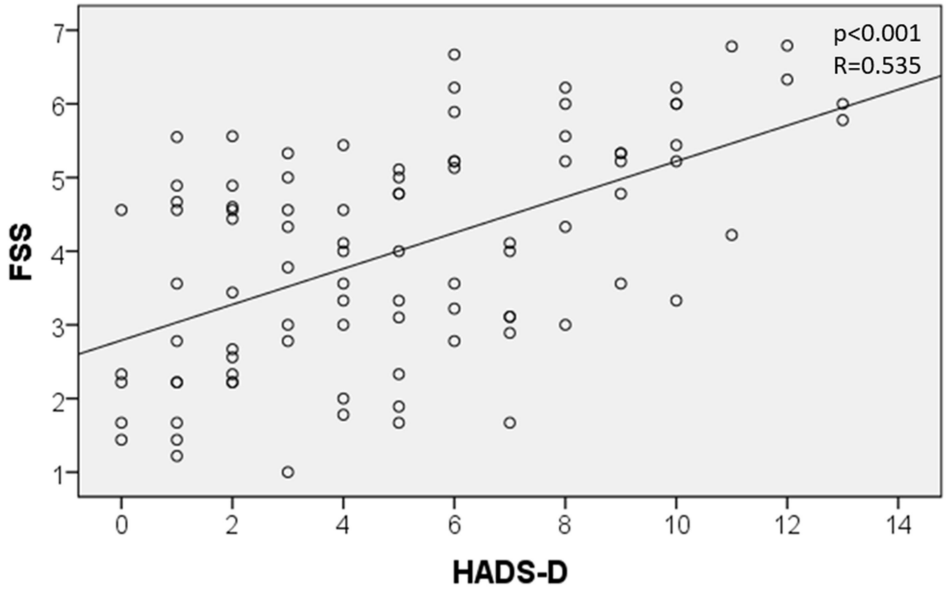

Figure 3.11. Scatter plot showing correlation between FSS and HADS-D. HADS-D, Hospital Anxiety and Depression Scale – depression subscale; FSS, Fatigue Severity Scale.

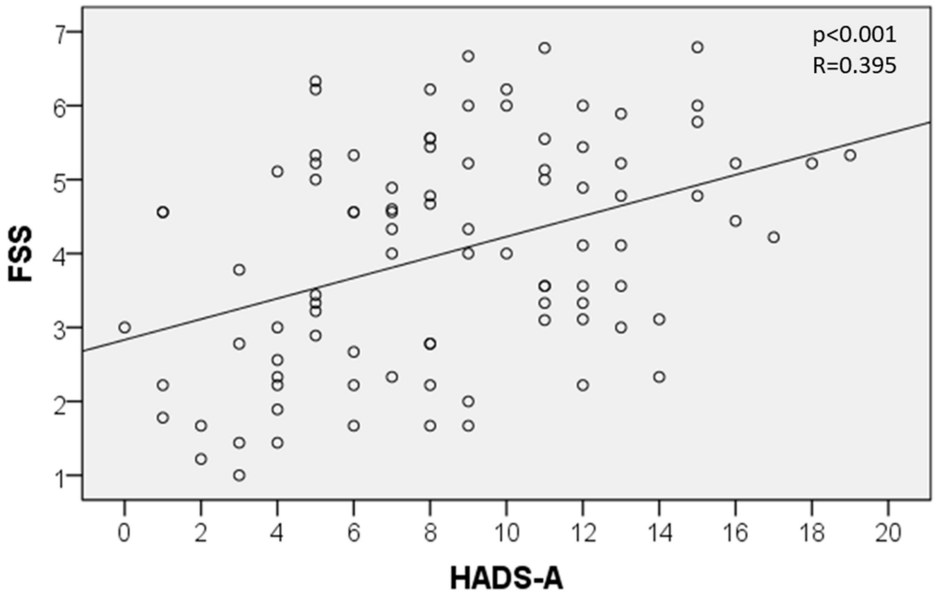

Figure 3.12. Scatter plot showing correlation between FSS and HADS-A. HADS-A, Hospital Anxiety and Depression Scale – anxiety subscale; FSS, Fatigue Severity Scale.

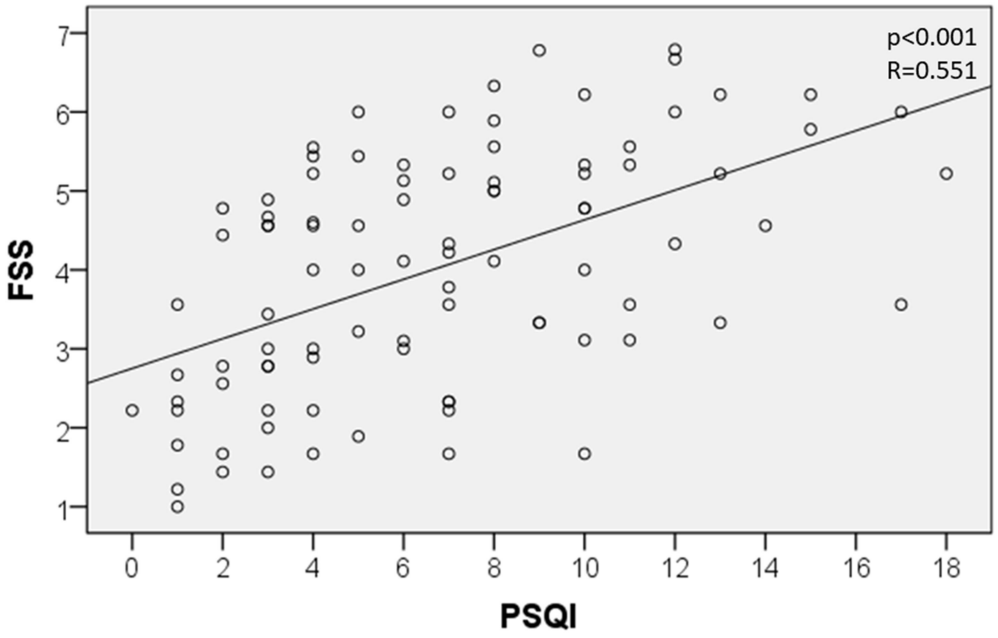

Figure 3.13. Scatter plot showing correlation between FSS and PSQI. FSS, Fatigue Severity Scale; PSQI, Pittsburgh Sleep Quality Index.

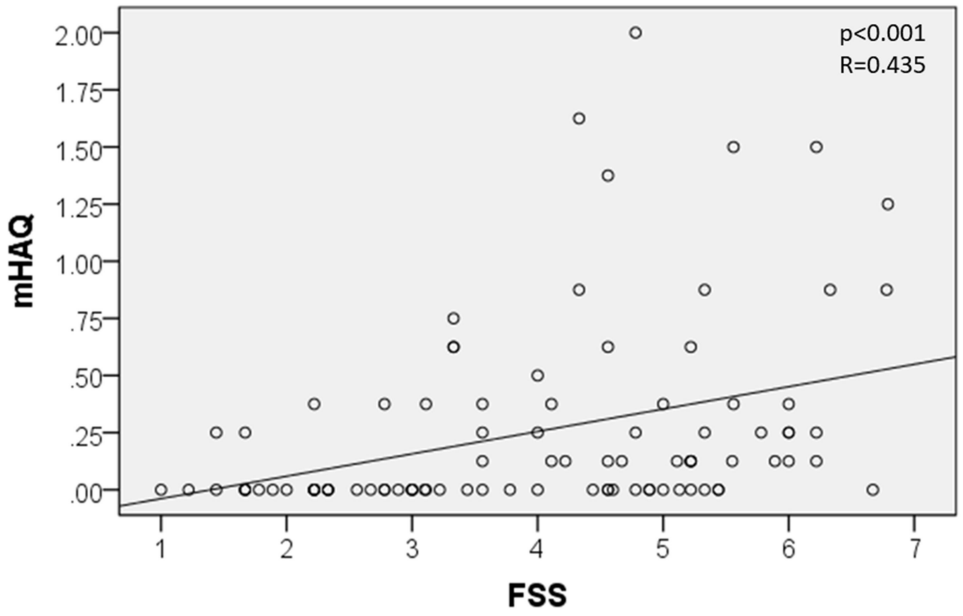

Figure 3.14. Scatter plot showing correlation between mHAQ and FSS. FSS, Fatigue Severity Scale; mHAQ, modified Health Assessment Questionnaire.

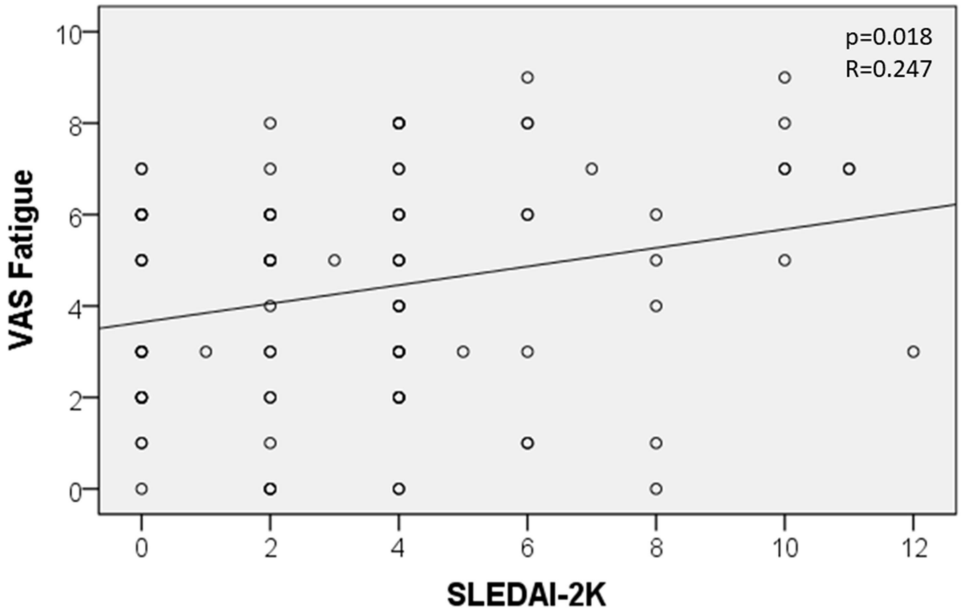

Figure 3.15. Scatter plot showing correlation between VAS Fatigue and SLEDAI-2K. SLEDAI-2K, Systemic Lupus Erythematosus Disease Activity Index 2000; VAS, Visual Analogue Scale.

In order to analyse the relationship between fatigue and categorical variables, the Mann-Whitney U-test was used as the non-parametric test to compare the FSS and VAS fatigue level between the two groups (tables 3.16-3.17). The FSS score was significantly higher in patients who were receiving hydroxychloroquine (p=0.009) and were diagnosed with fibromyalgia (p=0.047). These relationships were confirmed when using VAS fatigue as the measure for fatigue.

Table 3.16. Table showing categorical variables, the median FSS and its interquartile range when the variable was present and when absent.

Variable		Median FSS	Interquartile range	p value (2-tailed)
Gender	Female	4.11	2.44	0.188
	Male	5.22	2.23	
Current sunscreen use	Present	4.45	2.58	0.878
	Absent	4.00	2.30	
Smoking	Smoker	4.47	2.52	0.844
	Non-smoker	4.11	2.44	
Regular exercise	Present	4.28	4.11	0.857
	Absent	3.08	2.22	
Current prednisolone	Present	4.56	2.61	0.172
	Absent	3.78	2.11	
Current hydroxychloroquine	Present	4.67	2.33	**0.009**
	Absent	3.33	2.01	
Current azathioprine	Present	3.89	2.11	0.957
	Absent	4.33	2.52	
Current methotrexate	Present	4.67	2.44	0.526
	Absent	4.11	2.44	
Current mycophenolate	Present	4.06	3.24	0.978
	Absent	4.17	2.44	
Current calcium supplementation	Present	4.17	2.72	0.617
	Absent	4.22	2.11	
Current vitamin D suppplementation	Present	3.95	2.58	0.814
	Absent	4.33	2.17	
Anti-Sm	Positive	3.61	2.74	0.195
	Negative	4.33	2.22	
Anti-SSA52	Positive	3.44	2.56	0.092
	Negative	4.56	2.50	
Anti-SSA60	Positive	3.56	2.22	0.173
	Negative	4.56	2.74	
Anti-SSB	Positive	4.56	1.78	0.713
	Negative	4.17	2.69	
Anti-RNP	Positive	3.22	3.11	0.162
	Negative	4.56	2.17	
Raynauds syndrome	Present	4.33	2.28	0.387
	Absent	4.00	2.89	
Fibromyalgia	Present	5.33	3.72	**0.047***
	Absent	4.00	2.35	
Anti-phospholipid syndrome	Present	3.00	3.11	0.721
	Absent	4.22	2.38	
Sjogren's syndrome	Present	4.08	1.43	0.945
	Absent	4.17	2.44	
Rheumatoid Arthritis	Present	2.33	2.33	0.420
	Absent	4.22	2.38	

*one-tailed test. The p values were obtained by using the Mann-Whitney U-test.

anti-RNP, anti-ribonucleoprotein; anti-Sm, anti-Smith; anti-SSA, anti-Sjogren's syndrome related antigen A; anti-SSB, anti-Sjogren's syndrome related antigen B.

Table 3.17. Table showing categorical variables, the median VAS fatigue and its interquartile range when the variable was present and when absent.

Variable		Median VAS Fatigue	Interquartile range	p value (2-tailed)
Gender	Female	5.0	4	0.651
	Male	5.0	2	
Current sunscreen use	Present	5.0	4	0.416
	Absent	4.0	4	
Smoking	Smoker	5.5	3	0.369
	Non-smoker	5.0	4	
Regular exercise	Present	5.0	4	0.380
	Absent	5.0	4	
Current prednisolone	Present	6.0	5	0.120
	Absent	4.0	4	
Current hydroxychloroquine	Present	6.0	4	**0.003**
	Absent	3.0	3	
Current azathioprine	Present	4.5	4	0.634
	Absent	5.0	4	
Current methotrexate	Present	4.5	5	0.720
	Absent	5.0	4	
Current mycophenolate	Present	5.0	6	0.927
	Absent	5.0	4	
Current calcium supplementation	Present	4.5	5	0.927
	Absent	5.0	3	
Current vitamin D suppplementation	Present	5.0	4	0.518
	Absent	5.0	4	
Anti-Sm	Positive	4.0	4	0.337
	Negative	5.0	5	
Anti-SSA52	Positive	5.0	4	0.726
	Negative	5.0	3	
Anti-SSA60	Positive	5.0	4	0.645
	Negative	5.0	3	
Anti-SSB	Positive	5.0	3	0.290
	Negative	4.5	4	
Anti-RNP	Positive	3.0	4	0.205
	Negative	5.0	3	
Raynauds syndrome	Present	5.0	4	0.113
	Absent	4.0	4	
Fibromyalgia	Present	8.0	7	**0.042***
	Absent	5.0	4	
Anti-phospholipid syndrome	Present	5.0	6	0.785
	Absent	5.0	4	
Sjogren's syndrome	Present	5.5	2	0.281
	Absent	5.0	4	
Rheumatoid Arthritis	Present	2.0	2	0.281
	Absent	5.0	4	

*one-tailed test. The p values were obtained by using the Mann-Whitney U-test.

anti-RNP, anti-ribonucleoprotein; anti-Sm, anti-Smith; anti-SSA, anti-Sjogren's syndrome related antigen A; anti-SSB, anti-Sjogren's syndrome related antigen B.

3.2.6 ASSOCIATION BETWEEN DISEASE ACTIVITY AND OTHER VARIABLES

Spearman's correlation test was used to analyse the relationship between SLEDAI-2K and other continuous variables since SLEDAI-2K was not normally distributed (supplementary table S3). SLEDAI-2K was significantly positively correlated with mHAQ ($p<0.001$), VAS pain ($p=0.002$), HADS-D ($p=0.028$), PSQI ($p=0.014$) and anti-dsDNA titre ($p<0.001$) (figures 3.16-3.19). It was negatively correlated with disease duration ($p=0.028$) (figure 3.20), haemoglobin ($p=0.016$), C3 ($p<0.001$) and C4 ($p=0.001$).

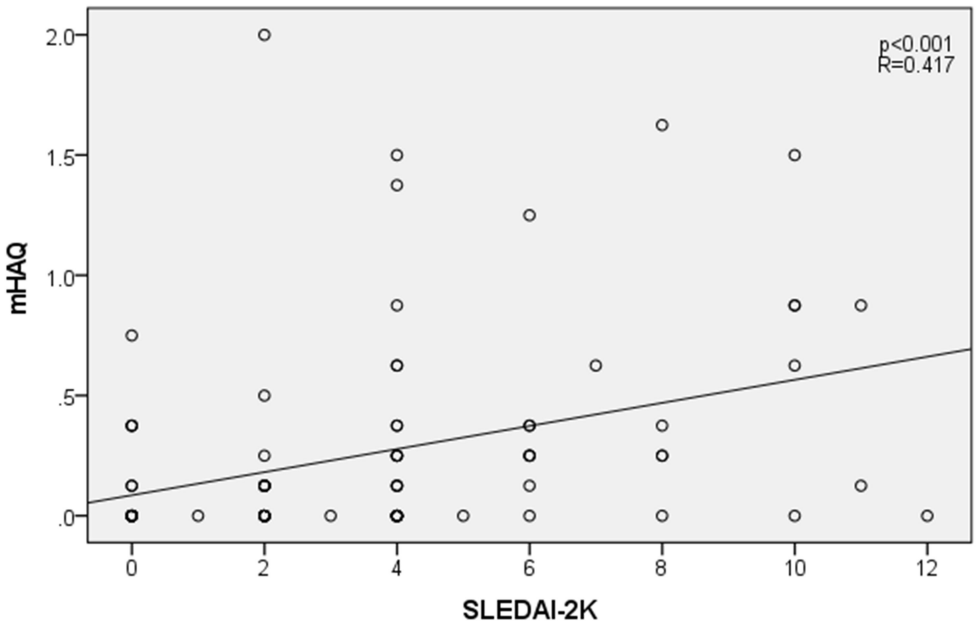

Figure 3.16. Scatter plot showing the correlation between mHAQ and SLEDAI-2K.
mHAQ, modified Health Assessment Questionnaire; SLEDAI-2K, Systemic Lupus Erythematosus Disease Activity Index 2000.

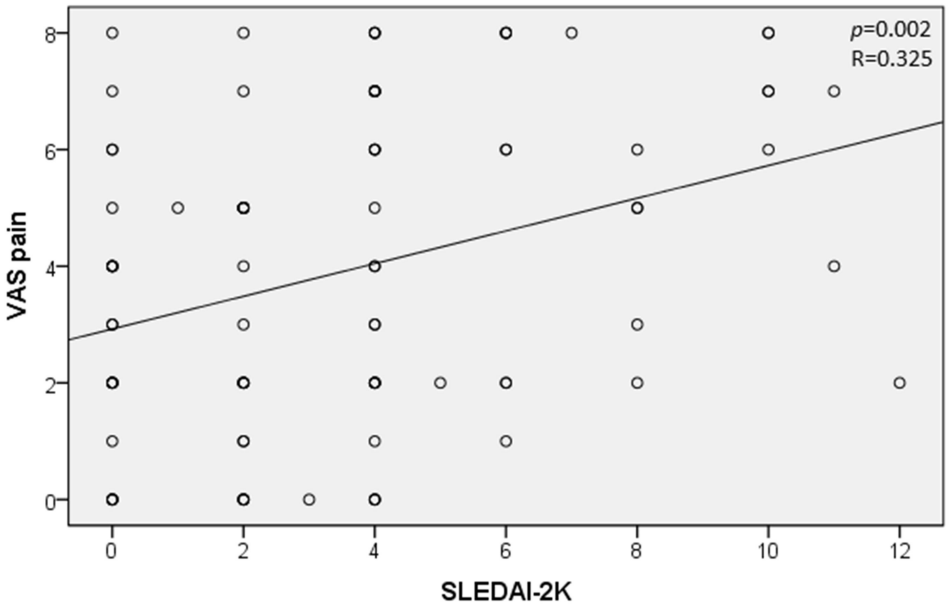

Figure 3.17. Scatter plot showing the correlation between VAS pain and SLEDAI-2K. SLEDAI-2K, Systemic Lupus Erythematosus Disease Activity Index 2000; VAS, Visual analogue scale.

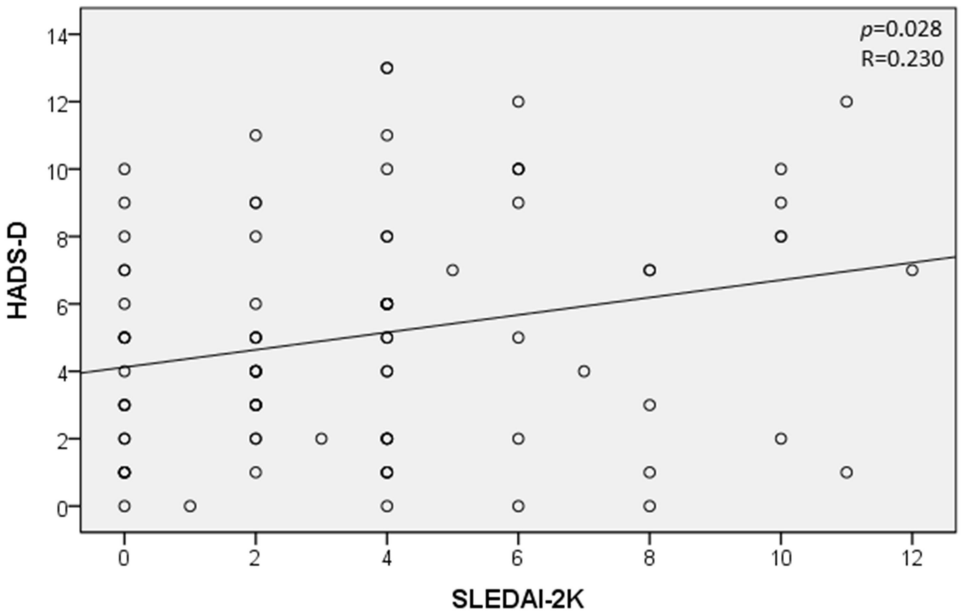

Figure 3.18. Scatter plot showing the correlation between HADS-D and SLEDAI-2K. HADS-D, Hospital Anxiety and Depression Scale – depression subscale; SLEDAI-2K, Systemic Lupus Erythematosus Disease Activity Index 2000.

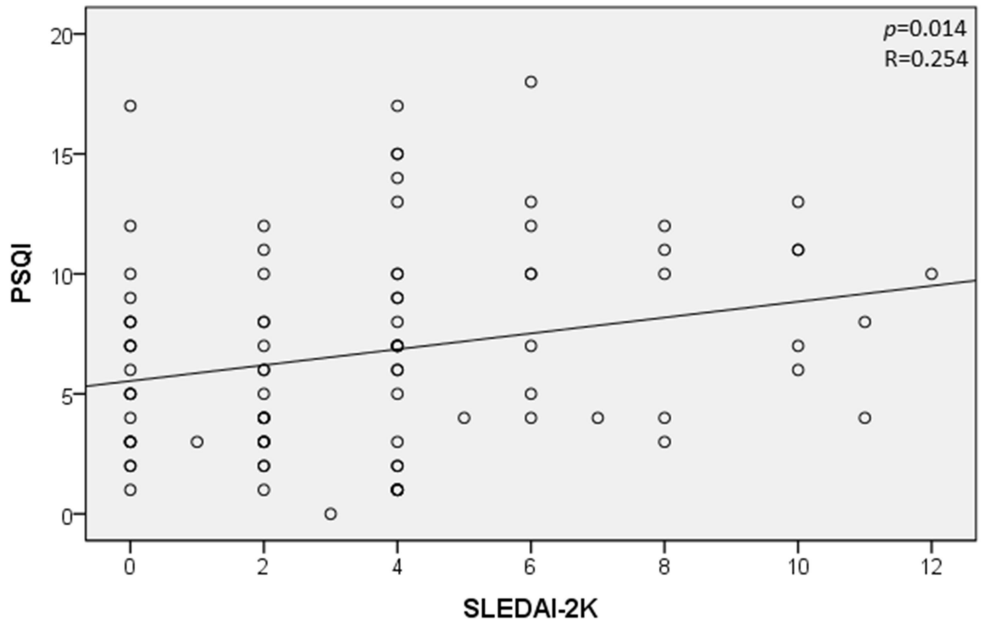

Figure 3.19. Scatter plot showing the correlation between PSQI and SLEDAI-2K.
PSQI, Pittsburgh Sleep Quality Index; SLEDAI-2K, Systemic Lupus Erythematosus Disease Activity Index 2000.

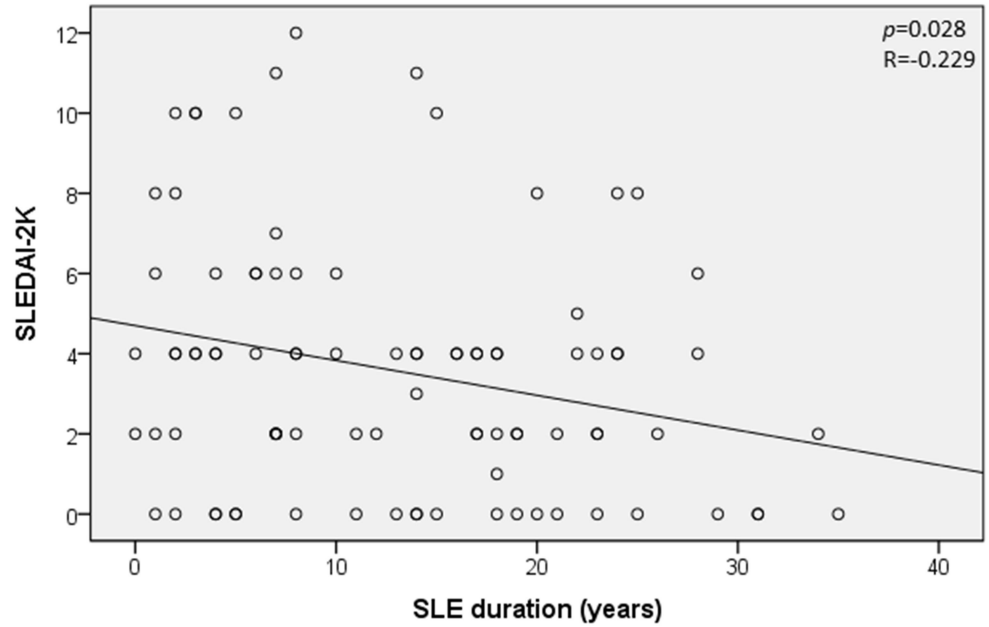

Figure 3.20. Scatter plot showing the correlation between SLEDAI-2K and SLE duration in years. SLEDAI-2K, Systemic Lupus Erythematosus Disease Activity Index 2000.

In order to analyse the relationship between SLEDAI-2K and categorical variables, the Mann-Whitney U-test was used to compare SLEDAI-2K between the two groups (table 3.18). SLEDAI-2K was significantly lower in patients who were receiving mycophenolate mofetil ($p=0.022$).

Table 3.18. Table showing categorical variables, the median SLEDAI-2K and its interquartile range when the variable was present and when absent.

Variable		Median SLEDAI-2K	Interquartile range	p value (2-tailed)
Gender	Female	4.0	3	0.626
	Male	4.0	6	
Current sunscreen use	Present	4.0	3	0.465
	Absent	4.0	5	
Smoking	Smoker	4.0	3	0.490
	Non-smoker	4.0	5	
Regular exercise	Present	4.0	4	0.219
	Absent	2.0	4	
Current prednisolone	Present	4.0	5	0.162
	Absent	4.0	4	
Current hydroxychloroquine	Present	3.0	4	0.809
	Absent	4.0	5	
Current azathioprine	Present	4.0	5	0.937
	Absent	4.0	4	
Current methotrexate	Present	4.0	6	0.359
	Absent	4.0	4	
Current mycophenolate	Present	0.0	3	**0.022**
	Absent	4.0	4	
Current calcium supplementation	Present	4.0	4	0.201
	Absent	2.5	5	
Current vitamin D suppplementation	Present	3.0	2	0.807
	Absent	4.0	6	
Anti-Sm	Positive	4.0	4	0.535
	Negative	4.0	4	
Anti-SSA52	Positive	3.0	4	0.513

	Negative	4.0	4	
Anti-SSA60	Positive	2.0	4	0.548
	Negative	4.0	4	
Anti-SSB	Positive	4.0	6	0.947
	Negative	4.0	3	
Anti-RNP	Positive	4.0	11	0.574
	Negative	4.0	12	
Raynauds syndrome	Present	4.0	5	0.086
	Absent	2.0	4	
Osteoporosis/ Osteopaenia	Present	2.0	4	0.129
	Absent	4.0	4	
Hypertension	Present	2.0	4	0.244
	Absent	4.0	4	
Hyperlipidaemia	Present	4.0	4	0.518
	Absent	4.0	4	
Diabetes Mellitus	Present	4.0	4	0.551
	Absent	4.0	5	
Fibromyalgia	Present	4.0	3	0.500
	Absent	3.0	4	
Anti-phospholipid syndrome	Present	4.0	2	0.988
	Absent	4.0	5	
Sjogren's syndrome	Present	2.0	9	0.730
	Absent	4.0	3	
Rheumatoid Arthritis	Present	2.0	2	0.473
	Absent	4.0	4	

The p values were obtained by using the Mann-Whitney U-test.

anti-RNP, anti-ribonucleoprotein; anti-Sm, anti-Smith; anti-SSA, anti-Sjogren's syndrome related antigen A; anti-SSB, anti-Sjogren's syndrome related antigen B.

3.2.7 ASSOCIATION BETWEEN SLEEP QUALITY AND OTHER VARIABLES

Spearman's correlation test was used to analyse the relationship between PSQI and other continuous variables since PSQI was not normally distributed (supplementary table S4). Sleep quality measured by PSQI was significantly positively correlated with VAS pain (p<0.001), HADS-D (p<0.001), HADS-A (p<0.001), mHAQ (p<0.001) and age at disease diagnosis (p=0.035) (figures 3.21-3.24). It was negatively correlated with eGFR (p=0.044) (figure 3.25). In order to analyse the relationship between PSQI and categorical variables, the Mann-Whitney U-test was used (table 3.19).

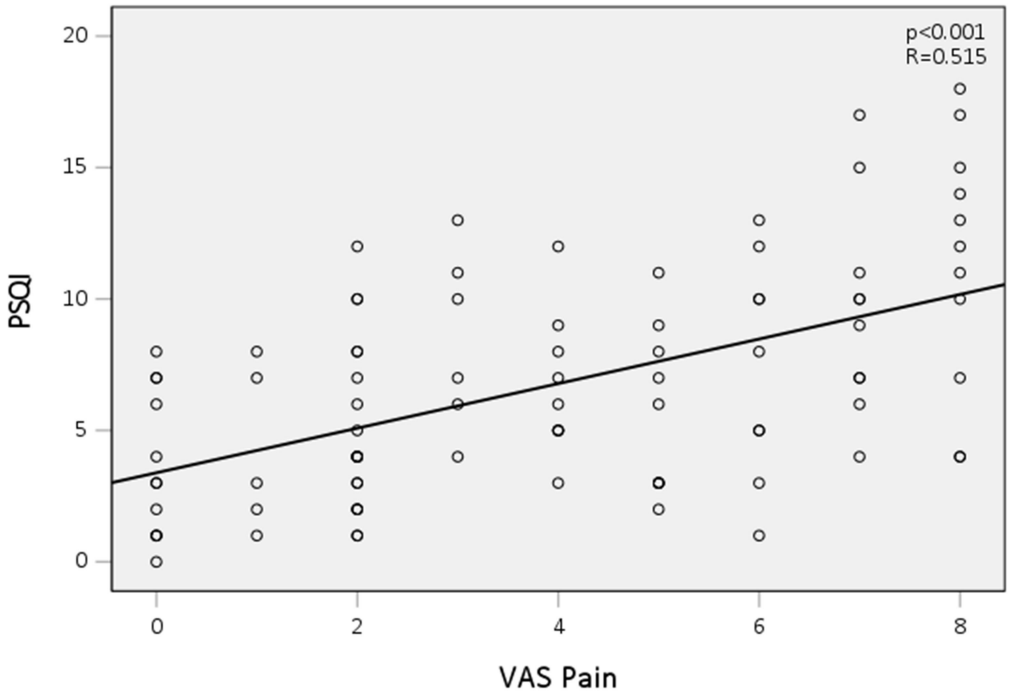

Figure 3.21. Scatter plot showing the correlation between PSQI and VAS pain. PSQI, Pittsburgh Sleep Quality Index; VAS, Visual analogue scale.

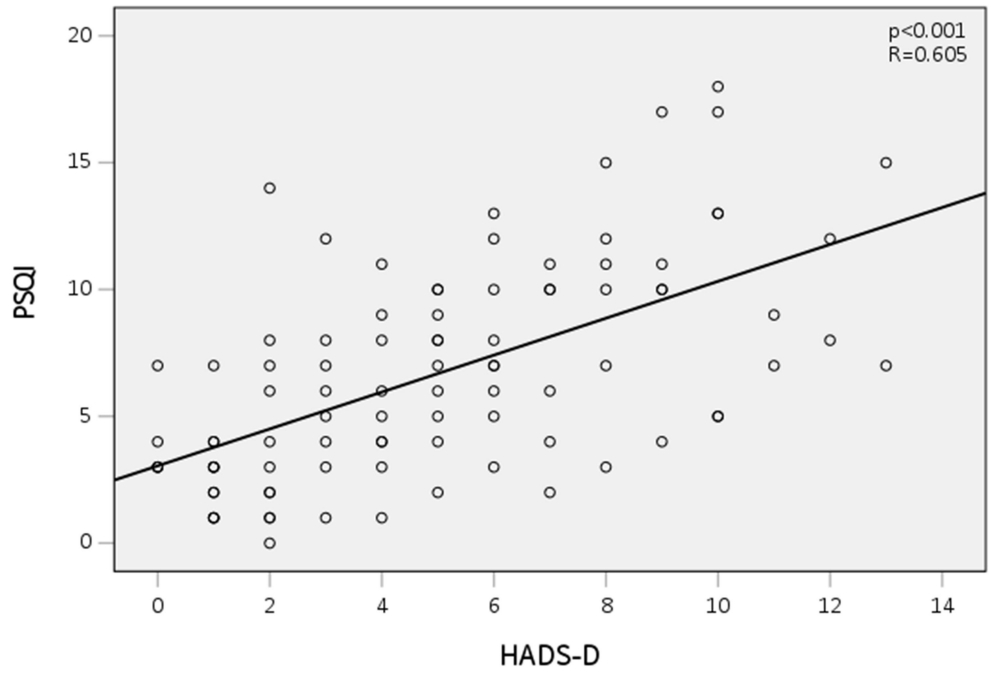

Figure 3.22. Scatter plot showing the correlation between PSQI and HADS-D. HADS-D, Hospital Anxiety and Depression Scale – depression subscale; PSQI, Pittsburgh Sleep Quality Index.

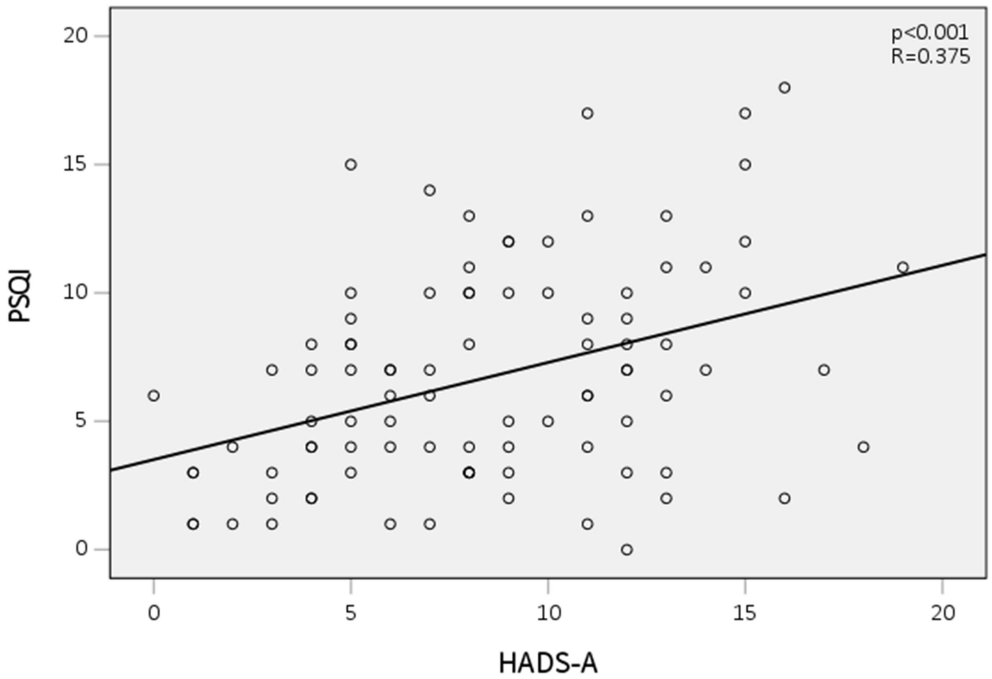

Figure 3.23. Scatter plot showing the correlation between PSQI and HADS-A. HADS-A, Hospital Anxiety and Depression Scale – anxiety subscale; PSQI, Pittsburgh Sleep Quality Index.

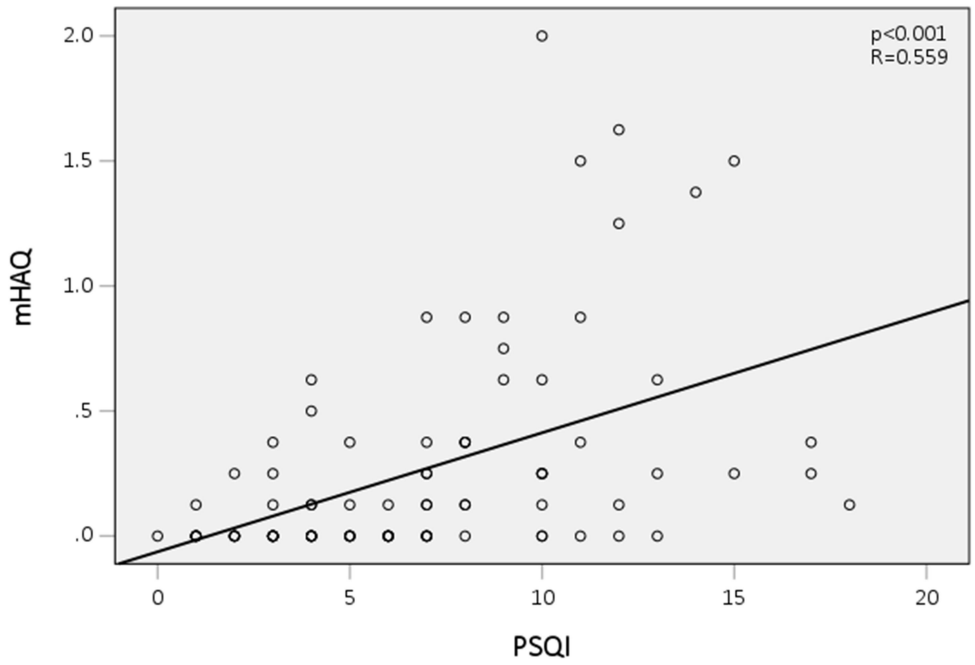

Figure 3.24. Scatter plot showing the correlation between mHAQ and PSQI. mHAQ, modified Health Assessment Questionnaire; PSQI, Pittsburgh Sleep Quality Index.

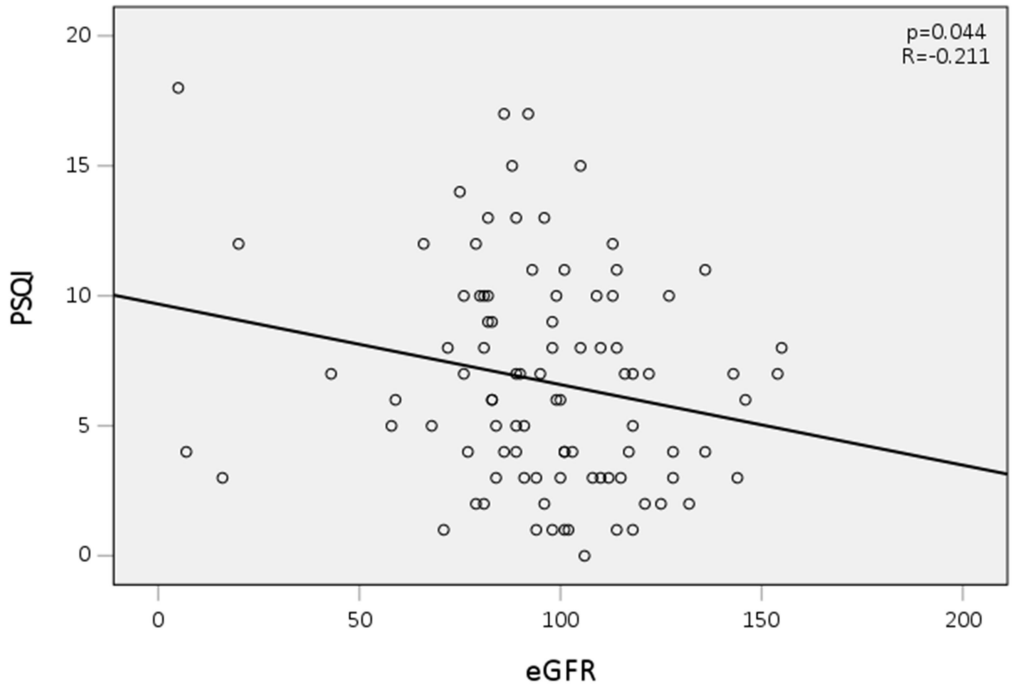

Figure 3.25. Scatter plot showing the correlation between PSQI and eGFR (ml/min/1.73m^2). eGFR, estimated glomerular filtration rate; PSQI, Pittsburgh Sleep Quality Index.

Table 3.19. Table showing categorical variables, the median PSQI and its interquartile range when the variable was present and when absent.

Variable		Median PSQI	Interquartile range	p value (2-tailed)
Gender	Female	6.0	7	0.300
	Male	8.0	2	
Current sunscreen use	Present	6.5	5	0.742
	Absent	6.0	7	
Smoking	Smoker	6.0	10	0.938
	Non-smoker	6.0	7	
Regular exercise	Present	6.5	6	0.522
	Absent	6.0	6	
Current prednisolone	Present	7.0	7	0.144
	Absent	6.0	6	
Current hydroxychloroquine	Present	6.0	7	0.626
	Absent	6.0	6	
Current azathioprine	Present	6.5	7	0.961
	Absent	6.0	7	
Current methotrexate	Present	7.5	2	0.266
	Absent	6.0	7	
Current mycophenolate	Present	9.5	10	0.208
	Absent	6.0	7	
Current calcium supplementation	Present	6.0	7	0.513
	Absent	7.0	6	
Current vitamin D suppplementation	Present	6.0	7	0.750
	Absent	7.0	6	
Raynauds syndrome	Present	5.0	7	0.725
	Absent	7.0	7	
Osteoporosis/ Osteopaenia	Present	5.5	5	0.060
	Absent	7.0	6	
Hypertension	Present	7.0	5	0.637
	Absent	6.0	7	
Hyperlipidaemia	Present	7.0	6	0.861
	Absent	6.0	7	
Diabetes Mellitus	Present	7.0	4	0.457
	Absent	6.0	7	
Fibromyalgia	Present	9.0	9	0.130
	Absent	6.0	7	
Anti-phospholipid syndrome	Present	5.0	6	0.256
	Absent	7.0	7	
Sjogren's syndrome	Present	6.5	4	0.893
	Absent	6.0	7	
Rheumatoid Arthritis	Present	7.0	1	0.582
	Absent	6.0	7	

The p values were obtained by using the Mann-Whitney U-test.

3.2.8 ASSOCIATION BETWEEN FUNCTIONAL DISABILITY AND OTHER VARIABLES

The relationship between functional disability measured by mHAQ and other continuous and categorical variables was assessed by Spearman's correlation test (supplementary table S5) and Mann-Whitney U-test respectively (table 3.20). Functional disability measured by mHAQ was significantly positively correlated with VAS pain (p<0.001), HADS-D (p<0.001), HADS-A (p=0.009) and age at disease diagnosis (p=0.001) (figures 3.26-3.29). It was negatively correlated with disease duration (p=0.023) and haemoglobin (p=0.038) (figure 3.30-3.31). Moreover, mHAQ was significantly lower in females (p=0.015). It was higher in patients who were receiving azathioprine (p=0.046) and in patients who were also diagnosed with fibromyalgia (p=0.022).

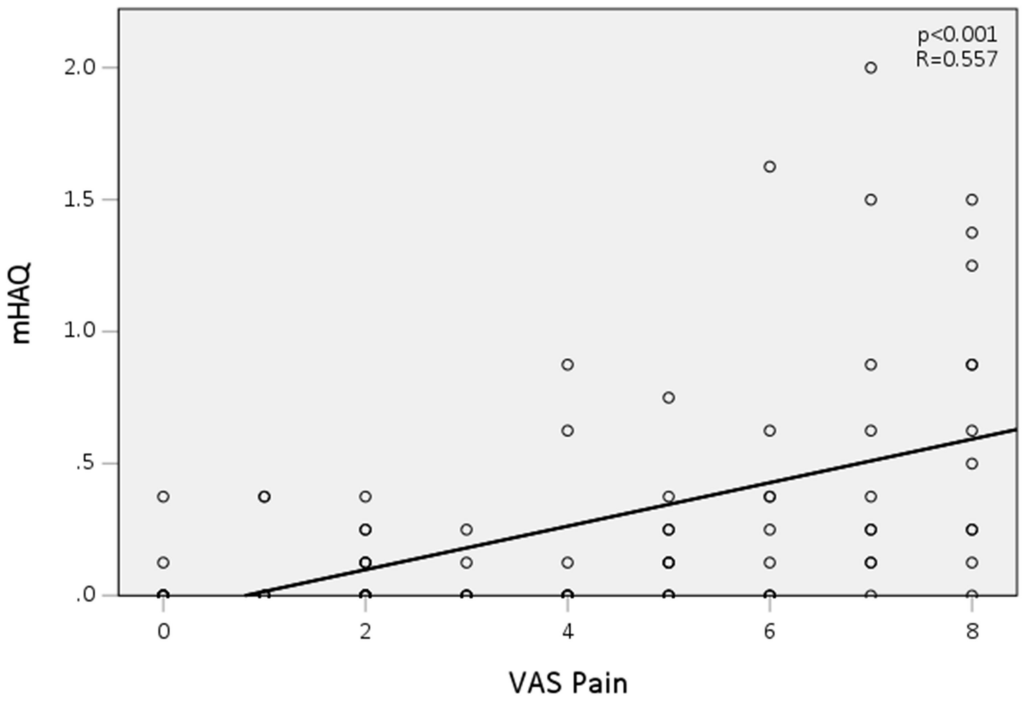

Figure 3.26. Scatter plot showing the correlation between mHAQ and VAS pain. mHAQ, modified Health Assessment Questionnaire; VAS, visual analogue scale.

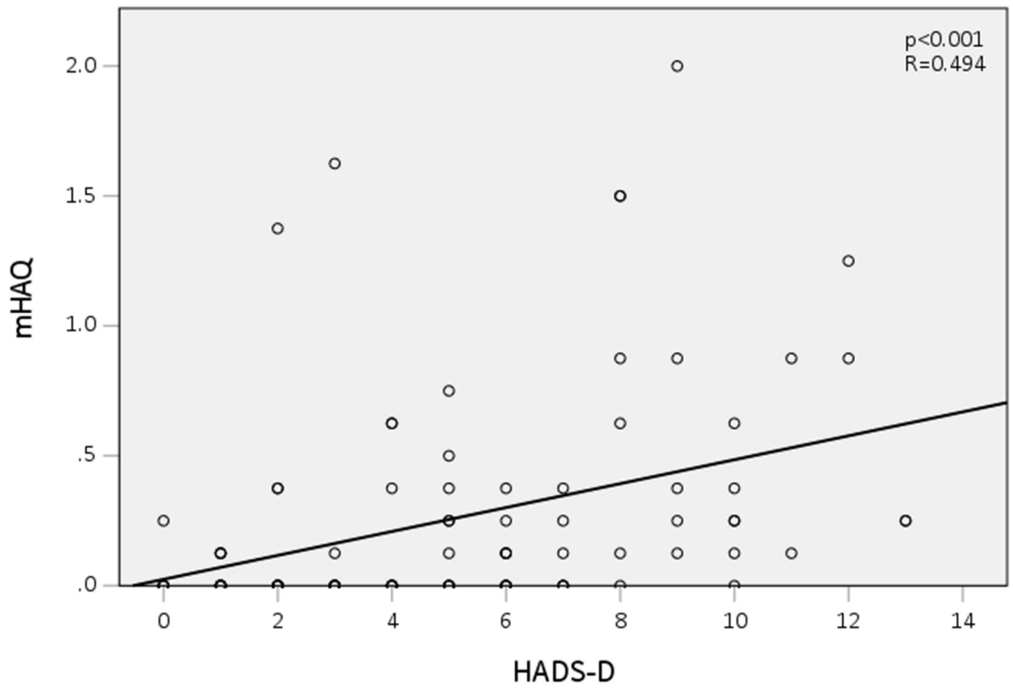

Figure 3.27. Scatter plot showing the correlation between mHAQ and HADS-D. HADS-D, Hospital Anxiety and Depression Scale – depression subscale; mHAQ, modified Health Assessment Questionnaire.

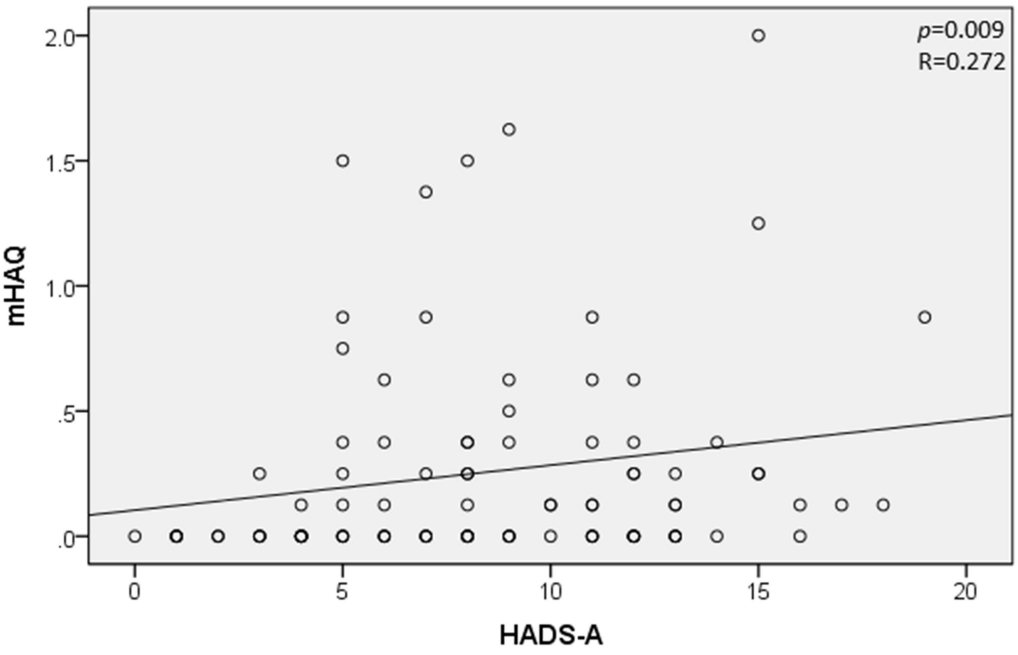

Figure 3.28. Scatter plot showing the correlation between mHAQ and HADS-A. HADS-A, Hospital Anxiety and Depression Scale – anxiety subscale; mHAQ, modified Health Assessment Questionnaire.

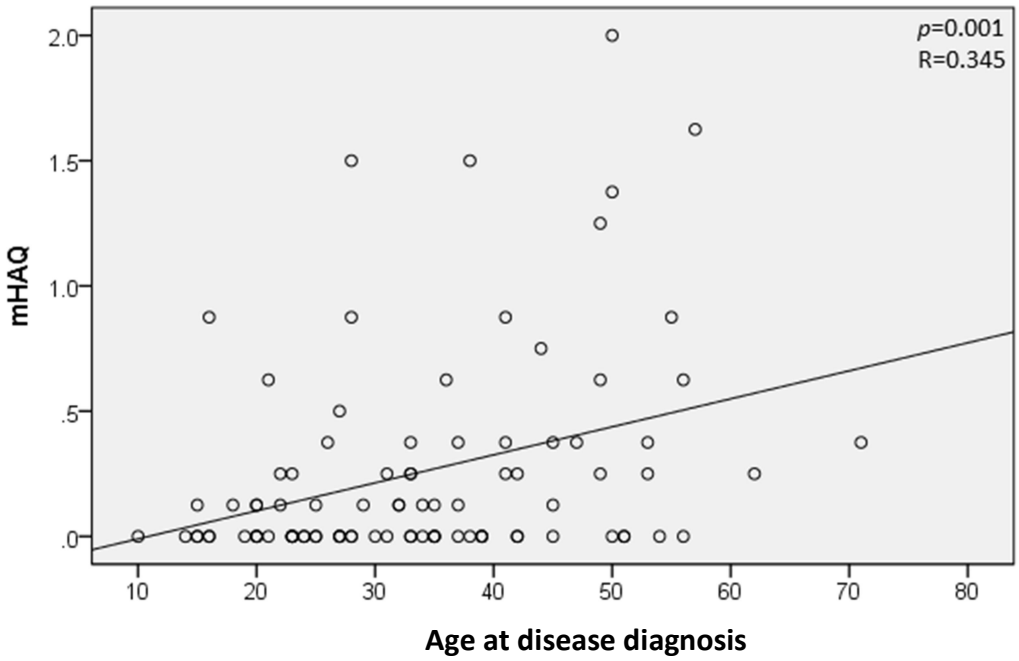

Figure 3.29. Scatter plot showing the correlation between mHAQ and age at disease diagnosis (years). mHAQ, modified Health Assessment Questionnaire.

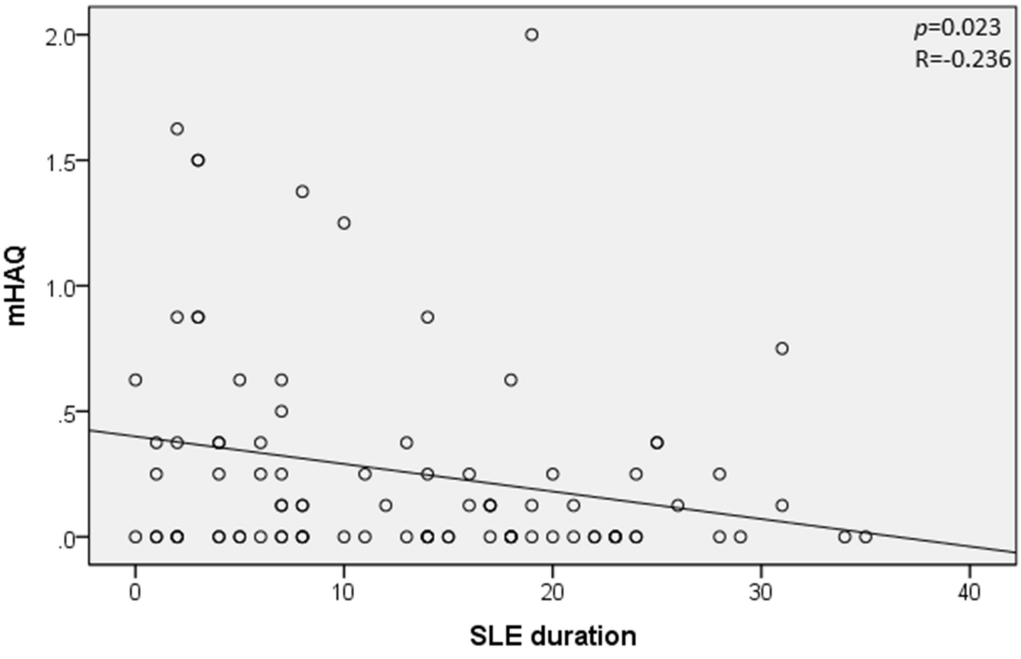

Figure 3.30. Scatter plot showing the correlation between mHAQ and duration of SLE (years). mHAQ, modified Health Assessment Questionnaire.

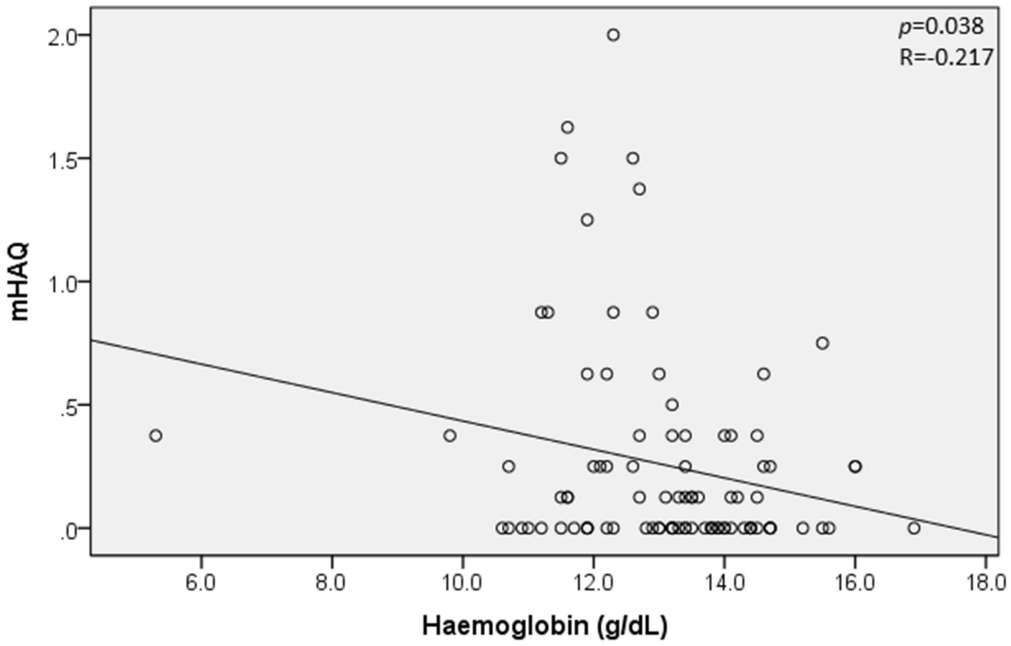

Figure 3.31. Scatter plot showing the correlation between mHAQ and haemoglobin (g/dL). mHAQ, modified Health Assessment Questionnaire.

Table 3.20. Table showing categorical variables, median mHAQ and its interquartile range when the variable was present and when absent.

Variable		Median mHAQ	Interquartile range	p value (2-tailed)
Gender	Female	0.000	0.250	**0.015**
	Male	0.375	0.500	
Current sunscreen use	Present	0.125	0.250	0.686
	Absent	0.063	0.375	
Smoking	Smoker	0.063	0.688	0.861
	Non-smoker	0.125	0.375	
Regular exercise	Present	0.125	0.375	0.272
	Absent	0.000	0.281	
Current prednisolone	Present	0.125	0.375	0.142
	Absent	0.000	0.250	
Current hydroxychloroquine	Present	0.000	0.375	0.995
	Absent	0.125	0.250	
Current azathioprine	Present	0.250	0.469	**0.046**
	Absent	0.000	0.250	
Current methotrexate	Present	0.125	0.500	0.579
	Absent	0.063	0.375	
Current mycophenolate	Present	0.000	0.188	0.310
	Absent	0.125	0.375	
Current calcium supplementation	Present	0.125	0.375	0.685
	Absent	0.063	0.250	
Current vitamin D suppplementation	Present	0.125	0.375	0.911
	Absent	0.063	0.281	
Raynauds syndrome	Present	0.125	0.375	0.269
	Absent	0.000	0.250	
Osteoporosis/ Osteopaenia	Present	0.000	0.156	0.149
	Absent	0.125	0.375	
Hypertension	Present	0.000	0.281	0.585
	Absent	0.125	0.375	
Hyperlipidaemia	Present	0.125	0.875	0.465
	Absent	0.125	0.313	
Diabetes Mellitus	Present	0.000	0.375	0.706
	Absent	0.125	0.375	
Fibromyalgia	Present	0.375	1.000	**0.022**
	Absent	0.000	0.250	
Anti-phospholipid syndrome	Present	0.000	0.250	0.409
	Absent	0.125	0.375	
Sjogren's syndrome	Present	0.125	0.625	0.885
	Absent	0.125	0.375	
Rheumatoid Arthritis	Present	0.000	0.125	0.385
	Absent	0.125	0.375	

The p values were obtained by using the Mann-Whitney U-test.

3.2.9 ASSOCIATION BETWEEN DAMAGE AND OTHER VARIABLES

The relationship between damage measured by SDI and other continuous variables was assessed by Spearman's correlation test since SDI was not normally distributed (supplementary table S6). Mann-Whitney U-test was used to assess the relationship between SDI and categorical variables (supplementary table S7).

Damage was significantly positively correlated with age (p=0.007), disease duration (p=0.001), current prednisolone dose (p=0.035), CRP (p=0.026) and urine PCR (p=0.030) (figures 3.32-3.35). Moreover, SDI was significantly higher in patients who were currently receiving prednisolone (p=0.011) or azathioprine (p=0.014) and patients who suffered from osteopaenia/osteoporosis (p<0.001), hypertension (p=0.009), diabetes (p=0.012) and anti-phospholipid syndrome (p=0.020) (figure 3.36). SDI was also significantly higher in patients with a history of renal (p=0.018), neurological (p=0.005), haematological (p=0.040) and cardiac (p=0.025) manifestations at any point in the disease course.

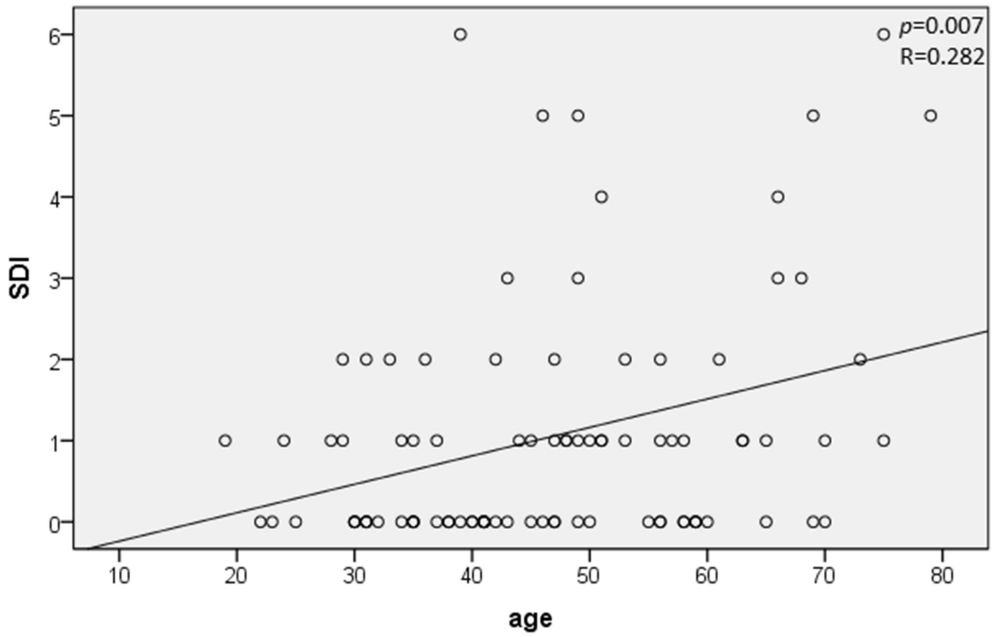

Figure 3.32. Scatter plot showing the correlation between SDI and age (years). SDI, Systemic Lupus International Collaborating Clinics/American College of Rheumatology damage index.

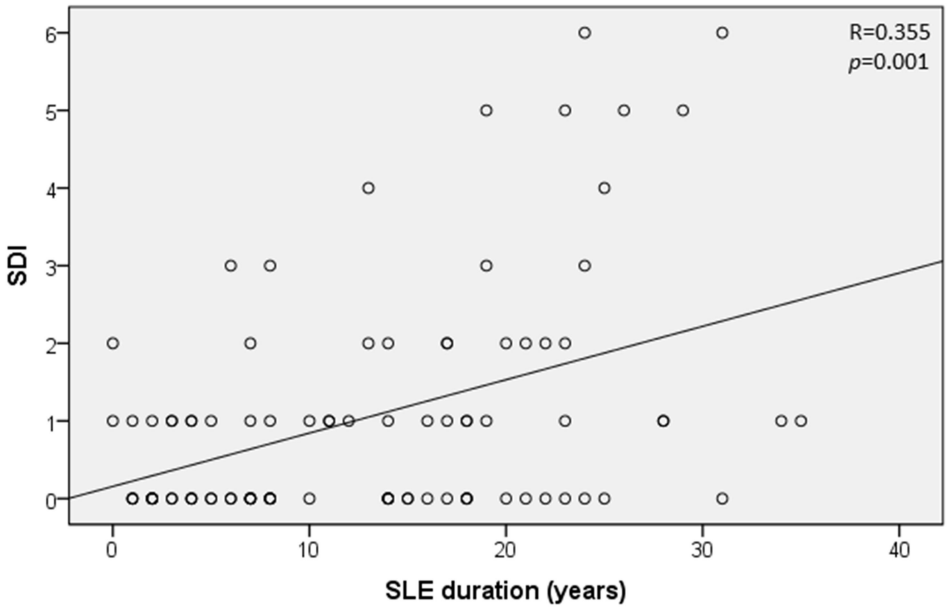

Figure 3.33. Scatter plot showing the correlation between SDI and disease duration. SDI, Systemic Lupus International Collaborating Clinics/American College of Rheumatology damage index.

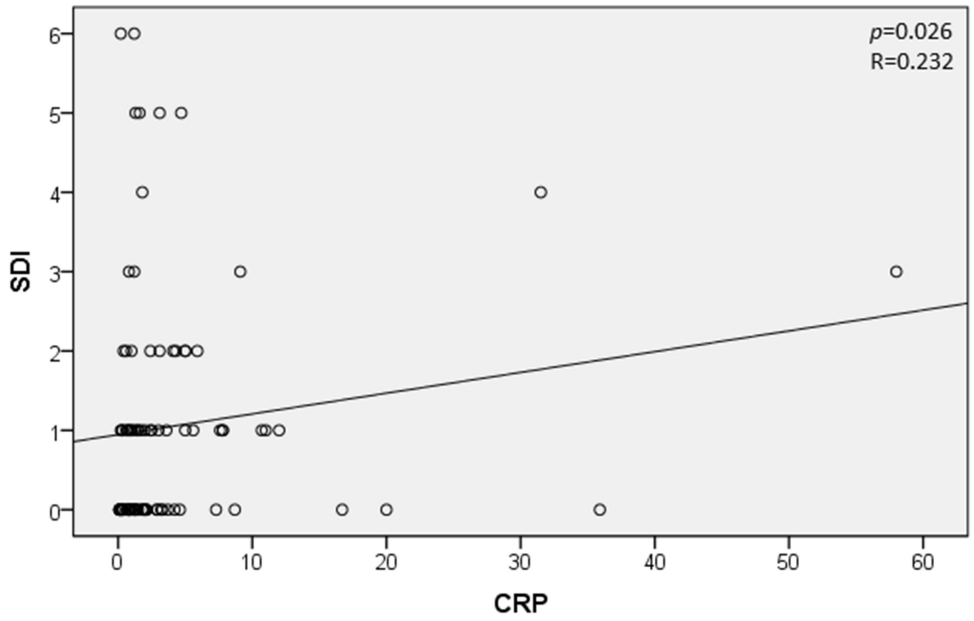

Figure 3.34. Scatter plot showing the correlation between SDI and CRP (mg/l). CRP, C reactive protein; SDI, Systemic Lupus International Collaborating Clinics/American College of Rheumatology damage index.

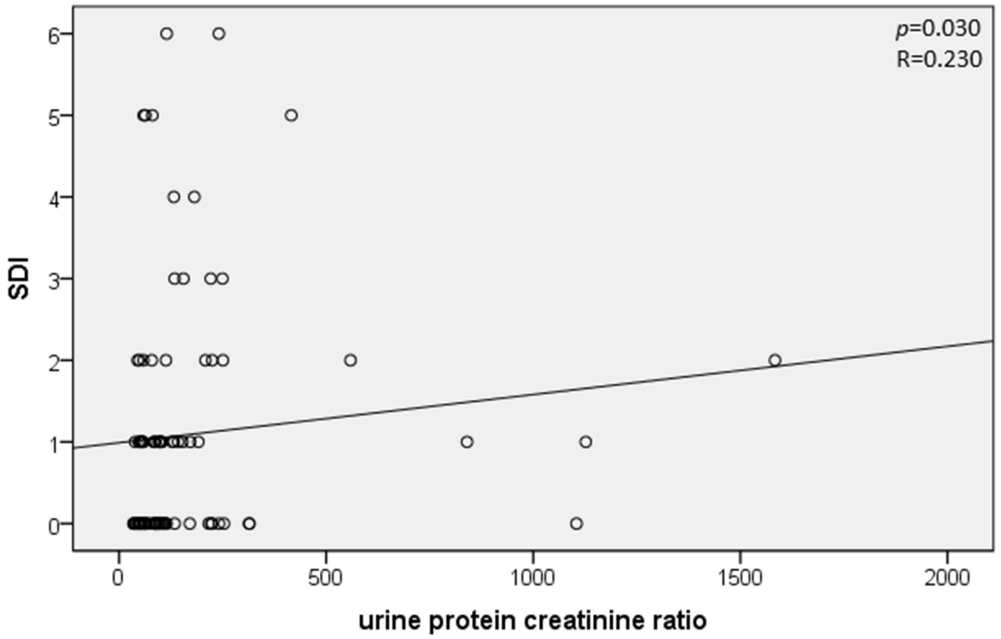

Figure 3.35. Scatter plot showing the correlation between SDI and urine protein creatinine ratio (mg/g). SDI, Systemic Lupus International Collaborating Clinics/American College of Rheumatology damage index.

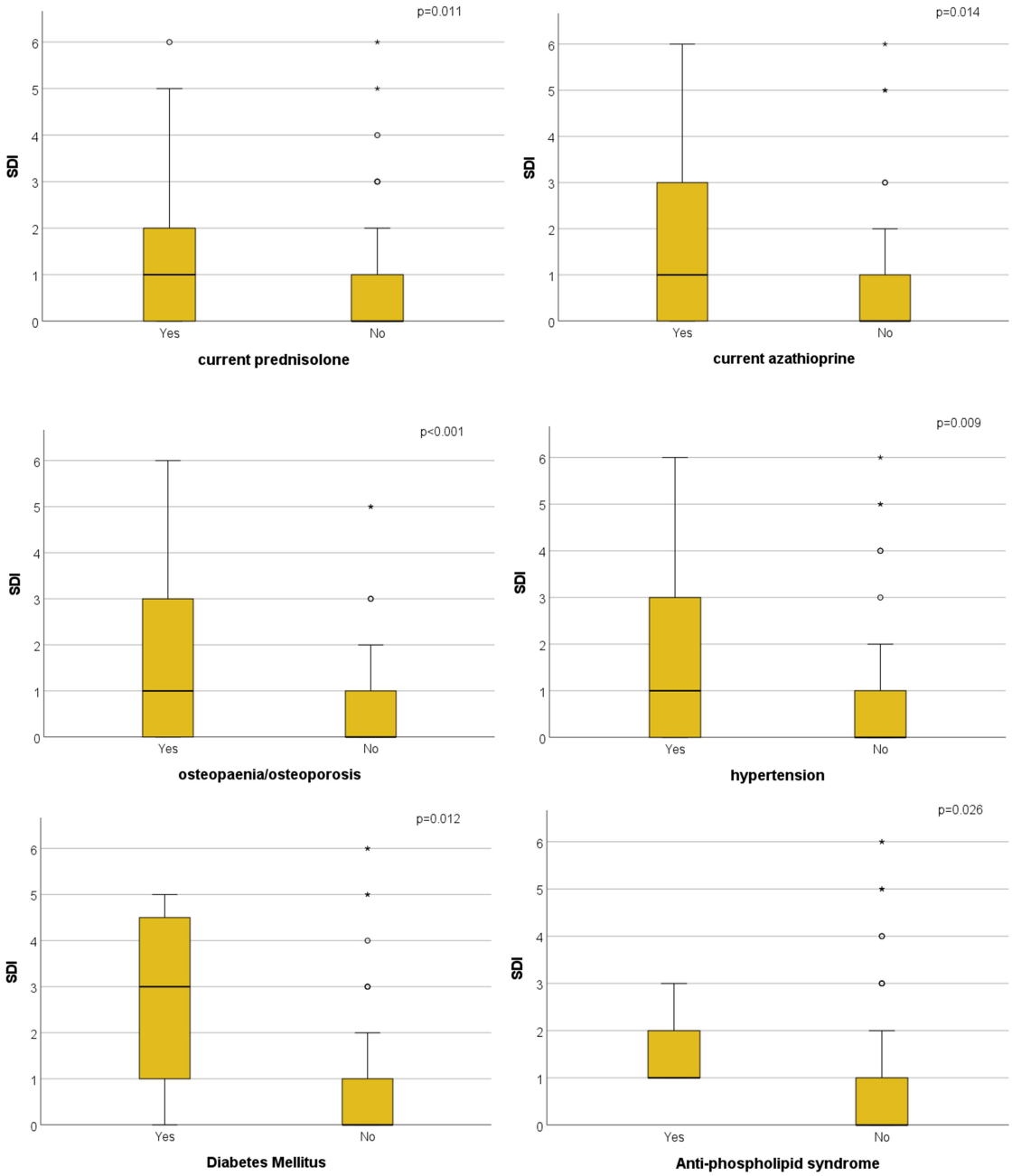

Figure 3.36. Box plots showing SDI in the presence and absence of current prednisolone, current azathioprine, osteopaenia/osteoporosis, hypertension, diabetes mellitus and anti-phospholipid syndrome.

SDI, Systemic Lupus International Collaborating Clinics/American College of Rheumatology damage index.

3.2.10 Association between Age at Disease Diagnosis and other variables

The relationship between age at disease diagnosis and other continuous variables was assessed using Pearson's correlation test when both variables were normally distributed and Spearman's correlation test when one of the variables was not (table 3.21). The independent samples t-test was used to assess the relationship with categorical variables since age at disease diagnosis was normally distributed (table 3.22).

A lower mean age at diagnosis was noted in patients with renal (p=0.003) and neuropsychiatric manifestations (p=0.032). For the other organ manifestations there was no significant difference in age at disease diagnosis. A lower mean age at disease diagnosis was also noted in patients who were anti-Sm (p=0.044) and anti-RNP (p=0.003) positive. Age at SLE diagnosis was significantly higher in males (p=0.016).

Table 3.21. Correlation of several continuous variables with age at disease diagnosis.

Variable	R value	P value (2-tailed)
BMI	0.187*	0.075
Current prednisolone dose	-0.156*	0.138
Current hydroxychloroquine dose	-0.075*	0.475
Current vitamin D dose	-0.020*	0.852
Current calcium dose	0.013*	0.900
eGFR	-0.131*	0.214
Urine PCR	0.204*	0.056
VAS Pain	0.181*	0.084
HADS-D	0.090*	0.392
HADS-A	-0.152	0.148
Haemoglobin	-0.051	0.627
Calcium (corrected)	0.186*	0.077
CRP	0.178*	0.089
ESR	0.154*	0.144
C3	-0.049	0.645
C4	-0.112	0.287
Anti-dsDNA titre	-0.077*	0.468

Pearson's correlation test was used when both variables had a normal distribution. Spearman's correlation test was used when one of the variables was not normally distributed (R value marked with *).

Anti-dsDNA, anti-double-stranded deoxyribonucleic acid; BMI, body mass index; C3, complement 3; C4, complement 4; CRP, C-reactive protein; eGFR, estimated glomerular filtration rate; ESR, erythrocyte sedimentation rate; HADS-A, Hospital Anxiety and Depression Scale – anxiety subscale; HADS-D, Hospital Anxiety and Depression Scale – depression subscale; PCR, protein creatinine ratio; VAS, Visual Analogue Scale.

Table 3.22. Table showing categorical variables, the mean age at SLE diagnosis and standard deviation (S.D.) when the variable was present and when absent.

Variable		Mean age at disease diagnosis (years)	S.D.	p value (2-tailed)
Gender	Female	32.92	12.288	**0.016**
	Male	45.00	15.044	
Current sunscreen use	Present	31.85	11.764	0.138
	Absent	35.83	13.658	
Smoking	Smoker	28.86	8.717	0.115
	Non-smoker	34.73	13.285	
Regular exercise	Present	34.79	14.929	0.553
	Absent	33.17	11.228	
Anti-Sm	Positive	29.97	13.472	**0.044**
	Negative	35.85	12.470	
Anti-SSA52	Positive	31.65	10.528	0.247
	Negative	34.97	13.922	
Anti-SSA60	Positive	32.06	11.274	0.301
	Negative	34.95	13.807	
Anti-SSB	Positive	32.38	7.985	0.536
	Negative	34.08	13.575	
Anti-RNP	Positive	28.48	9.691	**0.001**
	Negative	37.00	13.689	
Constitutional manifestations	Present	31.98	12.775	0.114
	Absent	36.25	12.661	
Mucocutaneous manifestations	Present	32.62	12.364	0.065
	Absent	38.83	13.866	
Renal manifestations	Present	27.25	10.203	**0.003**
	Absent	36.16	12.920	
Neurological manifestations	Present	23.86	7.426	**0.032**
	Absent	34.66	12.864	
Haematological manifestations	Present	33.38	12.359	0.403
	Absent	36.62	15.693	
Cardiac manifestations	Present	36.38	9.102	0.561
	Absent	33.60	13.149	
Respiratory manifestations	Present	37.75	14.392	0.181
	Absent	33.01	12.427	
Raynauds syndrome	Present	30.70	13.068	0.054
	Absent	35.95	12.346	
Fibromyalgia	Present	39.56	12.739	0.160
	Absent	33.22	12.765	
Anti-phospholipid syndrome	Present	34.86	15.700	0.828
	Absent	33.75	12.677	
Sjogren's syndrome	Present	38.00	6.055	0.510
	Absent	33.65	13.045	
Rheumatoid Arthritis	Present	46.00	4.583	0.095
	Absent	33.43	12.830	

The p values were obtained by using the independent samples t-test. anti-RNP, anti-ribonucleoprotein; anti-Sm, anti-Smith; anti-SSA, anti-Sjogren's syndrome related antigen A; anti-SSB, anti-Sjogren's syndrome related antigen B.

3.2.11 ANCOVA REGRESSION

When collecting a large number of variables, one of the biggest challenges is the selection of valuable data. This is data that can be used in prediction models. A model that could explore the relationship of multiple continuous and categorical variables to the dependent variable, the ANCOVA (analysis of co-variance) regression model was selected for serum 25-hydroxyvitamin D and fatigue as the dependent variables. For each dependent variable, the ANCOVA assumptions of normality, absence of outliers, homogeneity of variances, homogeneity of regression slopes and linearity were checked prior to carrying out ANCOVA analysis (Field, 2005). These assumptions were met in most instances. In the case of fatigue a bimodal distribution was noted within the subpopulations. The ANCOVA regression model was still deemed to be the most appropriate model in this case and it was used with the normality assumption violated.

The regression model, relating serum 25-hydroxyvitamin D to 5 predictors identified BMI and vitamin D daily dose as significant predictors since their p values were below the 0.05 level of significance. The Parsimonious model was established by using a backward procedure. Using this model, the variable with the highest *p* value was eliminated each time the linear regression was done. The 2 predictor model explained 19.3% of the total variation in the serum 25-hydroxyvitamin D (Tables 3.23-3.25).

Table 3.23. ANCOVA regression model with 25-hydroxyvitamin D as the dependent variable, and its relationship to 5 predictors.

Source	Sum of Squares	df	Mean Square	F	p value
Corrected Model	1921.650	5	384.330	5.217	<0.001
Intercept	4776.610	1	4776.610	64.838	<0.001
BMI	521.176	1	521.176	7.074	0.009
Daily dose of vitamin D	919.173	1	919.173	12.477	0.001
eGFR	127.651	1	127.651	1.733	0.192
Daily dose of calcium	221.425	1	221.425	3.006	0.087
Current sunscreen use	1.134	1	1.134	0.015	0.902
Error	6335.600	86	73.670		
Total	95249.000	92			
Corrected Total	8257.250	91			

R Squared = 0.233

BMI, body mass index; estimated glomerular filtration rate.

Table 3.24. ANCOVA regression model with 25-hydroxyvitamin D as the dependent variable, and the 2 identified significant predictors.

Source	Sum of Squares	df	Mean Square	F	p value
Corrected Model	1594.084	2	797.042	10.646	<0.001
Intercept	7389.619	1	7389.619	98.703	<0.001
BMI	545.285	1	545.285	7.283	0.008
Daily dose of vitamin D	1043.798	1	1043.798	13.942	<0.001
Error	6663.166	89	74.867		
Total	95249.000	92			
Corrected Total	8257.250	91			

R Squared = 0.193

BMI, body mass index.

Table 3.25. Parameter estimates obtained on ANCOVA regression model, with 25-hydroxyvitamin D as the dependent variable.

Parameter	B	Std. Error	t	p value	95% Confidence Interval	
					Lower Bound	Upper Bound
Intercept	39.377	3.964	9.935	<0.001	31.502	47.253
BMI	-0.372	0.138	-2.699	0.008	-0.646	-0.098
Daily dose of vitamin D	0.003	0.001	3.734	<0.001	0.001	0.004

BMI, body mass index.

The regression co-efficient (B) for BMI (-0.372) indicated that for every 1 unit increase in BMI, the serum 25-hydroxyvitamin D was expected to decrease by 0.372, given that other effects were kept constant. The regression co-efficient for daily dose of vitamin D (0.003) indicated that for every 1 unit increase in vitamin D daily dose, serum 25-hydroxyvitamin D was expected to increase by 0.003, given that other effects were kept constant.

The 5 predictor model explained 23.3% of the total variation in serum 25-hydroxyvitamin D. When 3 non-significant predictors were removed, the Parsimonious 2 predictor model explained 19.3% of the total variation in serum 25-hydroxyvitamin D. This implied that there are other significant predictors not included in the study (for example skin type, sun exposure) which would explain the remaining 80.7% of the total variation in serum 25-hydroxyvitamin D.

Similarly, regression models relating fatigue (measured by FSS and VAS fatigue) to 10 predictors were made as shown below (Tables 3.26-3.31). These both identified HADS-

D and VAS pain as significant predictors since their p values were below the 0.05 level of significance. The Parsimonious model was again established by using a backward procedure. These 2 predictor models explain 41.6% (FSS model) and 48.3% (VAS fatigue model) of the total variation in the level of fatigue. This implies that there are other significant predictors not included in the study which explain the remaining 58.4% and 51.7% of the level of fatigue respectively.

Table 3.26. ANCOVA regression model with FSS as the dependent variable, and its relationship to 10 predictors.

Source	Sum of Squares	df	Mean Square	F	p value
Corrected Model	100.064	11	7.697	5.579	<0.001
Intercept	1.341	1	1.341	0.972	0.327
VAS pain	12.325	1	12.325	8.933	0.004
HADS-D	6.913	1	6.913	5.011	0.028
HADS-A	0.562	1	0.562	0.407	0.525
Haemoglobin	1.765	1	1.765	1.279	0.262
PSQI	2.057	1	2.057	1.491	0.226
Serum 25-hydroxyvitamin D	0.184	1	0.184	0.133	0.716
SLEDAI-2K	1.125	1	1.125	0.815	0.369
Current hydroxychloroquine	2.639	1	2.639	1.913	0.171
Fibromyalgia	0.066	1	0.066	0.048	0.827
Regular exercise	0.045	1	0.045	0.033	0.857
Error	107.618	78			
Total	1691.707	92			
Corrected Total	207.682	91			

R Squared = 0.482

HADS-A, Hospital Anxiety and Depression Scale – anxiety subscale; HADS-D, Hospital Anxiety and Depression Scale – depression subscale; FSS, Fatigue Severity Scale; PSQI, Pittsburgh Sleep Quality Index; SLEDAI-2K, Systemic Lupus Erythematosus Disease Activity Index 2000; VAS, Visual Analogue Scale.

Table 3.27. ANCOVA regression model with FSS as the dependent variable, and the 2 identified significant predictors.

Source	Sum of Squares	df	Mean Square	F	p value
Corrected Model	86.404	2	43.202	31.704	<0.001
Intercept	117.797	1	117.797	86.445	<0.001
VAS pain	24.044	1	24.044	17.645	<0.001
HADS-D	26.131	1	26.131	19.176	<0.001
Error	121.279	89	1.363		
Total	1691.707	92			
Corrected Total	207.682	91			

R Squared = 0.416

FSS, Fatigue Severity Scale; HADS-D, Hospital Anxiety and Depression Scale – depression subscale; VAS, Visual Analogue Scale.

Table 3.28. Parameter estimates obtained on ANCOVA regression model, with FSS as the dependent variable.

Parameter	B	Std. Error	t	p value	95% Confidence Interval	
					Lower Bound	Upper Bound
Intercept	2.301	0.247	9.298	<0.001	1.809	2.793
VAS pain	0.214	0.051	4.201	<0.001	0.113	0.316
HADS-D	0.173	0.040	4.379	<0.001	0.095	0.252

FSS, Fatigue Severity Scale; HADS-D, Hospital Anxiety and Depression Scale – depression subscale; VAS, Visual Analogue Scale.

Table 3.29. ANCOVA regression model with VAS fatigue as the dependent variable, and its relationship to 10 predictors.

Source	Sum of Squares	df	Mean Square	F	p value
Corrected Model	300.344	13	23.103	7.120	<0.001
Intercept	0.003	1	0.003	0.001	0.974
VAS pain	27.420	1	27.420	8.451	0.005
HADS-D	33.518	1	33.518	10.330	0.002
HADS-A	4.906	1	4.906	1.512	0.223
PSQI	2.029	1	2.029	0.625	0.581
Haemoglobin	0.995	1	0.995	0.307	0.431
Serum 25-hydroxyvitamin D	0.003	1	0.003	0.001	0.977
SLEDAI-2K	0.545	1	0.545	0.168	0.683
Current hydroxychloroquine	0.004	1	0.004	0.001	0.972
Fibromyalgia	0.994	1	0.994	0.306	0.581
Regular exercise	10.900	1	10.900	3.359	0.071
Error	253.091	78	3.245		
Total	2310.000	92			
Corrected Total	553.435	91			

R Squared = 0.543

HADS-A, Hospital Anxiety and Depression Scale – anxiety subscale; HADS-D, Hospital Anxiety and Depression Scale – depression subscale; PSQI, Pittsburgh Sleep Quality Index; SLEDAI-2K, Systemic Lupus Erythematosus Disease Activity Index 2000; VAS, Visual Analogue Scale.

Table 3.30. ANCOVA regression model with VAS fatigue as the dependent variable, and the 2 identified significant predictors.

Source	Sum of Squares	df	Mean Square	F	p value
Corrected Model	267.501	2	133.750	41.631	<0.001
Intercept	40.663	1	40.663	12.657	0.001
VAS pain	71.834	1	71.834	22.359	<0.001
HADS-D	83.596	1	83.596	26.020	<0.001
Error	285.934	89	3.213		
Total	2310.000	92			
Corrected Total	553.435	91			

R Squared = 0.483

HADS-D, Hospital Anxiety and Depression Scale – depression subscale; VAS, Visual Analogue Scale.

Table 3.31. Parameter estimates obtained on ANCOVA regression model, with VAS fatigue as the dependent variable.

Parameter	B	Std. Error	t	p value	95% Confidence Interval	
					Lower Bound	Upper Bound
Intercept	1.352	0.380	3.558	0.001	0.597	2.107
VAS pain	0.371	0.078	4.729	<0.001	0.215	0.526
HADS-D	0.310	0.061	5.101	<0.001	0.189	0.431

HADS-D, Hospital Anxiety and Depression Scale – depression subscale; VAS, Visual Analogue Scale.

3.2.12 GENERALISED LINEAR MODEL

The generalised linear model for gamma distributed dependent variables was used to explore the relationship of multiple continuous and categorical variables to the dependent variables mHAQ, SDI and PSQI that had a right skewed distribution.

A generalised linear model relating functional disability measured by mHAQ to 12 predictors identified VAS pain, SLEDAI-2K and age at disease diagnosis as significant predictors, since their p values were below 0.05 (Table 3.32-3.33). The Parsimonious model was again established by using a backward elimination procedure. The regression co-efficient (B) for VAS pain, SLEDAI-2K and age at disease diagnosis indicated that for every 1 unit increase in these variables, mHAQ was expected to increase by 0.046, 0.030 and 0.010 respectively, given that other effects were kept constant.

Table 3.32. Generalised linear model for gamma distribution with mHAQ as the dependent variable, and its relationship to 12 predictors.

Parameter	B	Standard Error	Wald Chi-Square	df	p value
(Intercept)	0.074	0.404	0.034	1	0.854
[gender=male]	0.085	0.150	0.321	1	0.571
[gender=female]	0	.	.	.	.
[azathioprine=yes]	0.020	0.078	0.062	1	0.803
[azathioprine=no]	0	.	.	.	.
[Fibromyalgia=yes]	0.193	0.172	1.259	1	0.262
[Fibromyalgia=no]	0	.	.	.	.
Age at diagnosis	0.008	0.004	3.687	1	0.055
Disease duration	0.001	0.006	0.064	1	0.801
Haemoglobin	-0.021	0.026	0.679	1	0.410
VAS Pain	0.041	0.014	8.401	1	0.004
PSQI	0.010	0.014	0.488	1	0.485
HADS-D	0.015	0.015	1.022	1	0.312
HADS-A	0.002	0.011	0.028	1	0.868
FSS	-0.047	0.048	0.986	1	0.321
SLEDAI-2K	0.028	0.015	3.328	1	0.068
(Scale)	0.375	0.073			

FSS, Fatigue Severity Scale; HADS-A, Hospital Anxiety and Depression Scale – anxiety subscale; HADS-D, Hospital Anxiety and Depression Scale – depression subscale; mHAQ, modified Health Assessment Questionnaire; PSQI, Pittsburgh Sleep Quality Index; SLEDAI-2K, Systemic Lupus Erythematosus Disease Activity Index 2000; VAS, Visual Analogue Scale.

Table 3.33. Generalised linear model for gamma distribution with mHAQ as the dependent variable, and the 3 identified significant predictors.

Parameter	B	Standard error	Wald Chi-Square	df	p value
(Intercept)	-0.241	0.092	6.909	1	0.009
Age at diagnosis	0.010	0.003	8.787	1	0.003
VAS pain	0.046	0.018	6.537	1	0.011
SLEDAI-2K	0.030	0.014	4.335	1	0.037
(Scale)	0.430	0.083			

mHAQ, modified Health Assessment Questionnaire; SLEDAI-2K, Systemic Lupus Erythematosus Disease Activity Index 2000; VAS, Visual Analogue Scale.

A generalised linear model for gamma distributed dependent variables relating damage measured by SDI to 14 predictors identified SLE duration as a significant predictor since its p value was below 0.05 (Table 3.34-3.35). The Parsimonious model was established by using a backward procedure. The regression co-efficient (B) for SLE duration indicated that for every 1 unit increase, SDI was expected to increase by 0.063, given that other effects were kept constant.

Table 3.34. Generalised linear model for gamma distribution with SDI as the dependent variable, and its relationship to 14 predictors.

Parameter	B	Standard Error	Wald Chi-Square	df	p value
(Intercept)	0.895	0.740	1.462	1	0.227
[anti-phospholipid syndrome=yes]	0.190	0.410	0.215	1	0.643
[anti-phospholipid syndrome=no]	0	.	.	.	.
[osteopaenia=yes]	0.955	0.485	3.873	1	0.049
[osteopaenia=no]	0	.	.	.	.
[Hypertension=yes]	0.188	0.383	0.240	1	0.624
[Hypertension=no]	0	.	.	.	.
[Diabetes Mellitus=yes]	1.255	0.736	2.912	1	0.088
[Diabetes Mellitus=no]	0	.	.	.	.
[Renal manifestations=yes]	0.538	0.580	0.860	1	0.354
[Renal manifestations=no]	0	.	.	.	.
[Neurological manifestations=yes]	-0.057	0.376	0.023	1	0.880
[Neurological manifestations=no]	0	.	.	.	.
[Haematological manifestations=yes]	-0.404	0.646	0.391	1	0.532
[Haematological manifestations=no]	0	.	.	.	.
[Cardiac manifestations=yes]	0.264	0.427	0.381	1	0.537
[Cardiac manifestations=no]	0	.	.	.	.
[Current azathioprine=yes]	0.068	0.414	0.027	1	0.869
[Current azathioprine=no]	0	.	.	.	.
Age	0.008	0.010	0.634	1	0.426
SLE duration	0.014	0.020	0.441	1	0.507
Daily dose of prednisolone	0.070	0.049	2.029	1	0.154
CRP	0.010	0.018	0.347	1	0.556
Urine protein creatinine ratio	-0.002	0.001	4.870	1	0.027
(Scale)	0.234	0.047			

CRP, C reactive protein; SDI, Systemic Lupus International Collaborating Clinics/American College of Rheumatology damage index.

Table 3.35. Generalised linear model for gamma distribution with SDI as the dependent variable, and the identified significant predictor.

Parameter	B	Standard Error	Wald-Chi Square	df	p value
(Intercept)	1.076	0.214	25.387	1	<0.001
SLE duration	0.063	0.016	15.248	1	<0.001
(Scale)	0.313	0.062			

SDI, Systemic Lupus International Collaborating Clinics/American College of Rheumatology damage index.

A regression model relating sleep quality measured by PSQI to 6 predictors identified HADS-D, VAS pain and eGFR as significant predictors since their p values were below 0.05 (Tables 3.36-3.37). The Parsimonious model was again established by using a backward procedure.

Table 3.36. Generalised linear model for gamma distribution with PSQI as the dependent variable, and its relationship to 6 predictors.

Parameter	B	Standard Error	Wald Chi-Square	df	p value
(Intercept)	2.639	1.204	4.801	1	0.028
HADS-D	0.475	0.149	10.177	1	0.001
HADS-A	0.096	0.090	1.141	1	0.286
VAS Pain	0.439	0.132	11.050	1	0.001
eGFR	-0.024	0.009	6.626	1	0.010
Age at diagnosis	0.035	0.022	2.530	1	0.112
SLEDAI-2K	0.078	0.103	0.579	1	0.447
(Scale)	.250	.0356			

eGFR, estimated glomerular filtration rate; HADS-A, Hospital Anxiety and Depression Scale – anxiety subscale; HADS-D, Hospital Anxiety and Depression Scale – depression subscale; PSQI, Pittsburgh Sleep Quality Index; SLEDAI-2K, Systemic Lupus Erythematosus Disease Activity Index 2000; VAS, Visual Analogue Scale.

Table 3.37. Generalised linear model for gamma distribution with PSQI as the dependent variable, and the 3 identified significant predictors.

Parameter	B	Standard Error	Wald Chi-Square	df	p value
(Intercept)	3.893	1.082	12.937	1	<0.001
HADS-D	0.605	0.117	26.735	1	<0.001
VAS Pain	0.457	0.138	10.932	1	0.001
eGFR	-0.021	0.009	5.275	1	0.022
(Scale)	0.263	0.037			

eGFR, estimated glomerular filtration rate; HADS-D, Hospital Anxiety and Depression Scale – depression subscale; PSQI, Pittsburgh Sleep Quality Index; VAS, Visual Analogue Scale.

3.2.13 SUMMARY OF RESULTS

On univariate analysis, serum 25-hydroxyvitamin D level was significantly correlated with vitamin D (R=0.471, p<0.001) and calcium supplementation (R=0.285, p=0.006). ANCOVA analysis identified that serum 25-hydroxyvitamin D is significantly dependent on BMI (p=0.008) and vitamin D daily dose (p<0.001).

Fatigue measured by FSS was significantly positively correlated with PSQI (R=0.551, p<0.001), VAS pain (R=0.536, p<0.001), HADS-D (R=0.535, p<0.001), mHAQ (R=0.435, p<0.001), HADS-A (R=0.395, p<0.001) and current hydroxychloroquine dose (R=0.214, p=0.040). These relationships were confirmed when using VAS fatigue as the measure for fatigue. In addition to the above, a positive significant relationship was noted between SLEDAI-2K and VAS fatigue (R=0.247, p=0.018). Moreover, FSS score was significantly higher in patients who were receiving hydroxychloroquine (p=0.009) and were diagnosed with fibromyalgia (p=0.047). These results were also confirmed when using VAS fatigue as the measure for fatigue. ANCOVA analysis showed that fatigue (measured by both FSS and VAS fatigue) was significantly dependent on HADS-D (p<0.001) and VAS pain (p<0.001).

SLEDAI-2K was significantly positively correlated with mHAQ (R=0.417, p<0.001), VAS pain (R=0.325, p=0.002), HADS-D (R=0.230, p=0.028), PSQI (R=0.254, p=0.014) and anti-dsDNA titre (R=0.478, p<0.001). It was negatively correlated with disease duration (R=-0.229, p=0.028), haemoglobin (R=-0.251, p=0.016), C3 (R=-0.441, p<0.001) and C4 (R=-0.333, p=0.001). Moreover, SLEDAI-2K was significantly lower in patients who were receiving mycophenolate mofetil (p=0.022).

The results show that sleep quality measured by PSQI was significantly positively correlated with HADS-D (R=0.605, p<0.001), VAS pain (R=0.515, p<0.001), HADS-A (R=0.375, p<0.001), mHAQ (R=0.559, p<0.001) and age at disease diagnosis (R=0.220, p=0.035). It was negatively correlated with eGFR (R=-0.211, p=0.044). The generalised linear model showed that PSQI was dependent on HADS-D (p<0.001), VAS pain (p=0.001) and decreased eGFR (p=0.022).

Functional disability measured by mHAQ was significantly positively correlated with VAS pain (R=0.557, p<0.001), HADS-D (R=0.494, p<0.001), HADS-A (R=0.272, p=0.009), and age at disease diagnosis (R=0.345, p=0.001). It was negatively correlated with disease duration (R=-0.236, p=0.023) and haemoglobin (R=-0.217, p=0.038). Moreover, mHAQ was significantly lower in females (p=0.015). The mHAQ was significantly higher in patients who were receiving azathioprine (p=0.046) and in patients who were also diagnosed with fibromyalgia (p=0.022). On analysis in the generalised linear model, mHAQ was found to be significantly dependent on VAS pain (p=0.011), age at disease diagnosis (p=0.003) and SLEDAI-2K (p=0.037).

Damage measured by SDI was significantly positively correlated with age (R=0.282, p=0.007), disease duration (R=0.355, p=0.001), current prednisolone dose (R=0.220, p=0.035), CRP (R=0.232, p=0.026) and urine PCR (R=0.230, p=0.030). Moreover, SDI was significantly higher in patients who were currently receiving prednisolone (p=0.011) or azathioprine (p=0.014) and patients who suffered from osteopaenia/osteoporosis (p<0.001), hypertension (p=0.009), diabetes (p=0.012) and anti-phospholipid syndrome (p=0.020). SDI was also significantly higher in patients with a history of renal (p=0.018), neurological (p=0.005), haematological (p=0.040) and

cardiac (p=0.025) manifestations at any point in the disease course. Moreover, the generalised linear model identified disease duration (p<0.001) as the strongest predictor.

In addition, the results showed that there was a significant lower mean age at SLE diagnosis in patients with a history of renal (p=0.003) or neuropsychiatric manifestations (p=0.032). This was also the case in patients who were positive for anti-Sm (p=0.044) or anti-RNP antibodies (p=0.001). Age at SLE diagnosis was significantly higher in males (p=0.016).

3.3 Prospective Cohort study including SLE patients with Vitamin D Deficiency or Insufficiency

The results of 31 patients, who were followed up for one year after receiving treatment with vitamin D3, were analysed. These included 13 SLE patients with vitamin D deficiency and 18 patients with vitamin D insufficiency.

3.3.1 Patient Demographics

90.3% of SLE patients included in the prospective part of the research were female. 30 were Caucasian and 1 patient was of Asian ethnicity. The mean age was 47.9 years (S.D. 13.7 years) and the mean duration of SLE was 14.1 years (S.D. 8.0 years). The mean serum 25-hydroxyvitamin D level at baseline was 21.7ng/ml. Figure 3.37 shows a histogram of the frequency of serum 25-hydroxyvitamin D level at baseline. Out of the 31 patients included in this part of the research, 11 patients were receiving calcium and vitamin D3 supplements at baseline at a mean dose of 764mg and 527IU respectively. 7 patients were receiving prednisolone at baseline at a mean dose of 6.2mg daily. Out of the latter, 3 patients remained on the same prednisolone dose throughout the course of the study, 2 patients had a higher dose than baseline in their 6 month assessment (by 1.25mg and 1.5mg respectively) but had stopped prednisolone by 12 months, and 2 patients had a lower dose in both assessments when compared to baseline. One patient was started on prednisolone 2.5mg daily in the first 6 months but this was stopped completely before the 12 month assessment.

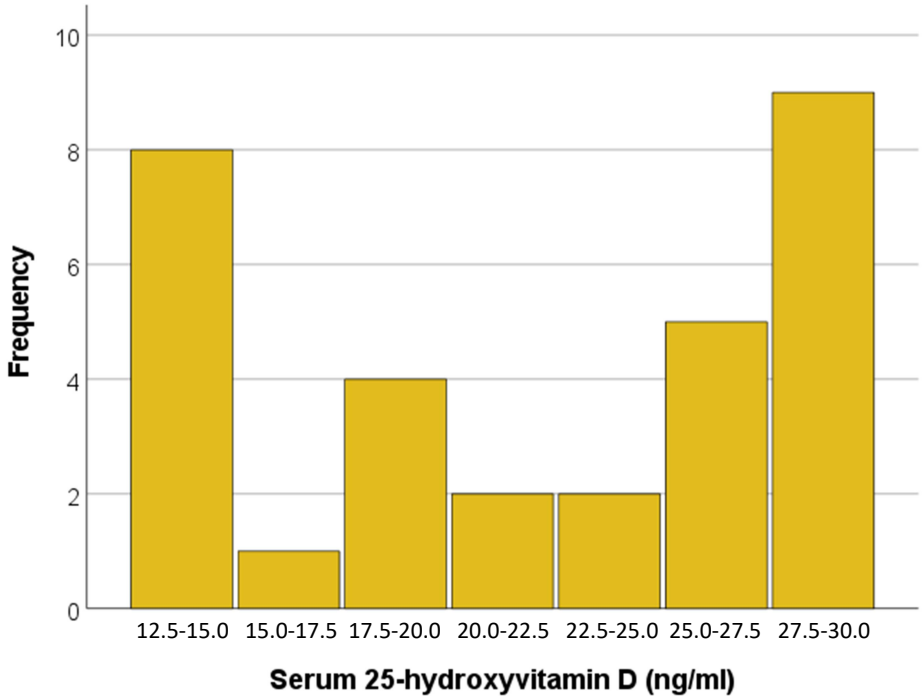

Figure 3.37. Histogram showing frequency of serum 25-hydroxyvitamin D at baseline.

Following 6 months of vitamin D3 supplementation, the target serum 25-hydroxyvitamin D (≥30ng/mL) was achieved in 83.9%. In the remaining patients, 9.7% were insufficient for vitamin D (20-29ng/mL) and 6.4% still had vitamin D deficiency (<20ng/mL). At 12 months, 35.5% had achieved the target level of vitamin D, 54.8% had vitamin D insufficiency and 9.7% had vitamin D deficiency. An assessment of adherence to vitamin D3 supplements was made based on the quantity of vitamin D3 boxes collected. All the boxes required to follow the recommended regime were not picked up by 64.5% of the participants. The baseline clinical characteristics of the cohort are summarised in table 3.38.

Table 3.38. Clinical characteristics of the cohort that was followed up in the prospective study.

Characteristics	**Values**
Age, mean (S.D.) years	47.9 (13.7)
Female sex , n/N (%)	28/31 (90.3)
Caucasian race, n/N (%)	30/31 (96.8)
Disease duration, mean (S.D.) years	14.1 (8.0)
Age at SLE diagnosis, mean (S.D.) years	33.8 (13.5)
BMI, mean (S.D.) kg/ m^2	29.8 (8.1)
Current smoker, n/N (%)	3/31 (9.7)
Ex-smoker, n/N (%)	8/31 (25.8)
Current use of sunscreen, n/N (%)	19/31 (61.3)
Regular exercise, n/N (%)	17/31 (54.8)
Any co-morbidity, n/N (%)	26/31 (83.9)
Osteopaenia/osteoporosis, n/N (%)	8/31 (25.8)
Hypertension, n/N (%)	8/31 (25.8)
Hyperlipidaemia on treatment, n/N (%)	2/31 (6.5)
Diabetes mellitus, n/N (%)	4/31 (12.9)
Fibromyalgia, n/N (%)	4/31 (12.9)
Anti-phospholipid syndrome, n/N (%)	3/31 (9.7)
Sjogren's syndrome, n/N (%)	2/31 (6.5)
Rheumatoid arthritis, n/N (%)	2/31 (6.5)
Current prednisolone, n/N (%)	7/31 (22.6)
Current hydroxychloroquine, n/N (%)	18/31 (58.1)
Current azathioprine, n/N (%)	5/31 (16.1)
Current methotrexate, n/N (%)	4/31 (12.9)
Current calcium supplementation, n/N (%)	11/31 (35.5)
Current vitamin D supplementation, n/N (%)	11/31 (35.5)
SLEDAI 2K, median (IQR)	4 (2)
SDI, median (IQR)	0 (1)

Mean and standard deviation are given for normally distributed variables. Median and range are given for variables that are not normally distributed. BMI, body mass index;

IQR, interquartile range; S.D., standard deviation; SDI, Systemic Lupus International Collaborating Clinics/American College of Rheumatology damage index; SLEDAI-2K, Systemic Lupus Erythematosus Disease Activity Index 2000.

3.3.2 TESTING FOR NORMALITY OF DATA

Testing for normality of continuous variables was carried out to indicate whether parametric or non-parametric tests were to be utilised during data analysis. The normality of the distribution of continuous variables was tested for by the K-S test. Table 3.39 shows the K-S results. For those variables that had a p value obtained by the K-S test higher than 0.05, the null hypothesis was accepted; meaning that they were normally distributed. The other continuous variables, whose p value obtained was less than 0.05, were not normally distributed.

Table 3.39. Kolmogorov-Smirnov test results for continuous variables in the 31 patients included in the prospective study.

Variable	Baseline		6 months		12 months	
	Test Statistic	P value	Test Statistic	P value	Test Statistic	P value
Age	0.132	0.179				
Age at disease diagnosis	0.098	0.200				
Disease duration	0.108	0.200				
BMI	0.131	0.186	0.147	0.085	0.169	0.024
Daily dose of hydroxychloroquine	0.274	<0.001	0.278	<0.001	0.295	<0.001
Daily dose of prednisolone	0.463	<0.001	0.441	<0.001	0.487	<0.001
Daily dose of calcium	0.380	<0.001	0.428	<0.001	0.428	<0.001
Daily dose of Vitamin D	0.360	<0.001	0.296	<0.001	0.269	<0.001
SLEDAI-2K	0.214	0.001	0.201	0.003	0.228	<0.001
Clinical SLEDAI-2K	0.355	<0.001	0.361	<0.001	0.421	<0.001
SDI	0.307	<0.001	0.307	<0.001	0.298	<0.001
FSS	0.104	0.200	0.124	0.200	0.185	0.008
VAS Fatigue	0.211	0.001	0.111	0.200	0.151	0.069
VAS Pain	0.155	0.057	0.141	0.120	0.217	0.001
HADS-D	0.135	0.157	0.176	0.016	0.155	0.057
HADS-A	0.148	0.081	0.116	0.200	0.123	0.200
PSQI	0.140	0.129	0.160	0.043	0.142	0.114
mHAQ	0.264	<0.001	0.249	<0.001	0.286	<0.001
Haemoglobin	0.082	0.200	0.086	0.200	0.086	0.200
eGFR	0.086	0.200	0.104	0.200	0.133	0.176
Serum calcium	0.097	0.200	0.118	0.200	0.076	0.200
Serum 25-hydroxyvitamin D	0.180	0.012	0.180	0.012	0.113	0.200
C3	0.139	0.135	0.091	0.200	0.114	0.200
C4	0.097	0.200	0.114	0.200	0.082	0.200
Anti-dsDNA titre	0.204	0.002	0.169	0.025	0.174	0.018

Anti-dsDNA, anti-double-stranded deoxyribonucleic acid; BMI, body mass index; C3, complement 3; C4, complement 4; eGFR, estimated glomerular filtration rate; FSS, Fatigue Severity Scale; HADS-A, Hospital Anxiety and Depression Scale – anxiety subscale; HADS-D, Hospital Anxiety and Depression Scale – depression subscale;

mHAQ, modified Health Assessment Questionnaire; PSQI, Pittsburgh Sleep Quality Index; SDI, Systemic Lupus International Collaborating Clinics/American College of Rheumatology damage index; SLEDAI-2K, Systemic Lupus Erythematosus Disease Activity Index 2000; VAS, Visual Analogue Scale.

3.3.3 COMPARING CONTINUOUS VARIABLES AT BASELINE, AFTER 6 MONTHS AND AFTER 12 MONTHS OF VITAMIN D3 TREATMENT

The paired samples t-test and Wilcoxon signed ranks test were used to compare continuous variables before and after vitamin D3 supplementation. The latter test is a non-parametric alternative of the former. Both tests were carried out when one value was normally distributed and the other was not. The null hypothesis specified that the scores before and after treatment, varied marginally and was accepted if the p value was greater than 0.05. The alternative hypothesis stated that the scores varied significantly and was accepted if the p value was less than 0.05. Table 3.40 shows the p values obtained on comparing the continuous variables at baseline to those at 6 and 12 months of vitamin D3 supplementation.

Table 3.40. Results obtained with the paired samples t-test and Wilcoxon signed ranks test for continuous variables at baseline and after 6 and 12 months of vitamin D3 supplementation.

Variable	Time	Mean	S.D.	Median	Inter-quartile range	p value Paired T-test	p value Wilcoxon signed ranks test
BMI (kg/m^2)	Baseline	29.84	8.121	27.9	10.2		
	6 months	29.53	7.410	27.1	10.0	0.271	
	12 months	29.26	7.704	27.1	11.4	0.604	0.272
SLEDAI-2K	Baseline	3.55	2.998	4.0	2.0		
	6 months	3.06	2.707	2.0	3.0		0.324
	12 months	2.45	2.158	2.0	4.0		**0.028**
Clinical SLEDAI-2K	Baseline	1.97	2.614	0.0	4.0		
	6 months	1.39	2.076	0.0	2.0		0.258
	12 months	0.90	1.620	0.0	2.0		**0.024**
SDI	Baseline	0.65	0.798	0.0	1.0		
	6 months	0.65	0.798	0.0	1.0		1.000
	12 months	0.68	0.871	0.0	1.0		0.317
FSS	Baseline	4.10	1.514	4.11	2.77		
	6 months	3.95	1.623	4.22	2.66	0.465	
	12 months	3.70	1.852	2.89	3.11	0.071	0.117
VAS Fatigue	Baseline	4.19	2.428	5.0	4.0		
	6 months	4.84	2.647	5.0	4.0	0.196	0.171
	12 months	4.06	2.707	5.0	4.0	0.763	0.755
VAS Pain	Baseline	3.74	2.966	4.0	6.0		
	6 months	3.97	3.027	4.0	4.0	0.603	
	12 months	3.35	3.115	2.0	5.0	0.276	0.341
HADS-D	Baseline	4.81	3.674	4.0	5.0		
	6 months	5.55	3.767	4.0	7.0	0.084	0.089
	12 months	4.48	4.186	3.0	6.0	0.389	
HADS-A	Baseline	8.58	4.703	9.0	7.0		
	6 months	8.26	4.389	9.0	7.0	0.499	
	12 months	8.29	4.776	7.0	8.0	0.605	
PSQI	Baseline	6.66	4.218	7.0	5.0		
	6 months	6.52	4.396	6.0	6.0	0.943	0.961
	12 months	6.16	4.352	5.0	6.0	0.289	
mHAQ	Baseline	0.226	0.357	0.13	0.25		

	6 months	0.290	0.402	0.13	0.63		0.146
	12 months	0.282	0.444	0.00	0.50		0.111
Haemoglobin (12/0-15.5g/dL)	Baseline	13.29	1.344	13.4	1.9		
	6 months	13.43	1.323	13.6	1.9	0.321	
	12 months	13.45	1.407	13.2	1.8	0.303	
eGFR (>60mls/min/ 1.73 m^2)	Baseline	102.16	16.625	101	20		
	6 months	98.26	17.699	99	19	0.155	
	12 months	100.13	20.268	100	23	0.476	
Corrected calcium (2.05-2.60 mmol/l)	Baseline	2.30	0.092	2.29	0.11		
	6 months	2.28	0.085	2.28	0.10	0.139	
	12 months	2.29	0.092	2.29	0.14	0.647	
25-hydroxyvitamin D (30-100ng/ml)	Baseline	21.74	6.444	23	15		
	6 months	32.61	6.396	33	6		**<0.001**
	12 months	28.32	8.368	27	11	**0.001**	**0.001**
C3 (900-1800mg/l)	Baseline	1074.8	253.032	1090	391		
	6 months	1042.0	231.402	1018	307	0.090	
	12 months	1095.7	244.181	1055	373	0.291	
C4 (100-400mg/l)	Baseline	230.58	91.787	228	147		
	6 months	230.35	98.207	215	97	0.973	
	12 months	227.87	93.202	214	123	0.659	
Anti-dsDNA titre (0-100IU/ml)	Baseline	172.26	184.217	93.4	307.9		
	6 months	154.99	151.173	111.6	286.9		**0.032**
	12 months	154.11	153.276	100.5	229.5		**0.045**
HCQ daily dose (mg)	Baseline	200.00	186.190	200	400		
	6 months	187.10	185.727	200	400		0.414
	12 months	187.10	178.404	200	400		0.157
Prednisolone daily dose (mg)	Baseline	1.403	2.850	0.0	0.0		
	6 months	1.550	2.973	0.0	2.5		0.144
	12 months	0.937	2.459	0.0	0.0		0.068
Calcium daily dose (mg)	Baseline	270.97	431.427	0.0	400		
	6 months	161.29	278.900	0.0	400		0.068
	12 months	190.32	328.993	0.0	400		0.109
Vitamin D daily dose (IU)	Baseline	187.1	330.396	0	200		
	6 months	2723.7	1475.368	2000	2000		**<0.001**
	12 months	2485.3	1917.331	2000	800		**<0.001**

The p values have been obtained on comparing the continuous variables at 6 and 12 months with those at baseline. Standard values of laboratory tests are shown in brackets. Anti-dsDNA, anti-double-stranded deoxyribonucleic acid; BMI, body mass

index; C3, complement 3; C4, complement 4; eGFR, estimated glomerular filtration rate; FSS, Fatigue Severity Scale; HADS-A, Hospital Anxiety and Depression Scale – anxiety subscale; HADS-D, Hospital Anxiety and Depression Scale – depression subscale; mHAQ, modified Health Assessment Questionnaire; PSQI, Pittsburgh Sleep Quality Index; S.D., standard deviation; SDI, Systemic Lupus International Collaborating Clinics/American College of Rheumatology damage index; SLEDAI-2K, Systemic Lupus Erythematosus Disease Activity Index 2000; VAS, Visual Analogue Scale.

The alternative hypothesis, stating that the variables changed significantly from baseline to 6 months of vitamin D3 supplementation was accepted for serum 25-hydroxyvitamin D, anti-dsDNA titre, and daily dose of vitamin D. The alternative hypothesis, stating that the variables changed significantly from baseline to 12 months of vitamin D3 supplementation was accepted for SLEDAI-2K, clinical SLEDAI-2K, serum 25-hydroxyvitamin D, anti-dsDNA titre, and daily dose of vitamin D. These results are displayed in the error bar graphs (figure 3.38).

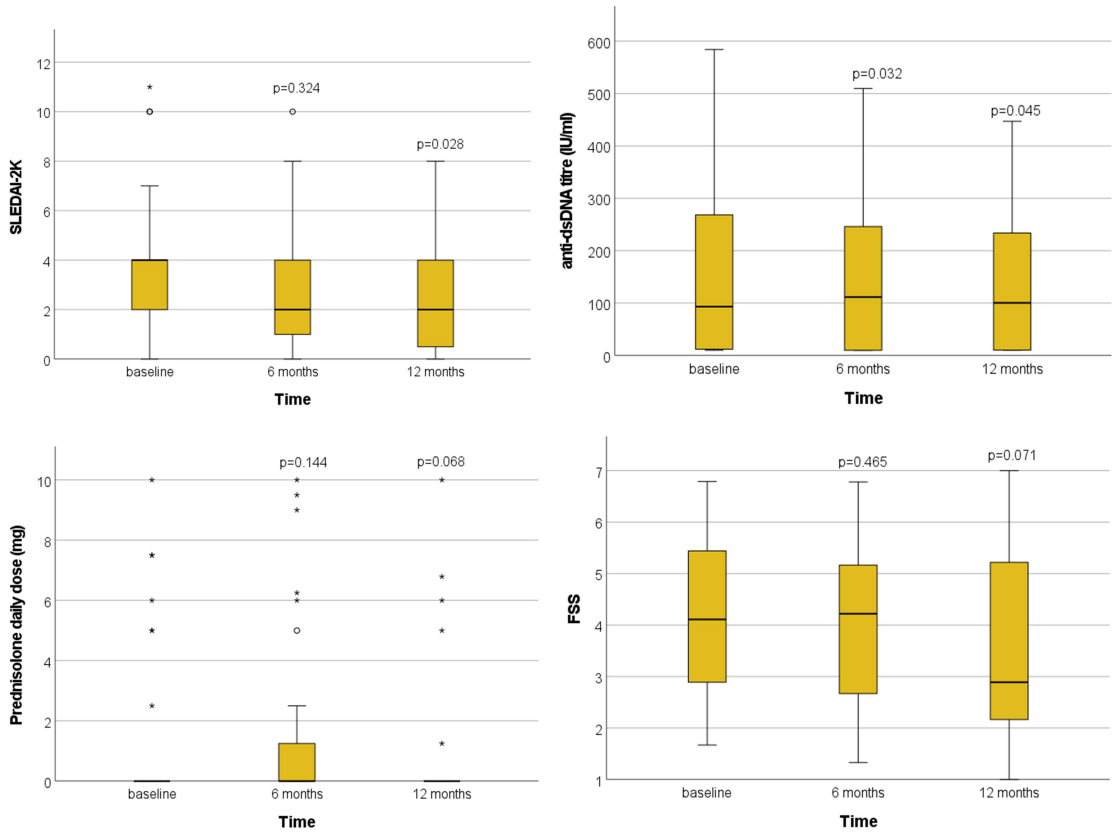

Figure 3.38. Box plots comparing results at baseline, 6 months and 12 months for SLEDAI-2K, anti-dsDNA titre, prednisolone daily dose and FSS.

Anti-dsDNA, anti-double-stranded deoxyribonucleic acid; FSS, Fatigue Severity Scale; SLEDAI-2K, Systemic Lupus Erythematosus Disease Activity Index 2000.

3.3.4 Testing for Normality of Data for Vitamin D Deficient patients

The K-S test was once again used to test for normality of the distribution of continuous variables, when analysing the 13 patients who had vitamin D deficiency at baseline. For those variables that had a p value obtained by the K-S test higher than 0.05, the null hypothesis was accepted; meaning that they are normally distributed. The other continuous variables, whose p value obtained was less than 0.05, were not normally distributed. Table 3.41 shows the K-S test results.

Table 3.41. Kolmogorov-Smirnov test results for continuous variables in patients with vitamin D deficiency at baseline.

Variable	Baseline		6 months		12 months	
	Test Statistic	P value	Test Statistic	P value	Test Statistic	P value
SLEDAI-2K	0.224	0.075	0.197	0.178	0.302	0.002
Clinical SLEDAI-2K	0.278	0.007	0.392	<0.001	0.461	<0.001
SDI	0.283	0.005	0.283	0.005	0.258	0.018
FSS	0.143	0.200	0.163	0.200	0.150	0.200
VAS Fatigue	0.292	0.003	0.192	0.200	0.148	0.200
HADS-D	0.141	0.200	0.211	0.118	0.124	0.200
HADS-A	0.140	0.200	0.136	0.200	0.140	0.200
PSQI	0.212	0.112	0.191	0.200	0.194	0.195
mHAQ	0.311	0.001	0.329	<0.001	0.326	0.001
Haemoglobin	0.122	0.200	0.160	0.200	0.173	0.200
eGFR	0.168	0.200	0.167	0.200	0.186	0.200
Serum calcium	0.173	0.200	0.194	0.195	0.167	0.200
Serum 25-hydroxyvitamin D	0.375	<0.001	0.164	0.200	0.192	0.200
C3	0.184	0.200	0.242	0.036	0.161	0.200
C4	0.177	0.200	0.144	0.200	0.147	0.200
Anti-dsDNA titre	0.339	<0.001	0.320	0.001	0.299	0.002
Daily dose of hydroxychloroquine	0.288	0.004	0.295	0.003	0.288	0.004
Daily dose of prednisolone	0.468	<0.001	0.470	<0.001	0.502	<0.001
Daily dose of calcium	0.415	<0.001	0.456	<0.001	0.461	<0.001
Daily dose of Vitamin D	0.415	<0.001	0.339	<0.001	0.216	0.099

Anti-dsDNA, anti-double-stranded deoxyribonucleic acid; C3, complement 3; C4, complement 4; eGFR, estimated glomerular filtration rate; FSS, Fatigue Severity Scale; HADS-A, Hospital Anxiety and Depression Scale – anxiety subscale; HADS-D, Hospital Anxiety and Depression Scale – depression subscale; mHAQ, modified Health Assessment Questionnaire; PSQI, Pittsburgh Sleep Quality Index; SDI, Systemic Lupus International Collaborating Clinics/American College of Rheumatology damage index; SLEDAI-2K, Systemic Lupus Erythematosus Disease Activity Index 2000; VAS, Visual Analogue Scale.

3.3.5 COMPARING CONTINUOUS VARIABLES AT BASELINE, AFTER 6 MONTHS AND AFTER 12 MONTHS OF VITAMIN D3 TREATMENT IN THE COHORT OF VITAMIN D DEFICIENT PATIENTS

The paired samples t-test and Wilcoxon signed ranks test were used to compare continuous variables before and after vitamin D3 supplementation in the cohort of vitamin D deficient patients at baseline. Table 3.42 shows the results of the continuous variables at baseline and after 6 and 12 months of vitamin D3 supplementation.

Table 3.42. Results obtained with the paired samples t-test and Wilcoxon signed ranks test for continuous variables at baseline, after 6 months and after 12 months of vitamin D3 supplementation in the cohort of vitamin D deficient patients at baseline.

Variable	Time	Mean	S.D.	Median	Inter-quartile range	p value Paired T-test	p value Wilcoxon signed ranks test
SLEDAI-2K	Baseline	3.62	3.355	3.0	4.0		
	6 months	2.00	2.345	2.0	4.0	**0.030**	
	12 months	1.77	2.088	2.0	2.0	**0.046**	0.062
Clinical SLEDAI-2K	Baseline	2.46	2.727	2.0	4.0		
	6 months	1.00	1.915	0.0	2.0		**0.042**
	12 months	0.77	1.536	0.0	1.0		0.079
SDI	Baseline	0.69	0.751	1.0	1.0		
	6 months	0.69	0.751	1.0	1.0		1.000
	12 months	0.77	0.927	1.0	1.0		0.317
FSS	Baseline	3.78	1.681	3.33	3.17		
	6 months	3.43	1.718	2.89	2.50	0.265	
	12 months	3.71	1.798	4.22	3.00	0.830	
VAS Fatigue	Baseline	3.85	2.512	5.0	5.0		
	6 months	5.00	2.614	5.0	4.0	0.209	0.194
	12 months	4.23	2.774	5.0	4.0	0.620	0.573
HADS-D	Baseline	5.23	3.586	5.0	5.0		
	6 months	5.85	4.140	4.0	7.0	0.426	
	12 months	4.77	3.833	5.0	7.0	0.495	
HADS-A	Baseline	7.85	4.634	9.0	9.0		
	6 months	8.00	4.546	9.0	9.0	0.867	
	12 months	8.00	5.180	7.0	10.0	0.896	
PSQI	Baseline	6.38	4.312	6.0	6.0		
	6 months	6.92	4.838	6.0	6.0	0.495	
	12 months	5.69	4.231	4.0	5.0	0.239	
mHAQ	Baseline	0.212	0.428	0.00	0.25		
	6 months	0.288	0.511	0.00	0.50		0.202
	12 months	0.221	0.399	0.00	0.31		0.783
Haemoglobin (12/0-15.5g/dL)	Baseline	13.18	1.414	13.4	2.2		
	6 months	13.45	1.437	13.0	2.2	0.306	
	12 months	13.46	1.251	13.2	1.0	0.264	
eGFR	Baseline	99.54	17.775	99	19		

(>60mls/min/ 1.73 m^2)	6 months	94.08	21.180	101	24	0.201	
	12 months	93.92	23.347	94	21	0.237	
Corrected calcium (2.05-2.60 mmol/l)	Baseline	2.31	0.113	2.29	0.20		
	6 months	2.31	0.0971	2.30	0.15	0.870	
	12 months	2.29	0.108	2.27	0.16	0.371	
25-hydroxyvitamin D (30-100ng/ml)	Baseline	14.92	2.722	13.0	6.0		
	6 months	32.62	5.026	33.0	8.0	**<0.001**	**0.001**
	12 months	27.00	7.724	27.0	9.0	**<0.001**	**0.002**
C3 (900-1800mg/l)	Baseline	1141.85	297.661	1090	547		
	6 months	1083.08	247.888	1021	275	0.110	0.142
	12 months	1120.92	243.441	1063	419	0.532	
C4 (100-400mg/l)	Baseline	225.38	89.057	257	151		
	6 months	221.69	77.310	215	84	0.675	
	12 months	212.31	73.724	210	109	0.137	
Anti-dsDNA titre (0-100IU/ml)	Baseline	127.45	194.223	15.3	196.4		
	6 months	116.81	174.366	10.0	191.5		0.110
	12 months	109.54	161.386	14.3	168.8		0.114
HCQ daily dose (mg)	Baseline	169.23	179.743	200	400		
	6 months	184.62	190.815	200	400		0.317
	12 months	169.23	179.743	200	400		1.000
Prednisolone daily dose (mg)	Baseline	1.92	3.702	0.00	3.75		
	6 months	2.19	4.171	0.00	4.50		0.180
	12 months	1.29	3.220	0.00	0.00		0.180
Calcium daily dose (mg)	Baseline	307.69	521.954	0.00	750		
	6 months	153.85	315.213	0.00	250		0.180
	12 months	192.31	383.974	0.00	250		0.317
Vitamin D daily dose (IU)	Baseline	246.15	417.563	0	600		
	6 months	2687.15	1157.099	2000	2000		**0.001**
	12 months	2395.62	1552.949	2000	2429	**<0.001**	**0.003**

The p values have been obtained on comparing the continuous variables at 6 and 12 months with those at baseline. Standard values of laboratory tests are shown in brackets. Anti-dsDNA, anti-double-stranded deoxyribonucleic acid; C3, complement 3; C4, complement 4; eGFR, estimated glomerular filtration rate; FSS, Fatigue Severity Scale; HADS-A, Hospital Anxiety and Depression Scale – anxiety subscale; HADS-D, Hospital Anxiety and Depression Scale – depression subscale; mHAQ, modified Health Assessment Questionnaire; PSQI, Pittsburgh Sleep Quality Index; S.D., standard deviation; SDI, Systemic Lupus International Collaborating Clinics/American College of

Rheumatology damage index; SLEDAI-2K, Systemic Lupus Erythematosus Disease Activity Index 2000; VAS, Visual Analogue Scale.

The alternative hypothesis, stating that the variables changed significantly from baseline to 6 months and from baseline to 12 months was accepted for SLEDAI-2K, serum 25-hydroxyvitamin D and daily dose of vitamin D. It was also accepted for the decrease in clinical SLEDAI-2K from baseline to 6 months.

3.3.6 TESTING FOR NORMALITY OF DATA FOR VITAMIN D INSUFFICIENT PATIENTS

The K-S Test was carried out to test for normality of the distribution of continuous variables, when analysing the 18 patients with vitamin D insufficiency at baseline (table 3.43).

Table 3.43. Kolmogorov-Smirnov test results for continuous variables in patients with vitamin D insufficiency at baseline.

Variable	Baseline		6 months		12 months	
	Test Statistic	P value	Test Statistic	P value	Test Statistic	P value
SLEDAI-2K	0.207	0.040	0.192	0.078	0.199	0.058
Clinical SLEDAI-2K	0.403	<0.001	0.332	<0.001	0.387	<0.001
SDI	0.320	<0.001	0.320	<0.001	0.320	<0.001
FSS	0.100	0.200	0.157	0.200	0.216	0.026
VAS Fatigue	0.147	0.200	0.162	0.200	0.151	0.200
HADS-D	0.163	0.200	0.148	0.200	0.222	0.019
HADS-A	0.153	0.200	0.116	0.200	0.128	0.200
PSQI	0.155	0.200	0.132	0.200	0.114	0.200
mHAQ	0.222	0.019	0.209	0.036	0.252	0.004
Haemoglobin	0.110	0.200	0.168	0.192	0.136	0.200
eGFR	0.107	0.200	0.152	0.200	0.120	0.200
Serum calcium	0.136	0.200	0.110	0.200	0.141	0.200
Serum 25-hydroxyvitamin D	0.217	0.024	0.231	0.012	0.124	0.200
C3	0.131	0.200	0.139	0.200	0.129	0.200
C4	0.096	0.200	0.154	0.200	0.110	0.200
Anti-dsDNA titre	0.192	0.077	0.184	0.111	0.159	0.200
Daily dose of hydroxychloroquine	0.322	<0.001	0.288	<0.001	0.254	0.003
Daily dose of prednisolone	0.468	<0.001	0.432	<0.001	0.482	<0.001
Daily dose of calcium	0.359	<0.001	0.407	<0.001	0.406	<0.001
Daily dose of Vitamin D	0.326	<0.001	0.304	<0.001	0.342	<0.001

Anti-dsDNA, anti-double-stranded deoxyribonucleic acid; C3, complement 3; C4, complement 4; eGFR, estimated glomerular filtration rate; FSS, Fatigue Severity Scale; HADS-A, Hospital Anxiety and Depression Scale – anxiety subscale; HADS-D, Hospital Anxiety and Depression Scale – depression subscale; mHAQ, modified Health Assessment Questionnaire; PSQI, Pittsburgh Sleep Quality Index; SDI, Systemic Lupus International Collaborating Clinics/American College of Rheumatology damage index; SLEDAI-2K, Systemic Lupus Erythematosus Disease Activity Index 2000; VAS, Visual Analogue Scale.

3.3.7 COMPARING CONTINUOUS VARIABLES AT BASELINE, AFTER 6 MONTHS AND AFTER 12 MONTHS OF VITAMIN D3 TREATMENT IN THE COHORT OF VITAMIN D INSUFFICIENT PATIENTS

The paired samples t-test and Wilcoxon signed ranks test were used to compare continuous variables before and after vitamin D3 supplementation in the cohort of 18 patients with vitamin D insufficiency at baseline (table 3.44).

Table 3.44. Results obtained with the paired samples t-test and Wilcoxon signed ranks test for continuous variables at baseline, after 6 months and after 12 months of vitamin D3 supplementation in the cohort of vitamin D insufficient patients at baseline.

Variable	Time	Mean	S.D.	Median	Inter-quartile range	p value Paired T-test	p value Wilcoxon signed ranks test
SLEDAI-2K	Baseline	3.50	2.813	4.0	3.0		
	6 months	3.83	2.749	4.0	4.0	0.623	0.522
	12 months	2.94	2.127	3.0	2.0	0.336	0.380
Clinical SLEDAI-2K	Baseline	1.61	2.547	0.0	4.0		
	6 months	1.67	2.196	0.0	4.0		0.854
	12 months	1.00	1.715	0.0	2.0		0.187
SDI	Baseline	0.61	0.850	0.0	1.0		
	6 months	0.61	0.850	0.0	1.0		1.000
	12 months	0.61	0.850	0.0	1.0		1.000
FSS	Baseline	4.33	1.383	4.39	2.19		
	6 months	4.31	1.490	4.61	2.58	0.960	
	12 months	3.69	1.941	2.84	3.62	**0.039**	0.052
VAS Fatigue	Baseline	4.44	2.406	5.0	4.0		
	6 months	4.72	2.740	4.5	5.0	0.629	
	12 months	3.94	2.733	4.0	5.0	0.319	
HADS-D	Baseline	4.50	3.808	4.0	6.0		
	6 months	5.33	3.581	4.5	5.0	0.105	
	12 months	4.28	4.522	2.5	6.0	0.620	0.611
HADS-A	Baseline	9.11	4.813	9.5	8.0		
	6 months	8.44	4.395	9.0	6.0	0.199	
	12 months	8.50	4.605	8.0	7.0	0.238	
PSQI	Baseline	6.67	4.270	7.0	6.0		
	6 months	6.22	4.166	6.0	7.0	0.415	
	12 months	6.50	4.528	6.5	7.0	0.729	
mHAQ	Baseline	0.236	0.309	0.13	0.38		
	6 months	0.292	0.318	0.19	0.63		0.404
	12 months	0.326	0.480	0.06	0.63		0.108
Haemoglobin (12/0-15.5g/dL)	Baseline	13.38	1.325	13.4	2.1		
	6 months	13.42	1.277	13.7	2.0	0.792	
	12 months	13.44	1.546	13.4	3.2	0.748	
eGFR	Baseline	104.06	15.991	101.5	21		

(>60mls/min/ 1.73 m^2)	6 months	101.28	14.600	99.0	26	0.455	
	12 months	104.61	17.013	104.5	20	0.878	
Corrected calcium (2.05-2.60 mmol/l)	Baseline	2.29	0.076	2.29	0.10		
	6 months	2.26	0.070	2.26	0.10	0.102	
	12 months	2.30	0.082	2.30	0.13	0.825	
25-hydroxyvitamin D (30-100ng/ml)	Baseline	26.67	2.612	27.5	4.0		
	6 months	31.89	7.283	33.0	6.0		**0.022**
	12 months	29.28	8.897	28.5	12.0	0.243	0.266
C3 (900-1800mg/l)	Baseline	1026.44	211.021	1054	296		
	6 months	1012.39	221.134	971	417	0.503	
	12 months	1077.44	250.093	1029	381	**0.032**	
C4 (100-400mg/l)	Baseline	234.33	96.091	219.5	149		
	6 months	236.61	112.703	214.5	125	0.821	
	12 months	239.11	105.706	228.5	146	0.575	
Anti-dsDNA titre (0-100IU/ml)	Baseline	204.63	174.969	154.2	285.5		
	6 months	182.57	130.182	179.4	214.7	0.227	
	12 months	186.30	143.031	186.0	256.8	0.438	
HCQ daily dose (mg)	Baseline	222.22	192.676	300	400		
	6 months	188.89	187.519	200	400		0.180
	12 months	200.00	181.497	200	400		0.157
Prednisolone daily dose (mg)	Baseline	1.028	2.076	0.0	0.63		
	6 months	1.236	2.237	0.0	2.50		0.180
	12 months	0.681	1.786	0.0	0		0.180
Calcium daily dose (mg)	Baseline	244.44	366.578	0.0	400		
	6 months	166.67	258.957	0.0	400		0.180
	12 months	188.89	294.836	0.0	400		0.180
Vitamin D daily dose (IU)	Baseline	144.44	254.887	0	200		
	6 months	2750.00	1701.297	2000	2000		**<0.001**
	12 months	25550.00	2185.043	2000	275		**<0.001**

The p values have been obtained on comparing the continuous variables at 6 and 12 months with those at baseline. Standard values of laboratory tests are shown in brackets. Anti-dsDNA, anti-double-stranded deoxyribonucleic acid; C3, complement 3; C4, complement 4; eGFR, estimated glomerular filtration rate; FSS, Fatigue Severity Scale; HADS-A, Hospital Anxiety and Depression Scale – anxiety subscale; HADS-D, Hospital Anxiety and Depression Scale – depression subscale; mHAQ, modified Health Assessment Questionnaire; PSQI, Pittsburgh Sleep Quality Index; S.D., standard deviation; SDI, Systemic Lupus International Collaborating Clinics/American College of

Rheumatology damage index; SLEDAI-2K, Systemic Lupus Erythematosus Disease Activity Index 2000; VAS, Visual Analogue Scale.

The alternative hypothesis, stating that the variables changed significantly from baseline to 6 months of vitamin D3 supplementation was accepted for serum 25-hydroxyvitamin D ($p=0.022$) and daily dose of vitamin D ($p<0.001$). The alternative hypothesis, stating that the variables changed significantly from baseline to 12 months of vitamin D3 supplementation was accepted for FSS ($p=0.039$), C3 ($p=0.032$) and daily dose of vitamin D ($p<0.001$).

3.3.8 COMPARING CONTINUOUS VARIABLES AT BASELINE AND AFTER 6 MONTHS OF VITAMIN D3 TREATMENT IN THE COHORT OF PATIENTS WHO OBTAINED TARGET SERUM 25-HYDROXYVITAMIN D AT 6 MONTHS

The analysis was repeated by excluding patients who did not achieve the target level of serum 25-hydroxyvitamin D (30ng/ml) after 6 months of vitamin D3 supplementation. Thus 26 patients were included in this analysis. Testing for normality of the continuous variables was first carried out using the K-S test (table 3.45).

Table 3.45. Kolmogorov-Smirnov test results for continuous variables in the cohort that achieved target level of serum 25-hydroxyvitamin D (≥30ng/ml) at 6 months.

Variable	Baseline		6 months	
	Test Statistic	P value	Test Statistic	P value
SLEDAI-2K	0.243	<0.001	0.184	0.023
Clinical SLEDAI-2K	0.398	<0.001	0.394	<0.001
FSS	0.121	0.200	0.132	0.200
VAS Fatigue	0.223	0.002	0.137	0.200
VAS Pain	0.184	0.023	0.126	0.200
HADS-D	0.154	0.113	0.179	0.032
HADS-A	0.140	0.200	0.135	0.200
PSQI	0.137	0.200	0.150	0.137
mHAQ	0.301	<0.001	0.326	<0.001
Serum 25-hydroxyvitamin D	0.184	0.024	0.175	0.039
C3	0.137	0.200	0.115	0.200
C4	0.092	0.200	0.151	0.130
Anti-dsDNA titre	0.176	0.038	0.152	0.127

Anti-dsDNA, anti-double-stranded deoxyribonucleic acid; C3, complement 3; C4, complement 4; FSS, Fatigue Severity Scale; HADS-A, Hospital Anxiety and Depression Scale – anxiety subscale; HADS-D, Hospital Anxiety and Depression Scale – depression subscale; mHAQ, modified Health Assessment Questionnaire; PSQI, Pittsburgh Sleep Quality Index; SLEDAI-2K, Systemic Lupus Erythematosus Disease Activity Index 2000; VAS, Visual Analogue Scale.

The paired samples t-test and Wilcoxon signed ranks test were used to compare continuous variables before and after vitamin D3 supplementation (table 3.46).

Table 3.46. Results obtained with the paired samples t-test and Wilcoxon signed ranks test for continuous variables at baseline and after 6 months of vitamin D3 supplementation in the cohort who achieved target serum 25-hydroxyvitamin D at 6 months.

Variable	Time	Mean	S.D.	Median	Inter-quartile range	p value Paired T-test	p value Wilcoxon signed ranks test
SLEDAI-2K	Baseline	3.50	3.076	3.5	2.0		0.751
	6 months	3.31	2.724	2.5	3.0		
Clinical SLEDAI-2K	Baseline	1.62	2.467	0.0	4.0		0.596
	6 months	1.31	2.035	0.0	3.0		
FSS	Baseline	3.87	1.482	3.56	3.06	0.600	
	6 months	3.75	1.567	3.56	2.66		
VAS Fatigue	Baseline	3.88	2.321	5.0	3.0	0.188	0.144
	6 months	4.62	2.547	5.0	3.0		
VAS Pain	Baseline	3.27	2.822	3.0	6.0	0.700	0.757
	6 months	3.46	2.760	3.5	5.0		
HADS-D	Baseline	4.46	3.679	3.5	5.0	0.056	0.062
	6 months	5.36	3.741	4.0	5.0		
HADS-A	Baseline	8.27	4.771	8.5	8.0	0.350	
	6 months	7.88	4.546	9.0	7.0		
PSQI	Baseline	6.23	4.003	7.0	5.0	0.606	
	6 months	5.96	3.965	5.5	5.0		
mHAQ	Baseline	0.115	0.162	0.00	0.25		0.194
	6 months	0.183	0.272	0.00	0.28		
C3 (900-1800mg/l)	Baseline	1040.0	246.278	988.5	304	0.312	
	6 months	1020.5	233.276	971.0	297		
C4 (100-400mg/l)	Baseline	214.31	89.895	205.0	129	0.764	
	6 months	216.65	98.753	197.5	92		
Anti-dsDNA titre (0-100IU/ml)	Baseline	200.02	188.437	142.85	337.25	0.126	**0.046**
	6 months	179.64	152.580	153.00	271.10		
Serum 25-hydroxyvitamin D (30-100ng/ml)	Baseline	21.54	6.507	22.5	15.0		**<0.001**
	6 months	34.77	3.892	33.5	6.0		

The p values have been obtained on comparing the continuous variables at 6 and 12 months with those at baseline. Standard values of laboratory tests are shown in brackets. Anti-dsDNA, anti-double-stranded deoxyribonucleic acid; C3, complement 3; C4, complement 4; FSS, Fatigue Severity Scale; HADS-A, Hospital Anxiety and Depression Scale – anxiety subscale; HADS-D, Hospital Anxiety and Depression Scale –

depression subscale; mHAQ, modified Health Assessment Questionnaire; PSQI, Pittsburgh Sleep Quality Index; S.D., standard deviation; SLEDAI-2K, Systemic Lupus Erythematosus Disease Activity Index 2000; VAS, Visual Analogue Scale.

The alternative hypothesis, stating that the variables vary significantly from baseline to 6 months of vitamin D3 supplementation was accepted for serum anti-dsDNA titre and serum 25-hydroxyvitamin D.

3.3.9 COMPARING CONTINUOUS VARIABLES AT BASELINE AND AFTER 12 MONTHS OF VITAMIN D3 TREATMENT IN THE COHORT OF PATIENTS WHO OBTAINED TARGET SERUM 25-HYDROXYVITAMIN D AT 12 MONTHS

The analysis was repeated by excluding patients who did not achieve the target level of serum 25-hydroxyvitamin D (30ng/ml) after 12 months of vitamin D3 supplementation. 11 patients were included in this analysis. Testing for normality of the continuous variables was first carried out using the K-S test (table 3.47).

Table 3.47. Kolmogorov-Smirnov test results for continuous variables in the cohort that achieved normal level of serum 25-hydroxyvitamin D (≥30ng/ml) at 12 months.

Variable	Baseline		12 months	
	Test Statistic	P value	Test Statistic	P value
SLEDAI-2K	0.248	0.058	0.288	0.011
Clinical SLEDAI-2K	0.320	0.002	0.482	<0.001
FSS	0.142	0.200	0.171	0.200
VAS Fatigue	0.216	0.162	0.199	0.200
VAS Pain	0.159	0.200	0.244	0.067
HADS-D	0.177	0.200	0.264	0.031
HADS-A	0.198	0.200	0.177	0.200
PSQI	0.181	0.200	0.200	0.200
mHAQ	0.369	<0.001	0.377	<0.001
Serum 25-hydroxyvitamin D	0.264	0.031	0.173	0.200
C3	0.119	0.200	0.140	0.200
C4	0.114	0.200	0.150	0.200
Anti-dsDNA titre	0.162	0.200	0.184	0.200

Anti-dsDNA, anti-double-stranded deoxyribonucleic acid; C3, complement 3; C4, complement 4; FSS, Fatigue Severity Scale; HADS-A, Hospital Anxiety and Depression Scale – anxiety subscale; HADS-D, Hospital Anxiety and Depression Scale – depression subscale; mHAQ, modified Health Assessment Questionnaire; PSQI, Pittsburgh Sleep Quality Index; SLEDAI-2K, Systemic Lupus Erythematosus Disease Activity Index 2000; VAS, Visual Analogue Scale.

The paired samples t-test and Wilcoxon signed ranks test were used to compare continuous variables before and after vitamin D3 supplementation (table 3.48).

Table 3.48. Results obtained with the paired samples t-test and Wilcoxon signed ranks test for continuous variables at baseline and after 12 months of vitamin D3 supplementation in the cohort who achieved target serum 25-hydroxyvitamin D at 12 months.

Variable	Time	Mean	S.D.	Median	Inter-quartile range	p value Paired T-test	p value Wilcoxon signed ranks test
SLEDAI-2K	Baseline	4.18	3.545	4.0	4.0	0.127	0.140
	12 months	2.27	1.794	2.0	3.0		
Clinical SLEDAI-2K	Baseline	2.18	2.892	0.0	4.0		0.140
	12 months	0.55	1.293	0.0	0.0		
FSS	Baseline	4.27	1.261	4.56	2.33	**0.011**	
	12 months	3.33	1.930	2.89	3.78		
VAS Fatigue	Baseline	4.73	1.794	5.0	3.0	0.501	
	12 months	4.27	3.349	5.0	7.0		
VAS Pain	Baseline	3.82	2.639	4.0	5.0	0.071	
	12 months	2.64	2.335	2.0	3.0		
HADS-D	Baseline	3.27	2.687	3.0	4.0	0.167	0.146
	12 months	2.55	3.328	1.0	3.0		
HADS-A	Baseline	6.45	3.698	5.0	6.0	0.370	
	12 months	5.82	3.573	7.0	6.0		
PSQI	Baseline	6.64	2.908	7.0	6.0	**0.004**	
	12 months	4.73	2.611	4.0	4.0		
mHAQ	Baseline	0.08	0.128	0.00	0.13		0.131
	12 months	0.15	0.229	0.00	0.25		
C3 (900-1800mg/l)	Baseline	1014.18	203.124	1011	259	0.988	
	12 months	1014.64	154.938	1042	213		
C4 (100-400mg/l)	Baseline	204.36	84.492	201	135	0.663	
	12 months	199.82	65.124	211	81		
Anti-dsDNA titre (0-100IU/ml)	Baseline	243.15	200.697	217.8	438.6	**0.027**	
	12 months	198.88	167.210	190.4	344.3		
Serum 25-hydroxyvitamin D (30-100ng/ml)	Baseline	23.45	6.138	27.0	11.0	**<0.001**	**0.003**
	12 months	37.27	5.293	37.0	6.0		

The p values have been obtained on comparing the continuous variables at 6 and 12 months with those at baseline. Standard values of laboratory tests are shown in brackets. Anti-dsDNA, anti-double-stranded deoxyribonucleic acid; C3, complement 3; C4, complement 4; FSS, Fatigue Severity Scale; HADS-A, Hospital Anxiety and

Depression Scale – anxiety subscale; HADS-D, Hospital Anxiety and Depression Scale – depression subscale; mHAQ, modified Health Assessment Questionnaire; PSQI, Pittsburgh Sleep Quality Index; S.D., standard deviation; SLEDAI-2K, Systemic Lupus Erythematosus Disease Activity Index 2000; VAS, Visual Analogue Scale.

The alternative hypothesis, stating that the variables vary significantly from baseline to 12 months of vitamin D3 supplementation was accepted for FSS, PSQI, serum anti-dsDNA titre and serum 25-hydroxyvitamin D.

3.3.10 Comparing categorical variables at baseline and after Vitamin D3 treatment

The Chi Squared test and the Fisher's exact test were used to assess change from baseline to 6 and 12 months of vitamin D treatment for categorical variables (table 3.49 and 3.50). The latter test was used when at least one of the values in any of the cells of the contingency table was less than 5. The null hypothesis specified that there was a marginal percentage change after the intervention for the categorical variable and was accepted if the p value exceeded the 0.05 level of significance. The alternative hypothesis specified that there was a significant percentage change after the intervention for the categorical variable and was accepted if the p value was less than 0.05.

Table 3.49. Results obtained when using the Chi Squared test or Fisher's exact test to compare categorical variables at baseline and after 6 months of vitamin D3 treatment.

Variable		Number of patients at baseline	Number of patients at 6 months	Pearson Chi Square value	p value (2-tailed)
Current sunscreen use	Present	19	19	0.000	1.000
	Absent	12	12		
Smoking	Smoker	3	4		1.000*
	Non-smoker	28	27		
Regular exercise	Present	17	16	0.000	1.000
	Absent	14	15		
Current prednisolone	Present	7	8	0.088	0.767
	Absent	24	23		
Current hydroxychloroquine	Present	18	17	0.066	0.798
	Absent	13	14		
Current azathioprine	Present	6	6	0.000	1.000
	Absent	25	25		
Current methotrexate	Present	4	4		1.000*
	Absent	27	27		
Current calcium supplementation	Present	11	9	0.295	0.587
	Absent	20	22		
Current vitamin D suppplementation	Present	11	30		**<0.001***
	Absent	20	1		

The p values marked with * indicate that Fisher's exact test has been used.

Table 3.50. Results obtained when using the Chi Squared test or Fisher's exact test to compare categorical variables at baseline and after 12 months of vitamin D3 treatment.

Variable		Number of patients at baseline	Number of patients at 12 months	Pearson Chi Square value	p value (2-tailed)
Current sunscreen use	Present	19	16	0.590	0.442
	Absent	12	15		
Smoking	Smoker	3	4		1.000*
	Non-smoker	28	27		
Regular exercise	Present	17	16	0.065	0.799
	Absent	14	15		
Current prednisolone	Present	7	4		0.508*
	Absent	24	27		
Current hydroxychloroquine	Present	18	18	0.000	1.000
	Absent	13	13		
Current azathioprine	Present	6	6	0.000	1.000
	Absent	25	25		
Current methotrexate	Present	4	4		1.000*
	Absent	27	27		
Current calcium supplementation	Present	11	9	0.295	0.587
	Absent	20	22		
Current vitamin D suppplementation	Present	11	27		**<0.001**
	Absent	20	4		

The p values marked with * indicate that Fisher's exact test has been used.

The results showed that there were no significant differences in all categorical variables, with the exception of the number of patients receiving vitamin D supplementation, as was expected ($p<0.001$).

3.3.11 SUMMARY OF RESULTS

In summary, on analysing the whole cohort of 31 patients, disease activity measured by clinical SLEDAI-2K and SLEDAI-2K was noted to improve significantly at 12 months (p=0.024, p=0.028). The improvement in SLEDAI-2K and clinical SLEDAI-2K was also statistically significant at 6 months when analysing the cohort of 13 patients with vitamin D deficiency at baseline (p=0.030, p=0.042).

On analysing the whole cohort, a reduction in prednisolone dosage and in the level of fatigue was noted after 12 months, however statistical significance was not achieved (p=0.068, p=0.071). On carrying out a subgroup analysis that included only the patients who achieved the target serum 25-hydroxyvitamin D of ≥30ng/ml at 12 months, a statistical significant improvement in FSS and PSQI was noted after 12 months of vitamin D3 supplementation (p=0.011, p=0.004).

3.4 INTERFERON SIGNATURE GENE EXPRESSION

RNA extracted from blood samples taken from 89 patients at baseline, and 31 patients following 6 months of vitamin D3 supplementation, had a good concentration (mean 64.0ng/µl, range 8.1-280.3ng/µl) and purity defined by 260/280 ratio (mean 1.99, range 1.76-2.18) (Qiagen® 2010). The expression of 12 IFN signature genes (*CCL2, CXCL1, IFI35, IFIT1, IFITM1, IFIT3, MX1, OAS1, STAT1, SOCS1, SOCS3, STAT2*) and 3 housekeeping genes (HPRT1, RPL13A, TBP) was measured in the extracted RNA. The MFI results obtained were then normalised by first subtracting the average background signal for each gene; and then dividing by the geometric mean of the result of the reference genes.

3.4.1 RELATIONSHIP OF IFN SIGNATURE GENE EXPRESSION WITH SEVERAL VARIABLES AT BASELINE

The relationship between the expression of the 12 IFN signature genes and other variables assessed in the cross-sectional cohort study was analysed. Testing for normality of MFI results and of IFN signature gene expression score was carried out using the K-S test (table 3.51).

Table 3.51. Kolmogorov-Smirnov test results for normalised MFI for the 12 IFN signature genes and for IFN signature gene expression score.

Normalised MFI	Test Statistic	P value
IFI35	0.138	<0.001
OAS1	0.204	<0.001
CCL2	0.262	<0.001
MX1	0.140	<0.001
IFITM1	0.297	<0.001
STAT2	0.158	<0.001
CXCL1	0.160	<0.001
IFIT3	0.216	<0.001
SOCS3	0.185	<0.001
IFIT1	0.178	<0.001
STAT1	0.168	<0.001
SOCS1	0.166	<0.001
IFN signature gene expression score	0.161	<0.001

IFN, interferon; MFI, median fluorescence intensity.

For all genes the p value obtained was less than 0.05. Thus the alternative hypothesis stating that the normalised MFI are not normally distributed, was accepted. The median and interquartile range of the normalised MFI obtained for each of the 12 genes studied are specified in Table 3.52.

Table 3.52. Descriptive statistics of the normalised MFI of the 12 IFN signature genes and of the IFN signature gene expression score.

Normalised MFI	Median	Interquartile Range
IFI35	1.76	2.34
OAS1	8.69	19.13
CCL2	0.13	0.28
MX1	4.03	7.55
IFITM1	65.79	74.43
STAT2	1.79	1.71
CXCL1	0.82	0.58
IFIT3	22.30	47.58
SOCS3	0.54	0.45
IFIT1	22.77	44.28
STAT1	9.49	6.95
SOCS1	0.33	0.33
IFN signature gene expression score	1.89	2.29

IFN, interferon; MFI, median fluorescence intensity.

The relationship of the MFI of the 12 IFN signature genes and the IFN signature gene expression score with the other variables assessed in the study was analysed. For continuous variables, Spearman's correlation test was used. Table 3.53 shows the results. The Bonferroni correction was used since multiple genes were tested. Using this method, a significance level for the p value of 0.004 was established for the MFI of the 12 IFN signature genes. The usual p value of 0.05 was used as a cut off to establish level of significance for the IFN signature gene expression score.

Table 3.53. Correlation of several variables with the normalised MFI of the 12 IFN signature genes and with the IFN signature gene expression score.

		IFI35	*OAS1*	*CCL2*	*MX1*	*IFITM1*	*STAT2*	*CXCL1*	*IFIT3*	*SOCS3*	*IFIT1*	*STAT1*	*SOCS1*	**IFN score**
Age	p value	0.312	0.227	0.394	0.240	0.082	0.388	0.535	0.142	0.385	0.095	0.496	0.106	0.139
	R value	-0.108	-0.130	-0.394	-0.127	-0.185	-0.093	-0.067	-0.157	-0.093	-0.178	-0.073	-0.177	-0.158
SLE duration	p value	0.251	0.146	0.551	0.267	0.124	0.357	0.648	0.159	0.368	0.321	0.198	0.031	0.140
	R value	-0.123	-0.156	-0.066	-0.120	-0.164	-0.099	-0.049	-0.151	-0.097	-0.106	-0.138	-0.236	-0.158
Age of disease onset	p value	0.895	0.845	0.667	0.681	0.583	0.959	0.922	0.659	0.919	0.369	0.820	0.915	0.743
	R value	-0.014	-0.021	-0.047	-0.045	-0.059	-0.006	0.011	-0.047	-0.011	-0.096	0.024	-0.012	-0.035
SLEDAI-2K	p value	**0.001**	**<0.001**	0.088	**0.001**	**0.001**	0.011	0.070	**0.001**	0.088	**0.001**	0.005	0.012	**0.001**
	R value	0.332	0.385	0.186	0.349	0.349	0.269	0.193	0.345	0.182	0.349	0.294	0.271	0.346
SDI	p value	0.625	0.785	0.655	0.880	0.134	0.826	0.327	0.758	0.041	0.718	0.875	0.574	0.999
	R value	-0.052	0.030	0.049	0.016	0.160	-0.024	0.105	0.033	0.217	0.039	-0.017	-0.062	0.000
FSS	p value	0.958	0.812	0.368	0.846	0.794	0.993	0.273	0.970	0.977	0.754	0.944	0.728	0.966
	R value	-0.006	0.026	-0.099	-0.021	-0.028	-0.001	-0.118	-0.004	-0.003	-0.034	0.008	0.039	0.005
VAS fatigue	p value	0.299	0.654	0.384	0.220	0.522	0.440	0.120	0.529	0.609	0.376	0.396	0.472	0.464
	R value	-0.111	-0.048	-0.096	-0.133	-0.069	-0.083	-0.166	-0.068	-0.055	-0.095	-0.091	0.080	-0.079
VAS pain	p value	0.876	0.545	0.950	0.913	0.930	0.522	0.285	0.969	0.365	0.928	0.532	0.470	0.810
	R value	0.017	0.065	0.007	0.012	0.009	0.069	-0.115	0.004	-0.097	-0.010	0.067	0.080	0.026
HADS-D	p value	0.532	0.917	0.099	0.638	0.735	0.425	0.040	0.875	0.515	0.808	0.645	0.913	0.404
	R value	-0.067	0.011	-0.180	-0.051	-0.036	-0.086	-0.219	0.017	0.070	-0.026	-0.049	0.012	-0.090
HADS-A	p value	0.256	0.357	0.188	0.385	0.773	0.296	0.349	0.471	0.968	0.331	0.243	0.148	0.259
	R value	-0.122	-0.099	-0.144	-0.094	-0.031	-0.113	-0.100	-0.077	0.004	-0.104	-0.125	-0.159	-0.121

PSQI	p value	0.646	0.670	0.059	0.324	0.356	0.432	0.155	0.843	0.669	0.598	0.887	0.832	0.473
	R value	-0.049	-0.046	-0.206	-0.107	-0.099	-0.085	-0.152	-0.021	-0.046	-0.057	-0.015	-0.024	-0.077
mHAQ	p value	0.558	0.282	0.684	0.536	0.897	0.918	0.735	0.718	0.883	0.823	0.819	0.968	0.721
	R value	0.063	0.116	-0.045	0.067	-0.014	0.011	-0.036	0.039	0.016	0.024	0.025	0.004	0.038
Haemoglobin	p value	0.279	0.444	0.274	0.459	0.079	0.448	0.849	0.358	0.905	0.382	0.247	0.222	0.349
	R value	-0.116	-0.083	-0.120	-0.080	-0.187	-0.082	-0.020	-0.099	0.013	-0.094	-0.124	-0.135	-0.100
Urine PCR	p value	0.900	0.614	0.686	0.913	0.909	0.662	0.154	0.911	0.088	0.718	0.323	0.682	0.648
	R value	-0.014	-0.056	0.045	-0.012	0.013	0.048	0.155	-0.012	0.185	-0.040	0.108	0.046	0.050
Serum 25-hydroxyvitamin D	p value	0.462	0.859	0.007	0.933	0.148	0.652	0.089	0.489	0.179	0.735	0.407	0.045	0.997
	R value	-0.079	-0.019	0.289	-0.009	0.155	0.049	-0.181	0.074	-0.144	0.036	0.089	0.219	0.000
CRP	p value	0.008	0.012	0.410	0.019	0.005	0.039	0.106	0.006	**<0.001**	0.014	0.007	0.169	**0.002**
	R value	0.278	0.265	0.091	0.251	0.298	0.221	0.172	0.290	0.429	0.258	0.283	0.152	0.317
ESR	p value	**0.001**	0.006	0.015	**0.003**	**0.001**	0.005	0.800	**0.002**	0.129	**0.002**	**0.003**	0.096	**0.003**
	R value	0.349	0.295	0.264	0.316	0.358	0.299	0.027	0.330	0.163	0.319	0.316	0.184	0.313
C3	p value	0.074	0.112	0.011	0.029	0.017	0.017	0.015	0.031	0.312	0.079	0.005	0.009	**0.012**
	R value	-0.190	-0.171	-0.274	-0.234	-0.253	-0.254	-0.257	-0.229	-0.108	-0.187	-0.296	-0.285	-0.264
C4	p value	0.046	0.048	0.052	0.046	0.307	0.032	0.036	0.050	0.428	0.062	0.037	0.064	**0.021**
	R value	-0.212	-0.212	-0.212	-0.215	-0.110	-0.229	-0.222	-0.209	-0.085	-0.198	-0.222	-0.203	-0.244
Anti-dsDNA titre	p value	0.288	0.139	0.375	0.161	0.051	0.488	0.048	0.082	0.028	0.072	0.144	0.052	**0.040**
	R value	0.114	0.159	0.098	0.152	0.208	0.075	0.210	0.185	0.234	0.192	0.156	0.213	0.218

P values have been obtained using Spearman's correlation test. Using the Bonferroni correction for multiple gene testing a significance level for the p value of 0.004 was established for the MFI of the 12 IFN signature genes. The p value of 0.05 was used as a cut off to establish level of significance for the IFN signature gene expression score. Statistically significant results are shown in bold. Anti-dsDNA, anti-double-stranded deoxyribonucleic acid; C3, complement 3; C4, complement 4; FSS, Fatigue Severity Scale; HADS-A, Hospital Anxiety and Depression Scale – anxiety subscale; HADS-D, Hospital Anxiety and

Depression Scale – depression subscale; MFI, median fluorescence intensity; mHAQ, modified Health Assessment Questionnaire; PCR, protein creatinine ratio; PSQI, Pittsburgh Sleep Quality Index; SDI, Systemic Lupus International Collaborating Clinics/American College of Rheumatology damage index; SLEDAI-2K, Systemic Lupus Erythematosus Disease Activity Index 2000; VAS, Visual Analogue Scale.

The table shows a significant positive correlation between the expression of many of the genes analysed as well as the IFN signature gene expression score, and SLEDAI-2K. In keeping with this there is a significant positive correlation between the IFN signature gene expression score and anti-dsDNA titre, ESR and CRP; and a significant negative correlation between IFN signature gene expression score and complement levels. No significant correlation was noted between gene expression and other variables including FSS, VAS fatigue, VAS pain, HADS-D, HADS-A, PSQI, mHAQ, SDI and serum 25-hydroxyvitamin D.

In order to analyse the relationship between the IFN signature gene expression score and categorical variables, the Mann-Whitney U-test was used since the former was not normally distributed (table 3.54).

Table 3.54. Table showing categorical variables, median IFN signature gene expression score and interquartile range when the variable was present and when absent.

Variable		Median IFN signature gene expression score	Interquartile range	p value (2-tailed)
Gender	Female	1.89	2.20	0.552
	Male	1.08	3.03	
Smoking	Smoker	2.21	2.13	0.478
	Non-smoker	1.79	2.40	
Anti-Sm	Positive	2.66	2.88	**0.039**
	Negative	1.79	2.04	
Anti-SSA52	Positive	2.28	2.63	0.066
	Negative	1.45	1.95	
Anti-SSA60	Positive	2.52	2.46	**0.011**
	Negative	1.34	1.67	
Anti-SSB	Positive	2.88	2.24	0.101
	Negative	1.69	2.18	
Anti-RNP	Positive	2.67	2.96	**0.043**
	Negative	1.46	1.96	
Raynauds syndrome	Present	2.36	2.52	0.189
	Absent	1.44	2.33	
Fibromyalgia	Present	1.42	1.90	0.399
	Absent	2.01	2.29	
Anti-phospholipid syndrome	Present	1.27	1.33	0.266
	Absent	1.92	2.34	

The p values shown were obtained using the Mann-Whitney U-test.

anti-RNP, anti-ribonucleoprotein; anti-Sm, anti-Smith; anti-SSA, anti-Sjogren's syndrome related antigen A; anti-SSB, anti-Sjogren's syndrome related antigen B; IFN, interferon.

There was a significantly increased IFN signature gene expression score in patients who were positive for anti-Sm, anti-SSA60 and anti-RNP antibodies.

3.4.2 CHANGE IN IFN SIGNATURE GENE EXPRESSION WITH VITAMIN D SUPPLEMENTATION IN VITAMIN D DEFICIENT AND INSUFFICIENT PATIENTS

The K-S test was used to test for normality of distribution of the MFI of the 12 IFN signature genes and IFN signature gene expression score at baseline and after 6 months of vitamin D supplementation (table 3.55). For those variables that had a p value obtained by the K-S test higher than 0.05, the null hypothesis was accepted; meaning that they were normally distributed. The other continuous variables, whose p value obtained was less than 0.05, were not normally distributed.

Table 3.55. Kolmogorov- Smirnov test results for the MFI of the 12 IFN signature genes and the IFN signature gene expression score obtained in 31 vitamin D deficient and insufficient patients.

Gene	Baseline		6 months	
	Test Statistic	P value	Test Statistic	P value
IFI35	0.182	0.012	0.136	0.167
OAS1	0.217	0.001	0.255	<0.001
CCL2	0.189	0.008	0.160	0.055
MX1	0.141	0.166	0.166	0.039
IFITM1	0.354	<0.001	0.380	<0.001
STAT2	0.213	0.001	0.109	0.200
CXCL1	0.115	0.200	0.163	0.041
IFIT3	0.234	<0.001	0.188	0.008
SOCS3	0.231	<0.001	0.107	0.200
IFIT1	0.207	0.002	0.253	<0.001
STAT1	0.192	0.006	0.090	0.200
SOCS1	0.212	0.001	0.127	0.200
IFN signature gene expression score	0.170	0.027	0.124	0.200

IFN, interferon; MFI, median fluorescence intensity.

The paired samples t-test and Wilcoxon signed ranks test were used to compare MFI for the 12 genes and the IFN signature gene expression score before and after vitamin D3 supplementation (table 3.56). The latter test is a non-parametric alternative of the former. Both tests were carried out when one value was normally distributed and the other was not. The Bonferroni correction was used since multiple genes were tested. Using this method, a significance level for the p value of 0.004 was established for the MFI of the 12 IFN signature genes. The usual p value of 0.05 was used as a cut off to establish level of significance for the IFN signature gene expression score.

Table 3.56. Results obtained with the paired samples t-test and Wilcoxon signed ranks test for the MFI of the 12 IFN signature genes and IFN signature gene expression score at baseline and after 6 months of vitamin D3 supplementation.

Gene	Time	Mean normalised gene MFI / IFN score	S.D.	Median normalised gene MFI / IFN score	Inter-quartile range	p value Paired T-test	p value Wilcoxon signed ranks test
IFI35	Baseline	2.41	1.777	1.81	1.81	0.281	0.465
	6 months	2.09	1.293	1.73	2.20		
OAS1	Baseline	11.30	9.887	8.23	16.58		0.039
	6 months	8.49	8.583	4.00	10.88		
CCL2	Baseline	0.12	0.124	0.10	0.12	0.259	0.517
	6 months	0.09	0.081	0.09	0.10		
MX1	Baseline	4.77	3.794	4.02	5.64	0.248	0.179
	6 months	3.88	3.375	2.73	4.98		
IFITM1	Baseline	99.26	223.724	40.11	51.90		0.629
	6 months	79.78	124.278	34.30	26.06		
STAT2	Baseline	1.97	1.200	1.76	1.18	0.227	0.644
	6 months	1.71	0.893	1.64	1.54		
CXCL1	Baseline	0.96	0.470	0.84	0.67	0.629	0.309
	6 months	0.89	0.666	0.74	0.68		
IFIT3	Baseline	29.41	38.866	18.25	31.21		0.405
	6 months	23.43	23.875	13.27	29.18		
SOCS3	Baseline	0.67	0.442	0.53	0.38	0.506	0.766
	6 months	0.61	0.306	0.59	0.54		
IFIT1	Baseline	27.19	28.622	15.99	40.24		0.586
	6 months	25.67	35.779	11.26	30.31		
STAT1	Baseline	9.88	5.963	9.13	5.51	0.200	0.504
	6 months	8.57	3.752	8.96	6.20		
SOCS1	Baseline	0.36	0.352	0.24	0.27	0.023	0.008
	6 months	0.20	0.141	0.18	0.16		
IFN signature gene expression score	Baseline	2.67	1.703	2.28	1.98	0.165	0.405
	6 months	2.22	1.323	1.99	1.77		

IFN, interferon; MFI, median fluorescence intensity; S.D., standard deviation.

The mean normalised MFI for all 12 IFN signature genes decreased from baseline to 6 months especially *OAS1* and *SOCS1* genes (p=0.039, p=0.008), however these results are not statistically significant in view of the Bonferroni correction. The latter was applied due to multiple gene testing and the p value of 0.004 was established as the cut-off for the level of significance for the 12 IFN signature gene MFI. The null hypothesis, stating that the MFI for the 12 genes and the IFN signature gene expression score did not vary significantly from baseline to 6 months of vitamin D3 supplementation, was accepted.

3.4.3 CHANGE IN IFN SIGNATURE GENE EXPRESSION WITH VITAMIN D SUPPLEMENTATION IN PATIENTS WHO DID NOT HAVE AN INCREASE IN PREDNISOLONE DOSAGE

The analysis was repeated after 3 patients who had an increase in prednisolone dosage from baseline to 6 months were excluded (table 3.57).

Table 3.57. Results obtained with the paired samples t-test and Wilcoxon signed ranks test for the MFI of the 12 IFN signature genes and IFN signature gene expression score at baseline and after 6 months of vitamin D3 supplementation in patients who did not have any increase in prednisolone dosage from baseline to 6 months.

Gene	Time	Mean normalised gene MFI / IFN score	S.D.	Median normalised gene MFI / IFN score	Inter-quartile range	p value Paired T-test	p value Wilcoxon signed ranks test
IFI35	Baseline	2.35	1.830	1.62	1.73	0.249	0.361
	6 months	1.98	1.250	1.58	2.19		
OAS1	Baseline	11.07	10.190	6.67	17.22		0.032
	6 months	7.89	8.146	3.89	10.25		
CCL2	Baseline	0.12	0.129	0.10	0.11		0.493
	6 months	0.09	0.080	0.08	0.10		
MX1	Baseline	4.94	3.938	4.03	6.48	0.070	
	6 months	3.59	3.145	2.50	4.38		
IFITM1	Baseline	102.22	235.883	37.80	51.12		0.471
	6 months	66.59	100.414	31.95	25.44		
STAT2	Baseline	1.94	1.253	1.74	0.95	0.184	0.517
	6 months	1.62	0.833	1.61	1.30		
CXCL1	Baseline	1.00	0.465	0.86	0.62	0.492	0.203
	6 months	0.90	0.688	0.72	0.63		
IFIT3	Baseline	29.97	40.854	17.30	36.78		0.349
	6 months	22.26	23.367	11.78	29.89		
SOCS3	Baseline	0.69	0.460	0.58	0.40	0.263	0.414
	6 months	0.60	0.308	0.59	0.47		
IFIT1	Baseline	27.34	29.744	11.60	40.77		0.349
	6 months	22.14	30.778	9.08	27.97		
STAT1	Baseline	9.81	6.274	8.84	6.45	0.173	0.428
	6 months	8.27	3.740	8.79	6.32		
SOCS1	Baseline	0.36	0.371	0.22	0.30	0.018	0.005
	6 months	0.18	0.108	0.18	0.14		
IFN signature gene expression score	Baseline	2.69	1.775	2.17	2.20	0.083	0.230
	6 months	2.10	1.185	1.96	1.63		

IFN, interferon; MFI, median fluorescence intensity; S.D., standard deviation.

In this cohort, statistical significant reduction in the normalised MFI of the SOCS1 gene was almost achieved (p=0.005). In view of the Bonferroni correction for multiple gene testing the established significance level for the p value for the MFIs was 0.004.

3.4.4 Change in IFN Signature Gene Expression with Vitamin D supplementation in patients who achieved target Serum 25-hydroxyvitamin D at 6 months

Statistical analysis was carried out on the cohort of 26 patients who achieved target serum 25-hydroxyvitamin D of ≥30ng/ml (table 3.58).

Table 3.58. Results obtained with the paired samples t-test and Wilcoxon signed ranks test for the MFI of the 12 IFN signature genes and IFN signature gene expression score at baseline and after 6 months of vitamin D3 supplementation in patients who achieved target serum 25-hydroxyvitamin D at 6 months.

Gene	Time	Mean normalised gene MFI / IFN score	S.D.	Median normalised gene MFI / IFN score	Inter-quartile range	p value Paired T-test	p value Wilcoxon signed ranks test
IFI35	Baseline	2.60	1.853	1.99	2.51	0.346	0.600
	6 months	2.27	1.334	2.25	2.09		
OAS1	Baseline	12.03	10.083	9.15	17.38		0.069
	6 months	9.34	8.943	4.27	11.95		
CCL2	Baseline	0.13	0.133	0.11	0.13	0.286	0.563
	6 months	0.10	0.086	0.09	0.11		
MX1	Baseline	5.17	3.824	4.44	6.52	0.244	
	6 months	4.26	3.424	3.34	4.61		
IFITM1	Baseline	112.19	243.43	45.81	53.38		0.527
	6 months	89.74	134.27	38.00	34.23		
STAT2	Baseline	2.12	1.227	1.79	1.62	0.194	0.563
	6 months	1.79	0.904	1.76	1.42		
CXCL1	Baseline	0.99	0.459	0.86	0.54	0.744	
	6 months	0.94	0.714	0.80	0.84		
IFIT3	Baseline	32.96	41.423	21.23	35.71		0.476
	6 months	25.96	25.150	16.58	30.64		
SOCS3	Baseline	0.71	0.469	0.58	0.40	0.366	0.581
	6 months	0.63	0.311	0.65	0.55		
IFIT1	Baseline	30.91	29.488	22.77	39.63		0.677
	6 months	29.16	37.985	13.47	31.49		
STAT1	Baseline	10.50	6.182	9.49	5.63	0.176	0.459
	6 months	8.88	4.002	9.22	6.58		
SOCS1	Baseline	0.329	0.237	0.28	0.28	0.010	
	6 months	0.207	0.146	0.19	0.15		
IFN signature gene expression score	Baseline	2.86	1.782	2.41	2.28	0.205	
	6 months	2.38	1.368	2.04	1.75		

IFN, interferon; MFI, median fluorescence intensity; S.D., standard deviation.

The results of this cohort were similar to the results obtained when the whole cohort of 31 patients was analysed (section 3.4.2). The p value obtained for the reduction in normalised MFI for the IFN signature genes was not below the established significance level of 0.004 due to the Bonferroni correction. As a result, the null hypothesis stating that the MFI for the 12 genes and the IFN signature gene expression score, did not vary significantly from baseline to 6 months of vitamin D3 supplementation, was accepted.

3.4.5 COMPARING CHARACTERISTICS OF PATIENTS WHO HAD A DECREASE IN IFN SIGNATURE GENE EXPRESSION SCORE WITH THOSE WHO DID NOT

The characteristics of the 17 patients who had a decrease in IFN signature gene expression score from baseline to 6 months were compared with those who did not have a decrease in score (12 patients). For continuous variables the independent samples t-test was used for normally distributed variables and the Mann-Whitney U-test was used as the non-parametric alternative (table 3.59).

Table 3.59. Results obtained with the independent samples t-test and Mann-Whitney U-test (denoted by *) for continuous variables in patients who had a decrease in IFN signature gene expression score and those who did not.

Variable	IFN score decrease	Baseline			6 months		
		Mean/ Median	S.D./ IQR	p value	Mean/ Median	S.D./ IQR	p value
SLEDAI-2K	Yes	3.00	4.00	0.635*	2.00	4.00	**0.015***
	No	4.00	4.00		4.00	6.00	
Clinical SLEDAI-2K	Yes	0.00	4.00	0.845*	0.00	2.00	0.078*
	No	2.00	4.00		2.00	4.00	
Serum 25-hydroxyvitamin D	Yes	25.00	14.00	0.982*	33.00	11.00	0.756*
	No	21.75	16.00		32.50	5.00	
FSS	Yes	4.32	1.47	0.471	4.10	1.64	0.690
	No	3.91	1.54		3.85	1.58	
PSQI	Yes	6.12	4.17	0.430	6.00	6.00	0.490*
	No	7.42	4.48		5.00	8.00	
mHAQ	Yes	0.13	0.31	0.963*	0.25	0.63	0.707*
	No	0.13	0.25		0.06	0.47	
Anti-dsDNA	Yes	15.30	185.55	**0.032***	10.00	180.75	**0.028***
	No	154.15	356.30		191.00	196.20	
C3	Yes	1099.94	233.12	0.788	1056.12	200.66	0.910
	No	1074.08	278.46		1045.83	284.43	
C4	Yes	255.76	93.08	0.148	251.71	111.66	0.272
	No	206.58	78.62		210.67	70.48	

Mean and S.D. are given to describe variables that are normally distributed. Median and IQR are given to describe variables that are not normally distributed (p value denoted by *).

Anti-dsDNA, anti-double-stranded deoxyribonucleic acid; C3, complement 3; C4, complement 4; FSS, Fatigue Severity Scale; IFN, interferon; IQR, interquartile range; mHAQ, modified Health Assessment Questionnaire; PSQI, Pittsburgh Sleep Quality

Index; S.D., standard deviation; SLEDAI-2K, Systemic Lupus Erythematosus Disease Activity Index 2000.

Anti-dsDNA titre was significantly lower at baseline and at 6 months in the cohort that had a decrease in IFN signature gene expression score from baseline to 6 months. SLEDAI-2K was noted to decrease from baseline to 6 months in the group who had a decrease in IFN signature gene expression score. On the other hand, the group in whom IFN signature gene expression score increased, did not have a change in median SLEDAI-2K from baseline to 6 months. This was reflected in a significant difference in SLEDAI-2K at 6 months (p=0.015).

Categorical variables were compared between the group who had a decrease in IFN signature gene expression score and the group that did not. The Chi Squared test and the Fisher's exact test were used in this analysis (table 3.60). The latter test was used when at least one of the values in any of the cells of the contingency table was less than 5. No significant difference in categorical variables were noted between the two groups.

Table 3.60. Results obtained when using the Chi Squared test or Fisher's exact test to compare categorical variables in patients who had a decrease in IFN signature gene expression score and in those who did not.

Variable		Number of patients with decrease in IFN score	Number of patients with increase in IFN score	Pearson Chi Square value	p value (2-tailed)
Anti-phospholipid syndrome	Present	1	2		0.553*
	Absent	16	10		
Mucocutaneous manifestations	Present	15	9		0.622*
	Absent	2	3		
Joint manifestations	Present	17	11		0.414*
	Absent	0	1		
Renal manifestations	Present	6	1		0.187*
	Absent	11	11		
Neurological manifestations	Present	4	0		0.121*
	Absent	13	12		
Haematological manifestations	Present	15	11		1.000*
	Absent	2	1		
Cardiac manifestations	Present	1	0		1.000*
	Absent	16	12		
Respiratory manifestations	Present	3	1		0.622*
	Absent	14	11		
Constitutional manifestations	Present	12	6	1.266	0.260
	Absent	5	6		
Anti-Sm	Present	2	3		0.622*
	Absent	15	9		
Anti-SSA52	Present	5	3		1.000*
	Absent	12	9		
Anti-SSA60	Present	7	3		0.449*
	Absent	10	9		
Anti-SSB	Present	3	1		0.622*
	Absent	14	11		
Anti-RNP	Present	3	2		1.000*
	Absent	13	10		
Current hydroxychloroquine	Present	12	5	2.426	0.119
	Absent	5	7		
Current azathioprine	Present	4	1		0.370*
	Absent	13	11		
Current methotrexate	Present	2	1		1.000*
	Absent	15	11		
Current prednisolone	Present	4	3		1.000*
	Absent	13	9		

The p values marked with * indicate that Fisher's exact test has been used.
IFN, interferon.

3.4.6 SUMMARY OF RESULTS

On analysing the correlation between the expression of the IFN signature genes and continuous variables collected in the cross-sectional cohort study, a significant positive correlation was found between the expression of many genes analysed and SLEDAI-2K. In keeping with this a significant positive correlation was found between the IFN signature gene expression score and SLEDAI-2K (R=0.346, p=0.001), anti-dsDNA titre (R=0.218, p=0.040), ESR (R=0.313, p=0.003) and CRP (R=0.317, p=0.002); and a significant negative correlation between IFN signature gene expression score and C3 (R=-0.264, p=0.012) and C4 (R=-0.244, p=0.021). In the cross-sectional cohort study it was also noted that there was a significantly increased IFN signature gene expression score in patients who were positive for anti-Sm (p=0.039), anti-SSA60 (p=0.011) and anti-RNP antibodies (p=0.043).

The expression of the IFN signature genes at baseline was compared with that after 6 months of vitamin D3 supplementation in the patients who participated in the prospective study. To eliminate the potential confounding effect caused by an increase in prednisolone dose, the results were re-analysed after excluding 3 patients who had an increase in prednisolone dosage from baseline to 6 months. The results showed a decrease in the normalised MFI for all 12 IFN signature genes particularly in the *OAS1* and *SOCS1* genes (p=0.032, p=0.005). However, these results are not statistically significant since the Bonferroni correction was applied and a cut off of <0.004 was established as the statistical significant p value. The mean IFN signature gene expression score decreased from 2.69 to 2.10 (p=0.083). On comparing the characteristics of the patients who had a decrease in IFN signature gene expression

score on vitamin D3 supplementation, with those that did not, a significantly lower SLEDAI-2K at 6 months was noted in the former group (p=0.015).

3.5 VDR POLYMORPHISMS

The study by J Grech Meli (2020) described the BsmI, FokI, ApaI, TaqI VDR polymorphisms in 59 SLE patients that have been included in the cross-sectional cohort study described in section 2.3 (table 3.61). The relationship between the VDR polymorphisms and the variables measured in the cross-sectional cohort study have been analysed.

Table 3.61. Frequencies of the different genotypes for the four VDR polymorphisms in 59 SLE patients.

Genotype	BsmI	FokI	ApaI	TaqI
Homozygous wild type	22	7	10	23
Heterozygous	29	27	39	29
Homozygous minor	8	25	10	6

3.5.1 Relationship of VDR Polymorphisms with continuous variables in Cross-sectional Cohort study

The normality of the continuous variables for the 59 patients in whom VDR polymorphisms have been assessed was found by means of the K-S test (table 3.62).

Table 3.62. Kolmogorov-Smirnov test results for continuous variables in the 59 patients in whom VDR polymorphisms had been assessed.

Variable	Test Statistic	P value
SLEDAI-2K	0.195	<0.001
SDI	0.275	<0.001
FSS	0.097	0.032
Serum 25-hydroxyvitamin D	0.081	0.180
C3	0.089	0.071
C4	0.066	0.200
Anti-dsDNA titre	0.229	<0.001
IFN signature gene expression score	0.161	<0.001

Anti-dsDNA, anti-double-stranded deoxyribonucleic acid; C3, complement 3; C4, complement 4; FSS, Fatigue Severity Scale; IFN, interferon; SDI, Systemic Lupus International Collaborating Clinics/American College of Rheumatology damage index; SLEDAI-2K, Systemic Lupus Erythematosus Disease Activity Index 2000; VDR, vitamin D receptor.

The p value obtained for serum 25-hydroxyvitamin D, C3 and C4 was higher than 0.05. The null hypothesis was accepted, meaning that they were normally distributed. The

other continuous variables, whose p value obtained was less than 0.05, were not normally distributed.

The relationship between VDR polymorphisms and serum 25-hydroxyvitamin D, C3 and C4 was analysed using one-way ANOVA. Homogeneity of variances was first checked using Levene's test. Since the p value was greater than 0.05, variances were all found to be equal and the one-way ANOVA test could be used (table 3.63). The Kruskal Wallis Test was used for variables that were not normally distributed. Results are shown in table 3.64.

Table 3.63. Table showing mean results for serum 25-hydroxyvitamin D, C3 and C4 for the various genotypes for the 4 VDR polymorphisms.

Variable		BsmI	FokI	ApaI	TaqI
Mean serum 25-hydroxyvitamin D (ng/ml)	Homozygous wild type	31.41	32.57	32.10	30.61
	Heterozygous	32.59	29.52	31.85	32.97
	Homozygous minor	30.50	34.20	31.7	32.50
	P value	0.835	0.215	0.996	0.685
Mean C3 (mg/l)	Homozygous wild type	964.5	1038.7	1008.7	971.2
	Heterozygous	1032.7	939.6	987.4	995.0
	Homozygous minor	911.13	1032.7	985.9	1024.2
	P value	0.356	0.314	0.967	0.874
Mean C4 (mg/l)	Homozygous wild type	219.23	214.1	243.4	235.9
	Heterozygous	237.48	210.8	233.5	227.5
	Homozygous minor	227.69	249.8	189.4	190.8
	P value	0.766	0.357	0.409	0.632

P values have been obtained using the one-way ANOVA test.

C3, complement 3; C4, complement 4; VDR, vitamin D receptor.

Table 3.64. Table showing median results for SLEDAI-2K, SDI, FSS anti-dsDNA titre and IFN signature gene expression score for the various genotypes for the 4 VDR polymorphisms.

Variable		BsmI	FokI	ApaI	TaqI
Median SLEDAI-2K	Homozygous wild type	4	4	4	4
	Heterozygous	2	4	2	2
	Homozygous minor	4	2	4	4
	P value	**0.031**	0.273	0.266	0.438
Median SDI	Homozygous wild type	0	0	0	1
	Heterozygous	1	1	1	0
	Homozygous minor	1	0	1	1
	P value	0.189	0.730	0.324	0.642
Median FSS	Homozygous wild type	3.78	4.33	3.72	4.11
	Heterozygous	4.56	4.78	4.56	4.44
	Homozygous minor	5.23	3.56	4.45	5.18
	P value	0.236	0.354	0.561	0.263
Median anti-dsDNA titre (IU/ml)	Homozygous wild type	144.6	135.6	116.3	96.9
	Heterozygous	74.6	154.8	91.0	91.0
	Homozygous minor	125.9	66.1	62.8	158.8
	P value	0.257	0.395	0.539	0.999
Median IFN score	Homozygous wild type	1.92	1.09	1.69	1.59
	Heterozygous	1.79	2.46	2.13	2.36
	Homozygous minor	1.51	1.46	1.11	1.52
	P value	0.887	0.188	0.361	0.715

P values have been obtained using the Kruskal Wallis test.

Anti-dsDNA, anti-double-stranded deoxyribonucleic acid; FSS, Fatigue Severity Scale; IFN, interferon; SDI, Systemic Lupus International Collaborating Clinics/American College of Rheumatology damage index; SLEDAI-2K, Systemic Lupus Erythematosus Disease Activity Index 2000; VDR, vitamin D receptor.

There was a significant difference in SLEDAI-2K for the different genotypes for BsmI polymorphism. Mann-Whitney U-test was carried out for pairwise comparisons (table 3.65).

Table 3.65. Median and interquartile range for SLEDAI-2K when making pairwise comparisons of the various genotypes for BsmI polymorphism.

Genotype	Median	IQR	P value
Homozygous wild type versus	4	5	0.098
Heterozygous	2	4	
Homozygous wild type versus	4	5	0.322
Homozygous minor	4	2	
Heterozygous versus	2	4	**0.010**
Homozygous minor	4	2	
Homozygous wild type and Heterozygous	3	4	**0.046**
versus Homozygous minor	4	2	

The p values have been obtained using the Mann-Whitney U-test.
IQR, interquartile range; SLEDAI-2K, Systemic Lupus Erythematosus Disease Activity Index 2000.

It was thus concluded that the patients who were homozygous minor for BsmI had a significantly higher SLEDAI-2K compared to those that were heterozygous. This was

also the case when comparing SLEDAI-2K of the homozygous minor with the combined homozygous wild type and heterozygous groups.

3.5.2 Relationship of VDR Polymorphisms with categorical variables in the Cross-sectional Cohort study

The relationship between the genotype for the four VDR polymorphisms and categorical variables (including co-morbidities, organ manifestations and autoantibody status) was established by using the Chi Squared test provided that less than 20% of the expected counts in the contingency table were less than 5 and all individual expected counts were 1 or greater. The Freeman-Halton extension of Fisher's exact test was used as an alternative (table 3.66-3.69).

Table 3.66. Results obtained when using the Chi Squared test or Fisher's exact test to compare categorical variables for the different genotypes for BsmI VDR polymorphism.

Variable		BsmI Homozygous wild type	BsmI Heterozygous	BsmI Homozygous minor	Pearson Chi Square/ Fisher's exact test value	p value
Fibromyalgia	Present	2	4	2	1.491	0.441*
	Absent	20	25	6		
Raynauds	Present	8	17	3	2.853	0.240
	Absent	14	12	5		
Mucocutaneous manifestations	Present	18	25	7	0.359	0.884*
	Absent	4	4	1		
Lupus nephritis	Present	3	11	2	3.702	0.144*
	Absent	19	18	6		
Neurological manisfestations	Present	0	4	1	3.520	0.160*
	Absent	22	25	7		
Haematological manifestations	Present	18	26	6	1.597	0.430*
	Absent	4	3	2		
Cardiac manifestations	Present	0	3	1	2.878	0.244*
	Absent	22	26	7		
Respiratory manifestations	Present	1	7	2	4.145	0.117*
	Absent	21	22	6		
Anti-dsDNA	Positive	19	27	7	1.046	0.603*
	Negative	3	2	1		
Anti-Sm	Positive	7	15	2	3.133	0.209
	Negative	14	13	6		
Anti-SSA52	Positive	8	12	1	2.315	0.314
	Negative	13	17	7		
Anti-SSA60	Positive	10	14	1	3.546	0.206
	Negative	11	15	7		
Anti-SSB	Positive	6	2	1	4.160	0.092*
	Negative	15	27	7		
Anti-RNP	Positive	6	13	2	1.905	0.386
	Negative	15	15	5		

The p values marked with * indicate that Fisher's exact test has been used.
Anti-dsDNA, anti-double-stranded deoxyribonucleic acid; anti-RNP, anti-ribonucleoprotein; anti-Sm, anti-Smith; anti-SSA, anti-Sjogren's syndrome related antigen A; anti-SSB, anti-Sjogren's syndrome related antigen B; VDR, vitamin D receptor.

Table 3.67. Results obtained when using the Chi Squared test or Fisher's exact test to compare categorical variables for the different genotypes for FokI VDR polymorphism.

Variable		FokI Homozygous wild type	FokI Heterozygous	FokI Homozygous minor	Pearson Chi Square/ Fisher's exact test value	p value
Fibromyalgia	Present	1	4	3	0.349	1.000*
	Absent	6	23	22		
Raynauds	Present	2	16	10	3.067	0.216
	Absent	5	11	15		
Mucocutaneous manifestations	Present	5	25	20	2.970	0.182*
	Absent	2	2	5		
Lupus nephritis	Present	2	5	9	2.016	0.365
	Absent	5	22	16		
Neurological manisfestations	Present	1	3	1	1.615	0.516*
	Absent	6	24	24		
Haematological manifestations	Present	7	23	20	1.235	0.605*
	Absent	0	4	5		
Cardiac manifestations	Present	0	1	3	1.448	0.608*
	Absent	7	26	22		
Respiratory manifestations	Present	2	3	5	1.778	0.385*
	Absent	5	24	20		
Anti-dsDNA	Positive	6	24	23	0.713	0.851*
	Negative	1	3	2		
Anti-Sm	Positive	3	12	9	0.477	0.868*
	Negative	4	14	15		
Anti-SSA52	Positive	4	8	9	1.883	0.381*
	Negative	3	19	15		
Anti-SSA60	Positive	3	12	10	0.141	1.000*
	Negative	4	15	14		
Anti-SSB	Positive	3	4	2	4.309	0.113*
	Negative	4	23	22		
Anti-RNP	Positive	5	8	8	4.013	0.134
	Negative	2	18	15		

The p values marked with * indicate that Fisher's exact test has been used.
Anti-dsDNA, anti-double-stranded deoxyribonucleic acid; anti-RNP, anti-ribonucleoprotein; anti-Sm, anti-Smith; anti-SSA, anti-Sjogren's syndrome related antigen A; anti-SSB, anti-Sjogren's syndrome related antigen B; VDR, vitamin D receptor.

Table 3.68. Results obtained when using the Chi Squared test or Fisher's exact test to compare categorical variables for the different genotypes for ApaI VDR polymorphism.

Variable		ApaI Homozygous wild type	ApaI Heterozygous	ApaI Homozygous minor	Pearson Chi Square/ Fisher's exact test value	p value
Fibromyalgia	Present	0	4	4	6.131	**0.032***
	Absent	10	35	6		
Raynauds	Present	5	19	4	0.274	0.872
	Absent	5	20	6		
Mucocutaneous manifestations	Present	8	34	8	0.980	0.644*
	Absent	2	5	2		
Lupus nephritis	Present	2	11	3	0.366	0.917*
	Absent	8	28	7		
Neurological manisfestations	Present	0	3	2	2.245	0.374*
	Absent	10	36	8		
Haematological manifestations	Present	7	36	7	5.343	0.056
	Absent	3	3	3		
Cardiac manifestations	Present	0	4	0	1.078	0.598
	Absent	10	35	10		
Respiratory manifestations	Present	0	8	2	2.299	0.353*
	Absent	10	31	8		
Anti-dsDNA	Positive	9	35	9	0.321	1.000
	Negative	1	4	1		
Anti-Sm	Positive	3	19	2	3.709	0.188*
	Negative	7	18	8		
Anti-SSA52	Positive	4	16	1	3.614	0.164*
	Negative	6	22	9		
Anti-SSA60	Positive	4	20	1	5.976	0.065*
	Negative	6	18	9		
Anti-SSB	Positive	3	5	1	1.950	0.553*
	Negative	7	33	9		
Anti-RNP	Positive	4	16	1	4.012	0.160*
	Negative	6	20	9		

The p values marked with * indicate that Fisher's exact test has been used.
Anti-dsDNA, anti-double-stranded deoxyribonucleic acid; anti-RNP, anti-ribonucleoprotein; anti-Sm, anti-Smith; anti-SSA, anti-Sjogren's syndrome related antigen A; anti-SSB, anti-Sjogren's syndrome related antigen B; VDR, vitamin D receptor.

Table 3.69. Results obtained when using the Chi Squared test or Fisher's exact test to compare categorical variables for the different genotypes for TaqI VDR polymorphism.

Variable		TaqI Homozygous wild type	TaqI Heterozygous	TaqI Homozygous minor	Pearson Chi Square/ Fisher's exact test value	p value
Fibromyalgia	Present	4	0	3	11.451	**0.002***
	Absent	19	29	3		
Raynauds	Present	11	15	1	2.481	0.289
	Absent	12	14	5		
Mucocutaneous manifestations	Present	20	23	6	1.230	0.664*
	Absent	3	6	0		
Lupus nephritis	Present	5	9	2	0.834	0.692
	Absent	18	20	4		
Neurological manisfestations	Present	0	5	0	4.478	0.093*
	Absent	23	24	6		
Haematological manifestations	Present	20	26	4	2.315	0.327*
	Absent	3	3	2		
Cardiac manifestations	Present	2	2	0	0.418	1.000*
	Absent	21	27	6		
Respiratory manifestations	Present	3	6	1	0.656	0.879*
	Absent	20	23	5		
Anti-dsDNA	Positive	21	26	6	0.374	1.000*
	Negative	2	3	0		
Anti-Sm	Positive	8	14	2	1.187	0.628*
	Negative	14	14	4		
Anti-SSA52	Positive	8	12	1	1.308	0.534
	Negative	14	17	5		
Anti-SSA60	Positive	8	16	1	3.811	0.149
	Negative	14	13	5		
Anti-SSB	Positive	4	4	1	0.463	0.878*
	Negative	18	25	5		
Anti-RNP	Positive	7	13	1	2.193	0.378
	Negative	14	15	5		

The p values marked with * indicate that Fisher's exact test has been used.
Anti-dsDNA, anti-double-stranded deoxyribonucleic acid; anti-RNP, anti-ribonucleoprotein; anti-Sm, anti-Smith; anti-SSA, anti-Sjogren's syndrome related antigen A; anti-SSB, anti-Sjogren's syndrome related antigen B; VDR, vitamin D receptor.

A significant relationship was found between ApaI and TaqI homozygous minor and fibromyalgia. This relationship was studied further by means of Odds ratio (table 3.70-3.73). The results showed that there was a higher prevalence of fibromyalgia in the patients who were homozygous minor for ApaI or TaqI polymorphisms.

Table 3.70. Odds ratio results for the presence of fibromyalgia and ApaI homozygous wildtype versus heterozygous and homozygous minor.

Variable		ApaI Homozygous wild type	ApaI Heterozygous and Homozygous minor	Odds ratio	95% CI	P value
Fibromyalgia	Present	0	8	0.232	0.012-4.361	0.329
	Absent	10	41			

CI, confidence interval.

Table 3.71. Odds ratio results for the presence of fibromyalgia and ApaI homozygous minor versus heterozygous and homozygous wildtype.

Variable		ApaI Homozygous minor	ApaI Heterozygous and Homozygous wildtype	Odds ratio	95% CI	P value
Fibromyalgia	Present	4	4	7.500	1.474-38.156	**0.015**
	Absent	6	45			

CI, confidence interval.

Table 3.72. Odds ratio results for the presence of fibromyalgia and TaqI homozygous wildtype versus heterozygous and homozygous minor.

Variable		TaqI Homozygous wildtype	TaqI Heterozygous and Homozygous minor	Odds ratio	95% CI	P value
Fibromyalgia	Present	4	3	2.246	0.453-11.134	0.322
	Absent	19	32			

CI, confidence interval.

Table 3.73. Odds ratio results for the presence of fibromyalgia and TaqI homozygous minor versus heterozygous and homozygous wildtype.

Variable		TaqI Homozygous minor	TaqI Heterozygous and Homozygous wildtype	Odds ratio	95% CI	P value
Fibromyalgia	Present	3	4	12.000	1.799-80.051	**0.010**
	Absent	3	48			

CI, confidence interval.

3.5.3 Relationship of VDR Polymorphisms with IFN Signature Gene response in Prospective study

Out of the 31 patients included in the prospective study, VDR polymorphisms data was available for 18 patients. The Freeman-Halton extension of Fisher's exact test was used to compare the genotype of the 4 VDR polymorphisms between the patients who had a decrease in IFN signature gene expression score from baseline to 6 months (11 patients) with those patients who had an increase in score (7 patients) (table 3.74).

Table 3.74. Results obtained when using Fisher's exact test to compare number of patients with an increase/decrease in IFN signature gene expression score for the different VDR polymorphisms.

VDR polymorphism		Number of patients with decrease in IFN score	Number of patients with increase in IFN score	Fisher's exact test value	p value (2-tailed)
BsmI	Homozygous wildtype	2	4	4.100	0.150
	Heterozygous	5	3		
	Homozygous minor	4	0		
FokI	Homozygous wildtype	1	0	2.780	0.316
	Heterozygous	5	6		
	Homozygous minor	5	1		
ApaI	Homozygous wildtype	0	1	3.978	0.155
	Heterozygous	7	6		
	Homozygous minor	4	0		
TaqI	Homozygous wildtype	4	3	1.418	0.647
	Heterozygous	4	4		
	Homozygous minor	2	0		

IFN, interferon.

No significant difference was noted in the number of IFN signature gene expression responders between the different genotypes for the four VDR polymorphisms.

3.5.4 SUMMARY OF RESULTS

In the cross-sectional cohort study, it was noted that there was a significantly higher SLEDAI-2K (median 4) in the patients who were homozygous for the minor allele for BsmI compared to the rest (median 3; p=0.046). An increased prevalence of fibromyalgia was found in SLE patients who were homozygous minor for the ApaI or TaqI polymorphisms (OR=7.50, CI 1.47-38.16, p=0.015 and OR=12.00, CI 1.80-80.05, p=0.010 respectively). No relationship was found between BsmI, FokI, ApaI and TaqI genotypes and damage, fatigue, serum 25-hydroxyvitamin D, IFN signature gene expression score, organ manifestations, autoantibodies and other variables measured.

On comparing the number of IFN signature gene responders with vitamin D3 treatment in the prospective part of the research, no significant difference was noted between the genotypes for the four VDR polymorphisms. However, in this analysis data was available for only 18 patients.

3.6 IN VITRO EFFECT OF CALCITRIOL SUPPLEMENTATION ON EXPRESSION OF IFN SIGNATURE GENES, *IRF8* AND *IRF7* IN PRIMARY CELL CULTURE

Isolated PBMCs from a SLE patient and control were differentiated to (i) dendritic cells and (ii) macrophages. Half of the cultures were supplemented with calcitriol and the rest were left untreated. RNA extraction was carried out from all samples at baseline, 24 hours and 48 hours following calcitriol supplementation. RNA obtained had a good concentration (mean 41.9ng/µl, range 19.3-84.2ng/µl) and purity defined by 260/280 ratio (mean 1.9, range 1.71-1.98). The expression of the 12 IFN signature genes (*IFITM1, IFI35, IFIT1, IFIT3, STAT1, MX1, OAS1, STAT2, SOCS1, CCL2, CXCL1, SOCS3*), *IRF8* and *IRF7* was measured in the extracted RNA.

3.6.1 MICROSCOPY

Microscopy was carried out on the isolated PBMCs from the control and SLE patient (figure 3.39). Further microscopy was carried out during several stages in DC and macrophage differentiation (figures 3.40-3.41). The characteristic elongation was noted in DCs (images 3.40A and 3.41A).

A B

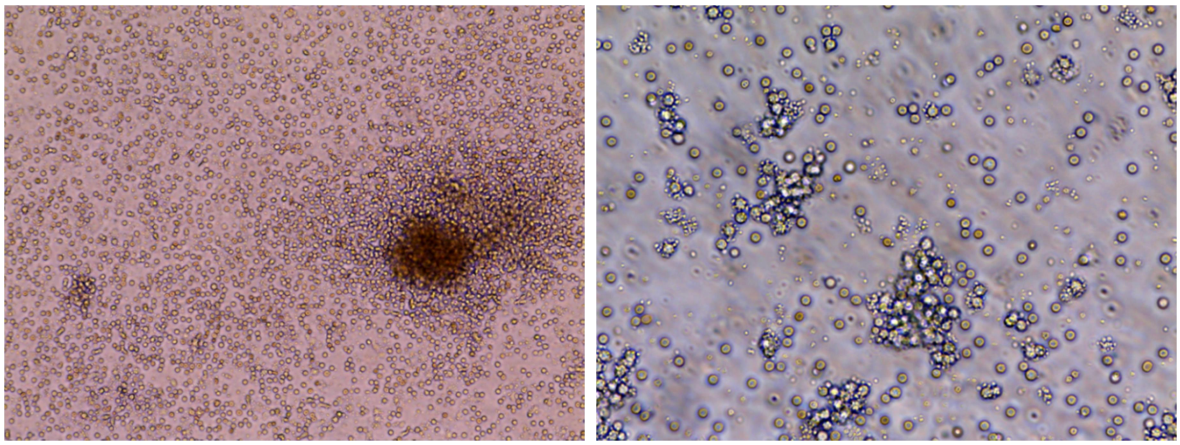

Figure 3.39. Microscope images of isolated peripheral blood mononuclear cells at 20X (image A) and 40X (image B) magnification.

A B

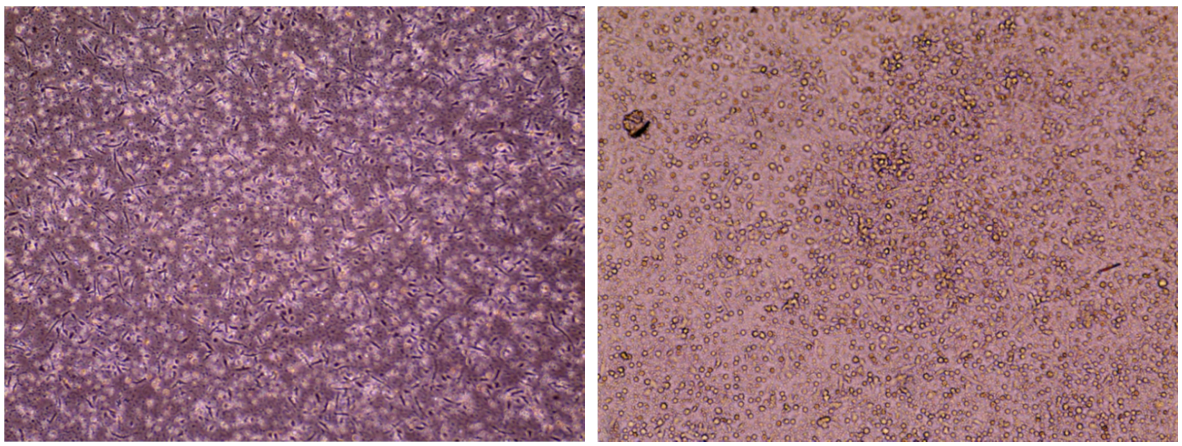

Figure 3.40. Microscope images at 20X magnification obtained on day 2 of dendritic cell differentiation (image A) and macrophage differentiation (image B).

A B

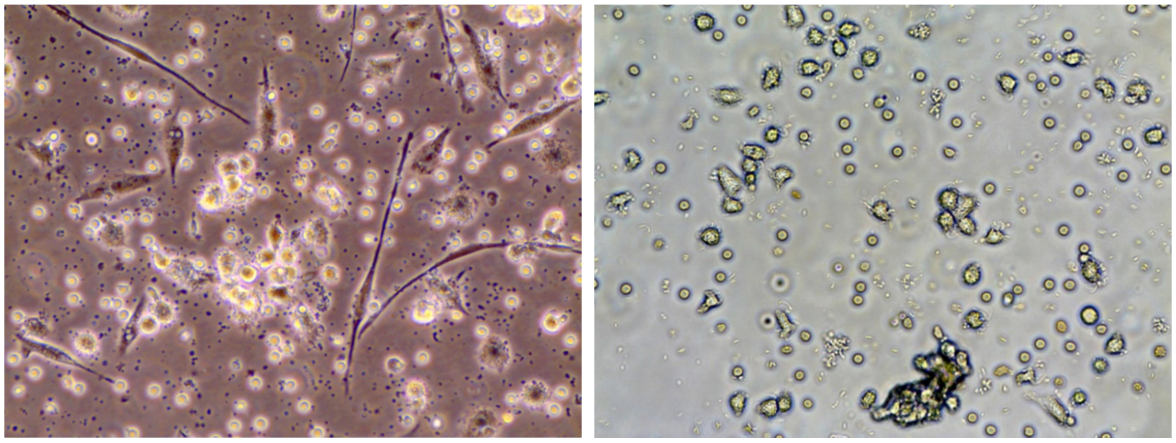

Figure 3.41. Microscope images at 40X magnification obtained on day 7 of dendritic cell differentiation (image A) and macrophage differentiation (image B).

3.6.2 CELL COUNT AND VIABILITY

The Countess™ automated cell counter was used to measure cell count and viability for isolated PBMCs and differentiated DCs and macrophages. For each sample these were checked twice. The results obtained are shown in table 3.75.

Table 3.75. Cell count and viability results.

Cell type	Cell count	Viability (% live cells)
Isolated PBMCs from SLE patient	$4.36x10^7$/ml	72%
	$4.20x10^7$/ml	78%
Isolated PBMCs from control	$4.18x10^7$/ml	69%
	$4.24x10^7$/ml	76%
Differentiated DCs from SLE patient	1.58 $x10^6$/ml	65%
	1.32 x 10^6/ml	73%
Differentiated DCs from control	$1.99x10^6$/ml	61%
	$1.75x10^6$/ml	75%
Differentiated macrophages from SLE patient	9.45 x 10^6/ml	82%
	9.46 x 10^6/ml	68%
Differentiated macrophages from control	$2.76x10^6$/ml	76%
	$3.11x10^6$/ml	73%

DCs, dendritic cells; PBMCs, peripheral blood mononuclear cells.

3.6.3 FLOW CYTOMETRY

Flow cytometry carried out on isolated PBMCs from SLE patient and control revealed the presence of monocytes expressing CD14 and lymphocytes which did not express CD14 or CD83 (figure 3.42). Few cells expressed CD83 in the isolated PBMCs.

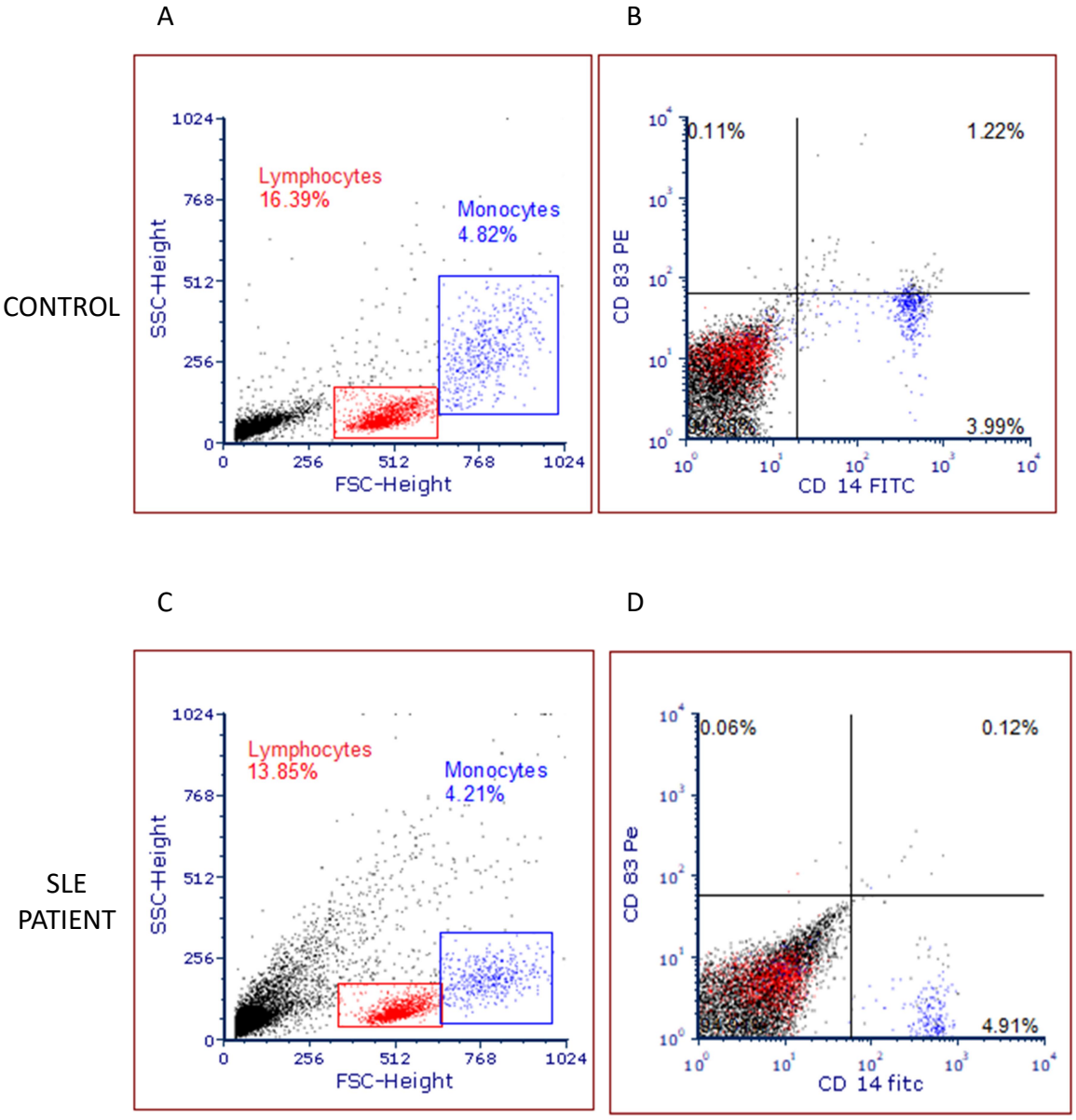

Figure 3.42. Figure showing flow cytometry results for PBMCs isolated from the control (images A and B) and the SLE patient (images C and D). Graphs A and C display the side scatter vs forward scatter for the control and SLE patient respectively. Graphs B and D display the CD83 vs CD14 for the control and SLE patient respectively.

CD, cluster of differentiation; FITC, fluorescein isothiocyanate; FSC, forward scatter; PE, phycoerythrin; SSC, side scatter.

Flow cytometry was also carried out following DC and macrophage differentiation. A high proportion of cells expressing CD83 in cultures differentiated to dendritic cells from both SLE patient and control were noted (figure 3.43). On the other hand, in both cultures differentiated to macrophages, a high proportion of cells expressing CD14 were present (figure 3.44).

A B

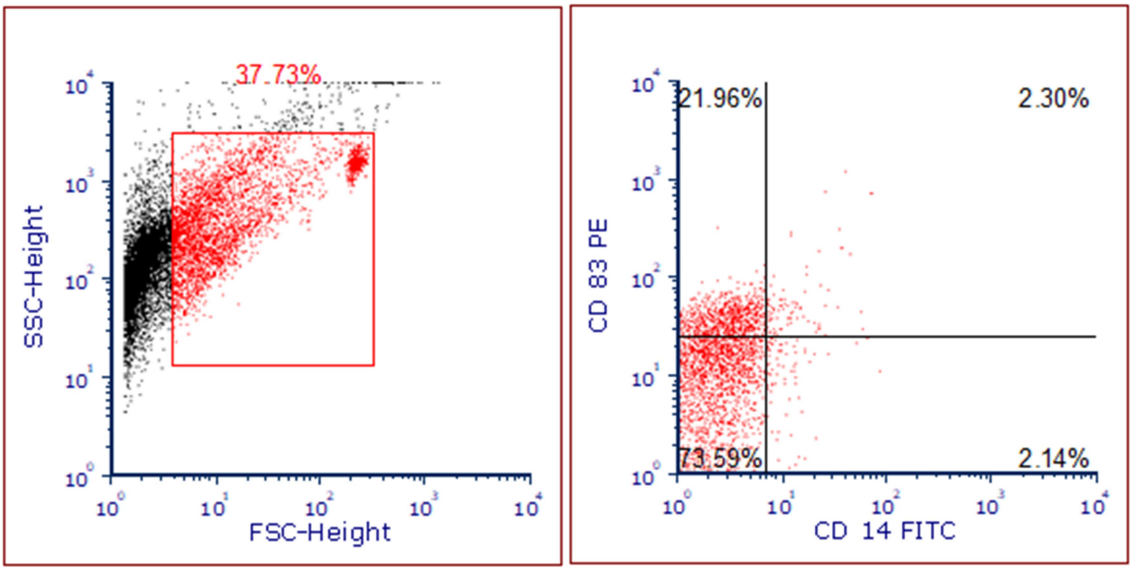

Figure 3.43. Figure showing flow cytometry results obtained after PBMCs obtained from the SLE patient underwent 7 days of differentiation to dendritic cells. Graph A shows the side scatter vs forward scatter and graph B shows CD83 vs CD14.

CD, cluster of differentiation; FITC, fluorescein isothiocyanate; FSC, forward scatter; PE, phycoerythrin; SSC, side scatter.

A B

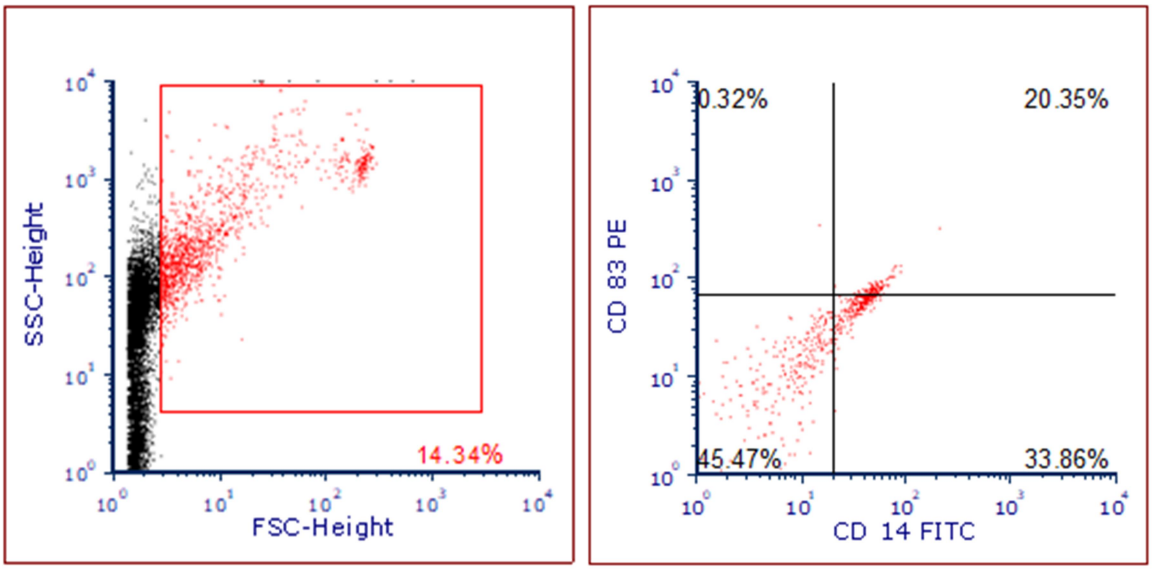

Figure 3.44. Figure showing flow cytometry results obtained after PBMCs obtained from the SLE patient underwent 7 days of differentiation to macrophages. Graph A shows the side scatter (SSC) vs forward scatter (FSC) and graph B shows CD83 vs CD14. CD, cluster of differentiation; FITC, fluorescein isothiocyanate; FSC, forward scatter; PE, phycoerythrin; SSC, side scatter.

3.6.4 COMPARING GENE EXPRESSION IN TREATED AND UNTREATED SAMPLES AT 24 AND 48 HOURS

The normalised MFI for the 14 genes analysed and the IFN signature gene expression score at 24 and 48 hours for all the cell cultures were tested for normality by the K-S test (table 3.76).

Table 3.76. Kolmogorov-Smirnov test results obtained for the normalised MFI for the genes analysed and for the IFN signature gene expression score for all the SLE patient and control cell cultures.

Variable	Time (hrs)	Patient DC culture		Control DC culture		Patient macrophage culture		Control macrophage culture	
		Test Statistic	P value	Test Statistic	P value	Test Statistic	P value	Test Statistic	P value
IFI35	**24**	0.168	0.200	0.284	0.141	0.267	0.141	0.215	0.200
	48	0.176	0.200	0.237	0.200	0.150	0.200	0.163	0.200
OAS1	**24**	0.191	0.200	0.263	0.200	0.266	0.143	0.155	0.200
	48	0.256	0.131	0.186	0.200	0.232	0.200	0.194	0.200
CCL2	**24**	0.213	0.200	0.285	0.140	0.225	0.200	0.173	0.200
	48	0.258	0.200	0.172	0.200	0.221	0.200	0.191	0.200
MX1	**24**	0.240	0.196	0.125	0.200	0.117	0.200	0.181	0.200
	48	0.248	0.160	0.283	0.200	0.378	0.003	0.247	0.200
SOCS1	**24**	0.318	0.017	0.202	0.200	0.256	0.184	0.313	0.123
	48	0.216	0.200	0.231	0.200	0.204	0.200	0.393	0.200
IFITM1	**24**	0.179	0.200	0.343	0.026	0.311	0.039	0.261	0.164
	48	0.188	0.200	0.260	0.200	0.200	0.200	0.165	0.200
STAT2	**24**	0.142	0.200	0.256	0.200	0.222	0.200	0.259	0.172
	48	0.267	0.097	0.289	0.200	0.197	0.200	0.198	0.200
CXCL1	**24**	0.342	0.013	0.194	0.200	0.302	0.053	0.332	0.200
	48	0.288	0.130	0.267	0.200	0.223	0.200	0.268	0.200
IFIT3	**24**	0.202	0.200	0.188	0.200	0.301	0.054	0.319	0.057
	48	0.249	0.200	0.198	0.200	0.163	0.200	0.305	0.085
SOCS3	**24**	0.195	0.200	0.246	0.200	0.212	0.200	0.170	0.200
	48	0.304	0.028	0.275	0.200	0.236	0.200	0.173	0.200
IFIT1	**24**	0.156	0.200	0.171	0.200	0.163	0.200	0.199	0.200
	48	0.160	0.200	0.154	0.200	0.172	0.200	0.235	0.200
STAT1	**24**	0.212	0.200	0.319	0.056	0.318	0.031	0.293	0.071
	48	0.227	0.200	0.257	0.200	0.329	0.010	0.327	0.023
IRF7	**24**	0.208	0.200	0.167	0.200	0.182	0.200	0.169	0.200
	48	0.295	0.039	0.233	0.200	0.176	0.200	0.157	0.200
IRF8	**24**	0.274	0.079	0.151	0.200	0.322	0.027	0.168	0.200
	48	0.199	0.200	0.320	0.105	0.271	0.129	0.219	0.200
IFN score	**24**	0.203	0.200	0.168	0.200	0.217	0.200	0.162	0.200
	48	0.260	0.119	0.149	0.200	0.132	0.200	0.189	0.200

DC, dendritic cell; IFN, interferon; MFI, median fluorescence intensity.

The normalised MFI (for the 14 genes analysed) obtained by Quantigene analysis on the extracted RNA from the samples treated with calcitriol was compared with that obtained in the untreated samples from both SLE patient and control (tables 3.77-3.78). The Bonferroni correction was used since multiple genes were tested. Using this method, a significance level for the p value of 0.004 was established for the MFI of the 14 genes. A statistically significant lower gene expression of *MX1* (at 24 hours), *IFIT3* (at 24 hours) and *STAT1* (at 48 hours) were noted in the treated SLE patient DC culture compared to the respective untreated culture. No significant differences were noted in the control DC cultures. In the SLE patient macrophage cultures, a statistically significant lower gene expression in the treated samples was noted for *IFI35, OAS1* and *CCL2* at 24 hours. Similarly, a lower gene expression for the treated control macrophage culture was noted for *IFIT3* and *STAT1* at 24 hours.

Table 3.77. Mean normalised MFI and standard deviation for the treated and untreated SLE patient and control DC cultures for the 14 genes analysed.

Gene	Time (hrs)	SLE Patient DC culture					Control DC culture				
		Mean MFI (untreated)	S.D. (untreated)	Mean MFI (treated)	S.D. (treated)	p value	Mean MFI (untreated)	S.D. (untreated)	Mean MFI (treated)	S.D. (treated)	p value
IFI35	24	0.338	0.007	0.289	0.018	0.005	0.357	0.029	0.283	0.029	0.173
	48	0.205	0.014	0.246	0.026	0.046	0.387	0.029	0.253	0.018	0.010
OAS1	24	0.269	0.032	0.251	0.062	0.667	0.547	0.117	0.399	0.057	0.122
	48	0.263	0.003	0.293	0.137	0.651	0.517	0.067	0.313	0.036	0.031
CCL2	24	0.005	0.002	0.011	0.005	0.082	0.015	0.006	0.120	0.030	0.004
	48	0.003	0.001	0.003	0.002	0.721	0.006	0.004	0.021	0.006	0.108
MX1	24	0.195	0.014	0.127	0.013	**<0.001**	0.076	0.013	0.083	0.013	0.541
	48	0.158	0.028	0.132	0.010	0.090	0.048	0.005	0.053	0.001	0.260
SOCS1	24	1.607	0.191	1.100	0.451	0.036*	1.418	0.101	1.066	0.226	0.070
	48	1.374	0.164	1.165	0.109	0.070	1.446	0.253	1.212	0.157	0.339
IFITM1	24	3.904	0.644	3.242	0.733	0.246	3.406	0.739	2.644	0.064	0.100*
	48	3.510	0.136	2.820	0.671	0.139	3.247	0.206	2.130	0.008	0.005
STAT2	24	0.192	0.028	0.156	0.034	0.175	0.226	0.016	0.202	0.058	0.535
	48	0.146	0.045	0.168	0.020	0.355	0.144	0.045	0.212	0.024	0.154
CXCL1	24	0.007	0.004	0.009	0.002	0.229*	0.021	0.013	0.016	0.006	0.604
	48	0.006	0.004	0.014	0.011	0.383	0.014	0.009	0.036	0.017	0.144
IFIT3	24	0.037	0.003	0.020	0.004	**<0.001**	0.086	0.018	0.122	0.014	0.054
	48	0.014	0.006	0.019	0.009	0.363	0.059	0.008	0.058	0.017	0.943
SOCS3	24	0.710	0.284	0.549	0.253	0.437	0.564	0.211	0.481	0.229	0.668
	48	0.641	0.314	0.564	0.219	0.571*	0.550	0.268	0.601	0.237	0.841
IFIT1	24	0.046	0.003	0.025	0.012	0.022	0.031	0.008	0.042	0.016	0.342
	48	0.039	0.005	0.030	0.007	0.105	0.024	0.007	0.015	0.007	0.239

STAT1	24	1.026	0.082	0.631	0.217	0.025	1.138	0.207	0.967	0.019	0.225
	48	0.622	0.017	0.464	0.036	**<0.001**	0.833	0.042	0.564	0.045	0.006
IRF8	24	0.306	0.003	0.253	0.077	0.295	0.437	0.071	0.424	0.014	0.782
	48	0.240	0.006	0.244	0.082	0.925	0.308	0.065	0.302	0.057	0.927
IRF7	24	0.141	0.064	0.111	0.040	0.438	0.154	0.045	0.140	0.042	0.708
	48	0.110	0.048	0.098	0.029	0.881*	0.132	0.057	0.125	0.065	0.902

P values comparing gene expression between the respective treated and untreated samples at 24 and 48 hours, have been obtained using two-tailed independent samples t-test for normally distributed variables and Mann-Whitney U-test (marked by *) as the non-parametric alternative. Using the Bonferroni correction for multiple gene testing, a significance level for the p value of 0.004 was established. Statistically significant results are shown in bold.

DC, dendritic cell; MFI, median fluorescent intensity; S.D., standard deviation.

Table 3.78. Mean normalised MFI and standard deviation for the treated and untreated SLE patient and control macrophage cultures for the 14 genes analysed.

Gene	Time (hrs)	SLE Patient Macrophage culture					Control Macrophage culture				
		Mean MFI (untreated)	S.D. (untreated)	Mean MFI (treated)	S.D. (treated)	p value	Mean MFI (untreated)	S.D. (untreated)	Mean MFI (treated)	S.D. (treated)	p value
IFI35	24	0.326	0.007	0.171	0.027	**<0.001**	0.207	0.005	0.112	0.051	0.054
	48	0.192	0.027	0.144	0.030	0.087	0.140	0.156	0.141	0.085	0.994
OAS1	24	0.192	0.019	0.091	0.013	**<0.001**	0.297	0.065	0.112	0.059	0.015
	48	0.105	0.034	0.054	0.018	0.048	0.320	0.112	0.175	0.104	0.160
CCL2	24	36.612	0.873	25.679	3.373	**0.003**	6.868	0.236	2.332	1.549	0.011
	48	44.133	8.226	37.318	3.978	0.200	10.349	0.361	4.564	2.492	0.006
MX1	24	0.190	0.014	0.143	0.017	0.012	0.321	0.038	0.240	0.113	0.388
	48	0.133	0.041	0.139	0.032	0.857*	0.205	0.077	0.198	0.071	0.907
SOCS1	24	0.214	0.031	0.217	0.035	0.906	0.066	0.020	0.045	0.009	0.197
	48	0.123	0.035	0.251	0.062	0.019	0.084		0.051	0.003	0.011
IFITM1	24	6.023	0.154	7.502	1.946	0.229*	5.121	0.630	3.632	1.206	0.171
	48	6.038	1.198	7.924	1.890	0.195	3.772	0.726	2.631	1.103	0.246
STAT2	24	0.251	0.051	0.210	0.040	0.279	0.413	0.061	0.335	0.134	0.480
	48	0.191	0.084	0.217	0.014	0.558	0.368	0.228	0.380	0.162	0.936
CXCL1	24	1.194	0.455	0.486	0.188	0.035	0.037	0.015	0.021	0.007	0.310
	48	1.744	0.916	1.067	0.366	0.228	0.065		0.062	0.036	0.942
IFIT3	24	0.208	0.029	0.091	0.007	0.016	0.299	0.012	0.065	0.032	**<0.001**
	48	0.100	0.066	0.126	0.045	0.565	0.376	0.184	0.166	0.074	0.095
SOCS3	24	1.428	0.703	1.660	0.588	0.654	0.147	0.106	0.084	0.053	0.316
	48	1.478	0.844	1.794	0.691	0.608	0.099	0.097	0.085	0.052	0.806
IFIT1	24	0.048	0.018	0.032	0.015	0.258	0.055	0.007	0.033	0.019	0.204
	48	0.036	0.025	0.033	0.011	0.817			0.039	0.021	

STAT1	24	3.378	0.039	1.191	0.136	0.057*	7.965	0.106	2.131	1.436	**0.003**
	48	1.959	0.056	0.817	0.021	0.057*	12.363	0.439	3.793	1.288	0.095*
IRF8	24	0.472	0.026	0.200	0.016	0.057*	0.788	0.112	0.361	0.201	0.041
	48	0.553	0.035	0.241	0.041	**<0.001**	0.786	0.556	0.436	0.241	0.260
IRF7	24	0.167	0.067	0.124	0.041	0.336	0.204	0.087	0.130	0.074	0.303
	48	0.127	0.069	0.129	0.058	0.963	0.255	0.112	0.198	0.088	0.523

P values comparing gene expression between the respective treated and untreated samples at 24 and 48 hours, have been obtained using two-tailed independent samples t-test for normally distributed variables and Mann-Whitney U-test (marked by *) as the non-parametric alternative. Using the Bonferroni correction for multiple gene testing, a significance level for the p value of 0.004 was established. Statistically significant results are shown in bold.

MFI, median fluorescent intensity; S.D., standard deviation.

The IFN signature gene expression score computed from the normalised MFI of the 12 IFN signature genes for the treated samples was compared with that of the untreated samples at 24 and 48 hours (figure 3.45, supplementary table S8). The usual p value of 0.05 was used as a cut off to establish level of significance for the IFN signature gene expression score.

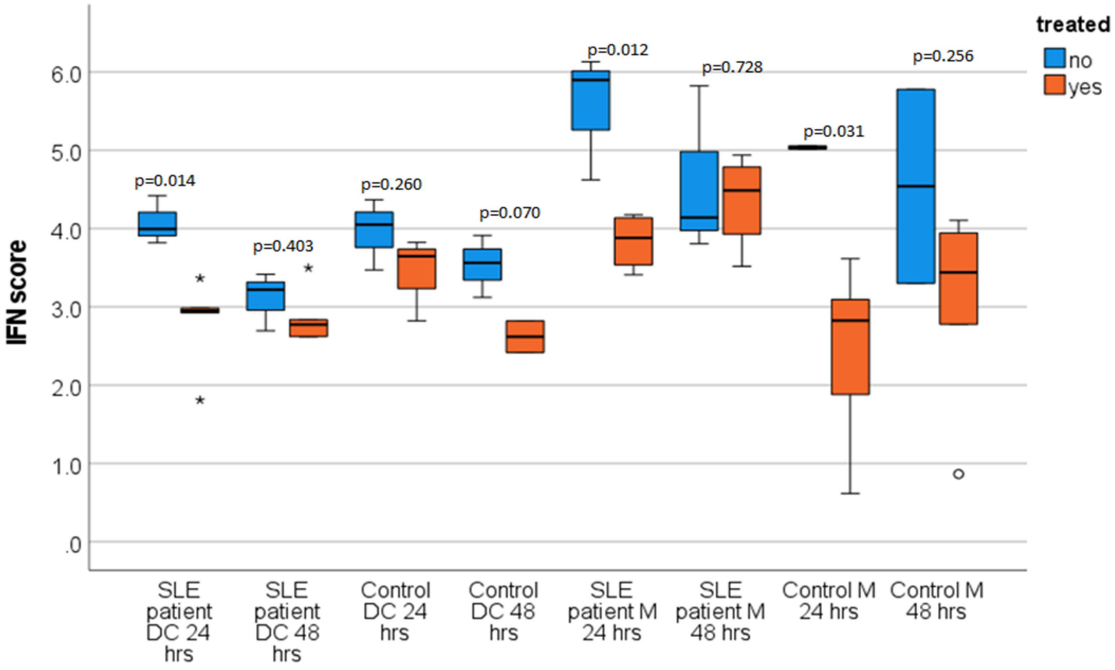

Figure 3.45. Box plot showing results obtained when comparing IFN score obtained in various cell cultures that were untreated and treated with calcitriol. DC, dendritic cell; IFN, interferon; M, macrophage.

A significant lower IFN signature gene expression score was noted at 24 hours in SLE patient dendritic cell and macrophage cell cultures and in control macrophage cell cultures treated with calcitriol when compared with respective untreated samples. On comparing *IRF8* expression in treated and untreated cell cultures, a significantly lower *IRF8* expression was noted in treated SLE patient macrophage cell culture at 48 hours

(figure 3.46, table 3.78). No difference was noted between treated and untreated DC cultures (table 3.77). No difference in *IRF7* expression was noted in treated and untreated cultures for all samples (figure 3.47, table 3.77-3.78).

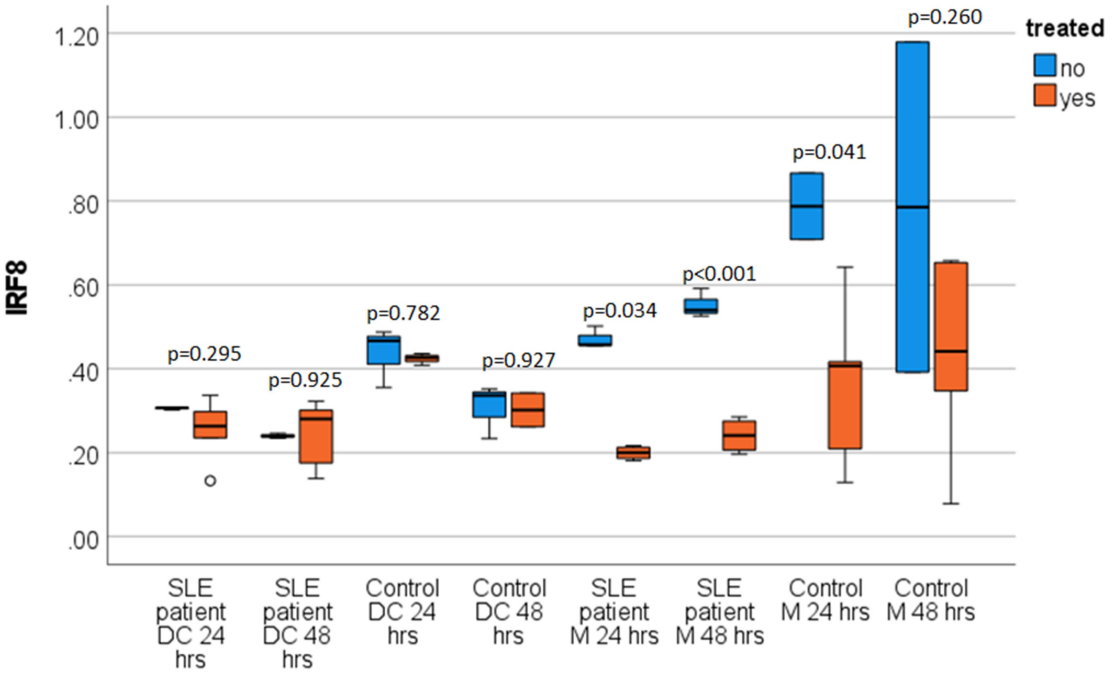

Figure 3.46. Box plot showing results obtained when comparing normalised median fluorescence intensity for *IRF8* obtained in various cell cultures that were untreated and treated with calcitriol. DC, dendritic cell; M, macrophage.

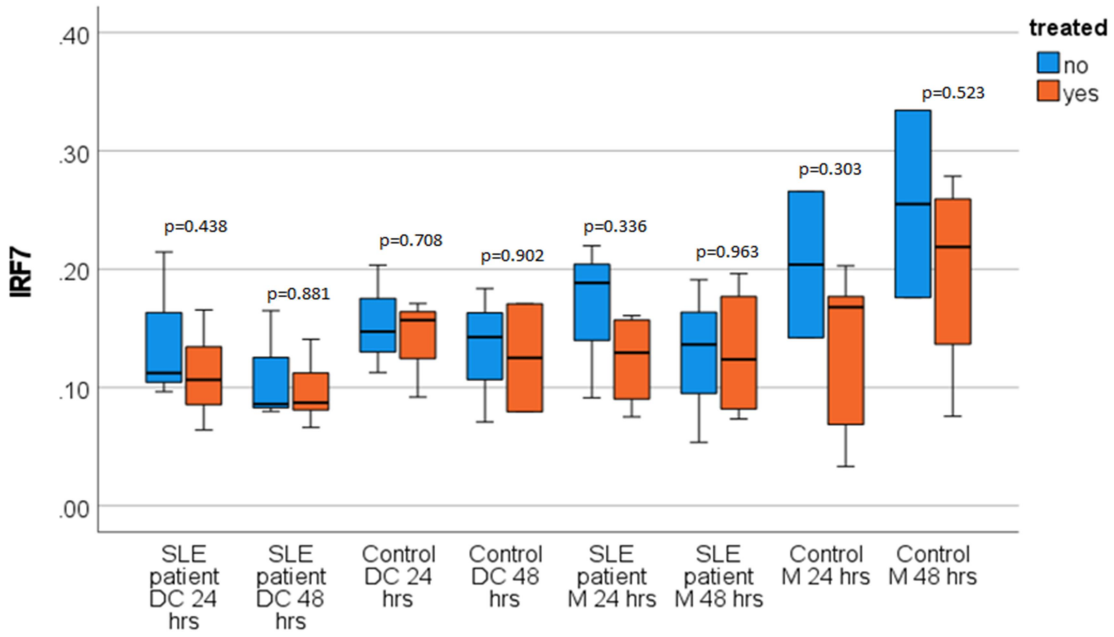

Figure 3.47. Box plot showing results obtained when comparing normalised median fluorescence intensity for *IRF7* obtained in various cell cultures that were untreated and treated with calcitriol. DC, dendritic cell; M, macrophage.

3.6.5 COMPARING GENE EXPRESSION IN TREATED SAMPLES AT 24 AND 48 HOURS TO BASELINE

The gene expression for the 14 genes analysed and the IFN signature gene expression score in samples treated with calcitriol were compared with baseline gene expression. The normalised MFI for the 14 genes analysed and the IFN signature gene expression score at baseline and at 24 and 48 hours for all the treated cell cultures were tested for normality by the K-S test (table 3.79).

Table 3.79. Kolmogorov-Smirnov test results obtained for the normalised MFI for the genes analysed and for the IFN signature gene expression score for the SLE patient and control cell cultures at baseline and at 24/48 hours for treated samples.

Variable	Time (hrs)	Patient DC culture		Patient macrophage culture		Control macrophage culture	
		Test Statistic	P value	Test Statistic	P value	Test Statistic	P value
IFI35	**24**	0.250	0.112	0.258	0.126	0.236	0.200
	48	0.181	0.200	0.127	0.200	0.169	0.200
OAS1	**24**	0.165	0.200	0.211	0.200	0.188	0.200
	48	0.183	0.200	0.267	0.098	0.196	0.200
CCL2	**24**	0.179	0.200	0.184	0.200	0.137	0.200
	48	0.199	0.200	0.201	0.200	0.259	0.123
MX1	**24**	0.303	0.017	0.177	0.200	0.192	0.200
	48	0.316	0.010	0.337	0.008	0.228	0.200
SOCS1	**24**	0.329	0.006	0.147	0.200	0.225	0.200
	48	0.232	0.176	0.228	0.200	0.268	0.200
IFITM1	**24**	0.247	0.120	0.196	0.200	0.203	0.200
	48	0.245	0.127	0.244	0.176	0.142	0.200
STAT2	**24**	0.126	0.200	0.218	0.200	0.273	0.080
	48	0.238	0.149	0.333	0.010	0.243	0.180
CXCL1	**24**	0.285	0.088	0.184	0.200	0.240	0.200
	48	0.245	0.200	0.195	0.200	0.258	0.200
IFIT3	**24**	0.364	0.002	0.267	0.098	0.202	0.200
	48	0.306	0.046	0.137	0.200	0.265	0.146
SOCS3	**24**	0.254	0.097	0.203	0.200	0.267	0.098
	48	0.241	0.142	0.159	0.200	0.273	0.080
IFIT1	**24**	0.124	0.200	0.172	0.200	0.185	0.200
	48	0.140	0.200	0.182	0.200	0.197	0.200
STAT1	**24**	0.270	0.057	0.229	0.200	0.241	0.193
	48	0.338	0.004	0.256	0.132	0.204	0.200
IRF7	**24**	0.169	0.200	0.197	0.200	0.280	0.065
	48	0.213	0.200	0.182	0.200	0.251	0.200
IRF8	**24**	0.185	0.200	0.291	0.045	0.184	0.200
	48	0.184	0.200	0.224	0.200	0.144	0.200
IFN score	**24**	0.167	0.200	0.247	0.165	0.119	0.200
	48	0.234	0.169	0.182	0.200	0.177	0.200

DC, dendritic cell; IFN, interferon; MFI, median fluorescence intensity.

The normalised MFI (for the 14 genes analysed) obtained by Quantigene analysis on the extracted RNA from the samples treated with calcitriol at 24 and 48 hours respectively were compared with that obtained at baseline (table 3.80). The Bonferroni correction was used since multiple genes were tested. Using this method, a significance level for the p value of 0.004 was established for the MFI of the 14 genes. The usual p value of 0.05 was used as a cut off to establish level of significance for the IFN signature gene expression score. A statistically significant decrease in the expression of STAT1 at 24 hours and IFIT1 at 48 hours were noted in the SLE patient DC culture. A significant reduction in the expression of *IRF8* at 48 hours and IFIT3 at 24 hours were noted in patient and control macrophage culture respectively. A significant decrease in IFN signature gene expression score was noted at 24 and 48 hours in SLE patient DC culture and at 24 hours in the control macrophage culture.

Table 3.80. Mean normalised MFI for the 14 genes analysed and mean IFN signature gene expression score at baseline and at 24 and 48 hours for the cultures treated with calcitriol for the SLE patient DC and macrophage cultures and the control macrophage culture.

Variable	Time (hrs)	Patient DC culture			Patient macrophage culture			Control macrophage culture		
		Mean MFI	S.D.	p value	Mean MFI	S.D.	P value	Mean MFI	S.D.	P value
IFI35	**0**	0.248	0.040		0.177	0.035		0.274	0.069	
	24	0.289	0.018	0.128	0.171	0.027	0.799	0.112	0.051	0.008
	48	0.246	0.026	0.945	0.144	0.030	0.208	0.141	0.085	0.063
OAS1	**0**	0.172	0.047		0.140	0.064		0.354	0.081	
	24	0.251	0.062	0.072	0.091	0.013	0.178	0.112	0.059	0.032
	48	0.293	0.137	0.138	0.054	0.018	0.040	0.175	0.104	0.045
CCL2	**0**	0.012	0.004		29.267	1.756		6.396	1.408	
	24	0.011	0.005	0.938	25.679	3.373	0.093	2.332	1.549	0.010
	48	0.003	0.002	0.111	37.318	3.978	0.011	4.564	2.492	0.233
MX1	**0**	0.311	0.042		0.155	0.043		0.312	0.067	
	24	0.127	0.013	0.016*	0.143	0.017	0.645	0.240	0.113	0.365
	48	0.132	0.010	0.016*	0.139	0.032	0.343*	0.198	0.071	0.066
SOCS1	**0**	1.179	0.426		0.248	0.055		0.105	0.026	
	24	1.100	0.451	0.730*	0.217	0.035	0.381	0.045	0.009	0.021
	48	1.165	0.109	0.947	0.251	0.062	0.934	0.051	0.003	0.068
IFITM1	**0**	5.926	1.881		6.603	1.113		4.415	1.530	
	24	3.242	0.733	0.021	7.502	1.946	0.453	3.632	1.206	0.449
	48	2.820	0.671	0.010	7.924	1.890	0.284	2.631	1.103	0.101
STAT2	**0**	0.189	0.036		0.195	0.038		0.447	0.009	
	24	0.156	0.034	0.201	0.210	0.040	0.598	0.335	0.134	0.209
	48	0.168	0.020	0.350	0.217	0.014	0.343*	0.380	0.162	0.512
CXCL1	**0**	0.009	0.004		0.254	0.150		0.076	0.023	
	24	0.009	0.002	0.791	0.486	0.188	0.103	0.021	0.007	0.053
	48	0.014	0.011	0.449	1.067	0.366	0.015	0.062	0.036	0.597
IFIT3	**0**	0.129	0.008		0.127	0.031		0.191	0.026	
	24	0.020	0.004	0.036*	0.091	0.007	0.062	0.065	0.032	**0.003**
	48	0.019	0.009	0.057*	0.126	0.045	0.954	0.166	0.074	0.607
SOCS3	**0**	1.697	0.616		1.078	0.896		0.476	0.218	
	24	0.549	0.253	0.006	1.660	0.588	0.319	0.084	0.053	0.085
	48	0.564	0.219	0.006	1.794	0.691	0.253	0.085	0.052	0.085
IFIT1	**0**	0.054	0.008		0.028	0.005		0.042	0.019	
	24	0.025	0.018	0.004	0.032	0.015	0.662	0.033	0.019	0.548
	48	0.030	0.007	**0.002**	0.033	0.011	0.477	0.039	0.021	0.845
STAT1	**0**	1.777	0.1750		1.334	0.305		7.652	2.893	
	24	0.631	.217	**<0.001**	1.191	0.136	0.425	2.131	1.436	0.010
	48	0.464	0.036	0.016*	0.817	0.021	0.015	3.793	1.288	0.037
IRF7	**0**	0.202	0.084		0.112	0.041		0.156	0.077	
	24	0.111	0.040	0.068	0.124	0.041	0.709	0.130	0.074	0.651
	48	0.098	0.029	0.084	0.129	0.058	0.648	0.198	0.088	0.540
IRF8	**0**	0.163	0.041		0.507	0.054		1.041	0.204	

	24	0.253	0.077	0.068	0.200	0.016	0.029*	0.361	0.201	0.008
	48	0.244	0.082	0.100	0.241	0.041	**<0.001**	0.436	0.241	0.020
IFN score	**0**	4.715	0.557		3.620	0.743		4.966	0.548	
	24	2.807	0.585	**0.002**	3.837	0.363	0.626	2.407	1.182	**0.014**
	48	2.870	0.364	**0.001**	4.358	0.611	0.176	3.027	1.314	0.055

P values comparing gene expression between the respective baseline and treated samples at 24 and 48 hours, have been obtained using the independent samples t-test for normally distributed variables, and Mann-Whitney U-test (marked by *) as the non-parametric alternative. DC, dendritic cell; IFN, interferon; MFI, median fluorescence intensity; S.D., standard deviation.

3.6.6 COMPARING GENE EXPRESSION BETWEEN THE TWO CELL CULTURE TYPES

The gene expression for the 14 genes analysed and the IFN signature gene expression score in untreated SLE patient dendritic cell culture samples were compared with the respective macrophage culture samples. The normalised MFI for the 14 genes analysed and the IFN signature gene expression in the untreated SLE patient DC and macrophage cultures were tested for normality by the K-S test (table 3.81).

Table 3.81. Kolmogorov-Smirnov test result obtained for the MFI obtained for the genes analysed and for the IFN score calculated for the untreated SLE patient cell cultures.

Variable	Test Statistic	P value
IFI35	0.208	0.024
OAS1	0.107	0.200
CCL2	0.278	0.001
MX1	0.228	0.008
SOCS1	0.273	<0.001
IFITM1	0.158	0.200
STAT2	0.150	0.200
CXCL1	0.246	0.005
IFIT3	0.189	0.072
SOCS3	0.196	0.043
IFIT1	0.129	0.200
STAT1	0.194	0.047
IRF7	0.160	0.194
IRF8	0.159	0.200
IFN score	0.109	0.200

IFN, interferon; MFI, median fluorescence intensity.

The normalised MFI for the 14 genes analysed and the IFN signature gene expression score for the two untreated SLE patient cell culture types were compared using independent samples t-test for normally distributed variables and the Mann-Whitney U-test as the non-parametric alternative (figure 3.48, supplementary table S9). Once again the Bonferroni correction was used and a significance level for the p value of 0.004 was established for the MFI of the 14 genes.

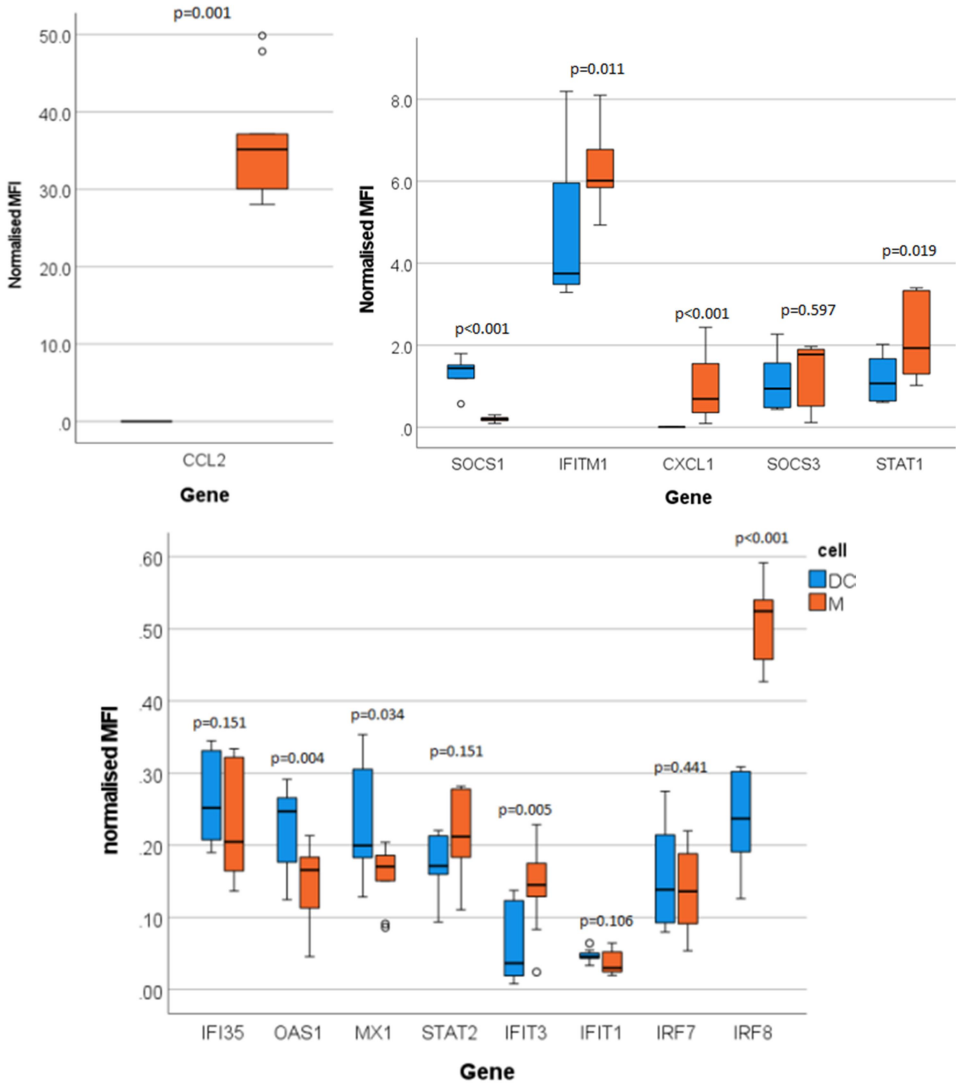

Figure 3.48. Box plots showing results obtained when comparing normalised MFI for the 14 genes analysed in untreated SLE patient cultures. DC, dendritic cell; M, macrophage; MFI, median fluorescence intensity.

The expression of *SOCS1* was significantly higher in the DC culture, while that of *CCL2, CXCL1* and *IRF8* was higher in the macrophage culture. There was no significant difference in the IFN signature gene expression score between the 2 untreated SLE patient cultures.

3.6.7 COMPARING GENE EXPRESSION BETWEEN UNTREATED CELL CULTURES FROM SLE PATIENT AND CONTROL

The normalised MFI obtained by Quantigene analysis for the 14 genes analysed and the IFN signature gene expression score in untreated SLE patient DC culture samples were compared with the respective control DC culture samples. The same was done for macrophage culture samples. The normalised MFI for the 14 genes analysed and the IFN signature gene expression in the untreated SLE patient and control DC and macrophage cultures were tested for normality by the K-S test (table 3.82).

Table 3.82. Kolmogorov-Smirnov test results obtained for the MFI obtained for the genes analysed and for the IFN score calculated for the untreated SLE patient and control DC and macrophage cultures.

Variable	DC culture		Macrophage culture	
	Test Statistic	P value	Test Statistic	P value
IFI35	0.214	0.134	0.156	0.200
OAS1	0.261	0.023	0.152	0.200
CCL2	0.213	0.200	0.259	0.056
MX1	0.181	0.200	0.190	0.200
SOCS1	0.140	0.200	0.189	0.200
IFITM1	0.189	0.200	0.161	0.200
STAT2	0.192	0.200	0.221	0.183
CXCL1	0.253	0.047	0.181	0.200
IFIT3	0.112	0.200	0.167	0.200
SOCS3	0.128	0.200	0.239	0.110
IFIT1	0.127	0.200	0.226	0.200
STAT1	0.127	0.200	0.306	0.009
IRF7	0.172	0.200	0.146	0.200
IRF8	0.181	0.200	0.250	0.076
IFN score	0.113	0.200	0.200	0.200

DC, dendritic cell; IFN, interferon; MFI, median fluorescence intensity.

The gene expression and IFN signature gene expression score for the untreated DC cultures and macrophage cultures were compared between the SLE patient and control by using the independent samples t-test and the Mann-Whitney U-test for normally distributed and non-normally distributed variables respectively (tables 3.83 – 3.84). A significance level for the p value of 0.004 was used for the MFI of the 14 genes in view of the Bonferroni correction.

Table 3.83. Results obtained when comparing gene expression in the untreated dendritic cell cultures in SLE patient and control.

Gene MFI	SLE patient DC culture		Control DC culture		P value
	Mean	S.D.	Mean	S.D.	
IFI35	0.271	0.074	0.372	0.052	0.021
OAS1	0.266	0.021	0.532	0.087	0.004*
CCL2	0.004	0.002	0.012	0.007	0.054
MX1	0.177	0.028	0.062	0.018	**<0.001**
SOCS1	1.490	0.204	1.432	0.173	0.603
IFITM1	3.707	0.469	3.327	0.493	0.201
STAT2	0.169	0.042	0.185	0.054	0.587
CXCL1	0.006	0.003	0.017	0.011	0.100*
IFIT3	0.025	0.014	0.072	0.019	**0.001**
SOCS3	0.675	0.270	0.557	0.216	0.421
IFIT1	0.043	0.006	0.027	0.008	0.004
STAT1	0.824	0.228	0.985	0.214	0.235
IRF7	0.126	0.053	0.143	0.048	0.557
IRF8	0.273	0.036	0.372	0.093	0.036
IFN score	3.595	0.612	3.748	0.448	0.631

P values have been obtained using the independent samples t-test for normally distributed variables and Mann-Whitney U-test (marked by *) as the non-parametric alternative. DC, dendritic cell; IFN, interferon; MFI, median fluorescence intensity; S.D., standard deviation.

Table 3.84. Results obtained when comparing gene expression in the untreated macrophage cultures from the SLE patient and the control.

Gene MFI	SLE patient macrophage culture		Control macrophage culture		P value
	Mean	S.D.	Mean	S.D.	
IFI35	0.259	0.076	0.174	0.098	0.158
OAS1	0.148	0.054	0.308	0.076	0.004
CCL2	40.373	6.659	8.608	2.025	**<0.001**
MX1	0.162	0.042	0.263	0.083	0.032
SOCS1	0.168	0.058	0.072	0.017	0.028
IFITM1	6.031	0.764	4.446	0.956	0.019
STAT2	0.221	0.071	0.390	0.139	0.033
CXCL1	1.469	0.714	0.046	0.020	0.012
IFIT3	0.154	0.074	0.337	0.115	0.015
SOCS3	1.453	0.695	0.123	0.087	0.006
IFIT1	0.042	0.021	0.055	0.007	0.437
STAT1	2.668	0.778	10.164	2.553	0.011*
IRF7	0.147	0.065	0.230	0.087	0.121
IRF8	0.512	0.052	0.787	0.328	0.071
IFN score	5.070	1.002	4.788	1.050	0.679

P values have been obtained using the independent samples t-test for normally distributed variables and Mann-Whitney U-test (marked by *) as the non-parametric alternative.

IFN, interferon; MFI, median fluorescence intensity; S.D., standard deviation.

On comparing the DC cultures MX1 had a higher expression and IFIT3 had a lower expression in the SLE patient DC culture compared to the control. CCL2 gene expression was higher in the SLE patient macrophage culture compared to the control. The IFN signature gene expression score for both DC and macrophage cultures was not significantly different between the SLE patient and control.

3.6.8 SUMMARY OF RESULTS

The differentiated DC and macrophage cultures from both SLE patient and control were characterised by microscopy and flow cytometry. The DCs in the DC cultures were noted to have the characteristic elongation on microscopy and a high proportion of cells expressed CD83 in the DC cultures. On the other hand, the macrophages in the macrophage cultures had a circular appearance and a high proportion of cells expression CD14 in the differentiated macrophage cultures.

On comparing the gene expression between untreated cell cultures and those treated with calcitriol, it was noted that the IFN signature gene expression score was significantly lower at 24 hours in the SLE patient DC and macrophage cell cultures and in control macrophage cell cultures in the treated samples ($p=0.014$, $p=0.012$, $p=0.031$ respectively). *IRF8* expression was noted to be lower in treated macrophage cell cultures in both SLE patient and control. This was statistically significant for the SLE patient macrophage cell culture at 48 hours ($p<0.001$). No difference in *IRF7* expression was noted between all treated and untreated cell cultures.

A statistically significant decrease in the IFN signature gene expression score was noted at 24 and 48 hours in treated SLE patient DC culture compared to baseline ($p=0.002$, $p=0.001$ respectively). Similarly, a significant decrease was noted in the treated control macrophage culture from baseline to 24 hours ($p=0.014$). A decrease in IRF8 expression was noted in the treated SLE patient and control macrophage culture compared to baseline. This was statistically significant for the SLE patient macrophage culture at 48 hours ($p<0.001$). No significant changes were noted for *IRF7* expression.

On comparing the gene expression between untreated SLE patient DC and macrophage cultures, *SOCS1* gene was noted to have a higher expression in the DC cultures and other genes had a higher expression in the macrophage cultures (*CCL2, CXCL1,* and *IRF8*). This showed the clear difference between the two cell culture populations. However, there was no significant difference in IFN signature gene expression score between the two cell cultures. On comparing the IFN signature gene expression score between untreated SLE patient DC cultures and untreated control DC cultures, no significant difference was noted. The same was observed for untreated SLE patient and control macrophage cultures.

CHAPTER 4 – DISCUSSION AND CONCLUSIONS

4.1 DISCUSSION

The research described included different studies carried out in both a clinical and laboratory setting. The primary aim of the various studies was to research the effect of vitamin D in SLE on several clinical and biochemical parameters. Hence the potential benefit of vitamin D supplementation in SLE was studied.

4.1.1 TRANSLATION, VALIDATION AND CROSS-CULTURAL ADAPTATION OF THE QUESTIONNAIRES

This study consisted of the development of a validated Maltese translation of the FSS, PSQI and mHAQ questionnaires. The Maltese translations underwent psychometric assessment, including reliability and internal consistency testing in a cohort of Maltese SLE patients. All the statements in the Maltese translations of the FSS, PSQI and mHAQ showed adequate reliability and good internal consistency, with Cronbach's alpha values of 0.877, 0.859 and 0.897 respectively. These values are similar to those quoted in the original publications of the questionnaires (0.89 for FSS, 0.83 for PSQI and 0.85 for mHAQ) (Krupp et al., 1989; Buysse et al., 1989; Pincus et al., 1983). The validity of the Maltese translation of the FSS was shown in its statistically significant correlation with VAS fatigue ($r=0.809$, $p<0.001$).

4.1.2 CROSS-SECTIONAL COHORT STUDY OF SLE PATIENTS IN MALTA

This study has been the first population based research on SLE done in Malta. It has estimated the prevalence of SLE in Malta to be 29.3 patients per 100,000. This has been computed by using the identified 107 SLE patients over the age of 18, who fulfilled the SLICC classification criteria for SLE. In addition, the Maltese population of adults, above 18 years of age, is estimated at 365,000, (National Statistics Office, Malta, 2016). This study has estimated the incidence of SLE in Malta to be 1.48 patients per 100,000 per year. This was determined using the mean number of newly diagnosed adult SLE patients from 2012 to 2016 per year, which were 5.4. The reported prevalence and incidence rates in other European countries (Pons-Estel et al., 2010; Bertsias et al., 2013) are similar to those obtained for Malta in this study.

Similar to other studies on SLE, the male to female ratio of SLE patients was 1:10 (Pons-Estel et al., 2010). Most of the SLE cases studied were 20 to 40 years of age at diagnosis. The organ involvement during the course of the disease was similar to the results obtained in other studies, including the Euro-Lupus cohort, which was a ten year European prospective research (Cervera et al., 2002). Of note, in the described local research, the frequency of neuropsychiatric manifestations was lower. This is possibly due to the fact that the information on organ involvement was collected retrospectively using the medical file and by obtaining a history from the patients. The demographic data of the local cohort, including age, gender, age at SLE diagnosis, organ manifestations, smoking status, disease activity and SDI, was similar to the cohort described in the National Spanish Registry of SLE patients (Rúa-Figueroa et al., 2014). A younger age at diagnosis was noted in patients with renal and

neuropsychiatric manifestations. This has also been shown in other studies (Livingstone et al., 2011; Aggarwal and Srivastava, 2015). Males had an older age at diagnosis than females, and patients who had anti-Sm and anti-RNP antibodies had a younger age at disease diagnosis. This is in keeping with findings from other publications (Boddaert et al., 2004; Ni et al., 2009).

A SLE cohort study in Crete, also an island in the Mediterranean, found higher incidence (7.4 per 100,000 persons/year) and prevalence rates (123.4 per 100,000) (Gergianaki et al., 2017). A reason for this difference could be the community-based approach of the study by Gergianaki et al., whereby milder cases could have been detected. In fact on comparison with our data, a lower prevalence of nephritis (13%), neuropsychiatric SLE (7.8%) and anti-dsDNA antibodies (23%) were reported. The female-to-male ratio (13:1) was comparable to the Maltese data with mean (±SD) age at SLE diagnosis being higher at 43 (±15) years in the study from Crete (compared to 33.1 (±13.3) years).

In the local cohort of SLE patients, overlap with Sjogren's syndrome was diagnosed in 3.7%. This has been established by using the clinical diagnoses documented in the medical notes by the rheumatologist. This is significantly lower than that in other studies. A cross-sectional study by Gianordoli et al. (2023) found that the prevalence of Sjogren's syndrome in SLE was 23% and 35% according to the American-European Consensus Group (AECG 2002) and the American-European classification criteria of 2016 (ACR/EULAR 2016) respectively. This suggests that Sjogren's has been underdiagnosed in our cohort particularly since the prevalence of anti-SSA52, anti-SSA60 and anti-SSB was 33.6%, 37.4% and 13.1% respectively.

A large proportion of SLE patients, 60.8% were noted to be overweight or obese. This is below that found in the Maltese general population, which is 70% (Cuschieri et al., 2016; Agius et al., 2021). A reason for this discrepancy is the female predominance of the SLE cohort, since in the study by Cuschieri at al. a high BMI (≥25kg/m^2) was more prevalent in males (76.28%) compared to females (63.06%). In addition, the presence of sarcopaenia in SLE patients, particularly those with active disease, could have resulted in a lower BMI for the degree of adiposity. Glucocorticoids received regularly by 45.8% of the cohort, contributed to obesity in SLE patients. This is supported by the positive correlation between the daily dosage of prednisolone and BMI (R=0.177, p=0.046).

Fewer SLE patients were current smokers (18.7%) in comparison to the Maltese general population (estimated to be around 24%) (Calleja et al., 2008; Cuschieri et al., 2019; Agius et al., 2021). This could be because the patients with SLE were more health conscious due to their concurrent medical problems. Co-morbidities were common. In particular, 9.3% had fibromyalgia. In the general population the prevalence of fibromyalgia is estimated to be around 2% (Wolfe et al., 1995). Other studies have also reported an increased prevalence of fibromyalgia in SLE patients (Haliloglu et al., 2014). The role of the anti-N-methyl-D-aspartate receptor antibodies in the underlying pathogenesis of fibromyalgia with SLE has been suggested by a case-control study (Park et al., 2017).

Diabetes was present in 8.4% of the SLE cohort. This is slightly less that the estimated prevalence of diabetes in adults (aged 25 to 64 years) in Malta that was found to be 10.39% in a cross-sectional study (Cuschieri et al., 2016). A reason for this discrepancy

is the different gender and age characteristics of the SLE cohort, particularly since the study by Cuschieri et al. found a higher prevalence of diabetes in males. The described local prevalence of diabetes in SLE patients is higher than that found in a Spanish SLE cohort (5%) (Rúa-Figueroa et al., 2014). Differences in dietary habits and genetic differences could explain this disparity.

Antimalarials, such as hydroxychloroquine, are highly recommended in the management of SLE since they minimise acute exacerbations of the disease, decrease the development of lupus nephritis and subsequent damage, and enable the use of less steroids (Gordon et al., 2018; Bruce et al., 2015; Costedoat-Chalumeau et al., 2013; The Canadian Hydroxychloroquine Study Group, 1991; Williams et al., 1994; Meinao et al., 1996). However, in this study 39.3% of SLE patients were not being treated with hydroxychloroquine. A significant proportion of the SLE cohort (20.6%) had received hydroxychloroquine in the past, despite not receiving it at the time of the study. The drug had been stopped for a number of reasons. The study showed that a significant proportion of patients required better control of their disease activity. These include 3.3% who had a SLEDAI-2K score of >10, and 15.0% of the SLE cohort who were receiving prednisolone at a dose of 7.5mg daily or higher. These patients could potentially benefit from biological drugs, including belimumab and anifrolumab. At the time of the cross-sectional cohort study (data collected in 2016-2017), only one patient was receiving a biological drug, namely rituximab. Since then more local SLE patients have been started on rituximab and belimumab.

Patients with a lower SLE duration had a higher disease activity, and consequently a higher functional disability measured by mHAQ. Prospective studies have also

demonstrated the improvement of disease activity with time (Joo et al., 2015; Nossent et al., 2010). Survivor bias could have contributed to this observation. Patients with a longer duration of SLE, had more damage. This is in keeping with results from other prospective studies and is due to the accumulation of damage due to the disease itself and its therapy; mostly atherosclerosis, infections and malignancies (Joo 2015, Nossent 2010). Disease activity was found to influence functional disability as measured by mHAQ. A relationship between HAQ (Health Assessment Questionnaire) and both SDI and SLEDAI was shown in other studies (Fortin et al., 1998; Bjork et al., 2015). The lack of correlation between mHAQ and damage ($R=-0.007$, $p=0.946$) could be related to the limited cohort size and the use of mHAQ rather than HAQ, since the latter is more detailed.

Fatigue is a very common symptom in SLE, and it has been described in up to 90% of patients (Zonana-Nacach et al., 2000). In the cross-sectional cohort study, SLE patients had a high prevalence of fatigue; over half of the cohort had a high level of fatigue as defined by a FSS of greater than 3.7. The mean FSS in the SLE cohort was 4.02; that in the normal healthy adult population is 2.3 (Krupp et al., 1989). The strongest predictive factors for fatigue, as determined by ANCOVA analysis, were pain ($p<0.001$) and depression ($p<0.001$). This is in keeping with the results from another cross-sectional cohort study that concluded that depression, pain and stress were the strongest independent predictors for fatigue in SLE (Azizoddin et al., 2019). Other factors found to have a positive correlation with fatigue (including anxiety, sleep quality and disease activity) could contribute in an indirect way. For example, disease activity could contribute towards fatigue through its effect on pain and depression. Poor sleep quality had a positive correlation with fatigue, and its main predictive factors were also

pain and depression. Anxiety could also contribute through its close relationship with depression and poor sleep quality. The higher level of fatigue noted in patients on a higher dose of hydroxychloroquine most likely reflects the higher uncontrolled disease activity present in these patients. The study confirmed the multi-factorial nature of fatigue and the importance of establishing the underlying cause in order to be able to treat accordingly (Arnaud et al., 2021).

Vitamin D deficiency and insufficiency were noted to be common. Only 14.1% of SLE patients did not need vitamin D3 supplementation. This is in keeping with other studies showing that vitamin D deficiency was more common in SLE patients than in the general population, possibly related to avoidance of sun exposure and renal impairment (Kamen et al., 2008). BMI was found to be an independent predictor of vitamin D level on ANCOVA analysis ($p=0.008$). This relationship has been demonstrated in other studies in the general population and is believed to be related to the decreased vitamin D bioavailability due to the body fat that serves as a storage reservoir for fat soluble vitamin D (Lagunova et al., 2009).

Functional disability measured by mHAQ was highly dependent on VAS pain ($p=0.011$), SLEDAI-2K ($p=0.037$) and older age at disease diagnosis ($p=0.003$) as determined by the use of a generalised linear model with gamma distribution. In addition, factors noted to have a significant correlation with mHAQ were fatigue ($R=0.435$, $p<0.001$), PSQI ($R=0.559$, $p<0.001$), depression ($R=0.494$, $p<0.001$), anxiety ($R=0.272$, $p=0.009$) and haemoglobin ($R=-0.217$, $p=0.038$). The mean mHAQ was higher in patients with fibromyalgia ($p=0.022$) and in patients receiving azathioprine ($p=0.046$). The latter likely reflects a higher disease activity in this cohort of patients. The results highlight

the negative impact of these factors on the patients' quality of life and the importance of addressing these factors in clinical practice.

Anxiety and depression were also highly prevalent; a HADS-A score of above 10 was noted in 35.9% and HADS-D was above 10 in 6.5%. Other studies, including a meta-analysis have also shown a high frequency of anxiety and depression in SLE (Zhang et al., 2017). Moreover, 55.4% had poor sleep quality (PSQI >5) (Omachi et al., 2011). The strongest predictive factors for poor sleep quality were depression ($p<0.001$), pain ($p=0.001$) and low eGFR ($p=0.022$) as determined by generalised linear model analysis. However, it also had a positive correlation with anxiety ($R=0.375$, $p<0.001$), fatigue ($R=0.551$, $p<0.001$) and disease activity ($R=0.254$, $p=0.014$). The results are similar to another smaller cross-sectional study on 19 SLE patients which showed a poorer sleep quality in SLE compared to the control group and poor sleep quality in SLE was also associated with pain and depression (Cervilla et al., 2020). Poor sleep quality has also been associated with chronic kidney disease (Iliescu et al., 2004; Guo et al., 2015).

In the cross-sectional cohort study no relationship was noted between serum 25-hydroxyvitamin D and (i) fatigue and (ii) SLEDAI-2K. However, the level of evidence provided by a cross-sectional cohort study is limited. More robust evidence is provided by prospective studies. For this reason, the prospective study in which vitamin D insufficient/deficient patients were supplemented with vitamin D3 was carried out.

4.1.3 PROSPECTIVE COHORT STUDY OF SLE PATIENTS WITH VITAMIN D DEFICIENCY OR INSUFFICIENCY

In the prospective study 31 SLE patients with vitamin D deficiency/insufficiency were treated with vitamin D3 and followed up for one year. Vitamin D3 at a dose of 8000IU daily for 8 weeks in vitamin D deficiency and 4 weeks in insufficiency, followed by a maintenance dose of 2000IU daily was observed to be safe. At this dose none of the patients developed hypercalcaemia. The study showed a statistically significant improvement in SLEDAI-2K after 12 months of vitamin D supplementation (p=0.028). The prednisolone dose and FSS at 12 months were noted to decrease but statistical significance was not achieved on analysing the whole cohort (p=0.068, p=0.071). The cohort included patients mostly with a mild phenotype (median SLEDAI-2K of 4 at baseline) and this could have masked further potential benefits of vitamin D3 supplementation in more severe cases.

Adherence to vitamin D3 treatment was analysed according to the amount of vitamin D3 supplements (provided for free) collected by each patient during the course of the study. 64.5% of patients did not acquire all the boxes required to take the advised dosage. Although the participants could have got hold of the treatment from an alternative source, the results indicate that a significant proportion of participants were not completely adherent especially between 6 and 12 months. In addition, the target serum 25-hydroxyvitamin D was achieved by 83.9% after 6 months, but this decreased to 35.5% after 12 months of supplementation. Even though more patients achieved serum 25-hydroxyvitamin D >30ng/ml at 6 months than at 12 months, fatigue and disease activity improvement were more apparent after 12 months of

supplementation. This indicates that for maximal benefits to be noted, Vitamin D needs to be supplemented for a period of more than 6 months. Other prospective studies failed to show a relationship between vitamin D and disease activity due to a shorter duration (Terrier B et al., 2012; Aranow et al., 2015; Karimzadeh et al., 2017; Al-Kushi et al., 2018). The levels of functional disability, depression and anxiety were noted to remain stable during the course of the study.

In view of the multi-factorial aetiology of fatigue, changes in factors that could influence it, such as changes in anti-depressants and lifestyle changes, could have affected the measured level of fatigue. The study participants were not recruited in the Summer months in order to avoid the possible seasonal effect of higher baseline vitamin D levels due to higher sunlight exposure and increased fatigue due to a higher temperature.

4.1.4 INTERFERON SIGNATURE GENE EXPRESSION

In this study, the expression of 12 IFN signature genes in RNA extracted from white blood cells in SLE patients was determined simultaneously, by using QuantiGene Plex technology. This was measured at baseline in the cross-sectional cohort study and after 6 months of vitamin D3 supplementation in the vitamin D insufficient/deficient patients who participated in the prospective study and were supplemented with vitamin D3.

In the cross-sectional cohort study the expression of six IFN signature genes assessed, as well as the IFN signature gene expression score, significantly correlated positively

with disease activity. This is in keeping with the results from other studies in SLE (Nikpour et al., 2008; Petri et al., 2009). However, there was no significant correlation with fatigue, depression, anxiety, sleep quality, functional disability and serum 25-hydroxyvitamin D. This is the first study to analyse the relationship between fatigue measured by FSS and the expression of 12 IFN signature genes. In another cross-sectional case-control study, the relationship betwen fatigue, measured by Multidimensional Fatigue Inventory, and the expression of three IFN signature genes was analysed and no correlation was found (Kellner et al., 2010).

There was a significantly increased IFN signature gene expression score in patients who were positive for anti-Sm, anti-SSA60 and anti-RNP antibodies. This finding has also been noted in other studies and suggests the presence of a subgroup of SLE patients defined by increased IFN signature gene expression in whom these autoantibodies are present (Kirou et al., 2005; Hubbard et al., 2022).

In the prospective study, vitamin D3 supplementation resulted in the decrease in the expression of all 12 IFN signature genes assessed especially in *OAS1* and *SOCS1* genes (p=0.032, p=0.005). Since the Bonferroni correction for multiple gene testing was applied, the results were not deemed statistically significant. Three patients who were receiving a higher prednisolone dosage compared to baseline were excluded in this analysis to eliminate its potential confounding effect. The IFN signature gene expression score also decreased from baseline to 6 months but did not achieve statistical significance (p=0.083), likely due to the small size of the sample. The characteristics of the SLE patients who had a decrease in IFN signature gene expression score with vitamin D supplementation were compared with those who did not. The

only significant difference that was noted was a lower SLEDAI-2K at 6 months in patients in whom the IFN signature gene expression score decreased with vitamin D treatment.

This study has measured gene expression in RNA extracted from white blood cells obtained on centrifugation of whole blood. Future studies measuring IFN signature gene expression in various white blood cell subtypes (such as PBMCs) would provide further detail.

4.1.5 VDR POLYMORPHISMS

The study found that SLE patients who were homozygous for the minor allele for BsmI had a significantly higher SLEDAI-2K compared to the other patients who were homozygous for the wild type allele and heterozygous (p=0.046). This contrasts with the results of the study by Azab et al. (2016) in which no relationship was found between BsmI gene variants and disease activity. On the other hand, another case-control study showed a significant association between ApaI, BsmI, and FokI homozygous wild type genotypes and higher SLE disease activity (Emerah and El-Shal, 2013). Few studies have looked into this relationship and further larger studies are required.

The study identified a higher prevalence of fibromyalgia in the patients who were homozygous minor for ApaI or TaqI polymorphisms. No other studies looking into this relationship have been identified and the only two studies looking into the relationship between fibromyalgia and VDR polymorphisms only analysed FokI polymorphisms

(Marasli et al., 2016; Khalil et al., 2021) and no relationship was found. Further studies looking into VDR polymorphisms in fibromyalgia patients who do not have SLE, are required to confirm the relationship found in this study.

An attempt was made to compare the genotypes for the four VDR polymorphisms between the patients who had a decrease in IFN signature gene expression score with vitamin D3 supplementation with those who did not. However, this was inconclusive since the sample size available for this analysis was small.

4.1.6 IN VITRO EFFECT OF CALCITRIOL SUPPLEMENTATION ON EXPRESSION OF IFN SIGNATURE GENES, *IRF8* AND *IRF7* IN PRIMARY CELL CULTURE

The in vitro experiment demonstrated a significant decrease in IFN signature gene expression in SLE patient DC and macrophage cultures and in control macrophage cultures when treated with calcitriol (1,25-dihydroxycholecalciferol). The results are in keeping with the hypothesis that the effect of vitamin D to improve disease activity in vivo is mediated by the influence of the VDR receptor in suppressing the expression of the IFN signature genes in dendritic cells and other cells belonging to the immune system. In this experiment the expression of all identified 12 IFN signature genes that are overexpressed in SLE, was measured and used to calculate the IFN signature gene expression score (Arasappan et al., 2011).

The effect of calcitriol on the expression of *IRF8* was studied since *IRF8* is known to have a VDR binding site (Ramagopalan SV et al., 2010) and the IRF8 protein regulates

expression of genes stimulated by IFN-alpha (including IFN signature genes). The in vitro study demonstrated decreased *IRF8* expression in macrophage cell cultures treated with calcitriol. This was not the case in the DC cultures, likely due to lower *IRF8* expression in the DC cultures ($p<0.001$). The suppression of *IRF8* expression by 1,25-dihydroxycholecalciferol could represent one of the mechanisms by which the latter results in decreased IFN signature gene expression. A literature search did not identify any other studies on the effect of vitamin D on the *IRF8* expression in SLE. An in vitro study by Parnell et al. (2019) showed that *IRF8* expression was decreased with calcipotriol in tolerogenic DCs differentiated from monocytes obtained from healthy individuals. Further in vivo studies on the effect of vitamin D3 supplementation on *IRF8* expression are required.

The expression of *IRF7* was also studied in order to be able to compare the effect of calcitriol on its expression with that of *IRF8*. *IRF7* also belongs to the IRF family, having a similar role to *IRF8* but it does not have a VDR binding site. No effect on the expression of *IRF7* with calcitriol was noted.

In the in vitro experiment the cultured macrophages and DCs were differentiated from monocytes in PBMCs isolated from whole blood. A limitation of the experiment is that the differentiated cells were not physically isolated from the other PBMCs. During the experiment it was noted that the other cell types (mainly lymphocytes) had a shorter life span. On comparing the gene expression between the two untreated cell culture types, some genes had a higher expression in the differentiated DC culture and others had a higher expression in the untreated macrophage culture. This showed that the two cultures had a distinctly different cell population. No significant difference was

noted between the IFN signature gene expression score in the untreated SLE patient and control DC cultures, as well as between the untreated macrophage cultures from the SLE patient and control. This could have been the case because the SLE patient recruited for this experiment had a low disease activity. Control DC cultures were not available for RNA extraction at baseline due to lack of sufficient ImmunoCult™ DC differentiation medium. However untreated control DC cultures at 24 and 48 hours were available for RNA extraction to enable comparison with samples treated with calcitriol.

In this experiment dendritic cells were differentiated from monocytes using ImmunoCult™ DC Culture Kit since dendritic cells have a very low frequency in blood (usually about 0.2% of PBMCs). It is therefore recommended to differentiate dendritic cells from monocytes, rather than isolate DCs from PBMCs, unless large volumes of blood are available (usually by using "buffy coats" prepared from donated peripheral blood) (Nair 2012).

4.1.7 STRENGTHS OF THE RESEARCH

All the data collected in the patient interviews, including the measurement of SLEDAI-2K, and the laboratory work carried out in relation to the measurement of IFN signature gene expression were carried out by the principal researcher. This provided the advantage that no inter-observer variability was introduced in the study.

The evaluation of fatigue is complex and a number of questionnaires have been utilised to measure fatigue in SLE (Neuberger 2003). The questionnaire used to

measure fatigue in this study, the FSS, was deemed as the tool of choice to assess fatigue in SLE by the Ad Hoc Committee on Systemic Lupus Erythematosus Response Criteria for Fatigue (2007). In view of the multi-factorial nature of fatigue, multiple variables (including depression, anxiety, sleep quality, haemoglobin and pain) have been assessed to take into consideration confounding factors.

All patients included in the prospective interventional study were not commenced on any new DMARDs or biological agents and did not have any dosage increase of the DMARDs that they were receiving, to eliminate the confounding effect of medications on the outcomes measured. In two cases there was a slight increase in the prednisolone dose by 1.25mg and 1.5mg daily from baseline to 6 months. In another case a patient was started on prednisolone 2.5mg daily during the first 6 months. However, prednisolone was stopped before 12 months in all three patients, hence eliminating the effect of prednisolone on comparing the measured variables from baseline to 12 months.

In this study, a loading dose of vitamin D3 was given initially and serum 25-hydroxyvitamin D was measured after 3 months of supplementation, with adjustment of vitamin D3 dosage when necessary, in order to enable more patients to reach the target serum 25-hydroxyvitamin D. The percentage of patients achieving this target at 6 months (83.9%) was higher than in other prospective studies that involved vitamin D supplementation (Aranow et al., 2015). This enabled more evident changes in IFN signature gene expression score and other measured variables after 6 months of supplementation.

The effect of vitamin D3 on IFN signature gene expression was assessed after 6 months of supplementation in the prospective interventional study, since the study by Aranow et al. (2015) concluded that a 12 week duration was too short to observe an effect on IFN gene expression. The prospective study was extended for a period of 12 months since other prospective studies with a shorter duration (Terrier et al., 2012; Aranow et al., 2015; Karimzadeh et al., 2017; Al-Kushi et al., 2018) had failed to show a significant relationship between vitamin D supplementation and disease activity. In addition, the possible influence of seasonality on fatigue was minimised by using a 12 month study period.

This is the first study to assess the relationship of vitamin D in SLE with the expression of all 12 IFN signature genes that are overexpressed in SLE, as established by meta-analysis (Arasappan et al., 2011). Other studies have analysed IFN signature gene expression by measuring the expression of up to three genes (Ritterhouse et al., 2011; Mandal et al., 2014; Aranow et al., 2015; Abdel Galil et al., 2018). The expression of all 12 IFN signature genes has also been measured in the in-vitro cell culture experiment, which used primary cell cultures derived from a SLE patient and healthy control, as opposed to the use of cell lines.

4.1.8 LIMITATIONS OF THE RESEARCH

Although the study on the translation of FSS, PSQI and mHAQ was able to show statistically significant reliability, internal consistency and validity, the sample size was limited. Another limitation was that it did not assess the ability of the Maltese

translations to measure alterations in fatigue, sleep quality and functional disability with time. The study on the psychometric testing of the questionnaires included only SLE patients. As expected, this cohort was a skewed sample with 95% being females. In addition this cohort included 20 bilingual SLE patients, whose educational, lifestyle and socio-economic characteristics are potentially different from those SLE patients who did not understand English and preferred using the Maltese version of the questionnaires during the cross-sectional cohort study and prospective interventional study. In order to carry out psychometric testing of the translated questionnaires, the 20 participants were asked to fill in the English versions after completing the Maltese versions. This could have introduced an element of bias since the participants could have recalled their response to the Maltese translation. This was minimised by allowing a time frame of 4 to 7 days between the completion of the two translations. The role of the Maltese translations of FSS, PSQI and mHAQ in other conditions and in the general population needs to be evaluated further.

A possible limitation of the cross-sectional cohort study is that the estimated prevalence and incidence rates could be slightly under-estimated, even though SLE patients in Malta were identified using multiple sources. In addition, any fatalities of newly diagnosed cases in the five year period used to estimate the incidence rate were not taken into account. The diagnosis of co-morbidities including fibromyalgia, Sjogren's syndrome, hypertension and hyperlipidaemia relied on the documentation on the medical notes by the rheumatologist caring for the patient, and was not based on validated criteria. This could have resulted in an overestimation of the prevalence of fibromyalgia due to symptoms (such as fatigue and pain) common to both fibromyalgia and SLE. Even though multiple variables (including depression, anxiety,

sleep quality, haemoglobin and pain) have been assessed to account for the multifactorial aetiology of fatigue, other factors potentially influencing fatigue and sleep quality, such as the presence of sleep apnoea, have not been taken into account. Physical activity has been assessed by asking the patients if they carried out regular exercise. Further detail on this aspect could have been obtained by using validated self-report questionnaires such as the modifiable activity questionnaire (Kriska et al., 1990).

Serum 25-hydroxyvitamin D was measured in this study, and not the active form 1,25-dihydroxyvitamin D. The latter is not commonly assessed due to variations in its serum level and since it does not provide information on vitamin D status (Holick 2009). The hydroxylation of 25-hydroxyvitamin D to 1,25-dihydroxyvitamin D is decreased by medications including hydroxychloroquine, potentially decreasing the metabolically active form. In addition to the factors influencing vitamin D level considered in this research (such as BMI and vitamin D supplementation), other potentially influencing factors have not been taken into account. This includes skin type, which could not be assessed since 97.8% of the cross-sectional cohort study participants were Caucasian. Even though the relationship between current sunscreen use and serum 25-hydroxyvitamin D was studied, the sun protection factor used, body area applied and degree of sunlight exposure was not taken into account. In view of the limited size of the cohort, the relationship of vitamin D level and other co-morbidities, such as coeliac disease and other causes of malabsorption, could not be studied.

In the prospective interventional study, target serum 25-hydroxyvitamin D dropped from 83.9% at 6 months to 35.5% at 12 months, suggesting decreased adherence

especially in the last 6 months of the study. This could be related to the fact that the provided free vitamin D3 supplements could only be collected from one pharmacy. Furthermore serum 25-hydroxyvitamin D was not checked in the interval between 6 and 12 months and patients were not contacted to ensure adherence during this interval. The decreased adherence, as well as the limited sample size, could have resulted in a type 2 error when comparing the variables measured at 12 months to baseline, including sleep quality and fatigue. These were noted to be significantly improved on sub-group analysis of patients who achieved target 25-hydroxyvitamin D level at 12 months ($p=0.004$, $p=0.011$ respectively).

Since this was an open-label study, the prospective study lacked a placebo group. As a result, an element of bias could have been introduced by the assessor or by the participants, especially in completing the questionnaires. This is not the case, however, with the laboratory investigations, such as the measurement of the anti-dsDNA titre. A stronger level of evidence would be provided by double blind randomised controlled trials on vitamin D3 treatment in SLE.

The scope of the study was to assess the effect of vitamin D supplementation in deficient/insufficient SLE patients. The results cannot be extrapolated to patients who do not have SLE or those that have other conditions. In addition, the effect of vitamin D supplementation in SLE patients with serum 25-hydroxyvitamin D ≥30ng/ml was not studied. Potential benefits of vitamin D supplementation in SLE patients with serum 25-hydroxyvitamin D ≥30ng/ml and optimal target serum 25-hydroxyvitamin D are still unknown.

The in vitro cell culture experiment was limited by the fact that it was a case study and included cell cultures derived from one patient with SLE and one healthy control. The results are preliminary findings and the study needs to be extended to include more patients and controls.

4.1.9 RECOMMENDATIONS FOR FURTHER RESEARCH

The research described in this thesis has identified aspects that need to be studied further. Recommendations for further research include:

a) Double blind randomised controlled trials on vitamin D3 treatment in SLE having a higher target serum 25-hydroxyvitamin D than the one used in the prospective study being described (having a target of $\geq$30ng/ml), to establish whether further benefit would be obtained from a higher target.

b) Larger studies comparing characteristics between SLE patients who have a decrease in IFN signature gene expression score with vitamin D3 supplementation, with those who do not. These include the genotypes for the four VDR polymorphisms.

c) Further larger studies on VDR polymorphisms and disease activity in SLE to confirm the finding that SLE patients homozygous for the minor allele for BsmI have a higher disease activity.

d) Studies on VDR polymorphisms in patients with fibromyalgia who do not suffer from SLE, since this research suggested that a higher prevalence of fibromyalgia

was present in the patients who were homozygous minor for ApaI or TaqI polymorphisms.

e) Further in vitro studies including samples from multiple SLE patients and in vivo studies on the effect of vitamin D3 supplementation on *IRF8* expression in various white blood cell subtypes in SLE.

f) Evaluation of the Maltese FSS, PSQI and mHAQ translations in other medical conditions and in the general population.

4.1.10 RECOMMENDATIONS IN CLINICAL PRACTICE

The research has identified that vitamin D deficiency in SLE is common. Regular monitoring of serum 25-hydroxyvitamin D is thus recommended. When found to be <30ng/ml, vitamin D3 supplementation is recommended in view of potential benefits in SLE, including improvement in disease activity, fatigue and sleep quality.

A number of common unmet needs in SLE patients have been identified and need to be addressed in clinical practice. These include fatigue, anxiety, poor sleep quality, obesity and uncontrolled disease activity. Fatigue has a multi-factorial aetiology and when present, the underlying cause needs to be identified and treated. The management of SLE is complex and a holistic approach is necessary to tackle various issues that impact quality of life. In addition to the unmet needs highlighted in this research, several other issues need to be tackled during the rheumatology consultation. These include tackling osteoporosis, fertility, cardiovascular risk assessment, vaccinations and monitoring drug side effects. It is ideal that SLE patients

are managed in a dedicated specialised lupus clinic, focussed on addressing these multiple issues.

4.2 CONCLUSIONS

The main conclusions are:

a) The Maltese translations of the FSS, PSQI and mHAQ developed in this study have been validated since they manifested good reliability and internal consistency in a cohort of patients with SLE. This enables them to be used in research and clinical practice (appendix 4).

b) The prevalence and incidence of SLE in Malta have been estimated enabling prospective local projects related to the treatment of SLE. The prevalence and incidence of SLE in Malta have been estimated to be 29.3 per 100,000 and 1.48 per 100,000 per year respectively.

c) In SLE patients, a high prevalence of fatigue (56.5% with FSS >3.7), anxiety (35.9% with HADS-A ≥11), uncontrolled disease activity (24.0% with SLEDAI-2K ≥6), obesity (60.8% with BMI ≥25), poor sleep quality (55.4% with PSQI >5) and vitamin D deficiency and insufficiency (42.4%) were noted. The study has shown the need to address these issues in clinical practice since they are very common.

d) Functional disability correlated significantly with fatigue, disease activity, sleep quality, pain, depression and anxiety. The study has highlighted the importance of

addressing these unmet needs in the management of SLE patients, since they have an impact on the patients' degree of disability and thus their quality of life.

e) The strongest predictive factors for fatigue and poor sleep quality were pain and depression. Other factors that had a positive correlation with fatigue and poor sleep quality included anxiety and disease activity. As a result, fatigue and poor sleep quality had a strong positive correlation. Since the aetiology of fatigue is multi-factorial the underlying causes need to be identified and treated in patients complaining of fatigue (Mertz et al. 2020).

f) In the cross-sectional cohort study, BMI was found to be an independent predictor of vitamin D, but there was no significant relationship between vitamin D and fatigue, disease activity, damage or sleep quality.

g) The prospective open-label study showed that the treatment of vitamin D deficiency/insufficiency resulted in an improved disease activity. Another possible benefit is the improvement of fatigue and sleep quality. A reduction in prednisolone dosage was also noted but statistical significance was not achieved. No effect on functional disability was noted.

h) The IFN signature gene expression score correlated positively with disease activity but no significant correlation was found with fatigue, depression, anxiety, sleep quality, functional disability, damage and serum 25-hydroxyvitamin D in the cross-sectional cohort study.

i) The expression of all 12 IFN signature genes and the IFN signature gene expression score decreased with vitamin D3 supplementation in the prospective study but

statistical significance was not achieved due to the Bonferroni correction for multiple gene testing.

j) In the in vitro experiment, calcitriol treatment of DC and macrophage cultures derived from a SLE patient and of control macrophage cultures resulted in a significant decrease in IFN signature gene expression score.

k) Calcitriol supplementation resulted in a decreased *IRF8* expression in the macrophage cultures in the in vitro experiment. This could be one of the mechanisms by which vitamin D causes decreased expression of the IFN signature genes.

l) SLE patients who were homozygous for the minor allele for BsmI had a significantly higher SLEDAI-2K; and a higher prevalence of fibromyalgia in SLE patients was noted in those who had the homozygous minor genotype for ApaI or TaqI polymorphisms.

In conclusion, the research carried out supports the regular screening of patients with SLE for vitamin D deficiency in clinical practice. Treatment of vitamin D deficiency in SLE results in an improved disease activity and has other possible benefits including improvement of fatigue and sleep quality. This is likely due to the reduction of IFN signature gene expression mediated by VDR bound to 1,25-dihydroxyvitamin D.

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APPENDICES

Appendix 1 – Information Sheet for Participants (English and Maltese)

Appendix 2 – Consent Form (English and Maltese)

Appendix 3 – Pro forma for data collection

Appendix 4 – Questionnaires (English and Maltese)

Appendix 5 – Approvals

Appendix 6 – Funding

Appendix 7 – Supplementary Tables

APPENDIX 1 – INFORMATION SHEET FOR PARTICIPANTS

Characterisation of the relationship between fatigue, vitamin D level, disease activity and interferon signature gene expression in Systemic Lupus Erythematosus: A population based study

My name is Dr Rosalie Magro and I am a rheumatology trainee working within the Rheumatology Department at Mater Dei Hospital. I am currently reading for a PhD degree at University of Malta.

I am currently conducting a research study with the aim of studying several factors in systemic lupus erythematosus (SLE) patients. All SLE patients in Malta will be invited to participate. This is the first time that such a study on patients with systemic lupus erythematosus in Malta will be carried out.

It is important that before taking part in this study you read the following information so that you can make an informed decision as to whether you want to participate or not.

Do you have to take part?

Participation is voluntary and it is up to you to decide whether or not to take part in any aspect of the study. If you decide to take part, you are still free to withdraw at any time. A decision to withdraw at any time, or a decision not to take part, will not have an effect on your medical care.

Participation in this study will help in the discovery of more knowledge on the condition; and thus potentially improved treatment.

What will happen if you decide to take part?

If you decide to participate in this study you will be asked to participate in a semi-structured face-to-face interview that will take around 20 minutes. You will then be asked to fill in a questionnaire which will take around 15 minutes. These will be related to your past history of systemic lupus erythematosus and your current symptoms. Moreover, I will need your permission to view your medical records and take some blood tests.

One of the blood tests will measure your vitamin D level and if this is found to be low, you will be advised to take vitamin D supplementation. If this is the case, you will then be advised to re-check the vitamin D level by means of a blood test after 3 months. You will then be invited to participate once again in the study after 6 months and after one year from the initial interview. This will consist of a similar interview, questionnaire and taking blood tests.

If you would like to participate in the study you will first be asked to fill in a consent form.

Will your taking part in this study be kept confidential?

All data collected will be treated in greatest confidence and full anonymity of all participants will be assured throughout the study. None of the participants will be individually identified or revealed at any time during the course of the study or after.

The data collected will be stored in a secure place and used solely for research purposes.

Who has reviewed the study?

The University Research Ethics Committee have reviewed and approved the study. The Data Protection Officer, Chairman of the Department of Medicine and your caring consultant have given their approval for the study to take place.

Contact for further information

If there is anything that is not clear or you would like more information, please feel free to contact me by email on lupusresearchmalta@gmail.com. If you would like to be informed on the results of this study you can contact me on the above email and I will let you know of the results once the study is completed.

Thank you for your assistance and cooperation.

Rosalie Magro MD MRCP(UK)

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FULJETT TA' INFORMAZZJONI GĦALL-PARTEĊIPANTI

Characterisation of the relationship between fatigue, vitamin D level, disease acivity and interferon signature gene expression in Systemic Lupus Erythematosus: A population based study

Jisimni Dr Rosalie Magro u jiena tabiba li qed nitħarreg fir-rewmatoloġija u naħdem fi ħdan id-Dipartiment tar-Rewmatoloġija fl-isptar Mater Dei. Bħalissa qedha nistudja għal grad ta' PhD fl-Universita' ta' Malta.

Bħalissa qedha nagħmel riċerka bl-iskop li nistudja diversi fatturi f'pazjenti li jbatu bil-kundizzjoni 'systemic lupus erythematosus' (SLE). Il-pazjenti kollha li jbatu mill-SLE f'Malta se jigu mistiedna biex jieħdu sehem. Din hija l-ewwel darba li studju ta' din in-natura fuq il-SLE se jsir f'Malta.

Huwa importanti li qabel tieħu sehem f'dan l-istudju taqra l-informazzjoni li ġejja biex tkun tista tieħu deċiżjoni nfurmata fuq jekk tridx tieħu sehem.

Tista' ma tieħux sehem?

Il-parteċipazzjoni tiegħek hija voluntarja u huwa f'idejk biex tiddeċiedi jekk tridx tieħu sehem f'dan l-istudju. Jekk tiddeċiedi li trid tieħu sehem, xorta waħda inti liberu/a li tirtira meta trid int. Tista tiddeċiedi li tirtira mill-istudju fi kwalunkwe stadju, jew tiddeċiedi li ma tieħux sehem, mingħajr ma jkun hemm l-ebda effett fuq il-kura medika li tirċievi.

Il-parteċipazzjoni tiegħek f'dan l-istudju tgħin fl-iskoperta ta' iktar taghrif fuq il-kundizzjoni; u għal potenzjalment kura aħjar.

X'jiġri jekk tiddeċiedi li trid tieħu sehem?

Jekk tiddeċiedi li trid tieħu sehem f'dan l-istudju se tkun mistieden għal intervista wiċċ imb'wiċċ li se tieħu xi għoxrin minuta. Imbagħad se tkun mitlub timla kwestjonarju li jieħu madwar kwarta. Dawn ser ikunu dwar l-istorja tieghek tal-SLE u s-sintomi li għandek bħalissa. Ser niġi bzonn ukoll il-permess tiegħek biex nara l-istorja medika tiegħek u biex isirulek xi testijiet tad-demm.

Wieħed mit-testijiet tad-demm se jkejjel il-livell ta' vitamina D, u jekk dan jinstab baxx intuk il-parir li tieħu pinnoli li jissuplixxu l-vitamina D. F'dan il-kaz, ntuk il-parir li terġa tiċċekja l-livell tal-vitamina D billi tieħu test tad-demm wara 3 xhur. Imbagħad nergaw nistednuk biex tipparteċipa f'dan l-istudju wara 6 xhur u wara sena mill-ewwel intervista. Dan jerġa jikkonsisti f'intervista simili, kwestjonarju u testijiet tad-demm.

Jekk tixtieq tieħu sehem f'dan l-istudju se tkun mitlub timla' l-formola tal-kunsens.

Il-parteċipazzjoni tiegħek f'dan l-istudju se tinzamm kunfidenzjali?

L-informazzjoni kollha miġbura se tinzamm bl-iktar mod kunfidenzjali u anonimu possibli tul l-istudju kollu. L-ebda parteċipant mhu se jkun identifikat jew żvelat individualment waqt jew wara li jsir dan l-istudju. L-informazzjoni miġbura se tinżamm f'post sigur u se tintuża biss ghall-iskop tar-riċerka.

Min ta' l-permess li jsir dan l-istudju?

Il-'University Research Ethics Committee' irreveda u ta' l-approvazzjoni tiegħu biex isir dan l-istudju. L-ufficjal tad-'data protection', ic-'Chairman' tad-Dipartiment tal-Mediċina u l-konsulent tiegħek taw il-permess li jsir dan l-istudju.

Kuntatt ghall-iktar informazzjoni

Jekk hemm xi ħaga li mhix ċara jew tixtieq iktar informazzjoni, jekk jogħġbok ikkuntatjani permezz tal-ittra eletronika fuq lupusresearchmalta@gmail.com. Jekk tixtieq li tkun infurmat dwar ir-riżultat ta' dan l-istudju ikkuntatjani permezz tal-ittra elettronika u ninfurmak dwar ir-riżultati ġaladarba l-istudju jkun lest.

Grazzi tal-għajnuna u l–koperazzjoni.

Rosalie Magro MD MRCP(UK)

Resident Specialist in General Medicine

Higher Specialist Trainee in Rheumatology

Department of Rheumatology,

Mater Dei Hospital

Msida MSD 2090

APPENDIX 2 – CONSENT FORM

Dear participant,

As explained in the information sheet I am carrying out a study on systemic lupus erythematosus in Malta. Your participation will consist of a 20 minute interview and filling in a questionnaire which will take around 15 minutes. Moreover, I will need your permission to view your medical records and take some blood tests.

One of the blood tests will measure your vitamin D level and if this is found to be low, you will be advised to take vitamin D supplementation. If this is the case, you will then be advised to re-check the vitamin D level by means of a blood test after 3 months. You will then be invited to participate once again in the study after 6 months and after one year from the initial interview. This will consist of a similar interview, questionnaire and taking blood tests.

Participation is voluntary and refusal to take part in this study will not affect your medical care. The information that is collected is confidential and according to the 'Data Protection Act'.

Your participation is greatly appreciated.

I hereby give my consent to take part in this study.

Name and Signature of Participant _____

Dr Rosalie Magro Signature _____ Date _____

FORMOLA TAL-KUNSENS

Għażiż/a Parteċipant/a,

Bħal ma hemm spjegat fuq il-karta ta' informazzjoni, qegħda nagħmel studju fuq il-kundizzjoni 'systemic lupus erythematosus' f'Malta. Il-parteċipazzjoni tiegħek tikkonsisti f'intervista li se ddum xi għoxrin minuta u billi tirrispondi kwestjonarju li jieħu xi ħmistax-il minuta. Ser niġi bżonn ukoll il-permess tiegħek biex nara il-'file' mediku tiegħek u biex nieħodlok xi testijiet tad-demm.

Wieħed mit-testjiet tad-demm se jkejjel il-livell ta vitamin D. Jekk dan jinstab li hu baxx, intuk il-parir li tieħu pinnoli li jissuplixxu l-vitamina D. F'dan il-każ, ntuk il-parir li terġa tiċċekja il-livell tal-vitamina D billi tieħu test tad-demm wara 3 xhur. Imbagħad nerġaw nistednuk biex tipparteċipa f'dan l-istudju wara 6 xhur u wara sena mill-ewwel intervista. Dan jerġa jikkonsisti f'intervista simili, kwestjonarju u testijiet tad-demm.

Il-parteċipazzjoni tiegħek hija waħda volontarja u inti tista' tirrifjuta li tieħu sehem f'dan l-istudju mingħajr ma taffettwa l-kura medika li inti tirċievi. L-informazzjoni li ħa niġbor hija kunfidenzjali u skond id-'Data Protection Act'.

Il-parteċipazzjoni tiegħek hija apprezzata ħafna.

Jiena nagħti l-permess tiegħi biex nieħu sehem f'dan l-istudju.

Isem u Firma tal-Parteċipant _____

Dr Rosalie Magro Firma _____ Data _____

APPENDIX 3 – PRO FORMA FOR DATA COLLECTION

Case no:_____ Date: _____

SLICC Classification criteria for SLE

Criteria	***SLICC criteria* (2012) (Petri et al, 2012)**	
Skin	1. Acute cutaneous lupus (lupus malar rash [do not count if malar discoid], bullous lupus, toxic epidermal necrolysis variant of SLE, maculopapular lupus rash, photosensitive lupus rash), or sub-acute cutaneous lupus (nonindurated psoriaform and/or annular polycyclic lesions that resolve without scarring) 2. Chronic cutaneous lupus (classic discoid rash: localized or generalized, hypertrophic [verrucous] lupus, lupus panniculitis [profundus], mucosal lupus, lupus erythematosus tumidus, chilblains lupus, discoid lupus/lichen planus overlap 3. Non-scarring alopecia	
Ulcers	4. Oral or nasal ulcers	
Synovitis	5. Inflammatory synovitis in ≥2 joints: a. characterized by swelling or effusion, or b. tenderness and ≥30 minutes of morning stiffness	
Serositis	6. Serositis: any of a. Typical pleurisy lasting >1 day, or pleural effusions, or pleural rub b. Typical pericardial pain (pain with recumbency improved by sitting forward) for >1 day, or pericardial effusion, or pericardial rub, or pericarditis by electrocardiography	
Renal disorder	7. Any of: a. Urine protein/creatinine (or 24 hr urine protein) representing ≥500 mg of protein/24 hr, or b. red blood cell casts	

Neurological disorder	8. Any of: a. seizures, b. psychosis, c. mononeuritis multiplex, d. myelitis, e. peripheral or cranial neuropathy, f. cerebritis (acute confusional state)	
Hematologic disorder	9. Haemolytic anaemia 10. Leukopenia (<4000/mm3), or lymphopaenia (<1000/mm3) at least once 11. Thrombocytopenia (<100,000/mm3) at least once	
Immunologic disorder	12. Anti-dsDNA above laboratory reference range (except ELISA: twice above laboratory reference range) 13. Anti-Sm 14. Antiphospholipid antibody, SLE anticoagulant, false-positive test for syphilis 15. Anticardiolipin (at least twice normal or medium-high titre), or anti-b2 glycoprotein 1 16. Low complement: low C3, or low C4, or low CH50 17. Direct Coombs test in the absence of haemolytic anaemia	
Antinuclear antibody	18. ANA above laboratory reference range	
Diagnosis of SLE	Either the biopsy-proven lupus nephritis in the presence of ANA or anti-dsDNA as a “stand alone” criterion, OR four criteria with at least one of the clinical and one of the immunologic/ANA criteria	

Age: _____ D.O.B.:_____

Weight: _____ Height: _____

Nationality: _____ Are all your grandparents Maltese? Y / N

Date of diagnosis of SLE: _____

Associated conditions

Anti-phospholipid syndrome	
Sjogren's syndrome	
Raynauds phenomenon	
Fibromyalgia	

Other rheumatological conditions:_____

Other medical conditions:_____

Organ involvement

Organ	**Yes**	**No**	**Specify**	**Date (if organ is involved)**
Skin				
Joints				
Kidneys				
Nervous system				
Haematological				
Cardiac				
Respiratory				
Other				

Serology

ANA (<1/80)			Anti-SSA 52 (Ro) (0-1)	
dsDNA (0-20U/ml)			Anti-SSA 60 (Ro) (0-1)	
Anti-Sm (0-1)			Anti-SSB(La) (0-1)	
			Anti-RNP (0-1)	

Family history of SLE: Yes (specify:_____) / No

Current main symptoms:

General		**Mucocutaneous**		**Neurological**		**Musculoskeletal**	
Fever		Rash		Seizure		Joint pains	
Weight loss		Alopecia		Headache		Joint swelling	
Fatigue		Mucosal ulcers		Numbness		Myalgia	
Anorexia		Raynaud's		Muscle weakness			
SOB							
Chest pain							

_____

_____

Drug History:

Lupus related drugs	Date started

Other drugs	

Calcium / Vitamin D supplements:

_____

If on HCQ, dates of ophthalmology reviews:

_____

Past DMARD history	**Date started**	**Date stopped**	**Reason why stopped**

SLEDAI-2K (Tick box if manifestation is present at time of visit or in proceeding 10 days.)

Manifestation			**Wt**
Seizure	Recent onset. Exclude metabolic, infectious or drug cause		8
Psychosis	Altered ability to function in normal activity due to severe disturbance in perception of reality; including hallucinations, bizarre, disorganized, or catatonic behavior. Excluded uremia and drug causes.		8
Organic Brain Syndrome	Altered mental function with impaired orientation or memory. Include clouding of consciousness with reduced capacity to focus plus at least 2 of the following: perceptual disturbance, incoherent speech, insomnia or daytime drowsiness, or increased or decreased psychomotor activity. Exclude metabolic, infectious or drug causes.		8
Visual Disturbance	Retinal changes of SLE. Include cytoid bodies, retinal hemorrhages, serious exudate or hemorrhages in the choroids, or optic neuritis. Exclude hypertension, infection, or drug causes.		8
Cranial Nerve Disorder	New onset of sensory or motor neuropathy involving cranial nerves.		8
Lupus Headache	Severe persistent headache: may be migrainous, but must be non-responsive to narcotic analgesia.		8
CVA	New onset of cerebrovascular accident(s). Exclude arteriosclerosis		8
Vasculitis	Ulceration, gangrene, tender finger nodules, periungual, infarction, splinter hemorrhages, or biopsy or angiogram proof of vasculitis.		8
Arthritis	>2 joints with pain and signs of inflammation (i.e.		4

	tenderness, swelling, or effusion).		
Myositis	Proximal muscle aching/weakness, associated with elevated CK/aldolase or EMG changes or a biopsy showing myositis.		4
Urinary casts	Heme-granular or red blood cell casts		4
Haematuria	>5 red blood cells/high power field. Exclude stone, infection or other cause.		4
Proteinuria	>0.5 gm/24 hours		4
Pyuria	>5 white blood cells/high power field. Exclude infection.		4
Rash	Inflammatory type rash.		2
Alopecia	Abnormal, patchy or diffuse loss of hair.		2
Mucosal Ulcers	Oral or nasal ulcerations		2
Pleurisy	Pleuritic chest pain with pleural rub or effusion, or pleural thickening.		2
Pericarditis	Pericardial pain with at least 1 of the following: rub, effusion, or ECG confirmation		2
Low Complement	Decrease in CH50, C3, or C4 below the lower limit of normal for testing lab		2
Increased DNA binding	Increased DNA binding by Farr assay or above normal range for testing laboratory		2
Fever	>38^{o}C. Exclude infectious cause.		1
Thrombocytopaenia	<100 x 10^{9}/L platelets. Exclude drug causes.		1
Leukopenia	<3 x 10^{9}/L white blood cells. Exclude drug causes.		1

SLEDAI-2K score: _____

SLICC/ACR Damage Index (Tick manifestation if present for at least 6 months; repeat episodes must occur at least 6 months apart to score 2)

Ocular (either eye by clinical assessment)	
Any cataract ever	0, 1
Retinal change or optic atrophy	0, 1
Neuropsychiatric	
Cognitive impairment (e.g. memory deficit, difficulty with calculation, poor concentration, difficulty in spoken or written language, impaired performance level) **or** major psychosis	0, 1
Seizures requiring therapy for 6 months	0, 1
Cerebrovascular accident ever (score 2 if > 1)	0, 1, 2
Cranial or peripheral neuropathy (excluding optic)	0, 1
Transverse myelitis	0, 1
Renal	
Estimated or measured glomerular filtration rate < 50%	0, 1
Proteinuria > 3,5 g/24 h	0, 1
or End-stage renal disease (regardless of dialysis or transplantation)	**or 3**
Pulmonary	
Pulmonary hypertension (right ventricular prominence, or loud P2)	0, 1
Pulmonary fibrosis (physical and radiographical)	0, 1
Shrinking lung (radiograph)	0, 1
Pleural fibrosis (radiograph)	0, 1
Pulmonary infarction (radiograph0	0, 1
Cardiovascular	
Angina **or** coronary artery bypass	0, 1
Myocardial infarction ever (score 2 if > 1)	0, 1, 2
Cardiomyopathy (ventricular dysfunction)	0, 1
Valvular disease (diastolic murmur, or systolic murmur >3/6)	0, 1
Pericarditis for 6 months **or** pericardiectomy	0, 1
Peripheral vascular	

Claudication for 6 months	0, 1
Minor tissue loss (pulp space)	0, 1
Significant tissue loss ever (e.g. loss of digit or limb) (score 2 if >1 site)	0, 1, 2
Venous thrombosis with swelling, ulceration **or** venous stasis	0, 1
Gastrointestinal	
Infarction or resection of bowel bellow duodenum, spleen, liver or gallbladder ever, for any cause (score 2 if >1 site)	0, 1, 2
Mesenteric insufficiency	0, 1
Chronic peritonitis	0, 1
Stricture **or** upper gastrointestinal tract surgery ever	0, 1
Chronic pancreatitis	0, 1
Musculoskeletal	
Muscle atrophy or weakness	0, 1
Deforming or erosive arthritis (including reversible deformities, excluding avascular necrosis)	0, 1
Osteoporosis with fracture or vertebral collapse (excluding avascular necrosis)	0, 1
Avascular necrosis (score 2 if > 1)	0, 1, 2
Osteomyelitis	0, 1
Tendon rupture	0, 1
Skin	
Scarring chronic alopecia	0, 1
Extensive scarring of panniculum other than scalp and pulp space	0, 1
Skin ulceration (excluding thrombosis for > 6 months)	0, 1
Premature gonadal failure	0, 1
Diabetes (regardless of treatment)	0, 1
Malignancy (exclude dysplasia) (score 2 if > 1 site)	0, 1,2

SLICC/ACR damage index score: _____

Smoking history: Lifelong non-smoker / Smoker / Ex-smoker

Sunscreen:

Do you use sunscreen? Y / N

If yes, how frequently do you use it:

- More than once a day ____
- Once a day ____
- Few times per week ____
- Once a week ____
- Less than once a week ____

When do you use sunscreen?

- Only in Summer ____
- Throughout the year ____

Physical activity:

Do you carry out regular exercise? Y / N

If yes, what exercise do you do? _____

Duration:

- Less than 15 minutes ____
- 15 – 30 minutes ____
- More than 30 minutes ____

Frequency:

- Daily ____
- 4-6 times per week ____
- 2-3 times per week ____
- Once a week ____
- Less than once a week ____

APPENDIX 4 – QUESTIONNAIRES

FATIGUE SEVERITY SCALE

Please circle the number between 1 and 7 which you feel best fits the following statements. This refers to your usual way of life within the last week. 1 indicates "strongly disagree" and 7 indicates "strongly agree."

Read and circle a number.	Strongly disagree			⇨			Strongly agree
1. My motivation is lower when I am fatigued.	1	2	3	4	5	6	7
2. Exercise brings on my fatigue.	1	2	3	4	5	6	7
3. I am easily fatigued.	1	2	3	4	5	6	7
4. Fatigue interferes with my physical functioning.	1	2	3	4	5	6	7
5. Fatigue causes frequent problems for me.	1	2	3	4	5	6	7
6. My fatigue prevents sustained physical functioning.	1	2	3	4	5	6	7
7. Fatigue interferes with carrying out certain duties and responsibilities.	1	2	3	4	5	6	7
8. Fatigue is among my most disabling symptoms.	1	2	3	4	5	6	7
9. Fatigue interferes with my work, family, or social life.	1	2	3	4	5	6	7

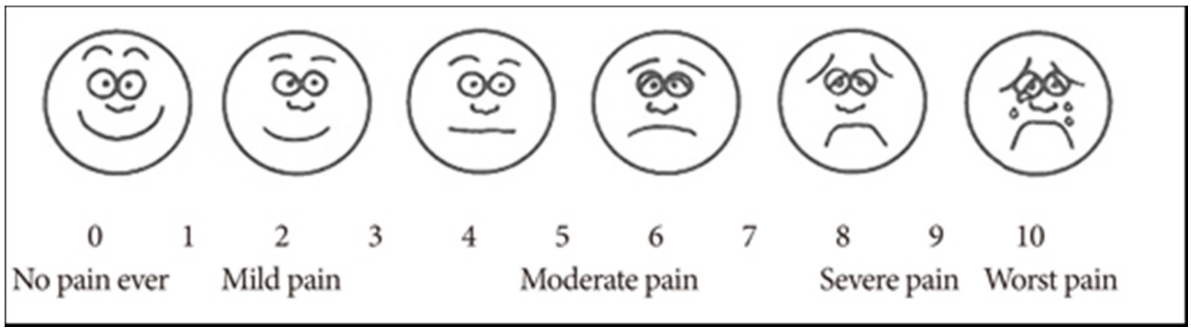
VISUAL ANALOGUE SCALE FOR FATIGUE

Please circle the number which describes your <u>current</u> global fatigue with 0 being no fatigue (energetic) and 10 being worst possible fatigue.

no fatigue ⇨		mild fatigue ⇨			moderate fatigue ⇨			severe fatigue		
0	1	2	3	4	5	6	7	8	9	10

VISUAL ANALOGUE SCALE FOR PAIN

Please circle the number which describes your current level of pain with 0 being no pain ever and 10 being worst pain.



HOSPITAL ANXIETY AND DEPRESSION SCALE (HADS)

Tick the box beside the reply that is closest to how you have been feeling in the past week.

I feel tense or wound up:	
Most of the time	
A lot of the time	
From time to time, occasionally	
Not at all	
I still enjoy the things I used to enjoy:	
Definitely as much	
Not quite so much	
Only a little	
Hardly at all	
I get a sort of frightened feeling as if something awful is about to happen:	
Very definitely and quite badly	
Yes, but not too badly	
A little, but it doesn't worry me	
Not at all	
I can laugh and see the funny side of things:	
As much as I always could	
Not quite so much now	
Definitely not so much now	
Not at all	

I feel as if I am slowed down:	
Nearly all the time	
Very often	
Sometimes	
Not at all	
I get a sort of frightened feeling like 'butterflies' in the stomach:	
Not at all	
Occasionally	
Quite often	
Very Often	
I have lost interest in my appearance:	
Definitely	
I don't take as much care as I should	
I may not take quite as much care	
I take just as much care as ever	
I feel restless as I have to be on the move:	
Very much indeed	
Quite a lot	
Not very much	
Not at all	

Worrying thoughts go through my mind:	
A great deal of the time	
A lot of the time	
From time to time, but not too often	
Only occasionally	
I feel cheerful:	
Not at all	
Not often	
Sometimes	
Most of the time	
I can sit at ease and feel relaxed:	
Definitely	
Usually	
Not Often	
Not at all	

I look forward with enjoyment to things:	
As much as I ever did	
Rather less than I used to	
Definitely less than I used to	
Hardly at all	
I get sudden feelings of panic:	
Very often indeed	
Quite often	
Not very often	
Not at all	
I can enjoy a good book or radio or TV program:	
Often	
Sometimes	
Not often	
Very seldom	

PITTSBURGH SLEEP QUALITY INDEX

The following questions relate to your usual sleep habits during the past month only. Your answers should indicate the most accurate reply for the majority of days and nights in the past month. Please answer all questions.

During the past month,

1. When have you usually gone to bed? _____
2. How long (in minutes) has it taken you to fall asleep each night? _____
3. When have you usually gotten up in the morning? _____
4. How many hours of actual sleep do you get at night? (This may be different than the number of hours you spend in bed) _____

Please check the one best response below.

	Not during the past month	Less than once a week	Once or twice a week	Three or more times a week
5. During the past month, how often have you had trouble sleeping because you				
A. Cannot get to sleep within 30 minutes				
B. Wake up in the middle of the night or early morning				
C. Have to get up to use the bathroom				
D. Cannot breathe comfortably				
E. Cough or snore loudly				
F. Feel too cold				
G. Feel too hot				
H. Have bad dreams				

I. Have pain				
J. Other reason (s), please describe, including how often you have had trouble sleeping because of this reason(s)				
6. During the past month, how would you rate your sleep quality overall	Very good	Fairly good	Fairly bad	Very bad
7. During the past month, how often have you taken medicine (prescribed or "over the counter") to help you sleep?				
8. During the past month, how often have you had trouble staying awake while driving, eating meals, or engaging in social activity?				
9. During the past month, how much of a problem has it been for you to keep up enthusiasm to get things done	No problem at all	Only a very slight problem	Somewhat of a problem	A very big problem

MODIFIED HEALTH ASSESSMENT QUESTIONNAIRE

Please tick the one best answer for your abilities.

At this moment, are you able to:	**Without ANY difficulty**	**With SOME difficulty**	**With MUCH difficulty**	**UNABLE to do**
Dress yourself, including tying shoelaces and doing buttons?				
Get in and out of bed?				
Lift a full cup or glass to your mouth?				
Walk outdoors on flat ground?				
Wash and dry your entire body?				
Bend down to pick up clothing from the floor?				
Turn faucets/taps on and off?				
Get in and out of a car?				

MALTESE TRANSLATIONS OF THE QUESTIONNAIRES

FATIGUE SEVERITY SCALE

Jekk jogħġbok indika kemm taqbel ma' kull sentenza billi timmarka b'ċirku t-tweġiba bejn 1 u 7. Dawn jirreferu għall-ħajja normali tiegħek f'din l-aħħar ġimgħa. 1 jindika "ma naqbel xejn" u 7 jindika "naqbel ħafna".

Aqra u mmarka numru b'ċirku.	Ma naqbel xejn			⇨			Naqbel ħafna
1. Il-motivazzjoni tiegħi hija iktar baxxa meta nkun għajjien(a) ħafna.	1	2	3	4	5	6	7
2. L-eżerċizzju jġib fuqi għajja kbira.	1	2	3	4	5	6	7
3. Ngħajja malajr.	1	2	3	4	5	6	7
4. L-għajja ttelifni milli nagħmel xogħol fiżiku.	1	2	3	4	5	6	7
5. L-għajja ta' spiss toħloqli problemi.	1	2	3	4	5	6	7
6. L-għajja ma tħallinix nagħmel xogħol fiżiku fit-tul.	1	2	3	4	5	6	7
7. L-għajja ttellifni milli naqdi wħud mid-dmirijiet u r-responsabbiltajiet tiegħi.	1	2	3	4	5	6	7
8. L-għajja hija fost l-iktar sintomi li jtellfuni f'ħajti.	1	2	3	4	5	6	7
9. L-għajja ttellifni f'xogħoli, mal-familja, jew fil-ħajja soċjali tiegħi.	1	2	3	4	5	6	7

VISUAL ANALOGUE SCALE FOR FATIGUE

Jekk jogħġbok għamel ċirku man-numru li jiddiskrivi l-għajja inġenerali li għandek bħalissa. In-numru 0 jindika l-ebda għajja (enerġetiku/a) u 10 jindika l-agħar għajja possibli.

l-ebda għajja	⇨	ftit għajja	⇨	għajja moderata	⇨	ħafna għajja
0 1	2	3	4	5	6 7	8 9 10

VISUAL ANALOGUE SCALE FOR PAIN

Jekk jogħġbok immarka b'ċirku in-numru li jiddiskrivi l-ammont ta' uġigħ li għandek bħalissa; in-numru 0 jindika l-ebda uġigħ u 10 jindika l-agħar uġigħ possibli.

L-ebda uġigħ | Ftit uġigħ | Uġigħ moderat | Uġigħ qawwi | L-agħar uġigħ possibli

HOSPITAL ANXIETY AND DEPRESSION SCALE

Immarki il-kaxxa ħdejn ir-risposta li l-iktar tindika kif ħassejtek f'din l-aħħar ġimgħa.

Inħoss it-tensjoni u l-anzjetà:	
Il-ħin kollu	
Ħafna mill-ħin	
Minn ħin għall-ieħor	
Qatt	
Għadni nieħu pjaċir nagħmel l-affarijiet li kont nagħmel qabel:	
Żgur daqs qabel	
Ftit inqas minn qabel	
Ftit biss	
Kważi xejn	
Inħossni mbeżża qisu ser jiġri xi ħaġa kerha:	
Inħossu ħafna u ħażin ħafna	
Iva, imma mhux daqstant	
Ftit, iżda ma jinkwetanix	
Lanqas xejn	
Niċċajta u nidħak u nara l-aspett inqas serju ta' l-affarijiet:	
L-aktar li nista' possibbli	
Mhux daqstant issa	
Żgur li le, issa	
Lanqas xejn	

Inħossni qiegħed/a inċedi:	
Il-ħin kollu	
Ta' spiss	
Xi kultant	
Qatt	
Inħoss sens ta' biżgħa u nħoss tferfir fl-istonku:	
Lanqas xejn	
Xi kultant	
Ta' spiss	
Spissi ħafna	
Tlift kull interess ta' kif inżomm persunti:	
Qatt ma nagħti kas	
Ma nagħtix kas daqskemm suppost	
Jista' jkun li ma tantx nagħti kas	
Nieħu ħsieb kemm nista'	
Inħossni bla kwiet, qisni għandi nibqa' sejjer il-ħin kollu:	
Ħafna, ħafna	
Mhux ħażin	
Ftit li xejn	
Lanqas xejn	

Ħsibijiet ta’ nkwiet jgħaddu minn moħħi:	
Il-ħin kollu	
Parti kbira tal-ħin	
Minn ħin għall-ieħor, imma mhux spiss	
Xi kultant	
Inħossni ferħan/a:	
Qatt	
Mhux dejjem	
Xi kultant	
Kważi il-ħin kollu	
Kapaċi noqgħod bilqegħda komdu u nħossni rilassat/a:	
Dejjem	
Sikwit	
Mhux ta’ spiss	
Qatt	

Inħares bil-ferħ lejn l-affarijiet:	
Ħafna bħal qabel	
Ftit inqas minn qabel	
Ħafna inqas minn qabel	
Ftit li xejn	
Kultant inħossni ‘ma nafx fejn se nagħti rasi’:	
Dejjem	
Ta’ spiss	
Mhux ta’ spiss	
Qatt	
Nieħu gost naqra ktieb tajjeb jew nisma’ r-radju jew nara programm tat-Televixin:	
Spiss	
Xi kultant	
Mhux dejjem	
Rari	

PITTSBURGH SLEEP QUALITY INDEX

Il-mistoqsijiet li ġejjin huma dwar id-drawwiet tal-irqad tiegħek matul l-aħħar xahar biss. It-tweġibiet tiegħek għandhom jindikaw ir-risposta l-aktar eżatta għall-maġġoranza tal-jiem u l-iljieli f'dan l-aħħar xahar. Jekk jogħġbok wieġeb il-mistoqsijiet kollha.

Fl-aħħar xahar,

5. Fi x'ħin normalment dħalt torqod? _____
6. Kemm domt (f'minuti) biex marret għajnejk bik? _____
7. Fi x'hin normalment qomt filgħodu?_____
8. Kemm-il siegħa jirnexxielek torqod bil-lejl? (It-tweġiba tista tkun differenti minn kemm tqatta siegħat fis-sodda.) _____

Jekk jogħġbok immarka l-aħjar risposta:

	Qatt f'dan l-aħħar xahar	Inqas minn darba fil-ġimgħa	Darba jew darbtejn fil-ġimgħa	Tlett darbiet jew iktar fil-ġimgħa
5. Matul dan l-aħħar xahar, kemm-il darba kellek diffikulta' biex torqod minħabba li				
A. Ma tistax torqod fl-ewwel nofs siegħa				
B. Tqum f'nofs ta' lejl jew filgħodu kmieni				
C. Ikollok tqum biex tuża l-kamra tal-banju				
D. Ma jirnexxielekx tieħu nifs komdu				
E. Tisgħol jew tonħor jgħajjat				
F. Tħoss ħafna bard				

G. Tħoss ħafna sħana				
H. Toħlom ikrah				
I. Tkun muġugħ(a)				
J. Għal xi raġuni(jiet) oħra. Jekk jogħġbok semmihom u inkludi kemm-il darba kellek diffikulta' biex toroqd minħabba f'dawn ir-raġunijiet.				
6. Matul dan l-aħħar xahar, kollox ma kollox, kif tikkunsidra li rqadt?	Tajjeb ħafna	Pjuttost tajjeb	Pjuttost ħażin	Ħażin ħafna
7. Matul dan l-aħħar xahar, kemm-il darba ħadt mediċina (bir-riċetta jew mingħajr riċetta tat-tabib) biex tgħinek torqod?				
8. Matul dan l-aħħar xahar, kemm-il darba batejt biex tibqa' mqajjem/mqajma waqt li kont qed issuq, tiekol ikla jew tissoċjalizza ma' ħaddieħor?				
9. Matul dan l-aħħar xahar, kemm kienet diffiċli żżomm l-entużjażmu biex tagħmel dak li għandek tagħmel?	Ma kienetx problema	Problema zgħira ħafna	Pjuttost problema	Problema kbira ħafna

MODIFIED HEALTH ASSESSMENT QUESTIONNAIRE

Jekk jogħġbok immarka l-aħjar tweġiba skond l-abbiltajiet tiegħek.

Bħalissa, tista:	**Mingħajr EBDA diffikulta'**	**B'xi FTIT diffikulta'**	**B'ĦAFNA diffikulta'**	**MA NISTAX nagħmilha**
Tilbes waħdek, inkluż taqfel iż-żarbun u l-buttuni?				
Tidħol u tqum mis-sodda?				
Terfa' kikkra jew tazza mimlija biex tixrob?				
Timxi barra fil-wita'?				
Tinħasel u tixxotta ġismek kollu?				
Titbaxxa biex tiġbor il-ħwejjeg mill-art?				
Tiftaħ u tagħlaq vit?				
Tidħol u toħrog minn karozza?				

APPENDIX 5 – APPROVALS

The following pages include the approvals obtained to carry out this research from:

- University Research Ethics Committee (ref no 54/2016);
- Chairman of the Department of Medicine at Mater Dei Hospital, Prof S Fava;
- Rheumatology Consultants at Mater Dei Hospital, Prof A Borg, Dr F Camilleri, Dr B Coleiro, Dr PJ Cassar and Dr C Mercieca;
- Chief Executive Officer at Mater Dei Hospital, Mr Ivan Falzon;
- Data Protection Officer at Mater Dei Hospital, Ms Sharon Young;
- Prof DJ Buysse, principal author of the PSQI;
- GL Assessment Ltd for permission to use HADS.

L-UNIVERSITÀ TA' MALTA

Msida – Malta
Skola Medika
Sptar Mater Dei

UNIVERSITY OF MALTA

Msida – Malta
Medical School
Mater Dei Hospital

Ref No: **54/2016**

Thursday, 20th October 2016

Dr Rosalie Magro

Dear Dr Rosalie Magro,

Please refer to your application submitted to the Research Ethics Committee in connection with your research entitled:

Characterisation of the relationship between fatigue, vitamin D level, disease activity and interferon gene expression in Systemic Lupus Erythematosus: A population based study

The University Research Ethics Committee granted ethical approval for the above mentioned protocol.

Yours sincerely,

Dr. Mario Vassallo
Chairman
Research Ethics Committee

Email: umms@um.edu.mt • ***Web:*** http://www.um.edu.mt/ms

[redacted]

Endorsed

18th March 2016.

Chairman Department of Medicine,

Mater Dei Hospital,

Msida MSD 2090

Prof. Stephen Fava
MD MRCP(U.K) MPhil, FACP
FEFIM, FRCP (Lond) PhD(Exeter)
Consultant Physician, Diabetologist & Endocrinologist
Mater Dei Hospital

Prof S Fava,

I would like to obtain your permission to carry out a research project entitled "Characterisation of the relationship between fatigue and Vitamin D level in Systemic Lupus Erythematosus: A population based study on lupus patients in Malta". The primary aim of the study will be the characterisation of the relationship between vitamin D and fatigue in SLE. Moreover the Maltese SLE patients will be characterised, including their vitamin D receptor gene polymorphisms.

I am thus seeking your approval to carry out this study, prior to applying for the approval of the Data Protection Officer and the University Research Ethics Committee. This will enable me to submit a proposal for my research at the University of Malta.

Yours Sincerely,

Rosalie Magro

Resident Specialist in General/Internal Medicine

Higher Specialist Trainee in Rheumatology

[redacted]

24th May 2016.

Rheumatology Consultants,

Department of Medicine,

Mater Dei Hospital,

Msida MSD 2090.

Prof Borg,

I would like to obtain your permission in order to include your patients in a research project entitled "Characterisation of the relationship between fatigue, vitamin D level, disease activity and interferon gene expression in Systemic Lupus Erythematosus: A population based study". The primary aim of the study will be the characterisation of the relationship between vitamin D and fatigue in SLE. The effect of vitamin D supplementation on fatigue, disease activity and interferon gene expression, in SLE patients who are deficient or insufficient, will be established. Moreover the Maltese SLE patients will be characterised, including the prevalence of fatigue and vitamin D deficiency.

I have obtained the approval of Prof Stephen Fava, Mr Ivan Falzon and the Data Protection Officer to carry out this study and I shall also be applying for the approval of the University Research Ethics Committee. This will enable me to submit a proposal for my research at the University of Malta.

Yours Sincerely,

Rosalie Magro

Resident Specialist in General/Internal Medicine

Higher Specialist Trainee in Rheumatology

Agree fully

Prof. Borg
Lead Clinician
Rheumatology
23.5.16

24th May 2016.

Rheumatology Consultants,

Department of Medicine,

Mater Dei Hospital,

Msida MSD 2090.

Dr Camilleri,

I would like to obtain your permission in order to include your patients in a research project entitled "Characterisation of the relationship between fatigue, vitamin D level, disease activity and interferon gene expression in Systemic Lupus Erythematosus: A population based study". The primary aim of the study will be the characterisation of the relationship between vitamin D and fatigue in SLE. The effect of vitamin D supplementation on fatigue, disease activity and interferon gene expression, in SLE patients who are deficient or insufficient, will be established. Moreover the Maltese SLE patients will be characterised, including the prevalence of fatigue and vitamin D deficiency.

I have obtained the approval of Prof Stephen Fava, Mr Ivan Falzon and the Data Protection Officer to carry out this study and I shall also be applying for the approval of the University Research Ethics Committee. This will enable me to submit a proposal for my research at the University of Malta.

Yours Sincerely,

Rosalie Magro

Resident Specialist in General/Internal Medicine

Higher Specialist Trainee in Rheumatology

Dr. Franco Camilleri

24/5/16

[redacted]

24th May 2016.

Rheumatology Consultants,

Department of Medicine,

Mater Dei Hospital,

Msida MSD 2090.

Dr Cassar,

I would like to obtain your permission in order to include your patients in a research project entitled "Characterisation of the relationship between fatigue, vitamin D level, disease activity and interferon gene expression in Systemic Lupus Erythematosus: A population based study". The primary aim of the study will be the characterisation of the relationship between vitamin D and fatigue in SLE. The effect of vitamin D supplementation on fatigue, disease activity and interferon gene expression, in SLE patients who are deficient or insufficient, will be established. Moreover the Maltese SLE patients will be characterised, including the prevalence of fatigue and vitamin D deficiency.

I have obtained the approval of Prof Stephen Fava, Mr Ivan Falzon and the Data Protection Officer to carry out this study and I shall also be applying for the approval of the University Research Ethics Committee. This will enable me to submit a proposal for my research at the University of Malta.

Yours Sincerely,

PJ CASSAR
2115

Rosalie Magro

Resident Specialist in General/Internal Medicine

Higher Specialist Trainee in Rheumatology

[redacted]

[redacted]

[redacted]

24th May 2016.

Rheumatology Consultants,

Department of Medicine,

Mater Dei Hospital,

Msida MSD 2090.

Dr Coleiro,

I would like to obtain your permission in order to include your patients in a research project entitled "Characterisation of the relationship between fatigue, vitamin D level, disease activity and interferon gene expression in Systemic Lupus Erythematosus: A population based study". The primary aim of the study will be the characterisation of the relationship between vitamin D and fatigue in SLE. The effect of vitamin D supplementation on fatigue, disease activity and interferon gene expression, in SLE patients who are deficient or insufficient, will be established. Moreover the Maltese SLE patients will be characterised, including the prevalence of fatigue and vitamin D deficiency.

I have obtained the approval of Prof Stephen Fava, Mr Ivan Falzon and the Data Protection Officer to carry out this study and I shall also be applying for the approval of the University Research Ethics Committee. This will enable me to submit a proposal for my research at the University of Malta.

Yours Sincerely,

Rosalie Magro

Resident Specialist in General/Internal Medicine

Higher Specialist Trainee in Rheumatology

I approve + consent that my patients may be approached to take part in this study.

M Coleiro

24/05/16

24th May 2016.

Rheumatology Consultants,

Department of Medicine,

Mater Dei Hospital,

Msida MSD 2090.

Dr Mercieca,

I would like to obtain your permission in order to include your patients in a research project entitled "Characterisation of the relationship between fatigue, vitamin D level, disease activity and interferon gene expression in Systemic Lupus Erythematosus: A population based study". The primary aim of the study will be the characterisation of the relationship between vitamin D and fatigue in SLE. The effect of vitamin D supplementation on fatigue, disease activity and interferon gene expression, in SLE patients who are deficient or insufficient, will be established. Moreover the Maltese SLE patients will be characterised, including the prevalence of fatigue and vitamin D deficiency.

I have obtained the approval of Prof Stephen Fava, Mr Ivan Falzon and the Data Protection Officer to carry out this study and I shall also be applying for the approval of the University Research Ethics Committee. This will enable me to submit a proposal for my research at the University of Malta.

Yours Sincerely,

Rosalie Magro

Resident Specialist in General/Internal Medicine

Higher Specialist Trainee in Rheumatology

Approved 26/5/16.

Gmail **Rosalie Magro <** **>**

permission for research study

2 messages

Rosalie Magro < **>** Thu, May 5, 2016 at 1:23 PM
To:

Mr Falzon,

I would like to obtain your permission to carry out a research project entitled "Characterisation of the relationship between fatigue, vitamin D level, disease activity and interferon gene expression in Systemic Lupus Erythematosus: A population based study". I shall be submitting my research proposal for a MPhil degree at the University of Malta. I am attaching my research proposal.

The study will include collecting demographic data, medical history and results by using iSOFT, medical notes and by interviewing the patients. Moreover the patients will be asked to fill in a questionnaire and have some blood tests.

I have already obtained the approval of Prof Fava and have been cleared from the MDH data protection point of view. I will also apply for approval from the University Research Ethics Commitee to carry out this study.

Thank You

Best Regards,
Rosalie Magro
Resident Specialist in General/Internal Medicine
Higher Specialist Trainee in Rheumatology

mobile no:

Fatigue and Vitamin D.pdf
1069K

Falzon Ivan at MDH-Health Thu, May 5, 2016 at 5:19 PM
To: Rosalie Magro <
Cc: Satariano Banavage Karen at MDH-Health <

Proceed in line with hospital protocols regulating these projects.

Ivan Falzon
Chief Executive Officer | TeaMDH

SPTAR MATER DEI

T
M
E

Mater Dei Hospital, Triq tal-Qroqq, Msida, Malta MSD 2090 | Tel +356 2545 0000 | www.materdeihospital.org.mt

RE: request for data protection clearance

Caruana Simon at MDH-Health on behalf of Data Protection at MDH
Sent:24 March 2016 13:15
To: Aquilina Rosalie at MDH-Health
Cc: Aquilina Graziella at MDH-Health; Buhagiar Nadine at MDH-Health

Dear Dr Aquilina

Good Afternoon
On the basis of the documentation you submitted, from the MDH data protection point of view you have been cleared to proceed with your study provided that you obtain approval from MDH CEO and the University Ethics Committee.
Please contact Ms. Nadine Buhagiar on 2545 5334 or Ms. Graziella Aquilina on 2545 5346 to present a copy of your approvals and fill in the appropriate Data Protection Form.
Remember that in no way should you retain any personal details you obtain from your research and this should be destroyed at the end of your study.
All medical records are to be viewed at the Medical Records Department MDH.
You are requested to submit a copy of your findings to this office at the end of your study.
Regards
Sharon Young
Data Protection Officer
Mater Dei Hospital

From: Aquilina Rosalie at MDH-Health
Sent: 24 March 2016 13:08
To: Data Protection at MDH
Subject: RE: request for data protection clearance

Mr Simon Caruana,

Many thanks for your reply. I am attaching the approval from the Chairman of the Department of Medicine, Prof Fava. I am also attaching the requested questionnaire in Maltese and English, the participant consent form in Maltese and English, the participant information letter in Maltese and English.

I am attaching once again my UREC proposal form and the proforma on which data will be collected.

Kindly confirm that nothing else is required from my end in order to obtain data protection clearance.

Thank you

Best Regards,
Rosalie Magro
Resident Specialist in General/Internal Medicine
HST Rheumatology

Mobile no: [redacted]

From: Caruana Simon at MDH-Health on behalf of Data Protection at MDH

Sent: 14 March 2016 15:22
To: Aquilina Rosalie at MDH-Health
Cc: Young Sharon at MDH-Health
Subject: RE: request for data protection clearance

Dear Ms Aquilina

Please:

- Seek approval from the Chair of the respective department where the rheumatology clinic forms part e.g.: if it falls under the pain clinic, you have to seek approval from Dr Gatt.

If you are interviewing any participants you should also:

- Provide us the questionnaire in Maltese and English
- Provide us participant consent form in Maltese and English. Another separate form should be available in case of participants under the age of 18
- Provide us information letter for the participant in Maltese and English

Regards

Simon Caruana
F/Sharon Young

From: Aquilina Rosalie at MDH-Health
Sent: 14 March 2016 14:55
To: Data Protection at MDH
Subject: request for data protection clearance

To whom it may concern,

I would like to carry out a research project entitled "Characterisation of the relationship between fatigue and Vitamin D level in Systemic Lupus Erythematosus: A population based study on lupus patients in Malta". My supervisor is Prof A Borg and I shall be submitting my research proposal for a MPhil degree at the University of Malta.

The study will include collecting demographic data, medical history and results by using iSOFT, medical notes and by interviewing the patients. This will be inputted in a standard proforma for each patient (attached).

The data collected from the proforma will be inputted in a spreadsheet in the form of case numbers and will not including the patient name or id number at this stage. The data will then be assessed and any conclusions will be drawn.

I will also apply for approval from the University Research Ethics Commitee to carry out this study (UREC proposal form attached).

Thus I would like to obtain data protection clearance to perform this study.

Thank You

Best Regards,

Rosalie Magro
Resident Specialist in General/Internal Medicine
HST Rheumatology

Mobile no: [redacted]

Gmail Rosalie Magro <[redacted]>

permission to use and translate the PSQI

Buysse, Daniel · [redacted] Tue, May 3, 2016 at 3:59 PM
To: Rosalie Magro · [redacted]
Cc: "Gasiorowski, Mary" <[redacted]>

Dear Rosalie,

You have my permission to use the PSQI for your research study. You can find the instrument, scoring instructions, the original article, links to available translations, and other useful information at www.sleep.pitt.edu under the Instruments tab. For future reference, please note that you can request permission to use the PSQI at https://docs.google.com/forms/d/11-TdSqe2gOtbY-DxtWcsMG-Wt4YvZnOaChybLqED8wY/viewform?c=0&w=1.

The PSQI has been translated into many languages. A list of available translations is on the website indicated above. We would prefer that you use existing translations of the PSQI rather than create another translation if at all possible. This makes it easier to standardize studies and publications, and ensures a consistent approach to translation.

If your requested language is not available, you can request a new translation. However, any new translation of the PSQI, must undergo a rigorous linguistic validation procedure. Please contact MAPI Research Trust with any questions regarding translations or copies of existing translations:

http://www.mapi-trust.org/services/questionnairelicensing/cataloguequestionnaires/155-psqi

or PROinformation@mapi-trust.org.

Please be sure to cite the 1989 paper in any publications that result.

Question 10 is not used in scoring the PSQI. This question is for informational purposes only, and may be omitted during data collection per requirements of the particular study.

Good luck with your research.

Sincerely,

Daniel J. Buysse, M.D.

Professor of Psychiatry and Clinical and Translational Science

University of Pittsburgh School of Medicine

E-1127 WPIC

3811 O'Hara St.

Pittsburgh, PA 15213

T:

F:

From: Rosalie Magro [ ]
Sent: Tuesday, May 03, 2016 8:38 AM
To: Buysse, Daniel
Subject: permission to use and translate the PSQI

Dr Buysse,

I would like to obtain your permission to use the Pittsburgh Sleep Quality Index in my study entitled "Characterisation of the relationship between fatigue, Vitamin D level, disease activity and interferon gene expression in Systemic Lupus Erythematosus: A population based study". Moreover, I also would like to obtain your permission to translate, validate and perform cross-cultural adaptation of the PSQI into the Maltese Language.

Thank You

Best Regards,

Rosalie Magro

Resident Specialist in General/Internal medicine

Mater Dei Hospital, Malta

INVOICE

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University of Malta Faculty of Medicine & Science
Black A Level O
Mater Dei Hospital
Msida
MDS 2090
Malta

Deliver To:

Rosalie Magro
Faculty of Medicine & Science
Black A Level O
Mater Dei Hospital
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APPENDIX 6 – FUNDING

The following page contains the letter from Prof G LaFerla, Dean, Faculty of Medicine and Surgery with the details of the funding granted by the Faculty of Medicine and Surgery for the research.

L-UNIVERSITÀ TA' MALTA
Msida - Malta
SKOLA MEDIKA
Sptar Mater Dei

UNIVERSITY OF MALTA
Msida - Malta
MEDICAL SCHOOL
Mater Dei Hospital

Professor G LaFerla
PhD, MD, MRCS LRCP, FRCS (Ed), FRCSRCPS (Glas), FRCS (Eng), FEBS
Dean, Faculty of Medicine and Surgery
Head, Department of Surgery
Consultant Surgeon

Tel: (00 356) 2340 1874
(00 356) 2340 1137
Fax: (00 356) 2340 1210
e-mail: godfrey.laferla@um.edu.mt

10th January 2017

Dr Rosalie Magro
[redacted]

Dear Dr Magro,

I refer to your request regarding financial support for your studies and I am pleased to inform you that the Faculty of Medicine and Surgery is in a position to grant funding for your research towards your PhD entitled "Characterisation of the relationship between fatigue, vitamin D level, disease activity and interferon gene expression in Systemic Lupus Erythematosus: A population based study".

You are therefore eligible to 10,000 euro per year over a period of three years or the equivalent on a pro-rata basis if your research is being undertaken on a part-time basis, up to a maximum of 30,000 euro subject to:

- material and services, such as laptops, which can be funded through other sources are not covered; and
- a signed declaration, endorsed by your PhD principal supervisor, that the material requested is not being funded by other sources.

I would hope that the funds approved will be well used and to the maximum benefit and I hope you will be successful in achieving your desired goal.

The Faculty would appreciate that on the submission of your thesis, an acknowledgement is made of the financial support you have received from the Faculty alone or in combination with other grants.

Furthermore, these funds are being made available on the condition that you obtain the PhD degree within the stipulated time and according to University of Malta regulations. Should this condition not be met, the amount utilised is to be reimbursed in full.

To use the funds approved above, kindly liaise with the Office of the Dean for the relevant paperwork to be raised and which should be in conformity with the University Financial regulations and requires the Dean's approval so as to be processed.

I wish you every success in your endeavour.

Yours sincerely,

Professor Godfrey LaFerla
Dean, Faculty of Medicine and Surgery

cc Professor Andrew Borg – Principal Supervisor
Mr Mark Debono – Director Finance (MDSCF99-01)
Professor Josanne Vassallo – Chair, Faculty Research Funding Committee

APPENDIX 7 – SUPPLEMENTARY TABLES

Table S1. Correlation of several continuous variables with FSS in the cross-sectional cohort study.

Variable	R value	P value (2-tailed)
Age	-0.085	0.421
Disease duration	-0.142	0.177
Age at disease diagnosis	-0.034	0.748
BMI	0.029	0.781
Current prednisolone dose	0.185	0.078
Current hydroxychloroquine dose	0.214	**0.040**
Current vitamin D dose	-0.042	0.694
Current calcium dose	-0.110	0.295
SLEDAI-2K	0.130	0.217
SDI	-0.190	0.069
VAS Pain	0.536	**<0.001**
HADS-D	0.535	**<0.001**
HADS-A	0.395	**<0.001**
PSQI	0.551	**<0.001**
Haemoglobin	0.045	0.667
Calcium (corrected)	-0.048	0.650
CRP	-0.018	0.863
ESR	-0.063	0.556
C3	0.146	0.165
C4	0.159	0.129
Anti-dsDNA titre	-0.004	0.970
eGFR	0.001	0.995
mHAQ	0.435	**<0.001**

The p values shown were obtained by using Spearman's correlation test. Anti-dsDNA, anti-double-stranded deoxyribonucleic acid; BMI, body mass index; C3, complement 3;

C4, complement 4; CRP, C-reactive protein; eGFR, estimated glomerular filtration rate; ESR, erythrocyte sedimentation rate; FSS, Fatigue Severity Scale; HADS-A, Hospital Anxiety and Depression Scale – anxiety subscale; HADS-D, Hospital Anxiety and Depression Scale – depression subscale; mHAQ, modified Health Assessment Questionnaire; PSQI, Pittsburgh Sleep Quality Index; SDI, Systemic Lupus International Collaborating Clinics/American College of Rheumatology damage index; SLEDAI-2K, Systemic Lupus Erythematosus Disease Activity Index 2000; VAS, Visual Analogue Scale.

Table S2. Correlation of several continuous variables with VAS fatigue in the cross-sectional cohort study.

Variable	R value	P value (2-tailed)
Age	-0.075	0.480
Disease duration	-0.108	0.305
Age at disease diagnosis	-0.035	0.740
BMI	-0.089	0.398
Current prednisolone dose	0.161	0.126
Current hydroxychloroquine dose	0.241	**0.021**
Current vitamin D dose	0.113	0.285
Current calcium dose	0.007	0.947
SLEDAI-2K	0.247	**0.018**
SDI	-0.101	0.336
VAS Pain	0.585	**<0.001**
HADS-D	0.589	**<0.001**
HADS-A	0.377	**<0.001**
PSQI	0.485	**<0.001**
Haemoglobin	-0.044	0.677
Calcium (corrected)	-0.064	0.541
CRP	-0.084	0.426
ESR	-0.078	0.464
C3	-0.011	0.919
C4	-0.020	0.846
Anti-dsDNA titre	0.076	0.472
eGFR	0.014	0.894
mHAQ	0.360	**<0.001**

The p values shown were obtained by using Spearman's correlation test.

Anti-dsDNA, anti-double-stranded deoxyribonucleic acid; BMI, body mass index; C3, complement 3; C4, complement 4; CRP, C-reactive protein; eGFR, estimated

glomerular filtration rate; ESR, erythrocyte sedimentation rate; HADS-A, Hospital Anxiety and Depression Scale – anxiety subscale; HADS-D, Hospital Anxiety and Depression Scale – depression subscale; mHAQ, modified Health Assessment Questionnaire; PSQI, Pittsburgh Sleep Quality Index; SDI, Systemic Lupus International Collaborating Clinics/American College of Rheumatology damage index; SLEDAI-2K, Systemic Lupus Erythematosus Disease Activity Index 2000; VAS, Visual Analogue Scale.

Table S3. Spearman's correlation coefficient (R value) and the respective p values of several continuous variables with SLEDAI-2K in the cross-sectional cohort study.

Variable	R value	P value (2-tailed)
Age	-0.025	0.816
Disease duration	-0.229	**0.028**
Age at disease diagnosis	0.105	0.319
BMI	-0.168	0.109
Current prednisolone dose	0.199	0.057
Current hydroxychloroquine dose	-0.046	0.663
Current vitamin D dose	-0.074	0.481
Current calcium dose	0.081	0.442
SDI	0.003	0.974
mHAQ	0.417	**<0.001**
VAS Pain	0.325	**0.002**
HADS-D	0.230	**0.028**
HADS-A	0.036	0.733
PSQI	0.254	**0.014**
Haemoglobin	-0.251	**0.016**
Calcium (corrected)	-0.070	0.506
CRP	0.142	0.176
ESR	0.198	0.060
C3	-0.441	**<0.001**
C4	-0.333	**0.001**
Anti-dsDNA titre	0.478	**<0.001**
eGFR	0.157	0.134
Urine PCR	-0.106	0.321

Anti-dsDNA, anti-double-stranded deoxyribonucleic acid; BMI, body mass index; C3, complement 3; C4, complement 4; CRP, C-reactive protein; eGFR, estimated glomerular filtration rate; ESR, erythrocyte sedimentation rate; HADS-A, Hospital

Anxiety and Depression Scale – anxiety subscale; HADS-D, Hospital Anxiety and Depression Scale – depression subscale; mHAQ, modified Health Assessment Questionnaire; PSQI, Pittsburgh Sleep Quality Index; SDI, Systemic Lupus International Collaborating Clinics/American College of Rheumatology damage index; SLEDAI-2K, Systemic Lupus Erythematosus Disease Activity Index 2000; VAS, Visual Analogue Scale.

Table S4. Correlation of several continuous variables with PSQI in the cross-sectional cohort study.

Variable	R value	P value (2-tailed)
Age	0.172	0.101
Disease duration	-0.138	0.189
Age at disease diagnosis	0.220	**0.035**
BMI	0.072	0.495
Current prednisolone dose	0.172	0.101
Current hydroxychloroquine dose	-0.020	0.851
Current vitamin D dose	0.003	0.979
Current calcium dose	-0.110	0.296
SDI	-0.071	0.502
VAS Pain	0.515	**<0.001**
HADS-D	0.605	**<0.001**
HADS-A	0.375	**<0.001**
Haemoglobin	-0.126	0.230
Calcium (corrected)	0.060	0.568
CRP	0.129	0.219
ESR	0.093	0.380
C3	0.186	0.075
C4	0.099	0.347
Anti-dsDNA titre	0.087	0.408
eGFR	-0.211	**0.044**
Urine PCR	-0.092	0.393
mHAQ	0.559	**<0.001**

The p values shown were obtained using Spearman's correlation test.

Anti-dsDNA, anti-double-stranded deoxyribonucleic acid; BMI, body mass index; C3, complement 3; C4, complement 4; CRP, C-reactive protein; eGFR, estimated glomerular filtration rate; ESR, erythrocyte sedimentation rate; HADS-A, Hospital

Anxiety and Depression Scale – anxiety subscale; HADS-D, Hospital Anxiety and Depression Scale – depression subscale; mHAQ, modified Health Assessment Questionnaire; PSQI, Pittsburgh Sleep Quality Index; SDI, Systemic Lupus International Collaborating Clinics/American College of Rheumatology damage index; VAS, Visual Analogue Scale.

Table S5. Correlation of several continuous variables with mHAQ using Spearman's correlation test in the cross-sectional cohort study.

Variable	R value	P value (2-tailed)
Age	0.198	0.059
Disease duration	-0.236	**0.023**
Age at disease diagnosis	0.345	**0.001**
BMI	-0.001	0.993
Current prednisolone dose	0.184	0.079
Current hydroxychloroquine dose	0.006	0.954
Current vitamin D dose	0.060	0.567
Current calcium dose	-0.013	0.901
SDI	-0.007	0.946
VAS Pain	0.557	**<0.001**
HADS-D	0.494	**<0.001**
HADS-A	0.272	**0.009**
Haemoglobin	-0.217	**0.038**
Calcium (corrected)	-0.078	0.458
CRP	0.125	0.234
ESR	0.057	0.589
C3	0.060	0.572
C4	0.034	0.746
Anti-dsDNA titre	0.194	0.064
eGFR	0.010	0.925
Urine PCR	-0.156	0.145

Anti-dsDNA, anti-double-stranded deoxyribonucleic acid; BMI, body mass index; C3, complement 3; C4, complement 4; CRP, C-reactive protein; eGFR, estimated glomerular filtration rate; ESR, erythrocyte sedimentation rate; HADS-A, Hospital Anxiety and Depression Scale – anxiety subscale; HADS-D, Hospital Anxiety and Depression Scale – depression subscale; mHAQ, modified Health Assessment

Questionnaire; PCR, protein creatinine ratio; SDI, Systemic Lupus International Collaborating Clinics/American College of Rheumatology damage index; VAS, Visual Analogue Scale.

Table S6. Correlation of several continuous variables with SDI using Spearman's correlation test in the cross-sectional cohort study.

Variable	R value	P value (2-tailed)
Age	0.282	**0.007**
Disease duration	0.355	**0.001**
Age at disease diagnosis	0.043	0.687
BMI	0.043	0.687
Current prednisolone dose	0.220	**0.035**
Current hydroxychloroquine dose	-0.106	0.316
VAS Pain	-0.158	0.133
HADS-D	-0.040	0.707
HADS-A	-0.131	0.213
Haemoglobin	0.020	0.852
CRP	0.232	**0.026**
ESR	0.145	0.172
C3	0.023	0.830
C4	0.062	0.557
Anti-dsDNA titre	0.194	0.063
eGFR	-0.082	0.437
Urine PCR	0.230	**0.030**

Anti-dsDNA, anti-double-stranded deoxyribonucleic acid; BMI, body mass index; C3, complement 3; C4, complement 4; CRP, C-reactive protein; eGFR, estimated glomerular filtration rate; ESR, erythrocyte sedimentation rate; HADS-A, Hospital Anxiety and Depression Scale – anxiety subscale; HADS-D, Hospital Anxiety and Depression Scale – depression subscale; PCR, protein creatinine ratio; SDI, Systemic Lupus International Collaborating Clinics/American College of Rheumatology damage index; VAS, Visual Analogue Scale.

Table S7. Table showing categorical variables, median SDI and its interquartile range when the variable was present and when absent in the cross-sectional cohort study.

Variable		Median SDI	Interquartile range	p value (2-tailed)
Gender	Female	0.0	1	0.203
	Male	1.0	2	
Current sunscreen use	Present	0.0	1	0.500
	Absent	1.0	1	
Smoking	Smoker	0.0	1	0.195
	Non-smoker	1.0	2	
Regular exercise	Present	1.0	2	0.729
	Absent	0.5	1	
Current prednisolone	Present	1.0	2	**0.011**
	Absent	0.0	1	
Current hydroxychloroquine	Present	0.0	1	0.105
	Absent	1.0	2	
Current azathioprine	Present	1.0	3	**0.014**
	Absent	0.0	1	
Current methotrexate	Present	1.0	1	1.000
	Absent	0.5	2	
Current mycophenolate	Present	0.5	3	0.855
	Absent	1.0	1	
Current calcium supplementation	Present	1.0	2	0.313
	Absent	0.0	1	
Current vitamin D suppplementation	Present	1.0	2	0.062
	Absent	0.0	1	
Constitutional manifestations	Present	0.0	1	**0.017**
	Absent	1.0	2	
Mucocutaneous manifestations	Present	1.0	1	0.937
	Absent	0.5	2	

Renal manifestations	Present	1.0	2	**0.018**
	Absent	0.0	1	
Neurological manifestations	Present	2.0	1	**0.005**
	Absent	0.0	1	
Haematological manifestations	Present	1.0	2	**0.040**
	Absent	0.0	1	
Cardiac manifestations	Present	1.5	2	**0.025**
	Absent	0.0	1	
Respiratory manifestations	Present	1.0	2	0.258
	Absent	0.0	1	
Raynauds syndrome	Present	1.0	1	0.628
	Absent	1.0	2	
Osteoporosis/ Osteopaenia	Present	1.0	3	**<0.001**
	Absent	0.0	1	
Hypertension	Present	1.0	3	**0.009**
	Absent	0.0	1	
Hyperlipidaemia	Present	1.0	5	0.162
	Absent	0.0	1	
Diabetes Mellitus	Present	3.0	4	**0.012**
	Absent	0.0	1	
Fibromyalgia	Present	1.0	1	0.698
	Absent	0.0	2	
Anti-phospholipid syndrome	Present	1.0	1	**0.026**
	Absent	0.0	1	
Sjogren's syndrome	Present	1.5	5	0.503
	Absent	1.0	1	
Rheumatoid Arthritis	Present	0.0	1	0.488
	Absent	1.0	2	

The p values were obtained by using the Mann-Whitney U-test.

Table S8. Mean IFN signature gene expression score and its standard deviation for the treated and untreated SLE patient and control dendritic cell and macrophage cultures.

Primary cell culture	Time (hours)	Mean score (untreated)	S.D. (untreated)	Mean score (treated)	S.D. (treated)	p value
SLE patient	24	4.079	0.309	2.807	0.585	**0.014**
dendritic cell	48	3.110	0.372	2.870	0.364	0.403
Control	24	3.963	0.455	3.432	0.536	0.260
dendritic cell	48	3.533	0.396	2.620	0.285	0.070
SLE patient	24	5.549	0.811	3.837	0.363	**0.012**
macrophage	48	4.591	1.079	4.358	0.611	0.728
Control	24	5.035	0.029	2.407	1.182	**0.031**
macrophage	48	4.540	1.750	3.027	1.314	0.256

P values comparing the score between the respective treated and untreated samples at 24 and 48 hours have been obtained using the two-tailed independent samples t-test.

IFN, interferon; S.D., standard deviation.

Table S9. Results obtained when comparing gene expression in the two untreated cell cultures (dendritic cells versus macrophages) from the SLE patient.

Gene MFI	SLE patient DC culture		SLE patient macrophage culture		P value
	Mean	S.D.	Mean	S.D.	
IFI35	0.262	0.061	0.226	0.074	0.151*
OAS1	0.228	0.058	0.145	0.055	0.004
CCL2	0.006	0.004	36.010	7.575	**0.001***
MX1	0.231	0.076	0.159	0.040	0.034*
SOCS1	1.366	0.331	0.200	0.067	**<0.001**
IFITM1	4.594	1.617	6.260	0.908	0.011
STAT2	0.177	0.391	0.210	0.059	0.151
CXCL1	0.007	0.004	0.983	0.827	**<0.001***
IFIT3	0.060	0.053	0.143	0.060	0.005
SOCS3	1.084	0.667	1.303	0.757	0.597*
IFIT1	0.047	0.009	0.037	0.017	0.106
STAT1	1.206	0.530	2.135	0.918	0.019*
IRF7	0.156	0.074	0.133	0.056	0.441
IRF8	0.229	0.067	0.510	0.050	**<0.001**
IFN score	4.043	0.804	4.490	1.141	0.324

P values have been obtained using the independent samples t-test for normally distributed variables, and Mann-Whitney U-test (marked by *) as the non-parametric alternative. DC, dendritic cell; IFN, interferon; MFI, median fluorescence intensity; S.D., standard deviation.