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A source of New Diagnostic Techniques for Asthma in Ireland

This thesis is submitted to National University of Ireland, Galway
For the degree of Doctor of Philosophy (PhD)
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June 2017

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Dedication

I would like to dedicate this thesis to my good friend Reverend Father Sean Quigley, in thanks for his prayers, friendship and support. He gave me a knowledge of Irish literature, music and history, and he helped me and my family in difficult times. I remain grateful for all that he has done and for his continuing readiness to encourage and advise me.
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DECLARATION

I hereby certify that the submitted dissertation is my own work and I have not obtained a degree elsewhere on the basis of the research presented in this submitted work.

Katrina Hutchinson

June 2017
Glossary of Abbreviations

- % predicated FEV₁ = percent predicated based on normative values for healthy age and BMI matched subjects
- 4-AAP = 4-amino-antipyrine
- 4-CP = 4-chlorophenol
- 1,25D = 1,25-dihydroxyvitamin D (C₂₇H₄₄O₃)
- 25OHD = 25-hydroxyvitamin D (C₂₇H₄₄O₂)
- AAR = allergic rhinitis + allergic asthma
- ABI = Applied Biosystems
- ACQ = asthma control questionnaire
- ACT = asthma control test
- AD = allelic discrimination
- ADAM33 = a disintegrin and metalloprotease domain-containing protein 33
- ADP = adenosine diphosphate
- AERD = aspirin-exacerbated respiratory disease
- AIF = assay information file
- ALP = alkaline phosphatase
- AMP = antimicrobial peptide
- ANOVA = analysis of variance
- ANSA = 8-anilo-1-naphalensulfonic acid
- Apal = a restriction enzyme isolated from Acetobacter pasteurianus
- APCs = antigen presenting cells
- AR = allergic rhinitis
- ASM = airway smooth muscle
- ATAQ = asthma therapy assessment questionnaire
- ATP = adenosine triphosphate
- ATS = American Thoracic Society
- BAL = bronchoalveolar lavage
- BASO = basophils
- BCG = Bromcresol Green
- BMI = body mass index
- BsmI = a restriction enzyme isolated from Bacillus stearothermophilus
- CAMP = cathelicidin antimicrobial peptide
- C-ACT = childhood asthma control test
- CBC = complete blood count
- CCR5 = C-C chemokine receptor type 5
- CD = cluster of differentiation
- CDC = Centres of Disease Control
- CE = cholesterol esterase
- CE-marked = "Conformité Européene" - marked
- cGMP = cyclic guanosine monophosphate
- CHOD = Cholesterol oxidase
- CI = confidence interval
- CLCA1 = chloride channel regulator 1
- CLIA = chemiluminescent immunoassay
• CLSI EP = Clinical and Laboratory Standards Institute evaluation protocol
• CMIA = chemiluminescent microparticle immunoassay
• COPD = chronic obstructive pulmonary disease
• COX-2 = cyclooxygenase-2
• CpG = regions of DNA where a cytosine nucleotide is followed by a guanine nucleotide (5’-C-phosphate-G-3’)
• CRP = C-reactive protein
• CRTAM = class I MHC–restricted T cell–associated molecule gene
• CV = coefficient of variation
• CYP24A1 = 24-hydroxylase
• CYP27A1 and CYP2R1 = 25-hydroxylase
• CYP27B1 = 1α-hydroxylase
• DAP = dihydroxyacetone phosphate
• DBD = DNA-binding domain
• DBRCT = double-blind, randomised, placebo controlled trial
• DC = dendritic cell
• DEQAS = Vitamin D External Quality Assessment Scheme
• DNA = deoxyribonucleic acid
• ECP = eosinophil cationic protein
• ED = emergency department
• EDTA= ethylenediaminetetraacetic acid
• ELISA = enzyme-linked immunosorbent assays
• EO = eosinophils
• EPX = eosinophil protein-X
• ERS = European Respiratory Society
• FAM™dye = fluorescein
• FBC = full blood count
• FceR = high-affinity IgE receptor
• FeNO = fraction of exhaled nitric oxide
• FEV1 = forced expiratory volume in 1 second
• FGF23 = fibroblast growth factor 23
• FIV1 = forced inspiratory volume in one second
• FokI = a restriction enzyme isolated from Flavobacterium okeanokoites
• FoxP3 = forkhead box P3
• FVC = forced vital capacity
• G6P = glucose-6-phosphate
• G6PDH = glucose-6-phosphate dehydrogenase
• GCS = glucocorticoids
• GERD = gastro eosophagael reflux disease
• GINA = global initive for asthma
• GK = glycerol kinase
• GM-CSF = granulocyte macrophage colony-stimulating factor
• GP = general practitioner
• GPO = glycerol phosphate oxidase
• GTP = guanosine triphosphate
• GWAS = genome-wide association studies
• hCAP-18 = human cathelicidin antimicrobial peptide-18
• HDL = high density lipoprotein
• HEDTA = (2-Hydroxyethyl)ethylenediaminetriacetic acid (C₃₀H₂₈N₂O₇)
• HK = hexokinase
• H₂O₂ = hydrogen peroxide
• HPLC-UV = high performance liquid chromatographic with ultraviolet
• HRP = horseradish peroxidase
• hs-CRP = high sensitivity C reactive protein
• ICS = inhaled corticosteroid
• ID-LC-MS/MS = isotope dilution liquid chromatography tandem mass spectrometry
• IDM = information data manager
• IFN γ = interferon gamma
• IG = immature granulocytes
• IgA = immunoglobulin A
• IgE = immunoglobulin E
• IL = interleukin
• IL1RL1 = interleukin 1 receptor-like 1
• INAB = Irish National Accreditation Board
• IOM = Institute of Medicine
• IQR = interquartile range
• ISAAC = International Study of Asthma and Allergies in Childhood
• ISO = International Organization for Standardization
• IU = international unit
• IVD = in vitro diagnostic product
• JCTLM = Joint Committee for Traceability in Laboratory Medicine
• kcal = kilocalorie
• kDa = kilodaltons
• LABA = long-acting β-agonist
• L-Arg = L-arginine
• LBD = ligand-binding domain
• LC-MS = liquid chromatography coupled with mass spectrometry
• LDL = low density lipoprotein
• LP-PLA₂ = lipoprotein-associated phospholipase A2
• LPS = lipopolysaccharides
• LTE₄ = urinary leukotrienes
• LTRA = leukotriene receptor antagonist
• LYMPH = lymphocytes
• M₁ and M₂ = macrophages type 1 and type 2
• MONO = monocytes
• MGB = minor groove binder
• µg/l = micrograms per litre
• MKPK = mitogen-activated protein kinase
• ml = millilitre
• mPAQLQ = mini-Paediatric Asthma Quality of Life Questionnaire
• NAD⁺ = adenine dinucleotide
• NADH = nicotinamide adenine dinucleotide
- NCCLS = National Committee for Clinical Laboratory Standards
- NEUT = neutrophils
- NF-kB = nuclear factor kappa-light-chain-enhancer of activated B cells
- NFQ = non-fluorescent quencher
- ng/ml = nanograms per millilitre
- NIST SRM 972 = National Institute of Standards and Technology Standard Reference material 2972
- NK = natural killer
- NLRP3 = NACHT (neuronal apoptosis inhibitor protein), LRR (leucine-rich repeat) and PYD (PYRIN domain) domains-containing protein 3 (NALP3) also known by cryopyrin is a protein that in humans is encoded by the NLRP3 gene
- NO = nitric oxide
- NR = nuclear receptor
- NRBC = nucleated red blood cells
- O₂ = oxygen
- OCS = oral corticosteroid
- OD = optical density
- OR = odds ratio
- OSAS = obstructive sleep apnoea syndrome
- PBMCs = peripheral blood mononuclear cells
- PDE = phosphodiesterase
- PEF = peak expiratory flow
- PGE2 = prostaglandin E2
- pg/ml = picogram per millilitre
- PL = placebo
- PLT = platelets
- PMT = photo multiplier
- p-NPP = p-nitrophenyl phosphate
- PO₄ = phosphate
- POD = peroxidase
- POSTN = peristin
- PPAR = peroxisome proliferator activated receptor
- PTH = parathyroid hormone
- RANTES = Regulated on Activation, Normal T Cell Expressed and Secreted
- RBC = red blood cells
- RCT = randomised controlled trial
- RFLPs = restriction fragment length polymorphisms
- RIA = radioimmunoassay
- RIQAS = Randox International Quality Assessment Scheme
- RLU = relative light units
- RNA = ribonucleic acid
- RNASE3 = Ribonuclease III
- ROI = region of interest
- RORC = retinoid-related orphan receptor C
- ROS = reactive oxygen species
- RSV = respiratory syncytial virus
• RTI = respiratory tract infection
• RT-PCR = real-time polymerase chain reaction
• RXR = retinoid X receptor
• SABA = short-acting beta-agonists
• SCIT = subcutaneous immunotherapy
• SD(s) = standard deviation(s)
• SDS = Safety data Sheets
• SERPINB2 = serpin peptidase inhibitor clade B
• SEV = standard elution volume
• SIT = specific immunotherapy
• SNP = single nucleotide polymorphism
• SOD = superoxide dismutases – enzymes which catalyse the dismutation of the superoxide radical
• SR = steroid resistant
• SS = steroid sensitive
• SST = serum separator tubes
• TAG = triacylglycerol
• TaqI = a restriction enzyme isolated from the bacterium Thermus aquaticus
• TCR = T cell receptor
• TEa = allowable total error
• TGF = transforming growth factor
• Th = T helper
• TLR = the toll-like receptor
• Tm = melting temperature
• TNF-α = tumor necrosis factor alpha
• T-reg = regulatory T cells
• u = standard uncertainty
• uc = combined uncertainty
• ubr = between-run variation
• UOM = uncertainty of measurement
• UNG = uracil-N-glycosylase
• UV = ultraviolet
• UVB = ultraviolet-B radiation
• uwr = within-run variation
• VDBP = vitamin D binding protein
• VDD = vitamin D deficiency
• VDI = vitamin D insufficiency
• VDR = vitamin D receptor
• VDRE = vitamin D response element
• VDS = vitamin D sufficient
• VDSP = Vitamin D Standardisation Programme
• VIC®dye = fluorescent dye (chemical structure is currently not publicly available)
• VIDA study = Vitamin D assessment study
• VIDSun = vitamin D + Sun questionnaire
• WBCs = white blood cells
Abstract

The objective of this thesis is to set up new diagnostic methods for the study of asthma and related respiratory conditions in an Irish clinical laboratory, and to deepen our knowledge of the pathophysiology of this illness.

Asthma currently affects 300 million individuals worldwide. Asthma healthcare costs continue to spiral upwards, incurring an estimated €500 million in direct and indirect costs to the Irish economy each year. Epidemiological studies have linked the illness with vitamin D deficiency (VDD). Like VDD, it is associated with the season and outdoor sun exposure, and both conditions are highly prevalent in Ireland, a country which enjoys little sunshine. Vitamin D receptor (VDR) polymorphisms have been associated with asthma and allergy susceptibility. A deeper understanding of the pathobiology of asthma and its association with VDD might help to provide a step towards personalised medicine, and also towards the diagnosis and treatment of asthma and associated respiratory conditions.

This pilot project was developed under the guidance of Dr Yury Rochev of NUIG, and it was awarded an employment-based postgraduate programme grant from the Irish Research Council. It was carried out at Biomnis Ireland. Biomnis is an independent sector clinical pathology diagnostic laboratory which uses the latest techniques and methodologies, and which is accredited by INAB to the ISO 15189 standard. Clinical supervision was provided by Dr John Faul in James Connolly Hospital.

Total serum 25OHD, IgE, calcium, hsCRP, phosphate, IgA, magnesium, alkaline phosphatase and PTH levels were measured on an Abbott Architect ci8200 (ABBOTT, Abbott Park, IL, USA). ECP was analysed on the IDM Phadia 250 (Phadia AB, Uppsala, Sweden). The FBC was determined using an automated analyser Sysmex XE-2100D. DNA extraction was performed on Maxwell 16 System (Promega Corporation, Madison, WI, USA). RT-PCR testing was conducted on Applied Biosystems Real-Time PCR System 7500 Fast (Applied Biosystems, Foster City, CA, USA). Tests for IL10, IL17a, VDR and CAMP were
analysed on DS2 automated ELISA (DYNEX Technologies, Chantilly, VA, USA).

Patients’ samples from cross sectional studies of vitamin D status in Irish patients and healthy volunteers were used.

Our team also analysed the samples from pilot double-blind, randomised, placebo-controlled trials of vitamin D supplementation in Irish adults with asthma, and in Irish asthmatic children. The Ethics Committees of the James Connolly Hospital and the National Children’s Hospital approved the studies.

During the project we successfully completed verification of the total 25OHD assays on Abbott Architect. The new tests for ECP, IL10, IL17a, VDR, CAMP and for 4 SNPs were set up for diagnostic and research testing.

We found associations between vitamin D levels (25OHD) and airway obstruction in adults’ asthma and BMI in healthy Irish adults. A negative association was noted between 25OHD and IgE levels in paediatric asthmatics. In general we did not observe any significant benefit of vitamin D supplementation in asthmatic children. However improvement in asthma control was noticeable in some patients with specific genotypes.

We have shown an association of TaqI and Apal polymorphisms of the VDR gene with a susceptibility to uncontrolled asthma in paediatric patients. Also, we have demonstrated that the patients with TC for TaqI, and CC and CT genotypes for Apal, have a significantly low level of IL-10 and an increased WBC (neutrophils in particular), and that they were associated with poor asthma control. The results of our pilot study in adult asthma were in line with those for asthmatic children.

Vitamin D’s role in respiratory disorders has not yet been fully investigated. Research in this area is still at an early stage, but the preliminary data seem encouraging. Further and more extensive studies, using a larger sample, will be necessary to confirm our findings, to examine links between vitamin D and VDR polymorphisms in specific asthma phenotypes, and to investigate the possibility of using VDR polymorphisms as biomarkers for susceptibility to asthma.
Chapter 1
Thesis Overview

1.1. Introduction.
The aim of this thesis is to set up new diagnostic methods in a clinical laboratory for asthma and related respiratory illnesses in Ireland. This could help improve diagnosis, treatment and management of the patients, and possibly also prevent frequent exacerbations and developments of these conditions.

Asthma is a common chronic respiratory disease that is characterised by airway inflammation, airway hyper-responsiveness, airflow obstruction, and a clinical course that varies according to season. It remains one of the world’s most prevalent diseases \(^{1,2}\) with an annual estimated impact of $20 billion on the world’s economy. Per capita, Ireland has the world’s fourth highest prevalence of asthma, which affects one-eighth of the population; it is estimated that 470,000 people suffer from the condition. The reasons for this remain obscure. One in five Irish schoolchildren have asthma symptoms. Asthma healthcare costs rise steadily, imposing on the Irish economy an estimated €500 million annually in both direct and indirect costs.\(^3\) For these reasons, asthma biology, the testing of novel asthma treatments, and the discovery of asthma biomarkers are all priorities in health research. A simple definition of a biomarker is: a molecule produced by the body that aids the detection and/or management of a disease.

The main question asked by this thesis is whether vitamin D or its receptor, or its receptor’s genetic variants, can be used as potential biomarkers for asthma.

1.2. Can vitamin D be a biomarker for asthma?
The prevalence of asthma has expanded by an average of 50% each decade since the 1950s, and while genetic factors contribute to the spread of the condition, environmental conditions also play a role.\(^3\) There is no single cause of this change, but some factors that appear to be
associated with an increase in asthma include “Western life style,” urbanisation, and changes in both diet and activity. Vitamin D deficiency is also highly prevalent in industrialised countries and (in common with asthma) is associated with indoor living, poor diet, obesity and low socioeconomic status. In addition, a number of studies demonstrate a strong association between low serum vitamin D levels and poor asthma control.\textsuperscript{5-9}

Vitamin D receptor (VDR) polymorphisms have been associated with asthma and with allergy susceptibility. VDR and vitamin D modulate T cell differentiation and may also act as immunomodulators. VDR genes are encoded on chromosome 12q, in close proximity to some asthma susceptibility genes. Recent studies in paediatric and adult asthma with vitamin D supplementation have shown conflicting results, suggesting that while some asthmatics might benefit from vitamin D supplementation, the conditions of others might worsen.\textsuperscript{10,11} One possible explanation for this pattern is that asthma phenotypes differ, so that people’s response to vitamin D supplementation might vary because of differences in the underlying disease. Alternatively, VDR polymorphism might change significantly from one patient to another, so that serum levels of vitamin D may need to be interpreted with respect to individual polymorphisms.

We are hypothesise that a combination of (i) asthma phenotype, (ii) serum vitamin D level, and (iii) VDR polymorphism might act as a biomarker and predict the benefit/risk for vitamin D supplementation. In this chapter we explore whether the idea that a combined score including vitamin D level, asthma phenotype, and VDR polymorphisms has any support in the current literature. We also hypothesise how such a score might be used as a clinical predictor for some asthmatic patients. This approach might help to provide a step towards individualised medicine, and towards the diagnosis and treatment of asthma. This introduction forms the background to the research and hypotheses outlined in the subsequent chapters.
1.3. **Vitamin D metabolism.**

The main source of vitamin D in humans is in sun-exposed skin. Solar ultraviolet B rays (UVB) photolyses 7-dehydrocholesterol in the skin to pre-vitamin D3, which is then converted to cholecalciferol (vitamin D3).\(^{12}\) Cholecalciferol from the skin and ergocalciferol (vitamin D2) from diet are hydroxylated in hepatocytes to 25-hydroxyvitamin D (25OHD), which is then stored. (Figures 1.1. and 1.2.)

A study by Blum *et al.*\(^ {13}\) reported a positive correlation between 25OHD concentration in serum and adipose tissue, suggesting that fat tissue is a storage site for 25-hydroxyvitamin D. 25OHD is the storage form of vitamin D, which reliably indicates systemic vitamin D status.\(^ {14}\) The second hydroxylation of 25OHD to its biologically active form 1a,25(OH)2D3 in the kidney is stimulated by parathyroid hormone (PTH), which controls calcium-phosphate homeostasis and is inhibited by fibroblast growth factor 23 (FGF23).\(^ {15}\) Studies show that the enzyme (1a-hydroxylase or CYP27B1) which is responsible for converting 25OHD into active 1a,25(OH)2D3 is also present in different immune cells. These include monocytes and other macrophages, T-cells, B-cells, dendritic cells, and cells in tissues vulnerable to asthma – for example, lung fibroblasts, airway epithelial cells, airway smooth muscle cells, and pulmonary alveolar macrophages.\(^ {16,17}\) Many new Vitamin D functions have been discovered recently, and they supplement the “classical” functions, as shown in Figure 1.1.
Vitamin D signalling is believed to occur through the binding of 1α,25(OH)2D3 to the VDR with the formation of a heterodimer with retinoid X receptor (RXR), and the consequent regulation of gene expression – through the binding of this heterodimer to the genomic sequences known as vitamin D response elements (VDREs). VDREs are important nuclear transcription factors that make it possible first to bind the DNA directly, and then to turn on or off particular genes.\textsuperscript{17} It has been suggested that the pleiotropic effects of vitamin D in humans are caused by hydroxylation of 25OHD in extrarenal sites,\textsuperscript{18} and over 200 genes were reported have significant changes in expression in response to calcitriol stimulation.\textsuperscript{19} (Figure 1.2.)
Vitamin D3 is mostly produced in the skin by the photolytic reactions from 7-dehydrocholesterol. The active form of vitamin D (1α,25(OH)2D3) is produced by two enzymatic activation steps from vitamin D3. CYP27A1 and CYP2R1 genes encode enzymes with 25-hydroxylase activity that catalyses the C-25 hydroxylation of vitamin D3. A final activation enzyme encoded by CYP27B1 subsequently catalyses the rate-limiting C-1 hydroxylation step in 1α,25(OH)2D3 synthesis. The later enzyme is tightly-regulated in the kidney by calcium homeostatic signals, but it is also strongly induced in many cells of the immune system. The active form of vitamin D, 1α,25(OH)2D3 (grey triangles), is then transported to target cells by the vitamin D binding protein (VDBP), or else it is metabolically inactivated by the 24-hydroxylase enzyme (encoded by the CYP24A1 locus). In vitamin D target cells, 1α,25(OH)2D3 translocates to the nucleus and binds to the VDR. The ligand/receptor complex binds vitamin D response element (VDRE), which is located in the promoter region of target genes. The DNA-bound complex interacts with nuclear coregulators and it alters the rate of gene transcription. For example, five genes having a VDRE that are transcriptionally regulated by vitamin D stimulation are shown (white rectangle).

Adapted from Bossé Y et al. 2009.
1.4. Methods for vitamin D testing.

The adequacy of vitamin D levels is generally assessed by measuring the serum or plasma level of 25OHD. This is the principal circulating storage form, and altered concentrations are correlated with secondary hyperparathyroidism, skeletal diseases such as rickets, and many chronic illnesses. We have demonstrated an association between 25OHD levels and the severity of airway obstruction in Irish patients with moderate asthma, as will be shown in chapter 4 of this thesis.

Various methods have been developed to assess circulating serum 25OHD concentrations. This is described in more detail in chapter 3, but the following paragraph provides a brief account.

Earlier routine methods based on competitive protein binding have been replaced by radioimmunoassay (RIA) and by chemiluminescent immunoassay (CLIA). Two main analytical approaches are commercially available: competitive immunoassays and chemical methods. The immunoassays include RIA, enzyme immunoassay, CLIA, electrochemiluminescence immunoassay, chemiluminescent microparticle immunoassay (CMIA) and competitive protein binding assay. The chemical methods are based on chromatographic separation followed by non-immunological direct detection. They include direct high performance liquid chromatographic with ultraviolet (HPLC-UV) detection or liquid chromatography coupled with mass spectrometry (LC-MS). The main difference between these methods is the ability of HPLC to quantify separately 25(OH)D2 and 25(OH)D3 and other vitamin D metabolites.

As mentioned above, 1,25(OH)2D3 is the biologically active form of vitamin D, but it is an imperfect measure of vitamin D status. The half life of circulating 1,25(OH)2D3 is no more than 4-6 hours. Circulating levels of 1,25(OH)2D3 are a thousand times less than 25OHD (i.e., pmol/L instead of nmol/L). Vitamin D deficient patients experience a decrease in intestinal calcium absorption which, for a time, lowers ionized calcium. The calcium sensor in the parathyroid glands recognises the signal, which leads to an increase in the production and secretion of the PTH. This hormone regulates calcium metabolism by increasing mobilisation of
calcium from the skeleton, tubular reabsorption of calcium in the kidney, and the renal production of 1,25(OH)2D. Accordingly, as a patient becomes vitamin D deficient the increase in PTH levels results in normal or elevated levels of 1,25(OH)2D. The 1,25(OH)2D assay is therefore of no value as a measure of vitamin D status. Nonetheless, the 1,25(OH)2D assay has been used effectively in helping the diagnosis of various inherited and acquired disorders in calcium metabolism which affect alterations in the renal or extra renal production of 1,25(OH)2D. For these reasons we did not measure this form of vitamin D in our study.

1.5. Epidemiological studies: vitamin D and asthma.

Metabolism of Vitamin D in skin is primarily affected by factors that influence sun exposure (such as latitude, season, time spent outdoors, clothing and sunscreen), but also by age, the level of melanin in the skin, and body fat content. Important sources of vitamin D dietary intake are oily fish, fortified grains, dairy products and supplements. VDD is conventionally measured by using serum levels of 25OHD. It is based on skeletal effects, and the American Institute of Medicine of the National Academy of Sciences currently defines it as a serum 25OHD below 50 nmol/L. However the concept of vitamin D sufficiency (>50 nmol/L) has provoked widespread controversy, mostly due to uncertainty concerning 25OHD ”sufficient” levels for general health. At present there is still no defined consensus on the optimal vitamin D levels for non-skeletal health, although it is likely that numerous chronic conditions might be associated with VDD.

VDD is widespread in many industrialised countries, and low serum 25OHD levels have also been associated with asthma and asthma severity. Although VDD has been linked to allergies and with a raised serum IgE in children, this is not so evident in adults. Furthermore, it appears that the effects of VDD vary from one population to another. For example, one study of the development of asthma in childhood indicated that a low serum 25OHD level was associated with increased rates of atopy and bronchial hyper-responsiveness in boys, but not in girls. In another study of the development of adult asthma, only
men without allergic rhinitis had an increased risk of developing the condition in association with VDD. This suggests that the effects of VDD in asthma are not easily explained by serum levels of 25OHD alone, but that they are highly dependent on the clinical phenotype. Over 70% of patients with severe asthma are obese, and obesity is closely related to lower vitamin D levels. But studies of large groups of patients indicate an association between low 25OHD levels and low pulmonary function – independent of obesity.

Recent studies of VDD include a significant proportion of black subjects. Black patients generally have lower 25OHD levels, but also their asthma can respond differently to anti-asthma therapies such as corticosteroids and long-acting beta-2-agonist (LABA) – and perhaps even vitamin D supplementation. In terms of responses to vitamin D supplementation therapy, a study of the safety of LABA revealed that the death rate in African-Americans was greater than in Caucasian subjects. For example, in the VIDA study almost one third of subjects were black, and although there was no overall effect of vitamin D supplementation, there was a trend whereby the rate of first asthma exacerbations in non-black subjects was reduced by half. This protective effect may be due to immune modulation and/or an increased response to inhaled corticosteroids (ICS), but only in specific patients. At the same time, we know that ICS may be responsible for increasing the metabolism of Vitamin D that can cause VDD. In asthmatics who receive glucocorticoid medication, more research will be needed to confirm the importance of 25OHD measurements to determine if there is a sufficient level of Vitamin D in the system for enhanced ICS and for immunomodulatory effects.

Therefore, while a number of epidemiological studies suggest that there is an important relation between VDD and asthma, the question arises whether this information is useful in the management of asthmatic patients. We hypothesise that more detailed information about vitamin D levels, VDR polymorphisms and clinical phenotypes could improve patient diagnosis and help guide the use of vitamin D supplements for patients with asthma.
1.6. **Asthma phenotypes, biomarkers and vitamin D.**

We use the term phenotype as meaning the observable characteristics of an organism that result from its genetic composition and its interactions with the environment. Clinical phenotypes are particularly important in asthma for several reasons. Almost 40% of adult asthmatics are atopic, and they are significantly affected by exposure to allergens and the use of anti-allergy therapy. Other factors that condition asthma control include bacterial and viral infections of the airway, susceptibility to irritants (such as tobacco smoke and pollution), diet (through food allergy and the intake of magnesium), physical activity, obesity, and medication (including beta-blockers and aspirin). We argue that airway inflammation, particularly when combined with airway hyperresponsiveness, can lead to several forms of airway obstruction and can cause the symptoms of wheezing, coughing, chest tightness, and shortness of breath. Airway inflammation seems to be the main cause of the pathogenesis of asthma.

One theory holds that asthma is caused by an imbalance between CD4+ T helper1 (Th1) and T helper2 (Th2). A Th1 response is mainly associated with cell-mediated immunity, and its Th2 counterpart is involved in antibody-mediated immune response. Atopic asthmatic inflammation is distinguished by increased Th2 activity. It is marked by increased IgE production, mediated by the Th2 cytokines interleukin-4 (IL-4) and IL-5. IgE production by B cells is triggered by IL-4 and IL-13, and IL-5 is responsible for eosinophil maturation. Also, IL-13 facilitates the development of mucus gland hyperplasia and airway hyperresponsiveness. Interferon-gamma (IFN-γ) suppresses the production of IgE. (Figure 1.3.)
Figure 1.3. Immunopathology in asthma.
Allergens are presented to naive T-helper cells (Th0) via antigen presenting cells (APCs). This results in a differentiation into Th1, Th17 and Th2 cells and the release of cytokines that are specific to these cells. Th2 cytokines mediate airway eosinophil and mast cell recruitment, B-cell IgE isotype class switching, and mucus secretion. Allergen specific B-cells change from IgM-producing to IgE-producing cells. Th17 cells produce Interleukin-17, which mediates airway neutrophilia by inducing the production of chemokines. (TCR = T cell receptor, FceR = high-affinity IgE receptor).
Adapted from Nanzer AM et al. 2014.

The immunopathology of asthma is very complicated. The majority of cytokines involved in pathogenesis of the illness cannot be used as routine biomarkers because changes in their concentration might be tissue-dependent.
At present the diagnosis of asthma in clinical practice is based on patients’ symptoms, clinical history, and spirometry values, as recomended by the Global Initiative for Asthma (GINA). (Figure S1.1. and Table S1.1 – in supplementary material for Chapter 1.)
Due to the heterogeneity of asthma and phenotypical differences the range of clinical biomarkers is very limited, and the source of new biomarkers is a prominent area of medical research.
Szefler SJ et al. carried out a comprehensive search of the literature to identify studies of existing asthma biomarkers. They classified the biomarkers as either core (required in future studies), supplemental (used according to study aims and standardised), or emerging (requiring validation and standardisation). Only one biomarker, multiallergen screening, was identified as a core atopic asthma outcome. Complete blood counts to measure total eosinophils, fractional exhaled nitric oxide (FeNO), sputum eosinophils, urinary leukotrienes, and total and allergen-specific IgE were recommended as supplemental measures. However it should be emphasised that these traditional biomarkers do not clearly represent different asthma phenotypes. Comprehensive identification of specific cytokines may assist in understanding the underlying mechanisms in the pathophysiology of airway inflammation. The most traditional asthma biomarkers are presented in Table 1.1.

Table 1.1. Recommendations for classifying asthma biomarker outcome measures for national Institutes of health-initiated clinical research for adults and children.

<table>
<thead>
<tr>
<th>Characterization of study population for prospective clinical trials (e, baseline information)</th>
<th>Prospective clinical trial efficacy/effectiveness outcomes</th>
<th>Observational study outcomes*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Core outcomes</td>
<td>Serologic multiallergen screen (IgE) to define atopic status (also for observational studies)</td>
<td>None</td>
</tr>
</tbody>
</table>

*Observational study designs include cohort, case-control, cross-sectional, retrospective reviews; genome-wide association studies (GWAS); and secondary analysis of existing data.

†The substance(s) being analysed. **Taken from Szefler SJ et al. 2012
In recent years new biomarkers have been developed with the aim of reflecting Th2 responses in asthma. Woodruff et al. singled out periostin (POSTN) in particular, but also serpin peptidase inhibitor clade B (SERPINB2), chloride channel regulator 1 (CLCA1), as epithelial genes that were induced in asthma. All of them were directly regulated by IL-13 in vitro. The investigators divided patients into Th2-high and Th2-low groups, which differed clinically from one another and were associated with different levels of IL-5 and IL-13. They also noticed that the Th2-high group had significantly lower Th1 cytokines IL-12 and interferon gamma. Clinically this group showed markedly higher atopy and higher eosinophil levels in bronchoalveolar lavage (BAL) and in peripheral blood. Based on this immunophenotyping, the choice of therapy can be important. For example, the treatment with inhaled corticosteroids did not improve the lung function of Th2-low asthmatics. The researchers observed a detrimental effect of corticosteroids during the first month of treatment based on results of forced expiratory volume in 1 second (FEV1).

Table 1.2. Asthma phenotypes according to their cytokine profiles divided into Th2-high and Th2-low asthma.*

<table>
<thead>
<tr>
<th>T cell signature</th>
<th>Phenotype</th>
<th>Biomarkers</th>
<th>Therapy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Th2-high</td>
<td>Early-onset allergic</td>
<td>Specific IgE, skin prick testing</td>
<td>Corticosteroids, anti-IgE</td>
</tr>
<tr>
<td></td>
<td>Late-onset (eosinophilic)</td>
<td>IL-5, IL-13, Sputum Eosinophilia, periostin</td>
<td>Anti-IL-5, anti-IL-13 (?), Poor response to</td>
</tr>
<tr>
<td></td>
<td>Exercise induced</td>
<td>Mast cells</td>
<td>corticosteroids.</td>
</tr>
<tr>
<td></td>
<td>Aspirin-exacerbated</td>
<td>Blood Eosinophilia 50%</td>
<td>Aspirin desensitization;</td>
</tr>
<tr>
<td></td>
<td>respiratory disease (AERD)</td>
<td></td>
<td>Leukotriene-modifying agents.</td>
</tr>
<tr>
<td>Th2-low</td>
<td>Obesity related</td>
<td>Mast cells, adiponectin, Th1 cytokines</td>
<td>Weight loss, peroxisome proliferator</td>
</tr>
<tr>
<td></td>
<td>Neutrophilic</td>
<td>Th17, Sputum neutrophilia</td>
<td>activated receptor (PPAR) agonists +</td>
</tr>
</tbody>
</table>

*Adapted from Nanzer AM et al. 2014.

Recently different immuopheonoypes have been suggested by other researchers. Choy et al. stressed the need to identify and develop non-invasive biomarkers of Th17 pathway activity, which they believe is strongly associated with adult asthma. They have described Th17-high
and Th2-17-low asthmatic phenotypes. They proposed the existence of a Th17-permissive environment when Th2 activity is suppressed by targeted therapy of corticosteroids, or in the absence of Th2 activity (true Th2-low). In some patients they suggested that Th17-high asthma may represent a transition or movement away from Th2-mediated disease. They proposed that it could be more valuable to target IL-13 and IL-17 in patients who have either a Th2 or Th17 signature than single Th2 or Th17 inhibition.

These studies show how different asthmatic “immunophenotypes” respond differently to conventional asthma therapy. The studies also noticed the lack of specific diagnostic markers in some asthma phenotypes. For example, the absence of Th2-17-low biomarkers is seen in obesity-related asthma and neutrophilic asthma. From all these investigations we can hypothesise why some medications do not seem to be effective in treating asthma.

In our study we lay particular emphasis on the use of vitamin D in diagnostic and treatment. And we can suggest that (i) the failure to detect any benefit from vitamin D supplements in asthma might be due to the selection of the wrong patient population and (ii) vitamin D might have an effect, but only in certain sub-populations. Patient selection might be the key to clarify if vitamin D can be useful for therapy for specific phenotypes, and if 25OHD testing can be helpful in diagnostic and monitoring of the treatments. A good example might be a steroid-resistant asthma where vitamin D might have a role as a useful biomarker. Studies have shown that vitamin D induces regulatory T (Treg) cells to secrete IL-10 in steroid resistant patients who have impaired IL-10 induction by glucocorticoids. Vitamin D may inhibit genes involved in steroid resistance and change chemokine expression in human airway smooth muscle cells. Based on these findings we can hypothesise that an anti-inflammatory effect of vitamin D may have a therapeutic role against steroid resistance in asthma. Not only can anti-inflammatory functions of vitamin D be beneficial for asthma, but its immunosuppressive functions can be important. It has been shown that 1,25(OH)2D3 can affect human immune responses by regulating FOXP3 expression in CD4+ T cells.
through direct VDR binding to the FOXP3 gene (which is vital for the inhibitory function of Treg cells).  

The next section describes the immunomodulatory effects of vitamin D in asthma in more detail.

1.7. Vitamin D as immunomodulator in asthma.

Vitamin D has a range of immunomodulatory effects. It is not only a hormone, but 1,25(OH)2D3 also has a paracrine role in different cells, including monocytes, T cells, B cells and dendritic cells. All these cells can metabolise vitamin D through CYP27B1, and they can activate vitamin D through 1α-hydroxylase activity.  

The presence and expression of this enzyme in significant quantities in various lung cells, including lung epithelium and inflammatory cells, alveolar macrophages, dendritic cells, and lymphocytes, are essential factors for cell functions. 

Some studies show that these cells are involved in inducing the expression of vitamin D related bactericidal proteins such as cathelicidin and CD14 cytokines. (In the course of this project the author set up a cathelicidin test (CAMP) in Biomnis Ireland for research use.) It has been confirmed that these cytokines and bacteriocidal peptides may affect not only bacterial colonisation of the airways but also airway inflammation. 

In the pre-antibiotic era sun exposure was used in the treatment of patients with tuberculosis in Ireland and elsewhere. Now we can say why: the antimicrobial activity of macrophages against Mycobacterium tuberculosis is increased by cholecalciferol.  

The effect is controlled by the upregulation of cathelicidin through the hCAP-18 gene and the induction of the defensin β2 gene. It has been shown that the active form of vitamin D (1α,25(OH)2D3) inhibits the expression of important inflammatory cytokines, including IL-1, IL-6, tumour necrosis factor-alpha (TNF-α), IL-8, and IL-12 in monocytes. It also contributes to apoptosis, and it inhibits the maturation of bone-marrow-derived mast-cell precursors. The inhibition of mast-cell differentiation seems to be dose-dependent.  

Studies have indicated that vitamin D affects some B-cell functions by inhibiting plasma-cell differentiation and immunoglobulin secretion (IgG
and IgM), through the apoptosis of activated B cells and through memory B-cell generation. Cholecalciferol inhibits the proliferation of some lymphocytes and their differentiation. As has been noted above, the effect of 1,25(OH)2D3 on Th-mediated cytokines varies, enhancing and suppressing secretion under different conditions. Vitamin D receptor expression appears to be at its lowest level in naive T cells and increases with the maturation of T cells. Vitamin D inhibits the synthesis of the Th1 cytokines, IFN-γ and IL-2. It also inhibits the production of IL-17, which is involved in neutrophil-driven responses and produced by Th17. Furthermore, it enhances the production IL-4 and IL-13 that lead to stimulation of Th2 differentiation. It seems that the overall effect is a shift in the expression of cytokines from a Th1 towards a Th2 type response.

A few studies have shown that the vitamin D-stimulated processes contribute to tolerance and the induction of tolerogenic dendritic cells. This process involves the development of CD4+CD25+Foxp3+Treg cells, the activation of T-cells and antigen receptor signalling, and the production of anti-inflammatory cytokines – including IL-10. Important findings have identified 1,25(OH)2D3-regulated transcripts that influence dendritic cell function which modify the gene expression profiles of these cells. It has been shown that 1,25(OH)2D3-treated human dendritic cells can convert CD4 T cells into IL-10-secreting Treg cells. This leads to a suppression of T cell proliferation. It seems as if vitamin D can establish homeostasis between regulatory and suppressor T cell functions and can modulate inflammatory processes – as reported in a review article by Chambers and Hawrylowicz.

Investigations are under way as to the suitability of the microbial pattern-recognition receptor called Toll-like receptor 9 (TLR9) as a potential clinical marker of vitamin D-induced IL-10+ Tregs. Binding TLR9 with its agonist CpG (cytosine-phosphate-guanine) oligonucleotide halted IL-10 production, indicating a control mechanism that suppresses Treg. (During our project we set up two further tests, IL-10 and IL-17a, in Biomnis Ireland for use in subsequent research.)
IL-10 was identified in 1989 as a cytokine synthesis inhibitory factor which has a down-regulatory effect on inflammatory responses. The IL-10 gene is located on chromosome 1 (1q31-32) in the human genome, and it consists of 5 exons and 4 introns. It inhibits the production of various cytokines, chemokines, chemokine receptors and inflammatory enzymes. As many studies have shown, IL-10 has a protective role in asthma. Low levels of IL-10 have been associated with susceptibility to the disease, but on the other hand overexpression of IL-10 in regulatory T cells can suppress airway hyper-responsiveness.\textsuperscript{57} The IL-10 gene promoter polymorphisms were associated with mild asthma.\textsuperscript{59}

As mentioned above, the active form of vitamin D can influence both innate and adaptive immunity. Its actions affect adaptive immunity through antigen-presenting cells (APCs) and T cells, promoting peripheral tolerance through the inhibition of inflammatory processes and by inducing Treg cells.\textsuperscript{60}

These findings indicate that vitamin D is an important regulator of immune functions. Numerous cells express the vitamin D receptor, and they are direct and indirect targets of vitamin D. (Table 1.3.)

\textbf{Table 1.3.} Response of various target cells to vitamin D in asthma.

<table>
<thead>
<tr>
<th>Target Cells of vitamin D</th>
<th>Effector function of vitamin D</th>
</tr>
</thead>
<tbody>
<tr>
<td>T cells</td>
<td>Inhibit T cell proliferation</td>
</tr>
<tr>
<td>Th1</td>
<td>Inhibit Th1 cytokine release</td>
</tr>
<tr>
<td>Th2</td>
<td>Conflicting evidence for enhancement and inhibition of Th2 responses</td>
</tr>
<tr>
<td>Th17</td>
<td>Inhibit Th17 cytokine release</td>
</tr>
<tr>
<td>B cells</td>
<td>Nonlinear association between serum vitamin D levels and IgE</td>
</tr>
<tr>
<td></td>
<td>Enhanced production of immunomodulatory cytokine IL-10</td>
</tr>
<tr>
<td>Regulatory T cells</td>
<td>Induces IL-10 synthesis</td>
</tr>
<tr>
<td></td>
<td>Enhance forkhead FOXP3 cells</td>
</tr>
<tr>
<td></td>
<td>Enhance transforming growth factor (TGF-β) synthesis</td>
</tr>
<tr>
<td>Dendritic cells</td>
<td>Renders monocyte derived dendritic cells more immature and tolerogenic</td>
</tr>
<tr>
<td></td>
<td>Induces IL-10 synthesis</td>
</tr>
<tr>
<td>Lung bronchial smooth muscle cells</td>
<td>Inhibit cytokine synthesis and release</td>
</tr>
<tr>
<td></td>
<td>Decrease lung inflammation</td>
</tr>
<tr>
<td></td>
<td>Inhibit bronchial smooth muscle cell proliferation and remodeling</td>
</tr>
<tr>
<td>Mast cells</td>
<td>Inhibit the differentiation, maturation, and homing of mast cells to allergic airways</td>
</tr>
<tr>
<td>Macrophages</td>
<td>Reduces infiltration</td>
</tr>
<tr>
<td></td>
<td>Inhibits M1 activation</td>
</tr>
<tr>
<td></td>
<td>Enhances M2</td>
</tr>
<tr>
<td></td>
<td>Increases antimicrobial activity</td>
</tr>
</tbody>
</table>
Vitamin D also regulates effector T cell cytokine production, which plays an important role in asthma pathogenesis. Furthermore, Cantorna et al.\textsuperscript{60} proposed that in the development of two cell types, NKT and CD8αα T (which inhibit autoimmunity) VDR expression is necessary. Future studies in human patients are needed to examine these potential benefits and functions of vitamin D in asthma pathogenesis. They could lead us to a greater understanding of the importance of 25OHD sufficiency in different asthma phenotypes and in different populations.

1.8. Vitamin D receptor and genetic studies.

The VDR is a member of the nuclear receptor (NR) superfamily. As a heterodimer with one of the three retinoid X receptor (RXR) isotypes, it binds to 1,25(OH)\textsubscript{2}D\textsubscript{3} with high affinity. VDR regulates the transcription through binding to the promoter region of DNA in controlled target genes. Thereby it mediates the biological effects of its ligand. The DNA-bound heterodimer recruits different transcription coregulators. If ligands are not present, or if antagonists are present, corepressors are normally recruited to the target genes. Agonist ligands bring about a change in the structure of the NR, and this allows interaction with coactivators. Recruitment of coactivators results both in histone acetylation (which prepares target gene promoters through decondensation of the chromatin), and also establishes a link with the basal transcriptional mechanism. Various proteins with a wide range of structures and functions have been identified as VDR coregulators. (Figure 1.4.)
Figure 1.4. The mechanism of VDR’s action. The heterodimers of 1,25(OH)2D, VDR and RXR bind to VDREs. This binding is accompanied by the formation of large complexes that can either help the expression of the targeted gene (coactivators), or inhibit its expression (cosuppressors). Different types of coactivator complexes expose the gene for transcription by acetylating the histones that otherwise conceal the DNA. Alternatively they bridge the gap between the VDREs and the initiation complex, stimulating transcription by activating the RNA polymerase. Co-repressors block this process in part by histone deacetylation – although other modifications of these histones, such as methylation, have been described. These processes can lead to changes in the production of different cytokines. In the case of decreased expression of VDR there is an altered production of different cytokines, as shown in this figure.

Adapted from Bikle D, 2010.

VDR shares the main structural characteristics of NRs. It consists of a highly conserved DNA-binding domain (DBD), a ligand-binding domain (LBD), and a linking region that connects both of these domains. The N-terminal domain (NTD) of VDR is very short, and its function is still imperfectly understood. VDR is an indiscriminate NR, and its deregulation may result in severe diseases such as asthma, cancers, rickets, autoimmunity disorders (multiple sclerosis, rheumatoid arthritis, and type I diabetes).

As mentioned above, asthma is a complex disorder which has many genetic and environmental causes. Numerous genomewide studies for screening gene polymorphisms in asthma have revealed some susceptibility loci; among them are regions
2q, 5q, 6p, 11q, 12q, and 13q. Several polymorphisms are associated with asthma risk in IL-10, RANTES, TGFB1, ADAM33, CCR5, TLR-10, IL-4, IL-13, IL-8, IL-18, TNF, TLR-4, and others. We hypothesise that, in addition to other genes that are known to increase the risk of asthma, the role of the VDR gene is also important. We further suggest that a greater understanding of VDR polymorphisms and their relation to asthma pathogenesis might shed light on better control, diagnostic and treatment.

The VDR gene, which is located on chromosome 12q in humans, was cloned in 1988. It consists of 9 exons, with at least 6 isoforms of exon1, and it spans 60–70 kb of the genomic sequence. (Figure 1.5.)

![VDR Gene and VDR Polymorphisms](image)

**Figure 1.5.** VDR gene and VDR polymorphisms associated with asthma. (TaqI, FokI, BsmI, Tru9I, Apal)

As mentioned above, VDR is a vital immune system regulator. It interacts with target-cell nuclei to perform a range of biological functions, including apoptosis, calcium and phosphorous homeostasis, and cell differentiation. Poon et al. have shown that some VDR gene SNPs (single nucleotide polymorphisms) were associated with asthma. The studies of VDR polymorphisms still have many limitations. Some are based on only a small number of patients, and the selection of asthma phenotypes remains imprecise. But even from the studies that have been made we can draw some preliminary conclusions. For example, the
association between VDR polymorphisms and asthma that has been observed in some studies appears to be more evident in girls than in boys.\textsuperscript{70} It is known that many important features of asthma and allergy are affected by gender. These include age of symptom onset, disease severity, and lung growth and development. Twin studies provide evidence that sex influences the heritability of asthma. There is considerable evidence of the modification of genotype-phenotype correlation by gender. This includes the gender-specific effects of VDR polymorphisms on skeletal growth and bone mineral density. It also seems that in other immune-mediated conditions, such as Type 1 diabetes mellitus, gender influences the effects of VDR polymorphisms. Together with the effects of VDR on the development of allergy and asthma, they may be hormonally-mediated; more research is needed because some studies were gender-specific.\textsuperscript{71}

It is known from many genome- and transcriptome-wide studies that vitamin D signalling alters numerous inflammatory responses at different levels. It regulates the expression of genes that produce pro-inflammatory mediators, including cyclooxygenases and 5-lipoxygenase, it interferes with transcription factors, such as NF-κB (that regulate the expression of inflammatory genes), and it activates signalling cascades (e.g., MAP kinases).\textsuperscript{72} Some studies indicate an association of asthma with SNPs between intron 2 and exon 9 in the VDR gene. The mechanism explaining this association remains unclear. But according to Bossé \textit{et al.}, the presence of some genetic variants alters VDR expression so that the highly-expressed haplotype is associated with asthma, and is over-transmitted. Accordingly, the low expressed haplotype was under-transmitted. These VDR haplotypes were studied and named as high-risk/high-expressed haplotypes, and low-risk/low-expressed haplotypes. The authors also described their important finding that the low IL10-producing haplotype was associated with asthma in their patients. They outlined a two-gene model using two different polymorphisms that predicted the risk of asthma – one in IL-10 - rs1800871 and the other in VDR - rs1544410.\textsuperscript{72}
Researchers have devoted much attention to the association between VDR polymorphisms and the risk of asthma, but the published results have proved to be conflicting. Tizaoui et al. used a meta-analysis approach in their study of the impact of TaqI, Bsml, Apal, and FokI VDR polymorphisms on asthma risk. They found significant associations between some polymorphisms and asthma risk – such as TaqI and Bsml in a co-dominant model. FokI polymorphism was only partially associated with asthma risk, but subgroup analyses showed that there was a significant association with asthma risk after modification for gender and age. On the other hand, they found no significant association between Apal polymorphism and asthma risk. From these studies we can conclude that the selection of the sample population is extremely important and adjustments for sex and age are necessary.

VDR polymorphisms have been associated not only with asthma risk but also with asthma susceptibility in different populations. For example, Papadopoulou et al. found an association of the minor TaqI genotype with asthma in Cypriot teenagers. They also observed that this polymorphism may contribute to asthma susceptibility – primarily under conditions of normal 25OHD levels. Iordanidou et al. show an association of VDR polymorphism Apal 'a' allele with improved asthma control in children. It is possible that some polymorphisms can act as a protection against asthma.

It is essential to understand the molecular mechanisms of VDR gene variants to enhance our awareness of their role in the pathogenesis of asthma and the possibility of therapeutic interventions (including vitamin D supplementation) for asthma in different populations.

Not only is genetical modification important in the study of asthma, but epigenetics influences many genes, among them those that are involved in inflammation and immune response. DNA methylation and histone modifications may form part of allergic processes and asthma. They may also have an impact on disease heritability, and may be associated with asthma phenotypes and environmental exposure. Some researchers have proposed that vitamin D and VDR may act as epigenetic regulators. Together with chromatin modification enzymes they are involved in post-
translational modification of histones in asthma and other chronic lung diseases. It has been shown that VDR, along with histone modification enzymes, may be involved in methylation, demethylation, acetylation, deacetylation, etc., of various epigenetically regulated target genes. Bégin and Nadeau point out that asthma is much more prevalent in the offspring of mothers than of fathers who suffer from asthma. This could be partly explained by parental imprinting – a process by which some genes are epigenetically silenced during gametogenesis in a parent-of-origin-specific manner. This results in only one allele being expressed for the imprinted loci. Another explanation may be that the pattern results from a direct modification of the foetal immune system by the mother’s atopic phenotype in utero.\textsuperscript{76}

Recent research has shown the importance of vitamin D, VDR and VDR polymorphisms in the environmental deregulation of the cellular and molecular functions that are involved in asthma.\textsuperscript{77} (Figure 1.6.) But much more work is needed to link specific immunological and clinical asthma phenotypes with 25OHD levels and VDR polymorphisms. Future studies may enable us to explore the use of Vitamin D and its metabolites, VDR, and VDR gene variants as potential biomarkers for asthma and bring us closer to personalised medicine.

From numerous studies of the association of vitamin D deficiency with asthma we can hypothesise that serum vitamin D levels (25OHD) might help to establish the severity of asthma and airway inflammation, and perhaps give an indication of the correct treatment, possibly with vitamin D supplementation. One of the aims of this dissertation is to explore the association between VDR SNPs and asthma susceptibility in Irish patients and to examine the possibility of using vitamin D as a potential clinical biomarker and a therapeutic agent for asthma for selected patients (as covered in chapters 4, 5 and 6).
Figure 1.6. Role of vitamin D, VDR and VDR polymorphisms in environmenal deregulation of molecular and cellular functions involved in asthma. Environmental agents such as cigarette smoke, oxidants, ozone, and aldehydes activate or inhibit vitamin D receptor and affect different molecular and cellular targets as a result of vitamin D-mediated deregulation. Adapted from Sundar I et al. 2011.


- The introduction outlined the clinical importance of the development of new diagnostic methods for different asthma phenotypes, and also the possibility of using vitamin D as a clinical biomarker and therapeutic agent for specific patients.
- Chapter 2 is devoted to the instrumentation and methods that we used in this project. We also outline the setting up of new clinical tests for research and diagnostics in a medical laboratory.
- Chapter 3 describes the validation of 25OHD method on Abbott architect analyser. Comprehensive comparative studies were performed with gold standard method. Reference interval examinations of 25OHD were conducted in healthy Irish volunteers.
• Chapter 4 studies the association between 25OHD levels and airway obstruction in adult Irish asthmatics within a single level of asthma severity. An association study was performed with 25OHD levels and other biomarkers of allergic and systemic inflammation.

• Chapter 5 is dedicated to a supplementation study of vitamin D in paediatric uncontrolled asthmatics. We studied the effects of vitamin D3 supplementation on different asthma parameters, including lung function, biomarkers of inflammation/allergy, and subjective asthma symptoms.

• Chapter 6 continues the theme developed in Chapter 5 and investigates the association between VDR polymorphisms and uncontrolled paediatric asthma. We examined the impact of polymorphisms in asthma susceptibility in relation to vitamin D status, IgE and other biochemical and immunological indices; and we studied the possibility of using these polymorphisms as potential biomarkers for asthma.

• Finally, in Chapter 7 we assess the outcomes of our research and we discuss possible future studies that might build on our work.
1.10. References for Chapter 1.


52. Matheu V, Bäck O, Mondoc E, Issazadeh-Navikas S. Dual effects of vitamin D-induced alteration of TH1/TH2 cytokine expression: enhancing IgE production and decreasing airway eosinophilia in


**1.11. Supplementary information for Chapter 1**

**Figure S1.1.** Flowchart for diagnosis of asthma in clinical practice as per Global Initiative for Asthma. Global Strategy for Asthma Management and Prevention, 2016. Accessed in May 2017. Taken from: [www.ginasthma.org](http://www.ginasthma.org).

#### Box 1.2. Diagnostic criteria for asthma in adults, adolescents, and children 6–11 years

#### 1. History of variable respiratory symptoms
- **Wheeze, shortness of breath, chest tightness, and cough**
  - Descriptors may vary between cultures and by age. E.g., children may be described as having heavy breathing.
  - Generally more than one type of respiratory symptom (in adults, isolated cough is seldom due to asthma).
  - Symptoms occur variably over time and in intensity, together with variable expiratory airflow limitation.

#### 2. Confirmed variable expiratory airflow limitation

<table>
<thead>
<tr>
<th>Diagnostic Feature</th>
<th>Criteria for Making the Diagnosis of Asthma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Documented excessive variability in lung function* (one or more of the tests below) AND documented airway limitation†</td>
<td>The greater the variations, or the more occasions excess variation is seen, the more confident the diagnosis.</td>
</tr>
<tr>
<td>Positive bronchodilator (BD) reversibility test* (more likely to be positive if BD medication is withheld before test: SABA ≥4 hours, LABA ≥15 hours)</td>
<td>Adults: increase in FEV1 of &gt;12% and &gt;200 mL from baseline, 10–15 minutes after 200–400 mcg salbutamol or equivalent (greater confidence if increase is &gt;15% and &gt;400 mL). Children: increase in FEV1 of &gt;12% predicted.</td>
</tr>
<tr>
<td>Excessive variability in twice-daily PEF over 2 weeks*</td>
<td>Adults: average daily diurnal PEF variability &gt;10%** Children: average daily diurnal PEF variability &gt;13%**</td>
</tr>
<tr>
<td>Significant increase in lung function after 4 weeks of anti-inflammatory treatment</td>
<td>Adults: increase in FEV1 by &gt;12% and &gt;200 mL (PEF** by &gt;20%) from baseline after 4 weeks of treatment, outside respiratory infections.</td>
</tr>
<tr>
<td>Positive exercise challenge test*</td>
<td>Adults: fall in FEV1 of &gt;10% and &gt;200 mL from baseline Children: fall in FEV1 of &gt;12% predicted, or PEF &gt;15%</td>
</tr>
<tr>
<td>Positive bronchial challenge test (usually only performed in adults)</td>
<td>Fall in FEV1 from baseline of &gt;20% with standard doses of methacholine or histamine, or &gt;15% with standardized hyperventilation, hypertonic saline, or mannitol challenge.</td>
</tr>
<tr>
<td>Excessive variation in lung function between visits* (less reliable)</td>
<td>Adults: variation in FEV1 of &gt;12% and &gt;200 mL between visits. Outside of respiratory infections Children: variation in FEV1 of &gt;12% in FEV1, or &gt;15% in PEF** between visits (may include respiratory infections).</td>
</tr>
</tbody>
</table>

BD: bronchodilator (short-acting SABA or rapid-acting LABA); FEV1: forced expiratory volume in 1 second; LABA: long-acting beta-agonists; PEF: peak expiratory flow (highest of three readings); SABA: short-acting beta-agonist. See Box 1-4 for diagnosis in patients already taking controller treatment.

*These tests can be repeated during symptoms or in the early morning. **Daily diurnal PEF variability is calculated from twice daily PEF as (day’s highest minus day’s lowest) / mean of day’s highest and lowest, and averaged over one week. †For FEV1, use the same meter each time, as FEV1 may vary by up to 20% between different meters. **Reversibility may be lost during severe exacerbations or viral infections. ††Bronchodilator reversibility is not present at initial presentation, the next step depends on the availability of other tests and the urgency of the need for treatment. In a situation of clinical urgency, asthma treatment may be commenced and diagnostic testing arranged within the next few weeks (Box 1-4, p.22), but other conditions that can mimic asthma (Box 1-3) should be considered, and the diagnosis of asthma confirmed as soon as possible.
Chapter 2
Materials and methods.

2.1. Introduction.

It is well-known that setting up new methods in a clinical laboratory for respiratory conditions, including asthma, is a difficult undertaking. Due to the complexities and heterogeneity of asthma, not many biochemical, immunological or genetic tests are routinely used by clinicians. However, recent research and publications open new horizons for asthma diagnostic. In the course of research for this dissertation we have set up a range of different diagnostic and research techniques that can be used by clinicians in Ireland (and elsewhere). This chapter contains details of the equipment for the tests, sample preparation, and the principles behind the methods we have applied in our laboratory. All analyses, unless otherwise stated, were carried out by the author in Biomnis Ireland laboratories, Dublin.

The aim of this dissertation has been to apply already-existing techniques for research and diagnostics that are available on the market, and to find clinical uses for them. Our objective has been to improve patient care. Most of the tests have been intensively validated by manufacturers. In our clinical laboratory our challenge was to adapt their new methods for routine use on equipment that had already been purchased. Some tests, such as ECP, are routinely used by respiratory clinicians in other countries for evaluation of airway inflammation. We are the first laboratory in Ireland to set up this method for diagnostic and research application. As already mentioned, Ireland’s situation in relation to asthma differs from that of most other countries.

Significant effort has been devoted to the verification of the Abbott 25OH vitamin D methods. The results will be outlined in Chapter 3 of this thesis.
2.2. **25OHD test on DiaSorin Liaison Chemiluminescence analyser.**

2.2.1. **Principles.**

DiaSorin Liaison Chemiluminescence analyser is a fully automated standby equipment and was used in our laboratory for measurement of different hormone levels.

The LIAISON 25OHD is a direct competitive chemiluminescent immunoassay (CLIA) for quantitative determination of total 25OHD in serum or plasma for the assessment of vitamin D sufficiency. During the first incubation 25OHD is dissociated from its binding protein and binds to the specific antibody on the solid phase. After 10 minutes the tracer (vitamin D, linked to an isoluminol derivative) is added. After a second 10 minutes incubation, the unbound material is removed with a wash cycle. Subsequently, the starter reagents are added to initiate a flash chemiluminescent reaction. The light signal is measured by a photomultiplier in the form of relative light units (RLU) and is inversely proportional to the concentration of 25OHD present in calibrators, controls or samples. (Figure 2.1.)
2.2.2. Measuring principle.

- After the last wash cycle has been completed, the reaction module is transported into the measuring chamber.
- When the first cavity of the reaction module reaches the position under the injection head, starter reagent 1 is injected into the first cavity.
- After a pump delay time of 2.55 secs the starter reagent 2 is injected into the same cavity to start the chemiluminescence reaction.
- After the measuring delay time of 0.1 sec, the measuring signal is obtained and integrated over the measuring period of 3.0 secs. (Figure 2.2.)
The chemically emitted light is measured by a selected highsensitive, low-noise photo multiplier (PMT). The linear measuring range of the photo multiplier is 300 - 650 nm. The light peak of the chemiluminescence is emitted at a wavelength of 420 nm.

- The PMT operates as an ultra-fast photon counter. The pulses are amplified by a rapid electronic amplifier. A circuit, which suppresses the PMT signal-noise, is also implemented in the PMT box.

- The relative light units (RLU) are used as units of measurement for the raw data, which are then multiplied by the RLU factor, that allows the compensation of the inevitable, individual fluctuations of the cathode sensitivity of the PMT.\(^1\)
2.2.3. Reagents requirements.

Table 2.1. LIAISON® 25 OH Vitamin D reagents are supplied ready for use.

| Reagent Integral: Magnetic Particles (2.4 mL) | Magnetic particles coated with antibody against 25 OH vitamin D, protein, phosphate buffer, <0.1% sodium azide. |
| Assay Buffer (28.0 mL) | Buffer with 10% ethanol, surfactants and 0.1% ProClin® 300 (ProClin® 300 is a registered trademark of Rohm and Haas Co.) |
| Conjugate (4.5 mL) | 25OH vitamin D conjugated to an isoluminol derivative, in phosphate buffer with 10% ethanol, EDTA, surfactant and preservatives. |
| Calibrator 1 (1.0 mL) | Human serum, Tris buffer, <0.1% sodium azide and 25OH vitamin D. The calibrator concentrations (ng/mL) are referenced to standard preparations containing highly purified 25OH vitamin D. |
| Calibrator 2 (1.0 mL) | Human serum, Tris buffer, <0.1% sodium azide and 25OH vitamin D. The calibrator concentrations (ng/mL) are referenced to standard preparations containing highly purified 25OH vitamin D. |
| Number of Tests | 100 |

Additional materials:
LIAISON® 25 OH Vitamin D TOTAL Control Set
LIAISON® 25 OH Vitamin D TOTAL Specimen Diluent
LIAISON® Cleaning Kit

2.2.4. Specimen collection and preparation; storage and stability.

The human serum, EDTA plasma, lithium heparin plasma may be used. Fasting samples are recommended, but are not required. Blood is collected aseptically by venepuncture and allowed to clot, and the serum separated from the clot as soon as possible. Specimens may be stored in glass or plastic vials. No additives or preservatives are required to maintain the integrity of the sample. Grossly haemolysed or lipemic samples as well as samples containing particulate matter or exhibiting
obvious microbial contamination are not tested. If the assay is performed within 120 hours of sample collection, the samples are kept at 2-8°C; otherwise they are stored frozen (-20°C or below). If the samples are stored frozen, thawed samples must be well-mixed before testing. Repeated freeze-thaw cycles must be avoided and air bubbles must be removed before assaying. The minimum volume required is 250 μL specimen for the first test, and 25 μL more for each additional LIAISON® 25OH Vitamin D TOTAL test.

2.2.5. Calibration, calibration interval and stability.

Data reduction is performed using a master curve with 2-point recalibration. The starting point of data reduction is the master curve, stored in the analyser. To compensate for differences between reagent lots, different analysers and environmental conditions, the assay calibration was run and validated according to the protocol given below. The measuring signals of the calibrators allow the shift of all master curve points to a working curve, corresponding to the actual conditions during measurement. See the following example. (Figure 2.3.)

- The stored master curve is generally defined with 10 master curve base points.
- Two calibrators with defined concentration values are measured. These measured signals (RLU) are compared with the master curve signal of the corresponding calibrator concentrations.
- The relative difference between the measured RLU and the master RLU of the calibrators is calculated, and a linear extrapolation is performed between the recalculated RLU (Y-axis) and the logarithmic (Log) concentrations (X-axis).
- Based on appropriate compensation factors, a re-adjustment of the master curve points is made in order to achieve the working curve by a “cubic spline function”.
- The predefined master curve is adjusted to a new instrument-specific curve using the two calibrators supplied in the reagent
integral via the bar codes on the reagent integral label. Renewed calibration is required:

- With each new lot of reagents (Reagent Integral or Starter Reagents).
- Every 7 days.
- After each servicing of the LIAISON® Analyser.
- If quality controls are out of the acceptable range.

**Figure 2.3.** Calibration concept on Liaison: example. Adapted from LIAISON Operation manual (DiaSorin, Dietzenbach, Germany).
2.2.6. **Measuring range.**

The DiaSorin LIAISON® 25OH Vitamin D TOTAL Assay measures between 4.0 and 150 ng/mL. Values below 4.0 ng/mL are reported as <4.0 ng/mL. The highest reportable value without dilution is 150 ng/mL. To convert results to SI units: ng/mL × 2.5 = nmol/L.

2.2.7. **Assay procedure.**

Each test parameter is identified by the barcode on the reagent integral. In case of a malfunction of the barcode reader, the relevant data can be entered manually.

The analyser operations are as follows:

- Dispense the sample, calibrator or control into the reaction module.
- Dispense the magnetic particles and assay buffer into the reaction module.
- Incubate.
- Dispense the tracer into the reaction module.
- Incubate.
- Wash with Wash/System liquid.
- Add the starter reagents and measure the light emitted.

Immunoassay results can be affected by temperature fluctuations. We were aware of variations in our laboratory environment; more frequent use of controls and subsequent recalibration were performed whenever necessary.

2.2.8. **Quality control.**

Quality control was analysed once per day before running patients’ samples, according to internal SOP requirements. The LIAISON® 25OH Vitamin D TOTAL Control Set was in use according to the manufacturer’s recommendations. Appropriate value ranges were established. Whenever controls lay outside the expected ranges, the calibration was repeated and the controls were retested.
The range of concentrations of each control was reported on the manufacture certificate of analysis and indicated the limits established by DiaSorin.

2.2.9. Interpretation of results.

The LIAISON® Analyser automatically calculates the concentration of 25OH vitamin D in the sample, and this concentration is expressed in ng/mL. Conversion was performed to SI units: ng/mL × 2.5 = nmol/L.

2.2.10. Limitations of the procedure.

Bacterial contamination of samples or repeated freeze-thaw cycles may affect the test results. The antibody that is utilised in this assay can demonstrate cross-reactivity to many dihydroxylated metabolites of vitamin D; in humans, these compounds are naturally present in picomolar concentrations.

2.2.11. Specific performance characteristics.

The LIAISON 25OH vitamin D method was extensively validated by the manufacturers. All the information below was provided by DiaSorin (DiaSorin, Dietzenbach, Germany). Our internal validation for Liaison 25OHD method was carried out at an earlier stage of the author's work and is not presented in this thesis.

2.2.11.1. Functional sensitivity.

The functional sensitivity, defined as the dose concentration at which the %CV exceeds 20%, was evaluated according to CLSI EP17-A. Samples were prepared at nominal concentrations of 2-14 ng/mL and assayed in multiple runs to determine mean concentration and %CV. Sample concentration was plotted against %CV and a regression was prepared to determine the functional sensitivity. The derived functional sensitivity from the regression equation is ≤4.0 ng/mL.
2.2.11.2. Method comparison.

A total of 155 samples was tested by LIAISON® 25OH Vitamin D TOTAL and by (DiaSorin 25OH Vitamin D RIA). The resulting regression equation was: LIAISON® = 0.99 (RIA) + 2.4; R = 0.97. (Figure 2.4.)

![Method Correlation: TOTAL LIAISON v. RIA](image)

Figure 2.4. Method comparison between LIAISON and RIA for total 25OHD. Taken from LIAISON 25OHD kit insert (DiaSorin, Dietzenbach, Germany).

2.2.11.3. Recovery test.

High concentration samples were mixed with low concentration samples in ratios of 1:2, 1:1, and 2:1. The observed values were then compared to the expected values to determine the % recovery. The mean recovery is 102.

2.2.11.4. Precision.

Assay imprecision was evaluated according to CLSI EP5-A2. Nine serum samples and four plasma samples, containing different concentrations of analyte, were assayed in duplicate in two assays per day over 20 operating days to determine the repeatability and reproducibility of the assay (i.e. within- and between-assay variability). (Table 2.2.)
Table 2.2. Precision study for LIAISON® 25 OH vitamin D TOTAL*

<table>
<thead>
<tr>
<th>Serum Repeatability</th>
<th>S1</th>
<th>S2</th>
<th>S3</th>
<th>S4</th>
<th>S5</th>
<th>S6</th>
<th>S7</th>
<th>S8</th>
<th>S9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of determinations</td>
<td>80</td>
<td>80</td>
<td>80</td>
<td>80</td>
<td>80</td>
<td>80</td>
<td>80</td>
<td>80</td>
<td>80</td>
</tr>
<tr>
<td>Mean (ng/mL)</td>
<td>7.2</td>
<td>14.7</td>
<td>21.7</td>
<td>35.0</td>
<td>73.0</td>
<td>62.7</td>
<td>93.6</td>
<td>115</td>
<td>128</td>
</tr>
<tr>
<td>Standard Deviation (ng/mL)</td>
<td>0.40</td>
<td>0.63</td>
<td>0.86</td>
<td>1.01</td>
<td>2.33</td>
<td>1.95</td>
<td>3.03</td>
<td>4.79</td>
<td>6.08</td>
</tr>
<tr>
<td>Coefficient of variation (%)</td>
<td>5.5</td>
<td>4.2</td>
<td>4.0</td>
<td>2.9</td>
<td>3.2</td>
<td>3.1</td>
<td>3.2</td>
<td>4.2</td>
<td>4.8</td>
</tr>
</tbody>
</table>

* Taken from LIAISON 25OHD kit insert (DiaSorin, Dietzenbach, Germany).

2.2.11.5. Trueness.

The assay Linearity has been verified by the dilution and recovery tests. Dilution Test: 5 samples were diluted with LIAISON® 25 OH vitamin D TOTAL specimen diluent and analysed. The results were analysed as a linear regression of the expected vs. observed values. The resulting regression equation is: Observed = Expected 0.99 +0.1; R² = 0.99. (Figure 2.5.)

![Linearity Study](image)

Figure 2.5. Linearity study for LIAISON® 25OH vitamin D TOTAL. Taken from LIAISON 25OHD kit insert (DiaSorin, Dietzenbach, Germany).
2.2.11.6. Interfering substances.

Controlled studies of potentially interfering substances showed that the assay performance was not affected by haemolysis (up to 200 mg/dL haemoglobin), lipemia (up to 549 mg/dL triglycerides), bilirubinaemia (up to 20 mg/dL bilirubin) or cholesterolemia (up to 259 mg/dL cholesterol). Note: the effect of heterophilic antibodies on the assay performance has not been evaluated.

2.2.11.7. Specificity.

Data on the cross-reactivity of the antiserum used in this assay were obtained by spiking up to 100 ng/mL of the potential cross-reactant and assaying. The cross-reactivity of each compound, normalised to 25 OH-vitamin D₃, is listed below. (Table 2.3.)

Table 2.3. Specificity study for LIAISON® 25 OH vitamin D TOTAL.

<table>
<thead>
<tr>
<th>Steroid</th>
<th>% Cross-reactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>25 OH vitamin D₂</td>
<td>104%</td>
</tr>
<tr>
<td>25 OH vitamin D₃</td>
<td>100%</td>
</tr>
<tr>
<td>vitamin D₂</td>
<td>&lt;1%</td>
</tr>
<tr>
<td>vitamin D₃</td>
<td>&lt;1%</td>
</tr>
<tr>
<td>1,25-(OH)₂ vitamin D₂</td>
<td>40%</td>
</tr>
<tr>
<td>1,25-(OH)₂ vitamin D₃</td>
<td>17%</td>
</tr>
<tr>
<td>3-epi-25 OH vitamin D₃</td>
<td>&lt;1%</td>
</tr>
</tbody>
</table>

Taken from LIAISON 25OHD kit insert (DiaSorin, Dietzenbach, Germany).
2.3. **Biochemical and immunological tests on Abbott Diagnostics Architect ci8200 analyser.**

The Architect ci8200 System is a fully automated Clinical Chemistry and Immunoassays analyser that includes the latest advancements in assay technology, software and modularity. It is easy to use, troubleshoot and maintain. The Architect ci8200 uses photometric, potentiometric, and CMIA (chemiluminescent microparticle immunoassay) technology to measure analyte concentrations in samples. The Architect ci8200 System contains two modules c and i (chemistry and immunoassays). At Biomnis Ireland we have a two module systems ci8200 and one stand-alone immunoassays module.

All the patients’ samples for total IgE, IgA, calcium, magnesium, phosphate, total protein, albumin, ALP, HDL, LDL, total cholesterol, triglycerides, hs-CRP, PTH, glucose and vitamin D (25OHD) were analysed on the serum aliquots stored at -80°C on Abbott Architect ci8200.

**2.3.1. 25OHD and PTH tests on Abbott Architect ci8200 analyser.**

In our studies we used two generations of the ARCHITECT 25-OH vitamin D assays. Chapter 3 of this thesis contains detailed information on the 25OHD methods. PTH and both generations of 25OHD assay use chemiluminescent microparticle immunoassays (CMIA) technology.

**2.3.1.1. CMIA principle.**

CMIA is a technology used to determine the presence of antigens, antibodies, and analytes in samples.

**2.3.1.2. The reactants necessary for CMIA method include**

- Paramagnetic microparticles coated with a capture molecule (antigen or antibody) specific for the analyte being measured.
- Acridinium-labeled conjugate.
- Pre-trigger solution and trigger solution.
2.3.1.3. CMIA reaction sequence.

A CMIA reaction sequence is the order of interactions between the analyte present in the sample and the reactants. A sequence is specific to the assay protocol. The Architect Intact PTH assay is a two-step sandwich immunoassay.

The example of two-step reaction sequence illustrates the basic principles of a reaction:

1. The pipettor dispenses microparticles (paramagnetic microparticles coated with capture molecules) into the sample in the reaction vessel. The vortexer mixes the reaction mixture.
2. The reaction mixture incubates, and the analyte present in the sample binds to the corresponding capture molecules on the microparticles forming the immune complex.
3. A magnet attracts the paramagnetic microparticles (bound to the specific analyte) to a wall of the reaction vessel. The wash zone manifold washes the reaction mixture to remove unbound materials. Further processing can now take place.
4. The pipettor dispenses a chemiluminescent acridinium-labelled conjugate. The conjugate binds to the immune complex to complete the reaction mixture.
5. The reaction mixture incubates.
6. The wash zone manifold washes the reaction mixture to remove unbound materials.
7. The pre-trigger nozzle dispenses pre-trigger solution (hydrogen peroxide) and the CMIA optical system takes a background read. Pre-trigger performs the following functions:
   - Creates an acidic environment to prevent early release of energy (light emission).
   - Helps to keep microparticles from clumping.
   - Splits acridinium dye off the conjugate bound to the microparticle complex. This action prepares the acridinium dye for the next step.
8. The trigger nozzle dispenses trigger solution (sodium hydroxide) to the reaction mixture. The acridinium undergoes an oxidative reaction when
exposed to peroxide and an alkaline solution. This reaction causes the chemiluminescent reaction to occur. N-methylacridone forms and releases energy (light emission) as it returns to its ground state.

9. The CMIA optical system measures the chemiluminescent emission (activated read) over a predefined time period to quantitate the analyte concentration.²

The 25OHD method is one-step immunoassays, and will be covered in more detail in chapter 3.

2.3.2. **Measurement of total IgE, IgA, calcium, total protein, albumin, ALP, HDL, LDL, total cholesterol, triglycerides, hs-CRP, glucose on Architect cSystem.**

2.3.2.1. **cSystem principles of operation.**

The cSystem uses the photometric method to measure sample absorbance for the quantitation of analyte concentration. Photometric technology is the measurement of the amount of light a sample absorbs. It involves passing a beam of light through a sample and measuring the intensity of light that reaches a detector. Beer’s Law establishes the mathematical relationship between the absorbance of the solution and the concentration of the analyte. The absorbance of the solution changes as the reaction progresses, and measurements are taken when either all the reactant is depleted and the reaction is stable (end-point assays), or when the reactant concentration reaches a stable rate (rate assays).

2.3.2.2. **Principle of total IgE method.**

The Abbott Architect Quantia IgE reagent is a suspension of polystyrene latex particles coated with mouse anti-human IgE. When a sample containing IgE is mixed with the latex reagent and the reaction buffer is included in the kit, agglutination occurs. The degree of agglutination is directly proportionate to the concentration of IgE in the sample and is determined by measuring the decrease of transmitted light by the aggregation. Linearity of IgE is 25 IU/ml to 10000 IU/mL with automatic rerun capability.
2.3.2.3. Principle of IgA method.

The IgA assay is an immunoturbidimetric procedure that measures increasing sample turbidity caused by the formation of insoluble immune complexes when the antibody to IgA is added to the sample. A sample containing IgA is incubated with a buffer and a sample blank determination is performed before the addition of the IgA antibody. In the presence of an appropriate antibody in excess, the IgA concentration is measured as a function of turbidity. Reportable range is from 0.05 g/L to 6.3 g/L.

2.3.2.4. Principle of Total Calcium method.

The Abbott Architect Total Calcium assay was used for the quantitation of total calcium in human serum. Arsenazo-III dye reacts with calcium in an acid solution to form a blue-purple complex. The colour developed is measured at 660 nm and is proportional to the calcium concentration in the sample.

\[
\text{Ca}^{2+} + \text{Arsenazo III} \rightarrow \text{Ca-Arsenazo III complex (purple)}
\]

The linearity of calcium is 0.50 – 6.00 mmol/L.

2.3.2.5. Principle of Total Protein method.

The Abbott Architect Total Protein assay was used for the quantitation of total protein in human serum. Polypeptides containing at least two peptide bonds react with biuret reagent. In alkaline solution, cupric ion forms a coordination complex with protein nitrogen, with very little difference between albumin and globulin on a protein-nitrogen basis. Total protein is linear up to 184g/L.

2.3.2.6. Principle of Albumin method.

The Abbott Architect Albumin BCG (bromcresol green) assay was used for the quantitation of albumin in human serum. The Albumin BCG procedure is based on the binding of bromcresol green with albumin to produce a coloured complex. The absorbance of the
complex at 628 nm is directly proportional to the albumin concentration in the sample. Albumin BCG is linear up to 105 g/L.

2.3.2.7. Principle of ALP method.

The Abbott Architect Alkaline Phosphatase assay was used for the quantitation of alkaline phosphatase in serum. Human alkaline phosphatase consists of a group of at least five tissue-specific isoenzymes which catalyse the hydrolysis of phosphate mono-esters at alkaline pH.

Several substrates have been used to measure alkaline phosphatase activity such as glycerophosphate, phenyl phosphate, and p-nitrophenyl phosphate. Bowers and McComb improved the method of Bessey et al. to include a kinetic measurement. Tietz et al. optimised this method to include a chelated metal-ion buffer of zinc, magnesium, and HEDTA. The Abbott Alkaline Phosphatase procedure is a modification of this method.

Alkaline phosphatase in the sample catalyses the hydrolysis of colourless p-nitrophenyl phosphate (p-NPP) to give p-nitrophenol and inorganic phosphate. At the pH of the assay (alkaline), the p-nitrophenol is in the yellow phenoxide form. The rate of absorbance increase at 404 nm is directly proportional to the alkaline phosphatase activity in the sample. Optimised concentrations of zinc and magnesium ions are present to activate the alkaline phosphatase in the sample.

\[
p – \text{nitrophenyl phosphate} + \text{H}_2\text{O} \xrightarrow{\text{ALP}} \text{phosphate} + p – \text{nitrophenol}.
\]

The p-nitrophenol released is directly proportional to the catalytic ALP activity. It is determined by measuring the increase in absorbance at 404 nm. Alkaline phosphatase is linear up to 2,200 U/L.

2.3.2.8. Principle of HDL method.

The Abbott Architect ultra HDL assay was used for the quantification of high density lipoprotein HDL cholesterol in serum.
The Ultra HDL assay is a homogeneous method for directly measuring HDL cholesterol concentrations in serum or plasma without the need for off-line pre-treatment or centrifugation steps. The method uses a two-reagent format and it depends on the properties of a unique detergent. This method is based on accelerating the reaction of cholesterol oxidase with non-HDL unesterified cholesterol and dissolving HDL cholesterol selectively, using a specific detergent. In the first reagent, non-HDL unesterified cholesterol is subject to an enzyme reaction, and the peroxide generated is consumed by a peroxidase reaction with DSBmT(4-sulphobutyl)-m-toluidine-disodium, yielding a colourless product. The second reagent consists of a detergent (capable of solubilising HDL cholesterol), cholesterol esterase, and chromogenic coupler to develop colour for the quantitative determination of HDL cholesterol. Ultra HDL is linear up to 4.66 mmol/L.

2.3.2.9. Principle of LDL method.

The Abbott MULTIGENT Direct LDL assay was used for the quantification of low density lipoprotein LDL cholesterol in serum. It is a homogeneous method for directly measuring LDL levels without the need for off-line pretreatment or centrifugation steps. The method is in a two-reagent format and it depends on the properties of a unique detergent. This detergent solubilises only the non-LDL particles. The cholesterol released is consumed by cholesterol esterase and cholesterol oxidase in a non-colour-forming reaction. A second detergent solubilises the remaining LDL particles, and a chromogenic coupler allows for colour formation. The enzyme reaction with LDL in the presence of the coupler produces colour that is proportional to the amount of LDL cholesterol present in the sample.

2.3.2.10. Principle of Total Cholesterol method.

The Abbott Cholesterol assay was used for the quantification of total Cholesterol in serum.
Enzymatic, colourimetric method with cholesterol esterase (CE) hydrolyses cholesterol esters to form free cholesterol and fatty acids. Cholesterol oxidase (CHOD) then catalyses the oxidation of cholesterol to form cholest-4-ene-3-one and \( \text{H}_2\text{O}_2 \). In the presence of peroxidase (POD), the hydrogen peroxide formed effects the oxidative coupling of phenol and 4-amino-antipyrine (4-AAP) to form a red-coloured quinoneimine dye.

\[
\begin{align*}
\text{CE} & : \text{Cholesterol Esters} + \text{H}_2\text{O} \rightarrow \text{Cholesterol} + \text{Fatty Acids} \\
\text{CHOD} & : \text{Cholesterol} + \text{O}_2 \rightarrow \text{Cholest}-4\text{-ene}-3\text{-one} + \text{H}_2\text{O}_2 \\
2 \text{H}_2\text{O}_2 + 4\text{-AAP} + \text{Phenol} & \rightarrow \text{Quinoneimine Dye} + 4 \text{H}_2\text{O} \\
\end{align*}
\]

The colour intensity of the red quinoneimine dye formed is directly proportional to the cholesterol concentration. It is determined by measuring the increase in absorbance at 500 nm.

The test range is: 0 – 18.26 mmol/L.

### 2.3.2.11. Principle of Triglycerides method.

The Abbott Triglyceride assay was used for the quantitation of triglyceride in serum.

Triglycerides are enzymatically hydrolysed by lipase to free fatty acids and glycerol. The glycerol is phosphorylated by adenosine triphosphate (ATP) with glycerol kinase (GK) to produce glycerol-3-phosphate and adenosine diphosphate (ADP). Glycerol-3-phosphate is oxidised to dihydroxyacetone phosphate (DAP) by glycerol phosphate oxidase (GPO), producing hydrogen peroxide (\( \text{H}_2\text{O}_2 \)). In a colour reaction catalysed by peroxidase, the \( \text{H}_2\text{O}_2 \) reacts with 4-aminoantipyrine (4-AAP) and 4-chlorophenol (4-CP) to produce a red coloured dye. The absorbance of this dye is proportional to the concentration of triglyceride present in the sample.

The triglycerides test is linear up to 16.05 mmol/L.
2.3.2.12. Principle of hs-CRP method.

The Abbott High Sensitive C-Reactive Protein (hs-CRP) assay was used for the quantitative analysis of C-reactive protein in serum. It is an immunoturbidimetric method. When an antigen-antibody reaction occurs between CRP in a sample and polyclonal anti-C-reactive protein antibody, which has been adsorbed to latex particles, agglutination results. This agglutination is detected as an absorbance change, with the magnitude of the change being proportional to the quantity of CRP in the sample. The actual concentration is then determined by interpolation from a calibration curve prepared from calibrators of known concentration. The increase in absorbance at 572 nm is proportional to the CRP concentration. The reportable range for hs-CRP is 0.1 – 160.0 mg/L.

2.3.2.13. Principle of glucose method.

The Abbott Glucose assay was used for the quantitative determination of glucose in fluoride plasma. Hexokinase (HK) catalyses the phosphorylation of glucose by adenosine triphosphate (ATP) to form glucose-6-phosphate (G6P) and adenosine diphosphate (ADP). To follow the reaction, a second enzyme, glucose-6-phosphate dehydrogenase (G6PDH), is used to catalyse oxidation of glucose-6-phosphate by adenine dinucleotide (NAD\(^+\)) to form nicotinamide adenine dinucleotide (NADH).

\[
\text{Glucose} + \text{ATP} \xrightarrow[HK]{\text{D-glucose-6-phosphate} + \text{ADP}} \\
\text{G6P} + \text{NAD}^+ \xrightarrow[\text{G6PDH}]{\text{D-6-phosphogluconate} + \text{NADH} + \text{H}^+}
\]

The concentration of the NADH formed is directly proportional to the glucose concentration. It is determined by measuring the increase in absorbance at 340 nm. Glucose plasma is linear from 0.28 to 44.4 nmol/L.
2.4. **ECP (eosinophil cationic protein) measurement on Phadia 250.**

2.4.1. **Test principle ImmunoCAP ECP.**

The ECP test was set up on Phadia 250 (Phadia AB, Uppsala, Sweden), using fluoroenzymeimmunoassay. Phadia 250 is a fully automated analyser and is ideal for our busy laboratory, delivering 400 - 2,000 results per week. Phadia 250 is designed for allergy and autoimmunity testing with high capacity (throughput: 60 results per hour) and continuous random access. All reagents are stored on board and have 28-day storage of calibrations curves. The analyser is connected to our Laboratory Automation Systems and has on board dilution protocols. The ImmunoCAP ECP technology is based on an extremely high total binding capacity per mg cellulose, in combination with an optimal amount of cellulose in each solid phase. This ensures binding of all relevant antibodies, regardless of antibody affinity, and gives low non-specific binding. The ImmunoCAP solid phase consists of a cellulose derivative enclosed in a capsule. The hydrophilic, highly-branched polymer provides an ideal microenvironment for antigens, binding them irreversibly while maintaining their native structure. 4

The ECP test is designed as a sandwich immunoassay. (Figure 2.6.)
Figure 2.6. Test principle ImmunoCAP ECP:

a) Anti ECP, covalently coupled to the solid phase, reacts with the ECP in the patient serum sample.
b) After washing, enzyme-labelled antibodies against ECP are added to form a complex.
c) After incubation, unbound enzyme-anti-ECP is washed away, and the bound complex is then incubated with a developing agent.
d) After stopping the reaction, the fluorescence of the eluate is measured. The fluorescence is directly proportional to the concentration of ECP in the serum sample. Adapted from Phadia 250 Operation manual (Phadia AB, Uppsala, Sweden)
2.4.2. **Measurement of ECP in clinical practice.**

Eosinophil Cationic Protein (ECP) also known as ribonuclease 3 is a basic protein located in the eosinophil primary matrix.\(^5\) In humans, the ECP is encoded by the *RNASE3* gene.\(^6\) ECP is released during degranulation of eosinophils. This protein is related to inflammation and asthma because these conditions cause increased levels of ECP in the body. There are three glycosylated forms of ECP, and consequently ECP has a range of molecular weights from 18-22 kDa.\(^7\)

ImmunoCAP ECP measures the level of ECP in serum.\(^8\) Eosinophils are the cells chiefly responsible for producing the inflammation associated with asthma. When eosinophils in the airway are activated, they undergo degranulation, causing airway epithelial damage. This can lead to chronic inflammatory disease of the airway. Asthmatic patients with eosinophilic inflammation have elevated levels of ECP in serum and in other body fluids such as bronchial alveolar fluid and (induced) sputum. A high level of serum ECP indicates inflammation, which is a risk factor for asthmatic patients.\(^9\)\(^-\)\(^11\)

Asthma therapy consists of suppressing chronic and persistent airway inflammation. Measuring ECP in a serum sample is an objective and direct way of estimating the severity of the airway inflammation and of following the course of the disease.\(^12\) (We used this test for research purposes although it has not been endorsed in the latest guidelines.)

As per manufacturer’s recommendations, ECP serum measurements can be used:

- For monitoring inflammation in asthma
- For guiding corticosteroid treatment in asthma
- To find non-compliant patients

2.4.3. **Expected test values.**

Normal adult values show a geometric mean of 5.5 µg/l and a 95th percentile of 13.3 µg/l. Values over 15 µg/l should be considered elevated. However, patients should be their own control during treatment follow-up. (Studies performed at Phadia AB, Uppsala, Sweden.)
2.4.4. Specimen collection and preparation.

Our laboratory has established our own procedure for serum sampling. Parameters such as blood collection tube, coagulation time and temperature are kept within specified limits, since they affect the concentration of released ECP in serum samples. The clotting is an important step of the process. During this process ECP is released by the eosinophils that are activated by the inflammation.

- Blood is collected by venepuncture using a serum-gel tube with clot activator.
- After collection, the tube is gently inverted several times.
- ECP is released by clotting for 60 to 120 minutes at room temperature 20-24°C.
- The tube is centrifuged at 1000–1300 x g for 10 minutes at room temperature.
- Serum is decanted into a new tube.
- Serum samples are stored at 2 °C to 8 °C if assayed within five days after collection, or at –20 °C if assayed later.

2.4.5. Measuring range and dilution.

The measuring range for undiluted serum is: 2-200 µg/l. Sample dilution is usually not required. For determination of values higher than 200 µg ECP/l, samples can be diluted with ImmunoCAP IgE/ECP/Tryptase Sample Diluent.

2.4.6. Materials

2.4.6.1. Materials provided by Phadia AB.

Reagents are packaged in separate units, each purchased separately. All units are required to perform an assay, though, calibrators are not required for additional assays while the stored curve is valid. The expiration date and storage temperature for each of the units are stated on the outer label (Tables: 2.4.; 2.5.; 2.6. and 2.7.).
### Table 2.4. ImmunoCAP ECP Conjugate 50.
(Fluoroenzymeimmunoassay for 2 x 50 determinations)

<table>
<thead>
<tr>
<th>ECP Conjugate 50</th>
<th>2 vials</th>
<th>Ready for use</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Galactosidase-anti-ECP</td>
<td></td>
<td>Stored at 2 - 8 °C until expiration date</td>
</tr>
<tr>
<td>(mouse monoclonal antibodies)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Approximately 1.5 µg/ml</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sodium azide 0.05%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Table 2.5. ImmunoCAP ECP Calibrator Strip.

<table>
<thead>
<tr>
<th>ECP Calibrator Strip</th>
<th>1 strip, contains one calibration curve</th>
<th>Ready for use</th>
</tr>
</thead>
<tbody>
<tr>
<td>(human ECP in buffer)</td>
<td></td>
<td>Stored at 2 - 8 °C until expiration date</td>
</tr>
<tr>
<td>Concentration 2; 5; 15; 100 and 200 µg/l</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sodium azide 0.05%; 0.2 ml</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Table 2.6. ImmunoCAP ECP.

<table>
<thead>
<tr>
<th>ImmunoCAP Anti-ECP (a-ECP)</th>
<th>Carriers of 16 ImmunoCAP</th>
<th>Ready for use</th>
</tr>
</thead>
<tbody>
<tr>
<td>(mouse monoclonal antibodies) 16</td>
<td></td>
<td>Stored at 2 - 8 °C until expiration date</td>
</tr>
<tr>
<td>Preservative &lt;0.003%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Table 2.7. Development Solution, Stop Solution and Washing Solution

<table>
<thead>
<tr>
<th>Development Solution</th>
<th>6 bottles</th>
<th>Ready for use</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-Methylumbelliferyl-β-D-galactoside 0.01%</td>
<td></td>
<td>Stored at 2 - 8 °C until expiration date</td>
</tr>
<tr>
<td>Preservative* &lt;0.0010%; 11 ml/17 ml</td>
<td></td>
<td><strong>Not frozen</strong></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Stop Solution</th>
<th>6 bottles</th>
<th>Ready for use</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium carbonate 4%, 119 ml</td>
<td></td>
<td>Stored at 2 - 8 °C until expiration date</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Washing Solution</th>
<th>2 bottles</th>
<th>Needs to be prepared as per operation manual</th>
</tr>
</thead>
</table>
2.4.6.2. **Materials required but not provided by Phadia AB.**

- Measuring cylinder 1000 ml
- Purified water

2.4.7. **Calibration.**

ImmunoCAP ECP Calibrator Strip is run in duplicate to obtain a full calibration curve. The curve can be stored. One ImmunoCAP ECP curve control strip in duplicate is needed to evaluate subsequent assays against the stored curve. (Figure 2.7)

The ECP calibrators are calibrated against pure ECP prepared according to Peterson, Jörnvall and Venge. ¹³

---

**Figure 2.7.** Example of ECP calibration curve on Phadia 250.
2.4.8. **Quality controls.**

ImmunoCAP ECP Controls are also provided and analysed before every samples batch and they are treated in the same way as patient samples.

2.4.9. **Performance characteristics.**

All the studies were performed at Phadia AB, Uppsala, Sweden.

2.4.9.1. **Precision.**

From Table 2.8. the following mean coefficients of variation have been obtained: each sample was assayed in 4 replicates on 18 totally different occasions using the same lot of reagents. The results have been obtained with Phadia 250 using stored calibration curves.

**Table 2.8.** Precision study for ECP on Phadia analyser. Table presented from ECP kit insirt (Phadia AB, Uppsala, Sweden).

<table>
<thead>
<tr>
<th>Sample level µg/l</th>
<th>Coefficients of variation (%)</th>
<th>Within assay</th>
<th>Between assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 – 5</td>
<td></td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>5 – 100</td>
<td></td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>100 – 200</td>
<td></td>
<td>4</td>
<td>5</td>
</tr>
</tbody>
</table>

2.4.9.2. **Sensitivity.**

The detection limit is <0.5 µg/l.

2.4.9.3. **Specificity.**

Cross reaction was tested with the following results:

EPX (*eosinophil* protein-X) <0.1%.
2.5. **ELISA methods for identification of Human IL-10, VDR, IL-17A and CAMP.**

### 2.5.1. **Instrumentation.**

The DS2 Automated ELISA Processing System (DYNEX Technologies, Chantilly, VA 20151, USA) was used to set up Human IL-10, Vitamin D Receptor, IL-17A and CAMP tests. The DS2 is a computer-controlled microplate processing system that fully automates the following steps of microplate ELISA assays (Figure 2.8.):

a) Sample distribution  
b) Reagent addition  
c) Incubation  
d) Plate washing  
e) Signal detection\textsuperscript{14}

### 2.5.2. **ELISAkitassays principle.**

All 4 ELISA tests were manufactured in Ireland by ELISAkitassays (Damastown, Dublin 15, Ireland) and all use the same improved ultra-fast protocol.\textsuperscript{14}

The ELISAkitassays FIRELISA ELISA (enzyme-linked immunosorbent assays) assay kits are designed for the quantitative measurement of analytes in a wide variety of samples.

ELISAkitassays have developed the FIRELISA range of sensitive, fast and reliable ELISA Assays. The kits use a quantitative Sandwich-ELISA technique and each kit comes with highly specific antibodies pre-coated on a 96-well microtiter plate.

The micro ELISA plates provided in the kits has been pre-coated with an antibody specific to IL-10, VDR, IL-17A and CAMP.

Standards or samples are added to the appropriate micro ELISA plate wells and are combined with the specific antibody. Then a biotinylated detection antibody specific for each analyte and Avidin-Horseradish Peroxidase (HRP) conjugate is added to each micro plate well in succession and incubated. The plate is washed to remove all non-specific
binding that may have occurred. The substrate solution is added to each well. Only those wells that contain specific analyte, biotinylated detection antibody and Avidin-HRP conjugate will exhibit a change in colour. The enzyme-substrate reaction is terminated by the addition of a sulphuric acid solution and the change in the optical density (OD) is measured spectrophotometrically at a wavelength of 450 nm ± 2 nm. The OD value is in proportion to the concentration of measured analyte. The concentration of IL-10, VDR, IL-17A and CAMP in the samples is calculated by comparing the OD of the samples to the standard curve.

2.5.2.1. Overview of ELISA workflow.

The reagents, samples and standards are prepared and equilibrated to room temperature.

100μL standard or sample is added to each well
Incubated @ 37°C for 120 minutes

Liquid is removed. 100μL Detection Reagent A is added
Incubated @ 37°C for 60 minutes

Wash plate x 3
100μL Detection Reagent B is added
Incubated @ 37°C for 60 minutes
90μL Substrate Solution is added
Incubated @ 37°C for 15-30 minutes

50μl Stop solution is added to each well

OD is measured at 450nm

Figure 2.8. ELISA workflow on DS2 analyser:
*Adapted from www.biolegend.com

2.5.3. FIRELISA Kit contents.

Table 2.9. FIRELISA Kits used.

<table>
<thead>
<tr>
<th>Products</th>
<th>Size (assays)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. FIRELISA Human Interleukin-10 Kit</td>
<td>96</td>
</tr>
<tr>
<td>2. FIRELISA Human Vitamin D Receptor Kit</td>
<td>96</td>
</tr>
<tr>
<td>3. FIRELISA Human CAMP Kit</td>
<td>96</td>
</tr>
<tr>
<td>4. FIRELISA Human Interleukin-17A</td>
<td>96</td>
</tr>
</tbody>
</table>

Each kit contains reagents for 96 assays in a 96 well plate including:
Table 2.10.  FIRELISA Kit contents.

<table>
<thead>
<tr>
<th>Item</th>
<th>Component</th>
<th>Qty</th>
<th>Item</th>
<th>Component</th>
<th>Qty</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Standard</td>
<td>2 vials</td>
<td>7</td>
<td>Detection reagent A</td>
<td>120µl</td>
</tr>
<tr>
<td>2</td>
<td>Sample Diluent</td>
<td>20ml</td>
<td>8</td>
<td>Detection reagent B</td>
<td>120µl</td>
</tr>
<tr>
<td>3</td>
<td>ELISA Strip plate coated with monoclonal antibodies</td>
<td>12w×8s</td>
<td>9</td>
<td>Stop Solution</td>
<td>10ml</td>
</tr>
<tr>
<td>4</td>
<td>Concentrated Wash Buffer (25×)</td>
<td>30ml</td>
<td>10</td>
<td>Substrate</td>
<td>10ml</td>
</tr>
<tr>
<td>5</td>
<td>Assay Diluent A</td>
<td>10ml</td>
<td>11</td>
<td>Plate Sealer</td>
<td>5 pieces</td>
</tr>
<tr>
<td>6</td>
<td>Assay Diluent B</td>
<td>10ml</td>
<td>12</td>
<td>Manual</td>
<td>1</td>
</tr>
</tbody>
</table>

2.5.4.  Specific characteristics: assay range, sensitivity, sample types and serum ranges.

2.5.4.1.  Specific characteristics for IL-10.
Wide Assay range: 15.6 pg/ml → 1000 pg/ml  
Sensitivity: 7.80 pg/ml

2.5.4.2.  Specific characteristics for IL-17A.
Wide assay range for IL-17A: 15.6 pg/ml → 1000 pg/ml.  
Sensitivity: 7.80 pg/ml

2.5.4.3.  Specific characteristics for VDR.
Wide Assay range: 0.625 ng/ml → 40 ng/ml  
Sensitivity: 0.16 ng/ml

2.5.4.4.  Specific characteristics for CAMP.
Wide Assay range: 125 pg/ml → 8000 pg/ml  
Sensitivity: 44 pg/ml  
Sample types: serum, plasma, saliva, urine, cell culture supernatant, tissue samples and other related supernatants can be used. We performed analysis only on human serum samples.

2.5.4.5.  Serum ranges.

- CAMP levels range in serum samples: 0.76-1.47 ng/mL
- IL-17α levels range in serum samples: 23-580 pg/mL
- IL-10 levels range in serum samples: 8.7-32 pg/mL
• VDR levels range in serum samples: 130-230 ng/mL

2.5.5. **Sample preparation.**

Serum separator tubes (SST) were used for sample collection. All samples were left to clot for 30 minutes before centrifugation for 15 minutes at approximately 1000 × g. Serum was removed and stored at -80°C until analysis.

2.5.6. **Protocol for IL-10, IL-17A, CAMP and VDR reagents and standard preparation.**

After removal from storage at 2-8°C, the kit equilibrated for 30 minutes at ambient temperature before use.

30 mL of wash buffer concentrate was diluted into distilled water to prepare 750 mL of wash buffer.

Standard was prepared within 15 minutes before use. Standard was centrifuged at 10,000×g for 1 minute, and reconstituted with 1.0ml of sample diluent. After it dissolved fully, it was mixed thoroughly with a pipette. This reconstitution produced a stock solution of 1000 pg/ml for IL-10 and IL-17A, a stock solution of 8000 pg/ml for CAMP and 40 ng/mL for VDR. The serial dilutions were made as needed as indicated in the supplementary Information for Chapter 2 (Figures: S2.1, S2.2. and S2.3.). The undiluted stock serves as the standard with the highest concentration. The sample diluent serves as the zero blank (0 pg/mL and 0 ng/mL).

The reagents A and B diluted to the working concentration using assay diluent A and B (1:100), respectively. For the standard preparation procedure for FIRELISA Human IL-17A, IL-10, VDR and CAMP, refer to Figures: S2.1. S2.2. and S2.3.

2.5.7. **Assays procedure on DS2 Automated ELISA Processing System.**

All reagents should reach room temperature and should be mixed well before the testing. For ELISA workflow on DS2 analyser see Figure 2.8.
2.5.7.1. Sample addition.
100μL of standard, blank, or sample per well is added. The blank well is added with sample diluent. Solutions are added to the bottom of the micro ELISA plate well. They are mixed gently and incubated for 120 minutes at 37°C.

2.5.7.2. Detection reagent A.
The liquid removed from each well. 100μL of detection reagent A working solution added to each well and mixed gently. It is then incubated for 1 hour at 37°C.

2.5.7.3. Wash.
Each well is aspirated and washed, and the process is repeated three times. Washing is carried out by filling each well with wash buffer (approximately 400μL).
Complete removal of liquid at each step is essential. After the last wash, the remaining wash buffer is completely removed by aspirating.

2.5.7.4. Detection reagent B.
100μL of Detection Reagent B working solution is added to each well and incubated for 60 minutes at 37°C.

2.5.7.5. Wash.
The wash process is repeated five times as conducted in step 2.5.7.3.

2.5.7.6. Substrate.
90μL of Substrate Solution is added to each well and incubated for 15-30 minutes at 37°C. The reaction time is about 30 minutes.

2.5.7.7. Stop.
50μL of stop solution is added to each well.

2.5.7.8. OD measurement.
The optical density (OD value) is determined at 450 nm wavelength filter by reading the absorbance of each well containing fluid.
After the experiment, all reagents are stored according to the specified storage temperature respectively until their expiry.

2.5.8. Data analysis.

All the data are stored on DS2 software. In the curve fit operation procedure, DS-Matrix software constructs a standard curve graph using OD values from standards of known concentrations. (Figure 2.9.) From the graph, the concentrations of test samples are determined by finding the concentration that corresponds to a given OD reading. Concentration data are always plotted on the X-axis, and OD data are always plotted on the Y-axis. (Figures 2.10., 2.11., 2.12. and 2.13.) Standards of increasing concentrations are included in the assay template, to use in the curve fit operation. The data are linearised by plotting the log of the analyte concentrations versus the log of the OD, and the best fit line is determined by regression analysis.

![Figure 2.9. An example of the curve fit concentration entry on DS2 analyser.](image)
Figure 2.10. A standard curve FIREILSA Human IL-17A on DS2. Concentration plotted on the X-axis, and OD data is on the Y-axis.

Linear Regression:
Log/Log with data extrapolation
Y = 10^(-1.486 + 0.562 log(X))
R-Squared = 0.931
Figure 2.11. A standard curve FIREILSA Human IL-10 on DS2. Concentration is plotted on the X-axis, and OD data are on the Y-axis.
Figure 2.12. A standard curve FIREILSA Human VDR on DS2. Concentration plotted on the X-axis, and OD data are on the Y-axis.
Figure 2.13. A standard curve FIREILSA Human CAMP on DS2.
Concentration plotted on the X-axis, and OD data are on the Y-axis.
2.6. **Automated DNA Purification on the Maxwell® 16 Instrument.**

The DNA purification was performed using the automated isolation of genomic DNA from human whole blood samples on the Maxwell® 16 Blood DNA Purification system. (Promega Corporation, Madison, WI 53711-5399 USA).

2.6.1. **Principle.**

The Maxwell® 16 IVD Instrument provides automated nucleic acid purification from up to 16 samples, using lysis to release nucleic acid and binding nucleic acid to paramagnetic particles as the primary separation principle. It has two modes for different elution volumes. We used the Standard Elution Volume (SEV) mode. This mode used for the Maxwell® 16 Blood DNA Purification System allows elution in a volume of 300μl. The Maxwell® 16 IVD Instrument provides high-purity nucleic acid that is compatible with standard amplification methods.

The automated steps performed by the Maxwell® 16 IVD Instrument include:

- Sample lysis in the presence of a chaotropic agent, detergent and/or alcohol
- Binding of nucleic acids to paramagnetic particles
- Washing of the nucleic acid bound to the particles away from other cellular components
- Elution of the product
- The instrument contains a touch screen for navigating, programming and running the instrument. It has a UV lamp to aid with decontamination.

2.6.2. **Reagents.**

2.6.2.1. **MAXWELL® 16 Blood DNA cartridges.**

Each reagent cartridge contains lysis buffer, MagneSil®PMPs (magnetic silica particles), and a series of five wash buffers containing ethanol and isopropanol. Each reagent cartridge comes sealed and is ready for use.
2.6.2.2. **Elution buffer.**

The elution buffer is supplied in a 20ml bottle and it is also ready for use. The Maxwell® 16 DNA Purification system, all reagent cartridges and elution buffer are stored at room temperature (15-30°C) until the expiry date on the product label. Products were not used after the expiration date.

2.6.3. **Samples preparation.**

Whole blood samples collected in EDTA treated tubes were used. Only the tubes with EDTA were used because some anticoagulant (eg., Heparin) are strong inhibitors of Taq Polymerase, and so could alter the efficiency of the amplification reaction later. Blood samples were stored at -20°C until processed.

2.6.4. **Experimental procedure.**

The Maxwell® 16 Blood DNA Purification System automates nucleic acid purification from up to 16 samples using cell lysis and binding of magnetized silica particles to nucleic acid as the primary separation principle. The automated extraction steps are performed in sequence:

- WBC sample lysis in the presence of a chaotropic agent and detergent.
- Binding of nucleic acids to magnetised silica particles.
- Washing of the bound particles away from other cellular components.
- Elution of nucleic acids into a formulation that can be added directly to standard PCR.

The total yield of genomic DNA from whole blood samples depends on the sample volume and the number of white blood cells/ml. Each cartridge supplied in the Maxwell® 16 Blood DNA Purification System is designed for purification of genomic DNA from 300μl of whole blood, assuming an average number of white blood cells in the range of $4.2 \times 10^6$ to $12 \times 10^6$/ml whole blood (values for a normal healthy adult).
The appropriate processing protocol on the Maxwell® 16 instrument was used. As shown in Figure 2.14, the samples are placed into the reagent cartridges, and the cartridges are then placed onto the Maxwell® 16 instrument platform for extraction. The temperature of the samples is regulated by a heating system, which is controlled by the system protocol. After the extraction, the eluate is transferred from the blue elution tubes into the labelled eppendorf and stored at -20°C. The extracted DNA was used for PCR amplification. 16


2.7.1. Instrumentation.

In Biomnis Ireland we used the Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). It is a 96-well, five-colour platform that uses fluorescent-based Polymerase Chain Reaction (PCR) reagents to provide:

- Quantitative detection of target nucleic acid sequences (targets) using real-time analysis,
- Qualitative detection of targets using post-PCR (endpoint) analysis,
• Qualitative analysis of the PCR product (achieved by melt-curve analysis that occurs post-PCR).

The 7500 Fast system collects raw fluorescence data at different points depending on the type of run that the instrument performs.

**Table 2.11.** Type of run on 7500 Fast Real-Time PCR System.

<table>
<thead>
<tr>
<th>Run Type</th>
<th>Data Collection Point</th>
</tr>
</thead>
<tbody>
<tr>
<td>Real-Time runs</td>
<td>Standard Curve</td>
</tr>
<tr>
<td></td>
<td>Relative standard Curve</td>
</tr>
<tr>
<td></td>
<td>Comparative Ct</td>
</tr>
<tr>
<td>Post-PCR (endpoint) runs</td>
<td>Genotyping</td>
</tr>
<tr>
<td></td>
<td>Presence/Absence</td>
</tr>
</tbody>
</table>

Regardless of run type, a data collection point (read) on the 7500 Fast system consists of three phases:

1. **Excitation:** The instrument illuminates all wells of the reaction plate within the instrument, exciting the fluorophores in each reaction.
2. **Emission:** The instrument optics collect the residual fluorescence emitted from the wells of the reaction plate. The resulting image collected by the device consists only of light that corresponds to the range of emission wavelengths.
3. **Collection:** The instrument assembles a digital representation of the residual fluorescence collected over a fixed time interval. The 7500 software stores the raw fluorescence image for analysis.

After a run, the 7500 software uses region of interest (ROI), optical, dye and background calibrations to determine the location and density of the fluorescence in each read, the dye associated with each fluorescent signal, and the significance of the signals. In our studies we used Real-Time PCR System 7500 Fast for the genotyping testing for 4 SNPs.
2.7.2. Allelic Discrimination / Genotyping assays principle.

An Allelic Discrimination (AD) assay is a multiplexed (more than one primer/probe pair per reaction), end-point (data are collected at the end of the PCR process) assay that detects variants of a single nucleic acid sequence. The presence of two primer/probe pairs in each reaction allows genotyping of the two possible variants at the single nucleotide polymorphism (SNP) site in a target template sequence. The actual quantity of the target sequence is not determined.

For each sample in an AD assay, a unique pair of fluorescent dye detectors is used – for example, two TaqMan® MGB probes that target an SNP site. One fluorescent dye detector is a perfect match to the wild type (allele 1) and the other fluorescent dye detector is a perfect match to the mutation (allele 2).

The Allelic Discrimination assay classifies unknown samples as:
- Homozygotes (samples having only allele 1 or allele 2)
- Heterozygotes (samples having both allele 1 and allele 2)

A genotyping assay detects variants of a single nucleic acid sequence, without quantifying the target. The presence of two probes in each reaction allows genotyping of the two possible variants at the SNP site in a target sequence.

Each Taqman® SNP Genotyping assays consists of a single, ready to use tube containing:
- Two sequence specific primers for amplifying the polymorphism of interest.
- Two allele specific Taqman® MGB probes for detecting the alleles of the specific polymorphism of interest. (Tables 2.13 and 2.14.)

Each allele specific Taqman® MGB probe has:
- A reporter dye at its 5’ end
- VIC® dye, linked to the 5’ end of the Allele 1 probe
- FAM™ dye, linked to the 5’ end of the Allele 2 probe
The Allele 1 VIC® dye-labeled probe corresponds to the first nucleotide inside the square brackets of the context sequence in the assay information file (AIF) shipped with each order. The Allele 2 FAM™ dye-labeled probe corresponds to the second nucleotide.

- A minor groove binder (MGB) which increases the melting temperature (Tm) for a given probe length.
- A non-fluorescent quencher (NFQ) at its 3’ end, allowing for detection of the reporter dye fluorescent with greater sensitivity.

**During PCR**

- Each TaqMan® MGB probe anneals specifically to its complementary sequence between the forward and reverse primer sites.
- When the oligonucleotide probe is intact, the proximity of the quencher dye to the reporter dye quenches the reporter signal.
- AmpliTaq Gold® DNA polymerase extends the primers bound to the genomic DNA template.
- AmpliTaq Gold® DNA polymerase (with its 5´ nuclease activity) cleaves probes that are hybridised to the target sequence.
- Cleavage of the probes hybridised to the target sequence separates the quencher dye from the reporter dye, resulting in an increased fluorescence by the reporter. The fluorescence generated by PCR amplification indicates which alleles are present in the sample. (Figure 2.15.)

In TaqMan® assays, fluorescence from nonspecifically bound probes is reduced because nucleotide mismatches between a probe and a sequence lessen the chances that the probe will be cleaved. The probe’s short length means that a one base pair mismatch has a greater negative effect on the binding. The mismatched probe does not bind tightly to the allele, allowing the AmpliTaq® Gold DNA polymerase to displace the probe without cleaving the dye.17
2.7.3. **TaqMan® SNP Genotyping Assays products.**

The assays were purchased from ABI (Applied Biosystems), and they were applied using a Real Time PCR 7500 Fast System. For this study we chose 4 SNPs: (Table 2.12 and 2.14).
Table 2.12. TaqMan® SNP Genotyping Assays data sheet.

<table>
<thead>
<tr>
<th>Tube Position</th>
<th>Assay ID*</th>
<th>RS Number*</th>
</tr>
</thead>
<tbody>
<tr>
<td>A01</td>
<td>C___28977635_10</td>
<td>rs7975232</td>
</tr>
<tr>
<td>B01</td>
<td>C___1747362_10</td>
<td>rs1800871</td>
</tr>
<tr>
<td>C01</td>
<td>C___2404008_10</td>
<td>rs731236</td>
</tr>
<tr>
<td>D01</td>
<td>C___2404009_20</td>
<td>rs757343</td>
</tr>
</tbody>
</table>

For more information, see sections 2.7.3.1. – 2.7.3.4.

Table 2.13. TaqMan® SNP Genotyping Assay details.

<table>
<thead>
<tr>
<th>Part Number</th>
<th>Product</th>
<th>Reporter / Quencher</th>
<th>Volume (µL)</th>
<th>Formulation</th>
<th>No. of Reactions (5µL Reaction Size)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4351379</td>
<td>TaqMan® SNP Genotyping Assays, Human, SM</td>
<td>Allele 1: VIC/MGB-NFQ</td>
<td>188</td>
<td>40x</td>
<td>1,500</td>
</tr>
</tbody>
</table>

Table 2.14. TaqMan® SNP Genotyping Assay sequence.

<table>
<thead>
<tr>
<th>Assay ID</th>
<th>Context Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>C___1747362_10</td>
<td>AGTGAGCAAAACTGAGGCACAGAGAT[A/G]TTACATCACCTGTACAAGGGTACAC</td>
</tr>
<tr>
<td>C___2404008_10</td>
<td>TGGACAGGCGGTCTGTGATGGCCTGGCAGGCGGTACCTGTACAAGGGTACAC</td>
</tr>
<tr>
<td>C___2404009_20</td>
<td>CTCACCTCTAACCAGCGGAAGGGT[C/T]AAGGGTCACTGCACATGCAACCTCCAA</td>
</tr>
<tr>
<td>C___28977635_10</td>
<td>AAGGGCAGGAGCTCTAGCTGGGGG[C/A]CCTGACTGCTCAATCCACACCACCC</td>
</tr>
</tbody>
</table>

Table 2.15. TaqMan® SNP Genotyping Universal Master Mix.

<table>
<thead>
<tr>
<th>Cat No</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>4440042</td>
<td>TaqMan® Universal Master Mix II, with UNG Mini-Pack (1 x 1 mL)</td>
</tr>
</tbody>
</table>

TaqMan reagents use a fluorogenic probe to enable detection of a specific PCR product as it accumulates during PCR cycles.

Advantages.

- Specificity is increased with the addition of a fluorogenic probe.
- Multiplex capability is provided.
Preformulated assays are optimised to run under universal thermal cycling conditions.

2.7.3.1. **SNP: rs731236.**

The assay number is C_2404008_10. This Vitamin D Receptor variant is commonly known as TaqI (A>G) or (T>C) and is located in exon 9 (allele A is often referred to as allele T, and allele G is often referred to as allele t).

2.7.3.2. **SNP: rs7975232.**

The assay number is C_28977635_10. This Vitamin D Receptor variant is commonly known as ApaI (C>T) and is located in intron 8 (allele C is often referred to as allele A, and allele T is often referred to as allele a).

2.7.3.3. **SNP: rs757343.**

The assay number is C__2404009_20. This Vitamin D Receptor polymorphism is known as Tru9I (C > T) and is located in intron 8. All these three VDR polymorphisms studied are close to the 3’-UTR region of the gene, the ligand-binding domain.

2.7.3.4. **SNP: rs1800871.**

The assay number is C_1747362_10. This IL-10 gene functional polymorphism −819 (A > G) is located in the promoter region.

2.7.4. **Genotyping experiment procedure.**

Genotyping of Taq-I, Apa I, Tru 9 and rs1800871 was performed using TaqMan® SNP Genotyping Assay. This consists of a predesigned mix of unlabelled polymerase chain reaction (PCR) primers and the TaqMan® minor groove binding group (MGB) probe (FAM™ and VIC® dye-labelled). All TaqMan SNP Genotyping Assays are designed to work with TaqMan® Universal PCR MasterMix. (Table 2.15.) This contains DNA polymerase, dNTPs and optimised mix components and it uses the same thermal conditions. It was genotyped using Real-Time PCR in which one probe labelled with VIC® detects the allele 1 and the other probe labelled with FAM™ detects allele 2. Real Time PCR was performed using 5 µl
TaqMan® Genotyping Master Mix, 0.25 μl TaqMan® SNP Genotyping Assay (TaqMan probes) (40×), 3.75 μl Dnase Free Water and 1 μl DNA (1-10 ng), to bring the final reaction volume to 10 μl. The Real Time PCR thermal conditions consisted of the following: Initial denaturing at 95°C for 10 mins; 40 cycles of 95°C for 15 secs (denaturing) and 60°C for 1 min (annealing/extension). (Figure 2.16.)

*Figure 2.16.* Presentation of Run stages on Real Time PCR 7500 Fast System.

The progress of the run was checked in real time during the run, and was periodically viewed in all three available plots from the 7500 software for potential problems.

The amplification plot displays as the instrument collects fluorescence data during a run. The plot shows normalised dye fluorescence (ΔRn) as a function of the cycle number. The figure below (Figure 2.17.) shows the Amplification Plot screen as it appears during a run.
Figure 2.17. Amplification Plot for rs731236 and rs7975232 SNPs on Real Time PCR 7500 Fast System.

An initial review of the experiment results was performed in the Allelic Discrimination Plot, which contrasts the normalised reporter fluorescence (Rn) for the allele-specific probes of the SNP assay. The Allelic Discrimination plot displays clusters for the three possible genotypes (Allele 1 homozygous (red), Allele 2 homozygous (blue), Heterozygous Allele1/ Allele 2 (green), and a cluster for negative controls (black)). Allele A is plotted on the X-axis, and Allele G is plotted on the Y-axis. (Figure 2.18.)

When analysing the results, all controls were verified as having the correct genotype and the patient’s results were screened for flags generated during the run.
Figure 2.18. The Allelic Discrimination plot for rs731236 SNPs on Real Time PCR 7500 Fast System. Allele A is plotted on the X-axis, and Allele G is plotted on the Y-axis.

All materials were used in the TaqMan® SNP Genotyping Assay (ABI) in accordance with the manufacturer’s instructions and the information on the Applied Biosystems web site http://www.appliedbiosystems.com.
2.8. FBC testing on automated analyser Sysmex XE-2100D.

The full blood count in Biomnis Ireland was performed on the Sysmex XE-2100D. (Sysmex America, Inc., Mundelein, IL 60060 USA).

Knowing the proportions of the different components (neutrophils, lymphocytes, monocytes, red blood cells, basophils, haemoglobin and reticulocytes) of peripheral blood is vital in distinguishing between diseased and normal blood.

The Sysmex XE-2100 System is a fully automated haematology analyser which produces the results for 32 parameters of blood samples. It is easy to use and has intuitive software menus with on-board help key for rapid troubleshooting.

The Sysmex XE-2100 utilises the fluorescent flow cytometry and hydrodynamic focusing technologies. Using a diode laser bench, Sysmex fluorescent flow cytometry provides the sensitivity needed for measuring and differentiating cell types in both whole blood and body fluid samples. The Sysmex XE-2100 quantitates the standard five part differential, immature granulocytes (IG) (metamyelocytes, myelocytes and promyelocytes), nucleated red blood cells (NRBC), reticulocyte count, immature reticulocyte fraction and “optical” fluorescent platelet count. The combination of side scatter (the inner complexity of the cell), forward scatter (volume) and fluorescence intensity of nucleated cells gives a concise yet precise image of each cell detected in the peripheral blood. A well-defined physical description of the different leucocyte populations (clusters) is obtained. (Figure 2.19.)

Abnormal and immature cells, with their larger nuclear volume, show a much higher fluorescence intensity than normal cells, and they are easily distinguishable in the DIFF scattergram.

The technologies used enable the XE-2100 to classify normal WBC, RBC and PLT populations from abnormal ones.\textsuperscript{18}

In our study we used only EDTA samples for FBC analysis.
Figure 2.19. Example of scattergram from Sysmex® XE. Taken from Sysmex® XE-Series Automated Haematology Systems Interpretation Guide, website: www.sysmex.com.
2.9. References for Chapter 2.

2.10. Supplementary information for Chapter 2

2.10.1. Dilution protocol for IL-10, IL-17, CAMP and VDR standard preparation.

<table>
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<th>Dilution Protocol</th>
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<tr>
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<td>No. 3</td>
<td>500μl Standard No. 2 + 500μl Sample Diluent</td>
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<td>500μl Standard No. 5 + 500μl Sample Diluent</td>
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<td>No. 7</td>
<td>500μl Standard No. 6 + 500μl Sample Diluent</td>
</tr>
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<td>0pg/ml</td>
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<td>500μl Sample Diluent</td>
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</tbody>
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Figure S2.1. Standard preparation procedure for FIRELISA Human IL-17A and IL-10.
Taken from ELISAkitassays.com Technical Manual: www.elisakitassays.com
Figure S2.2. Standard preparation procedure for FIRELISA VDR. Taken from ELISAkitassays.com Technical Manual: www.elisakitassays.com

<table>
<thead>
<tr>
<th>Concentration (ng/ml)</th>
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<th>Description</th>
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<tr>
<td>10</td>
<td>No. 3</td>
<td>500 µl</td>
<td>500 µl Standard No. 2 + 500 µl Sample Diluent</td>
</tr>
<tr>
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<td>500 µl</td>
<td>500 µl Standard No. 3 + 500 µl Sample Diluent</td>
</tr>
<tr>
<td>2.5</td>
<td>No. 5</td>
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<td>No. 7</td>
<td>500 µl</td>
<td>500 µl Standard No. 6 + 500 µl Sample Diluent</td>
</tr>
<tr>
<td>0</td>
<td>No. 8</td>
<td>500 µl</td>
<td>500 µl Sample Diluent</td>
</tr>
</tbody>
</table>

Stock solution

- 40ng/ml
- 20ng/ml
- 10ng/ml
- 5ng/ml
- 2.5ng/ml
- 1.25ng/ml
- 0.62ng/ml
- 0.31ng/ml
Figure S2.3. Standard preparation procedure for FIRELISA CAMP.
Taken from ELISAkitaAssays.com Technical Manual: www.elisakitassays.com
Chapter 3


### 3.1 Introduction.

Recent studies have shown that VDD is not only associated with bone and severe liver and kidney disease; it also has important implications in many chronic illnesses, including cancer, diabetes mellitus, hypertension and asthma.¹,²

As mentioned in Chapter 1, vitamin D is a fat-soluble steroid prohormone that is mainly produced photochemically in the skin from 7-dehydrocholesterol. Two forms of vitamin D are biologically important – vitamin D3 (Cholecalciferol) and vitamin D2 (Ergocalciferol).

Both vitamins D3 and D2 can be absorbed from food and can be found in vitamin supplements, but it is estimated that only 10-20% of vitamin D is supplied through food.³ Vitamin D is metabolised to the active hormone 1,25(OH)2-vitamin D (Calcitriol) through two hydroxylation reactions. The first of these occurs in the liver, converting vitamin D into 25-hydroxyvitamin D. The second converts 25OHD form into the biologically active 1,25(OH)2D, and it occurs in the kidneys and in many other cells.

Most cells express the vitamin D receptor, and about 3% of the human genome is regulated by the vitamin D endocrine system.³ The vitamin D level is generally assessed by measuring the serum or plasma level of 25OHD. This has a half-life of 2–3 weeks, and it is not influenced by any changes in calcium and parathyroid hormone levels.⁴ As was mentioned previously, this major storage and circulating form is a reliable indicator of vitamin D status.⁵ Low 25OHD concentrations are associated with secondary hyperparathyroidism, skeletal diseases such as rickets, and many chronic illnesses.²,⁶
A panel from the Institute of Medicine (IOM) of the American National Academy of Sciences decided that, on the basis of skeletal anomalies, VDD can be defined as a serum 25OHD below 50 nmol/L. A newly-proposed definition of vitamin D sufficiency (>50 nmol/L) has proved to be controversial, mainly because it has a major impact on the clinical evaluation of vitamin D insufficiency. There is no current consensus on the optimal vitamin D levels for non-musculoskeletal health; therefore it is important to establish reference intervals among the general population – specifically in different seasons.

Vitamin D tests are now widely included as a part of routine laboratory work. A significant increase in laboratory testing for 25OHD has resulted in the development and the implementation of new automated diagnostic approaches to keep pace with the volume of demand. In our laboratory we have looked for a rapid, reliable, fully-automated and cost-saving assay that would improve turnaround time for our clients.

Numerous methods have been developed for measuring serum and plasma 25OHD concentrations. The first routine methods for assessing 25OHD were based on competitive protein binding. These have been supplanted by radioimmunoassay (RIA) and chemiluminescent immunoassay (CLIA), which form the fundamental principles of several methods that are commercially available. Two main analytical techniques normally employed in the laboratories are competitive immunoassays and chemical methods. Among the immunoassays are the following: RIA, CLIA, enzyme immunoassay, electrochemiluminescence immunoassay, chemiluminescent microparticle immunoassay (CMIA) and competitive protein binding assay. The chemical methods are based on chromatographic separation, followed by non-immunological direct detection. They include direct high performance liquid chromatographic with ultraviolet (HPLC-UV) detection, and liquid chromatography combined with mass spectrometry (LC-MS). The principal difference between these two methods is the ability of HPLC and LC-MS to quantify 25(OH)D2 and 25(OH)D3 separately. Recently a C3-epimeric form of 25(OH)D3 has been detected which is unresolved in some LC-MS/MS assays but may add to the total result of 25OHD. The epimer, however,
has been found primarily in neonatal samples and it has been suggested that it does not add significantly to overall measured 25OHD concentration.\textsuperscript{11,12}

The Joint Committee for Traceability in Laboratory Medicine (JCTLM) recognises isotope dilution liquid chromatography tandem mass spectrometry (ID-LC-MS/MS) as the reference method for vitamin D. Mass Spectrometry is highly sensitive and specific and it can analyse several related analytes in a single run, with a potential for cost savings. The introduction of commercial validated assay kits, traceable standards and participation in external quality assessment schemes has substantially improved assay quality for LC-MS/MS determination of 25(OH)D\textsubscript{3}. However, the initial capital purchase of these instruments can be costly, they require highly-trained staff, and they are currently best suited to larger laboratories.

Within the last few decades \textit{in vitro} diagnostic companies have introduced new automated immunoassays for the measurement of 25(OH)D\textsubscript{3}, thus improving the laboratory's ability to cope with increasing demand. Recent publications have outlined the limitations of these immunoassays. Some have poor antibody specificity with cross-reactivity to other metabolites of Vitamin D, as well as a problematic extraction of the 25OHD form from the vitamin D-binding protein (DBP). Some of them also interact with matrix substances such as lipids, and there are notable variations in 25OHD determination between different assays.\textsuperscript{13-16} Until recently standardisation and harmonisation between the various marketed vitamin D assays was poor. The introduction of a new traceable reference standard by NIST SRM 972 (American National Institute of Standards and Technology Standard Reference material 2972), however, has improved both validation and calibration of 25OHD assays across platforms.

In our laboratory a Vitamin D test was run and accredited to ISO 15189 on the DiaSorin Liaison instrument. When Abbott Architect 25OHD kit CE marked became available it had already been extensively validated by the manufacturers, so a full validation was not indicated. In 2016 a new
generation of the Abbott reagents came onto the market, standardised to NIST (SRM 2972).

The purpose of our verification was to confirm that the assay was analysed in our laboratory to the manufacturer’s standards, to establish any clinical difference between results obtained using the Liaison, Architect and liquid chromatography-tandem mass spectrometry (LC-MS/MS) methods, to calculate the uncertainty of measurement for the assay, to run External Quality Assessment samples, and to compare the reference interval study results with current 25OHD recommendations.

3.2. Materials and methods.

3.2.1 Abbott Architect 25OHD immunoassay method.

The Architect 25OH Vitamin D assay (3L52) is a delayed 1-step chemiluminescence microparticle immunoassay (CMIA) involving automated online pre-treatment with flexible assay protocols, which are known as Chemiflex. (Figure 3.1.) It uses a polyclonal sheep anti-vitamin D IgG antibody coated microparticles and a biotinylated vitamin D antibiotin IgG acridinium-labelled conjugate complex for the quantitative determination of 25OH vitamin D2 and D3 in human serum and plasma. The measuring range for the assays is 32.5 to 240 nmol/L. The new generation of the Architect 25OHD assay (5P02) is also a delayed 1-step competitive immunoassay, but is notably improved. (Figure 3.2.) Conjugate is added after microparticles have incubated with the sample, but there is no wash before this addition of conjugate, which fills the vacant sites on the microparticle antibody. The pre-treatment, ANSA (8-anilo-1-naphalensulfonic acid) in assay specific diluent is added at the same time as microparticle (Rabbit Monoclonal Anti-Human Vitamin D IgG) reagent. The avidity and stability of microparticles antibody for vitamin D is much higher than in the old generation. The acridinium-labelled conjugate is also an improved, much simpler and more stable conjugate than the previous assay. The crossreactivity between the assays is similar: with 25OHD3 it is 105%, 25OHD2 – 54%, and it is very low with 3-epi-25OHD3 – 1.3%. The new generation measuring range is 8.5 to 389.8 nmol/L. Both assays are VDSP (Vitamin D Standardisation
Programme) certified and they successfully passed the performance criterion of ±5% mean bias to the Centres of Disease Control (CDC) and University of Ghent Vitamin D2 and D3 Reference Method with an overall imprecision of <10% over the concentration range of 22-275 nmol/L for total 25-hydroxyvitamin D.

Figure 3.1. 25OH Vitamin D “Old” Assay (3L52) principle.*
3.2.2 DiaSorin Liaison 25OHD immunoassay method.

The DiaSorin Liaison used in our laboratory for total 25OHD is a direct competitive chemiluminescent immunoassay (DiaSorin, Dietzenbach, Germany) with inter- and intra-assay coefficient of variation (CV) of 6.8% and 7% respectively. This method is reported to have relative crossreactivities with 25OHD3, 25OHD2, and 3-epi-25OHD3 of 100%, 104%, and less than 1%, respectively. The analytical measuring range is 10 to 375 nmol/L. Detailed information on the method was provided in Chapter 2.

3.2.3 Total 25OHD LC-MS/MS method.

The 25-hydroxyvitamin D levels were measured by liquid chromatography tandem mass spectrometry (LC-MS/MS) on an AB SCIEX API 4000 analyser (Applied Biosystems Life Technologies, Foster City, CA, USA) at St. James’s Hospital, Dublin. The vitamin D assay was developed by Chromsystems GmbH (Munich, Germany; Catalogue No. 62000) for analysis by mass chromatography. The reportable results for this test.
included the concentrations of 25OHD2, 25OHD3, and total 25OHD (calculated as the sum of 25OHD2 and 25OHD3). The method has been shown to provide results that are in close agreement with the American National Institute of Standards and Technology (NIST) target values for 25OHD2 and 25OHD3 for Standard Reference Material 972, levels I to III. The analytical measuring range is 1.5 to 624 nmol/L.

The within-run coefficient of variation was 4.1% at 60.7 nmol/L for vitamin D2 and 3.4% at 27.7 nmol/L for vitamin D3. The between run precision was 5.8% at 42.6 nmol/L and 5.7% at 95.8 nmol/L for vitamin D2, 6.1% at 41.6 nmol/L, and 5.0% at 98.2 nmol/L for vitamin D3.

### 3.2.4. Other methods.

For the reference intervals study all serum measures of total calcium, albumin, alkaline phosphatases, phosphate, total protein, bilirubin, alanine transaminase, aspartate aminotransferase, gamma-glutamyl transferase, urea and creatinine, glucose, cholesterol, HDL and LDL-cholesterol were performed, using commercially available diagnostic kits on the Abbott Architect ci8200 (Abbott Laboratories, Abbott Park, IL, USA). The mean between-run and within-run CVs for these assays ranged between 1% and 5%. More detailed information on the methods was provided in Chapter 2.

### 3.2.5. Statistical softwares.

We used EP Evaluator Release 9 software (Data Innovations, LLC), GraphPad Prism 5, version 5.01 and Microsoft Excel 2010 for the statistical evaluation of the results.

### 3.2.6 Validation samples.

We used only serum samples for 25OHD determination. All the samples were treated according to our preanalytical procedure: after sampling, they were spun at +4°C at 3500G, aliquoted within 1 hour, and kept frozen at −20°C until determination, as it has been shown that 25OHD is particularly stable. For the examination of reference intervals, samples were obtained from healthy Irish participants as part of their routine health and lifestyle screen. Method comparison studies were carried out on
samples from patients (age range, 5 - 80 years) suffering from different diseases, specifically osteoporosis and asthma. As mentioned above, the epimer form is not discussed because it is not relevant to our study.\textsuperscript{11,12} The values for total 25OHD varied from 2.5 to 223 nmol/L. We used serum specimens submitted to a laboratory for the measurement of total 25OHD from three Dublin hospitals (James Connolly, The Adelaide and The Meath, and St. James’s). As vitamin D deficiency is very common in Ireland, we fortified some volunteers from the patients with softgel capsules of 2,000 IU cholecalciferol for 15 weeks daily (Best Formulations \textregistered, California, USA). All supplemented subjects signed an institutional review board-approved written informed consent form. The hospitals’ Research Ethics committees approved the study. Abbott 25OHD Quality Controls and Technopath Immunoassays Controls were used for the precision and uncertainty of measurement study.

### 3.3 Validation protocol.

We evaluated the precision based on guidance from the National Committee for Clinical Laboratory Standards (NCCLS) Protocol EP5-A2.\textsuperscript{18} For between-run precision, 3 levels of Technopath Immunoassays Controls were analysed for twenty days, using one instrument and the same lot of reagents. A method of comparison was assessed between patient sample results run on the DiaSorin Liaison, AB SCIEX API 4000 liquid chromatography tandem mass spectrometer and the Architect, using EP Evaluator software (release 9, build 4-457). The two-instrument comparison method (based on allowable total error) was used to determine clinical equivalence of the results from the instruments. Clinical equivalence was calculated as follows: two methods are seen as clinically equivalent if the difference between them is less than the allowable total error for each specimen. It does not matter if there is a bias, provided that the bias is within allowable error. All methods were run according to the manufacturer’s instructions.

We established uncertainty of measurement for both the old and new generations of the Abbott method, based on our internal standard operation procedure, using calculations from the source listed in the
Abbott provided us with their uncertainty data, but these were irrelevant to our study and we did not factor them into our calculations. The within-run variation (uwr) was calculated from the mean and standard deviation (SD) of repeated measurements of a single sample in single run. The standard uncertainty (u) was calculated using the formula: $u = \frac{s}{\sqrt{n}}$, where $s =$ the SD, and $n =$ the number of measurements. The between-run variation (ubr) was calculated from the mean, and the SD obtained from the daily control runs. To calculate the total uncertainty of measurement ($u_c$) for 25OHD we used summation in quadrature (root sum of the squares) of the standard uncertainties:

Combined uncertainty: $u_c = \sqrt{(u_{wr})^2 + (u_{br})^2}$.

To calculate the 95% confidence interval (CI), this combined uncertainty was multiplied by a “coverage factor” of 2. The result is known as the “expanded uncertainty”.

To calculate Uncertainty of Measurement (UOM), one sample, with a 25OHD concentration of 50 nmol/L, was run 20 times to calculate within-run precision. For between-run precision, controls with a concentration of 67 and 57 nmol/L, for the old and new generation respectively, were run daily for more than 30 days. We calculated an expanded uncertainty with $k = 2$. (Table 3.1.) The precision protocols differed for the old and new assays, due to differences in lot of the quality controls used and in the number of samples analysed.

Serum samples from 210 healthy individuals were assayed to assess reference intervals for 25OHD and to verify the reference interval used with the Liaison method. All the patients were carefully screened for any chronic illnesses by Biomnis’s nurse during a “Health and Lifestyle” screening programme. The samples were tested for total calcium, albumin, ALP, PO4, urea, creatinine and liver enzymes. We studied only patients whose results were entirely within the reference ranges. Body mass index (BMI) was calculated using participants’ weight and height. We also acquired information from healthy adults relating to their age, sex, race, medications taken, and vitamin D supplements. Reference
interval data were analysed according to the National Committee for Clinical Laboratory Standards guideline for determining reference values.21

Data were analysed using EP Evaluator Nonparametric Method (CLSI C28-A) 21 of calculating a Reference Interval. This method makes no assumption about the shape of population distribution. The central 95% of the data were taken as the reference interval for the 25OHD.

3.4 Results.

For the between-run precision, Stockl et al. 22 suggest a maximum CV of 10% for 25OHD analysis. The manufacturers claim 4.6% for their low control, 3.0% for medium, and 2.8% for high level. Our instrument showed a CV of 6.2% for our 40 nmol/L low control, 3.9% for the 67 nmol/L for medium and 4% for 100 nmol/L high level for Architect 25OHD old assay (3L52). For the new generation of the reagents (5P02) we showed a CV of 5.6% for our 36 nmol/L low control, 5% for 57 nmol/L medium and 5% for 113 nmol/l high level.

The Instrument comparison data were analysed for Clinical Equivalence by EP Evaluator using the Two Instrument Comparison module. An Allowable Total Error (TEa) was used in accordance with the paper listed in the references.22 The paper’s calculations were “derived from the evaluation criteria set by the Advisory Panel of DEQAS”,23 using a common proficiency testing programme, i.e., 46%. The difference between the Liaison and Architect instruments for 36 of 37 (97.3%) samples was within TEa.22 Specimens were compared over a range 15.9 to 136 nmol/L. The average Error Index (Y-X)/TEa was -0.22 with a range of -1.02 to 0.86. The largest Error Index occurred at a concentration of 83.6 nmol/L. Deming regression analysis yielded a slope of 0.85 (95% CI, 0.79-0.91) and y-intercept of 3.11 nmol/L (95% CI, 0.07-6.16). (Figure 3.3. a)

We also analysed 69 specimens for 25OHD (old 3L52) by Architect at Biomnis Ireland and LC-MS/MS method in St. James’s Hospital, Dublin. The difference between the methods was within allowable total error for 67 of 69 samples (97.1%). Specimens were compared over a range 27.8
to 170 nmol/L. The average Error Index (Y-X)/TEa was 0.19 with a range of -0.52 to 1.63. The largest Error Index occurred at a concentration of 44.6 nmol/L. Deming regression analysis yielded a slope of 0.96 (95% CI, 0.88-1.04) and y-intercept of 6.73 nmol/L (95% CI, 1.11-12.35). (Figure 3.3. b)

The new generation of 25OHD (5P02) was compared with the LC-MS/MS method using 100 patients’ samples. Only 6 of them had a significant 25(OH)D2 level. It should be noted that only total 25OHD levels were evaluated. Specimens were compared over a range 2.5 to 223 nmol/L. The difference between the two methods was within allowable error for 99 of 100 specimens (99%). The average Error Index (Y-X)/TEa was -0.24 with a range of -0.88 to 4.70. The largest Error Index occurred at a concentration of 2.5 nmol/L. Deming regression analysis yielded a slope of 0.88 (95% CI, 0.847-0.92) and y-intercept of -1.20 nmol/L (95% CI, -3.52 -1.12). (Figure 3.4. b) We identify the range of data for which the difference is significant. Using the Paired Sample Wilcoxon Signed Rank test we demonstrated that for a concentration less than 30 nmol/L the difference between two methods was not significant (p = 0.05). However the difference between two methods was significant for a concentration greater than 30 nmol/L.

The difference between the new and old generation of 25OHD Architect assays for 45 of 47 (96%) samples was within Allowable Total Error. Specimens were compared over a range of 21 to 162 nmol/L. The average Error Index (Y-X)/TEa was -0.45 with a range of -1.23 to 0.82. The largest Error Index occurred at a concentration of 55 nmol/L. Deming regression analysis yielded a slope of 0.87 (95% CI, 0.8 - 0.96) and y-intercept of -4.9 nmol/L (95% CI, -10.93 - 1.15). (Figure 3.4. a) Accordingly, the old and new methods were shown not to be statistically equivalent (95% CI of slope does not include unity). However, all the instruments and methods produced clinically equivalent results. The results covered the medical decision points of 30, 40, 50 and 125 nmol/L. (Table 3.2.) These decision points are based on the cutoffs for deficiency, inadequacy, adequacy and risk of excess.24-26 (Figures 3.3. and 3.4.)
Figure 3.3. Two instrument comparisons between (a) Liaison and old Architect 25OHD (3L52) and (b) old Architect 25OHD method and LC-MS/MS.

The regression line for 37 samples (a), calculated according to Deming, is indicated (25OHD old Architect nmol/L = 0.85 x (25OHD Liaison) nmol/L + 3.11 nmol/L, $r^2 = 0.98$). The regression line for 69 samples (b), calculated according to Deming, is indicated (25OHD old Architect nmol/L = 0.96 x (25OHD MS) nmol/L + 6.73 nmol/L, $r^2 = 0.94$). The difference between the methods was within total allowable error (TEa) of 46%. The instruments produced clinically equivalent results. The results covered the medical decision points (MDPs) of 30, 40, 50 and 125 nmol/L.
Figure 3.4. Two instrument comparisons between (a) the new and old generation of Architect 25OHD methods and (b) the new Architect 25OHD and LC-MS/MS(MS).

The regression line for 47 samples (a), calculated according to Deming, is indicated (25OHD new generation nmol/L = 0.87 x (25OHD old generation) nmol/L - 4.9 nmol/L, r^2 = 0.95). The regression line for 100 samples (b), calculated according to Deming, is indicated (25OHD new generation nmol/L = 0.88 x (total 25OHD MS) nmol/L - 1.2 nmol/L, r^2 = 0.98). The difference between the two methods was within total allowable error (TEa) of 46%. The methods produced clinically equivalent results. The results covered the medical decision points (MDPs) of 30, 40, 50 and 125 nmol/L.
Both Abbott methods showed an excellent within-run precision, giving an uncertainty of 0.335 for the old generation and 0.266 for the new one. The between-run uncertainties were 0.28 and 0.36, with expanded uncertainties 0.87 and 0.98 (k = 2) for (old 3L52) and (new 5P02) respectively. At a 25OHD concentration of 67 nmol/L the uncertainty of measurement was 0.87 nmol/L or 1.3% for the old generation. We can also say that we are 95% confident that the vitamin D result is between 66 and 68 nmol/L for this value. For the new generation of Abbott reagents, at a 25OHD concentration of 57 nmol/L the uncertainty of measurement was 0.98 nmol/L or 1.72%, and we are 95% confident that the 25OHD result is between 56 and 58 nmol/L. (Table 3.1.)

**Table 3.1.** Uncertainty of measurement (UOM) for total 25OHD Architect methods.

<table>
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<th>TEST</th>
<th>UNITS</th>
<th>N</th>
<th>VALUE</th>
<th>uwr</th>
<th>urb</th>
<th>UOM (k=2) Absolute value (nmol/L)</th>
<th>UOM (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>25OHD (3L52,OLD)</td>
<td>nmol/L</td>
<td>172</td>
<td>67</td>
<td>0.335</td>
<td>0.28</td>
<td>0.87</td>
<td>1.30</td>
</tr>
<tr>
<td>25OHD (5P02,NEW)</td>
<td>nmol/L</td>
<td>66</td>
<td>57</td>
<td>0.266</td>
<td>0.36</td>
<td>0.98</td>
<td>1.72</td>
</tr>
</tbody>
</table>

n = number of samples, uwr = within-run uncertainty, urb = between-run uncertainty, k = coverage factor.

The new generation of the reagent shows an acceptable level of performance in RIQAS Immunoassay external quality assessment (Standard Deviation Index = -0.21(< 2)).

To establish our reference range, we analysed serum samples from 210 healthy adults (Irish Caucasians, age range, 29-65 years) for 25OHD on Abbott Architect. Participants currently using medications that modulate or influence vitamin D metabolisms were excluded. Medical history, including consumption of alcohol and tobacco, was assessed by the nurse.
The distribution of results for 25OHD was non-gaussian. The calculated reference interval based on the central 95% of the data was 24 – 111 nmol/L for total 25OHD. The median, 10th, and 90th percentiles of total 25OHD for all subjects were 49, 23, and 100 nmol/L respectively. We observed no statistically significant differences as a result of sex or age. (Figure 3.5.) But significant negative correlation was found between BMI and 25OHD levels (r^2 = 0.06, p = 0.0005) (Figure 3.6). We need to point out that a high proportion of our patients were overweight (mean for BMI = 29.6 kg/m^2).

Examples of the EP Evaluator Reports are presented as Figures S3.1 and S3.2 and in Table S3.1.

**Figure 3.5.** Reference interval data and distribution for 25OHD with the old generation of Abbott reagents. Results are from 210 healthy volunteers who provided serum samples for the study. No significant difference was observed in sex or age. All adults were Irish Caucasian. Distribution shows the non-gaussian trend in the data, and median value for all subjects was for total 25OHD 49 nmol/L.
Figure 3.6. Correlation of patients’ body mass index with 25OHD serum concentrations for 210 healthy adults. (BMI mean = 29.6 kg/m² ± 4.4; slope = -1.19; 95% confidence interval: -1.84 to -0.53; p = 0.0005, r² = 0.06).

Table 3.2. The current decision intervals or cutoffs for 25OHD used at our institution based on dietary reference intakes²⁴-²⁶ are as follows:

- Increased risk of deficiency: < 30 nmol/L
- Increased risk of inadequacy: < 40 nmol/L
- Adequacy: > 50 nmol/L
- Increased risk of excess: > 125 nmol/L

Based on IOM newly proposed definition of vitamin D sufficiency (>50 nmol/L), 51% of the healthy volunteers in our study had vitamin D deficiency.

Example of patients’ reports for 25OHD in Biomnis Ireland from Abbott Architect method and LC-MS/MS (Biomnis France) provided in Figure S3.3.
3.5 Discussion.

In this study, we verified the Abbott Architect total 25OHD vitamin D assays on the i2000SR platform. The old generation kit presented an excellent CV, and a comparison study with Diasorin Liaison and LC-MS/MS shows clinically equivalent results. Although the old generation Architect Vitamin D assay performed adequately there was potential to improve reagent and calibrator stability, improve alignment to LC-MS/MS (especially at high levels), and improve low end precision. The new generation of the Architect Vitamin D assay (5P02) shows many advantages when compared to the old one (3L52). These include: improved calibration stability from 7 to 30 days, one position used on the reagent carousel vs two, 200 tests analysed per hour vs 100, increased reagent on board stability from 14 to 21 days, and improved precision at 20% CV at about 6 nmol/L vs 20 nmol/L.\(^2\) (Table 3.3.)

Table 3.3. Improvement summary New ARCHITECT Vitamin D assay (5P02) vs old Vitamin D (3L52) assay.

<table>
<thead>
<tr>
<th></th>
<th>25OHD (3I52) OLD</th>
<th>25OHD (5P02) NEW</th>
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<td>On board stability</td>
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<tr>
<td>VDSP (Vitamin D Standardization Program) certified</td>
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Although the Deming slope value obtained for the new generation assay indicates that the old assay aligns well with the LC-MS/MS method, it should be pointed out that the Correlation Coefficient for the new
generation assay was higher (0.98). In addition, the correlation with the new assay also showed a lower intercept compared to the old assay and only one concentration value outside the TEa at a very low concentration of 2.5 nmol/L. This sample concentration falls below the Limit of Quantitation (6 nmol/L)\(^2\) of the assay. Overall, the correlation plot demonstrated a well-aligned distribution across and beyond the medically relevant range.

The new assay is standardised to NIST SRM 2972 and VDSP, certified from Centres of Disease Control and Prevention (CDP). For our busy clinical laboratory the new improved generation of 25OHD assays is a major advantage. We have significantly improved the turn-around-time for our patients for total 25OHD assays.

Our results are in line with a recent publication by Cavalier E et al.\(^2\) which provides a clinical and analytical evaluation of the same Abbott method. This study also shows a comparison of this method with a VDSP-traceable LC-MS/MS in six different populations and addressed cross-reactivity with vitamin D2.

Here we wish to point out a limitation of our investigations. We did not perform cross-reactivity studies because only a small number (6%) of the samples had elevated 25(OH)D2 levels. Therefore we could not confirm Abbott cross-reactivity data. It is well recognised that mass spectrometry can resolve vitamin D2 completely from D3, allowing quantitation of the D2 form. However, immunoassays only partially detect vitamin D2, with varying degrees of cross-reactivity. This may not be important in Ireland, due to the limited food sources of vitamin D2 and the predominant use of vitamin D3 in supplementation.

Our laboratory is accredited to the ISO 15189, and ISO 15189, 5.6.2 requires that “The laboratory shall determine the uncertainty of results where relevant and possible”. Uncertainty of Measurement provides a quantitative estimate of the quality of a test result, and therefore is a core element of a quality system for clinical laboratories.\(^3\) We have estimated the between-run uncertainty and expanded uncertainty for total 25OHD Architect methods.\(^1\) Measurement uncertainty must be taken into account for an optimal interpretation of the measured values. This means
that the “true” vitamin D concentration of a patient whose measured value is 50 nmol/L, for example, could be between 45 and 55 nmol/L, with a maximum analytical CV of 10%. We argue that measurement uncertainty must be taken into account at any cut-off level, and if we want to ensure that a measured 25OHD concentration is really >50 nmol/L, a value of at least 55 – 65 nmol/L should be targeted. We can say from our results that we have achieved an “ideal uncertainty” of measurement because our calculated UOM % of 1.3 and 1.72 % is < 0.25xCVw (2.5).\textsuperscript{22}

The results from our reference interval study advocate the use of 25OHD guidelines based on disease states, as opposed to reference intervals based on an apparently healthy population.

We are convinced that the strength of our study lies in the careful selection of participants without any chronic conditions who had normal results for calcium, phosphate, renal and liver function. A limitation is that, for financial reasons, we did not measure PTH levels. It is important to point out that we found negative correlation between 25OHD and BMI. We appreciate that obesity is a growing problem in Ireland and it is possible that it is one of the major factors in vitamin D “deficiency”.

There have been several hypotheses to explain the negative correlation between BMI and 25OHD levels. Some of them speculated that individuals with a higher BMI may engage in fewer outdoor activities due to their restricted mobility. They may also expose less skin to the sun, since they tend to cover themselves more than leaner individuals, thereby limiting endogenous production of 25OHD in the skin.\textsuperscript{31,32} It also may be explained by the storage of 25OHD in adipose tissue because of its high lipid solubility, leading to lower bioavailability in individuals with a higher BMI.\textsuperscript{33,34} More studies are needed to explain the relationship between high BMI and low 25OHD levels in the Irish population. We suggest that normal levels of 25OHD should take BMI into account.

It is also critical to note that these studies were conducted during the winter in Dublin, Ireland (latitude, 53°N). A minimum energy of 20 kJ/cm\textsuperscript{2} is needed to produce cutaneous vitamin D3, and during the winter months in Ireland such an exposure level cannot be achieved, even during (rare) sunny conditions.\textsuperscript{35} Thus our patients were at their lowest annual vitamin...
D status. Adequate levels of >50 nmol/L were achieved only in 49% of healthy Irish volunteers. This percentage would be considerably worse if the target range was raised to 75-100 nmol/L as suggested by some groups. The calculated reference interval median for total 25OHD for our subjects was only 49 nmol/L. Some clinicians may consider that vitamin D supplementation, without prior expensive determination, should be initiated in people at a higher risk of deficiency, based on our findings. But we emphasise the importance of patients’ baseline vitamin D status. Patients with chronic illnesses may have different vitamin D metabolisms, and after supplementation it would be important to achieve sufficient levels of 25OHD to prescribe the correct dosage and achieve optimal health outcome.

Along with others, we believe that dominant 25OHD levels may be crucial, because they affect local tissue concentrations of the active vitamin D form. Serum 25OHD levels up to 120 nmol/L may be necessary to achieve optimal immune function. It has been claimed that the anti-inflammatory benefit of vitamin D was seen only in patients whose 25OHD reached >100 nmol/L. There were no beneficial effects when vitamin D status fell to below 100 nmol/L.

3.6 Conclusions.

- From our findings we conclude that, in Ireland, and especially during the winter months, vitamin D testing can be important for general health screening.
- We found negative correlation between 25OHD and BMI.
- More studies are needed to explain the relationship between high BMI and low 25OHD levels in the Irish population.
- More study is also warranted to establish reference values that truly represent the healthy Irish population, including seasonal variations and taking BMI into account.
- The new generation of the 25OHD assay was confirmed to be useful, cost-effective, reliable, accurate, rapid, and a good indicator for 25OHD measurement.
• From 2016 a new generation of 25OHD was INAB accredited to ISO 15189 on Abbott Architect in Biomnis Ireland.

The next chapter will study the importance of 25OHD levels in Irish asthmatics.
3.7. References for Chapter 3.


23. DEQAS Vitamin D External Quality Assessment Scheme.  
http://www.deqas.org/.
27. RIQAS Evaluation of Performance.  


3.8. Supplementary information for Chapter 3.

3.8.1. Examples of EP Evaluator reports:

Figure S3.1. Example of EP Evaluator report of two instrument comparison for 25OHD between Old Generation Abbott Architect method and LC-MS/MS.
Figure S3.2. Example of EP Evaluator report of alternate method comparison for 25OHD between New Generation Abbott Architect method and LC-MS/MS.
Table S3.1. Example of experimental results in EP Evaluator report of alternate method comparison for 25OHD between New Generation Abbott Architect method and LC-MS/MS.

![EP Evaluator Report](image)

### Alternate (Quantitative) Method Comparison

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Values with an "X" were excluded from the calculations. Outliers "O" were also excluded.
3.8.2. Examples of patients’ reports for 25OHD levels.

![Biochemistry Table](image)

**Biochemistry**

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</table>

25-OH-VITAMIN D CUTOFFS BASED ON DIETARY REFERENCE INTAKES

- Increased risk of deficiency: < 30 nmol/L
- Increased risk of inadequacy: < 40 nmol/L
- Adequacy: > 50 nmol/L
- Increased risk of excess: > 125 nmol/L


Methods: ABC - Architect - Biomnis Ireland

---

**25 OH calciférol profile (Vit D3 + D2) by LCMSMS**

<p>| | | |</p>
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<tr>
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<tr>
<td>i.e</td>
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<tr>
<td>i.e</td>
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</table>

*Figure S3.3.* Example of patients’ reports for 25OHD in Biomnis Ireland from Abbott Architect method and LC-MS/MS (Biomnis France).
Chapter 4

A Study of association between 25OHD level and Airway Obstruction in Irish Asthmatics.*


4.1 Aims and objectives of the chapter.

A number of studies have demonstrated an association between low serum 25-hydroxyvitamin D (25OHD) concentration and asthma severity, but such links might be explained by lower sun exposure in patients with more severe airway disease. We wondered whether an association exists between low serum 25OHD levels and airway obstruction within a single level of asthma severity. In addition, several inflammatory disorders that are important in human asthma have also been associated with VDD, including obesity and systemic and allergic inflammation. Serum IgE is closely linked to VDD in children, as has been shown in our studies of paediatric asthma. In a general population, a relationship exists between VDD and adiposity and between 25OHD and hs-CRP.

We therefore hypothesised that observed associations between serum 25OHD concentrations and airway obstruction might be partly explained by the presence of obesity and other chronic inflammatory processes. In particular we wanted to study patients with a uniform skin phototype because serum 25OHD levels are dependent on it. Ireland has a high prevalence of VDD, especially during winter time, due to low levels of sunlight (average of 3.5 hours of sunshine each day) and poor dietary intake of oily fish and other vitamin D containing foods. Since VDD is not generally believed to be more common in asthma, and since replacement is not known to improve lung function in adults, Irish patients with asthma are neither routinely screened nor treated with vitamin D supplements. We decided to explore an association of serum 25OHD
levels and other serum markers of inflammation in Irish patients with moderate persistent asthma and a uniform skin phototype (Fitzpatrick Skin Phototypes I and II).

4.2. Materials and methods.

4.2.1. Participants.

Eligible adult asthmatic subjects (aged ≥18y) with moderate persistent asthma, who attended the Asthma Clinic in James Connolly Hospital, Blanchardstown, Dublin, Ireland, were invited to participate in a study of factors contributing to the development of airway obstruction in asthma in autumn/winter. All clinical tests and investigations were performed by hospital staff under the supervision of two respiratory consultants, Dr Faul and Dr Cormican.

All subjects were white Caucasians with Fitzpatrick skin phototype I and II and a diagnosis of asthma for at least 12 months substantiated by a reversibility of forced expiratory volume in 1 sec (FEV₁) or by a positive histamine bronchial provocation challenge. All had moderate persistent asthma based on the following criteria: (i) symptoms of recurrent episodes of wheezing, cough, shortness of breath, or a combination of these, despite treatment with medium-dose to high-dose inhaled corticosteroids plus long-acting beta agonist (LABA) twice a day; (ii) an Asthma Control Questionnaire (five-question version: ACQ5) of greater than 3.0; (iii) at least one asthma exacerbation within the previous 2 years requiring systemic corticosteroid therapy.

Skin phototype was determined using the Fitzpatrick classification scale.¹¹

We excluded (i) patients with a recent (less than 1 year) diagnosis of asthma and patients with recent systemic corticosteroid therapy (less than 6 months prior to enrolment); (ii) patients who took vitamin D supplements and those with a diagnosis of osteoporosis, liver, renal and celiac disease, or cystic fibrosis; and (iii) patients who were current smokers or who took anticonvulsants (or other medications thought to increase the metabolism of vitamin D). All subjects signed an institutional review board-approved written informed consent form and underwent
blood draw followed by computerised spirometry. The James Connolly Hospital Research Ethics Committee approved the study.

4.2.2. Biochemistry.

Blood samples were collected and centrifuged, aliquoted, and frozen to -70°C until required. Total serum 25-hydroxyvitamin D level was measured by using competitive chemiluminescence immunoassays (DiaSorin, Dietzenbach, Germany) with inter-assay coefficient of variation (CV) of 6.8%.

Total 25OHD is currently considered the best circulating biomarker of vitamin D status as it reflects total vitamin D$_2$ and D$_3$ from dietary and supplemental intake, as well as cutaneous inputs resulting from ultraviolet B radiation. 25OHD levels were dichotomised, based on the most recent approach of the North American Institute of Medicine 2011 report, which is in line with the positions of the Food Safety Authority of Ireland and the European Food Safety Authority. As mentioned above, we should point out that 25OHD level is a measure of risk, not a diagnostic of deficiency: a level below 30 nmol/L indicates “risk of deficiency”; between 30-50 nmol/L is the range of adequacy for the population.

Serum total IgE, total calcium, magnesium and hs-CRP were assessed using an Abbott Architect ci8200 analyser; the inter-assay CVs for these assays ranged between 1 and 5.6%. Eosinophil Cationic Protein was measured applying ImmunoCAP Technology on the IDM Phadia 250. All these diagnostic methods have been outlined above in Chapter 2.

4.2.3. Lung function and BMI.

Spirometry was performed with a computerised spirometer (Jaeger, Masterscreen PFTpro, Würzburg, Germany) and measures were taken 20 minutes after administration of a short-acting bronchodilator (albuterol 80 micrograms). The best of three measures of FEV$_1$; forced vital capacity (FVC), and FEV$_1$/FVC values were recorded for analysis. Spirometry is not covered in detail in this dissertation because its concern is with the biochemical and genetical aspects of asthma. BMI was calculated from measured height and weight.

\[ \text{Body mass index} = \frac{\text{Weight (kg)}}{\text{Height (m$^2$)}} \]
4.2.4. Statistical analyses.

We hypothesized that any observed association between 25OHD and airway obstruction might be explained by the presence of confounding variables such as allergy, systemic inflammation, or obesity. We therefore conducted multiple linear regression analysis to address the influence of confounding variables. Since a minimum of 20 subjects is generally required for each variable, we recruited more than 80 subjects to avoid type 1 statistical error (where an effect is not detected due to low sample size). Since 25OHD levels are skewed, we converted values to log scale. We treated each potential confounding variable as a continuous variable by using hs-CRP, IgE and ECP levels, and BMI as measures of systemic inflammation, allergy, and obesity respectively. We employed multiple linear regression analysis and Spearman correlation to determine whether a relationship exists between FEV$_1$/FVC, hs-CRP, BMI, serum IgE, ECP, and log$_{10}$ serum 25OHD levels. P < 0.05 was considered statistically significant.

All analyses were performed using Graphpad prism (Graphpad, San Diego, CA, USA) and Microsoft Excel (Microsoft Inc., Redmond, WA, USA) statistical software.

4.3. Results.

4.3.1 Baseline characteristics.

187 subjects with asthma were eligible (from 2500) to enrol for the study because they had skin phototype I and II, moderate persistent asthma, and they had not taken oral corticosteroid therapy within the prior 6 months. A total of 100 patients agreed to participate and all completed the study. The average age was 53 years (95% confidence intervals: 50 to 56 years). Male and female participants were similar in terms of age, BMI and 25OHD levels, although there was a trend towards higher FVC (101 (95, 106)%) and FEV$_1$/FVC (0.76 (0.73, 0.8)) and lower serum magnesium (0.95 (0.89, 1.02) mmol/L) in female subjects compared to males (95 (91, 99)%, 0.73 (0.69, 0.76) and 1.08 (0.95, 1.2) mmol/L) respectively, but these differences did not reach significance. (Table 4.1.)
Normal reference ranges are provided in Table S5.1.

**Table 4.1.** Demographics of the subjects who completed the study. Results are expressed as average (95% confidence intervals). The $p$ value indicates the level of statistical significance of differences between the groups (t test).

<table>
<thead>
<tr>
<th></th>
<th>Male (n = 47)</th>
<th>Female (n = 53)</th>
<th>$p$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>51 (46, 56)</td>
<td>55 (51, 59)</td>
<td>0.21</td>
</tr>
<tr>
<td>BMI kg/m$^2$</td>
<td>29 (27, 31)</td>
<td>29 (26.5, 31.5)</td>
<td>0.64</td>
</tr>
<tr>
<td>25OHD nmol/L</td>
<td>41 (34, 48)</td>
<td>38 (31, 45)</td>
<td>0.58</td>
</tr>
<tr>
<td>FVC % predicted</td>
<td>95 (91, 99)</td>
<td>101 (95, 106)</td>
<td>0.1</td>
</tr>
<tr>
<td>FEV$_1$ % predicted</td>
<td>86 (80, 92)</td>
<td>92 (85, 99)</td>
<td>0.2</td>
</tr>
<tr>
<td>FEV$_1$/FVC</td>
<td>0.73 (0.69, 0.76)</td>
<td>0.76 (0.73, 0.79)</td>
<td>0.15</td>
</tr>
<tr>
<td>ECP µg/L</td>
<td>48 (32, 64)</td>
<td>38 (25, 51)</td>
<td>0.3</td>
</tr>
<tr>
<td>Calcium mmol/L</td>
<td>2.28 (2.26, 2.31)</td>
<td>2.26 (2.21, 2.3)</td>
<td>0.38</td>
</tr>
<tr>
<td>Magnesium mmol/L</td>
<td>1.08 (0.95, 1.20)</td>
<td>0.95 (0.89, 1.02)</td>
<td>0.08</td>
</tr>
<tr>
<td>hs-CRP mg/L</td>
<td>4.8 (2.3, 7.2)</td>
<td>5.3 (3.5, 7.1)</td>
<td>0.72</td>
</tr>
<tr>
<td>IgE IU/ml</td>
<td>144 (73, 216)</td>
<td>142 (10, 295)</td>
<td>0.98</td>
</tr>
</tbody>
</table>

4.3.2. **Study outcomes.**

Approximately one fifth (19%) of subjects were allergic (having a serum IgE > 200 IU/ml and skin test positivity to one or more of a range of common indoor and outdoor allergens). Almost half (46%) were obese (BMI > 30kg/m$^2$).

The overall level of 25OHD < 30 nmol/L was observed in 45%. The average ± standard deviation (95th percentile) serum 25OHD level was 41 ± 25 (52) nmol/L in men and 38 ± 24 (51) nmol/L in women. There was a significant positive relationship between log$_{10}$ serum 25OHD concentrations and FEV$_1$/FVC ($p < 0.01$). (Figure 4.1.)
Figure 4.1.  Plot of log_{10} serum vitamin D levels (nmol/L). \( \log_{10} 25\text{OHD} \) and (a) FEV\(_1\)/FVC \((r^2 = 0.07, p < 0.01)\)*, (b) ECP µg/L \((r^2 = 0.03, p = 0.09)\), (c) IgE IU/ml \((r^2 = 0.01, p = 0.42)\), (d) hs-CRP mg/L \((r^2 = 0.015, p = 0.23)\) and (e) BMI kg/m\(^2\) \((r^2 = 0.001, p = 0.78)\) in 100 Caucasian adults with moderate persistent asthma.

*Denotes statistically significant (Linear regression).

There were no significant relationships between log_{10} serum 25OHD and ECP, IgE or hs-CRP levels. We performed multiple linear regression using BMI, hs-CRP, ECP and IgE in order to determine whether these variables might influence the strength of our observed association. (Table 4.2.)

The observed association between log_{10} serum 25OHD concentrations and FEV\(_1\)/FVC remained significant \((p = 0.012)\), but the association was not significantly influenced by any or all of the potential confounding variables of BMI, hs-CRP, ECP, IgE.
Table 4.2. Log₁₀ serum 25OHD concentration (nmol/L) and FEV₁/FVC, BMI, ECP, IgE, hs-CRP (in 100 Irish Caucasian adults with moderate persistent asthma.) * Denotes statistically significant (Linear regression).

<table>
<thead>
<tr>
<th></th>
<th>$r^2$</th>
<th>$p$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>FEV₁/FVC</td>
<td>0.07</td>
<td>&lt; 0.01*</td>
</tr>
<tr>
<td>BMI kg/m²</td>
<td>0.001</td>
<td>0.78</td>
</tr>
<tr>
<td>ECP µg/L</td>
<td>0.03</td>
<td>0.09</td>
</tr>
<tr>
<td>hs-CRP mg/L</td>
<td>0.015</td>
<td>0.23</td>
</tr>
<tr>
<td>IgE IU/ml</td>
<td>0.01</td>
<td>0.42</td>
</tr>
</tbody>
</table>

4.4. Discussion.

To our surprise we found that serum 25OHD levels in Caucasian adults with moderate persistent asthma were associated with airway obstruction, but this association is not explained by the presence of obesity, or allergic and systemic inflammation. In our previous study of healthy Irish adults we observed a significant association between BMI and 25OHD levels. The precise mechanism by which a low 25OHD level might worsen airway obstruction is unclear. In this study, the effect is not closely linked to allergy (as measured by IgE and ECP), although it should be noted that only 20% of our population were atopic. While these data regarding the impact of allergy should be interpreted with caution, our findings are supported by other studies that suggest that the association between VDD and asthma in adults does not appear to be dictated by the presence of allergy. For example, in one large study of the development of adult asthma in Caucasians, VDD was associated with an increased risk of developing asthma, but only in men without allergic rhinitis, indicating that some of the pathways involved are not allergy-based and that vitamin D may be linked to non-atopic asthma. Our findings are in contrast to our studies of childhood asthma (chapter 5), which describe a significant link
between 25OHD and serum IgE levels. The fact that only 20% of our adult patients were atopic suggests that most had a severe asthma phenotype that is not allergy-based. In support of this idea, recent studies of German and Chinese adults with newly diagnosed asthma also showed an association between airway obstruction and VDD, but not with serum IgE.\textsuperscript{16,17}

We examined whether the presence of obesity explains the link between 25OHD and airway obstruction. Obesity appears to be important in the pathogenesis of asthma, and the association between asthma and obesity is stronger in non-atopic than in atopic individuals.\textsuperscript{18-20} While genetic, developmental, lung mechanical, immunological and behavioural factors have been implicated as playing a causal role between asthma and obesity, recent evidence suggests that asthma associated with obesity can develop through a pathway involving NLRP3, IL-1β and IL-17 production from pulmonary IL-17+ ILC3 cells.\textsuperscript{21}

IL-17 is an important feature of airway pathophysiology in asthma. Vitamin D treatment significantly inhibits IL-17 production \textit{in vitro}; thus we expected that an association between airway obstruction and 25OHD might be explained by alterations in inflammatory pathways that are seen in obesity.\textsuperscript{22} (We set up an IL-17a test in our laboratory which could be used for future investigations.) In our sample population the association between 25OHD and airway obstruction is not significantly explained by the presence of obesity.

Chronic systemic inflammation (reflected by hs-CRP) is related to VDD\textsuperscript{6} and some studies describe reductions in serum hs-CRP after vitamin D supplementation.\textsuperscript{23} Our observed association between 25OHD levels and airway obstruction is not explained by hs-CRP, although the degree of systemic inflammation in this cohort of patients was low (none had a hs-CRP level greater than 25). It is possible that VDD causes airway obstruction in the presence of high levels of inflammation during exacerbations of asthma.

In earlier studies, 25OHD was also significantly associated with a lower FEV\textsubscript{1}, a higher BMI, and greater sputum eosinophilia across a range of asthma severities.\textsuperscript{2} This study goes further: our data support the idea that
VDD affects airway obstruction and that low 25OHD levels might be important in airway biology even within a single level of disease severity. We find that a strong relationship exists between FEV\textsubscript{1}/ FVC and 25OHD levels in asthmatic adults, and the effect is independent of the presence of potential confounders such as obesity, allergy and systemic inflammation. Our study does not rule out the possibility of reverse causation: that asthma states may have caused lower 25OHD by virtue of less sun exposure or avoidance of dairy products.

A limitation of the study is that there was no control group and we made no estimate of sunlight exposure or a measure of vitamin D intake from diet or supplements. Risk of VDD remains highly prevalent in Irish asthmatic patients, but many studies of Vitamin D supplementation in asthma show very controversial and in some cases detrimental effects on airway obstruction.\textsuperscript{24,25}

In the next chapters, 5 and 6, we will examine the effects of vitamin D supplementation in paediatric asthma.
4.5. **Conclusions.**

- In summary, low serum 25OHD levels are positively associated with the severity of airway obstruction in Irish Caucasian adults with moderate persistent asthma. This link is not explained by differences in asthma severity or skin phototype.

- From our study of 100 patients we did not observe any significant association between 25OHD and markers of allergy (IGE), systemic inflammation (hsCRP), airway inflammation (ECP) and obesity (BMI). Nor did we identify any significant difference between the sexes.

- However there was a significant association between FEV₁/ FVC and 25OHD levels in our adult patients.

- The connection between airway obstruction and low 25OHD levels in this study of Irish asthmatic adults supports the idea that 25OHD levels might be important for airway obstruction, but the effect is independent of some of the commoner inflammatory pathways involved in the pathogenesis of asthma. Future and more extensive studies will be necessary to understand the pathophysiological mechanism of this relationship.

Having looked in this chapter at the association between 25OHD levels and adult asthma, we will now turn to the study of 25OHD levels and vitamin D supplementation in Irish paediatric asthmatics.
4.6. References for Chapter 4.


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4.7. Supplementary information for Chapter 4.

Table S4.1. Reference ranges for the measured parameters

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Normal Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>FEV1 (%)</td>
<td>≥ 100%</td>
</tr>
<tr>
<td>FVC (%)</td>
<td>≥ 80%</td>
</tr>
<tr>
<td>FEV1/FVC (%)</td>
<td>≥ 70%</td>
</tr>
<tr>
<td>25OHD nmol/L</td>
<td>50 – 125 nmol/L</td>
</tr>
<tr>
<td>IgE (IU/mL)</td>
<td>&lt; 100 UI/mL</td>
</tr>
<tr>
<td>ECP (ug/L)</td>
<td>&lt; 15 ug/L</td>
</tr>
<tr>
<td>BMI kg/m²</td>
<td>&lt; 18.5 kg/m² : underweight</td>
</tr>
<tr>
<td></td>
<td>18.5 – 25 kg/m² : normal range</td>
</tr>
<tr>
<td></td>
<td>25.0 - 30 kg/m²: overweight</td>
</tr>
<tr>
<td></td>
<td>&gt; 30.0 kg/m²: obese*</td>
</tr>
<tr>
<td>CRP (mg/L)</td>
<td>&lt; 5 mg/L</td>
</tr>
<tr>
<td>Total Ca (mmol/L)</td>
<td>2.1 - 2.6 nmol/L</td>
</tr>
<tr>
<td>Mg (mmol/L)</td>
<td>0.65-1.05 mmol/L</td>
</tr>
</tbody>
</table>

Ranges for 25OHD, CRP, total Ca and Mg have been established in the Biomnis Ireland laboratory. Other ranges have been provided by the manufacturers. The reference for lung function is as per Figure S6.5 in supplementary material for Chapter 6.

Chapter 5

A pilot study of 25OHD level and Vitamin D3 supplementation for uncontrolled childhood asthma.*


5.1. Introduction.

This chapter describes our study of vitamin D3 supplementation in Irish asthmatic children, and the results of supplementation on different subjective and objective asthma parameters – including lung function, biochemical markers (such as 25OHD), and other biomarkers of allergy and inflammation.

Most of our work was presented in the paper mentioned above, but it did not include some of the author’s findings, e.g., the negative correlation between 25OHD levels and total IgE at the baseline of the trial. These findings are presented below.

It should be pointed out that for this project the author set up a range of new diagnostic tests in Biomnis Ireland for IL-10, IL-17A, CAMP and VDR. In addition, other laboratory investigations were also carried out for this study, including tests for 25OHD, FBC, calcium, phosphate, Mg, CRP and ECP. All these methods were already routinely performed in Biomnis Ireland.

5.2. Aims and objectives of the chapter.

As has previously been discussed, many recent investigations have highlighted the potential mechanisms whereby vitamin D may influence asthma. Some research has suggested that childhood asthma/allergy and the severity of the illness may be dependent on vitamin D levels. This hypothesis is based on studies that show a strong association between 25OHD levels and asthma/allergy in children, in comparison to adults.1-3 Studies of vitamin D supplementation give controversial results and indicate varying outcomes. For example, vitamin D supplementation in neonates may increase the risk of developing allergic disease.4,5 Some
research has demonstrated beneficial results, while in other cases there was no apparent advantage. A recent meta-analysis of vitamin D supplementation in children with asthma concluded, perhaps in a quite vague form, that “the available very low to low quality evidence does not confirm or rule out beneficial effects of vitamin D supplementation in children with asthma”.6

As a result, we wanted to study the effects of vitamin D3 supplementation on different asthma parameters, including lung function, biomarkers of inflammation/allergy, and subjective asthma symptoms in uncontrolled paediatric Irish asthmatics.

5.3. Methods.

The trial was conducted in winter in the National Children’s Hospital, Dublin. Parental/guardian consent and institutional review board approval had already been secured.

5.3.1. Subjects.

Children with asthma were recruited from paediatric respiratory outpatient clinics. Our criteria for inclusion were: Caucasians aged 6–16 with previous diagnosis of asthma, who were on antiasthmatic pharmacotherapy with uncontrolled asthma at baseline according to the Global Initiative for Asthma 2011 guidelines. Exclusion criteria included conditions/medications that influence vitamin D metabolism or absorption. More information on recruitment of subjects and on the study design is provided in the paper that forms a basis to this chapter.

5.3.2. Study design.

The hospital carried out a parallel, randomised, double-blind, placebo (PL)-controlled trial involving two clinic visits. Recruitment occurred between November 2013 and January 2014, with a follow-up 15 weeks later. All assessments were conducted at baseline and follow-up by a qualified dietician (Dr. Conor Kerley). Physicians who were not involved in data gathering generated the allocation sequence. Subjects were randomised to vitamin D3 (D3) or placebo groups. Parents/guardians were directed to administer their children with one softgel daily to
accompany their food. The vitamin D dose was 2000 international units (IU). We conducted this trial at high latitude during winter, aware that diet, supplement use, and – in particular – exposure to ultraviolet B radiation contribute to vitamin D status.

We assessed behaviour relating to vitamin D status with the VIDSun Questionnaire at baseline and end-point. Supplement compliance was also measured. We specifically wanted to study the known uncontrolled asthmatics on previously established diverse anti-asthma pharmacotherapy. Adherence to anti-asthma pharmacotherapy was examined by a clinical nurse specialist at baseline and end-point. All physiological functions, such as lung functions, were assessed by respiratory physiologists. All the above were carried out under the supervision of three respiratory and paediatric consultants, Drs Greally, Coghlan and Elnazir. Laboratory testing was conducted at Biomnis Ireland, St. James’s Hospital and Tallaght Hospital, Dublin.

5.3.3. Outcomes.

We used a combination of biochemical and immunological tests and objective and subjective asthma parameters. Spirometry was performed in accordance with the American Thoracic Society/European Respiratory Society Task Force, using the spirometry module of the V-max Encore System (Carefusion). The results were expressed as a percentage of predicted values.

Subjective asthma control and quality-of-life scores were obtained by combining the childhood asthma control test (C-ACT), the Global Initiative for Asthma score (GINA score), and the mini-Paediatric Asthma Quality of Life Questionnaire (mPAQLQ).

5.3.4. Biochemistry and immunology.

Atopic status was determined by radioallergosorbent blood test in Tallaght Hospital. Additional blood was sent to Biomnis Ireland for future analysis, and some blood was centrifuged, aliquoted, and frozen to -80°C until required. EDTA whole blood samples were stored for future genetic testing.
We assessed vitamin D status by measuring total serum 25-hydroxyvitamin D, which is currently considered the best circulating biomarker of vitamin D status. 25OHD levels were measured by LC-MS/MS on an AB SCIEX API 4000 liquid chromatography–tandem mass spectrometer (Applied Biosystems Life Technologies, Foster City, CA, USA). We also analysed 25OHD levels on Abbott Architect at Biomnis Ireland. Both methods have already been outlined above, in Chapter 3. Based on the most recent Institute of Medicine recommendations, we dichotomised 25OHD levels: <50 nmol/l indicates VDD and >50 nmol/l - vitamin D sufficiency (VDS). Serum measures of parathyroid hormone, albumin, total calcium, alkaline phosphatase, phosphate, total IgE, immunoglobulin A, and high-sensitivity C-reactive protein were measured using commercially available diagnostic kits on a biochemical autoanalyser Abbott Architect ci8200 (Abbott Laboratories, Abbott Park, IL, USA). The mean between-run and within-run CVs for these assays ranged between 1% and 6%. All these methods have been outlined in Chapter 2.

Reference ranges for the measured parameters are provided in Table S.5.1. Eosinophil cationic protein was analysed using ImmunoCAP Technology by fluorescent enzyme immunoassays on the IDM Phadia 250 with a minimum detectable level of 2 ug/l (normal range: < 13 ug/l) and between-run CV < 7%.

Serum levels of IL-10, Cathelicidin antimicrobial peptide, IL-17A and VDR were determined by human enzyme-linked immunosorbent assay method (ELISA kit assays, Damastown, Dublin 15). Intra- and inter-assay CVs were <8% for these tests. All assays were analysed with kits of the same lot number. All methods were run according to manufacturer’s instructions. Again, see above, Chapter 2.
5.3.5. Statistics.

For this pilot study we did not perform a sample size calculation. Although our study group was small it was very specific. We wanted to assess the potential effects of vitamin D supplementation on lung function, biochemical and immunological parameters and subjective asthma control/quality of life. Four independent groups of children were examined, employing the following baseline 25OHD level: VDD + supplementation, VDS + supplementation, VDD + PL, and VDS + PL. (Table 5.4)

A Shapiro–Wilk test was carried out to check the normality assumption for all the variables across all the groups of subjects. For the majority of variables data were not normally distributed, and therefore we used nonparametric tests.

Mann–Whitney U-tests were applied to compare the differences between D3 vs. PL groups at baseline, as well as the changes between baseline and end-point.

Nonparametric ANOVA tests were performed to detect differences between the four independent groups, as described above. If differences were detected, the Mann–Whitney U-test was performed for pairwise comparisons. The results were expressed as median (Q1–Q3). p < 0.05 was considered statistically significant.

Statistical analysis was performed using a software package SPSS (SPSS, version 18; Chicago, IL, USA).

5.4. Results.

Respiratory consultants at Tallaght Hospital, Dublin screened 500 children attending paediatric respiratory clinics. 75 children were eligible, but consent was received for only 51 of them. These were recruited, and baseline assessments were completed.

Of these 51 subjects, seven children refused blood testing. Therefore, 44 children (24 male, mean 8.7 years, mean BMI 19.9 kg/m2) completed all baseline measures. Mean 25OHD was 51 nmol/l (range: 24–80 nmol/l) and 22 children were VDD, while 22 were VDS. Both 25OHD and VIDSun
score were significantly higher in VDS vs. VDD patients ($p < 0.001$ and $p = 0.012$, respectively). (Table 5.1.)

Consistent with previous reports, a significant (but not strong) negative correlation was found between IgE and 25OHD levels ($p = 0.023$) in all patients studied (Figure 5.1).

![Figure 5.1.](image1)

**Figure 5.1.** Relationship between serum 25OHD levels and serum total IgE ($r^2 = 0.12, p = 0.023^*$) in 44 uncontrolled asthmatic children. * Denotes statistically significant.

There was a greater lung function in VDS vs. VDD for both FEV1% ($p = 0.03$) and FVC% ($p = 0.03$). (Table 5.1) Although the VDS group showed clinically preferable results on C-ACT, mPAQLQ, ECP, and hsCRP compared with the VDD group, none of these differences were statistically significant – with the exception of IgE. (Figure 5.2.)

![Figure 5.2.](image2)

**Figure 5.2.** Average of Total IgE level ($\pm$ SD) in VDS and VDD groups of 44 uncontrolled asthmatic children according to 25OHD levels of < 50 and >50 nmol/L ($p = 0.005^*$). * Denotes statistically significant.
Because of the small size of our study group it should be stressed that we were particularly interested in the effect of vitamin D supplementation on different asthma parameters. To confirm our findings for correlation studies, as above, a larger sample size would have been beneficial.

It should be pointed out that tests for VDR and IL-17A were set up by the author but the results were inconclusive. In the case of VDR, very low values were noted in the patients’ samples, while in the case of IL-17A there was a remarkably wide discrepancy between different samples. More investigations will be needed to understand the reason for these results. Accordingly the data are not presented in this dissertation.

**Table 5.1** Baseline data for VDD and VDS groups (44 asthmatics).

<table>
<thead>
<tr>
<th></th>
<th>&lt;50nmol/L</th>
<th>&gt;50nmol/L</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>N (patients)</td>
<td>22</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>25(OH)D (nmol/L)</td>
<td>39.6 ± 7.4</td>
<td>62.2 ± 8.2</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>IgE (IU/L) (median, IQR)</td>
<td>548 (239-1275)</td>
<td>80.2 (28.5-326)</td>
<td>0.005*</td>
</tr>
<tr>
<td>P-ACT (0-27)</td>
<td>17 ± 5</td>
<td>17 ± 5</td>
<td>0.42</td>
</tr>
<tr>
<td>mPAQLQ (0-91)</td>
<td>64 ± 18</td>
<td>73 ± 17</td>
<td>0.05</td>
</tr>
<tr>
<td>VIDSun</td>
<td>3 ± 1</td>
<td>4 ± 1</td>
<td>0.012*</td>
</tr>
<tr>
<td>FEV1%</td>
<td>93.6 ± 13.1</td>
<td>101.9 ± 15.1</td>
<td>0.03*</td>
</tr>
<tr>
<td>FVC%</td>
<td>88.9 ± 13.9</td>
<td>96.1 ± 9.2</td>
<td>0.03*</td>
</tr>
</tbody>
</table>

Data shown as mean±SD. * Denotes statistically significant.

Of the 51 recruits, 12 children dropped out (mean demographic values were similar between trial completers and drop-outs: age 7.9y; BMI = 15.7kg/m²; FVC% = 94.2; FEV1% = 94.5; 25(OH)D = 57.6 nmol/L).
<table>
<thead>
<tr>
<th>Table 5.2</th>
<th>Baseline demographic and clinical data.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Placebo</td>
</tr>
<tr>
<td>Male/female</td>
<td>13 /9</td>
</tr>
<tr>
<td>Age (years)</td>
<td>7 (7 - 10)</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>130 (119 -144)</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>18.2 (16 to 20)</td>
</tr>
<tr>
<td>Atopic n (%)</td>
<td>19 (86)</td>
</tr>
<tr>
<td>Subjects with passive smoking n (%)</td>
<td>11 (50)</td>
</tr>
<tr>
<td>Vaccinated against influenza n (%)</td>
<td>5 (23)</td>
</tr>
<tr>
<td>Worse in Winter n (%)</td>
<td>19 (86)</td>
</tr>
<tr>
<td>Medications</td>
<td></td>
</tr>
<tr>
<td>SABA n (%)</td>
<td>20 (90)</td>
</tr>
<tr>
<td>LABA n (%)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>ICS n (%)</td>
<td>19 (86)</td>
</tr>
<tr>
<td>LTRA</td>
<td>7 (32)</td>
</tr>
<tr>
<td>Co-morbidities</td>
<td></td>
</tr>
<tr>
<td>s</td>
<td>6</td>
</tr>
<tr>
<td>Eczema</td>
<td>2</td>
</tr>
<tr>
<td>Rhinitis</td>
<td>2</td>
</tr>
<tr>
<td>Hayfever</td>
<td>1</td>
</tr>
<tr>
<td>GERD</td>
<td></td>
</tr>
<tr>
<td>Subjective scores</td>
<td></td>
</tr>
<tr>
<td>C-ACT score</td>
<td>17 (14.3 - 19)</td>
</tr>
<tr>
<td>GINA Score</td>
<td>3.2 (2 - 4)</td>
</tr>
<tr>
<td>mPAQLQ score</td>
<td>5.4 (3.8 - 6)</td>
</tr>
<tr>
<td>Pulmonary function</td>
<td></td>
</tr>
<tr>
<td>FEV₁ %</td>
<td>96 (90 - 104)</td>
</tr>
<tr>
<td>FVC%</td>
<td>93 (85 - 98)</td>
</tr>
<tr>
<td>FEV₁/FVC</td>
<td>94 (89 - 97)</td>
</tr>
<tr>
<td>Asthma control*</td>
<td></td>
</tr>
<tr>
<td>Controlled n (%)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Partly controlled n (%)</td>
<td>8 (36)</td>
</tr>
<tr>
<td>Uncontrolled n (%)</td>
<td>14 (64)</td>
</tr>
<tr>
<td>VIDSUn score</td>
<td>3 (3 - 4)</td>
</tr>
<tr>
<td>Biochemistry</td>
<td></td>
</tr>
<tr>
<td>25OHDD (nmol/L)</td>
<td>51 (39 - 64)</td>
</tr>
<tr>
<td>CAMP (pg/mL)</td>
<td>548 (324 - 3071)</td>
</tr>
<tr>
<td>PTH (pg/mL)</td>
<td>36 (29 - 43)</td>
</tr>
<tr>
<td>Ca²⁺ (mmol/L)</td>
<td>2.3 (2.4 - 2.4)</td>
</tr>
<tr>
<td>ALP (U/L)</td>
<td>219 (180 - 257)</td>
</tr>
<tr>
<td>PO₄ (mmol/L)</td>
<td>1.6 (1.5 - 1.7)</td>
</tr>
<tr>
<td>IgE (IU/ml)</td>
<td>399 (106 - 679)</td>
</tr>
<tr>
<td>Eosinophils (x10⁹/L)</td>
<td>0.6 (0.3 - 0.8)</td>
</tr>
<tr>
<td>IgA (g/L)</td>
<td>1.2 (1.1 - 1.5)</td>
</tr>
<tr>
<td>ECP (µg/L)</td>
<td>62 (29.8 - 94.4)</td>
</tr>
<tr>
<td>hsCRP (mg/L)</td>
<td>0.8 (0.5 - 1.5)</td>
</tr>
<tr>
<td>IL-10 (pg/mL)</td>
<td>110 (86 - 150)</td>
</tr>
</tbody>
</table>

Data shown are median (Q1 to Q3). p values were derived from Mann-Whitney test.

* Asthma control according to GINA classification at baseline.
39 patients completed the supplementation trial. There was no statistical difference in the demographics or baseline characteristics between the two intervention groups at baseline (Table 5.2) – except that serum hsCRP was significantly elevated in the PL group, and that there was a trend toward higher serum IgE and ECP in PL vs. D3.

During the trial there was no change in medication use, and supplement compliance was high in both groups (>85%). The supplements were well tolerated, without any adverse effects. After 15 weeks of vitamin D3 supplementation there was a marked increase in 25OHD in the D3 group compared with PL, but there was no change in VIDSun score. We observed a trend toward increased airway obstruction (FEV1%, FVC%) in D3 compared with PL, but there was no difference relating to C-ACT, mPAQLQ, or GINA scores.

There was a noticeable increase in ALP in the PL group compared with D3, but no change in other biomarkers of bone metabolism (PTH, Ca2+, PO₄). Although notably fewer school days were missed because of asthma in the D3 group, there were no differences regarding respiratory tract infection (RTI) incidence, GP visits, or antibiotic/steroid courses. A significant decrease was noticed in hsCRP in the PL compared with the D3 group, and there was no change in relation to IgE, eosinophils, IgA, or ECP. (Table 5.3)
Table 5.3 Absolute difference in medians after 15 weeks of vitamin D or placebo.

<table>
<thead>
<tr>
<th>Subjective scores</th>
<th>PL (n=22)</th>
<th>D₃ (n=17)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-ACT Score</td>
<td>3.5 (0 - 5)</td>
<td>2 (-2 - 4)</td>
<td>0.34</td>
</tr>
<tr>
<td>GINA Score</td>
<td>-1 (-2 - 0)</td>
<td>-0.5 (-2 - 0)</td>
<td>0.9</td>
</tr>
<tr>
<td>mPAQLQ Score</td>
<td>0.9 (-0.3 - 1.5)</td>
<td>0.5 (-0.2 - 0.8)</td>
<td>0.56</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Pulmonary function</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>FEV₁ %</td>
<td>2.5 (-4.3 - 6.5)</td>
<td>-4 (-6.3 - -1)</td>
<td>0.06</td>
</tr>
<tr>
<td>% Decreased (%)</td>
<td>45</td>
<td>81</td>
<td>-</td>
</tr>
<tr>
<td>% Increased (%)</td>
<td>55</td>
<td>13</td>
<td>-</td>
</tr>
<tr>
<td>% did not change</td>
<td>0</td>
<td>6</td>
<td>-</td>
</tr>
<tr>
<td>FVC%</td>
<td>0 (-5 - 4.5)</td>
<td>-2.5 (-8.3 - 3)</td>
<td>0.36</td>
</tr>
<tr>
<td>FEV₁/FVC</td>
<td>1 (-1 - 3)</td>
<td>-2.5 (-5 - 1.3)</td>
<td>0.15</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Biochemistry</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>25OHD (nmol/L)</td>
<td>0.5 (-3.5 - 11)</td>
<td>41.5 (24.5 - 72.8)</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>CAMP (pg/mL)</td>
<td>-56 (-94 - -1130)</td>
<td>-97 (-191 - 31)</td>
<td>0.49</td>
</tr>
<tr>
<td>ALP (U/L)</td>
<td>16 (6 - 27)</td>
<td>-3.5 (-17 - 15.5)</td>
<td>0.037*</td>
</tr>
<tr>
<td>hsCRP (mg/L)</td>
<td>-0.4 (-0.9 - 0.2)</td>
<td>0.05 (-0.06 - 0.9)</td>
<td>0.04*</td>
</tr>
<tr>
<td>IL-10 (pg/mL)</td>
<td>-17 (-26 - -27)</td>
<td>-13 (-25 - 3)</td>
<td>0.42</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Diary</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>School days missed due to asthma</td>
<td>5 (2 - 10)</td>
<td>1 (0 - 5)</td>
<td>0.04*</td>
</tr>
<tr>
<td>Infection rate</td>
<td>1 (1 - 2.5)</td>
<td>1 (0 - 2)</td>
<td>-</td>
</tr>
<tr>
<td>GP visits</td>
<td>1 (0 - 1.9)</td>
<td>0 (0 - 2)</td>
<td>-</td>
</tr>
<tr>
<td>Antibiotic courses</td>
<td>0.5 (0 - 1)</td>
<td>0 (0 - 1)</td>
<td>-</td>
</tr>
<tr>
<td>Steroid courses</td>
<td>0 (0 - 1)</td>
<td>0 (0 - 1)</td>
<td>-</td>
</tr>
</tbody>
</table>

Data shown are median (Q1 to Q3). p values were derived from the Mann-Whitney test. Abbreviations: 25OHD =25-hydroxyvitamin D; C-ACT = childhood asthma control test; FEV₁ = percentage of predicted forced expiratory volume in 1 second; FVC% = percentage of predicted forced vital capacity; GINA = global initiative for asthma; hsCRP = high sensitivity C reactive protein; IL-10 = interleukin10; CAMP = cathelicidin antimicrobial peptide; mPAQLQ = mini paediatric quality of life questionnaire; GP = general practitioner; * Denotes statistically significant.

We stratified results by baseline 25OHD level (i.e., VDD vs. VDS) because any detrimental effect of low vitamin D status may be more apparent when VDD and VDS groups are compared. (Table 5.4).
Table 5.4 Absolute difference in medians after 15 weeks of D3 or PL stratified by baseline 25OHD level.

<table>
<thead>
<tr>
<th></th>
<th>PL 50nmol/L</th>
<th>PL &gt;50nmol/L</th>
<th>D3 &lt;50nmol/L</th>
<th>D3 &gt;50nmol/L</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>8</td>
<td>14</td>
<td>6</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>Age (y)</td>
<td>7.5</td>
<td>7</td>
<td>11.5</td>
<td>8</td>
<td>0.56</td>
</tr>
<tr>
<td>Baseline BMI (kg/m²)</td>
<td>18 (15-21)</td>
<td>19 (16-20)</td>
<td>20 (18-21)</td>
<td>21 (18-22)</td>
<td>0.59</td>
</tr>
</tbody>
</table>

**Subjective scores**

<table>
<thead>
<tr>
<th></th>
<th>PL &lt;50nmol/L</th>
<th>PL &gt;50nmol/L</th>
<th>D3 &lt;50nmol/L</th>
<th>D3 &gt;50nmol/L</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-ACT</td>
<td>3.5 (0.3-5.5)</td>
<td>3.5 (0-5)</td>
<td>0 (-6.5-5)</td>
<td>2 (-0.5-3.5)</td>
<td>0.79</td>
</tr>
<tr>
<td>GINA Score</td>
<td>0 (-0.5-0.5)</td>
<td>-2 (-3-0.3)</td>
<td>-1 (-2-0.8)</td>
<td>-0.5 (-1.8-0)</td>
<td>0.31</td>
</tr>
<tr>
<td>mPAQLQ</td>
<td>1.2 (0.7-1.5)</td>
<td>0.3 (-0.9-1.5)</td>
<td>0.6 (-0.02-0.8)</td>
<td>0.5 (-0.08-0.9)</td>
<td>0.63</td>
</tr>
</tbody>
</table>

**Pulmonary function**

<table>
<thead>
<tr>
<th></th>
<th>PL &lt;50nmol/L</th>
<th>PL &gt;50nmol/L</th>
<th>D3 &lt;50nmol/L</th>
<th>D3 &gt;50nmol/L</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>FEV1%</td>
<td>4.5 (-0.8-6)</td>
<td>0.5 (-4.8-7)</td>
<td>-3.5 (-6.8-1)</td>
<td>-4 (-5.5-2)</td>
<td>0.52</td>
</tr>
<tr>
<td>FVC%</td>
<td>2 (-3.5-5.4)</td>
<td>-2 (-4.8-3.8)</td>
<td>0.5 (-4.5-1.8)</td>
<td>-5.5 (-8.8-4.5)</td>
<td>0.78</td>
</tr>
<tr>
<td>FEV1/FVC</td>
<td>3 (0.8-4.5)</td>
<td>0.5 (-1-1.8)</td>
<td>-3.5 (-4-2.3)</td>
<td>0 (-5-1.8)</td>
<td>0.023**</td>
</tr>
</tbody>
</table>

**Asthma Control**

<table>
<thead>
<tr>
<th></th>
<th>PL &lt;50nmol/L</th>
<th>PL &gt;50nmol/L</th>
<th>D3 &lt;50nmol/L</th>
<th>D3 &gt;50nmol/L</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Partly Uncontrol-led</td>
<td>2</td>
<td>6</td>
<td>4</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>8</td>
<td>2</td>
<td>7</td>
<td></td>
</tr>
</tbody>
</table>

**Laboratory**

<table>
<thead>
<tr>
<th></th>
<th>PL &lt;50nmol/L</th>
<th>PL &gt;50nmol/L</th>
<th>D3 &lt;50nmol/L</th>
<th>D3 &gt;50nmol/L</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>25OHD (nmol/L)</td>
<td>-1 (-4.5-11)</td>
<td>1 (-2.5-10)</td>
<td>71 (43-78)</td>
<td>31 (18.5-54)</td>
<td>0.001**</td>
</tr>
<tr>
<td>ALP (U/L)</td>
<td>11 (-0.3-32.3)</td>
<td>24 (13-27)</td>
<td>-2 (-33-1)</td>
<td>-5 (-12-21)</td>
<td>0.96</td>
</tr>
</tbody>
</table>

**Diary**

<table>
<thead>
<tr>
<th></th>
<th>PL &lt;50nmol/L</th>
<th>PL &gt;50nmol/L</th>
<th>D3 &lt;50nmol/L</th>
<th>D3 &gt;50nmol/L</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>School days missed</td>
<td>8 (5.3-10)</td>
<td>4.5 (0.5-8.8)</td>
<td>5 (0.8-10.8)</td>
<td>0.5 (0-3.5)</td>
<td>0.03**</td>
</tr>
<tr>
<td>No. steroid courses</td>
<td>1 (0-2)</td>
<td>0 (0-0.75)</td>
<td>1 (0.3-1.8)</td>
<td>0 (0-0)</td>
<td>0.05</td>
</tr>
</tbody>
</table>

Data shown are median (Q1 to Q3). * Asthma control according to GINA classification at baseline. p values were derived from independent samples Kruskal-Wallis test.

Abbreviations: BMI = body mass index; C-ACT = childhood asthma control test; Ca²⁺ = calcium; FEV1 = percentage of predicted forced expiratory volume in 1 second; FVC% = percentage of predicted forced vital capacity; GINA = global initiative for asthma; mPAQLQ = mini paediatric asthma quality of life questionnaire; ** Denotes statistically significant.
Levels of 25OHD increased significantly in all supplemented patients, specifically in the VDD group, compared with all other groups. There was no change in VIDSun score, or in Asthma Control Questionnaires (C-ACT, mPAQLQ, or GINA). There was no significant difference in biomarkers of bone metabolism (PTH, Ca2+, ALP, PO4), or between markers of allergy (IgE), immunity (eosinophils, IgA), airway inflammation (ECP), and systemic inflammation (hsCRP). In the case of pulmonary function, we observed apparent adverse effects of vitamin D3 supplementation in both the VDD and VDS groups. Insignificant decreases in FEV1% and FVC% in both supplementation groups were accompanied by a significant decrease in FEV1/FVC in the supplemented VDD group only. The VDS group who received vitamin D3 supplements missed significantly fewer days of school. They also had fewer steroid requirements compared with all other groups, but there was no difference regarding RTI incidence, GP visits, or antibiotic usage. On the basis of the questionnaires, a few patients had a marked improvement in their asthma symptoms.

5.5. Discussion.

For 15 weeks we investigated the effects of vitamin D3 supplementation (2000 IU/day) compared with PL among a small sample of urban, Caucasian, mostly atopic children who were receiving appropriate medication but whose asthma was uncontrolled. Our trial failed to demonstrate any significant benefit. At the baseline we did observe a trend toward greater lung function, higher quality of life, and lower inflammation and level of atopy in the VDS vs. VDD group. We also noticed a significant increase in median 25OHD level with vitamin D3 supplementation compared with PL. To our surprise, we observed a trend toward decreased pulmonary function with vitamin D supplementation compared with PL. We should emphasise that at the baseline lung function was higher in the vitamin D group compared with the PL (FEV1 105 vs. 96%; p = 0.19), leaving little or no room for improvement in lung function after supplementation. It would be difficult to make a statement about the negative effects of vitamin D on lung function in our paediatric
patients. Also, previous reports have noted no effect of supplementary vitamin D on pulmonary function in children with asthma. The evidence remains unsatisfactory, but we suspect that some detrimental effects of supplementation with vitamin D3 on lung function can be explained by an allergic response in some children to vitamin D3 capsules made by Best Formulations®, CA, USA. In such a case their asthma symptoms could be worsened.

Some studies have suggested positive associations between 25OHD levels and pulmonary function in childhood asthmatics, but few vitamin D intervention trials have assessed pulmonary function. Supplemental vitamin D has been found to increase the rate of FEV1 change in adults. This is also the case after asthma exacerbation. In our trials we did not detect any significant benefit to lung function or to quality-of-life scores (C-ACT, mPAQLQ, or GINA score). This is in line with previous trials which noted no effect of vitamin D supplementation to ACT in either adults or children. But some (20%) of our children showed an improvement in asthma symptoms.

Our findings suggest that daily supplementation with 2000 IU vitamin D3 may not improve asthma symptoms in all atopic Caucasian children, but may be of benefit in some cases. In support of this idea, diary analysis revealed significantly fewer days of school missed in the D3 group compared with PL group. Upon stratification by baseline 25OHD, this effect was confined to the VDS group who took supplements. Children in this group missed fewer school days – without any significant improvement in their symptoms, RTI incidence, or antibiotic/steroid use. As we showed above, vitamin D sufficient children at the baseline were less allergic compared to the VDD group, and it is possible that they did not develop an allergic reaction to supplementation.

Previous studies suggested that vitamin D has a broad protective effect against RTIs, and that the effect appears stronger in asthmatics. Several plausible mechanisms might explain how vitamin D can affect RTIs. One of these relates to the immunosuppressive action of Vitamin D on the cells producing pro-inflammatory cytokines. (We did not analyse these markers during our study because they did not form part of our
research project, but future work will be necessary in this area.) Such action can lead to some improvement of asthma symptoms, as has been reported previously.\textsuperscript{22} Another possibility is that vitamin D increases the production of antimicrobial peptides such as cathelicidin (CAMP). But our data do not support this (see below).

We did not observe any effect on IgA levels, suggesting that vitamin D had no impact on mucosal immunity. In agreement with other researchers,\textsuperscript{23} we found negative associations between 25OHD levels and total IgE in all studied patients at the baseline. We did not notice any improvement in the level of atopy in our vitamin D supplemented patients, based on the results of serum eosinophil count, IgE or ECP levels. The majority of our patients were atopic, but our group was heterogenous and also included non-atopic children (about 20%). In comparison to our study, previous reports demonstrated decreased sputum eosinophils among non-atopic asthmatics with sputum eosinophilia,\textsuperscript{24} and also a decreased serum IgE after supplementary vitamin D combined with anti-asthma therapy (subcutaneous immunotherapy).\textsuperscript{9} Possible explanations for the discrepancies between our findings and other studies include differing vitamin D dosing, different analysis (whole blood eosinophils vs sputum), inclusion of non-atopic as well as atopic asthmatics, and co-administration of subcutaneous immunotherapy in other studies.

Although there was a notable decrease in hsCRP in the PL group compared with the D3 group (-1 vs. +1 mg/l; \( p = 0.04 \)), this may not be clinically significant because all the subjects always displayed low, normal values for hsCRP, and at the baseline the PL had a higher hsCRP level. Vitamin D is known to trigger production of CAMP, and vitamin D supplementation increases CAMP in healthy children.\textsuperscript{25} There is a lack of data on the links between cathelicidin and vitamin D in asthma. In our study CAMP decreased in both groups, but it did so to a greater extent in the vitamin D3 supplemented group. It should be noted that the baseline for CAMP was higher in the vitamin D group. Our preliminary results do not support a role for vitamin D in increasing cathelicidin levels and in decreasing RTIs in children with asthma.
IL-10 is an anti-inflammatory and immunosuppressive cytokine, which is inversely associated with asthma severity, and its production appears to be influenced by vitamin D supplementation. We noticed that, similarly to CAMP, IL-10 decreased in both groups. Our biochemical observations did not confirm a potential anti-inflammatory effect of vitamin D supplementation in paediatric asthma. A larger sample of patients would be necessary to carry this investigation further. There was no difference between the groups regarding PTH, Ca2+, or PO4. However ALP, a marker of osteoblastic activity, was significantly increased in the PL group compared with D3. This suggests an adverse effect of VDD on bone health for children with asthma who receive standard therapies. It is important to monitor the level of sufficiency of 25OHD in paediatric asthmatics for skeletal health, but to be aware of the unknown detrimental effects of vitamin D excess.

Being a hormone, the active form of vitamin D (calcitriol) can be responsible for adverse outcomes at a low or a high level. In consequence, a U-shaped curve of 25OHD level has been reported regarding serum IgE level, paediatric lung function, and wheeze. More study is necessary to confirm any benefit of vitamin D supplementation in atopic and non-atopic asthma.

It has been proposed that genetic differences regarding the vitamin D metabolic pathway (e.g., vitamin D Receptors, or vitamin D-binding protein) may influence the effect of vitamin D on asthmatic parameters. An observational study among children with asthma found that specific vitamin D receptor polymorphisms were associated with P-ACT score. This leads us to believe that genetic factors may be important in paediatric asthma. We will examine some of these genetic factors (SNPs in VDR gene) in Chapter 6 of this thesis.
5.6. **Trial strengths.**

We utilised the gold standard study design and assessed objective, subjective, and biochemical markers of asthma. We studied a well-characterised group of urban, Caucasian, mostly atopic children with diagnosed asthma. The PL and vitamin D groups were well matched at baseline, and there were no significant differences in their demographics. Although differences in prescribed pharmacological treatment and effectiveness are important in asthma research, there was no difference between our two study groups at baseline, follow-up medications, or compliance. Vitamin D trials can be influenced by various factors; these include fluctuations in sun exposure (and hence 25OHD levels), the different quality of vitamin D assays, compliance with the medication, and differences in vitamin D supplementation doses. The fact that we conducted this trial in winter at a high latitude, when vitamin D photosynthesis is minimal, helped counteract these factors. Although we purposely did not restrict patients’ diet or supplemental vitamin D intake, we did ask that they would not change their behaviour during the trial. The same vitamin D behaviour was evident in both groups throughout the trial, as was confirmed with VIDSun scores.

We utilised two different methods for total 25OHD measurement, including the current gold standard assay (liquid chromatography–tandem mass spectrometry). All laboratory testing in Biomnis Ireland was performed by the author.

Compliance, as assessed by parents’ diary data and capsule counts, was high (>85%). We utilised a moderate dose of vitamin D3 (2000 IU/day). As expected, because of our trial design and good compliance with the supplements, serum 25OHD increased significantly in the vitamin D3 group, but did not change in the PL group.

5.7. **Trial limitations.**

Our sample size was small, it was exclusively Caucasian, and there were 12 dropouts (24%). These dropdowns had similar baseline characteristics to the children who completed the study, and there are no statistically
significant differences in several important characteristics (age, BMI, FVC, FEV1, 25OHD; p > 0.5 for all).
Baseline lung function values were high in both groups (96% vs. 105%), leaving little room for improvement. In consequence, we are unable to comment on the effect of vitamin D supplementation among children with poor lung function. At baseline in the intervention group, we observed a standard deviation of 15.9 with regard to FEV1%. With a significance level of 0.05, a sample of either 82 or 100 would be required at 80% or 90% power, respectively, to demonstrate a 10% difference in FEV1%.
Most observational studies of vitamin D analyse and report total vitamin D status as 25OHD. As mentioned previously, there are 2 major forms of vitamin D: 25(OH)D2 (vitamin D2, ergocalciferol, produced from plants), and 25(OH)D3 (vitamin D3, cholecalciferol, synthesised in the human skin or coming from animal sources). These may have different effects. 25(OH)D3 contributes 80–90% of the total 25OHD concentration in humans, even at high latitude, and it is thought to be more potent than 25(OH)D2.29 However a study of over 2200 children in England demonstrated an inverse association between 25(OH)D2 and both flexural dermatitis and wheezing, but a positive association with FEV1 and FVC. In direct contrast, 25(OH)D3 was positively associated with both flexural dermatitis and wheezing but not associated with lung function.30 From these studies we can hypothesise that vitamin D2 could be more beneficent in the treatment of paediatric asthma.
The author’s colleagues recently published detailed reviews of vitamin D mechanisms and vitamin D supplementation in asthma.25,31 Most association studies show the potential benefits of vitamin D in asthma, but studies of vitamin D supplementation have demonstrated little or no benefit.9-11,19,20,31 Our pilot study is in agreement with this previous research. Our observations also raise the possibility that vitamin D supplementation in uncontrolled paediatric asthma might have negative effects in some patients. This, too, is consistent with existing data.31 Although 25OHD is considered to be the best circulating biomarker of vitamin D status, there are many important intermediate forms in the vitamin D metabolic pathway. We did not measure levels of vitamin D-
binding protein or 1,25(OH)D, or other metabolites. We did measure vitamin D receptors, but we found that the levels were undetectable in most of our patients.

24-hydroxylase (CYP24A1) is responsible for catabolism of both 25OHD and 1,25(OH)D. It is found in most tissues in the body and it is rapidly induced by 1,25D. It also catalyses the synthesis of 24,25(OH)D and 1,24,25D from 25OHD and 1,25(OH)D respectively. Recently the 24,25(OH)D form in particular has emerged as a potentially important marker, which might contribute to response to supplementation and genetic variations. It seems as if vitamin D supplementation may raise 24,25(OH)D, causing increased catabolism of both 25OHD and 1,25(OH)D. This could potentially explain the detrimental effects of high dose vitamin D supplementation.

We used fixed vitamin D dosing, but it might be more appropriate to choose vitamin D dosage according to baseline 25OHD levels.

25OHD has a half-life of approximately 15 days, and is possible that our intervention period (15 weeks) may have been too brief to result in steady state 25OHD levels. A longer intervention period duration might be more appropriate to vitamin D supplementation studies; this would take account of seasonal variations in infections, allergies, asthma, and vitamin D status.
5.8. Conclusions.

- In the course of this research a wide range of biochemical tests was performed by the author, who also set up new diagnostic methods in her clinical laboratory, including IL-10 and CAMP.

- Our research confirmed earlier findings of a negative association between 25OHD levels and total IgE in all studied paedriatic asthmatic patients at the baseline.

- Like earlier researchers, we observed a trend towards greater lung function, higher quality of life and lower inflammation in vitamin D sufficient children, compared to those who were VDD at the beginning of the trial.

- This study of vitamin D supplementation in uncontrolled paediatric asthma in Ireland examined the effect of supplements on 25OHD levels, lung function, biomarkers of systemic and allergic inflammation, and subjective and objective asthma parameters. We did not confirm positive outcomes in asthmatic patients after vitamin D3 supplementation. Accordingly our preliminary results do not support the use of supplementary vitamin D3 in all Caucasian, atopic children with uncontrolled asthma.
5.9. Future studies.

- Further research will be needed to understand the precise role of vitamin D in paediatric asthma. In particular it will be necessary to explore more extensively the mechanisms whereby, in our study, vitamin D sufficient children presented with milder asthma symptoms in comparison with those who were VDD. It will be important to understand the mechanisms whereby VDR, vitamin D and its metabolites are involved in the immunomodulation of different cell types and cytokines – and in particular of IgE.

- The sample size in this trial was quite small and therefore the conclusions reached must be tentative. Bearing this in mind, a decreased pulmonary function with vitamin D supplementation was observed. Careful selection of patients would be important in any future study.

- In general, caution is warranted before widespread clinical use of vitamin D for asthmatics. More extensive studies are necessary, with larger sample sizes, on different asthma phenotypes (both atopic and non-atopic). Such research should include not merely vitamin D3 supplementation, but also vitamin D2 and simple sun exposure.

- More biochemical markers for research and diagnostic should be set up in clinical laboratories, including pro-inflammatory cytokines and various transcription factors that play a role in the pathophysiology of asthma.

The next chapter will be a continuation of this topic and will investigate the importance of genetic polymorphisms in vitamin D receptor and IL-10 in our sample group.
5.10. References for Chapter 5.


Table S5.1. Reference ranges for the measured parameters

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Normal Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>FEV1 (%)</td>
<td>≥ 100%</td>
</tr>
<tr>
<td>FVC (%)</td>
<td>≥ 80%</td>
</tr>
<tr>
<td>FEV1/FVC (%)</td>
<td>≥ 70%</td>
</tr>
<tr>
<td>25OHD nmol/L</td>
<td>50 – 125 nmol/L</td>
</tr>
<tr>
<td>IgE (IU/mL)</td>
<td>&lt; 100 UI/mL</td>
</tr>
<tr>
<td>ECP (ug/L)</td>
<td>&lt; 15 ug/L</td>
</tr>
<tr>
<td>IL-10 (pg/mL)</td>
<td>8.7 - 32 pg/mL</td>
</tr>
<tr>
<td>CAMP (pg/mL)</td>
<td>760 - 1470 pg/mL</td>
</tr>
<tr>
<td>IgA (g/L)</td>
<td>0.6-4 g/L</td>
</tr>
<tr>
<td>CRP (mg/L)</td>
<td>&lt; 5 mg/L</td>
</tr>
<tr>
<td>Total Ca (mmol/L)</td>
<td>2.1 - 2.6 nmol/L</td>
</tr>
<tr>
<td>PO4 (mmol/L)</td>
<td>0.7-1.4 mmol/L</td>
</tr>
<tr>
<td>PTH (pg/mL)</td>
<td>15-68 pg/mL</td>
</tr>
<tr>
<td>ALP (U/L)</td>
<td>40-150 IU/L</td>
</tr>
<tr>
<td>Mg (mmol/L)</td>
<td>0.65-1.05 mmol/L</td>
</tr>
<tr>
<td>WBC (x10^9/L)</td>
<td>4-10 x 10^9/L</td>
</tr>
<tr>
<td>EO (x10^9/L)</td>
<td>0.05-0.5 x10^9/L</td>
</tr>
<tr>
<td>Lymph (x10^9/L)</td>
<td>1-3 x 10^9/L</td>
</tr>
<tr>
<td>Neut (x10^9/L)</td>
<td>2-7 x 10^9/L</td>
</tr>
</tbody>
</table>

Ranges for 25OHD, CRP, Total Ca, PO4, PTH, ALP, Mg, WBC, EO, Lymph and Neut have been established in the Biomnis Ireland Laboratory. Other ranges have been provided by the manufacturers. The reference for lung function is as per table S6.5.
Chapter 6

A pilot study of Vitamin D Receptor TaqI Gene’s Variant in Exon 9 and Apal in Intron 8 in uncontrolled paediatric asthma* 


6.1. Introduction.

This chapter continues the theme developed in Chapter 5. In addition, the supplementary materials include the author’s preliminary work on her study of VDR SNPs in a cohort of asthmatic adults. (See below, supplementary material, 6.10.2.)

Asthma’s prevalence in Ireland has already been mentioned. The results of recent studies imply that VDR genetic variants may impact on lung function and allergic or systemic inflammation in this condition. VDR polymorphisms have been associated with asthma and with allergy susceptibility.1,2 The vitamin D receptor belongs to the nuclear hormone receptor superfamily and is a ligand-activated transcription factor. It is widely expressed in human lungs throughout the full epithelial layer, as is 1a-hydroxylase,3 which is responsible for the formation of the active vitamin D metabolite, 1a-calcitriol (1a,25(OH)2D3).

Beyond its classic functions in calcium homeostasis, vitamin D plays an important role in the regulation of both adaptive and innate immune functions, in hormone secretion, in the regulation of the proliferation and differentiation of many cell types.4 It may influence airway remodelling,5 such as subepithelial fibrosis, increased smooth muscle mass, and epithelial alterations.

Vitamin D deficiency is very common worldwide, particularly in children.6,7 Many medical paediatric conditions are also associated with low vitamin D, including asthma.8,9 As was shown in Chapter 5, in our recent study of Vitamin D supplementation in uncontrolled paediatric asthmatics in Ireland, 50% of the children were vitamin D deficient (25OHD levels <50 nmol/L).10 The results of our study with Vitamin D supplementation in our paediatric patients proved disappointing. We hypothesise that VDR
genetic variants can be the reason for specific responses to supplementation, and that they may influence the effects of Vitamin D’s actions. It is possible that these polymorphisms are responsible for different baseline levels of Vitamin D in our patients, and that the dosage needed may vary considerably from one patient to another. In the recent meta-analysis of case–control studies, Tizaoui et al. showed that VDR polymorphisms contribute to asthma susceptibility and risk. Han et al. have suggested that VDR polymorphisms could be developed as biomarkers for asthma susceptibility. The association between genetic variants of VDR and paediatric asthma has been studied in different ethnic groups. The most studied single nucleotide polymorphisms (SNPs) are those located in the last exon 9 (TaqI) of the gene and in the last intron 8 (ApaI). These genetic variants may influence the transcription of the target genes by influencing VDR’s RNA stability and translation efficiency. But the exact mechanism of action is still under investigation. Over 900 genes may be transcribed by VDR, and the widespread expression of the receptor is extremely important for a variety of human tissues – because it allows vitamin D act as a pleotropic agent. In a recent review of genetic association studies Jolliffe et al. have suggested that variation in VDR is a more important determinant of phenotype for various chronic illnesses, including asthma, than circulating 25OHD concentrations.

6.2. Aims and objectives of the study.

In this study we had three aims:

• first, to determine the VDR gene variants TaqI in exon 9 (T/C) (rs731236), ApaI(C/T) (rs7975232) and Tru9I(C/T) (rs757343) in intron 8 (C/T), and rs1800871(A/G) (IL-10 gene functional polymorphism) in uncontrolled paediatric asthmatics, and also in healthy volunteers in Ireland;

• secondly, to investigate the impact of those polymorphisms that differed significantly between patients and control in asthma susceptibility and also in relation to vitamin D status, IgE and other biochemical and immunological indices;
and finally, to examine the possibility of using these polymorphisms as potential biomarkers for asthma.

6.3. Methods and participants.

The study was carried out at the National Children’s Hospital, Tallaght, James Connolly Hospital Dublin, and Biomnis Ireland, after receiving institutional review board approval from the National Children’s Hospital, Tallaght and James Connolly hospitals, and after having obtained consent from parents, guardians and the healthy adults who were involved.

6.3.1. Subjects.

Asthmatic children were recruited from paediatric respiratory out-patient clinics for a vitamin D supplementation study (the trial was registered at ClinicalTrials.gov. Identifier: NCT02428322).10 Our 44 subjects were Caucasian, aged 6-16, and established on anti-asthmatic medication with previous diagnosis of uncontrolled asthma according to the Global Initiative for Asthma 2011 guidelines.18 The healthy 57 subjects had no personal or family history of asthma or other respiratory illnesses, or bone, articular, renal or any other chronic diseases. We excluded participants with conditions/medications that influence vitamin D metabolism or absorption.

6.3.2. Study design.

Asthmatic subjects participated in the vitamin D supplementation study mentioned above (see chapter 5).10 For this study of VDR polymorphisms we also recruited healthy volunteers. We examined two restriction fragment length polymorphisms (RFLPs) in the VDR gene in both groups. In addition we studied the relationship between the polymorphisms and different biomarkers in a cohort of uncontrolled paediatric asthmatics. Subjective and objective asthma measures were also analysed in relation to genotypes.

Vitamin D status is affected by diet, supplement use and exposure to ultraviolet B radiation, and (as mentioned already) our study was carried out at a high latitude during winter 2013-14, at a time of low skin vitamin
D production. Behaviour relating to vitamin D status was examined using a VIDSun questionnaire given to asthmatics. It should be pointed out that all the paediatric patients were known uncontrolled asthmatics on established anti-asthma therapy. A clinical nurse specialist assessed adherence to anti-asthma medication. Spirometry was carried out according to the American Thoracic Society/European Respiratory Society Task Force, with the spirometry module of the V-max Encore System (Carefusion). Example of normal spirometry report presented in Figure S6.5. Results were presented as a percentage of predicted values. Subjective asthma control and quality of life scores were described in Chapter 5.

6.3.3. Laboratory methods.

6.3.3.1. Biochemistry and FBCs.

Venous blood was collected into vacutainer tubes, one containing EDTA and the other without additive. Whole blood with EDTA was analysed for full blood count using an automated analyser Sysmex XE-2100D (Sysmex, Mundelein, IL 60060 USA) on the day of collection, and samples were kept for DNA extraction. Additional blood without additive was centrifuged, aliquoted, and frozen to -80°C until further analysis. We assessed vitamin D status by measuring total serum 25-hydroxyvitamin D, the best biomarker of vitamin D status. 25OHD levels were analysed on Abbott Architect ci8200 (Abbott Laboratories, Abbott Park, IL, USA) using chemiluminescent microparticle immunoassay method with between-run and within-run CVs < 6%. The assay is Vitamin D Standardisation Programme certified. It successfully passed the performance criterion of ±5% mean bias of the Centres of Disease Control and the University of Ghent Vitamin D2 and D3 Reference Method with an overall imprecision of <10% over the concentration range of 22-275 nmol/L for total 25OHD. (This has been discussed already in Chapter 3.) We divided 25OHD levels into two groups, based on the most up-to-date Institute of Medicine recommendations, according to which <50nmol/L indicates vitamin D deficiency and >50nmol/L indicates vitamin D sufficiency.
Serum concentrations of intact parathyroid hormone, albumin, total calcium, alkaline phosphatases, phosphate, total IgE, immunoglobulin A, and high sensitivity C reactive protein were measured using commercially available diagnostic kits on the automated analyser Abbott Architect ci8200. The between-run and within-run CVs for these assays ranged between 1% and 6%.

Eosinophil cationic protein was analysed on the Phadia 250, using fluorescent enzymeimmunoassays (ImmunoCAP Technology) with a between-run CV <7 % and minimum detectable level of 2 ug/L (normal range: <13  ug/L).

Serum levels of IL-10 and CAMP were determined by human enzyme-linked immunosorbent assay method (ELISA kit assays, Damastown, Dublin 15). Intra- and interassay CVs were less than 8% for these tests. All assays were analysed with kits of the same lot number.

6.3.3.2. Genotyping of TaqI, ApaI, Tru9I and rs1800871 polymorphisms.

All molecular genetic studies were carried out by the author in the Molecular Laboratory in Biomnis Ireland. DNA isolation was performed on Maxwell 16 System (Promega Corporation, Madison, WI, USA). The outcome of this technique was high molecular weight DNA (>20kb) that had no traces of RNA contamination and had a 260/280 absorbance ratio >1.7. The isolated DNA was stored at -20°C until it was required for analysis.

Based on a candidate gene approach, we selected for this study three single-nucleotide polymorphisms of the VDR gene and one functional polymorphism of the IL-10, which had already been described in current literature as having a functional impact on gene expression and function. All fourth polymorphisms were widely studied in different populations in relation to various medical conditions, including asthma.\textsuperscript{1,2,11-13}

Genotyping of TaqI (rs731236, assay number C_2404008_10), Apal (rs7975232, assay number C_28977635_10), Tru9I (rs757343, assay number C_2404009_20) and rs1800871 (assay number C_1747362_10) was performed using TaqMan® SNP Genotyping Assay. This consists of
a predesigned mix of unlabelled polymerase chain reaction primers and the TaqMan® minor groove binding group probe (FAM™ and VIC® dye-labeled). TaqMan SNP Genotyping Assays are developed to work with TaqMan® Universal PCR MasterMix. This contains DNA polymerases, dNTPs and optimised mix components and it exploits the same thermal conditions. Genotyping was preformed with Real-Time PCR in which one probe labelled with VIC® recognizes the allele 1, while the other probe labelled with FAM™ recognizes the allele 2. Real Time PCR was carried out using 5 μl TaqMan® Genotyping Master Mix, 0.25 μl TaqMan® SNP Genotyping Assay (TaqMan probes) (40×), 3.75 μl Dnase Free Water and 1 μl DNA (1-10 ng). This was intended to bring the final reaction volume to 10 μl. The thermal conditions of Real Time PCR were: initial denaturing at 95°C for 10 min; 40 cycles of 95°C for 15 sec (denaturing) and 60°C for 1 min (annealing/extension). Approximately 10% of the samples were selected randomly for confirmation of the previous results, and 100% of them matched. The genotyping success rates for two SNPs were >99%. SDS 2.3 software was used for allelic discrimination (Applied Biosystems).

All the materials were used in the TaqMan® SNP Genotyping Assay (ABI), in compliance with the manufacturer’s instructions and with the information supplied on the Applied Biosystems website http://www.appliedbiosystems.com.

6.4. Statistical analysis.

Allele and genotype frequencies were calculated by direct counting. The χ² and Fisher’s exact tests were used to compare frequencies between cases and controls, and also for Hardy-Weinberg equilibrium determination. In investigating genotypic associations, odd ratios (OR) were reported for the allelic distribution in the study groups. For group comparisons for serum 25OHD levels and other biomarkers we used the t-test and Kruskal–Wallis test, followed by Dunn’s multiple comparison test. Mean for lung function measurements, 25OHD, IL-10 and other markers’ values of genotypes in groups were compared with one-way ANOVA and Tukey’s multiple comparisons test. P value less 0.05 counts
as significant. The software used for the statistical analysis was GraphPad Prism 5, Version 5.01. For the haplotype analysis we used the following programme from the website: https://www.snpstats.net/snpstats/start.htm.

6.5 Results.

The frequencies of VDR Tru9I and rs1800871 polymorphisms did not vary significantly between asthmatics and controls (Figures S6.2 and S6.4) in comparison with two other polymorphisms (Figures S6.1 and S6.3). For this reason we did not perform further analysis of VDR Tru9I and rs1800871 polymorphisms in our patients. The frequencies of VDR TaqI T and C alleles were 0.63 and 0.37 in cases and 0.8 and 0.2 in controls. The genotypic frequencies of VDR TaqI were 40%, 47% and 13% in cases, and 60%, 40% and 0% in controls for TT, TC and CC genotypes respectively. For VDR ApaI gene variant the frequencies of T and C alleles were 0.52 and 0.48 in cases, and 0.26 and 0.74 in controls. The genotypic frequencies of this polymorphism were 28%, 50% and 24% in cases, and 9%, 35% and 56% in controls for TT, TC and CC genotypes respectively. We found that the distribution of T and C alleles and genotype frequencies varied significantly between asthmatics and controls for both polymorphisms (p value < 0.05). (Tables 6.1; 6.2).
Table 6.1. Genotypic association analysis of VDR RFLPs (restriction fragment length polymorphisms) Taq1 and Apa1 between paediatric asthmatic patients and control individuals.

<table>
<thead>
<tr>
<th>Enzyme analysis</th>
<th>Patients (44)</th>
<th>Controls (57)</th>
<th>Multiple comparison p value</th>
<th>$\chi^2$</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>TaqI Genotypes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TT</td>
<td>17 (38 %)</td>
<td>34 (60 %)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CT</td>
<td>21 (48%)</td>
<td>23 (40 %)</td>
<td></td>
<td>10.25</td>
<td>0.006*</td>
</tr>
<tr>
<td>CC</td>
<td>6 (14 %)</td>
<td>0 (0 %)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TT/CT</td>
<td>17/21</td>
<td>34/23</td>
<td>0.21@</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TT/CC</td>
<td>17/6</td>
<td>34/0</td>
<td>0.003@</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CT/CC</td>
<td>21/6</td>
<td>23/0</td>
<td>0.025@</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apal Genotypes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TT</td>
<td>11 (25 %)</td>
<td>5 (9 %)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CT</td>
<td>23 (52%)</td>
<td>20 (35 %)</td>
<td></td>
<td>18.82</td>
<td>0.0001*</td>
</tr>
<tr>
<td>CC</td>
<td>10 (23 %)</td>
<td>32 (56 %)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TT/CT</td>
<td>11/23</td>
<td>5/20</td>
<td>0.38@</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TT/CC</td>
<td>11/10</td>
<td>5/32</td>
<td>0.002@</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CT/CC</td>
<td>23/10</td>
<td>20/32</td>
<td>0.007@</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Data are given as numbers and percentages. *p<0.05 is considered significant. @ Fisher’s exact test.

Table 6.2. Allelic association analysis of VDR RFLPs Taq1 (T>C) and Apa1 (C>T) between uncontrolled paediatric asthmatic patients and control individuals.

<table>
<thead>
<tr>
<th>Enzyme analysis</th>
<th>Patients (44)</th>
<th>Controls (57)</th>
<th>OR (95 % CI)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>TaqI (Allelic association)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T</td>
<td>57(63 %)</td>
<td>91 (80 %)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>33(37 %)</td>
<td>23 (20 %)</td>
<td>2.37(1.27-4.45)</td>
<td>0.007*</td>
</tr>
<tr>
<td>ApaI (Allelic association)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T</td>
<td>47(52 %)</td>
<td>30 (26 %)</td>
<td>2.93(1.62-5.3)</td>
<td>0.0004*</td>
</tr>
<tr>
<td>C</td>
<td>43(48 %)</td>
<td>84 (74 %)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Data are given as numbers and percentages. OR=odds ratio; 95% CI (in parentheses) * Denotes statistically significant
The alleles’ frequencies were significantly different, as was shown by the 37% prevalence of C allele (TaqI) and the 52% prevalence of T allele (ApaI) in asthmatic patients versus 20% and 26% in the controls (P = 0.007, P = 0.0004). Children carrying the C allele for TaqI are 2.37 times more likely to develop asthma (OR = 2.37, 95% CI (1.27–4.45)) and children carrying the risk T allele for ApaI are 2.93 times more susceptible for asthma development (OR = 2.93, 95% CI (1.62-5.3)) than healthy individuals.

Both cases and controls were in Hardy-Weinberg equilibrium for both ApaI and TaqI: p > 0.2 in three analyses, with the exception for TaqI in healthy individuals. The two SNPs were in linkage disequilibrium in cases (D = 1.000, r² = 0.633 ) but not in controls (D = 0.596, r² = 0.25). ApaI C allele was linked to TaqI C, and ApaI T to TaqI T in asthmatic children.

In relation to polymorphisms study in uncontrolled asthmatics, we found no association between genotypes and lung function, serum 25OHD levels and other biomarkers including IgE, ECP, CAMP and hsCRP – except IL-10 and white blood cells count. (Table S6.1. and S6.2.) IL-10 levels were significantly low in asthmatics with TC genotype for TaqI (p <0.003) and significantly high in patients with TT genotype for ApaI polymorphism (p < 0.005). (Tables 6.3, 6.4; Figures 6.1, 6.2) WBC was significantly high in patients with TC and CC genotypes for TaqI and significantly low in TT genotype for ApaI. (Tables 6.5, 6.6; Figures 6.3, 6.4)
Table 6.3. Relationship between serum IL-10 and TaqI (T>C) VDR RFLP (restriction fragment length polymorphism) genotypes in uncontrolled paediatric asthmatics.

<table>
<thead>
<tr>
<th>Serum IL-10 pg/mL</th>
<th>Sig.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
</tr>
<tr>
<td>TaqI RFLP</td>
<td></td>
</tr>
<tr>
<td>TT</td>
<td>16</td>
</tr>
<tr>
<td>TC</td>
<td>20</td>
</tr>
<tr>
<td>CC</td>
<td>6</td>
</tr>
</tbody>
</table>

n = number of subjects; SD = standard deviation; p = value is for the Kruskal-Wallis on the overall effect; Sig. refers to difference between means of homozygotes and heterozygotes (CC vs. TC), of the homozygotes and heterozygotes (TT vs. TC); ns = non-significant. * Denotes statistically significant.

Table 6.4. Relationship between serum IL-10 and ApaI (C>T) VDR RFLP (restriction fragment length polymorphism) genotypes in uncontrolled paediatric asthmatics.

<table>
<thead>
<tr>
<th>Serum IL-10 pg/mL</th>
<th>Sig.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
</tr>
<tr>
<td>ApaI RFLP</td>
<td></td>
</tr>
<tr>
<td>TT</td>
<td>10</td>
</tr>
<tr>
<td>CT</td>
<td>22</td>
</tr>
<tr>
<td>CC</td>
<td>10</td>
</tr>
</tbody>
</table>

n = number of subjects; SD = standard deviation; p = value is for the Kruskal-Wallis on the overall effect; Sig. refers to difference between means of homozygotes and heterozygotes (CC vs. CT), of the homozygotes and heterozygotes (TT vs. CT); ns = non-significant. * Denotes statistically significant.
Figure 6.1. Average IL-10 levels in uncontrolled asthmatic children according to TaqI genotypes. (Tukey's Multiple Comparison Test: TT vs TC, p < 0.05*).

Figure 6.2. Average IL-10 levels in uncontrolled asthmatic children according to ApaI genotypes. (Tukey's Multiple Comparison Test: CC vs TT and for TT vs TC, p < 0.05*).

Table 6.5. Relationship between WBC and TaqI (T>C) VDR RFLP (restriction fragment length polymorphism) genotypes in uncontrolled paediatric asthmatics.

<table>
<thead>
<tr>
<th>WBC (x10^9/L)</th>
<th>Sig.</th>
<th>TaqI RFLP</th>
<th>n</th>
<th>Mean</th>
<th>SD</th>
<th>p value</th>
<th>CC vs.TT</th>
<th>CC vs.TC</th>
<th>TT vs.TC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>TT</td>
<td>17</td>
<td>7.61</td>
<td>1.35</td>
<td>p = 0.009*</td>
<td>P&lt;0.05*</td>
<td>P&lt;0.05*</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TC</td>
<td>21</td>
<td>9.04</td>
<td>2.05</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>CC</td>
<td>6</td>
<td>9.82</td>
<td>1.15</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

n = number of subjects; SD = standard deviation; p = value is for the Kruskal-Wallis on the overall effect; Sig. refers to difference between means of homozygotes and heterozygotes (CC vs.TC), of the homozygotes and heterozygotes (TT vs. TC); ns = non-significant. * Denotes statistically significant.
Table 6.6. Relationship between WBC and Apal (C>T) VDR RFLP (restriction fragment length polymorphism) genotypes in uncontrolled pediatric asthmatics.

<table>
<thead>
<tr>
<th>WBC (x10^9/L)</th>
<th>Sig.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apa-I RFLP</td>
<td>n</td>
</tr>
<tr>
<td>TT</td>
<td>12</td>
</tr>
<tr>
<td>CT</td>
<td>24</td>
</tr>
<tr>
<td>CC</td>
<td>8</td>
</tr>
</tbody>
</table>

n = number of subjects; SD = standard deviation; p = value is for the Kruskal-Wallis on the overall effect; Sig. refers to difference between means of homozygotes and heterozygotes (CC vs. CT), of the homozygotes and heterozygotes (TT vs. CT); ns = non-significant. * Denotes statistically significant.

Figure 6.3. Average WBC levels in uncontrolled asthmatic children according to TaqI genotypes. (Tukey's Multiple Comparison Test: TT vs TC and for TT vs CC, p < 0.05*).

Figure 6.4. Average WBC levels in uncontrolled asthmatic children according to Apal genotypes. (Tukey's Multiple Comparison Test: TT vs CC, p < 0.05*).
There was a trend toward greater Neutrophils count, respectively \((p = 0.05)\) for TaqI, and \((p = 0.08)\) for patients with CC genotype for Apal. Only two of our children were obese \((\text{BMI} > 30 \text{kg/m}^2)\) and both of them were VDD \((25\text{OHD level} < 50 \text{nmol/L})\) at the baseline. After the supplementation, an improvement in asthma condition was observed only in the patient with TT genotype for TaqI and Apal polymorphisms. The other child, who had TC genotypes for both polymorphisms, registered no improvement.

Our haplotype analysis for two polymorphisms showed that TT and CC haplotypes were significantly associated with asthma risk \((\text{OR} 40.26 \ (95\% \text{ CI}: \ (5.27 - 307.79), \ p < 0.001, \text{ and OR} 43.74 \ (95\% \text{ CI}: \ (4.87 - 393.20), \ p < 0.001, \text{ respectively}). \ (\text{Tables S6.3. and S6.4.})\)

6.6. Discussion.

We studied 25OHD levels and asthma symptom control in relation to TaqI and Apal VDR polymorphisms in Irish children with uncontrolled asthma and different asthma phenotypes. We found a significant association between TaqI and Apal polymorphisms and susceptibility to uncontrolled paediatric asthma. We also observed lower serum levels of IL-10 and increased WBC and neutrophils in children with specific genotypes for these polymorphisms.

The VDR gene is known as a pleiotropic gene, and is associated with numerous conditions – such as autoimmune, inflammatory, and allergic diseases, including asthma. The gene maps to chromosome 12q13.11, it contains nine exons with at least six isoforms of exon 1, it encodes a 427 amino acid protein, and it spans 63.5 kb.

The VDR binds to its ligand the active form of vitamin D \((1,25\text{-dihydroxyvitamin D3})\). It belongs to the nuclear receptors family of trans-acting transcriptional regulatory factors and it shows a sequence similarity to the thyroid’s and steroids’ hormone receptors.

The Genetic Association’s studies on VDR polymorphisms and asthma are conflicting, and the role of VDR polymorphisms remains unclear.\(^1,11,23\)

One explanation for such a discrepancy between these studies may be differences in ethnicity and geographic location.
TaqI and ApaI VDR’s SNPs are named after the corresponding restriction enzymes used in restriction fragment length polymorphism analysis. Both of these polymorphisms were relatively well-studied in different asthmatic populations. We examined these polymorphisms in Irish uncontrolled paediatric asthmatics. The results of our work on their associations with asthma agree with other studies \(^{12,24}\) in which TaqI polymorphism has been linked with asthma in paediatric patients. We also found an association between ApaI polymorphism and asthma susceptibility. These findings agree with studies by Saadi et al. \(^{25}\) and Iordanidou et al. \(^{2}\) the latter showed that ApaI ‘a’ allele was associated with improved asthma control in children.

The TaqI (rs731236, c.1056T>C, p.Ile352Ile) is a synonymous polymorphism at codon 352 (isoleucine) in exon 9 of the gene, and this T>C alteration does not result in amino acid sequence change.\(^{26,27}\) The ApaI (rs7975232, c.1025-49G>T) is located in the intron 8 of the VDR gene.\(^ {15}\) The two tested polymorphisms do not cause any structural changes of the VDR protein, but they are linked with other functional SNPs and may take part in a complex gene network enhancing or inhibiting the expression of VDR target genes.

The Apal and TaqI polymorphisms are located at the 3’ end of the gene and are near the regulatory 3’ untranslated region (3’-UTR) of mRNA. This indicates that they have the potential to alter splicing regulation. When the Apal and TaqI SNPs are found in specific haplotypes, they affect VDR mRNA stability and the rate of transcription, and this may result in altered protein expression.\(^ {26-30}\) For example, in our study both SNPs were in linkage disequilibrium in paediatric asthmatics, but not in healthy volunteers. Our haplotype analysis shows that the most frequent haplotype in our population is TC (44%), and is not significantly associated with asthma. The frequency of the TT haplotype was found to be notably higher (p<0.0001) in cases (50%) than in controls (12%). In similar fashion, CC haplotypes were found markedly (p<0.001) more frequently in cases (35%) than controls (6%). More work will be needed to confirm our findings and the possibility of using haplotype testing as a biomarker in paediatric asthma.
Alternatively, epigenetic modifications in the VDR gene can suppress VDR transcription. In a study on tuberculosis susceptibility in lymphoblastoid cell lines, Andraos et al. have demonstrated that the TaqI variant resides on a CpG island and the C allele is always methylated. They also showed that there are interactions between TaqI polymorphism, methylation levels, ethnicity, and tuberculosis susceptibility. Consequently, we can hypothesise that this SNP may serve as a marker of methylation for other functional polymorphisms in the VDR gene or in nearby genes. TaqI SNP is located in the exon 9 which encodes the ligand-binding region of the VDR. The DNA methylation and histone modifications in these regions can change the chromatin state from an open to a closed conformation. It could lead to transcriptional repression of these genes. The expression of genes involved in Vitamin D metabolism are deregulated in various chronic diseases, and these changes may be partially accredited to epigenetic modifications.

The most consistent and significant environmental risk factor for the development of childhood onset asthma, as identified in many epidemiological studies, is exposure to tobacco smoke. It has often been claimed that in utero tobacco smoke exposure – smoking by the mother previous to pregnancy, and even smoking by the grandmother – is linked to the occurrence of asthma in children or grandchildren, respectively. Parental smoking affects the methylation of CpGs in relation to numerous genes investigated in DNA collected from buccal cells of children. Over a quarter of the asthmatic children in our study live with a parent who smokes, but the distribution of the genotypes for TaqI SNP did not vary significantly between these children and those of non-smokers. We did not observe any principal differences in subjective and objective asthma parameters or in biomarkers between these groups. A special and larger study may be needed to confirm an association between TaqI polymorphism and passive smoking in asthmatic children.

Like some other researchers, we have found negative associations in our paediatric patients between 25OHD level and total IgE. (See Chapter 5) In contrast to other studies, we did not see any significant association between TaqI and ApaI polymorphisms and IgE in our paediatric
asthmatics. But we should point out that 80% of our children were atopic, with elevated IgE level. On the other hand, in our pilot study of adult asthma we noticed an association of the TT genotype for Apal SNP with a significant increase in IgE. (See supplementary material for Chapter 6.) As we showed above, TaqI and Apal polymorphisms are in linkage disequilibrium (non-random association of alleles at different loci in the population studied) in paedriatic patients (because the T allele of Apal is linked to the T allele for TaqI). We can hypothesise that these polymorphisms can lead to the stimulation not only of IL-10 expression in TT homozygeous patients, but also of IL-4. Such a pattern could explain increased IgE levels in adult asthmatics with TT genotype for Apal. This would have to be confirmed by studying larger sample groups and analysing other cytokines, including IL-4 and IL-13.

In agreement with many other studies,\textsuperscript{1,11} we found no associations between genotypes and serum 25OHD levels. Interestingly, IL-10 levels were significantly low in uncontrolled asthmatics with TC and CT genotypes for TaqI and Apal polymorphisms. WBC was significantly high with a trend toward a higher Neutrophils count in patients with TC and CC genotypes for both SNPs. IL-10 is a cytokine that shows mainly suppressive effects on innate immunity, but it has also a stimulatory effect on adaptive immunity. IL-10 is widely expressed among innate and adaptive immune cells – including monocytes, macrophages, dendritic cells, B cells, CD8\(^+\) and regulatory T cells, \(T_h 1\), \(T_h 2\) and \(T_h 17\) cells.\textsuperscript{38} IL-10 restricts the ability of antigen presenting cells to promote the differentiation and proliferation of CD4\(^+\) T cells, and it influences the initiation and progress of adaptive T cell responses. IL-10 also inhibits the expression of numerous pro-inflammatory cytokines, thus further suppressing the ability of effector T cells to prolong inflammatory responses.\textsuperscript{39} 1\(\alpha\),25(OH)\(_2\)D3 induces the expression of IL-10 in different cells of the immune system. Thereby it helps bring about the known immunosuppressive effects of Vitamin D.\textsuperscript{40-42} Matilainen \textit{et al.} showed that the effect of 1\(\alpha\),25(OH)\(_2\)D3 on the expression of IL-10 is achieved through cyclic recruitment of VDR to Vitamin D response elements within a promoter region of the IL-10 gene.\textsuperscript{43}
The result in up-regulation of IL-10 by vitamin D is to suppress the innate immune response, in order to avoid the effects of long-lasting inflammation – such as tissue damage and development of chronic illnesses.\(^{44}\)

Based on our findings we can hypothesise that patients with specific genotypes for TaqI and Apal polymorphisms have suppressed IL-10 production due to a decrease in expression of VDR. This can lead to the deregulation of innate immune responses and to the continuation of inflammatory processes. The increased levels of the neutrophils and WBC in patients with these SNPs support this interpretation. It may be possible to use these genotypes as predictive biomarkers of chronic asthma.

Half of our patients were vitamin D deficient, and low levels of 25OHD may be responsible for the suppression of their IL-10, due to insufficient production of the active form 1α,25(OH)2D3. This active form of Vitamin D has potent effects on both innate and adaptive immune responses, as has been shown by many studies.\(^{45}\) The sufficient 25OHD levels may be vital, since they influence local tissue concentrations of the active vitamin D metabolite.\(^{46,47}\) Ojaimi et al. have suggested that serum 25OHD levels as high as 120 nmol/L may be necessary for optimal immune function.\(^{48}\) This could explain why in our Vitamin D supplementation study\(^ {10}\) we did not observe any beneficial effects of vitamin D on asthma parameters and biochemical markers of allergic and systemic inflammation in our patients. Only 20% of VDD subjects achieved 25OHD levels >120 nmol/L after 15 weeks of supplementation with 2000 units each day. In the VDS group 63% of the patients achieved these levels. It can partially explain why the VDS group who received vitamin D3 supplements for 15 weeks had significantly fewer days of school missed and fewer steroid requirements compared with all other groups.\(^ {10}\)

We could not make any conclusions regarding the genotypes' effects on different asthma parameters and biomarkers after Vitamin D3 supplementation, due to the small number of children in the VDD and VDS groups. But we would like to point out the importance of phenotypical differences in our asthmatic patients. Two of the children (both female)
from our study were obese and VDD. One of them improved significantly after vitamin D supplementation without achieving 25OHD level over 120 nmol/L, and her genotype was TT for TaqI and ApaI polymorphisms. But the other patient, who was non-atopic and had heterozygous genotypes for both SNPs, did not improve after supplementation.

We hypothesise that in obesity-related asthma, genotypical investigation can be used to predict a beneficial response to vitamin D treatment. And we furthermore hypothesise that any benefit from vitamin D supplements can be achieved only in patients who have a specific genotype with a particular asthma phenotype. Patient selection might be the key to clarify whether vitamin D can be useful for enhancing asthma therapy.

We have to admit that the main limitation of our study is our limited sample size, but in a small country it is difficult to recruit a sufficient number of paediatric patients with uncontrolled asthma.

In this dissertation the author explored only two VDR polymorphisms, TaqI and ApaI, without including other VDR SNPs, such as FokI and BsmI. We also limited our study to the IL-10 measurements, and we did not analyse other cytokines due to financial reasons. But ours is a pioneering work in Irish paedriatic asthmatic research, and we hope that it will open new horizons for future studies in this area.

6.7. Conclusions.

- In summary, in this present study we have revealed an association of TaqI and ApaI polymorphisms of the VDR gene with the susceptibility to uncontrolled asthma in a cohort of paediatric Irish patients.
- Also, we have shown that the patients with TC for TaqI, and CC and CT genotypes for ApaI had a significantly low level of IL-10 and increased WBC (neutrophils in particular). This is a novel finding of our research.
- In our study we were the first to observe that TT and CC haplotypes for both polymorphisms were significantly associated with asthma in Irish children and could be potential biomarkers for paediatric asthma.

- The results of our hypothesis-generating investigation, based on the evidence of 44 cases of uncontrolled Irish paediatric asthmatics, are consistent with an important role for vitamin D in childhood asthma. Furthermore we suggest the possibility of using these polymorphisms as predictive biomarkers of chronic asthma.
- Further and more extensive functional studies will be necessary to confirm our findings in order to elucidate the underlying mechanisms in asthma that are related to vitamin D and VDR polymorphisms in specific asthma phenotypes.
- It would have been desirable to confirm our findings of the SNPs by performing direct sequencing by Sanger, but we could not do so for financial reasons.
- More research is warranted, focused not only on the identification of risk polymorphisms involved in development of asthma, but also on the mechanism by which they lead to the development and course of the disease.
- We propose that subsequent studies might examine the links between vitamin D, VDR polymorphisms, and other inflammation-related genes in larger asthmatic populations.
6.9 References for chapter 6.


37. Sharief S, Jariwala S, Kumar J, Muntner P, Melamed ML. Vitamin D levels and food and environmental allergies in the United States:


6.10. Supplementary information for Chapter 6.

6.10.1. The Allelic Discrimination plots for VDR SNPs and for IL-10 SNP.

**Figure S6.1.** The Allelic Discrimination plot for TaqI (rs731236) SNPs on Real Time PCR 7500 Fast System: a) data distribution for asthmatics and b) for control.
Figure S6.2. The Allelic Discrimination plot for rs757343 SNPs on Real Time PCR 7500 Fast System: a) data distribution for asthmatics (38 patients had CC genotype and 8 had CT) and b) for control (36 subjects had CC genotype and 9 had CT).
Figure S6.3. The Allelic Discrimination plot for rs7975232 SNPs on Real Time PCR 7500 Fast System: a) data distribution for asthmatics and b) for control.
Figure S6.4. The Allelic Discrimination plot for rs1800871 SNPs on Real Time PCR 7500 Fast System: a) data distribution for asthmatics (33 patients had GG genotype and 13 had AG) and b) for control (27 subjects had GG genotype and 12 had AG).
Table S6.1. Table of parameters analysed for TaqI (T>C) genotype before supplementation. Data shown as are mean +/- SD. * Denotes statistically significant.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>TaqI genotypes</th>
<th>P-value (ANOVA)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TT n=18</td>
<td>TC n=21</td>
</tr>
<tr>
<td>25(OH)D (nmol/L)</td>
<td>46.9 +/- 13.5</td>
<td>52.6 +/- 15.2</td>
</tr>
<tr>
<td>FEV1/FVC</td>
<td>89 +/- 9</td>
<td>92 +/- 8</td>
</tr>
<tr>
<td>FEV1(%)</td>
<td>99.2 +/- 13.8</td>
<td>102.4 +/- 15.6</td>
</tr>
<tr>
<td>FVC(%)</td>
<td>94.9 +/- 7.3</td>
<td>95 +/- 15.6</td>
</tr>
<tr>
<td>IgE (IU/mL)</td>
<td>580.5 +/- 538.7</td>
<td>527.2 +/- 737.0</td>
</tr>
<tr>
<td>ECP (ug/L)</td>
<td>51.7 +/- 49.6</td>
<td>67.7 +/- 46.1</td>
</tr>
<tr>
<td>IL-10 (pg/mL)</td>
<td>134.7 +/- 48</td>
<td>94.1 +/- 25.6</td>
</tr>
<tr>
<td>CAMP (pg/mL)</td>
<td>2220 +/- 5650</td>
<td>5320 +/- 7955</td>
</tr>
<tr>
<td>IgA (g/L)</td>
<td>1.27 +/- 0.44</td>
<td>1.18 +/- 0.40</td>
</tr>
<tr>
<td>CRP (mg/L)</td>
<td>1.07 +/- 1.27</td>
<td>1.89 +/- 3.47</td>
</tr>
<tr>
<td>Total Ca (mmol/L)</td>
<td>2.46 +/- 0.09</td>
<td>2.46 +/- 0.07</td>
</tr>
<tr>
<td>PO4 (mmol/L)</td>
<td>1.64 +/- 0.2</td>
<td>1.54 +/- 0.2</td>
</tr>
<tr>
<td>PTH (pg/mL)</td>
<td>34.29 +/- 14.83</td>
<td>34.41 +/- 13.26</td>
</tr>
<tr>
<td>ALP (U/L)</td>
<td>229.71 +/- 54.82</td>
<td>213.93 +/- 65.83</td>
</tr>
<tr>
<td>Mg (mmol/L)</td>
<td>0.94 +/- 0.07</td>
<td>0.93 +/- 0.06</td>
</tr>
<tr>
<td>WBC (x10^9/L)</td>
<td>7.61 +/- 1.35</td>
<td>9.04 +/- 2.05</td>
</tr>
<tr>
<td>EO (x10^9/L)</td>
<td>0.48 +/- 0.30</td>
<td>0.5 +/- 0.35</td>
</tr>
<tr>
<td>Lymph (x10^9/L)</td>
<td>3.01 +/- 0.8111</td>
<td>3.53 +/- 1.12</td>
</tr>
<tr>
<td>Neut (x10^9/L)</td>
<td>3.45 +/- 1.36</td>
<td>4.30 +/- 1.7</td>
</tr>
</tbody>
</table>
**Table S6.2.** Table of parameters analysed for Apal (C>T) genotype before supplementation. Data shown are mean +/- SD. * Denotes statistically significant.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Apal genotypes</th>
<th>P-value (ANOVA)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TT n=12</td>
<td>CT n=24</td>
</tr>
<tr>
<td>25(OH)D (nmol/L)</td>
<td>50.9 +/- 13.3</td>
<td>50.2 +/- 15.8</td>
</tr>
<tr>
<td>FEV1/FCV</td>
<td>89 +/- 10</td>
<td>92 +/- 8</td>
</tr>
<tr>
<td>FEV1 (%)</td>
<td>100.4 +/- 17.6</td>
<td>101.7 +/- 12</td>
</tr>
<tr>
<td>FVC (%)</td>
<td>93.5 +/- 9</td>
<td>95.7 +/- 10</td>
</tr>
<tr>
<td>IgE (IU/mL)</td>
<td>553 +/- 575</td>
<td>528 +/- 710</td>
</tr>
<tr>
<td>ECP (ug/L)</td>
<td>53 +/- 44</td>
<td>54 +/- 39</td>
</tr>
<tr>
<td>IL-10 (pg/mL)</td>
<td>154.8 +/- 45.5</td>
<td>95.3 +/- 25.4</td>
</tr>
<tr>
<td>CAMP (pg/mL)</td>
<td>984 +/- 1399</td>
<td>5248 +/- 8273</td>
</tr>
<tr>
<td>IgA (g/L)</td>
<td>1.30 +/- 0.45</td>
<td>1.20 +/- 0.40</td>
</tr>
<tr>
<td>CRP (mg/L)</td>
<td>0.9 +/- 1.2</td>
<td>1.97 +/- 3.34</td>
</tr>
<tr>
<td>Ca^2+ (mmol/L)</td>
<td>2.44 +/- 0.1</td>
<td>2.48 +/- 0.07</td>
</tr>
<tr>
<td>PO4 (mmol/L)</td>
<td>1.60 +/- 0.2</td>
<td>1.57 +/- 0.18</td>
</tr>
<tr>
<td>PTH (pg/mL)</td>
<td>31.32 +/- 17.69</td>
<td>35.04 +/- 12.81</td>
</tr>
<tr>
<td>ALP (U/L)</td>
<td>230.5 +/- 56.7</td>
<td>210.2 +/- 67.2</td>
</tr>
<tr>
<td>Mg (mmol/L)</td>
<td>0.93 +/- 0.06</td>
<td>0.93 +/- 0.06</td>
</tr>
<tr>
<td>WBC (x10^9/L)</td>
<td>7.35 +/- 1.33</td>
<td>8.8 +/- 2.04</td>
</tr>
<tr>
<td>EO (x10^9/L)</td>
<td>0.47 +/- 0.31</td>
<td>0.47 +/- 0.35</td>
</tr>
<tr>
<td>Lymph (x10^9/L)</td>
<td>2.94 +/- 0.79</td>
<td>3.40 +/- 1.1</td>
</tr>
<tr>
<td>Neut (x10^9/L)</td>
<td>3.25 +/- 1.49</td>
<td>4.21 +/- 1.54</td>
</tr>
</tbody>
</table>
Figure S6.5. Example of normal spirometry report.
Normal spirometry: FEV1/FVC ≥ 70%, FVC ≥ 80% of predicted; restrictive ventilatory pattern (RVP): FEV1/FVC ≥ 70%, FVC < 80%; obstructive ventilatory pattern (OVP): FEV1/FVC < 70%, FVC ≥ 80%; mixed ventilatory pattern (MVP): FEV1/FVC < 70%, FVC < 80%.*

Table S6.3. Haplotypes frequencies for VDR TaqI and ApaI polymorphisms.

<table>
<thead>
<tr>
<th>TaqI (SNP1)</th>
<th>Apal (SNP2)</th>
<th>Freq</th>
<th>OR (95% CI)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>T</td>
<td>C</td>
<td>0.46</td>
<td>1.00</td>
<td>---</td>
</tr>
<tr>
<td>T</td>
<td>T</td>
<td>0.26</td>
<td>40.26 (5.27 - 307.79)</td>
<td>6e-04</td>
</tr>
<tr>
<td>C</td>
<td>C</td>
<td>0.17</td>
<td>43.74 (4.87 - 393.20)</td>
<td>0.0011</td>
</tr>
<tr>
<td>C</td>
<td>T</td>
<td>0.11</td>
<td>0.53 (0.08 - 3.38)</td>
<td>0.51</td>
</tr>
</tbody>
</table>

Global haplotype association p-value: <0.0001

Data show haplotype association with response (asthma) in asthmatic cases and in control. n = total number studied.

Table S6.4. Haplotypes frequencies for VDR TaqI and ApaI polymorphisms for asthmatics (cases) and Control.

<table>
<thead>
<tr>
<th>TaqI (SNP1)</th>
<th>Apal (SNP2)</th>
<th>Total</th>
<th>Control (freq) (n = 57)</th>
<th>Cases (freq) (n = 44)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T</td>
<td>C</td>
<td>0.46</td>
<td>0.68</td>
<td>0.1287</td>
</tr>
<tr>
<td>T</td>
<td>T</td>
<td>0.26</td>
<td>0.12</td>
<td>0.4963</td>
</tr>
<tr>
<td>C</td>
<td>C</td>
<td>0.17</td>
<td>0.06</td>
<td>0.3485</td>
</tr>
<tr>
<td>C</td>
<td>T</td>
<td>0.11</td>
<td>0.14</td>
<td>0.0265</td>
</tr>
</tbody>
</table>

The haplotype analysis performed using the programme from the website: https://www.snpstats.net/snpstats/start.htm.
6.10.2. A preliminary work: A pilot study of Vitamin D Receptor TaqI and ApaI Gene Variants in adult asthma.

We have already started work on a new pilot study of the association of Vitamin D Receptor Polymorphisms with adult asthma, 25OHD level and other biomarkers of allergy, immunity and systemic inflammation.

14 adult asthmatics (7 male; 9 atopic; mean age = 36y, mean BMI = 28kg/m², 25OHD = 52nmol/L) and 56 (34 male; 6 atopic; mean age = 46y, mean BMI = 25kg/m², mean 25OHD = 54nmol/L) healthy volunteers were studied. (Table S6.5.) Although our study groups had significant differences in some parameters, as shown below, they matched for 25OHD levels. We investigated 2 VDR polymorphisms TaqI and Apal in Irish asthmatic adults.

Table S6.5. Demographics of the subjects who completed the study. Results are expressed as average (95% confidence intervals). The $p$ value indicates the level of statistical significance of differences between the groups (t test).

<table>
<thead>
<tr>
<th></th>
<th>Asthma (n = 14)</th>
<th>Control (n = 56)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td>7 male (50%)</td>
<td>34 male (61%)</td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>36 (29, 43)</td>
<td>46 (43, 49)</td>
<td>0.008*</td>
</tr>
<tr>
<td>BMI kg/m²</td>
<td>28 (25, 31)</td>
<td>25 (24, 26)</td>
<td>0.035*</td>
</tr>
<tr>
<td>25OHD nmol/L</td>
<td>52 (35, 70)</td>
<td>54 (47, 62)</td>
<td>0.48</td>
</tr>
<tr>
<td>FEV₁ % predicted</td>
<td>94.8 (80.8, 100.8)</td>
<td>109 (103.5, 114.5)</td>
<td>0.001*</td>
</tr>
<tr>
<td>IL-10 pg/mL</td>
<td>31 (14, 49)</td>
<td>125 (102, 148)</td>
<td>0.0001*</td>
</tr>
<tr>
<td>WCC x10⁹/L</td>
<td>6.2 (5.6, 6.8)</td>
<td>6.5 (6.2, 6.8)</td>
<td>0.5</td>
</tr>
<tr>
<td>Neutrophils x10⁹/L</td>
<td>3.3 (2.8, 3.8)</td>
<td>3.6 (3.3, 3.9)</td>
<td>0.41</td>
</tr>
<tr>
<td>Eosinophils x10⁹/L</td>
<td>0.2 (0.15, 0.25)</td>
<td>0.18 (0.15, 0.21)</td>
<td>0.46</td>
</tr>
<tr>
<td>hs-CRP mg/L</td>
<td>2.3 (0.6, 4.0)</td>
<td>4.3 (0.9)</td>
<td>0.59</td>
</tr>
<tr>
<td>IgE IU/ml</td>
<td>192 (66, 318)</td>
<td>81 (40, 122)</td>
<td>0.027*</td>
</tr>
</tbody>
</table>
In this study the frequencies of VDR TaqI T and C alleles were 0.54 and 0.46 in cases and 0.8 and 0.2 in controls. The genotypic frequencies of VDR TaqI were 22%, 64% and 14% in cases, and 61%, 39% and 0% in controls for TT, TC and CC genotypes respectively. For VDR Apal gene variant the frequencies of T and C alleles were 0.68 and 0.32 in cases, and 0.26 and 0.74 in controls. The genotypic frequencies of this polymorphism were 43%, 50% and 7% in cases, and 9%, 34% and 57% in controls for TT, TC and CC genotypes respectively. The alleles’ frequencies were significantly different, as is shown by 46% prevalence of C allele (TaqI) and 68% prevalence of T allele (ApaI) in asthmatic patients versus 19.6% and 26% in the controls (P = 0.006, P = <0.0001). Adults carrying the C allele for TaqI are 3.55 times more likely to develop asthma (OR = 3.55, 95% CI (1.48-8.52)) and subjects carrying the risk T allele for ApaI are 6 times more susceptible to asthma development (OR = 6.04, 95% CI (2.5 to 14.85)) than healthy individuals. (Tables S6.6. and S6.7.)

Table S6.6. Genotypic association analysis of VDR RFLPs (restriction fragment length polymorphisms) Taq1 (T>C) and Apa1(C>T) between asthmatic patients and control individuals.

<table>
<thead>
<tr>
<th>Enzyme analysis</th>
<th>Patients (14)</th>
<th>Controls (56)</th>
<th>$\chi^2$</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>TaqI Genotypes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TT</td>
<td>3 (21.4 %)</td>
<td>34 (60.7 %)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CT</td>
<td>9 (64.3%)</td>
<td>22 (39.3 %)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>2 (14.3%)</td>
<td>0 (0 %)</td>
<td>12.85</td>
<td>0.002*</td>
</tr>
<tr>
<td>Apal Genotypes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TT</td>
<td>6 (43 %)</td>
<td>5 (9 %)</td>
<td>14.9</td>
<td>0.001*</td>
</tr>
<tr>
<td>CT</td>
<td>7 (50 %)</td>
<td>19 (34 %)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>1 (7 %)</td>
<td>32 (57 %)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Data are given as numbers and percentages. *p<0.05 is considered significant.
Table S6.7.  Allelic association analysis of VDR RFLPs (restriction fragment length polymorphisms) Taq1 (T>C) and Apa1(C>T) between asthmatic patients and control individuals.

<table>
<thead>
<tr>
<th>Enzyme analysis</th>
<th>Patients (14)</th>
<th>Controls (56)</th>
<th>OR (95 % CI)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>TaqI Allelic association</td>
<td>15 (54 %)</td>
<td>90 (80.4 %)</td>
<td>3.55(1.48-8.52)</td>
<td>0.006*</td>
</tr>
<tr>
<td>ApaI Allelic association</td>
<td>19 (68 %)</td>
<td>29 (26 %)</td>
<td>6.04 (2.5 to 14.85)</td>
<td>&lt;0.0001*</td>
</tr>
</tbody>
</table>

Data are given as numbers and percentages. OR = odds ratio; 95% CI (in parentheses) * Denotes statistically significant

CT haplotype was significantly associated with asthma risk (OR 9.38 (95 % CI: 2.39 - 36.86), p = 0.002). (Tables S6.8. and S6.19.)

Table S6.8.  Haplotypes frequencies for VDR Taq1 and Apal polymorphisms for asthmatics (cases) and Control.

<table>
<thead>
<tr>
<th>Haplotype frequencies estimation</th>
<th>TaqI (SNP1)</th>
<th>Apal (SNP2)</th>
<th>Total</th>
<th>Control (freq.) Healthy adults (n = 56)</th>
<th>Cases (freq.) adults asthma (n = 14)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T</td>
<td>C</td>
<td>0.61</td>
<td>0.68</td>
<td>0.32</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>T</td>
<td>0.20</td>
<td>0.14</td>
<td>0.46</td>
<td></td>
</tr>
<tr>
<td>T</td>
<td>T</td>
<td>0.14</td>
<td>0.12</td>
<td>0.22</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>C</td>
<td>0.05</td>
<td>0.06</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>
Table S6.9. Haplotype association with response (asthma) for VDR TaqI and ApaI polymorphisms.

<table>
<thead>
<tr>
<th>TaqI (SNP1)</th>
<th>Apal (SNP2)</th>
<th>Frequency</th>
<th>OR (95% CI)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>T</td>
<td>C</td>
<td>0.6082</td>
<td>1.00</td>
<td>---</td>
</tr>
<tr>
<td>C</td>
<td>T</td>
<td>0.201</td>
<td><strong>9.38 (2.39 - 36.86)</strong></td>
<td>0.0021*</td>
</tr>
<tr>
<td>T</td>
<td>T</td>
<td>0.1418</td>
<td>2.56 (0.69 - 9.51)</td>
<td>0.16</td>
</tr>
<tr>
<td>C</td>
<td>C</td>
<td>0.049</td>
<td>0.00</td>
<td>1</td>
</tr>
</tbody>
</table>

Global haplotype association p-value: 0.0005*

Data show haplotype association with response (asthma) in asthmatic cases and in control. n = total number studied. * Denotes statistically significant.

The haplotype analysis performed using the programme from the website: [https://www.snpstats.net/snpstats/start.htm](https://www.snpstats.net/snpstats/start.htm).

We also observed that the asthmatics with only TC genotype for both polymorphisms had significantly lower FEV1% compared to controls (p<0.05). (Figures S6.6 and S6.7.)

There were no significant differences between genotypes for 25OHD level, BMI, or inflammatory biomarkers. Asthmatics with TC+CC genotypes for Apal had significantly lower IgE level (p<0.05). (Figure S6.8.)
Figure S6.6. Average FEV1% levels in asthmatic adults and healthy control with TC genotype for TaqI polymorphism. (t-test: TC vs TC between asthmatics and control, p < 0.016*).

Figure S6.7. Average FEV1% levels in asthmatic adults and healthy control with TC genotype for ApaI polymorphism. (t-test: TC vs TC between asthmatics and control, p < 0.04*).
In summary, we found that the distribution of T and C alleles and genotype frequencies varied significantly between asthmatics and controls for both polymorphisms (p value < 0.05).

Our preliminary data are promising, and they are in line with our study carried out on paediatric asthmatics in this dissertation.

Our data suggest that TaqI and ApaI polymorphisms are more common in asthmatics. It is possible that the ApaI polymorphism is associated with the atopic asthma phenotype in adults. More extensive studies are warranted to investigate the importance of these polymorphisms in asthma in Ireland, and also mechanisms by which they may influence the development and course of the disease.

**Figure S6.8.** Average IgE levels in asthmatic adults with TC+CC genotypes vs TT for Apa polymorphism. (t-test: TC+CC vs TT in asthmatics, p < 0.012*).
Chapter 7
Overall conclusions and suggestions for future research

7.1. Introduction.

This chapter consists of a summary of the work described in this dissertation, its clinical outcome, and its impact in the Irish context. Also it suggests future studies that might be carried out in this area. The background to this dissertation is the fact that, as already mentioned, asthma and VDD are highly prevalent in Ireland. The author specifically focused on VDD, because it is known that diet and a lack of sun exposure (a well-known feature of Irish life) contribute to 25OHD insufficiency in Ireland. This research has demonstrated an association between VDD and the severity of airway obstruction in adult patients suffering from moderate asthma.

Asthma follows a polygenic mode of inheritance, and genetic susceptibility factors may be due to variations in the response to vitamin D and VDD. VDR SNPs have been associated with asthma in different populations, and it has been observed that these polymorphisms may contribute to asthma risk.

In the course of this project the author established new molecular diagnostic tests for VDR’s gene polymorphisms to question the relevance of this hypothesis in the Irish context.

The underpinning research was highly interdisciplinary, involving several research centres in Galway and Dublin: the Biomnis Ireland diagnostic facility (Clinical Chemistry, Immunology, and Molecular Diagnostic Departments), the School of Chemistry at NUI Galway, the Asthma Research Centre at James Connolly Hospital, Dublin, and St. James’s Hospital, Dublin. It has addressed significant limitations of the biochemical diagnostics of asthma and related respiratory illnesses, and also the understanding of the pathophysiology and genetics of this condition.

This study combined tests which are routinely used in clinical laboratories, as well as others that were specifically set up by the author for her research on Irish asthmatic patients. For example, Biomnis Ireland is the
only provider in this country of ECP tests for the evaluation of airway inflammation. Other tests, such as VDR SNPs, are still only at the research stage. The author was the first to set up these methods for diagnostic and research application in a clinical laboratory in Ireland. She was able to link her research for this dissertation to her professional practice.

Initial work on novel diagnostic methods included the clinical application of previously developed tests by different manufacturers and their adaptation to medical and research use. Among them are 25-hydroxyvitamin D assays on Abbott Architect ci8200 (ABBOTT, Abbott Park, IL, USA); Eosinophil Cationic Protein on Phadia 250 (Phadia AB, Uppsala, Sweden); Human Interleukin-10, Vitamin D Receptor, Interleukin-17A, and Cathelicidin antimicrobial peptide tests on DS2 Automated ELISA Processing System (DYNEX Technologies, Chantilly, VA, USA); the single nucleotide polymorphisms of VDR’s and IL-10’s genes using TaqMan® SNP Genotyping Assays on Applied Biosystems Real-Time PCR-7500 Fast (Applied Biosystems, Foster City, CA, USA).

Biomnis availed of its long relationship with Abbott Diagnostic, and verification of the 25OHD method on Abbott Architect was performed when assays became obtainable. The availability of a new generation of tests enabled the author’s team to achieve a superior turnaround time, with corresponding cost effectiveness.

A grant from the IRC gave the author the opportunity to participate in clinical studies on respiratory patients in James Connolly Hospital and Tallaght Hospital, Dublin. Because of our findings of high levels of VDD in asthmatics, Irish respiratory clinicians familiar with our work are able to carry out appropriate treatment with vitamin D supplementation in selected patients. Some of these clinicians now collaborate with Biomnis in scientific studies of VDD and in treating it with supplementation in asthma and other related illnesses, specifically sleep apnoea syndrome.

(Two papers on this subject are provided in appendices.)

Based on this study, Biomnis Ireland now provides a range of new diagnostic markers for respiratory conditions. The fact that these conditions include asthma is particularly significant in the Irish context.
7.2. Conclusion.

A new range of biochemical markers was successfully set up by the author for diagnostic and research use in the Biomnis Ireland laboratory. The verification of a new generation of the 25OHD assay was performed and confirmed to be useful, cost-effective, reliable, accurate, rapid, and a good indicator for 25OHD measurement. Since 2016 a new generation of 25OHD has been INAB accredited to ISO 15189 on Abbott Architect in Biomnis Ireland.

In our research we found a high level of obesity and a negative association between 25OHD levels and body mass index in our healthy Irish cohort but not in asthmatics.

In the study of adult asthma, it emerged that low serum 25OHD levels are positively associated with severity of airway obstruction in Irish Caucasian adults who suffer from moderate persistent asthma. This association was independent of some of the commoner inflammatory pathways involved in the pathogenesis of asthma.

In the study of vitamin D supplementation in uncontrolled paediatric asthmatics we did not confirm positive outcomes in our patients, as some other researchers had done. We did confirm earlier findings of a negative association between 25OHD levels and total IgE in paediatric asthma.

In the course of this research the author set up a range of new genetic testings in her clinical laboratory, including VDR’s and IL-10’s gene polymorphisms. We have shown an association of TaqI and Apal SNPs of the VDR gene with the susceptibility to asthma in cohorts of Irish patients. Novel findings in our study of paediatric asthma were that patients with TC for TaqI, and CC and CT genotypes for Apal had a significantly low level of IL-10 and increased WBC (neutrophils in particular).

In addition, we were the first to observe that TT and CC haplotypes for both polymorphisms were significantly associated with asthma in Irish children, and that they could be potential biomarkers for paediatric asthma.
In the case of our study of TaqI and ApaI SNPs in adult asthma, we revealed that CT haplotype was significantly associated with asthma. Our haplotype analysis in paediatric and adult asthma led us to believe that the disease pathophysiology and its molecular mechanisms differ between adults and children, and probably require different approaches in diagnosis and treatment.

In the case of genotypes studies in adult asthma our novel observation was that the TT genotype for ApaI SNP was associated with a significant increase in IgE in our patients.

In summary, our data suggest that TaqI and ApaI polymorphisms are more common in paediatric and adult asthma. It is possible that the ApaI polymorphism is associated with the atopic asthma phenotype in adult patients. Based on our research we suggest the possibility of using VDR SNPs tests as potential biomarkers for asthma susceptibility and asthma risk in Irish asthmatics.

7.3. Impact: altered clinical practice / application of new diagnostic tests.

Research on the mechanisms of the aetiology and pathobiology of asthma undertaken in Biomnis Ireland, under the direction of Dr. Yury Rochev at the School of Chemistry at NUI Galway and the Asthma Research Centre at James Connolly Hospital Dublin, has resulted in the application of new diagnostic methods available to clinicians.

As mentioned briefly in Chapter 1, Biomnis is a leading independent sector clinical pathology diagnostic laboratory that uses the latest techniques and methodologies, and it is accredited by INAB to the ISO 15189 standard. Biomnis Ireland’s research collaboration with clinical consultants and NUI Galway, combined with financial sponsorship from the Irish Research Council (IRC), has strengthened its already-existing involvement in the research and development market for new diagnostic markers in Irish health care, specifically for respiratory illnesses. It processes approximately 1.5 million tests per annum, and it performs referral work for nearly every HSE hospital in Ireland.
A strong partnership with NUI Galway, resulting from the author’s research, and also a partnership with clinicians, has led to an extension of existing laboratory tests for medical use, thereby expanding Irish expertise and jobs. As has been pointed out above, the author has been able to link creatively her research and her practical work. These developments represent (a) the creation of a new research and development sector for the company, and (b) the adoption of new diagnostic methods into its portfolio.

7.3.1. **Significance and consequences.**

In collaboration with Abbott Diagnostic and St. James’s Hospital, extensive verification of a new vitamin D method was performed in Biomnis and successfully accredited, and this is now used for medical testing. An extended range of novel biomarkers is now also available for clinicians. A few hundred patients from three hospitals in Dublin were tested with new genetic and immunological methods that were set up for research use, and important medical conclusions were drawn. In the course of research on this project we have established links between vitamin D levels and (a) obesity in healthy adults and (b) lung function in asthmatics; also we have emphasised the importance of Vitamin D receptor polymorphisms in asthma and the possibility of using VDR SNPs tests as biomarkers for asthma susceptibility.

7.3.2. **Attribution.**

This work was led by NUI Galway PhD student Katrina Hutchinson MD, the Senior Clinical Biochemist in Biomnis Ireland, directed by her academic supervisor Dr. Yury Rochev from NUI Galway School of Chemistry, guided by her employment mentor, Medical Director and Consultant Chemical Pathologist Dr. Mike Louw, and by Consultant Respiratory Physician Dr. John Faul, and sponsored by an employment-based PhD award from the IRC.
7.3.3. Impact description.

Biomnis Ireland was established in 1991 and for many years it has been the leading independent provider of medical laboratory testing services throughout Ireland. The company offers its clients accredited testing services which use the most sophisticated diagnostic equipment and techniques in all fields of medical pathology.

In 2013 a new Employment Based Postgraduate Scholarship (€96,000) by the Irish Research Council was awarded to the author at NUI Galway for her PhD project: “A Source of New Diagnostic Techniques for Asthma in Ireland”.

 Until recently the Irish branch of Biomnis lacked research facilities and expertise, in comparison to its much larger French counterpart. But due to this grant it has been able to exploit the dramatic expansion of research opportunities in Ireland. The grant has also enabled the author and other members of the Biomnis staff to be trained in new techniques – specifically in molecular diagnostic. During the past few years other grants have been secured – for example, €7,300 from Tallaght Hospital for the study of paediatric asthma.

Due to the impact of our research projects with NUI Galway, James Connolly, St. James’s and Tallaght hospitals, Biomnis is now deeply involved in research and development and in setting up new diagnostic methods. Our research is recognised both nationally and internationally.

Diagnostic companies, including Abbott, Roche Diagnostics and Thermo Fisher Scientific have given the author extensive support in the setting of new methodologies, and in training and expertise.

The work carried out for this dissertation forms a central part of linking research with the business development of Biomnis, and thereby it strengthens links between academia, hospitals, and private laboratories. Governments, insurance agencies and others who provide and pay for healthcare have become more aware of the value of diagnostics, such as early detection and prevention. They appreciate that these can lead to more effective treatment, improved health, and a reduction in the cost of
healthcare. The “Source of New Diagnostic Techniques for Asthma in Ireland” project was directed specifically towards achieving these aims. From a wide range of standpoints – primarily the nation’s health, but also its finances, economic expansion, the creation of jobs, and the development of new areas of expertise in clinical diagnostics in Ireland – research that would help contain the spread of asthma should be given a high priority. Biomnis Ireland plays a disproportionately large role in this effort, and the author provides the company’s principal link with academia. Our research in the area shows promising lines of development. Due to this project, the new range of diagnostic markers for respiratory conditions is now available in Biomnis Ireland. A few hundred patients have already benefited from our new methodologies. We plan to build on our achievement and to secure more support from government agencies and other sources to further develop our research.
7.3.4. Evidence of impact.

corroborates the claims made in the case study.

2. Oral presentation “A randomised, double-blind, placebo-controlled of
   vitamin D for Irish children with asthma: baseline data”.

   http://www.biomnis.ie/utilities/news/15-08
   06/Biomnis_Ireland_Vitamin_D_Sleep_Apnoea_Poster_at_the_IFCC-EFLM_EuroMedLab_2015_Congress.aspx

4. Presentation at the Biodetection & Biosensors meeting October 2016
   in Cambridge, UK.
   http://www.biomnis.ie/Libraries/Scientific_Resources_Documents/Vitamin_D_Receptor_VDR_Polymorphisms_in_the_Uncontrolled_Paediatric_Asthma.sflb.ashx

corroborates the link between underpinning research and impact.

6. Oral presentation “Verification of Abbott 25-OH-Vitamin D assays on
   the Architect System” at Labcon conference March 2017 in Galway.

7.4. Further studies.

Our research has shown the importance (and also the lack of
understanding) of the molecular mechanisms that are involved in
paediatric and adult asthma.

Our observation of the connection between airway obstruction and low
25OHD levels in Irish adult asthmatics reinforces the argument that
25OHD levels could be important in the pathogenesis of asthma. More
extensive studies are necessary to understand the pathophysiological
mechanism of this relationship.
On the basis of our work on paediatric asthmatic patients, further research is needed to understand vitamin D’s role in paediatric asthma. Above all, it will be necessary to explore when vitamin D supplementation will benefit uncontrolled paediatric asthmatic patients. Larger and carefully-selected patient groups (with specific phenotypes) will be essential in any future studies.

More biomarkers for research and diagnostic need to be established, including pro- and anti-inflammatory cytokines, different transcription factors, and genetic testing (including testing for various SNPs that are involved in the development of asthma). The development of more biomarkers which could be tested on our patients might assist us in understanding the processes whereby TaqI, Apal and other polymorphisms are associated with the production of various cytokines. This could help explain the molecular mechanisms regulating the production of IgE and other immunoglobulins in different chronic illnesses. Further research is warranted to confirm our findings on the importance of VDR gene polymorphisms in asthma in Ireland. There is also a need to investigate the ways in which they may influence the development and course of the disease, and also the possibility of using them as biomarkers for asthma susceptibility.
APPENDIX 1 : PUBLICATIONS & CONFERENCE PROCEEDINGS

First author Publications


Submitted articles


Other Publications


Conference proceedings


1. Serum Vitamin D Is Significantly Inversely Associated with Disease Severity in Caucasian Adults with Obstructive Sleep Apnea Syndrome.

2. Vitamin D Improves Selected Metabolic Parameters but not Neuropsychological or Quality of Life Indices in OSA: A Pilot Study.
Serum Vitamin D Is Significantly Inversely Associated with Disease Severity in Caucasian Adults with Obstructive Sleep Apnea Syndrome

Conor P. Kerley, BS;1,2 Katrina Hutchinson, MD;1,3 Kenneth Bolger;2 Aisling McGowan;1 John Faul, MD;1 Liam Cormican, MD1

1Respiratory and Sleep Diagnostics Department, Connolly Hospital, Blanchardstown, Dublin, Ireland; 2School of Medicine and Medical Sciences, University College Dublin, Belfield, Dublin, Ireland; 3Bonnins Ireland, Dublin, Ireland; *NCBES, National University of Ireland, Galway, Ireland

Study Objectives: To evaluate vitamin D (25(OH)D) levels in obstructive sleep apnea syndrome (OSAS) and possible relationships to OSAS severity, sleepiness, lung function, nocturnal heart rate (HR), and body composition. We also aimed to compare the 25(OH)D status of a subset of OSAS patients compared to controls matched for important determinants of both OSAS and vitamin D deficiency (VDD).

Methods: This was a cross-sectional study conducted at an urban, clinical sleep medicine outpatient center. We recruited newly diagnosed, Caucasian adults who had recently undergone nocturnal polysomnography. We compared body mass index (BMI), body composition (bioelectrical impedance analysis), neck circumference, sleepiness (Epworth Sleepiness Scale), lung function, and vitamin D status (serum 25-hydroxyvitamin D (25(OH)D)) across OSAS severity categories and non-OSAS subjects. Next, using a case-control design, we compared measures of serum 25(OH)D from OSAS cases to non-OSAS controls who were matched for age, gender, skin pigmentation, sleepiness, season, and BMI.

Results: 106 adults (77 male; median age = 54.5; median BMI = 34.3 kg/m²) resident in Dublin, Ireland (latitude 53°N) were recruited and categorized as non-OSAS or mild/moderate/severe OSAS. 98% of OSAS cases had insufficient 25(OH)D (< 75 nmol/L), including 72% with VDD (< 50 nmol/L). 25(OH)D levels decreased with OSAS severity (P = 0.003). 25(OH)D was inversely correlated with BMI, percent body fat, AHI, and nocturnal HR. Subsequent multivariate regression analysis revealed that 25(OH)D was independently associated with both AHI (P = 0.016) and nocturnal HR (P = 0.0419). Our separate case-control study revealed that 25(OH)D was significantly lower in OSAS cases than matched, non-OSAS subjects (P = 0.001).

Conclusions: We observed widespread vitamin D deficiency and insufficiency in a Caucasian, OSAS population. There were significant, independent, inverse relationships between 25(OH)D and AHI as well as nocturnal HR, a known cardiovascular risk factor. Further, 25(OH)D was significantly lower in OSAS cases compared to matched, non-OSAS subjects. We provide evidence that 25(OH)D and OSAS are related, but the role, if any, of replenishment has not been investigated.

Keywords: vitamin D, obstructive sleep apnea, obesity, apnea-hypopnea index, nutrition, diet, sunshine

Citation: Kerley CP, Hutchinson K, Bolger K, McGowan A, Faul J, Cormican L. Serum vitamin D is significantly inversely associated with disease severity in Caucasian adults with obstructive sleep apnea syndrome. SLEEP 2016;39(2):293–300.

Significance
We demonstrate that vitamin D deficiency/insufficiency is almost universal in a cohort with obstructive sleep apnea syndrome (OSAS). Further, we observed significant, independent, inverse relationships between vitamin D levels and both OSAS severity and nocturnal heart rate, a known cardiovascular risk factor. Although previous studies have found a link between vitamin D levels and OSAS, this could be due to confounding. We compared relationships between vitamin D levels and OSAS severity. We also compared the difference between vitamin D levels in OSAS cases and controls matched for important determinants of OSAS and vitamin D, including BMI, age, gender and sleepiness. Prospective and/or randomized trials are warranted to fully assess the effect, if any, of vitamin D in OSAS.

INTRODUCTION
Obstructive sleep apnea syndrome (OSAS) represents a major public health problem.1 One major risk factor for OSAS is obesity, which is reported in up to 70% of cases. The incidence and/or severity of OSAS also appears related to ethnicity, winter season, and lack of physical activity.2–4 Additionally, OSAS has been associated with multiple metabolic disturbances including excess systemic inflammation, hyperglycemia, hyperlipidemia, cardiovascular disease, and increased bone loss.5,6

Vitamin D receptors and enzymes have been discovered in most cell types and tissues leading to the realization that vitamin D exerts non-skeletal, pleiotropic effects in multiple organs.7 However, vitamin D deficiency (VDD) remains highly prevalent worldwide.8 Similar to OSAS, VDD is associated with adiposity, dark skin pigmentation, winter season, and physical inactivity. The non-skeletal consequences of VDD are not well understood; however, VDD has been associated with similar metabolic disturbances as OSAS, including elevated systemic inflammation, impaired glucose metabolism, dyslipidemia, and bone deformities as well as many of the comorbidities associated with OSAS, including cardiovascular disease.9

Heart rate (HR) parameters provide important information regarding cardiovascular regulatory mechanisms and are mainly affected by the sympathetic nervous system (SNS). One of the most important effects of OSAS is SNS activation,8 and numerous studies have noted HR perturbations in OSAS.9 Elevated HR has been associated with low 25-hydroxyvitamin D (25(OH)D) in both cross-sectional10 and prospective11 studies. However, there is a lack of studies regarding HR and 25(OH)D in OSAS.

Recently there has been interest in the idea that vitamin D could be important for sleep disorders.12–14 Several cross-sectional studies have reported a high prevalence of VDD in OSAS,15–18 which is more pronounced in severe OSAS19,20 and associated with abnormal glucose metabolism.21,22 These studies suggest a possible link between OSAS and VDD but are limited by the lack of controls13 and the inclusion of controls who had undergone polysomnography16 or were not matched for important determinants of OSAS and VDD such as BMI, age, gender, and degree of sleepiness.20

In this cross-sectional study, we examined the prevalence of vitamin D insufficiency and deficiency among urban adults.
with newly diagnosed, untreated OSAS. We also wanted to assess relationships between 25(OH)D and OSAS severity, sleepiness, lung function, nocturnal HR, and body composition. Finally, we wanted to compare vitamin D levels from selected OSAS cases with non-OSAS subjects matched for important determinants of OSAS and VDD.

METHODS

Subjects/Study Population
We conducted a cross-sectional study among consecutive Caucasian, urban adults who had been referred to the Sleep Clinics of Connolly Hospital, Blanchardstown, Dublin, Ireland. All subjects underwent overnight polysomnography (PSG) and were recruited after signing an institutional ethics committee approved consent form. We excluded participants currently using multivitamin or vitamin D supplements or medications that modulate vitamin D metabolism, and those with disorders known to influence vitamin D metabolism/absorption.

Sleep Indices
The presence or absence of OSAS was based on the results of full, in-house, nocturnal PSG (SomnoScreen Plus), which were conducted and analyzed at the Sleep Laboratory of Connolly Hospital. PSG data were analyzed manually by a respiratory physiologist using computer software (Somnomedics Domino Software) according to Irish Sleep Society/AASM guidelines. An obstructive apnea was defined as a drop in airflow to ≤90% of baseline for ≥10s as recorded by the oronasal sensor with continued respiratory effort. A hypopnea was defined as a drop in airflow ≥30% from baseline as recorded with the nasal cannula for ≥10s accompanied by ≥3% oxygen desaturation. A decrease in SpO2 ≥3% was considered to represent an oxygen desaturation. The average number of apneas and hypopneas per hour of sleep was defined as the apnea-hypopnea index (AHI). The diagnosis and severity of OSAS was based on the definitions recommended by the American Academy of Sleep Medicine as follows: non-OSAS (AHI < 5), mild OSAS (5 ≤ AHI < 15), moderate OSAS (15 ≥ AHI < 30) and severe OSAS (AHI ≥30). The non-OSAS group comprised subjects with snoring or insomnia but without OSAS as confirmed by PSG. Consenting and eligible, non-OSAS subjects for the case-control study were matched in a paired fashion to individual OSAS cases in terms of important determinants of both OSAS and VDD, including age, gender, skin pigmentation, sleepiness, season, and BMI. We evaluated daytime sleepiness with the Epworth Sleepiness Scale (ESS), which is a widely used, validated tool.

Pulmonary Function Testing
Values for percentage of predicted forced expiratory volume in 1 second (FEV1%) and percentage of predicted forced expiratory volume (FVC%) were obtained according to ATS/ERS recommended techniques by an experienced respiratoryphysiologist. The results were compared with predicted normal values from the European Community for Coal and Steel/European Respiratory Society.

Anthropometry
Body mass index (BMI) was calculated from measured height and weight. Neck circumference was measured by a trained dietician (CPK). We estimated total fat mass (FM), fat free mass (FFM), and trunk fat with bioelectrical impedance analysis (BIA) using the Tanita Segmental Body Composition Analyzer, Model BC-418 (Tanita Corporation, Tokyo, Japan), which correlates highly with DEXA measurements (r = 0.82–0.87).28

Vitamin D Analysis
Blood samples were collected, centrifuged, aliquoted, and frozen to −80°C, which does not affect 25(OH)D. Circulating levels of total 25(OH)D are considered the most reliable measure of overall vitamin D status since they reflect vitamin D2 + D3 contributions from all sources.27 25(OH)D was measured using competitive chemiluminescence immunoassays (DiaSorin, Dietzenbach, Germany) with inter-assay coefficient of variation of 6.8%. Internal quality control was determined using kit controls of 2 different concentrations.

Values were reported as nmol/L. Here we use the 2011 Endocrine Society guidelines to define serum 25(OH)D status.22 whereby 25(OH)D < 50 nmol/L equates to VDD, while < 75 nmol/L equates to vitamin D insufficiency (VDI) and > 75 nmol/L denotes vitamin D sufficiency (VDS).

Skin pigmentation, which is a major influencer of vitamin D photosynthesis,7 was assessed using the 6 point Fitzpatrick scale.21

Statistical Methods
The Shapiro-Wilk test was performed to check the data for normality. The majority of variables were not normally distributed. Therefore we utilized Kruskal-Wallis, one-way, nonparametric, ANOVA tests to detect differences between non-OSAS subjects and OSAS severities and if differences were detected, the Mann-Whitney test was performed for pairwise comparisons. We used Spearman rho derived from bivariate correlation analysis to assess relevant correlations. Associations between variables on univariate regression analysis with P values < 0.05 were entered into multivariable models to determine the independent correlations to AHI and HR. Paired t-tests were used to compare differences between OSAS cases and matched, non-OSAS subjects. All analyses were performed using SPSS statistical software (version 20.0. Armonk, NY: IBM Corp). Results were expressed as median ± interquartile range (IQR). All P values reported are 2-tailed with statistical significance set at < 0.05.

RESULTS
We recruited 106 untreated Caucasian adults who had recently undergone nocturnal PSG. Table 1 displays all parameters and results of the non-OSAS, control group, and the OSAS subjects grouped by severity. Severe OSAS was overrepresented compared to mild and moderate OSAS, reflecting the typical demographics of our clinic population. Similarly, males were overrepresented compared to females, reflecting OSAS epidemiology. The age profile of the 4 groups was similar. Patients with severe OSAS group had significantly lower FEV1% and FVC% than other groups, as well as a more adverse anthropometric profile (higher BMI, percentage body fat, FM, trunk fat, neck circumference, but significantly lower FFM) and higher...
Vitamin D and OSAS—Kerley et al.

...nocturnal mean HR. Median ESS scores were only slightly elevated in severe OSAS compared to other groups but the difference was statistically significant. As expected PSG variables increased across OSAS severity classes (AHI, total apnea number, total hypopnea number, desaturation index). Although the severe OSAS group had less total sleep time and less deep sleep than other groups, there was no difference in measures of nocturnal mean HR.

There were statistically significant, inverse correlations between 25(OH)D and anthropometric variables including BMI, total percentage body fat, and total FM (Figure 1D). Further, there were statistically significant, inverse correlations between 25(OH)D and OSAS variables including AHI (Figure 1B), desaturation index, time spent below 90% oxygen saturation, and average nocturnal HR (Figure 1C). Conversely, there was a positive correlation between 25(OH)D and baseline oxygen saturation. There was no association between 25(OH)D and age, FFM, neck circumference, lung function, ESS score, or PSG variables not listed in Table 2.

Many factors influencing 25(OH)D status, AHI, and HR are related, and correlations between variables may be due to confounding. Therefore, we conducted regression analysis to determine if there were independent association between variables of interest.

### Table 1—Demographic and physiologic characteristics of subjects by OSAS severity.

<table>
<thead>
<tr>
<th></th>
<th>Non-OSAS</th>
<th>Mild</th>
<th>Moderate</th>
<th>Severe</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>31</td>
<td>22</td>
<td>18</td>
<td>35</td>
</tr>
<tr>
<td>Age</td>
<td>53 ± 19</td>
<td>54 ± 19</td>
<td>57 ± 17</td>
<td>55.5 ± 17</td>
</tr>
<tr>
<td>Male, n (%)</td>
<td>16 (50)</td>
<td>17 (77)</td>
<td>12 (67)</td>
<td>28 (80)</td>
</tr>
<tr>
<td>Current smoker</td>
<td>1 (3)</td>
<td>6 (27)</td>
<td>5 (28)</td>
<td>12 (34)</td>
</tr>
<tr>
<td>Ex-smoker</td>
<td>5 (16)</td>
<td>0 (0)</td>
<td>1 (6)</td>
<td>2 (6)</td>
</tr>
<tr>
<td>FEV1%</td>
<td>94 ± 21</td>
<td>100 ± 26</td>
<td>98 ± 15</td>
<td>83 ± 25</td>
</tr>
<tr>
<td>FVC%</td>
<td>99 ± 21</td>
<td>105.5 ± 23.3</td>
<td>99 ± 11.3</td>
<td>87 ± 29</td>
</tr>
<tr>
<td>FEV1:FVC%</td>
<td>81 ± 7</td>
<td>82 ± 11</td>
<td>81 ± 5</td>
<td>79 ± 10</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>32 ± 8</td>
<td>31 ± 8</td>
<td>33 ± 7</td>
<td>39 ± 8</td>
</tr>
<tr>
<td>Fat%</td>
<td>34 ± 12</td>
<td>32 ± 9</td>
<td>33 ± 14</td>
<td>49 ± 28</td>
</tr>
<tr>
<td>FM (kg)</td>
<td>30 ± 13</td>
<td>33 ± 12</td>
<td>30 ± 14</td>
<td>71 ± 12</td>
</tr>
<tr>
<td>Trunk fat (kg)</td>
<td>37 ± 10</td>
<td>35 ± 11</td>
<td>34 ± 9</td>
<td>42 ± 9</td>
</tr>
<tr>
<td>NC</td>
<td>41 ± 5</td>
<td>39 ± 1</td>
<td>41 ± 3</td>
<td>48 ± 7</td>
</tr>
<tr>
<td>FFM (kg)</td>
<td>58 ± 14</td>
<td>69 ± 14</td>
<td>58 ± 20</td>
<td>52 ± 9</td>
</tr>
<tr>
<td>ESS</td>
<td>12 ± 6</td>
<td>13 ± 7</td>
<td>2 ± 5</td>
<td>13 ± 9</td>
</tr>
<tr>
<td>EDS, n (%)</td>
<td>24 (77)</td>
<td>14 (64)</td>
<td>11 (61)</td>
<td>23 (66)</td>
</tr>
<tr>
<td>AHI</td>
<td>2 ± 2</td>
<td>10 ± 4</td>
<td>22 ± 9</td>
<td>59 ± 26</td>
</tr>
<tr>
<td>Total apnea number</td>
<td>0.3 ± 0.9</td>
<td>8 ± 17</td>
<td>22 ± 26</td>
<td>107 ± 183</td>
</tr>
<tr>
<td>Total hypopnea number</td>
<td>2 ± 2</td>
<td>54 ± 29</td>
<td>129 ± 65</td>
<td>196 ± 186</td>
</tr>
<tr>
<td>Baseline O₂</td>
<td>94 ± 2</td>
<td>94 ± 2</td>
<td>94 ± 2</td>
<td>93 ± 3</td>
</tr>
<tr>
<td>Time below 90% O₂</td>
<td>0 ± 0</td>
<td>1 ± 5</td>
<td>7 ± 24</td>
<td>38 ± 6</td>
</tr>
<tr>
<td>Min O₂</td>
<td>90 ± 2</td>
<td>87 ± 4</td>
<td>84 ± 8</td>
<td>74 ± 13</td>
</tr>
<tr>
<td>Desaturation index</td>
<td>2 ± 0.2</td>
<td>10 ± 5</td>
<td>20 ± 19</td>
<td>51 ± 29</td>
</tr>
<tr>
<td>Mean nocturnal HR</td>
<td>60 ± 15</td>
<td>58 ± 10</td>
<td>64 ± 10</td>
<td>68 ± 17</td>
</tr>
<tr>
<td>TST (min)</td>
<td>432 ± 62</td>
<td>418 ± 70</td>
<td>424 ± 79</td>
<td>411 ± 138</td>
</tr>
<tr>
<td>Snore index</td>
<td>78 ± 115</td>
<td>73 ± 83</td>
<td>165 ± 492</td>
<td>329 ± 304</td>
</tr>
<tr>
<td>25(OH)D (nmol/L)</td>
<td>60 ± 33</td>
<td>40 ± 22</td>
<td>3 ± 30</td>
<td>37 ± 26</td>
</tr>
<tr>
<td>VDD (%)</td>
<td>32</td>
<td>61</td>
<td>61</td>
<td>82</td>
</tr>
<tr>
<td>VDI (%)</td>
<td>50</td>
<td>39</td>
<td>39</td>
<td>15</td>
</tr>
<tr>
<td>VDS (%)</td>
<td>18</td>
<td>0</td>
<td>0</td>
<td>3</td>
</tr>
</tbody>
</table>

Data are displayed as median ± IQR. *P values derived from independent samples Kruskal-Wallis test. 25(OH)D, 25-hydroxyvitamin D; AHI, apnea-hypopnea index; BMI, body mass index; EDS, excessive daytime sleepiness; ESS, Epworth sleepiness scale; FEV1, forced expiratory volume in 1 second; FFM, fat free mass; FM, fat mass; FVC, forced vital capacity; HR, heart rate; LLNS, lifelong non-smoker; NC, neck circumference; O₂, oxygen; PLM, periodic leg movements; REM, rapid eye movement; TST, total sleep time; VDD, vitamin D deficiency; VDI, vitamin D insufficiency; VDS, vitamin D sufficiency.
ESS score (β = 0.22; P = 0.028), 25(OH)D (β = 0.34; P = 0.001), and several measures of adiposity were significantly associated with AHI in univariate regression analysis. Therefore we selected the most significant adiposity association (BMI) and entered BMI, ESS score, and 25(OH)D level into a multivariate model. These variables explained 56.7% of the variance in AHI in this cohort. However, only BMI (β = 0.42; P < 0.0001) and 25(OH)D (β = −0.22; P = 0.016) were significant independent predictors of AHI, while ESS score was of borderline significance (β = −0.19; P = 0.065).

Similarly, 25(OH)D level (β = 0.35; P < 0.0001) as well as several measures of both adiposity and sleep were significantly associated with HR in univariate regression analysis. We selected the most significant associations—BMI for adiposity and AHI for sleep variables—and entered these values into a multivariate model which revealed that these variables explained 46.4% of the variance in mean nocturnal HR in this cohort. However, only 25(OH)D level could be considered a significant independent predictor of HR (β = −0.24; P = 0.019), while BMI was of borderline significance (β = 0.21; P = 0.057). AHI was not associated with HR after adjusting for 25(OH)D level and BMI (β = 0.17; P = 0.14).

**DISCUSSION**

In the present study, we investigated the relation of 25(OH)D levels to OSAS indices, lung function, mean nocturnal HR, and body composition. 25(OH)D levels were highest in non-OSAS cases vs. non-OSAS subjects and the proportion of subjects with VDD was markedly higher in severe OSAS compared to non-OSAS subjects.

**Case-Control Study**

To further assess if there was any relationship between OSAS and 25(OH)D, we wanted to examine 25(OH)D levels between a separate cohort of OSAS cases and non-OSAS subjects after matching for potential confounders: age, gender, skin pigmentation, sleepiness, season, and BMI (Table 3).

Nocturnal HR was significantly higher in OSAS vs. matched, non-OSAS subjects, while FVC% was significantly lower. Additionally, 25(OH)D levels were significantly lower in OSAS cases vs. non-OSAS subjects and the proportion of subjects with VDD was markedly higher in severe OSAS compared to non-OSAS subjects.

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subjects and decreased with increasing OSAS severity. Of the 75 OSAS cases, there was only one subject who had sufficient vitamin D (1.5%), while 6 of 31 non-OSAS subjects (18%) were VDS. Further, the proportion of subjects with VDD was markedly higher in severe OSAS (82%) compared to mild OSAS, moderate OSAS, and non-OSAS subjects (61%, 61%, and 32%, respectively). Our results agree with previous cross-sectional reports of widespread VDD in OSAS, whereby levels were lowest in severe OSAS.15–18

We conducted this study at high latitude in a country with limited sun exposure (Dublin, Ireland, 53°N). It is perhaps not surprising that a mostly obese cohort with OSAS living in such an environment exhibited decreased vitamin D levels. However, in a recent cohort study carried out in a representative Irish population, it was observed that 40.1% were VDD and 75.6% were VDS.24 In this context, we report a very high prevalence of VDD (71%) and VDI (99%) in OSAS cases, which is particularly pronounced in severe OSAS cases (82% VDD).

25(OH)D was inversely associated with numerous OSAS parameters as documented with PSG, including AHI, total apnea number, total hypopnea number, baseline oxygen concentration as well as time spent below 90% oxygen saturation.

### Table 2—Correlations between 25(OH)D and anthropometric, demographic, and polysomnographic measures.

<table>
<thead>
<tr>
<th></th>
<th>Spearman rho</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMI</td>
<td>-0.27</td>
<td>0.01</td>
</tr>
<tr>
<td>Fat%</td>
<td>-0.22</td>
<td>0.048</td>
</tr>
<tr>
<td>FM</td>
<td>-0.18</td>
<td>0.09</td>
</tr>
<tr>
<td>Trunk fat (kg)</td>
<td>0.27</td>
<td>0.04</td>
</tr>
<tr>
<td>AHI</td>
<td>-0.40</td>
<td>0.0001</td>
</tr>
<tr>
<td>Total apnea</td>
<td>-0.25</td>
<td>0.01</td>
</tr>
<tr>
<td>Total hypopnea</td>
<td>-0.43</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Baseline O2</td>
<td>0.22</td>
<td>0.03</td>
</tr>
<tr>
<td>Time below 90% O2</td>
<td>-0.28</td>
<td>0.009</td>
</tr>
<tr>
<td>Min O2</td>
<td>0.38</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Desaturation Index</td>
<td>-0.41</td>
<td>0.0001</td>
</tr>
<tr>
<td>Mean nocturnal HR</td>
<td>-0.33</td>
<td>0.001</td>
</tr>
<tr>
<td>Snore index</td>
<td>-0.28</td>
<td>0.007</td>
</tr>
</tbody>
</table>

Spearman rho and P value derived from bivariate correlation analysis. AHI, apnea-hypopnea index; BMI, body mass index; FM, fat mass; HR, heart rate; O2, oxygen.

### Table 3—Characteristics of OSAS subjects vs. matched, non-OSAS subjects.

<table>
<thead>
<tr>
<th></th>
<th>Non-OSAS</th>
<th>OSAS</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>18</td>
<td>18</td>
<td>–</td>
</tr>
<tr>
<td>Age</td>
<td>55 ± 9</td>
<td>57 ± 12</td>
<td>0.38</td>
</tr>
<tr>
<td>Male, n (%)</td>
<td>12 (67)</td>
<td>12 (67)</td>
<td>–</td>
</tr>
<tr>
<td>AHI</td>
<td>2 ± 1</td>
<td>36 ± 21</td>
<td>&lt;0.0000001</td>
</tr>
<tr>
<td>PSG Diagnosis, n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Snoring</td>
<td>9 (50)</td>
<td>9 (50)</td>
<td>–</td>
</tr>
<tr>
<td>Insomnia</td>
<td>9 (50)</td>
<td>9 (50)</td>
<td>–</td>
</tr>
<tr>
<td>ESS</td>
<td>11 ± 6</td>
<td>12 ± 6</td>
<td>0.29</td>
</tr>
<tr>
<td>EDS n (%)</td>
<td>13</td>
<td>10</td>
<td>–</td>
</tr>
<tr>
<td>Average HR</td>
<td>58 ± 19.4</td>
<td>70 ± 20</td>
<td>0.045</td>
</tr>
<tr>
<td>BMI kg/m²</td>
<td>33 ± 7</td>
<td>34 ± 7</td>
<td>0.34</td>
</tr>
<tr>
<td>Fat%</td>
<td>37 ± 9</td>
<td>37 ± 11</td>
<td>0.27</td>
</tr>
<tr>
<td>FM</td>
<td>36 ± 14</td>
<td>38 ± 11</td>
<td>0.33</td>
</tr>
<tr>
<td>FFM</td>
<td>60 ± 11</td>
<td>60 ± 19</td>
<td>0.22</td>
</tr>
<tr>
<td>NC</td>
<td>40 ± 0.6</td>
<td>42 ± 5</td>
<td>0.07</td>
</tr>
<tr>
<td>FEV %</td>
<td>94 ± 12</td>
<td>90 ± 20</td>
<td>0.17</td>
</tr>
<tr>
<td>FVC %</td>
<td>103 ± 15</td>
<td>96 ± 16</td>
<td>0.09</td>
</tr>
<tr>
<td>FEV/FVC %</td>
<td>76 ± 11</td>
<td>78 ± 9</td>
<td>0.46</td>
</tr>
<tr>
<td>Skin type</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Skin type 1, n (%)</td>
<td>1 (6)</td>
<td>1 (6)</td>
<td>–</td>
</tr>
<tr>
<td>Skin type 2, n (%)</td>
<td>16 (88)</td>
<td>16 (88)</td>
<td>–</td>
</tr>
<tr>
<td>Skin type 3, n (%)</td>
<td>1 (6)</td>
<td>1 (6)</td>
<td>–</td>
</tr>
<tr>
<td>25(OH)D (nmol/L)</td>
<td>51 ± 25</td>
<td>36 ± 15</td>
<td>0.02</td>
</tr>
<tr>
<td>VDD, n (%)</td>
<td>8 (44)</td>
<td>16 (89)</td>
<td>–</td>
</tr>
<tr>
<td>VDI, n (%)</td>
<td>7 (39)</td>
<td>2 (11)</td>
<td>–</td>
</tr>
<tr>
<td>VDS, n (%)</td>
<td>3 (17)</td>
<td>0</td>
<td>–</td>
</tr>
</tbody>
</table>

Data are displayed as mean ± SD. P values derived from paired t-tests. ESS, Epworth sleepiness scale; AHI, apnea-hypopnea index; HR, heart rate; BMI, body mass index; NC, neck circumference; LLNS, lifelong non-smoker; FEV1, forced expiratory volume in 1 second; FVC, forced vital capacity; 25(OH)D, 25-hydroxyvitamin D.
minimal oxygen concentration, and desaturation index. The inverse relationship between 25(OH)D and AHI was still present after multivariate analysis suggesting an independent association. An inverse correlation between 25(OH)D and various OSAS measures on PSG has been reported previously by some authors\textsuperscript{3,4,10} but not others.\textsuperscript{11,12} However, in one of these studies,\textsuperscript{13} 25(OH)D was associated with OSAS according to multivariate logistic regression analysis. It is noteworthy that the studies that did not report an association between 25(OH)D and AHI reported higher mean 25(OH)D levels compared to the 25(OH)D levels we and others\textsuperscript{14} observed among OSAS cohorts. In this context, it is possible that VDD is more strongly associated with OSAS compared to VDI.

An interesting and novel finding of this study is the independent, inverse relationship between 25(OH)D and mean nocturnal HR. This is consistent with previous cross-sectional\textsuperscript{10} and prospective\textsuperscript{11} studies in non-OSAS adults. Further, low 25(OH)D levels are associated with cardiac autonomic dysfunction.\textsuperscript{22} In this context, it is noteworthy that previous trials have demonstrated that vitamin D can significantly reduce HR.\textsuperscript{23,24} Elevated HR has been associated with metabolic syndrome, type 2 diabetes, as well as CVD and mortality.\textsuperscript{25} The possibility that vitamin D repletion offers opportunity to reduce HR and morbidity in OSAS cannot be discounted. However, our preliminary findings require confirmation.

Here, 25(OH)D was inversely associated with BMI and percentage body fat, and there was a trend to an inverse relationship with FM. These observations are consistent with previous reports in OSAS.\textsuperscript{3,4,10,13,14} It is well established that there is an inverse association between 25(OH)D and BMI. There was a suggestion that low 25(OH)D could lead to weight gain,\textsuperscript{26} potentially through elevated parathyroid hormone (PTH) promoting fat accumulation through increased calcium influx into adipocytes, which would theoretically enhance lipogenesis and inhibit lipolysis.\textsuperscript{27} Support for this theory comes from murine work showing that vitamin D regulates energy expenditure and enhances fatty acid oxidation.\textsuperscript{28} However, most vitamin D supplementation trials have demonstrated little impact on FM,\textsuperscript{29} and a recent bi-directional Mendelian randomization analysis of multiple cohorts concluded that higher BMI leads to lower 25(OH)D and any effects of lower 25(OH)D increasing BMI are likely minor.\textsuperscript{30} Indeed, there is consensus that the bioavailability of vitamin D (either from dietary sources or ultraviolet irradiation) is truly decreased in obesity. Although it has traditionally been assumed that sequestration of 25(OH)D by adipocytes explained this association, it has recently been demonstrated that volumetric dilution offers a superior explanation.\textsuperscript{31}

We failed to demonstrate an association between sleepiness and 25(OH)D. This is in contrast to cross-sectional reports among varied ethnicities.\textsuperscript{32,33} The discrepancy regarding associations between sleepiness and 25(OH)D may be race dependent.\textsuperscript{34} Nevertheless, our results do not support a relationship between vitamin D levels and sleepiness, at least not in Caucasians with untreated OSAS. This raises the intriguing possibility that race modifies the relationship between vitamin D level and excessive daytime sleepiness. Similarly, although epidemiologic data demonstrate a positive association between 25(OH)D level and pulmonary function in healthy adults and those with asthma or COPD,\textsuperscript{35} we did not observe an association here. The lack of association might be explained by the presence of OSAS and/or excess adiposity.

Our subsequent analysis of OSAS cases and non-OSAS subjects matched for important determinants of 25(OH)D and OSAS revealed that 25(OH)D levels were significantly decreased in OSAS. This observation is consistent with most previous reports,\textsuperscript{36-38,39} but not others.\textsuperscript{40}

In the present study, all patients and non-OSAS subjects were Caucasian and resided in Dublin, Ireland. All of the 25(OH)D assays were conducted using the same batch of commercial assays and were performed concomitantly by the same biochemist in the same laboratory, thereby reducing biochemical variability. The strength of the case-control analysis was in comparing vitamin D levels in patients with OSAS to subjects without OSAS confirmed by PSG who were matched for important determinants of both VDD and OSAS.

This study has some limitations. We included a relatively small sample size and did not assess sun exposure, dietary habits or metabolic biomarkers, such as PTH, lipids, or glycemic indices. Further, we did not assess polymorphisms in the vitamin D metabolism pathway (vitamin D receptor, 25-hydroxylase and 1α-hydroxylase). It is well known that variants in this pathway are associated with perturbed vitamin D metabolism and clinical outcomes. The cross-sectional nature of our study and other studies relating vitamin D to OSAS makes it impossible to infer that VDD predisposes to OSAS or vice versa.

In our cohort, BMI did not fully explain the association between AHI and 25(OH)D. This is supported by the largest report of 25(OH)D levels in OSAS to date, which reported no association with obesity.\textsuperscript{15} Therefore, an alternative explanation regarding low 25(OH)D in OSAS is needed. It is possible that 25(OH)D level reflects healthy behaviors such as outdoor exercise or fish consumption. However, there is an idea that OSAS is an inflammatory disorder.\textsuperscript{3} Therefore, it is possible the VDD either predisposes to or exacerbates OSAS through an upregulation of inflammatory pathways. It is also plausible that VDD may predispose to OSAS through mediation by myopathy, inflammatory rinitis, and/or tonsillar hypertrophy as first suggested by McCarty and colleagues.\textsuperscript{32} On the other hand, it is possible that the chronic low-grade inflammation accompanying untreated OSAS degrades vitamin D stores. 25(OH)D may be rapidly depleted by acute stress leading to the suggestion that 25(OH)D acts as an acute phase reagent.\textsuperscript{37} This hypothesis is further supported by a recent report demonstrating markedly increased 25(OH)D levels after only 7 days of CPAP use in male OSAS subjects.\textsuperscript{38}

VDD has been associated with similar metabolic disturbances as OSAS, including elevated systemic inflammation, impaired glucose metabolism, dyslipidemia, and bone deformities, as well as many of the comorbidities associated with OSAS, including cardiovascular disease.\textsuperscript{3} Specific to OSAS, previous studies have demonstrated that 25(OH)D levels were inversely correlated with multiple metabolic parameters including HbA1c, HOMA-IR, fasting insulin, fasting glucose, total cholesterol, and triglyceride levels, as well as incidence of insulin resistance, type 2 diabetes, and metabolic syndrome.\textsuperscript{39,40} Further there is interventional data suggesting that vitamin D
supplementation may have benefits regarding inflammation, 
glycemic indices, and lipids.  
Although, effects of vitamin D supplementation are inconsistent and controversial, these observations raise the intriguing possibility that reduced bioavailability and activity of 25(OH)D in clinical settings such as obesity or OSAS may facilitate the emergence of insulin resistance, systematic inflammation, dyslipidemia, and other OSAS-related morbidities.

CONCLUSION
We report a high prevalence of VDD in OSAS and that 25(OH)D supplementation are warranted to determine the effect, if any, on core symptoms of OSAS and related metabolic disturbances (e.g. inflammation, hyperglycemia, hyperlipidemia).

ABBREVIATIONS
25(OH)D, 25-hydroxyvitamin D  
AHI, apnea-hypopnea index  
BM, body mass index  
EDS, excessive daytime sleepiness  
ESS, Epworth sleepiness scale  
FEV1%, percentage of predicted forced expiratory volume in 1 second  
FVC%, percentage of predicted 
FFM, fat free mass  
FM, fat mass  
FVC, forced vital capacity  
HR, heart rate  
LLNS, lifelong non-smoker  
NC, neck circumference  
O2, oxygen  
OSAS, obstructive sleep apnea syndrome  
PLM, periodic leg movements  
PSG, polysomnography  
PTh, parathyroid hormone  
REM, rapid eye movement  
TST, total sleep time  
VDD, vitamin D deficiency  
VDI, vitamin D insufficient  
VDS, vitamin D sufficient

REFERENCES
Vitamin D Improves Selected Metabolic Parameters but not Neuropsychological Quality of Life Indices in OSA: A Pilot Study

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Study Objectives: Our group and others have reported a high rate of vitamin D deficiency in obstructive sleep apnea (OSA), where vitamin D levels (25(OH)D) correlate negatively with OSA severity and some of its associated metabolic alterations. Data regarding vitamin D supplementation in OSA are lacking. We wanted to evaluate the effect of vitamin D3 supplementation on OSA symptoms and metabolic parameters.

Methods: We conducted a pilot, double-blind, randomized, placebo-controlled trial of daily supplementation with 4,000 IU vitamin D3 (D3) or placebo (PL). We studied 19 Caucasian adults (14 male, mean age 55.7 years, mean body mass index [BMI] 30.2 kg/m2) with OSA. Fifteen patients were stable on continuous positive airways pressure (CPAP) therapy, whereas four were CPAP naïve. Assessments were completed at baseline and after 15 weeks of supplementation. Outcome included sleepiness (Epworth Sleepiness Scale), quality of life (Sleep Apnea Quality of Life Inventory), fatigue (fatigue severity scale) and neuropsychological function (trail making test and Connor’s Continuous Performance Test II). In addition, we assessed biochemical indices of vitamin D status (25(OH)D, calcium), inflammation (high sensitivity C-reactive protein, and lipoprotein-associated phospholipase A2), lipids (total cholesterol [low-density and high-density lipoprotein]) and glycemic indices (fasting glucose, oral glucose tolerance test).

Results: There was no change in BMI, medication, or CPAP usage. Although there was no change in neuropsychological or quality of life indices, we observed a significant increase in 25(OH)D (p = 0.00001) and significant decreases in both low-density lipoprotein (p = 0.04) and lipoprotein-associated phospholipase A2 (p = 0.037) as well as trends toward decreased fasting glucose (p = 0.02) and increased high-density lipoprotein (p = 0.07) in the D3 group compared to PL.

Conclusions: Vitamin D3 supplementation increased vitamin D levels and decreased metabolic markers compared to placebo. Larger trials are required.

Keywords: inflammation; lipids; obstructive sleep apnea; vitamin D

INTRODUCTION

Obstructive sleep apnea (OSA) is strongly associated with obesity, which is reported in up to 70% of cases. OSA incidence and/or severity is also related to ethnicity,1,2 winter season,3 and lack of physical activity.4 Additionally, OSA has been associated with multiple metabolic disturbances including excess systemic inflammation, hyperglycemia, hyperlipidemia, cardiovascular disease, and increased bone loss.5

Vitamin D could, at least partially, mediate these relationships. Indeed, recently there has been interest in the idea that vitamin D could be important for sleep disorders.6-8 A recent study suggested that the association between lower 25(OH)D and OSA was largely confounded by larger BMI and neck circumference.9 In contrast, our group and others10-14 have reported a high rate of vitamin D deficiency in OSA, where vitamin D levels (25(OH)D) are lower in severe OSA10,11,13 and inversely correlate with some of its associated metabolic alterations, including abnormal glucose metabolism11,12 as well as nocturnal heart rate.13 Vitamin D receptors are located systemically and recent advances have the potential of vitamin D to influence a wide variety of organs.15

There is preliminary evidence that vitamin D supplementation may attenuate some metabolic disturbances through anti-inflammatory, anti-hyperglycemic, and anti-hyperlipidemic roles, although these potential effects are inconsistent and controversial.

A recent comprehensive review regarding the link between vitamin D metabolism and sleep medicine concluded that controlled studies are needed to further explore the relationship...
between inadequate vitamin D and daytime neurocognitive impairment, OSA and associated morbidity, particularly cardiovascular disease. 

Further, vitamin D supplementation may have roles regarding sleep quality, 

sleepiness, fatigue, poor mood, and neuropsychological function. In this context, it is noteworthy that a 2010 case study reported resolution of hypersomnia following vitamin D supplementation in a vitamin D deficient, African American woman.

To our knowledge, there are no reports regarding the effect of vitamin D supplementation in OSA. In this pilot study, we examined the effect of short-term vitamin D supplementation among urban adults with OSA, in terms of quality of life, neuropsychological function, and cardiovascular biomarkers.

METHODS

Subjects/Study Population

This pilot intervention study was conducted at Connolly Hospital, Blanchardstown (Dublin 15, Ireland, latitude, 53°N) after institutional review board approval.

Inclusion criteria included previous OSA diagnosis by polysomnography (PSG), Caucasian race, and stable medical regimen including with or without CPAP for 6 mo or longer. Exclusion criteria included mixed sleep apnea, history of coronary artery disease or diabetes or hypoglycemic agents and syndromes/therapy known to interfere with vitamin D metabolism, including inflammatory bowel disease, as well as renal/liver diseases and use of anticonvulsants, multivitamins, or vitamin D. At baseline, informed consent was obtained.

Study Design

This pilot study was designed as a parallel, randomized, double-blind, placebo-controlled trial and involved two clinic visits (Figure 1). Recruitment occurred between November 2013 and January 2014, with follow-up 15 w later. All assessments were conducted at both baseline and follow-up in an identical manner at the same time of morning with the same researcher (CPK). Further, all assessments were conducted in the same windowless room with identical lighting and temperature conditions. Following completion of day 1 presupplementation assessments, subjects were randomized to vitamin D3 (D3) or placebo (PL) groups using an online randomization program.

Assessments

PSG was conducted on all study participants as previously described. At each time point, subjects reported to our research facility after an overnight fast, had blood drawn, and consumed 75 g of glucose. Next, a battery of self-report questionnaires was completed in the same order followed by neuropsychological testing and a second blood sample was taken 2 h after glucose ingestion (Figure 1). For those established on CPAP, compliance data were downloaded at baseline and endpoint. For those not established on CPAP, therapy was not initiated during this study.

The battery of self-report questionnaires included validated questionnaires regarding sleep quality sleepiness (Epworth Sleepiness Scale), quality of life (Sleep Apnea Quality of Life Inventory), fatigue (Fatigue Severity Scale), and mood (Beck Depression Inventory).

The neuropsychological testing included trail-making forms A and B and two subscales of the Repeatable Battery for the Assessment of Neuropsychological Status (R-BANS) (semantic fluency and coding). Subjects also completed the computerized Conner's Continuous Performance test. Additionally, all participants completed the Wechsler test of adult reading at baseline only.

Biochemistry

Venous blood was analyzed locally for full blood count, glucose, hemoglobin A1c, and lipids (total cholesterol, low-density lipoprotein [LDL], high-density lipoprotein [HDL], triglycerides). Additional blood was centrifuged with isolated serum aliquoted and frozen to −80°C until required for further analysis.

Circulating levels of total 25-hydroxyvitamin D (25(OH)D) are considered the most reliable measure of overall vitamin D status because they reflect vitamin D2 + D3, contributions from all sources (i.e., diet, supplements, and sun exposure. 25(OH)
D was measured using the Architect 25(OH)D chemiluminescent microparticle immunoassay (CMIA) with interassay coefficient of variation of 3.5% and functional sensitivity less than 20 nmol/L. 25(OH)D, parathyroid hormone, insulin, high sensitivity C-reactive protein, and lipoprotein-associated phospholipase A2 (Lp-PLA2) were analyzed on Abbott Architect ci8200 instrument (Abbott Laboratories, Abbott Park, IL, USA). The interassay coefficient of variation for these assays ranged between 1% and 5.6%. Lp-PLA2 activity was assessed using an enzyme-linked immunoassay (PLAC test,DiaDexus, Inc., San Francisco, CA, USA). The range of detection is 10 – 400 nmol/min/mL, with a clinical sensitivity less than 10 nmol/min/mL. Low Lp-PLA2 activity is indicated with values of 151 nmol/min/mL or less, with medium values falling between 152–194 nmol/min/mL and high values indicated by a result of 195 nmol/min/mL or higher.

Supplements
At baseline, each subject was provided with a consecutively numbered bottle containing 120 softgel capsules of vitamin D3 (cholecalciferol) or identical placebo in a double-blind, randomized fashion. A physician not involved in data gathering (JF) generated the allocation sequence, while a dietitian and nutrition researcher enrolled subjects (CPK). Subjects were instructed to ingest one softgel daily with food. The vitamin D dose was 4,000 IU daily, which corresponds to the tolerable upper intake limit in Europe.

Diet, supplement use, and particularly exposure to ultraviolet B radiation contribute to vitamin D status. Therefore, we conducted this trial at high altitude during winter season when skin vitamin D synthesis is minimal. Further, we advised recruits not to change dietary/supplemental behaviors during the trial. We assessed behavior relating to vitamin D status with the VIDSun questionnaire at baseline and endpoint, which contains questions relating to BMI, skin type, sun exposure, and supplement use. Supplement compliance was assessed with a diary and by counting the remaining softgels at the follow-up visit.

Statistical Analysis
For this pilot study we did not conduct a sample size calculation. To our knowledge, this pilot study represents the only vitamin D supplementation trial in OSA and therefore we recruited a convenience sample to preliminary assess the effect, if any, of vitamin D3 supplementation in OSA. We used two-tailed, unpaired t-tests to compare baseline demographics between the two groups. We used two-tailed, paired t-tests to compare changes within groups and two-tailed, unpaired t-tests to compare changes between groups. All analyses were performed using a software package (SPSS, version 18; SPSS, Inc., Chicago, IL). Results were expressed as mean ± standard deviation. We defined statistical significance as p < 0.05.

RESULTS
We screened 97 adults attending sleep clinics of Connolly Hospital. Of these 97, 40 were eligible and 26 provided consent. These 26 subjects were recruited and completed baseline assessments. Of the 26, there were 7 dropouts for miscellaneous reasons (Figure 1). Baseline demographics for the 19 subjects who completed the study are presented in Table 1. The mean pretreatment 25(OH)D level for all patients was 37.2 nmol/L (range, 14.5 to 86.5 nmol/L) and there was no significant difference in baseline 25(OH)D levels between the PL and D3 groups. Further, there were no significant differences regarding age, BMI, sleepiness (Egbert Sleepiness Scale score), education level, or reading score between the D3 and PL group; however, there were more males in the PL group and more CPAP users in the D3 group.

Throughout the study, there was no change in medication or CPAP compliance in either group. In addition, there was no change in dietary habit or use of medication, alcohol, tobacco, or caffeine. Compliance with the supplements was high in both groups (93%) and there were no adverse effects.

Results before and after supplementation and comparisons between groups are displayed in Table 2 (biochemical indices), Table 3 (neuropsychological indices), and Table 4 (self-reported questionnaire scores).

There was a significant increase in 25(OH)D, which was accompanied by a significant decrease in LDL and Lp-PLA2 in the vitamin D3 group compared to placebo. Additionally, there was a trend toward decreased fasting glucose and increased HDL in the D3 group (Table 2).

We did not detect any differences in neuropsychological indices (Table 3) or quality of life scores (Table 4), except significantly improved fatigue in the vitamin D group.

DISCUSSION
To our knowledge, this is the first report of vitamin D supplementation in OSA. We investigated the effect of vitamin D3 supplementation (4,000 IU/day) for 15 w compared to placebo among a small sample (n = 19) of urban, Caucasian adults with definite OSA as diagnosed by PSG. Consistent with previous studies, we observed a high rate of vitamin D deficiency (89%) in our small OSA cohort. In addition to significantly increased 25(OH)D in the vitamin D supplementation group (n = 10), there were significant reductions in both LDL and Lp-PLA2 compared to placebo. Additionally, there was a trend toward decreased fasting glucose and increased HDL in the D3 group.

Interest in the potential role of vitamin D in sleep disorders has grown recently. We and others have observed widespread vitamin D OSAs. Interestingly, 25(OH)D levels have been shown to be inversely correlated with OSA severity and with metabolic alterations associated with OSA, including abnormal glucose metabolism and nocturnal heart rate.

A 2014 meta-analysis concluded that “Patients with OSA appear to have increased dyslipidemia (high total cholesterol, LDL, TG, and low HDL).” Further, a large 2015 cross-sectional study demonstrated an independent association between OSA and elevated LDL. The association between OSA and dyslipidemia may mediate some of the excess cardiovascular morbidity and mortality risk associated with OSA. Further, a
2014 mendelian randomization study concluded that interventions that increased HDL may have an effect on coronary artery disease, but that interventions that decrease LDL have a stronger effect on coronary artery disease. CPAP represents the current gold standard treatment option for OSA and results in numerous biochemical and physiological benefits. However, the effect of CPAP on lipidemia is controversial. Two 2014 meta-analyses provided conflicting evidence. One meta-analysis concluded that “treatment for OSA seems to improve dyslipidemia (decrease in total cholesterol and LDL, and increase in HDL).” However, a second meta-analysis concluded that “CPAP did not alter TG, LDL, or HDL levels, suggesting that CPAP may have no clinically important effect on lipid metabolism.” Therefore, non-CPAP approaches to lipid lowering in OSA are warranted. Although the association between vitamin D and cholesterol profiles is controversial, previous reports have demonstrated significantly negative correlations between serum LDL cholesterol and 25(OH)D. We provide evidence that vitamin D supplementation can modestly but significantly decrease serum LDL while maintaining serum HDL concentration in OSA over a 15 w period with no change in lifestyle or dietary intake. There was only a single subject on lipid-lowering therapy (Atorvastatin) in this pilot study. Interestingly, vitamin D supplementation decreased both total and LDL cholesterol in this subject (−0.3 and −0.44 mmol, respectively) despite concurrent lipid-lowering therapy, which can be expected to dilute any benefit.

Lp-PLA2 is a biomarker that may be viewed as a potential link between the pathogenic effects of oxidized LDL cholesterol and plaque vulnerability. Although there is a lack of data regarding Lp-PLA2 and OSA, a recent cross-sectional study of 50 male Turkish subjects with newly diagnosed OSA demonstrated a moderate linear relationship between arousal index and Lp-PLA2 levels. Because the arousal index is an important index of sleep fragmentation and the restorative quality of sleep, this association may contribute to the increased cardiovascular risk association with frequent arousal in OSA. Further, several epidemiology studies have shown an association between Lp-PLA2 and both cardiovascular and cerebrovascular events. We are not aware of any reports linking vitamin D and Lp-PLA2. The relationship between vitamin D and inflammation has been controversial. However, in vitro evidence suggests that vitamin D has potent anti-inflammatory properties. Further, there are reports of decreased production of several proinflammatory markers including tumor necrosis factor-α, interferon γ, and interleukins 2,12,17, and 2,1 but increased production of anti-inflammatory interleukin-10 has been reported with vitamin D supplementation in humans. Here, we observed a significant 19-point decrease in Lp-PLA2 after vitamin D supplementation compared to placebo.
### Table 2—Changes in biochemical indices.

<table>
<thead>
<tr>
<th></th>
<th>Placebo</th>
<th>Vitamin D</th>
<th>p*</th>
</tr>
</thead>
<tbody>
<tr>
<td>25(OH)D (nmol/L)</td>
<td>Baseline: 41.4 ± 22</td>
<td>Endpoint: 43.0 ± 21.4</td>
<td>Change: 1.6</td>
</tr>
<tr>
<td></td>
<td>(18.9 to 86.5)</td>
<td>(20.2 to 83.9)</td>
<td></td>
</tr>
<tr>
<td>Fasting glucose</td>
<td>Baseline: 5.4 ± 1.1</td>
<td>Endpoint: 5.9 ± 1.9</td>
<td>Change: 0.5</td>
</tr>
<tr>
<td></td>
<td>(4.3 to 7.3)</td>
<td>(4.6 to 10.8)</td>
<td></td>
</tr>
<tr>
<td>2 h blood glucose</td>
<td>Baseline: 7.8 ± 3.3</td>
<td>Endpoint: 7.3 ± 2.7</td>
<td>Change: -0.5</td>
</tr>
<tr>
<td></td>
<td>(4.8 to 13.2)</td>
<td>(4.8 to 12.4)</td>
<td></td>
</tr>
<tr>
<td>Glucose excursion</td>
<td>Baseline: 1.5 ± 3.6</td>
<td>Endpoint: 0.6 ± 4.7</td>
<td>Change: -0.9</td>
</tr>
<tr>
<td></td>
<td>(~6.0 to 6.3)</td>
<td>(~10.8 to 5.7)</td>
<td></td>
</tr>
<tr>
<td>HbA1c</td>
<td>Baseline: 40.3 ± 8.7</td>
<td>Endpoint: 38.4 ± 8.9</td>
<td>Change: -1.9</td>
</tr>
<tr>
<td></td>
<td>(31.0 to 57.0)</td>
<td>(30.0 to 51.0)</td>
<td></td>
</tr>
<tr>
<td>Insulin</td>
<td>Baseline: 19.3 ± 20.9</td>
<td>Endpoint: 13.0 ± 10.4</td>
<td>Change: -6.3</td>
</tr>
<tr>
<td></td>
<td>(6.9 to 69.0)</td>
<td>(3.6 to 31.8)</td>
<td></td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>Baseline: 4.4 ± 1.1</td>
<td>Endpoint: 4.2 ± 0.9</td>
<td>Change: -0.2</td>
</tr>
<tr>
<td></td>
<td>(3.1 to 6.3)</td>
<td>(3.3 to 5.9)</td>
<td></td>
</tr>
<tr>
<td>LDL</td>
<td>Baseline: 2.4 ± 0.8</td>
<td>Endpoint: 2.5 ± 0.7</td>
<td>Change: 0.1</td>
</tr>
<tr>
<td></td>
<td>(1.4 to 3.9)</td>
<td>(1.7 to 3.9)</td>
<td></td>
</tr>
<tr>
<td>HDL</td>
<td>Baseline: 1.2 ± 0.2</td>
<td>Endpoint: 1.1 ± 0.1</td>
<td>Change: -0.1</td>
</tr>
<tr>
<td></td>
<td>(0.9 to 1.7)</td>
<td>(0.9 to 1.3)</td>
<td></td>
</tr>
<tr>
<td>CRP</td>
<td>Baseline: 2.7 ± 2.7</td>
<td>Endpoint: 2.5 ± 2.2</td>
<td>Change: -0.2</td>
</tr>
<tr>
<td></td>
<td>(0.3 to 8.4)</td>
<td>(0.1 to 7.0)</td>
<td></td>
</tr>
<tr>
<td>Lp-PLA2</td>
<td>Baseline: 173.0 ± 33.6</td>
<td>Endpoint: 172.0 ± 34.7</td>
<td>Change: -1.0</td>
</tr>
<tr>
<td></td>
<td>(100.0 to 207.0)</td>
<td>(119.0 to 207.0)</td>
<td></td>
</tr>
</tbody>
</table>

Data are presented as mean ± standard deviation (range). *p* values derived from one-tailed, unpaired t-tests comparing the change after placebo to change after vitamin D. 25(OH)D = 25-hydroxyvitamin D; CRP = C-reactive protein; HbA1c = glycated haemoglobin; HDL = high-density lipoprotein; LDL = low-density lipoprotein; Lp-PLA2 = Lipoprotein-associated phospholipase A2; PTH = parathyroid hormone; TAG = triglycerides.

### Table 3—Changes in neuropsychological indices.

<table>
<thead>
<tr>
<th></th>
<th>Placebo</th>
<th>Vitamin D</th>
<th>p*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Semantic fluency</td>
<td>Baseline: 17 ± 3</td>
<td>Endpoint: 17 ± 34</td>
<td>Change: 0</td>
</tr>
<tr>
<td></td>
<td>(12 to 22)</td>
<td>(11 to 22)</td>
<td></td>
</tr>
<tr>
<td>Coding</td>
<td>Baseline: 35 ± 16</td>
<td>Endpoint: 41 ± 13</td>
<td>Change: 6</td>
</tr>
<tr>
<td></td>
<td>(5 to 65)</td>
<td>(24 to 66)</td>
<td></td>
</tr>
<tr>
<td>Trail making A (sec)</td>
<td>Baseline: 35 ± 16</td>
<td>Endpoint: 35 ± 18</td>
<td>Change: 0</td>
</tr>
<tr>
<td></td>
<td>(22 to 72)</td>
<td>(16 to 60)</td>
<td></td>
</tr>
<tr>
<td>Trail making B (sec)</td>
<td>Baseline: 89 ± 53</td>
<td>Endpoint: 101 ± 52</td>
<td>Change: 11</td>
</tr>
<tr>
<td></td>
<td>(33 to 209)</td>
<td>(22 to 184)</td>
<td></td>
</tr>
<tr>
<td>CPT II Omission T-score</td>
<td>Baseline: 71 ± 53</td>
<td>Endpoint: 71 ± 45</td>
<td>Change: 0</td>
</tr>
<tr>
<td></td>
<td>(42 to 209)</td>
<td>(42 to 161)</td>
<td></td>
</tr>
<tr>
<td>CPT II Omission percentile</td>
<td>Baseline: 61 ± 32</td>
<td>Endpoint: 65 ± 34</td>
<td>Change: 4</td>
</tr>
<tr>
<td></td>
<td>(22 to 99)</td>
<td>(22 to 99)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(38 to 76)</td>
<td>(33 to 59)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(13 to 99)</td>
<td>(5 to 83)</td>
<td></td>
</tr>
</tbody>
</table>

Data are presented as mean ± standard deviation (range). *p* values derived from one-tailed, unpaired t-tests comparing the change after placebo to change after vitamin D. CPT II = Conner’s Continuous Performance Test.

Patients with OSA demonstrate cognitive dysfunction both subjectively and objectively.15 A recent comprehensive review concluded that controlled studies are needed to further explore the relation between inadequate vitamin D and daytime neurocognitive impairment in OSA.7 There is much interest in the potential of vitamin D regarding cognition, with a recent meta-analysis demonstrating that low 25(OH)D predicts executive dysfunctions, especially on mental shifting, information updating, and processing speed.18 It is has been suggested that hypoxemia contributes more to cognitive dysfunction in OSA.
than frequent arousals or daytime sleepiness.36 However, this suggestion is controversial. Considering that 90% of the vitamin D supplementation group were CPAP users, any potential benefit of vitamin D supplementation in OSA could have been diluted. Therefore, although we performed very detailed neuropsychological assessments and observed no cognitive benefit here, our small sample and the use of CPAP mean that this pilot study cannot rule out a potential benefit of vitamin D supplementation in OSA.

Our pilot study has several strengths. We utilized the gold standard study design and assessed objective, subjective, and biochemical markers of OSA. We studied a well-characterized group of urban, Caucasian adults with definite OSA. The placebo and vitamin D groups were well matched at baseline with no significant differences in demographics. In the current study, all patients were Caucasian and resided in Dublin, Ireland. All of the 25(OH)D assays were conducted using the same batch of commercial assays and were performed concomitantly by the same biochemist in the same laboratory, thereby reducing biochemical variability. Vitamin D trials can be influenced by a number of factors, including fluctuations in sun exposure and hence 25(OH)D levels, variable quality of vitamin D assays, compliance with the intervention, and provision of inadequate vitamin D supplementation doses. To overcome these factors, we conducted this trial over the winter season at high latitude (53°N) when vitamin D photosynthesis is minimal. Although we purposely did not restrict nonprotocol dietary or supplemental vitamin D intake, we did ask that such behaviors were not altered during the trial. Identical vitamin D behaviors were evident in both groups throughout the trial as confirmed with VIDSun scores. Compliance, as assessed by diary data and capsule counts, was high (93%). We utilized a moderate-high dose of vitamin D3 (4,000 IU/day). In unison with our trial design and high compliance with the supplements, serum 25(OH)D increased significantly in the vitamin D3 group, but did not change in the placebo group.

Our pilot study also has several important limitations. Although there was no difference in 25(OH)D levels at baseline, the mean 25(OH)D was higher in the placebo group. Twenty-two percent of subjects were vitamin D sufficient at baseline, which is of note because supplementation appears most beneficial to those with vitamin D deficiency. Further, as expected all recruits in the intervention arm had increases in 25(OH)D but three of the placebo group (33%) also experienced increases in 25(OH)D (+10 to +22 nmol/L). Although we recruited an exclusively Caucasian sample, the sample was very small (n = 19) and heterogeneous in terms of CPAP usage. However, we ensured that CPAP was neither commenced nor discontinued throughout the trial. Further, we downloaded CPAP compliance data at baseline and endpoint, observing the CPAP usage did not change and ensuring that CPAP was carefully controlled during the study. An additional limitation is the lack of a power calculation and primary endpoint. However, the value of this pilot is to act as a hypothesis-generating basis for future work. It is possible that the statistical significance of our observations would be increased with a large sample size and a more homogeneous population.

**CONCLUSIONS**

In conjunction with a significant increase in 25(OH)D levels, this pilot study suggests that vitamin D supplementation has the potential to improve relevant biomarkers of cardiometabolic health in OSA. It can be hypothesized that this effect may translate into clinical benefit over the long term due to decreased morbidity and mortality. Vitamin D replenishment warrants further investigation as an adjunct therapeutic strategy in OSA.

**ABBREVIATIONS**

25(OH)D, 25-hydroxyvitamin D  
AHI, apnea-hypopnea index  
BDI, Beck depression inventory  
BMI, body mass index  
CAD, coronary artery disease

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**Table 4—Changes in self-reported questionnaire score.**

<table>
<thead>
<tr>
<th>Questionnaire</th>
<th>Placebo</th>
<th>Vitamin D</th>
<th>p*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ESS (0 to 24)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>Endpoint</td>
<td>Mean Change</td>
<td>Baseline</td>
</tr>
<tr>
<td>10 ± 6</td>
<td>7 ± 5</td>
<td>-3</td>
<td>11 ± 5</td>
</tr>
<tr>
<td>(1 to 19)</td>
<td>(0 to 16)</td>
<td></td>
<td>(3 to 19)</td>
</tr>
<tr>
<td><strong>FSS (0 to 63)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>Endpoint</td>
<td>Mean Change</td>
<td>Baseline</td>
</tr>
<tr>
<td>46 ± 7</td>
<td>34 ± 8</td>
<td>-10</td>
<td>53 ± 7</td>
</tr>
<tr>
<td>(29 to 58)</td>
<td>(0 to 52)</td>
<td></td>
<td>(39 to 62)</td>
</tr>
<tr>
<td><strong>SAQI total score (0 to 7)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>Endpoint</td>
<td>Mean Change</td>
<td>Baseline</td>
</tr>
<tr>
<td>0.5 ± 0.2</td>
<td>0.5 ± 0.2</td>
<td>0</td>
<td>1.5 ± 0.3</td>
</tr>
<tr>
<td>(0.3 to 0.7)</td>
<td>(0.2 to 0.7)</td>
<td></td>
<td>(1.1 to 2)</td>
</tr>
<tr>
<td><strong>VIDSun score (0 to 7)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>Endpoint</td>
<td>Mean Change</td>
<td>Baseline</td>
</tr>
<tr>
<td>2 ± 1</td>
<td>2 ± 1</td>
<td>0</td>
<td>3 ± 1</td>
</tr>
<tr>
<td>(1 to 4)</td>
<td>(1 to 4)</td>
<td></td>
<td>(2 to 6)</td>
</tr>
<tr>
<td><strong>BDI (0 to 63)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>Endpoint</td>
<td>Mean Change</td>
<td>Baseline</td>
</tr>
<tr>
<td>16 ± 10</td>
<td>12 ± 9</td>
<td>-4</td>
<td>14 ± 7</td>
</tr>
<tr>
<td>(1 to 37)</td>
<td>(0 to 28)</td>
<td></td>
<td>(2 to 23)</td>
</tr>
</tbody>
</table>

Data are presented as mean ± standard deviation (range). *p values derived from two-tailed, unpaired t-tests comparing the change after placebo to change after vitamin D3. BDI = Beck Depression Inventory; ESS = Epworth Sleepiness Scale; FSS = Fatigue Severity Scale; SAQI = sleep apnea quality of life inventory; VIDSun = vitamin D + Sun questionnaire.
CPT II, Conner’s Continuous Performance Test
CRP, C-reactive protein
ESS, Epworth Sleepiness Scale
HbA1c, glycated haemoglobin
HDL, high density lipoprotein
LDL, low density lipoprotein
LLNS, lifelong non-smoker
LP-PLA2, lipoprotein-associated phospholipase A2
OSA, obstructive sleep apnoea
PSG, polysomnography
PTH, parathyroid hormone
SAQLI, sleep apnea quality of life inventory
TAG, triacylglycerol
VDD, vitamin D deficiency
VDSun, vitamin D + Sun questionnaire
WTAR, Wechsler test of adult reading

REFERENCES

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