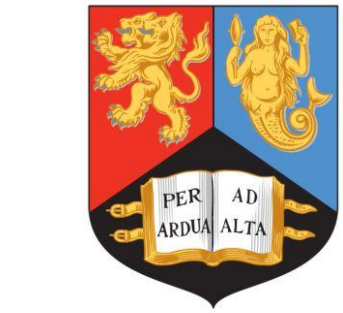
MICRORNAS AND IMMUNOMODULATION BY VITAMIN D

By

DANYANG LI



UNIVERSITY OF BIRMINGHAM

A thesis submitted to the University of Birmingham for the degree of DOCTOR OF PHILOSOPHY

Institute of Metabolism and Systems Research
College of Medical and Dental Sciences
University of Birmingham
January 2019

UNIVERSITY OF
BIRMINGHAM

University of Birmingham Research Archive

e-theses repository

This unpublished thesis/dissertation is copyright of the author and/or third parties. The intellectual property rights of the author or third parties in respect of this work are as defined by The Copyright Designs and Patents Act 1988 or as modified by any successor legislation.

Any use made of information contained in this thesis/dissertation must be in accordance with that legislation and must be properly acknowledged. Further distribution or reproduction in any format is prohibited without the permission of the copyright holder.

Abstract

The active form of vitamin D, 1,25$(OH)_2$D3, plays well-established roles in calcium regulation and bone formation. 1,25$(OH)_2$D3 is also thought to exert immunoregulatory effects upon cells of the innate (dendritic cell) and adaptive (T cell) immune systems, that may impact health and disease. In recent years, the role of 1,25$(OH)_2$D3 has been implicated in autoimmune diseases such as rheumatoid arthritis (RA). 1,25$(OH)_2$D3 brings about genetic and epigenetic changes within immune cells, the latter which may include effects of microRNAs (miRNAs); small non-coding RNAs with an important regulatory role.

To study the role of 1,25$(OH)_2$D3 on miRNAs in RA, we utilised n=20 (RA) and n=7 (reactive arthritis, ReA) matched patient serum and synovial fluid (SF) samples to derive measurements of vitamin D metabolite concentrations by LC-MS/MS, vitamin D binding protein abundance by ELISA, and circulating miRNA expression by qPCR. To study the role of 1,25$(OH)_2$D3 on miRNAs in healthy immune cells, we also generated *in vitro* models of dendritic cells (DC) and CD4+ T cells, treated with 1,25$(OH)_2$D3 or vehicle at different stages of development. An unbiased array approach was then used to screen 372 miRNAs closely related to inflammation in the DC and T cell models. Bioinformatic analyses were used to identify predicted gene targets of significantly regulated miRNAs in both DC and T cells.

Results showed that 1,25$(OH)_2$D3 in SF was low or undetectable in 13/20 RA and 4/7 ReA samples. MiR-146a and miR-155 was up-regulated in RA SF compared to serum, but did not significantly correlate with RA disease markers. In DC, miR-155 but not miR-146a was up-regulated by LPS-induced cell maturation in the presence

or absence of 1,25$(OH)_2$D3. Global down-regulation of miRNAs was observed after either short or long-term treatment of DC with 1,25$(OH)_2$D3. This was due, in part, to suppression of expression for miRNA processing genes. In contrast to DC, global miRNA down-regulation was not observed in T cells treated with 1,25$(OH)_2$D3. Notably, MiR-155 was up-regulated by cell activation but not 1,25$(OH)_2$D3, and miR-212-3p was up-regulated by activation and 1,25$(OH)_2$D3.

Together, these results suggest that any 1,25$(OH)_2$D3 generated within the synovial microenvironment may be restricted to the cells involved in immunoregulation within this tissue. The role of miR-146a and miR-155 in immune cells is still unclear; it is unlikely that these miRNAs are actively mediating gene silencing to cause inflammation within the local environment, but rather they are maintained as a reserve of miRNAs not associated with their target gene. Global down-regulation of miRNAs following 1,25$(OH)_2$D3 in DC, but not T cells, suggests a role for 1,25$(OH)_2$D3-mediated miRNA down-regulation as opposed to decreased miRNA synthesis. Coupled with bioinformatic tools and gene ontology analysis, there is potential to identify novel roles for 1,25$(OH)_2$D3-responsive miRNAs in the prediction and pathogenesis of inflammatory disease.

Acknowledgements

Thank you to the Medical Research Council for funding this PhD Studentship research project.

Firstly, I would like to express my sincerest gratitude to my PhD supervisors Professor Martin Hewison and Professor Karim Raza, for their continued advice and support throughout my PhD. Professor Hewison has been an outstanding supervisor and his experience, patience and approachability has been paramount to the success of this project. Furthermore his enthusiasm, motivation and immense knowledge has inspired me to continue pursuing a career in academia.

Professor Raza has been a source of inspiration by providing fresh insights on my project and encouraging me to think critically from a wider perspective. The clinical chapter of this thesis would not have been possible without his input and sample sourcing.

I would also like to thank a number of academics at the University of Birmingham for their assistance and contributions to this project, in particular Dr Louisa Jeffery and Dr Carl Jenkinson from the Institute of Metabolism and Systems Research, and Dr Peter Nightingale from the Institute of Translational Medicine. Additionally thank you to Dr Dean Larner and Amadeo Muñoz García for their input at various stages of data collection and analysis.

I feel extremely fortunate to have a group of wonderful colleagues within the department who have been a joy to work with. A massive thank you to my friends

with whom I have laughed through the highs and been supported through the lows of my PhD – you guys are amazing.

Finally, this acknowledgement would not be complete without a heartfelt thank you to my family for their unwavering love, advice and support. From the bottom of my heart, I am thankful for everything. I hope I have made you all proud.

CHAPTER 1.

TABLE OF CONTENTS, LIST OF FIGURES AND LIST OF TABLES

1.1 Table of contents

1.2 List of figures and tables

2. General Introduction

3. Materials and Methods

In Chapter 9. Appendices

5. Vitamin D and miRNAs in dendritic cells

6. Vitamin D and miRNAs in T cells

Abbreviations	
1,25(OH)$_2$D3	1,25D/1-alpha-dihydroxyvitamin D3/calcitriol
24,25(OH)$_2$D3	24,25-dihydroxyvitamin D3
25(OH)D	25-hydroxyvitamin D
3-epi-25(OH)D3	C-3 epimer of 25-hydroxyvitamin D3
APC	antigen presenting cell
CYP	cytochrome P oxidase
DBP	vitamin D binding protein
DC	dendritic cell
DMP1	dentin matrix protein 1
DNA	deoxyribonucleic acid
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
FACS	fluorescence-activated cell sorting
FBS	foetal bovine serum
FGF23	fibroblast growth factor 23
GM-CSF	granulocyte macrophage-colony stimulating factor
HLA	human leukocyte antigen
HPLC	high-performance liquid chromatography
IBD	inflammatory bowel disease
IFN-γ	interferon gamma
IgE	immunoglobulin E
IL	Interleukin
KO	Knockout
LC/MS-MS	liquid chromatography-mass spectrometry
LPS	Lipopolysaccharide
miRNA	microRNA
mRNA	messenger RNA
MS	multiple sclerosis
ng/mL	nanogram/millilitre
nmol/L	nanomolar/litre
PAMPs	pathogen-associated molecular pattern
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PFA	Paraformaldehyde
pg/mL	picogram/millilitre
PHEX	phosphate-regulating gene with homologies to endopeptidases on the X chromosome
PMA	phorbol 12-myristate 13-acetate
PTH	parathyroid hormone
qRT-PCR	quantitative reverse transcription-polymerase chain reaction

RA	rheumatoid arthritis
ReA	reactive arthritis
RXR	retinoid X receptor
SD	standard deviation
SF	synovial fluid
SLE	systemic lupus erythematosus
T cell	T lymphocyte
T1D	type 1 diabetes
TGF-β	transformation growth factor beta
Th1	interleukin-1 secreting T cells
Th17	interleukin-17 secreting T cells
Th2	interleukin-2 secreting T cells
TLR	toll-like receptor
TNF-α	tumour necrosis factor-alpha
Treg	T regulatory cell
UVB	ultraviolet B
VDR	vitamin D receptor
VDRE	vitamin D response element
α-CD28	anti-human CD3 monoclonal antibody
α-CD3	anti-human CD28 monoclonal antibody

CHAPTER 2.

GENERAL INTRODUCTION

2.1 Vitamin D metabolism, signalling and endocrine regulation

Vitamin D is a fat-soluble secosteroid and an essential vitamin with important physiological roles in the human body including the regulation of mineral homeostasis and bone metabolism. The discovery of vitamin D and its link to rickets in 1924 was a key advance in medical history [1], and to this day vitamin D continues to be well studied with respect to its classical effects on the skeleton, but also in relation to many other diseases. Vitamin D can be found in two main forms; ergocalciferol (vitamin D2) and cholecalciferol (vitamin D3), both of which are able to fulfil the actions of vitamin D. Vitamin D3 can be obtained as dietary intake from foods such as oily fish, or from *de novo* epidermal synthesis from the conversion of 7-dehydrocholesterol to vitamin D3 by UV irradiation [2]. Epidermal production of vitamin D3 by UV light varies depending on the season of the year, geographic location and social patterns of an individual [3]. Conversely, vitamin D2, which is produced by irradiation of the molecule ergosterol in plant sources, can be used to fortify food as a 'vegetarian' source of vitamin D. The chemical structure of vitamin D2 was characterised in 1931 [4], followed by vitamin D3 in 1937 [5].

Both vitamin D2 and D3 are inactive forms of vitamin D, and are required to undergo metabolic conversion to become active; this process is mediated by cytochrome P450 (CYP) mixed-function oxidase enzymes located in the endoplasmic reticulum (ER) or mitochondria [6]. Prior to these metabolic steps, parental vitamin D is first bound to vitamin D binding protein (DBP) and transported via the bloodstream to the liver, where it undergoes hydroxylation at carbon position 25 to form 25-hydroxyvitamin D3 (25(OH)D3/calcidiol). This reaction appears to be mediated by the

enzyme CYP2R1, located in the ER and in which mutations in the *CYP2R1* gene have been linked to low circulating levels of 25(OH)D3 [7]. However, other enzymes such as CYP27A1 may also participate in 25-hydroxylase activity [8]. 25(OH)D3 is the major circulating vitamin D metabolite and has a half-life of 14-20 days [9], thus circulating concentrations of 25(OH)D3 are routinely used to measure vitamin D status. After 25-hydroxylation, 25(OH)D3 is then transported to the kidneys via DBP, where the endocytic receptors megalin and cubilin present in proximal tubule cells aid reabsorption of the DBP-25(OH)D3 complex from the glomerular filtrate, and subsequent conversion of 25(OH)D3 to the hormonally active 1,25-dihydroxyvitamin D_3 (1,25$(OH)_2$D3/calcitriol) by hydroxylation of 25(OH)D3 at the carbon 1 position [10]. This conversion is mediated by the enzyme 25-hydroxyvitamin D-1α-hydroxylase (1α-hydroxylase), also known as CYP27B1 [11]. Mutations in the *CYP27B1* gene are associated with low serum concentrations of 1,25$(OH)_2$D3 and the genetic disease hereditary vitamin D resistant rickets type 1 [12]. The vitamin D uptake/synthesis, transport and metabolic pathways are summarised in **Figure 2.1**.

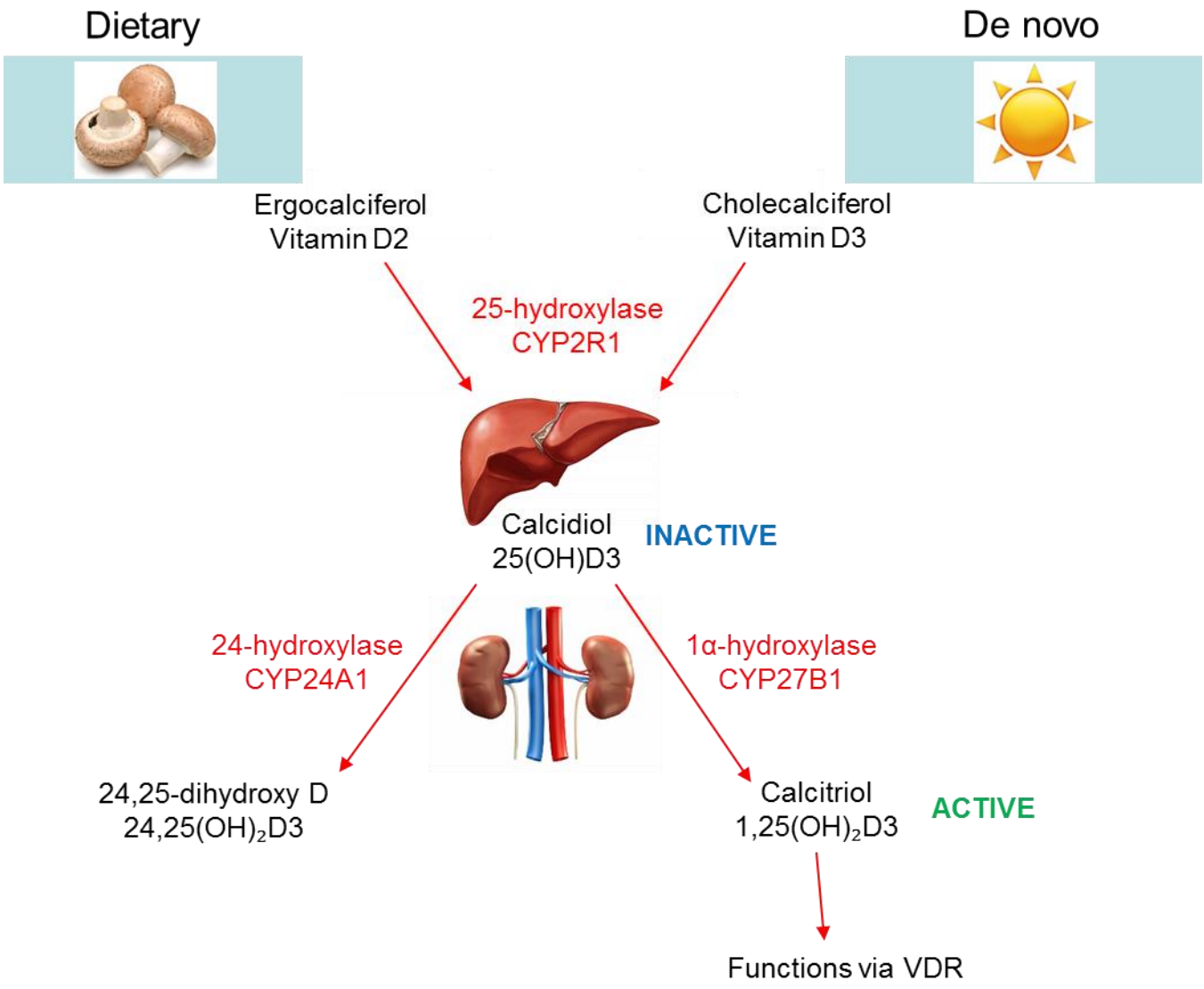


Figure 2.1. The vitamin D metabolic pathway

Vitamin D can be obtained from fortified dietary intake, or direct conversion of 7-dehydrocholesterol mediated by UVB. The molecule undergoes several hydroxylation reactions to become the active form, 1,25-dihydroxy D3 responsible for the reported effects of vitamin D.

The active form of vitamin D, $1,25(OH)_2D3$, functions as a steroid hormone and exerts its biological effects by binding to the intracellular vitamin D receptor (VDR) located in the nuclei of target cells [13]. VDR is a member of the steroid

hormone receptor superfamily of transcription regulatory proteins [14]. Upon binding 1,25$(OH)_2$D3, the VDR conventionally forms a heterodimer with the retinoid X receptor (RXR) and this dimer complex binds to hormone response element DNA sequences within target genes (see **Figure 2.2**) [13]. These vitamin D response element (VDRE) sequences targeted by VDR-1,25$(OH)_2$D3 are frequently located within proximal gene promoter regions, but may also be found in more distal regions of genes [15]. The process of interaction between the VDR and VDRE involves alteration in chromatin structure [16] and recruitment of accessory proteins (co-activators and co-repressors) that facilitate function of the VDR-RXR heterodimer [17, 18], which acts as a transcription factor that can then modulate target gene expression [13, 15]. Mutations in the *VDR* gene are associated with elevated serum concentrations of 1,25$(OH)_2$D3, due to impaired VDR-mediated feedback regulation of this metabolite, and the disease hereditary vitamin D resistant rickets type 2 [19].

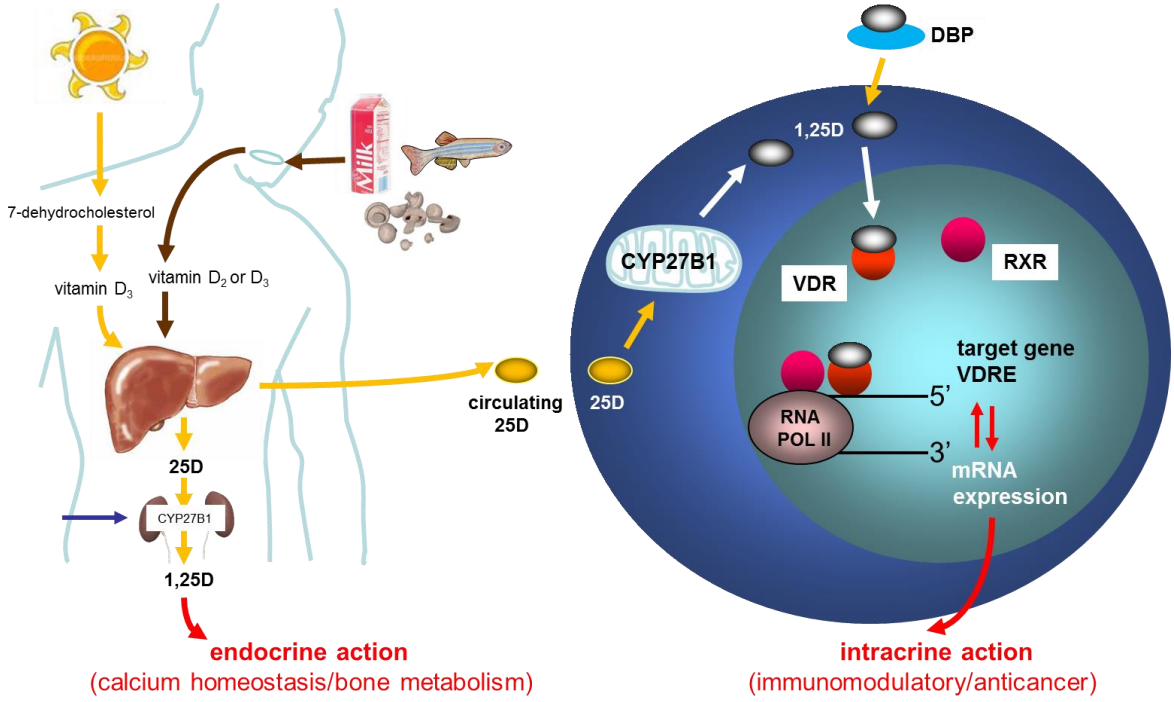


Figure 2.2. The endocrine and intracrine systems for vitamin D

Active $1,25(OH)_2D3$ (1,25D) exerts intracrine effects on cells of the immune system. Circulating 25(OH)D3 (25D) is mostly bound to DBP, which aids conversion to $1,25(OH)_2D3$ (1,25D3) in renal proximal tubules to generate systemic $1,25(OH)_2D3$ which acts on distal target tissues (endocrine action). In other cells such as macrophages, 25(OH)D3 is internalized and converted to $1,25(OH)_2D3$ locally before acting on VDR within the same cell (intracrine action).

The VDR is almost ubiquitously expressed throughout the body and thus, $1,25(OH)_2D3$ potentially has diverse actions in a wide range of biological processes beyond its classical roles in mineral homeostasis and bone metabolism. To maintain homeostasis in the setting of potent VDR-$1,25(OH)_2D3$ activity, the vitamin D pathway is tightly regulated. This occurs primarily via altered expression of CYP enzymes that catabolise $1,25(OH)_2D3$ (and other vitamin D metabolites) to less active catabolites [20, 21]. The most well characterised catabolic pathway for vitamin

D involves the enzyme vitamin D-24-hydroxylase (24-hydroxylase) also known as CYP24A1, which catalyses conversion of 1,25$(OH)_2$D3 to various intermediates leading to the catabolic end product calcitroic acid [22]. These reactions act as a negative feedback loop which regulates vitamin D metabolite concentrations and limits potential toxicity to 1,25$(OH)_2$D3; mutations in the *CYP24A1* gene are associated with idiopathic infantile hypercalcemia due to impaired catabolism of serum 1,25$(OH)_2$D3 and inappropriate serum elevation of this metabolite [23].

The metabolism and function of vitamin D is regulated by well characterised endocrine mechanisms, involving the interplay of positive and negative feedback regulation by parathyroid hormone (PTH) and fibroblast growth factor 23 (FGF23). Osteocytes in bone synthesise and secrete FGF23; this process is under positive regulation by 1,25$(OH)_2$D3 and serum phosphorus levels. The negative regulatory mechanism of FGF23 is not as well understood but is thought to involve dentin matrix protein 1 (DMP1) and phosphate-regulating gene with homologies to endopeptidases on the X chromosome (PHEX). Conversely, FGF23 can also inhibit the synthesis of 1,25$(OH)_2$D3 and the secretion of PTH, which is produced by the parathyroid glands [24].

The main action of PTH besides bone remodelling includes increasing calcium reabsorption in renal tubules both directly and indirectly via stimulating renal 1,25$(OH)_2$D3 production [25]. The parathyroid glands act as a ‘calciostat’ to increase or inhibit the production of PTH depending on the extracellular concentration of calcium [26]. Additionally, the parathyroid glands closely regulate serum phosphate levels, where elevated serum phosphate stimulates the secretion of PTH in a feedback fashion [27, 28]. Phosphate is taken up from circulation by cells via

sodium-phosphate cotransporters, where it is used for a variety of functions including DNA synthesis, intracellular signalling and, more recently established, vitamin D regulation. Serum phosphate levels are dependent on the balance between dietary intestinal absorption and urinary excretion, with around 30% of intestinal phosphate absorption occurring in a $1,25(OH)_2D3$-regulated manner [29]. $1,25(OH)_2D3$ is able to increase the efficiency of phosphorus absorption by up to 80% [30]. Renal phosphate reabsorption plays a key role in maintaining phosphate homeostasis, and is under tight control by PTH and FGF23 regulation [31]. As a result of this tightly regulated process, serum PTH levels in healthy vitamin D-sufficient adults have the capacity to act as a biological readout of serum 25(OH)D activity independent of DBP concentrations [32]. This bone-parathyroid-kidney axis ultimately acts to regulate the production of renal $1,25(OH)_2D3$ to ensure homeostasis. The relationship between PTH, FGF23 and $1,25(OH)_2D3$ is summarised in **Figure 2.3**.

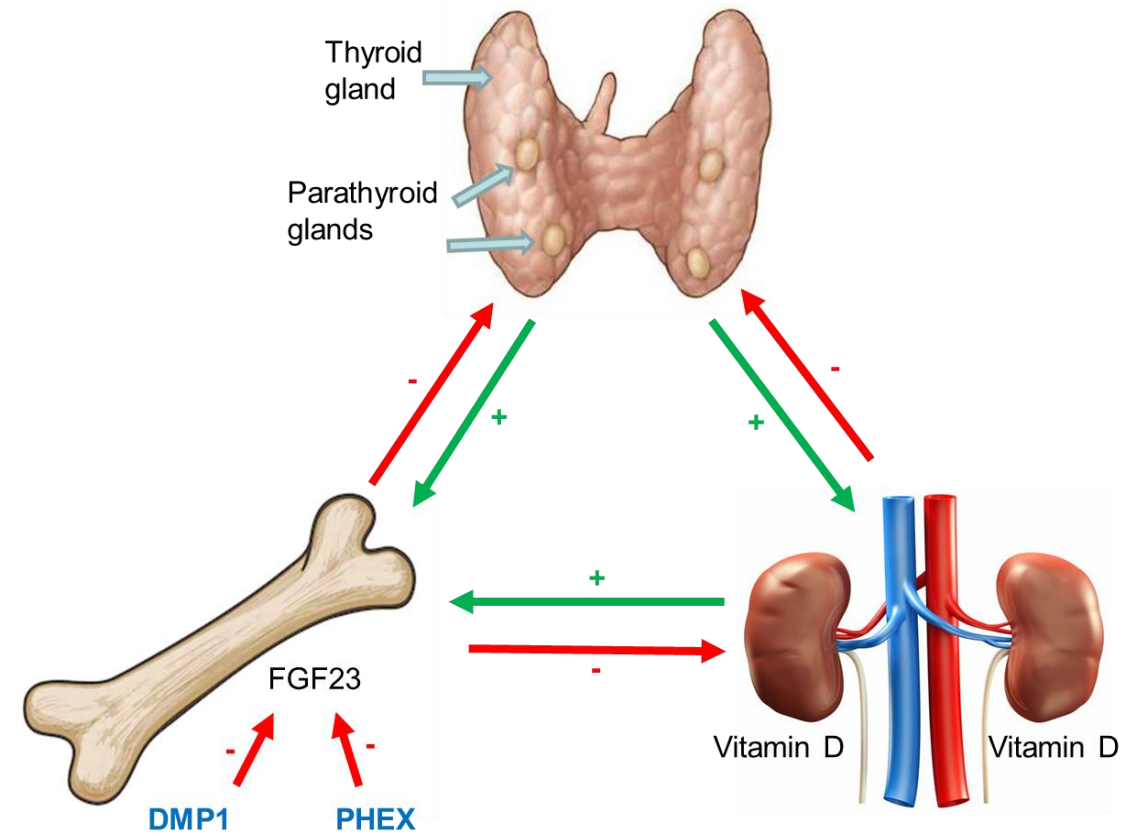


Figure 2.3. The relationship between PTH, FGF23 and vitamin D control

PTH is secreted by the parathyroid glands and is up-regulated by increased serum phosphate, and down-regulated by increased calcium and $1,25(OH)_2D3$ (vitamin D) and high FGF23. FGF23 expression is up-regulated by increased $1,25(OH)_2D3$ levels and down-regulated by DMP1 and PHEX. High FGF23 expression in turn down-regulates PTH secretion. $1,25(OH)_2D3$ production is up-regulated by PTH and down-regulated by high FGF23.

Over the past decade the generation of genomic datasets, in particular chromatin immunoprecipitation sequencing (ChIPseq) data, have been very informative in detailing the role of the VDR in genetic regulation. Key studies have shown the vast number of VDR binding sites and target genes thought to be within the genome, providing an appreciation for considerable range and complexity of vitamin D effects that may be mediated through effects on the transcriptome [33-35].

ChIPseq in human cell models have demonstrated between 1,000 and 10,000 genomic VDR binding sites, providing great potential for genome-wide effects of VDR targeting by 1,25$(OH)_2$D3 [36]. Of particular interest, recent ChIPseq data revealed the occurrence of VDR binding sites was linked to the development of different immune phenotypes [37]. Results from this study suggest that due to enriched numbers of VDR binding sites within genes involved in immune regulation, the immunomodulatory functions of vitamin D may be amongst its most important actions.

2.2 Vitamin D transport and bioavailability

In circulation, the majority of vitamin D metabolites including 25(OH)D3 and 1,25$(OH)_2$D3 are bound to DBP, with smaller proportions of these metabolites bound to other abundant serum carrier proteins such as albumin [38]. In the kidney, binding to DBP facilitates the bioavailability of 25(OH)D3 for proximal tubule uptake and renal conversion to 1,25$(OH)_2$D3 [10]. A similar mode of uptake for vitamin D may also occur in several extra-renal tissues that express megalin [39]. However, in cells that do not express megalin or cubilin, it is unclear how vitamin D metabolites such as 25(OH)D3 or 1,25$(OH)_2$D3 are taken up by these tissues. This process may involve megalin-independent receptor-mediated uptake of DBP, but may also occur via non-receptor uptake of vitamin D metabolites not associated with DBP or other binding proteins – the so-called free hormone hypothesis [40]. Uptake of non DBP-bound or free 25(OH)D3 may be particularly important for immunomodulatory actions of vitamin D where because DBP binds 25(OH)D3 more strongly than 1,25$(OH)_2$D3,

uptake of 25(OH)D3 is central to the production of 1,25$(OH)_2$D3 that occurs in immune cells such as monocytes, macrophages and dendritic cells [41]. The amount of free or bioavailable 25(OH)D is dependent on the amount of DBP and other binding proteins in circulation, and has been postulated as a more physiologically relevant marker of vitamin D status [42]. However, in some studies free or bioavailable 25(OH)D3 does not appear to have any advantages over conventional total serum 25(OH)D3 [43]. Expression of DBP and albumin remains stable despite seasonal variations in the amount of DBP-bound 25(OH)D and free 25(OH)D, which also correlates with variations in total 25(OH)D levels [44]. However, DBP expression has also been reported to vary according to sex [45] and race [46]. In the latter study, decreased serum concentrations of DBP in African and African-American subjects were associated with increased free or bioavailable 25(OH)D3 [46], although this observation has not been confirmed in subsequent studies [47]. Altered DBP and free/bioavailable 25(OH)D3 in different racial groups has been proposed to be due to single nucleotide polymorphic variations in the DBP gene (*Gc*) that may affect the level of DBP [46], and/or the affinity of 25(OH)D3 binding [48].

Around 90% of 25(OH)D is bound to DBP in circulation, with another 10-15% bound to albumin, leaving <1% of 25(OH)D that is unbound and 'free' in circulation [40]. Nevertheless, due to the lipophilic nature of 25(OH)D3 it is suggested that some of its biological actions are exerted through the small fraction of 25(OH)D3 that is not bound to DBP or albumin and is therefore able to diffuse across cell membranes [49, 50]. It is unclear whether this mode of action for 25(OH)D3 is common to most cell types, and whether a similar mechanism is observed for other vitamin D metabolites such as 1,25$(OH)_2$D3, which has lower binding affinity for DBP

compared to 25(OH)D3 [51]. The 'free hormone' hypothesis is not unique to the actions of vitamin D; this mechanism has been described for the uptake of other steroid hormones [52]. Extra-renal actions of vitamin D are highly dependent on the availability of 25(OH)D for cell-specific conversion to $1,25(OH)_2D3$, thus seasonal variations in 25(OH)D may result in differential effects on immune cells at different times of the year. Since DBP binds 25(OH)D3 with higher affinity than it binds $1,25(OH)_2D3$, it is important to consider the impact of bound versus free 25(OH)D3 in extra-renal vitamin D-mediated responses. An alternative name has been proposed for free 25(OH)D3, termed 'bioavailable' 25(OH)D3 which includes 25(OH)D3 in circulation that is not bound to DBP but is associated with albumin. This component of circulating 25(OH)D3, which makes up around 10% of all serum 25(OH)D3, has gained popularity in clinical studies as an alternative to free 25(OH)D although, unlike free 25(OH)D3, bioavailable 25(OH)D3 cannot be measured directly in serum [53]. Studies have shown that the 25(OH)D2 form of 25(OH)D has a lower affinity for DBP compared to 25(OH)D3, as a result of structural differences at carbon position 24. Lower binding affinity for 25(OH)D2 results in faster clearance of this metabolite from circulation, thereby reducing levels available for conversion to $1,25(OH)_2D$ [54, 55]. Because of this it has been suggested that supplementation with vitamin D2 is less effective than vitamin D3 in maintaining serum levels of 25(OH)D.

2.3 Vitamin D-sufficiency and -deficiency

The vitamin D 'status' of any given individual is normally referred to by serum or plasma levels of 25(OH)D3, even though this is not the active form of vitamin D. In

contrast to 1,25$(OH)_2$D3, measurement of 25(OH)D3 is relatively straightforward, with this analysis being common to many core assay services at hospitals around the world. Despite this, the question still remains as to what is the optimal serum concentration of 25(OH)D3? In 2011, the Institute of Medicine (now the National Academy of Medicine) in North America carried out a consultation that resulted in a recommended serum 25(OH)D3 concentration of 20 ng/mL (50 nmol/L), although they emphasized that this only related to the calcium and bone effects of vitamin D [56]. Other organisations have proposed alternative targets for optimal 25(OH)D3 status. In the USA, the Endocrine Society recommended an optimal 25(OH)D3 serum level of 30 ng/mL (75 nmol/L), with levels between 50-75 nmol/L being classed as vitamin D 'insufficient' [57]. In the UK, the Science Advisory Council on Nutrition (SACN) recommended serum 25(OH)D3 greater than 10 ng/mL (25 nmol/L) but again, this was purely for calcium and bone effects of vitamin D [58]. Overall the cut-off value for vitamin D deficiency has been the topic of much debate, although current accepted consensus by medical experts define vitamin D deficiency as a serum 25(OH)D3 concentration of below 20 ng/mL [59]. A normal serum 25(OH)D3 range can be between 20-100 ng/mL, however 25(OH)D3 levels <30 ng/mL are classed as sub-optimal for general health [60].

2.4 Vitamin D status and human health

The importance of vitamin D as a regulator of calcium homeostasis and bone metabolism is well established [61-63], but in recent years there has been growing interest in the extra-skeletal effects of vitamin D [64], particularly with regard to

human disease. There have been many association studies that have reported the link between vitamin D status and a variety of disease manifestations including cancers [65-68] and autoimmune diseases such as rheumatoid arthritis [69-73]. However other studies have questioned whether low vitamin D levels are a consequence, as opposed to the cause of, these diseases [61]. Despite this, the overarching proposal is that deficiency of vitamin D is very likely to be a leading factor in the onset and progression of multiple diseases. A list of well-published extra-skeletal diseases with regard to their links with vitamin D status is summarised in **Table 2.1**.

Extra-skeletal diseases associated with vitamin D deficiency	Role of vitamin D in disease manifestation
Breast cancer, prostate cancer	Vitamin D is a potent inhibitor of tumour cell proliferation [65-68, 74].
Cardiovascular diseases (CVD)	Vitamin D down-regulates genes encoding pro-inflammatory markers [75-77]. Low serum $1,25(OH)_2D3$ is associate with vascular dysfunction, a leading predictor of CVD [78, 79].
Muscle myopathies	Vitamin D reduces muscle inflammation [80]. Low serum $1,25(OH)_2D3$ raises PTH excessively [81, 82].
Alzheimer's Disease	Vitamin D is associated with neuroprotective functions and reduced cognitive decline [83-85].
Type 1 Diabetes (T1D)	Polymorphisms in genes encoding CYP2R1[86], VDBP [87], VDR [88] and CYP27B1 [89, 90] is associated with increased susceptibility to T1D.
Multiple sclerosis (MS)	Higher vitamin D status is correlated with reduced brain lesions and severity of MS symptoms [91-95].
Systemic lupus erythematosus (SLE)	Low vitamin D status is associated with loss of tolerance of immune response leading to worsened SLE symptoms [96-98].
Pre-eclampsia	Vitamin D exerts effects on molecular pathways implicated in the development of pre-eclampsia. Maternal deficiencies lead to increased risk of pre-eclampsia [99-103].
Rheumatoid arthritis (RA)	Vitamin D levels are inversely associated with RA disease progress and activity [69-73, 104].
Inflammatory bowel disease (IBD)	Low vitamin D status is associated with worsened IDB severity and disease duration [105-108]. VDR gene polymorphisms are associated with greater susceptibility to IBD [109].

Table 2.1. Common extra-skeletal diseases associated with vitamin D status

Vitamin D status has also been implicated in pregnancy outcomes, with maternal serum 25(OH)D3 levels positively correlated with a reduced risk of the hypertensive disease pre-eclampsia [101, 110]. Target serum levels for 25(OH)D3 and 1,25$(OH)_2$D3 vary at different stages of life. Circulating levels of 1,25$(OH)_2$D3 in pregnant women averages 130 pg/mL and can be as high as 300-400 pg/mL compared to a range of 16-56 pg/mL in healthy non-pregnant women [111]. This dramatic increase in 1,25$(OH)_2$D3 occurs very early in pregnancy, and the function of this is still unclear as most skeletal development within the foetus takes place much later in gestation, but is thought to relate to hormonal changes [112].

Whilst some published studies have suggested that high dose vitamin D supplementation has limited benefits to general health [113-115] and disease outcomes [116-119], with one study suggesting that a serum 25(OH)D concentration of >50 ng/mL has no additional benefits compared to levels between 30-50 ng/mL [80], there have been some notable examples of successful vitamin D supplementation trials in improving disease outcomes [96, 120-124]. For example, vitamin D supplementation in patients with multiple sclerosis significantly improved cognitive function and decreased mental decline compared to non-supplemented control patients [121]. Vitamin D supplementation also decreased the rate of recurrence of rheumatic disease [122]. Despite the controversy in literature regarding the effectiveness of vitamin D supplementation in disease outcomes, none of these trials have associated vitamin D supplementation with an increased risk of disease, and research is mostly positive regarding the benefits of vitamin D supplementation.

The upper normal limit of serum 25(OH)D3 has also been debated, with previous studies suggesting a serum concentration of over 55 ng/mL as potentially dangerous [125-127], with very high levels of supplementation causing hypercalcaemia. However later studies dispelled this limit and showed that vitamin D toxicity did not occur unless serum 25(OH)D3 levels were above 150 ng/mL, after which symptoms of hypercalcemia manifested [128]. Based on the current literature, vitamin D intoxication is defined as being above a total 25(OH)D3 serum concentration of 150 ng/mL [128, 129]. In contrast to the uncertainties concerning circulating concentrations of 25(OH)D3, the range for serum levels of $1,25(OH)_2D3$ are clearer. $1,25(OH)_2D3$ concentrations are subject to much tighter regulation, with a normal reference range between 39-59 pg/mL in healthy individuals [130]. The current paradigm of these strict limits lies in the tight regulation of renal CYP27B1 activity amongst other regulatory factors [131]. Excessive serum $1,25(OH)_2D3$ in sarcoidosis patients has been linked to worsened disease severity, perhaps due to the overall burden of the disease triggering further $1,25(OH)_2D3$ production [132].

Despite the biological activity of $1,25(OH)_2D3$, this form of vitamin D is not routinely used to measure vitamin D status. This is due, in part, to the secondary hyperparathyroidism associated with vitamin D deficiency, which elevates circulating levels of $1,25(OH)_2D3$ [133]. However, serum concentrations of $1,25(OH)_2D3$ are an important indicator of diseases including hereditary vitamin D-resistant rickets types 1 and 2 (where $1,25(OH)_2D3$ is respectively suppressed or elevated), and chronic renal failure, provided no other nutritional deficiencies are present [133].

2.5 Quantification of vitamin D metabolites

Methods of quantifying 25(OH)D levels in patients have come a long way since the first assay to quantify 25(OH)D was performed using DBP as a competitive binder [134]. The major limitation of this assay was the detection of not only 25(OH)D in serum, but also other metabolites including 24,25$(OH)_2$D3. Since then more advanced methods of analysing 25(OH)D metabolite concentrations have been developed, notably the development of radioimmunoassays (Diasorin™) in 1985 [135] and high-performance liquid chromatography (HPLC) [136], both of which are now also known to have limitations affecting their detection accuracy. The major limitation of the Diasorin method was, like the DBP assay method, the detection of other metabolites, which meant that levels of 25(OH)D were usually overestimated by around 15%. Such an overestimation can be crucial for defining the line between vitamin D sufficiency and deficiency. The HPLC method for quantification of vitamin D removed assay interference by separating out 25(OH)D from other vitamin D metabolites, but it was a long and expensive procedure requiring large sample volumes and was therefore not suitable for large-throughput analysis in clinical settings. The current gold standard for measuring vitamin D status uses liquid chromatography-tandem mass spectrometry (LC/MS-MS) which combines the separation capabilities of HPLC with the detection specificity of mass spectrometry, to accurately measure 25(OH)D3 and other vitamin D metabolites in patient serum [137, 138]. This technique is more accurate, and requires relatively small volumes of serum or plasma. Recent developments in analytical chemistry mean that it is now possible to accurately differentiate between 25(OH)D2 and 25(OH)D3 in serum, as well as other metabolites including 1,25$(OH)_2$D3 [139, 140].

2.6 Vitamin D and the immune system

Perhaps the best example of an extra-skeletal role for vitamin D is its potent ability to regulate the function of immune cells. A link between vitamin D and the immune system was first proposed based on two key observations: 1) activated cells from both the innate and adaptive immune systems express VDR and respond to $1,25(OH)_2D3$; 2) activated innate immune cells such as macrophages and DC express CYP27B1 and are able to synthesise $1,25(OH)_2D3$ (see **Figure 2.4**). Initially these observations were considered to be either pathological features or potential mechanisms for therapeutic targeting. For example, over-active macrophage production of $1,25(OH)_2D3$ in granulomatous diseases such as sarcoidosis was linked to the pathological hypercalcemia that is occasionally observed for this type of disorder [141, 142]. In a similar fashion, the expression of VDR in cells of the immune system suggested potential therapeutic use of $1,25(OH)_2D3$ as treatment for diseases such as leukaemia [143]. In the case of the latter, this led to the development of analogues of $1,25(OH)_2D3$ that retained ability to regulate monocyte function, whilst minimising the established hypercalcaemic effects of $1,25(OH)_2D3$ [144]. However, in more recent years it has become clear that vitamin D may have an important role in regulating normal immune responses, by modulating cells from both the innate and adaptive immune systems. The remainder of this section of the thesis introduction will detail the mechanisms associated with immunoregulation by vitamin D. However, specific details on the effects of vitamin D on DC and T cells are provided in the thesis chapters dedicated to these cell types (**Chapters 5** and **6** respectively). The interactions between vitamin D and the innate and adaptive immune systems are summarised in **Figure 2.5**.

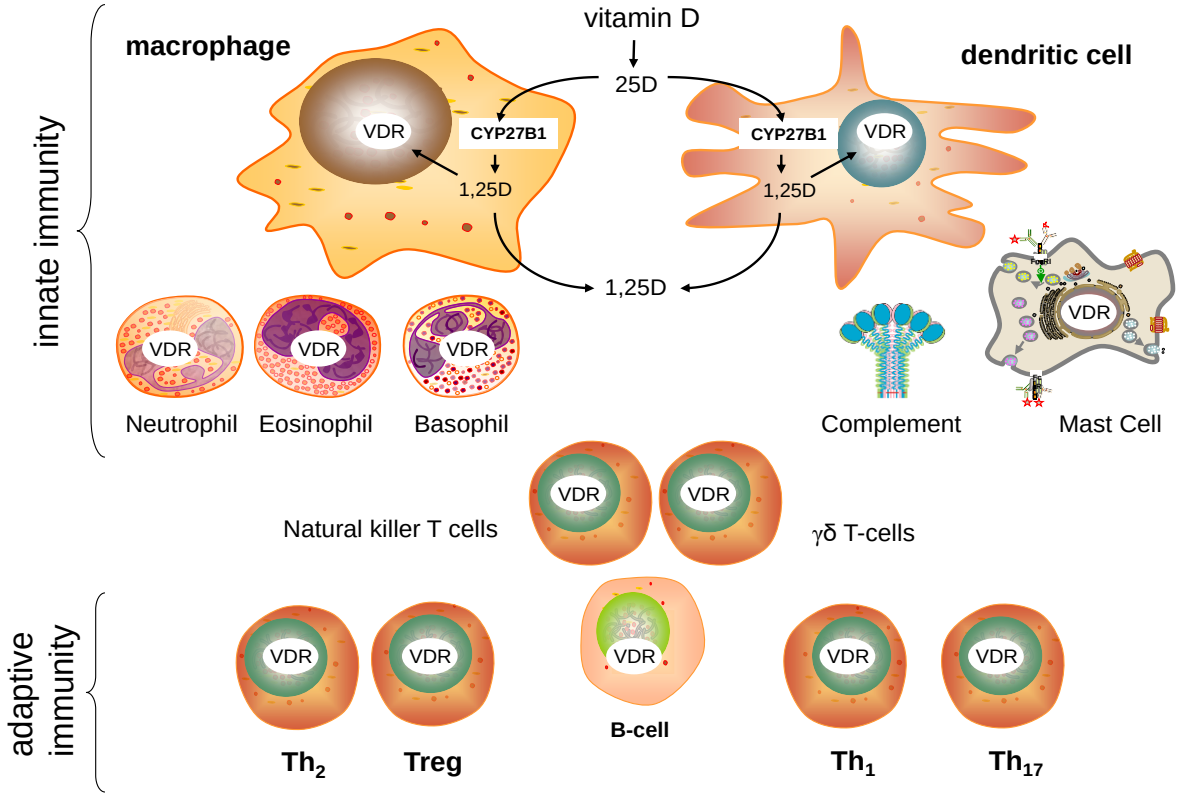


Figure 2.4. Expression of VDR and CYP27B1 in cells from the innate and adaptive immune systems

Immune cells including macrophages and dendritic cells of the innate immune system express VDR and CYP27B1, hence are able to activate 25(OH)D3 (25D) to 1,25$(OH)_2$D3 (1,25D). VDR has also been found in cells of the adaptive immune system including T cell subsets and B cells, meaning these cells are all capable of responding to 1,25$(OH)_2$D3 in a VDR-dependent manner.

Monocytes/macrophages

Monocytes and macrophages constitute key members of the innate immune response with the ability to recognise foreign pathogen-associated molecular patterns (PAMPs) via pattern recognition receptor (PRR) such as toll-like receptors (TLRs), thus providing the first line of defence against pathogens and enhancing the antimicrobial properties of these immune cells. Effects of 1,25$(OH)_2$D3 on monocytes and macrophages include its potent anti-mycobacterial activity [145].

Further research found that monocytes and macrophages also express the enzyme CYP27B1, thus are able to produce $1,25(OH)_2D3$ within immune-active tissues independent of renal CYP27B1 [11, 146, 147]. Macrophage CYP27B1 behaves differently to renal CYP27B1; CYP27B1 produced by macrophages can be up-regulated by the presence of immune stimuli such as IFN-γ and Lipopolysaccharide (LPS). Conversely, $1,25(OH)_2D3$ produced by monocytes and macrophages is not subject to the same regulatory mechanisms as renal CYP27B1 in that elevated calcium or $1,25(OH)_2D3$ levels do not suppress CYP27B1 production [148, 149]. Instead, IFN-ɣ and LPS stimulate up-regulation of CYP27B1, via the *JAK/STAT* or *MAPK* signalling pathways [148, 150].

$1,25(OH)_2D3$ promotes antibacterial activity responses following activation of TLRs as part of innate immune responses to infection. Liu *et al*. [145] showed that activation of the TLR-2 pathway in macrophages resulted in up-regulation of *CYP27B1* and *VDR* expression, leading to intracrine synthesis of $1,25(OH)_2D3$, $1,25(OH)_2D3$-*VDR-RXR* heterodimer binding to *VDRE* and subsequent transcription of antimicrobial genes such as cathelicidin. Wang *et al.* [151] showed that in addition to this, $1,25(OH)_2D3$ induced expression of CD14, a TLR co-receptor and monocyte marker.

Dendritic cells (DC)

In contrast to stimulatory effects on innate immune function, $1,25(OH)_2D3$ tends to inhibit the adaptive immune system, but some of these effects can be mediated via cells from the innate immune system such as the antigen presenting

dendritic cells (DC). In antigen presenting cells (APC) such as DC, 1,25$(OH)_2$D3 decreases cell maturation and thereby changes antigen presentation to T cells [152, 153]. In DC, 1,25$(OH)_2$D3 down-regulates expression of the maturation marker CD80/CD86 [154-156] and CD83 [157], indicating that 1,25(OH)2D3 promotes DC differentiation way from an antigen presentation phenotype. 1,25$(OH)_2$D3 also induces maintenance of tolerogenic DC [158-160]. DC stimulated with 1,25$(OH)_2$D3 show up-regulated IL-10 production and inhibition of pro-inflammatory cytokine release, such as TNF-α, IFN-γ and IL-12 [154, 161, 162]. Thus 1,25$(OH)_2$D3 is able to maintain an immature-like DC phenotype [163], thereby further reducing stimulation of T cells. The role of vitamin D in DC immunomodulation is described in further detail in **Chapter 5** of this thesis.

Neutrophils

Neutrophils are granulocyte cells that also constitute part of the innate immune system, and they have been shown to express functional VDR at a level similar to that found in monocytes [164]. However neutrophils lack the CYP27B1 required to convert 25(OH)D to 1,25$(OH)_2$D3 and subsequent intracrine stimulation of cathelicidin expression. It has been demonstrated that CD14 cell surface marker is augmented in neutrophils in the presence of 1,25$(OH)_2$D3, with this mechanism likely to involve 1,25$(OH)_2$D3-mediated modulation of the target genes *trappin-2/elafin/SKALP* [164]. The anti-microbial activity of neutrophils is driven by the presence of granules containing anti-microbial agents; neutrophil development and granulopoiesis requires the down-regulation of *RXRα* [165, 166]. The exact

mechanism of this action and the role of $1,25(OH)_2D3$ in this response is not well understood, although studies suggest that $1,25(OH)_2D3$ may alter the neutrophil inflammatory process in a VDR-dependent manner [167]. $1,25(OH)_2D3$ up-regulates the production of IL-8 in neutrophils following LPS stimulus [168] and suppresses neutrophil activation [169].

Eosinophils/Basophils

Eosinophils and basophils, along with mast cells, are involved in the pathogenesis of allergic disease in individuals with allergen-specific immunoglobulin E (IgE). Upon activation, these cells release or synthesise a host of immunomodulatory mediators such as histamines, cytokines and chemokines [170]. Eosinophils make up around 5% of circulating leukocytes in blood, with the majority found residing in bone marrow, thymus and lymph organs. Basophils make up less than 1% of leukocytes in healthy blood, and are rapidly recruited to sites of inflammation. Eosinophils and basophils both express VDR, and serum 25(OH)D3 levels have been reported to be inversely correlated with blood eosinophil counts in human [171-173] and mouse [174] models of allergic disease, leading to excessive eosinophil activation, overproduction of IgE and exacerbated allergic reactions. The precise molecule mechanism of $1,25(OH)_2D3$ in eosinophils and basophils is still unknown. Epidemiological studies have suggested that vitamin D is protective against allergic diseases by prolonging eosinophil survival at non-inflamed sites [175], whilst other studies have not demonstrated this link [176].

Mast cells

The activation of mast cells forms the acute phase of an allergic reaction. Mast cells express TLR-2 and their activation is triggered by the crosslinking of IgE with specific allergens, and primarily leads to the release of histamines and TNF-α cytokine, followed by the release of prostaglandins [170, 177]. The expression of VDR in these cells provides potential for $1,25(OH)_2D3$ to dampen mast cell activation by suppressing IgE and reducing inflammatory cytokine release [178]. Mast cells can actively convert 25(OH)D3 to $1,25(OH)_2D3$ via intrinsic CYP27B1 activity [179], and exposure to $1,25(OH)_2D3$ in culture up-regulates mast cell expression of VDR [180]. Furthermore, it has been demonstrated that $1,25(OH)_2D3$ is required to maintain mast cell stability as shown by excessive mast cell activation under vitamin D-deficient conditions. In mast cells of *Vdr* knockout mice, $1,25(OH)_2D3$ can induce IL-10 secretion and suppress inflammation, demonstrating that $1,25(OH)_2D3$ can act upon mast cells in a VDR-independent fashion [181]. These studies highlight the wide range of potential anti-inflammatory actions of $1,25(OH)_2D3$ in different innate immunity cells.

T cells

In the adaptive immune system, numerous studies have shown $1,25(OH)_2D3$ also plays a key role in mediating T cell immunity, where $1,25(OH)_2D3$ added *in vitro* acts to suppress immune responses [182] by stimulating an increase in numbers of Treg cells [158, 159]. Vitamin D also exerts inhibitory actions by suppressing the development of Th1 and Th17 cells, resulting in decreased production of pro-

inflammatory cytokines IFN-У, IL-2 and IL-17 [183]. Further studies have reported $1,25(OH)_2D3$ induces changes in T helper (Th) cell cytokine secretions, or differential expression of co-stimulatory molecules upon T cell activation [184]. One of the ways in which $1,25(OH)_2D3$ suppresses T cell activity is by mediating a shift from Th1 to Th2 phenotype and reducing their proliferation [185, 186]; this Th cell polarisation shift inhibits pro-inflammatory IFN-У producing Th1 cells, and augments IL-10 producing Th2 cells. High doses of $1,25(OH)_2D3$ in culture also acts on CD4+ T cells by suppressing their activation [187]. Similarly in murine models, the effect of $1,25(OH)_2D3$ on T cells includes inhibition of IFN-У and IL-17 secretion and reduced proliferation, and induction of Tregs and IL-4 secretion [188]. The immunomodulatory role of vitamin D on T cell subset function is described in greater detail in **Chapter 6** of this thesis.

B cells

B cells play an important role in the adaptive immune response by secreting antibodies after differentiation into plasma cells, as well as being involved in antigen presentation and cytokine secretion. They are also responsible for the production of autoreactive antibodies in autoimmune disease. B cells express VDR but despite their importance in immune function there have been limited and conflicting reports on the action of $1,25(OH)_2D3$ on B cells. Both indirect and direct effects of $1,25(OH)_2D3$ on B cells have been reported, with indirect effects of $1,25(OH)_2D3$ thought to inhibit B cell function via the impairment of CD4+ T cell signalling [189]. Conversely, direct effects of $1,25(OH)_2D3$ may involve the inhibition of IgE production

by B cells [190], blocking B cell differentiation and proliferation [191, 192] and inducing apoptosis [193], suggesting $1,25(OH)_2D3$ plays an important role in maintaining B cell homeostasis by controlling the abundance of plasma cells and therefore autoreactive antibodies produced. Indeed, deficiency of $1,25(OH)_2D3$ in serum appears to be a contributing factor in the development and severity of B cell-mediated systemic lupus erythematosus (SLE) [194], further defining the important role of $1,25(OH)_2D3$ in B cell homeostasis.

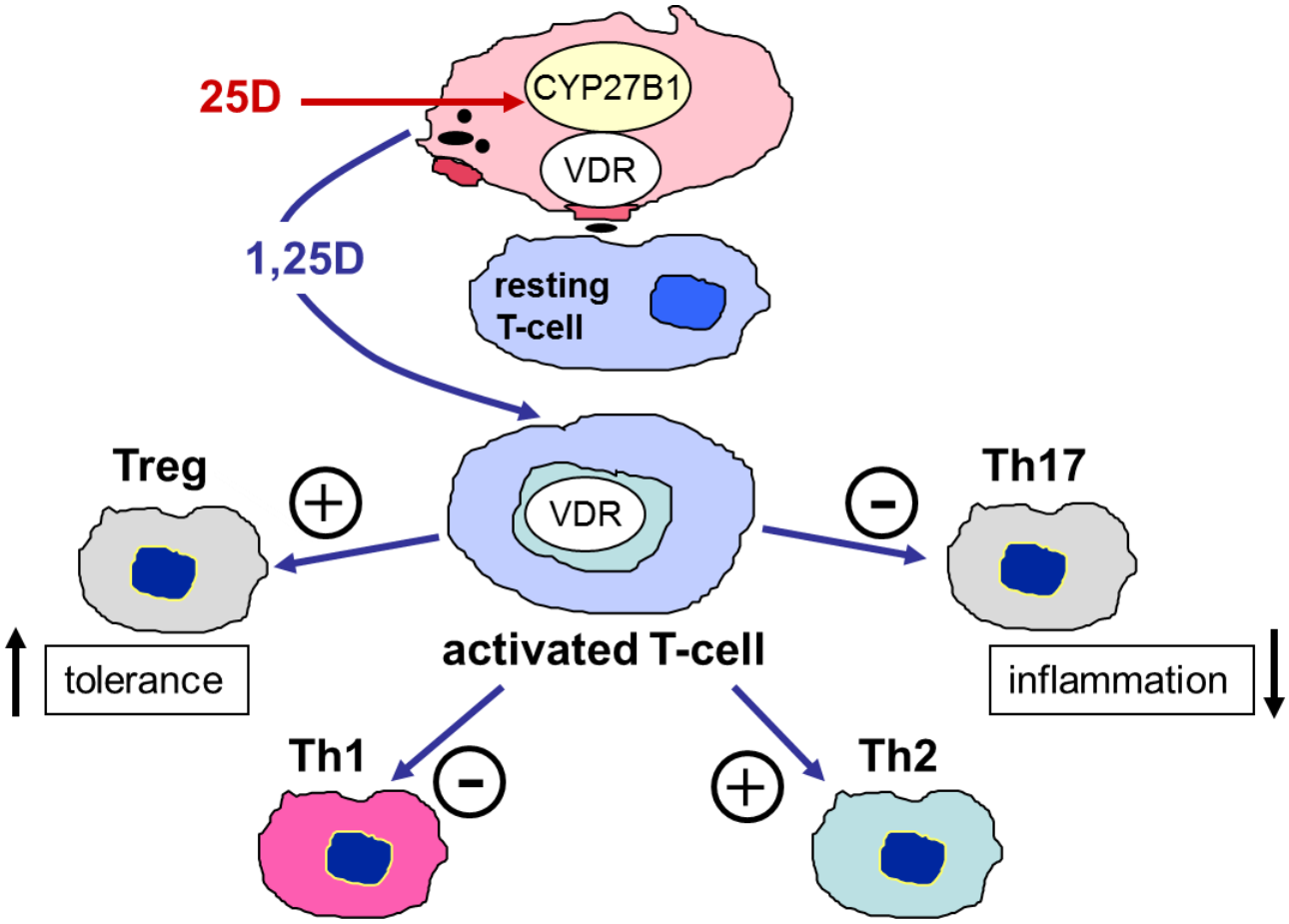


Figure 2.5. The relationship between vitamin D and the innate and adaptive immune systems

Vitamin D acts via the VDR intrinsically expressed by cells of the innate and adaptive immune systems. Monocytes and macrophages also express CYP27B1 allowing the synthesis of 1,25$(OH)_2$D3 from 25(OH)D. Active vitamin D, when bound to the VDR, elicits anti-inflammatory effects on cells of the adaptive immune system including T cell subsets. Treg and Th2 cell activity is up-regulated and primed to release anti-inflammatory cytokines, whilst Th1 and Th17 fates, which favour a pro-inflammatory fate, are suppressed.

Overall the active form of vitamin D acts to suppress immune responses through decreased T cell activation, decreased DC maturation, and increased DC tolerogenesis. Together, these molecular changes suggest that 1,25$(OH)_2$D3 has great potential to regulate inflammation in the innate and adaptive immune system.

2.7 Vitamin D and microRNAs

Introduction to microRNAs

The first endogenous microRNA (miRNA) was discovered in *C. elegans* in 1993, where scientists found that *lin-4* was essential in controlling post-embryonic development via the negative regulation of LIN-14 protein [195]. This led to the concept that small endogenous molecules were capable of functioning in a regulatory fashion but in non-protein encoding roles. This challenged the previous central dogma that protein translation only involved a single direction of genetic information flow via mRNAs. It was only in the early 2000s that miRNAs were classed as distinct biological regulators [196], with their link to vitamin D emerging almost a decade later [197, 198].

It is now known that miRNAs are small single-stranded non-coding RNAs that are highly conserved amongst eukaryotes [199]. Around 16-22 nucleotides in length, they play a host of different functions which ultimately involve regulating gene expression at the post-transcriptional level [200]. MiRNAs are functionally similar to siRNAs, and exert their function by base pairing to target mRNAs to inhibit gene translation and, in some cases, destabilizing mRNA transcripts, thereby ultimately

suppressing protein synthesis of the miRNA target transcript. Understanding the mechanism of action of miRNAs has provided the tools for prediction of many potential miRNA targets in the human genome. It is predicted that over 40% of protein coding genes in the human body are regulated by miRNAs [201, 202], with many of these miRNAs acting in immune regulation. In recent years, dysregulation of a number of miRNAs has been linked to a range of diseases in humans, including but not limited to autoimmune diseases.

To date, over 2500 mature miRNAs have been experimentally validated in the human genome (miRBase21) and due to the important regulatory role they play it is likely that at least some of these will be dysregulated in disease, thus placing miRNAs at the forefront of disease-based therapeutic research. The relationship between miRNAs and the immune system has only recently been discovered, and research is still ongoing to decipher this link. Multiple studies have shown a strong link between miRNAs and their role in immune cell regulation and development, via modulating gene expression or other epigenetic regulations. As miRNAs are found ubiquitously, it is likely that miRNAs target genes required for immune cell differentiation and survival, hence any dysregulated miRNA activity may also impact upon cells of the immune system. Multiple miRNAs may be responsible for targeting a single RNA sequence, hence it is often difficult to pinpoint gene dysregulation as a result of a single miRNA. The level of gene repression is also dependent on the amount of mRNA and the availability of miRNAs [203].

miRNA expression and function

MiRNAs are first transcribed within the cell nucleus by RNA Pol II and III to form the primary miRNA (pri-miRNA), a long double-stranded structure with a hairpin loop on the end. The pri-miRNA is further cleaved in the nucleus to leave the 60-70 nucleotide hairpin loop structure known as the precursor miRNA (pre-miRNA) [204]. This step requires the action of the microprocessor complex made up of DROSHA and its co-factor DGCR8. DGCR8 is responsible for guiding DROSHA to cleave the pre-miRNA, leaving a 2 nucleotide overhang on the 3' end. The pre-miRNA is exported out of the nucleus into the cytoplasm by exportin-5, a nuclear transport receptor that is dependent on Ran and GTP [205]. In the cytoplasm the pre-miRNA undergoes further cleavage mediated by TRBP and DICER, an RNAse III nuclease enzyme which generates a 22 nucleotide double-stranded RNA complex comprising the passenger and guide strand. DICER cleaves the 5' end of the pre-miRNA by recognising the 3' end, leading to the production of both 3' and 5' end miRNAs [206]. It is thought that TRBP alone does not contribute to pre-miRNA processing, but instead it is important for forming part of the RNA-induced silencing complex (RISC) [207]. The miRNA duplex is then incorporated onto Ago2, the passenger strand is degraded, and the guide strand remains bound to the Ago2-RISC complex as the mature miRNA. The mature miRNA guides RISC to its target mRNA, where it exerts its silencing actions via mRNA cleavage, translational repression or mRNA deadenylation [208]. A diagram of this mechanism and proteins involved is shown in **Figure 2.6**.

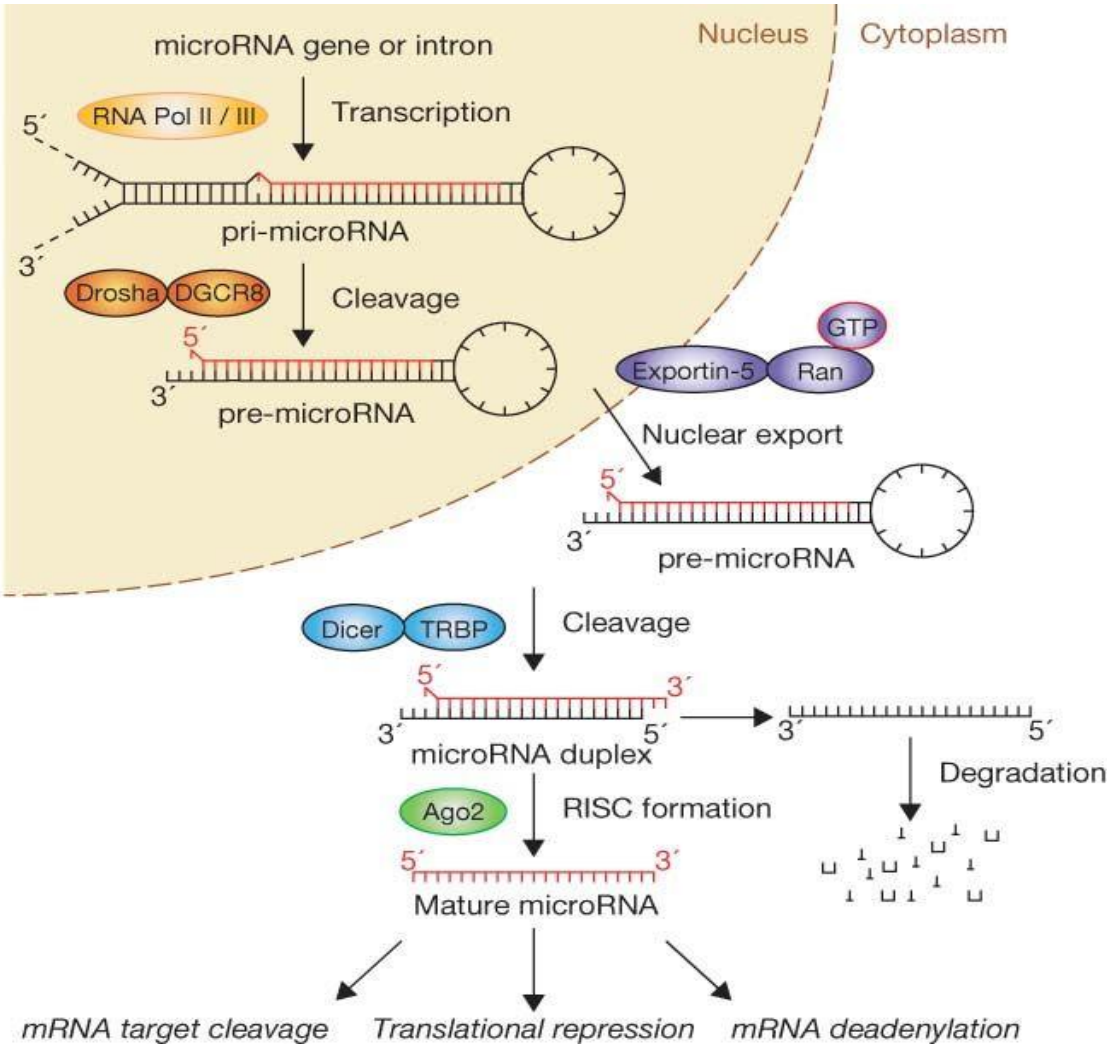


Figure 2.6. Mechanism of miRNA synthesis in humans

Pri-miRNA cleavage by DROSHA and DGCR8 takes place in the nucleus, and pre-miRNA cleavage by DICER and TRBP takes place in the cytoplasm. The mature miRNA is incorporated into RISC and responsible for subsequent silencing actions on target mRNA. Image cited and adapted from Nature Cell Biology [209].

The exact mechanism by which miRNAs process their target genes for degradation is more complex than previously thought; studies have suggested this process is not only mediated by complementary base pairing which triggers Ago protein slicer activity, but also involves other processes such as mRNA de-capping, deadenylation and exonuclease digestion [210, 211]. Others have suggested

miRNAs mediate translational repression of their target mRNA through the accumulation of target mRNAs in processing bodies (p-bodies), which are not involved in the translational process themselves [212]. Nucleotides 2-7 of the 5' end of a miRNA, also known as the seed strand which is highly conserved amongst vertebrates, have been shown to be necessary for miRNAs to complementary base pair to their target mRNA [203, 213]. Conversely, the 3' end of a miRNA is not generally required to be complementary to the mRNA. Whilst mRNA non-complementarity to the 3' region of miRNAs does not appear to hinder miRNA function, it is likely that mRNA complementarity to both the 5' and the 3' ends greatly enhances miRNA-mediated gene silencing.

Pre-miRNAs can arise from exons or introns of gene transcripts, the former of which is retained, and the latter spliced out. It has been shown that a large majority of mammalian miRNAs are found within intron regions of protein-coding genes. These miRNA host genes encode proteins that are involved in a wide range of biological functions from metabolism to cell cycle control [214]. This finding suggests that miRNAs located in intron regions of their host genes may be involved in their transcriptional regulation.

2.8 A role for miRNAs in the immunomodulatory actions of vitamin D

It is well established that vitamin D influences immune function by acting as a transcriptional regulator in conjunction with the VDR [215, 216]. Over the years, key studies have emerged from the analysis of vitamin D-regulated transcription on immune function [217-220], detailing the important role of vitamin D-mediated

transcriptional modulation on both the innate and adaptive immune system. In addition to transcription, it is clear that vitamin D can also influence epigenetic mechanisms, including modulating activity of the *VDR* promoter [221, 222], *CYP24A1* [223, 224] and *CYP27B1* [225-227] by DNA methylation or histone acetylation. Additionally, the VDR protein can directly interact with co-activator and co-repressor proteins which are in contact with chromatin modifiers such as histone acetyltransferases (HATs), which in turn interact with the VDR/RXR heterodimer to induce transcriptional activation [228]. These studies suggest that the vitamin D system is highly dependent on epigenetic modifications of its receptor and associated enzyme coding genes.

One prominent epigenetic target for vitamin D is miRNAs. Extensive studies have demonstrated that various miRNAs can be dysregulated in a variety of cell types from cancer cells [229, 230] to fibroblast-like synoviocytes in rheumatoid arthritis [231, 232], although far fewer studies have been dedicated to exploring the role of vitamin D on miRNA modulation in these cells. The majority of studies involving vitamin D and miRNA modulation have been performed using cell lines. A summary of published vitamin D-mediated miRNAs in different cell types is shown in **Table 2.2**.

Cell type	miRNAs regulated by vitamin D
Osteoblast	miR-125 [233], miR-146a [234], miR-637, miR-1228 [63]
Monocyte	miR-21 [235], miR-146a, miR-130a [236]
Colon cancer (SW480-ADH, HCT116)	miR-346 [237], miR-22 [238]
Dendritic cell	miR-378, miR-155 [239, 240], miR-29a, miR-146a, miR-193a, miR-387, miR-770 [241]
Pancreatic	miR-326 [242]
Prostate cancer (LNCaP)	miR-29a, miR-1915, miR-663, miR-134, miR-542 [243], miR-98 [244], miR-100, miR-125b
Lung cancer (A549)	miR-17 [229], miR-298 [245], miR-27b [245], let-7a [246]
Pulmonary fibroblast	miR-27b [245]
Ovarian cancer (OCa)	miR-498 [247, 248]
Breast cancer (MCF-7)	miR-125b [249, 250]
Cervical cancer (SiHa)	DICER, miR-22, miR-296-3p, miR-498 [251]
Kidney (NRK-52)	miR-125b [252]
Bladder cancer (253J)	94 miRNAs [253]
PBMC	miR-589, miR-601, miR-573, miR-138, miR-926, miR-423, mIR-484 [254], miR-181, miR-130, miR-135b, miR0146a [255]
HUVEC	miR-181c, miR-15a, miR-20b, miR-411, miR-659, miR-126, miR-510 [256]
Melanocytes (MeWo)	miR-302c, miR-520c [257], miR-125b, miR-27b [258]
Myeloid leukaemia (HL60, U937)	miR-181a [198]

Table 2.2. List of published vitamin D-regulated miRNAs in different cell types

MiRNAs play particularly important roles in vitamin D-mediated immune responses. Studies using immune-challenged monocytes have shown that miR-21 suppresses expression of *CYP27B1* which leads to inhibition of cathelicidin (*CAMP*), an anti-bacterial protective response normally induced by intracrine vitamin D signalling [235]. Validated gene targets of miR-21 include *BCL-2* and *PTEN*, which are involved in the vitamin D antimicrobial response [259]. Other vitamin D metabolism genes such as *CYP24A1* have also been reported to be targeted by miRNAs including miR-125b [250]. MiR-125b [258] as well as miR-27b and miR-298 have been suggested to target the VDR [260].

In the experimental *HeLa* cell models for cancer, calcitriol binding to the VDR has been shown to directly up-regulate *DICER* expression through targeting its *VDRE* region [251]. This stimulation, in turn, up-regulated the expression of a subset of miRNAs including miR-22, miR296-3p and miR-498 found to be involved in anti-tumorigenesis. The importance of vitamin D-mediated effects on gene expression beyond VDR-dependent mechanisms was further elucidated by a study using human osteoblasts, which found that differential regulation of multiple miRNAs following 10nM $1,25(OH)_2D3$ in culture coincided with gene regulation in a VDR-independent manner [63]. This study provides strong evidence that the effects of $1,25(OH)_2D3$ are not restricted to classical VDR-mediated transcriptional responses.

A recent study in human adipocytes found the expression of miR-146a and miR-155, two of the most well-profiled miRNAs in inflammation, to be up-regulated by TNF-α, with $1,25(OH)_2D3$ preventing this up-regulation [261]. NF-kB signalling was found to be the driving force behind the induction of these miRNAs, suggesting the mechanism of regulation by $1,25(OH)_2D3$ was due to the ability of $1,25(OH)_2D3$ to deactivate NF-kB signalling. MiR-146a and miR-155 up-regulation by TNF-α and down-regulation by $1,25(OH)_2D3$ was also observed in transgenic mouse models of inflammation, indicating a ubiquitous regulatory role of these miRNAs across species.

The key role that miR-155 plays in all aspects of immune cell function suggests exogenous targeting of this miRNA in particular could ameliorate disease, by preventing over-expression of the miRNA which in turn prevents aberrant silencing of its target genes. Thus $1,25(OH)_2D3$ could be a potential therapeutic treatment to modify expression of miR-155 in immune cells. There have already

been studies showing down-regulated miR-155 expression following 1,25$(OH)_2$D3 treatment in immune cells [262, 263].

Other miRNAs have also shown potential as immunomodulatory targets for vitamin D. MiR-22 has been reported to be induced by 1,25$(OH)_2$D3 in colon cancer cells, with its induction regulating gene expression in a VDR-dependent manner [238]. MiR-125b, which regulates VDR via a repression mechanism [197] has been reported to be one of many miRNAs down-regulated in cancer. Logically, the de-repression of VDR in cancer should augment the capacity for response to 1,25$(OH)_2$D3, and by extension, its immunomodulatory properties.

MiRNAs act within tissues but also circulate in serum. Circulating miRNA expression has been linked to the concentration of serum 25(OH)D in pregnancy, diabetes and pregnancy-related pre-eclampsia [99, 264], although studies on the link between serum 25(OH)D and miRNAs are limited. The link between 1,25$(OH)_2$D3 and miRNAs has been more widely studied, however results are still varied and controversial. A pilot study comparing miRNA expression in 1,25$(OH)_2$D3-supplemented groups versus non-supplemented groups found the expression of let-7a, a predicted target of VDR, to be significantly elevated in the 1,25$(OH)_2$D3 supplementation group. Others have found the entire let-7 miRNA family elevated in the presence of 1,25$(OH)_2$D3 [246], reaffirming the relationship between let-7, the VDR and vitamin D. It remains very likely that other miRNAs may be important targets for the immunomodulatory effects of 1,25$(OH)_2$D3, with implications in both health and autoimmune disease.

2.9 Project aims and objectives

The central hypothesis for this Ph.D. project is that microRNAs (miRNAs) play a pivotal role in mediating innate and adaptive immune responses to vitamin D, and this may be particularly important in the autoimmune disease rheumatoid arthritis (RA). To test this hypothesis the project involved three specific aims:

i) To use RA patient serum and synovial fluid to investigate the relationship between circulating vitamin D metabolites and miRNAs in RA disease activity (**Chapter 4**).

ii) To use primary cultures of human cells to investigate the role of miRNAs as mediators of the innate immune effects of $1,25(OH)_2D3$ on DC (**Chapter 5**).

iii) To use primary cultures of human cells to investigate the role of miRNAs as mediators of the adaptive immune effects of $1,25(OH)_2D3$ on T cells (**Chapter 6**).

CHAPTER 3.

MATERIALS AND METHODS

3. Introduction

The aim of the inflammatory arthritis (RA) patient section of this PhD project (**Chapter 4**) was to use serum and SF to quantify the concentration of 25(OH)D2, 25(OH)D3, 3-epi-25(OH)D3, 24,25$(OH)_2$D3 and 1,25$(OH)_2$D3 in order to correlate metabolite concentration with RA disease activity markers CRP (C-reactive protein), ESR (erythrocyte sedimentation rate), SJC (swollen joint count) and TJC (tender joint count). These vitamin D metabolites were selected for analysis as they are intermediate or catabolic products that arise from the vitamin D synthesis pathway. The biological significance of the metabolite 3-epi-25(OH)D3 has been debated in literature, however since it has the ability to bind VDR to potentially give rise to genetic and epigenetic modifications [265], we have focused on this metabolite along with the active form 1,25$(OH)_2$D3.

The rationale for including reactive arthritis (ReA) patient samples in **Chapter 4** was to use these as a control cohort, since ReA does not have the inflammatory progression associated with RA. The listed RA disease activity markers were measured either during structured clinical assessment, or by routine hospital assays service (Birmingham, UK). The expression of miR-146a and miR-155, two major inflammatory miRNAs, was quantified in the RA patient samples and correlated with vitamin D metabolites and patient disease activity markers. **Figure 3.1** shows a schematic representation of the human samples used in **Chapter 4** and the methodologies used to analyse these samples.

The aim of *in vitro* studies in **Chapter 5** and **Chapter 6** was to develop cell culture models to investigate: 1) the effects of 1,25$(OH)_2$D3 on DC differentiation and

miRNA expression; 2) the effects of 1,25$(OH)_2$D3 on T cell phenotype and miRNA expression. **Figure 3.2** shows a schematic representation of the DC and T cell models used in **Chapter 5** and **Chapter 6** respectively, and the methodology used to analyse these models.

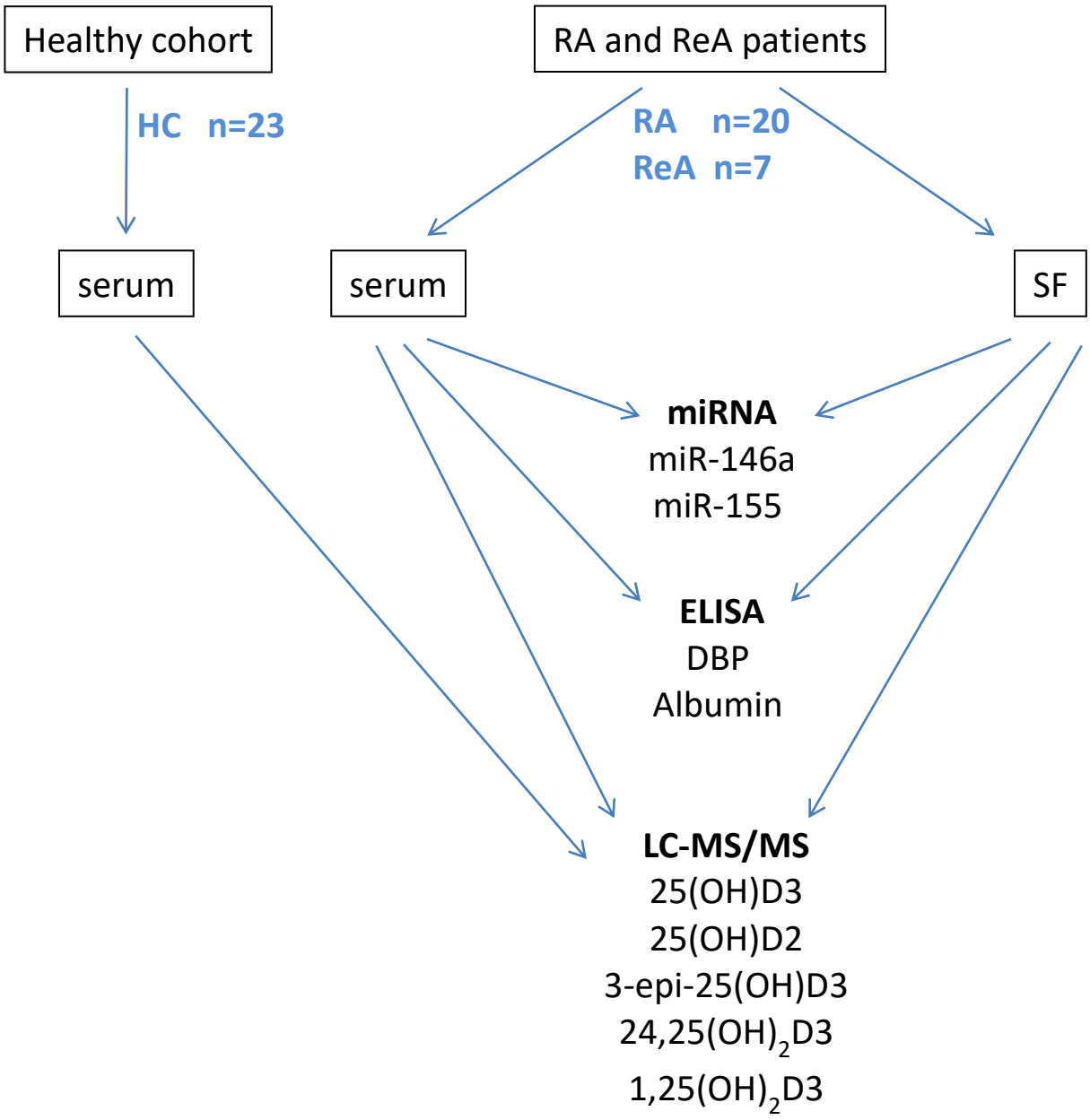


Figure 3.1. Workflow for patient studies
Paired RA (n=20) and ReA (n=7) serum and SF were obtained from patients according to existing ethical agreements. Serum from n=23 heathy cohort was used as disease-free controls. Methodologies used to analyse these samples were: miRNA isolation, LC-MS/MS and ELISA. RA=rheumatoid arthritis; ReA=reactive arthritis.

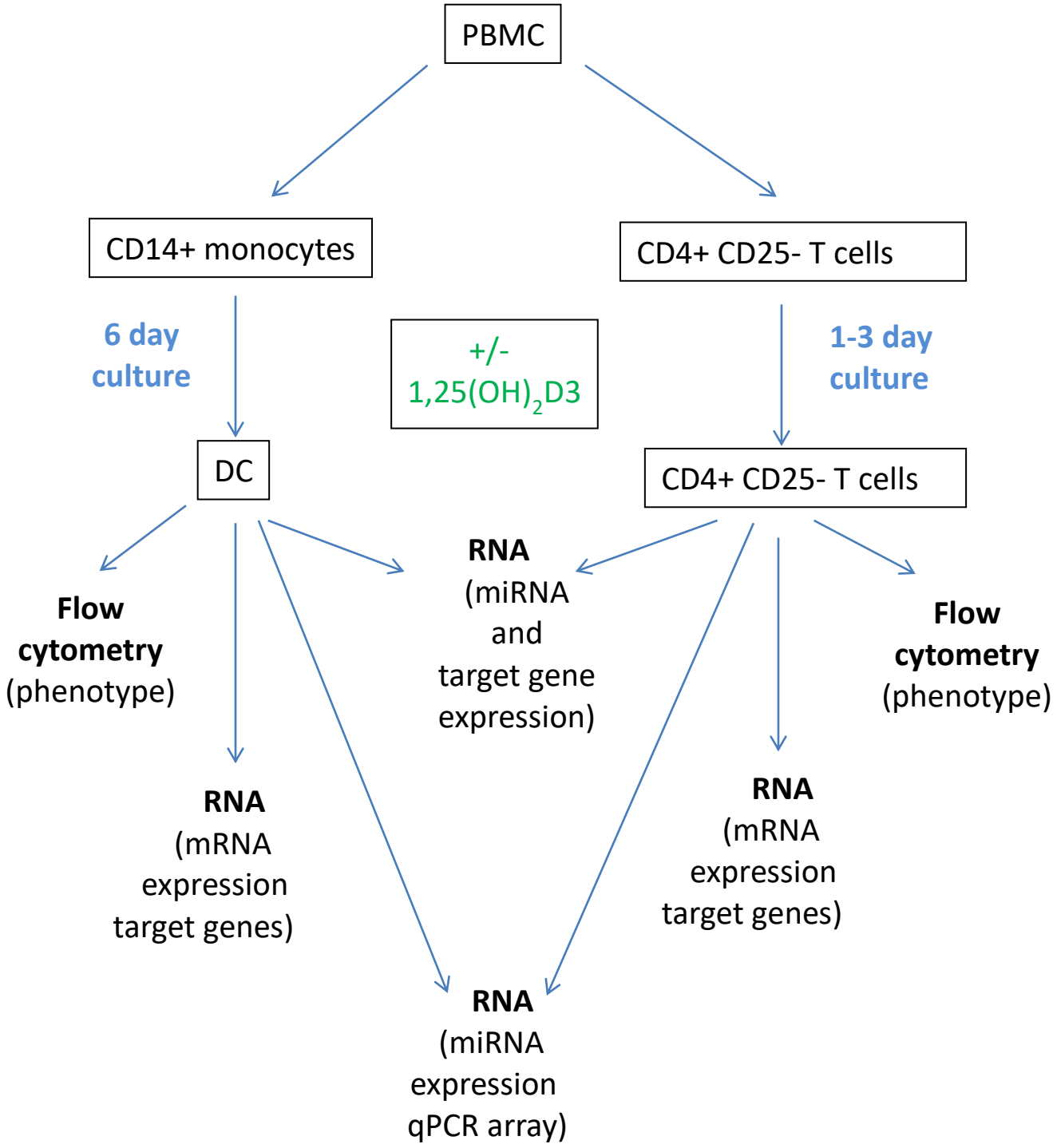


Figure 3.2. Workflow for *in vitro* studies
Both DC and T cell cultures were derived from healthy donor peripheral blood mononuclear cells (PBMC). CD14+ monocytes were cultured over 6 days into DC; CD4+ CD25- T cells were cultured over 3 days. Both cell models were left treated or untreated with $1,25(OH)_2D3$. Methodologies used to analyse these models were: flow cytometry, mRNA expression and miRNA expression (target and array profiling).

3.1 Primary cell culture

3.1.1 Ethics

Healthy human peripheral blood mononuclear cells (PBMC) were obtained from fully anonymised blood cones obtained from the National Blood Service, Birmingham, UK, in accordance with ethical agreement ERN_14-0446.

3.1.2 Isolation of PBMC from whole blood

PBMC were isolated from whole blood leukocyte cones from healthy donors. 10mL whole blood was diluted with 40mL phosphate buffered saline (PBS), and pipetted drop-wise into two 50mL falcon tubes containing 15mL LymphoPrep Separation Media (Stem Cell Technologies) in each tube. The preparation was separated by density gradient centrifugation at 2700rpm (revolutions per minute) for 20 minutes, with acceleration set at 9 and brake set at 0 so as not to disturb the pellet. The resulting preparation separated the blood into layers allowing the PBMC layer to be isolated using a Pasteur pipette. Following isolation of this layer, PBS was then added to a total of 50mL, and the preparation was centrifuged a further 3 times for 5 minutes each, re-suspending the resulting pellet in 50mL PBS following each centrifugation. Centrifuge settings were set at 2200rpm, 1100rpm and 1500rpm respectively for successive centrifugation runs to separate the pellet based on density. The final preparation was re-suspended in ice-cold MACS (magnetic-activated cell sorting) buffer following a cell count using a haemocytometer, to achieve around 50million PBMCs per mL. MACS buffer was made up of the

following: 1.25g bovine serum albumin (BSA; Thermo Fisher Scientific), 1mL Ethylenediaminetetraacetic acid (EDTA; Amresco) and PBS (Gibco, Life Technologies) to a total of 250mL, and stored on ice until required.

3.1.3 Isolation of CD14+ monocytes

CD14+ monocytes were isolated from PBMC using the EasySep Monocyte Isolation Kit (Stem Cell Technologies) as per manufacturer's instructions. The principle behind immunomagnetic negative selection involved labelling unwanted cells with antibody complexes and magnetic particles which were separated from the cells of interest using an EasySep magnet. Labelled unwanted cells were stuck to the side of the tube whilst cells of interest were collected by pouring into a fresh tube. 1mL of PBMC re-suspended at 50 million cells per mL was aliquoted into a fresh tube and 50µL Monocyte Isolation Cocktail was added and incubated for 5 minutes at room temperature. 50µL D-magnetic particles was then added and incubated for 5 minutes at room temperature. The preparation was made up to 10mL with MACS buffer and placed in the EasySep magnet for 5 minutes. The preparation was swiftly tipped into a fresh 15ml tube in one smooth motion with the old tube still in the magnet, leaving isolated CD14+ cells in the new tube. The yield of CD14+ monocytes achieved from around 500 million PBMC preparations was around 50 million. The resulting monocytes were transferred to a new tube, and PBS added up to 15mL. Following centrifugation at 1500rpm for 5 minutes, the pellet was re-suspended in Roswell Park Memorial Institute (RPMI) 1640 Medium (Gibco, Life Technologies) supplemented with 5% L-glutamine (Sigma Aldrich) and 10% foetal

bovine serum (FBS; Biosera) to achieve 2 million CD14+ monocytes per mL. These cells were processed immediately for purity analysis by flow cytometry, or seeded into cell culture plates and differentiated into dendritic cells (DC) for *in vitro* analyses.

3.1.4 Induction of monocyte-derived DC phenotypes

To study the effects of $1,25(OH)_2D3$ on DC phenotype and miRNA expression, a model system for DC differentiation was devised (**Figure 3.3**). CD14+ monocytes derived in **3.1.3** were differentiated for 5 days with granulocyte macrophage-stimulating colony factor (GM-CSF; 800U/mL, Berlex Laboratories) and Interleukin-4 (IL-4; 400U/mL), in the presence or absence of 10nM $1,25(OH)_2D3$ (Enzo Life Sciences; diluted in RPMI 1640 medium from 50µg/mL stock), at 37°C and 5% CO_2. Fresh medium supplemented with GM-CSF and IL-4 was added on day 2 and day 5 of culture. Immature DC (iDC) were matured on day 6 with 1µg/mL Lipopolysaccharide from *E. coli* (LPS; Sigma Aldrich) in the presence and absence of $1,25(OH)_2D3$ (10nM), and incubated for a further 24 hours. This gave rise to 6 distinct DC populations, which were harvested at day 7 of culture, for downstream studies in flow cytometry and gene quantification (see **Figure 3.2**).

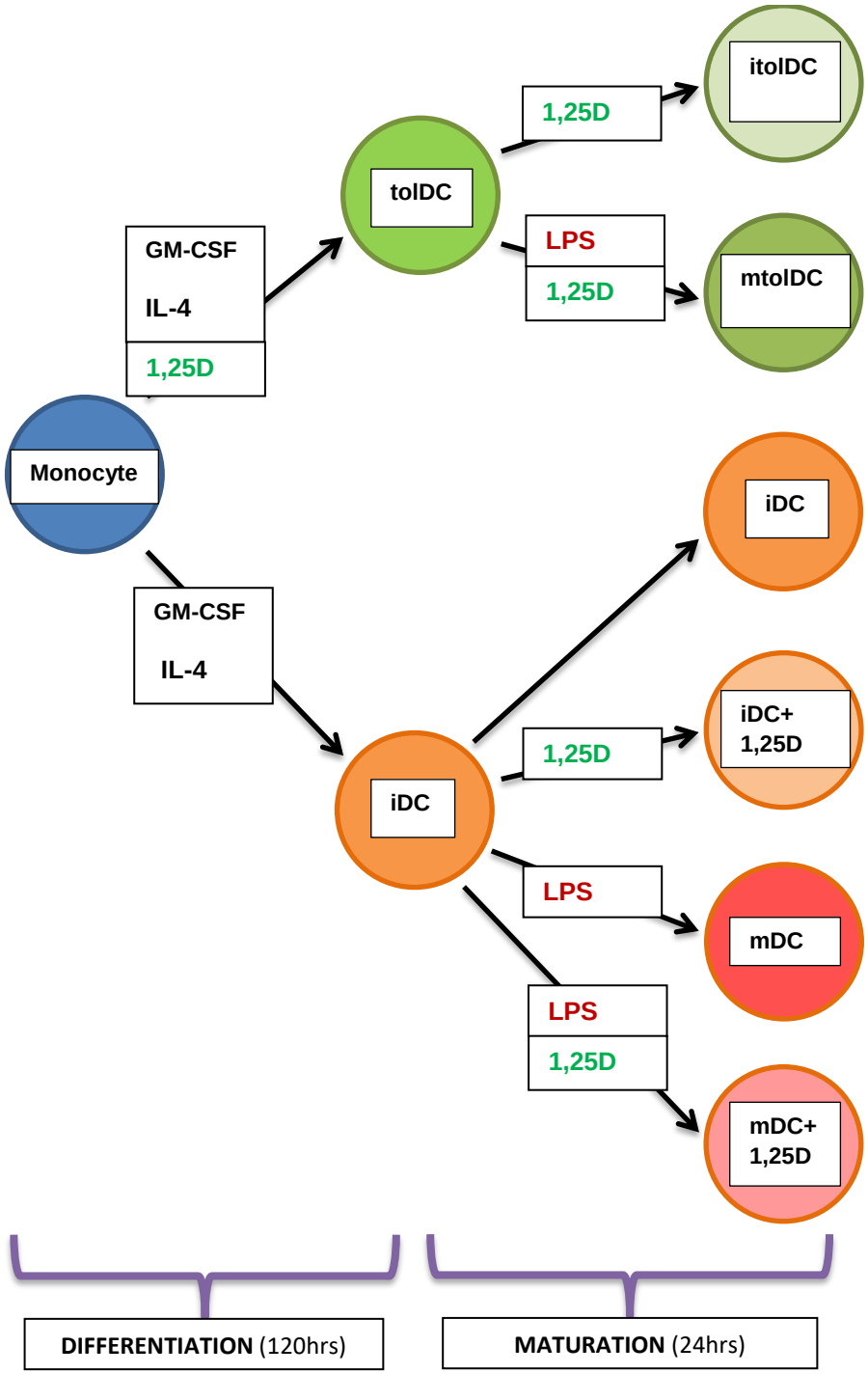


Figure 3.3. Induction of variable DC phenotypes with lipopolysaccharide (LPS) and 1,25(OH)$_2$D3 (1,25D)
itolDC – immature tolerogenic dendritic cell with 1,25D; mtolDC – mature tolerogenic dendritic cell with 1,25D; iDC – immature dendritic cell; mDC – mature dendritic cell; iDC+1,25D – immature dendritic cell with 1,25D; mDC+1,25D – mature dendritic cell with 1,25D. Monocytes were derived from whole blood leukocyte cones from healthy donors (n = 6).

3.1.5 Isolation of naïve CD4+ CD25- T cells

Naïve CD4+ CD25- T cells were also isolated from PBMC using the EasySep Human CD4+ T cell Enrichment Kit (Stem Cell Technologies) as per manufacturer's directions. The principle of the isolation process was based firstly on the addition of CD4+ enrichment cocktail to the PBMC to enrich the CD4+ population and maximise yield, followed by the addition of CD25 magnetic particles for the negative selection of CD25+ cells. The yield of T cells from 500 million PBMC was around 75 million. The resulting CD4+ CD25- T cells were centrifuged at 1500rpm for 5 minutes, and the pellet was re-suspended in 1mL CellGro GMP DC Medium (CellGenix), to achieve 1 million T cells per mL. T cells were either processed for purity analysis by flow cytometry or seeded in cell culture plates for *in vitro* analysis of cell phenotype and miRNA expression.

3.1.6 Naïve CD4+ T cell activation

18.75µL anti-CD3 (1:200 dilution from 1mg/mL stock) and 37.5µL anti-CD28 (1:100 dilution from 0.5mg/mL stock) plate-coating antibody was added to 3.75mL PBS. 250µL of this antibody cocktail was plated per well in a 24-well culture plate, and incubated at 37^{o}C for 2 hours to coat the wells. After 2 hours residual, non-coated antibodies were removed by two washes; first in 500µL PBS then in 300µL serum free media. To activate T cells with an inflammatory phenotype, a Th17 cytokine cocktail was also added to the wells pre-coated with anti-CD3 and anti-CD28. The Th17 cytokine cocktail was made up in the following concentrations and volumes: 0.65µL IL-2 (1:10000 dilution); 1.3µL transforming growth factor β (TGF-β)

(1:5000); 2.6µL IL-1β (1:2500); 13µL IL-6 (1:500) and 6.5µL IL-23 (1:1000), to make a total of 6.5mL in serum free media. 500µL of cocktail was added per well, followed by 500µL of CD4+ T cells obtained from **3.1.5**. The resulting CD4+ T cell culture was incubated at 37°C for 20 hours to allow for T cell activation.

3.1.7 T cell 1,25(OH)$_2$D3 time-course study

Following 20 hours incubation with the Th17 cytokine cocktail, the resulting activated CD4+ T cells were treated with either 1,25(OH)$_2$D3 (10nM) or vehicle (0.1% ethanol). The T cells were then harvested after 0hr, 2hrs, 10hrs, 24hrs, 48hrs or 72hrs of treatment with 1,25(OH)$_2$D3 or vehicle. Unstimulated (no anti-CD3/CD28 or Th17 cocktail) naïve CD4+ T cells were also harvested at the 0 hour time point as an additional control cell population. All harvested cells were transferred to 1.5mL Eppendorf tubes (Axygen), centrifuged at 1500rpm for 5 minutes, and the supernatant discarded. Cell pellets were re-suspended in 100µL RNALater (Invitrogen) and transferred to a -80°C freezer for RNA isolation at a later date. A schematic summarising the culture and treatment of CD4+ CD25- T cells is shown in **Figure 3.4.**

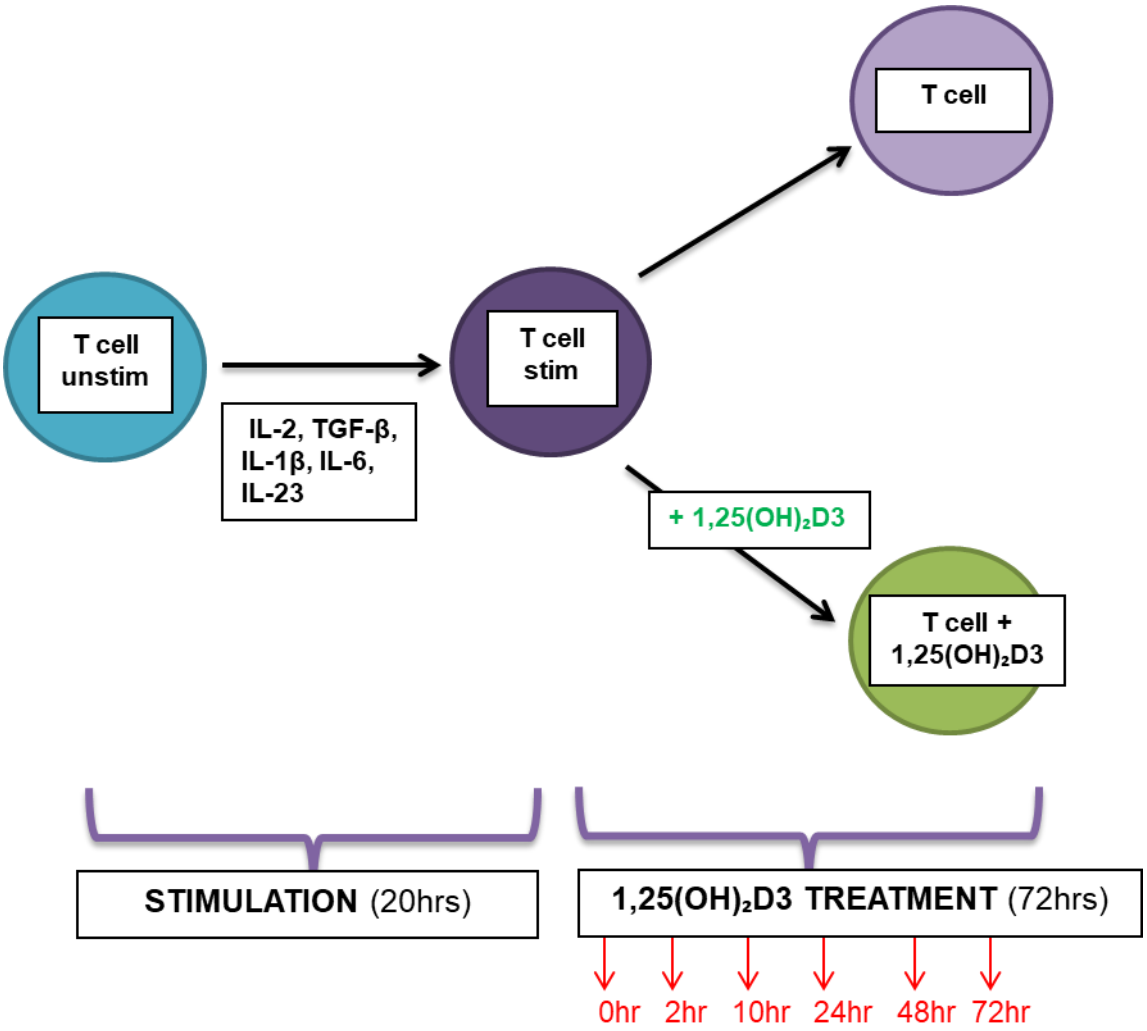


Figure 3.4. Induction of CD4+ CD25- T cells with cytokine cocktail and $1,25(OH)_2D3$ (1,25D)
T cell unstim – unstimulated naïve CD4+ CD25- T cell; T cell stim – CD4+ T cell activated with Th17 cytokine cocktail; T cell UT – untreated activated CD4+ T cell (vehicle); T cell +1,25D – activated CD4+ T cell+1,25D (10nM). CD4+ CD25- T cells were isolated from PBMC derived from whole blood leukocyte cones of healthy donors (n = 4). Anti-CD3 and anti-CD28 antibodies were coated onto an empty culture plate for 2 hours at 37^{O}C, prior to addition of T cells and cytokines for stimulation. Th17 cytokine cocktail was added to promote CD4+ T cell differentiation into a pro-inflammatory Th17 phenotype. 1,25D was added at a final concentration of 10nM per well. T cells were harvested at the following time points after 1,25D treatment: 0hr (control), 2hrs, 10hrs, 24hrs, 48hrs, and 72hrs.

3.2 Flow cytometry

Flow cytometry to detect surface and intracellular antigens was performed on all DC and T cell populations. Approximately 50,000 cells were used from each sample for flow cytometry processing. Cells in culture were transferred directly to 5mL flow cytometry tubes (Axygen, UK) and washed once with 2mL PBS. Culture plates were wrapped in foil and incubated on ice shortly prior to transferring to flow cytometry tubes, to allow the cells to detach from the plate. Unless otherwise stated, all tubes were washed following staining, in 2mL PBS and centrifuged at 1500rpm for 5 minutes. Following each centrifugation, the wash solution was discarded by carefully tipping the tubes in one rapid motion, and any remaining liquid was dabbed onto paper towels. All washing and staining stages were carried out in the absence of light. Stained cells were stored at 4°C wrapped in foil before running on a DakoCyAn FACS Machine (Beckman Coulter, High Wycombe, UK).

A full list of antibodies, cytokines, volumes used and product numbers is shown in **Table 3.1**. Rat IgG antibodies conjugated with matching fluorophores were used for the isotype control stain, and compensation colours were also added.

Antibody	Conjugated fluorophore	Volume used (µL)	Product number
CD83	PE	3	556855
CD80	PE	3	557227
CD14	PerCP	3	345786
HLA-DR	FITC	2	555811
CD209	APC	3	Miltenyi Biotech 130-092-871
CD11c	APC	3	559877
CD68	PE	3	564944
CD3	FITC	2	345763
CD3	PeCy-7	3	563423
CD3	PE	3	555340
CD3	PerCP	3	345766
CD4	FITC	2	555346
CD4	PE	3	555347
CD4	PerCP	3	345770
CD4	APC	3	555349
CD45-RO	FITC	2	555492
CD86	FITC	2	555657
CD127	PeCy7	3	25-1278-42
IL-17	PE	2	12-7178-42
IL-10	PE	2	559337
CD4	e450	2	48-0048-42
FoxP3	APC	3	17-4776-42

Table 3.1. List of antibodies for flow cytometry

Flow cytometry antibodies, volumes used and product codes. All antibodies were purchased from BD Pharmingen unless otherwise stated. Fluorophores used were FITC, Fluorescein isothiocyanate conjugate; PE, Phycoerythrin; PerCP, Peridinin-chlorophyll-protein Complex Conjugate; APC, Allophycocyanin conjugate; Pe Cy7, Phycoerythrin Cy-7 tandem conjugate.

3.2.1 DC and T cell surface antigen staining

Approximately 50,000 cells were transferred directly from culture plates into flow cytometry tubes, washed once with PBS and centrifuged at 1500rpm for 5 minutes. After removing the supernatant, each sample was re-suspended by gentle vortex in 100µL LIVE/DEAD Fixable Near-IR Dead Cell Stain (1µL stock in 1mL PBS; Life Technologies) to stain for dead cells. Samples were incubated in the dark on ice for 20 minutes, and then washed with PBS and the supernatant discarded. Fluorescent cell surface staining antibodies were added to the sample, to a total of 100µL per sample made up with PBS (for antibody volumes used see **Table 3.1**). Following 30 minutes incubation on ice, samples were washed again and the resulting pellet was re-suspended in 200µL PBS for immediate flow cytometry analysis, or fixed as below for subsequent analysis at a later date.

3.2.2 Cell fixation with Paraformaldehyde (PFA)

Cells were re-suspended in 400µL 4% paraformaldehyde (PFA; diluted in PBS) for analysis at a later date, and incubated in the dark for 10 minutes at room temperature. Following a PBS wash, the cell pellet was re-suspended in 200µL PBS and kept at 4°C until analysis. Samples were kept for no more than 24 hours to minimise any decrease in antibody fluorescence quality due to ambient light exposure.

3.2.3 CD4+ T cell intracellular staining of CTLA-4 expression (using the Saponin method)

CD4+ CD25- T cells were stimulated for 5 hours with phorbol myristate acetate (PMA) (50ng/mL, Sigma Aldrich) and ionomycin (1µM, Sigma Aldrich) to mimic T cell activation by CD3 and CD28 and induce intracellular CTLA-4 expression. After 1 hour, Brefeldin A (5µg/mL, Sigma Aldrich) was added to block protein secretion. Cells were incubated at 37°C for a further 4 hours. Cells were transferred to 5mL tubes, washed in PBS, and dead cells stained with 100µL LIVE/DEAD Fixable Near-IR Dead Cell Stain. Following 20 minutes incubation on ice, samples were washed once in PBS, then fixed in 400µL 3% paraformaldehyde (PFA) and incubated at room temperature for 10 minutes. Samples were then washed with PBS and the resulting pellet was processed for permeabilisation. To permeabilise cells, samples were washed in 1mL 0.1% Saponin (diluted from stock concentration of 5%) to permeabilise the cells. Supernatant was discarded and cells were re-suspended in 50µL normal goat serum prepared in 0.1% Saponin for 30 minutes to block background signals. Staining antibodies were then added to samples for 30 minutes, followed by two washes with PBS. The samples were re-suspended in 200µL PBS for FACS analysis of intracellular CTLA-4 expression.

3.2.4 CD4+ T cell intracellular staining of FoxP3 expression (using the eBioscience FoxP3 method)

CD4+ CD25- T cells were washed once in PBS and the supernatant was discarded. The pellet was re-suspended in 500µL eBioscience fix buffer (prepared as 1 part fixation/permeabilisation concentrate: 3 parts fixation/permeabilisation

diluent) to fix the cells. Cells were incubated at 4°C for 45 minutes then washed with PBS. Following the PBS wash, cells were washed with 1mL 1Xpermeabilisation buffer (prepared as 1 part 10X permeabilisation buffer stock: 9 parts PBS). All buffers were made up from fresh stock and used immediately. Staining antibodies were added and samples were incubated at 4°C for 45 minutes. A wash in diluted permeabilisation buffer followed by a wash in PBS was then performed, and the final pellet was re-suspended in 200µL PBS for flow cytometry.

3.3 DC and T cell gene expression analysis

3.3.1 RNA isolation

Each stage of RNA isolation and centrifugation was carried out at room temperate (18°C) unless otherwise stated. All surfaces and equipment were wiped down with RNAse Zap (Invitrogen) and 100% ethanol prior to RNA isolation to minimise degradation of sample material by RNAses.

To generate RNA for downstream mRNA analysis, RNA isolation was carried out using TRIzol Reagent (Thermo Fisher) following manufacturer's protocol for human samples, consisting of cell lysis stage, RNA precipitation stage, RNA wash stage and solubilisation stage. Cell lysis stage: 1 million cells per culture well were transferred into 1.5mL Eppendorf tubes and centrifuged for 5 minutes at 1500rpm to pellet the cells. The media was discarded and 750µL TRIzol was added to lyse the cells, pipetting up and down to homogenise the cells. After 5 minutes incubation, 100µL chloroform was added per sample and incubated for a further 5 minutes.

Samples were centrifuged at 10,000rpm for 15 minutes at 4°C. The upper aqueous phase containing RNA was transferred to fresh tubes, being careful not to disturb the lower phase. RNA precipitation stage: 300µL isopropanol was added per sample and incubated for 10 minutes. RNA was precipitated by centrifugation at 10,000rpm for 10 minutes at 4°C. RNA wash stage: supernatant was discarded and the pellet containing total RNA was re-suspended in 750µL 75% ethanol. Samples were centrifuged at 7500rpm for 5 minutes at 4°C. Supernatant was discarded and the pellet was left to air-dry for 10 minutes. Solubilisation stage: the RNA pellet was re-suspended in 50µL RNAse-free water, incubated at 60°C for 10 minutes, and stored at -80°C for future use.

To generate RNA for downstream miRNA analysis, RNA was isolated from cultured cells using the mirVana miRNA Isolation Kit (Invitrogen), as per manufacturer's instructions to preserve miRNA recovery (following the Organic Extraction protocol). Cell culture plates were incubated briefly on ice to allow cells to detach from the plate. Cells were harvested directly from the plate by repeated pipetting and transferred to 1.5mL Eppendorf tubes (Axygen). To maximise recovery of material, the wells were also washed with ice-cold PBS and contents were transferred into the same tubes, which were then centrifuged at 2000rpm for 5 minutes. The resulting cell pellets were either re-suspended in 100µL RNALater and stored at -20°C, or underwent immediate processing for RNA isolation. Around 500,000 cells were harvested from each well for RNA isolation. The volume of Lysis Buffer was adjusted depending on cell number, with the lower recommended volume used for fewer cell numbers. For 500,000 cells, 400µL of Lysis Buffer was used to lyse the cells by vigorous pipetting. After adding Acid-Phenol:chloroform to aid the

removal of DNA and vortexing for 60 seconds, cells were centrifuged at 10,000rpm for 10 minutes instead of 5 minutes as stated in the protocol, as it was found 5 minutes was insufficient for a defined aqueous layer to form. Following centrifugation, 360µL of the aqueous layer was aspirated and transferred to a new collection tube. Following the wash stages, filter cartridges were transferred into new collection Eppendorf tubes and 50µL pre-heated elution solution was pipetted onto the filter. Eluate was collected from the cartridges and 1µL of RNAse inhibitor (Invitrogen) added to each sample to minimise RNA degradation over time. All RNA concentrations (ng/µL) and purities were measured on a NanoDrop 2000 Spectrophotometer (Thermo Fisher) and their values noted for later calculations. The ratio of absorbance at 260nm and 280nm was used to assess the purity of RNA, with values of ~2.0 considered pure for RNA. If the 260/280 ratio was below 1.8, this indicated the presence of proteins that absorb strongly at 280nm. The process of RNA extraction was repeated for these samples, and all RNA was stored at -80°C.

3.3.2 Reverse transcription

Reverse transcription of isolated RNA to synthesise cDNA was performed using the miScript II RT Kit (Qiagen) as per manufacturer's instructions. Reverse transcription was performed in 8-strip capped PCR tubes (Life Technologies), and prepared on ice to ensure enzymes did not denature. The concentration of template RNA was adjusted to 400ng/µL for each sample by calculation, and varying volumes of RNAse free water was added to make a total volume of 12µL per reaction tube. A

master mix was then prepared, ensuring 10% excess was made up. Each reaction contained the following master mix reagents:

4µL 5x miScript HiFlex Buffer

2µL 10x miScript Nucleics mix

2µL miScript Reverse Transcriptase mix

8µL of master mix was pipetted into each reaction already containing 400ng/µL RNA, to make a total of 20µL per reaction. The samples were spun briefly in a mini centrifuge to evenly mix all the reagents. cDNA was synthesised using a ThermoCycler on the following settings: 37°C for 60 minutes, 95°C for 5 minutes to deactivate the transcriptase, followed by a 4°C hold. Stock cDNA was diluted 1:3 with RNAse free water (Applied Biosystems) prior to qRT-PCR, and stored at -20°C. 40µL RNAse free water was added to each 20µL cDNA reaction.

3.3.3 qRT-PCR analysis of mRNA expression

All qRT-PCR reactions were performed with 10µL reaction volumes on 96-well semi-skirted PCR plates (Applied Biosystems) unless otherwise stated. All reagents were thawed on ice in the dark due to the light sensitivity of SYBR Green in the master mix, and plates were prepared on ice. A clear plastic cover plate (Applied Biosystems) was placed over the prepared plate and edges firmly sealed. A master mix was prepared for each target gene, ensuring there was 10% excess made up. Each 10µL qPCR reaction was made up of the following components:

5µL 2x TaqMan Universal PCR Master Mix (Applied Biosystems)

1µL RNAse free water (Applied Biosystems)

2µL primer assay (Applied Biosystems)

2µL diluted cDNA

Prior to running the qPCR, the plate was centrifuged at 1200rpm for 2 minutes to ensure all reagents were evenly distributed in each well. Plates were run on an ABI 7500 qPCR machine (Applied Biosystems) using the Standard TaqMan Protocol for Quantitative Ct, defined as the cycle number required for the fluorescent signal to cross the threshold and exceed background level. Each run took approximately 90 minutes. 18S rRNA (Applied Biosystems) was used as the endogenous housekeeping gene. Delta Ct (ΔCt) values were obtained and analysed. Initial Ct values for each mRNA transcript were converted to ΔCt using 18S rRNA ΔCt values. Data in results are shown as raw ΔCt values rather than fold-change in gene expression.

For candidate qPCR data, target Ct (threshold cycle) values were obtained as the cycle number at which the amplification curve crossed a threshold line (automatically calculated). This value is the relative abundance of the target being measured in the sample. Samples were run in duplicate, and the mean Ct value used for subsequent calculations. Target Ct was normalised against the housekeeping Ct to give ΔCt; the standard value used to show relative gene expression.

3.4 DC and T cell candidate miRNA expression analysis

Preliminary studies on miRNA expression were performed following analysis of mRNA expression in DC and T cell populations. RNA isolation and reverse transcription was performed as described in **3.3.1** and **3.3.2**. 7 candidate miRNAs were chosen due to reported associations with inflammation. qPCR was performed using the miScript SYBR Green PCR Kit (Qiagen). A master mix was prepared for each target, ensuring there was 10% excess made up. Each 10µL qPCR reaction was made up of the following components:

1µL 10x miScript Universal Primer

1µL miScript primer assay (Qiagen)

5µL 2x QuantiTect SYBR Green PCR Master Mix

1µL RNAse free water (Applied Biosystems)

2µL diluted cDNA

qPCR plates were run on an ABI 7500 qPCR machine (Applied Biosystems) using the SYBR Green Protocol for Quantitative Ct. Cycling stage conditions were set as per the Qiagen miScript Kit Handbook recommendations: 95°C for 15 minutes initially to activate HotStarTaq DNA Polymerase, then 40 cycles of 94°C for 15 seconds, 55°C for 30 seconds, 70°C for 34 seconds. The run ended with a melt curve stage pre-determined by the machine protocol. Each run took approximately 140 minutes. RNU-6B was used as the endogenous housekeeping gene. A list of probes and primers used for mRNA and miRNA targets is shown in **Table 3.2**.

Gene	Reporter	Gene expression assay
18S	VIC	4319413E
CTLA-4	FAM	Hs00175480_m1
IFN-y	FAM	Hs00989291_m1
CYP24A1	FAM	Hs00167999_m1
CYP27B1	FAM	Hs01096154_m1
VDR	FAM	Hs00118624_CE
IL-10	FAM	Hs00961622_m1
DROSHA	FAM	Hs00203008_m1
DICER1	FAM	Hs00229023_m1
DGCR8	FAM	Hs00987082_m1
RAN	FAM	Hs01044225_g1
TARBP2	FAM	Hs00998379_m1
XPO5	FAM	Hs00382453_m1
AGO2	FAM	Hs01085579_m1

miRNA	Reporter	Assay ID
RNU-6B	SYBR	Hs_RBU6-2_1
miR-21	SYBR	hsa-miR-21-5p
miR-29a	SYBR	hsa-miR-29a-3p
miR-145	SYBR	hsa-miR-145-5p
miR-146a	SYBR	hsa-miR-146a-5p
miR-155	SYBR	hsa-miR-155-5p
miR-627	SYBR	hsa-miR-627-5p
let-7i	SYBR	hsa-let-7i-5p

Table 3.2. List of mRNA and miRNA probes and primers

List of gene and candidate miRNA primers for qPCR, reporter assay and assay ID.

3.5 MiScript HC qPCR array analysis of miRNA expression

To provide a comprehensive unbiased analysis of miRNA expression in DC and T cells, PCR array analyses were carried out. MiScript HC miRNA PCR Array plates (Qiagen, Manchester, UK) were used, with each plate containing 372 primers and probes for human miRNAs most closely linked to inflammation, as well as 12 endogenous control primers. One PCR array plate was used to profile miRNA expression for each cell treatment. For studies of DC miRNAs, n=6 separate donor replicates were used, each incorporating 4 cell treatments. For T cell studies, n=4

separate donor replicates were used for 1,25$(OH)_2$D3 and vehicle treated cells for 24 hour and 72 hour treatment periods.

3.5.1 Reverse transcription for miRNA PCR array

Reverse transcription to synthesise cDNA for the PCR array analyses was performed using RNA isolated in **3.3.1**, in conjunction with the miScript RT II Kit (Qiagen) as described below. Reverse transcription was performed in 8-strip capped PCR tubes, and prepared on ice to ensure enzymes did not denature. The concentration of template RNA was adjusted to 100ng/µL for each sample by calculation from values obtained with the NanoDrop 2000 Spectrophotometer (Thermo Fisher), and varying volumes of RNAse free water was added to make a total volume of 6µL per reaction tube. The master mix was then prepared, ensuring 10% excess. Each reaction contained:

2µL 5x miScript HiSpec Buffer

1µL 10x miScript Nucleics mix

1µL miScript Reverse Transcriptase mix

4µL of master mix was pipetted into each reaction already containing 100ng/µL RNA, to make a total of 10µL per reaction. The samples were spun briefly in a mini centrifuge to mix all the reagents. cDNA was synthesised using a Thermo Cycler on the following settings: 37°C for 60 minutes, 95°C for 5 minutes to deactivate the transcriptase enzyme, followed by a 4°C hold.

Prior to pre-amplification of the cDNA to increase the quantity of cDNA, 10µL stock cDNA was diluted with 40µL RNAse free water. Pre-amplification was performed immediately afterwards.

3.5.2 Pre-amplification of cDNA for miRNA array

cDNA generated in **3.5.1** was processed for pre-amplification to increase the quantity of specific cDNA targets for downstream gene expression analysis, using the miScript PreAMP PCR Kit (Qiagen, UK). 5µL of 20-fold diluted cDNA was used per reaction as suggested by the manufacturers' protocol. The master mix was prepared at room temperature, mixed gently then stored on ice until use. Each reaction contained:

5µL 5x miScript PreAMP Buffer

2µL HotStarTaq DNA Polymerase

5µL miScript PreAMP Primer Mix

7µL RNAse free water

1µL miScript PreAMP Universal Primer

20µL of master mix was pipetted into each reaction tube, and 5µL diluted template cDNA added to make a total of 25µL. The samples were spun briefly in a mini centrifuge to mix all the reagents. cDNA was pre-amplified using a Thermo Cycler programmed according to the 384-plex pre-amplification reaction setting:

1) Initial activation step (to activate HotStarTaq Polymerase): 95°C for 15 minutes

2) 3-step cycling (denaturation, annealing, extension): 94°C for 30 seconds, 55°C for 60 seconds, and 70°C for 60 seconds (repeated for 2 cycles).

3) 2-step cycling (denaturation, annealing/extension): 94°C for 30 seconds, 60°C for 3 minutes (repeated for 10 cycles).

After the run ended, pre-amplified cDNA was diluted appropriately for use in downstream miScript HC arrays. A 20-fold dilution of 25µL pre-amplified cDNA in 475µL RNAse free water was performed as suggested in the protocol.

3.5.3 qPCR of miScript HC miRNA qPCR arrays

All miScript HC qPCR miRNA array plates were run on a 7900HT Fast Real-Time PCR machine (Applied Biosystems) using the SYBR Green Protocol for Quantitative Ct. Cycling stage conditions were set as per the miScript HC PCR Array Handbook recommendations: 95°C for 15 minutes initially to activate HotStarTaq, then 40 cycles of 94°C for 15 seconds, 55°C for 30 seconds, 70°C for 30 seconds. The run ended with a 4°C holding stage.

Pre-amplified and diluted cDNA generated in **3.5.2** was used for mature miRNA expression profiling. All individual components of the reaction were thawed at room temperature and mixed gently. A reaction mix was prepared as below, making around 15% excess:

2255µL 2x QuantiTect SYBR Green PCR Master Mix

451µL 10x miScript Universal Primer

1694µL RNAse free water

110µL diluted template cDNA

Reagents were mixed gently but thoroughly, and transferred into an RT_2 PCR Array Loading Reservoir (Qiagen). 10µL reaction mix was added into each well of the miScript PCR Array plate using a multichannel pipette. The plate was sealed and centrifuged at 1000rpm for 2 minutes at room temperature. The 7900HT Fast Real-Time PCR (Applied Biosystems) was programmed as follows, as suggested by the miScript HC PCR Array Handbook (Qiagen):

1) PCR initial activation step: 95°C for 15 minutes

2) 3-step cycling (Denaturation, Annealing, Extension): 94°C for 15 seconds, 55°C for 30 seconds, and 70°C for 30 seconds (repeated for 40 cycles). The full list of 372 miRNAs profiled in the arrays, and the array plate layout, is shown in **Table 3.3**.

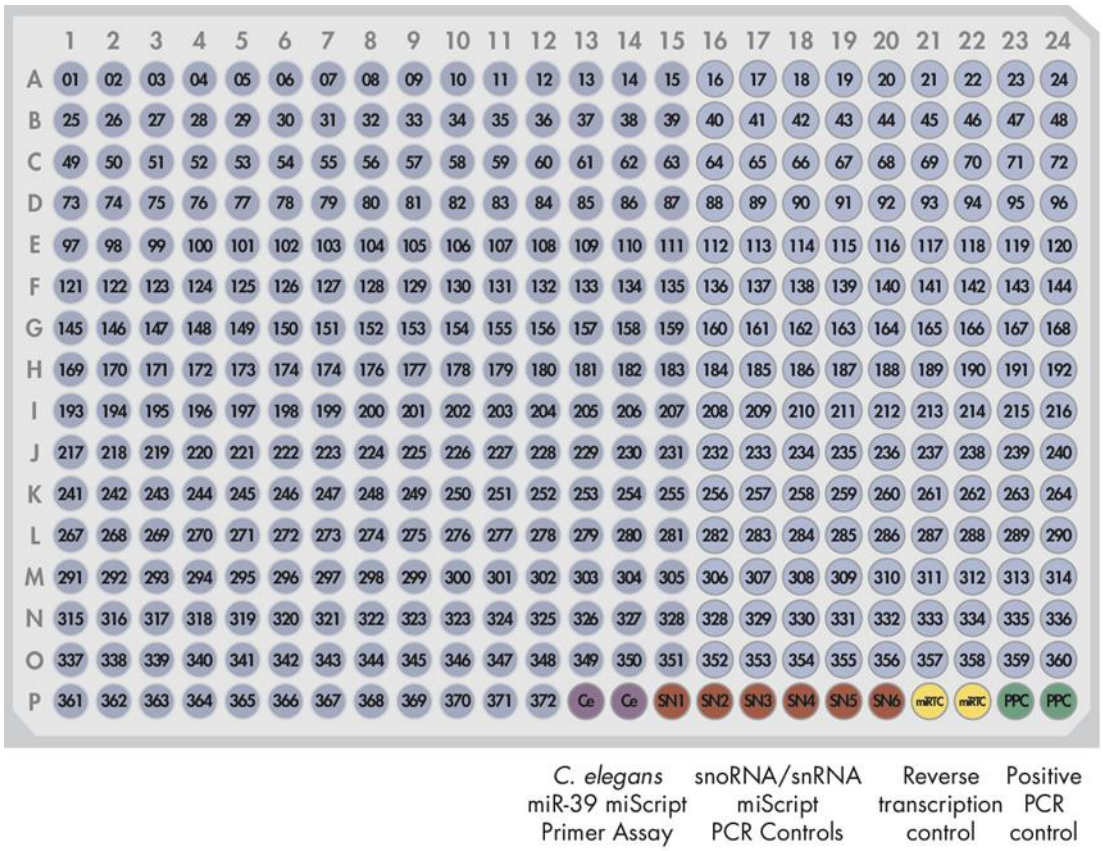


Table 3.3.1. miScript miRNA PCR Array Human miRBase Profiler HC plate layout

Diagram shows array plate layout of 372 miRNAs closely related to inflammation, and control wells consisting of *C. elegans* miR-39 endogenous normalisation control, snRNA PCR normalisation controls, reverse transcription performance control and PCR performance positive controls. Image sourced from the miScript miRNA PCR Array Handbook (Qiagen, Manchester, UK).

Well	miRNA	Well	miRNA	Well	miRNA	Well	miRNA
A01	hsa-let-7a-5p	C01	hsa-miR-132-3p	E01	hsa-miR-181d-5p	G01	hsa-miR-205-3p
A02	hsa-let-7b-5p	C02	hsa-miR-132-5p	E02	hsa-miR-182-5p	G02	hsa-miR-206
A03	hsa-let-7c-5p	C03	hsa-miR-1324	E03	hsa-miR-182-3p	G03	hsa-miR-208a-3p
A04	hsa-let-7d-5p	C04	hsa-miR-133a-3p	E04	hsa-miR-183-5p	G04	hsa-miR-208b-3p
A05	hsa-let-7d-3p	C05	hsa-miR-133b	E05	hsa-miR-183-3p	G05	hsa-miR-20a-5p
A06	hsa-let-7e-5p	C06	hsa-miR-134-5p	E06	hsa-miR-184	G06	hsa-miR-20a-3p
A07	hsa-let-7f-5p	C07	hsa-miR-135a-5p	E07	hsa-miR-185-5p	G07	hsa-miR-20b-5p
A08	hsa-let-7g-5p	C08	hsa-miR-135b-5p	E08	hsa-miR-186-5p	G08	hsa-miR-20b-3p
A09	hsa-let-7g-3p	C09	hsa-miR-136-5p	E09	hsa-miR-186-3p	G09	hsa-miR-21-5p
A10	hsa-let-7i-5p	C10	hsa-miR-137	E10	hsa-miR-187-3p	G10	hsa-miR-21-3p
A11	hsa-miR-1-3p	C11	hsa-miR-138-5p	E11	hsa-miR-187-5p	G11	hsa-miR-210-3p
A12	hsa-miR-100-5p	C12	hsa-miR-139-3p	E12	hsa-miR-18a-5p	G12	hsa-miR-211-5p
A13	hsa-miR-101-3p	C13	hsa-miR-139-5p	E13	hsa-miR-18a-3p	G13	hsa-miR-212-3p
A14	hsa-miR-101-5p	C14	hsa-miR-140-3p	E14	hsa-miR-18b-5p	G14	hsa-miR-214-3p
A15	hsa-miR-103a-3p	C15	hsa-miR-140-5p	E15	hsa-miR-18b-3p	G15	hsa-miR-215-5p
A16	hsa-miR-105-5p	C16	hsa-miR-141-3p	E16	hsa-miR-190a-5p	G16	hsa-miR-216a-5p
A17	hsa-miR-106a-3p	C17	hsa-miR-142-3p	E17	hsa-miR-1908-5p	G17	hsa-miR-217
A18	hsa-miR-106b-5p	C18	hsa-miR-142-5p	E18	hsa-miR-191-5p	G18	hsa-miR-218-5p
A19	hsa-miR-106b-3p	C19	hsa-miR-143-3p	E19	hsa-miR-191-3p	G19	hsa-miR-218-1-3p
A20	hsa-miR-107	C20	hsa-miR-143-5p	E20	hsa-miR-1914-3p	G20	hsa-miR-219a-5p
A21	hsa-miR-10a-5p	C21	hsa-miR-144-3p	E21	hsa-miR-192-5p	G21	hsa-miR-22-3p
A22	hsa-miR-10a-3p	C22	hsa-miR-144-5p	E22	hsa-miR-192-3p	G22	hsa-miR-22-5p
A23	hsa-miR-10b-5p	C23	hsa-miR-145-5p	E23	hsa-miR-193a-3p	G23	hsa-miR-221-3p
A24	hsa-miR-10b-3p	C24	hsa-miR-145-3p	E24	hsa-miR-193a-5p	G24	hsa-miR-221-5p
B01	hsa-miR-1180-3p	D01	hsa-miR-146a-5p	F01	hsa-miR-193b-3p	H01	hsa-miR-222-3p
B02	hsa-miR-1207-5p	D02	hsa-miR-146b-5p	F02	hsa-miR-193b-5p	H02	hsa-miR-222-5p
B03	hsa-miR-122-5p	D03	hsa-miR-147a	F03	hsa-miR-194-5p	H03	hsa-miR-223-3p
B04	hsa-miR-122-3p	D04	hsa-miR-148a-3p	F04	hsa-miR-195-5p	H04	hsa-miR-223-5p
B05	hsa-miR-1224-3p	D05	hsa-miR-148b-3p	F05	hsa-miR-195-3p	H05	hsa-miR-224-5p
B06	hsa-miR-1224-5p	D06	hsa-miR-149-5p	F06	hsa-miR-196a-5p	H06	hsa-miR-224-3p
B07	hsa-miR-124-3p	D07	hsa-miR-150-5p	F07	hsa-miR-196a-3p	H07	hsa-miR-23a-3p
B08	hsa-miR-124-5p	D08	hsa-miR-151a-3p	F08	hsa-miR-196b-5p	H08	hsa-miR-23a-5p
B09	hsa-miR-125a-3p	D09	hsa-miR-151a-5p	F09	hsa-miR-197-3p	H09	hsa-miR-23b-3p
B10	hsa-miR-125a-5p	D10	hsa-miR-152-3p	F10	hsa-miR-199b-3p	H10	hsa-miR-23b-5p
B11	hsa-miR-125b-5p	D11	hsa-miR-153-3p	F11	hsa-miR-199a-5p	H11	hsa-miR-24-3p
B12	hsa-miR-126-3p	D12	hsa-miR-154-5p	F12	hsa-miR-199b-5p	H12	hsa-miR-25-3p
B13	hsa-miR-126-5p	D13	hsa-miR-155-5p	F13	hsa-miR-19a-3p	H13	hsa-miR-25-5p
B14	hsa-miR-1265	D14	hsa-miR-155-3p	F14	hsa-miR-19a-5p	H14	hsa-miR-26a-5p
B15	hsa-miR-127-3p	D15	hsa-miR-15a-5p	F15	hsa-miR-19b-3p	H15	hsa-miR-26b-5p
B16	hsa-miR-127-5p	D16	hsa-miR-15b-5p	F16	hsa-miR-200a-3p	H16	hsa-miR-27a-3p
B17	hsa-miR-128-3p	D17	hsa-miR-15b-3p	F17	hsa-miR-200a-5p	H17	hsa-miR-27a-5p
B18	hsa-miR-1284	D18	hsa-miR-16-5p	F18	hsa-miR-200b-3p	H18	hsa-miR-27b-3p
B19	hsa-miR-129-1-3p	D19	hsa-miR-17-5p	F19	hsa-miR-200c-3p	H19	hsa-miR-27b-5p
B20	hsa-miR-1290	D20	hsa-miR-181a-5p	F20	hsa-miR-200c-5p	H20	hsa-miR-28-3p
B21	hsa-miR-129-2-3p	D21	hsa-miR-181a-3p	F21	hsa-miR-202-3p	H21	hsa-miR-28-5p
B22	hsa-miR-129-5p	D22	hsa-miR-181b-5p	F22	hsa-miR-203a-3p	H22	hsa-miR-296-3p
B23	hsa-miR-130a-3p	D23	hsa-miR-181c-5p	F23	hsa-miR-204-5p	H23	hsa-miR-298

Table 3.3.2. miScript miRNA PCR Array miRNA list

List of 372 mature miRNAs profiled, plus 8 endogenous controls and 4 quality controls.

Well	miRNA	Well	miRNA	Well	miRNA	Well	miRNA
I01	hsa-miR-29a-3p	K01	hsa-miR-346	M01	hsa-miR-483-5p	O01	hsa-miR-605-5p
I02	hsa-miR-29a-5p	K02	hsa-miR-34a-5p	M02	hsa-miR-484	O02	hsa-miR-606
I03	hsa-miR-29b-3p	K03	hsa-miR-34a-3p	M03	hsa-miR-485-5p	O03	hsa-miR-608
I04	hsa-miR-29b-2-5p	K04	hsa-miR-34b-3p	M04	hsa-miR-486-3p	O04	hsa-miR-622
I05	hsa-miR-29c-3p	K05	hsa-miR-34b-5p	M05	hsa-miR-486-5p	O05	hsa-miR-626
I06	hsa-miR-300	K06	hsa-miR-34c-3p	M06	hsa-miR-488-3p	O06	hsa-miR-639
I07	hsa-miR-301a-3p	K07	hsa-miR-34c-5p	M07	hsa-miR-489-3p	O07	hsa-miR-643
I08	hsa-miR-302a-3p	K08	hsa-miR-361-5p	M08	hsa-miR-490-3p	O08	hsa-miR-649
I09	hsa-miR-302a-5p	K09	hsa-miR-363-3p	M09	hsa-miR-491-5p	O09	hsa-miR-652-3p
I10	hsa-miR-302b-3p	K10	hsa-miR-363-5p	M10	hsa-miR-493-3p	O10	hsa-miR-661
I11	hsa-miR-302b-5p	K11	hsa-miR-365b-3p	M11	hsa-miR-495-3p	O11	hsa-miR-7-5p
I12	hsa-miR-302c-3p	K12	hsa-miR-370-3p	M12	hsa-miR-497-5p	O12	hsa-miR-708-5p
I13	hsa-miR-30a-5p	K13	hsa-miR-372-3p	M13	hsa-miR-498	O13	hsa-miR-708-3p
I14	hsa-miR-30b-5p	K14	hsa-miR-373-3p	M14	hsa-miR-499a-3p	O14	hsa-miR-720
I15	hsa-miR-30c-5p	K15	hsa-miR-373-5p	M15	hsa-miR-499a-5p	O15	hsa-miR-744-5p
I16	hsa-miR-30c-1-3p	K16	hsa-miR-374a-5p	M16	hsa-miR-500a-5p	O16	hsa-miR-765
I17	hsa-miR-30d-5p	K17	hsa-miR-374c-5p	M17	hsa-miR-504-5p	O17	hsa-miR-770-5p
I18	hsa-miR-30d-3p	K18	hsa-miR-375	M18	hsa-miR-505-3p	O18	hsa-miR-802
I19	hsa-miR-30e-5p	K19	hsa-miR-376a-3p	M19	hsa-miR-506-3p	O19	hsa-miR-885-5p
I20	hsa-miR-30e-3p	K20	hsa-miR-376b-3p	M20	hsa-miR-508-5p	O20	hsa-miR-888-5p
I21	hsa-miR-31-5p	K21	hsa-miR-376c-3p	M21	hsa-miR-509-3p	O21	hsa-miR-9-5p
I22	hsa-miR-31-3p	K22	hsa-miR-377-3p	M22	hsa-miR-511-5p	O22	hsa-miR-9-3p
I23	hsa-miR-32-5p	K23	hsa-miR-377-5p	M23	hsa-miR-512-5p	O23	hsa-miR-920
I24	hsa-miR-32-3p	K24	hsa-miR-378a-3p	M24	hsa-miR-513a-5p	O24	hsa-miR-924
J01	hsa-miR-320a	L01	hsa-miR-378a-5p	N01	hsa-miR-514a-3p	P01	hsa-miR-92a-3p
J02	hsa-miR-320b	L02	hsa-miR-379-5p	N02	hsa-miR-517b-3p	P02	hsa-miR-92a-1-5p
J03	hsa-miR-323b-5p	L03	hsa-miR-381-3p	N03	hsa-miR-518b	P03	hsa-miR-92b-3p
J04	hsa-miR-324-3p	L04	hsa-miR-382-5p	N04	hsa-miR-519d-3p	P04	hsa-miR-92b-5p
J05	hsa-miR-324-5p	L05	hsa-miR-383-5p	N05	hsa-miR-520f-3p	P05	hsa-miR-93-5p
J06	hsa-miR-325	L06	hsa-miR-409-3p	N06	hsa-miR-520g-3p	P06	hsa-miR-93-3p
J07	hsa-miR-326	L07	hsa-miR-410-3p	N07	hsa-miR-522-3p	P07	hsa-miR-95-3p
J08	hsa-miR-328-3p	L08	hsa-miR-411-5p	N08	hsa-miR-524-5p	P08	hsa-miR-96-5p
J09	hsa-miR-330-3p	L09	hsa-miR-421	N09	hsa-miR-532-5p	P09	hsa-miR-98-5p
J10	hsa-miR-331-5p	L10	hsa-miR-423-3p	N10	hsa-miR-539-5p	P10	hsa-miR-99a-5p
J11	hsa-miR-335-5p	L11	hsa-miR-423-5p	N11	hsa-miR-542-3p	P11	hsa-miR-99a-3p
J12	hsa-miR-335-3p	L12	hsa-miR-424-5p	N12	hsa-miR-542-5p	P12	hsa-miR-99b-5p
J13	hsa-miR-337-5p	L13	hsa-miR-424-3p	N13	hsa-miR-549a	P13	cel-miR-39-3p
J14	hsa-miR-338-3p	L14	hsa-miR-425-5p	N14	hsa-miR-551b-3p	P14	cel-miR-39-3p
J15	hsa-miR-338-5p	L15	hsa-miR-425-3p	N15	hsa-miR-567	P15	SNORD61
J16	hsa-miR-339-3p	L16	hsa-miR-4258	N16	hsa-miR-570-3p	P16	SNORD68
J17	hsa-miR-33a-5p	L17	hsa-miR-429	N17	hsa-miR-574-3p	P17	SNORD72
J18	hsa-miR-33a-3p	L18	hsa-miR-431-5p	N18	hsa-miR-575	P18	SNORD95
J19	hsa-miR-33b-5p	L19	hsa-miR-432-5p	N19	hsa-miR-580-3p	P19	SNORD96A
J20	hsa-miR-340-5p	L20	hsa-miR-433-3p	N20	hsa-miR-581	P20	RNU6-6P
J21	hsa-miR-340-3p	L21	hsa-miR-449a	N21	hsa-miR-583	P21	miRTC
J22	hsa-miR-342-3p	L22	hsa-miR-451a	N22	hsa-miR-588	P22	miRTC
J23	hsa-miR-342-5p	L23	hsa-miR-454-3p	N23	hsa-miR-589-3p	P23	PPC
J24	hsa-miR-345-5p	L24	hsa-miR-455-5p	N24	hsa-miR-600	P24	PPC

Gender	Age (years)	Hb	fasting glucose	Total cholesterol	HDL	LP(a)	GH	cortisol	SHBG	IGF-1	FSH	LH	Testosterone	DHEAS	CRP
F	51	15.2	4.7	5.8	1.22	104	0.58	392	66.3	19.3	60.8	42.5	0.40	1.64	n/a
F	32	11.7	4.5	3.2	0.85	<49	7.67	735	147	23.0	3.50	2.80	0.50	1.83	n/a
M	21	17.2	5.1	5.2	2.35	57	0.11	426	15.8	42.0	1.90	2.80	17.4	10.1	n/a
M	33	n/a	5.3	4.1	1.44	91	<0.07	311	28.8	20.5	5.00	4.10	10.4	6.30	n/a
F	26	12.8	4.2	3.5	1.37	<49	3.04	344	53.6	23.0	2.90	11.0	1.20	4.56	< 3
M	20	14.9	5.1	4.2	1.31	62	2.85	662	21.9	39.3	0.70	4.10	11.6	10.2	< 3
F	48	12.8	5.0	4.8	1.81	107	0.11	226	64.7	19.1	2.70	2.50	0.50	2.34	< 3
M	48	14.2	5.2	6.0	1.30	<57	1.62	235	29.1	16.3	4.90	4.50	17.8	7.60	< 3
F	47	11.8	4.0	4.7	1.82	432	0.24	147	72.9	16.5	9.40	13.0	0.90	2.26	< 3
F	49	13.3	4.4	4.8	1.24	244	3.13	270	17.4	22.0	7.70	17.1	0.50	3.25	< 3
F	32	11.7	4.4	4.8	2.39	<57	0.39	174	225	13.6	1.90	1.20	0.80	2.05	< 3
F	39	13.4	4.7	n/a	1.27	123	3.73	292	58.9	15.1	15.1	6.90	0.70	6.08	< 3
F	53	13.3	5.7	5.5	1.10	450	0.17	344	61.9	22.0	61.3	41.7	0.60	3.91	< 3
F	60	12.3	4.2	5.4	1.84	<57	0.52	183	48.4	11.8	80.7	34.9	0.40	3.22	< 3
F	67	12.0	4.7	5.6	2.35	1003	0.12	218	187	9.20	74.5	44.5	0.20	0.41	< 3
M	67	n/a	4.2	4.4	1.60	2151	0.92	390	79.5	18.4	5.10	4.90	20.3	1.27	< 3
F	68	13.6	4.8	4.1	1.05	<61	0.19	180	38.3	11.4	84.1	50.8	0.90	0.65	< 3
M	65	14.0	5.1	n/a	1.51	1257	0.32	143	98.3	17.7	3.70	4.20	21.1	2.31	< 3
F	73	12.8	4.7	5.4	2.22	536	0.43	392	92.0	8.60	27.1	10.2	0.40	2.14	< 3
M	46	14.4	4.6	5.7	1.02	72	0.08	277	26.8	n/a	2.30	2.30	12.6	6.47	4
F	48	11.5	4.8	4.8	1.70	110	0.34	156	68.2	22.1	10.2	6.60	0.50	1.43	< 3
M	51	15.3	4.6	4.9	1.00	<61	<0.05	200	21.2	24.7	12.2	6.40	11.4	5.09	< 3
M	61	14.8	5.0	6.3	1.54	159	0.17	301	43.5	15.5	8.60	6.80	12.0	3.28	5

Table 3.4. Healthy control patient profiles

Serum from n=23 healthy controls (HC) were used for age-matched disease-free comparison. Samples were collected in accordance with ethics guidelines. Patients were mixed for age and sex. N/A refers to missing data. HDL, high-density lipoprotein; LP(a), lipoprotein(a); GH, growth hormone; SHBG, sex hormone binding globulin; IGF-1, insulin-like growth factor-1; FSH, follicle-stimulating hormone; LH, luteinising hormone; DHEAS, dehydroepiandrosterone sulphate; CRP, C-reactive protein.

Gender	Age (years)	Duration of arthritis	TJC (28)	SJC (28)	ESR	CRP	RA or ReA diagnosis	Medication (DMARDs, biologics, steroids, NSAIDs)
M	40	5 years	4	4	33	35	RA	etanercept, prednisolone
F	19	6 months	25	7	42	28	RA	NSAID
M	75	1 year	23	19	41	23	RA	NSAID
F	33	1 year	6	3	19	15	RA	nil
F	40	16 years	0	15	29	<5	RA	nil
F	56	2 years	17	4	61	8	RA	methotrexate, hydroxychloroquine
F	43	7 months	3	2	20	<5	RA	NSAID
M	59	10 years	8	18	n/a	108	RA	nil
F	41	6 weeks	28	18	54	40	RA	NSAID
F	67	6 years	5	8	86	84	RA	methotrexate, hydroxychloroqine
F	73	18 years	9	10	7	7	RA	methotrexate, NSAID
F	68	12 weeks	6	4	97	77	RA	NSAID
M	82	11 weeks	5	1	35	60	RA	nil
M	58	1 year	5	15	12	6	RA	nil
F	78	1 year	10	15	31	8	RA	nil
F	74	4 months	5	9	34	<5	RA	nil
F	39	13 weeks	11	6	81	59	RA	nil
M	39	4 years	1	2	28	30	RA	etanercept, prednisolone
F	48	16 days	8	6	4	102	RA	NSAID
F	49	14 days	9	8	6	<5	RA	nil
M	23	6 weeks	1	1	n/a	<5	ReA	NSAID
M	23	10 weeks	1	2	2	6	ReA	NSAID
M	28	10 weeks	1	2	4	<5	ReA	nil
M	18	8 weeks	1	1	33	36	ReA	NSAID
M	28	19 weeks	1	1	5	5	ReA	nil
M	23	3 weeks	0	1	5	<5	ReA	NSAID
M	32	7 weeks	1	1	n/a	38	ReA	NSAID

Table 3.5. RA and ReA patient clinical data
Data includes age of patient, disease duration, and disease activty markers tender joint count out of 28 joints (TJC28); swollen joint count out of 28 joints (SJC28); erythrocyte sedimentation rate (ESR); C-reactive protein (CRP). NSAID=non-steroidal anti-inflammatory drug. N/A refers to missing data.

3.6 Profiling serum and SF in rheumatoid arthritis

Matched serum and SF samples from n=20 patients with persistent rheumatoid arthritis (RA; defined according to fulfilment of the 2010 classification criteria for RA) and n=7 patients with resolving post-infective reactive arthritis (ReA) were obtained with patient consent according to existing ethical agreements (Reference 12/WM/0258). Both RA and ReA patients had arthritis and active inflammation at the time of presentation and sample collection in clinic. RA patients continued to have ongoing inflammatory disease whereas ReA patients had a form of reactive arthritis that spontaneously resolved. Patient ages ranged from 18-82yrs. Patients were clinically assessed for various disease activity markers; these included erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), and the 28 joint tender joint count (TJC) and swollen joint count (SJC). Disease-free age and sex-matched subjects were used as healthy control (HC) group for serum analyses. Healthy control demographics are shown in **Table 3.4**, and demographics and clinical assessments for RA and ReA patients are shown in **Table 3.5**. Both serum and SF samples were used to quantify various vitamin D metabolites, DBP and albumin and also to quantify circulating levels of miRNAs.

3.6.1 MicroRNA isolation from serum and SF

Serum and SF miRNA isolation was performed on all matched RA and ReA samples using the miRNeasy Serum/Plasma Kit (Qiagen) to maximise miRNA recovery. 200µL serum or SF was used as starting material, and RNA extraction was carried out following the manufacturer's protocol. All steps were performed at room

temperature unless otherwise stated. 1mL QIAzol Lysis Reagent was added per sample and vortexed to mix. The sample was incubated for 5 minutes and 3.5µL reconstituted miRNeasy Serum/Plasma Spike-In Control (at $1.6x10^8$ copies/µL) was added per sample. 200µL chloroform was added and incubated for 5 minutes, followed by centrifugation at 10,000rpm for 15 minutes at 4°C. The aqueous layer was transferred to a fresh tube and 800µL 100% ethanol added. Samples were spun by two centrifugations at 8000rpm for 15 seconds using a RNeasy MinElute spin column (supplied in kit), followed by 3 washes with wash buffer (supplied) on the same centrifuge settings. All flow-through was discarded following each centrifugation step. 14µL RNAse-free water was added to the tube and samples were spun at 10,000rpm for 1 minute to elute the RNA. After the final step, RNA eluate contained the spike-in control at $4x10^7$ copies/µL. RNA was stored at -80°C.

3.6.2 Reverse transcription for circulating miRNAs

Reverse transcription to synthesise cDNA was performed using the miScript II RT Kit (Qiagen) as described below. Reverse transcription was performed in 8-strip capped PCR tubes and prepared on ice. The master mix was prepared, ensuring 10% excess was made up. Each reaction contained:

4µL 5x miScript HiSpec Buffer (miScript II RT Kit, Qiagen)

2µL 10x miScript Nucleics mix (miScript II RT Kit, Qiagen)

2µL miScript Reverse Transcriptase mix (miScript II RT Kit, Qiagen)

10.5µL RNAse free water (Invitrogen)

18.5µL of master mix was pipetted into each reaction, and 1.5µL RNA (containing Spike-In control) was added to a total of 20µL per reaction. The samples were spun briefly in a mini centrifuge to evenly distribute the reagents. Samples were processed using an ABI GeneAmp PCR System 2700 (Applied Biosystems) on the following settings: 37°C for 60 minutes, 95°C for 5 minutes to deactivate the transcriptase, followed by a 4°C hold. Stock cDNA was diluted in 200µL RNAse free water, to bring the Spike-In control to $2.7x10^5$ copies/µL. Samples were stored at -20°C prior to qRT-PCR.

3.6.3 Quantification of circulating miRNAs by qPCR

For serum and SF miRNAs, qRT-PCR reactions were performed using 25µL reaction volumes on 96-well semi-skirted PCR plates (Applied Biosystems). All reagents were thawed on ice in the dark, and plates were prepared at room temperature. A clear plastic cover plate (Applied Biosystems) was placed over the prepared plate and edges firmly sealed to minimise contamination of the samples. Prior to running the qPCR, the plate was centrifuged at 1200rpm for 2 minutes to ensure all reagents were evenly distributed in each well. 96-well plates were run on an ABI 7500 Real Time PCR System (Applied Biosystems, serial no. 275000090), using the SYBR Green protocol for miRNA targets. Targets were defined at the plate set-up stage, and cycling conditions were set according to manufacturer's recommendations. ΔCt values were obtained and analysed, to determine miRNA expression profiles in serum and SF. Ce_miR_39_1 (Qiagen) was used as the endogenous housekeeping gene to calculate ΔCt values.

Each 25µL reaction was made up of the following components:

12.5µL 2x QuantiTect SYBR Green PCR Master Mix (Qiagen)

2.5µL 10x miScript Universal Primer (Qiagen)

2.5µL Primer Assay (Qiagen)

6.5µL RNAse free water (Invitrogen)

24µL master mix was pipetted for each reaction, and 1µL diluted cDNA was then added. 96-well plates were run on an ABI7500 Real-Time PCR Cycler programmed on the following settings:

1) 95°C for 15 minutes (to activate HotStarTaq DNA Polymerase)

2) 3-step cycling (Denaturation, Annealing, Extension): 94°C for 15 seconds, 55°C for 30 seconds, 70°C for 34 seconds (fluorescence data collection performed at this step) (Repeated for 40 cycles)

After the run, Raw Ct values were extracted to calculate the mean Ct and ΔCt value for each reaction.

3.6.4 Quantification of serum and SF vitamin D metabolites by LC-MS/MS

Liquid-chromatography tandem mass-spectrometry (LC-MS/MS) was used to quantify the following vitamin D metabolites in serum and SF; 25(OH)D3, 3-epi-25(OH)D3, 25(OH)D2, 24,25$(OH)_2$D3 and 1,25$(OH)_2$D3 as described previously [266-268] with slight modifications as follows: 200µL serum or SF was used as starting material for vitamin D analyte expression. Due to limited sample volumes

this was adapted for the current protocol; for sample volumes less than 200µL the maximum available volume was used and the rest made up to 200µL with LC-MS grade water (Thermo Fisher). 20µL of vitamin D internal standard was then added to each sample. LC-MS grade methanol (Greyhound Chromatography, Merseyside, UK), isopropanol (Sigma Aldrich) and LC-MS grade water were then added to precipitate the proteins. The table below summarises the volume of reagents added to calibrant samples and patient samples.

	Calibrant samples	Patient samples
LC-MS grade methanol	30µL	80µL
Isopropanol	50µL	50µL
LC-MS grade water	100µL	150µL

Internal reference standards for the following vitamin D metabolites were used for analysis: 25(OH)D2, 25(OH)D3, 3-epi-25(OH)D3, 24,25$(OH)_2$D3 and 1,25$(OH)_2$D3 (Sigma Aldrich). Stock solutions were diluted in methanol to prepare standard curves with concentrations between 5–100ng/mL. External vitamin D calibrators and quality controls were purchased from Chromsystems (Am Haag, Germany).

Samples were transferred to 96-well SLE plates (Phenomenex, Macclesfield, UK) and prepared for analysis by protein precipitation, using isopropanol to remove proteins from the sample that may interfere with downstream LC-MS/MS analysis. Samples were extracted using a novel supportive liquid-liquid extraction (SLE) method instead of liquid-liquid extraction (LLE), to allow for faster sample preparation at lower costs without compromising analyte recovery [268]. Samples were derivatized to enable the analysis of compounds that otherwise cannot be readily monitored, using 4-(2-(6,7-dimethoxy-4-methyl-3-oxo-3,4-dihydroquinoxalinyl)ethyl)-1,2,4-triazoline-3,5-dione (DMEQ-TAD) as previously described [269] with the following modifications: 30µL 0.1mg/mL DMEQ dissolved in ethyl acetate was added twice for each sample, with 30 minutes incubation at room temperature after the first addition, and 60 minutes after the second addition. Samples were eluted each time by applying a vacuum (5Hg) until all the solution was eluted. Afterwards 40µL pure ethanol was added to each sample, and samples were dried under nitrogen for 10 minutes. Samples were then reconstituted in 125µL 50/50 methanol/water, and stored at -80°C until analysis.

Analysis of serum by LC-MS/MS was performed by Dr C Jenkinson (Institute of Metabolism and Systems Research, Birmingham, UK) on a Waters ACQUITY ultra

performance liquid chromatography (uPLC) coupled to a Waters Xevo TQ-S mass spectrometer (Waters, Manchester, UK). The LC-MS/MS method has been validated on US Food and Drug Administration guidelines for analysis of these metabolites as previously described [268]. In brief, ionisation was perfomed in electrospray ionisation (ESI) mode, with the mass spectrometer operating in positive ion mode. Multiple reaction monitoring (MRM) mode was used to quantify vitamin D metabolites, with a total run time of 8 minutes per sample. The capillary voltage was 3.88KV and desolvation temperature was 500°C. Chromatography separation was perfromed using a Lux Cellulose-3 chiral column (100mm, 2mm, 3µm) at 60°C, and a 0.2µm inline filter was added to prevent contamination and blockage of the column. The mobile phase was made up of methanol/water/0.1% formic acid at a flow rate of 330µL/min. Current assay perfomance data for measuring vitamin D metabolites in human serum have been established previously [268], and all vitamin D metabolites measured in the current analysis were within reference range of previous literature [270-272].

Utilising LC-MS/MS to quantify vitamin D metabolites in RA SF was a novel analysis in this field, hence sample preparation comparisons were performed before runing on the LC-MS/MS to check the recovery from SF. Due to the viscosity of SF it was a concern that the fibrous components may interfere with recovery. A normal sample was compared with one that had been sonicated to reduce the viscosity, and one that had Hyaluronidase enzyme added to break down the fibres. Sonication did not affect internal standard recovery after running on the LC-MS/MS, and the hyaluronidase approach was decided against as it was unknown how it would affect LC-MS/MS analysis. Based on the analyte:internal standard ratios obtained from this

sample run, the actual measurments did not differ based on the approach, hence it was decided that samples would be prepared for LC-MS/MS without prior sonication or enzyme additon.

3.6.5 Quantification of DBP and albumin by enzyme-linked immunosorbent assay (ELISA)

In serum and SF, DBP and albumin were quantified using a VDBP ELISA kit (Immundiagnostik, Germany) and a human serum albumin ELISA kit (Thermo Fisher, UK). In these assays, the plate wells were pre-coated with antibodies against the protein of interest. All reagents supplied were diluted to the appropriate concentrations as stated in the manufacturers' protocols. For serum and SF samples, the suggested dilution was 1:40,000 for quantifying DBP and at least 1:500,000 for quantifying albumin, although it was found the optimum dilution factor for albumin was 1:1,000,000 due to the abundance of albumin in serum. Samples were serially diluted with dilution reagent provided by the kits. 100µL of the final dilution was used per well for analysis, following the manufacturers' protocols. All steps were carried out at room temperature.

3.6.6 DBP and albumin calibrant quality check

DBP and albumin concentrations in serum and SF were quantified by ELISA. Internal standards were provided in ELISA kits and were used to calculate accuracy of all other sample values. Known calibrant concentrations were plotted against their

optical density (OD) values with interpolation of control lines, to determine the accuracy of sample data measurements shown in **Figure 3.5**. Calibrant concentrations were within the normal range after interpolating the control line, confirming DBP measurements for test samples were reliable. Similarly, calibrant OD values for albumin were plotted to produce a standard curve used to determine the OD values for test samples.

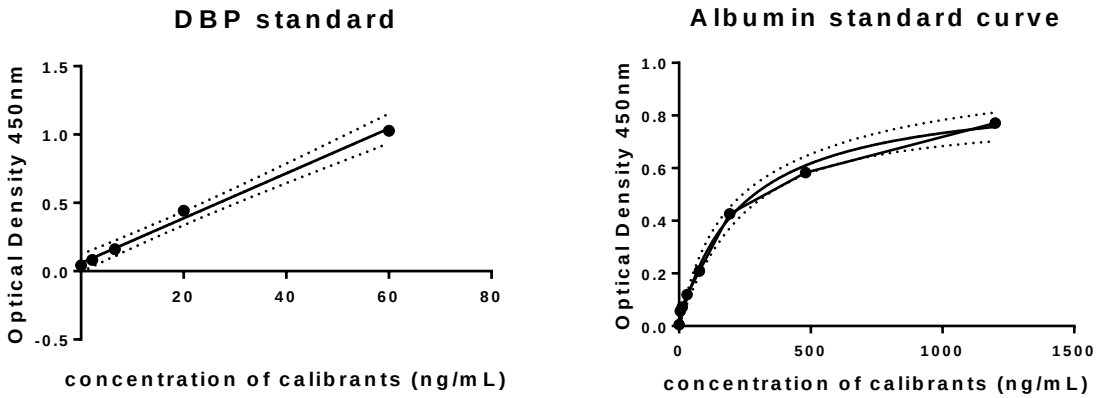


Figure 3.5. DBP and albumin ELISA calibration

ELISA was used to measure vitamin D binding protein (DBP) and albumin concentration in serum and SF. Optical density at 450nm wavelength was plotted against known concentration of calibrant. Interpolation of linear control line (DBP) and standard curve (albumin) gives an idea of how reliable the sample data will be.

The full list of reagents used in this thesis is shown in **Table 3.6**.

molecular biology		
Product	**Company**	**Catalogue number/product code**
MirVana miRNA Isolation Kit with Phenol	Life Technologies	AM1561
miScript II RT kit	Qiagen	218161
miScript SYBR Green PCR Kit	Qiagen	218075
miScript PreAMP PCR kit	Qiagen	331451
MBHS-3001Z miScript PreAmp Pathway HC Primer mix	Qiagen	331242
miScript miRNA HC PCR Array (MIHS-3001ZE)	Qiagen	331223
RT2 PCR Array Loading Reservoir	Qiagen	338162
RNAse Free water	ThermoFisher	BP2484-100
RNALater	Invitrogen	AM7020
Vitamin D binding Protein (VDBP) ELISA	Immunodiagnostik	K2314
Albumin Human ELISA Kit	Invitrogen	EHALB
miRNeasy Serum/Plasma Kit	Qiagen	217184

Table 3.6.1. Reagents used for molecular biology studies

Full list of reagents and catalogue numbers for molecular biology studies.

flow cytometry		
Product	**Company**	**Catalogue number/product code**
Live/Dead Fixable Near-IR Dead Cell Stain Kit	Invitrogen	L10119
Saponin	Sigma Aldrich	84510
FOXP3 Permeabilisation Buffer Set (concentrate and diluent)	eBioscience	Fixation/Permeabilisation Diluent: 00-5223-56
		Fixation/Permeabilisation Concentrate: 00-5123-43
FOXP3 Staining Buffer Set	eBioscience	Permeabilisation Buffer 10x: 00-8333-56
		Fixation/Permeabilisation Diluent: 00-5223-56
Phorbol 12-myristate 13-acetate (PMA)	Sigma Aldrich	16561-29-8
Ionomycin (calcium salt from Streptomyces conglobatus, 1mM stock in DMSO)	Sigma Aldrich	10634-1MG
Brefeldin A	Sigma Aldrich	B7651-5MG
Actinomycin D	Sigma Aldrich	A1410-2MG

Table 3.6.2. Reagents used for flow cytometry studies

Full list of reagents and catalogue numbers for flow cytometry studies.

cell culture		
Product	**Company**	**Catalogue number/product code**
Lymphoprep	Stem Cell Technologies	7851
Bovine Serum Albumin (BSA)	ThermoFisher	BP9702-100
Ethylenediaminetetraacetic acid (EDTA)	Amresco	E522-100ML
PBS tablets	Gibco (Life Technologies)	18912-014
EasySep Human CD4+ T-cell Enrichment Kit	Stem Cell Technologies	19052
EasySep Monocyte Isolation Kit	Stem Cell Technologies	19059
EasySep Magnet	Stem Cell Technologies	18000
RPMI 1640 Medium + Glutamine	Gibco (Life Technologies)	21875-034
CellGro GMP DC medium	CellGenix	0020801-0500
Fetal Bovine Serum (FBS)	Biosera	30502
Penicillin and Streptomycin	ThermoFisher	15070063
Anti-CD3 cytokine (OKT3, 1mg/ml)	UoB stocks	30816
Anti-CD28 cytokine (CD28 Pure)	BD Pharmingen	348040
GM-CSF (500mcg/ml)	Berlex Laboratories	NDC 50419-050-14
Interleukin-4 (IL-4) Recombinant Human Protein	ThermoFisher	PHC0044
Interleukin-2 (IL-2) Recombinant Human Protein	ThermoFisher	PHC0026
Transforming Growth Factor-beta (TGF-β) Recombinant Human Protein	ThermoFisher	PHG9204
Interleukin-1β (IL-1β) Recombinant Human Protein	ThermoFisher	PHC0815
Interleukin-6 (IL-6) Recombinant Human Protein	ThermoFisher	PHC0065
Lipopolysaccharide from *E. coli*	Sigma Aldrich	L4391-1MG
1α,25-Dihydroxyvitamin D3 50µg	Enzo Life Sciences	BML-DM200-0050

Table 3.6.3. Reagents used for cell culture studies

Full list of reagents and catalogue numbers for cell culture studies.

3.7 Statistics and calculations

Unless otherwise stated, all data are shown as mean +/- standard deviation (SD), and statistical significance defined as $p<0.05$. For paired data comparing 2 treatment groups, normality was first assessed using the D'Agostino-Pearson omnibus normality test. Parametric data was compared using a paired samples Student's *t*-test. In the case of non-parametric paired data, Wilcoxon matched-pairs signed rank test was performed, calculated as two-tailed test with 95% confidence intervals.

For paired data comparing 3 treatment groups, data was first assessed for Gaussian distribution. All data passed the Gaussian distribution test, and repeated-measures (RM) one-way ANOVA with the Greenhouse-Geisser sphericity correction was performed. This was then corrected for post-hoc multiple comparisons using Kruskal-Wallis testing.

For paired data with over 3 categories of comparison, paired repeated-measures (RM) ANOVA was performed, and data was tested for sphericity using the Greenhouse-Geisser test. In the case of sphericity, *p*-values from the Huynh-Feldt correction test were used instead. The Bonferroni or Tukey method was used for post-hoc multiple-comparison testing. This was to adjust for multiple comparisons corrections, to account for erroneous inferences caused by too many parameters in different comparisons.

For any unpaired data comparisons, non-parametric data was assessed using the unpaired Kolmogorov-Smirnov test with descriptive statistics.

For miRNA qPCR array data, the raw Ct value obtained for each individual miRNA was normalised against the mean of 6 endogenous reference genes (SNORD61, SNORD68, SNORD72, SNORD95, SNORD96A and RNU6-6P). Non-parametric repeated measures (RM) ANOVA was performed across all treatment conditions for individual donors, with post-hoc Kruskal-Wallis multiple comparisons test performed to assess the significance of each treatment condition compared to all other conditions.

For ELISA assays, absorption was determined immediately after adding acidic solution to stop the reaction, on a WALLAC Victor3 1420 Multilabel Counter plate reader set at 450nm wavelength. 690nm and 550nm wavelengths were used as references for DBP and albumin measurements, respectively. The 450nm values were subtracted from the reference values, and a dose-response graph of optical density (OD at 450nm) against standard concentration was generated, producing a linear line or standard curve. Values depending on this line or curve were interpolated to derive all other sample values for DBP or Albumin. All derived values were multiplied by the dilution factor to calculate the final concentration of BDP and Albumin in serum and SF samples.

Estimations of free and bioavailable 25(OH)D were performed using mathematical algorithms adapted from the original calculations [48] to incorporate *Gc* polymorphic variations in DBP binding affinity and DBP concentration. Levels of free 25(OH)D in serum and SF were calculated using this novel extra-cellular steady state (eSS) mathematical model [273].

Pathway analysis of miRNA gene targets was performed using Cytoscape v.6.3.1 in conjunction with publicly available miRNA repository database MiRBase, to identify experimentally validated miRNA gene targets. Using this software, over-representation was tested for using the hypergeometric test, with further Benjamini and Hochberg False Discovery Rate correction. ClueGo v.2.3.3 gene ontology (GO) analysis was then performed as an extension to the Cytoscape software, to identify those genes with enriched biological processes. Results were visualised in gene networks and statistical significance of enriched genes in the network was calculated by Bonferroni step-down, with only genes showing $p<0.05$ included in the network. This workflow to study miRNA gene targets has been set up and described previously [274-277].

3.8 Software

Data from flow cytometry was analysed using FlowJo Version 10 (Tree Star, Ashland, OR). Data analyses were performed on GraphPad PRISM Version 6.07 software (San Diego, CA, USA). Cytoscape v3.1.6, miRTarBase and ClueGo were used for bioinformatics analyses to identify miRNA targets.

CHAPTER 4. VITAMIN D AND MICRORNAS IN RHEUMATOID ARTHRITIS

Parts of this chapter have been published as:

Danyang Li; Louisa E. Jeffery; Carl Jenkinson; Stephanie R. Harrison; Rene F. Chun; John S. Adams; Karim Raza; Martin Hewison. Serum and synovial fluid vitamin D metabolites and rheumatoid arthritis. The Journal of Steroid Biochemistry and Molecular Biology [278]

Martin Hewison; Stephanie R. Harrison; **Danyang Li**; Louisa E. Jeffery; Karim Raza. Vitamin D, autoimmune disease and rheumatoid arthritis. Calcified Tissues International CTIN (Review). Submitted to Springer Journals Editorial Office 12/12/2018

4.1.1 Rheumatoid arthritis: diagnosis, prevalence and pathogenesis

Rheumatoid arthritis (RA) is a systemic inflammatory disease characterised by the symmetrical onset of multiple painful swollen joints, often leading to permanent joint destruction, loss of mobility and increased risk of mortality [279]. There is currently no cure for RA, although many treatments are available that aim to slow disease progression and reduce the severity of symptoms. As with most chronic diseases early diagnosis and intervention is vital for the best response to treatment [280].

RA affects 1% of the worldwide population, with an annual prevalence of 2-4 cases per 10,000 adults [281]. Difficulty in the diagnosis of early RA lies in the symptom similarity with other types of inflammatory arthritis, for example presence of joint pain, swelling and stiffness (hallmarks of inflammatory arthritis) which can also be seen in other conditions such as seronegative spondyloarthropathies and connective tissue diseases, and the presence of shared abnormalities on laboratory tests such as elevated C-reactive protein (CRP), a systemic non-specific marker of inflammation. However elevated CRP can be helpful when taken in combination with other disease markers, and together can be used to classify patients as having RA (according to 2010 classification criteria for RA).

Clinically the diagnosis of RA is defined by the European League Against Rheumatism (EULAR) Classification Criteria updated in 2010, which replaced the previous criteria established in 1987. Amongst the 1987 classification criteria are the presence of bony erosions shown on patient X-rays; this has since been removed as a classification criterion due to their presence only at late stages of the disease, by which time aggressive forms of therapy are usually required. The new set of criteria

allows for earlier detection of RA and thus more effective treatment. The new classification criteria are composed of a points-based system with multiple categories (**Table 4.1**), where a score of ≥6 allows patients to be classified as having RA. Patients may fulfil these criteria immediately or cumulatively.

JOINT DISTRIBUTION (0-5)	**Score**
1 large joint	0
2-10 large joints	1
1-3 small joints	2
4-10 small joints	3
>10 joints (at least 1 small joint)	5
SEROLOGY (0-3)	
Neg. RF and neg. ACPA	0
Low pos. RF or low pos. ACPA	2
High pos. RF or high pos. ACPA	3
SYMPTOM DURATION (0-1)	
<6 weeks	0
≥6 weeks	1
ACUTE PHASE REACTANTS (0-1)	
Normal CRP and normal ESR	0
Abnormal CRP or abnormal ESR	1

Table 4.1. The 2010 EULAR Classification Criteria for RA diagnosis

Table created from criteria defined by the American College of Rheumatology (EULAR). Patients are reviewed using a scoring system, with a score of ≥6 indicating RA diagnosis. RF, rheumatoid factor; ACPA, anti-citrullinated protein antibody; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; pos, positive; neg, negative.

RA is amongst the most common of the autoimmune rheumatic diseases but its pathogenesis and aetiology remains complex and the subject of ongoing study. Due to the systemic nature of RA, inflammation is likely to involve a host of immune cells of the innate and adaptive systems, including T cells and DC. In the initial stages of disease onset, up-regulated peptide citrullination triggers activation of dendritic cells which present foreign antigens to other immune cells [282]. Following this, the activation of B cells and subsequent secretion of antibodies promotes further immune cell autoreactivity, which is further augmented by the repeated activation of innate immune cells leading to loss of tolerance [283]. Persistent inflammation, coupled with the activation of bone-resorbing osteoclasts by macrophages, and by activated T and B cells expressing RANKL, and synovial cell hyperplasia, leads to irreversible cartilage damage in the joint space as well as other secondary conditions such as vasculitis [284]. RA synovial fibroblast cells play a key effector role in the inflammatory process and pathogenesis of RA [285]; these cells appear to be intrinsically active which results in the increased production of matrix metalloproteinases (MMPs) that contribute to permanent joint destruction [286].

Mature DC present in the RA synovial tissue and synovial fluid (SF) may mediate the inflammatory process of RA disease. Studies have shown these cells to be potent stimulators of T cells and subsequent inflammatory cytokine production by Th1 subsets [287-289]. In turn, cytokines present in the inflamed joint synovium further facilities DC maturation leading to chronic inflammation. In RA, synovial tissue is dramatically infiltrated by large numbers of DC which go on to present self-antigens to autoreactive CD4+ T cells, leading to the subsequent activation of B cells and the production of autoantibodies. DC are thus likely to be key players of

autoimmunity in RA, supported by the presence of pro-inflammatory cytokines in the SF of RA joints which may stimulate DC activation. Cells that are induced by DC include Th1 and Th17 [290] populations that have been well-implicated in models of autoimmune disease. Activated Th1 cells have been found to preferentially accumulate in synovial joints of RA patients [291, 292]. DC are a source of cytokines that influence the immune pathway of Th cells. Amongst these cytokines, DC release IL-12 which induces the Th1 pathway and leads to the production of IFN-γ [293]; IL-23 can induce the Th17 pathway to release IL-17 [294]. Together these cytokines contribute to the active inflammation that is characteristic of RA disease.

In autoimmunity, an imbalance of pro- versus anti-inflammatory cytokines is usually the driving force behind disease-related inflammation. It is therefore important to understand the function of cytokines and their signalling networks in the pathogenesis of autoimmune disease. The interaction between pro-inflammatory cytokines plays a pivotal role in the process of RA inflammation, and mainly involves the actions of inflammatory cytokines such as IL-1β, IL-6, TNF-α, and IL-17 [295]. These cytokines activate genes associated with inflammatory responses, leading to dysregulation of immune activity and associated tissue damage. In the context of synoviocytes, it has been suggested that RA pathogenesis involves impaired synoviocyte programmed cell death (apoptosis), leading to the destruction of articular cartilage [296].

Other studies have shown that the elevated presence of several RA markers including rheumatoid factor (RF) and anti-cyclic citrullinated peptide (anti-CCP) are good early predictors of future RA development [297]; these are also key variables within the 2010 ACR/EULAR criteria set for RA classification. The search for other

RA diagnostic biomarkers has been the focus of more recent research, with the aim being to improve the relative ease of detection using non-invasive methods, where markers enable disease progression to be monitored [298]. Often in the pre-RA stage of disease before the appearance of clinical symptoms, increased blood biomarker expression can be the only indication or predictor of future disease onset. In the context of RA, a meta-analysis of fourteen studies was performed comparing the accuracy of the established biomarker anti-CCP with that of a potential new biomarker anti-MCV (mutated citrullinated vimentin antibodies) in predicting RA. Data from these studies concluded that serological levels of anti-MCV were equally as effective at predicting RA as anti-CCP [299]. When anti-CCP and anti-MCV are tested together, the diagnostic accuracy for early RA may be as high as 98% [300] compared to 95% specificity of anti-CCP alone [301]. The culmination of these studies confirms the importance of using multiple markers to accurately diagnose RA, and that other unstudied markers, including the use of miRNAs, may also have diagnostic potential.

4.1.2 Vitamin D deficiency and RA disease activity

Initial interest in the potential link between vitamin D and RA was prompted by the established anti-inflammatory properties of vitamin D in many other autoimmune diseases including Type 1 Diabetes and Multiple Sclerosis [302] (see **Chapter 2**). Meanwhile other studies have presented evidence that RA patients frequently present with low vitamin D status [73, 303, 304]. To further strengthen the link between vitamin D and RA, the presence of VDR has been detected in synovial cells

[305], indicating that these cells have the potential to respond to 1,25$(OH)_2$D3. Other studies have demonstrated a significant correlation between low vitamin D status and RA disease risk and activity [71, 306] although others have not [307]. The debate regarding vitamin D deficiency as a cause or effect of RA disease etiology is ongoing.

Multiple studies have analysed the link between RA disease activity and vitamin D deficiency, including studies in African-American [73], Mediterranean [304] and European [308] populations. Some of these studies found significant correlations between RA disease activity and vitamin D status whereas others found only trends towards this correlation. The conclusions from these studies varied widely, perhaps due to differences in the number of patients recruited, geographic location, length of RA disease and, to a degree, the definition of vitamin D deficiency in the patients. For example in the African-American cohort [73], vitamin D deficiency was defined as a serum 25(OH)D concentration of <15 ng/mL (25 nmol/L), compared to the more generally agreed 20 ng/mL (50 nmol/L). Whilst this difference may appear small, studies have shown the amount of vitamin D required to increase vitamin D status to above 20 ng/mL is around 5,000 IU (125 µg)/day [309].

A meta-analysis performed six years ago concluded that there was an inverse association between vitamin D status (serum 25(OH)D) and the risk of developing RA [310]. This study was however unable to establish a definitive correlation between vitamin D status and RA disease activity due to the limited study numbers and the heterogeneity of patients. Since this meta-analysis was performed, many more studies have focused on the association of vitamin D and RA disease activity, allowing for a more recent meta-analysis to be performed [311]. Results from this

study concluded that RA patients generally exhibited lower serum 25(OH)D levels compared to healthy controls, and that serum 25(OH)D was inversely correlated with RA disease activity [311]. Despite the inclusion of many more studies than used in the previous report, the current meta-analysis had some limitations. For example, due to the relatively long serum half-life of 25(OH)D compared to other vitamin D metabolites, even a transient change in serum 25(OH)D levels, such as by exposure of skin to UV-light, can markedly elevate 25(OH)D in serum leading to elevated vitamin D status. This issue has also been raised in previous studies that measured 25(OH)D in serum, thus the validity of using this metabolite alone to define vitamin D status in disease studies is open to debate.

A large-scale observational study performed in 2017, the COMORA study, evaluated the link between vitamin D and RA disease activity in patients across fifteen countries worldwide [312]. They observed that vitamin D insufficiency was present in over half of the RA cohort across all fifteen countries, confirming that low vitamin D status is prevalent amongst RA patients. This varied widely between different countries, with the lowest prevalence of low serum vitamin D in RA at 36% and the highest at 71%, which could potentially be explained by latitude and availability to sun exposure [312]. Interestingly this study found vitamin D deficiency and RA prevalence was also evident in countries with plenty of sun exposure. Possible explanations for this could be the avoidance of sunlight in hotter climates, or lower synthesis of parental vitamin D in the epidermis of people with darker skin pigmentation that is more common to indigenous populations in sunnier countries [313].

The COMORA study also observed that non vitamin D-supplemented patients had lower vitamin D status, and that low vitamin D was inversely correlated with the RA disease activity (DAS28) score (p<0.001). However, it is worth remembering that the COMORA study is an observational study, and hence it still has the limitations of all other observational association studies. One major limitation of the COMORA study is that the methods and amounts of vitamin D supplementation were not uniform across cohorts, therefore doses were not optimised. Other studies have also addressed the link between vitamin D status and RA, reaching similar conclusions to that observed in the COMORA study, that vitamin D deficiency inversely correlates with RA disease activity [314, 315]. In particular, a strong negative correlation between 25(OH)D levels and DAS28 was found, with significant inverse correlations between 25(OH)D and CRP and ESR also observed [314, 315]. This correlation was seen in both the northern and southern hemisphere, underlining the importance of vitamin D in RA disease activity globally.

The genetic components of the vitamin D receptor (VDR) and RA have also been studied with regard to disease development and activity. For example, polymorphisms of the *VDR* gene have been studied in the context of RA prevalence and disease activity [316-318]. Certain *VDR* genotypes may be associated with the outcome of RA; experimental studies have found the *FokI* polymorphism of *VDR* occurred significantly more frequently in RA patients compared to disease-free controls [319], and the *bb* genotype of the *BsmI* polymorphism of *VDR* was associated with reduced disease activity [320, 321]. However other studies have found no significant role of *VDR* polymorphisms in RA disease susceptibility or activity [322-324]. A meta-analysis performed in 2015 reviewed the association

between *VDR* gene polymorphisms and RA disease and found that only *TaqI* and *FokI* polymorphisms contributed significantly to increased risk and activity of RA [325]. The role of distinct receptor variants and the influence on RA disease susceptibility is an area that requires further research.

Whilst the majority of literature on vitamin D and RA has been focused on the correlation between serum 25(OH)D and disease activity in established RA, more research is required regarding the risks of vitamin D deficiency at early or even pre-established RA stages. Significant inverse correlations between serum 25(OH)D levels and markers of RA disease activity in new-onset RA have been found [326, 327]. In addition, low serum $1,25(OH)_2D3$ has recently been shown to correlate with disease activity in early RA patients [328]. As the physiologically active form of vitamin D, $1,25(OH)_2D3$ status may provide an alternative to 25(OH)D as a marker for predicting the development of RA in patients with early arthritis, although the relatively short half-life in serum means that repeated analysis of this form of vitamin D may be required. Collectively, these studies have underlined the potential importance of maintaining sufficient vitamin D levels in RA patients, especially at early disease stages when they may be most responsive to therapies. A summary of these studies showing associations between vitamin D status and RA disease activity is shown in **Table 4.2**. A PubMed (NCBI) advanced database search was performed with all experimental studies within the last 20 years (1998-2018) included, with RA patients from different geographical locations. All reported studies measured vitamin D status as the level of serum 25(OH)D, with the exception of a recent Danish study which measured $1,25(OH)_2D3$ instead as they justified this active metabolite was more physiologically relevant in RA disease status [329]. Overall the outcomes of

these studies were contradictory, with some observing a significant correlation between low vitamin D status and worsened RA disease activity [302, 304, 315, 330-337] whilst others found only some associations [104, 338-341] or no significant link at all [73, 342-351]. Despite this, a common theme amongst the studies that included a healthy control cohort was that 25(OH)D levels were consistently lower in RA patients compared to disease-free controls [71, 104, 302, 304, 332, 333, 339, 343, 348, 352-356]. However, serum vitamin D status may simply be a marker of RA disease progression rather than a contributory factor in the pathogenesis of RA. To determine whether improved serum 25(OH)D levels are beneficial to patients with RA disease has required studies to investigate the effects of vitamin D supplementation, and these are detailed in **Table 4.2**.

Study details (year, lead author)	Population size and ethnicity	Study outcome
1998, Oelzner [357]	RA=96, Germany	High RA disease activity was associated with altered vitamin D metabolism
2006, Cutolo [315]	RA=118, HC=75, Estonia and Italy	Serum 25(OH)D status correlated negatively with DAS28
2010, Craig [73]	RA=266, African American	25(OH)D insufficiency was not significantly associated with DAS28, VAS or SJC
2010, Haque [330]	RA=62, USA	25(OH)D deficiency in active RA was negatively correlated with DAS28 and VAS. This correlation was not found in patients in remission
2010, Rossini [304]	RA=1191, HC=1019, Italy	25(OH)D levels inversely correlated with patient DAS28. RA patients commonly displayed vitamin D deficiency
2011, Braun-Moscovici [71]	RA=85, Israel	The majority of RA patients were vitamin D deficient, despite the levels of sunshine being above average in this country
2011, Turhanoglu [331]	RA=65, HC=40, Turkey	Serum 25(OH)D levels were significantly inversely correlated with CRP and DAS28 in RA patients
2012, Kostoglou-Athanassiou [302]	RA=44, HC=44, Greece	Vitamin D deficiency was highly prevalent in RA patients compared to healthy controls. 25(OH)D status was negatively correlated with DAS28, CRP and ESR.
2012, Attar [358]	RA=100, HC=100, Saudi Arabia	Serum 25(OH)D levels in RA patients were not significantly different to those in healthy controls, although significantly lower 25(OH)D levels was associated with poorer response to treatment in RA
2012, Heidari [342]	RA=108, HC=239, Iran	No significant correlations were found between vitamin D status and disease activity in RA
2013, Atwa [343]	RA=55, HC=40, Egypt	Serum 25(OH)D levels were significantly lower in RA patients compared to healthy controls, however 25(OH)D was not correlated with DAS28, CRP or ESR
2013, Sabbagh [332]	RA=39, HC=56	25(OH)D was significantly lower in RA patients compared to controls. Low 25(OH)D correlated with significantly increased risk of higher disease activity in RA
2013, Furuya [344]	RA=4793, Japan	25(OH)D deficiency was not correlated with RA risk or activity
2013, Higgins [338]	RA=126, New Zealand	25(OH)D status was not correlated with DAS28, although there was a

		significant inverse relationship between 25(OH)D levels and patient VAS scores
2013, Haga [352]	RA=302, Denmark	25(OH)D3 deficiency was detected in 33.4% of the RA patients. Patients with severe vitamin D deficiency <15 nmol/L were all positive for RF and had very high DAS28 scores
2014, Yazmalar [345]	RA=71, HC=70, Turkey	No significant correlations were found between 25(OH)D levels and DAS28 in RA patients
2014, Sahebari [346]	RA=99, HC=68, Iran	There was no significant correlation between serum 25(OH)D levels and DAS28 in RA patients
2014, Gheita [353]	RA=63, HC=62, Egypt	25(OH)D levels were significantly lower in RA compared to controls, with 50.8% RA cohort being classed as vitamin D deficient.
2014, Sharma [333]	RA=80, HC=80	90% of RA patients were vitamin D deficient, compared with 70% of healthy controls. Serum 25(OH)D levels in RA were significantly lower than HC. Low serum 25(OH)D was correlated inversely with high DAS28
2014, Hong [339]	RA=130, HC=80	RA patients had lower 25(OH)D levels compared to HC, and low 25(OH)D3 was associated with higher disease activity in RA patients
2014, Hiraki [359]	Pre-RA=166, HC=490	25(OH)D levels were not significantly lower in RA compared to HC
2014, Cote [347]	RA=120, HC=1341, USA	25(OH)D status was not associated with increased risk of RA
2015, Raczkiewicz [348]	RA=97, Poland	76.3% RA patients were vitamin D deficient, however vitamin D status was not correlated with RA disease activity
2015, Matsumoto [354]	RA=181, HC=186, Japan	Serum 25(OH)D levels were lower in RA patients compared to HC. 25(OH)D was not significantly correlated with DAS28
2015, Azzeh [334]	RA=102, Saudi Arabia	25(OH)D levels were significantly inversely correlated with DAS28 in RA patients
2015, Brance [355]	RA=34, HC=41, Argentina	RA patients had significantly lower 25(OH)D levels than HC, although 25(OH)D status was not correlated with disease activity
2015, Cen [349]	RA=116, China	Serum 25(OH)D levels was not correlated with RA disease activity
2016, Cecchetti [104]	RA=894, HC=861, multiple countries	COMEDRA study. RA patients had more prevalent vitamin D deficiency compared to HC. Vitamin D levels were inversely correlated with CRP but not ESR, in RA patients
2016, Pakchotanon [350]	RA=239, Thailand	No significant associations were found between 25(OH)D levels and RA disease activity

2016, Wang [335]	Early RA=154, HC=60, China	Serum 25(OH)D was inversely correlated with RA disease activity
2016, Zakeri [340]	RA=66, Iran	Serum 25(OH)D in RA patients was inversely correlated with TJC, SJC and VAS, but not ESR
2017, Mateen [356]	RA=100, HC=50	RA patients had lower serum 25(OH)D compared to HC
2017, Hajjaj-Hassouni [336]	RA=1413, 15 countries	Low 25(OH)D status was associated with increased disease activity in RA
2017, Vojinovic [341]	RA=625, HC=276, 13 European countries	Serum 25(OH)D levels in RA patients were significantly lower compared to HC. Inverse correlations were found between 25(OH)D and CRP in RA patients
2018, Herly [329]	RA=160, Denmark	$1,25(OH)_2D3$ was inversely correlated with CRP in RA patients
2018, de la Torre Lossa [351]	RA=100, Ecuador	No significant correlations were found between 25(OH)D levels and RA disease activity
2018, Khoja [337]	RA=41, HC=41	Serum 25(OH)D in RA patients was inversely correlated with DAS28

Table 4.2. Summary of studies on the relationship between vitamin D status and RA disease

DAS28, disease activity score of 28 joints; SJC, swollen joint count; TJC, tender joint count; RA, rheumatoid arthritis; HC, healthy control; VAS, visual analogue scale; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; RF, rheumatoid factor

4.1.3 Vitamin D supplementation and RA disease activity

There are multiple observational studies on the association between dietary intake of vitamin D and RA symptoms, progression and disease activity. Strong associations have been made between vitamin D status and RA disease activity, however contradictions in the literature mean a definitive conclusion cannot be drawn. The majority of existing studies have correlated the effect of existing dietary vitamin D intake in RA patients against disease activity, however often these patients are still vitamin D deficient or insufficient. As a result, these studies have not found significant correlations between dietary vitamin D and RA disease activity [73, 341, 360, 361].

Randomised control vitamin D supplementation trials for RA patients have also been reported; these trials have noted associations between RA disease activity and vitamin D supplementation dosage and are summarised in **Table 4.3**. Some studies reported a positive effect of vitamin D supplementation on RA disease activity markers such as SJC and TJC [362-364] as well as a significant increase in serum vitamin D levels [364]. Buondonno *et al*. [365] found that $1,25(OH)_2D3$ combined with methotrexate significantly reduced inflammation in early RA patients compared to using methotrexate alone, however these patients received a very high dose of $1,25(OH)_2D3$ and results could not be replicated in another study on established RA patients given a lower dose of vitamin D [366]. Yang *et al.* [367] reported that RA patients in remission who had vitamin D deficiency were significantly more likely to have recurring RA compared to those with sufficient vitamin D levels, however vitamin D supplementation did not reduce recurrence rates. Similarly other studies have not reported a significant effect of vitamin D supplementation on different RA

outcome measures [307, 366] although it is difficult to directly compare results due to varying study design, supplementation quantity and the type of medication already used by patients; all of which are factors that may impact on trial outcomes.

Study	Study participants (number + medications)	Treatment groups and dosage	Summary of key findings
Andjelkovic et al. 1999 [362]	RA=19 (on standard DMARDs)	2 µg/day oral alphacalcidiol (vitamin D analogue) in 2 groups; moderate + highly active RA	CRP, SJC, TJC were all significantly decreased after 3 months' treatment. RF and CRP were decreased but this was not statistically significant
Gopinath et al. 2011 [363]	RA=121 (on triple DMARDs)	500 IU $1,25(OH)_2D3$ given in n=59 vitamin D group, and not given in n=62 control group	Patients supplemented with $1,25(OH)_2D3$ reported significantly higher pain relief after 3 months, compared to control group
Salesi et al. 2012 [366]	RA=117 (on methotrexate)	50,000 IU 25(OH)D or placebo given. Randomized double-blind trial	Vitamin D supplemented group did not show improvement in outcome measures compared to the placebo group
Dehghan et al. 2014 [307]	RA=80	Patients randomly allocated to receive vitamin D supplement or placebo, in a double-blind trial	The DAS28 score was not significantly different between the treatment group and the placebo group
Yang J et al. 2015 [367]	RA=377 RA remission	Patients were divided into normal vitD group and vitD-deficient group based on their baseline levels. VitD deficient group were subdivided to receive +/- vitamin D.	The RA recurrence rate was significantly lower in the normal vitD group compared to the deficient –vitD subgroup. There was no difference in recurrence rates between the deficient –vitD subgroup and the deficient +vitD subgroup
Buondonno et al. 2017 [365]	Early RA=39 (treatment naïve), HC=31	Patients received standard methotrexate treatment +/- single dose 300,000 IU $1,25(OH)_2D3$, in a randomised double-blind trial	Significantly reduced numbers of pro-inflammatory IL-23 was found in the group that received methotrexate + $1,25(OH)_2D3$, compared to the methotrexate-only group
Chandrashekara et al. 2017 [364]	RA = 73 (on DMARDs)	Patients received 60,000 IU/week of vitamin D supplements for 6 weeks, then 60,000 IU/month for 3 months	Supplementation of RA patients led to significantly lower CRP score compared to pre-supplementation score. Serum vitamin D also increased from 10.05 to 24.77 ng/mL

Table 4.3. Vitamin D supplementation trials in rheumatoid arthritis

CRP, C-reactive protein; SJC, swollen joint count; TJC, tender joint count; RF, rheumatoid factor; DAS28, disease activity score of 28 joints; RA, rheumatoid arthritis; vitD, vitamin D; IL-23, interleukin-23; IU, international units; HC, healthy control; DMARD, disease-modifying anti-rheumatic drug

In addition, a large-scale association study aimed to identify the link between serum 25(OH)D levels and RA disease activity compared to healthy controls. The study involved 1191 RA patients (of whom over half were not taking vitamin D supplements) and 1019 healthy non-supplemented controls [304]. The authors found that serum 25(OH)D deficiency (<20 ng/mL) was similarly prevalent in non-supplemented healthy controls compared to supplemented RA patients, and that despite supplementation one-third of RA patients remained vitamin D-deficient. Higher serum 25(OH)D levels were however inversely correlated with RA disease activity, suggesting that vitamin D deficiency is common amongst RA patients.

The role of vitamin D supplementation in the recurrence of RA in patients in remission has also recently been studied. RA patients in remission who are vitamin D sufficient have a lower risk of disease recurrence and lower disease activity, compared to those who are vitamin D deficient. However the risk of recurrence is not significantly different with and without vitamin D supplementation in patients with RA who are already deficient [367]. This study suggests the earlier vitamin D supplementation is given, the better the prognosis and more likely the chances of recurrence is prevented, thus cementing the importance of maintaining a sufficient vitamin D profile in healthy individuals who may be at risk of RA.

4.1.4 Immunological mechanisms of vitamin D in RA

Vitamin D has the potential to exert potent anti-inflammatory effects on cells of the immune system (see **Chapter 2**). This may be particularly important in RA, where an initial step in development of RA involves the activation of T cells leading to a pro-inflammatory Th1 or Th17 phenotype. The Th1 response involves subsequent

activation, proliferation and recruitment of synovial and pro-inflammatory cells, leading to further pro-inflammatory cytokine secretion and excess antibody production [305]. With this in mind, it is possible for vitamin D to modulate immune responses associated with RA at a variety of different facets of the disease.

In vitro, 1,25$(OH)_2$D3 has been shown to antagonise RA-related pro-inflammatory processes in synoviocytes, by promoting p53 apoptosis mechanism in *MH7A* cells [296]. Interestingly, the up-regulation in apoptosis was only seen when 1,25$(OH)_2$D3 was applied in conjunction with TNF-α, a master regulator of inflammation normally associated with worsening RA pathogenesis on its own. 1,25$(OH)_2$D3 is known to induce production of tolerogenic dendritic cells (tolDC) which have immunosuppressive functions, suggesting 1,25$(OH)_2$D3-induced tolDC could be a viable treatment for RA in future. The immunosuppressive functions of tolDC, including induction of antigen-specific regulatory T cells (Treg), are critically dependent on the actions of TNF-α [368], further supporting the TNF-α and 1,25$(OH)_2$D3 synergy theory. However, anti-TNF-α therapy is a highly effective method of RA treatment [369, 370], involving suppression of pro-inflammatory cytokines and leading to TNF-α effects being antagonised [371]. The idea that 1,25$(OH)_2$D3 can have potent anti-inflammatory effects even in the presence of TNF-α is still poorly understood. The current proposed mechanism for TNF-α and 1,25$(OH)_2$D3 synergy is that an inflammatory environment may first be required to stimulate the conversion of 25(OH)D3 to 1,25$(OH)_2$D3. 1,25$(OH)_2$D3 then acts back on inflammatory cells to reduce inflammation [84]. In this way, high levels of TNF-α provide a trigger for the negative feedback system regulated by 1,25$(OH)_2$D3. Hence previously established methods of anti-TNF-α therapies must be reconsidered in RA

patients when used in tandem with tolDC therapy due to potential adverse counter-effects. In RA the function of Tregs is impaired as a result of down-regulated *CTLA-4* expression; *in vitro* studies have shown the 1,25$(OH)_2$D3 analogue TX527 can promote a CD4+ Treg profile by up-regulating *CTLA-4* expression, leading to favoured migration to sites of inflammation [372, 373]. So far there are no studies that have been able to replicate this Treg effect using 1,25$(OH)_2$D3, but given the positive data from epidemiological studies and clinical trials, the use of 1,25$(OH)_2$D3 holds promise as a viable therapy in RA. Cartilage erosion in joint spaces involves the action of matrix metalloproteinases (MMPs), and 1,25$(OH)_2$D3 is thought to be a regulatory factor in MMP expression [374]. The pathophysiology of RA has been associated with pro-inflammatory cytokines including IL-1β and IL-6. Although direct regulation was not seen in this study [374], others have found 1,25$(OH)_2$D3 inhibits IL-1β-induced expression of MMP and reduces migration and invasion of synovial fibroblast cells to the inflamed joint space [375].

In murine models of RA, mice with knockout of the *Cyp27b1* gene displayed exacerbated articular damage, decreased synoviocyte apoptosis and increased TNF-α secretion; these effects were reversed after administration of 1,25$(OH)_2$D3 [296]. The authors suggested 1,25$(OH)_2$D3 therefore prevented RA pathogenesis by inducing synoviocyte apoptosis. Despite this, the high doses of 1,25$(OH)_2$D3 administered to the mice (1 μg/kg body weight) must be taken into consideration when interpreting the results. Such high doses are rarely replicated in human trials due to risks associated with vitamin D toxicity. In mice, tolDC can significantly inhibit RA disease activity and progression, which coincides with an up-regulation of IL-10 producing CD4+ T cells and decreased numbers of pro-inflammatory Th17 cells

[376]. Murine models of inflammatory disease mimicking human RA, when supplemented with 1,25$(OH)_2$D3, prevented the progression of more severe disease [377, 378]. *Vdr* knockout mice with chronic arthritis have significantly more synovial joint damage when compared to mice with normal *Vdr* function, suggesting that VDR signalling plays an important role in limiting the inflammatory phenotype in RA mouse models [379].

1,25$(OH)_2$D3 production has been shown at sites of RA inflammation including the SF of arthritic joints [380]; furthermore, VDR can be found in synovial tissues. The local production of 1,25$(OH)_2$D3 in an autocrine fashion has also been well documented in other cell types including bone [381, 382] and keratinocytes [383]. The presence of VDR has been reported in cells from SF, indicating that these cells have the capacity to synthesise and respond to 1,25$(OH)_2$D3 [384]. Indeed, previous studies have highlighted the aberrant synthesis of 1,25$(OH)_2$D3 from 25(OH)D3 in SF macrophages of RA patients [385, 386]. 1,25$(OH)_2$D3 metabolism in RA synovial macrophages is under autocrine control, by promoting its own catabolism via the intrinsic up-regulation of *CYP24A1* in a VDR-dependent manner [387].

4.1.5 RA pathogenesis and miRNAs

To better understand the mechanisms that underpin the immune dysregulation associated with RA, several studies have characterised the transcriptomic changes that occur in different types of immune cells associated with RA [388-391]. However, in recent years there has been increasing attention paid to the role of epigenetic factors in diseases such as RA. This includes DNA methylation [392-395] and

histone acetylation [396], and how these mechanisms may influence the immune dysregulation associated with RA disease onset and progression. Another key component of epigenetic regulation is non-coding RNA i.e. RNA that is not translated but which nevertheless modulates protein expression via a variety of mechanisms [397-399]. Non-coding RNAs include transfer RNAs (tRNA), ribosomal RNAs (rRNA) and small RNAs such as miRNAs, all of which are abundant and functionally important. The different types of non-coding RNAs and their functions in regulation of gene expression are summarised in **Figure 4.1**.

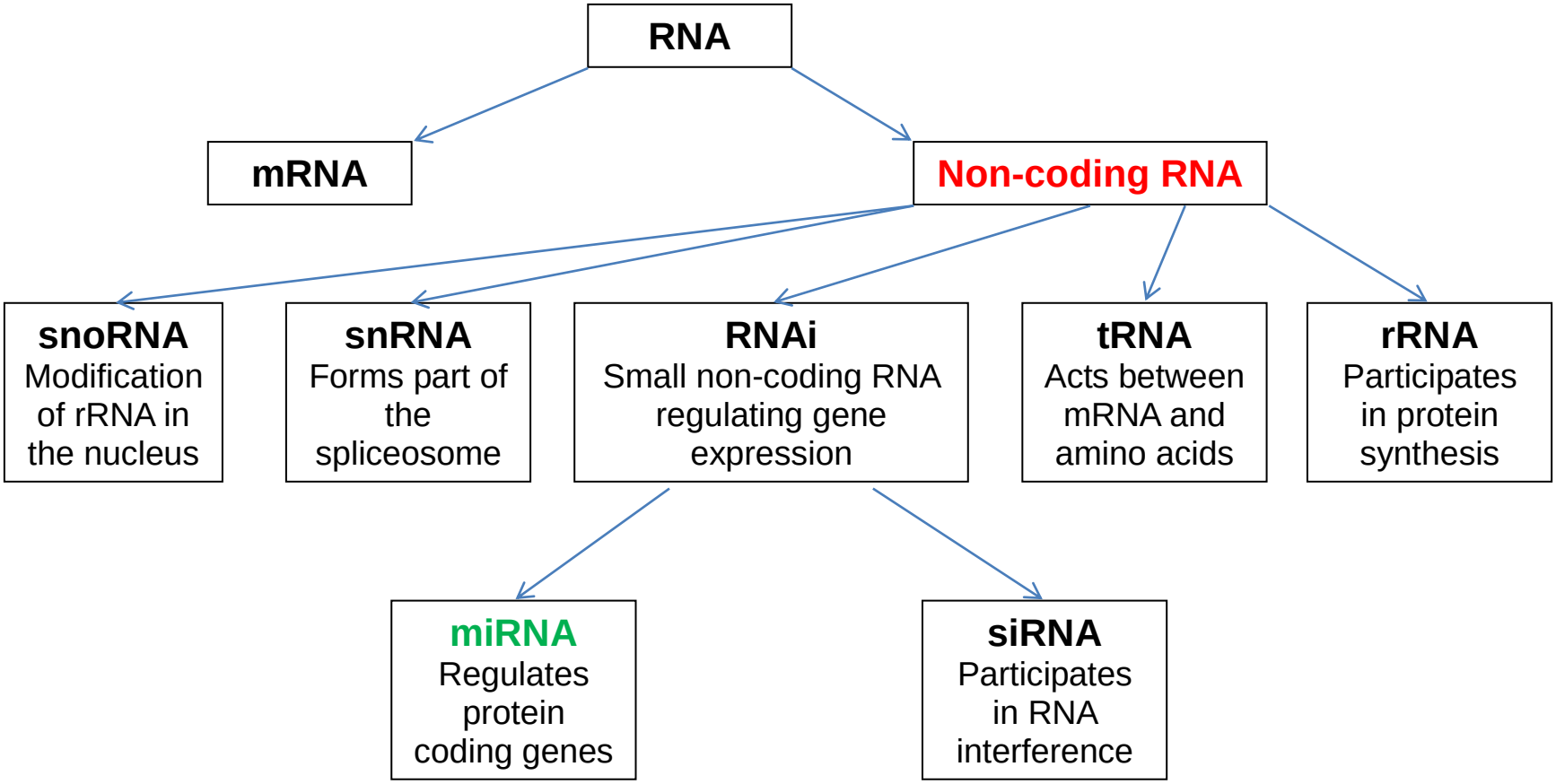


Figure 4.1. **Types of non-coding RNAs and their functions in gene expression**

RNA, ribonucleic acid; mRNA, messenger RNA; snoRNA, small nucleolar RNA; snRNA, small nuclear RNA; RNAi, RNA interference; miRNA, microRNA; siRNA, small interfering RNA

Prominent amongst the non-coding RNAs are miRNAs. As detailed in **Chapter 2, page 38**, miRNAs are small non-coding RNAs that modulate the stability and translation of mRNA. MiRNAs have been shown to play an important role in immune function (see **Chapter 2**, **page 42**) Studies have provided compelling evidence of miRNA dysregulation in the pathogenesis of RA [232, 400-402]. Of over two thousand miRNAs currently validated in humans, the role of two miRNAs in particular, miR-146a and miR-155, have been major focuses of RA-related research [403, 404].

In vitro studies on the dysregulation of cellular miRNAs in RA have been centred mainly on the role of miRNAs in immunoregulatory processes such as Th17 differentiation, B cell activation and inflammatory cytokine production. IL-6 and IL-1β are important inflammatory cytokines associated with RA pathogenesis, and studies have linked differential miRNA expression with the production of these cytokines in RA. Up-regulated miR-221 expression has been documented in synovial cells of RA patients compared to healthy controls, and is associated with increased production of MMPs in the joint space, and the increased production of IL-6 and IL-1β [405, 406]. Up-regulated expression of miR-18a, miR-22, miR-203 and miR-346 in RA synovial cells have also been linked to the increased production of IL-6 and IL-1β [407]. MiR-125b is known to participate in the role of B cell differentiation and TNF production, and RA patients with low synovial miR-125b expression have worse disease outcomes compared to patients with higher miR-125b expression [408]. The over-expression of miRNAs in the PBMC of RA patients compared to disease-free individuals has also been a key theme in miRNA-related RA research. MiR-146a plays an important role in regulating the NF-kB pathway. Significantly higher

expression of miR-146a in the PBMC of RA patients have been found compared to non-inflammatory osteoarthritis (OA) patients, as well as a correlation with higher disease activity [409, 410]. Higher circulating miR-155 expression has also been observed in RA versus OA PMBC, but not in RA versus healthy PMBC [411], and another study found that high miR-155 expression in RA correlated with CRP, DAS28 and levels of TNF-α [412]. In contrast, miR-21 expression was significantly lower in RA patients compared to healthy controls, and this dysregulation is thought to cause an imbalance of Th17 versus Treg cells leading to persistent inflammation [413]. In addition, polymorphisms in several miRNAs have been associated with a higher risk of developing RA; a polymorphism found in miR-499 which regulates genes encoding proteins in the TNF-α pathway has been found as an independent risk factor for joint erosions associated with RA, as well as higher DAS28 scores, elevated CRP, ESR and RF [414, 415]. A polymorphism in miR-146a in RA patient has also been associated with higher DAS28 scores and increased disease risk [415-417].

MiRNAs can exist as circulating miRNAs, and this has also been a key focus of the link between miRNAs and RA [418-420]. Inflammatory miRNAs miR-146a and miR-155 are up-regulated in the serum and SF of RA patients compared to healthy controls [404, 421, 422]; this up-regulation has been observed along with up-regulated ESR and anti-CCP, and correlated positively with RA disease activity [423]. However the role of miR-155 in RA pathogenesis is still contradictory; an *in vitro* study found miR-155 was up-regulated in RA patients but suggested the up-regulation could offer a protection factor against joint inflammation by silencing its target gene *IKBKE* [411]. These contradictory results could be explained by the multifunctional role of miR-155 in a wide range of actions including in both the

activation of T cells and suppression of Tregs, making it difficult to pinpoint specific gene targets of miR-155. Furthermore the actions of miR-155 are likely to be very different between *in vitro* and *in vivo* studies, as extracellular factors may contribute to its regulation [424]. MiR-16 and miR-223 have also been well studied in the context of circulating miRNAs in RA. Over-expression of both miRNAs have been found in the SF of RA patients compared to the SF of OA patients who lack the inflammatory phenotype associated with RA [425, 426], with serum miR-223 positively correlating with CRP and RA disease activity [427]. Additionally, miR-16 and miR-223 are highly expressed in established RA serum compared to early RA serum, marking these miRNAs as potential markers of disease progression. Hence all the above miRNAs may be viable targets for the attenuation of RA-related inflammation and joint destruction.

Due to the relative abundance and ease of detection of miRNAs in most tissues of the body, miRNAs have been proposed as potential novel biomarkers for the diagnosis and monitoring of diseases. MiRNAs appear to fit the criteria used to define suitable biomarkers which include: high tissue specificity [426], sensitivity to detection [428] and non-invasive detection methods [429]. In addition, miRNAs in circulation are protected from endogenous RNAse degradation [430, 431] and are resistant to degradation by changes in pH and boiling and freeze-thaw cycles, making them an ideal marker for detection in research. The role of circulating miRNAs as novel biomarkers of RA therefore requires further research before diagnostic use in a clinical setting. As described earlier in this chapter, low vitamin D status has been implicated in the activity and progression of RA. The immunological mechanisms involved in the inflammatory process are likely to involve dysregulated

miRNA expression, and vitamin D may be acting upon select miRNAs to mediate inflammation in RA.

4.2 Chapter outline

The aims of experiments presented in this chapter were as follows:

1) To investigate the link between serum vitamin D status and disease activity in RA patients, but to extend analysis to include multiple vitamin D metabolites beyond 25(OH)D i.e. to assess the vitamin D metabolome.

2) To compare the serum vitamin D metabolome in RA patients and patients with another form of arthritis - Reactive Arthritis (ReA).

3) To compare the serum vitamin D metabolome in RA and ReA patients with the serum metabolome from aged-matched healthy controls.

4) To compare the serum vitamin D metabolome in RA and ReA patients with the SF vitamin D metabolome using paired samples.

5) To quantify circulating levels of two miRNAs - miR-146a and miR-155 in serum and SF of RA and ReA patients.

6) To compare the serum and SF vitamin D metabolome with RA disease activity markers and levels of miR-146a and miR-155.

4.3 Results

4.3.1 Serum vitamin D metabolites in RA, ReA and HC

Data in **Figure 4.2** showed that of the 5 serum vitamin D metabolites analysed, only 3-epi-25(OH)D3 was significantly different in serum from RA (median 0.788 with 95% confidence limits 0.573-0.997, $p<0.01$) relative to healthy controls (HC) (median 1.17 with 95% confidence limits 0.832-1.59). Comparing ReA serum metabolites with HC serum metabolites, 3-epi-25(OH)D3 (median 0.361 with 95% confidence limits 0.197-1.69, $p<0.05$) and 25(OH)D2 (median 0.315 with 95% confidence limits 0.247-0.452, $p<0.01$) were significantly lower in ReA serum compared to HC serum. 25(OH)D3, 24,25$(OH)_2$D3 and 1,25$(OH)_2$D3 were not significantly different between HC and ReA serum. Data for serum vitamin D metabolites in RA, ReA and HC were unaffected by donor sex, age, or duration of disease (**Figures 4.20-4.22**). RA disease activity markers were also not significantly different between genders (**Figure 4.23**).

4.3.2 Serum versus SF vitamin D metabolites in RA and ReA

With the exception of 25(OH)D2, all vitamin D metabolites showed significantly lower concentrations in SF compared to paired serum from RA patients (**Figure 4.3A**). However, only SF 25(OH)D3 ($p<0.0001$) and 3-epi-25(OH)D3 ($p=0.0053$) showed significant linear correlation with serum levels of the same metabolites (**Figure 4.3B**). In patients with ReA, 25(OH)D3, 24,25$(OH)_2$D3 and 1,25$(OH)_2$D3 were statistically lower in SF compared to paired serum (**Figure 4.4A**), whilst only

25(OH)D3 (p=0.0002) and 24,25$(OH)_2$D3 (p=0.0002) in ReA SF correlated significantly with the same metabolites in serum (**Figure 4.4B**).

Correlating 25(OH)D3 concentration with other metabolites in RA serum and SF, 25(OH)D3 showed significant positive correlation with 3-epi-25(OH)D3 in both RA serum and SF (**Figure 4.5A; 4.5B**), whilst 25(OH)D3 correlated with 24,25$(OH)_2$D3 and 1,25$(OH)_2$D3 in serum only. The relationship between 24,25$(OH)_2$D3 and 1,25$(OH)_2$D3 showed a positive correlation between these metabolites in RA serum and HC serum, but not RA SF (**Figure 4.24**).

4.3.3 Serum and SF vitamin D metabolites and disease activity in RA

Vitamin D metabolites 25(OH)D3, 25(OH)D2, 3-epi-25(OH)D3, 24,25$(OH)_2$D3 and 1,25$(OH)_2$D3 in RA serum and SF were correlated with RA disease markers CRP (C-reactive protein), ESR (erythrocyte sedimentation rate), SJC (swollen joint count) and TJC (tender joint count). Serum 25(OH)D2 correlated directly with serum levels of CRP (**Figure 4.6**). Serum 25(OH)D2 also correlated with SJC (**Figure 4.8**). In SF, 25(OH)D3 and 3-epi-25(OH)D3 both correlated inversely with SJC (**Figure 4.8**). Other metabolites did not show significant correlation with RA disease markers in serum or SF. The relationship between serum or SF 25(OH)D3, 25(OH)D2, 3-epi-25(OH)D3, 24,25$(OH)_2$D3 and 1,25$(OH)_2$D3 and markers of RA disease (CRP, ESR, SJC and TJC) in RA patients is shown in full in **Figures 4.6-4.9**.

4.3.4 Serum and SF vitamin D binding protein, albumin and free vitamin D

The abundance of DBP and albumin in RA and ReA serum and SF samples was quantified to gain insight into the role of DBP and albumin in mediating free and bioavailable vitamin D in an inflammatory context. Concentrations of DBP were significantly lower in SF (median 33 with 95% confidence limits 24.37-43.19) vs paired serum (median 117 with 95% confidence limits 95.38-149.8) in both RA (median 85.66 with 95% confidence limits 68.48-103.1, $p<0.0001$) and ReA (median 35.82 with 95% confidence limits 19.25-77.98, $p=0.0156$) patients (**Figure 4.10**). Albumin concentrations were also significantly lower in RA SF (median 1.423 with 95% confidence limits 1.398-1.712) vs paired serum (median 2.22 with 95% confidence limits 2.054-2.602, $p=0.0005$). Albumin concentration was significantly higher in ReA SF compared to RA SF ($p=0.0195$); this was not significant in ReA serum ($p=0.61$).

Based on the quantification of DBP and albumin levels and total 25(OH)D3 values it was possible to calculate free 25(OH)D3 (not bound to DBP or albumin) and bioavailable 25(OH)D3 (not bound to DBP) for serum and SF samples (**Figure 4.25**). These values showed no significant difference between RA and ReA serum. However, in contrast to the decreased total serum 25(OH)D3 observed for SF versus paired serum (see **Figure 4.3**), free 25(OH)D3 levels were significantly higher in RA SF compared to RA serum ($p=0.0046$). Bioavailable 25(OH)D3 showed no significant variations in RA vs ReA for either serum or SF.

Correlating DBP and albumin with RA disease markers, serum DBP was positively correlated with CRP and inversely correlated with SJC, but only in the SF of RA patients (**Figure 4.11**). None of the other disease markers showed significant

correlation with DBP concentrations in either serum or SF. Albumin was not significantly correlated with any RA disease markers (**Figure 4.12**).

4.3.5 Serum and SF miRNAs in RA and ReA

The differential expression of specific inflammatory miRNAs, in particular miR-146a and miR-155, has previously been reported in RA patient serum and SF [432, 433]. Current data showed no significant difference in miR-146a expression between RA and ReA serum vs SF (**Figure 4.13**). MiR-155 expression was significantly higher in the SF of RA, but not ReA, patients compared to matched serum (p=0.0348). The relationship between the expression of these miRNAs and RA disease activity was assessed by linear correlation analysis with RA disease markers CRP, ESR, SJC and TJC, in both serum and SF of RA patients. Neither miR-146a nor miR-155 showed statistically significant correlations with any of the disease markers (**Figure 4.15**). The relationship between serum and SF miRNAs and vitamin D metabolites was also assessed and all correlation graphs shown in **Figures 4.16-4.19**, in both RA and ReA patient cohorts. MiR-155 was significantly correlated (p=0.0287) with 3-epi-25(OH)D3 in RA serum (**Figure 4.16**). There was a significant inverse correlation between serum miR-146a and serum 25(OH)D2 (p=0.0012) but only in ReA patients (**Figure 4.18**). Similarly, serum miR-155 also showed a significant inverse correlation with serum 25(OH)D2 (p=0.0011) only in ReA patients (**Figure 4.19**).

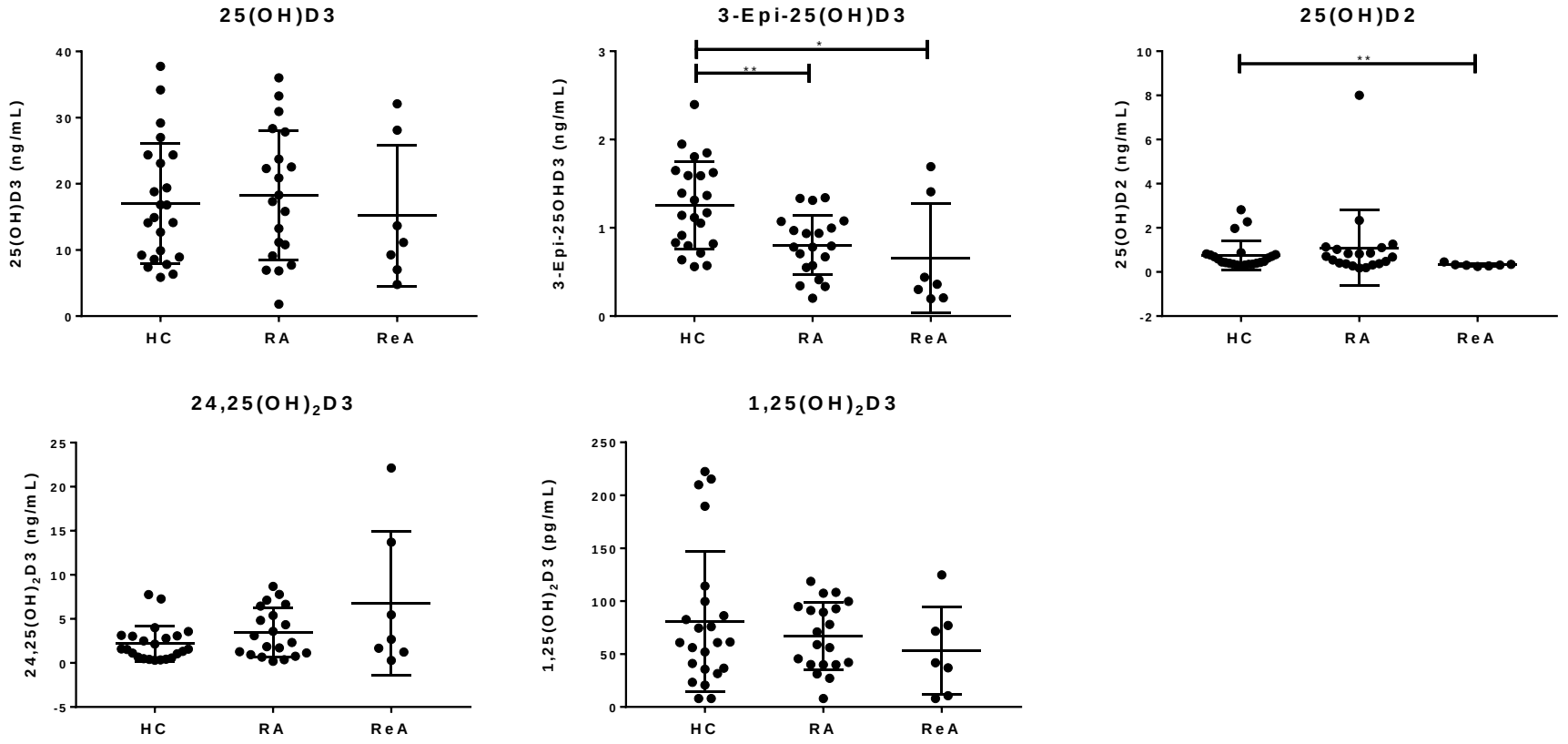


Figure 4.2. Rheumatoid arthritis and the serum vitamin D metabolome
Serum from n=20 persistent RA (RA) patients, n=7 reactive arthritis (ReA) patients and n=23 healthy controls (HC) were analysed by LC-MS/MS for: 25(OH)D3 (ng/mL); 3-epi-25(OH)D3 (ng/mL); 25(OH)D2 (ng/mL); 24,25$(OH)_2$D3 (ng/mL); 1,25$(OH)_2$D3 (pg/mL). * = significantly different HC vs RA or ReA, *p<0.05. Mann-Whitney t-test for unpaired samples was performed for statistical analysis.

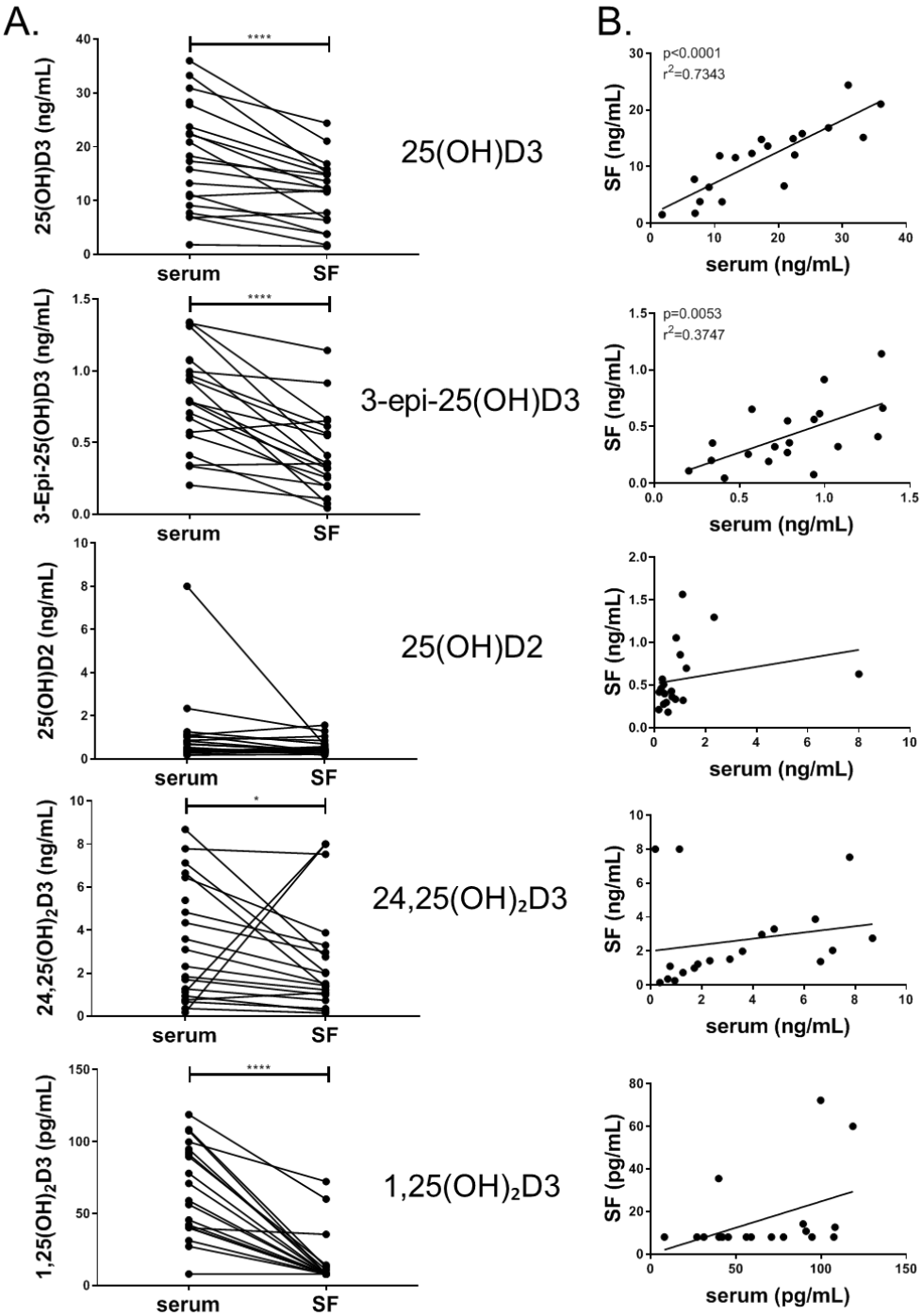


Figure 4.3. Serum vs SF vitamin D metabolites in patients with persistent RA

A) Comparison of serum and synovial fluid (SF) concentrations for: 25(OH)D3; 3-epi-25(OH)D3; 25(OH)D2; 24,25$(OH)_2$D3 (ng/mL); 1,25$(OH)_2$D3 (pg/mL). B) Correlation of serum and SF concentrations for: 25(OH)D3; 3-epi-25(OH)D3; 25(OH)D2; 24,25$(OH)_2$D3 (all ng/mL); 1,25$(OH)_2$D3 (pg/mL). n=20 matched for serum and SF (One-way ANOVA). Linear regression was performed by best-fit values ± SD. Coefficient of determination (r^2) values are shown for correlations that were statistically significant, *p<0.05.

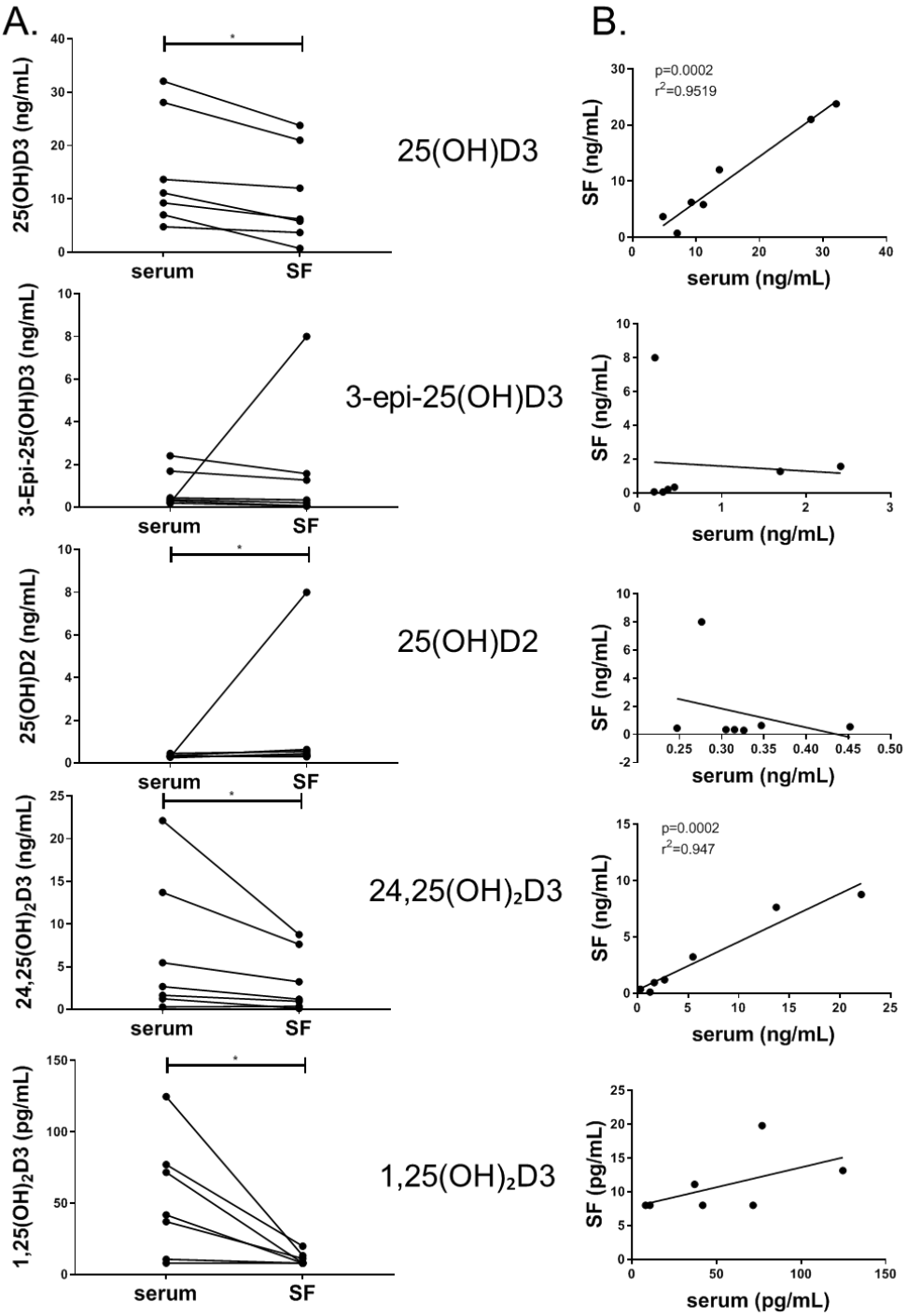


Figure 4.4. Serum vs SF vitamin D metabolites in patients with reactive arthritis (ReA)

A) Comparison of serum and synovial fluid (SF) concentrations for: 25(OH)D3; 3-epi-25(OH)D3; 25(OH)D2; 24,25$(OH)_2$D3 (ng/mL); 1,25$(OH)_2$D3 (pg/mL). B) Correlation of serum and SF concentrations for: 25(OH)D3; 3-epi-25(OH)D3; 25(OH)D2; 24,25$(OH)_2$D3 (all ng/mL); 1,25$(OH)_2$D3 (pg/mL). n=7 matched for serum and SF (One-way ANOVA). Linear regression was performed by best-fit values ± SD. Coefficient of determination (r^2) values are shown for correlations that were statistically significant, **p*<0.05.

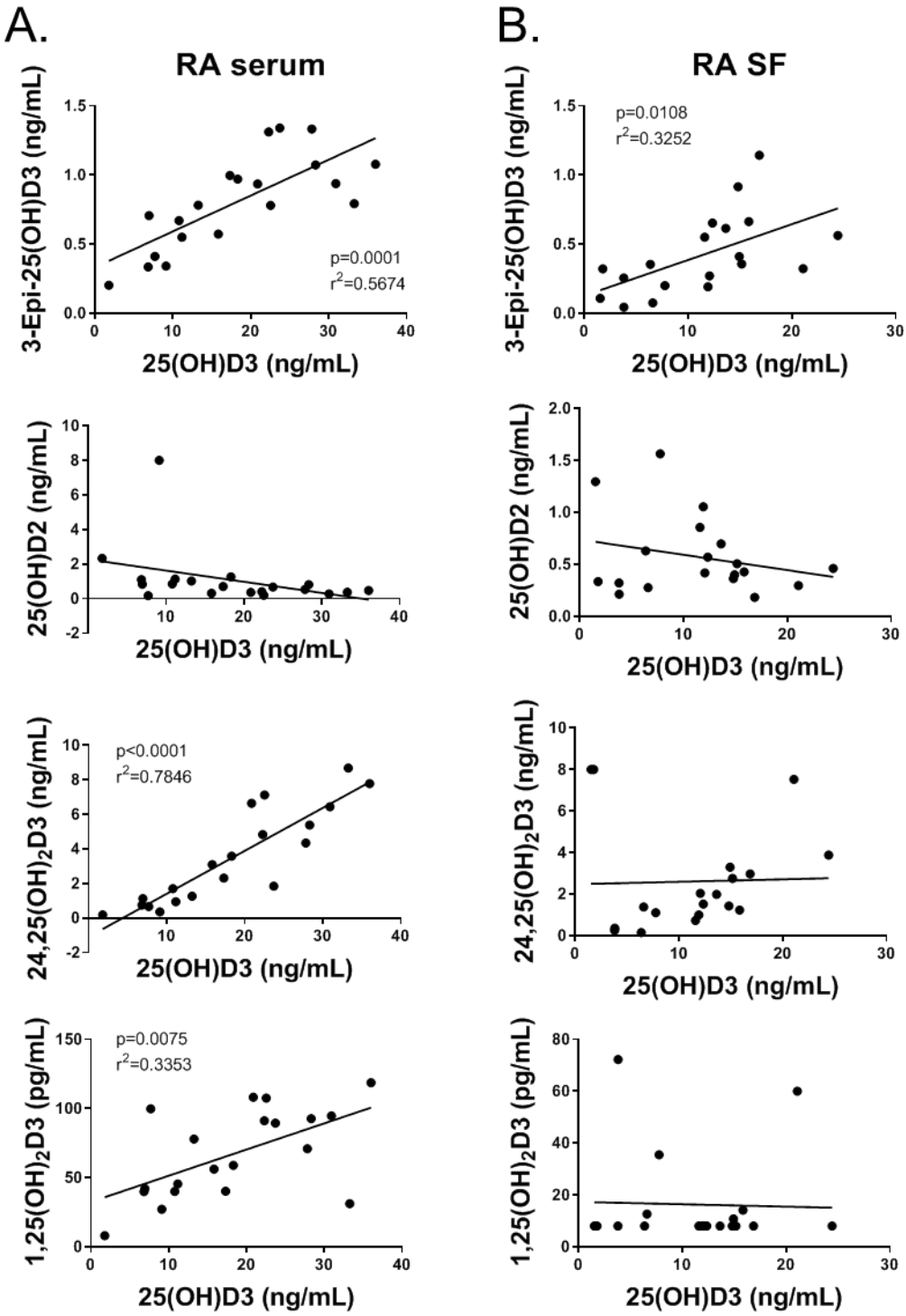


Figure 4.5. Vitamin D metabolite correlations in RA patient serum and SF
Correlation between 25(OH)D3 and other vitamin D metabolites from persistent rheumatoid arthritis (RA) patients in A) serum; B) SF. Linear regression was performed by best-fit values ± SD. Coefficient of determination (r^2) values are shown for correlations that were statistically significant, **p*<0.05.

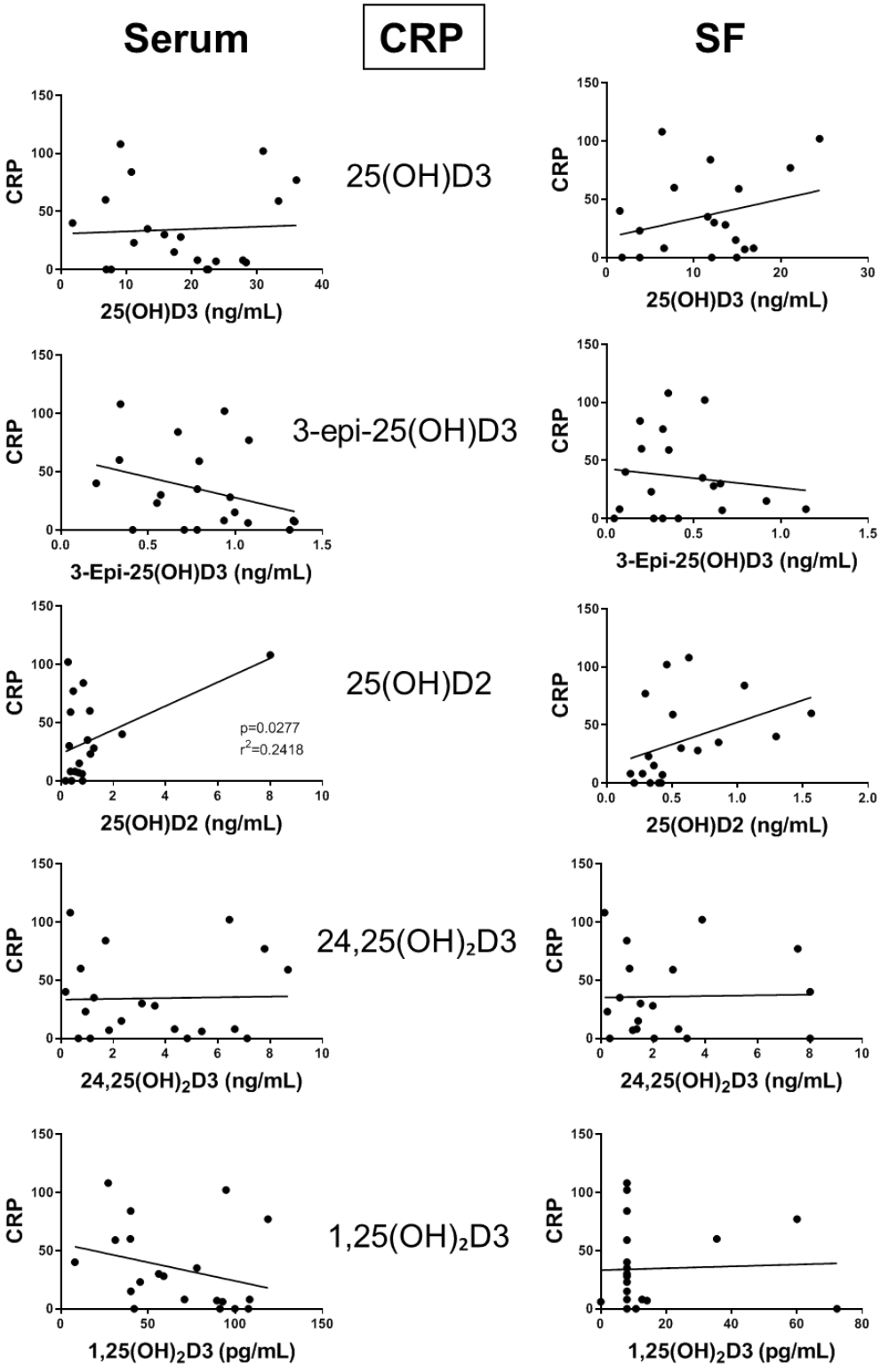


Figure 4.6. Correlation of the RA disease activity marker CRP with serum/SF vitamin D metabolites

Correlation of C-reactive protein (CRP) with 25(OH)D3; B) 3-epi-25(OH)D3; C) 25(OH)D2; D) $24,25(OH)_2D3$; E) $1,25(OH)_2D3$ in serum and SF from RA patients. Linear regression was performed by best-fit values ± SD. Coefficient of determination (r^2) values are shown for correlations that were statistically significant, **p*<0.05.

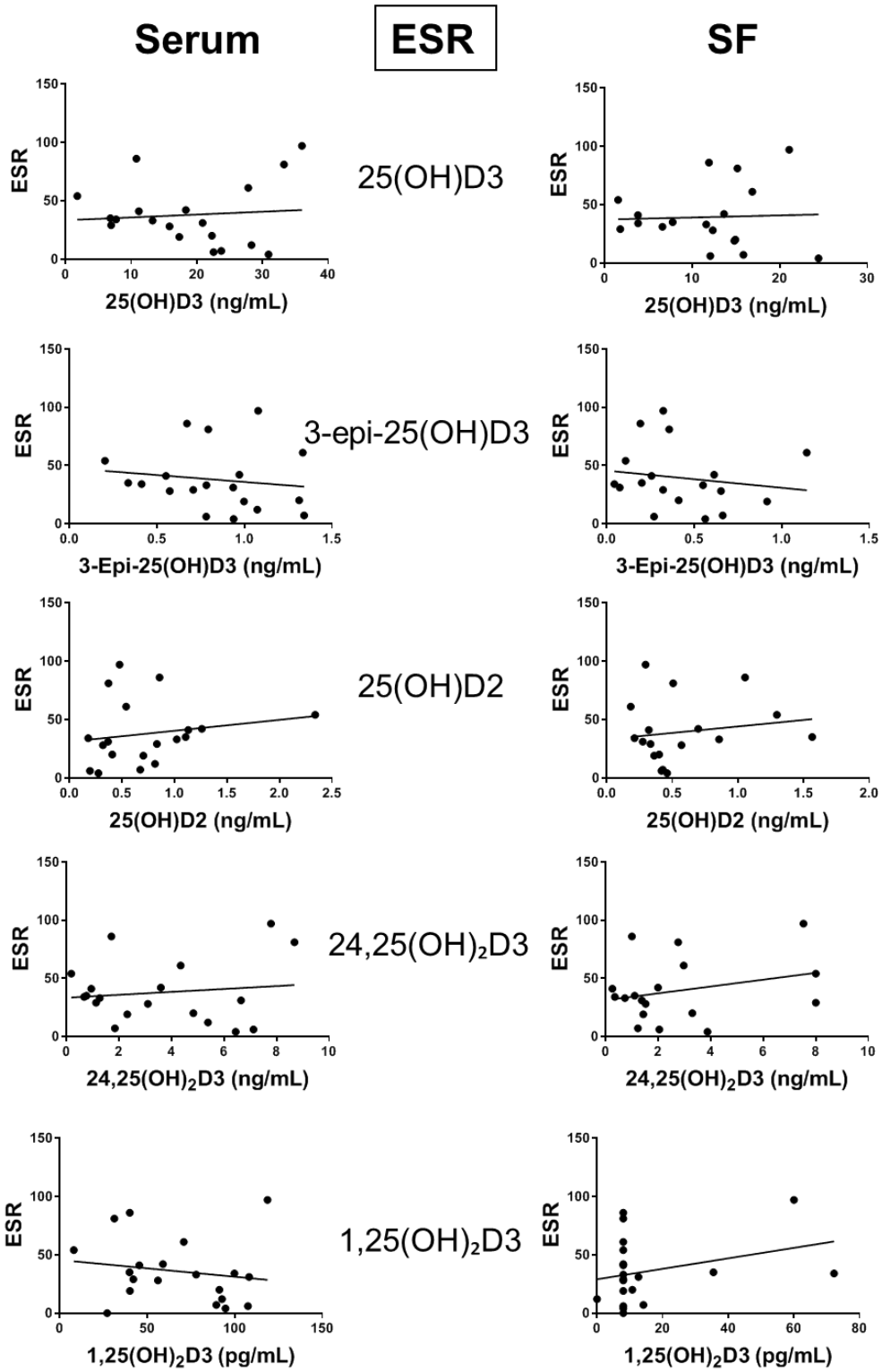


Figure 4.7. Correlation of the RA disease activity marker ESR with serum/SF vitamin D metabolites

Correlation of erythrocyte sedimentation rate (ESR) with 25(OH)D3; B) 3-epi-25(OH)D3; C) 25(OH)D2; D) 24,25$(OH)_2$D3; E) 1,25$(OH)_2$D3 in serum and SF from RA patients. Linear regression was performed by best-fit values ± SD. Coefficient of determination (r^2) values are shown for correlations that were statistically significant, **p*<0.05.

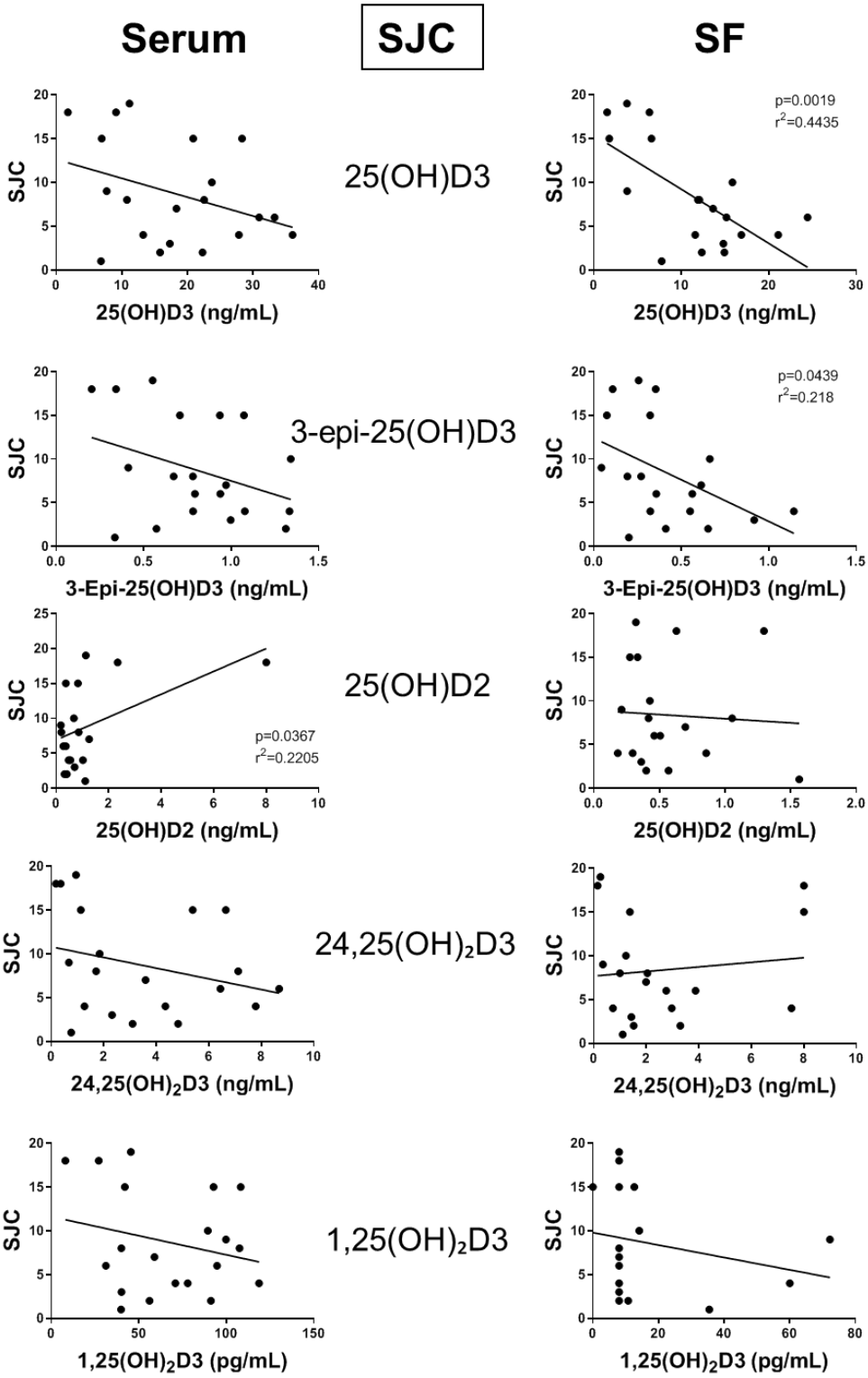


Figure 4.8. Correlation of the RA disease activity marker SJC with serum/SF vitamin D metabolites

Correlation of swollen joint count (SJC) with 25(OH)D3; B) 3-epi-25(OH)D3; C) 25(OH)D2; D) 24,25$(OH)_2$D3; E) 1,25$(OH)_2$D3 in serum and SF from RA patients. Linear regression was performed by best-fit values ± SD. Coefficient of determination (r^2) values are shown for correlations that were statistically significant, **p*<0.05.

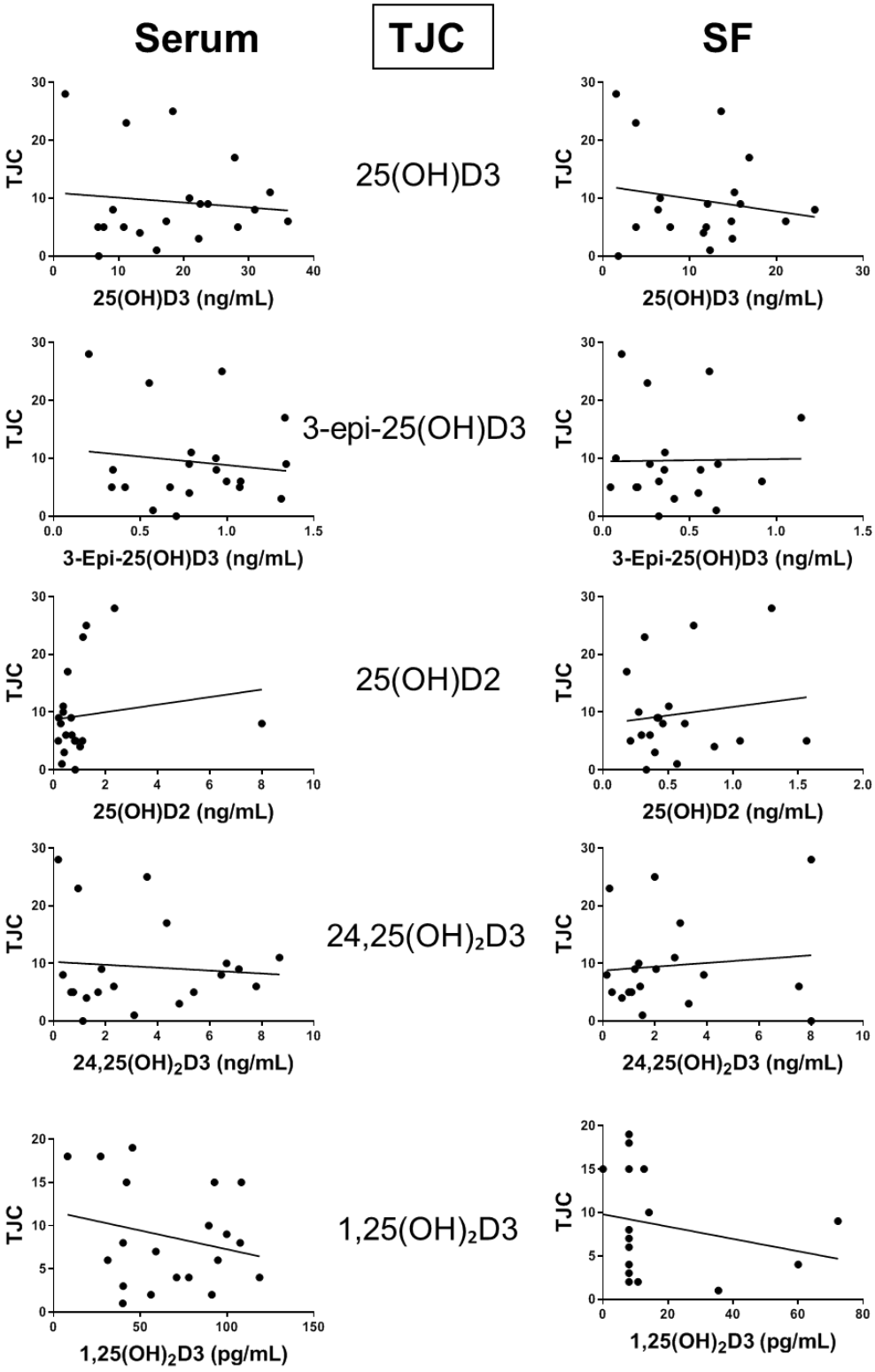


Figure 4.9. Correlation of the RA disease activity marker TJC with serum/SF vitamin D metabolites

Correlation of tender joint count (TJC) with 25(OH)D3; B) 3-epi-25(OH)D3; C) 25(OH)D2; D) 24,25$(OH)_2$D3; E) 1,25$(OH)_2$D3 in serum and SF from RA patients. Linear regression was performed by best-fit values ± SD. Coefficient of determination (r^2) values are shown for correlations that were statistically significant, *p<0.05.

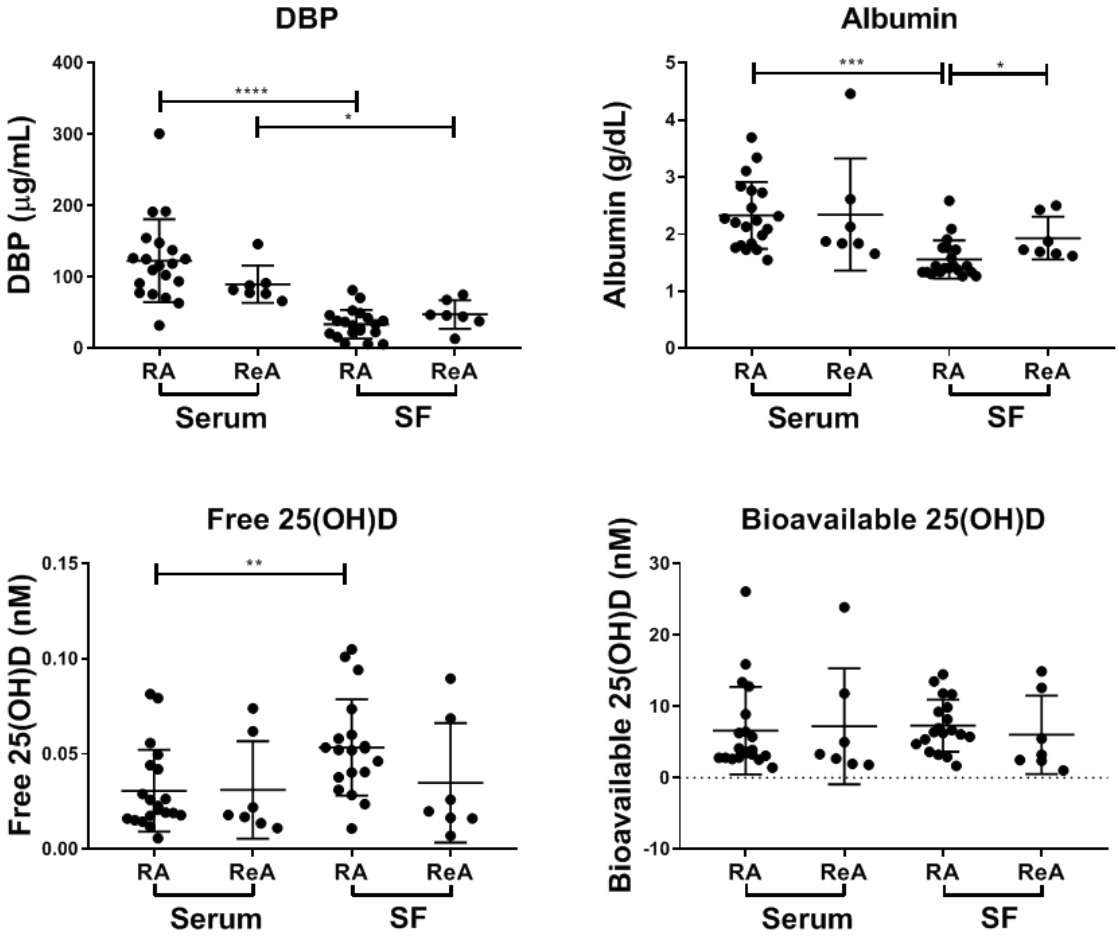


Figure 4.10. DBP, albumin and free versus bioavailable 25(OH)D in serum and SF
Serum and SF from n=20 RA and n=7 ReA patients were used to measure concentrations of DBP and albumin, and calculate free and bioavailable 25(OH)D. Statistical significance is taken as *p<0.05. Wilcoxon matched-pairs signed rank test was performed for matched serum vs SF samples. Mann-Whitney unpaired *t*-test was performed for non-matched samples.

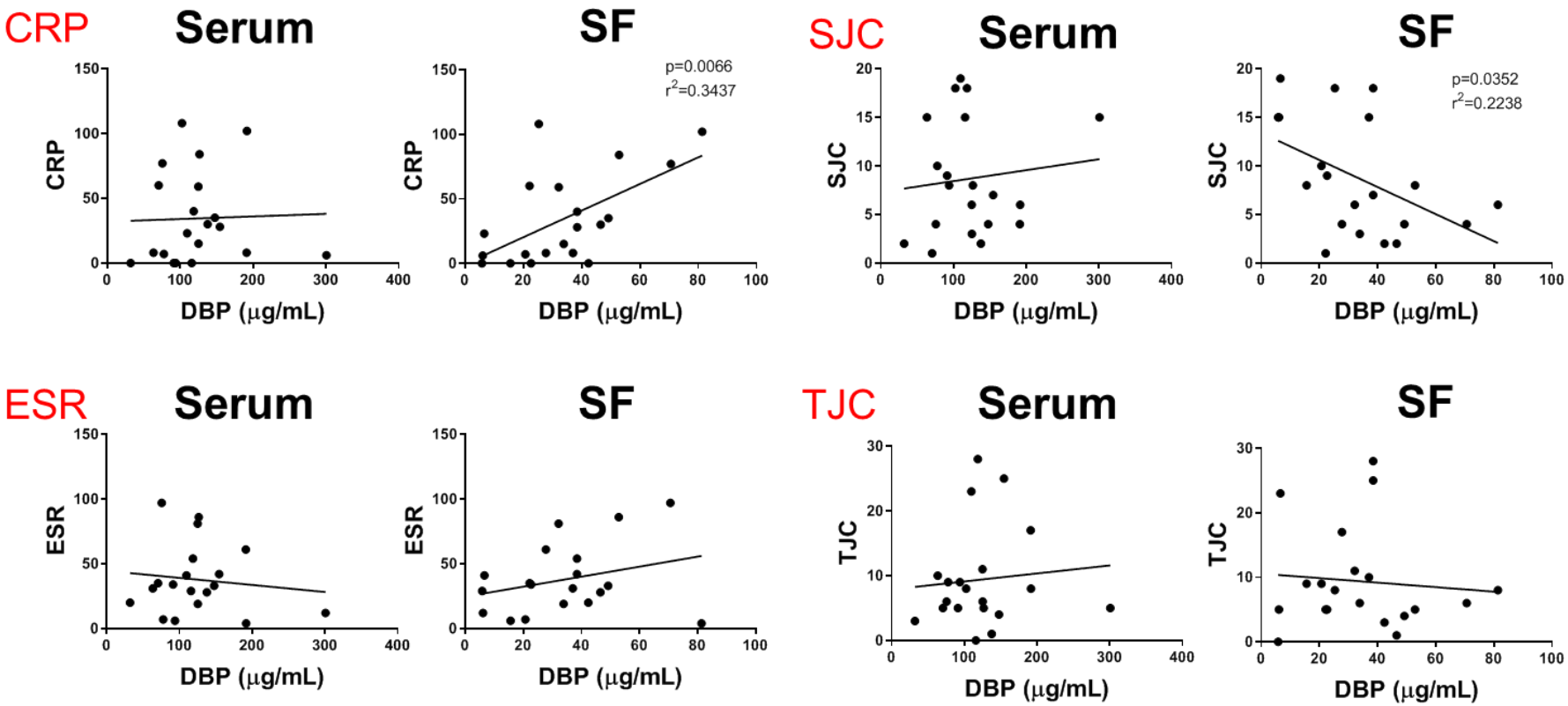


Figure 4.11. Correlations between DBP and RA disease activity markers in RA serum and SF
CRP, ESR, SJC and TJC measurements in matched RA serum and SF were correlated against concentrations of DBP. Linear regression was performed by best-fit values ± SD. Coefficient of determination (r^2) values are shown for correlations that were statistically significant, **p*<0.05.

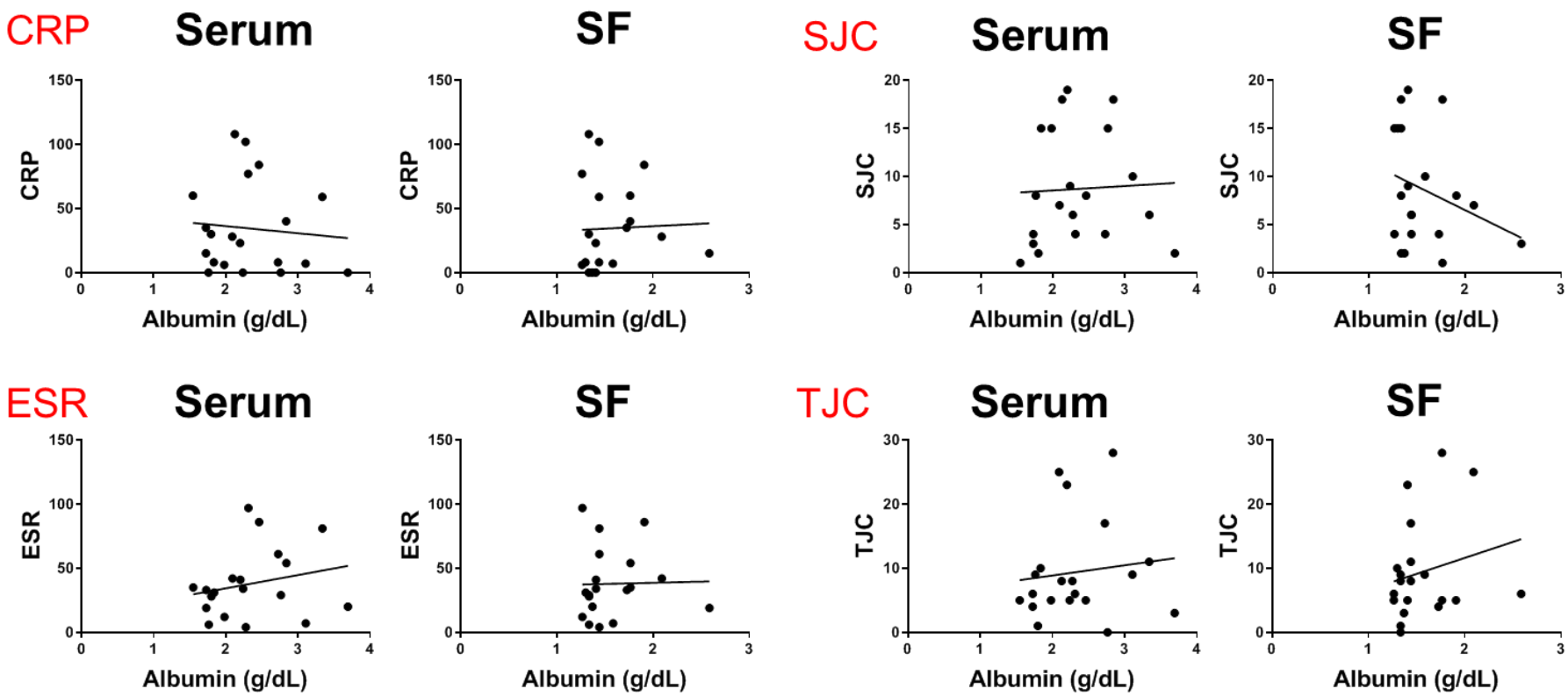


Figure 4.12. Correlations between albumin and RA disease activity markers in RA serum and SF
CRP, ESR, SJC and TJC measurements in matched RA serum and SF were correlated against concentrations of albumin. Linear regression was performed by best-fit values ± SD. Coefficient of determination (r^2) values are shown for correlations that were statistically significant, *p<0.05.

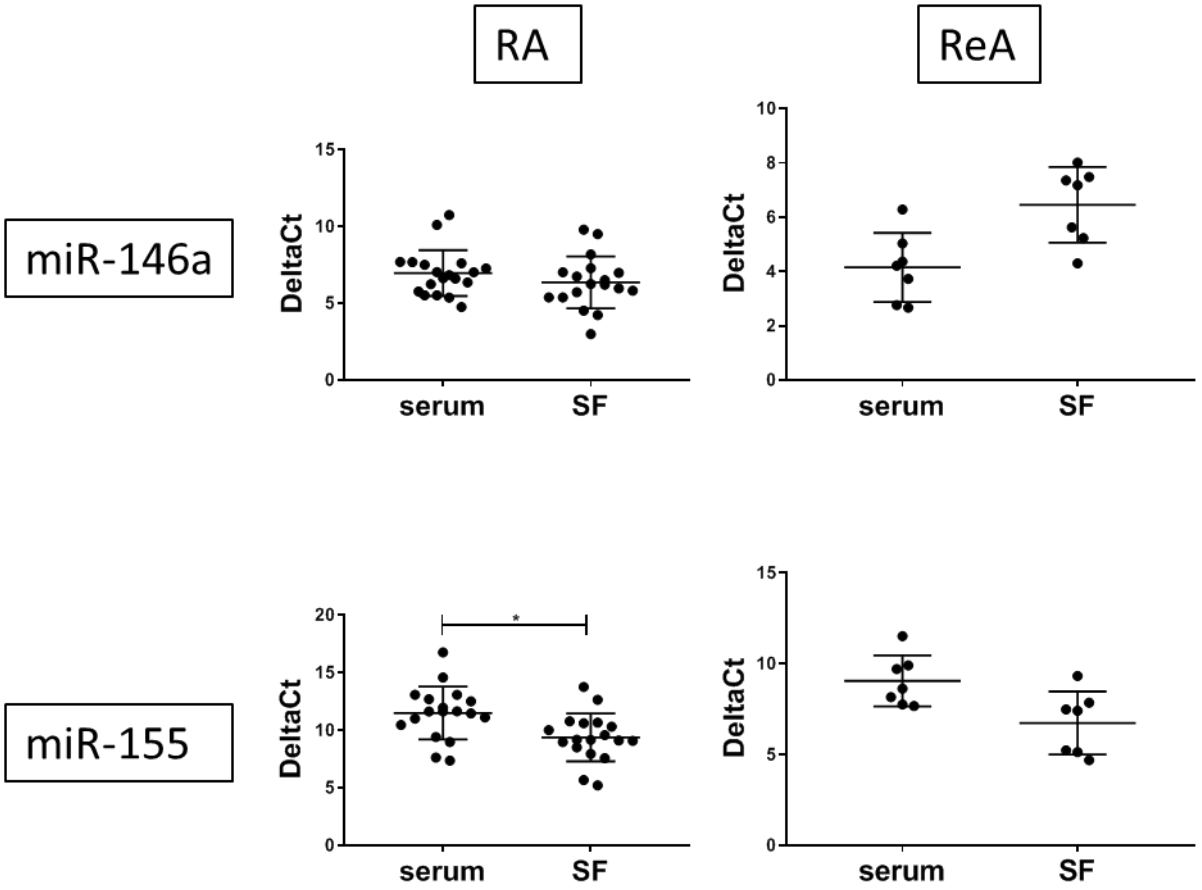


Figure 4.13. miR-146a and miR-155 expression in serum and SF
MiR-146a and miR-155 expression in RA (n=20) and ReA (n=7) matched serum vs SF. Serum vs SF comparison performed by Wilcoxon test for paired samples. Significance is taken as *p<0.05 and shown where determined.

miR-146a

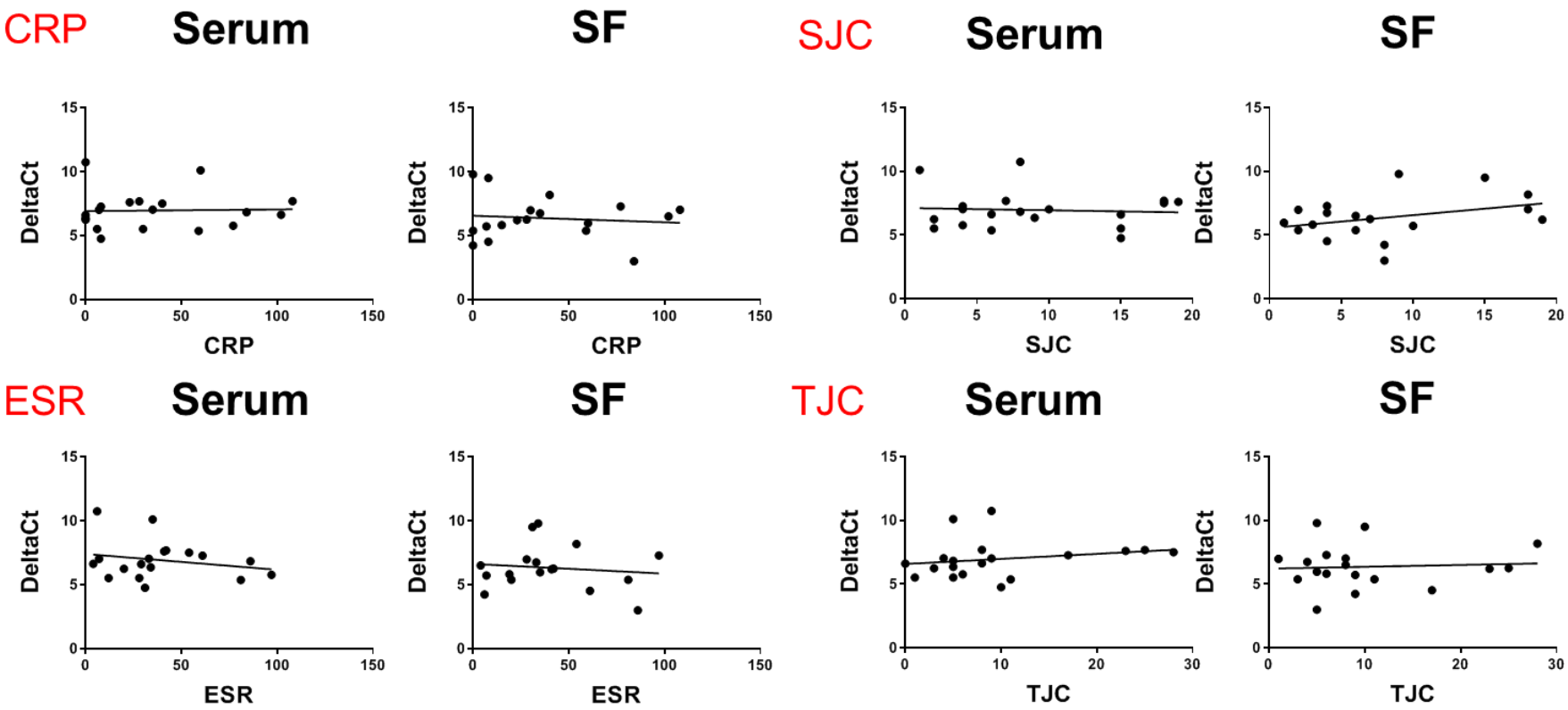


Figure 4.14. miR-146a expression vs disease activity markers, in serum and SF
CRP, ESR, SJC and TJC measurements in matched RA serum and SF were correlated against miR-146a expression. Linear regression was performed by best-fit values ± SD. Coefficient of determination (r^2) values are shown for correlations that were statistically significant, *p<0.05.

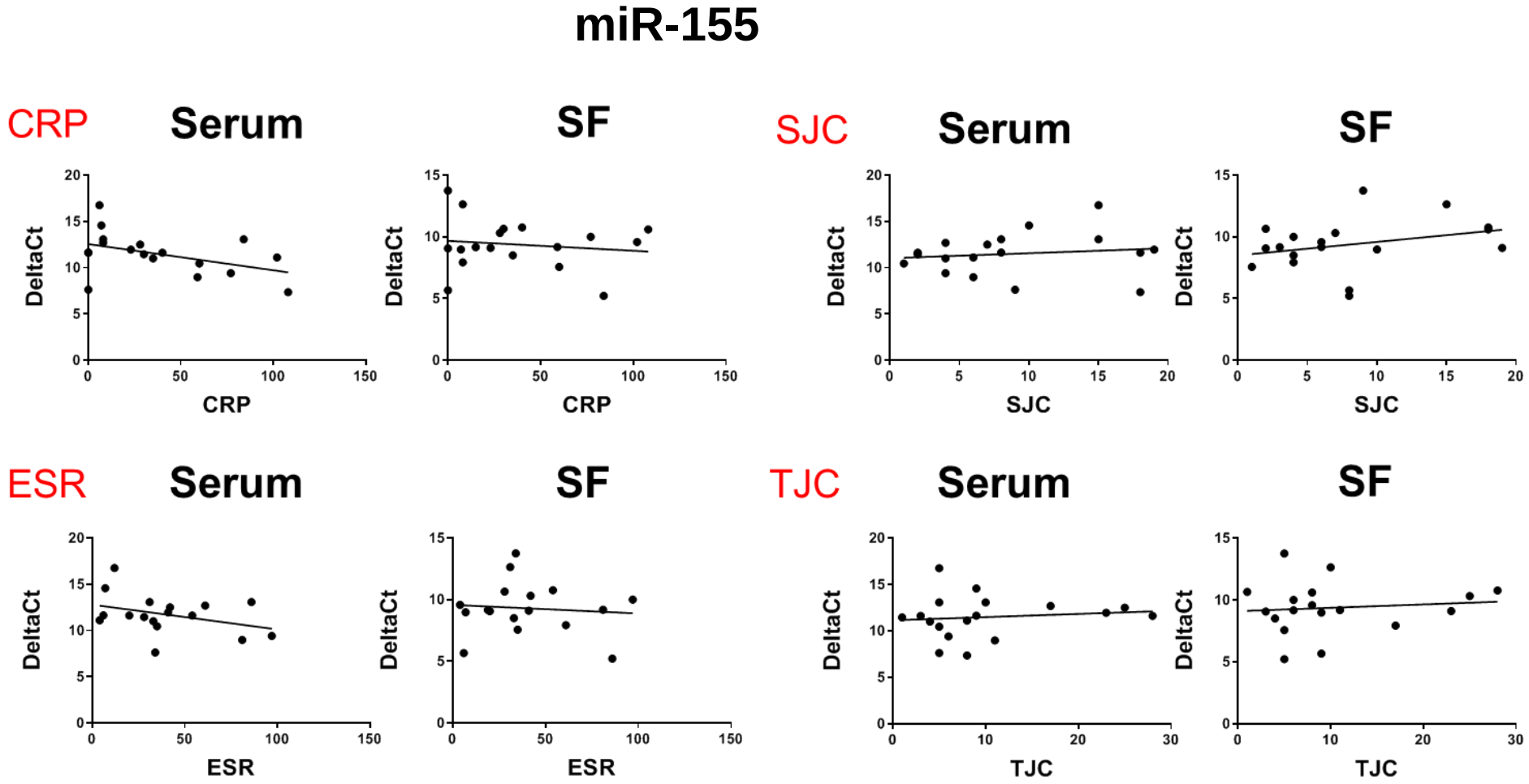


Figure 4.15. miR-155 expression vs disease activity markers, in serum and SF
CRP, ESR, SJC and TJC measurements in matched RA serum and SF were correlated against miR-155 expression. Linear regression was performed by best-fit values ± SD. Coefficient of determination (r^2) values are shown for correlations that were statistically significant, **p*<0.05.

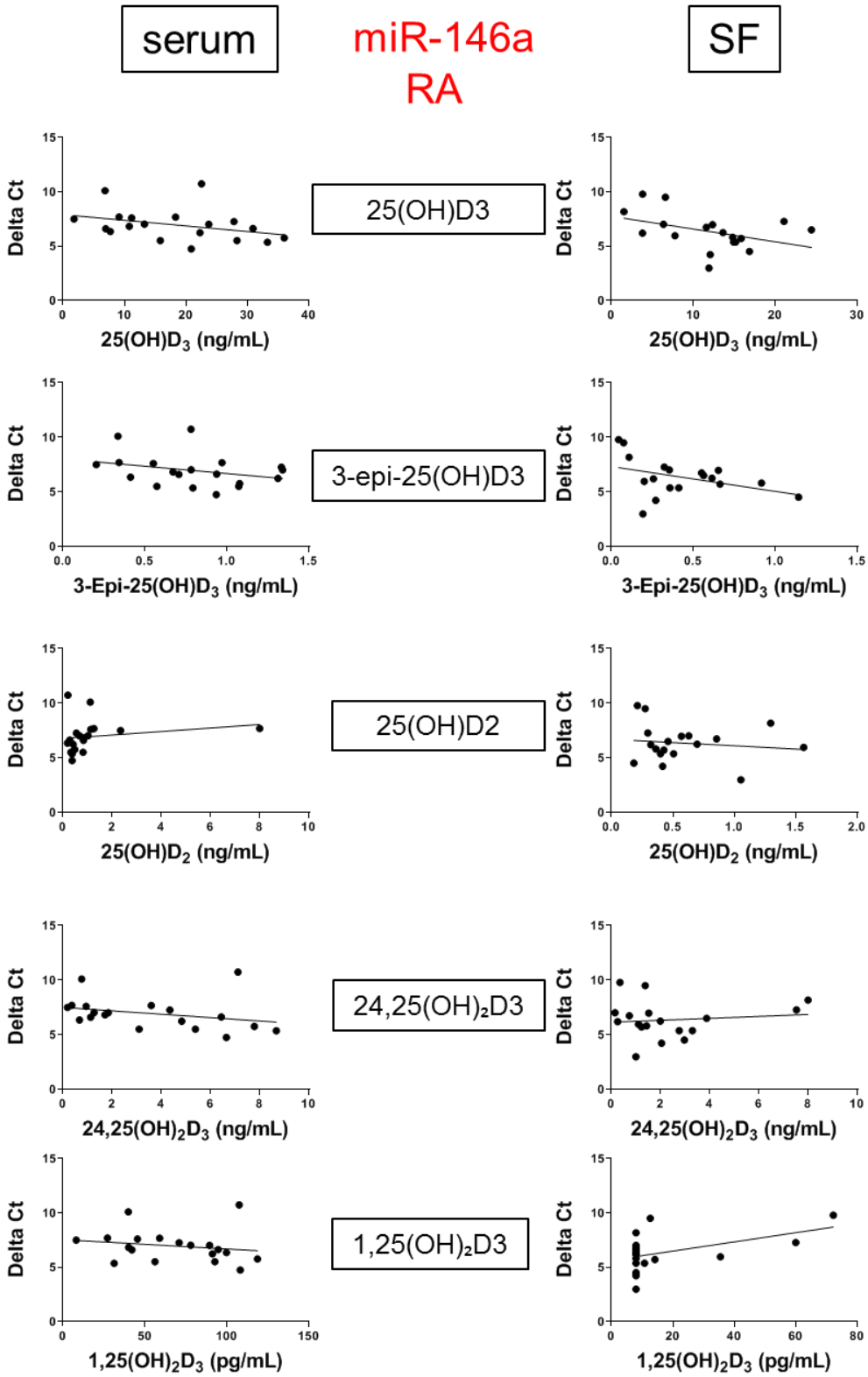


Figure 4.16. miR-146a expression vs vitamin D metabolites, in RA serum and SF
Correlation of miR-146a with A) 25(OH)D3; B) 3-epi-25(OH)D3; C) 25(OH)D2; D) $24,25(OH)_2D3$; E) $1,25(OH)_2D3$ in serum and SF from RA patients. Linear regression was performed by best-fit values ± SD. Coefficient of determination (r^2) values are shown for correlations that were statistically significant, **p*<0.05.

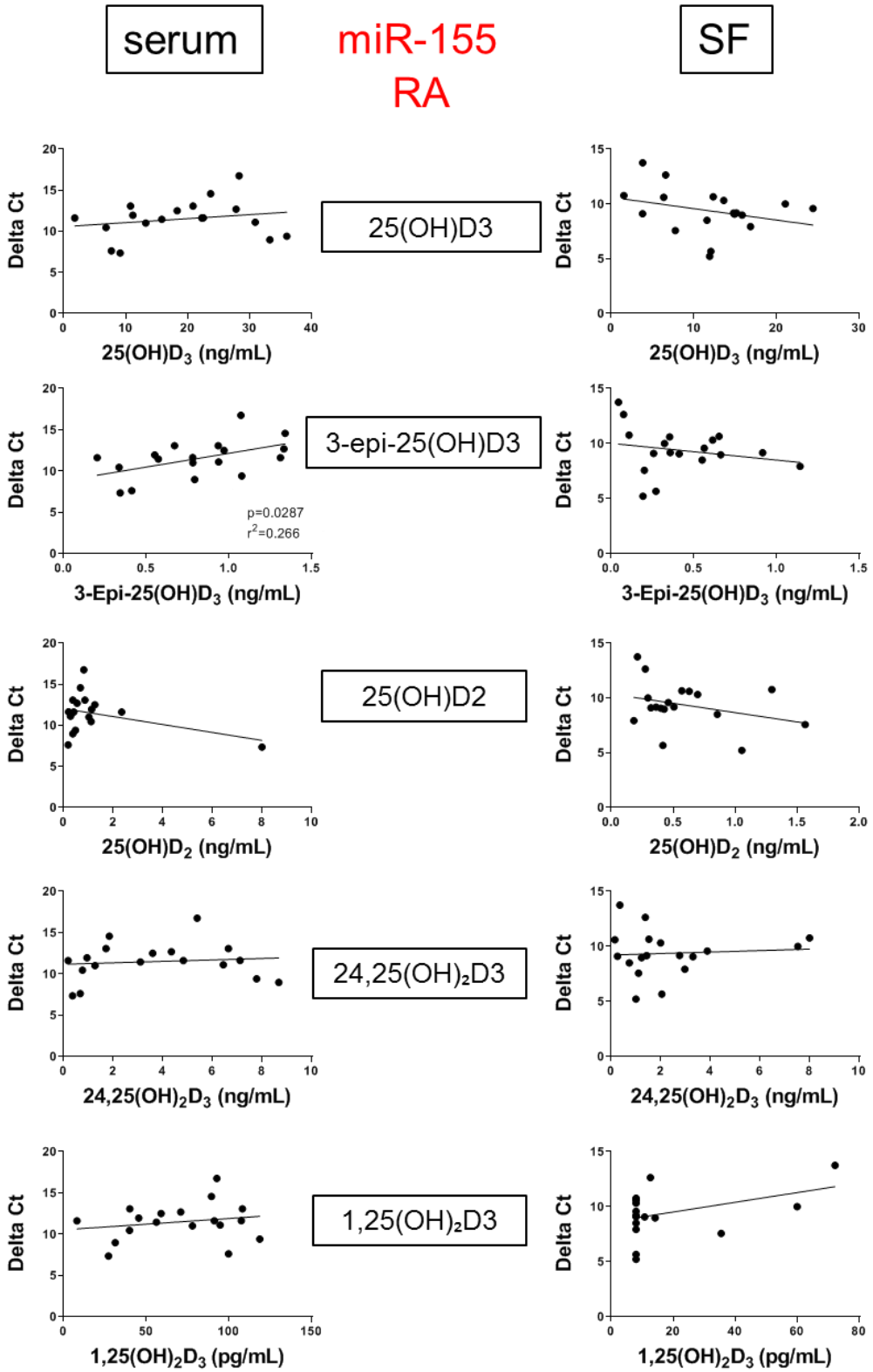


Figure 4.17. miR-155 expression vs vitamin D metabolites, in RA serum and SF
Correlation of miR-155 with A) 25(OH)D3; B) 3-epi-25(OH)D3; C) 25(OH)D2; D) 24,25$(OH)_2$D3; E) 1,25$(OH)_2$D3 in serum and SF from RA patients. Linear regression was performed by best-fit values ± SD. Coefficient of determination (r^2) values are shown for correlations that were statistically significant, **p*<0.05.

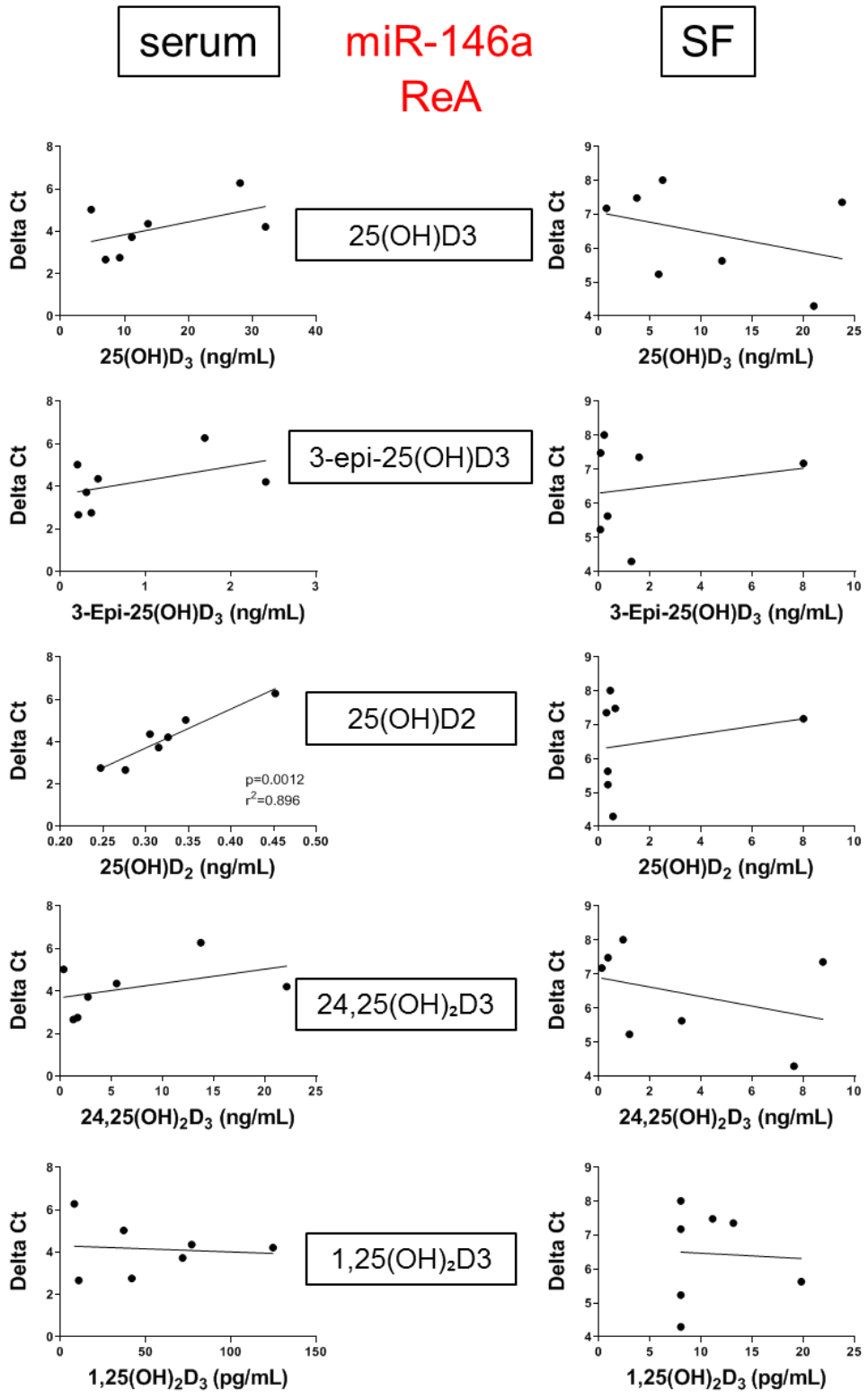


Figure 4.18. miR-146a expression vs vitamin D metabolites, in ReA serum and SF
Correlation of miR-146a with A) 25(OH)D3; B) 3-epi-25(OH)D3; C) 25(OH)D2; D) $24,25(OH)_2D3$; E) $1,25(OH)_2D3$ in serum and SF from ReA patients. Linear regression was performed by best-fit values ± SD. Coefficient of determination (r^2) values are shown for correlations that were statistically significant, *p<0.05.

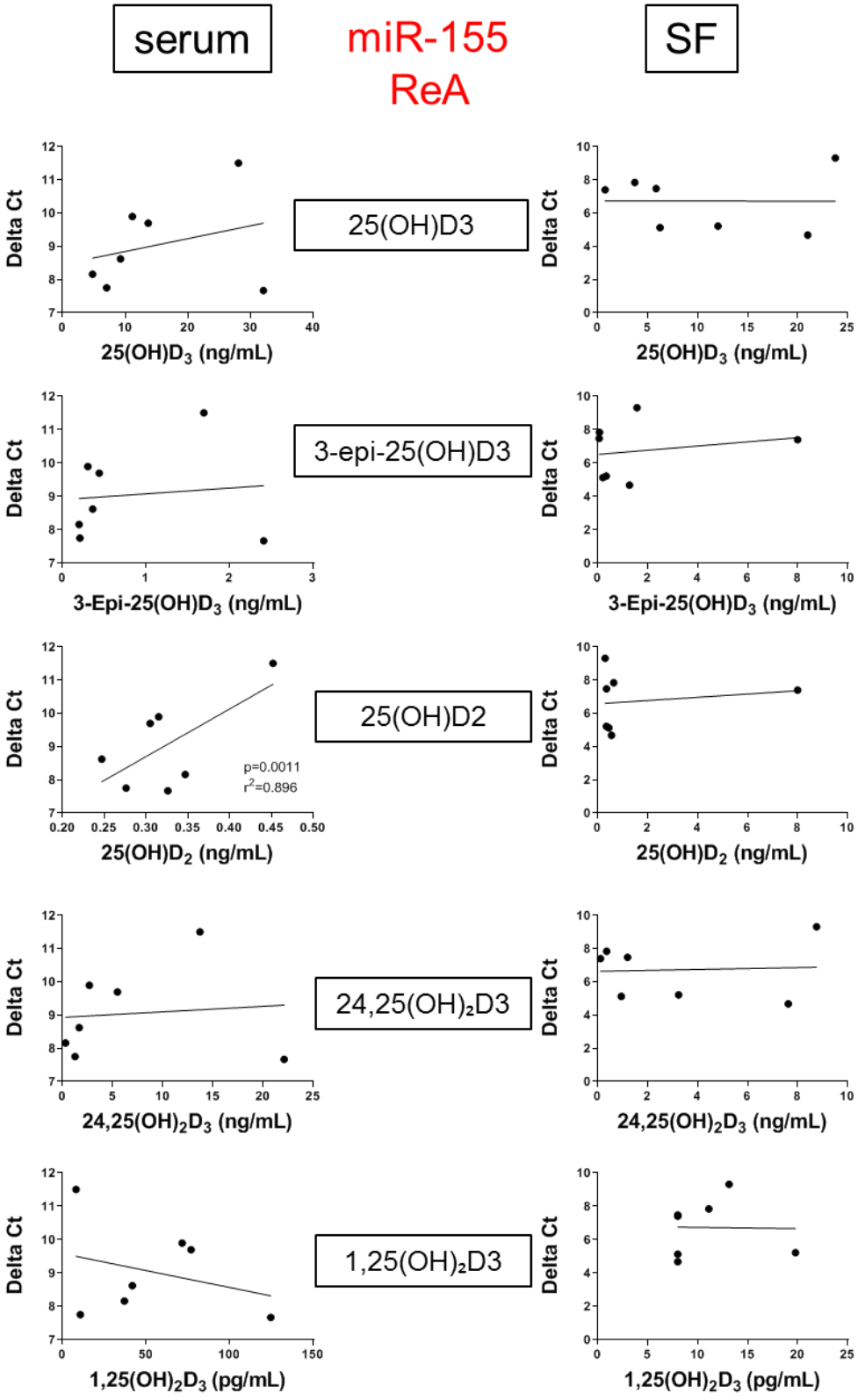


Figure 4.19. miR-155 expression vs vitamin D metabolites, in ReA serum and SF
Correlation of miR-155 with A) 25(OH)D3; B) 3-epi-25(OH)D3; C) 25(OH)D2; D) $24,25(OH)_2D3$; E) $1,25(OH)_2D3$ in serum and SF from ReA patients. Linear regression was performed by best-fit values ± SD. Coefficient of determination (r^2) values are shown for correlations that were statistically significant, *p<0.05.

4.4 Discussion

4.4.1 The serum and SF vitamin D metabolome in health and inflammation

The aim of the work carried out in this chapter was to determine if vitamin D metabolites other than 25(OH)D, including active 1,25$(OH)_2$D3, could be better suited to predicting the activity of RA. Current literature has shown that low serum concentrations of 25(OH)D3 are a feature of inflammatory diseases such as RA [104, 311, 341, 434] although this is not a universal observation [104]. Consistent with the latter report, data from the current study showed no significant difference between serum 25(OH)D3 concentration in RA or ReA serum compared to HC serum (**Figure 4.2**). However, it is important to recognize that in the cohorts analysed in our study, the median serum concentrations for 25(OH)D3 were relatively low for all three of the groups we studied (14.9 ng/mL, 17.8 ng/mL, 11.1 ng/mL for HC, RA, ReA respectively). Only 10 of the RA/ReA patients and 7 HC subjects, had serum levels of 25(OH)D3 >20 ng/mL (50 nmol/L) which is the vitamin D-sufficiency level proposed by the Institute of Medicine [56]. Only 6 of all 47 samples had serum 25(OH)D3 levels >30 ng/mL (75 nmol/L), the optimal levels recommended by the US Endocrine Society [435]. Interestingly, 16 of the total 47 samples measured (34%) had serum 25(OH)D3 levels <10 ng/mL (25 nmol/L), the target level for minimum vitamin D status recommended by the UK Science Advisory Council on Nutrition [58], indicating that severe vitamin D-deficiency was common to all of the groups. As such, increased rates of 25(OH)D deficiency in RA patients were unlikely to be observed. With these observations in mind it is clear that larger studies are required to better understand the scale of low vitamin D status in RA patients from the UK, but the data

in this study also suggest that measurement of serum 25(OH)D alone has limited value in assessing diseases such as RA.

Notably the one vitamin D metabolite that was significantly lower in serum from RA and ReA patients relative to healthy controls was 3-epi-25(OH)D3. The physiological importance of C-3 epimerisation has yet to be elucidated, but all of the vitamin D metabolites can be converted to 3-epi forms with varying degrees of activity [436]. The 3-epi form of $1,25(OH)_2D3$ can bind to the VDR and induce transcription [437], but the impact of 3-epi-25(OH)D3 and other 3-epi vitamin D metabolites on human physiology and diseases such as RA is still unclear. It was previously thought that 3-epi-25(OH)D3 was only present in serum from neonates, however the use of recent improved LC-MS/MS detection methods has found 3-epi-25(OH)D3 present in most healthy adults [438, 439], at levels which vary between 0.1% and 25% of 25(OH)D3 in the same sample [438]. These studies also found the concentration of 3-epi-25(OH)D3 was positively correlated with levels of 25(OH)D3; similarly strong posititve correlations between both serum and SF 25(OH)D3 with 3-epi-25(OH)D3 were also observed in RA patient samples (**Figure 4.5A; 4.5B**). Additionally, due to the almost identical molecular structure of 25(OH)D3 and 3-epi-25(OH)D3 and the direct correlation in concentration between the two molecules, 3-epi-25(OH)D3 levels could affect measurements of 25(OH)D3 if insensitive detection methods are used. Accounting for 3-epi-25(OH)D3 when measuring vitamin D status is therefore important to avoid misclassification of patients as vitamin D sufficient [440]. The precise function of 3-epi-25(OH)D3 in vitamin D physiology is still unclear, and interpretation of data is complicated by the fact that levels of this metabolite are closely linked to serum 25(OH)D3.

In addition to a lower concentration of 3-epi-25(OH)D3 in RA and ReA serum compared to HC serum, a lower serum concentration of 25(OH)D2 in ReA patients compared to HC patients was also observed (**Figure 4.2**). The basis for this is unclear as, unlike 25(OH)D3, 25(OH)D2 is derived exclusively from dietary or supplementation sources, and the most likely explanation is that this simply reflects the small number of samples in the ReA cohort, as well as the possibility that some of the subjects were taking vitamin D2 supplements. Although levels of serum 24,25$(OH)_2$D3 and 1,25$(OH)_2$D3 were not significantly different between HC, RA or ReA cohorts, it was important to measure their concentrations in serum. As the active metabolite, 1,25$(OH)_2$D3 levels may be a more physiologically relevant measure of vitamin D status in health and disease as opposed to 25(OH)D currently measured (this is discussed in more detail in **4.4.2**). The physiological significance of 24,25$(OH)_2$D3 remains to be elucidated, however 24-hydroxylase activity may still have a significant impact on the analysis of 25(OH)D (and 1,25$(OH)_2$D3) levels [441]. In particular, genetic variations in *CYP24A1* expression are known to influence serum levels of 1,25$(OH)_2$D3 by suppressing its catabolism.

4.4.2 Vitamin D metabolites in serum versus SF

A key observation in the current study is the lower vitamin D metabolite concentrations in SF versus paired serum samples (**Figure 4.3; Figure 4.4**). For 25(OH)D3 the mean ratio of serum/SF concentrations was 1.74, whilst for 1,25$(OH)_2$D3 this was 6.07. Similar observations have been reported previously for 25(OH)D3 [380, 442], although levels of 25(OH)D3 in serum and SF in those studies varied considerably. By contrast, previous analysis of 1,25$(OH)_2$D3 in RA patients

reported similar [443] or modestly suppressed [380] concentrations in SF relative to serum. One possible explanation for the significantly lower levels of SF $1,25(OH)_2D3$ in the current study could be the use of more reliable LC-MS/MS technology for quantification of $1,25(OH)_2D3$, compared to the relatively non-specific radioreceptor [443] or radioimmunoassays [380] used to measure $1,25(OH)_2D3$ in the previous studies. Previous studies also reported strong correlations between serum and SF $1,25(OH)_2D3$, and between SF 25(OH)D3 and SF $1,25(OH)_2D3$ [380], which was not observed in the current study. The inference from previous studies is that inflammatory factors promote enhanced extra-renal synthesis of $1,25(OH)_2D3$ via immune cell activity of 1α-hydroxylase [385]. Based on this model, enhanced vitamin D status (higher serum levels of 25(OH)D3), leads to increased SF 25(OH)D3, and this in turn promotes SF production of $1,25(OH)_2D3$. Data in the current study do not appear to support this mechanism, with $1,25(OH)_2D3$ being below the lower limit of LC-MS/MS quantification (<8.00 pg/mL) in 13/20 RA SF samples, and 4/7 ReA samples. This may reflect the efficacy of the anti-inflammatory therapies currently used to manage RA, with several of the treatments used in the current study being potential inhibitors of the 1α-hydroxylase enzyme involved in SF synthesis of $1,25(OH)_2D3$ [385]. However, it is also possible that synthesis of $1,25(OH)_2D3$ within SF is tightly regulated and limited to the immediate immune microenvironment, for example the local actions of $1,25(OH)_2D$ on synoviocytes in the inflamed joint space. This would maximize anti-inflammatory responses to $1,25(OH)_2D3$, whilst limiting the potential for spill-over of $1,25(OH)_2D3$ into the general circulation.

$1,25(OH)_2D3$ was directly correlated with 25(OH)D3 in RA serum in the current study (**Figure 4.5A**); a previous study also showed that levels of these metabolites

were linked in serum, and suggested this was likely reflecting the efficiency of the vitamin D hydroxylases [444]. Whilst availability of 25(OH)D3 is necessary for $1,25(OH)_2D3$ synthesis, it is mainly 1α-hydroxylase and 24-hydroxylase activities that define the concentration of $1,25(OH)_2D3$ although these enzymes cannot be measured in serum. The same correlation between 25(OH)D3 and $1,25(OH)_2D3$ observed in serum was not seen in RA SF. This may be due to differential regulation of 1α-hydroxylase and 24-hydroxylase activities in synovial local environment versus the kidney (the main sources of circulating $1,25(OH)_2D3$), but could also be due to the lower concentration and activity of vitamin D hydroxylases in SF compared to serum [387, 445]. These studies also found levels of $1,25(OH)_2D3$ correlated with $24,25(OH)_2D3$ via the self-promoted up-regulation of 24-hydroxylase activity by synovial fibroblasts, likely in a VDR-dependent fashion.

Synovial fibroblasts and active macrophages within the SF environment can produce $1,25(OH)_2D3$ *in vivo* in an inflammatory RA setting [380, 446]; these studies proposed that local synthesis of $1,25(OH)_2D3$ is important in immunoregulation within inflamed joints. Results from the current study differed from those found in the above studies; a significant positive correlation between $24,25(OH)_2D3$ and $1,25(OH)_2D3$ was found in RA serum but not in RA SF (**Figure 4.24**), perhaps due to the reduced presence of hydroxylases in SF compared to serum as mentioned previously [445]. A strong positive correlation between $24,25(OH)_2D3$ and $1,25(OH)_2D3$ in HC serum was also observed in the current study. Overall these results showed direct correlation between 25(OH)D3 with $24,25(OH)_2D3$, where a strong correlation between these two metabolites was also found in a previous study using LC/MS analysis on RA patient serum [269]. In RA serum, but not SF, 25(OH)D3 and

1,25$(OH)_2$D3, and 24,25$(OH)_2$D3 and 1,25$(OH)_2$D3 all showed direct correlation with each other, suggesting interplay between the different enzymes involved in vitamin D metabolism, and underlining the advantage of measuring the vitamin D metabolome rather than a single vitamin D metabolite.

4.4.3 Vitamin D metabolites and RA disease activity

In common with other previous studies, the current study was unable to obtain SF from control HC subjects due to the invasive nature of sample collection. It is therefore unclear whether decreased 1,25$(OH)_2$D3 is a feature of SF in general, or whether it is specific to SF affected by inflammatory disease. It is also unclear whether SF 1,25$(OH)_2$D3 is a more informative marker of vitamin D function than more conventionally measured 25(OH)D3. In SF, 1,25$(OH)_2$D3 did not appear to correlate with markers of RA disease activity (**Figures 4.6-4.9**), whereas SF 25(OH)D3 and 3-epi-25(OH)D3 were both inversely related to swollen joint count (SJC), an important parameter for assessing RA disease activity (**Figure 4.8**). One challenge with the interpretation of data in this chapter is that measures of inflammation were made at the 'whole patient' level (i.e. systemic inflammatory markers or total joint counts) rather than at the level of the individual joint from which SF was studied and in which levels of vitamin D metabolites were measured. Indeed, markers currently used to assess RA disease activity include factors such as CRP that are circulating measures of joint inflammation. Thus, the relevance of these markers to vitamin D activity in the SF of individual inflamed joints is debatable-especially in situations where the level of arthritis activity in the sampled joint is

different from that of other joints. In support of this, no significant correlations were observed between CRP and vitamin D metabolite concentrations in SF; CRP was statistically correlated with 25(OH)D2 in RA serum, but this was likely a false positive with regard to one outlier measurement. Previous studies also found no significant correlation between vitamin D metabolites and CRP levels [447, 448]. In this study those classified as vitamin D deficient in the former study (25(OH)D <20 ng/mL), with high CRP levels, produced higher levels of pro-inflammatory cytokines relative to those with sufficient vitamin D. Overall, data from the current study linking SF 25(OH)D3 rather than 1,25$(OH)_2$D3 with RA inflammation scores would endorse a proposed intracrine model for SF metabolism of 25(OH)D3 [41], with locally synthesised 1,25$(OH)_2$D3 acting at a cellular level to promote anti-inflammatory responses and inhibit disease markers independent of serum 1,25$(OH)_2$D3.

Regarding other markers of RA disease status, erythrocyte sedimentation rate (ESR) is commonly measured to assess the degree of inflammation on a systemic level. However, ESR is measured in blood and is an indicator of systemic inflammation, thus its levels can be raised in many other inflammatory conditions. Thus measuring ESR is a useful tool to obtain an overall indication of inflammation, but can be unreliable if not used in parallel with other, more specific, markers of RA activity. The current study found no significant correlation between 25(OH)D and ESR in serum (**Figure 4.7**), consistent with some previous studies [311, 346]. However other studies have found strong correlations between serum 25(OH)D3 and RA disease activity [69], although the study was limited by the sole use of ESR as a measure of RA disease activity. Another marker, DAS28, has also been correlated with vitamin D status, the advantage with this marker being that it accounts for ESR

as well as other parameters of disease activity such as the patient's global disease activity score (as measured using a visual analogue scale; VAS) [449]. In some studies DAS28 showed significant inverse correlation with 25(OH)D3 status in the serum of RA patients [450, 451], whereas previous studies correlating ESR or CRP alone did not, but this observation is not universal [70, 350]. Due to the inclusion of the patient's VAS scores and the patient's tender joint count when calculating DAS28, results are subjective and can be influenced by other comorbidities such as depression, thus influencing the final DAS28 score. Thus the controversy regarding the role of 25(OH)D3 specifically in RA disease activity remains unsolved, but overall there are suggestions that low 25(OH)D3 levels in serum and SF contribute to higher RA disease activity. Another factor to consider when correlating vitamin D metabolite concentrations with RA disease activity markers is the type of medication that is used by patients at the time of sample collection. Certain RA medications such as hydroxychloroquine may influence vitamin D levels and metabolism by inhibiting the conversion of 25(OH)D to $1,25(OH)_2D$ [452, 453]; these studies found patients treated with hydroxychloroquine showed marked improvements in other parameters including SJC but not ESR. Patients using other drugs such as prednisolone may also expect adverse effects on their vitamin D status compared to patients not on treatment or using non-steroidal anti-inflammatory drugs (NSAID). However the majority of patients in the current study were not taking medications or only using NSAIDs, which so far have not been reported to affect vitamin D status. Hence the adverse effects on vitamin D status are likely minimal when these patients are considered as a group. The data presented in this chapter highlight the importance of monitoring vitamin D status not only as a predictive measure of RA activity, but

also during the course of RA treatment, and should be accounted for when relating to RA disease activity.

4.4.4 DBP, albumin and free and bioavailable vitamin D

As well as studying the impact of vitamin D metabolites on RA disease activity, serum and SF levels of DBP and albumin were also measured. Interestingly SF DBP correlated inversely with SJC (**Figure 4.11**). This may simply reflect the association between DBP and SF concentrations of 25(OH)D3, but 25(OH)D3-independent DBP activities have been described, including effects as a macrophage-activation factor [454] with actin-binding capacity [455], that have the potential to influence RA disease activity. In common with most vitamin D metabolites and consistent with previous publications [442], concentrations of DBP in RA and ReA were lower in patient SF relative to serum. The current study also observed lower concentrations of albumin in SF (**Figure 4.10**) as previously reported [456].

The net effect of these observations is that calculated levels of 25(OH)D3 not bound to DBP or albumin (free 25(OH)D3), and 25(OH)D3 not bound to DBP (bioavailable 25(OH)D3), were the same for RA and ReA SF as paired serum values. Although this suggests that vitamin D binding proteins (DBP/albumin) are regulated in SF to maximise free or bioavailable 25(OH)D3, it is important to recognise that the values presented in the current study are calculated estimates of unbound 25(OH)D3. Direct measurements of free 25(OH)D3 as recently described in other studies [457] could not be performed in the current study, and it is possible that other factors present in SF may significantly enhance or impede protein binding of

25(OH)D3. Further studies are required to determine more accurately the proportions of bound and unbound 25(OH)D3 in SF from RA patients.

Cells such as macrophages [458] and DC [459] express 1α-hydroxylase, but do not appear to express the DBP receptor megalin [41]. In this setting, synthesis of $1,25(OH)_2D3$ and associated immune functions may be dependent on macrophage uptake of 25(OH)D3 that is not bound to DBP (free or bioavailable 25(OH)D3) [41]. Albumin is the most abundant protein in the human body, acting as a binding site for endogenous proteins and steroids including vitamin D derivatives. The proportion of 25(OH)D3 bound to albumin is exempt from free, but included in bioavailable, 25(OH)D3 calculations. This is due to the low binding affinity of albumin for steroid hormones, contributing to easier off-loading and bioavailability of 25(OH)D3 at target sites [460]. It is estimated that a significant proportion of steroid hormones are carried bound to albumin in circulation despite the low binding affinity. Due to the lipophilic nature of 25(OH)D3, it is thought that its biological actions are exerted through the unbound 25(OH)D that is able to diffuse across cell membranes – the so-called free hormone hypothesis [49, 50]. The current study was unable to demonstrate that free or bioavailable 25(OH)D3 are better correlates of inflammatory RA disease than total 25(OH)D3 (**Figure 4.26**). However, the fact that the limited inverse correlations with RA disease identified involved SF rather than serum further supports the view that circulating concentrations of vitamin D provide only a very limited perspective on the anti-inflammatory actions of vitamin D. This appears to be particularly true in diseases such as RA where tissue-specific concentrations of 25(OH)D3 may be crucial in defining the functional potential of vitamin D.

4.4.5 MiRNAs and RA disease activity

The present study found that both miR-146a and miR-155 were up-regulated in RA SF compared to RA serum, with the difference in expression being statistically significant for miR-155 (**Figure 4.13**) and almost significant for miR-146a (p=0.051). This is consistent with previous studies for miR-155 expression [420, 461, 462], and miR-146a [421, 463]. Mechanistic studies have identified gene targets for miR-146a such as *IL-1β* and *TRAF6*, with the latter being a key component for TNF-α signal transduction in the inflammatory activation pathway [464]. Murata *et al.* also showed that miR-146a and miR-155 was up-regulated in RA synovial tissue compared to healthy controls or synovium from osteoarthritis (OA) patients, suggesting that these two miRNAs play a key role in inflammation to regulate the condition of the joint space. Since OA does not have the level of inflammation typically associated with RA, it is likely that miR-155 contributes to another regulatory role in RA pathogenesis, further highlighting the potential role that miRNAs play in the development and maintenance of RA-associated inflammation. Murata *et al*. further showed the SF miRNA profile was similar to that of synovial tissues yet distinct to the miRNA profile in serum and plasma, suggesting synovial tissue cells themselves are the major sources of SF miRNAs. In contrast, serum miRNAs can be made up of secretions from many different tissues. This supports the previously stated idea of a localised environment of inflammatory regulation within the SF and surrounding inflamed joints, giving rise to a specific environment within the joint space.

Similar to the project data presented in this thesis, Murata *et al.* did not find a significant correlation between TJC and miR-146a expression in RA SF. Consistent with this, and other null findings [465], the present study did not find significant

correlations between any of the RA disease markers CRP, ESR, SJC and TJC with miR-146a or miR-155 expression in serum or SF. This could be due to a number of reasons. Firstly, despite the promising evidence of miRNAs as potential novel biomarkers of RA, the pathogenesis and progression of RA is complex and multifactorial. Each miRNA regulates multiple gene targets so it is difficult to define one specific gene dysregulated in RA [466], and even more difficult to identify the specific miRNA responsible for silencing that gene. This may account for the variability of data between studies depending on the abundance and expression of other miRNAs between individual patients. Additionally, whilst qPCR remains a sensitive and reproducible method to detect miRNA expression [467], caution must be taken when selecting an appropriate endogenous control that is stably expressed and not targeted by miRNAs. Other factors to consider include the sex of patients as well as environmental factors, which may all contribute to data variability seen between different studies. Furthermore, since many miRNAs are not exclusively linked to RA and are differentially expressed in other inflammatory diseases [468], miRNA expression studies should be analysed together with other parameters of RA disease activity such as TJC and ESR, in order to best interpret the role of miRNAs in RA. However, often in the early stages of RA disease the only indication of abnormality is the alteration in miRNA expression profile compared to healthy individuals. By studying miRNA profiles of early arthritis patients before the onset of classical RA symptoms and signs, and comparing expression to healthy individuals, it may be possible to predict which patients go on to develop RA in future.

4.4.6 Vitamin D metabolites in RA: the influence of patient sex, age and disease duration

The influence of sex on vitamin D metabolite concentrations has been debated in the context of both health and disease [469-471]. The present study did not find significant differences between RA patient sex and serum vitamin D metabolite concentrations, nor were there differences between sex-matched comparisons of RA patients and healthy controls (**Figure 4.20**). A previous study comparing vitamin D metabolite concentrations from sex-matched healthy control serum with RA serum also did not find any significant differences [472], although a similar study reported a significant decrease in serum 25(OH)D levels in female RA patients versus sex-matched controls [473]. Females have a higher risk and prevalence of RA compared to males, and it has been proposed that this may be due to the role of oestrogen in autoimmune regulation - termed the hormonal theory [474, 475]. However, males may have increased RA activity compared to females [476]. Male RA patients have been reported to exhibit higher TJC and CRP counts compared to age-matched female RA patients, indicating more severe RA symptoms and disease activity. This correlation has so far not been reported in female RA patients, which could perhaps be due to the synergistic actions of vitamin D with oestrogen to protect against autoimmune processes [474, 477]. The present study found no significant differences between male and female patients and markers of RA disease activity (**Figure 4.23**), although the numbers of patients in the study were low and there was an uneven number of each sex. However the majority of male RA patients in the previous study were vitamin D deficient [476]. Coupled with the lower serum 25(OH)D previously observed in female RA patients [473], it is unclear whether low

25(OH)D is a cause or effect of RA. It is most likely that RA disease activity is associated with low 25(OH)D levels rather than associated with patient sex. The current study also found no significant correlations between patient age and serum and SF vitamin D metabolite concentrations (**Figure 4.21**), or RA disease duration and vitamin D metabolite concentrations (**Figure 4.22**), similar to previous studies [472, 473]. Together, results from the current study suggest that vitamin D metabolite concentrations in RA patients are unlikely to be affected by patient age, sex or disease duration, meaning the variations in metabolites concentrations between healthy, RA and ReA serum and SF are more likely a result of disease pathophysiology.

4.5 Summary

The work detailed in this chapter firstly indicates that serum 25(OH)D3 provides only limited insight into the role of vitamin D in RA. Alternative serum metabolites such as 3-epi-25$(OH)_2$D3, and SF metabolites and, notably, lack of SF 1,25$(OH)_2$D3, may be more closely linked to RA disease activity. Secondly, the current study showed up-regulated expression of the inflammation-associated miRNAs miR-146a and miR-155 in RA SF compared to serum, however since there was a lack of correlation between miRNA expression and RA disease markers in serum or SF, it is unlikely that these miRNAs are actively mediating gene silencing to cause inflammation within the local environment; it is possible they are maintained as a reserve of miRNAs not associated with their target gene [478]. Hence the role of miR-146a and miR-155 in the pathogenesis of RA remains unclear. Despite this, past and current research indicates miRNAs show promising potential in the field of diagnostic biomarker and inflammation research. To date, one study has profiled all human miRNAs covered in the MiRBase database to create a miRNA expression profile specific to RA, however its study limitations include a small patient sample size and lack of heterogeneity [479]. Future work on the contribution of miRNAs to various aspects of RA pathogenesis is required and holds great potential.

Lastly, the best correlations of RA disease so far relate to 25(OH)D measurements rather than 1,25$(OH)_2$D3, which supports a synovium-specific intracrine model, with local SF conversion of 25(OH)D3 to 1,25$(OH)_2$D3 linking vitamin D with RA pathogenesis. However, the actual levels of 1,25$(OH)_2$D3 present within the SF appear to be very low or undetectable. This suggests that any

1,25$(OH)_2$D3 generated within the synovial microenvironment may be restricted to the cells involved in immunoregulation within this tissue. Collectively the data from this chapter of the thesis indicate that the role of vitamin D metabolites, especially active 1,25$(OH)_2$D3, in the synovium of RA patients is still far from clear and further investigation of the link between 1,25$(OH)_2$D3 and miRNAs is the focus of the next two chapters of this thesis.

CHAPTER 5. VITAMIN D AND MICRORNAS IN DENDRITIC CELLS

5.1.1 Dendritic cells and innate immunity

Dendritic cells (DC) are antigen-presenting cells (APC) of the innate immune system that are capable of capturing and presenting foreign antigens to T cells, thus serving as the translator from innate to adaptive immunity [480]. The innate immune system functions to provide a rapid but non-specific response to foreign antigens, whereas the adaptive immune system provides a latent but antigen-specific response to generate an immune memory. Neutrophils, macrophages, natural killer (NK) cells and DC are the most notable populations of innate immune cells, whereas the major players in adaptive immunity are T and B lymphocytes (T cells and B cells) [481]. Populations of innate and adaptive immune cells are present in the general circulation, but are also important in specific target tissues associated with immune regulation [482]. Although these tissue-resident immune cells are the best representation of normal and disease immune function, their availability in humans is often restricted based on tissue availability. In recent studies our group used T cells isolated from the SF of inflamed joints of RA patients to study their role in inflammatory response [483], but the relatively small number of cells available from these samples severely restricts the scope of possible experiments. In contrast, recent studies of DC have utilised the greater abundance of circulating immune cells to develop *in vitro* cell culture model systems using circulating populations of DC precursor monocytes, and a variant of this model forms the basis of the current chapter of this thesis.

In humans there are two main types of DC: 1) conventional myeloid or monocyte DC (moDC) located in lymphoid tissue which are closely related to monocytes and which stimulate CD4+ and CD8+ T cells upon detection of foreign

antigens [484]; 2) plasmacytoid DC (pDC) which are stimulated via TLR-7 and TLR-9 and produce IFN-α [97]. The origins and distinguishing markers of the DC subtypes are summarised in **Figure 5.1**. The work in this chapter focuses on the use of an *ex vivo* model of differentiation and activation of moDC, which, for simplicity's sake, are simply termed DC throughout the remainder of the chapter.

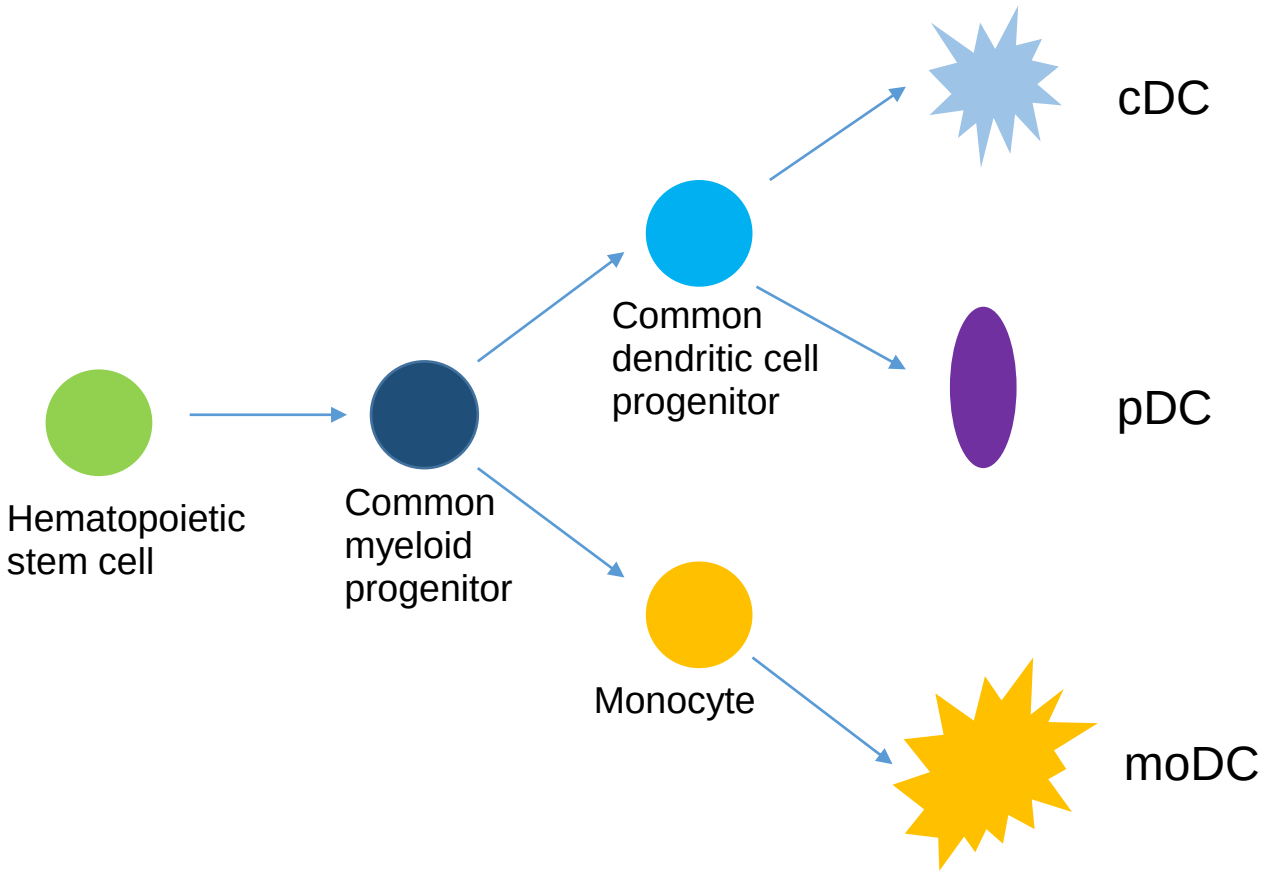


Figure 5.1. Origins of conventional, plasmacytoid and monocyte-derived DC

cDC and pDC arise from a common progenitor cell, whereas moDC are derived from monocytes. All DC types arise from a common myeloid progenitor.

DC maturation is an essential step for immune regulation and the subsequent activation of cells in the adaptive immune system. *In vivo*, immature DC (iDC) are recruited to sites of inflammation following an immune challenge, where they rapidly internalize foreign antigens, triggering their maturation and migration to lymphoid organs. During the journey from peripheral tissue to secondary lymphoid organs, they process foreign antigens and undergo a functional shift to a mature phenotype [485]. The process of maturation involves a series of functional and phenotypical changes that include the redistribution of major histocompatibility type II (MHC II) from endosomal compartments to the cell surface, the up-regulated surface expression of T cell co-stimulatory molecules such as cluster of differentiation (CD)80 and CD86, and the development of characteristic dendrites which facilitate DC migration from tissues to lymphoid organs [486]. Following these phenotypic changes, mature DC (mDC) present foreign antigens on their MHC II complex, for recognition by naïve CD4+ T cells and peptide binding by CD8+ T cells. The mDC maturation process can be influenced by a variety of factors including the inflammatory cytokine milieu of the local environment, which is able to determine different DC states and functions [487, 488]. In the absence of infection, DC exist in an immature steady state in peripheral tissues to maintain immune surveillance and tolerance, expressing constitutively low levels of MHC II but not co-stimulatory molecules [489, 490]. In this state they present self-antigens to T cells to maintain the production of induced Tregs, which are immunosuppressive and down-regulate the induction of effector T cells to prevent inappropriate immune responses [488].

Populations of iDC can be derived *in vitro* for experimental study by culturing PBMC-derived monocytes in the presence of granulocyte macrophage-colony

stimulating factor (GM-CSF) and interleukin-4 (IL-4) [491, 492]. *In vitro* DC were first identified in 1998 by Randolph *et al*. [493], who found that culturing mononuclear blood cells on a collagen matrix evoked cells that were capable of functions similar to conventional DC such as the ability to stimulate allogenic T cells. DC maturation *in vitro* can be triggered by the presence of immune activator molecules such as lipopolysaccharide (LPS), a ligand for toll-like receptor-4 (TLR-4). The differentiation from blood-derived monocytes to iDC in culture can be monitored by flow cytometry to study changes in surface marker expression as DC mature towards mDC. This includes down-regulation of the monocyte marker CD14 and an up-regulation of the T cell co-stimulatory molecules CD86 and CD80 [494]. Other major markers of DC maturation include up-regulation of the maturation marker CD83, the MHC II molecule HLA-DR, and CD209 (DC-SIGN). The latter can be used to distinguish sub-types of DC [495], where up-regulated expression of DC-SIGN is found in cultured DC in the presence of GM-CSF and IL-4. Myeloid DC are distinguished by CD11c marker whereas the plasmacytoid DC are distinguished by CD123 [496].

As well as immature and mature differentiation states, DC can also exist as tolerogenic DC (tolDC), that act to limit immune cell activation by promoting the induction of tolerogenic regulatory T cells (Tregs) [497]. There has been debate regarding whether tolDC status is a result of DC maturation (similar to the transition from iDC to mDC) or the result of a separate tolDC lineage [498, 499], with the most likely mechanism being the maturation status of the DC. In both mice and humans, the presence of the anti-inflammatory cytokine IL-10 has been shown to favour Tregs [500, 501], and IL-10 plays a key role in the differentiation of tolDC [502]. To facilitate T cell stimulation, DC normally produce a host of cytokines including IL-12.

As part of tolDC function, these cytokines are inhibited by IL-10 to impair T cell activation and thus prevent further production of pro-inflammatory cytokines and promote tolerance [503]. In experimental mouse models of tolDC function, iDC have been shown to express high amounts of TNF-α which mediate their maturation and induce CD4+ Treg activation [504]. In contrast, LPS-matured iDC shifted the balance of T cell differentiation to favour the inflammatory Th1 fate. Previous studies have also found differences in DC function depending on their maturation stimulus [505]. LPS-stimulated DC up-regulate the expression of genes that control inflammation, whereas TNF-α stimulated DC do not. These studies lead to the conclusion that the immunogenic versus tolerogenic fate of DC is decided by differences in DC stimulation signals [506]. The differences in DC fates by maturation is described in **Figure 5.2**.

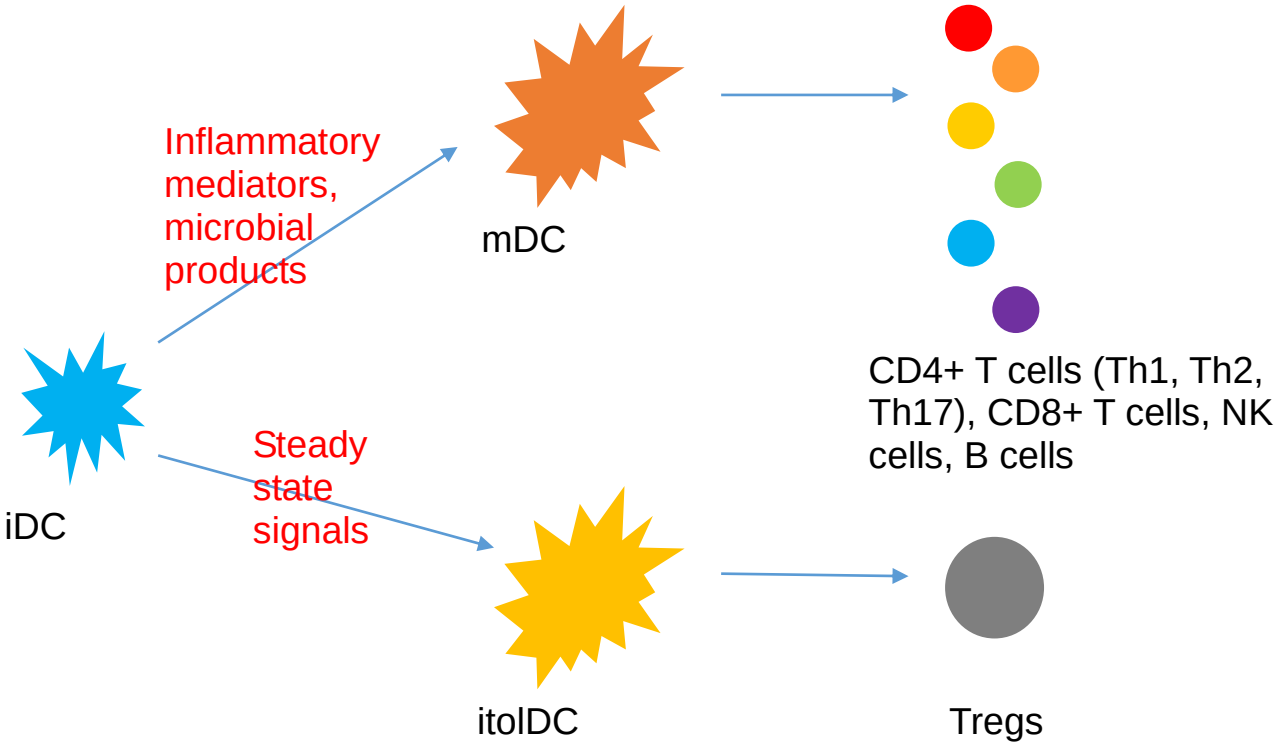


Figure 5.2. Differential DC fates upon maturation

The functional fate of immature DC (iDC) is influenced by the type of signals received by resting DC. Inflammatory signals produce a mature immunogenic DC capable of activating T cells, NK cells and B cells of the adaptive immune system, whilst steady state signals favour a tolerogenic fate to induce Tregs. itolDC, tolerogenic DC; mDC, mature DC.

5.1.2 Immunological actions of vitamin D on dendritic cells

Initial studies on the action of vitamin D in DC were prompted by the discovery of the vitamin D receptor (VDR) in purified tissue DC [507]. A later study then provided evidence that $1,25(OH)_2D3$ could act to suppress the numbers and function of antigen-presenting cells in populations of DC isolated from skin [508]. These studies involving isolated DC from lymphoid tissues gave rise to the idea that $1,25(OH)_2D3$ could affect DC function, but in recent years studies using monocyte-derived DC have further elucidated the role of vitamin D in DC. Now the actions of $1,25(OH)_2D3$ have been extensively linked to maintaining immature DC status [158,

459], promoting tolerogenic DC [509] and enhancing activation of Tregs [510]. *In vitro*, DC cultured in the presence of $1,25(OH)_2D3$ have decreased IL-12 [459] but increased IL-10 [511] production, exhibit down-regulated CD80/CD86 expression and are less capable of inducing T cell proliferation. Based on these observations it was then discovered that $1,25(OH)_2D3$ could promote tolerance via a tolDC phenotype [154]. $1,25(OH)_2D3$ has been shown to regulate DC maturation via a VDR-mediated mechanism, by inhibiting down-regulation of CD14 and maintaining an immature phenotype [512]. When DC are exposed to $1,25(OH)_2D3$ during monocyte to iDC differentiation, DC exhibit reduced ability to differentiate and mature, impairing their stimulatory capacity for T cells [155, 158, 513]. Furthermore, following maturation of DC with LPS in the presence of $1,25(OH)_2D3$, mDC exhibit down-regulated expression of CD80/CD86, CD83 and HLA-DR compared to mDC matured without $1,25(OH)_2D3$ [511, 514].

A seminal study in the field of DC-related vitamin D research compared the effect of $1,25(OH)_2D3$ on pDC versus mDC function. The authors found that $1,25(OH)_2D3$ preferentially regulated mDC, leading to a reduced capacity for mDC to induce inflammatory Th1 cell development [515]. Interestingly $1,25(OH)_2D3$ had no significant effects on the tolerogenic properties of pDC, despite both pDC and mDC expressing similar levels of VDR. It is hence likely that tolerogenic pDC may respond to $1,25(OH)_2D3$ via an alternate local intracrine mechanism. Local production of $1,25(OH)_2D3$ from its inactive precursor 25(OH)D3 is likely to be an important mechanism for the contribution of $1,25(OH)_2D3$ to immune regulation. A key study supported this idea by showing that monocyte-derived DC express the enzyme 1α-hydroxylase (CYP27B1), required for the conversion of 25(OH)D3 to $1,25(OH)_2D3$

[516]. These DC were able to intrinsically produce 1,25(OH)$_2$D3 following stimulation with LPS, with mature DC then producing 1,25(OH)$_2$D3 to suppress the differentiation of precursor cells in a paracrine fashion. Subsequent studies then demonstrated that the local conversion of 25(OH)D3 to 1,25(OH)$_2$D3 by DC was able to drive functional T cells responses, shown by the up-regulated expression of CTLA-4 to suppress immune responses [517]. The mechanism behind this response is described in detail later in this thesis (see **Chapter 6**).

The actions of 1,25(OH)$_2$D3 on DC have also been studied in mouse models. The ability of 1,25(OH)$_2$D3 to maintain DC tolerance was also endorsed by studies in mice underdoing pancreatic islet allografts, showing that mice treated with 1,25(OH)$_2$D3 had lower transplant rejection rates compared to untreated mice [283]. Importantly, this 1,25(OH)$_2$D3 response was likely due to reduced DC maturation or enhanced Treg induction. The ability of 1,25(OH)$_2$D3 to maintain an immature DC state is dependent on the expression of *VDR* in both mice (*Vdr*) and humans (*VDR*). *Vdr* knockout mice are not resistant to DC maturation and exhibit increased numbers of mDC and enlarged lymph nodes compared to wild-type mice expressing *Vdr* [157]. Surprisingly, *Vdr* knockout mice also exhibit reduced numbers of Th1 cells as opposed to the elevated levels predicted [518]. Together these studies show that DC differentiation can occur in the absence of the VDR, but 1,25(OH)$_2$D3-induced differentiation absolutely requires VDR. In the non-obese diabetic (NOD) mouse model for Type 1 diabetes, 1,25(OH)$_2$D3 can induce DC to develop a tolerogenic phenotype and subsequently induce an increased population of Tregs [519, 520]. In murine models fed a vitamin D-deficient diet, an overproduction of Th1 and Th17 inflammatory responses was seen, as well as a reduction in numbers of tolDC [521].

Vitamin D is able to reduce the inflammation by inhibiting the production of inflammatory cytokines and reducing Th1 and Th17 responses [522]. In *Cyp27b1* knockout mice deficient of 1α-hydroxylase, mice induced to develop inflammatory colitis showed exacerbated disease symptoms due to excessive IL-1 and IL-17 production in the colon, suggesting that vitamin D deficiency may produce similar effects [523].

In humans, exposure of mDC to 1,25$(OH)_2$D3 promotes the production of C-C motif chemokine ligand 22 (CCL22), a chemokine which attracts Tregs and suppresses the production of CCL17 [524, 525]. Currently there are two proposed pathways of mDC regulation by 1,25$(OH)_2$D. During the early stages of inflammation the chemokine IL-8, encoded by *CXCL8*, is up-regulated by 1,25$(OH)_2$D3 binding to the VDR, mediating direct binding to the CXCL cluster [526]. Further sequencing revealed that *CXCL8* is under the direct control of the nearby VDR binding site, so in this way 1,25$(OH)_2$D3-mediated VDR activation leads to the control of IL-8 expression, which is an important chemokine during the immune response [526]. At later stages of inflammation, 1,25$(OH)_2$D3 prevents an over-active immune response in mDC by decreasing translocation of the p65 subunit of NF-κB [525]. Others have found similar actions of 1,25$(OH)_2$D3 on repressing the transcription factor *NF-κB* [527], whose activity has been associated with the production of pro-inflammatory IL-12, CCL7, CCL22 and MHC II [527]. Furthermore, DC exposed to 1,25$(OH)_2$D3 show reduced chemotaxis to the chemokine receptor (CCR) 7 and CCL21 [524, 528], which are required for the migrations of DC to the draining lymph node.

The mechanisms of 1,25$(OH)_2$D3 with regard to monocyte to DC differentiation have been studied, with the consensus that 1,25$(OH)_2$D3 also exerts

anti-inflammatory effects at this early stage of DC development. The differentiation of monocytes to iDC is mediated by Type 1 Interferon including interferon-alpha (IFN-α), which is also a target of $1,25(OH)_2D3$ [529]. When $1,25(OH)_2D3$ was added to freshly isolated monocytes *in vitro*, the differentiation of IFN-mediated DC was inhibited. Furthermore, the presence of $1,25(OH)_2D3$ prevented iDC from maturing, as demonstrated by a lower expression of CD83. Others have found a similar ability of $1,25(OH)_2D3$ to redirect the fate of iDC; iDC differentiated from monocytes in the presence of 10nM $1,25(OH)_2D3$ showed up-regulated CD14 expression, thus $1,25(OH)_2D3$ acts on iDC to maintain their immature status and inhibit T cell stimulatory potential [158, 530].

5.1.3 MiRNAs and dendritic cells

The process of DC differentiation and maturation involves the regulation of expression of many genes, and an increasing body of literature suggests that some of these genes may also be regulated by miRNAs [153, 531, 532]. The expression of various cytokines and cell intrinsic factors can modify the miRNA expression profile in DC [532]. Depending on the signals received from the pro- or anti-inflammatory pathway, different stimuli are able to induce or down-regulate the expression of specific miRNAs that are linked to DC function. Following DC maturation, up-regulated expression of miR-155, let-7i, miR-22, miR-34a and miR-126 has been linked to a pro-inflammatory mDC phenotype associated with T cell activation, whilst up-regulated expression of miR-29, miR-146a, miR-148, miR-152 and miR-451 has been associated with anti-inflammatory tolDC phenotype [153]. Amongst these miRNAs, miR-155 and let-7i have been most strongly associated with DC maturation

and function. MiR-155 expression is strongly up-regulated following DC maturation [533, 534], which results in decreased expression of the transcription factor *PU.1* which regulates the mDC marker DC-SIGN [534]. MiR-155 has also been implicated in controlling cytokine release; inhibition of miR-155 expression in mDC leads to increased pro-inflammatory cytokine expression [535, 536] therefore the role of miR-155 appears to be able to promote and inhibit inflammation in DC.

Let-7i has also been associated with DC maturation and is thought an essential miRNA for the normal maturation of DC [537]. Let-7i was one of the first miRNAs to be discovered, deriving from the let-7 family of miRNA precursors involved in the post-transcriptional control of innate immune responses to pathogens. Immune-stimulated macrophages are able to down-regulate the expression of several members of the let-7 family to relieve suppression of IL-6 and IL-10 cytokines. To clarify, let-7 targets genes encoding IL-6 and IL-10 and thus down-regulation of let-7 enhances their expression. In this way let-7 can act as a tumour suppressor, amongst other immunosuppressive functions. In DC, let-7i has been shown to be up-regulated in response to DC maturation [537, 538]; Kuppusamy *et al*. indicated that the let-7 family of miRNAs may be important mediators of cell maturation and immune responses. Other studies support this idea; LPS-induced DC maturation was found to require the up-regulation of let-7i, where inhibition of let-7i expression prevented DC maturation shown by suppressed expression of CD80 and CD86 mDC surface markers, and impaired downstream T cell responses [539]. Dynamic regulation of miR-155 and let-7i are responsible for fine-tuning DC maturation and immune response by targeting and inhibiting suppressor of cytokine signalling 1 (SOCS1) [540]. *SOCS1* is a crucial gene that is up-regulated during DC

maturation [541] and controls immune responses via a negative feedback loop that regulates the intensity and duration of cytokine signalling. On the other hand, miR-155 targets *SOCS1* to block the negative feedback loop [542]. *SOCS1* is a confirmed target of both let-7i and miR-155, where over-expression of let-7i [537] and miR-155 [541] leads to excessively suppressed SOCS1 protein expression in DC and increased production of pro-inflammatory IL-12. In *miR-155*-deletion mice, the addition of $1,25(OH)_2D3$ suppresses inflammation and stimulates *SOCS1* by down-regulating miR-155 [240]. Together these studies indicate that miR-155 and let-7i play important roles in DC development and immune regulation.

Other miRNAs are associated with an anti-inflammatory phenotype, and act to prevent DC maturation by reducing MHC II expression or inhibiting the release of pro-inflammatory cytokines [543]. MiRNAs such as miR-148 and miR-146a fulfil this anti-inflammatory role; miR-146a impairs DC maturation by targeting components of the NF-κB pathway, as well as inhibiting the up-regulation of CD80/CD86 and HLA-DR expression on DC [544]. Other miRNAs such as miR-142 are down-regulated in mDC to promote an anti-inflammatory phenotype by increasing IL-6 expression [540]. MiR-146a suppresses IL-6 and IL-8 expression by silencing its target gene *IRAK1* which is a crucial component of the inflammatory IL-1 signalling pathway [545]. The other target of miR-146a, *TRAF6*, is linked to attenuation of signalling processes in DC [546].

As well as being influenced by cytokine expression, several miRNAs have been shown to modify the release of cytokines themselves. MiR-21, miR-10 and miR-148a all inhibit pro-inflammatory IL-12 production [543, 547, 548]. Other miRNAs have been associated with a tolDC. Gene sequencing revealed miR-30b,

miR-99a and miR-125a are up-regulated in tolDC compared to other DC phenotypes, both *in vitro* and *in vivo* [549]. In mice, differential expression of 391 miRNAs has been observed during the differentiation of iDC to mDC [549]. The method of DC activation is also an important consideration for the differential expression of miRNAs; for example LPS-induced mature DC show a distinct miRNA expression profile compared to other induction methods [550].

One miRNA can also control the expression of another miRNA. The expression of miR-155 has been linked to the expression of miR-142; increased miR-155 down-regulates miR-142, due to miR-155 targeting the *PU.1* promotor region which is necessary for miR-142 expression [540], whereas inhibiting miR-155 expression results in an up-regulation of miR-142 expression [551]. These studies in DC have provided miR-155 with the term 'master miRNA regulator', where the expression of other miRNAs only occurs in the presence or absence of miR-155 [551]. Following DC maturation, the presence of miR-155 allows the induction of miR-210-3p, whereas miR-445-3p is only induced in its absence, underlining a hierarchy of miRNA-mediated miRNA regulation [552].

5.2 Chapter outline

DC are initiators of adaptive immune responses and are thus important targets for the immunomodulatory actions of $1,25(OH)_2D3$ [152, 153]. The mechanisms by which $1,25(OH)_2D3$ influences DC differentiation, maturation and function have yet to be fully elucidated but include important changes in gene expression that have highlighted novel immunomodulatory mechanisms, including effects on cellular

metabolism [274, 553, 554]. However, it is also likely that epigenetics will play a role in mediating changes in DC differentiation and function [555]. The aim of studies in this chapter of the thesis were therefore to investigate the contribution of one component of epigenetic regulation – miRNAs – on the DC modulatory effects of $1,25(OH)_2D3$.

Previously, **Chapter 4** described miRNAs including miR-155 in an inflammatory autoimmune disease setting, as well as the correlation between the vitamin D metabolome with inflammatory markers and miRNA expression. It is therefore likely that miRNAs also play an important role in maintaining an appropriate level of immune response in healthy individuals, and are sensitive to immunomodulation by $1,25(OH)_2D3$. The journey from monocyte to DC bridges the innate and adaptive immune systems; a crucial stage for healthy immune response and a potential target for $1,25(OH)_2D3$ mediated regulation of miRNAs.

The work described in the current chapter tested the central hypothesis that **the ability of $1,25(OH)_2D3$ to modulate DC differentiation and function is associated with the specific regulation of miRNAs that influence DC immune function.** Distinct DC phenotypes were generated to study the effect of $1,25(OH)_2D3$ on miRNA expression at different stages of DC development. Candidate gene and miRNA expression was quantified in these DC populations, and unbiased analysis of miRNAs was carried out using PCR array analysis. Finally, gene ontology analyses were performed for differentially regulated miRNAs using a bioinformatics approach to predict miRNA targets related to immune function and regulation.

5.3 Results

5.3.1 Induction of distinct DC phenotypes

The basis for the studies forming this chapter involved the induction of 6 distinct DC phenotypes as detailed in Materials and Methods (**Chapter 3**). Monocytes were isolated from peripheral blood mononuclear cells in fresh whole blood and cultured in GM-CSF and IL-4 to stimulate differentiation of iDC and mDC. Both stages of differentiation and maturation involved the presence and absence of $1,25(OH)_2D3$. The 6 distinct DC populations generated were: immature tolerogenic DC (itolDC); mature tolerogenic DC (mtolDC); immature DC (iDC); iDC+/-$1,25(OH)_2D3$; mature DC (mDC); mDC+/-$1,25(OH)_2D3$ (see **Figure 3.3**). All 6 DC populations were used for candidate gene and miRNA expression analysis, and DC populations (iDC, mDC, itolDC and mDC+$1,25(OH)_2D3$) were used for cell surface antigen characterisation and unbiased analysis of miRNA expression (**Figure 5.3**).

5.3.2 Flow cytometry validation of monocyte-derived DC phenotypes

Flow cytometry was used to validate the projected DC phenotypes for iDC, mDC, itolDC and mDC+$1,25(OH)_2D3$ by quantifying specific cell surface markers. Representative flow cytometry plots showed that long-term treatment with $1,25(OH)_2D3$ to generate itolDC from iDC decreased HLA-DR and CD80 (**Figure 5.4A**), and CD68 (**Figure 5.4B**), whilst increasing CD14 (**Figure 5.4A**). Short-term treatment with $1,25(OH)_2D3$ to generate mDC+$1,25(OH)_2D3$ from mDC decreased HLA-DR and CD83 (**Figures 5.4A and 5.4B**), whilst increasing CD14 (**Figure 5.4A**).

Transition from iDC (no treatment during differentiation or maturation) to mDC (LPS treatment for maturation only) was associated with increased numbers of cells expressing HLA-DR (**Figure 5.4A**), CD209 and CD83 (**Figure 5.4B**). In addition to analysis of the number of cells expressing specific cell-surface markers, flow cytometry was also used to characterise the relative levels of expression for cell surface antigens using mean fluorescence intensity (MFI). Data shown in **Table 5.1** showed that long-term $1,25(OH)_2D3$ treatment (itolDC) suppressed HLA-DR (MFI 6.55), CD80 (MFI 4.88), CD83 (MFI 3.09) and CD86 (MFI 2.79) compared to iDC populations (HLA-DR MFI 199; CD80 MFI 6.15; CD83 MFI 3.57; CD86 MFI 6.41), and increased expression of CD14 (MFI 12.7 vs 7.42 in iDC).

Short-term treatment with $1,25(OH)_2D3$ (mDC vs mDC+$1,25(OH)_2D3$) suppressed expression of HLA-DR (MFI 250 vs 275 in mDC), CD80 (MFI 29.0 vs 63.1 in mDC), CD209 (MFI 1.01 vs 14.9 in mDC), CD83 (MFI 1.52 vs 7.2 in mDC) and CD86 (MFI 1.3 vs 44.9 in mDC). Similar to that observed with long-term $1,25(OH)_2D3$, CD14 expression was up-regulated by short term $1,25(OH)_2D3$ treatment (MFI 3.29 to 23.4).

Maturation by LPS (iDC vs mDC) increased expression of HLA-DR (MFI 199 to 277; iDC vs mDC), CD80 (MFI 6.15 to 63.1), CD209 (MFI 2.71 to 14.9), CD83 (MFI 3.57 to 7.2) and CD86 (MFI 6.41 to 44.9). As expected, iDC to mDC maturation led to the down-regulation of CD14 expression (MFI 7.42 to 3.29). Together these results confirm the different phenotypes of the different monocyte-derived DC and shows that the DC model was functional and responsive to LPS and $1,25(OH)_2D3$.

5.3.3 Effect of 1,25(OH)$_2$D3 on vitamin D-related gene expression in DC

Following on from phenotype characterisation by flow cytometry, all 6 DC populations were used to investigate expression of components of the vitamin D metabolism and signalling by assessing mRNA expression of *VDR*, *CYP24A1* and *CYP27B1* (see **Figure 5.5**). Data from these studies showed that all DC phenotypes expressed mRNA for *VDR*. Expression of *VDR* was higher in mDC vs iDC and mtolDC, and higher in mDC+1,25(OH)$_2$D3 vs mtolDC. Similar to *VDR*, expression of mRNA for *CYP27B1* was higher in mDC than iDC, and in mDC+1,25(OH)$_2$D3 vs iDC+1,25(OH)$_2$D3. The VDR target gene *CYP24A1* is potently induced by 1,25(OH)$_2$D3, but this was only statistically significant with long term 1,25(OH)$_2$D3 in the presence of LPS.

5.3.4 Effect of 1,25(OH)$_2$D3 on candidate miRNA expression in DC

Having shown that the different DC phenotypes express *VDR* and respond to treatment with 1,25(OH)$_2$D3 by changes in cell surface antigens and *CYP24A1* mRNA expression, the next stage of the project investigated possible changes in miRNA expression in the different DC types. qRT-PCR analysis was performed on 7 candidate miRNAs (miR-21, miR-29a, miR-145, miR-146a, miR-155, miR-627 and let-7i) closely related to immune cell function and inflammation according to previous literature [556-562]. In particular, miR-146a and miR-155 have been consistently linked to inflammatory disease risk and outcome. Of these, only miR-155 and let-7i showed any significant change in expression between the different DC populations. MiR-155 was up-regulated by LPS (mDC vs iDC; p=0.0311). MiR-155 was also up-

regulated in the presence of LPS and short-term 1,25$(OH)_2$D3 (mDC+1,25$(OH)_2$D3 vs iDC+1,25$(OH)_2$D3; p=0.0091). LPS treatment of long-term 1,25$(OH)_2$D3 cultured DC (mtolDC vs itolDC) also increased miR-155 (p=0.049) (**Figure 5.6E**). Let-7i expression was up-regulated by LPS but only in the presence of short-term 1,25$(OH)_2$D3 (mDC+1,25$(OH)_2$D3 vs iDC+1,25$(OH)_2$D3; p=0.0012) (**Figure 5.6G**). These results indicated that miRNAs are targets for 1,25$(OH)_2$D3 in DC, but also showed that analysis of candidate miRNAs based on previously reported literature may not be the most effective approach for determining DC miRNAs for 1,25$(OH)_2$D3. Therefore, in the next stage of the project we carried out unbiased analysis of miRNAs using PCR array analyses.

5.3.5 Array miRNA expression by LPS maturation and 1,25$(OH)_2$D3

Using mRNA isolated from DC cultures and previously analysed by qRT-PCR (**5.3.3** and **5.3.4**), qPCR miRNA arrays were performed to profile the expression of 372 validated human miRNAs most closely linked to immune function and inflammation. The miRNA arrays included the previously selected candidate miRNAs. Because of the significant costs associated with this, only 4 of the 6 DC phenotypes shown in **Figure 5.3** were processed for array analysis; the selected populations were itolDC, iDC, mDC, and mDC+1,25$(OH)_2$D3. This selection of DC phenotypes allowed us to carry out an unbiased analysis of miRNAs that would assess the effect of long-term 1,25$(OH)_2$D3 alone (iDC vs itolDC), short-term 1,25$(OH)_2$D3 when used in combination with LPS (mDC vs mDC+1,25$(OH)_2$D3), and LPS maturation alone (iDC vs mDC).

5.3.5.1 Preliminary analysis of differentially regulated miRNAs

Data from the qPCR arrays were initially obtained as delta Ct (ΔCt) values for each miRNA/PCR plate well. These raw PCR values were then used to carry out statistical analyses to assess changes in miRNA expression for: 1) long-term treatment with $1,25(OH)_2D3$ (iDC vs itolDC); 2) short-term $1,25(OH)_2D3$ in combination with LPS (mDC vs mDC+$1,25(OH)_2D3$); 3) LPS maturation alone (iDC vs mDC). Data was analysed using SABiosciences PCR Array Data Analysis software (SABiosciences) recommended for the arrays, and were initially visualised as representative PCR plate heat-maps for the PCR arrays based on log fold-change in miRNA expression for specific $1,25(OH)_2D3$ or LPS treated DC relative to untreated controls (**Figures 5.7A – 5.7C**).

Data were also visualised as scatter plots also showing log fold-change in miRNA expression. Only miRNAs with a fold-change >2 were shown as significant in the preliminary analyses (**Figures 5.8A-5.10A**). Preliminary analysis indicated that long-term $1,25(OH)_2D3$ (itolDC vs iDC) down-regulated expression of 193 miRNAs (>2 fold change). Short-term $1,25(OH)_2D3$ (mDC+$1,25(OH)_2D3$ vs mDC) down-regulated expression of 172 miRNAs, and up-regulated 2 miRNAs. LPS maturation (mDC vs iDC) down-regulated expression of 15 miRNAs and up-regulated the expression of 29 miRNAs; these miRNAs are visualised as red or green dots on the scatter plots (**Figures 5.8B-5.10B**). Huynh-Feldt statistical testing was then performed on all miRNAs showing >2 fold regulation in these DC populations, to identify miRNAs that showed statistically significant differences in expression following treatment with $1,25(OH)_2D3$ or LPS based on n=6 different donor DC populations. Only these significantly regulated miRNAs are listed in the figure table.

5.3.5.2 Huynh-Feldt significant miRNAs

Huynh-Feldt statistical analysis identified 8 miRNAs that were significantly down-regulated following long term 1,25$(OH)_2$D3 (**Figure 5.8B**) and 14 miRNAs that were significantly down-regulated following short-term 1,25$(OH)_2$D3 (**Figure 5.9B**). In contrast only 2 miRNAs were significantly regulated by maturation with LPS (**Figure 5.10B**); miR-155-5p was up-regulated (p=0.049) and miR-221-5p was down-regulated (p=0.001). All Huynh-Feldt significantly-regulated miRNAs are shown in full graphical format for long-term 1,25$(OH)_2$D3 (itolDC vs iDC) (**Figure 5.11**), short-term 1,25$(OH)_2$D3 (mDC+1,25$(OH)_2$D3 vs mDC) (**Figure 5.12**) and LPS maturation (mDC vs iDC) (**Figure 5.13**).

Interestingly, miR-155-5p was significantly up-regulated by LPS maturation in the array data (iDC to mDC) (**Figure 5.10B**), then down-regulated following short-term 1,25$(OH)_2$D3 (mDC to mDC+1,25$(OH)_2$D3) (**Figure 5.9B**). This highlights miR-155-5p as a potential regulator of inflammation, and a promising target for 1,25$(OH)_2$D3 based anti-inflammatory therapies.

5.3.6 Predicted targets for miRNAs: a bioinformatics approach

A full list of significantly regulated miRNAs as identified by Huynh-Feldt analysis is shown in **Table 5.2**, with different miRNAs regulated by LPS or 1,25$(OH)_2$D3 highlighted accordingly. Following the identification of these miRNAs from the qPCR arrays, a bioinformatics approach was used to identify their predicted target genes. Firstly, MiRTarBase database was used to find predicted gene targets

for all significantly regulated miRNAs. All gene targets on miRTarBase have been experimentally validated and ranked according to the number of research articles verifying the targets, then given a target prediction score out of 100. The top 5 target genes for each miRNA identified by Huynh-Feldt analysis in the current study were ranked and compiled in **Table 5.2**; target genes were ranked from most to least validated, and the established cut-off target prediction score was set at 95 and above to exclude experimentally 'weak' targets.

To further analyse and validate the functionality of select miRNA gene targets, bioinformatics approaches were used to identify predicted gene targets of these miRNAs. MiRBase miRNA database was combined with Cytoscape software to produce a list of significant (p<0.05) experimentally validated gene targets of miRNAs. By combining Cytoscape analysis with ClueGo gene ontology (GO) analysis, target genes were grouped according to enriched biological functions. This approach was used to analyse the expression of miR-155-5p due to its differential regulation in response to LPS and 1,25$(OH)_2$D3. Target genes of miR-155-5p were found to function in a variety of biological processes including immune regulation (**Figure 5.14A**), underlining the potential for miR-155 mediated regulation of the immune system. Additionally, gene ontology analysis was performed to produce networks to visualise miRNA gene regulation with regard to immune function. The first network analysed all miRNAs that were significantly regulated in response to all treatments; long term 1,25$(OH)_2$D3, short term 1,25$(OH)_2$D3 and LPS (**Figure 5.14B**) where gene targets were involved in immune regulation. **Figures 5.14C-5.14E** show networks created from miRNAs down-regulated by long term 1,25$(OH)_2$D3 treatment, miRNAs down-regulated by short term 1,25$(OH)_2$D3, and miRNAs regulated by LPS.

This bioinformatics approach identified many genes that were potentially targeted by 1,25$(OH)_2$D3-sensitive miRNAs, with some genes involved in regulation of immune responses, hence there is great potential for 1,25$(OH)_2$D3-mediated miRNA modulation for immune health and disease.

5.3.7 Effect of 1,25$(OH)_2$D3 on miRNA synthesis genes in DC

Due to the global miRNA down-regulation observed with long term 1,25$(OH)_2$D3 and short term 1,25$(OH)_2$D3 treatment, genes involved in the miRNA synthesis pathway were also quantified by qRT-PCR in all 6 DC populations, to study the effect of long and short term 1,25$(OH)_2$D3 and LPS maturation on gene expression. Genes analysed were *DROSHA, DICER1, AGO2, DGCR8, RAN, TARBP2* and *XPO5*, which encode proteins forming the key components of the miRNA synthesis machinery. LPS-induced DC maturation in the absence of 1,25$(OH)_2$D3 (iDC to mDC) significantly down-regulated the expression of 5 out of the 7 genes; these were *DROSHA* (p=0.0212), *DICER1* (p=0.0253), *DGCR8* (p=0.0334), *RAN* (p=0.0024) and *XPO5* (p=0.0071) (**Figure 5.15**). LPS-induced DC maturation in the presence of 1,25$(OH)_2$D3 (itolDC to mtolDC) down-regulated the expression of *DGCR8* (p=0.0087) and *RAN* (p=0.013). Conversely, already mature DC populations in the presence of 1,25$(OH)_2$D3 (mDC to mDC+1,25$(OH)_2$D3; mDC to mtolDC) did not significantly regulate the expression of any genes. Long term 1,25$(OH)_2$D3 treatment in the immature phenotype in the absence of LPS (iDC to itolDC) significantly down-regulated the expression of *DROSHA* (p=0.0266), *RAN* (p=0.0289), *TARBP2* (p=0.0368) and *XPO5* (p=0.0333). Short term 1,25$(OH)_2$D3

treatment in the absence of LPS (iDC to iDC+1,25$(OH)_2$D3) also significantly down-regulated the expression of *DROSHA* (p=0.0306), *DICER1* (p=0.0209) and *RAN* (p=0.0124). Interestingly, LPS or 1,25$(OH)_2$D3-driven regulation of *DROSHA* or *DICER1* only occurred with one or the other in culture, not both 1,25$(OH)_2$D3 and LPS together.

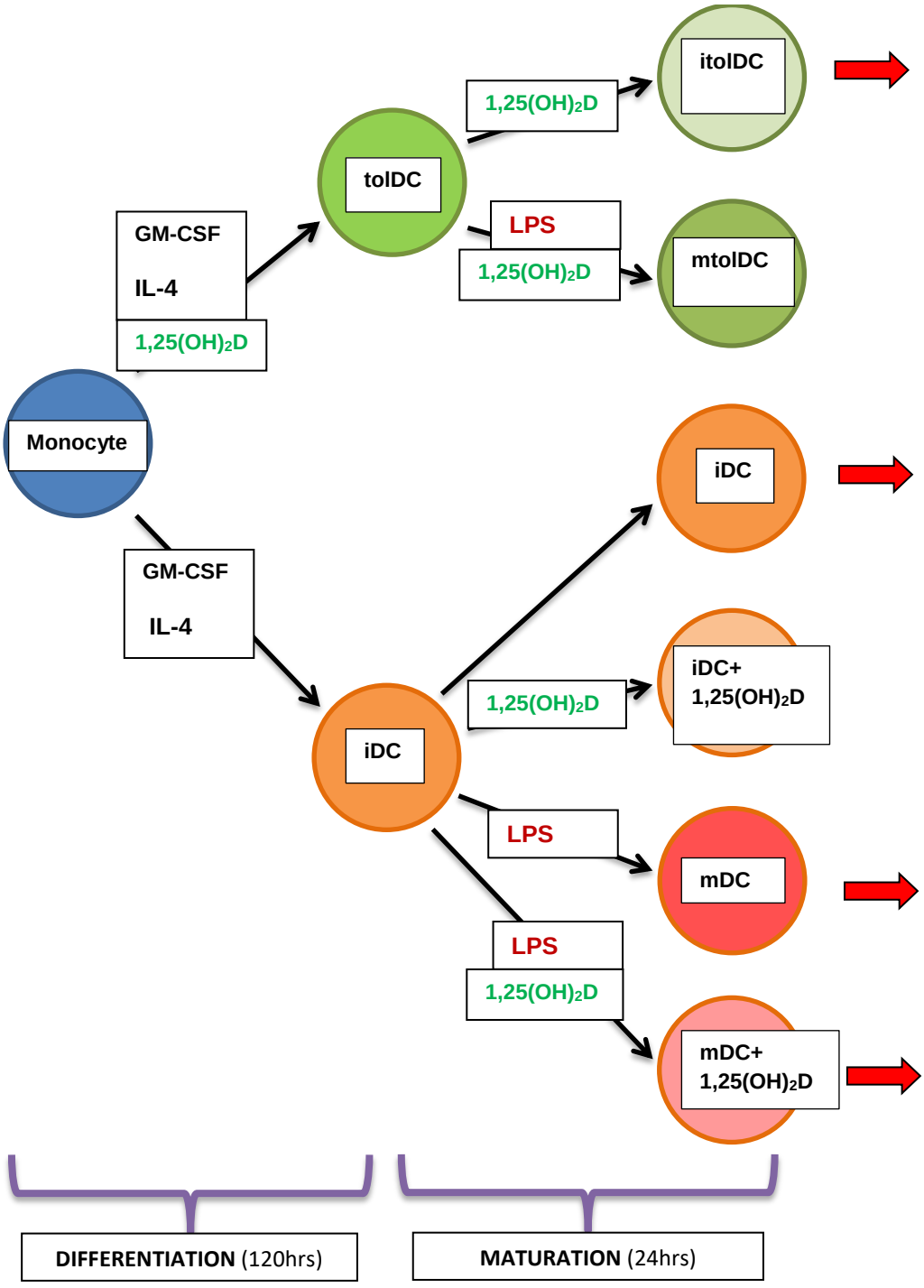
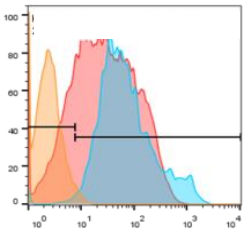
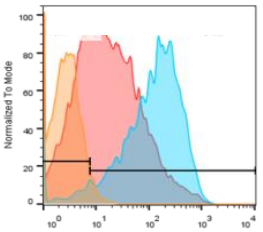


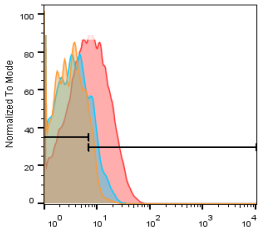
Figure 5.3. Induction of variable DC phenotypes with lipopolysaccharide (LPS) and 1,25-dihydroxyvitamin D ($1,25(OH)_2D3$)
itolDC – immature tolerogenic dendritic cell with $1,25(OH)_2D3$; mtolDC – mature tolerogenic dendritic cell with $1,25(OH)_2D3$; iDC – immature dendritic cell; mDC – mature dendritic cell; $1,25(OH)_2D3$ – immature dendritic cell with $1,25(OH)_2D3$; mDC+$1,25(OH)_2D3$ – mature dendritic cell with $1,25(OH)_2D3$. Monocytes were derived from whole blood leukocyte cones from healthy donors (n=6). All 6 phenotypes were used for candidate mRNA and miRNA analysis. The 4 cell phenotypes indicated were used for flow cytometry analysis and array miRNA analysis.

A

Long term $1,25(OH)_2D3$ (120hrs)





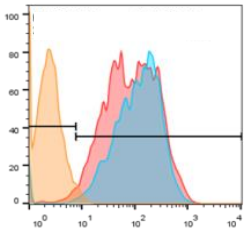


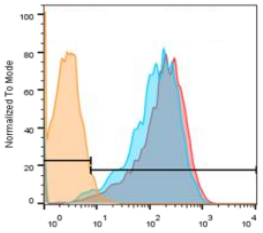
Isotype

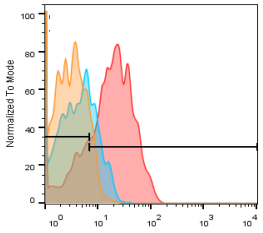
iDC

itolDC

Short term $1,25(OH)_2D3$ (24hrs)





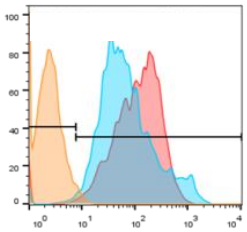


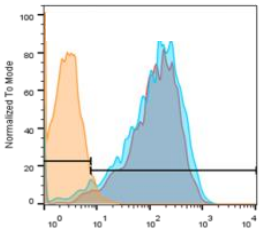
Isotype

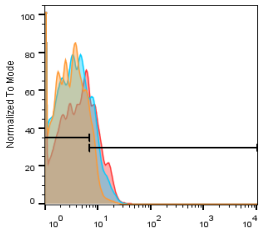
mDC

mDC+$1,25(OH)_2D3$

LPS (Maturation)







Isotype

iDC

mDC

HLA-DR

CD80

CD14

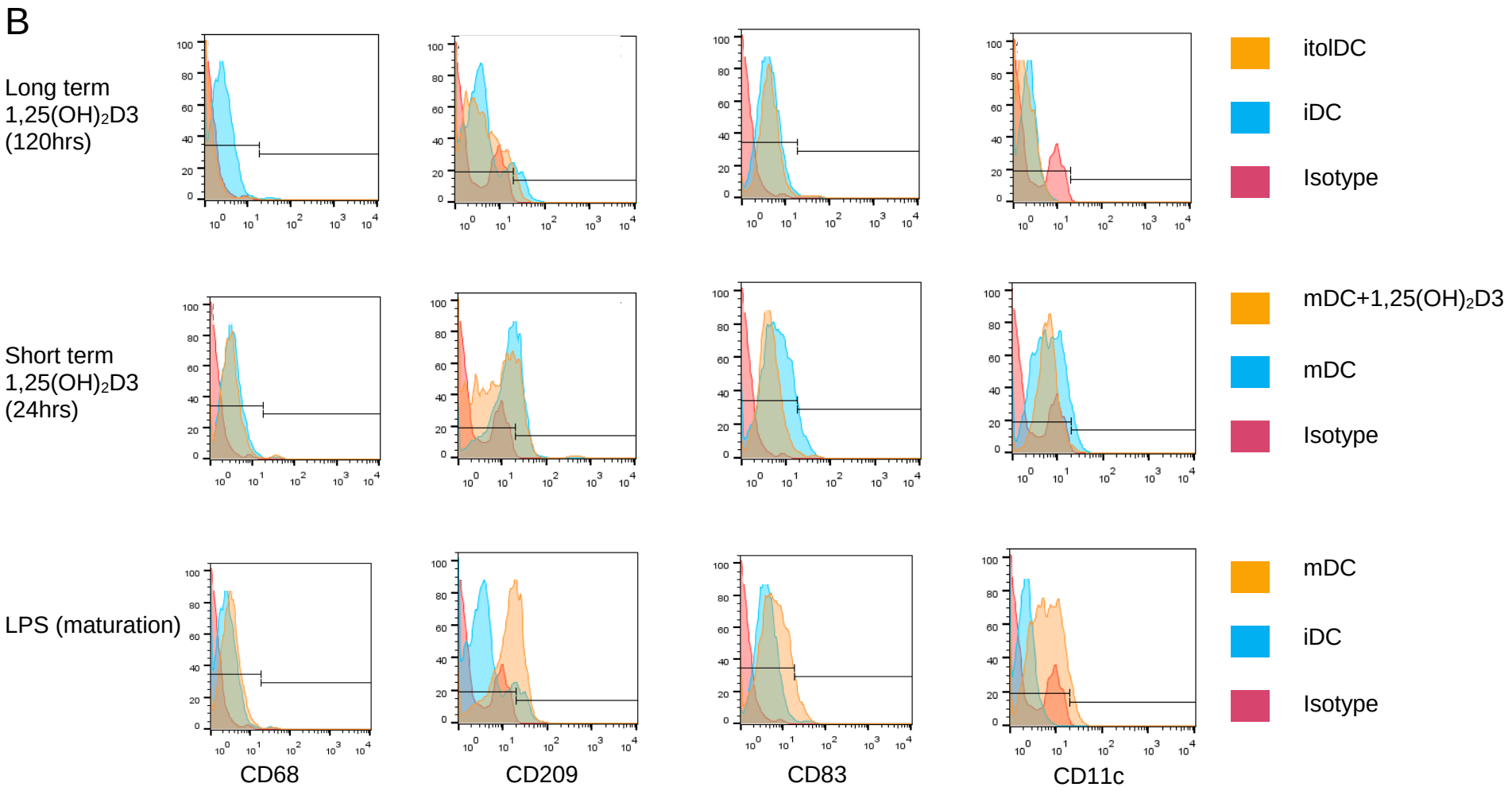


Figure 5.4 Flow cytometry staining validation of DC phenotype
Populations were stained for surface markers **A**) HLA-DR, CD80, CD14; **B**) CD68, CD209 (DC-SIGN), CD83 and CD11c. Isotype stain or a control population was used as the experimental control. Data is shown as histogram overlay plots, with the median fluorescence intensity (MTI) shown. Plots represent the mean expression pattern across all 6 donors.

Cell population	Median Fluorescence Intensity (MFI)							
	HLA-DR	**CD80**	**CD14**	**CD209**	**CD83**	**CD11c**	**CD86**	**CD40**
Isotype	3.64	3.02	2.18	1	1.56	3.35	3.46	11.5
iDC	199	6.15	7.42	2.71	3.57	326	6.41	9.5
itolDC	6.55	4.88	12.7	4.01	3.09	311	2.79	8.78
mDC	277	63.1	3.29	14.9	7.2	351	44.9	12.8
mDC+1,25(OH)2D3	250	29	23.4	1.01	1.52	306	1.3	8.58

Table 5.1. Expression of DC surface antigens by flow cytometry
Relative expression of DC surface antigens measured by flow cytometry are shown as median fluorescence intensity (MFI) values. iDC, immature DC; itolDC, long term 1,25$(OH)_2$D3; mDC, mature DC; mDC+1,25$(OH)_2$D3, short term 1,25$(OH)_2$D3

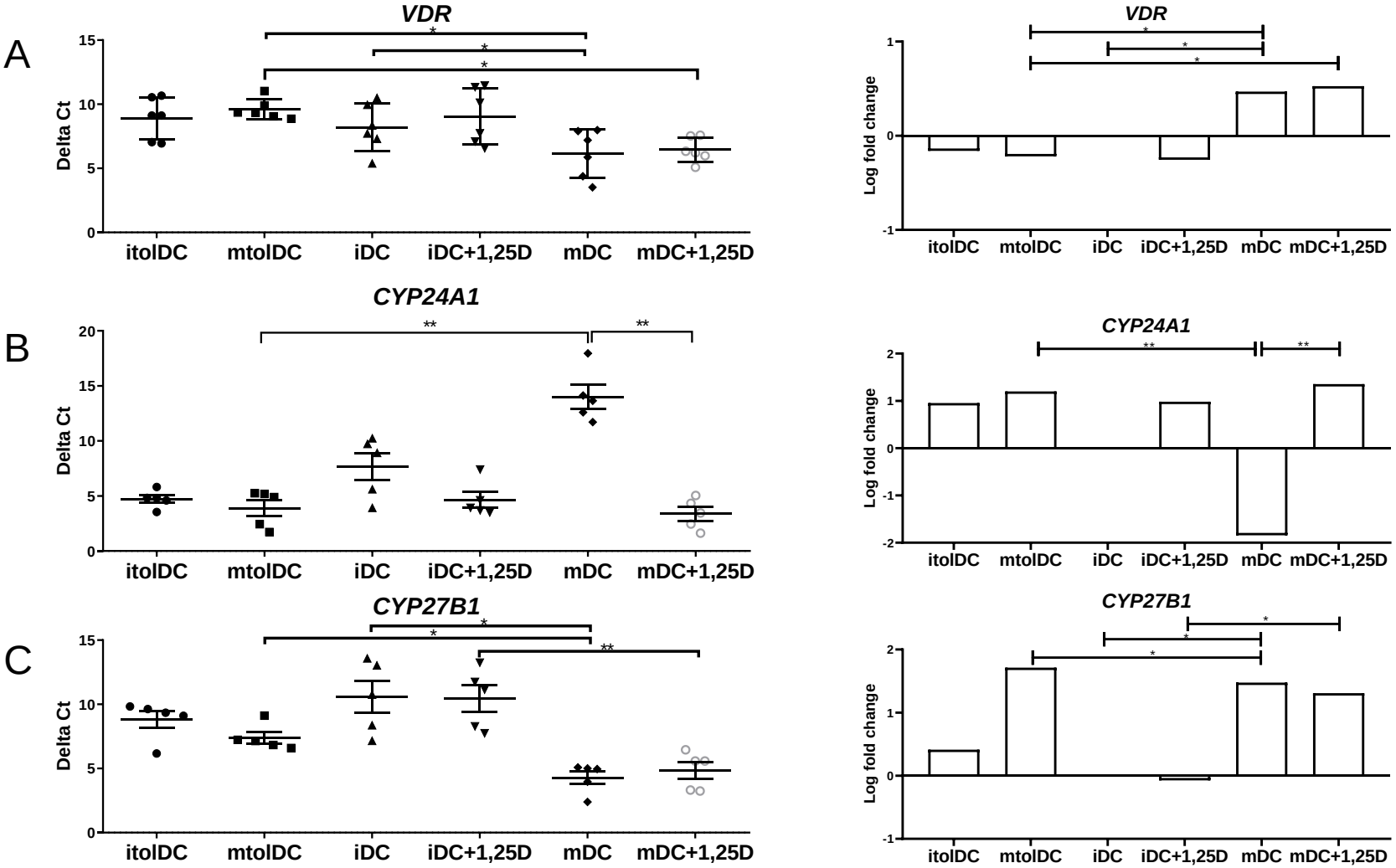


Figure 5.5. Expression of genes associated with vitamin D metabolism and function in different DC phenotypes
Data for qRT-PCR analysis of mRNA represents an individual donor (n=6). Data are shown as: mean ± SD raw Delta Ct (ΔCt) values from qRT-PCR (left panels), and fold-change in mRNA expression (right panels) of: A) *VDR*; B) *CYP24A1*; C) *CYP27B1* in each of the 6 different DC phenotypes shown in **Figure 5.3**.

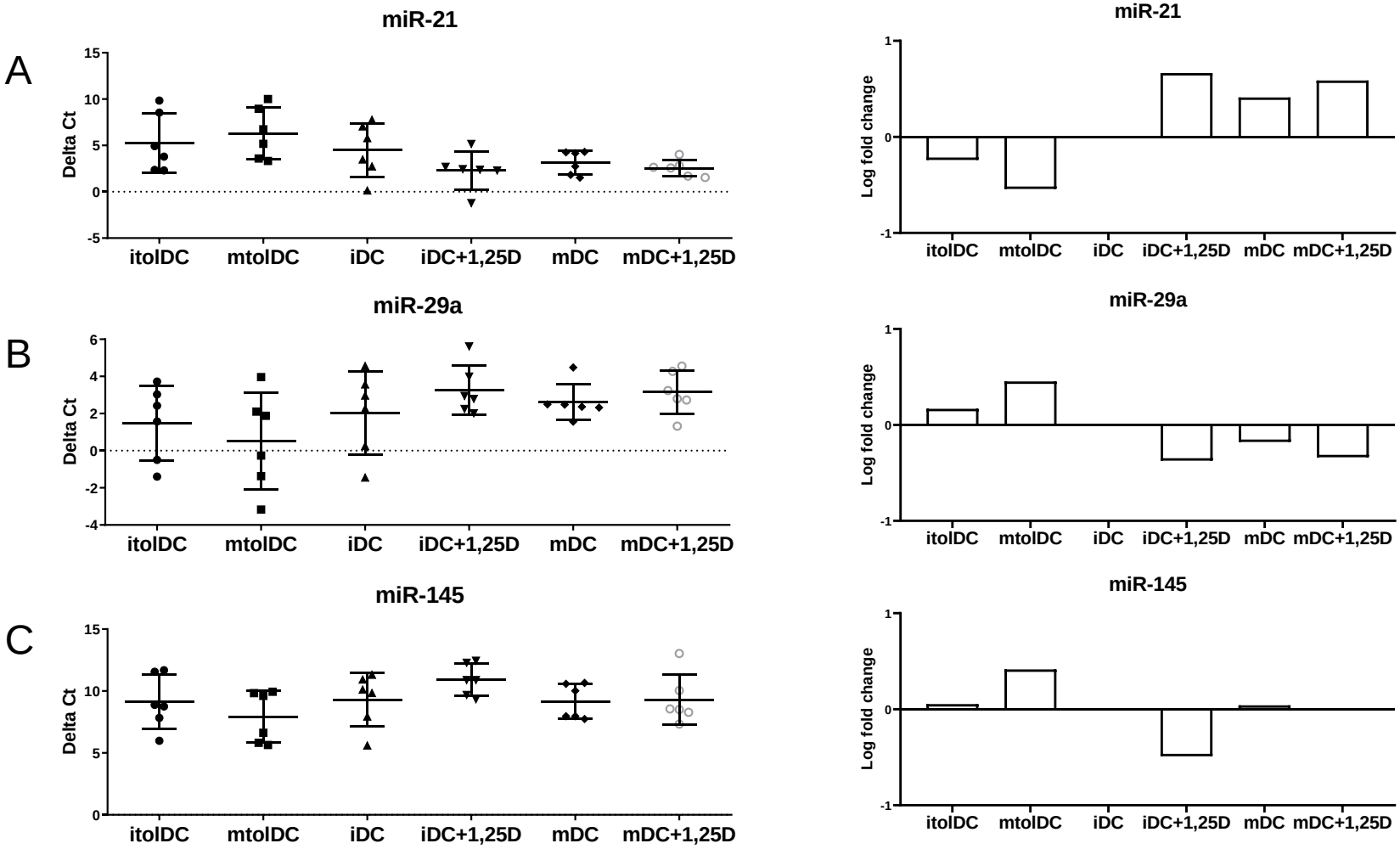
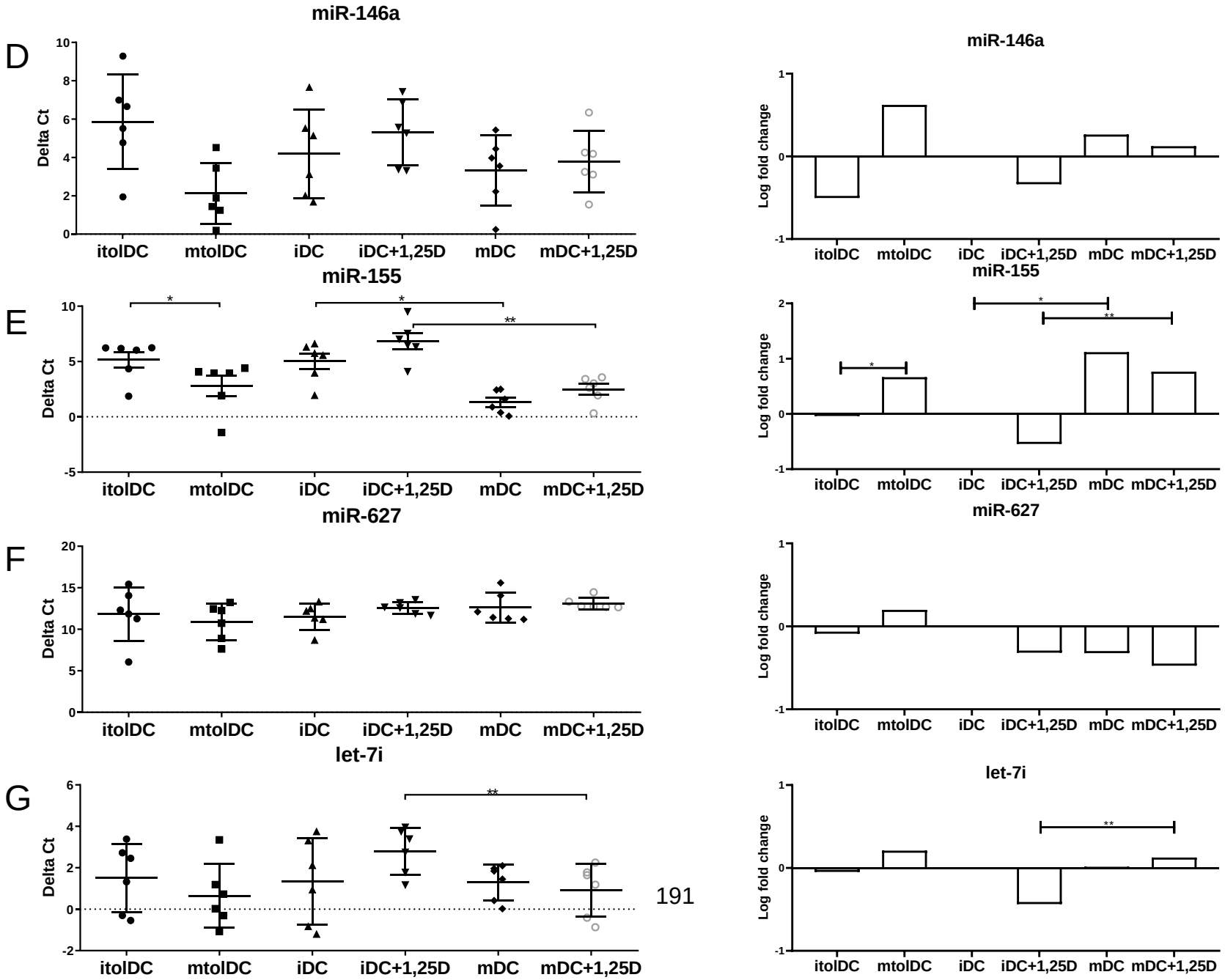


Figure 5.6. Expression of candidate miRNAs in different DC phenotypes
Data for qRT-PCR analysis of miRNA represents an individual donor (n=6). Data are shown as: mean ± SD raw ΔCt values from qRT-PCR (left panels), and fold-change in miRNA expression (right panels) of: A) miR-21; B) miR-29a; C) miR-145; D) miR-146a; E) miR-155; F) miR-627; G) let-7i, in each of the 6 different DC phenotypes shown in **Figure 5.3**.


D
miR-146a
Delta Ct
10
8
6
4
2
0
itolDC
mtolDC
iDC
iDC+1,25D
mDC
mDC+1,25D
miR-146a
Log fold change
1
0
-1
E
miR-155
Delta Ct
10
5
0
-5
*
*
**
miR-155
Log fold change
2
1
0
-1
F
miR-627
Delta Ct
20
15
10
5
0
miR-627
Log fold change
G
let-7i
Delta Ct
6
4
2
0
-2
**
let-7i
Log fold change

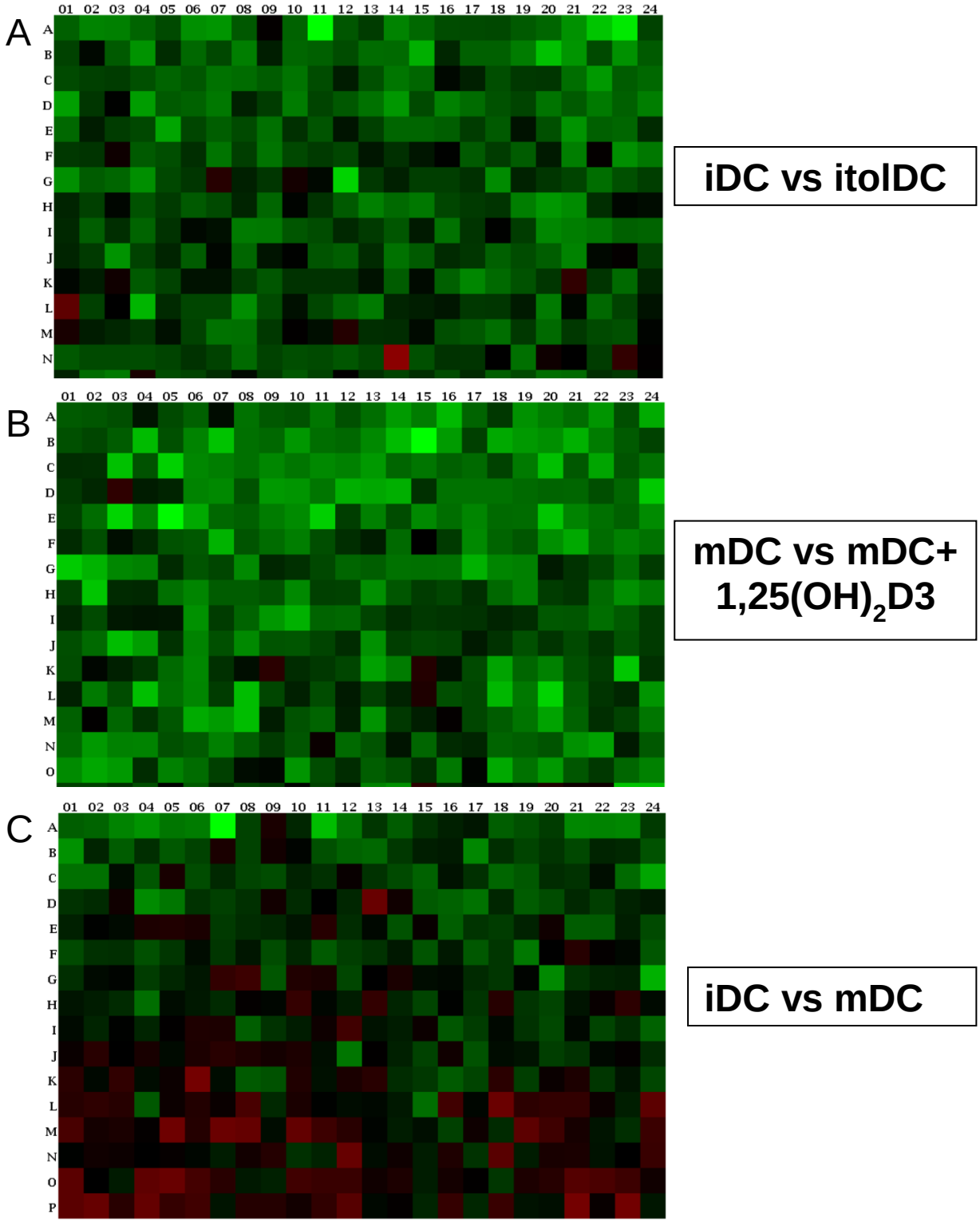
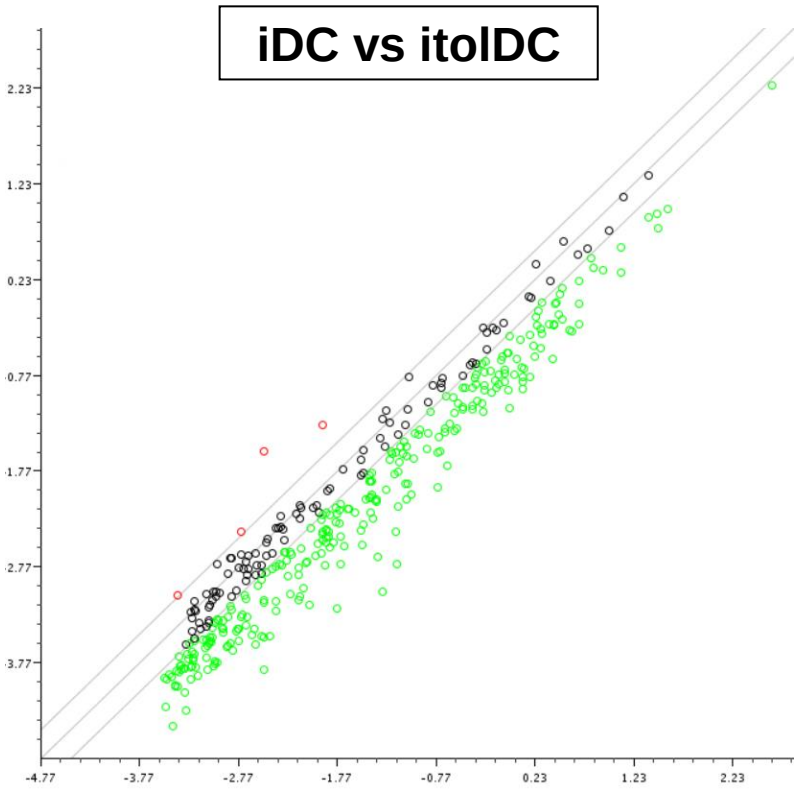


Figure 5.7. Array miRNA expression shown as representative heat-maps
Green and red plots represent downregulation and up-regulation of miRNA expression respectively, calculated as $\log_2$ fold change for 372 miRNAs. Heat-maps show miRNA expression change to treatment of DC with A) long term 1,25$(OH)_2$D3 (iDC vs itolDC); B) short term 1,25$(OH)_2$D3 (mDC vs mDC+1,25$(OH)_2$D3); C) LPS maturation (iDC vs mDC). Coordinates on the heat-map correspond to individual miRNAs profiled in the array, which are listed in **Table 3.3.2**.

A



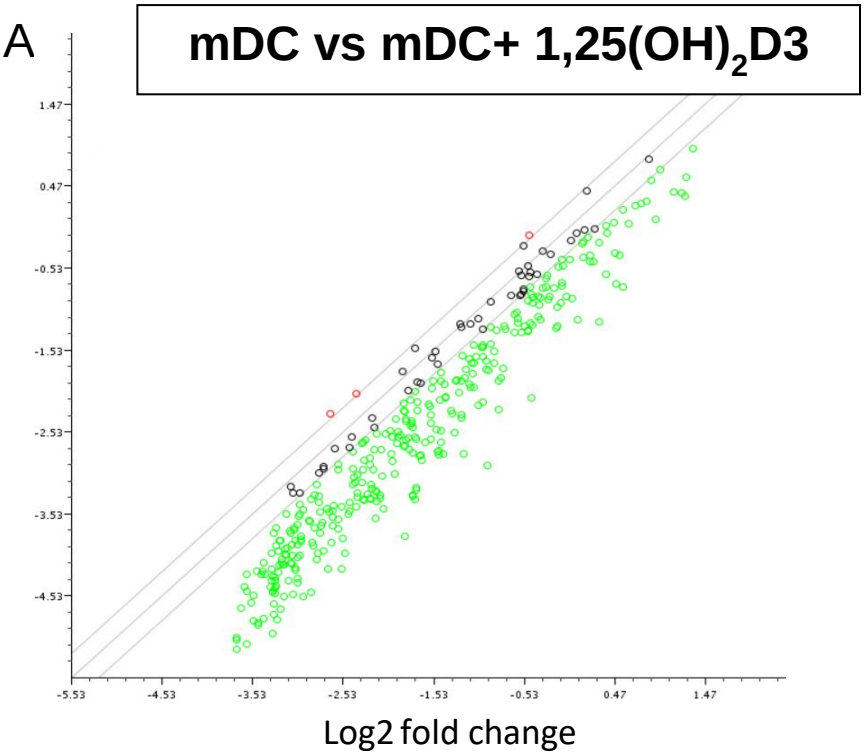
B

miRNAs	Long term 1,25D Expression change	*p* value
miR-1-3p	↓	0.005
miR-10b-5p	↓	0.029
miR-28-3p	↓	0.016
miR-28-5p	↓	0.037
miR-30e-3p	↓	0.025
miR-32-3p	↓	0.009
miR-340-3p	↓	0.031
miR-374c-5p	↓	0.021

Figure 5.8. qPCR array of miRNA expression in response to long term treatment of DC with 1,25(OH)$_2$D3

A) Scatter plot showing miRNA expression changes following long term 1,25(OH)$_2$D3 (10 nM, 120hrs) in itolDC (x-axis) vs iDC (y-axis) populations. Individual green and red dots represent downregulation and up-regulation of a single miRNA respectively, calculated as $\log_2$ fold change; B) Huynh-Feldt statistical analysis of statistically significant miRNAs differentially regulated following long term 1,25(OH)$_2$D3. Huynh-Feldt test was performed on normalised data, and significance taken as $p<0.05$.

A



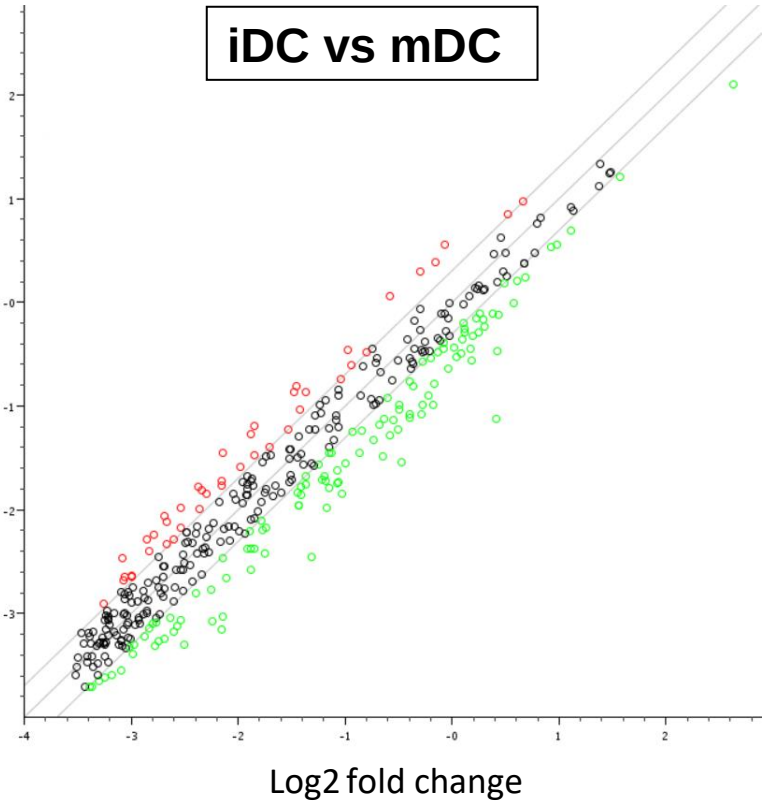
B

miRNAs	Short term 1,25D Expression change	*p* value
miR-125a-5p	↓	0.025
miR-1284	↓	0.027
miR-143-5p	↓	0.018
miR-152-3p	↓	0.015
miR-155-5p	↓	0.049
miR-155-3p	↓	0.004
miR-1914-3p	↓	0.049
miR-193a-5p	↓	0.001
miR-200c-5p	↓	0.022
miR-27b-5p	↓	0.036
miR-296-3p	↓	0.030
miR-377-5p	↓	0.026
miR-432-5p	↓	0.050
miR-506-3p	↓	0.030

Figure 5.9. qPCR array of miRNA expression in response to short term treatment of DC with 1,25(OH)$_2$D3

A) Scatter plot showing miRNA expression changes following short term 1,25(OH)$_2$D3 (10 nM, 24hrs) in mDC+1,25(OH)$_2$D3 (x-axis) vs mDC (y-axis) populations. Individual green and red dots represent downregulation and up-regulation of a single miRNA respectively, calculated as $\log_2$ fold change; B) Huynh-Feldt statistical analysis of statistically significant miRNAs differentially regulated following short term 1,25(OH)$_2$D3. Huynh-Feldt test was performed on normalised data, and significance taken as $p<0.05$.

A



B

miRNAs	LPS maturation	*p* values
	Expression change	
miR-155-5p	↑	0.049
miR-221-5p	↓	0.001

Figure 5.10. qPCR array of miRNA expression in response to maturation of DC with LPS

A) Scatter plot showing miRNA expression changes following LPS maturation (24hrs) in mDC (x-axis) vs iDC (y-axis) populations. Individual green and red dots represent downregulation and up-regulation of a single miRNA respectively, calculated as $\log_2$ fold change; B) Huynh-Feldt statistical analysis of statistically significant miRNAs differentially regulated following LPS. Huynh-Feldt test was performed on normalised data, and significance taken as $p<0.05$.

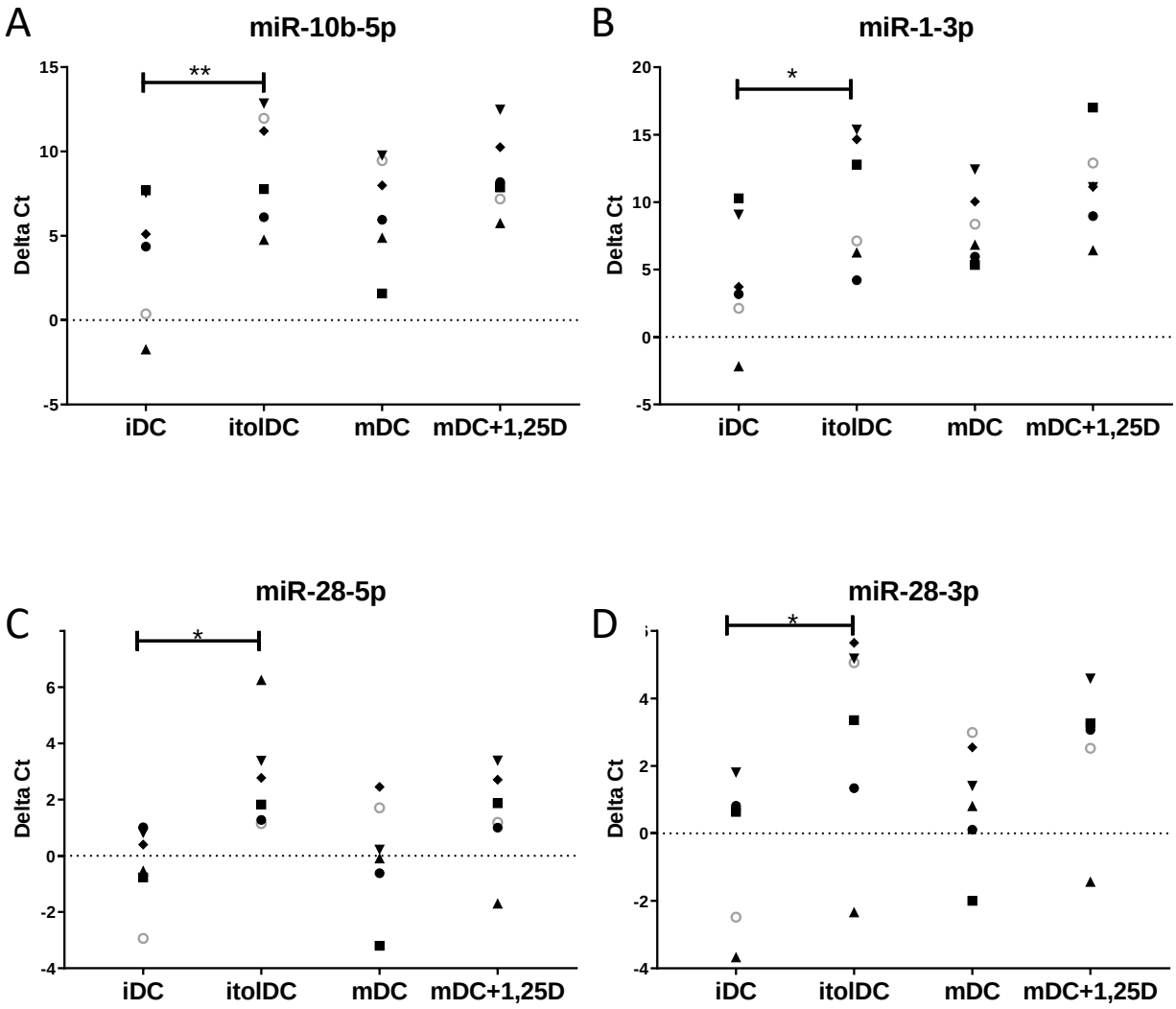
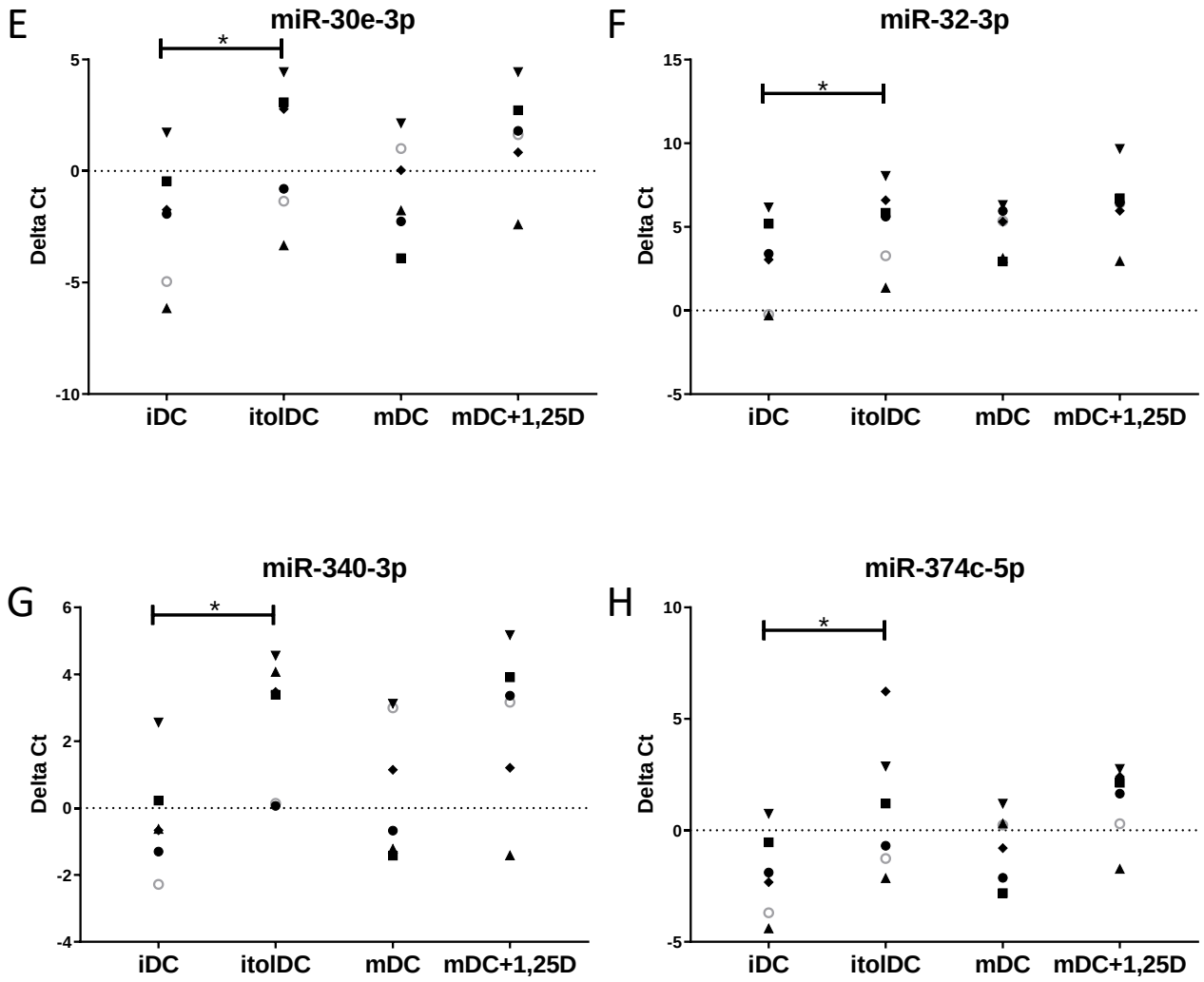


Figure 5.11. Graphical representation of significantly regulated qPCR array miRNAs, to long term 1,25(OH)$_2$D3 (120hrs)

qPCR array quantified expression changes in response to long term 1,25(OH)$_2$D3 for A) miR-10b-5p; B) miR-1-3p; C) miR-28-5p; D) miR-28-3p; E) miR-30e-3p; F) miR-32-3p; G) mir-340-3p; H) miR-374c-5p. Individual donors (n=6) are shown. 1,25(OH)$_2$D3 has been abbreviated to 1,25D. Repeated-measures ANOVA for paired data was used to calculate statistical significance, and significance was taken as *p<0.05.


E
miR-30e-3p
*
5
0
-5
-10
Delta Ct
iDC
itolDC
mDC
mDC+1,25D
F
miR-32-3p
*
15
10
5
0
-5
Delta Ct
iDC
itolDC
mDC
mDC+1,25D
G
miR-340-3p
*
6
4
2
0
-2
-4
Delta Ct
iDC
itolDC
mDC
mDC+1,25D
H
miR-374c-5p
*
10
5
0
-5
Delta Ct
iDC
itolDC
mDC
mDC+1,25D

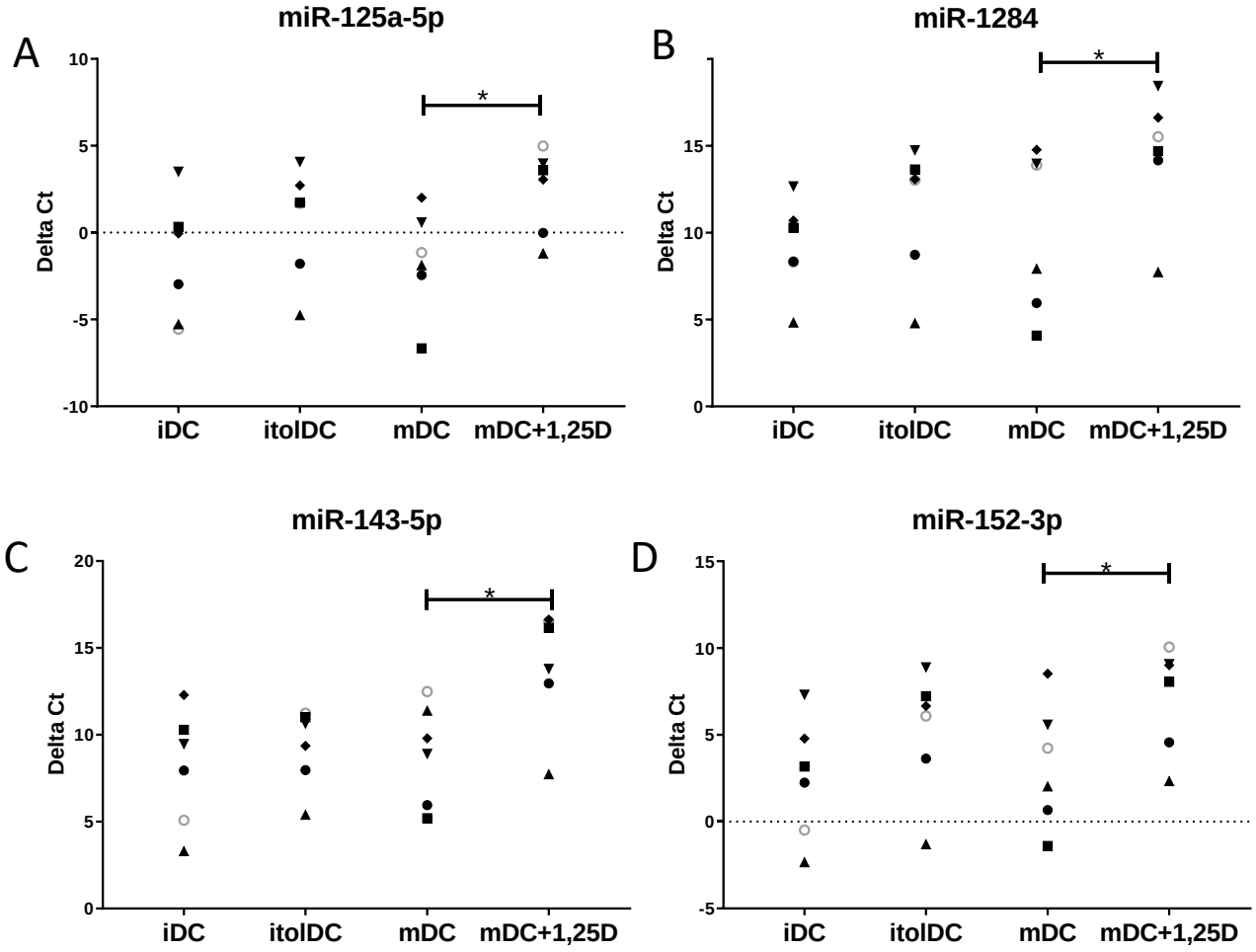
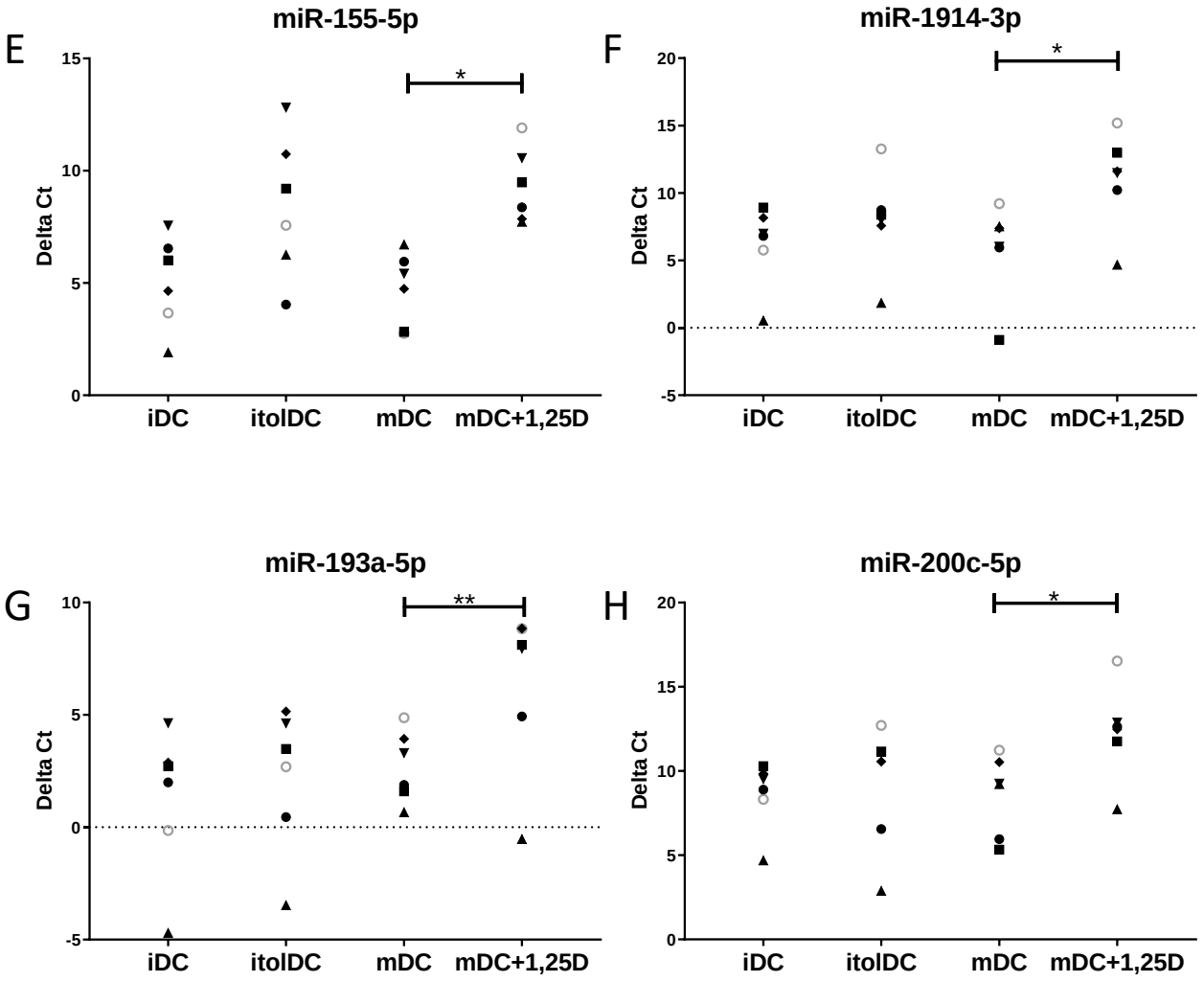
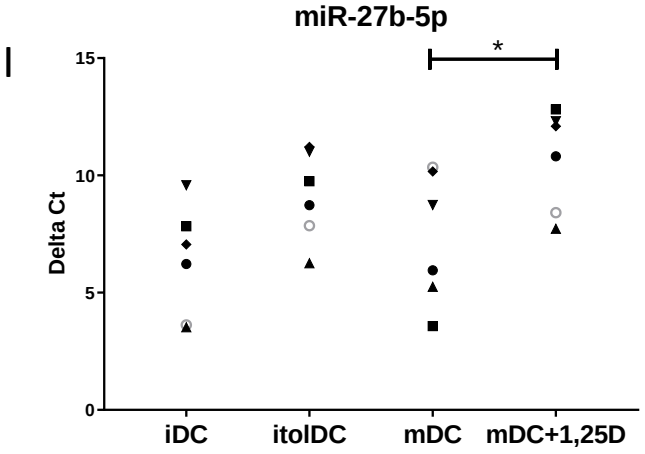
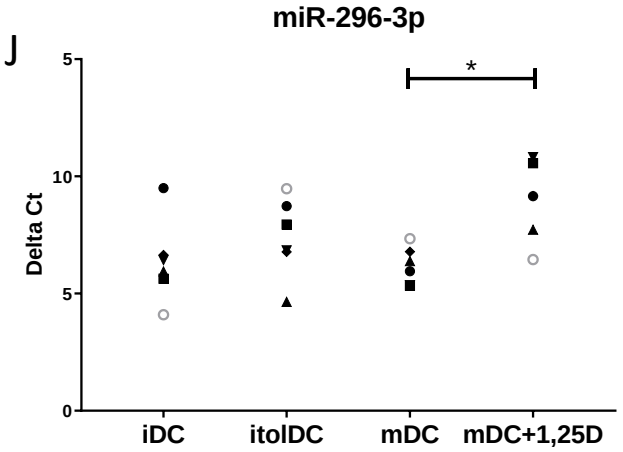


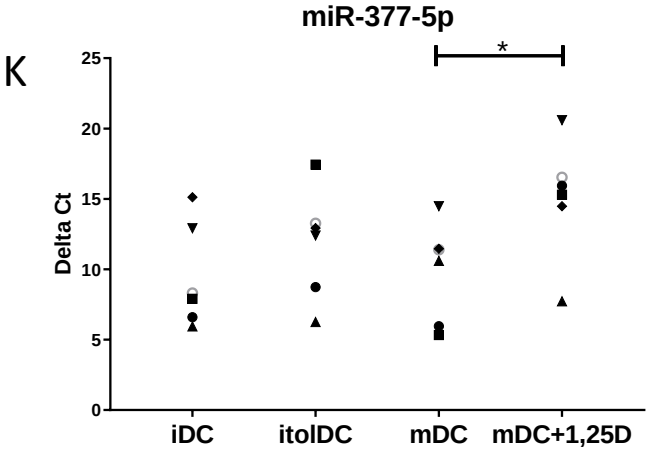
Figure 5.12. Graphical representation of significantly regulated qPCR array miRNAs, to short term 1,25(OH)$_2$D3 (24hrs)

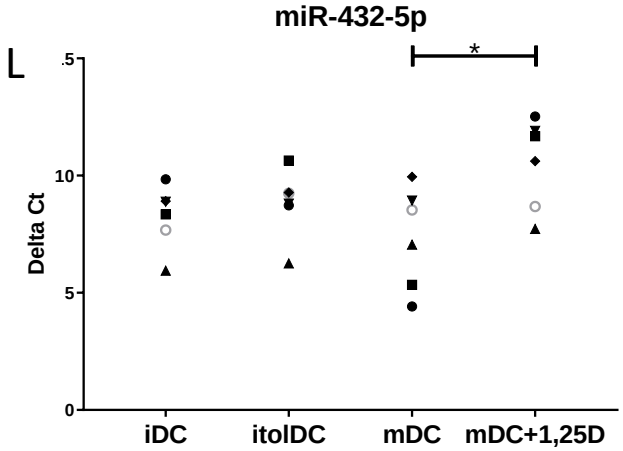
qPCR array quantified expression change in response to short term 1,25(OH)$_2$D3 for A) miR-125a-5p; B) miR-1284; C) miR-143-5p; D) miR-152-3p; E) miR-155-5p; F) miR-1914-3p; G) mir-193a-5p; H) miR-200c-5p; I) miR-27b-5p; J) miR-296-3p; K) miR-377-5p; L) miR-432-5p; M) miR-506-3p. Individual donors (n=6) are shown. 1,25(OH)$_2$D3 has been abbreviated to 1,25D. Repeated-measures ANOVA for paired data was used to calculate statistical significance, and significance was taken as *p<0.05.

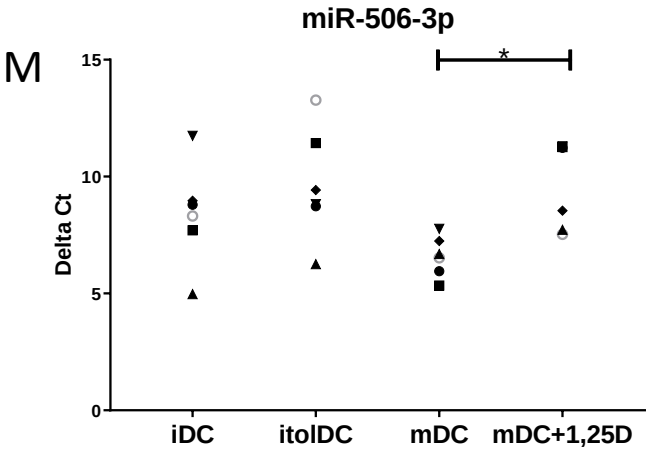

E
miR-155-5p
Delta Ct
15
10
5
0
*
iDC
itolDC
mDC
mDC+1,25D
F
miR-1914-3p
Delta Ct
20
15
10
5
0
-5
*
iDC
itolDC
mDC
mDC+1,25D
G
miR-193a-5p
Delta Ct
10
5
0
-5
**
iDC
itolDC
mDC
mDC+1,25D
H
miR-200c-5p
Delta Ct
20
15
10
5
0
*
iDC
itolDC
mDC
mDC+1,25D


I
miR-27b-5p
Delta Ct
15
10
5
0
*
iDC
itolDC
mDC
mDC+1,25D


J
miR-296-3p
Delta Ct
5
10
5
0
*
iDC
itolDC
mDC
mDC+1,25D


K
miR-377-5p
Delta Ct
25
20
15
10
5
0
*
iDC
itolDC
mDC
mDC+1,25D


L
miR-432-5p
Delta Ct
.5
10
5
0
*
iDC
itolDC
mDC
mDC+1,25D


M
miR-506-3p
Delta Ct
15
10
5
0
*
iDC
itolDC
mDC
mDC+1,25D

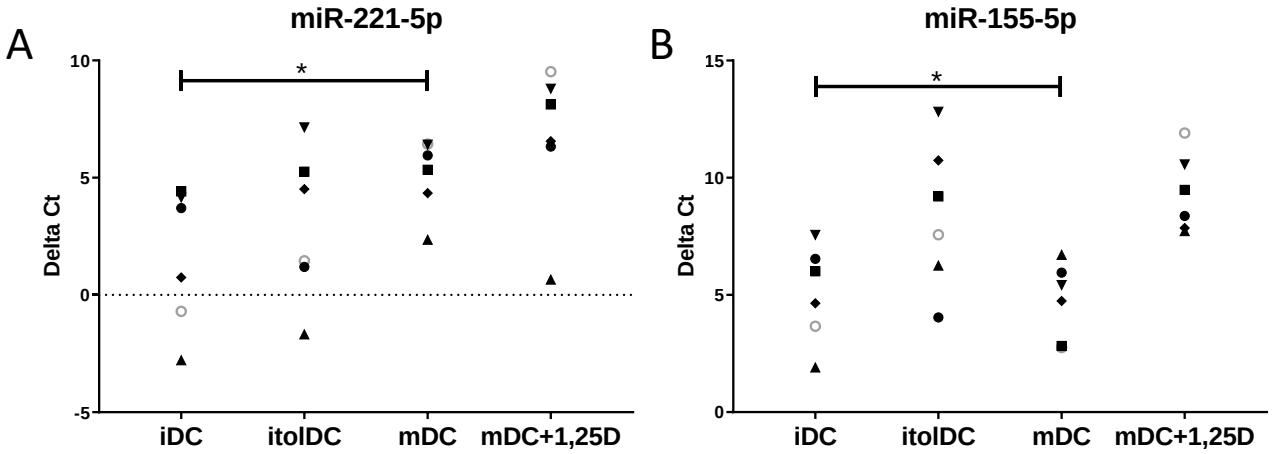


Figure 5.13. Graphical representation of significantly regulated qPCR array miRNAs, to LPS maturation (24hrs)

qPCR array quantified expression changes in response to LPS maturation for A) miR-221-5p; B) miR-155-5p. Individual donors (n=6) are shown. 1,25$(OH)_2$D3 has been abbreviated to 1,25D. Repeated-measures ANOVA for paired data was used to calculate statistical significance, and significance was taken as *p<0.05.

miRNAs	verified mRNA targets (miRTarBase)				
miR-1-3p	MET	TAGLN2	PTMA	GJA1	EDN1
miR-10b-5p	HOXD10	KLF4	BCL2L11	HOXB3	PTEN
miR-125a-5p	CDKN1A	TNFAIP3	CD34	TP53	ERBB2
miR-1284	KCNK10	EIF4A1	KIF13A	PMAIP1	TCTE1
miR-143-5p	COX2	GLCE	RAB12	GINS4	ZNF460
miR-152-3p	HLA-G	DNMT1	ADAM17	CD151	CD274
miR-155-5p	SOCS1	TAB2	PIK3R1	JARID2	APC
miR-155-3p	IRAK3	PTEN	CREBRF	ZNF460	UBE2K
miR-1914-3p	SEPT8	ZBTB46	SRM	BARHL1	TXNIP
miR-193a-5p	TP73	ERBB2	RBBP6	PCSK9	FGF19
miR-200c-5p	C2orf72	POLR2F	PKIA	HPS4	ACTR1A
miR-221-5p	MBD2	YOD1	HES7	C18orf25	MUC17
miR-27b-5p	COX20	PPP4R1L	G3BP1	DDX55	SNAP47
miR-28-3p	CTC1	VAV3	PER1	EIF4G2	MCTS1
miR-28-5p	CDKN1A	MPL	N4BP1	MAPK1	E2F6
miR-296-3p	KCNH1	FBXW7	GBA2	TFAP2A	KMT2D
miR-30e-3p	NFKBIA	KPNA2	EIF1	VAV3	MYC
miR-32-3p	ATAD5	COL23A1	VPS4A	C5orf24	RBPJ
miR-340-3p	AGBL5	SYNJ2BP	CNTN4	C18orf32	QTRT2
miR-374c-5p	E2F7	ANKRD33B	ZNF850	KPNA6	CNBP
miR-377-5p	LYRM4	GDE1	TFDP2	TRIM66	ZNF573
miR-432-5p	MECP2	RCOR1	NES	LRPPRC	BLCAP
miR-506-3p	CD151	VIM	CDK4	CDK6	YAP1

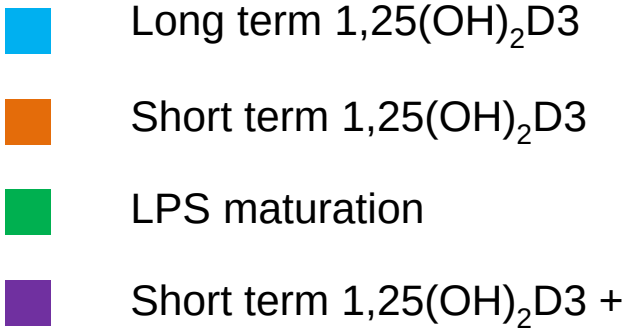
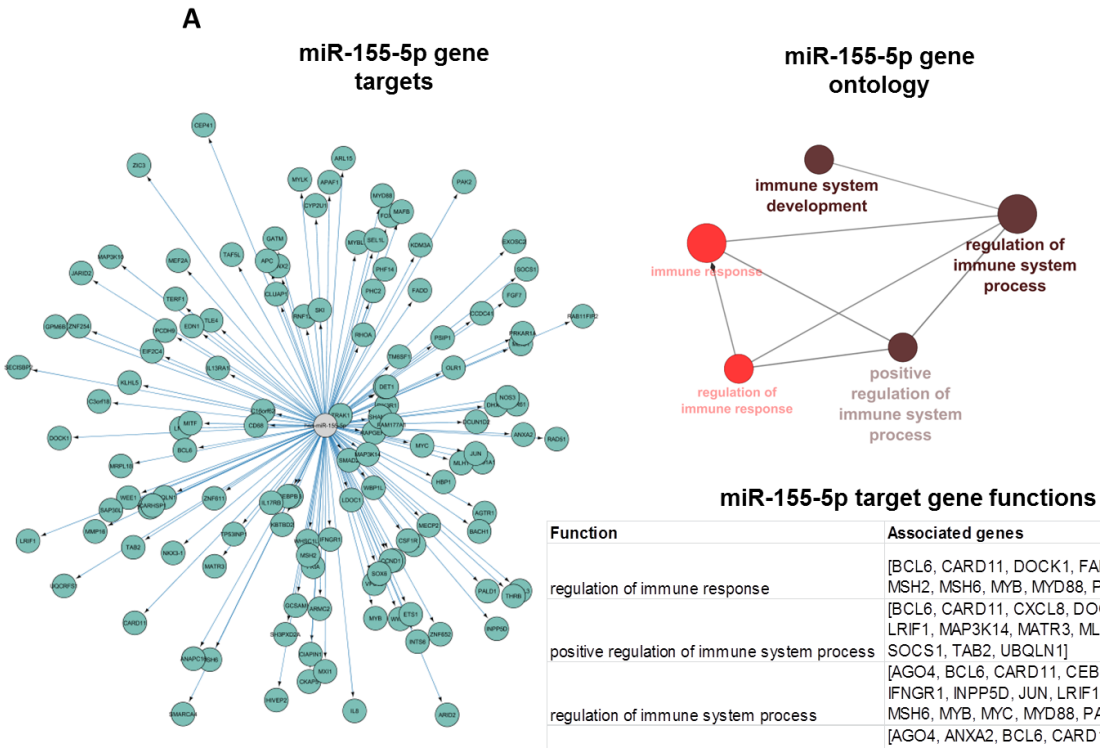


Table 5.2. Summary of DC miRNAs significantly regulated by 1,25(OH)$_2$D3 and their predicted mRNA targets

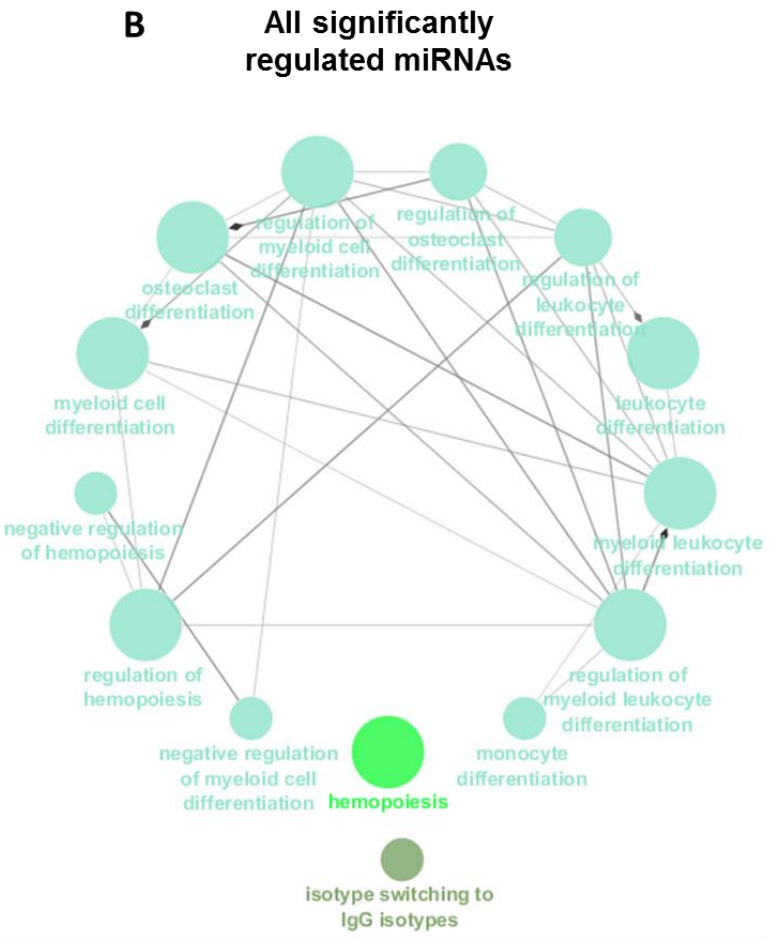
Table of significantly regulated miRNAs in response to long term 1,25(OH)$_2$D3, short term 1,25(OH)$_2$D3 and LPS. MiRNAs significantly regulated by each treatment is colour-coded accordingly. Statistical significance is taken as $p<0.05$ by Huynh-Feldt analysis. The top 5 experimentally validated mRNA targets for each significant miRNA as predicted by miRTarBase are shown. Only those with a target prediction score of 95/100 or above were included.

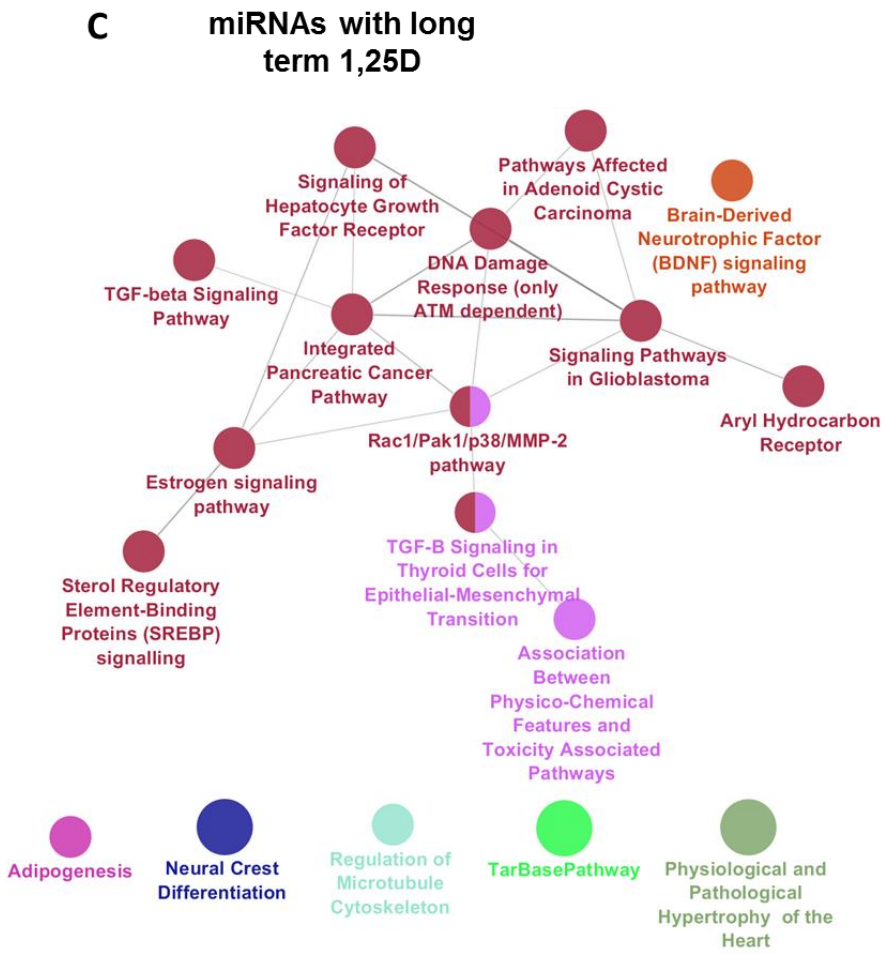


miR-155-5p target gene functions

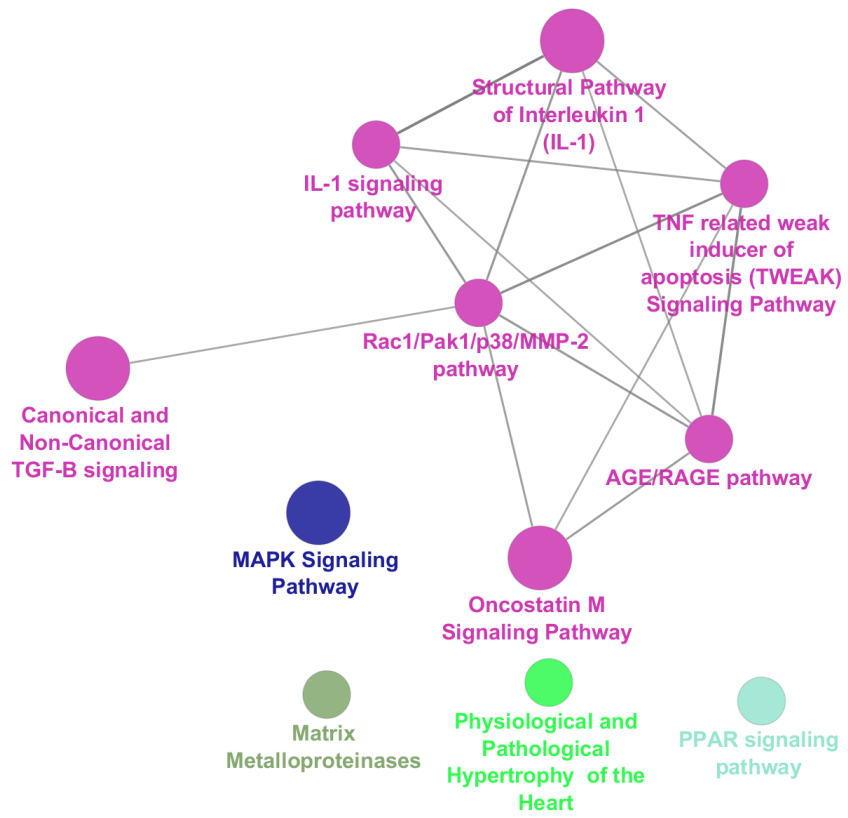
Function	Associated genes
regulation of immune response	[BCL6, CARD11, DOCK1, FADD, GCSAM, IFNGR1, INPP5D, JUN, LRIF1, MATR3, MLH1, MSH2, MSH6, MYB, MYD88, PAK2, PIK3R1, SOCS1, TAB2, UBQLN1]
positive regulation of immune system process	[BCL6, CARD11, CXCL8, DOCK1, EDN1, ETS1, FADD, FOXO3, GCSAM, INPP5D, JUN, LRIF1, MAP3K14, MATR3, MLH1, MSH2, MSH6, MYB, MYD88, PAK2, PIK3R1, RHOA, SOCS1, TAB2, UBQLN1]
regulation of immune system process	[AGO4, BCL6, CARD11, CEBPB, CXCL8, DOCK1, EDN1, ETS1, FADD, FOXO3, GCSAM, IFNGR1, INPP5D, JUN, LRIF1, MAFB, MAP3K14, MATR3, MEIS1, MLH1, MSH2, MSH6, MYB, MYC, MYD88, PAK2, PIK3R1, PRKAR1A, RHOA, SOCS1, TAB2, UBQLN1]
immune system development	[AGO4, ANXA2, BCL6, CARD11, CEBPB, CIAPIN1, CSF1R, ETS1, FADD, FOXO3, INPP5D, JARID2, JUN, LRIF1, MAFB, MEIS1, MLH1, MSH2, MSH6, MYB, MYC, PIK3R1, RUNX2, SH3PXD2A, SOCS1, SOX6]
immune response	[ANXA2, APAF1, BCL6, C16orf62, CARD11, CD68, CEBPB, CSF1R, CXCL8, DOCK1, EDN1, ETS1, FADD, GCSAM, IFNGR1, INPP5D, JUN, LRIF1, MAP3K14, MATR3, MLH1, MSH2, MSH6, MYB, MYD88, OLR1, PAK2, PIK3R1, RHOA, SOCS1, TAB2, TSPAN14, UBQLN1]

Figure 5.14. Gene Ontology analysis of significantly regulated miRNA targets
Cytoscape v3.6.1 gene prediction software was used in conjunction with ClueGo gene ontology software to identify experimentally validated gene targets of miRNAs and their biological functions. Networks show validated gene targets of A) miR-155-5p; B) all significantly regulated miRNAs with functions in immune regulation; C) miRNAs significantly downregulated by long term 1,25(OH)$_2$D3; D) miRNAs significantly downregulated by short term 1,25(OH)$_2$D3; E) miRNAs significantly regulated by LPS. For Cytoscape miRNA target prediction, all targets with a target prediction score below of 95% were excluded. For ClueGo gene ontology (GO) analysis, statistical significance of genes with enriched biological functions was taken as $p<0.05$ calculated by Bonferroni step-down. Where gene ontology is not shown, no statistically significant links were found. Colours and distance of nodes do not correspond to level of significance, and are for display purposes only.

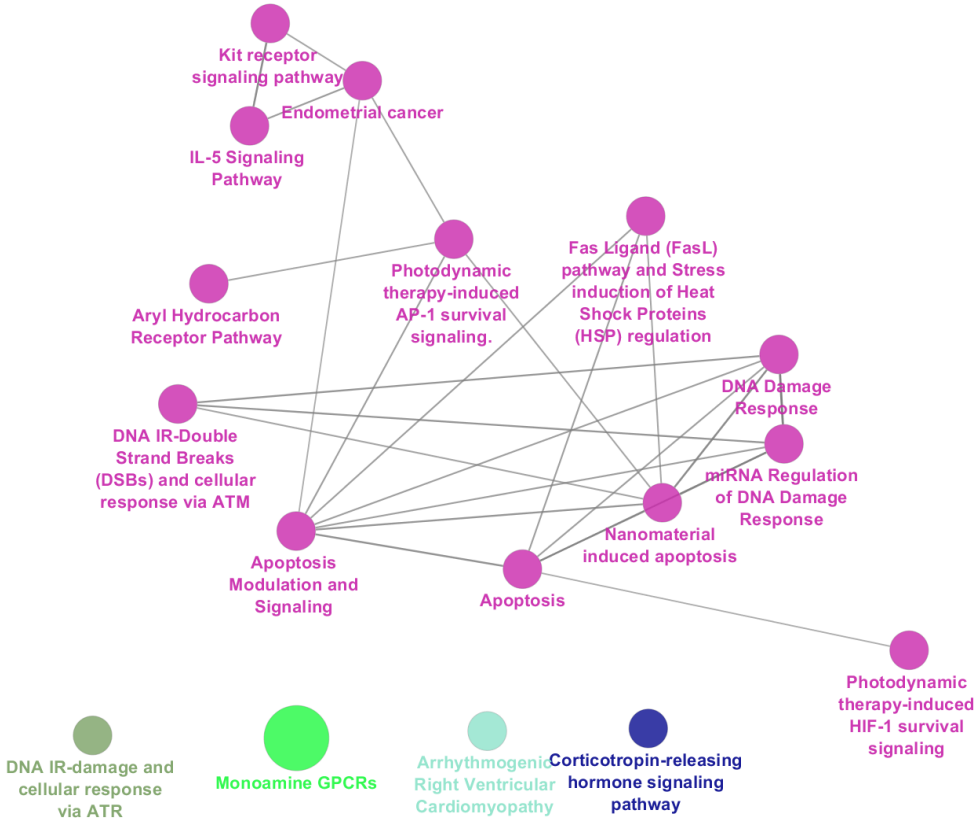

B
All significantly regulated miRNAs
regulation of myeloid cell differentiation
regulation of osteoclast differentiation
osteoclast differentiation
regulation of leukocyte differentiation
myeloid cell differentiation
leukocyte differentiation
negative regulation of hemopoiesis
myeloid leukocyte differentiation
regulation of hemopoiesis
regulation of myeloid leukocyte differentiation
negative regulation of myeloid cell differentiation
hemopoiesis
monocyte differentiation
isotype switching to IgG isotypes


C
miRNAs with long term 1,25D
Signaling of Hepatocyte Growth Factor Receptor
Pathways Affected in Adenoid Cystic Carcinoma
Brain-Derived Neurotrophic Factor (BDNF) signaling pathway
TGF-beta Signaling Pathway
DNA Damage Response (only ATM dependent)
Integrated Pancreatic Cancer Pathway
Signaling Pathways in Glioblastoma
Aryl Hydrocarbon Receptor
Rac1/Pak1/p38/MMP-2 pathway
Estrogen signaling pathway
TGF-B Signaling in Thyroid Cells for Epithelial-Mesenchymal Transition
Sterol Regulatory Element-Binding Proteins (SREBP) signalling
Association Between Physico-Chemical Features and Toxicity Associated Pathways
Adipogenesis
Neural Crest Differentiation
Regulation of Microtubule Cytoskeleton
TarBasePathway
Physiological and Pathological Hypertrophy of the Heart

D **miRNAs with short term 1,25D**



E **miRNAs matured by LPS**



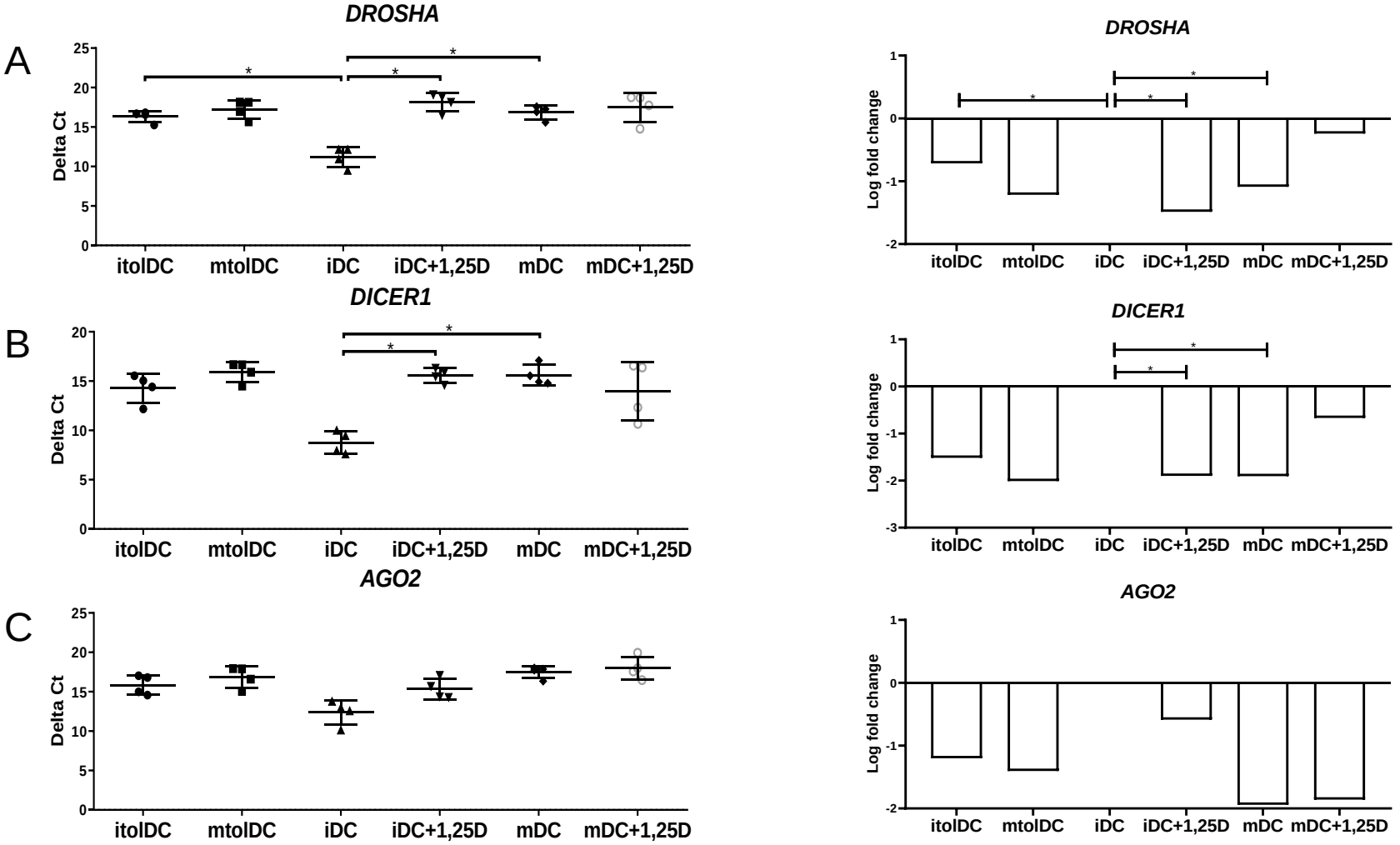
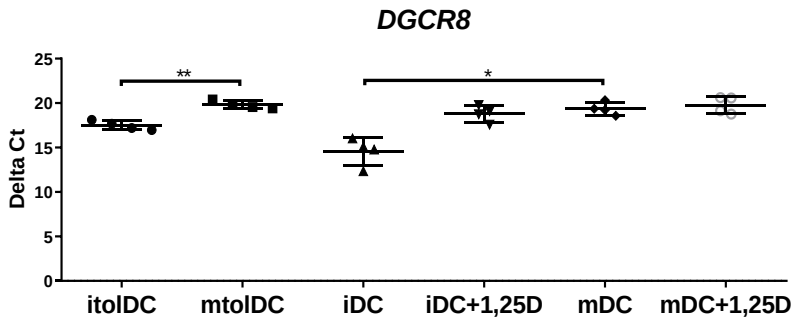
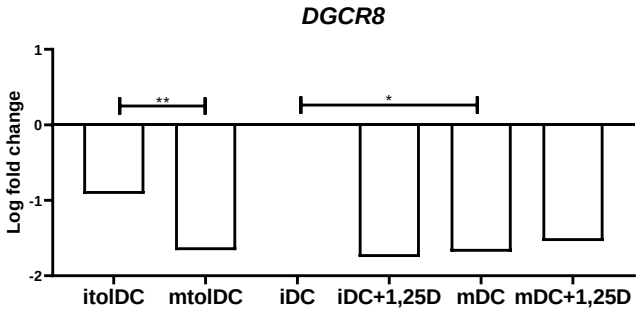


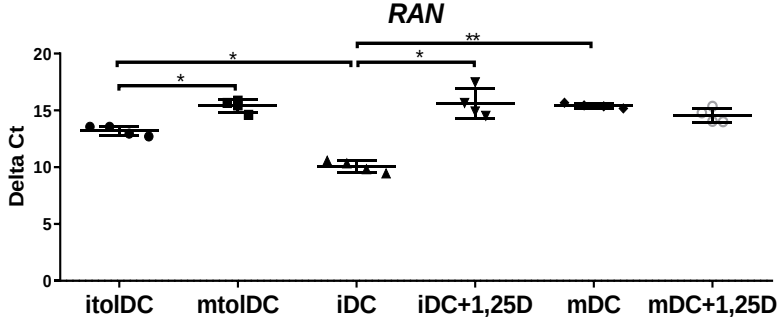
Figure 5.15. Expression of genes associated with miRNA synthesis in different DC phenotypes
Data for qRT-PCR analysis of mRNA represents an individual donor (n=4). Data is shown as: mean ± SD raw ΔCt values from qRT-PCR (left panels), and fold-change in mRNA expression (right panels) of: A) *DROSHA*; B) *DICER1*; C) *AGO2*; D) *DGCR8*; E) *RAN*; F) *TARBP2*; G) *XPO5*, in each of the 6 different DC phenotypes shown in **Figure 5.3**.

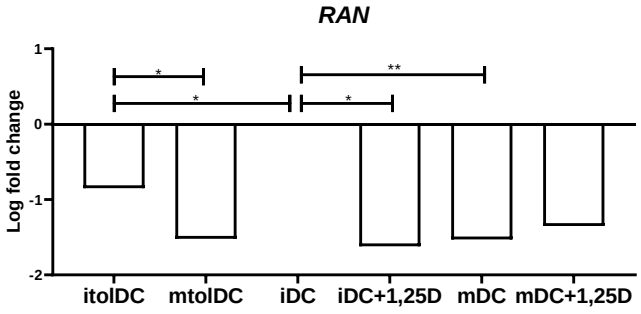
D


DGCR8
Delta Ct
25
20
15
10
5
0
**
*
itolDC
mtolDC
iDC
iDC+1,25D
mDC
mDC+1,25D

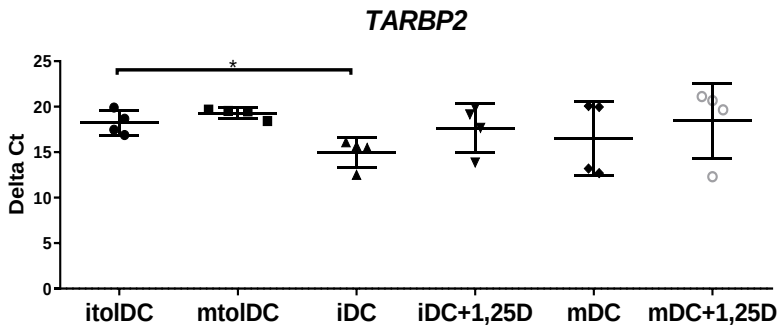

DGCR8
Log fold change
1
0
-1
-2
**
*
itolDC
mtolDC
iDC
iDC+1,25D
mDC
mDC+1,25D

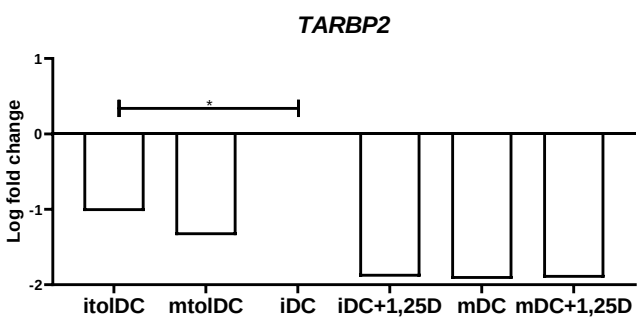
E


RAN
Delta Ct
20
15
10
5
0
*
*
*
**
itolDC
mtolDC
iDC
iDC+1,25D
mDC
mDC+1,25D

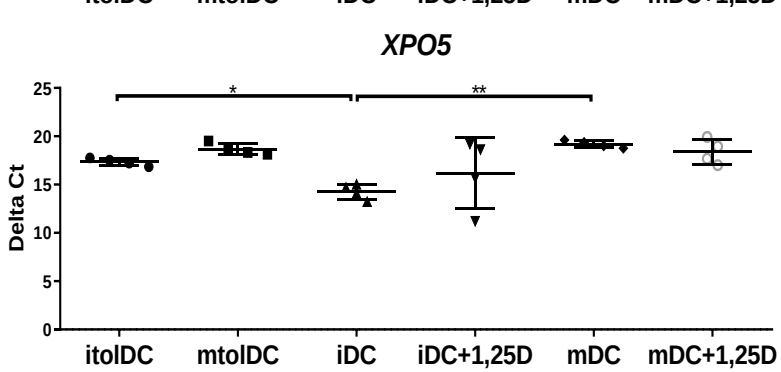

RAN
Log fold change
1
0
-1
-2
*
*
*
**
itolDC
mtolDC
iDC
iDC+1,25D
mDC
mDC+1,25D

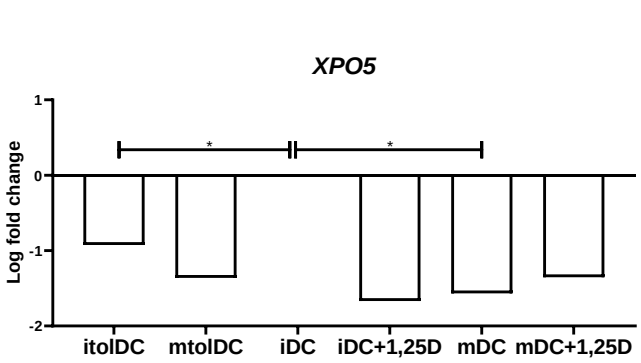
F


TARBP2
Delta Ct
25
20
15
10
5
0
*
itolDC
mtolDC
iDC
iDC+1,25D
mDC
mDC+1,25D


TARBP2
Log fold change
1
0
-1
-2
*
itolDC
mtolDC
iDC
iDC+1,25D
mDC
mDC+1,25D

G


XPO5
Delta Ct
25
20
15
10
5
0
*
**
itolDC
mtolDC
iDC
iDC+1,25D
mDC
mDC+1,25D


XPO5
Log fold change
1
0
-1
-2
*
*
itolDC
mtolDC
iDC
iDC+1,25D
mDC
mDC+1,25D

5.4 Discussion

The anti-inflammatory actions of $1,25(OH)_2D3$ have been well defined, with many studies showing a correlation between altered levels of $1,25(OH)_2D3$ and greater susceptibility to inflammatory disease via dysregulation of immune responses. The key driver to this finding was the discovery of vitamin D activating enzyme CYP27B1 and the vitamin D receptor (VDR) present in other non-calcium processing related cells including immune cells [563]. Thus immune cells that express the appropriate enzymes and receptors are capable of synthesising $1,25(OH)_2D3$ in an extra-renal fashion, leading to immunomodulatory actions similar to that produced by local cytokines [564], by inducing activation on downstream T cells and mediating further release of cytokines. Such cells capable of synthesising $1,25(OH)_2D3$ include macrophages and DC. Studies have shown $1,25(OH)_2D3$ to be capable of altering the function and morphology of DC to inhibit maturation [565] and induce tolerogenic status [524, 566].

5.4.1 $1,25(OH)_2D3$ on DC differentiation and maturation

$1,25(OH)_2D3$ has previously been shown to inhibit maturation of DC *in vitro*, as seen by down-regulation of CD80 and CD86 DC maturation marker expression [154, 156, 567]. The current study showed immature DC (iDC) treated with $1,25(OH)_2D3$ for 120hrs led to markedly down-regulated CD80 expression compared to untreated iDC (**Figure 5.4A**). Interestingly, treatment with $1,25(OH)_2D3$ for 24hrs (mDC vs mDC+$1,25(OH)_2D3$) did not produce such a significant effect on CD80 expression despite the fact mature DC have greater capacity for immunomodulation and

1,25$(OH)_2$D3 response [481]. However, the lack of a significant reduction in CD80 expression could be due to the short incubation time of 1,25$(OH)_2$D3 in the current study (24hrs); this may not have been sufficient time for the establishment and up-regulation of *VDR* to drive 1,25$(OH)_2$D3-mediated responses. Indeed, *VDR* expression was not significantly up-regulated following short term 1,25$(OH)_2$D3 treatment (**Figure 5.5A**), suggesting the immunomodulatory properties of 1,25$(OH)_2$D3 may be more apparent over a longer period of time.

Penna and Adorini previously studied the inhibitory effect of 1,25$(OH)_2$D3 on monocyte to iDC differentiation, and found that 1,25$(OH)_2$D3 prevented the differentiation of monocytes into iDC, as seen by down-regulated CD14 monocyte marker expression [163]. The current study did not profile CD14 expression in monocytes as the study focus was on the phenotypic changes in DC, but did find that both long term and short term 1,25$(OH)_2$D3 sustained expression of CD14 in iDC (**Figure 5.4A**). This observation was supported by previous studies also finding CD14+ monocyte marker sustenance following 1,25$(OH)_2$D3 exposure in iDC, suggesting the DC were promoted to differentiate away from the antigen presentation pathway [158, 568]. These studies found monocytes differentiated into iDC in the presence of 10nM 1,25$(OH)_2$D3 in culture, the same concentration used in the current study, showed CD14 expression remained elevated compared to untreated cells, whilst co-stimulatory molecule CD86 was suppressed. The current study also observed that LPS-induced DC maturation led to elevated expression of the DC activation markers CD83 and CD80, whereas subsequent addition of 1,25$(OH)_2$D3 resulted in the down-regulation of CD83 and CD80 expression; these findings were also observed in previous studies [157]. This inhibition of DC maturation by

1,25$(OH)_2$D3 leads to reduced antigen presentation and acts to dampen the pro-inflammatory immune response. All of the above actions of 1,25$(OH)_2$D3 act to maintain an immature DC phenotype, reduce antigen presentation and promote T cell hypo-responsiveness, thus leading to the strong anti-inflammatory properties of 1,25$(OH)_2$D3.

5.4.2 1,25$(OH)_2$D3 and the generation of tolerogenic DC

1,25$(OH)_2$D3 also promotes a tolerogenic DC status [158, 569]. This is possible due to the intracellular expression of the enzyme 1α-hydroxylase, allowing DC to establish and maintain a high local intracellular concentration of 1,25$(OH)_2$D3 required for immunomodulation [565]. *CYP27B1* and *VDR* gene expression is up-regulated as the DC undergoes maturation [459, 570]. Similarly, the current study also found a significant up-regulation of *CYP27B1* and *VDR* expression upon maturing iDC with LPS (**Figure 5.5**). Hewison *et al.* suggested the mechanism of up-regulating *CYP27B1* and *VDR* in mature DC phenotypes may be beneficial for inducing downstream T cell responses, due to overstimulation rendering the DC insensitive to further 1,25$(OH)_2$D3. In support of this mechanism, the current study found that *CYP27B1* and *VDR* expression was down-regulated in mature DC phenotypes following long term 1,25$(OH)_2$D3 treatment; this could be to prevent desensitisation to excess 1,25$(OH)_2$D3 as part of the negative feedback mechanism of 1,25$(OH)_2$D3 synthesis and regulation. Another mechanism proposed for this regulation suggested that excessive 1,25$(OH)_2$D3 limits 1α-hydroxylase activity by increasing FGF23 production [219] as well as self-regulation by inducing the

catabolic enzyme 24-hydroxylase [227], thus acting to maintain 1,25$(OH)_2$D3 levels within a normal range. Results from the current study appear to support this mechanism; consistent with down-regulated *CYP27B1* and *VDR* expression following long term 1,25$(OH)_2$D3, significant up-regulation of *CYP24A1* expression was also observed (**Figure 5.5B**). Other studies have shown 1,25$(OH)_2$D3 to induce *CYP24A1* expression and suppress *CYP27B1* expression in DC [571, 572], which was also observed in the current study. In addition, 1,25$(OH)_2$D3 is able to regulate the expression of its own receptor VDR in an autocrine fashion [573, 574]. The current accepted model for 1,25$(OH)_2$D3 regulation shows 1,25$(OH)_2$D3 to function in an autocrine or paracrine fashion in cells such as DC which express the 1α-hydroxylase enzyme. The 24-hydroxylase enzyme is also present in most cells expressing VDR. In this way DC are capable of responding to 1,25$(OH)_2$D3 to bring about molecular changes intracellularly, whilst regulating levels of 1,25$(OH)_2$D3 extracellularly to modulate downstream immune responses.

5.4.3 1,25$(OH)_2$D3 and immunomodulation by miRNAs in DC

The process of immunomodulation is thought to involve post-transcriptional epigenetic regulation, in particular by miRNAs due to the regulatory roles they play in biological processes including immune regulation [575, 576]. Several miRNAs including miR-155 [577], let-7 [578] and miR-146a [464] have been shown to be selectively or highly expressed in immune cells, with potential roles in regulating proliferation, maturation and differentiation of these cells [578].

5.4.3.1 1,25(OH)$_2$D3 and miR-155

The current study found that miR-155 expression was significantly up-regulated in DC following maturation with LPS (**Figure 5.6E**). Previous studies have also shown an association between miR-155 and DC maturation, where LPS induction of iDC leads to significantly up-regulated miR-155 expression [533, 535]. These studies proposed that maturation-induced miR-155 up-regulation in DC were due to down-regulated expression of genes in the *TIR/IL-1* pathway, of which genes are potential targets for miR-155. In this way miR-155 acts in a negative feedback loop to target *SOCS-1*, leading to down-regulated inflammatory cytokine production following an immune challenge to prevent excessive inflammation [579]. MiRNA quantification data in the current study appear to support this mechanism of regulation, although surprisingly miR-155 expression was up-regulated by LPS-induced maturation both in the presence and absence of 1,25(OH)$_2$D3. Given the anti-inflammatory nature of 1,25(OH)$_2$D3, a down-regulation of miR-155 expression in mDC following 1,25(OH)$_2$D3 treatment would have been expected, which was not the case in the current study. Perhaps this finding further highlights the importance of the negative feedback loop in tightly regulating miR-155 expression within baseline and maximum levels in the cell to prevent autoimmune response.

5.4.3.2 1,25(OH)$_2$D3 and miR-146a

Previous studies also found miR-146a expression to be up-regulated following LPS stimulus in DC [464]. The current study did not find such induction in LPS-matured DC (**Figure 5.6D**); however this disparity could be explained by the different

sources of LPS used to stimulate DC between both studies. Comparing the regulation of miR-146a expression in response to different sources of LPS stimulus in DC, purer forms of LPS such as those from *E. coli* as used to mature DC in the current study showed lower levels of miR-146a up-regulation compared to an alternative form of LPS as used by the previous study, which may explain why a significant induction of up-regulation of miR-146a in LPS-stimulated DC was not seen. The current study also found let-7i expression to be regulated after LPS maturation (**Figure 5.6G**). In DC, let-7i has been shown to be up-regulated in response to DC maturation [537, 538]; Kuppusamy *et al*. indicated that the let-7 family of miRNAs may be important mediators of cell maturation. Other studies support this idea; LPS-induced DC maturation was found to require the up-regulation of let-7i [539], where down-regulation of let-7i prevented DC maturation as shown by decreased expression of CD80 and CD86 mDC surface markers. Similarly, the current study also saw an induction of let-7i expression upon maturation of DC with LPS, but this was only significant in the presence of $1,25(OH)_2D3$ (**Figure 5.6G**).

5.4.3.3 $1,25(OH)_2D3$ and LPS

LPS stimulus, amongst other inflammatory stimuli such as cytokines, is able to induce the up-regulation or down-regulation of select miRNAs that control maturation processes and antigen presentation within DC, leading to modulations in the immune response [532, 580, 581]. Multiple studies have profiled miRNA expression changes before and after LPS-induced DC maturation, and found that miR-146a [582] and consistently miR-155 [535, 583, 584] was up-regulated during DC maturation,

suggesting that these miRNAs contributed to a pro-inflammatory pathway. Similarly, in the current study both candidate miRNA analyses and miRNA qPCR array analyses identified miR-155 expression as significantly up-regulated following LPS maturation in DC (**Figure 5.6E; Figure 5.10B**) however neither analysis identified miR-146a as being significantly regulated by LPS (**Figure 5.6D; Figure 5.10B**), in contrast to miR-146a up-regulation previously observed by Jin *et al*. [582]. This disparity could be due to a number of reasons. Firstly, differences in culture methodology between studies could attribute to differences in miRNA expression; Jin *et al.* cultured monocytes from derived PBMC with GM-CSF and IL-4 for only 3 days, compared to the standard 5 day culture used in the current study. The length of culture time is likely to affect miRNA expression as DC reach different levels of differentiation and maturity, with differential miRNA expression associated with each developmental stage. Furthermore, Jin *et al.* profiled miRNA expression by only standard qPCR methodology and data analysis, whilst the current study and a number of others performed array analyses of multiple miRNA panels; for example Ceppi *et al.* performed an array analysis of multiple miRNAs and found miR-155 but not miR-146a to be up-regulated during DC maturation. Likewise, in the current study let-7i expression by LPS maturation was originally identified as significantly up-regulated in the single candidate qPCR experiments (**Figure 5.6G**), but this was not detected as significant in the qPCR arrays (**Figure 5.10B**). This could be attributed to better detection and more advanced data analysis methods in array analyses compared to single qPCR miRNA analyses. Additionally, the use of multiple endogenous controls in the arrays leads to better data normalisation, hence overall

array data is likely to be more reliable at detecting true changes in miRNA expression compared to single miRNA analysis.

5.4.3.4 1,25(OH)$_2$D3 and other miRNAs

Following LPS maturation to DC in the current study, miR-221 down-regulation in DC was observed (**Figure 5.10B**); a previous study suggested that miR-221 was important for and contributed to the survival of iDC [539]. This study found decreased miR-221 in conjunction with up-regulated miR-155 expression following DC maturation correlated with the accumulation of p27 (*kip1*) expression and subsequent cell survival. Therefore, this could explain why upon DC maturation in the current study, miR-221 expression was down-regulated as it was no longer required. Based on these results it is possible that miR-221 and miR-155 are involved in regulation of apoptosis in DC. An additional role of miR-155 in DC has been proposed; one study suggested miR-155 was necessary for the normal function of mDC to activate T cells [585]. However, this study found that despite knocking down miR-155 expression in mDC, these mDC did not show decreased CD86 expression compared to mDC with functional miR-155, despite having impaired T cell activation. In contrast, another study found that miR-155-deficient mDC had impaired T cell activation that correlated with significantly reduced expression of CD86 [533]. The current study did not profile miRNA expression in miR-155-decficient DC but did find LPS maturation significantly up-regulated the expression of DC maturation markers including CD86.

Other miRNAs of interest with regard to DC differentiation, maturation and survival include miR-29, miR-148 and miR-451; all these miRNAs have previously been found to be up-regulated following DC maturation and play an anti-inflammatory role. Increased miR-29 expression has been correlated with inhibited pro-inflammatory cytokine secretion [586]. However, the vast majority of literature on candidate miRNA studies involves the role of miRNAs in DC function in BMDC, plasmacytoid DC or murine DC, thus were incomparable directly with the monocyte-derived human DC in the present study. There have been previous qPCR array analyses performed to quantify miRNA expression in human monocyte-derived DC; these studies identified miR-511 and miR-99b to be highly expressed in mDC [587], and inhibition of these miRNAs resulted in reduced CD80 and CD86 expression, suggesting these miRNAs are important for regulating the immune response. The qPCR arrays performed in the current study was limited to 372 miRNAs most associated with inflammation. Due to time and cost factors it was not feasible to profile the expression of all human miRNAs, and it is possible that other miRNAs with novel roles in immune regulation have yet to be identified.

5.4.4 Global miRNA expression and the miRNA synthesis pathway

It was interesting to observe the global down-regulation of miRNAs in DC following long term $1,25(OH)_2D3$, short term $1,25(OH)_2D3$ and LPS treatment. The significance of this finding was debated with regard to whether the down-regulation of miRNA expression was truly due to a $1,25(OH)_2D3$ or LPS effect, or if there was another intrinsic mechanism more likely to be responsible for the global miRNA

down-regulation observed. Since DROSHA and DICER1, amongst others, are crucial components of the pathway responsible for miRNA biogenesis, expression of the major miRNA synthesis genes was quantified in all DC phenotypes to identify any correlations between miRNA expression and these genes. *DROSHA, DICER1* and *RAN* expression were significantly down-regulated with short term $1,25(OH)_2D3$ treatment (iDC to iDC+$1,25(OH)_2D3$), and expression of *DROSHA, RAN, TARBP2* and *XPO5* was also down-regulated following long term $1,25(OH)_2D3$ (iDC to itolDC) (**Figure 5.15**). The down-regulation of these miRNA synthesis genes was originally thought to explain the majority of miRNAs similarly down-regulated with short term and long term $1,25(OH)_2D3$ in the qPCR arrays (**Figures 5.8-5.10**). Interestingly, significant down-regulation of their counterpart genes *DGCR8, XPO5* and *TARBP2* respectively was not observed, lending the possibility of these genes compensating for the diminished expression of their co-factors to maintain miRNA synthesis. Previous studies have shown the expression of DICER1 does not affect the function of its co-factor TARBP2 [588], but rather that TARBP2 is necessary for the recruitment of DICER1 and normal miRNA synthesis [589]. Additionally, AGO2, which is not associated with a co-functioning protein and acts at the end of the miRNA synthesis pathway, was not significantly regulated with long term $1,25(OH)_2D3$, short term $1,25(OH)_2D3$ or LPS in the current study. Hence the global down-regulation of miRNA expression observed is unlikely to be due to down-regulated miRNA synthesis genes. The importance of miRNAs for regulating DC differentiation has further been demonstrated by *DICER1* knockdown studies, which observed that suppressed miRNA biogenesis led to the up-regulation of CD14 marker in DC [590].

However, in the case of LPS-induced DC maturation in the absence of 1,25$(OH)_2$D3 (iDC to mDC) and its effect on the expression of miRNA synthesis genes, *DROSHA* and *RAN* expression was significantly down-regulated along with their associated co-factors *DGCR8* and *XPO5*. Similarly, LPS-induced maturation in DC in the presence of long term 1,25$(OH)_2$D3 (itolDC to mtolDC) also produced down-regulation of *DGCR8* and *RAN* expression (**Figure 5.15**). These findings would suggest a reduced synthesis of pre-miRNAs as well as reduced nuclear export of the pre-miRNA, leading to the overall down-regulated synthesis of mature miRNAs detected in DC. Perhaps in this way LPS and other cell stressors function to down-regulate the expression of miRNAs normally associated with preventing inappropriate immune activation [591]. Although many miRNAs in the current study were indeed down-regulated following LPS maturation in DC, miR-155 expression was up-regulated. If the global down-regulation of miRNAs was a result of reduced miRNA synthesis, one would expect all miRNAs to be down-regulated without exception. In addition, miRNA biogenesis-mediated miRNA down-regulation would target both the 5' and 3' end miRNA families for down-regulation, for example miR-155-5p and miR-155-3p, however this was not the case in the current study. Interestingly, further bioinformatics analysis identified genes encoding enzymes that hydrolyse the 5' end miRNAs were down-regulated, but enzymes that attach to the 3' end were unaffected, suggesting the global down-regulation of miRNAs was not linked miRNA synthesis machinery but rather involved select enzymatic targeting. Under normal settings the 5' region of a pre-miRNA is crucial for recognition and binding by DICER to synthesise the mature miRNA [592].

Additionally, the half-life of mature miRNAs in human immune cells can vary from 5 days to two weeks [593]. Relating this to miRNA biogenesis, even if expression of miRNA synthesis genes were down-regulated leading to reduced miRNA synthesis, it would take at least 5 days for the expression levels of miRNAs to fall by half and for significant changes to be detected, i.e. the time taken for the loss of *DICER* expression to take effect would most likely exceed the DC lifespan. This is supported by a study which found that blocking the function of miRNA processing enzymes such as DICER1 took up to 3 days for the effects to be translated onto miRNAs [593]. Since DC in the current study were cultured for 6 days, it is very unlikely that during this time the miRNAs would become unstable and significantly down-regulated. Instead, the current study proposes an alternative explanation for the global down-regulation of miRNAs observed, which suggests this effect may be independent of the DROSHA/DICER machinery. Indeed a previous study suggested the availability of DROSHA and DICER did not affect miRNA synthesis, but instead it was the abundance of Argonaute proteins that controlled levels of mature miRNAs [594]. Genes including *AGO2* which encodes the Argonaute proteins play a key post-transcriptional role in elevating levels of mature miRNAs in the cellular environment. Further studies are required to elucidate the role of miRNA biogenesis genes alone, and determine whether the function of one gene affects that of others, and what these implications are with regard to miRNA synthesis.

5.4.5 MiRNA targets identified by bioinformatics analysis

Bioinformatics coupled with gene ontology analysis of miRNA gene targets and functions in DC revealed that the majority of significantly regulated miRNAs targeted genes with roles in immune regulation. MiR-155 targets many genes with new and established roles in immune regulation, including *SOCS1, BCL6* and *PIK3R1* (**Table 5.2; Figure 5.14A**). Many of the miRNAs that were significantly regulated by both $1,25(OH)_2D3$ and LPS were shown to be over-represented for target genes with a role in immune regulation (**Figure 5.14B**). In contrast, miRNAs significantly regulated by long term $1,25(OH)_2D3$ targeted genes with a much wider variety of functions from adipogenesis to cell hypertrophy (**Figure 5.14C**). Some of these genes carry out more than one function, which makes it very difficult to identify a specific miRNA that targets each gene. These bioinformatics tools are helpful to identify miRNA gene targets and biological functions of these genes, as a first step for any future studies delineating the mechanistic actions of these miRNAs.

5.5 Summary

The mechanism of miRNA regulation with regard to DC differentiation and maturation remains unclear, but there is strong evidence to suggest that select miRNAs including miR-155 play key regulatory roles in the homeostasis and normal function of the innate immune system. The immunomodulatory effects of $1,25(OH)_2D3$ in DC bring about different phenotypical and functional changes depending on the stage of DC development, length of treatment time and the cytokine milieu.

The expression of many genes is altered during these changes, and it remains likely that these changes are facilitated by the differential expression of a host of miRNAs. In DC, miRNAs of particular interest with regard to inflammation and $1,25(OH)_2D3$-mediated immune modulation include miR-146a, miR-155 and to an extent let-7i. Furthermore, genes involved in the miRNA biogenesis pathway appear to be modulated by LPS-induced maturation, however the expression of these genes is unlikely to affect miRNA biogenesis in DC phenotypes. LPS-mediated down-regulation of these genes was not rescued by short term or long term $1,25(OH)_2D3$, suggesting the miRNA biogenesis machinery is stable and any immunomodulatory effects of $1,25(OH)_2D3$ are likely to act on mature miRNAs only. Future studies should involve quantifying expression of pre-miRNAs and pri-miRNAs in DC, in conjunction with miRNA biogenesis gene expression analysis and $1,25(OH)_2D3$ treatment, to elucidate the mechanism of $1,25(OH)_2D3$ on miRNA regulation.

CHAPTER 6. VITAMIN D AND MICRORNAS IN T CELLS

6.1.1 Introduction to T cells

T lymphocytes (T cells) are responder cells that form part of the adaptive immune response. They originate from bone marrow progenitor cells and migrate to the thymus where they mature and are exported to peripheral tissues. Here they recognise foreign antigens presented by antigen presenting cells (APC) such as dendritic cells. In this way peripheral T cells become activated and can stimulate the production of a host of cytokines and chemokines to mediate inflammation. The functional roles and mechanisms of T cells *in vivo* have largely been elucidated through mouse models [595, 596], however the interactions between T cells and pathogens that humans are exposed to in everyday life is thought to involve complex mechanisms [597].

T cells are distinguished from other immune cells by the expression of Cluster of Differentiation (CD) CD3 on their cell surface. Subsets of T cells are further classified by the expression of receptors on their cell surface; the two main classes are CD4+ and CD8+ T cells. CD4+ T cells, or T helper (Th) cells, are distinguished by the abundant expression of CD4 receptor. They coordinate immune responses by participating in cell-cell signalling to modulate the release of cytokines that go on to influence the behavior of downstream immune cells such as B cells. On the other hand, CD8+ T cells, or cytotoxic killer T (Tc) cells, express CD8 receptor and are involved in killing infected cells. In a healthy adult both CD4+ and CD8+ T cell subsets begin as naïve quiescent cells, and upon the detection of a foreign antigen presented by APC they become primed in secondary lymphoid tissues.

Multiple sub-phenotypes of CD4+ and CD8+ T cells have been discovered, each with specific roles in the immune response. CD4+ subsets include Th1, Th2, Th17 and T

regulatory (Treg) cells, each of which is associated with the production of a specific set of cytokines [598]. Th1 cells produce IFN-γ which creates an environment to activate CD8+ T cells, and Th1 cells are therefore associated with cellular immunity [599]. Th2 cells mainly secrete IL-4 and IL-10 which assists in the stimulation of antibody production by B cells, and Th2 cells are therefore associated with humoral immunity [599]. The Th17 subset is perhaps the most well documented with regards to inflammation, with the production of pro-inflammatory cytokines such as IL-17 and its interaction with IL-23 implicated in the basis of many autoimmune diseases including rheumatoid arthritis [600]. Finally, Tregs are characterised by the expression of CD4 and CD25 on their cell surface and FoxP3 intracellularly; these cells also secrete IL-10 as well as TGF-β and recognise self-antigens to prevent autoimmune responses [601]. Treg dysregulation has been implicated in malignancy, allergy and chronic infections. CD8+ subsets include Tc1 and Tc2 groups which are similar to Th1 and Th2 in terms of cytokine profile [602]. The subsets of T cells and their associated cytokines are summarised in **Figure 6.1**.

In addition to Th (CD4+) and Tc (CD8+) subsets, both types of T cells can also be divided into naïve and memory T cells. Memory T cells are found in the periphery and maintain long term immunity, having a protective role against previously encountered antigens in order to elicit a faster immune response [603]. Memory T cells develop from effector T cells after exposure to an antigen, however their origins are still debated. The focus of this chapter is on CD4+ T cells. Further details about specific T cell sub-groups are provided in the following sections of this chapter.

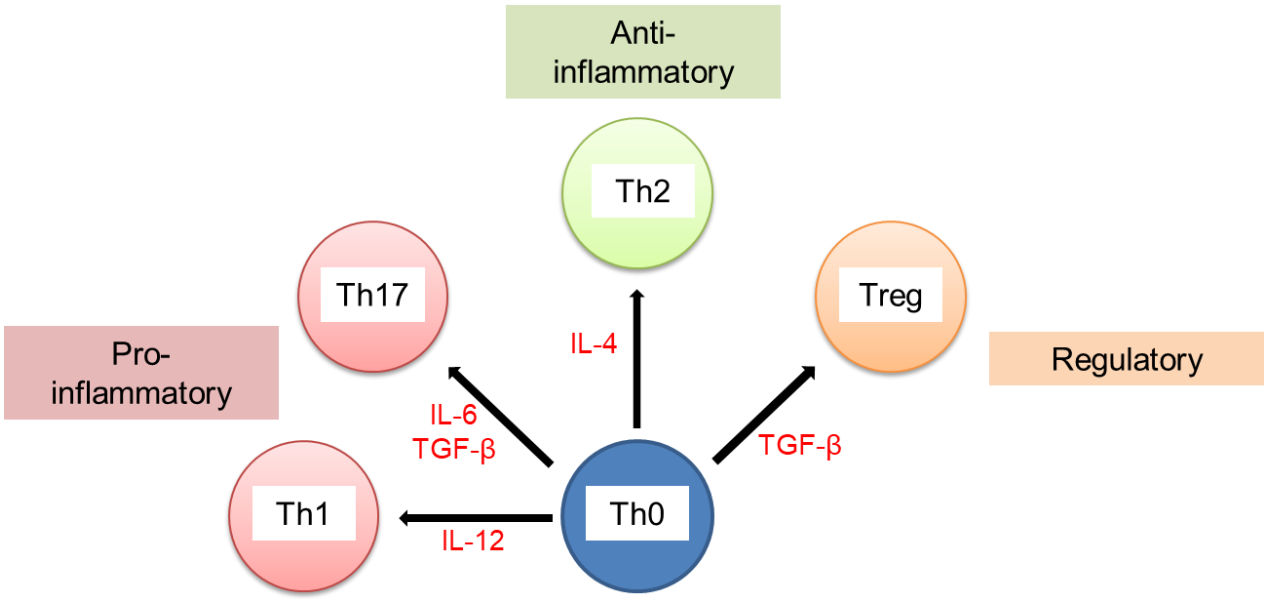


Figure 6.1. Subsets of CD4+ T lymphocytes

CD4+ T lymphocyte subsets include Th1, Th17, Th2 and Treg cells. Each subset is associated with cytokines which play a role in the pro-inflammatory, anti-inflammatory and regulation of immune responses. Following activation of naïve CD4+ T cells, these cells may differentiate into one of several lineages, defined by the cytokine signals in the environment. Th, T-helper; Treg, T-regulatory; IL, interleukin; TGF, transformation growth factor.

6.1.2 Conventional CD4+ CD25- T cells

Previously, the presence of cell surface CD25 was used to distinguish Treg cells from other classes of T cells, however activated effector T cells also express CD25. A proportion of CD4+ CD25- cells known as conventional T cells possess regulatory properties similar to CD4+ CD25+ Tregs [604]. Now FoxP3 expression is used as the most definitive marker to distinguish between differentiated Tregs as they express FoxP3 in high quantities whereas conventional CD4+ CD25- T cells express it at very low levels. A proportion of naïve CD4+ CD25- T cells expressing FoxP3 possess the capacity to convert to CD25+ Tregs after homeostatic expansion,

and can be selected for by IL-2 [605]. This process is thought to help maintain the Treg population in peripheral tissues [606].

When naïve CD4+ CD25- T cells are subject to an immune challenge, they up-regulate the expression of FoxP3 mediated by TGF-β, which promotes their transition to a Treg phenotype with immunosuppressive properties. These 'induced' Tregs are unresponsive to further signals via the T cell receptor (TCR) [607]. Furthermore, when peripheral naïve CD4+ CD25- T cells are stimulated with anti-CD3 and anti-CD28 and exposed to a Th17 cytokine cocktail of TGF-β, IL-6, IL-21 and IL-23, this encourages the generation of a pro-inflammatory Th17 lineage [608]. Th17 cells are a subset of CD4+ effector T cells that produce IL-17 and have a pro-inflammatory role; dysregulation in the form of over-activation of Th17 cells is a key feature of the pathogenesis of many autoimmune diseases such as rheumatoid arthritis (RA) [600]. In contrast, the presence of TGF-β alone during peripheral CD4+ CD25- naïve T cell differentiation mediates the development of induced regulatory T (iTreg) cells which have immunosuppressive functions [609].

The relocation of CTLA-4 from intracellular vesicles to the cell surface is important for T cells to mediate suppressor function. In naïve CD4+ CD25- T cells, CTLA-4 is localised in vesicles away from the cell surface, with no effect on T cell function. Upon TCR-mediated T cell activation, internal CTLA-4 is released and signals at the cell surface to mediate T cell suppressor functions [610]. In this way the localisation of CTLA-4 is able to mediate immune response in T cells. CTLA-4 at the cell surface is thought to be localised at the immune synapse, where it acts as a checkpoint control to determine whether a T cell responds to an antigen, and thus prevent autoimmune targeting of self-antigens [611]. Once CTLA-4 is internalised, it

can be recycled back to the plasma membrane or targeted for degradation by lysosomes. Those that are targeted for degradation can capture its ligands on the way, so in this way CTLA-4 controls ligand availability for CD28 and suppresses T cell responses.

6.1.3 Regulatory CD4+ CD25+ T cells

Tregs are a subset of CD4+ CD25+ T cells that possess immunosuppressive functions. This population was first discovered in mice by Asano and Sakaguchi, and was found to function in the maintenance of self-tolerance by down-regulating immune responses to self and non-self antigens [612, 613]. Initially they were identified in humans by their high expression of FoxP3 and CD25. It is now known that activated effector T cells also express CD25, however in mice high CD25 expression alone still remains a good cell surface discriminatory molecule for Tregs. Activated effector T cells also express many of the functional molecules of Tregs such as high CTLA-4 expression; hence it can be difficult to differentiate between the two T cell subtypes using these markers alone. Activated T cells can also express FoxP3 although this transient expression only lasts for a few days.

Tregs derived directly from the thymus are classed as natural Tregs, and T cells manipulated to differentiate into Tregs from peripheral cells are called induced/adaptive Tregs (iTreg) but possess the functions and characteristics of natural Tregs [606]. *In vivo*, TGF-β produced by natural Tregs is capable of inducing iTreg differentiation from naïve CD4+ T cells [614]. There are many classes of peripherally derived Tregs, of which some express FoxP3 and IL-10. Tregs mainly

express CD4, CD25 and FoxP3, and may be derived from the same lineage as naïve CD4+ T cells. Increased FoxP3 expression is a marker for naïve CD4+ T cell conversion to CD4+ Treg cell phenotype. Similarly, CTLA-4 is lowly expressed at rest and up-regulated during T cell activation/stimulation, with maximal expression at 48-72hrs post-activation [615]. CTLA-4 expression is critical for the Treg to function and prevent autoimmunity, and its expression is dependent on the expression of FoxP3 [616]; lack of CTLA-4 in Tregs leads to uncontrolled activation and expansion of naïve T cells [617-619]. The depletion of CD4+ CD25+ Tregs has been implicated in the development of many autoimmune diseases [613, 620, 621]. As a consequence, studies have focused on the effect of enhancing Treg numbers and function as a possible strategy for managing the pathogenesis of inflammation.

In mice, stimulation of CD4+ CD25+ FoxP3+ Tregs prevents transplant rejection [622]. In humans, CD4+ CD25+ FoxP3+ Tregs are actively recruited to sites of infection [623]. It is thought Tregs exert these immunosuppressive functions mainly through the production of cytokines and occasionally through cell-cell contact [624, 625], or even via down-regulating CD80/CD86 expression on DC [626, 627]. These studies summarise the critical role of Tregs in maintaining a healthy immune response.

6.1.4 Th17 cells

Th17 cells traditionally have roles in mediating responses to bacteria and fungi [628], however recently they have been implicated in the pathogenesis of autoimmune disease including inflammatory bowel disease and rheumatoid arthritis

[628]. *In vitro*, Th17 cells can be induced by first stimulating naïve CD4+ T cells with anti-CD3 and anti-CD28, followed by incubation in inflammatory Th17 cytokine cocktail consisting of IL-1β, IL-2, TGF-β, IL-6 and IL-23 [629, 630]. IL-4 and IFN-У cytokines released by Th1/Th2 subsets act to inhibit the differentiation of Th17 cells [631, 632], making the Th17 lineage distinct to that of Th1 and Th2. In mice, Th17 subsets can be induced by stimulating CD4+ T cells via the TCR, in the presence of TGF-β and IL-6. The presence of TGF-β is critical to drive Th17 differentiation both in mice [633, 634] and in humans [635].

The Th17 differentiation process involves three stages in humans: 1) differentiation stage mediated by TGF-β and IL-6; 2) self-amplification stage by IL-21; 3) survival and expansion stage by IL-23. Studies show TGF-β signalling is a critical driving force of CD4+ T cell commitment to Th17 lineage [634, 636]; the first study showed that TGF-β in the presence of IL-6 led to the generation of Th17 producing cells. The latter study confirmed the role of TGF-β signaling in differentiation to Th17 fate, and showed TGF-β up-regulated IL-23 receptor to enhance survival and further expansion of the lineage. High concentrations of TGF-β alone in the CD4+ T cell differentiation stage mediates generation of Treg cells from peripheral CD4+ CD25- naïve T cells, via the up-regulation of FoxP3 transcription factor [607]. These induced Tregs have potent immunosuppressive properties [607]. In contrast, low concentrations of TGF-β synergizing with IL-6 induces Th17 differentiation to induce IL-23 receptor expression [634, 637], thus the decision between CD4+ T cells differentiating into a Treg or Th17 fate is dependent on the cytokine environment and regulatory expression of FoxP3.

The second stage of Th17 differentiation requires IL-21, produced by Th17 cells, to act together with TGF-β to promote further Th17 differentiation in a self-amplification fashion [638, 639]. The final stage is mediated by IL-23 produced by APC, to maintain the Th17 population [634, 640]. Together this shows the importance of maintaining the cytokine balance for appropriate T cell differentiation. Interestingly, previous studies have shown that in order for APC to direct Th cell differentiation, a third activation signal is required [641]; only APC directly activated by PAMPs are capable of inducing the third stimulatory signal for T cells, whereas APC indirectly activated by inflammatory mediators are capable of CD4+ T cell expansion but not Th cell differentiation.

6.1.5 CD4+ T cell activation

The activation of T cells serves several main purposes in the adaptive immune response: promoting inflammatory cytokine production (involves Th1 and Th17 pathways); mediating B cell activation (involves Th2 cells); regulating immunosuppression (involves Tregs) and killing infected cells (involves cytotoxic T cells).

CD4+ T cells which have never seen an immune stimulus are classed as naïve T cells. Upon immune challenge they are activated and differentiate into different subtypes depending on the different cytokines present in the environment. Naïve CD4+ T cell activation requires a two-step process; the antigen-specific first step involves engagement of the highly variable T cell TCR with the peptide/MHC II (Major histocompatibility II) complex on APC, and the second step involves activation

of the CD28 receptor on naïve T cells with the co-stimulatory ligands CD80/CD86 on APC [642]. Following recognition and binding, in order for T cells to elicit a strong immune response, they also receive survival signals from inducible T cell co-stimulator (ICOS) and CD134/OX40 ligands on APC, which bind to their respective receptors on T cells. ICOS and OX40 ligands are only expressed by APC following pathogen recognition, to ensure inappropriate T cell activation does not occur. In the absence of a co-stimulatory signal, TCR binding to the MHC II complex acts as an off switch for T cell activation. The final stage to complete T cell activation, signal three, involves additional signals from cytokines. This stage is critical to determine the T cell subset that will differentiate and is dependent on the cytokine milieu. T cells will be pushed to differentiate into a Th1 fate in the presence of IL-12, Th2 fate in the presence of IL-4, and Th17 fate in the presence of IL-6 and IL-23. Each cell subset has its unique role in downstream immune responses, as they migrate away from the site of infection. Other factors such as chemokines and other cytokines at these sites will control the activation and proliferation of the newly differentiated T cell subset. A simplified schematic of T cell activation by APC is depicted in **Figure 6.2**.

CTLA-4 up-regulation is a marker of T cell activation; this receptor undergoes competitive binding with CD28 for the CD80/CD86 ligand on the surface of APC. In this way CTLA-4 acts as a pivotal checkpoint regulator to control immune signals and prevent T cell over-activation. A variety of mechanisms have been proposed for CTLA-4 mediated attenuation of T cell activation: 1) competitive binding of CTLA-4 to the CD80/CD86 co-stimulatory molecules on APC [643]; 2) selective recruitment of CTLA-4 in the immunological synapse [644]; 3) attenuation of TCR signalling by recruiting phosphatases [645, 646]; 4) reducing the contact period between T cells

and APC [647]. So far there is no definitive mechanism explaining CTLA-4 mediated inhibition of T cell signalling, although all the above theories are supported by strong experimental studies.

CTLA-4 is located intracellularly and its relocation to the cell surface is required for negatively regulating T cell activation; this attenuation is possible since CTLA-4 has a twenty-fold higher affinity for CD80/CD86 ligands than CD28 does [648]. Thus, it is most likely that CTLA-4 mediates T cell signal inhibition by competing with CD28 for the common ligands CD80/CD86. It has been reported that, following activation and translocation of CTLA-4 to the cell surface, CD4+ CD25- T cells see a rapid decline in surface CTLA-4 expression after just 4 hours, compared to much longer for memory T cells [649]. CTLA-4 therefore plays a critical role in the regulation of immune function but little is known about how its expression is controlled, although its expression appears to be modulated by $1,25(OH)_2D3$ [650, 651].

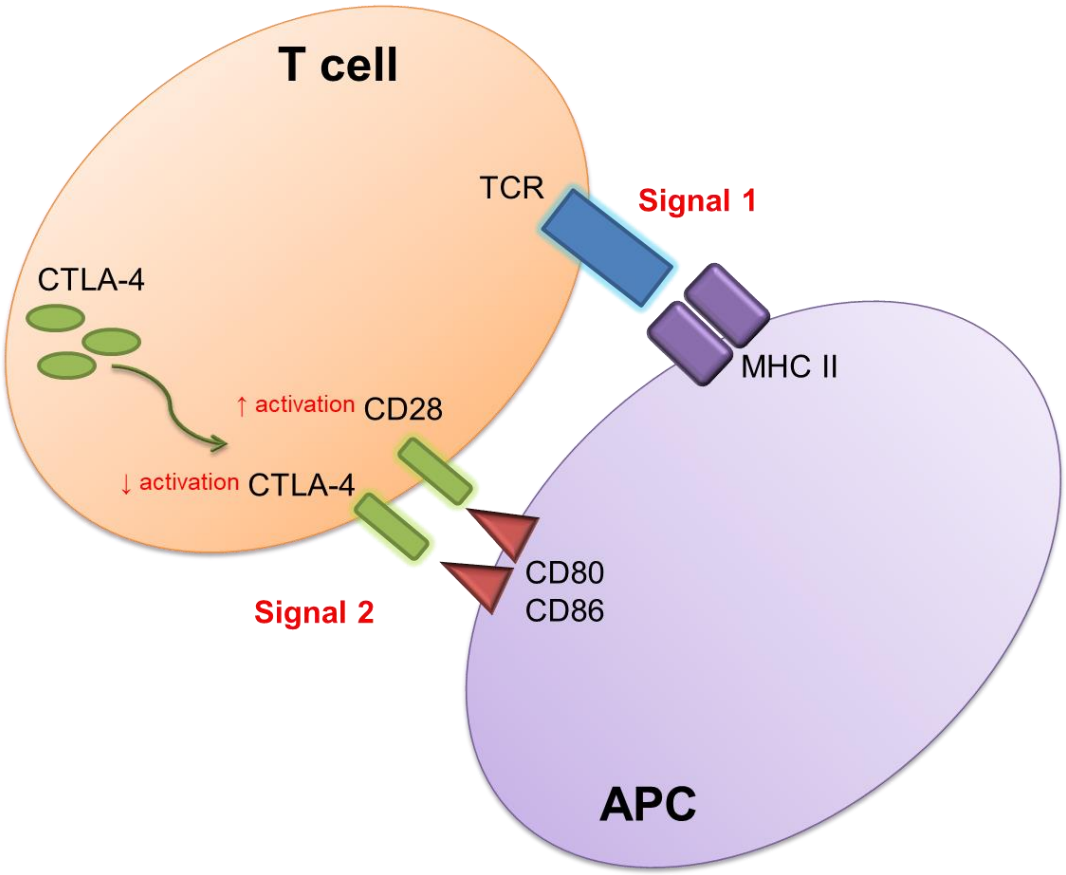


Figure 6.2. Schematic of T cell activation by APC

Activation of T cells requires 2 signals; TCR binding to the MHC II, and CD28 binding to co-stimulatory molecules CD80/CD86. Conversely CTLA-4 translocating to the cell surface and competitively binding to CD80/CD86 prevents T cell over-activation.

6.1.6 Immunological actions of 1,25(OH)$_2$D3 on CD4+ T cells

Evidence that CD4+ T cells express VDR [652, 653] led to the idea that T cells are able to participate in 1,25(OH)$_2$D3-mediated signalling at sites of inflammation. Since then synthesis of 1,25(OH)$_2$D3 has been observed for many cell types, notably those in the colon [523, 654], placenta [655] as well as APC [142], all of which are sites frequented by T cells. Research in mice has shown that inhibition of

1,25$(OH)_2$D3 production in immune cells led to reduced paracrine signalling to T cells and worsened autoimmune disease prognosis [656, 657]. In this way, paracrine signalling to T cells by 1,25$(OH)_2$D3 produced by APC has become a widely accepted mechanism for 1,25$(OH)_2$D3-mediated immune regulation [658].

As key effector cells of autoimmune disease, the roles of CD4+ T cells in the pathogenesis of multiple autoimmune diseases have been studied in detail [659-661]. In addition, the anti-inflammatory actions of 1,25$(OH)_2$D3 on CD4+ T cells have been implicated in the pathogenesis of autoimmune diseases. In the context of RA, impaired CD4+ T cell function has been found as a major cause of disease progression [662]. Previous studies have shown that in CD4+ CD25- T cells primed to differentiate into Th17 cells, 1,25$(OH)_2$D3 impairs pro-inflammatory IL-17 production [373, 663] and enhances CTLA-4 and FoxP3 expression which is associated with a more immunosuppressive Treg phenotype [650], underlining the immunoregulatory potential of 1,25$(OH)_2$D3 in the adaptive immune system. The potent immunosuppressive phenotype of these 1,25$(OH)_2$D3-induced CD4+ CD25- FoxP3+ Tregs has been confirmed by multiple studies [91, 372, 373]. Hence CD4+ T cells are potential targets for the protective biological effects of 1,25$(OH)_2$D3 via VDR signalling in both mice [664] and humans [665].

The actions of 1,25$(OH)_2$D3 on CD4+ CD25+ Tregs have also been studied in detail. Previous studies have positively correlated CD4+ CD25+ Treg cell function with serum vitamin D (25(OH)D3) status in patients with autoimmune disease [666, 667]; however this correlation was not found upon 1,25$(OH)_2$D3 supplementation in humans [667]. In mice, CD4+ CD25+ Tregs treated with 1,25$(OH)_2$D3 exhibited inhibited development of pro-inflammatory Th1 phenotype and promoted Th2 fate,

showing that Tregs can be direct targets of 1,25$(OH)_2$D3 to enhance their suppressive ability [186, 668]. One theory is that 1,25$(OH)_2$D3 acts as an on/off switch especially with regard to autoimmune disease prevention, however once T cells have matured and committed to a phenotype, 1,25$(OH)_2$D3 is no longer able to modulate these immune responses.

6.1.7 MiRNAs and CD4+ T cell activation, proliferation and function

The role of miRNAs in the normal functioning and dysregulation of the immune response has been well studied. Recent reports have suggested a role for select miRNAs in the development and function of CD4+ T cells, mediated by intracellular and extracellular signalling. It is thought that miRNAs function post-transcriptionally to target genes involved in mediating T cell proliferation, differentiation and apoptosis.

MiRNA expression during different T cell developmental stages can regulate the fate, function and development of CD4+ T cells [669]. Over-expression of miR-181 inhibits T cell development by targeting CD69 [670, 671] whilst over-expression of let-7 in CD4+ T cells results in reduced IL-10 levels which may have an anti-inflammatory effect [672]. T cell activation in healthy individuals has been associated with the induced expression of miR-21, miR-146a and miR-155, and the reduced expression of miR-31 [673]; up-regulated miR-155 expression occurred early after activation and miR-146a was up-regulated later. MiR-146a silences its target gene *STAT1*, which helps to maintain the immunosuppressive function of CD4+ FoxP3+ Tregs [674]. MiR-155 co-functioning with miR-221 is predicted to target *PIK3R1* to

inhibit excess CD4+ T cell proliferation and cytokine production [675]. MiR-21, induced by TCR activation, is thought to negatively modulate T cell activation by targeting genes involved in signal transduction [676] and supporting the survival of memory T cells [677], thereby helping to maintain an appropriate level of immune response. Regulation of CD4+ T cell apoptosis is another process mediated by miRNA targeted gene silencing; miR-15a and miR-16-1 targets *BCL2* which regulates the *BCL-2/BIM* apoptosis pathway [678, 679]. Previous global microarray analysis [680] and candidate miRNA analysis [681, 682] observed significant down-regulation of miR-16, miR-142, miR-150, miR-15 and let-7 expression following naïve CD4+ T cell activation, suggesting these miRNAs may target genes necessary for mediating the shift from naïve to activated T cells.

On the other hand, up-regulated expression of miRNAs has also been implicated in facilitating the inflammatory response. Induction of miR-214, a target of *PTEN*, leads to increased T cell proliferation [683]. IL-2 induces the expression of miR-182, a target of *FOXO1*, and the silencing of this gene acts as a switch for the conversion of resting state T cells to Th cell clonal expansion [684]. In Th1 cells, miR-17 and miR-19 function as a cluster to target *CREB1* and *PTEN* respectively, to enhance T cell proliferation, survival and cytokine production, and to prevent inducible Treg differentiation in humans [685, 686] and mice [687]. In CD4+ Tregs, the induction of miR-210, miR-24, miR-95 and miR-145 is necessary for immune homeostasis, with miR-95 regulating *FOXP3* indirectly and miR-145 regulating *CTLA-4* [688]. The immunoregulatory functions of Tregs may also be dependent on the high expression of miR-10a, which is induced by TGF-β and retinoic acid and targets *BCL-6* [689]. In this way miR-10a discourages the conversion from Treg to

inflammatory Th17 cell phenotype. In stimulated Tregs, the predicted miR-155 target *FOXOa3* is down-regulated, reducing T cell action via the *AKT* pathway [690].

The differential expression of these miRNAs at different stages of development may be in part due to the efficiency of T cell responses at each stage. More so it suggests that strict miRNA regulation at different developmental stages of T cells is important to maintain an appropriate level of immune response, and aberrant over-expression or down-regulation of their expression can have consequences on inappropriate gene expression.

6.2 Chapter outline

Lineage-specific differentiation of T cells is dependent on many factors including the cytokine milieu of the microenvironment [691], strength of TCR signals [692], and epigenetic modifications [693]. These factors must be tightly regulated; over-activation of T cells can lead to many problems including autoimmune diseases [694].

Previously, Jeffery *et al.* [650] showed that naïve CD4+ CD25- T cells stimulated in the presence of $1,25(OH)_2D3$ and Th17 polarising conditions are able to inhibit the production of pro-inflammatory cytokines such as IFN-γ, IL-17 and IL-21, as well as up-regulating expression of CTLA-4 and FoxP3 [373]. The authors utilised these conditions to generate cells for RNA sequencing to observe genome-wide changes in mRNA expression and found multiple patterns of gene regulation at early and late time points for incubation with $1,25(OH)_2D3$ (unpublished data). However, in addition to transcriptomic changes, multiple miRNAs are known to be involved in the

activation and differentiation of immune cells. In the current thesis, this has been demonstrated for the effects of 1,25$(OH)_2$D3 on DC from the innate immune system (see **Chapter 5**). We therefore hypothesised that **changes in miRNAs also play an important role in maintaining immune responses to 1,25$(OH)_2$D3 in the adaptive immune system**. Due to the role that Th17 cells play in the pathogenesis of autoimmune disease, studies in the current chapter sought to profile changes in gene and miRNA expression during T cell activation under Th17 inflammatory conditions, and how these changes may be differentially regulated in the presence of 1,25$(OH)_2$D3. Work described in this chapter aims to investigate the role of miRNAs in mediating CD4+ CD25- T cell responses to 1,25$(OH)_2$D3.

6.3 Results

6.3.1 Preliminary gene analysis in CD4+ T cell subsets

Preliminary studies to explore the effect of T cell activation and $1,25(OH)_2D3$ treatment on gene expression were performed by qPCR in Th0, Th1, Th17 and Treg cell subsets. These Th cell subsets were generated from naïve CD4+ cells under culture with different cytokines. Th0 populations were generated by incubating naïve CD4+ cells with IL-4 for 3 days. Th1 populations were generated by culturing CD4+ cells in Human Th1 differentiation media containing IL-12 (R&D Systems) on an anti-CD3 coated plate for 3 days. Tregs were isolated and expanded using Dynal CD4+ CD25+ Treg Kit (Thermo Fisher) with IL-2 for 5 days. The expression of *VDR, IL-10* and *IL-21* in these subsets and treatment groups was quantified and compared. *VDR* was expressed in all CD4+ T cell populations and following activation, expression was slightly increased but this was not statistically significant. The addition of $1,25(OH)_2D3$ did not significantly affect *VDR* expression (**Figure 6.3A**). *IL-10* expression was significantly up-regulated following T cell activation for all CD4+ T cell subtypes (**Figure 6.3B**), but only Th17 cells and Treg cells saw a further significant up-regulation of *IL-10* expression following $1,25(OH)_2D3$ treatment (p=0.0052). Although Th17 and Treg cells both showed a response to $1,25(OH)_2D3$, only Treg cells are sensitive to modulation by $1,25(OH)_2D3$. *IL-21* expression was also significantly up-regulated in Th0 (p=0.0007), Th1 (p=0.0245), Th17 (p=0.0246) and Treg (p=0.043) cell subsets after activation (**Figure 6.3C**) however *IL-21* was not significantly regulated by $1,25(OH)_2D3$ in any of the subsets. These results showed that Th17 and Treg cells show a significant response to $1,25(OH)_2D3$ in the form of

increased *IL-10* expression, hence subsequent studies were carried out using one of these T cell phenotypes: Th17 cells.

6.3.2 CD4+ T cell model of cytokine activation and 1,25(OH)$_2$D3 response

Previous literature has shown that for CD4+ T cells activated under Th17 inflammatory conditions, the addition of 1,25(OH)$_2$D3 promoted up-regulation of cell markers associated with an anti-inflammatory Treg phenotype [650]. Therefore, to explore if these changes were associated with gene regulation at specific time points following 1,25(OH)$_2$D3 treatment, a CD4+ T cell time course model for T cell activation and 1,25(OH)$_2$D3 treatment was utilised for gene and miRNA studies in this chapter.

Naïve CD4+ CD25- T cells from healthy donors were stimulated with plate-coated anti-CD3 and anti-CD28, then activated for 20 hours with a Th17 pro-inflammatory cytokine cocktail consisting of IL-2, TBG-β, IL-1β, IL-6 and IL-23 to encourage Th17 polarisation. The resulting activated Th17 cells were then incubated with 1,25(OH)$_2$D3 for up to 72 hours, and cells were harvested at regular time points for flow cytometric phenotype analysis and extraction of RNA for gene and miRNA expression studies. The model for induction and treatment of CD4+ Th17 cells is shown in **Figure 6.4**.

6.3.3 Flow cytometry analysis of CD4+ T cell purity

In order to validate the purity and functionality of the Th17 CD4+ T cell model shown in **Figure 6.4**, flow cytometry was used to quantify changes in T cell marker

expression. Naïve CD4+ CD25- T cells were isolated from PBMC and stained for CTLA-4, FoxP3, CD4 and the IL-7 receptor CD127. As expected low expression of the Treg markers CTLA-4 (MFI 15.0) and FoxP3 (MFI 33.9) were observed in resting naïve T cells, with levels of CTLA-4 being similar to PBMC controls (MFI 15.1) (**Figure 6.5A**), By contrast high expression of CD4 (MFI 100) and CD127 (MFI 150) compared to PBMC (MFI 18.3 and 17.9 respectively), distinguished the isolated T cells from PBMC mixed populations.

6.3.4 Flow cytometry analysis of CD4+ T cell activation and 1,25(OH)$_2$D3 response

Following activation of naïve CD4+ T cells with the Th17 cytokine cocktail, cell surface expression of CTLA-4 (MFI 28), FoxP3 (MFI 11.9) and IFN-γ (MFI 48.4) was up-regulated compared to the naïve T cells (CTLA-4 MFI 2.82; FoxP3 MFI 3.53; IFN-γ MFI 6.8). Treatment with 1,25(OH)$_2$D3 for 72 hours decreased IFN-γ (MFI 26.1) and increased CTLA-4 (MFI 262) expression, compared to activated vehicle-treated cells (IFN-γ MFI 48.4; CTLA-4 MFI 28) (**Figure 6.5B**). FoxP3 expression was also up-regulated following T cell activation (MFI 11.9) compared to naïve T cells (MFI 3.53), with 1,25(OH)$_2$D3 treatment resulting in only a modest further up-regulation of FoxP3 (MFI 14.7). Activation of CD4+ T cells also induced the expression of CD25 (MFI 40.9 compared to 1.91 in the unstimulated naïve population), showing that these previously CD4+ CD25- T cells now possessed a more anti-inflammatory phenotype. This is also shown in FACS plot format (**Figure 6.5C**); unstimulated naïve CD4+ T cells were mostly negative for FoxP3 and CTLA-4 as shown by the majority of cells stained in the lower left quadrant Q4 (left plot, 99.9%). Following

stimulation of CD4+ naïve T cells with a Th17 inflammatory cytokine cocktail, there was strong up-regulation of CTLA-4 compared to unstimulated T cells (central plot, population expressing CTLA-4 increased from 0.15% to 69.4% in Q1). Naïve T cell stimulation also mildly up-regulated the expression of FoxP3 (central plot, population expressing FoxP3 increased from ~0% to 1.27% in Q3). Stimulated cells were also analysed following $1,25(OH)_2D3$ treatment for 72 hours, and these cells showed up-regulation of both FoxP3 and CTLA-4, as shown by the increase in double-positive stained cells in the top right quadrant Q2 (right plot, 5.96% T cells + vehicle to 8.36% T cells +$1,25(OH)_2D3$).

To further characterise the effect of $1,25(OH)_2D3$ on activated T cells in an inflammatory setting, cytokine secretion was also analysed (**Figure 6.5D**). Treatment with $1,25(OH)_2D3$ treatment up-regulated CD25 (MFI 9978) and IL-10 (MFI 184) compared to vehicle controls (CD25 MFI 3437; IL-10 MFI 169). $1,25(OH)_2D3$ also down-regulated IL-17 (MFI 6054), IFN-γ (MFI 133), IL-21 (MFI 7934) and IL-2 (MIF 6.73) compared to vehicle controls.

Collectively these results confirmed that the CD4+ T cell model was responsive to Th17 activation and treatment with $1,25(OH)_2D3$, and also suggested that $1,25(OH)_2D3$ stimulated CD4+ T cells to overcome the Th17 inflammatory polarisation by up-regulating CTLA-4 expression and thus promote a Treg phenotype, despite the presence of the Th17 inflammatory cocktail. These observations further highlight the immunosuppressive properties of $1,25(OH)_2D3$ in CD4+ T cell regulation.

6.3.5 Effect of 1,25(OH)$_2$D3 on candidate gene expression in CD4+ T cells

To assess gene expression changes in activated CD4+ T cells in response to 1,25(OH)$_2$D3, a time course study was performed to profile mRNA expression at 0hr, 2hrs, 10hrs, 24hrs, 48hrs and 72hrs post-1,25(OH)$_2$D3 treatment. qPCR analysis was performed on two candidate genes associated with T cell inflammation. Firstly, *CTLA-4* expression did not significantly change in the vehicle-treated cells at different time points, compared to cells at the 0hr time point. *IFN-γ* expression was significantly up-regulated at the 24hr time point in the vehicle compared to unstimulated control (p=0.0425) (**Figure 6.6B**).

In response to 1,25(OH)$_2$D3, *CTLA-4* expression was significantly up-regulated at 2hrs, 24hrs, 48hrs and 72hrs following 1,25(OH)$_2$D3 treatment, compared to the vehicle-treated T cells at the same time points (**Figure 6.7A**). *IFN-γ* expression was only significantly down-regulated after 72 hours treatment with 1,25(OH)$_2$D3 (**Figure 6.7B**), although earlier time points showed a downward trend in expression. These results confirmed the anti-inflammatory effects of 1,25(OH)$_2$D3 on CD4+ CD25- T cells.

6.3.6 Effect of 1,25(OH)$_2$D3 on candidate miRNA expression in CD4+ T cells

To determine if the anti-inflammatory effects of 1,25(OH)$_2$D3 on CD4+ T cells shown in **Figure 6.7** were associated with regulation of miRNA expression, further qRT-PCR was performed for 7 candidate miRNAs (miR-21, miR-29a, miR-145, miR-146a, miR-155, miR-627 and let-7i) closely related to immune cell function and inflammation according to previous literature [556-562]. The unstimulated control at

the 0hr time point was compared to vehicle cells at each time point, to determine any miRNA expression changes in the absence of 1,25$(OH)_2$D3. Only miR-155 expression was significantly up-regulated at the 48hr and 72hr time points, in vehicle compared to unstimulated control cells (p=0.04) (**Figure 6.8E**).

Following the addition of 1,25$(OH)_2$D3, significant expression changes in expression were observed for 2 miRNAs; miR-155 (p=0.048) (**Figure 6.9E**) and miR-627 (p=0.02) (**Figure 6.9F**), which were significantly down-regulated by 1,25$(OH)_2$D3 at 2hrs treatment, compared to the vehicle-treated T cells at the same time point. These results indicated that although the candidate miRNAs have been linked to inflammatory immune function, they are not strongly responsive to 1,25$(OH)_2$D3 in CD4+ T cells. Further studies were therefore required for an unbiased analysis of miRNAs in these cells.

6.3.7 miRNA qPCR array response to 1,25$(OH)_2$D3

To identify other miRNAs potentially responding to 1,25$(OH)_2$D3, qPCR arrays were performed to profile expression of 372 human miRNAs closely linked to immune function and inflammation. Early (24hr) and late (72hr) time points for T cells treated with 1,25$(OH)_2$D3 were chosen to compare possible temporal effects of 1,25$(OH)_2$D3 on T cell miRNA expression. Based on the gene and candidate miRNA expression data obtained earlier in this study, it was predicted that these time points were the most likely to have significantly regulated miRNA expression in response to 1,25$(OH)_2$D3.

6.3.7.1 Effect of CD4+ T cell activation on miRNA expression

In the first instance, experiments were carried out to determine changes following activation of T cells to induce a Th17 inflammatory phenotype. After incubating naïve CD4+ T cells with the cytokine cocktail of IL-2, TGF-β, IL-1β, IL-6 and IL-23 for 20 hours, *CTLA-4* and *IFN-γ* gene expression was quantified by qPCR to assess changes in T cell phenotype (**Figure 6.10**). The T cell activation marker CTLA-4 (p=0.0103; 95% confidence interval 3.444-0.997; SD 0.7688) and inflammatory cytokine IFN-γ (p=0.0023; 95% confidence interval 8.039-4.076; SD 1.245) were both significantly up-regulated following stimulation, confirming that the previously naïve CD4+ T cells had been activated to an inflammatory phenotype. Following on from this, further qPCR quantification of 7 candidate miRNAs was performed to determine possible effects of T cell stimulation on miRNA expression. Of the candidate miRNAs quantified, only miR-155 expression was significantly up-regulated (p=0.0199; 95% confidence interval 8.263-1.459; SD 2.138) following cytokine stimulation (**Figure 6.11**).

Further studies were therefore carried out to produce an unbiased analysis of altered miRNA expression following T cell activation. Preliminary qPCR data were analysed using SABiosciences PCR Array Data Analysis software (SABiosciences) recommended for the arrays, and data visualized in heat maps and scatter plots. Only miRNAs with a fold change >2 were included in the preliminary analysis. Of the 372 miRNAs included in the qPCR array panel, 16 miRNAs were down-regulated and 128 up-regulated in response to cytokine stimulation of the T cells (>2-fold change based on mean of n=4 separate donor samples) (**Figure 6.12B**). Huynh-Feldt statistical analysis revealed that of the miRNAs showing > 2-fold change, 9 were

significantly down-regulated and 24 significantly up-regulated, with miR-155 amongst the latter (**Figure 6.12C**). All Huynh-Feldt significantly regulated miRNAs in response to T cell activation are shown in full graphical format in **Figure 6.13.**

6.3.7.2 Effects of short-term treatment with 1,25(OH)$_2$D3 (24hrs) on miRNA expression

Preliminary heat map and scatter plot visualisation data showed that of the 372 miRNAs screened following short term (24hr) culture of cytokine-stimulated T cells with 1,25(OH)$_2$D3, 6 miRNAs were up-regulated (>2-fold change) and 2 down-regulated (**Figure 6.14B**). Statistical analysis by Huynh-Feldt testing was then performed on all miRNAs to identify statistically significant changes to miRNA expression in response to short term 1,25(OH)$_2$D3. Further Huynh-Feldt analysis identified only 2 miRNAs that were significantly regulated by short term 1,25(OH)$_2$D3; miR-16-5p (p=0.004) and miR-212-3p (p=0.004) were both up-regulated (**Figure 6.14C**). Full details of the results for short term treatment of activated T cells with 1,25(OH)$_2$D3 are shown in **Figure 6.15**.

6.3.7.3 Effects of long-term treatment with 1,25(OH)$_2$D3 (72hrs) on CD4+ T cell miRNA expression

Preliminary data showed that of the 372 miRNAs analysed following long term (72hr) culture with 1,25(OH)$_2$D3, 16 miRNAs were up-regulated (>2-fold change) and 6 down-regulated (**Figure 6.16B**). Huynh-Feldt testing was then performed on all 372 miRNAs to identify statistically significant changes to miRNA expression in

response to long term 1,25(OH)$_2$D3. Further Huynh-Feldt statistical analysis revealed 9 miRNAs were significantly up-regulated (p<0.05) and 1 miRNA down-regulated, with that being miR-335-3p (p=0.008) (**Figure 6.16C**). All Huynh-Feldt significantly regulated miRNAs for long term 1,25(OH)$_2$D3 are shown in full graphical format in **Figure 6.17**.

6.3.8 Predicted targets for miRNAs: a bioinformatics approach

A full list of T cell miRNAs significantly regulated in response to cytokine activation or 24 or 72 hour treatment with 1,25(OH)$_2$D3, as calculated by Huynh-Feldt analysis is shown in **Table 6.1**. Following the identification of these miRNAs from the qPCR array analysis, a bioinformatics approach was carried out to identify potential target genes for each miRNA. The MiRTarBase database was initially used to identify predicted gene targets for all significantly regulated miRNAs. All gene targets on miRTarBase have been experimentally validated and ranked according to published research results verifying the targets, then given a target prediction score out of 100. The top 5 target genes for each miRNA identified in **Figures 6.12-6.17** were then ranked and listed (**Table 6.1B**); target genes were ranked from most to least validated, and the established cut-off target prediction score was set at 95 and above to exclude experimentally 'weak' targets.

To further analyse and validate the functionality of select miRNA gene targets, bioinformatic strategies were used to identify predicted gene targets for each of the miRNAs shown to be significantly regulated in the current study. MiRBase miRNA

database was combined with Cytoscape software to produce a list of significant (p<0.05) experimentally validated gene targets of miRNAs. By combining Cytoscape analysis with ClueGo gene ontology (GO) analysis, target genes were grouped according to enriched biological functions. This approach was used to analyse the expression of miR-132-3p, miR-132-5p, miR-147a, miR-424-3p, miR-424-5p, miR-212-3p and miR-155-5p which were selected from the miRNA list in **Table 6.1A** as they were regulated by more than one treatment i.e. a combination of short term 1,25$(OH)_2$D3, long term 1,25$(OH)_2$D3 and stimulation.

Using MiRBase, miR-132-3p was shown to target 14 genes (**Figure 6.18**). Gene ontology analysis showed that these genes were associated with cell processes related to cell proliferation and vesicle transportation, with *CDKN1A* being a common gene for all these biological processes. MiR-132-5p, miR-147a and miR-424-3p targeted a much smaller collection of genes (**Figure 6.19**) and none of these effects were significantly enriched in specific biological processes so, unlike miR-132-3p, we were unable to produce a gene ontology analysis for these miRNAs. MiR-212-3p and miR-424-5p targeted genes that were mainly associated with cell cycle regulation and apoptotic processes (**Figures 6.20-6.21**). Gene targets of miR-155-5p were of special interest due to the differential regulation of miR-155 expression in response to LPS and 1,25$(OH)_2$D3 in DC described in **Chapter 5**, and the implications of miR-155 as a master regulator of inflammation. Indeed miR-155 was found to target many genes which are involved in a plethora of enriched biological processes, indicating the widespread systemic role of miR-155 from regulation of the cell cycle to haematopoiesis (**Figure 6.22**). This network was simplified in order to focus on genes with functions in immune regulation; this identified well-studied genes

such as *SOCS1*, and lesser studied genes such as *CD68*. Overall, this bioinformatics analysis was a useful tool to identify miRNA gene targets from an experimentally validated database, and to identify genes linked to immune functions. These data were compared to previous unpublished data by other Birmingham group members, where CD4+ T cells polarised with Th17 cocktail was treated with $1,25(OH)_2D3$. The genes significantly regulated with long term (72hr) $1,25(OH)_2D3$ are shown in **Table 6.2**.

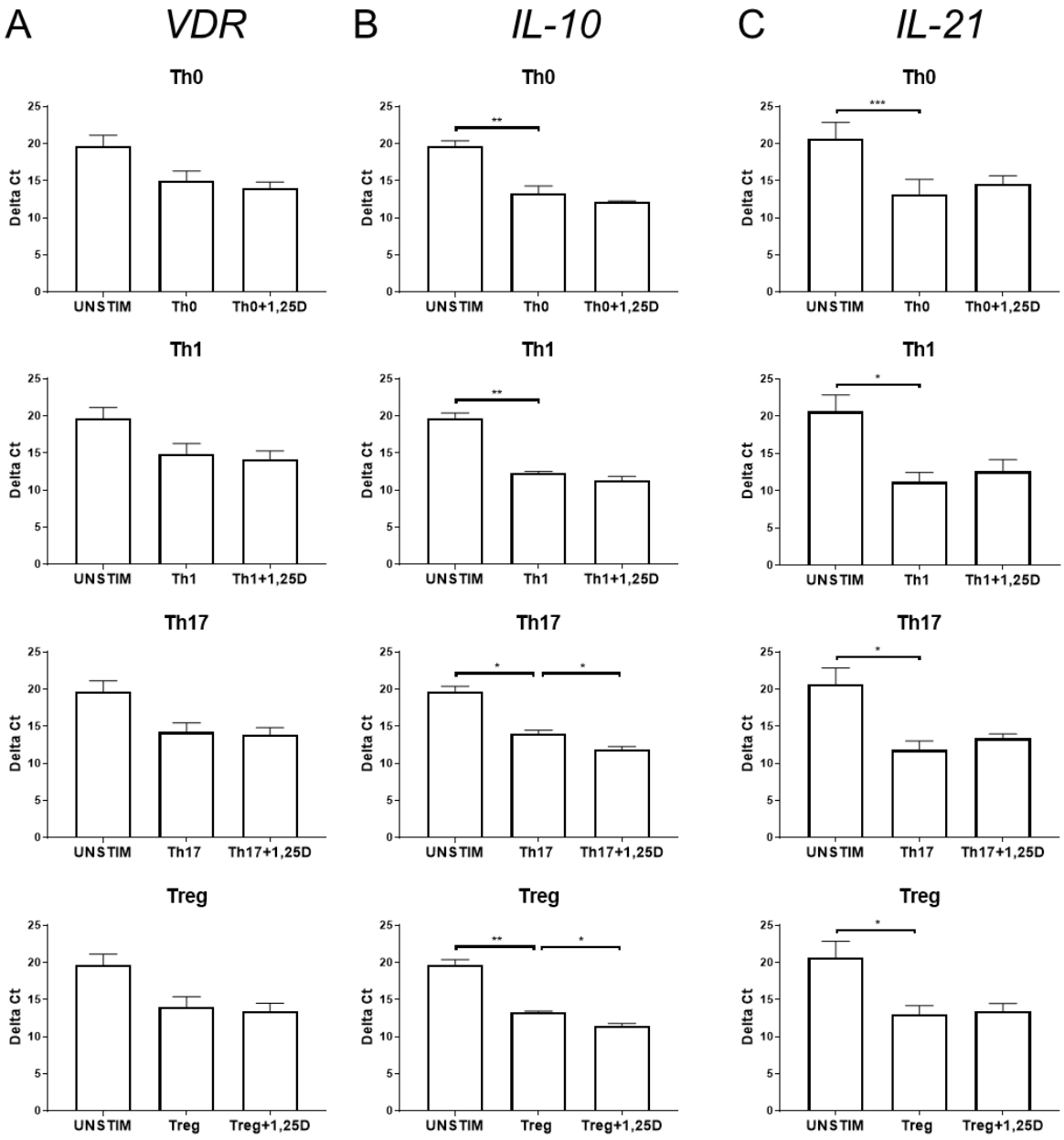


Figure 6.3. Gene expression analysis in CD4+ T cell subsets
qPCR gene expression analysis to study the effect of T cell activation and 1,25$(OH)_2$D3 treatment on gene expression in Th0, Th1, Th17 and Treg cell subsets. Genes quantified were A) *VDR*; B) *IL-10*; C) *IL-21*. Data is shown as delta Ct (ΔCt) so a higher value reflects a lower expression. Bars are shown as mean expression ± SD for n=3 individual samples. RM one-way ANOVA for paired values was performed for analysis of significance, and Tukey's multiple comparisons test was performed to identify differences between treatment groups. Statistical significance is taken as **p*<0.05.

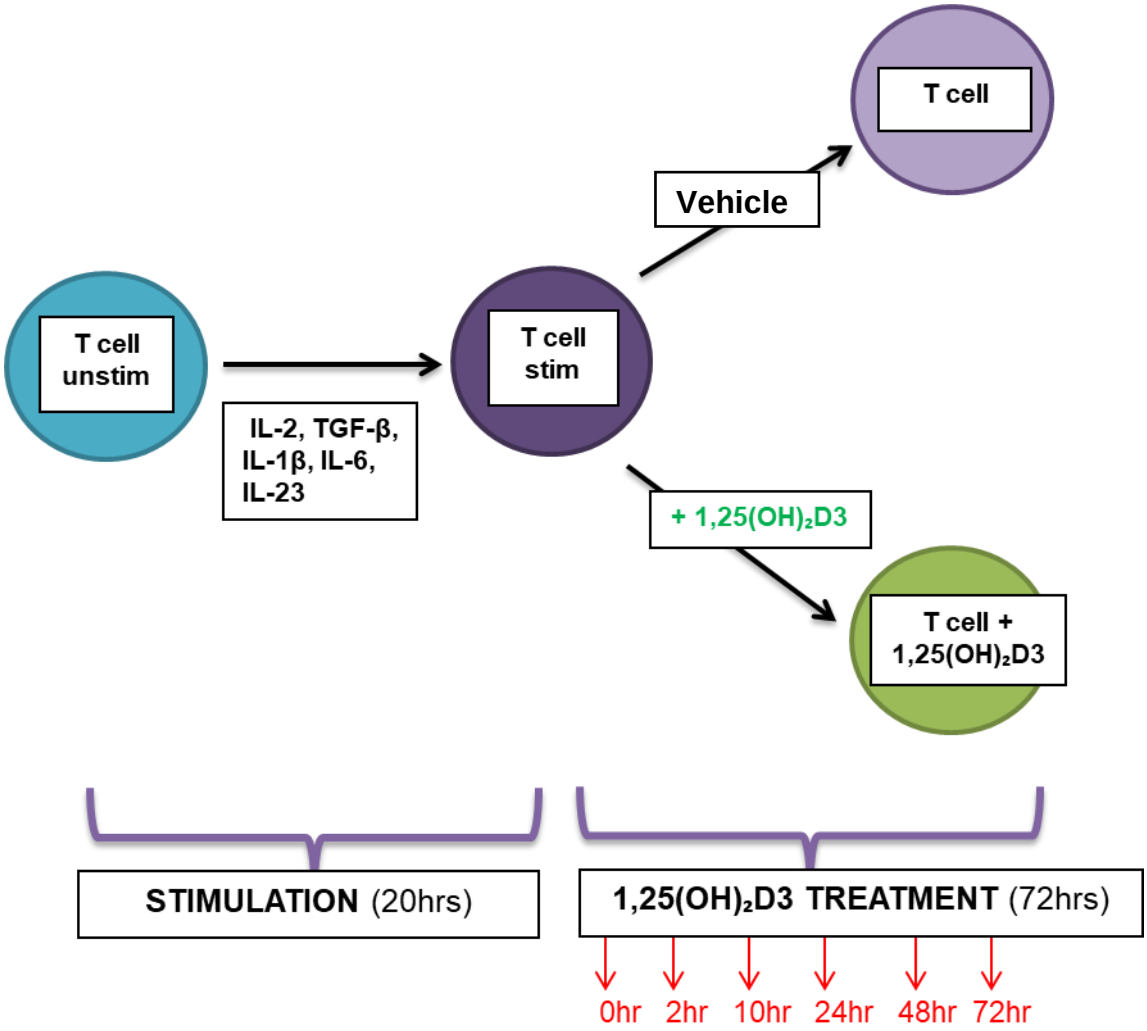
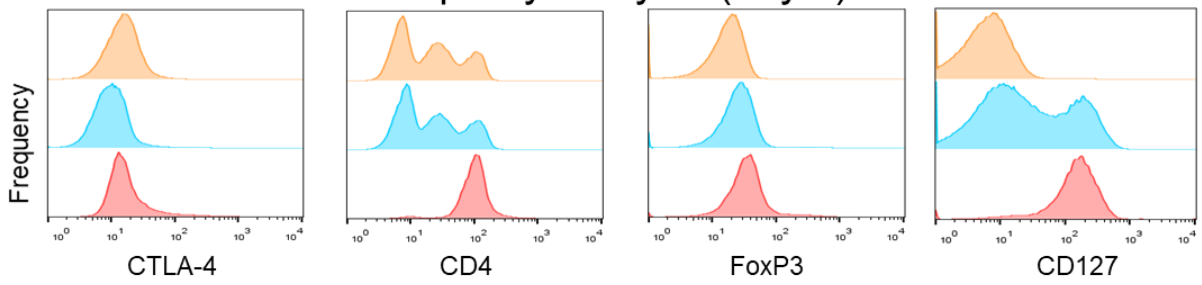


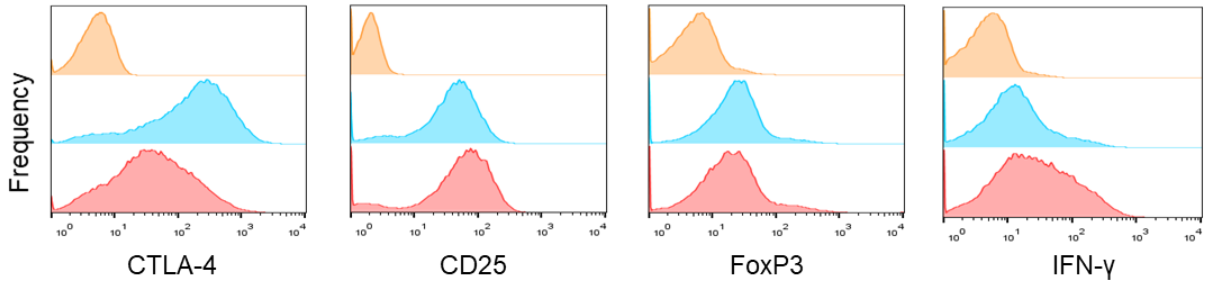
Figure 6.4. Induction of CD4+ CD25- T cells with cytokine cocktail and $1,25(OH)_2D3$
T cell unstim – unstimulated naïve CD4+ CD25- T cell; T cell stim – CD4+ T cell activated with Th17 cytokine cocktail; T cell –activated vehicle CD4+ T cell; T cell +$1,25(OH)_2D3$ – activated CD4+ T cell+$1,25(OH)_2D3$ (10nM). CD4+ CD25- T cells were isolated from PBMC derived from whole blood leukocyte cones of healthy donors (n=4). Anti-CD3 and anti-CD28 antibodies were coated onto an empty culture plate for 2 hours at 37^{O}C, prior to addition of T cells and cytokines for stimulation. Th17 cytokine cocktail was added to promote CD4+ T cell differentiation into a pro-inflammatory Th17 phenotype. $1,25(OH)_2D3$ or vehicle (Ethanol) was added at a final concentration of 10nM per well. T cells were harvested at the following time points after $1,25(OH)_2D3$ treatment: 0hr (control), 2hrs, 10hrs, 24hrs, 48hrs, 72hrs.

A CD4+ CD25- T cell purity analysis (day 0)



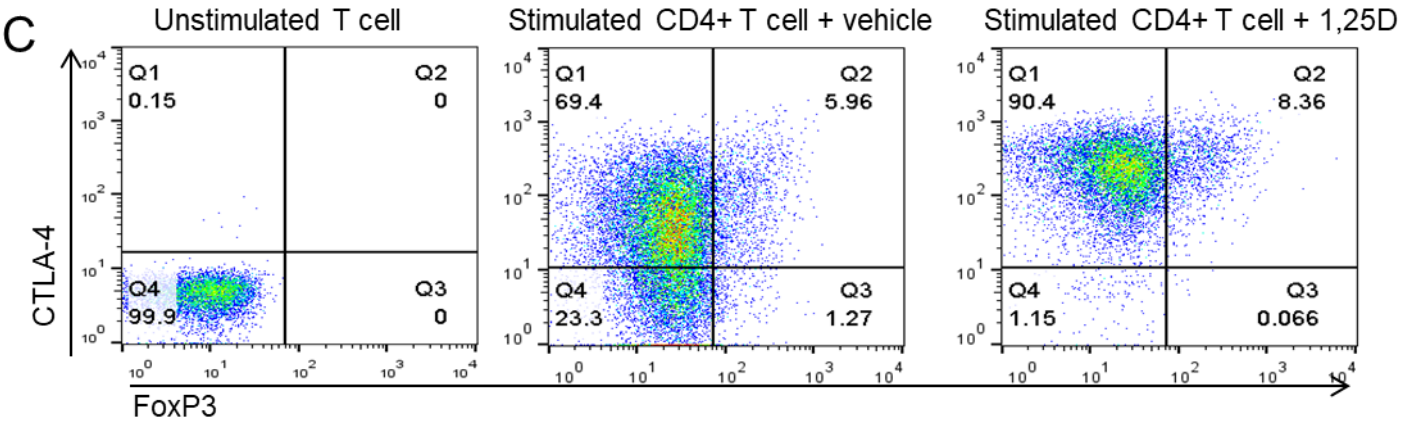
Cell population	Median Fluorescence Intensity (MFI)			
	CTLA-4	**CD4**	**FoxP3**	**CD127**
Isotype	15.1	18	17.4	4.4
PBMC	9.62	18.3	25.5	17.9
Naïve CD4+ CD25- T cell	15	100	33.9	150

B CD4+ T cell +/- 1,25D (day 4)



Cell population	Median Fluorescence Intensity (MFI)			
	CTLA-4	**CD25**	**FoxP3**	**IFN-γ**
Naïve CD4+ CD25- T cell	2.82	1.91	3.53	6.8
Activated CD4+ T cell + 1,25D (72hr)	262	56.9	14.7	26.1
Activated CD4+ T cell + vehicle (72hr)	28	40.9	11.9	48.4

C



D CD4+ T cell cytokine expression (day 5)

Cell population	Median Fluorescence Intensity (MFI)					
	CD25	**IL-17**	**IFN-γ**	**IL-21**	**IL-10**	**IL-2**
CD4+ T cell + 1,25D	9978	6054	133	7934	184	6.73
CD4+ T cell + vehicle	3437	10086	4610	9978	169	12.4
Isotype	2.9	295	86	1063	82	1

Figure 6.5. Flow cytometry validation of CD4+ T cell purity, activation and response to 1,25(OH)$_2$D3
CD4+ CD25- T cells were stained for intracellular and surface markers. FACS plots show A) intracellular expression of CTLA-4 and FoxP3 and surface expression of CD4 and CD127 in freshly isolated CD4+ CD25- naïve T cells; B) surface expression of CTLA-4, CD25, FoxP3 and IFN-γ in activated CD4+ T cells in the presence and absence of 1,25(OH)$_2$D3 after 72hrs, compared to unstimulated naïve T cells; C) Surface expression of CTLA-4 vs FoxP3 in unstimulated, vehicle and treated T cells after 72hrs; D) Median fluorescence intensity (MFI) values for 5 day CD4+ T cell cytokine expression. IgG Isotype stain conjugated to the matching fluorophores was used as the experimental control for all stains. Plots represent the mean expression pattern across all 4 donors.

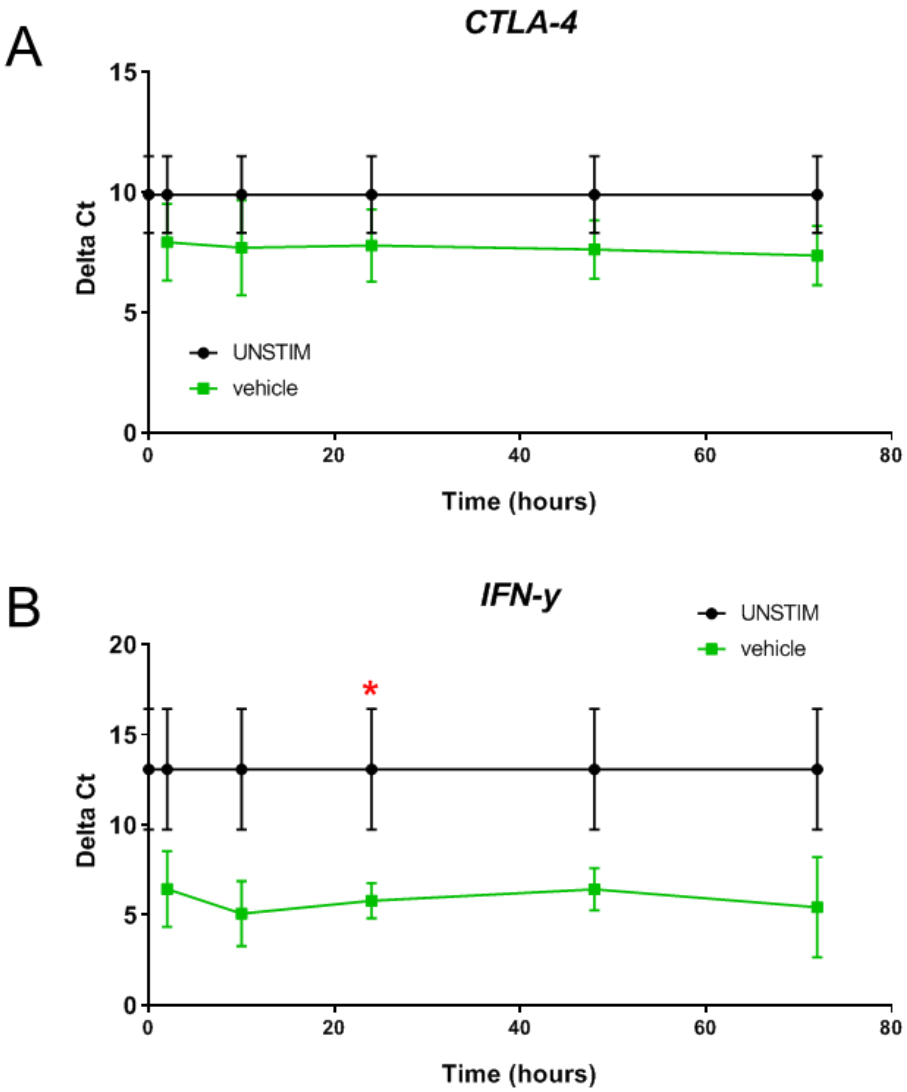


Figure 6.6. Gene expression time course for CD4+ T cell response over time
qPCR time course graphs showing T cell gene expression at 0hr, 2hrs, 10hrs, 24hrs, 48hrs and 72hrs in vehicle cells compared to the unstimulated control, for the genes A) *CTLA-4*; B) *IFN-Y*. Y-axis shows delta Ct (ΔCt) values of vehicle compared to unstimulated cells at each time point, so a higher ΔCt reflects a lower expression. Results for each time point are shown as individual response for n=4 individual samples. 2-way ANOVA with Sidak's multiple analysis test for paired values was performed for each time point. Statistical significance is taken as *p<0.05 and indicated where significant.

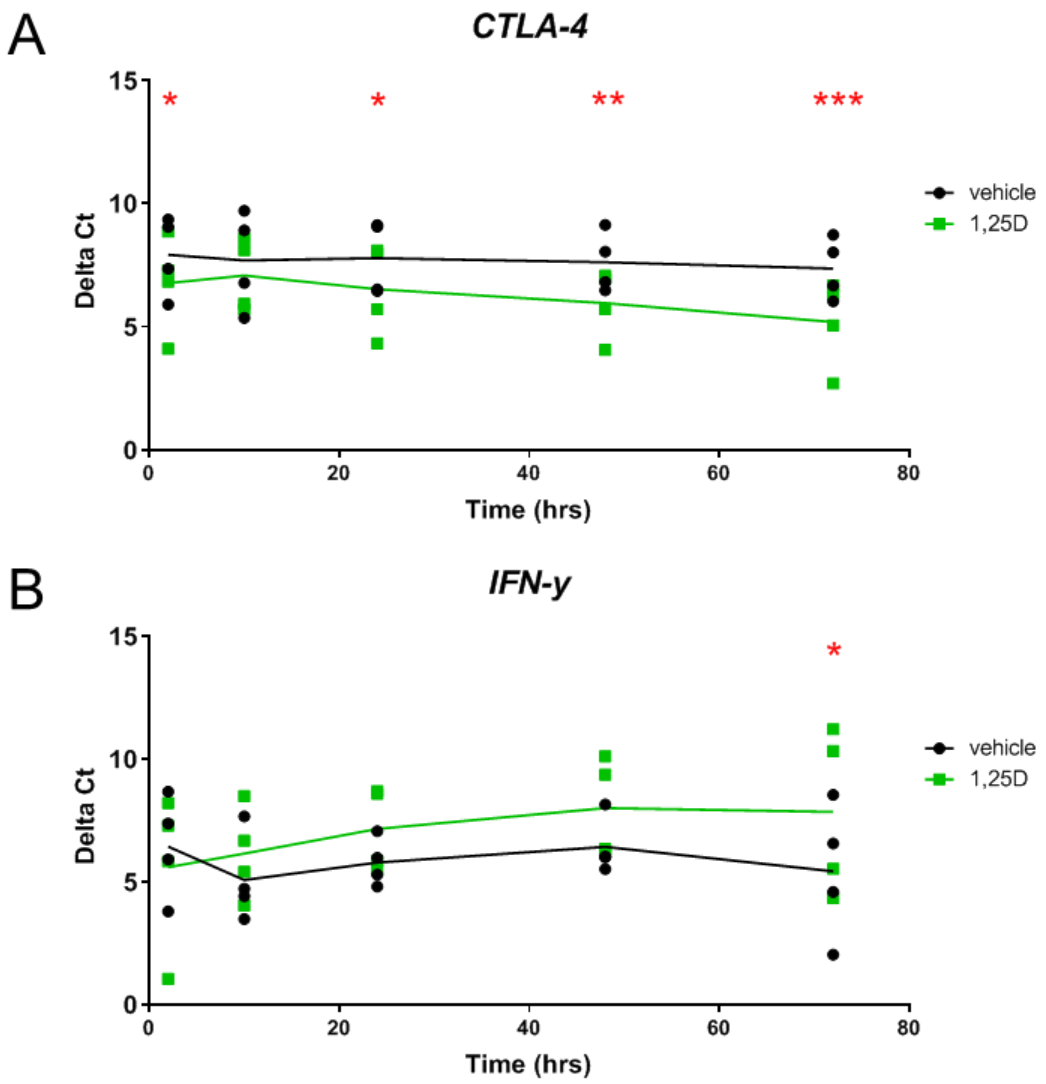


Figure 6.7. Gene expression time course for CD4+ T cell response to $1,25(OH)_2D3$ by qRT-PCR

qPCR time course graphs showing T cell gene expression at 0hr, 2hrs, 10hrs, 24hrs, 48hrs and 72hrs post incubation with $1,25(OH)_2D3$ (10nM), for the genes A) *CTLA-4*; B) *IFN- Y*. Y-axis shows delta Ct (ΔCt) values of vehicle compared to treated cells at each time point, so a higher ΔCt reflects a lower expression. Results for each time point are shown as individual response for n=4 individual samples. 0hr time point refers to 20hrs after T cell activation, but 0hr after $1,25(OH)_2D3$ treatment. 2-way ANOVA with Sidak's multiple analysis test for paired values was performed for each time point. Statistical significance is taken as $*p<0.05$ and indicated where significant.

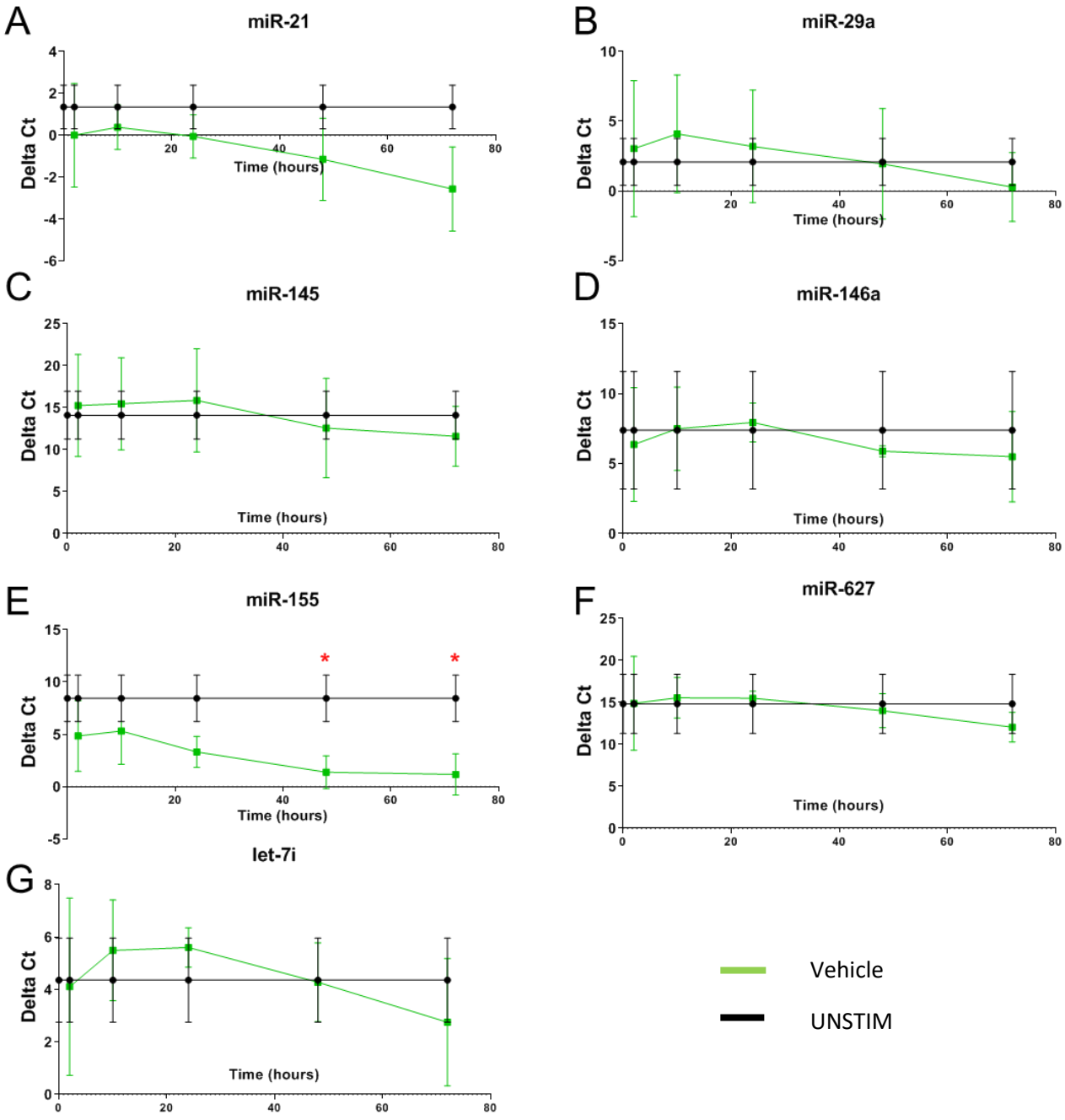


Figure 6.8. miRNA expression time course for CD4+ T cell response over time
qPCR time course graphs showing T cell miRNA expression at 0hr, 2hrs, 10hrs, 24hrs, 48hrs and 72hrs in vehicle cells compared to the unstimulated control, for the miRNAs A) miR-21; B) miR-29a; C) miR-145; D) miR-146a; E) miR-155; F) miR-627; G) let-7i. Y-axis shows delta Ct (ΔCt) values of vehicle compared to unstimulated cells at each time point, so a higher ΔCt reflects a lower expression. Results for each time point are shown as individual response for n=4 individual samples. 2-way ANOVA with Sidak's multiple analysis test for paired values was performed for each time point. Statistical significance is taken as *p<0.05 and indicated where significant.

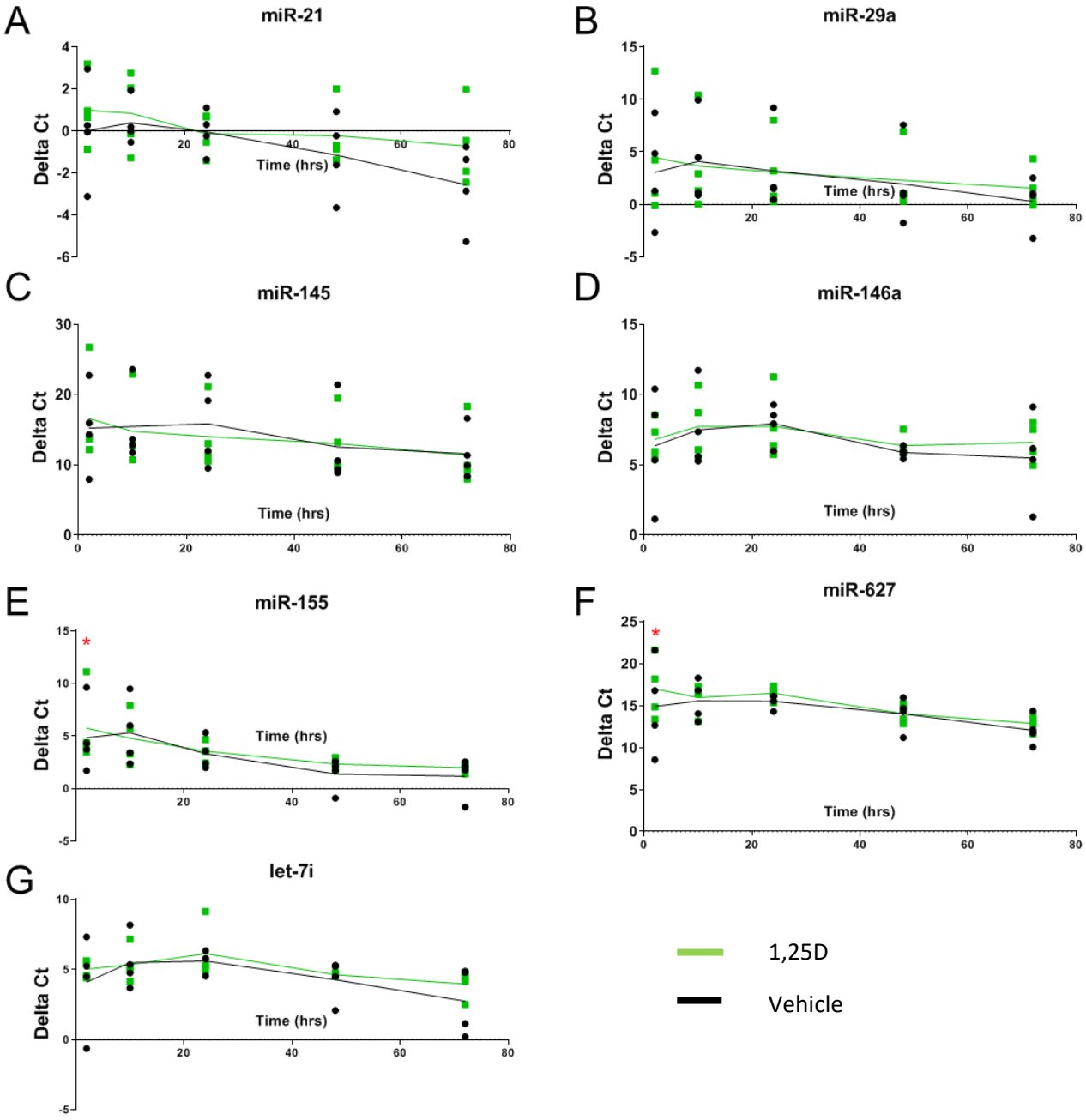


Figure 6.9. miRNA expression time course for CD4+ T cell response to 1,25(OH)$_2$D3 by qRT-PCR

qPCR time course showing miRNA expression in T cells at 0hr, 2hrs, 10hrs, 24hrs, 48hrs and 72hrs post incubation with 1,25(OH)$_2$D3 (10nM), for the miRNAs A) miR-21; B) miR-29a; C) miR-145; D) miR-146a; E) miR-155; F) miR-627; G) let-7i. Y-axis shows delta Ct (ΔCt) values of vehicle compared to treated cells at each time point, so a higher ΔCt reflects a lower expression. Results for each time point are shown as individual response for n=4 individual samples. 0hr time point refers to 20hrs after T cell activation, but 0hr after 1,25(OH)$_2$D3 treatment. 2-way ANOVA with Sidak's multiple analysis test for paired values was performed for each time point. Statistical significance is taken as *p<0.05 and indicated where significant.

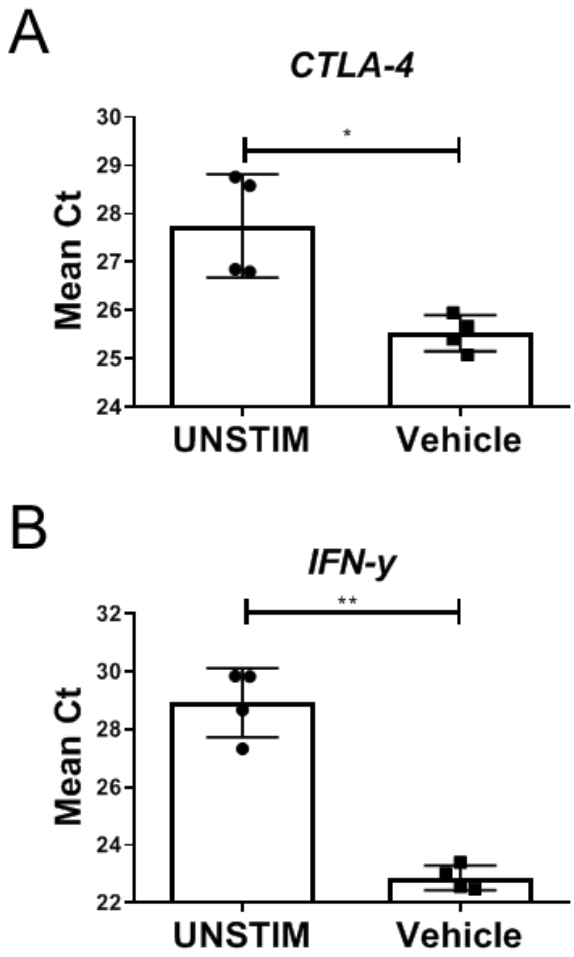


Figure 6.10. qPCR gene expression for CD4+ CD25- T cell response to Th17 cytokine cocktail stimulation (20hrs)
qPCR detected gene expression between unstimulated and Th17 cytokine cocktail stimulated (vehicle) T cells (n=4), for A) *CTLA-4*; B) *IFN-Y*. Stimulation was performed for 20hrs in the presence of pro-inflammatory Th17 cytokine cocktail: TGF-β, IL-1β, IL-2, IL-6, IL-23. Data is shown as mean normalised Ct, so a lower value corresponds to higher expression. Significance was calculated using paired two-tailed *t*-test, and significance taken as *p<0.05. Data is shown as mean ± SD.

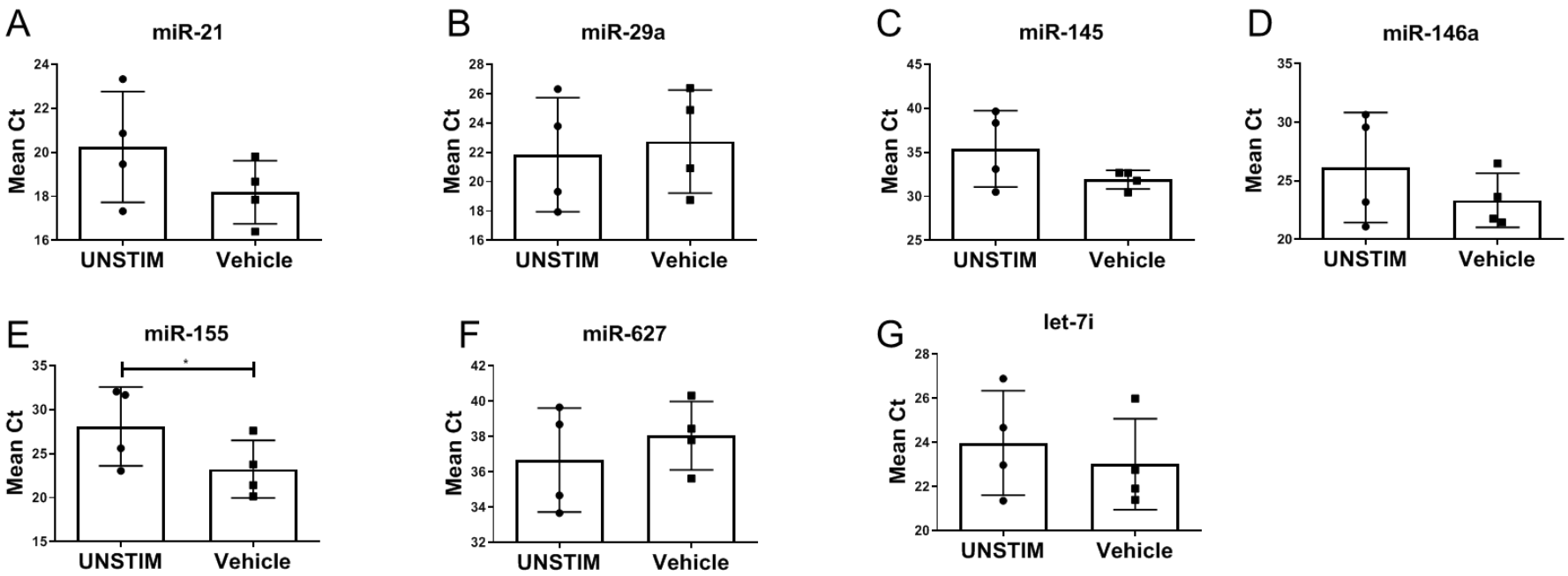
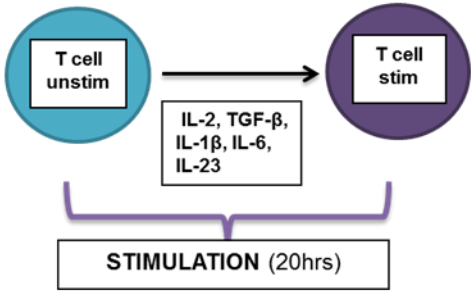


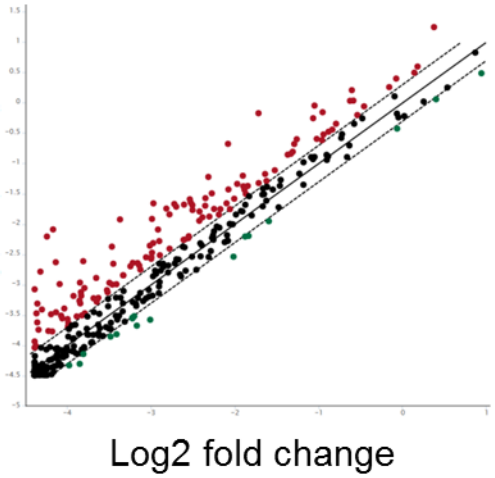
Figure 6.11. Candidate qPCR miRNA expression for CD4+ CD25- T cell response to Th17 cytokine cocktail stimulation
qPCR detected candidate miRNA expression between unstimulated and Th17 cytokine cocktail stimulated (vehicle) T cells (n=4), for A) miR-21; B) miR-29a; C) miR-145; D) miR-146a; E) miR-155; F) miR-627; G) let-7i. Stimulation was performed for 20hrs in the presence of pro-inflammatory Th17 cytokine cocktail: TGF-β, IL-1β, IL-2, IL-6, IL-23. Data is shown as mean normalised Ct, so a lower value corresponds to higher expression. Significance was calculated using paired two-tailed *t*-test, and significance taken as *$p<0.05$. Data is shown as mean ± SD.

Unstim vs stim

A



B



C

miRNAs	Stimulation Expression change	p values
miR-103a-3p	↑	0.049
miR-106a-3p	↑	0.020
miR-106b-5p	↑	0.027
miR-107	↑	0.012
miR-10a-5p	↓	0.049
miR-10b-5p	↓	0.026
miR-125b-5p	↓	0.047
miR-132-3p	↑	0.049
miR-132-5p	↑	0.009
miR-138-5p	↓	0.035
miR-147a	↑	0.004
miR-155-5p	↑	0.0001
miR-185-5p	↑	0.045
miR-196b-5p	↓	0.011
miR-20b-5p	↑	0.004
miR-212-3p	↑	0.002
miR-221-5p	↑	0.003
miR-330-3p	↑	0.001
miR-340-5p	↑	0.003
miR-342-3p	↓	0.041
miR-378a-3p	↑	0.049
miR-423-3p	↑	0.012
miR-424-3p	↑	0.017
miR-424-5p	↑	0.039
miR-431-5p	↓	0.004
miR-505-3p	↑	0.039
miR-744-5p	↑	0.013
miR-7-5p	↑	0.008
miR-93-5p	↑	0.016
miR-96-5p	↑	0.002
miR-98-5p	↑	0.002

Figure 6.12. qPCR array of miRNA expression in response to cytokine stimulation
A) Naïve CD4+ T cell activation with Th17 cytokine cocktail (20hrs) schematic; B) Scatter plot showing expression change of miRNAs following 20hrs stimulation with pro-inflammatory Th17 cytokine cocktail in CD4+CD25- T cells. Green and red represent downregulation and up-regulation of miRNA expression respectively, calculated as $\log_2$ fold change; C) Huynh-Feldt statistical analysis of statistically significant miRNAs differentially regulated following T cell activation. Huynh-Feldt test for sphericity was performed on normalised data, and significance taken as *p<0.05.

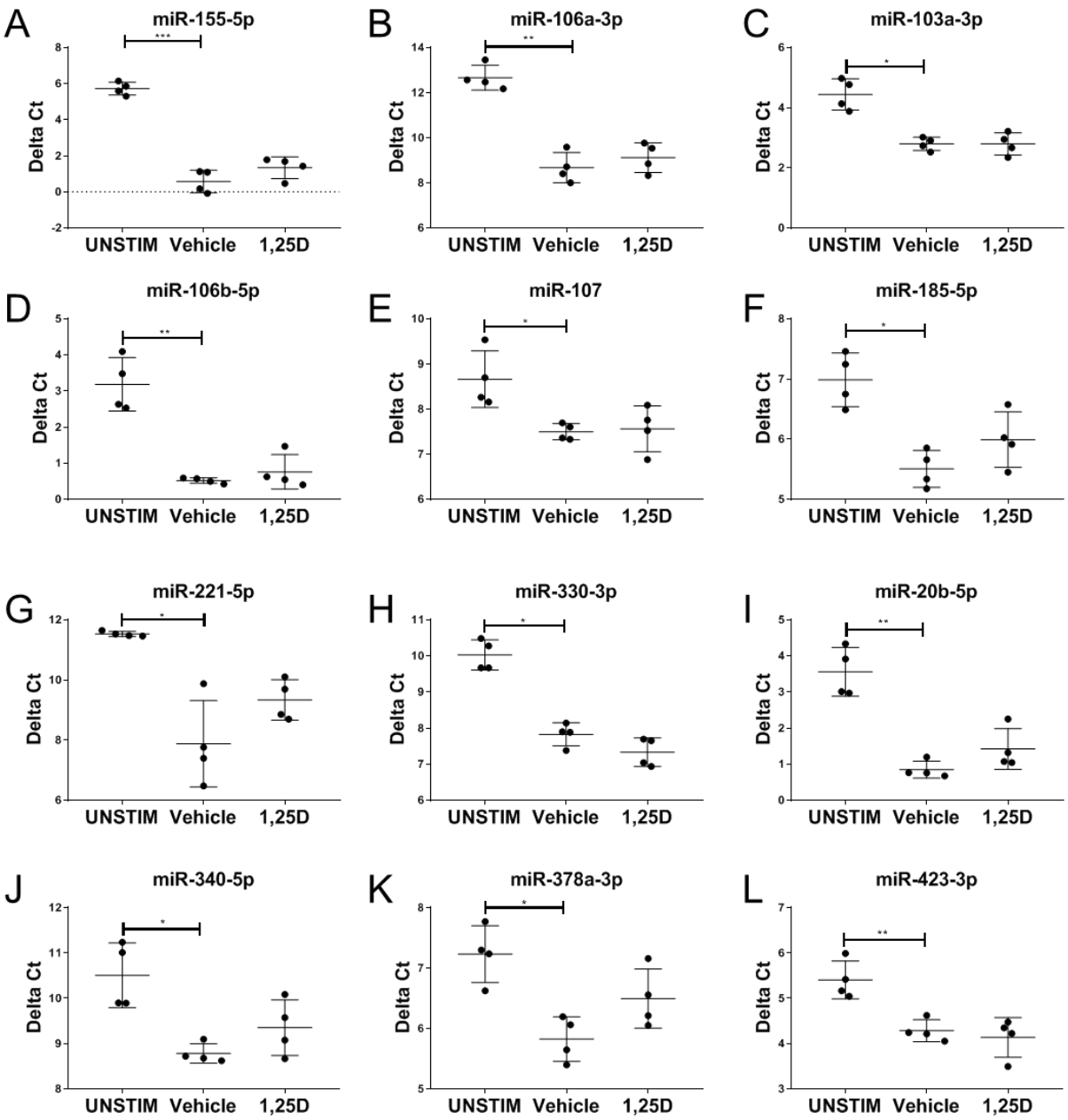


Figure 6.13. Graphical representation of significantly regulated qPCR array miRNAs, to Th17 cytokine cocktail stimulation (20hrs)

qPCR array quantified expression change in response to Th17 cytokine cocktail stimulation for A) miR-155-5p; B) miR-106a-3p; C) miR-103a-3p; D) miR-106b-5p; E) miR-107; F) miR-185-5p; G) mir-221-5p; H) miR-330-3p; I) miR-20b-5p; J) miR-340-5p; K) miR-378a-3p; L) miR-423-3p; M) miR-7-5p; N) miR-744-5p; O) miR-505-3p; P) miR-93-5p; Q) miR-96-5p; R) miR-98-5p; S) miR-10a-5p; T) miR-10b-5p; U) miR-125b-5p; V) miR-138-5p; W) miR-196b-5p; X) miR-342-3p; Y) miR-431-5p. UNSTIM, unstimulated naïve T cells; vehicle, stimulated T cells; 1,25D, stimulated T cells + 1,25D. Individual donors (n=4) are shown. Repeated-measures ANOVA for paired data was used to calculate statistical significance, and significance was taken as *p<0.05. Data is shown as mean ± SD.

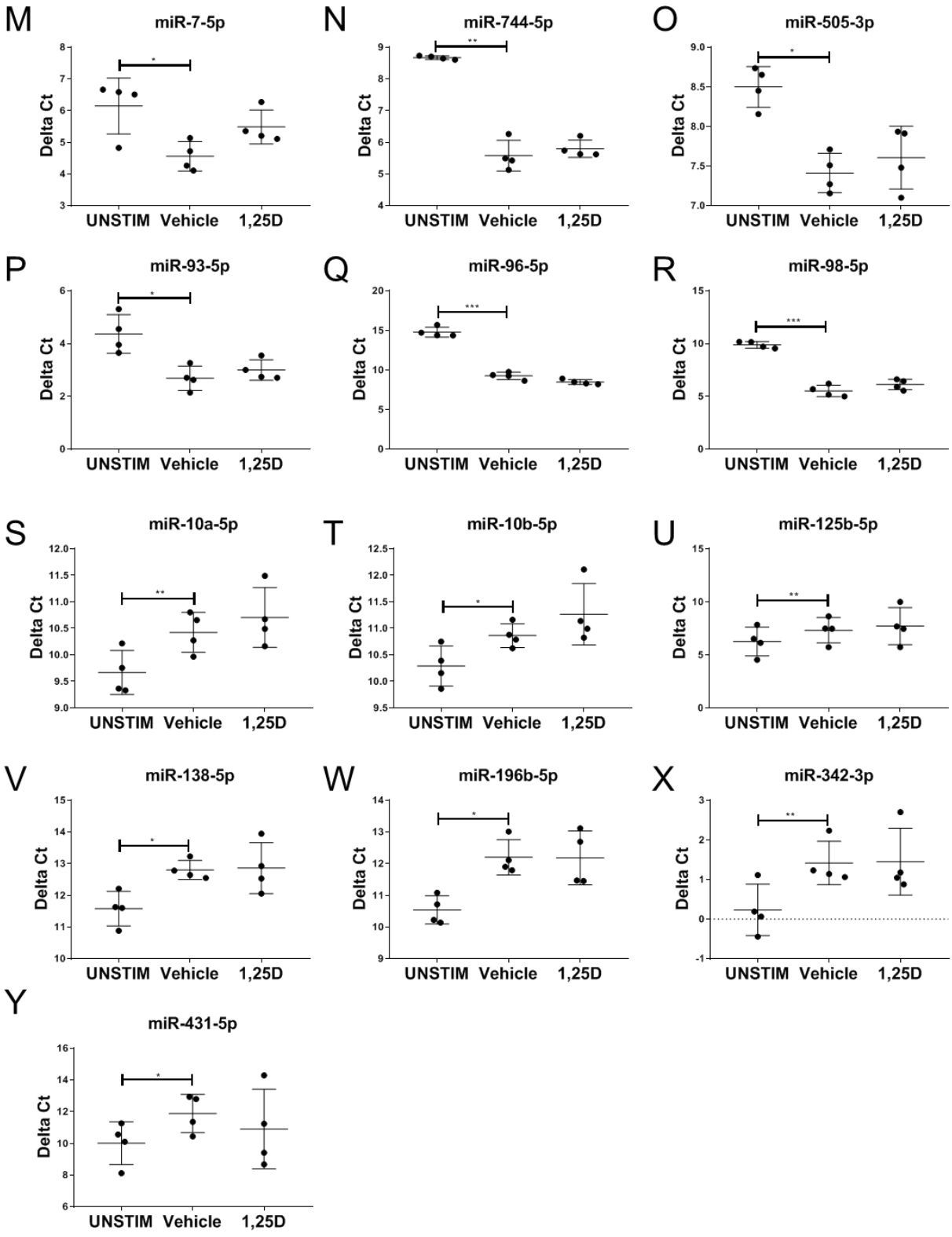
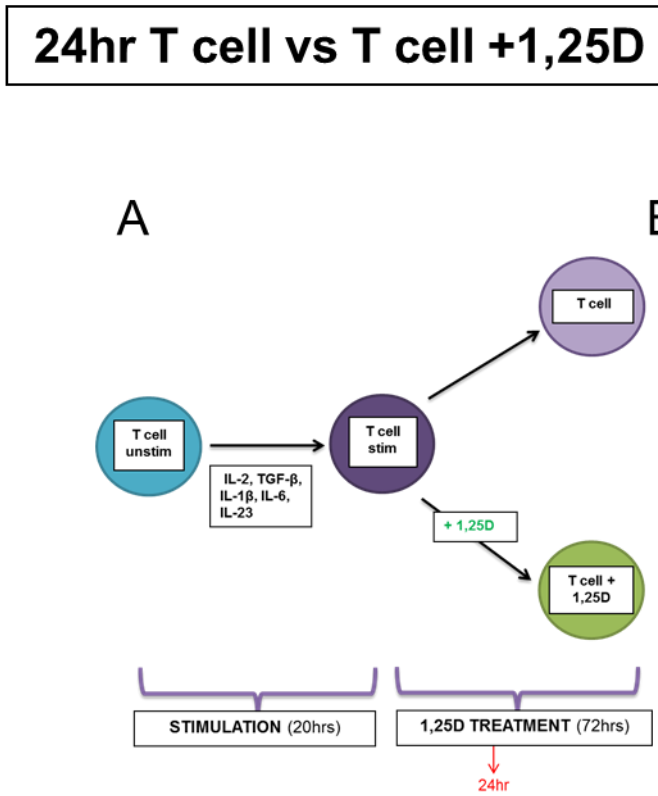
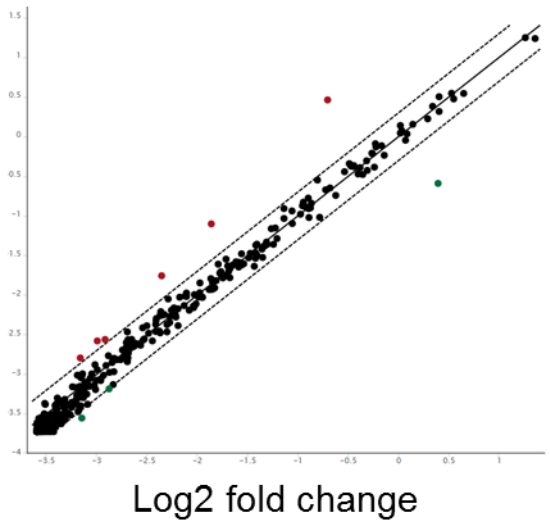


Figure 6.13. Continued





C

miRNAs	Short term 1,25D Expression change	*p* values
miR-16-5p	↑	0.004
miR-212-3p	↑	0.004

Figure 6.14. qPCR array of miRNA expression in response to short term 1,25(OH)$_2$D3 (10nM)
A) Short term 1,25(OH)$_2$D3 (24hrs) schematic; B) Scatter plot showing miRNA expression change following short term 1,25(OH)$_2$D3 (24hrs) in activated CD4+ CD25- T cell populations. Green and red represent downregulation and up-regulation of miRNA expression respectively, calculated as log$_2$ fold change; C) Huynh-Feldt statistical analysis of statistically significant miRNAs differentially regulated following short term 1,25(OH)$_2$D3. Huynh-Feldt test for sphericity was performed on normalised data, and significance taken as *p<0.05.

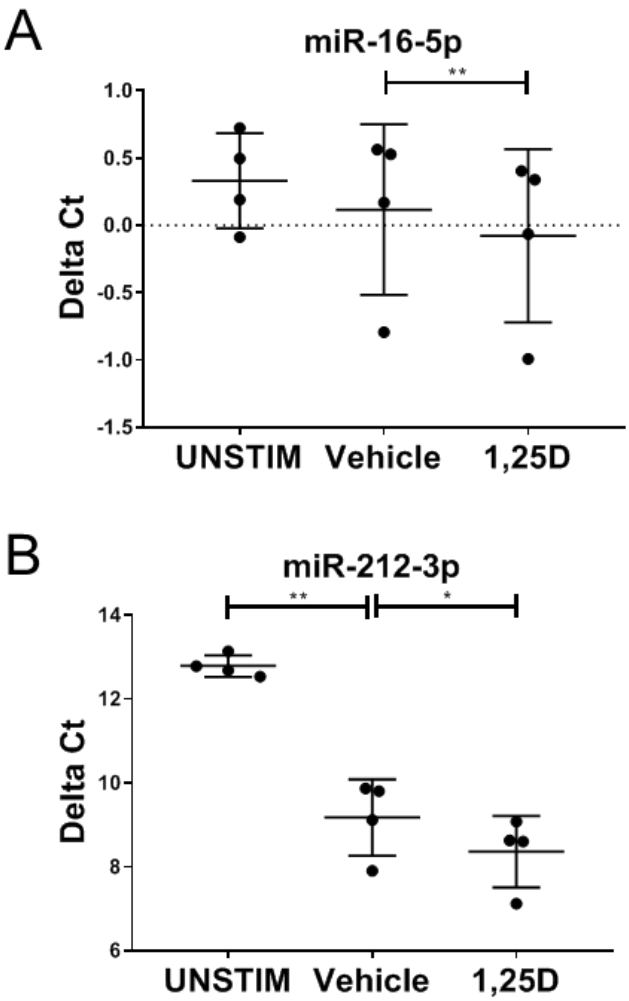
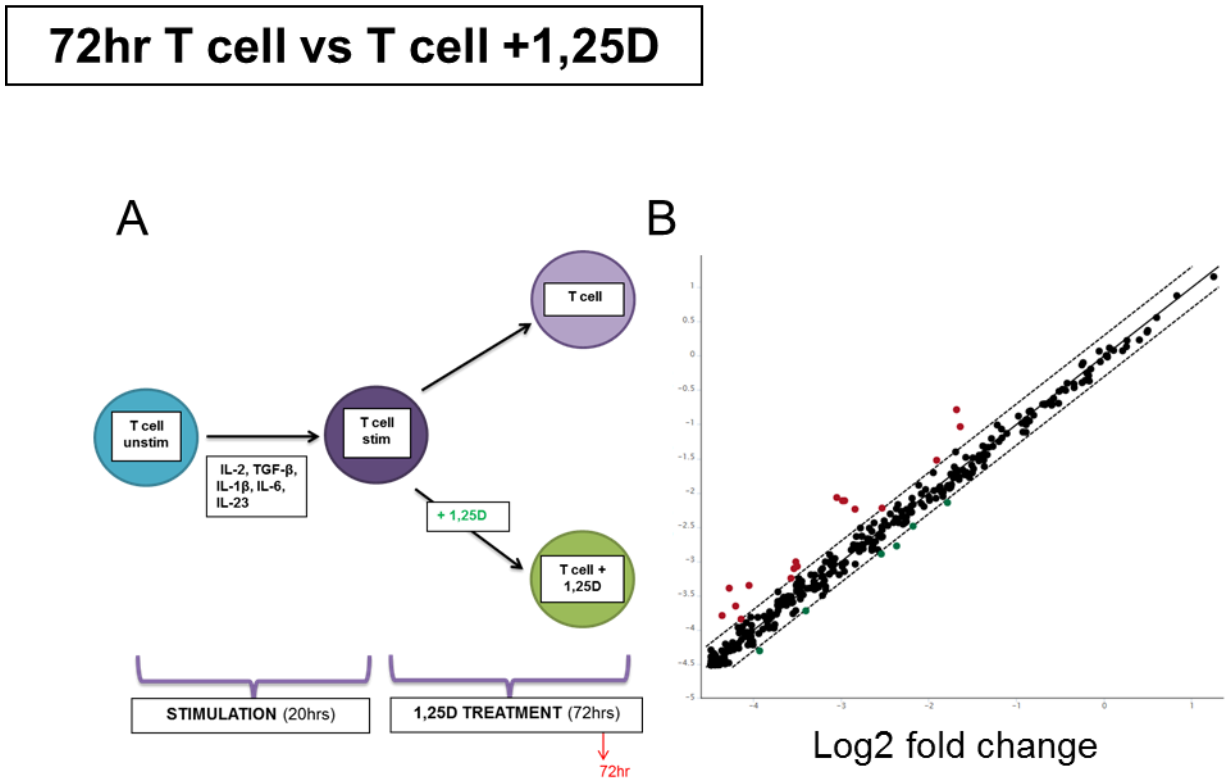


Figure 6.15. Graphical representation of significantly regulated qPCR array miRNAs, to short term 1,25(OH)$_2$D3 (24hrs)

qPCR array quantified expression changes in response to short term 1,25(OH)$_2$D3 for A) miR-16-5p; B) miR-212-3p. UNSTIM, unstimulated naïve T cells; vehicle, stimulated T cells; 1,25D, stimulated T cells + 1,25D. Individual donors (n=4) are shown. Repeated-measures ANOVA for paired data was used to calculate statistical significance, and significance was taken as *p<0.05. Data is shown as mean ± SD.



C

miRNAs	Long term 1,25D Expression change	*p* values
miR-129-1-3p	↑	0.018
miR-129-2-3p	↑	0.003
miR-132-3p	↑	0.049
miR-132-5p	↑	0.009
miR-137	↑	0.036
miR-147a	↑	0.004
miR-212-3p	↑	0.002
miR-335-3p	↓	0.008
miR-424-3p	↑	0.017
miR-424-5p	↑	0.039

Figure 6.16. qPCR array of miRNA expression in response to long term 1,25(OH)$_2$D3 (10nM)
A) Long term 1,25(OH)$_2$D3 (72hrs) schematic; B) Scatter plot showing miRNA expression change following long term 1,25(OH)$_2$D3 (72hrs) in activated CD4+ CD25- T cell populations. Green and red represent downregulation and up-regulation of miRNA expression respectively, calculated as log$_2$ fold change; C) Huynh-Feldt statistical analysis of statistically significant miRNAs differentially regulated following long term 1,25(OH)$_2$D3. Huynh-Feldt test for sphericity was performed on normalised data, and significance taken as *p<0.05.

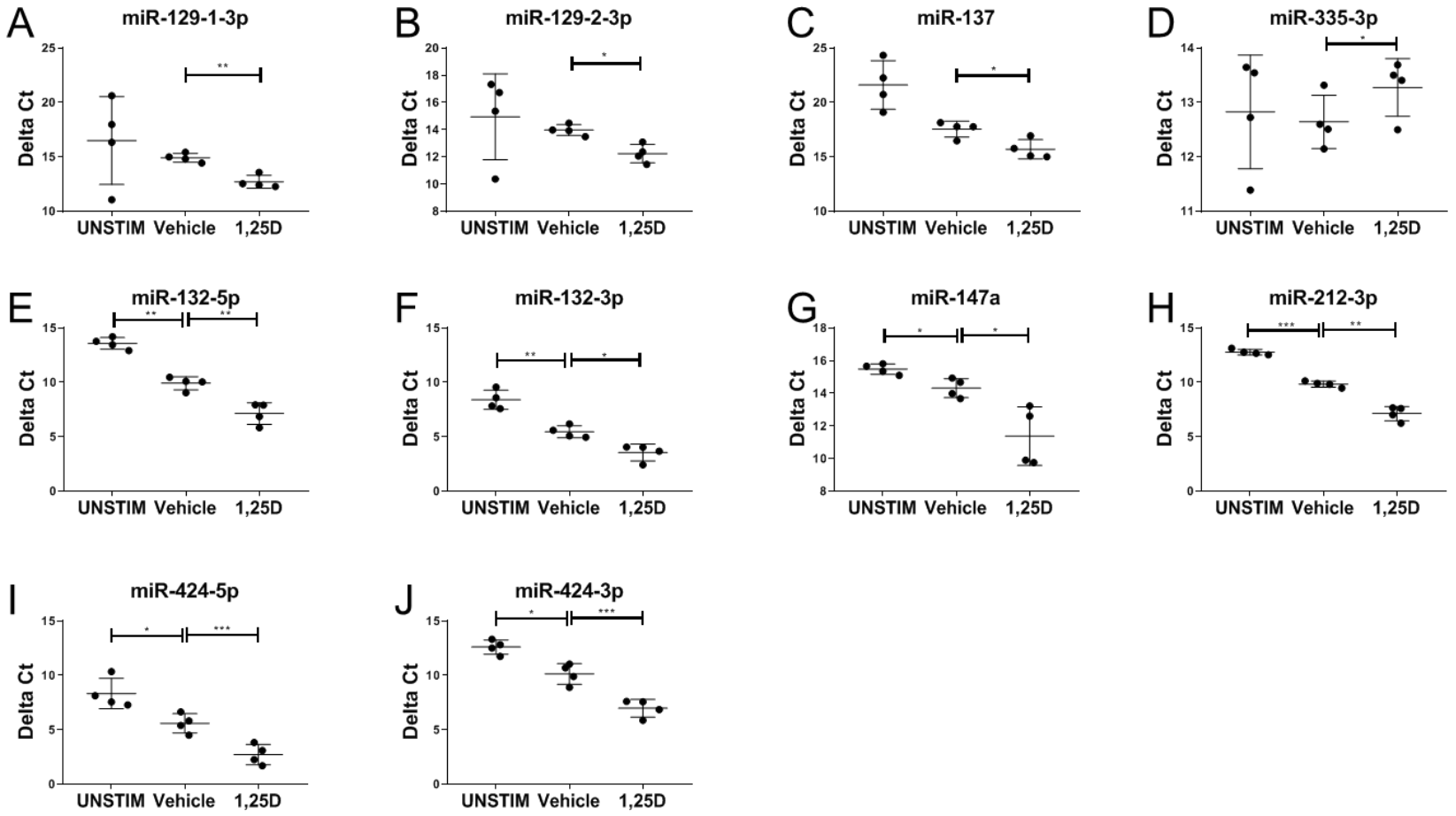


Figure 6.17. Graphical representation of significantly regulated qPCR array miRNAs, to long term 1,25(OH)$_2$D3 (72hrs)
qPCR array quantified expression changes in activated CD4+ T cells in response to long term 1,25(OH)$_2$D3 for A) miR-129-1-3p; B) miR-129-2-3p; C) miR-137; D) miR-335-3p; E) miR-132-5p; F) miR-132-3p; G) mir-147a; H) miR-212-3p; I) miR-424-5p; J) miR-424-3p. UNSTIM, unstimulated naïve T cells; vehicle, stimulated T cells; 1,25D, stimulated T cells + 1,25D. Individual donors (n=4) are shown. Repeated-measures ANOVA for paired data was used to calculate statistical significance, and significance was taken as *p<0.05. Data is shown as mean ± SD.

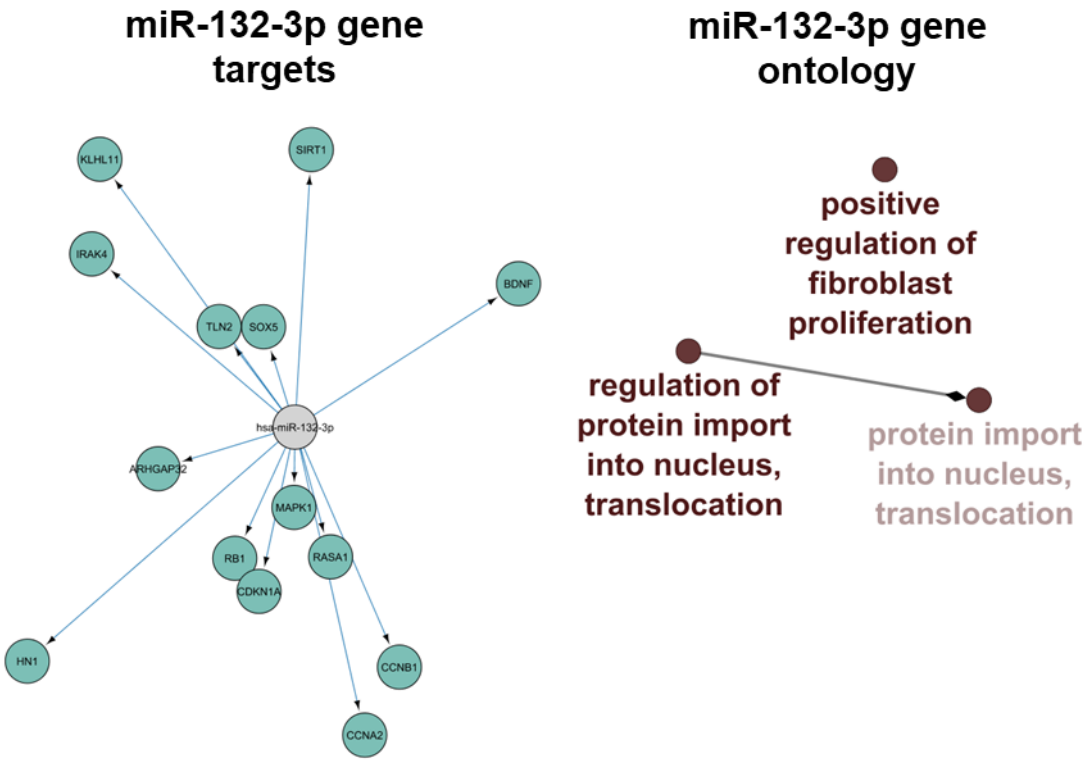
A

miRNAs	Stimulation	Long term 1,25D	Short term 1,25D	*p* values
	Expression change			
miR-103a-3p	↑			0.049
miR-106a-3p	↑			0.020
miR-106b-5p	↑			0.027
miR-107	↑			0.012
miR-10a-5p	↓			0.049
miR-10b-5p	↓			0.026
miR-125b-5p	↓			0.047
miR-129-1-3p		↑		0.018
miR-129-2-3p		↑		0.003
miR-132-3p	↑	↑		0.049
miR-132-5p	↑	↑		0.009
miR-137		↑		0.036
miR-138-5p	↓			0.035
miR-147a	↑	↑		0.004
miR-155-5p	↑			0.000
miR-16-5p			↑	0.004
miR-185-5p	↑			0.045
miR-196b-5p	↓			0.011
miR-20b-5p	↑			0.004
miR-212-3p	↑	↑	↑	0.002
miR-221-5p	↑			0.003
miR-330-3p	↑			0.001
miR-335-3p		↓		0.008
miR-340-5p	↑			0.003
miR-342-3p	↓			0.041
miR-378a-3p	↑			0.049
miR-423-3p	↑			0.012
miR-424-3p	↑	↑		0.017
miR-424-5p	↑	↑		0.039
miR-431-5p	↓			0.004
miR-505-3p	↑			0.039
miR-744-5p	↑			0.013
miR-7-5p	↑			0.008
miR-93-5p	↑			0.016
miR-96-5p	↑			0.002
miR-98-5p	↑			0.002

B

miRNAs	verified mRNA targets (MiRTarBase)				
miR-103a-3p	CDK2	DICER1	KLF4	CCNE1	CREB1
miR-106a-3p	CLIC4	KLHL15	DICER1	ZFN507	MLLT10
miR-106b-5p	ITCH	CDKN1A	BCL2L11	E2F1	PTEN
miR-107	CDK6	HIF1A	ARNT	BACE1	MYB
miR-10a-5p	HOXA1	MAP3K7	BTRC	EPHA4	USF2
miR-10b-5p	HOXD10	PPARA	NF1	KLF4	BCL2L11
miR-125b-5p	BMPR1B	EIF4EBP1	NKIRAS2	TP53	VDR
miR-129-1-3p	PDCD2	CRK	PRRG4	ZFN200	SOX4
miR-129-2-3p	SOX4	CCP110	CDK6	ZFN200	UBE2F
miR-132-3p	SIRT1	CDKN1A	HBEGF	MECP2	EP300
miR-132-5p	FOXO1	SNX5	YWHAG	REM1	NSUN2
miR-137	CDK6	CDC42	PXN	KDM1A	E2F6
miR-138-5p	VIM	RHOC	EZH2	IGF1R	SIRT1
miR-147a	MCM3	NDUFA4	PSMA3	VEGFA	CYP2S1
miR-155-5p	SOCS1	SMAD5	TAB2	APC	BACH1
miR-16-5p	BMI1	CDK1	ACVR2A	WT1	HMGA1
miR-185-5p	RHOA	CDC42	CDK6	CCNE1	SREBF1
miR-196b-5p	HOXB8	HOXC8	CD8A	COX3	BCL2
miR-20b-5p	STAT3	HIF1A	CDKN1A	ESR1	MYLIP
miR-212-3p	MECP2	TJP1	PEA15	PTCH1	AGO1
miR-221-5p	MBD2	PRNP	NUP50	MUC17	HES7
miR-330-3p	E2F1	CDC42	CD44	AGO1	VEGFA
miR-335-3p	ANK3	INSIG1	NECAP1	RPS21	ZFN460
miR-340-5p	MET	SMAD5	SUFU	LARP4	SYAP1
miR-342-3p	GEMIN4	DNMT1	FIGN	FIBP	IDH3B
miR-378a-3p	VEGFA	SUFU	TUSC2	GALNT7	GRB2
miR-423-3p	CDKN1A	KIF2C	RAP2C	TATDN2	ATXN7
miR-424-3p	LGALS3	ARID2	HUWE1	MAT2A	EIF5A
miR-424-5p	CCNF	CHEK1	KIF23	WEE1	ATF6
miR-431-5p	TBPL1	FEM1A	SOCS6	KREMEN1	PROX2
miR-505-3p	SRSF1	TAF9	DNAJB9	CD47	SERBP1
miR-744-5p	ARL15	AGPAT3	PPTC7	RASSF1	IER5
miR-7-5p	SNCA	PAK1	EGFR	FNG	ABCC1
miR-93-5p	TP53INP1	CDKN1A	STAT3	E2F1	MAPK9
miR-96-5p	FOXO1	MITF	KRAS	HTR1B	ADCY6
miR-98-5p	E2F1	E2F2	MYC	CASP3	TUSC2

Table 6.1. Summary of all significantly regulated miRNAs
A) Table of significantly regulated miRNAs in response to long term 1,25$(OH)_2$D3 (72hrs), short term 1,25$(OH)_2$D3 (24hrs) and Th17 cytokine cocktail stimulation (20hrs). Significance taken as $p<0.05$ by Huynh-Feldt analysis. B) Top 5 experimentally validated gene targets for each significant miRNA, as predicted by miRTarBase. Only those with a target prediction score of 95/100 or above were included.



miR-132-3p target gene functions

Function	Associated Genes
protein import into nucleus, translocation	[CDKN1A, MAPK1, SIRT1]
regulation of protein import into nucleus, translocation	[CDKN1A, MAPK1, SIRT1]
positive regulation of fibroblast proliferation	[CCNA2, CCNB1, CDKN1A]

Figure 6.18. Gene Ontology analysis of significantly regulated miRNA targets (miR-132-3p)
Cytoscape v3.6.1 gene prediction software was used in conjunction with ClueGo to identify experimentally validated gene targets of miRNAs and their cellular functions. Validated gene targets and functional gene links of miR-132-3p is shown. For Cytoscape miRNA target prediction, all targets with a target prediction score below of 95% were excluded. For ClueGo gene ontology (GO) analysis, statistical significance of genes with enriched biological functions was taken as $p<0.05$ calculated by Bonferroni step-down. Colours and distance of nodes do not correspond to level of significance, and are for display purposes only.

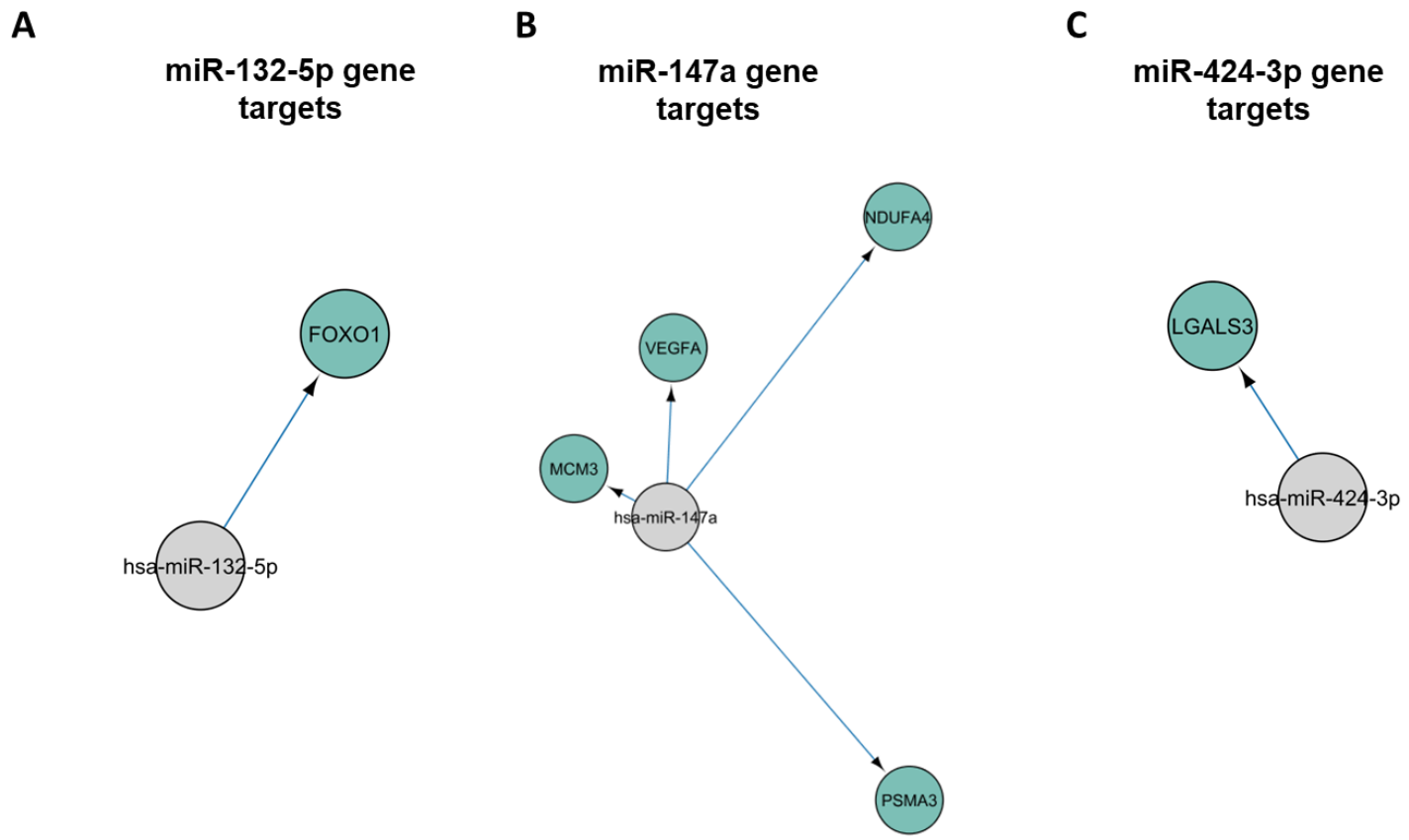
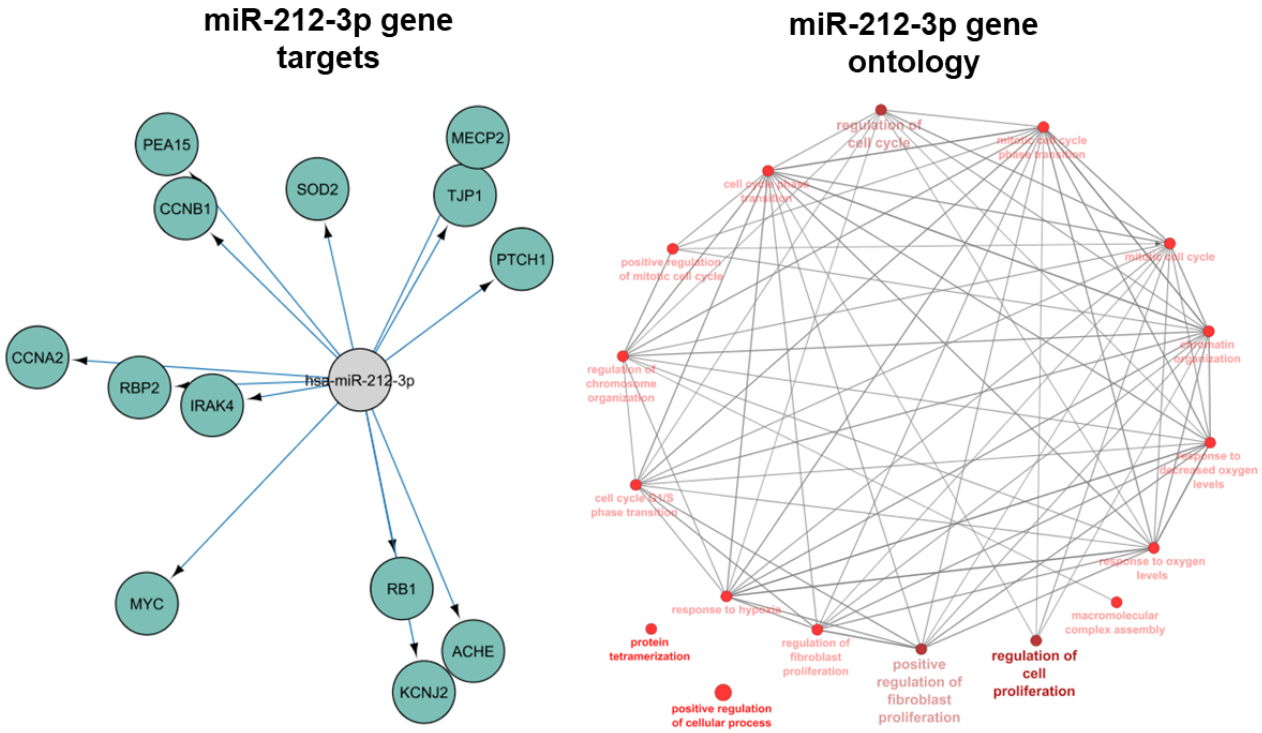


Figure 6.19. Gene Ontology analysis of significantly regulated miRNA targets (miR-132-5p, miR-147a, miR-424-3p)
Cytoscape v3.6.1 gene prediction software was used in conjunction with ClueGo to identify experimentally validated gene targets of miRNAs and their cellular functions. Validated gene targets of A) miR-132-5p; B) miR-147a; C) miR-424-3p. For Cytoscape miRNA target prediction, all targets with a target prediction score below of 95% were excluded. Where gene ontology is not shown, no statistically significant links were found. Colours and distance of nodes do not correspond to level of significance, and are for display purposes only.



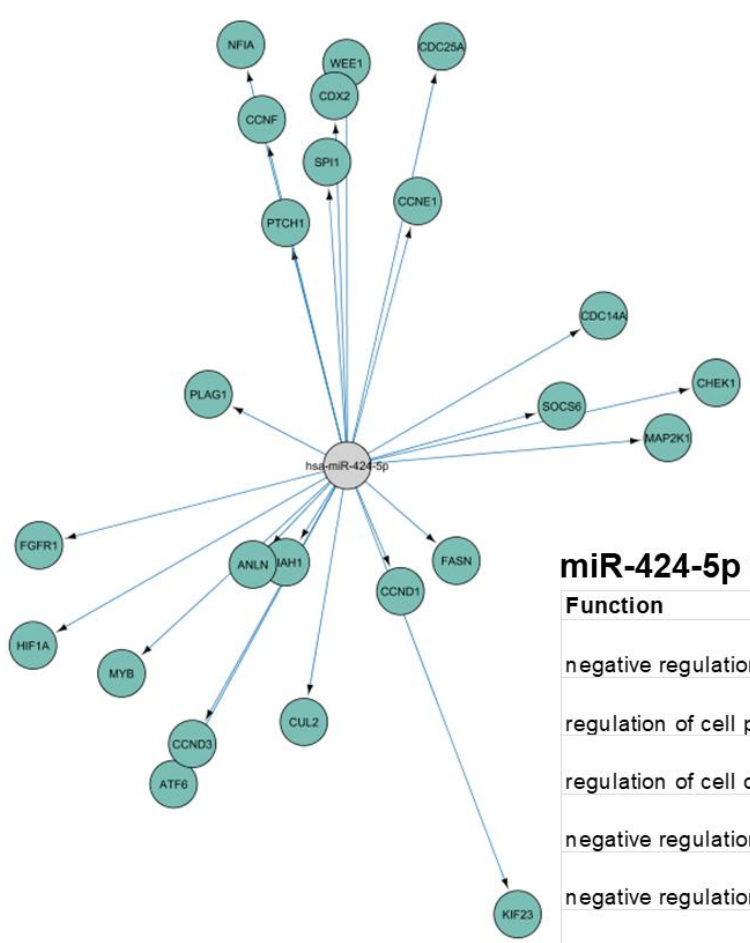
miR-212-3p target gene functions

Function	Associated genes
mitotic cell cycle	[CCNA2, CCNB1, MECP2, MYC, PTCH1, RB1]
response to hypoxia	[CCNA2, CCNB1, MECP2, MYC]
chromatin organization	[CCNA2, CCNB1, MECP2, MYC, RB1]
regulation of chromosome organization	[CCNB1, MECP2, MYC, RB1]
response to decreased oxygen levels	[CCNA2, CCNB1, MECP2, MYC]
regulation of cell proliferation	[CCNA2, CCNB1, IRAK4, MECP2, MYC, PTCH1, RB1, SOD2]
cell cycle phase transition	[CCNA2, CCNB1, MECP2, MYC, RB1]
mitotic cell cycle phase transition	[CCNA2, CCNB1, MECP2, MYC, RB1]
cell cycle G1/S phase transition	[CCNA2, CCNB1, MYC, RB1]
positive regulation of mitotic cell cycle	[CCNB1, MECP2, RB1]
regulation of fibroblast proliferation	[CCNA2, CCNB1, MYC]
positive regulation of fibroblast proliferation	[CCNA2, CCNB1, MYC]
protein tetramerization	[ACHE, KCNJ2, SOD2]
regulation of cell cycle	[CCNA2, CCNB1, MECP2, MYC, PEA15, PTCH1, RB1]
macromolecular complex assembly	[ACHE, CCNB1, KCNJ2, MECP2, MYC, RB1, SOD2]
response to oxygen levels	[CCNA2, CCNB1, MECP2, MYC]

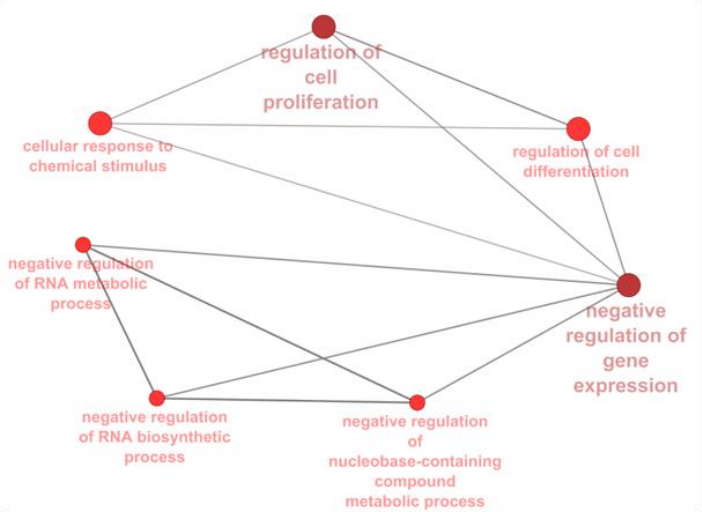
Figure 6.20. Gene Ontology analysis of significantly regulated miRNA targets (miR-212-3p)
Cytoscape v3.6.1 gene prediction software was used in conjunction with ClueGo to identify experimentally validated gene targets of miRNAs and their cellular functions. Validated gene targets and functional gene links of miR-212-3p is shown. For Cytoscape miRNA target prediction, all targets with a target prediction score below of 95% were excluded. For ClueGo gene ontology (GO) analysis, statistical significance of genes with enriched biological functions was taken as $p<0.05$ calculated by Bonferroni step-down. Colours and distance of nodes do not correspond to level of significance, and are for display purposes only.

21.

miR-424-5p gene targets



miR-424-5p gene ontology

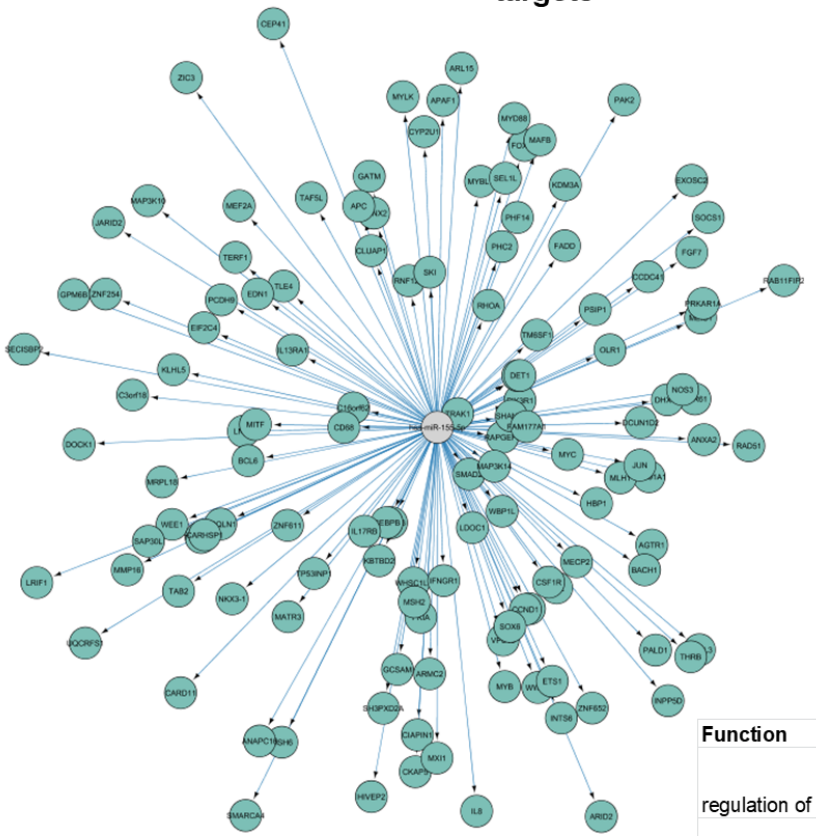


miR-424-5p target gene functions

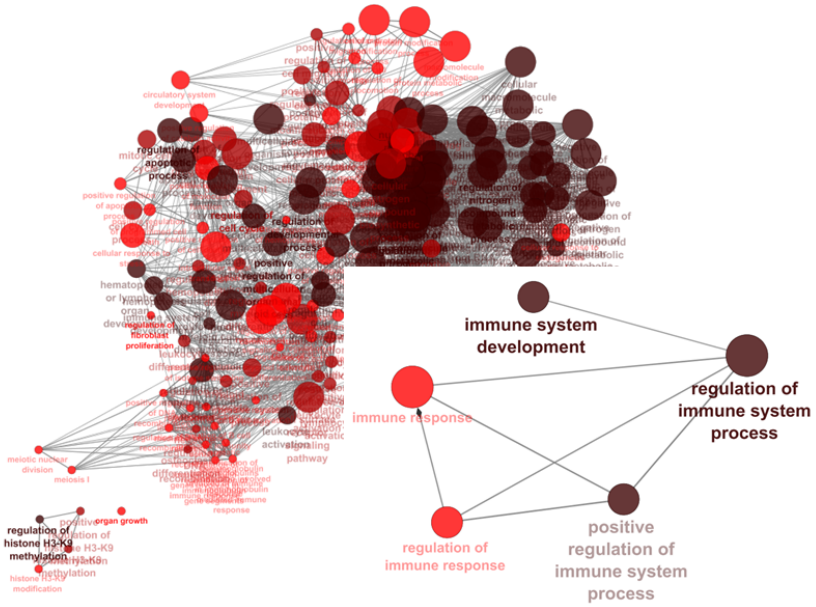
Function	Associated genes
negative regulation of gene expression	[CCND1, CCND3, CCNE1, CDX2, FGFR1, HIF1A, MAP2K1, MYB, NFIA, PLAG1, PTCH1, SPI1]
regulation of cell proliferation	[CCND1, CCND3, CDX2, CUL2, FASN, FGFR1, HIF1A, MAP2K1, MYB, PLAG1, PTCH1]
regulation of cell differentiation	[CCND1, CDX2, FASN, FGFR1, HIF1A, MAP2K1, MYB, PLAG1, PTCH1, SPI1]
negative regulation metabolic process	[CCND1, CCND3, CCNE1, CDX2, FGFR1, MYB, NFIA, PTCH1, SPI1]
negative regulation of RNA metabolic process	[CCND1, CCND3, CCNE1, CDX2, FGFR1, MYB, NFIA, PTCH1, SPI1]
cellular response to chemical stimulus	[ATF6, CCND1, CCND3, CCNE1, CUL2, FASN, FGFR1, HIF1A, MAP2K1, MYB, PTCH1, SOCS6, SPI1]
negative regulation of RNA biosynthetic process	[CCND1, CCND3, CCNE1, CDX2, FGFR1, MYB, NFIA, PTCH1, SPI1]

22.

miR-155-5p gene targets



miR-155-5p gene ontology



miR-155-5p target gene functions

Function	Associated genes
regulation of immune response	[BCL6, CARD11, DOCK1, FADD, GCSAM, IFNGR1, INPP5D, JUN, LRIF1, MATR3, MLH1, MSH2, MSH6, MYB, MYD88, PAK2, PIK3R1, SOCS1, TAB2, UBQLN1]
positive regulation of immune system process	[BCL6, CARD11, CXCL8, DOCK1, EDN1, ETS1, FADD, FOXO3, GCSAM, INPP5D, JUN, LRIF1, MAP3K14, MATR3, MLH1, MSH2, MSH6, MYB, MYD88, PAK2, PIK3R1, RHOA, SOCS1, TAB2, UBQLN1]
regulation of immune system process	[AGO4, BCL6, CARD11, CEBPB, CXCL8, DOCK1, EDN1, ETS1, FADD, FOXO3, GCSAM, IFNGR1, INPP5D, JUN, LRIF1, MAFB, MAP3K14, MATR3, MEIS1, MLH1, MSH2, MSH6, MYB, MYC, MYD88, PAK2, PIK3R1, PRKAR1A, RHOA, SOCS1, TAB2, UBQLN1]
immune system development	[AGO4, ANXA2, BCL6, CARD11, CEBPB, CIAPIN1, CSF1R, ETS1, FADD, FOXO3, INPP5D, JARID2, JUN, LRIF1, MAFB, MEIS1, MLH1, MSH2, MSH6, MYB, MYC, PIK3R1, RUNX2, SH3PXD2A, SOCS1, SOX6]
272 immune response	[ANXA2, APAF1, BCL6, C16orf62, CARD11, CD68, CEBPB, CSF1R, CXCL8, DOCK1, EDN1, ETS1, FADD, GCSAM, IFNGR1, INPP5D, JUN, LRIF1, MAP3K14, MATR3, MLH1, MSH2, MSH6, MYB, MYD88, OLR1, PAK2, PIK3R1, RHOA, SOCS1, TAB2, TSPAN14, UBQLN1]

Figure 6.21. Gene Ontology analysis of significantly regulated miRNA targets (miR-424-5p)
Cytoscape v3.6.1 gene prediction software was used in conjunction with ClueGo to identify experimentally validated gene targets of miRNAs and their cellular functions. Validated gene targets and functional gene links of miR-424-5p is shown. For Cytoscape miRNA target prediction, all targets with a target prediction score below of 95% were excluded. For ClueGo gene ontology (GO) analysis, statistical significance of genes with enriched biological functions was taken as $p<0.05$ calculated by Bonferroni step-down. Colours and distance of nodes do not correspond to level of significance, and are for display purposes only.

Figure 6.22. Gene Ontology analysis of significantly regulated miRNA targets (miR-155-5p)
Cytoscape v3.6.1 gene prediction software was used in conjunction with ClueGo to identify experimentally validated gene targets of miRNAs and their cellular functions. Validated gene targets and functional gene links of miR-155-5p is shown. For Cytoscape miRNA target prediction, all targets with a target prediction score below of 95% were excluded. For ClueGo gene ontology (GO) analysis, statistical significance of genes with enriched biological functions was taken as $p<0.05$ calculated by Bonferroni step-down. Colours and distance of nodes do not correspond to level of significance, and are for display purposes only.

Genes regulated by long term (72hr) 1,25(OH)2D3				
ABCA1	CTSB	IFNG	PLK3	TRIM56
ADGRE1	CXCR6	IL10RA	PLXNB2	TSPAN2
ADGRE5	CYTH4	IL23R	PLXNC1	UCP2
AGFG2	DDX26B	IL26	POU2AF1	VAV1
AHR	DENND1B	IL7R	PPFIBP1	VPS37B
ALOX5	DENND6B	IL9	PTPRJ	YWHAQ
APOL3	DMD	INPP5D	PTRF	ZC3H12C
ARHGAP5	DUSP2	IRAK2	RARRES3	ZFP36
ARHGEF1	DUSP5	IRF2BPL	RGS16	ZHX2
ARHGEF2	ECE1	ISG20	RGS3	ZSWIM6
BACH2	EFTUD1	ITGAM	SDR42E1	
BEND5	EHBP1L1	ITGAX	SERPINA1	
BTBD11	ELF4	JHDM1D-AS1	SERPINB1	
BTLA	EML2	JUNB	SERPINB6	
C18orf54	EPAS1	KDM1B	SERPINB9	
CA2	EPS8	KDM7A	SFT2D1	
CARD11	ETS1	LAG3	SH3TC1	
CCR7	EVI2B	LINC00649	SLC16A2	
CD200	EVL	LRP12	SLC2A3	
CD28	FAM53B	LRRC32	SLC5A3	
CD38	FAM89B	LRRC8A	SNN	
CD59	FBLN7	MALT1	SPECC1	
CDCA7L	FNBP1L	MAP4K4	ST3GAL1	
CHST3	FOSL2	NA	TAGAP	
CLMN	FYCO1	NAMPT	TIMP1	
COL6A3	G0S2	NAPSA	TMC6	
CRTAP	GADD45A	NFKBIA	TNFRSF8	
CSF1	GIT1	NKG7	TNFSF14	
CSRNP1	GPCPD1	NRIP1	TRAK1	
CTLA4	IDH2	PLEKHO2	TRAT1	

Table 6.2. Transcriptomics genes regulated by long term 1,25(OH)$_2$D3 (72hrs)
Unpublished data from University of Birmingham lab group showing list of genes significantly regulated by long term 1,25(OH)$_2$D3 (72hrs) in CD4+ T cells stimulated with Th17 polarising cocktail. Statistical significance of genes was taken as $p<0.05$.

6.4 Discussion

6.4.1 Overview

It is well established that CD4+ T cells play a critical role in the regulation of the immune system, by mediating a number of processes including pro- versus anti-inflammatory cytokine production, downstream (B cell) antibody secretion and Treg maintenance. These processes all require the complex regulation of gene expression at different stages of T cell development and function. Dysregulation in gene expression can lead to aberrant T cell signalling which has been implicated in many autoimmune diseases. The anti-inflammatory actions of $1,25(OH)_2D3$ are thought to act potently at specific stages of T cell development [191, 695]. These T cell effects of $1,25(OH)_2D3$ are primarily mediated via changes in *VDR* target gene expression. The transcriptomic effect of $1,25(OH)_2D3$ on T cells is clear [696, 697]. However, it is now clear that epigenetic mechanisms may also contribute to the actions of $1,25(OH)_2D3$, and the aim of this chapter of the thesis was to investigate the role of one particular epigenetic pathway, namely miRNAs.

6.4.2 CD4+ T cell subtypes and cytokine production with regards to activation and $1,25(OH)_2D3$

Activation of naïve CD4+ T cells has been associated with an up-regulation of IL-21, a pro-inflammatory cytokine which promotes T cell survival and is a contributing factor in the onset of autoimmune disease [698, 699]. IL-10 is also a key regulatory cytokine involved in the T cell immune response, and it is up-regulated in activated CD4+ T cells and acts to inhibit their proliferation [700-702]. In this way an

abundance of IL-10 maintains immune homeostasis by preventing excessive inflammation, hence IL-10 has an important role in T cell immunosuppression. In the current study, *IL-10* expression in naïve CD4+ T cells in response to activation showed an increase in all CD4+ T cell subtypes (Th0, Th1, Th17, Treg), although the expression of *IL-10* was further increased upon $1,25(OH)_2D3$ treatment in Th17 and Treg cell subtypes only (**Figure 6.3**). Previous studies also found increased gene expression and secretion of IL-10 in Treg cells after $1,25(OH)_2D3$ treatment [159, 703]. IL-10 is known to inhibit further CD4+ T cell proliferation [700, 704] by selective inhibition of the CD28 pathway [705], suggesting that Treg cell function is likely to be regulated by $1,25(OH)_2D3$-mediated cytokine secretion.

Similarly, in the current study CD4+ T cell activation led to an up-regulation in *IL-21* expression. There was also a trend for the down-regulation of *IL-21* expression in response to $1,25(OH)_2D3$, however unlike observations from previous studies [651, 706, 707] this was not statistically significant. Due to the well-established effects of $1,25(OH)_2D3$ on IL-21 secretion [159, 373], it is possible that a significant reduction in *IL-21* expression would have been observed with larger sample numbers. However, this observation also highlights the limited information gained from simply studying established T cell marker genes as targets for $1,25(OH)_2D3$. To address this, unpublished studies by other Birmingham group members have characterised genome-wide changes in mRNA expression in the same $1,25(OH)_2D3$-treated activated Th17 cell model described in this thesis. These transcriptomic data indicated that *IL-21* was not amongst the genes regulated by early or late $1,25(OH)_2D3$ treatment in this particular T cell model.

The current proposed mechanism for 1,25$(OH)_2$D3-mediated gene regulation in T cells is via 1,25$(OH)_2$D3 binding to VDR and translocation of the 1,25$(OH)_2$D3-VDR complex to target gene *VDRE* and regulation of gene expression. In the current study, *VDR* expression was not significantly up-regulated in response to 1,25$(OH)_2$D3 treatment in Tregs. Consistent with this, activated, but not resting T cells, express VDR [652], and this was confirmed in the current study by the approximate 50-fold higher levels of *VDR* mRNA observed for all different types of T cells following activation, with this response being unaffected by the addition of 1,25$(OH)_2$D3. An induction in *VDR* mRNA expression is not required to obtain a response to 1,25$(OH)_2$D3, since the VDR is already present.

The VDR has been demonstrated as essential for the regulation and control of IL-10 secretion by 1,25$(OH)_2$D3 [708], and in turn 1,25$(OH)_2$D3 has been shown to decrease *STAT3* phosphorylation in autoimmune models [709]. Interestingly, IL-10 acts primarily through the *STAT3* pathway to induce anti-inflammatory effects [710], however the *STAT3* pathway is also used by IL-6 which promotes a pro-inflammatory Th17 fate. The ability of IL-10 to mimic IL-6 signalling in the presence of excess IL-10 receptor suggests this signaling pathway is highly modulatory, and pro- versus anti-inflammatory outcomes are dependent on receptor expression rather than cytokine availability [711]. Hence 1,25$(OH)_2$D3 may act to reprogram T cells via controlling *STAT3* pathway activation. Future studies may explore the mechanism of 1,25$(OH)_2$D3 on components of the *STAT3* signalling pathway with regards to T cell function.

6.4.3 CD4+ CD25- T cells and cytokine production with regards to activation and $1,25(OH)_2D3$

Activation of naïve CD4+ T cells is associated with the increased production of pro-inflammatory cytokines including IFN-У, IL-17 and IL-21. It has been shown that naïve CD4+ T cells activated in the presence of $1,25(OH)_2D3$ are able to inhibit the production of these cytokines as well as up-regulate the expression of intracellular CTLA-4 and FoxP3 [159, 373], where CTLA-4 is later translocated to the cell surface to mediate immunosuppression. Flow cytometry data in the current study showed the up-regulated expression of intracellular CTLA-4 and FoxP3 in response to CD4+ T cell activation, and a further up-regulation in cell surface CTLA-4 expression in response to long term $1,25(OH)_2D3$ treatment (**Figure 6.5**). This result supports the current mechanism of CTLA-4 mediated immunoregulation, where CTLA-4 is thought to exert its actions once it has translocated to the cell surface where it directly competes with the CD28 ligand to attenuate signalling. $1,25(OH)_2D3$ is thought to then enhance the up-regulation of CTLA-4 to mediate further immunosuppression, underlining the immunoregulatory potential of $1,25(OH)_2D3$ in the adaptive immune system.

In naïve CD4+ CD25- T cells, the intracellular expression of CTLA-4 and FoxP3 was very low before stimulation, and the process of activation led to the up-regulation of both markers. Multiple other studies have found intracellular CTLA-4 to be constitutively expressed at low levels in resting T cells, and up-regulated in response to cytokine stimulation with maximal effects around 48-72 hours post-activation [649, 712, 713]. The current study did not measure CTLA-4 expression on the cell surface immediately following T cell activation due to expression being

undetectable in many previous studies [714-716], however one study found that naïve CD4+ T cells exhibited up-regulated CTLA-4 surface expression after just 2 hours post-stimulation [649], whereas previously only *CTLA-4* mRNA was found so soon after activation [717]. Perhaps the presence of CTLA-4 protein on the cell surface so early suggests a role for CTLA-4 in rapidly preventing inappropriate activation in the first instance, with later CTLA-4 expression helping to maintain this response [718].

Activation of CD4+ T cells has also been associated with the up-regulation of FoxP3 and CD25, both considered Treg cell markers [719]. In the current study, a proportion of activated CD4+ T cells would have been expected to express FoxP3 albeit at very low levels. The reason that FoxP3 expression was not observed as highly as expected following T cell stimulation may be due in part to the strength of the stimulation factors. The stimulation may not have been sufficiently strong through the CD28 receptor and therefore did not produce a sufficient amount of IL-2. IL-2 in the thymus promotes naïve T cell to Treg differentiation to prevent autoimmune response; in this way IL-2 enhances FoxP3 expression. Additionally, using anti-CD3 and anti-CD28 beads for stimulation may have been able to produce much stronger T cell stimulatory signals compared to anti-CD3/anti-CD28 plate-bound antibodies used in the current study, leading to more FoxP3 expression. Alternatively, culturing naïve T cells in a higher dose of IL-2 (200 IU/mL) would have produced more FoxP3 induction, and in theory could induce FoxP3 expression to high levels expressed by Tregs.

6.4.4 CD4+ CD25- T cell stimulation with Th17 cocktail is able to overcome inflammatory phenotype

The rationale for stimulating naïve CD4+ T cells with the Th17 inflammatory cytokines was to gain a further understanding of cytokine production and gene regulation during inflammation versus regulation. In the current study, the naïve CD4+ CD25- T cells were stimulated under pro-inflammatory conditions with Th17 cytokine cocktail consisting of IL-2, IL-1β, TGF-β, IL-6 and IL-23, to promote differentiation into a pro-inflammatory Th17 fate. Interestingly, it was found that under these inflammatory conditions the CD4+ regulatory phenotype was able to overcome the inflammatory Th17 phenotype, as shown by the strong induction of CTLA-4 expression and mild FoxP3 up-regulation, which are both associated with an immunosuppressive Treg phenotype (**Figure 6.5**). The induction of Treg phenotypes under pro-inflammatory conditions have also been reported in previous studies; CD4+ T cells stimulated with Th17 cocktail containing IL-2 in the presence of $1,25(OH)_2D3$ produced the highest levels of CTLA-4 and FoxP3 [651], as well as up-regulated IL-10 and suppression of inflammatory cytokines IL-17 and IFN-γ, underlining the potential for $1,25(OH)_2D3$ to suppress inflammation. Similarly, in the current study the addition of $1,25(OH)_2D3$ following T cell activation further enhanced the regulatory phenotype as shown by increased CTLA-4 and FoxP3 expression compared to activated vehicle T cells (**Figure 6.5C**). Results from the current study suggest that the Treg regulatory phenotype was 'strong' enough to overcome the Th17 polarising conditions. Since the dysregulation of Th17 lymphocytes are strongly implicated in the onset and pathogenesis of autoimmune disease like

rheumatoid arthritis [600, 720], this brings potential for 1,25(OH)$_2$D3-enhanced, Treg-mediated immunosuppression in health and disease.

6.4.5 1,25(OH)$_2$D3 exerts anti-inflammatory effects on Th17 cells over time

Previous studies found that 1,25(OH)$_2$D3 in T cell culture acts to regulate CD4+ differentiation and promote a Treg phenotype, as well as induce *CTLA-4* expression [721, 722]. In the current study, the expression of *CTLA-4* in activated T cells cultured under Th17 inflammatory conditions was significantly up-regulated in the presence of 1,25(OH)$_2$D3 compared to untreated T cells (**Figure 6.7A**). This difference was observed throughout the time course of 1,25(OH)$_2$D3 treatment, and was most apparent at 72 hours post-1,25(OH)$_2$D3.

Also in the current study, *IFN-γ* expression was most significantly down-regulated at the 72 hour post-1,25(OH)$_2$D3 time point in T cells (**Figure 6.7B**). This observation has been well established in previous studies showing that 1,25(OH)$_2$D3 down-regulates the production of *IFN-γ* in activated T cells [723-725], although no studies to date have looked at the 1,25(OH)$_2$D3-mediated regulation of *IFN-γ* over time. In the current study, the delayed down-regulation of *IFN-γ* after 1,25(OH)$_2$D3 treatment may be explained by the role of *IFN-γ* in mediating an early antimicrobial response. Following an immune challenge to T cells, the presence of 1,25(OH)$_2$D3 induces the release of IFN-γ, which up-regulates the production of antimicrobial cathelicidin to overcome pathogens [726]. This process occurs rapidly and only after the removal of pathogens does 1,25(OH)$_2$D3 mediate the down-regulation of *INF-γ* to prevent excess inflammatory responses. Another explanation for the delayed

response of IFN-γ to 1,25(OH)$_2$D3 may be to provide the T cells with ample time to establish VDR responses. Activated T cells express the CYP27B1 enzyme and are able to convert 25(OH)D3 in the local environment to 1,25(OH)$_2$D3 [721], hence they are also responsive to extracellular 1,25(OH)$_2$D3 responses via binding to the VDR. The current mechanism for 1,25(OH)$_2$D3-mediated *INF-γ* down-regulation involves the binding of 1,25(OH)$_2$D3 to VDR and *RXR* to inhibit production of the Th1 polarising cytokine IL-12 [727]. Normally IL-12 activates the *STAT4* signaling pathway for IFN-γ production. In this way VDR acts as a transcriptional modulator of the *IFN-γ* gene [728], with 1,25(OH)$_2$D3 able to control its expression and mediate inflammatory responses. These findings confirm the immunosuppressive and anti-inflammatory role of 1,25(OH)$_2$D3 in Th17 cells over time.

6.4.6 *CTLA-4* and *IFN-γ* expression is up-regulated by T cell activation

The constitutive expression of the immune suppressor protein CTLA-4 is required for the normal function of Tregs [650]. Following T cell activation, the majority of CTLA-4 molecules are transported to the cell surface via AP-2 mediated clathrin-coated pits and is thought to act at the immunological synapse for TCR engagement [729, 730]. In the current study, both *CTLA-4* and *IFN-γ* mRNA expression was up-regulated following T cell activation (**Figure 6.10**). Similarly, previous studies found that stimulation of naïve CD4+ T cells led to the up-regulation of inflammatory cytokines such as IFN-γ [731]. Previous studies also found that the expression of *CTLA-4* mRNA was detectable in naïve T cells following activation after just 1 hour [717], however surface CTLA-4 protein expression was not significantly

detectable until 48-72hrs post-activation [732], possibly to provide ample time for CTLA-4 localization and TCR engagement. This is necessary to prevent NF-AT translocation to the nucleus in order to inhibit transcription of the *IL-2* gene [733]. In this way CTLA-4 is thought to exert its immunosuppressive effects upon the immune response. Importantly, the presence of $1,25(OH)_2D3$ is able to enhance *CTLA-4* expression even under Th17 inflammatory conditions [373] (see **Figure 6.5**), suggesting a role for $1,25(OH)_2D3$-mediated regulation of anti-inflammatory gene expression in T cells. This effect was most significant after 72 hours of $1,25(OH)_2D3$. However, one limitation in the current study is the interpretation of CTLA-4 expression by flow cytometry on a whole population level. Single cell analysis is thought be a more sensitive detection method, particularly during early time points when surface expression of CTLA-4 is very low. Since only a small percentage of newly activated T cells express surface CTLA-4 expression [734], perhaps these are the T cells that form the most potent immune responses and are capable of modulation by $1,25(OH)_2D3$. Future studies could utilise analysis of CTLA-4 expression on newly activated T cells to study individual cell responses to activation, in order to identify such cells. Further improvements to the current study could involve quantifying the expression of AP-2 to study its relationship between intracellular and cell surface CTLA-4 expression and validating its role in CTLA-4 mediated T cell signal attenuation.

6.4.7 Candidate miRNAs miR-155 and miR-627 expression is down-regulated by 1,25(OH)$_2$D3

Intrinsic changes to gene expression are likely to be mediated by differential miRNA expression, as different miRNA expression profiles have been associated with different stages of CD4+ T cell development. Thus, any dysregulation of miRNAs that attenuate expression of target genes involved in T cell differentiation, proliferation and survival is likely to impact upon the normal function of these T cells at different time points. Taken together with changes to gene expression in response to T cell activation and 1,25(OH)$_2$D3 as shown earlier in this chapter, the aim of further studies in **Chapter 6** was to characterise the effects of 1,25(OH)$_2$D3 on T cell miRNA expression at different time points.

Candidate miRNA analyses of 1,25(OH)$_2$D3 effects on T cell miRNA expression showed that miR-155 and miR-627 were significantly down-regulated within 2 hours following 1,25(OH)$_2$D3 treatment (**Figure 6.9**). This down-regulation was not significant at later time points, and none of the other candidate miRNAs showed significant changes in response to 1,25(OH)$_2$D3 over time. Previous studies have implicated the role for miRNA-mediated gene regulation in controlling immune responses in T cells. Some studies have also suggested a role for 1,25(OH)$_2$D3 in further mediating epigenetic changes via the regulation of miRNA expression in T cells [98, 735, 736], sometimes in a negative feedback fashion such as the relationship between 1,25(OH)$_2$D3 and miR-155 [240]. These studies have shown that miRNAs can be up-regulated or down-regulated in response to 1,25(OH)$_2$D3, reflecting the complex regulatory roles of miRNAs in immune cells. In the present study we also expected to see an up-regulation of miR-627 expression following

1,25$(OH)_2$D3 in T cells, due to the proposed role of miR-627 to target *JMJD1A*, whose down-regulation leads to suppressed cell proliferation factors [737]. Indeed, the authors of that study found that 1,25$(OH)_2$D3 down-regulated miR-627 expression [737, 738] whereas the present study did not find this. However, studies on miR-627 are still relatively limited and its role is not fully understood, with contradicting studies reporting elevated levels of miR-627 in various disease compared to healthy controls, from cancer [739] to Parkinson's disease [740]. Thus it is likely that miR-627 targets other genes in addition to *JMJD1A*, and further studies are needed to elucidate the role of miR-627 in immune regulation and identify any other important gene targets. Perhaps future studies involving larger sample numbers will observe significant changes to this miRNA in response to 1,25$(OH)_2$D3.

6.4.8 MiR-212-3p and miR-16 is up-regulated by 1,25$(OH)_2$D3, and may be therapeutic targets in disease

Further unbiased array analysis of T cell miRNA expression identified more miRNAs were up-regulated in response to long term (72hr) 1,25$(OH)_2$D3 compared to short term (24hr) 1,25$(OH)_2$D3 treatment (**Figure 6.16; Figure 6.14**), with miR-212-3p being the only miRNA up-regulated by both long and short term 1,25$(OH)_2$D3. Interestingly, miR-212-3p which was significantly up-regulated by 1,25$(OH)_2$D3 was also significantly up-regulated by T cell activation (p=0.002). There are no studies to date on the regulation of this miRNA with respect to T cells and 1,25$(OH)_2$D3, however low miR-212-3p expression has been linked to increased risks of colorectal cancer [741] and rheumatoid arthritis [742], both diseases which are linked to excess pro-inflammatory cytokine release. The role of miR-212-3p in disease may be an

important area of research for future studies. Of the up-regulated miRNAs following short term 1,25$(OH)_2$D3 (24hrs), 6 of these miRNAs were also up-regulated in response to cytokine stimulation. These results suggest that select miRNAs are very responsive to modulation during CD4+ T cell activation, and 1,25$(OH)_2$D3 appeared to enhance this up-regulation. Of these miRNAs significantly up-regulated, it is likely that some may also be involved in the inflammatory process.

MiR-16 up-regulation following short term 1,25$(OH)_2$D3 was also observed in the present study. Previous studies have detailed the link between suppressed miR-16 expression and B cell lymphocytic leukemia [743, 744]. The importance of this miRNA in immune health is further validated by its role in inducing apoptosis by targeting *BCL2* [745, 746], to promote malignant B cell death. Thus it is possible that sufficient miR-16 expression also plays an important role in mediating T cell apoptosis, and the role of 1,25$(OH)_2$D3 in up-regulating its expression reinforces the immunomodulatory role of 1,25$(OH)_2$D3.

Another miRNA of interest is miR-146a. No published studies to date have found down-regulated miR-146a expression after 1,25$(OH)_2$D3 treatment specifically in CD4+ T cells, which the current study aimed to address. MiR-146a is an inflammation-associated miRNA consistently up-regulated following immune challenge [261, 747-749] and down-regulated following 1,25$(OH)_2$D3 treatment in disease states [238, 750]. Despite these promising studies, the current study did not observe a significant down-regulation of miR-146a in CD4+ T cells after 1,25$(OH)_2$D3 treatment, in the candidate studies or the unbiased array studies.

6.4.9 MiR-155 is induced by T cell activation but not by 1,25(OH)$_2$D3

As expected, activation of naïve CD4+ T cells induced the expression of miR-155 when profiled by both candidate qPCR and array qPCR (**Figure 6.11; Figure 6.12**), due to the established role of miR-155 in systemic inflammation [751-753]. Surprisingly, miR-155 expression was not significantly regulated following long term (72hr) or short term (24hr) 1,25(OH)$_2$D3 treatment. The work on DC described in **Chapter 5** along with previous literature [240, 754] found that in DC, miR-155 was down-regulated after 1,25(OH)$_2$D3, thus the same may be expected of miR-155 in T cells. There may be several explanations as to why the current study did not find miR-155 down-regulation with long term or short term 1,25(OH)$_2$D3 treatment. Whilst down-regulation of miR-155 expression after 1,25(OH)$_2$D3 was observed in candidate miRNA analysis (**Figure 6.11E**), that was only at a very early time point of 2 hours after 1,25(OH)$_2$D3 treatment. Therefore, perhaps 1,25(OH)$_2$D3-mediated regulation of miR-155 expression occurs very early in T cells, thus changes were not detected in the 'early' 24 hour array time point. The early regulation of miR-155 expression could be important for its mechanism of action; miR-155 normally targets *SOCS1* to mediate inflammation [240]. Perhaps rapid 1,25(OH)$_2$D3-induced down-regulation of miR-155 is needed to induce effects on *SOCS1* which in turn blocks the *JAK/STAT* signalling pathway of pro-inflammatory cytokines, leading to immediate anti-inflammatory responses. Secondly, it is plausible that 1,25(OH)$_2$D3 may not affect miR-155 expression depending on the cytokine milieu present during T cell differentiation. This theory has been suggested by a recent study which found 1,25(OH)$_2$D3 was able to suppress Th17 subtype differentiation with no effects on miRNA expression including miR-155 [755].

6.4.10 Gene ontology analysis reveals miRNAs with roles in immune regulation

Bioinformatics coupled with gene ontology analysis of miRNA gene targets and functions in T cells revealed the majority of significantly regulated miRNAs targeted genes with roles in cell proliferation and metabolism. MiR-155 targets many genes with new and established roles in immune regulation, such as *SOCS1, BCL6* and *PIK3R1* to name a few (see **Figure 6.22**). Some of these genes carry out more than one function, which makes it very difficult to identify a specific miRNA that targets each gene. These bioinformatics tools are helpful to identify miRNA gene targets and biological functions of these genes, as a first step for any future studies delineating the mechanistic actions of these miRNAs.

Previous unpublished transcriptomic data from other Birmingham group members showed genes significantly regulated by long term (72 hour) and short term (4 hour) $1,25(OH)_2D3$ treatment in CD4+ T cells polarised with the same T17 cocktail. There were 130 genes regulated by long term $1,25(OH)_2D3$, with *CTLA-4* amongst them. We compared these genes with those targeted by the miRNAs regulated by $1,25(OH)_2D3$ and found no common genes. The list of unpublished genes regulated by long term $1,25(OH)_2D3$ is shown in **Table 6.2**. We attempted to relate some of the anticipated transcriptome changes that may arise as a result of reported changes in miRNA expression with corresponding transcriptome changes within the RNAseq dataset. However limitations that complicate this potential approach is largely due to the broad array of genes targeted by each miRNA. As a result it is difficult to pinpoint one specific miRNA as the regulator of a single gene.

6.5 Summary

The studies reported in this chapter have firstly confirmed the CTLA-4 mediated immunoregulation in CD4+ T cells, demonstrated by the up-regulated expression of intracellular CTLA-4 and FoxP3 in response to T cell activation. $1,25(OH)_2D3$ further enhanced the up-regulation of CTLA-4, underlining the immunoregulatory potential of $1,25(OH)_2D3$ in the adaptive immune system. Naïve CD4+ CD25- T cells stimulated under Th17 polarising conditions were able to overcome the inflammatory Th17 phenotype in the presence of $1,25(OH)_2D3$, as shown by the strong induction of CTLA-4 in these cells. This suggested that the Treg regulatory phenotype was strong enough to exert its immunosuppressive effects on these T cells even under pro-inflammatory conditions. The expression of *CTLA-4* was further up-regulated after 72 hours incubation with $1,25(OH)_2D3$ compared to untreated T cells, with a simultaneous down-regulation of *IFN-γ* expression. The above results demonstrate that $1,25(OH)_2D3$ is able to exert immunosuppressive actions upon CD4+ T cells polarised under Th17 conditions.

It was proposed that miRNAs could be mediating these changes due to their established regulatory roles in literature. Results showed that miR-155 was up-regulated following T cell activation but was not significantly down-regulated following $1,25(OH)_2D3$ treatment, most likely due to the rapid actions of miR-155 that occurred too early to detect in the arrays. Interestingly, long term $1,25(OH)_2D3$, short term $1,25(OH)_2D3$ and T cell activation all up-regulated miR-212-3p expression. When interpreted with gene ontology analysis identifying gene targets of miR-212-3p with roles in cell proliferation and metabolism, it is likely that this miRNA has important

regulatory roles in and beyond the immune system. Similarly, targets of miR-155 included genes with new and established roles in the immune response. Taken together, miR-212-3p and miR-155 provide a useful starting point to identify other miRNAs with key regulatory roles in the adaptive immune system.

CHAPTER 7. SUMMARY AND FUTURE DIRECTIONS

Vitamin D deficiency has been implicated in a wide range of diseases from cancer [74, 756] to autoimmune conditions [96, 757], although whether vitamin D deficiency is causal or simply associated with these diseases is still unclear. What is clear is that vitamin D exerts potent anti-inflammatory actions upon cells including those in the innate and adaptive immune system such as dendritic cells (DC) and T cells [758]. This is thought to occur via epigenetic regulation by modulating the expression of miRNAs in these cells, leading to altered gene expression and downstream effects. Vitamin D has thus been proposed to be a modulator of miRNA expression. This thesis aimed to address the relationship between vitamin D and miRNA expression in cells of the human innate and adaptive immune system, from both a healthy and inflammatory disease perspective.

The principle aims of this thesis were to firstly investigate the relationship between vitamin D metabolites and miRNAs with respect to RA disease activity, as discussed in **Chapter 4**. Current analyses of vitamin D status relies on measuring inactive serum 25(O)D3 levels, thus another aim of this study was to identify if other vitamin D metabolites including 1,25$(OH)_2$D3 could be used as a better indicator of RA disease status. Interestingly, the actual levels of 1,25$(OH)_2$D3 generated locally within the SF appeared to be very low or undetectable. This suggests that any 1,25$(OH)_2$D3 generated within the synovial microenvironment may be restricted to the cells involved in immunoregulation within this tissue. Additionally, the current study showed up-regulated expression of the inflammation-associated miRNAs miR-146a and miR-155 in RA SF compared to serum, however since there was a lack of correlation between miRNA expression and RA disease markers in serum or SF, it is unlikely that these miRNAs are actively mediating gene silencing to cause

inflammation within the local environment; it is possible they are maintained as a reserve of miRNAs not associated with their target gene [478]. Hence the role of miR-146a and miR-155 in the pathogenesis of RA remains unclear, although future work on the contribution of miRNAs to various aspects of RA pathogenesis is required and holds great potential. There is scope for future studies to include the metabolite analysis of healthy control SF, in order to better establish whether the low $1,25(OH)_2D3$ observed in SF compared to serum is a cause or simply a result of disease aetiology. We did have access to limited SF from a healthy cohort of pregnant women, however due to the complex hormonal changes that occur in pregnancy we decided that this may interfere with $1,25(OH)_2D3$ readings. Hence any future recruitment of healthy controls should contain a mix of genders and ages. Overall, the role of vitamin D metabolites, especially active $1,25(OH)_2D3$, in the synovium of RA patients is still far from clear and the link between $1,25(OH)_2D3$ and miRNAs was further investigated in the rest of the thesis.

Studies in **Chapter 5** and **Chapter 6** investigated the role of miRNAs as mediators of the immunomodulatory effects of $1,25(OH)_2D3$ on the innate and adaptive immune system, respectively. The work undertaken in these chapters of the thesis have provided detailed insights into the role of $1,25(OH)_2D3$ on miRNA expression in monocyte-derived dendritic cells (DC) of the innate immune system and CD4+ T cells of the adaptive immune system, by profiling the expression of 372 miRNAs most closely related to inflammation and the regulation of their expression with regard to vitamin D treatment. The expression of many genes is altered during these changes, and it remains likely that these changes are facilitated by the differential expression of a host of miRNAs.

In DC, analysis of these data identified 8 miRNAs that were significantly down-regulated by long term (72hr) vitamin D treatment, and 14 miRNAs down-regulated by short term (24hr) vitamin D treatment. DC maturation by LPS saw the up-regulation of miR-155-5p and down-regulation of miR-221-5p. The mechanism of miRNA regulation with regard to DC differentiation and maturation remains unclear, but there is strong evidence to suggest that select miRNAs including miR-155 play key regulatory roles in the homeostasis and normal function of the innate immune system. Overall the array profiling data showed a global down-regulation of miRNAs following vitamin D treatment. Down-regulation of these genes were not rescued by short term or long term $1,25(OH)_2D3$, suggesting the miRNA biogenesis machinery was stable and any immunomodulatory effects of $1,25(OH)_2D3$ are likely to act on mature miRNAs only. The immunomodulatory effects of $1,25(OH)_2D3$ in DC bring about different phenotypical and functional changes depending on the stage of DC development, length of treatment time and the cytokine milieu. Future studies may involve quantifying expression of pre-miRNAs and pri-miRNAs in DC, in conjunction with miRNA biogenesis gene expression analysis and $1,25(OH)_2D3$ treatment, to elucidate the mechanism of $1,25(OH)_2D3$ on miRNA regulation. In addition it may be prudent to validate the gene targets of any miRNAs that were significantly regulated by $1,25(OH)_2D3$. This can be achieved by miRNA over-expression or knock-down studies in the DC phenotypes, which will induce or silence target gene expression. Using further qPCR or western blotting to validate gene and protein expression will allow us to identify any genes specifically targeted by these miRNAs, and the effect that $1,25(OH)_2D3$ may have in modulating their expression.

In T cells, studies have firstly confirmed CTLA-4 mediated immunoregulation in CD4+ T cells, which was further enhanced by 1,25$(OH)_2$D3, demonstrating the immunosuppressive role of 1,25$(OH)_2$D3 in the adaptive immune system. Furthermore, naïve T cells stimulated under Th17 polarising conditions were able to overcome the pro-inflammatory Th17 phenotype, suggesting that the Treg regulatory phenotype was strong enough to exert its immunosuppressive effects despite inflammatory conditions. Similar to DC, it was proposed that miRNAs could be mediating these changes. Results showed that miR-155 was up-regulated following T cell activation but was not significantly down-regulated following 1,25$(OH)_2$D3 treatment, most likely due to the rapid actions of miR-155 that occurred too early for detection. Interestingly, long term 1,25$(OH)_2$D3, short term 1,25$(OH)_2$D3 and T cell activation all up-regulated miR-212-3p expression. When interpreted with gene ontology analyses identifying gene targets of miR-212-3p with roles in cell proliferation and metabolism, it is likely that this miRNA has important regulatory roles in and beyond the immune system. Similarly, targets of miR-155 included genes with new and established roles in the immune response. Importantly, T cells treated with vitamin D did not display global down-regulation of miRNAs as previously observed with DC, suggesting this miRNA down-regulation was not likely due to mechanisms such as decreased miRNA synthesis and was likely to be mediated by 1,25$(OH)_2$D3. Taken together, miR-212-3p and miR-155 provide a useful starting point to identify other miRNAs with key regulatory roles in the adaptive immune system. Similar to **Chapter 5**, future studies should include further validation of miRNA gene targets, and how 1,25$(OH)_2$D3 may modulate their expression.

In summary, the work in this thesis has demonstrated that vitamin D has a broad range of genetic and epigenetic effects on cells of the immune system. In RA, other metabolites aside from 25(OH)D may be better markers of disease activity. In healthy immune cells, miRNAs appear as likely targets for the immunomodulatory actions of $1,25(OH)_2D3$, with effects on both the innate and adaptive immune system. Bioinformatics tools have identified predicted targets of significantly modulated miRNAs, some of which have roles in immune function. Despite the current data depicting an association between $1,25(OH)_2D3$ exposure and changes in miRNA expression associated with immune-function related gene targets, further studies are needed to provide definitive proof that the immunomodulatory effects of $1,25(OH)_2D3$ are truly mediated through these miRNAs. To do so would require additional experimental studies, for example using miRNA over-expression to silence the gene of interest to study the effect this has on immune cell function, phenotype and response to $1,25(OH)_2D3$.

Nonetheless, the focus of this thesis was to provide a theoretical overview of the complex roles of miRNAs in immune modulation, and how $1,25(OH)_2D3$ further modulates their expression. Data arising from this thesis has provided strong indications that the immunomodulatory effects of $1,25(OH)_2D3$ are mediated by miRNAs, as shown by significant miRNA expression changes in DC of the innate immune system and T cells of the adaptive immune system in response to $1,25(OH)_2D3$. Future studies may also focus on elucidating the mechanism of $1,25(OH)_2D3$-mediated miRNA modulation in other immune cells, to further understand the overarching role of miRNAs in health and disease.

CHAPTER 8. REFERENCES

1. Steenbock, H., *THE INDUCTION OF GROWTH PROMOTING AND CALCIFYING PROPERTIES IN A RATION BY EXPOSURE TO LIGHT.* Science, 1924. **60**(1549): p. 224-5.
2. DeLuca, H.F., *Overview of general physiologic features and functions of vitamin D.* Am J Clin Nutr, 2004. **80**(6 Suppl): p. 1689S-96S.
3. Webb, A.R., L. Kline, and M.F. Holick, *Influence of season and latitude on the cutaneous synthesis of vitamin D3: exposure to winter sunlight in Boston and Edmonton will not promote vitamin D3 synthesis in human skin.* J Clin Endocrinol Metab, 1988. **67**(2): p. 373-8.
4. Askew, F.A., et al., *The Distillation of Vitamin D.* Proceedings of the Royal Society. Series B., 1930. **107**: p. 76-90.
5. Wolf, G., *The Discovery of Vitamin D: The Contribution of Adolf Windaus.* The Journal of Nutrition, 2004. **134**(6): p. 1299-1302.
6. Omdahl, J.L., H.A. Morris, and B.K. May, *Hydroxylase enzymes of the vitamin D pathway: expression, function, and regulation.* Annu Rev Nutr, 2002. **22**: p. 139-66.
7. Cheng, J.B., et al., *Genetic evidence that the human CYP2R1 enzyme is a key vitamin D 25-hydroxylase.* Proc Natl Acad Sci U S A, 2004. **101**(20): p. 7711-5.
8. Zhu, J.G., et al., *CYP2R1 is a major, but not exclusive, contributor to 25-hydroxyvitamin D production in vivo.* Proc Natl Acad Sci U S A, 2013. **110**(39): p. 15650-5.
9. Jones, K.S., et al., *25(OH)D2 half-life is shorter than 25(OH)D3 half-life and is influenced by DBP concentration and genotype.* J Clin Endocrinol Metab, 2014. **99**(9): p. 3373-81.
10. Nykjaer, A., et al., *An endocytic pathway essential for renal uptake and activation of the steroid 25-(OH) vitamin D3.* Cell, 1999. **96**(4): p. 507-15.
11. Hewison, M., et al., *1alpha-Hydroxylase and the action of vitamin D.* J Mol Endocrinol, 2000. **25**(2): p. 141-8.
12. Miller, W.L., *Genetic disorders of Vitamin D biosynthesis and degradation.* J Steroid Biochem Mol Biol, 2016.
13. Haussler, M.R., et al., *Vitamin D receptor: molecular signaling and actions of nutritional ligands in disease prevention.* Nutr Rev, 2008. **66**(10 Suppl 2): p. S98-112.
14. Kishimoto, M., et al., *Nuclear receptor mediated gene regulation through chromatin remodeling and histone modifications.* Endocr J, 2006. **53**(2): p. 157-72.
15. Pike, J.W., M.B. Meyer, and K.A. Bishop, *Regulation of target gene expression by the vitamin D receptor - an update on mechanisms.* Rev Endocr Metab Disord, 2012. **13**(1): p. 45-55.
16. Carlberg, C. and M.J. Campbell, *Vitamin D receptor signaling mechanisms: Integrated actions of a well-defined transcription factor.* Steroids, 2013. **78**(2): p. 127-36.
17. Yamaoka, K., et al., *Multiple co-activator complexes support ligand-induced transactivation function of VDR.* Arch Biochem Biophys, 2007. **460**(2): p. 166-71.
18. Sanchez-Martinez, R., et al., *Vitamin D-dependent recruitment of corepressors to vitamin D/retinoid X receptor heterodimers.* Mol Cell Biol, 2008. **28**(11): p. 3817-29.
19. Malloy, P.J., et al., *Vitamin D receptor mutations in patients with hereditary 1,25-dihydroxyvitamin D-resistant rickets.* Mol Genet Metab, 2014. **111**(1): p. 33-40.
20. Bikle, D.D., *Vitamin D Metabolism, Mechanism of Action, and Clinical Applications.* Chem Biol, 2014. **21**(3): p. 319-329.
21. Haussler, M.R., et al., *The role of vitamin D in the FGF23, klotho, and phosphate bone-kidney endocrine axis.* Rev Endocr Metab Disord, 2012. **13**(1): p. 57-69.
22. Prosser, D.E. and G. Jones, *Enzymes involved in the activation and inactivation of vitamin D.* Trends Biochem Sci, 2004. **29**(12): p. 664-73.

23. Schlingmann, K.P., et al., *Mutations in CYP24A1 and idiopathic infantile hypercalcemia.* N Engl J Med, 2011. **365**(5): p. 410-21.
24. Bergwitz, C. and H. Juppner, *Regulation of phosphate homeostasis by PTH, vitamin D, and FGF23.* Annu Rev Med, 2010. **61**: p. 91-104.
25. Hodsman, A.B., et al., *Parathyroid hormone and teriparatide for the treatment of osteoporosis: a review of the evidence and suggested guidelines for its use.* Endocr Rev, 2005. **26**(5): p. 688-703.
26. Kemper, B., et al., *Parathyroid secretion: discovery of a major calcium-dependent protein.* Science, 1974. **184**(4133): p. 167-9.
27. Moallem, E., et al., *RNA-Protein binding and post-transcriptional regulation of parathyroid hormone gene expression by calcium and phosphate.* J Biol Chem, 1998. **273**(9): p. 5253-9.
28. Breslau, N.A., *Normal and abnormal regulation of 1,25-(OH)2D synthesis.* Am J Med Sci, 1988. **296**(6): p. 417-25.
29. Wilz, D.R., et al., *Plasma 1,25-(OH)2-vitamin D concentrations and net intestinal calcium, phosphate, and magnesium absorption in humans.* Am J Clin Nutr, 1979. **32**(10): p. 2052-60.
30. Heaney, R.P., et al., *Calcium absorption varies within the reference range for serum 25-hydroxyvitamin D.* J Am Coll Nutr, 2003. **22**(2): p. 142-6.
31. Murer, H., I. Forster, and J. Biber, *The sodium phosphate cotransporter family SLC34.* Pflugers Arch, 2004. **447**(5): p. 763-7.
32. Dastani, Z., et al., *In healthy adults, biological activity of vitamin D, as assessed by serum PTH, is largely independent of DBP concentrations.* J Bone Miner Res, 2013.
33. Ramagopalan, S.V., et al., *A ChIP-seq defined genome-wide map of vitamin D receptor binding: associations with disease and evolution.* Genome research, 2010. **20**(10): p. 1352-1360.
34. Seuter, S., A. Neme, and C. Carlberg, *Characterization of Genomic Vitamin D Receptor Binding Sites through Chromatin Looping and Opening.* PLOS ONE, 2014. **9**(4): p. e96184.
35. cui, m., et al., *Genome-wide identification of vitamin D receptor (VDR) binding sites in DNA from prostate epithelial cells (PEC) by chromatin immunoprecipitation-sequencing (ChIP-seq).* The FASEB Journal, 2010. **24**(1_supplement): p. 212.8-212.8.
36. Carlberg, C., *Genome-wide (over)view on the actions of vitamin D.* Frontiers in Physiology, 2014. **5**(167).
37. Singh, P.K., et al., *Integration of VDR genome wide binding and GWAS genetic variation data reveals co-occurrence of VDR and NF-κB binding that is linked to immune phenotypes.* BMC Genomics, 2017. **18**(1): p. 132.
38. Tsuprykov, O., et al., *Why should we measure free 25(OH) vitamin D?* J Steroid Biochem Mol Biol, 2018. **180**: p. 87-104.
39. Lundgren, S., et al., *Tissue distribution of human gp330/megalin, a putative Ca(2+)-sensing protein.* J Histochem Cytochem, 1997. **45**(3): p. 383-92.
40. Chun, R.F., et al., *Vitamin D and DBP: the free hormone hypothesis revisited.* J Steroid Biochem Mol Biol, 2014. **144 Pt A**: p. 132-7.
41. Chun, R.F., et al., *Vitamin D-binding protein directs monocyte responses to 25-hydroxy- and 1,25-dihydroxyvitamin D.* J Clin Endocrinol Metab, 2010. **95**(7): p. 3368-76.
42. Shieh, A., et al., *Effects of High-Dose Vitamin D2 Versus D3 on Total and Free 25-Hydroxyvitamin D and Markers of Calcium Balance.* J Clin Endocrinol Metab, 2016. **101**(8): p. 3070-8.
43. Srikanth, P., et al., *Associations of total and free 25OHD and 1,25(OH)2D with serum markers of inflammation in older men.* Osteoporos Int, 2016. **27**(7): p. 2291-300.

44. Olerod, G., et al., *The variation in free 25-hydroxy vitamin D and vitamin D-binding protein with season and vitamin D status.* Endocr Connect, 2017. **6**(2): p. 111-120.
45. Moller, U.K., et al., *Increased plasma concentrations of vitamin D metabolites and vitamin D binding protein in women using hormonal contraceptives: a cross-sectional study.* Nutrients, 2013. **5**(9): p. 3470-80.
46. Powe, C.E., et al., *Vitamin D-binding protein and vitamin D status of black Americans and white Americans.* N Engl J Med, 2013. **369**(21): p. 1991-2000.
47. Nielson, C.M., et al., *Free 25-hydroxyvitamin D: impact of vitamin D binding protein assays on racial-genotypic associations.* J Clin Endocrinol Metab, 2016: p. jc20161104.
48. Arnaud, J. and J. Constans, *Affinity differences for vitamin D metabolites associated with the genetic isoforms of the human serum carrier protein (DBP).* Hum Genet, 1993. **92**(2): p. 183-8.
49. Bikle, D.D., et al., *Free 1,25-dihydroxyvitamin D levels in serum from normal subjects, pregnant subjects, and subjects with liver disease.* J Clin Invest, 1984. **74**(6): p. 1966-71.
50. Bouillon, R., et al., *Influence of the vitamin D-binding protein on the serum concentration of 1,25-dihydroxyvitamin D3. Significance of the free 1,25-dihydroxyvitamin D3 concentration.* J Clin Invest, 1981. **67**(3): p. 589-96.
51. Bikle, D.D., et al., *Assessment of the free fraction of 25-hydroxyvitamin D in serum and its regulation by albumin and the vitamin D-binding protein.* J Clin Endocrinol Metab, 1986. **63**(4): p. 954-9.
52. Mendel, C.M., *The free hormone hypothesis: a physiologically based mathematical model.* Endocr Rev, 1989. **10**(3): p. 232-74.
53. Bhan, I., et al., *Bioavailable vitamin D is more tightly linked to mineral metabolism than total vitamin D in incident hemodialysis patients.* Kidney Int, 2012. **82**(1): p. 84-9.
54. Houghton, L.A. and R. Vieth, *The case against ergocalciferol (vitamin D2) as a vitamin supplement.* Am J Clin Nutr, 2006. **84**(4): p. 694-7.
55. Hollis, B.W., *Comparison of equilibrium and disequilibrium assay conditions for ergocalciferol, cholecalciferol and their major metabolites.* J Steroid Biochem, 1984. **21**(1): p. 81-6.
56. Ross, A.C., et al., *The 2011 report on dietary reference intakes for calcium and vitamin D from the Institute of Medicine: what clinicians need to know.* J Clin Endocrinol Metab, 2011. **96**(1): p. 53-8.
57. Holick, M.F., et al., *Evaluation, treatment, and prevention of vitamin D deficiency: an Endocrine Society clinical practice guideline.* J Clin Endocrinol Metab, 2011. **96**(7): p. 1911-30.
58. (SACN), T.S.A.C.o.N., *SACN vitamin D and health report.* Public Health England, 2016.
59. Holick, M.F., *VITAMIN D STATUS: MEASUREMENT, INTERPRETATION AND CLINICAL APPLICATION.* Annals of epidemiology, 2009. **19**(2): p. 73-78.
60. Ringe, J.D. and C. Kipshoven, *Vitamin D-insufficiency: An estimate of the situation in Germany.* Dermato-endocrinology, 2012. **4**(1): p. 72-80.
61. Autier, P., et al., *Vitamin D status and ill health: a systematic review.* The Lancet Diabetes & Endocrinology, 2014. **2**(1): p. 76-89.
62. Bell, T.D., M.B. Demay, and S.-A.M. Burnett-Bowie, *The biology and pathology of vitamin D control in bone.* Journal of Cellular Biochemistry, 2010. **111**(1): p. 7-13.
63. Lisse, T.S., et al., *Vitamin D activation of functionally distinct regulatory miRNAs in primary human osteoblasts.* J Bone Miner Res, 2013. **28**(6): p. 1478-88.
64. Christakos, S., et al., *Vitamin D: beyond bone.* Ann N Y Acad Sci, 2013. **1287**(1): p. 45-58.
65. Feldman, D., X.Y. Zhao, and A.V. Krishnan, *Vitamin D and prostate cancer.* Endocrinology, 2000. **141**(1): p. 5-9.

66. Holick, M.F., *Noncalcemic actions of 1,25-dihydroxyvitamin D3 and clinical applications.* Bone, 1995. **17**(2 Suppl): p. 107S-111S.
67. Garland, C.F., F.C. Garland, and E.D. Gorham, *Can colon cancer incidence and death rates be reduced with calcium and vitamin D?* Am J Clin Nutr, 1991. **54**(1 Suppl): p. 193s-201s.
68. Hanchette, C.L. and G.G. Schwartz, *Geographic patterns of prostate cancer mortality. Evidence for a protective effect of ultraviolet radiation.* Cancer, 1992. **70**(12): p. 2861-9.
69. Azzeh, F.S. and O.A. Kensara, *Vitamin D Is a Good Marker for Disease Activity of Rheumatoid Arthritis Disease.* Disease Markers, 2015. **2015**: p. 6.
70. Baykal, T., et al., *Is there an association between serum 25-hydroxyvitamin D concentrations and disease activity in rheumatoid arthritis?* Bratisl Lek Listy, 2012. **113**(10): p. 610-1.
71. Braun-Moscovici, Y., et al., *Vitamin D level: is it related to disease activity in inflammatory joint disease?* Rheumatol Int, 2011. **31**(4): p. 493-9.
72. Cen, X., et al., *Association between Serum 25-Hydroxyvitamin D Level and Rheumatoid Arthritis.* BioMed Research International, 2015. **2015**: p. 913804.
73. Craig, S.M., et al., *Vitamin D status and its associations with disease activity and severity in African Americans with recent-onset rheumatoid arthritis.* J Rheumatol, 2010. **37**(2): p. 275-81.
74. Ahonen, M.H., et al., *Prostate cancer risk and prediagnostic serum 25-hydroxyvitamin D levels (Finland).* Cancer Causes Control, 2000. **11**(9): p. 847-52.
75. Pilz, S., et al., *Association of vitamin D deficiency with heart failure and sudden cardiac death in a large cross-sectional study of patients referred for coronary angiography.* J Clin Endocrinol Metab, 2008. **93**(10): p. 3927-35.
76. Giovannucci, E., et al., *25-hydroxyvitamin D and risk of myocardial infarction in men: a prospective study.* Arch Intern Med, 2008. **168**(11): p. 1174-80.
77. Fiscella, K. and P. Franks, *Vitamin D, race, and cardiovascular mortality: findings from a national US sample.* Ann Fam Med, 2010. **8**(1): p. 11-8.
78. Mozos, I. and O. Marginean, *Links between Vitamin D Deficiency and Cardiovascular Diseases.* BioMed Research International, 2015. **2015**: p. 12.
79. Al Mheid, I., et al., *Vitamin D and cardiovascular disease: is the evidence solid?* European Heart Journal, 2013. **34**(48): p. 3691-3698.
80. Shuler, F.D., et al., *Sports health benefits of vitamin d.* Sports Health, 2012. **4**(6): p. 496-501.
81. Prabhala, A., R. Garg, and P. Dandona, *Severe myopathy associated with vitamin D deficiency in western New York.* Arch Intern Med, 2000. **160**(8): p. 1199-203.
82. Rasheed, K., P. Sethi, and E. Bixby, *Severe vitamin d deficiency induced myopathy associated with rhabydomyolysis.* North American journal of medical sciences, 2013. **5**(5): p. 334-336.
83. Littlejohns, T.J., et al., *Vitamin D and the risk of dementia and Alzheimer disease.* Neurology, 2014. **83**(10): p. 920-928.
84. Nissou, M.F., et al., *Additional clues for a protective role of vitamin D in neurodegenerative diseases: 1,25-dihydroxyvitamin D3 triggers an anti-inflammatory response in brain pericytes.* J Alzheimers Dis, 2014. **42**(3): p. 789-99.
85. Shen, L. and H.-F. Ji, *Vitamin D deficiency is associated with increased risk of Alzheimer's disease and dementia: evidence from meta-analysis.* Nutrition Journal, 2015. **14**(1): p. 76.
86. Ramos-Lopez, E., et al., *CYP2R1 (vitamin D 25-hydroxylase) gene is associated with susceptibility to type 1 diabetes and vitamin D levels in Germans.* Diabetes Metab Res Rev, 2007. **23**(8): p. 631-6.

87. Ongagna, J.C., M. Pinget, and A. Belcourt, *Vitamin D-binding protein gene polymorphism association with IA-2 autoantibodies in type 1 diabetes.* Clin Biochem, 2005. **38**(5): p. 415-9.
88. Nguyen, M., et al., *Vitamin D-resistant rickets and type 1 diabetes in a child with compound heterozygous mutations of the vitamin D receptor (L263R and R391S): dissociated responses of the CYP-24 and rel-B promoters to 1,25-dihydroxyvitamin D3.* J Bone Miner Res, 2006. **21**(6): p. 886-94.
89. Lopez, E.R., et al., *A promoter polymorphism of the CYP27B1 gene is associated with Addison's disease, Hashimoto's thyroiditis, Graves' disease and type 1 diabetes mellitus in Germans.* Eur J Endocrinol, 2004. **151**(2): p. 193-7.
90. Motohashi, Y., et al., *Vitamin D receptor gene polymorphism affects onset pattern of type 1 diabetes.* J Clin Endocrinol Metab, 2003. **88**(7): p. 3137-40.
91. Correale, J., M.C. Ysrraelit, and M.I. Gaitan, *Immunomodulatory effects of Vitamin D in multiple sclerosis.* Brain, 2009. **132**(Pt 5): p. 1146-60.
92. Skalli, A., et al., *Association of vitamin D status with multiple sclerosis in a case-control study from Morocco.* Revue Neurologique, 2018. **174**(3): p. 150-156.
93. Smolders, J., et al., *Vitamin D status is positively correlated with regulatory T cell function in patients with multiple sclerosis.* PLoS One, 2009. **4**(8): p. e6635.
94. Fitzgerald, K.C., et al., *Association of Vitamin D Levels With Multiple Sclerosis Activity and Progression in Patients Receiving Interferon Beta-1b.* JAMA Neurol, 2015: p. 1-8.
95. Harroud, A. and J.B. Richards, *Mendelian randomization in multiple sclerosis: A causal role for vitamin D and obesity?* Multiple Sclerosis, 2018. **24**(1): p. 80-85.
96. Abou-Raya, A., S. Abou-Raya, and M. Helmii, *The effect of vitamin D supplementation on inflammatory and hemostatic markers and disease activity in patients with systemic lupus erythematosus: a randomized placebo-controlled trial.* J Rheumatol, 2013. **40**(3): p. 265-72.
97. Banchereau, J. and V. Pascual, *Type I interferon in systemic lupus erythematosus and other autoimmune diseases.* Immunity, 2006. **25**(3): p. 383-92.
98. Chen, D.-J., et al., *Altered microRNAs expression in T cells of patients with SLE involved in the lack of vitamin D.* Oncotarget, 2017. **8**(37): p. 62099-62110.
99. Mulligan, M.L., et al., *Implications of vitamin D deficiency in pregnancy and lactation.* Am J Obstet Gynecol, 2010. **202**(5): p. 429.e1-9.
100. Fischer, D., et al., *Metabolism of vitamin D3 in the placental tissue of normal and preeclampsia complicated pregnancies and premature births.* Clin Exp Obstet Gynecol, 2007. **34**(2): p. 80-4.
101. Aghajafari, F., et al., *Association between maternal serum 25-hydroxyvitamin D level and pregnancy and neonatal outcomes: systematic review and meta-analysis of observational studies.* BMJ, 2013. **346**.
102. Bodnar, L.M., et al., *Maternal vitamin D deficiency increases the risk of preeclampsia.* J Clin Endocrinol Metab, 2007.
103. Bodnar, L.M., et al., *Maternal vitamin D deficiency increases the risk of preeclampsia.* J Clin Endocrinol Metab, 2007. **92**(9): p. 3517-22.
104. Cecchetti, S., et al., *Prevalence of vitamin D deficiency in rheumatoid arthritis and association with disease activity and cardiovascular risk factors: data from the COMEDRA study.* Clin Exp Rheumatol, 2016. **34**(6): p. 984-990.
105. Abreu, M.T., et al., *Measurement of vitamin D levels in inflammatory bowel disease patients reveals a subset of Crohn's disease patients with elevated 1,25-dihydroxyvitamin D and low bone mineral density.* Gut, 2004. **53**(8): p. 1129-36.
106. Froicu, M., et al., *A crucial role for the vitamin D receptor in experimental inflammatory bowel diseases.* Mol Endocrinol, 2003. **17**(12): p. 2386-92.
107. Gorham, E.D., et al., *Vitamin D and prevention of colorectal cancer.* J Steroid Biochem Mol Biol, 2005. **97**(1-2): p. 179-94.

108. Tajika, M., et al., *Risk factors for vitamin D deficiency in patients with Crohn's disease.* J Gastroenterol, 2004. **39**(6): p. 527-33.
109. Simmons, J.D., et al., *Vitamin D receptor gene polymorphism: association with Crohn's disease susceptibility.* Gut, 2000. **47**(2): p. 211-4.
110. Wagner, C.L., et al., *Vitamin D and Its Role During Pregnancy in Attaining Optimal Health of Mother and Fetus.* Nutrients, 2012. **4**(3): p. 208.
111. Kumar, R., et al., *Elevated 1,25-dihydroxyvitamin D plasma levels in normal human pregnancy and lactation.* J Clin Invest, 1979. **63**(2): p. 342-4.
112. Kovacs, C.S. and H.M. Kronenberg, *Maternal-fetal calcium and bone metabolism during pregnancy, puerperium, and lactation.* Endocr Rev, 1997. **18**(6): p. 832-72.
113. Rosjo, E., et al., *Vitamin D supplementation and systemic inflammation in relapsing-remitting multiple sclerosis.* J Neurol, 2015. **262**(12): p. 2713-21.
114. Sidbury, R., et al., *Randomized controlled trial of vitamin D supplementation for winter-related atopic dermatitis in Boston: a pilot study.* Br J Dermatol, 2008. **159**(1): p. 245-7.
115. Mitri, J., et al., *Effects of vitamin D and calcium supplementation on pancreatic beta cell function, insulin sensitivity, and glycemia in adults at high risk of diabetes: the Calcium and Vitamin D for Diabetes Mellitus (CaDDM) randomized controlled trial.* Am J Clin Nutr, 2011. **94**(2): p. 486-94.
116. Harris, S.S., A.G. Pittas, and N.J. Palermo, *A randomized, placebo-controlled trial of vitamin D supplementation to improve glycaemia in overweight and obese African Americans.* Diabetes Obes Metab, 2012. **14**(9): p. 789-94.
117. Muris, A.H., et al., *Immune regulatory effects of high dose vitamin D3 supplementation in a randomized controlled trial in relapsing remitting multiple sclerosis patients receiving IFNbeta; the SOLARIUM study.* J Neuroimmunol, 2016. **300**: p. 47-56.
118. Wejse, C., et al., *Vitamin D as supplementary treatment for tuberculosis: a double-blind, randomized, placebo-controlled trial.* Am J Respir Crit Care Med, 2009. **179**(9): p. 843-50.
119. Brekke, H.K. and J. Ludvigsson, *Vitamin D supplementation and diabetes-related autoimmunity in the ABIS study.* Pediatr Diabetes, 2007. **8**(1): p. 11-4.
120. Biancuzzo, R.M., et al., *Serum concentrations of 1,25-dihydroxyvitamin D2 and 1,25-dihydroxyvitamin D3 in response to vitamin D2 and vitamin D3 supplementation.* The Journal of clinical endocrinology and metabolism, 2013. **98**(3): p. 973-979.
121. Bhargava, P., et al., *Metabolic alterations in multiple sclerosis and the impact of vitamin D supplementation.* JCI Insight, 2017. **2**(19).
122. Franco, A.S., et al., *Vitamin D supplementation and disease activity in patients with immune-mediated rheumatic diseases.* Medicine, 2017. **96**(23): p. e7024-e7024.
123. Schleithoff, S.S., et al., *Vitamin D supplementation improves cytokine profiles in patients with congestive heart failure: a double-blind, randomized, placebo-controlled trial.* Am J Clin Nutr, 2006. **83**(4): p. 754-9.
124. *Vitamin D supplement in early childhood and risk for Type I (insulin-dependent) diabetes mellitus. The EURODIAB Substudy 2 Study Group.* Diabetologia, 1999. **42**(1): p. 51-4.
125. Holick, M.F., *High prevalence of vitamin D inadequacy and implications for health.* Mayo Clin Proc, 2006. **81**(3): p. 353-73.
126. Vieth, R., *The role of vitamin D in the prevention of osteoporosis.* Ann Med, 2005. **37**(4): p. 278-85.
127. Vieth, R., *Why the optimal requirement for Vitamin D3 is probably much higher than what is officially recommended for adults.* J Steroid Biochem Mol Biol, 2004. **89-90**(1-5): p. 575-9.
128. Jones, G., *Pharmacokinetics of vitamin D toxicity.* Am J Clin Nutr, 2008. **88**(2): p. 582s-586s.

129. Garland, C.F., et al., *Vitamin D supplement doses and serum 25-hydroxyvitamin D in the range associated with cancer prevention.* Anticancer Res. **31**(2): p. 607-11.
130. Haug, C., et al., *Subnormal serum concentration of 1,25-vitamin D in human immunodeficiency virus infection: correlation with degree of immune deficiency and survival.* J Infect Dis, 1994. **169**(4): p. 889-93.
131. Anderson, P.H., et al., *Determinants of circulating 1,25-dihydroxyvitamin D3 levels: the role of renal synthesis and catabolism of vitamin D.* J Steroid Biochem Mol Biol, 2004. **89-90**(1-5): p. 111-3.
132. Baughman, R.P., et al., *Clinical characteristics of patients in a case control study of sarcoidosis.* Am J Respir Crit Care Med, 2001. **164**(10 Pt 1): p. 1885-9.
133. Hollis, B.W., *Assessment and Interpretation of Circulating 25-Hydroxyvitamin D and 1,25-Dihydroxyvitamin D in the Clinical Environment.* Endocrinology and metabolism clinics of North America, 2010. **39**(2): p. 271-contents.
134. Horst, R.L. and B.W. Hollis, *Vitamin D Assays and Their Clinical Utility*, in *Vitamin D: Molecular Biology, Physiology, and Clinical Applications*, M.F. Holick, Editor. 1999, Humana Press: Totowa, NJ. p. 239-271.
135. Hollis, B.W., *Editorial: The determination of circulating 25-hydroxyvitamin D: no easy task.* J Clin Endocrinol Metab, 2004. **89**(7): p. 3149-51.
136. Jones, G., *Assay of vitamins D2 and D3, and 25-hydroxyvitamins D2 and D3 in human plasma by high-performance liquid chromatography.* Clin Chem, 1978. **24**(2): p. 287-98.
137. Guo, T., et al., *Simultaneous determination of 12 steroids by isotope dilution liquid chromatography-photospray ionization tandem mass spectrometry.* Clin Chim Acta, 2006. **372**(1-2): p. 76-82.
138. Enko, D., et al., *Method evaluation study of a new generation of vitamin D assays.* Biochemia Medica, 2015. **25**(2): p. 203-212.
139. Jenkinson, C., et al., *High throughput LC-MS/MS method for the simultaneous analysis of multiple vitamin D analytes in serum HHS Public Access.* J Chromatogr B Analyt Technol Biomed Life Sci, 2016. **1014**: p. 56-63.
140. Jenkinson, C., et al., *Analysis of multiple vitamin D metabolites by ultra-performance supercritical fluid chromatography-tandem mass spectrometry (UPSFC-MS/MS).* Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences, 2018. **1087-1088**(March): p. 43-48.
141. Adams, J.S., et al., *Metabolism of 25-hydroxyvitamin D3 by cultured pulmonary alveolar macrophages in sarcoidosis.* J Clin Invest, 1983. **72**(5): p. 1856-60.
142. Adams, J.S. and M.A. Gacad, *Characterization of 1 alpha-hydroxylation of vitamin D3 sterols by cultured alveolar macrophages from patients with sarcoidosis.* J Exp Med, 1985. **161**(4): p. 755-65.
143. DeLuca, H.F. and V.K. Ostrem, *Analogs of the hormonal form of vitamin D and their possible use in leukemia.* Prog Clin Biol Res, 1988. **259**: p. 41-55.
144. Bouillon, R., W.H. Okamura, and A.W. Norman, *Structure-function relationships in the vitamin D endocrine system.* Endocr Rev, 1995. **16**(2): p. 200-57.
145. Liu, P.T., et al., *Toll-like receptor triggering of a vitamin D-mediated human antimicrobial response.* Science, 2006. **311**(5768): p. 1770-3.
146. Hewison, M., et al., *Vitamin D and barrier function: a novel role for extra-renal 1 alpha-hydroxylase.* Mol Cell Endocrinol, 2004. **215**(1-2): p. 31-8.
147. Hewison, M., et al., *Extra-renal 25-hydroxyvitamin D3-1alpha-hydroxylase in human health and disease.* J Steroid Biochem Mol Biol, 2007. **103**(3-5): p. 316-21.
148. Stoffels, K., et al., *Immune regulation of 1alpha-hydroxylase in murine peritoneal macrophages: unravelling the IFNgamma pathway.* J Steroid Biochem Mol Biol, 2007. **103**(3-5): p. 567-71.

149. Esteban, L., M. Vidal, and A. Dusso, *1alpha-Hydroxylase transactivation by gamma-interferon in murine macrophages requires enhanced C/EBPbeta expression and activation.* J Steroid Biochem Mol Biol, 2004. **89-90**(1-5): p. 131-7.
150. Stoffels, K., et al., *Immune regulation of 1alpha-hydroxylase in murine peritoneal macrophages: unravelling the IFNgamma pathway.* The Journal of steroid biochemistry and molecular biology, 2007. **103**(3-5): p. 567-571.
151. Wang, T.T., et al., *Cutting edge: 1,25-dihydroxyvitamin D3 is a direct inducer of antimicrobial peptide gene expression.* J Immunol, 2004. **173**(5): p. 2909-12.
152. Sato, K. and S. Fujita, *Dendritic cells: nature and classification.* Allergol Int, 2007. **56**(3): p. 183-91.
153. Smyth, L.A., et al., *MicroRNAs affect dendritic cell function and phenotype.* Immunology, 2015. **144**(2): p. 197-205.
154. Penna, G. and L. Adorini, *1 Alpha,25-dihydroxyvitamin D3 inhibits differentiation, maturation, activation, and survival of dendritic cells leading to impaired alloreactive T cell activation.* J Immunol, 2000. **164**(5): p. 2405-11.
155. van Halteren, A.G., et al., *1alpha,25-dihydroxyvitamin D3 or analogue treated dendritic cells modulate human autoreactive T cells via the selective induction of apoptosis.* J Autoimmun, 2004. **23**(3): p. 233-9.
156. Pedersen, A.W., et al., *Phenotypic and functional markers for 1α,25-dihydroxyvitamin D3-modified regulatory dendritic cells.* Clinical & Experimental Immunology, 2009. **157**(1): p. 48-59.
157. Griffin, M.D., et al., *Dendritic cell modulation by 1alpha,25 dihydroxyvitamin D3 and its analogs: a vitamin D receptor-dependent pathway that promotes a persistent state of immaturity in vitro and in vivo.* Proc Natl Acad Sci U S A, 2001. **98**(12): p. 6800-5.
158. Piemonti, L., et al., *Vitamin D3 affects differentiation, maturation, and function of human monocyte-derived dendritic cells.* J Immunol, 2000. **164**(9): p. 4443-51.
159. Cantorna, M.T., et al., *Vitamin D and 1,25(OH)(2)D Regulation of T cells.* Nutrients, 2015. **7**(4): p. 3011-3021.
160. Szeles, L., et al., *1,25-dihydroxyvitamin D3 is an autonomous regulator of the transcriptional changes leading to a tolerogenic dendritic cell phenotype.* J Immunol, 2009. **182**(4): p. 2074-83.
161. Willheim, M., et al., *Regulatory effects of 1alpha,25-dihydroxyvitamin D3 on the cytokine production of human peripheral blood lymphocytes.* The Journal of clinical endocrinology and metabolism, 1999. **84**(10): p. 3739-3744.
162. Bartels, L.E., et al., *1,25-dihydroxyvitamin D3 and dexamethasone increase interleukin-10 production in CD4+ T cells from patients with Crohn's disease.* International immunopharmacology, 2007. **7**(13): p. 1755-1764.
163. Penna, G. and L. Adorini, *1 ,25-Dihydroxyvitamin D3 Inhibits Differentiation, Maturation, Activation, and Survival of Dendritic Cells Leading to Impaired Alloreactive T Cell Activation.* The Journal of Immunology, 2000. **164**(5): p. 2405-2411.
164. Takahashi, K., et al., *Human neutrophils express messenger RNA of vitamin D receptor and respond to 1alpha,25-dihydroxyvitamin D3.* Immunopharmacol Immunotoxicol, 2002. **24**(3): p. 335-47.
165. Tan, B.H., et al., *Macrophages acquire neutrophil granules for antimicrobial activity against intracellular pathogens.* J Immunol, 2006. **177**(3): p. 1864-71.
166. Taschner, S., et al., *Down-regulation of RXRα expression is essential for neutrophil development from granulocyte/monocyte progenitors.* Blood, 2007. **109**(3): p. 971-979.
167. Subramanian, K., P. Bergman, and B. Henriques-Normark, *Vitamin D Promotes Pneumococcal Killing and Modulates Inflammatory Responses in Primary Human Neutrophils.* J Innate Immun, 2017. **9**(4): p. 375-386.

168. Chen, L., M.S. Eapen, and G.R. Zosky, *Vitamin D both facilitates and attenuates the cellular response to lipopolysaccharide.* Scientific Reports, 2017. **7**: p. 45172.
169. Hirsch, D., et al., *Decreased anti-inflammatory responses to vitamin D in neonatal neutrophils.* Mediators of inflammation, 2011. **2011**: p. 598345-598345.
170. Metcalfe, D.D., et al., *Biomarkers of the involvement of mast cells, basophils and eosinophils in asthma and allergic diseases.* World Allergy Organization Journal, 2016. **9**(1): p. 7.
171. Souto Filho, J.T.D., et al., *Impact of vitamin D deficiency on increased blood eosinophil counts.* Hematol Oncol Stem Cell Ther, 2018. **11**(1): p. 25-29.
172. Wu, H.-Y., et al., *Serum 25-hydroxyvitamin D inversely associated with blood eosinophils in patients with persistent allergic rhinitis.* Asia Pacific allergy, 2017. **7**(4): p. 213-220.
173. Souto Filho, J.T.D., et al., *Impact of vitamin D deficiency on increased blood eosinophil counts.* Hematology/Oncology and Stem Cell Therapy, 2018. **11**(1): p. 25-29.
174. Gorman, S., et al., *Reversible control by vitamin D of granulocytes and bacteria in the lungs of mice: an ovalbumin-induced model of allergic airway disease.* PloS one, 2013. **8**(6): p. e67823-e67823.
175. Hiraguchi, Y., et al., *1,25-Dihydroxyvitamin D3 upregulates functional C-x-C chemokine receptor type 4 expression in human eosinophils.* Int Arch Allergy Immunol, 2012. **158 Suppl 1**: p. 51-7.
176. Wittke, A., et al., *Vitamin D receptor-deficient mice fail to develop experimental allergic asthma.* J Immunol, 2004. **173**(5): p. 3432-6.
177. Carlos, D., et al., *TLR2-dependent mast cell activation contributes to the control of Mycobacterium tuberculosis infection.* Microbes Infect, 2009. **11**(8-9): p. 770-8.
178. Baker, B., et al., *The role of 1a,25-dihydroxyvitamin D₃ in IgE-mediated mast cell activation (HYP4P.313).* The Journal of Immunology, 2015. **194**(1 Supplement): p. 123.12.
179. Yip, K.-H., et al., *Mechanisms of vitamin D_3 metabolite repression of IgE-dependent mast cell activation.* The Journal of allergy and clinical immunology, 2014. **133**(5): p. 1356-136514.
180. Liu, Z.Q., et al., *Vitamin D contributes to mast cell stabilization.* Allergy, 2017. **72**(8): p. 1184-1192.
181. Biggs, L., et al., *Evidence that vitamin D₃ promotes mast cell–dependent reduction of chronic UVB-induced skin pathology in mice.* The Journal of Experimental Medicine, 2010. **207**(3): p. 455.
182. Yang, S., et al., *Vitamin D deficiency suppresses cell-mediated immunity in vivo.* Arch Biochem Biophys, 1993. **303**(1): p. 98-106.
183. Speiser, D.E., et al., *Unmodified self antigen triggers human CD8 T cells with stronger tumor reactivity than altered antigen.* Proceedings of the National Academy of Sciences, 2008. **105**(10): p. 3849.
184. Ahmadpoor, P., et al., *Effect of active vitamin D on expression of co-stimulatory molecules and HLA-DR in renal transplant recipients.* Exp Clin Transplant, 2009. **7**(2): p. 99-103.
185. Mattner, F., et al., *Inhibition of Th1 development and treatment of chronic-relapsing experimental allergic encephalomyelitis by a non-hypercalcemic analogue of 1,25-dihydroxyvitamin D(3).* Eur J Immunol, 2000. **30**(2): p. 498-508.
186. Boonstra, A., et al., *1alpha,25-Dihydroxyvitamin d3 has a direct effect on naive CD4(+) T cells to enhance the development of Th2 cells.* J Immunol, 2001. **167**(9): p. 4974-80.
187. Konijeti, G.G., et al., *Vitamin D Supplementation Modulates T Cell-Mediated Immunity in Humans: Results from a Randomized Control Trial.* J Clin Endocrinol Metab, 2016. **101**(2): p. 533-8.

188. Cantorna, T.M., et al., *Vitamin D and 1,25(OH)2D Regulation of T cells.* Nutrients, 2015. **7**(4).
189. Muller, K., et al., *The role of monocytes and T cells in 1,25-dihydroxyvitamin D3 mediated inhibition of B cell function in vitro.* Immunopharmacology, 1991. **21**(2): p. 121-8.
190. Heine, G., et al., *1alpha,25-dihydroxyvitamin D3 inhibits anti-CD40 plus IL-4-mediated IgE production in vitro.* Eur J Immunol, 2002. **32**(12): p. 3395-404.
191. Lemire, J.M., et al., *1 alpha,25-dihydroxyvitamin D3 suppresses proliferation and immunoglobulin production by normal human peripheral blood mononuclear cells.* J Clin Invest, 1984. **74**(2): p. 657-61.
192. Chen, S., et al., *Modulatory effects of 1,25-dihydroxyvitamin d3 on human B cell differentiation.* J Immunol, 2007. **179**(3): p. 1634-47.
193. Heine, G., et al., *1,25-dihydroxyvitamin D3 promotes IL-10 production in human B cells.* European Journal of Immunology, 2008. **38**(8): p. 2210-2218.
194. Chen, S., et al., *Modulatory Effects of 1,25-Dihydroxyvitamin D₃ on Human B Cell Differentiation.* The Journal of Immunology, 2007. **179**(3): p. 1634.
195. Lee, R.C., R.L. Feinbaum, and V. Ambros, *The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14.* Cell, 1993. **75**(5): p. 843-54.
196. Lee, R.C. and V. Ambros, *An extensive class of small RNAs in Caenorhabditis elegans.* Science, 2001. **294**(5543): p. 862-4.
197. Mohri, T., et al., *MicroRNA regulates human vitamin D receptor.* Int J Cancer, 2009. **125**(6): p. 1328-33.
198. Wang, X., et al., *MicroRNAs181 regulate the expression of p27Kip1 in human myeloid leukemia cells induced to differentiate by 1,25-dihydroxyvitamin D3.* Cell Cycle, 2009. **8**(5): p. 736-41.
199. Krol, J., I. Loedige, and W. Filipowicz, *The widespread regulation of microRNA biogenesis, function and decay.* Nat Rev Genet, 2010. **11**(9): p. 597-610.
200. Raisch, J., A. Darfeuille-Michaud, and H.T.T. Nguyen, *Role of microRNAs in the immune system, inflammation and cancer.* World Journal of Gastroenterology : WJG, 2013. **19**(20): p. 2985-2996.
201. He, L. and G.J. Hannon, *MicroRNAs: small RNAs with a big role in gene regulation.* Nat Rev Genet, 2004. **5**(7): p. 522-31.
202. Friedman, R.C., et al., *Most mammalian mRNAs are conserved targets of microRNAs.* Genome Res, 2009. **19**(1): p. 92-105.
203. Doench, J.G. and P.A. Sharp, *Specificity of microRNA target selection in translational repression.* Genes Dev, 2004. **18**(5): p. 504-11.
204. Lee, Y., et al., *MicroRNA maturation: stepwise processing and subcellular localization.* Embo j, 2002. **21**(17): p. 4663-70.
205. Lund, E., et al., *Nuclear export of microRNA precursors.* Science, 2004. **303**(5654): p. 95-8.
206. Sheng, P., et al., *Dicer cleaves 5'-extended microRNA precursors originating from RNA polymerase II transcription start sites.* Nucleic Acids Research, 2018. **46**(11): p. 5737-5752.
207. Chendrimada, T.P., et al., *TRBP recruits the Dicer complex to Ago2 for microRNA processing and gene silencing.* Nature, 2005. **436**(7051): p. 740-4.
208. Kim, V.N., J. Han, and M.C. Siomi, *Biogenesis of small RNAs in animals.* Nat Rev Mol Cell Biol, 2009. **10**(2): p. 126-39.
209. Winter, J., et al., *Many roads to maturity: microRNA biogenesis pathways and their regulation.* Nature Cell Biology, 2009. **11**: p. 228.

210. Behm-Ansmant, I., et al., *mRNA degradation by miRNAs and GW182 requires both CCR4:NOT deadenylase and DCP1:DCP2 decapping complexes.* Genes & Development, 2006. **20**(14): p. 1885-1898.
211. Wu, L., J. Fan, and J.G. Belasco, *MicroRNAs direct rapid deadenylation of mRNA.* Proc Natl Acad Sci U S A, 2006. **103**(11): p. 4034-9.
212. Valencia-Sanchez, M.A., et al., *Control of translation and mRNA degradation by miRNAs and siRNAs.* Genes Dev, 2006. **20**(5): p. 515-24.
213. Lewis, B.P., C.B. Burge, and D.P. Bartel, *Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets*, in *Cell*. 2005: United States. p. 15-20.
214. Rodriguez, A., et al., *Identification of Mammalian microRNA Host Genes and Transcription Units.* Genome Research, 2004. **14**(10a): p. 1902-1910.
215. Adams, J.S., et al., *Substrate and enzyme trafficking as a means of regulating 1,25-dihydroxyvitamin D synthesis and action: the human innate immune response.* J Bone Miner Res, 2007. **22 Suppl 2**: p. V20-4.
216. Adams, J.S. and M. Hewison, *Extrarenal expression of the 25-hydroxyvitamin D-1-hydroxylase.* Arch Biochem Biophys, 2012. **523**(1): p. 95-102.
217. Adams, J.S. and M. Hewison, *Unexpected actions of vitamin D: new perspectives on the regulation of innate and adaptive immunity.* Nature Clinical Practice Endocrinology and Metabolism, 2008.
218. Bacchetta, J., et al., *Antibacterial responses by peritoneal macrophages are enhanced following vitamin d supplementation.* PLoS One, 2014. **9**(12): p. e116530.
219. Bacchetta, J., et al., *Fibroblast growth factor 23 inhibits extrarenal synthesis of 1,25-dihydroxyvitamin D in human monocytes.* J Bone Miner Res, 2013. **28**(1): p. 46-55.
220. Chun, R.F., et al., *Vitamin D-Binding Protein Directs Monocyte Responses to 25-Hydroxy- and 1,25-Dihydroxyvitamin D.* J Clin Endocrinol Metab.
221. Ayuso, T., et al., *Vitamin D receptor gene is epigenetically altered and transcriptionally up-regulated in multiple sclerosis.* PLOS ONE, 2017. **12**(3): p. e0174726.
222. Marik, R., et al., *DNA methylation-related vitamin D receptor insensitivity in breast cancer.* Cancer Biol Ther, 2010. **10**(1): p. 44-53.
223. Ramnath, N., et al., *Epigenetic regulation of vitamin D metabolism in human lung adenocarcinoma.* Journal of thoracic oncology : official publication of the International Association for the Study of Lung Cancer, 2014. **9**(4): p. 473-482.
224. Luo, W., et al., *Epigenetic regulation of vitamin D 24-hydroxylase/CYP24A1 in human prostate cancer.* Cancer Res, 2010. **70**(14): p. 5953-62.
225. Diesel, B., et al., *Vitamin D(3) metabolism in human glioblastoma multiforme: functionality of CYP27B1 splice variants, metabolism of calcidiol, and effect of calcitriol.* Clin Cancer Res, 2005. **11**(15): p. 5370-80.
226. Johnson, C.S., I. Chung, and D.L. Trump, *Epigenetic silencing of CYP24 in the tumor microenvironment.* J Steroid Biochem Mol Biol, 2010. **121**(1-2): p. 338-42.
227. Kim, M.S., et al., *1alpha,25(OH)2D3-induced DNA methylation suppresses the human CYP27B1 gene.* Mol Cell Endocrinol, 2007. **265-266**: p. 168-73.
228. Karlic, H. and F. Varga, *Impact of vitamin D metabolism on clinical epigenetics.* Clin Epigenetics, 2011. **2**(1): p. 55-61.
229. Borkowski, R., et al., *Genetic mutation of p53 and suppression of the miR-17 approximately 92 cluster are synthetic lethal in non-small cell lung cancer due to upregulation of vitamin D Signaling.* Cancer Res, 2015. **75**(4): p. 666-75.
230. Tsai, H.-P., et al., *Differential microRNA expression in breast cancer with different onset age.* PLOS ONE, 2018. **13**(1): p. e0191195.
231. Shi, D.-L., et al., *MicroRNA-27a Inhibits Cell Migration and Invasion of Fibroblast-Like Synoviocytes by Targeting Follistatin-Like Protein 1 in Rheumatoid Arthritis.* Molecules and cells, 2016. **39**(8): p. 611-618.

232. Hong, B.K., et al., *MicroRNA-143 and -145 modulate the phenotype of synovial fibroblasts in rheumatoid arthritis.* Exp Mol Med, 2017. **49**(8): p. e363.
233. Mizuno, Y., et al., *miR-125b inhibits osteoblastic differentiation by down-regulation of cell proliferation.* Biochem Biophys Res Commun, 2008. **368**(2): p. 267-72.
234. Cho, H.H., et al., *NF-kappaB activation stimulates osteogenic differentiation of mesenchymal stem cells derived from human adipose tissue by increasing TAZ expression.* J Cell Physiol, 2010. **223**(1): p. 168-77.
235. Liu, P.T., et al., *MicroRNA-21 targets the vitamin D-dependent antimicrobial pathway in leprosy.* Nat Med, 2012. **18**(2): p. 267-73.
236. Lutherborrow, M., et al., *Expression profiling of cytogenetically normal acute myeloid leukemia identifies microRNAs that target genes involved in monocytic differentiation.* Am J Hematol, 2011. **86**(1): p. 2-11.
237. Chen, Y., et al., *MicroRNA-346 mediates tumor necrosis factor alpha-induced downregulation of gut epithelial vitamin D receptor in inflammatory bowel diseases.* Inflamm Bowel Dis, 2014. **20**(11): p. 1910-8.
238. Alvarez-Diaz, S., et al., *MicroRNA-22 is induced by vitamin D and contributes to its antiproliferative, antimigratory and gene regulatory effects in colon cancer cells.* Hum Mol Genet, 2012. **21**(10): p. 2157-65.
239. Pedersen, A.W., et al., *Phenotypic and functional markers for 1alpha,25-dihydroxyvitamin D(3)-modified regulatory dendritic cells.* Clin Exp Immunol, 2009. **157**(1): p. 48-59.
240. Chen, Y., et al., *1,25-Dihydroxyvitamin D promotes negative feedback regulation of TLR signaling via targeting microRNA-155-SOCS1 in macrophages.* J Immunol, 2013. **190**(7): p. 3687-95.
241. Pedersen, A.W., et al., *Phenotypic and functional markers for 1alpha,25-dihydroxyvitamin D(3)-modified regulatory dendritic cells.* Clinical and experimental immunology, 2009. **157**(1): p. 48-59.
242. Sebastiani, G., et al., *Increased expression of microRNA miR-326 in type 1 diabetic patients with ongoing islet autoimmunity.* Diabetes Metab Res Rev, 2011. **27**(8): p. 862-6.
243. Kutmon, M., et al., *Integrative network-based analysis of mRNA and microRNA expression in 1,25-dihydroxyvitamin D3-treated cancer cells.* Genes Nutr, 2015. **10**(5): p. 35.
244. Ting, H.-J., et al., *Identification of microRNA-98 as a therapeutic target inhibiting prostate cancer growth and a biomarker induced by vitamin D.* The Journal of biological chemistry, 2013. **288**(1): p. 1-9.
245. Li, F., et al., *1alpha,25-Dihydroxyvitamin D3 prevents the differentiation of human lung fibroblasts via microRNA-27b targeting the vitamin D receptor.* Int J Mol Med, 2015. **36**(4): p. 967-74.
246. Guan, H., et al., *1,25-Dihydroxyvitamin D3 up-regulates expression of hsa-let-7a-2 through the interaction of VDR/VDRE in human lung cancer A549 cells.* Gene, 2013. **522**(2): p. 142-6.
247. Kasiappan, R., et al., *1,25-Dihydroxyvitamin D3 suppresses telomerase expression and human cancer growth through microRNA-498.* J Biol Chem, 2012. **287**(49): p. 41297-309.
248. Kasiappan, R., et al., *Vitamin D suppresses leptin stimulation of cancer growth through microRNA.* Cancer research, 2014. **74**(21): p. 6194-6204.
249. Tili, E., et al., *Modulation of miR-155 and miR-125b levels following lipopolysaccharide/TNF-alpha stimulation and their possible roles in regulating the response to endotoxin shock.* J Immunol, 2007. **179**(8): p. 5082-9.
250. Komagata, S., et al., *Human CYP24 catalyzing the inactivation of calcitriol is post-transcriptionally regulated by miR-125b.* Mol Pharmacol, 2009. **76**(4): p. 702-9.

251. Gonzalez-Duarte, R.J., et al., *Calcitriol increases Dicer expression and modifies the microRNAs signature in SiHa cervical cancer cells.* Biochem Cell Biol, 2015. **93**(4): p. 376-84.
252. Wang, L., et al., *Loss of miR-125b contributes to upregulation of CYP24 in uraemic rats.* Nephrology (Carlton), 2016. **21**(12): p. 1063-1068.
253. Ma, Y., et al., *1α,25(OH)2D3 differentially regulates miRNA expression in human bladder cancer cells.* The Journal of steroid biochemistry and molecular biology, 2015. **148**: p. 166-171.
254. Enquobahrie, D.A., et al., *Global maternal early pregnancy peripheral blood mRNA and miRNA expression profiles according to plasma 25-hydroxyvitamin D concentrations.* The journal of maternal-fetal & neonatal medicine : the official journal of the European Association of Perinatal Medicine, the Federation of Asia and Oceania Perinatal Societies, the International Society of Perinatal Obstetricians, 2011. **24**(8): p. 1002-1012.
255. Lutherborrow, M., et al., *Expression profiling of cytogenetically normal acute myeloid leukemia identifies MicroRNAs that target genes involved in monocytic differentiation.* American Journal of Hematology, 2010. **86**(1): p. 2-11.
256. Zitman-Gal, T., et al., *Vitamin D manipulates miR-181c, miR-20b and miR-15a in human umbilical vein endothelial cells exposed to a diabetic-like environment.* Cardiovasc Diabetol, 2014. **13**: p. 8.
257. Min, D., et al., *Downregulation of miR-302c and miR-520c by 1,25(OH)2D3 treatment enhances the susceptibility of tumour cells to natural killer cell-mediated cytotoxicity.* Br J Cancer, 2013. **109**(3): p. 723-30.
258. Essa, S., et al., *Signature of VDR miRNAs and epigenetic modulation of vitamin D signaling in melanoma cell lines.* Anticancer Res, 2012. **32**(1): p. 383-9.
259. Wickramasinghe, N.S., et al., *Estradiol downregulates miR-21 expression and increases miR-21 target gene expression in MCF-7 breast cancer cells.* Nucleic Acids Res, 2009. **37**(8): p. 2584-95.
260. Ekstrom, L., et al., *miRNA-27b levels are associated with CYP3A activity in vitro and in vivo.* Pharmacol Res Perspect, 2015. **3**(6): p. e00192.
261. Karkeni, E., et al., *Vitamin D limits inflammation-linked microRNA expression in adipocytes in vitro and in vivo: A new mechanism for the regulation of inflammation by vitamin D.* Epigenetics, 2018. **13**(2): p. 156-162.
262. Arboleda, J.F., G.J. Fernandez, and S. Urcuqui-Inchima, *Vitamin D-mediated attenuation of miR-155 in human macrophages infected to dengue virus: Implications for the cytokine response.* Infection, Genetics and Evolution, 2019.
263. Chen, Y., et al., *1,25-Dihydroxyvitamin D Promotes Negative Feedback Regulation of TLR Signaling via Targeting MicroRNA-155–SOCS1 in Macrophages.* The Journal of Immunology, 2013. **190**(7): p. 3687.
264. Enquobahrie, D.A., et al., *Global maternal early pregnancy peripheral blood mRNA and miRNA expression profiles according to plasma 25-hydroxyvitamin D concentrations.* J Matern Fetal Neonatal Med, 2011. **24**(8): p. 1002-12.
265. Molnár, F., et al., *1α,25(OH)2-3-Epi-Vitamin D3, a Natural Physiological Metabolite of Vitamin D3: Its Synthesis, Biological Activity and Crystal Structure with Its Receptor.* PLOS ONE, 2011. **6**(3): p. e18124.
266. Jenkinson, C., et al., *High throughput LC-MS/MS method for the simultaneous analysis of multiple vitamin D analytes in serum.* J Chromatogr B Analyt Technol Biomed Life Sci, 2016. **1014**: p. 56-63.
267. Tamblyn, J.A., et al., *Dysregulation of maternal and placental vitamin D metabolism in preeclampsia.* Placenta, 2017. **50**: p. 70-77.
268. Jenkinson, C., et al., *High throughput LC–MS/MS method for the simultaneous analysis of multiple vitamin D analytes in serum.* Journal of Chromatography B, 2016. **1014**: p. 56-63.

269. Kaufmann, M., et al., *Clinical utility of simultaneous quantitation of 25-hydroxyvitamin D and 24,25-dihydroxyvitamin D by LC-MS/MS involving derivatization with DMEQ-TAD.* J Clin Endocrinol Metab, 2014. **99**(7): p. 2567-74.
270. Chailurkit, L., W. Aekplakorn, and B. Ongphiphadhanakul, *Serum C3 epimer of 25-hydroxyvitamin D and its determinants in adults: a national health examination survey in Thais.* Osteoporos Int, 2015. **26**(9): p. 2339-44.
271. Muller, M.J. and D.A. Volmer, *Mass spectrometric profiling of vitamin D metabolites beyond 25-hydroxyvitamin D.* Clin Chem, 2015. **61**(8): p. 1033-48.
272. Wagner, D., et al., *The ratio of serum 24,25-dihydroxyvitamin D(3) to 25-hydroxyvitamin D(3) is predictive of 25-hydroxyvitamin D(3) response to vitamin D(3) supplementation.* J Steroid Biochem Mol Biol, 2011. **126**(3-5): p. 72-7.
273. Chun, R.F., et al., *Vitamin D Binding Protein and Monocyte Response to 25-Hydroxyvitamin D and 1,25-Dihydroxyvitamin D: Analysis by Mathematical Modeling.* PLoS ONE, 2012. **7**(1): p. e30773.
274. Munoz Garcia, A., et al., *Pathway analysis of transcriptomic data shows immunometabolic effects of vitamin D.* J Mol Endocrinol, 2018. **60**(2): p. 95-108.
275. Smoot, M.E., et al., *Cytoscape 2.8: new features for data integration and network visualization.* Bioinformatics, 2011. **27**(3): p. 431-2.
276. Bindea, G., et al., *ClueGO: a Cytoscape plug-in to decipher functionally grouped gene ontology and pathway annotation networks.* Bioinformatics, 2009. **25**(8): p. 1091-3.
277. Shannon, P., et al., *Cytoscape: a software environment for integrated models of biomolecular interaction networks.* Genome Res, 2003. **13**(11): p. 2498-504.
278. Li, D., et al., *Serum and synovial fluid vitamin D metabolites and rheumatoid arthritis.* The Journal of Steroid Biochemistry and Molecular Biology, 2019.
279. Sharp, J.T., et al., *The progression of erosion and joint space narrowing scores in rheumatoid arthritis during the first twenty-five years of disease.* Arthritis Rheum, 1991. **34**(6): p. 660-8.
280. Baumgartner, S.W., et al., *Etanercept (Enbrel) in patients with rheumatoid arthritis with recent onset versus established disease: improvement in disability.* J Rheumatol, 2004. **31**(8): p. 1532-7.
281. Wiles, N., et al., *Estimating the incidence of rheumatoid arthritis: trying to hit a moving target?* Arthritis Rheum, 1999. **42**(7): p. 1339-46.
282. Klareskog, L., et al., *A new model for an etiology of rheumatoid arthritis: smoking may trigger HLA-DR (shared epitope)-restricted immune reactions to autoantigens modified by citrullination.* Arthritis Rheum, 2006. **54**(1): p. 38-46.
283. Gregori, S., et al., *Regulatory T cells induced by 1 alpha,25-dihydroxyvitamin D3 and mycophenolate mofetil treatment mediate transplantation tolerance.* J Immunol, 2001. **167**(4): p. 1945-53.
284. Branimir, A. and M. Miroslav, *[Pathogenesis of rheumatoid arthritis].* Reumatizam, 2014. **61**(2): p. 19-23.
285. Philippe, L., et al., *TLR2 expression is regulated by microRNA miR-19 in rheumatoid fibroblast-like synoviocytes.* J Immunol, 2012. **188**(1): p. 454-61.
286. Karouzakis, E., et al., *Epigenetic control in rheumatoid arthritis synovial fibroblasts.* Nat Rev Rheumatol, 2009. **5**(5): p. 266-72.
287. Santiago-Schwarz, F., et al., *Dendritic cells (DCs) in rheumatoid arthritis (RA): progenitor cells and soluble factors contained in RA synovial fluid yield a subset of myeloid DCs that preferentially activate Th1 inflammatory-type responses.* J Immunol, 2001. **167**(3): p. 1758-68.
288. Rodriguez-Fernandez, J.L., *Antigen presentation by dendritic cells in rheumatoid arthritis.* Curr Top Med Chem, 2013. **13**(6): p. 712-9.
289. Thomas, R., et al., *Dendritic cells and the pathogenesis of rheumatoid arthritis.* J Leukoc Biol, 1999. **66**(2): p. 286-92.

290. Segura, E., et al., *Human inflammatory dendritic cells induce Th17 cell differentiation.* Immunity, 2013. **38**(2): p. 336-48.
291. Yamada, H., et al., *Preferential accumulation of activated Th1 cells not only in rheumatoid arthritis but also in osteoarthritis joints.* J Rheumatol, 2011. **38**(8): p. 1569-75.
292. Norii, M., et al., *Selective recruitment of CXCR3+ and CCR5+ CCR4+ T cells into synovial tissue in patients with rheumatoid arthritis.* Acta Med Okayama, 2006. **60**(3): p. 149-57.
293. Kawashima, M. and P. Miossec, *Heterogeneity of response of rheumatoid synovium cell subsets to interleukin-18 in relation to differential interleukin-18 receptor expression.* Arthritis Rheum, 2003. **48**(3): p. 631-7.
294. McKenzie, B.S., R.A. Kastelein, and D.J. Cua, *Understanding the IL-23-IL-17 immune pathway.* Trends Immunol, 2006. **27**(1): p. 17-23.
295. Smolen, J.S. and G. Steiner, *Therapeutic strategies for rheumatoid arthritis.* Nat Rev Drug Discov, 2003. **2**(6): p. 473-88.
296. Gu, X., et al., *1, 25-dihydroxy-vitamin D3 with tumor necrosis factor-alpha protects against rheumatoid arthritis by promoting p53 acetylation-mediated apoptosis via Sirt1 in synoviocytes.* Cell Death Dis, 2016. **7**(10): p. e2423.
297. Gossec, L., et al., *Relative clinical influence of clinical, laboratory, and radiological investigations in early arthritis on the diagnosis of rheumatoid arthritis. Data from the French Early Arthritis Cohort ESPOIR.* J Rheumatol, 2010. **37**(12): p. 2486-92.
298. Atzeni, F., et al., *Biomarkers in Rheumatoid Arthritis.* Isr Med Assoc J, 2017. **19**(8): p. 512-516.
299. Luime, J.J., et al., *Does anti-mutated citrullinated vimentin have additional value as a serological marker in the diagnostic and prognostic investigation of patients with rheumatoid arthritis? A systematic review.* Ann Rheum Dis, 2010. **69**(2): p. 337-44.
300. Bizzaro, N., et al., *Anti-cyclic citrullinated peptide antibody titer predicts time to rheumatoid arthritis onset in patients with undifferentiated arthritis: results from a 2-year prospective study.* Arthritis Research & Therapy, 2013. **15**(1): p. R16-R16.
301. Debaugnies, F., et al., *Anti-cyclic citrullinated peptide antibodies: a comparison of different assays for the diagnosis of rheumatoid arthritis.* Scand J Rheumatol, 2013. **42**(2): p. 108-14.
302. Kostoglou-Athanassiou, I., et al., *Vitamin D and rheumatoid arthritis.* Ther Adv Endocrinol Metab, 2012. **3**(6): p. 181-7.
303. Kerr, G.S., et al., *Prevalence of vitamin D insufficiency/deficiency in rheumatoid arthritis and associations with disease severity and activity.* J Rheumatol, 2011. **38**(1): p. 53-9.
304. Rossini, M., et al., *Vitamin D deficiency in rheumatoid arthritis: prevalence, determinants and associations with disease activity and disability.* Arthritis Res Ther, 2010. **12**(6): p. R216.
305. Marques, C.D., et al., *The importance of vitamin D levels in autoimmune diseases.* Rev Bras Reumatol, 2010. **50**(1): p. 67-80.
306. Gatenby, P., R. Lucas, and A. Swaminathan, *Vitamin D deficiency and risk for rheumatic diseases: an update.* Curr Opin Rheumatol, 2013. **25**(2): p. 184-91.
307. Dehghan, A., et al., *Role of vitamin D in flare ups of rheumatoid arthritis.* Z Rheumatol, 2014. **73**(5): p. 461-4.
308. Skaaby, T., et al., *Prospective population-based study of the association between vitamin D status and incidence of autoimmune disease.* Endocrine, 2015. **50**(1): p. 231-8.
309. Aloia, J.F., et al., *Vitamin D intake to attain a desired serum 25-hydroxyvitamin D concentration.* Am J Clin Nutr, 2008. **87**(6): p. 1952-8.
310. Song, G.G., S.C. Bae, and Y.H. Lee, *Association between vitamin D intake and the risk of rheumatoid arthritis: a meta-analysis.* Clin Rheumatol, 2012. **31**(12): p. 1733-9.

311. Lin, J., et al., *Serum Vitamin D Level and Rheumatoid Arthritis Disease Activity: Review and Meta-Analysis.* PLOS ONE, 2016. **11**(1): p. e0146351.
312. Hajjaj-Hassouni, N., et al., *Evaluation of Vitamin D Status in Rheumatoid Arthritis and Its Association with Disease Activity across 15 Countries: “The COMORA Study&#x201d.* International Journal of Rheumatology, 2017. **2017**: p. 8.
313. Harris, S.S., *Vitamin D and African Americans.* The Journal of Nutrition, 2006. **136**(4): p. 1126-1129.
314. Kostoglou-Athanassiou, I., et al., *Vitamin D and rheumatoid arthritis.* Therapeutic Advances in Endocrinology and Metabolism, 2012. **3**(6): p. 181-187.
315. Cutolo, M., et al., *Circannual vitamin d serum levels and disease activity in rheumatoid arthritis: Northern versus Southern Europe.* Clin Exp Rheumatol, 2006. **24**(6): p. 702-4.
316. Hitchon, C.A., et al., *Vitamin D receptor polymorphism rs2228570 (Fok1) is associated with rheumatoid arthritis in North American natives.* J Rheumatol, 2012. **39**(9): p. 1792-7.
317. Rass, P., et al., *Vitamin D receptor gene polymorphism in rheumatoid arthritis and associated osteoporosis.* Rheumatol Int, 2006. **26**(11): p. 964-71.
318. Cavalcanti, C.A.J., *Vitamin D receptor polymorphisms and expression profile in rheumatoid arthritis brazilian patients.* Molecular biology reports, 2016. **v. 43**(no. 1): p. pp. 41-51-2016 v.43 no.1.
319. Maalej, A., et al., *Association study of VDR gene with rheumatoid arthritis in the French population.* Genes Immun, 2005. **6**(8): p. 707-11.
320. Gómez-Vaquero, C., et al., *Influence of the BsmI polymorphism of the vitamin D receptor gene on rheumatoid arthritis clinical activity.* The Journal of Rheumatology, 2007. **34**(9): p. 1823.
321. Mosaad, Y.M., et al., *Vitamin D receptor gene polymorphism as possible risk factor in rheumatoid arthritis and rheumatoid related osteoporosis.* Human Immunology, 2014. **75**(5): p. 452-461.
322. Saad, M.N., et al., *Genetic Case-Control Study for Eight Polymorphisms Associated with Rheumatoid Arthritis.* PLOS ONE, 2015. **10**(7): p. e0131960.
323. Goertz, B., et al., *Vitamin D receptor genotypes are not associated with rheumatoid arthritis or biochemical parameters of bone turnover in German RA patients.* Clin Exp Rheumatol, 2003. **21**(3): p. 333-9.
324. Lee, C.K., et al., *Lack of relationship between vitamin D receptor polymorphism and bone erosion in rheumatoid arthritis.* J Korean Med Sci, 2001. **16**(2): p. 188-92.
325. Tizaoui, K. and K. Hamzaoui, *Association between VDR polymorphisms and rheumatoid arthritis disease: Systematic review and updated meta-analysis of case-control studies.* Immunobiology, 2015. **220**(6): p. 807-16.
326. Caraba, A., et al., *Vitamin D Status, Disease Activity, and Endothelial Dysfunction in Early Rheumatoid Arthritis Patients.* Disease Markers, 2017. **2017**: p. 7.
327. Raczkiewicz, A., et al., *AB0170 Vitamin D Deficiency Is Associated with Higher Disease Activity in Patients with Early Rheumatoid Arthritis.* Annals of the Rheumatic Diseases, 2016. **75**(Suppl 2): p. 954.
328. Herly, M., et al., *The D-vitamin metabolite 1,25(OH)2D in serum is associated with disease activity and Anti-Citrullinated Protein Antibodies in active and treatment naïve, early Rheumatoid Arthritis Patients.* Scandinavian Journal of Immunology, 2018. **0**(ja): p. e12704.
329. Herly, M., et al., *The D-vitamin metabolite 1,25(OH)2 D in serum is associated with disease activity and Anti-Citrullinated Protein Antibodies in active and treatment naive, early Rheumatoid Arthritis Patients.* Scand J Immunol, 2018. **88**(3): p. e12704.
330. Haque, U.J. and S.J. Bartlett, *Relationships among vitamin D, disease activity, pain and disability in rheumatoid arthritis.* Clin Exp Rheumatol, 2010. **28**(5): p. 745-7.

331. Turhanoglu, A.D., et al., *The relationship between vitamin D and disease activity and functional health status in rheumatoid arthritis.* Rheumatol Int, 2011. **31**(7): p. 911-4.
332. Sabbagh, Z., J. Markland, and H. Vatanparast, *Vitamin D status is associated with disease activity among rheumatology outpatients.* Nutrients, 2013. **5**(7): p. 2268-2275.
333. Sharma, R., et al., *Estimation of vitamin D levels in rheumatoid arthritis patients and its correlation with the disease activity.* J Assoc Physicians India, 2014. **62**(8): p. 678-81.
334. Azzeh, F.S. and O.A. Kensara, *Vitamin D Is a Good Marker for Disease Activity of Rheumatoid Arthritis Disease.* Dis Markers, 2015. **2015**: p. 260725.
335. Wang, Y., et al., *Serum Vitamin D Level is Inversely Associated With Anti-Cyclic Citrullinated Peptide Antibody Level and Disease Activity in Rheumatoid Arthritis Patients.* Archives of rheumatology, 2015. **31**(1): p. 64-70.
336. Hajjaj-Hassouni, N., et al., *Evaluation of Vitamin D Status in Rheumatoid Arthritis and Its Association with Disease Activity across 15 Countries: "The COMORA Study".* Int J Rheumatol, 2017. **2017**: p. 5491676.
337. Khoja, S.O., et al., *Associations of Vitamin D Levels and Vitamin D Receptor Genotypes with Patient-Reported Outcome/Disease Activity in Patients with Rheumatoid Arthritis.* Clin Lab, 2018. **64**(1): p. 51-58.
338. Higgins, M.K., et al., *The effect of vitamin D levels on the assessment of disease activity in rheumatoid arthritis.* Clinical Rheumatology, 2013. **32**(6): p. 863-867.
339. Hong, Q., et al., *Associations between serum 25-hydroxyvitamin D and disease activity, inflammatory cytokines and bone loss in patients with rheumatoid arthritis.* Rheumatology (Oxford), 2014. **53**(11): p. 1994-2001.
340. Zakeri, Z., et al., *Serum vitamin D level and disease activity in patients with recent onset rheumatoid arthritis.* Int J Rheum Dis, 2016. **19**(4): p. 343-7.
341. Vojinovic, J., et al., *European multicentre pilot survey to assess vitamin D status in rheumatoid arthritis patients and early development of a new Patient Reported Outcome questionnaire (D-PRO).* Autoimmun Rev, 2017. **16**(5): p. 548-554.
342. Heidari, B. and M.R. Hassanjani Roushan, *Rheumatoid arthritis and osteoporosis.* Caspian journal of internal medicine, 2012. **3**(3): p. 445-446.
343. Atwa, M.A., et al., *Serum 25-hydroxyvitamin D concentration in patients with psoriasis and rheumatoid arthritis and its association with disease activity and serum tumor necrosis factor-alpha.* Saudi Med J, 2013. **34**(8): p. 806-13.
344. Furuya, T., et al., *Prevalence of and factors associated with vitamin D deficiency in 4,793 Japanese patients with rheumatoid arthritis.* Clin Rheumatol, 2013. **32**(7): p. 1081-7.
345. Yazmalar, L., et al., *Seasonal disease activity and serum vitamin D levels in rheumatoid arthritis, ankylosing spondylitis and osteoarthritis.* African health sciences, 2013. **13**(1): p. 47-55.
346. Sahebari, M., et al., *25(OH) vitamin D serum values and rheumatoid arthritis disease activity (DA S28 ESR).* Caspian J Intern Med, 2014. **5**(3): p. 148-55.
347. Cote, J., et al., *Low vitamin D level is not associated with increased incidence of rheumatoid arthritis.* Rheumatol Int, 2014. **34**(10): p. 1475-9.
348. Raczkiewicz, A., et al., *Vitamin D status and its association with quality of life, physical activity, and disease activity in rheumatoid arthritis patients.* J Clin Rheumatol, 2015. **21**(3): p. 126-30.
349. Cen, X., et al., *Association between Serum 25-Hydroxyvitamin D Level and Rheumatoid Arthritis.* Biomed Res Int, 2015. **2015**: p. 913804.
350. Pakchotanon, R., et al., *The association between serum vitamin D Level and disease activity in Thai rheumatoid arthritis patients.* Int J Rheum Dis, 2016. **19**(4): p. 355-61.
351. de la Torre Lossa, P., et al., *Vitamin D is not useful as a biomarker for disease activity in rheumatoid arthritis.* Reumatol Clin, 2018.

352. Haga, H.J., et al., *Severe deficiency of 25-hydroxyvitamin D(3) (25-OH-D(3)) is associated with high disease activity of rheumatoid arthritis.* Clin Rheumatol, 2013. **32**(5): p. 629-33.
353. Gheita, T.A., et al., *Vitamin D status in rheumatoid arthritis patients: relation to clinical manifestations, disease activity, quality of life and fibromyalgia syndrome.* Int J Rheum Dis, 2016. **19**(3): p. 294-9.
354. Matsumoto, Y., et al., *Relationships between serum 25-hydroxycalciferol, vitamin D intake and disease activity in patients with rheumatoid arthritis--TOMORROW study.* Mod Rheumatol, 2015. **25**(2): p. 246-50.
355. Brance, M.L., et al., *Vitamin D levels and bone mass in rheumatoid arthritis.* Rheumatol Int, 2015. **35**(3): p. 499-505.
356. Mateen, S., et al., *Level of inflammatory cytokines in rheumatoid arthritis patients: Correlation with 25-hydroxy vitamin D and reactive oxygen species.* PLoS One, 2017. **12**(6): p. e0178879.
357. Oelzner, P., et al., *Relationship between disease activity and serum levels of vitamin D metabolites and PTH in rheumatoid arthritis.* Calcif Tissue Int, 1998. **62**(3): p. 193-8.
358. Attar, S.M., *Vitamin D deficiency in rheumatoid arthritis. Prevalence and association with disease activity in Western Saudi Arabia.* Saudi Med J, 2012. **33**(5): p. 520-5.
359. Hiraki, L.T., et al., *Circulating 25-hydroxyvitamin D level and risk of developing rheumatoid arthritis.* Rheumatology (Oxford, England), 2014. **53**(12): p. 2243-2248.
360. Lourdudoss, C., et al., *Are dietary vitamin D, omega-3 fatty acids and folate associated with treatment results in patients with early rheumatoid arthritis? Data from a Swedish population-based prospective study.* BMJ Open, 2017. **7**(6).
361. Merlino, L.A., et al., *Vitamin D intake is inversely associated with rheumatoid arthritis: results from the Iowa Women's Health Study.* Arthritis Rheum, 2004. **50**(1): p. 72-7.
362. Andjelkovic, Z., et al., *Disease modifying and immunomodulatory effects of high dose 1 alpha (OH) D3 in rheumatoid arthritis patients.* Clin Exp Rheumatol, 1999. **17**(4): p. 453-6.
363. Gopinath, K. and D. Danda, *Supplementation of 1,25 dihydroxy vitamin D3 in patients with treatment naive early rheumatoid arthritis: a randomised controlled trial.* Int J Rheum Dis, 2011. **14**(4): p. 332-9.
364. Chandrashekara, S. and A. Patted, *Role of vitamin D supplementation in improving disease activity in rheumatoid arthritis: An exploratory study.* Int J Rheum Dis, 2017. **20**(7): p. 825-831.
365. Buondonno, I., et al., *Vitamin D and immunomodulation in early rheumatoid arthritis: A randomized double-blind placebo-controlled study.* PLOS ONE, 2017. **12**(6): p. e0178463.
366. Salesi, M. and Z. Farajzadegan, *Efficacy of vitamin D in patients with active rheumatoid arthritis receiving methotrexate therapy.* Rheumatol Int, 2012. **32**(7): p. 2129-33.
367. Yang, J., et al., *Effect of vitamin D on the recurrence rate of rheumatoid arthritis.* Experimental and therapeutic medicine, 2015. **10**(5): p. 1812-1816.
368. Kleijwegt, F.S., et al., *Critical role for TNF in the induction of human antigen-specific regulatory T cells by tolerogenic dendritic cells.* J Immunol, 2010. **185**(3): p. 1412-8.
369. Seymour, H.E., et al., *Anti-TNF agents for rheumatoid arthritis.* Br J Clin Pharmacol, 2001. **51**(3): p. 201-8.
370. Ma, X. and S. Xu, *TNF inhibitor therapy for rheumatoid arthritis.* Biomedical reports, 2013. **1**(2): p. 177-184.
371. Feldmann, M. and R.N. Maini, *Anti-TNF alpha therapy of rheumatoid arthritis: what have we learned?* Annu Rev Immunol, 2001. **19**: p. 163-96.

372. Baeke, F., et al., *The vitamin D analog, TX527, promotes a human CD4+CD25highCD127low regulatory T cell profile and induces a migratory signature specific for homing to sites of inflammation.* J Immunol, 2011. **186**(1): p. 132-42.
373. Jeffery, L.E., et al., *1,25-Dihydroxyvitamin D3 and IL-2 combine to inhibit T cell production of inflammatory cytokines and promote development of regulatory T cells expressing CTLA-4 and FoxP3.* J Immunol, 2009. **183**(9): p. 5458-67.
374. Tetlow, L.C. and D.E. Woolley, *The effects of 1α,25-dihydroxyvitamin D(3) on matrix metalloproteinase and prostaglandin E(2) production by cells of the rheumatoid lesion.* Arthritis Research, 1999. **1**(1): p. 63-70.
375. Laragione, T., A. Shah, and P.S. Gulko, *The vitamin D receptor regulates rheumatoid arthritis synovial fibroblast invasion and morphology.* Mol Med, 2012. **18**: p. 194-200.
376. Stoop, J.N., et al., *Therapeutic effect of tolerogenic dendritic cells in established collagen-induced arthritis is associated with a reduction in Th17 responses.* Arthritis Rheum, 2010. **62**(12): p. 3656-65.
377. Cantorna, M.T., C.E. Hayes, and H.F. DeLuca, *1,25-Dihydroxycholecalciferol inhibits the progression of arthritis in murine models of human arthritis.* J Nutr, 1998. **128**(1): p. 68-72.
378. Lemire, J.M., A. Ince, and M. Takashima, *1,25-Dihydroxyvitamin D3 attenuates the expression of experimental murine lupus of MRL/l mice.* Autoimmunity, 1992. **12**(2): p. 143-8.
379. Zwerina, K., et al., *Vitamin D receptor regulates TNF-mediated arthritis.* Ann Rheum Dis, 2011. **70**(6): p. 1122-9.
380. Mawer, E.B., et al., *Evidence for nonrenal synthesis of 1,25-dihydroxyvitamin D in patients with inflammatory arthritis.* J Bone Miner Res, 1991. **6**(7): p. 733-9.
381. van Driel, M., et al., *Evidence for auto/paracrine actions of vitamin D in bone: 1alpha-hydroxylase expression and activity in human bone cells.* Faseb j, 2006. **20**(13): p. 2417-9.
382. Atkins, G.J., et al., *Metabolism of vitamin D3 in human osteoblasts: evidence for autocrine and paracrine activities of 1 alpha,25-dihydroxyvitamin D3.* Bone, 2007. **40**(6): p. 1517-28.
383. Huang, D.C., et al., *Targeted disruption of the 25-hydroxyvitamin D3 1alpha-hydroxylase gene in ras-transformed keratinocytes demonstrates that locally produced 1alpha,25-dihydroxyvitamin D3 suppresses growth and induces differentiation in an autocrine fashion.* Mol Cancer Res, 2002. **1**(1): p. 56-67.
384. Tetlow, L.C., et al., *Vitamin D receptors in the rheumatoid lesion: expression by chondrocytes, macrophages, and synoviocytes.* Ann Rheum Dis, 1999. **58**(2): p. 118-21.
385. Hayes, M.E., et al., *Synthesis of the active metabolite of vitamin D, 1,25(OH)2D3, by synovial fluid macrophages in arthritic diseases.* Ann Rheum Dis, 1989. **48**(9): p. 723-9.
386. Hayes, M.E., et al., *Inhibition by prostaglandin E1 and E2 of 1,25-dihydroxyvitamin D3 synthesis by synovial fluid macrophages from arthritic joints.* Ann Rheum Dis, 1992. **51**(5): p. 632-7.
387. Smith, S., et al., *Autocrine control of vitamin D metabolism in synovial cells from arthritic patients.* Annals of the Rheumatic Diseases, 1999. **58**(6): p. 372-378.
388. Teixeira, V.H., et al., *Transcriptome Analysis Describing New Immunity and Defense Genes in Peripheral Blood Mononuclear Cells of Rheumatoid Arthritis Patients.* PLOS ONE, 2009. **4**(8): p. e6803.
389. Cooles, F.A.H., et al., *Phenotypic and Transcriptomic Analysis of Peripheral Blood Plasmacytoid and Conventional Dendritic Cells in Early Drug Naïve Rheumatoid Arthritis.* Frontiers in immunology, 2018. **9**: p. 755-755.

390. Raychaudhuri, S., et al., *Five amino acids in three HLA proteins explain most of the association between MHC and seropositive rheumatoid arthritis.* Nat Genet, 2012. **44**(3): p. 291-6.
391. Law, S.C., et al., *T-cell autoreactivity to citrullinated autoantigenic peptides in rheumatoid arthritis patients carrying HLA-DRB1 shared epitope alleles.* Arthritis Res Ther, 2012. **14**(3): p. R118.
392. Richardson, B., et al., *Evidence for impaired T cell DNA methylation in systemic lupus erythematosus and rheumatoid arthritis.* Arthritis Rheum, 1990. **33**(11): p. 1665-73.
393. Karouzakis, E., et al., *DNA hypomethylation in rheumatoid arthritis synovial fibroblasts.* Arthritis Rheum, 2009. **60**(12): p. 3613-22.
394. Nakano, K., et al., *DNA methylome signature in rheumatoid arthritis.* Ann Rheum Dis, 2013. **72**(1): p. 110-7.
395. Nile, C.J., et al., *Methylation status of a single CpG site in the IL6 promoter is related to IL6 messenger RNA levels and rheumatoid arthritis.* Arthritis Rheum, 2008. **58**(9): p. 2686-93.
396. Avni, O., et al., *T(H) cell differentiation is accompanied by dynamic changes in histone acetylation of cytokine genes.* Nat Immunol, 2002. **3**(7): p. 643-51.
397. Anderson, P.H., et al., *RNAi-mediated silencing of CYP27B1 abolishes 1,25(OH)2D3 synthesis and reduces osteocalcin and CYP24 mRNA expression in human osteosarcoma (HOS) cells.* J Steroid Biochem Mol Biol, 2007. **103**(3-5): p. 601-5.
398. Bae, Y., et al., *miRNA-34c regulates Notch signaling during bone development.* Hum Mol Genet, 2012. **21**(13): p. 2991-3000.
399. Bosher, J.M., et al., *RNA interference can target pre-mRNA: consequences for gene expression in a Caenorhabditis elegans operon.* Genetics, 1999. **153**(3): p. 1245-56.
400. Ouboussad, L., et al., *Profiling microRNAs in individuals at risk of progression to rheumatoid arthritis.* Arthritis Research & Therapy, 2017. **19**(1): p. 288.
401. Singh, R.P. and B.H. Hahn, *Novel microRNAs in the pathogenesis of rheumatoid arthritis.* The Journal of Immunology, 2017. **198**(1 Supplement): p. 224.11.
402. Chen, X.-M., et al., *Role of Micro RNAs in the Pathogenesis of Rheumatoid Arthritis: Novel Perspectives Based on Review of the Literature.* Medicine, 2015. **94**(31): p. e1326-e1326.
403. Mookherjee, N. and H.S. El-Gabalawy, *High degree of correlation between whole blood and PBMC expression levels of miR-155 and miR-146a in healthy controls and rheumatoid arthritis patients.* J Immunol Methods, 2013. **400-401**: p. 106-10.
404. Stanczyk, J., et al., *Altered expression of MicroRNA in synovial fibroblasts and synovial tissue in rheumatoid arthritis.* Arthritis Rheum, 2008. **58**(4): p. 1001-9.
405. Yang, S. and Y. Yang, *Downregulation of microRNA221 decreases migration and invasion in fibroblastlike synoviocytes in rheumatoid arthritis.* Mol Med Rep, 2015. **12**(2): p. 2395-401.
406. Nakasa, T., et al., *A mini-review: microRNA in arthritis.* Physiol Genomics, 2011. **43**(10): p. 566-70.
407. Ceribelli, A., et al., *MicroRNAs in rheumatoid arthritis.* FEBS Lett, 2011. **585**(23): p. 3667-74.
408. Duroux-Richard, I., et al., *Circulating miRNA-125b is a potential biomarker predicting response to rituximab in rheumatoid arthritis.* Mediators Inflamm, 2014. **2014**: p. 342524.
409. Abou-Zeid, A., M. Saad, and E. Soliman, *MicroRNA 146a expression in rheumatoid arthritis: association with tumor necrosis factor-alpha and disease activity.* Genet Test Mol Biomarkers, 2011. **15**(11): p. 807-12.
410. Song, G.G., et al., *The association between susceptibility to inflammatory arthritis and miR-146a, miR-499 and IRAK1 polymorphisms. A meta-analysis.* Z Rheumatol, 2015. **74**(7): p. 637-45.

411. Long, L., et al., *Upregulated microRNA-155 expression in peripheral blood mononuclear cells and fibroblast-like synoviocytes in rheumatoid arthritis.* Clin Dev Immunol, 2013. **2013**: p. 296139.
412. Li, X., F. Tian, and F. Wang, *Rheumatoid arthritis-associated microRNA-155 targets SOCS1 and upregulates TNF-alpha and IL-1beta in PBMCs.* Int J Mol Sci, 2013. **14**(12): p. 23910-21.
413. Dong, L., et al., *Decreased expression of microRNA-21 correlates with the imbalance of Th17 and Treg cells in patients with rheumatoid arthritis.* J Cell Mol Med, 2014. **18**(11): p. 2213-24.
414. El-Shal, A.S., et al., *Association of microRNAs genes polymorphisms with rheumatoid arthritis in Egyptian female patients.* Joint, bone, spine : revue du rhumatisme, 2013. **80**(6): p. 626-631.
415. Hashemi, M., et al., *Association of pre-miRNA-146a rs2910164 and premiRNA-499 rs3746444 polymorphisms and susceptibility to rheumatoid arthritis.* Mol Med Rep, 2013. **7**(1): p. 287-91.
416. Li, K., et al., *Association of two polymorphisms rs2910164 in miRNA-146a and rs3746444 in miRNA-499 with rheumatoid arthritis: a meta-analysis.* Hum Immunol, 2014. **75**(7): p. 602-8.
417. Zhou, X., et al., *Is the microRNA-146a (rs2910164) polymorphism associated with rheumatoid arthritis? Association of microRNA-146a (rs2910164) polymorphism and rheumatoid arthritis could depend on gender.* Joint Bone Spine, 2015. **82**(3): p. 166-71.
418. Castro-Villegas, C., et al., *Circulating miRNAs as potential biomarkers of therapy effectiveness in rheumatoid arthritis patients treated with anti-TNFalpha.* Arthritis Res Ther, 2015. **17**: p. 49.
419. Vicente, R., et al., *Deregulation and therapeutic potential of microRNAs in arthritic diseases.* Nat Rev Rheumatol, 2016. **12**(4): p. 211-20.
420. Murata, K., et al., *Plasma and synovial fluid microRNAs as potential biomarkers of rheumatoid arthritis and osteoarthritis.* Arthritis Res Ther, 2010. **12**(3): p. R86.
421. Nakasa, T., et al., *Expression of microRNA-146 in rheumatoid arthritis synovial tissue.* Arthritis Rheum, 2008. **58**(5): p. 1284-92.
422. Pauley, K.M., et al., *Upregulated miR-146a expression in peripheral blood mononuclear cells from rheumatoid arthritis patients.* Arthritis Res Ther, 2008. **10**(4): p. R101.
423. Elsayed, H.M.A., et al., *MicroRNA-146a expression as a potential biomarker for rheumatoid arthritis in Egypt.* Egyptian Journal of Medical Human Genetics, 2017. **18**(2): p. 173-179.
424. Moran-Moguel, M.C., et al., *Rheumatoid Arthritis and miRNAs: A Critical Review through a Functional View.* Journal of Immunology Research, 2018. **2018**: p. 16.
425. Murata, K., et al., *Plasma and synovial fluid microRNAs as potential biomarkers of rheumatoid arthritis and osteoarthritis.* Arthritis Research & Therapy, 2010. **12**(3): p. R86.
426. Filková, M., et al., *Association of circulating miR-223 and miR-16 with disease activity in patients with early rheumatoid arthritis.* Ann Rheum Dis., 2014. **73**.
427. Filková, M., et al., *Association of circulating miR-223 and miR-16 with disease activity in patients with early rheumatoid arthritis.* Annals of the Rheumatic Diseases, 2014. **73**(10): p. 1898.
428. Simon, R., *Sensitivity, Specificity, PPV, and NPV for Predictive Biomarkers.* J Natl Cancer Inst, 2015. **107**(8).
429. Mayeux, R., *Biomarkers: Potential Uses and Limitations.* NeuroRx, 2004. **1**(2): p. 182-188.

430. Arroyo, J.D., et al., *Argonaute2 complexes carry a population of circulating microRNAs independent of vesicles in human plasma.* Proc Natl Acad Sci U S A, 2011. **108**(12): p. 5003-8.
431. Mitchell, P.S., et al., *Circulating microRNAs as stable blood-based markers for cancer detection.* Proc Natl Acad Sci U S A, 2008. **105**(30): p. 10513-8.
432. Ceribelli, A., et al., *MicroRNAs in rheumatoid arthritis.* FEBS letters, 2011. **585**(23): p. 3667-3674.
433. Chen, X.-M., et al., *Role of Micro RNAs in the Pathogenesis of Rheumatoid Arthritis: Novel Perspectives Based on Review of the Literature.* Medicine, 2015. **94**(31): p. e1326.
434. Lee, Y.H. and S.C. Bae, *Vitamin D level in rheumatoid arthritis and its correlation with the disease activity: a meta-analysis.* Clin Exp Rheumatol, 2016. **34**(5): p. 827-833.
435. Holick, M.F., et al., *Evaluation, treatment, and prevention of vitamin D deficiency: an Endocrine Society clinical practice guideline.* J Clin Endocrinol Metab, 2011. **96**(7): p. 1911-30.
436. Kamao, M., et al., *Measurement and characterization of C-3 epimerization activity toward vitamin D3.* Archives of Biochemistry and Biophysics, 2005. **436**(1): p. 196-205.
437. Messerlian, S., X. Gao, and R. St-Arnaud, *The 3-epi- and 24-oxo-derivatives of 1alpha,25 dihydroxyvitamin D(3) stimulate transcription through the vitamin D receptor.* J Steroid Biochem Mol Biol, 2000. **72**(1-2): p. 29-34.
438. Lensmeyer, G., et al., *The C-3 epimer of 25-hydroxyvitamin D(3) is present in adult serum.* J Clin Endocrinol Metab, 2012. **97**(1): p. 163-8.
439. Lutsey, P.L., et al., *The 25-hydroxyvitamin D(3) C-3 epimer: Distribution, correlates, and reclassification of 25-hydroxyvitamin D status in the population-based Atherosclerosis Risk in Communities Study (ARIC).* Clinica chimica acta; international journal of clinical chemistry, 2015. **442**: p. 75-81.
440. Strathmann, F.G., et al., *3-epi-25 hydroxyvitamin D concentrations are not correlated with age in a cohort of infants and adults.* Clinica chimica acta; international journal of clinical chemistry, 2012. **413**(1-2): p. 203-206.
441. Cashman, K.D., et al., *Significance of serum 24,25-dihydroxyvitamin D in the assessment of vitamin D status: a double-edged sword?* Clin Chem, 2015. **61**(4): p. 636-45.
442. Fairney, A., et al., *Vitamin D metabolites in synovial fluid.* Ann Rheum Dis, 1987. **46**(5): p. 370-4.
443. Inaba, M., et al., *Positive correlation between levels of IL-1 or IL-2 and 1,25(OH)2D/25-OH-D ratio in synovial fluid of patients with rheumatoid arthritis.* Life Sci, 1997. **61**(10): p. 977-85.
444. Pasquali, M., et al., *Calcitriol/calcifediol ratio: An indicator of vitamin D hydroxylation efficiency?* BBA Clinical, 2015. **3**: p. 251-256.
445. Hayes, M.E., et al., *Differential metabolism of 25-hydroxyvitamin D3 by cultured synovial fluid macrophages and fibroblast-like cells from patients with arthritis.* Ann Rheum Dis, 1992. **51**(2): p. 220-6.
446. Schäfer, J., *Vitamin D. A pluripotent steroid hormone: structural studies; molecular endocrinology and clinical applications. Proceedings of the Ninth Workshop on Vitamin D, Orlando, Florida (USA), May 28 — June 2, 1994. Herausgegeben von A. W. Norman, R. Bouillon and M. Thomasset. 966 Seiten, zahlr. Abbildungen und Tabellen. Walter de Gruyter, Berlin, New York, 1994. Preis: 470, — DM.* Food / Nahrung, 1995. **39**(4): p. 333-333.
447. Foroughi, M., et al., *Effect of Vitamin D Supplementation on C-reactive Protein in Patients with Nonalcoholic Fatty Liver.* International Journal of Preventive Medicine, 2014. **5**(8): p. 969-975.

448. Azizieh, F., K.O. Alyahya, and R. Raghupathy, *Association between levels of vitamin D and inflammatory markers in healthy women.* J Inflamm Res, 2016. **9**: p. 51-7.
449. Allen, A., S. Carville, and F. McKenna, *Diagnosis and management of rheumatoid arthritis in adults: summary of updated NICE guidance.* BMJ, 2018. **362**.
450. Hong, Q., et al., *Associations between serum 25-hydroxyvitamin D and disease activity, inflammatory cytokines and bone loss in patients with rheumatoid arthritis.* Rheumatology, 2014. **53**(11): p. 1994-2001.
451. Povoroznyuk, V., O. Synenky, and N. Balatska, *AB0369 Vitamin d status and disease activity in patients with rheumatoid arthritis.* Annals of the Rheumatic Diseases, 2017. **76**(Suppl 2): p. 1177-1177.
452. Dixon, J.S., M. Greenwood, and H.A. Bird, *How reliable is ESR as a measure of disease activity in rheumatoid arthritis treated with hydroxychloroquine?* Clin Rheumatol, 1988. **7**(2): p. 262-6.
453. O'Leary, T.J., et al., *The effects of chloroquine on serum 1,25-dihydroxyvitamin D and calcium metabolism in sarcoidosis.* N Engl J Med, 1986. **315**(12): p. 727-30.
454. Yamamoto, N. and V.R. Naraparaju, *Role of vitamin D3-binding protein in activation of mouse macrophages.* J Immunol, 1996. **157**(4): p. 1744-9.
455. Gomme, P.T. and J. Bertolini, *Therapeutic potential of vitamin D-binding protein.* Trends Biotechnol, 2004. **22**(7): p. 340-5.
456. Binette, J.P. and K. Schmid, *The Proteins of Synovial Fluid: A Study of the Alpha 1-Alpha 2 Globulin Ratio.* Arthritis Rheum, 1965. **8**: p. 14-28.
457. Nielson, C.M., et al., *Free 25-Hydroxyvitamin D: Impact of Vitamin D Binding Protein Assays on Racial-Genotypic Associations.* J Clin Endocrinol Metab, 2016. **101**(5): p. 2226-34.
458. Kreutz, M., et al., *1,25-dihydroxyvitamin D3 production and vitamin D3 receptor expression are developmentally regulated during differentiation of human monocytes into macrophages.* Blood, 1993. **82**(4): p. 1300-7.
459. Hewison, M., et al., *Differential regulation of vitamin D receptor and its ligand in human monocyte-derived dendritic cells.* J Immunol, 2003. **170**(11): p. 5382-90.
460. Nicholson, J.P., M.R. Wolmarans, and G.R. Park, *The role of albumin in critical illness.* British Journal of Anaesthesia, 2000. **85**(4): p. 599-610.
461. Wang, K., et al., *Comparing the MicroRNA Spectrum between Serum and Plasma.* PLoS ONE, 2012. **7**(7): p. e41561.
462. Churov, A.V., E.K. Oleinik, and M. Knip, *MicroRNAs in rheumatoid arthritis: Altered expression and diagnostic potential.* Autoimmunity Reviews, 2015. **14**(11): p. 1029-1037.
463. Nakamachi, Y., et al., *MicroRNA-124a is a key regulator of proliferation and monocyte chemoattractant protein 1 secretion in fibroblast-like synoviocytes from patients with rheumatoid arthritis.* Arthritis Rheum, 2009. **60**(5): p. 1294-304.
464. Taganov, K.D., et al., *NF-kappaB-dependent induction of microRNA miR-146, an inhibitor targeted to signaling proteins of innate immune responses.* Proc Natl Acad Sci U S A, 2006. **103**(33): p. 12481-6.
465. Castro-Villegas, C., et al., *Circulating miRNAs as potential biomarkers of therapy effectiveness in rheumatoid arthritis patients treated with anti-TNFα.* Arthritis Research & Therapy, 2015. **17**(1): p. 49.
466. Duroux-Richard, I., C. Jorgensen, and F. Apparailly, *What do microRNAs mean for rheumatoid arthritis?* Arthritis Rheum, 2012. **64**(1): p. 11-20.
467. Pritchard, C.C., H.H. Cheng, and M. Tewari, *MicroRNA profiling: approaches and considerations.* Nature reviews. Genetics, 2012. **13**(5): p. 358-369.
468. Chatzikyriakidou, A., et al., *The role of microRNA-146a (miR-146a) and its target IL-1R-associated kinase (IRAK1) in psoriatic arthritis susceptibility.* Scand J Immunol, 2010. **71**(5): p. 382-5.

469. Stadlmayr, A., et al., *Relations of vitamin D status, gender and type 2 diabetes in middle-aged Caucasians.* Acta Diabetol, 2015. **52**(1): p. 39-46.
470. Sharifi, N., et al., *Women may respond different from men to vitamin D supplementation regarding cardiometabolic biomarkers.* Experimental biology and medicine (Maywood, N.J.), 2016. **241**(8): p. 830-838.
471. Al-Horani, H., et al., *Nationality, Gender, Age, and Body Mass Index Influences on Vitamin D Concentration among Elderly Patients and Young Iraqi and Jordanian in Jordan.* Biochemistry research international, 2016. **2016**: p. 8920503-8920503.
472. Polasik, K., et al., *Vitamin D status in patients with rheumatoid arthritis: a correlation analysis with disease activity and progression, as well as serum IL-6 levels.* Acta Biochim Pol, 2017. **64**(4): p. 667-670.
473. Eissa, H.A., - *Relationship Between Vitamin D and Rheumatoid Arthritis Activity.* - Vitamins & Minerals, (- 3): p. - 1.
474. Clara, C. and M. Salvatore, *Vitamin D : Autoimmunity and Gender.* Current Medicinal Chemistry, 2017. **24**(24): p. 2671-2686.
475. Nussinovitch, U. and Y. Shoenfeld, *The role of gender and organ specific autoimmunity.* Autoimmun Rev, 2012. **11**(6-7): p. A377-85.
476. Kerr, G.S., et al., *Prevalence of Vitamin D Insufficiency/Deficiency in Rheumatoid Arthritis and Associations with Disease Severity and Activity.* The Journal of Rheumatology, 2011. **38**(1): p. 53.
477. Nashold, F.E., et al., *Estrogen controls vitamin D3-mediated resistance to experimental autoimmune encephalomyelitis by controlling vitamin D3 metabolism and receptor expression.* J Immunol, 2009. **183**(6): p. 3672-81.
478. La Rocca, G., et al., *In vivo, Argonaute-bound microRNAs exist predominantly in a reservoir of low molecular weight complexes not associated with mRNA.* Proceedings of the National Academy of Sciences of the United States of America, 2015. **112**(3): p. 767-772.
479. Wu, W., et al., *Comparison of microRNA expression profiles of Kashin-Beck disease, osteoarthritis and rheumatoid arthritis.* Scientific reports, 2017. **7**(1): p. 540-540.
480. Steinman, R.M. and H. Hemmi, *Dendritic cells: translating innate to adaptive immunity.* Curr Top Microbiol Immunol, 2006. **311**: p. 17-58.
481. Dudek, A.M., et al., *Immature, Semi-Mature, and Fully Mature Dendritic Cells: Toward a DC-Cancer Cells Interface That Augments Anticancer Immunity.* Front Immunol, 2013. **4**: p. 438.
482. Geremia, A., et al., *Innate and adaptive immunity in inflammatory bowel disease.* Autoimmun Rev, 2014. **13**(1): p. 3-10.
483. Jeffery, L.E., et al., *Decreased sensitivity to 1,25-dihydroxyvitamin D3 in T cells from the rheumatoid joint.* J Autoimmun, 2018. **88**: p. 50-60.
484. Dudziak, D., et al., *Differential antigen processing by dendritic cell subsets in vivo.* Science, 2007. **315**(5808): p. 107-11.
485. Cella, M., F. Sallusto, and A. Lanzavecchia, *Origin, maturation and antigen presenting function of dendritic cells.* Curr Opin Immunol, 1997. **9**(1): p. 10-6.
486. Cella, M., et al., *Inflammatory stimuli induce accumulation of MHC class II complexes on dendritic cells.* Nature, 1997. **388**(6644): p. 782-7.
487. Steinman, R.M. and J. Idoyaga, *Features of the dendritic cell lineage.* Immunol Rev, 2010. **234**(1): p. 5-17.
488. Mellman, I., *Dendritic cells: master regulators of the immune response.* Cancer Immunol Res, 2013. **1**(3): p. 145-9.
489. Lutz, M.B. and G. Schuler, *Immature, semi-mature and fully mature dendritic cells: which signals induce tolerance or immunity?* Trends Immunol, 2002. **23**(9): p. 445-9.
490. Mommaas, A.M., et al., *Distribution of HLA class II molecules in epidermal Langerhans cells in situ.* Eur J Immunol, 1995. **25**(2): p. 520-5.

491. Romani, N., et al., *Proliferating dendritic cell progenitors in human blood.* J Exp Med, 1994. **180**(1): p. 83-93.
492. Sallusto, F. and A. Lanzavecchia, *Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha.* J Exp Med, 1994. **179**(4): p. 1109-18.
493. Randolph, G.J., et al., *Differentiation of monocytes into dendritic cells in a model of transendothelial trafficking.* Science, 1998. **282**(5388): p. 480-3.
494. Delirezh, N., et al., *Generation of Mature Monocyte-Derived Dendritic Cells in the Presence of Heparin and Monocyte Conditioned Medium: Phenotypic and Functional Comparison.* Iranian Biomedical Journal, 2011. **15**(3): p. 79-84.
495. Cheong, C., et al., *Microbial stimulation fully differentiates monocytes to DC-SIGN/CD209(+) dendritic cells for immune T cell areas.* Cell, 2010. **143**(3): p. 416-429.
496. Osugi, Y., S. Vuckovic, and D.N. Hart, *Myeloid blood CD11c(+) dendritic cells and monocyte-derived dendritic cells differ in their ability to stimulate T lymphocytes.* Blood, 2002. **100**(8): p. 2858-66.
497. Maldonado, R.A. and U.H. von Andrian, *How tolerogenic dendritic cells induce regulatory T cells.* Adv Immunol, 2010. **108**: p. 111-65.
498. Hawiger, D., et al., *Dendritic cells induce peripheral T cell unresponsiveness under steady state conditions in vivo.* J Exp Med, 2001. **194**(6): p. 769-79.
499. Hawiger, D., et al., *Immunological unresponsiveness characterized by increased expression of CD5 on peripheral T cells induced by dendritic cells in vivo.* Immunity, 2004. **20**(6): p. 695-705.
500. Akbari, O., R.H. DeKruyff, and D.T. Umetsu, *Pulmonary dendritic cells producing IL-10 mediate tolerance induced by respiratory exposure to antigen.* Nat Immunol, 2001. **2**(8): p. 725-31.
501. Gandhi, R., et al., *Activation of the aryl hydrocarbon receptor induces human type 1 regulatory T cell-like and Foxp3(+) regulatory T cells.* Nat Immunol, 2010. **11**(9): p. 846-53.
502. Steinbrink, K., et al., *Induction of tolerance by IL-10-treated dendritic cells.* J Immunol, 1997. **159**(10): p. 4772-80.
503. Moore, K.W., et al., *Interleukin-10 and the interleukin-10 receptor.* Annu Rev Immunol, 2001. **19**: p. 683-765.
504. Morelli, A.E., et al., *Cytokine production by mouse myeloid dendritic cells in relation to differentiation and terminal maturation induced by lipopolysaccharide or CD40 ligation.* Blood, 2001. **98**(5): p. 1512-23.
505. Granucci, F., et al., *Transcriptional reprogramming of dendritic cells by differentiation stimuli.* Eur J Immunol, 2001. **31**(9): p. 2539-46.
506. Steinman, R.M., D. Hawiger, and M.C. Nussenzweig, *Tolerogenic dendritic cells.* Annu Rev Immunol, 2003. **21**: p. 685-711.
507. Brennan, A., et al., *Dendritic cells from human tissues express receptors for the immunoregulatory vitamin D3 metabolite, dihydroxycholecalciferol.* Immunology, 1987. **61**(4): p. 457-61.
508. Dam, T.N., et al., *The vitamin D3 analog calcipotriol suppresses the number and antigen-presenting function of Langerhans cells in normal human skin.* J Investig Dermatol Symp Proc, 1996. **1**(1): p. 72-7.
509. Nikolic, T. and B.O. Roep, *Regulatory multitasking of tolerogenic dendritic cells - lessons taken from vitamin d3-treated tolerogenic dendritic cells.* Front Immunol, 2013. **4**: p. 113.
510. Adorini, L. and G. Penna, *Induction of tolerogenic dendritic cells by vitamin D receptor agonists.* Handb Exp Pharmacol, 2009(188): p. 251-73.

511. van Halteren, A.G., et al., *Redirection of human autoreactive T-cells Upon interaction with dendritic cells modulated by TX527, an analog of 1,25 dihydroxyvitamin D(3).* Diabetes, 2002. **51**(7): p. 2119-25.
512. Ferreira, G.B., et al., *1alpha,25-Dihydroxyvitamin D3 and its analogs as modulators of human dendritic cells: a comparison dose-titration study.* J Steroid Biochem Mol Biol, 2013. **136**: p. 160-5.
513. Adorini, L., *Tolerogenic dendritic cells induced by vitamin D receptor ligands enhance regulatory T cells inhibiting autoimmune diabetes.* Ann N Y Acad Sci, 2003. **987**: p. 258-61.
514. Bartels, L.E., et al., *Human dendritic cell antigen presentation and chemotaxis are inhibited by intrinsic 25-hydroxy vitamin D activation.* Int Immunopharmacol, 2010. **10**(8): p. 922-8.
515. Penna, G., et al., *1,25-Dihydroxyvitamin D₃ Selectively Modulates Tolerogenic Properties in Myeloid but Not Plasmacytoid Dendritic Cells.* The Journal of Immunology, 2007. **178**(1): p. 145.
516. Fritsche, J., et al., *Regulation of 25-hydroxyvitamin D3-1 alpha-hydroxylase and production of 1 alpha,25-dihydroxyvitamin D3 by human dendritic cells.* Blood, 2003. **102**(9): p. 3314-6.
517. Jeffery, L., et al., *25-hydroxyvitamin D₃ conversion by dendritic cells and T cells drives 1,25-dihydroxyvitamin D₃ mediated anti-inflammatory CD4⁺ T cell responses.* Annals of the Rheumatic Diseases, 2011. **70**(Suppl 2): p. A45.
518. O'Kelly, J., et al., *Normal myelopoiesis but abnormal T lymphocyte responses in vitamin D receptor knockout mice.* J Clin Invest, 2002. **109**(8): p. 1091-9.
519. Sochorova, K., et al., *Paricalcitol (19-nor-1,25-dihydroxyvitamin D2) and calcitriol (1,25-dihydroxyvitamin D3) exert potent immunomodulatory effects on dendritic cells and inhibit induction of antigen-specific T cells.* Clin Immunol, 2009. **133**(1): p. 69-77.
520. Ferreira, G.B., et al., *1,25-Dihydroxyvitamin D3 promotes tolerogenic dendritic cells with functional migratory properties in NOD mice.* J Immunol, 2014. **192**(9): p. 4210-20.
521. Bruce, D., et al., *Converging pathways lead to overproduction of IL-17 in the absence of vitamin D signaling.* Int Immunol, 2011. **23**(8): p. 519-28.
522. Ooi, J.H., et al., *Vitamin D regulates the gut microbiome and protects mice from dextran sodium sulfate-induced colitis.* J Nutr, 2013. **143**(10): p. 1679-86.
523. Liu, N., et al., *Altered endocrine and autocrine metabolism of vitamin D in a mouse model of gastrointestinal inflammation.* Endocrinology, 2008. **149**(10): p. 4799-808.
524. Penna, G., et al., *1,25-Dihydroxyvitamin D3 selectively modulates tolerogenic properties in myeloid but not plasmacytoid dendritic cells.* J Immunol, 2007. **178**(1): p. 145-53.
525. Penna, G., et al., *Vitamin D receptor agonists in the treatment of autoimmune diseases: selective targeting of myeloid but not plasmacytoid dendritic cells.* J Bone Miner Res, 2007. **22 Suppl 2**: p. V69-73.
526. Ryynanen, J. and C. Carlberg, *Primary 1,25-dihydroxyvitamin D3 response of the interleukin 8 gene cluster in human monocyte- and macrophage-like cells.* PLoS One, 2013. **8**(10): p. e78170.
527. Maxwell, P.J., et al., *Potentiation of inflammatory CXCL8 signalling sustains cell survival in PTEN-deficient prostate carcinoma.* Eur Urol, 2013. **64**(2): p. 177-88.
528. Enioutina, E.Y., D. Bareyan, and R.A. Daynes, *TLR ligands that stimulate the metabolism of vitamin D3 in activated murine dendritic cells can function as effective mucosal adjuvants to subcutaneously administered vaccines.* Vaccine, 2008. **26**(5): p. 601-13.

529. Gauzzi, M.C., et al., *Suppressive effect of 1alpha,25-dihydroxyvitamin D3 on type I IFN-mediated monocyte differentiation into dendritic cells: impairment of functional activities and chemotaxis.* J Immunol, 2005. **174**(1): p. 270-6.
530. Corripio-Miyar, Y., et al., *1,25-Dihydroxyvitamin D3 modulates the phenotype and function of Monocyte derived dendritic cells in cattle.* BMC Veterinary Research, 2017. **13**(1): p. 390.
531. So, E.Y., T. Winchester, and T. Ouchi, *The screening of a microRNA expression during development of human macrophages and mouse dendritic cells.* Cancer Biology & Therapy, 2017. **18**(3): p. 152-157.
532. Turner, M.L., F.M. Schnorfeil, and T. Brocker, *MicroRNAs regulate dendritic cell differentiation and function.* J Immunol, 2011. **187**(8): p. 3911-7.
533. Dunand-Sauthier, I., et al., *Repression of arginase-2 expression in dendritic cells by microRNA-155 is critical for promoting T cell proliferation.* J Immunol, 2014. **193**(4): p. 1690-700.
534. Martinez-Nunez, R.T., et al., *MicroRNA-155 modulates the pathogen binding ability of dendritic cells (DCs) by down-regulation of DC-specific intercellular adhesion molecule-3 grabbing non-integrin (DC-SIGN).* J Biol Chem, 2009. **284**(24): p. 16334-42.
535. Ceppi, M., et al., *MicroRNA-155 modulates the interleukin-1 signaling pathway in activated human monocyte-derived dendritic cells.* Proc Natl Acad Sci U S A, 2009. **106**(8): p. 2735-40.
536. Zhou, H., et al., *miR-155 and its star-form partner miR-155* cooperatively regulate type I interferon production by human plasmacytoid dendritic cells.* Blood, 2010. **116**(26): p. 5885-94.
537. Zhang, M., et al., *Inhibition of microRNA let-7i depresses maturation and functional state of dendritic cells in response to lipopolysaccharide stimulation via targeting suppressor of cytokine signaling 1.* J Immunol, 2011. **187**(4): p. 1674-83.
538. Kuppusamy, K.T., et al., *Let-7 family of microRNA is required for maturation and adult-like metabolism in stem cell-derived cardiomyocytes.* Proc Natl Acad Sci U S A, 2015. **112**(21): p. E2785-94.
539. Lu, C., et al., *miR-221 and miR-155 regulate human dendritic cell development, apoptosis, and IL-12 production through targeting of p27kip1, KPC1, and SOCS-1.* Blood, 2011. **117**(16): p. 4293-303.
540. Sun, Y., et al., *PU.1-dependent transcriptional regulation of miR-142 contributes to its hematopoietic cell-specific expression and modulation of IL-6.* J Immunol, 2013. **190**(8): p. 4005-13.
541. Lu, C., et al., *miR-221 and miR-155 regulate human dendritic cell development, apoptosis, and IL-12 production through targeting of p27kip1, KPC1, and SOCS-1.* Blood, 2011. **117**(16): p. 4293-4303.
542. Li, Y.C., et al., *MicroRNA-mediated mechanism of vitamin D regulation of innate immune response.* J Steroid Biochem Mol Biol, 2014. **144 Pt A**: p. 81-6.
543. Liu, X., et al., *MicroRNA-148/152 impair innate response and antigen presentation of TLR-triggered dendritic cells by targeting CaMKIIalpha.* J Immunol, 2010. **185**(12): p. 7244-51.
544. Karrich, J.J., et al., *MicroRNA-146a regulates survival and maturation of human plasmacytoid dendritic cells.* Blood, 2013. **122**(17): p. 3001-9.
545. Bhaumik, D., et al., *MicroRNAs miR-146a/b negatively modulate the senescence-associated inflammatory mediators IL-6 and IL-8.* Aging, 2009. **1**(4): p. 402-411.
546. Park, H., et al., *MicroRNA-146a and microRNA-146b regulate human dendritic cell apoptosis and cytokine production by targeting TRAF6 and IRAK1 proteins.* J Biol Chem, 2015. **290**(5): p. 2831-41.

547. Lu, T.X., et al., *MicroRNA-21 limits in vivo immune response-mediated activation of the IL-12/IFN-gamma pathway, Th1 polarization, and the severity of delayed-type hypersensitivity.* J Immunol, 2011. **187**(6): p. 3362-73.
548. Xue, X., et al., *Microbiota downregulates dendritic cell expression of miR-10a, which targets IL-12/IL-23p40.* J Immunol, 2011. **187**(11): p. 5879-86.
549. Su, X., et al., *miRNomes of haematopoietic stem cells and dendritic cells identify miR-30b as a regulator of Notch1.* Nat Commun, 2013. **4**: p. 2903.
550. Riepsaame, J., et al., *MicroRNA-Mediated Down-Regulation of M-CSF Receptor Contributes to Maturation of Mouse Monocyte-Derived Dendritic Cells.* Front Immunol, 2013. **4**: p. 353.
551. Zheng, J., et al., *MicroRNA-23b promotes tolerogenic properties of dendritic cells in vitro through inhibiting Notch1/NF-kappaB signalling pathways.* Allergy, 2012. **67**(3): p. 362-70.
552. Dueck, A., et al., *A miR-155-dependent microRNA hierarchy in dendritic cell maturation and macrophage activation.* FEBS Lett, 2014. **588**(4): p. 632-40.
553. Vanherwegen, A.S., C. Gysemans, and C. Mathieu, *Vitamin D endocrinology on the cross-road between immunity and metabolism.* Mol Cell Endocrinol, 2017.
554. Ferreira, G.B., et al., *Vitamin D3 Induces Tolerance in Human Dendritic Cells by Activation of Intracellular Metabolic Pathways.* Cell Rep, 2015.
555. Tian, Y., L. Meng, and Y. Zhang, *Epigenetic Regulation of Dendritic Cell Development and Function.* Cancer J, 2017. **23**(5): p. 302-307.
556. Sen, C.K. and S. Roy, *MicroRNA 21 in tissue injury and inflammation.* Cardiovascular Research, 2012. **96**(2): p. 230-233.
557. Tang, B., et al., *MicroRNA-29a regulates lipopolysaccharide (LPS)-induced inflammatory responses in murine macrophages through the Akt1/ NF-kappaB pathway.* Exp Cell Res, 2017. **360**(2): p. 74-80.
558. Li, R., et al., *MiR-145 improves macrophage-mediated inflammation through targeting Arf6.* Endocrine, 2018. **60**(1): p. 73-82.
559. Roos, J., et al., *miR-146a-mediated suppression of the inflammatory response in human adipocytes.* Scientific Reports, 2016. **6**: p. 38339.
560. Ye, J., et al., *miR-155 Regulated Inflammation Response by the SOCS1-STAT3-PDCD4 Axis in Atherogenesis.* Mediators of Inflammation, 2016. **2016**: p. 14.
561. Padi, S.K.R., et al., *MicroRNA-627 Mediates the Epigenetic Mechanisms of Vitamin D to Suppress Proliferation of Human Colorectal Cancer Cells and Growth of Xenograft Tumors in Mice.* Gastroenterology, 2013. **145**(2): p. 437-446.
562. Wang, X., et al., *MicroRNA Let-7i negatively regulates cardiac inflammation and fibrosis.* Hypertension, 2015. **66**(4): p. 776-85.
563. Battault, S., et al., *Vitamin D metabolism, functions and needs: from science to health claims.* Eur J Nutr, 2013. **52**(2): p. 429-41.
564. Adams, J.S. and M. Hewison, *Update in vitamin D.* J Clin Endocrinol Metab, 2010. **95**(2): p. 471-8.
565. Baeke, F., et al., *Vitamin D: modulator of the immune system.* Curr Opin Pharmacol, 2010. **10**(4): p. 482-96.
566. Penna, G., et al., *Expression of the inhibitory receptor ILT3 on dendritic cells is dispensable for induction of CD4+Foxp3+ regulatory T cells by 1,25-dihydroxyvitamin D3.* Blood, 2005. **106**(10): p. 3490-7.
567. Almerighi, C., et al., *1Alpha,25-dihydroxyvitamin D3 inhibits CD40L-induced pro-inflammatory and immunomodulatory activity in human monocytes.* Cytokine, 2009. **45**(3): p. 190-7.
568. Landmann, R., et al., *Human monocyte CD14 is upregulated by lipopolysaccharide.* Infection and Immunity, 1996. **64**(5): p. 1762-1769.
569. Prietl, B., et al., *Vitamin D and Immune Function.* Nutrients, 2013. **5**(7): p. 2502-2521.

570. Kundu, R., et al., *Regulation of CYP27B1 and CYP24A1 hydroxylases limits cell-autonomous activation of vitamin D in dendritic cells.* Eur J Immunol, 2014. **44**(6): p. 1781-90.
571. Prosser, D.E. and G. Jones, *Enzymes involved in the activation and inactivation of vitamin D.* Trends in Biochemical Sciences, 2004. **29**(12): p. 664-673.
572. Zierold, C., H.M. Darwish, and H.F. DeLuca, *Two vitamin D response elements function in the rat 1,25-dihydroxyvitamin D 24-hydroxylase promoter.* J Biol Chem, 1995. **270**(4): p. 1675-8.
573. Costa, E.M., M.A. Hirst, and D. Feldman, *Regulation of 1,25-dihydroxyvitamin D3 receptors by vitamin D analogs in cultured mammalian cells.* Endocrinology, 1985. **117**(5): p. 2203-10.
574. Zella, L.A., et al., *Enhancers located within two introns of the vitamin D receptor gene mediate transcriptional autoregulation by 1,25-dihydroxyvitamin D3.* Mol Endocrinol, 2006. **20**(6): p. 1231-47.
575. Gracias, D.T. and P.D. Katsikis, *MicroRNAs: key components of immune regulation.* Adv Exp Med Biol, 2011. **780**: p. 15-26.
576. Wu, C.J. and L.F. Lu, *MicroRNA in Immune Regulation.* Curr Top Microbiol Immunol, 2017. **410**: p. 249-267.
577. O'Connell, R.M., et al., *MicroRNA-155 is induced during the macrophage inflammatory response.* Proc Natl Acad Sci U S A, 2007. **104**(5): p. 1604-9.
578. Tsitsiou, E. and M.A. Lindsay, *microRNAs and the immune response.* Current Opinion in Pharmacology, 2009. **9**(4): p. 514-520.
579. Du, F., et al., *MicroRNA-155 deficiency results in decreased macrophage inflammation and attenuated atherogenesis in apolipoprotein E-deficient mice.* Arterioscler Thromb Vasc Biol, 2014. **34**(4): p. 759-67.
580. Zhan, Y. and L. Wu, *Functional regulation of monocyte-derived dendritic cells by microRNAs.* Protein Cell, 2012. **3**(7): p. 497-507.
581. Busch, M. and A. Zernecke, *microRNAs in the regulation of dendritic cell functions in inflammation and atherosclerosis.* J Mol Med (Berl), 2012. **90**(8): p. 877-85.
582. Motta, V., et al., *Integrative Analysis of miRNA and Inflammatory Gene Expression After Acute Particulate Matter Exposure.* Toxicological Sciences, 2013. **132**(2): p. 307-316.
583. Holmstrom, K., et al., *Identification of a microRNA signature in dendritic cell vaccines for cancer immunotherapy.* Hum Immunol, 2010. **71**(1): p. 67-73.
584. Jin, P., et al., *Molecular signatures of maturing dendritic cells: implications for testing the quality of dendritic cell therapies.* J Transl Med, 2010. **8**: p. 4.
585. Rodriguez, A., et al., *Requirement of bic/microRNA-155 for normal immune function.* Science, 2007. **316**(5824): p. 608-11.
586. Brain, O., et al., *The intracellular sensor NOD2 induces microRNA-29 expression in human dendritic cells to limit IL-23 release.* Immunity, 2013. **39**(3): p. 521-36.
587. Tserel, L., et al., *MicroRNA Expression Profiles of Human Blood Monocyte-derived Dendritic Cells and Macrophages Reveal miR-511 as Putative Positive Regulator of Toll-like Receptor 4.* The Journal of Biological Chemistry, 2011. **286**(30): p. 26487-26495.
588. Kim, Y., et al., *Deletion of human tarbp2 reveals cellular microRNA targets and cell-cycle function of TRBP.* Cell Rep, 2014. **9**(3): p. 1061-74.
589. Chendrimada, T.P., et al., *TRBP recruits the Dicer complex to Ago2 for microRNA processing and gene silencing.* Nature, 2005. **436**(7051): p. 740-744.
590. Tserel, L., et al., *MicroRNA expression profiles of human blood monocyte-derived dendritic cells and macrophages reveal miR-511 as putative positive regulator of Toll-like receptor 4.* J Biol Chem, 2011. **286**(30): p. 26487-95.
591. Izzotti, A. and A. Pulliero, *The effects of environmental chemical carcinogens on the microRNA machinery.* Int J Hyg Environ Health, 2014. **217**(6): p. 601-27.

592. Park, J.E., et al., *Dicer recognizes the 5' end of RNA for efficient and accurate processing.* Nature, 2011. **475**(7355): p. 201-5.
593. Gantier, M.P., et al., *Analysis of microRNA turnover in mammalian cells following Dicer1 ablation.* Nucleic Acids Res, 2011. **39**(13): p. 5692-703.
594. Diederichs, S. and D.A. Haber, *Dual role for argonautes in microRNA processing and posttranscriptional regulation of microRNA expression.* Cell, 2007. **131**(6): p. 1097-108.
595. Cohen, I.R., *Activation of benign autoimmunity as both tumor and autoimmune disease immunotherapy: a comprehensive review.* J Autoimmun, 2014. **54**: p. 112-7.
596. Rosenberg, S.A., *Decade in review-cancer immunotherapy: entering the mainstream of cancer treatment.* Nat Rev Clin Oncol, 2014. **11**(11): p. 630-2.
597. Evans, C.J., et al., *Place and cause of death in centenarians: a population-based observational study in England, 2001 to 2010.* PLoS Med, 2014. **11**(6): p. e1001653.
598. Raphael, I., et al., *T cell subsets and their signature cytokines in autoimmune and inflammatory diseases.* Cytokine, 2015. **74**(1): p. 5-17.
599. Romagnani, S., *Th1/Th2 cells.* Inflamm Bowel Dis, 1999. **5**(4): p. 285-94.
600. Leipe, J., et al., *Role of Th17 cells in human autoimmune arthritis.* Arthritis Rheum, 2010. **62**(10): p. 2876-85.
601. Grant, C.R., et al., *Regulatory T-cells in autoimmune diseases: challenges, controversies and--yet--unanswered questions.* Autoimmun Rev, 2015. **14**(2): p. 105-16.
602. Salehi, Z., et al., *Differential Frequency of CD8+ T Cell Subsets in Multiple Sclerosis Patients with Various Clinical Patterns.* PloS one, 2016. **11**(7): p. e0159565-e0159565.
603. Thome, J.J.C., et al., *Spatial map of human T cell compartmentalization and maintenance over decades of life.* Cell, 2014. **159**(4): p. 814-828.
604. Coleman, M.M., et al., *The immunoregulatory role of CD4+FoxP3+CD25− regulatory T cells in lungs of mice infected with Bordetella pertussis.* FEMS Immunology & Medical Microbiology, 2012. **64**(3): p. 413-424.
605. Zelenay, S., et al., *Foxp3(+) CD25(–) CD4 T cells constitute a reservoir of committed regulatory cells that regain CD25 expression upon homeostatic expansion.* Proceedings of the National Academy of Sciences of the United States of America, 2005. **102**(11): p. 4091-4096.
606. Liang, S., et al., *Conversion of CD4(+) CD25(−) cells into CD4(+) CD25(+) regulatory T cells in vivo requires B7 costimulation, but not the thymus.* The Journal of Experimental Medicine, 2005. **201**(1): p. 127-137.
607. Chen, W., et al., *Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3.* J Exp Med, 2003. **198**(12): p. 1875-86.
608. Zhu, J., H. Yamane, and W.E. Paul, *Differentiation of effector CD4 T cell populations (*).* Annu Rev Immunol, 2010. **28**: p. 445-89.
609. Chen, W., et al., *Conversion of Peripheral CD4(+)CD25(−) Naive T Cells to CD4(+)CD25(+) Regulatory T Cells by TGF-β Induction of Transcription Factor Foxp3.* The Journal of Experimental Medicine, 2003. **198**(12): p. 1875-1886.
610. Tai, X., et al., *Basis of CTLA-4 function in regulatory and conventional CD4(+) T cells.* Blood, 2012. **119**(22): p. 5155-5163.
611. Sansom, D.M., *IMMUNOLOGY. Moving CTLA-4 from the trash to recycling.* Science (New York, N.Y.), 2015. **349**(6246): p. 377-378.
612. Asano, M., et al., *Autoimmune disease as a consequence of developmental abnormality of a T cell subpopulation.* J Exp Med, 1996. **184**(2): p. 387-96.
613. Sakaguchi, S., et al., *Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of*

self-tolerance causes various autoimmune diseases. J Immunol, 1995. **155**(3): p. 1151-64.
614. Li, M.O., S. Sanjabi, and R.A. Flavell, *Transforming growth factor-beta controls development, homeostasis, and tolerance of T cells by regulatory T cell-dependent and -independent mechanisms.* Immunity, 2006. **25**(3): p. 455-71.
615. Freeman, G.J., et al., *CTLA-4 and CD28 mRNA are coexpressed in most T cells after activation. Expression of CTLA-4 and CD28 mRNA does not correlate with the pattern of lymphokine production.* J Immunol, 1992. **149**(12): p. 3795-801.
616. Hori, S., T. Nomura, and S. Sakaguchi, *Control of regulatory T cell development by the transcription factor Foxp3.* Science, 2003. **299**(5609): p. 1057-61.
617. Jain, N., et al., *Dual function of CTLA-4 in regulatory T cells and conventional T cells to prevent multiorgan autoimmunity.* Proc Natl Acad Sci U S A, 2010. **107**(4): p. 1524-8.
618. Wing, K., et al., *CTLA-4 control over Foxp3+ regulatory T cell function.* Science, 2008. **322**(5899): p. 271-5.
619. Doyle, A.M., et al., *Induction of Cytotoxic T Lymphocyte Antigen 4 (Ctla-4) Restricts Clonal Expansion of Helper T Cells.* The Journal of Experimental Medicine, 2001. **194**(7): p. 893-902.
620. Suri-Payer, E., et al., *CD4+CD25+ T cells inhibit both the induction and effector function of autoreactive T cells and represent a unique lineage of immunoregulatory cells.* J Immunol, 1998. **160**(3): p. 1212-8.
621. Itoh, M., et al., *Thymus and autoimmunity: production of CD25+CD4+ naturally anergic and suppressive T cells as a key function of the thymus in maintaining immunologic self-tolerance.* J Immunol, 1999. **162**(9): p. 5317-26.
622. Joffre, O., et al., *Prevention of acute and chronic allograft rejection with CD4+CD25+Foxp3+ regulatory T lymphocytes.* Nat Med, 2008. **14**(1): p. 88-92.
623. Chen, X., et al., *CD4(+)CD25(+)FoxP3(+) regulatory T cells suppress Mycobacterium tuberculosis immunity in patients with active disease.* Clin Immunol, 2007. **123**(1): p. 50-9.
624. Shevach, E.M., *From vanilla to 28 flavors: multiple varieties of T regulatory cells.* Immunity, 2006. **25**(2): p. 195-201.
625. Collison, L.W., et al., *Regulatory T cell suppression is potentiated by target T cells in a cell contact, IL-35- and IL-10-dependent manner.* J Immunol, 2009. **182**(10): p. 6121-8.
626. Misra, N., et al., *Cutting edge: human CD4+CD25+ T cells restrain the maturation and antigen-presenting function of dendritic cells.* J Immunol, 2004. **172**(8): p. 4676-80.
627. Serra, P., et al., *CD40 ligation releases immature dendritic cells from the control of regulatory CD4+CD25+ T cells.* Immunity, 2003. **19**(6): p. 877-89.
628. Weaver, C.T., et al., *Th17: an effector CD4 T cell lineage with regulatory T cell ties.* Immunity, 2006. **24**(6): p. 677-88.
629. Bedoya, S.K., et al., *Isolation and th17 differentiation of naive CD4 T lymphocytes.* J Vis Exp, 2013(79): p. e50765.
630. Holderness, K., et al., *Validation of Th17 Cell Differentiation from Peripheral Blood CD4+ T Cells Through Assessment of mRNA Expression and Cytokine Secretion using Microplate Reading and Cellular Imaging.* The Journal of Immunology, 2016. **196**(1 Supplement): p. 209.12.
631. Park, H., et al., *A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17.* Nat Immunol, 2005. **6**(11): p. 1133-41.
632. Harrington, L.E., et al., *Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages.* Nat Immunol, 2005. **6**(11): p. 1123-32.

633. Bettelli, E., et al., *Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells.* Nature, 2006. **441**(7090): p. 235-8.
634. Veldhoen, M., et al., *TGFbeta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells.* Immunity, 2006. **24**(2): p. 179-89.
635. Yang, L., et al., *IL-21 and TGF-beta are required for differentiation of human T(H)17 cells.* Nature, 2008. **454**(7202): p. 350-2.
636. Mangan, P.R., et al., *Transforming growth factor-beta induces development of the T(H)17 lineage.* Nature, 2006. **441**(7090): p. 231-4.
637. Zhou, L., et al., *TGF-beta-induced Foxp3 inhibits T(H)17 cell differentiation by antagonizing RORgammat function.* Nature, 2008. **453**(7192): p. 236-40.
638. Korn, T., et al., *IL-21 initiates an alternative pathway to induce proinflammatory T(H)17 cells.* Nature, 2007. **448**(7152): p. 484-487.
639. Nurieva, R., et al., *Essential autocrine regulation by IL-21 in the generation of inflammatory T cells.* Nature, 2007. **448**(7152): p. 480-3.
640. Langrish, C.L., et al., *IL-23 drives a pathogenic T cell population that induces autoimmune inflammation.* J Exp Med, 2005. **201**(2): p. 233-40.
641. Sporri, R. and C. Reis e Sousa, *Inflammatory mediators are insufficient for full dendritic cell activation and promote expansion of CD4+ T cell populations lacking helper function.* Nat Immunol, 2005. **6**(2): p. 163-70.
642. Kalinski, P., et al., *T-cell priming by type-1 and type-2 polarized dendritic cells: the concept of a third signal.* Immunol Today, 1999. **20**(12): p. 561-7.
643. Thompson, C.B. and J.P. Allison, *The emerging role of CTLA-4 as an immune attenuator.* Immunity, 1997. **7**(4): p. 445-50.
644. Pentcheva-Hoang, T., et al., *B7-1 and B7-2 selectively recruit CTLA-4 and CD28 to the immunological synapse.* Immunity, 2004. **21**(3): p. 401-13.
645. Chuang, E., et al., *The CD28 and CTLA-4 receptors associate with the serine/threonine phosphatase PP2A.* Immunity, 2000. **13**(3): p. 313-22.
646. Marengere, L.E., et al., *Regulation of T cell receptor signaling by tyrosine phosphatase SYP association with CTLA-4.* Science, 1996. **272**(5265): p. 1170-3.
647. Schneider, H., et al., *Reversal of the TCR stop signal by CTLA-4.* Science, 2006. **313**(5795): p. 1972-5.
648. Linsley, P.S. and J.A. Ledbetter, *The role of the CD28 receptor during T cell responses to antigen.* Annu Rev Immunol, 1993. **11**: p. 191-212.
649. Jago, C.B., et al., *Differential expression of CTLA-4 among T cell subsets.* Clin Exp Immunol, 2004. **136**(3): p. 463-71.
650. Jeffery, L.E., et al., *Vitamin D Antagonises the Suppressive Effect of Inflammatory Cytokines on CTLA-4 Expression and Regulatory Function.* PLoS One, 2015. **10**(7): p. e0131539.
651. Jeffery, L.E., et al., *1,25-dihydroxyvitamin D(3) and interleukin-2 combine to inhibit T cell production of inflammatory cytokines and promote development of regulatory T cells expressing CTLA-4 and FoxP3.* Journal of immunology (Baltimore, Md. : 1950), 2009. **183**(9): p. 5458-5467.
652. Bhalla, A.K., et al., *Specific high-affinity receptors for 1,25-dihydroxyvitamin D3 in human peripheral blood mononuclear cells: presence in monocytes and induction in T lymphocytes following activation.* J Clin Endocrinol Metab, 1983. **57**(6): p. 1308-10.
653. Provvedini, D.M., et al., *1,25-dihydroxyvitamin D3 receptors in human leukocytes.* Science, 1983. **221**(4616): p. 1181-3.
654. Cross, H.S., T. Nittke, and E. Kallay, *Colonic vitamin D metabolism: implications for the pathogenesis of inflammatory bowel disease and colorectal cancer.* Mol Cell Endocrinol, 2011. **347**(1-2): p. 70-9.

655. O'Brien, K.O., et al., *Placental CYP27B1 and CYP24A1 expression in human placental tissue and their association with maternal and neonatal calcitropic hormones.* J Clin Endocrinol Metab, 2014. **99**(4): p. 1348-56.
656. Overbergh, L., et al., *Identification and immune regulation of 25-hydroxyvitamin D-1-alpha-hydroxylase in murine macrophages.* Clin Exp Immunol, 2000. **120**(1): p. 139-46.
657. Spach, K.M. and C.E. Hayes, *Vitamin D3 confers protection from autoimmune encephalomyelitis only in female mice.* J Immunol, 2005. **175**(6): p. 4119-26.
658. Hewison, M., *Vitamin D and immune function: autocrine, paracrine or endocrine?* Scand J Clin Lab Invest Suppl, 2012. **243**: p. 92-102.
659. Jeffery, L.E., et al., *Decreased sensitivity to 1,25-dihydroxyvitamin D3 in T cells from the rheumatoid joint.* Journal of Autoimmunity, 2018. **88**: p. 50-60.
660. Wucherpfennig, K.W. and D. Sethi, *T cell receptor recognition of self and foreign antigens in the induction of autoimmunity.* Semin Immunol, 2011. **23**(2): p. 84-91.
661. Hafler, D.A. and H.L. Weiner, *T cells in multiple sclerosis and inflammatory central nervous system diseases.* Immunol Rev, 1987. **100**: p. 307-32.
662. Cope, A.P., H. Schulze-Koops, and M. Aringer, *The central role of T cells in rheumatoid arthritis.* Clin Exp Rheumatol, 2007. **25**(5 Suppl 46): p. S4-11.
663. Chang, S.H., Y. Chung, and C. Dong, *Vitamin D suppresses Th17 cytokine production by inducing C/EBP homologous protein (CHOP) expression.* J Biol Chem, 2010. **285**(50): p. 38751-5.
664. Mayne, C.G., et al., *1,25-Dihydroxyvitamin D3 acts directly on the T lymphocyte vitamin D receptor to inhibit experimental autoimmune encephalomyelitis.* Eur J Immunol, 2011. **41**(3): p. 822-32.
665. Tiosano, D., et al., *The role of vitamin D receptor in innate and adaptive immunity: a study in hereditary vitamin D-resistant rickets patients.* J Clin Endocrinol Metab, 2013. **98**(4): p. 1685-93.
666. Khoo, A.L., et al., *Seasonal variation in vitamin D(3) levels is paralleled by changes in the peripheral blood human T cell compartment.* PLoS One, 2012. **7**(1): p. e29250.
667. Smolders, J., et al., *Vitamin D status is positively correlated with regulatory T cell function in patients with multiple sclerosis.* PLoS One, 2009. **4**(8): p. e6635.
668. Gorman, S., et al., *Topically applied 1,25-dihydroxyvitamin D3 enhances the suppressive activity of CD4+CD25+ cells in the draining lymph nodes.* J Immunol, 2007. **179**(9): p. 6273-83.
669. Muljo, S.A., et al., *Aberrant T cell differentiation in the absence of Dicer.* J Exp Med, 2005. **202**(2): p. 261-9.
670. Chen, C.Z., et al., *MicroRNAs modulate hematopoietic lineage differentiation.* Science, 2004. **303**(5654): p. 83-6.
671. Li, Q.J., et al., *miR-181a is an intrinsic modulator of T cell sensitivity and selection.* Cell, 2007. **129**(1): p. 147-61.
672. Swaminathan, S., et al., *Differential regulation of the Let-7 family of microRNAs in CD4+ T cells alters IL-10 expression.* J Immunol, 2012. **188**(12): p. 6238-46.
673. Teteloshvili, N., et al., *T-cell Activation Induces Dynamic Changes in miRNA Expression Patterns in CD4 and CD8 T-cell Subsets.* Microrna, 2015. **4**(2): p. 117-22.
674. Lu, L.F., et al., *Function of miR-146a in controlling Treg cell-mediated regulation of Th1 responses.* Cell, 2010. **142**(6): p. 914-29.
675. Grigoryev, Y.A., et al., *MicroRNA regulation of molecular networks mapped by global microRNA, mRNA, and protein expression in activated T lymphocytes.* J Immunol, 2011. **187**(5): p. 2233-43.
676. Carissimi, C., et al., *miR-21 is a negative modulator of T-cell activation.* Biochimie, 2014. **107**: p. 319-326.

677. Smigielska-Czepiel, K., et al., *Dual role of miR-21 in CD4+ T-cells: activation-induced miR-21 supports survival of memory T-cells and regulates CCR7 expression in naive T-cells.* PLoS One, 2013. **8**(10): p. e76217.
678. Marrack, P. and J. Kappler, *Control of T cell viability.* Annu Rev Immunol, 2004. **22**: p. 765-87.
679. Lorenzi, J.C., et al., *miR-15a and 16-1 are downregulated in CD4+ T cells of multiple sclerosis relapsing patients.* Int J Neurosci, 2012. **122**(8): p. 466-71.
680. Wu, H., et al., *miRNA profiling of naive, effector and memory CD8 T cells.* PLoS One, 2007. **2**(10): p. e1020.
681. Zhou, Q., et al., *miR-142-3p is involved in CD25+ CD4 T cell proliferation by targeting the expression of glycoprotein A repetitions predominant.* J Immunol, 2013. **190**(12): p. 6579-88.
682. Li, Z., et al., *IL-23 receptor regulation by Let-7f in human CD4+ memory T cells.* J Immunol, 2011. **186**(11): p. 6182-90.
683. Jindra, P.T., et al., *Costimulation-dependent expression of microRNA-214 increases the ability of T cells to proliferate by targeting Pten.* J Immunol, 2010. **185**(2): p. 990-7.
684. Stittrich, A.B., et al., *The microRNA miR-182 is induced by IL-2 and promotes clonal expansion of activated helper T lymphocytes.* Nat Immunol, 2010. **11**(11): p. 1057-62.
685. Jiang, S., et al., *Molecular dissection of the miR-17-92 cluster's critical dual roles in promoting Th1 responses and preventing inducible Treg differentiation.* Blood, 2011. **118**(20): p. 5487-97.
686. Lykken, E.A. and Q.J. Li, *microRNAs at the regulatory frontier: an investigation into how microRNAs impact the development and effector functions of CD4 T cells.* Immunol Res, 2011. **49**(1-3): p. 87-96.
687. Xiao, C., et al., *Lymphoproliferative disease and autoimmunity in mice with increased miR-17-92 expression in lymphocytes.* Nat Immunol, 2008. **9**(4): p. 405-14.
688. Fayyad-Kazan, H., et al., *MicroRNA profile of circulating CD4-positive regulatory T cells in human adults and impact of differentially expressed microRNAs on expression of two genes essential to their function.* J Biol Chem, 2012. **287**(13): p. 9910-22.
689. Takahashi, H., et al., *TGF-beta and retinoic acid induce the microRNA miR-10a, which targets Bcl-6 and constrains the plasticity of helper T cells.* Nat Immunol, 2012. **13**(6): p. 587-95.
690. Yamamoto, M., et al., *miR-155, a Modulator of FOXO3a Protein Expression, Is Underexpressed and Cannot Be Upregulated by Stimulation of HOZOT, a Line of Multifunctional Treg.* PLoS One, 2011. **6**(2): p. e16841.
691. Luckheeram, R.V., et al., *CD4(+)T Cells: Differentiation and Functions.* Clinical and Developmental Immunology, 2012. **2012**: p. 925135.
692. Tao, X., et al., *Strength of TCR signal determines the costimulatory requirements for Th1 and Th2 CD4+ T cell differentiation.* J Immunol, 1997. **159**(12): p. 5956-63.
693. Ashkar, S., et al., *Eta-1 (osteopontin): an early component of type-1 (cell-mediated) immunity.* Science, 2000. **287**(5454): p. 860-4.
694. Maddur, M.S., et al., *Th17 cells: biology, pathogenesis of autoimmune and inflammatory diseases, and therapeutic strategies.* Am J Pathol, 2012. **181**(1): p. 8-18.
695. Mahon, B.D., et al., *The targets of vitamin D depend on the differentiation and activation status of CD4 positive T cells.* J Cell Biochem, 2003. **89**(5): p. 922-32.
696. Zeitelhofer, M., et al., *Functional genomics analysis of vitamin D effects on CD4+ T cells in vivo in experimental autoimmune encephalomyelitis.* Proc Natl Acad Sci U S A, 2017. **114**(9): p. E1678-e1687.

697. Seuter, S., A. Neme, and C. Carlberg, *Epigenome-wide effects of vitamin D and their impact on the transcriptome of human monocytes involve CTCF.* Nucleic Acids Res, 2016. **44**(9): p. 4090-104.
698. Spolski, R., et al., *IL-21 signaling is critical for the development of type I diabetes in the NOD mouse.* Proceedings of the National Academy of Sciences, 2008. **105**(37): p. 14028.
699. McGuire, Helen M., et al., *A Subset of Interleukin-21+ Chemokine Receptor CCR9+ T Helper Cells Target Accessory Organs of the Digestive System in Autoimmunity.* Immunity, 2011. **34**(4): p. 602-615.
700. Ye, Z., et al., *IL-10 has a distinct immunoregulatory effect on naive and active T cell subsets.* J Interferon Cytokine Res, 2007. **27**(12): p. 1031-8.
701. Maynard, C.L. and C.T. Weaver, *Diversity in the contribution of IL-10 to T-cell-mediated immune regulation.* Immunological reviews, 2008. **226**: p. 219-233.
702. Brooks, D.G., et al., *IL-10 directly suppresses CD4 but not CD8 T cell effector and memory responses following acute viral infection.* Proceedings of the National Academy of Sciences, 2010. **107**(7): p. 3018.
703. Ma, Y.-h., et al., *Vitamin D Deficiency Contributes to the Reduction and Impaired Function of Naïve CD45RA(+) Regulatory T Cell in Chronic Heart Failure.* Journal of Immunology Research, 2015. **2015**: p. 547697.
704. Jankovic, D., D.G. Kugler, and A. Sher, *IL-10 production by CD4(+) effector T cells: a mechanism for self-regulation.* Mucosal immunology, 2010. **3**(3): p. 239-246.
705. Akdis, C.A. and K. Blaser, *Mechanisms of interleukin-10-mediated immune suppression.* Immunology, 2001. **103**(2): p. 131-136.
706. Bruce, D., et al., *Converging pathways lead to overproduction of IL-17 in the absence of vitamin D signaling.* International Immunology, 2011. **23**(8): p. 519-528.
707. Ferrari-Lacraz, S., et al., *IL-21 promotes survival and maintains a naive phenotype in human CD4+ T lymphocytes.* International Immunology, 2008. **20**(8): p. 1009-1018.
708. Froicu, M., Y. Zhu, and M.T. Cantorna, *Vitamin D receptor is required to control gastrointestinal immunity in IL-10 knockout mice.* Immunology, 2006. **117**(3): p. 310-318.
709. Olson, K.C., et al., *Vitamin D decreases STAT phosphorylation and inflammatory cytokine output in T-LGL leukemia.* Cancer Biology & Therapy, 2017. **18**(5): p. 290-303.
710. Murray, P.J., *The primary mechanism of the IL-10-regulated antiinflammatory response is to selectively inhibit transcription.* Proceedings of the National Academy of Sciences of the United States of America, 2005. **102**(24): p. 8686.
711. Jones, L.L., et al., *Differential T Cell Cytokine Receptivity and Not Signal Quality Distinguishes IL-6 and IL-10 Signaling during Th17 Differentiation.* The Journal of Immunology, 2016. **196**(7): p. 2973.
712. Wang, X.B., et al., *Regulation of surface and intracellular expression of CTLA-4 on human peripheral T cells.* Scand J Immunol, 2001. **54**(5): p. 453-8.
713. Chan, D.V., et al., *Differential CTLA-4 expression in human CD4(+) versus CD8(+) T cells is associated with increased NFAT1 and inhibition of CD4(+) proliferation.* Genes and immunity, 2014. **15**(1): p. 25-32.
714. Mir, M.A., *Chapter 1 - Introduction to Costimulation and Costimulatory Molecules*, in *Developing Costimulatory Molecules for Immunotherapy of Diseases*, M.A. Mir, Editor. 2015, Academic Press. p. 1-43.
715. Jain, N., et al., *Dual function of CTLA-4 in regulatory T cells and conventional T cells to prevent multiorgan autoimmunity.* Proceedings of the National Academy of Sciences, 2010. **107**(4): p. 1524.
716. Iida, T., et al., *Regulation of Cell Surface Expression of CTLA-4 by Secretion of CTLA-4-Containing Lysosomes Upon Activation of CD4⁺ T Cells.* The Journal of Immunology, 2000. **165**(9): p. 5062.

717. Lindsten, T., et al., *Characterization of CTLA-4 structure and expression on human T cells.* J Immunol, 1993. **151**(7): p. 3489-99.
718. Sansom, D.M., *CD28, CTLA-4 and their ligands: who does what and to whom?* Immunology, 2000. **101**(2): p. 169-77.
719. Alegre, M.L., et al., *Regulation of surface and intracellular expression of CTLA4 on mouse T cells.* J Immunol, 1996. **157**(11): p. 4762-70.
720. Gaffen, S.L., *Role of IL-17 in the Pathogenesis of Rheumatoid Arthritis.* Current rheumatology reports, 2009. **11**(5): p. 365-370.
721. Kongsbak, M., et al., *Vitamin D-binding protein controls T cell responses to vitamin D.* BMC Immunology, 2014. **15**(1): p. 35.
722. Hawthorne, G., et al., *Vitamin D supplementation in health and latent tuberculosis promotes regulatory T cell expression.* European Respiratory Journal, 2016. **48**(suppl 60).
723. Matsui, T., et al., *1,25-Dihydroxyvitamin D₃-regulated Expression of Genes Involved in Human T-Lymphocyte Proliferation and Differentiation.* Cancer Research, 1986. **46**(11): p. 5827.
724. Rigby, W.F., S. Denome, and M.W. Fanger, *Regulation of lymphokine production and human T lymphocyte activation by 1,25-dihydroxyvitamin D3. Specific inhibition at the level of messenger RNA.* J Clin Invest, 1987. **79**(6): p. 1659-64.
725. Reichel, H., et al., *1 alpha,25-Dihydroxyvitamin D3 inhibits gamma-interferon synthesis by normal human peripheral blood lymphocytes.* Proceedings of the National Academy of Sciences, 1987. **84**(10): p. 3385.
726. Fabri, M., et al., *Vitamin D is required for IFN-gamma-mediated antimicrobial activity of human macrophages.* Sci Transl Med, 2011. **3**(104): p. 104ra102.
727. Cippitelli, M. and A. Santoni, *Vitamin D3: a transcriptional modulator of the interferon-gamma gene.* Eur J Immunol, 1998. **28**(10): p. 3017-30.
728. Spanier, J.A., et al., *The Ifng Gene Is Essential for Vdr Gene Expression and Vitamin D₃-Mediated Reduction of the Pathogenic T Cell Burden in the Central Nervous System in Experimental Autoimmune Encephalomyelitis, a Multiple Sclerosis Model.* The Journal of Immunology, 2012. **189**(6): p. 3188.
729. Linsley, P.S., et al., *Intracellular trafficking of CTLA-4 and focal localization towards sites of TCR engagement.* Immunity, 1996. **4**(6): p. 535-43.
730. Egen, J.G. and J.P. Allison, *Cytotoxic T lymphocyte antigen-4 accumulation in the immunological synapse is regulated by TCR signal strength.* Immunity, 2002. **16**(1): p. 23-35.
731. Sun, S., et al., *Type I Interferon-mediated Stimulation of T Cells by CpG DNA.* The Journal of Experimental Medicine, 1998. **188**(12): p. 2335-2342.
732. Maszyna, F., et al., *Diversity of Clonal T Cell Proliferation Is Mediated by Differential Expression of CD152 (CTLA-4) on the Cell Surface of Activated Individual T Lymphocytes.* The Journal of Immunology, 2003. **171**(7): p. 3459.
733. Powell, J.D., et al., *Molecular regulation of interleukin-2 expression by CD28 co-stimulation and anergy.* Immunol Rev, 1998. **165**: p. 287-300.
734. Scheffold, A., et al., *High-sensitivity immunofluorescence for detection of the pro- and anti-inflammatory cytokines gamma interferon and interleukin-10 on the surface of cytokine-secreting cells.* Nat Med, 2000. **6**(1): p. 107-10.
735. Zeitelhofer, M., et al., *Functional genomics analysis of vitamin D effects on CD4+ T cells in vivo in experimental autoimmune encephalomyelitis .* Proceedings of the National Academy of Sciences of the United States of America, 2017. **114**(9): p. E1678-E1687.
736. Jorde, R., et al., *Plasma profile of microRNA after supplementation with high doses of vitamin D(3) for 12 months.* BMC Research Notes, 2012. **5**: p. 245-245.

737. Padi, S.K., et al., *MicroRNA-627 mediates the epigenetic mechanisms of vitamin D to suppress proliferation of human colorectal cancer cells and growth of xenograft tumors in mice.* Gastroenterology, 2013. **145**(2): p. 437-46.
738. Sun, M., et al., *Vitamin D Enhances the Efficacy of Irinotecan through miR-627-mediated Inhibition of Intratumoral Drug Metabolism.* Molecular cancer therapeutics, 2016. **15**(9): p. 2086-2095.
739. Shin, V.Y., et al., *A three-miRNA signature as promising non-invasive diagnostic marker for gastric cancer.* Mol Cancer, 2015. **14**: p. 202.
740. Hao, B., et al., *Bioinformatic analysis of microRNA expression in Parkinson's disease.* Mol Med Rep, 2015. **11**(2): p. 1079-84.
741. Meng, X., et al., *Genetic and epigenetic down-regulation of microRNA-212 promotes colorectal tumor metastasis via dysregulation of MnSOD.* Gastroenterology, 2013. **145**(2): p. 426-36.e1-6.
742. Balzano, F., et al., *MicroRNA Expression Analysis of Centenarians and Rheumatoid Arthritis Patients Reveals a Common Expression Pattern.* Int J Med Sci, 2017. **14**(7): p. 622-628.
743. Calin, G.A., et al., *Frequent deletions and down-regulation of micro- RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia.* Proc Natl Acad Sci U S A, 2002. **99**(24): p. 15524-9.
744. Calin, G.A., et al., *A MicroRNA signature associated with prognosis and progression in chronic lymphocytic leukemia.* N Engl J Med, 2005. **353**(17): p. 1793-801.
745. Cimmino, A., et al., *miR-15 and miR-16 induce apoptosis by targeting BCL2.* Proc Natl Acad Sci U S A, 2005. **102**(39): p. 13944-9.
746. Garzon, R., et al., *MicroRNA gene expression during retinoic acid-induced differentiation of human acute promyelocytic leukemia.* Oncogene, 2007. **26**(28): p. 4148-57.
747. Wang, S., et al., *MicroRNA-146a Feedback Suppresses T Cell Immune Function by Targeting Stat1 in Patients with Chronic Hepatitis B.* The Journal of Immunology, 2013. **191**(1): p. 293.
748. Ye, E.A. and J.J. Steinle, *miR-146a Attenuates Inflammatory Pathways Mediated by TLR4/NF-kappaB and TNFalpha to Protect Primary Human Retinal Microvascular Endothelial Cells Grown in High Glucose.* Mediators Inflamm, 2016. **2016**: p. 3958453.
749. Wu, S., et al., *miR-146a facilitates replication of dengue virus by dampening interferon induction by targeting TRAF6.* J Infect, 2013. **67**(4): p. 329-41.
750. Wang, G., et al., *Expression of miR-146a and miR-155 in the urinary sediment of systemic lupus erythematosus.* Clin Rheumatol, 2012. **31**(3): p. 435-40.
751. Rasmussen, T.K., et al., *Overexpression of microRNA-155 increases IL-21 mediated STAT3 signaling and IL-21 production in systemic lupus erythematosus.* Arthritis Res Ther, 2015. **17**: p. 154.
752. Kurowska-Stolarska, M., et al., *MicroRNA-155 as a proinflammatory regulator in clinical and experimental arthritis.* Proc Natl Acad Sci U S A, 2011. **108**(27): p. 11193-8.
753. Gracias, D.T., et al., *The microRNA miR-155 controls CD8(+) T cell responses by regulating interferon signaling.* Nat Immunol, 2013. **14**(6): p. 593-602.
754. Alhassan Mohammed, H., et al., *Immunoregulation of Inflammatory and Inhibitory Cytokines by Vitamin D3 in Patients with Inflammatory Bowel Diseases.* Scandinavian Journal of Immunology, 2017. **85**(6): p. 386-394.
755. Huang, J., et al., *1,25-Dihydroxyvitamin D(3) Does Not Affect MicroRNA Expression When Suppressing Human Th17 Differentiation.* Medical Science Monitor : International Medical Journal of Experimental and Clinical Research, 2017. **23**: p. 535-541.

756. Abdelbaset-Ismail, A., et al., *Vitamin D3 stimulates embryonic stem cells but inhibits migration and growth of ovarian cancer and teratocarcinoma cell lines.* J Ovarian Res, 2016. **9**: p. 26.
757. Becklund, B.R., D.W. Hansen, Jr., and H.F. Deluca, *Enhancement of 1,25-dihydroxyvitamin D3-mediated suppression of experimental autoimmune encephalomyelitis by calcitonin.* Proc Natl Acad Sci U S A, 2009. **106**(13): p. 5276-81.
758. Adams, J.S., et al., *Vitamin D in Defense of the Human Immune Response.* Ann N Y Acad Sci, 2007.

CHAPTER 9. APPENDICES

9.1 Publications arising from this thesis

Danyang Li, Louisa E. Jeffery, Carl Jenkinson, Stephanie R. Harrison, Rene F. Chun, John S. Adams, Karim Raza, Martin Hewison. Serum and synovial fluid vitamin D metabolites and rheumatoid arthritis. The Journal of Steroid Biochemistry and Molecular Biology [278]

Martin Hewison; Stephanie R. Harrison; **Danyang Li**; Louisa E. Jeffery; Karim Raza. Vitamin D, autoimmune disease and rheumatoid arthritis. Calcified Tissues International CTIN (Review). Submitted to Springer Journals Editorial Office 12/12/2018

9.2 Chapter 4 Figures 4.20-4.26

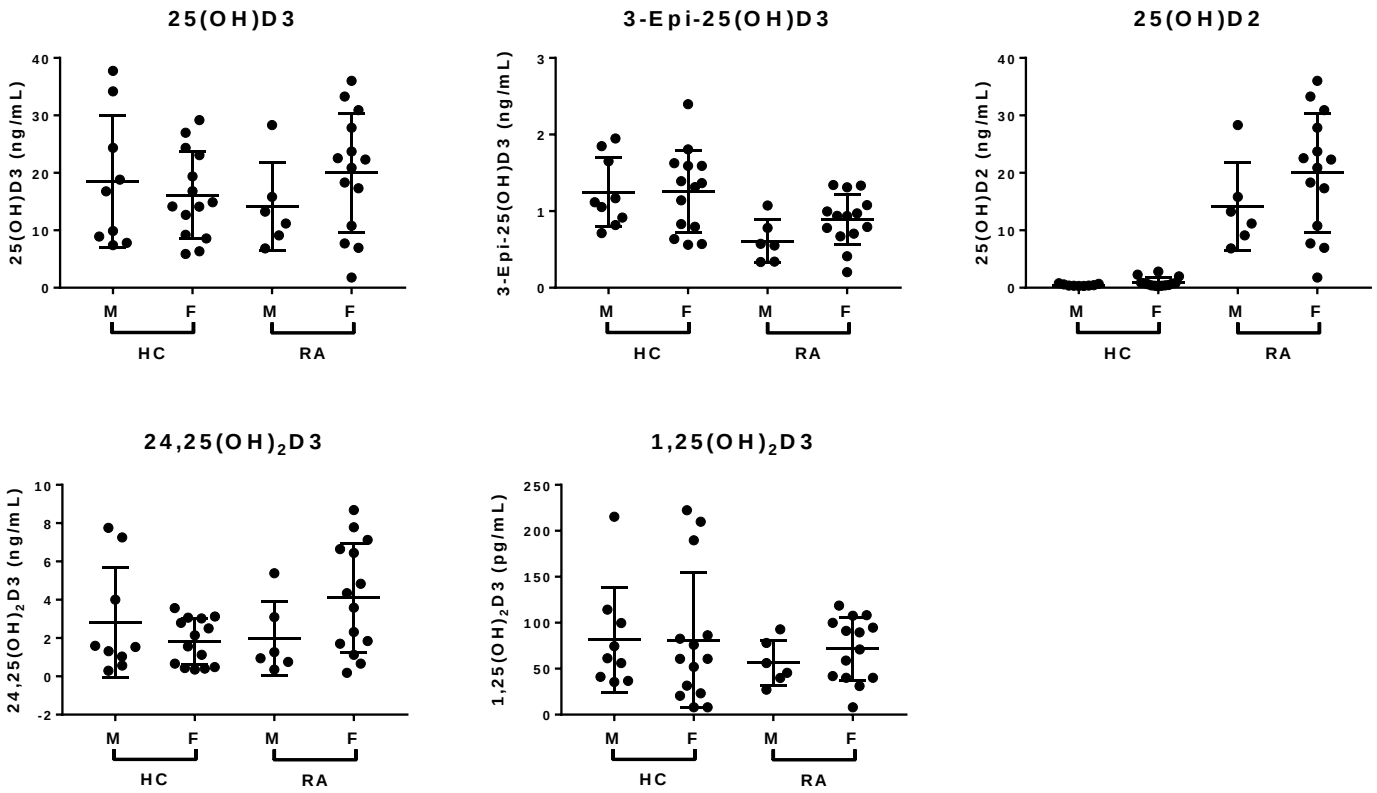


Figure 4.20. Relationship between serum vitamin D metabolites and patient sex
Serum vitamin D metabolites 25(OH)D3, 3-epi-25(OH)D3, 25(OH)D2, 24,25$(OH)_2$D3 (all ng/mL) and 1,25$(OH)_2$D3 (pg/mL) concentrations compared to patient sex. Unpaired *t*-test for non-parametric data was performed between male and females from the same cohort. Unpaired non-parametric Kolmogorov-Smirnov test was performed between sex-matched HC and RA patients. Significance taken as *p<0.05.

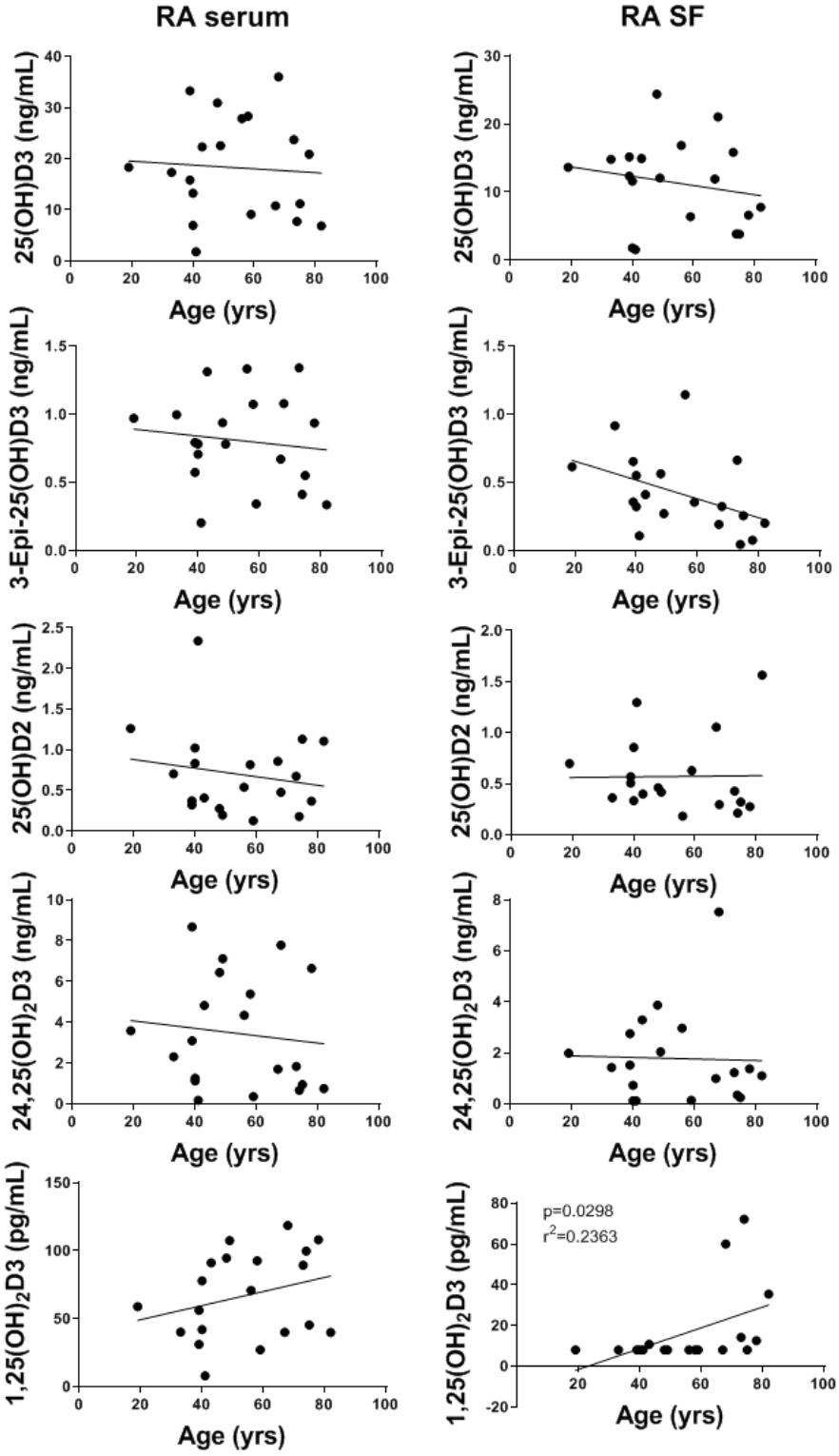


Figure 4.21. Relationship between serum and SF vitamin D metabolites and patient age

Serum and SF vitamin D metabolites 25(OH)D3, 3-epi-25(OH)D3, 25(OH)D2, $24,25(OH)_2D3$ (all ng/mL) and $1,25(OH)_2D3$ (pg/mL) concentrations correlated to patient age. Linear regression was performed by best-fit values ± SD. Coefficient of determination (r^2) values are shown for correlations that were statistically significant, **p*<0.05.

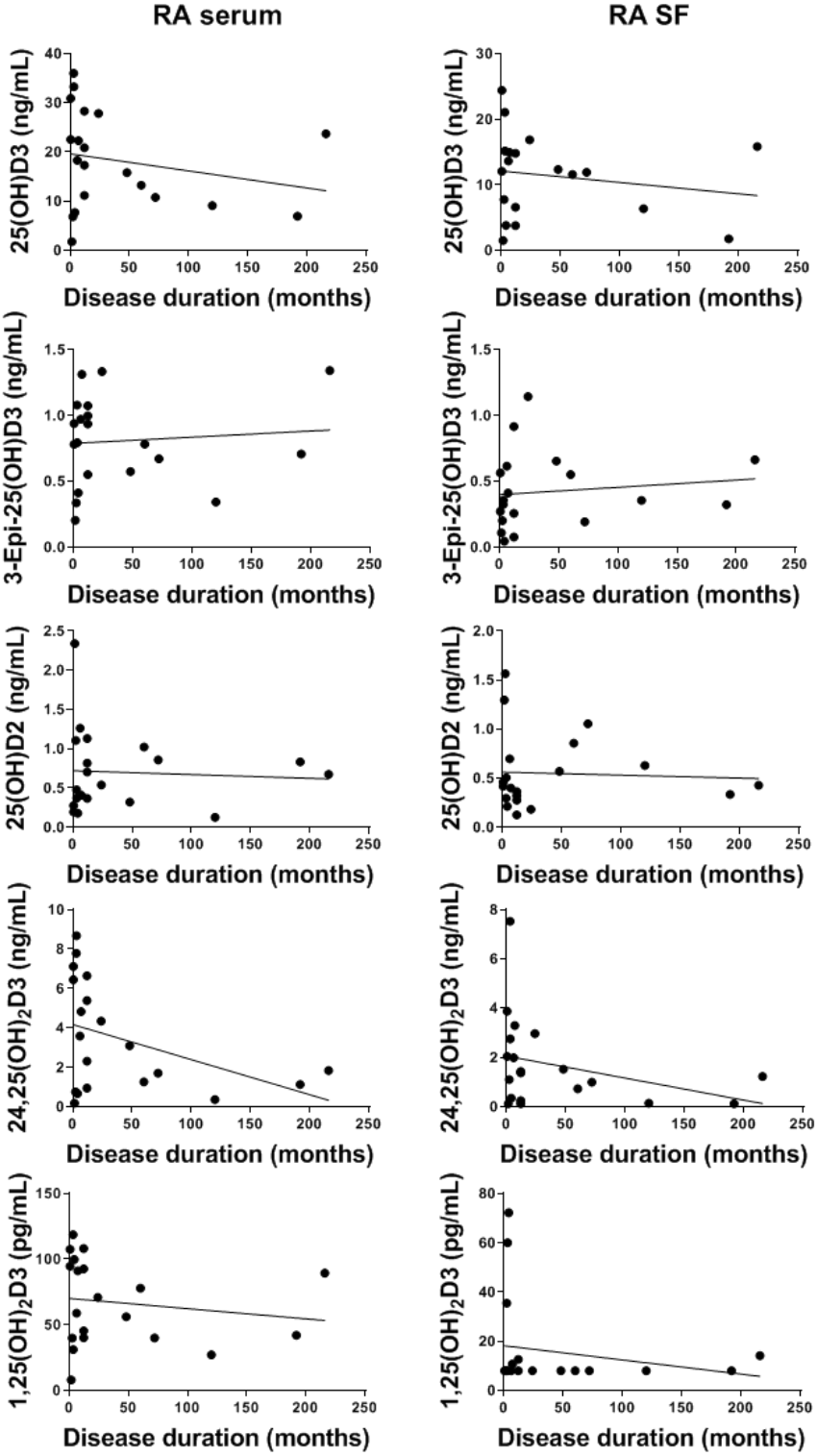


Figure 4.22. Relationship between serum and SF vitamin D metabolites and RA disease duration

Serum and SF vitamin D metabolites 25(OH)D3, 3-epi-25(OH)D3, 25(OH)D2, $24,25(OH)_2D3$ (all ng/mL) and $1,25(OH)_2D3$ (pg/mL) concentrations correlated to RA patient disease duration (months). Linear regression was performed by best-fit values ± SD. Coefficient of determination (r^2) values are shown for correlations that were statistically significant, **p*<0.05.

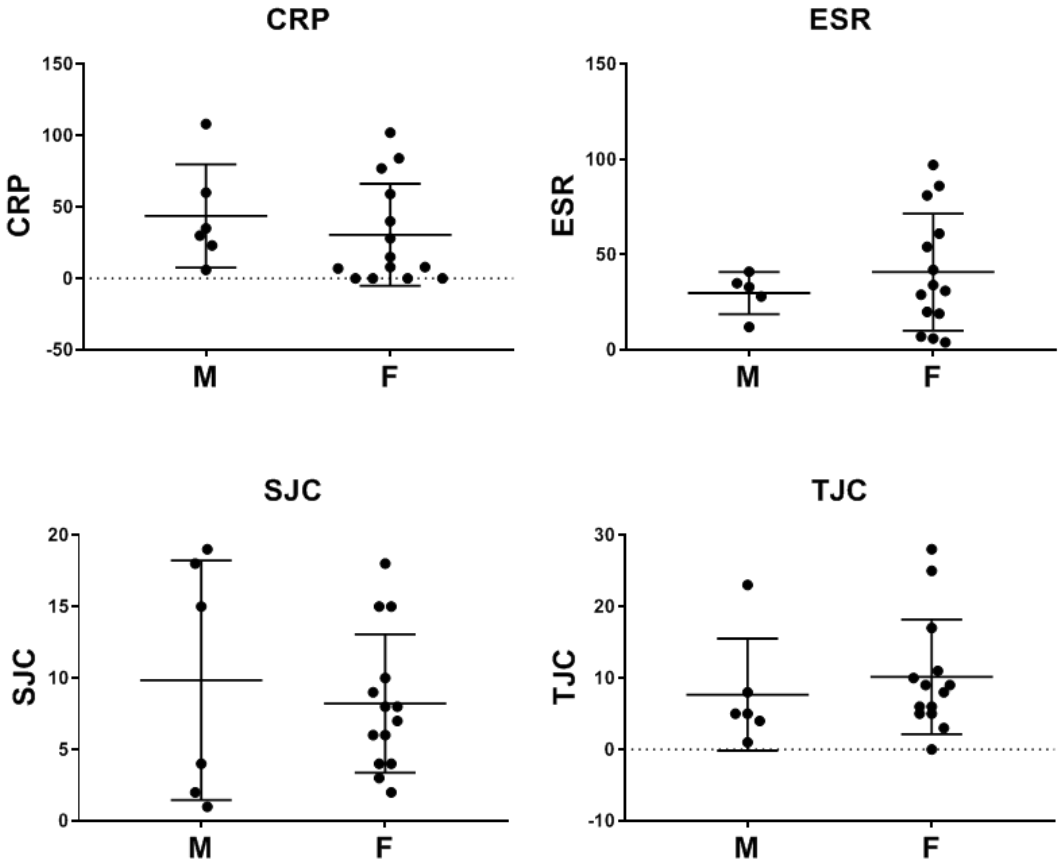


Figure 4.23. RA disease activity markers in male vs female RA
Comparisons between RA activity markers CRP, ESR, SJC and TJC in male vs female RA patients. Kolmogorov-Smirnov unpaired test for non-parametric data was performed between male and females. Significance taken as *p<0.05.

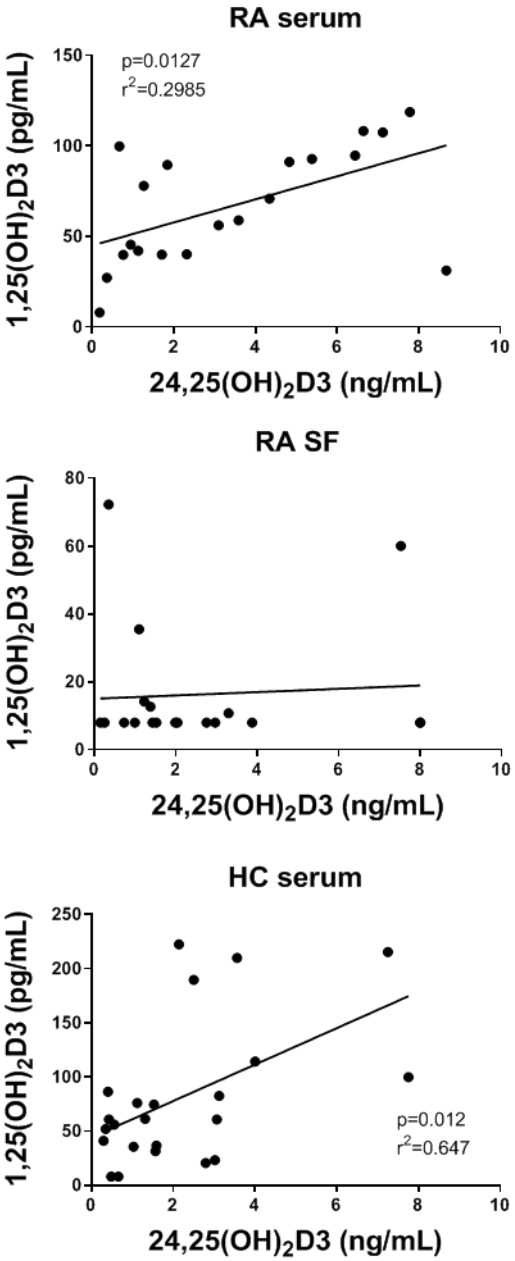


Figure 4.24. Vitamin D metabolite correlations in RA and HC

Correlation between 25(OH)D3 and other vitamin D metabolites in serum and synovial fluid (SF) from persistent rheumatoid arthritis (RA) patients. 25(OH)D3 vs A) 3-epi-25(OH)D3; B) 25(OH)D2; C) 24,25$(OH)_2$D3 (all ng/mL); D) 1,25$(OH)_2$D3 (pg/mL); E) linear correlation between 24,25$(OH)_2$D3 and 1,25$(OH)_2$D3 in RA; F) linear correlation between 24,25$(OH)_2$D3 and 1,25$(OH)_2$D3 in HC . Significance is taken as $*p<0.05$. Linear regression graphs performed by best-fit values ± SD. P and r^2 significance values shown where significance is determined.

			Serum			SF		
Patient	DBP (uM)	Albumin (uM)	Total 25(OH)D3 (nM)	free 25(OH)D3 (nM)	Bioavailable 25(OH)D3 (nM)	Total 25(OH)D3 (nM)	free 25(OH)D3 (nM)	Bioavailable 25(OH)D3 (nM)
M	0.96	249.04	37.59	0.02	2.64	32.46	0.04	6.07
F	0.75	301.50	51.28	0.02	4.13	37.29	0.05	9.85
M	0.13	202.61	32.07	0.02	3.68	10.89	0.05	6.39
F	0.66	372.85	47.50	0.03	3.90	40.17	0.06	13.50
F	0.11	192.39	21.16	0.01	2.80	6.06	0.03	3.63
F	0.54	207.73	74.16	0.03	6.26	45.38	0.09	11.80
F	0.83	197.50	59.96	0.08	26.10	39.29	0.06	6.94
M	0.49	192.39	23.92	0.02	2.81	18.34	0.04	4.71
F	0.75	254.25	10.83	0.01	1.43	7.29	0.01	1.66
F	1.03	275.16	30.76	0.02	3.42	32.81	0.04	6.26
F	0.40	228.32	64.26	0.05	13.37	42.25	0.11	14.50
F	1.38	182.21	93.74	0.08	15.94	54.14	0.05	5.73
M	0.43	254.25	20.66	0.02	2.56	23.79	0.05	8.20
M	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
F	0.72	187.30	55.35	0.06	8.91	17.33	0.03	3.22
F	0.44	202.61	20.72	0.01	2.83	10.13	0.02	2.91
F	0.63	207.73	85.97	0.04	12.79	40.00	0.07	9.24
M	0.91	192.39	41.73	0.02	3.25	33.86	0.05	5.38
F	1.59	207.73	80.23	0.03	5.74	63.51	0.05	6.66
F	0.30	192.39	58.71	0.04	6.44	31.81	0.10	11.70
M	0.92	360.29	75.48	0.07	11.81	56.98	0.07	14.90
M	0.90	269.91	29.34	0.02	5.00	15.59	0.02	3.23
M	0.26	238.66	18.75	0.01	1.96	2.29	0.01	1.01
M	0.74	249.04	13.33	0.01	1.80	10.98	0.02	2.49
M	1.33	243.85	24.64	0.02	3.31	17.16	0.02	2.37
M	0.87	233.48	86.91	0.06	23.92	64.02	0.09	12.60
M	1.47	349.52	35.98	0.02	2.70	31.75	0.03	5.48

Figure 4.25. Total vs free vs bioavailable 25(OH)D3 in RA and ReA serum and SF
Calculations of free (not bound to DBP or albumin) and bioavailable (not bound to DBP) 25(OH)D3 based on total 25(O)D3 concentrations, DBP and albumin, in RA and ReA serum and SF. DBP and albumin expressed as uM; total, free and bioavailable 25(OH)D3 expressed as nM. RA=blue (n=20); ReA=green (n=7)

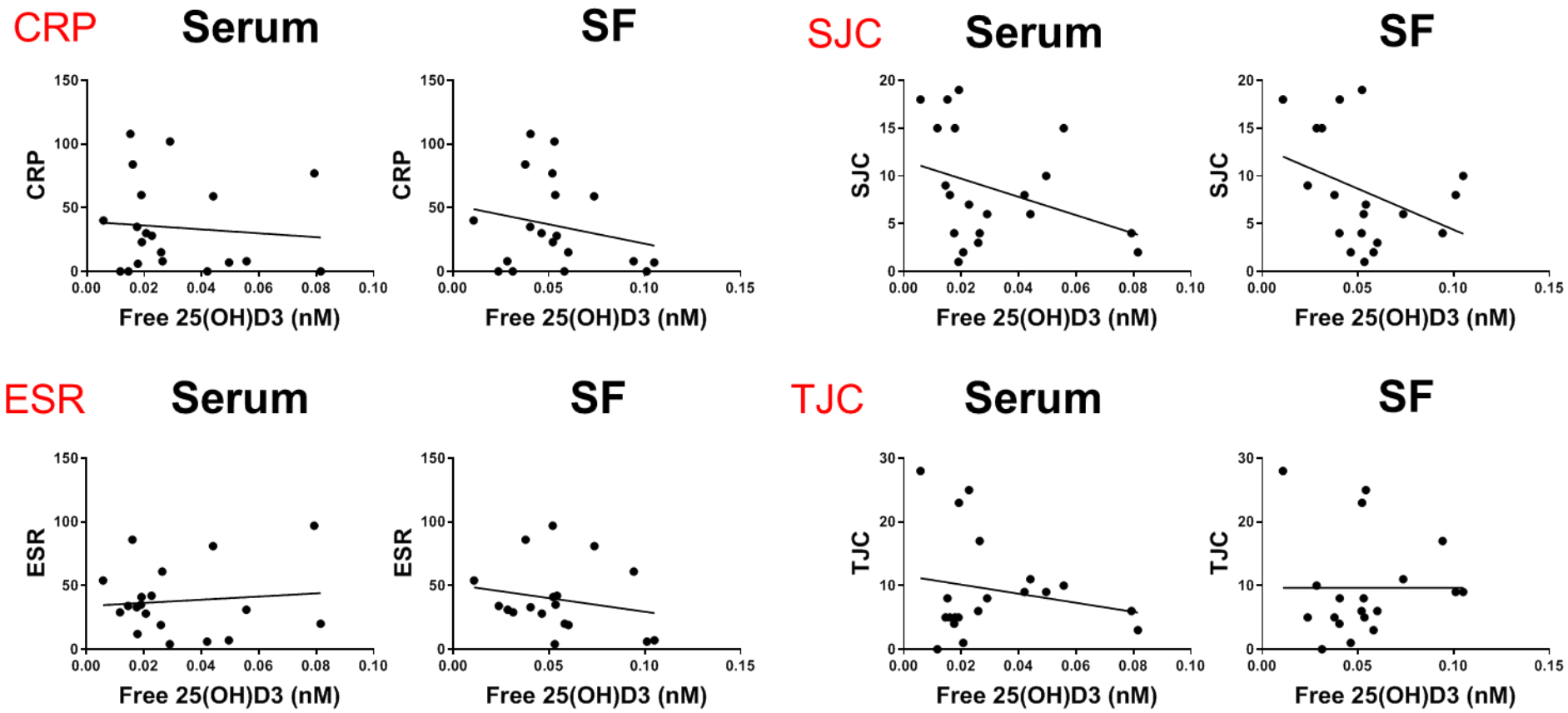


Figure 4.26A. Free 25(OH)D3 and RA disease activity markers
Correlations between free 25(OH)D3 and RA disease activity markers CRP, ESR, SJC and TJC. Linear regression was performed by best-fit values ± SD. Coefficient of determination (r^2) values are only shown for correlations that were statistically significant, *p<0.05.

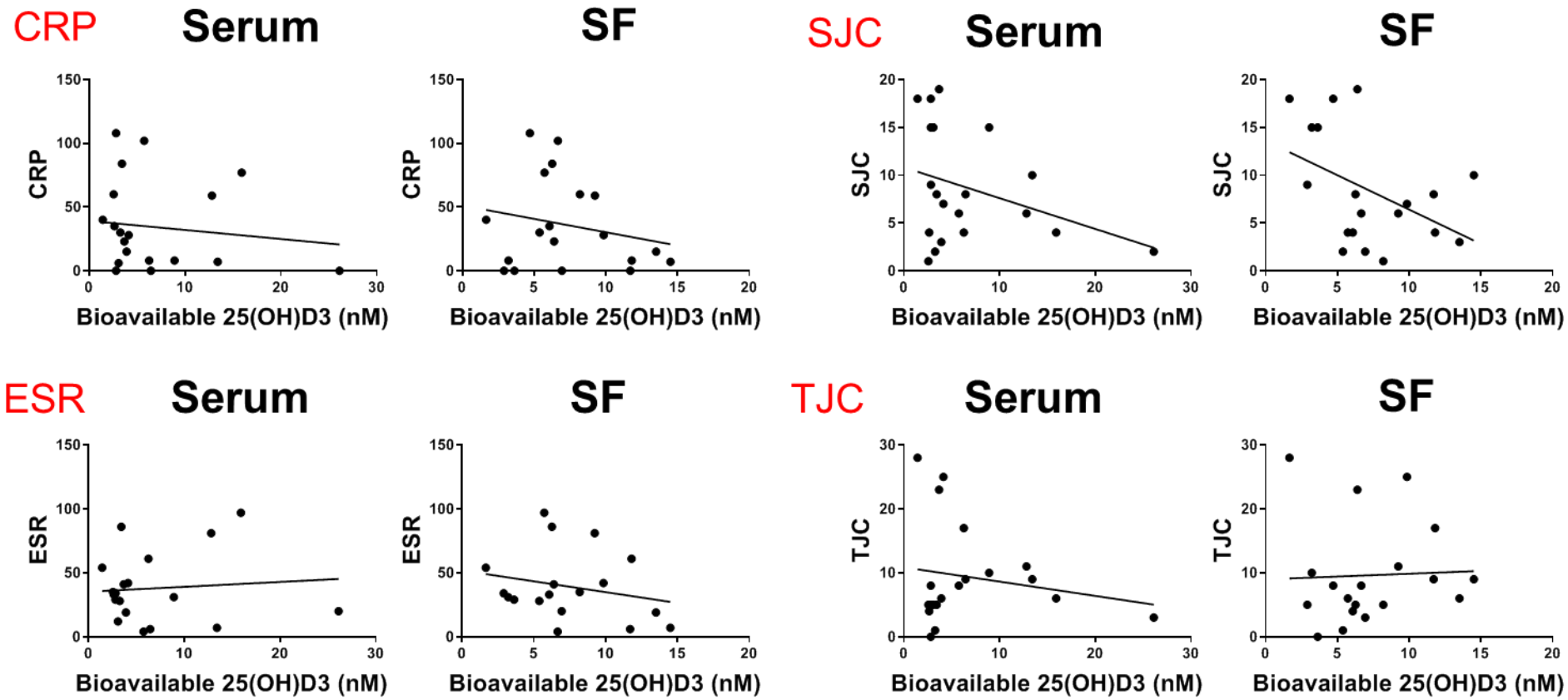


Figure 4.26B. Bioavailable 25(OH)D3 and RA disease activity markers
Correlations between bioavailable 25(OH)D3 with RA disease activity markers CRP, ESR, SJC and TJC. Linear regression was performed by best-fit values ± SD. Coefficient of determination (r^2) values are only shown for correlations that were statistically significant, *p<0.05.