School of Public Health

The Role of Vitamin D Status in the Bioenergetics of Inflammation

Emily Kathleen Calton

This thesis is presented for the Degree of
Doctor of Philosophy
of
Curtin University

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DECLARATION

To the best of my knowledge and belief this thesis contains no material previously published by any other person except where due acknowledgment has been made.

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university.

The research presented and reported in this thesis was conducted in accordance with the National Health and Medical Research Council National Statement on Ethical Conduct in Human Research (2007) – updated March 2014. The proposed research study received human research ethics approval from the Curtin University Human Research Ethics Committee (EC00262), Approval Number RDHS-13-15 and HR 72/2013.

Signature: 

Date: 01/07/2017

CV

Education and Awards

2016	**CUPSA Excellence Award** 1 of the top 8 PhD students at Curtin University in 2016
2014-present	**Doctor of Philosophy (Public Health)**
2014	**Sports Dietitian Course** Sports Dietitians Australia
2014	**David Kritchevsky Graduate Student Award** This International award recognises young investigators contributing to the field of nutrition knowledge to advance food and life science research
2012	**Bachelor of Science (Health Sciences) (Honours)(First Class)** Curtin University Member of the Vice Chancellor's list Recognition for being within the top 1% of Curtin University graduates Course weighted average 90.88% Curtin University Academic Performance Recognition
2011	**Postgraduate Diploma in Dietetics with Distinction** Curtin University Course weighted average 85.40% Curtin University Academic Performance Recognition
2010	**Bachelor of Science (Nutrition) with Distinction** Curtin University Course weighted average 80.98% Curtin University Academic Performance Recognition

Conference and Workshop Presentations

2016	Nutrition Society of Australia Annual Scientific Meeting, Melbourne, Australia Curtin University Postgraduate Student Association Workshop, Perth, Australia Australian Society for Medical Research Scientific Symposium, Perth, Australia Science on the Swan Conference, Perth, Australia Nurses Association of Short Stay Facilities Conference, Busselton, Australia
2014	Nutrition Society of Australia Annual Scientific Meeting, Tasmania, Australia
2013	Nutrition Society of Australia Annual Scientific Meeting, Brisbane, Australia Joint India and America conference in Nutrition (BBNC), India

Publications

2017

Calton, E. K., K. N. Keane, P. Newsholme, Y. Zhao, and M. J. Soares. 2017. "The impact of cholecalciferol supplementation on the systemic inflammatory profile: a systematic review and meta-analysis of high-quality randomized controlled trials." *Eur J Clin Nutr*. doi: 10.1038/ejcn.2017.67.

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Pannu, P. K., E. K. Calton, and M. J. Soares. 2016. "Calcium and Vitamin D in Obesity and Related Chronic Disease." *Adv Food Nutr Res* 77:57-100. doi: 10.1016/bs.afnr.2015.11.001.

2015

Calton, E. K., K. N. Keane, P. Newsholme, and M. J. Soares. 2015. "The impact of vitamin D levels on inflammatory status: A systematic review of immune cell studies." *PLoS ONE* 10 (11):e0141770. doi: 10.1371/journal.pone.0141770.

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Calton, E. K., V. S. Miller, and M. J. Soares. 2013. "Factors determining the risk of the metabolic syndrome: is there a central role for adiponectin?" *Eur J Clin Nutr* 67 (5):485-91. doi: 10.1038/ejcn.2013.1.

Future research vision

I would like to continue pursuing my research interests in exploring the influence of nutrition on energy metabolism, body composition and human health. I am interested in furthering my research skills and learning new research techniques, as well as expanding my collaboration network.

ACKNOWLEDGMENTS

It gives me a great pleasure to express my respect and sincere gratitude to those who have assisted me during my PhD program.

To my Supervisors, thank you for your unfaltering support throughout my degree. Thank you for being a sounding board for my ideas and providing clear guidance along the way. To my Primary Supervisor, Associate Professor Mario Soares, thank you for your mentorship. Your wisdom, dedication and encouragement were an inspiration to me. To Professor Philip Newsholme, thank you for sharing your expert knowledge in the field of metabolism and welcoming me into your laboratory. I have truly enjoyed our collaboration. Thank you to Dr Kevin Keane for your cell biology and bioenergetics expertise and constant support throughout my PhD studies.

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Thank you to my fellow PhD students and the Newsholme Laboratory Group, for your support and encouragement.

To the study participants, thank you for being willing to provide your time for this research.

Thank you to my family and friends for your unwavering support and belief in my success.

The skills I have developed along the way are highly valuable and the knowledge I have required is significant. I would like to thank you all, once again, for your support.

ABSTRACT

The research within this thesis was conducted through a collection of studies to investigate the role of vitamin D in modulating bioenergetics and inflammation in humans and human-derived cells. The research was undertaken through a narrative review, two systematic reviews, two cross-sectional study designs and an observational study design. The thesis begins with a narrative review exploring the link between bioenergetics and inflammation. The requirement for further studies to confirm vitamin D's immuno-modulatory action and role in bioenergetics is emphasised. The second publication of the thesis, a systematic review, found that the active metabolite 1,25-dihydroxy vitamin D [$1,25(OH)_2D$] exerts an anti-inflammatory effect in both human-derived cell lines and *ex vivo* peripheral blood mononuclear cells (PBMCs). However, there was a clear lack of studies examining the impact of 25-hydroxy vitamin D [25(OH)D] on inflammatory status. The third publication of the thesis, utilised a cross-sectional study design and found 25(OH)D to be a novel positive predictor of whole-body bioenergetics. The impact of *in vivo* 25(OH)D on *ex vivo* PBMC bioenergetics was investigated through use of a cross-sectional study design and an optimal 25(OH)D for PBMC bioenergetic function >50 nmol/L was suggested (publication four). To track the seasonal variation of 25(OH)D and its putative influence on whole-body bioenergetics, cellular bioenergetics and inflammatory status, an observational study was carried out (publication five). Seasonal improvements in 25(OH)D were associated with reduced systemic inflammation, whole-body bioenergetics and PBMC bioenergetics. The second systematic review of this thesis and final publication did not support a beneficial effect of cholecalciferol supplementation on the concentration of systemic inflammatory markers. However, sub-group analysis indicated a trend for lower CRP in studies that achieved 25(OH)D $\geq$ 80 nmol/L ($p=0.067$). Collectively, through a series of diverse approaches, the thesis suggests 25(OH)D may have modulatory effects on whole body and cellular bioenergetics. $1,25(OH)_2D$ appears to exert effects on inflammatory markers, however whether this translates to other areas of immune function was outside of the scope of this thesis. Future studies are needed to build on these findings and validate whether vitamin D has a causal role in inflammation and energy metabolism.

LIST OF PUBLICATIONS INCLUDED AS PART OF THE THESIS

1. Calton, E. K., K. N. Keane, and M. J. Soares. 2015. "The potential regulatory role of vitamin D in the bioenergetics of inflammation." *Curr Opin Clin Nutr Metab Care* 18 (4):367-73.
2. Calton, E. K., K. N. Keane, P. Newsholme, and M. J. Soares. 2015. "The impact of vitamin D levels on inflammatory status: A systematic review of immune cell studies." *PLoS ONE* 10 (11):e0141770. doi: 10.1371/journal.pone.0141770.
3. Calton, E. K., K. Pathak, M. J. Soares, H. Alfonso, K. N. Keane, P. Newsholme, N. K. Cummings, W. Chan She Ping-Delfos, and A. Hamidi. 2016. "Vitamin D status and insulin sensitivity are novel predictors of resting metabolic rate: a cross-sectional analysis in Australian adults." *Eur J Nutr* 55 (6):2075-80. doi: 10.1007/s00394-015-1021-z.
4. Calton, E. K., K. N. Keane, M. J. Soares, J. Rowlands, and P. Newsholme. 2016. "Prevailing vitamin D status influences mitochondrial and glycolytic bioenergetics in peripheral blood mononuclear cells obtained from adults." *Redox Biol* 10:243-250. doi: 10.1016/j.redox.2016.10.007.
5. Calton, E. K., K. N. Keane, R. Raizel, J. Rowlands, M. J. Soares, and P. Newsholme. 2017. "Winter to summer change in vitamin D status reduces systemic inflammation and bioenergetic activity of human peripheral blood mononuclear cells." *Redox Biol* 12:814-820. doi: http://doi.org/10.1016/j.redox.2017.04.009.
6. Calton, E. K., K. N. Keane, P. Newsholme, Y. Zhao, and M. J. Soares. 2017. "The impact of cholecalciferol supplementation on the systemic inflammatory profile: a systematic review and meta-analysis of high-quality randomized controlled trials." *Eur J Clin Nutr*. doi: 10.1038/ejcn.2017.67.

All of the above publications have been refereed and I warrant that I have obtained, where necessary, permission from the copyright owners to use any of my own published work in which the copyright is held by another party (Appendix A)

STATEMENT OF CONTRIBUTION OF OTHERS

My contribution to each individual publication has also been outlined in order to summarise and clearly identify the nature and extent of the intellectual input by myself and co-authors (Appendix B).

ADDITIONAL PUBLICATIONS AND CONFERENCES

PUBLICATIONS

1. Soares, M. J., K. Pathak, and E. K. Calton. 2014. "Calcium and vitamin D in the regulation of energy balance: where do we stand?" *Int J Mol Sci* 15 (3):4938-45. doi: 10.3390/ijms15034938.
2. Keane, K. N., E. K. Calton, V. F. Cruzat, M. J. Soares, and P. Newsholme. 2015. "The impact of cryopreservation on human peripheral blood leucocyte bioenergetics." *Clin Sci (Lond)* 128 (10):723-33. doi: 10.1042/cs20140725.
3. Pannu, P. K., E. K. Calton, and M. J. Soares. 2016. "Calcium and Vitamin D in Obesity and Related Chronic Disease." *Adv Food Nutr Res* 77:57-100. doi: 10.1016/bs.afnr.2015.11.001.
4. Keane, K. N., E. K. Calton, R. Carlessi, P. H. Hart, and P. Newsholme. 2017. "The bioenergetics of inflammation: insights into obesity and type 2 diabetes." *Eur J Clin Nutr*. doi: 10.1038/ejcn.2017.45.

CONFERENCES

Nutrition Society of Australia Annual Scientific Meeting, Tasmania, 2014
Australian Society for Medical Research Scientific Symposium, Perth, 2016
Nutrition Society of Australia Annual Scientific Meeting, Melbourne, 2016
Science on the Swan, Perth, 2016

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

$1,25(OH)_2D$	1,25-dihydroxy vitamin D
25(OH)D	25-hydroxy vitamin D
2-DG	2-deoxyglucose
AT	Adipose tissue
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
BHI	Bioenergetic health index
BMI	Body mass index
CCL5	Chemokine motif ligand 5
CEBPβ	Enhancer binding protein beta
CI	Confidence interval
CO_2	Carbon dioxide
CRP	C-reactive protein
CVD	Cardiovascular disease
COX-2	Cyclooxygenase 2
DEXA	Dual-energy X-ray absorptiometry
DHA	Docosahexaenoic acid
DNA	Deoxyribonucleic acid
EE	Energy expenditure
ERK1/2	Extracellular signal-regulated kinase1/2
ETC	Electron transport chain
FBG	Fasting blood glucose
FFAs	Free fatty acids
FFG	Forearm-fingertip gradient
FFM	Fat-free mass
FM	Fat mass
GM-CSF	Granulocyte macrophage colony-stimulating factor
H^+	Protons
H_2O	Water
HDL	High-density lipoprotein
H_2O_2	Hydrogen peroxide
hs-CRP	High sensitivity C-reactive protein

IFN-γ	Interferon gamma
IkBα	Inhibitor kappa-B alpha
IL-1β	Interleukin 1β
IL-1RA	Interleukin 1 receptor antagonist
IL-2	Interleukin 2
IL-4	Interleukin 4
IL-5	Interleukin 5
IL-6	Interleukin 6
IL-7	Interleukin 7
IL-8	Interleukin 8
IL-10	Interleukin 10
IL-12	Interleukin 12
IL-13	Interleukin 13
IL-15	Interleukin 15
IL-17	Interleukin 17
IL-18	Interleukin 18
IL-21	Interleukin 21
IPAQ	International physical activity questionnaire
iPTH	Intact serum parathyroid hormone
IR	Insulin resistance
IRS	Insulin receptor substrate
IS	Insulin sensitivity
IQR	Interquartile range
JAK	Janus kinase
JNK	Jun N-terminal Kinase
LCMS	Liquid chromatography mass spectrophotometry
LDL	Low-density lipoprotein
LPS	Lipopolysaccharide
MAPK	Mitogen activated protein kinase
McA	McAuley's index of insulin sensitivity
MCP	Macrophage chemotactic protein
MetS	Metabolic syndrome
MMP-9	Matrix metallopeptidase 9
mtDNA	Mitochondrial deoxyribonucleic acid

mtROS	Mitochondrial derived reactive oxygen species
mVDR	Membrane bound vitamin D receptor
NC	No change
NFκB	Nuclear factor kappa B
NS	Non-significant
nVDR	Nuclear vitamin D receptor
O_2	Oxygen
O_2^-	Superoxide
OCR	Oxygen consumption rate
PAI-1	Plasminogen activator inhibitor 1
PBMCs	Peripheral blood mononuclear cells
PICOS	Patients, intervention, comparator, outcomes, study design
PPR	Proton production rate
PRISMA	Preferred Reporting Items for Systematic Reviews and Meta-analyses
PTH	Parathyroid thyroid hormone
QUICKI	Quantitative insulin sensitivity check index
RCTs	Randomized controlled trials
RMR	Resting metabolic rate
ROS	Reactive oxygen species
RQ	Respiratory quotient
RXR	Retinoid X receptor
sCD40L	Soluble CD40 ligand
sICAM-1	Soluble intracellular adhesion molecule 1
SFA	Saturated fatty acids
SOCS	Suppressor of cytokine signalling
STAT	Signal transducer and activation of transcription
sTNFrII	Soluble tumour necrosis factor receptor type II
T2DM	Type 2 diabetes mellitus
TC	Total cholesterol
TCA	Tricarboxylic acid cycle
TG	Triglycerides
TGF-β	Transforming growth factor beta
TLR	Toll-like receptor

TNF-α	Tumour necrosis factor alpha
UCP	Uncoupling protein
UV	Ultraviolet
Vitamin D_2	Ergocalciferol
Vitamin D_3	Cholecalciferol
VDR	Vitamin D receptor
WMD	Weighted mean difference

THESIS OUTLINE

This thesis consists of an exegesis (Chapter 1) and six peer-reviewed papers that have been published (Chapter 1- Chapter 6).

Chapter 1 - Exegesis

The exegesis (Chapter 1) commences with a full explanatory overview and review of the literature outlining an emerging role for 25-hydroxy vitamin D [25(OH)D] and 1,25-dihydroxy vitamin D [1,25$(OH)_2$D] in the modulation of bioenergetics and inflammation. Key papers are reviewed to synthesize the key elements and knowledge gaps which underpin the overall research aim and the specific objectives of the thesis. The first peer-reviewed publication forms the next part of the exegesis and is a narrative review summarising the literature at the commencement of the PhD program which informed the thesis objectives (Chapter 1). This review provides an overview of bioenergetics and inflammation. Vitamin D, specifically metabolites 25(OH)D and 1,25$(OH)_2$D, are postulated to alter mitochondrial function and cellular bioenergetics and are suggested to be a potential therapeutic agent for inflammation of chronic disease. It is hypothesised that adequate circulating 25(OH)D would improve inflammatory cell bioenergetics and possibly lessen whole-body energetic demand, as part of its immunomodulatory role. Determining the precise circulating level of 25(OH)D that is critical to exerting extra-skeletal health effects, such as the modulation of inflammation, is identified as an important focus for future research. Circulating PBMCs are identified as a useful and novel model for studying metabolic dysfunction in chronic non-communicable disease states. The requirement for further studies to confirm vitamin D's immunomodulatory action and role in energy metabolism is emphasised. Next, the overall research aim and thesis objectives are identified. The exegesis finishes with a discussion of the main findings of the thesis whereby the findings of each separate publication are linked together, the significance of the thesis is identified and limitations of the thesis and future directions for research are presented.

The content of this chapter is partially covered by Paper 1:
Calton, E. K., K. N. Keane, and M. J. Soares. 2015. "The potential regulatory role of vitamin D in the bioenergetics of inflammation." *Curr Opin Clin Nutr Metab Care* 18 (4):367-73.

Chapter 2 - Vitamin D promotes an anti-inflammatory cytokine profile in human-derived immune cells

Although immunomodulating effects of $1,25(OH)_2D$ are frequently documented, no systematic review has examined the effect of $1,25(OH)_2D$, 25(OH)D or cholecalciferol on the expression and secretion of inflammatory markers by human-derived immune cells. Chapter 2, the second publication of the thesis, is a systematic review investigating the effect of $1,25(OH)_2D$, 25(OH)D and cholecalciferol on inflammatory marker expression and production in PBMCs and human-derived immune cell lines. These cells were chosen as they are utilised throughout the PhD thesis (Chapters 4 and 5) to study bioenergetics. Twenty-three studies (n=7 cell lines, n=16 *ex vivo* PBMCs) were retrieved in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA) guidelines. The majority of studies (n=22) used the active metabolite $1,25(OH)_2D$, two studies additionally used 25(OH)D and the remaining study utilised cholecalciferol. Four out of seven cell line studies demonstrated a clear anti-inflammatory effect whereby key inflammatory markers were supressed. These results were mirrored in fourteen of sixteen PBMC studies. Mechanisms for such effects included decreased protein expression of toll-like receptor (TLR)-2 and TLR-5; lower levels of phosphorylated p38 and p42/42; reduced expression of phosphorylated signal transducer and activation of transcription (STAT)-5 and decreased reactive oxygen species (ROS). Overall, a consistent anti-inflammatory effect of $1,25(OH)_2D$ was observed in studies of cell lines and human derived PBMCs. However, conclusions on whether 25(OH)D modulates inflammation were unable to be drawn, as few studies used this form of the vitamin. As it is likely that prevailing 25(OH)D levels are crucial since they influence local tissue concentrations of $1,25(OH)_2D$, investigation of the impact of 25(OH)D on modulating inflammation is required and occurs in Chapters 4, 5 and 6 of the thesis.

The content of this chapter is covered by Paper 2:
Calton, E. K., K. N. Keane, P. Newsholme, and M. J. Soares. 2015. "The impact of vitamin D levels on inflammatory status: A systematic review of immune cell studies." *PLoS ONE* 10 (11):e0141770. doi: 10.1371/journal.pone.0141770.

Chapter 3 - Vitamin D is a novel determinant of resting metabolic rate

Chapter 3, the third publication of the thesis explores the impact of 25(OH)D on whole body bioenergetics. One hundred and twenty-seven Caucasian Australian participants (n=45 men, n=82 women), had their resting metabolic rate (RMR) measured by indirect calorimetry and 25(OH)D was measured by the chemiluminescent immunoassay method. Blood samples were collected across all seasons. A parsimonious regression model was generated and explained 85.8% of RMR variance. 25(OH)D was found to be a novel positive predictor of RMR. The model predicted that for every 10 nmol/L increase in 25(OH)D, RMR would increase by 56.5 kJ/d, after accounting for all other significant predictors of RMR. As the study was a cross-sectional design, it was recommended that future human studies should investigate the causal associations between 25(OH)D and whole-body bioenergetics. Chapters 4 and 5 further investigate the association between 25(OH)D and bioenergetics.

The content of this chapter is covered by Paper 3:
Calton, E. K., K. Pathak, M. J. Soares, H. Alfonso, K. N. Keane, P. Newsholme, N. K. Cummings, W. Chan She Ping-Delfos, and A. Hamidi. 2016. "Vitamin D status and insulin sensitivity are novel predictors of resting metabolic rate: a cross-sectional analysis in Australian adults." *Eur J Nutr* 55 (6):2075-80. doi: 10.1007/s00394-015-1021-z.

Chapter 4 - *In vivo* 25(OH)D is associated with the bioenergetic profile of *ex vivo* peripheral blood mononuclear cells

To further explore the effect of 25(OH)D on bioenergetics, a cross-sectional study with 38 adults (n=16 males, n=22 females) was designed to investigate the impact of *in vivo* 25(OH)D on *ex vivo* PBMC bioenergetics (Chapter 4; publication 4). As findings from Chapter 2 demonstrate that $1,25(OH)_2D$ is an anti-inflammatory agent, the current

chapter hypothesised that inadequate 25(OH)D would promote an inflammatory state. Furthermore, it was postulated that both low 25(OH)D and an inflammatory state would reduce whole body insulin sensitivity (IS), and a dysfunctional PBMC bioenergetic response would occur. The resultant lower bioenergetic health index (BHI), an indicator of lowered mitochondrial health status, would drive an increase in whole body RMR. As a heightened inflammatory state is also energetically expensive, RMR would therefore increase. To examine this hypothesis, PBMCs from individual participants were isolated from whole blood, counted and freshly seeded for bioenergetic analysis (Mitochondrial Stress Test and Glycolysis Stress Test) using a Seahorse XF^{e}96 flux analyser, according to an established protocol. Whole body energy metabolism was measured using indirect calorimetry, and body composition assessed by dual-energy X-ray absorptiometry (DEXA). 25(OH)D was assessed using the Architect immunoassay method with blood collection occurring during winter months (July-September). Participants were grouped into three groups based on 25(OH)D status [Group 1 (< 50 nmol/L), Group 2 (50-75 nmol/L), and Group 3 (> 75 nmol/L)]. A multivariate general linear model adjusting for age, fat mass (FM), fat-free mass (FFM), parathyroid hormone (PTH) and IS was used.

The results described in this chapter suggested that *in vivo* circulating 25(OH)D, was inversely associated with *ex vivo* PBMC bioenergetics resulting in significantly greater bioenergetics responses in those with 25(OH)D < 50 nmol/L. It appeared that this effect may plateau above 50 nmol/L, since the group > 75 nmol/L showed no further change in these bioenergetic parameters.

RMR was positively associated with inflammatory cytokine tumour necrosis factor alpha (TNF-α) level, mimicking the increased energetic cost of an activated immune system. BHI, was directly related to IS and inversely to RMR, and markers of systemic inflammation. This potentially validates a scenario where attenuation of systemic inflammation, improved IS, reduced whole body energetic demand and improved BHI are inter-linked.

In this study, the quantitative insulin sensitivity check index (QUICKI), an indicator of IS, trended towards an inverse association with inflammatory macrophage chemotactic protein (MCP)-1 and IS was highest in the highest 25(OH)D group. As the links

between BHI, inflammatory markers and whole-body bioenergetics disappeared on adjustment for QUICKI, IS may be key to the interrelationships of bioenergetics responses and systemic inflammation.

The study did not find any differences in circulating inflammatory markers among the three 25(OH)D groups. This is surprising given the findings from Chapter 2 where cellular studies convincingly demonstrated an anti-inflammatory benefit following exposure to $1,25(OH)_2D$. Although the majority of these cellular studies tested the effects of $1,25(OH)_2D$, there is a close relationship between circulating 25(OH)D and its active metabolite as the immune cells can convert 25(OH)D to $1,25(OH)_2D$ provided they express the necessary enzyme complement. However, it must be acknowledged that measuring systemic cytokine concentrations does not provide information on cytokine release at the local tissue level. It is also likely group differences were not found because participants with acute inflammatory conditions were excluded.

Never-the-less, this is the first study to suggest there may be an effect of circulating 25(OH)D on PBMC bioenergetics. Taken together, these data indicate
a relationship between 25(OH)D and immune cell bioenergetic responses that should be further investigated. Chapter 5 further investigates this novel association.

The content of this chapter is covered by Paper 4:
Calton, E. K., K. N. Keane, M. J. Soares, J. Rowlands, and P. Newsholme. 2016. "Prevailing vitamin D status influences mitochondrial and glycolytic bioenergetics in peripheral blood mononuclear cells obtained from adults." *Redox Biol* 10:243-250. doi: 10.1016/j.redox.2016.10.007.

Chapter 5 - Winter to summer change in vitamin D status reduces systemic inflammation and bioenergetic activity of human peripheral blood mononuclear cells

Results described in Chapter 4 demonstrate that *in vivo* circulating 25(OH)D, was inversely associated with *ex vivo* PBMC cell bioenergetic responses resulting in significantly greater levels of these bioenergetics parameters in those with 25(OH)D <

50 nmol/L. This effect appeared to plateau above 50 nmol/L. Additionally, RMR was positively associated with inflammatory cytokine TNF-α, mimicking the increased energetic cost of an activated immune system. BHI, was directly related to IS but inversely to RMR, and markers of systemic inflammation. Taken together, attenuation of systemic inflammation may improve IS, reduce whole body energetic demand and improve bioenergetic function. Therefore, there may be an optimal 25(OH)D cut-off associated with cellular bioenergetic responses and inflammation that should be maintained year-round.

The 25(OH)D of humans and other animals is well known to rise in summer and decline in winter in response to seasonal variation in the intensity of solar UV light. However, there is a lack of reliable information to indicate whether seasonal variation in 25(OH)D positively or negatively influences health.

The impact of seasonal variation in 25(OH)D on cellular bioenenergetics and inflammation, and the potential flow through impact on whole-body bioenergetics, has not been previously described. In Chapter 5, it is hypothesised that a lower 25(OH)D in winter may promote greater inflammation and insulin resistance (IR); the attendant higher energetic cost would be reflected in a higher RMR and increased PBMC bioenergetic parameters. Consequently, a seasonal increase in 25(OH)D would reverse these effects and be most evident in those who started with the lowest 25(OH)D. To examine this hypothesis, whole-body bioenergetics and substrate utilisation were measured by indirect calorimetry. PBMCs obtained from the same participants were isolated from whole blood, counted and freshly seeded. Bioenergetic analysis (Mitochondrial Stress Test and Glycolysis Stress Test) was performed using the Seahorse XF^{e}96 flux analyser. 25(OH)D was assessed using the Architect immunoassay method. To examine the influence of initial 25(OH)D on bioenergetics and inflammation change, the study sample were divided into three groups based on cut offs for 25(OH)D of 50 nmol/L and 75 nmol/L. To examine the influence of final 25(OH)D achieved and absolute change in 25(OH)D on bioenergetics and inflammation change, participants were divided into tertiles.

25(OH)D increased by a median (IQR) of 14.40 (20.13) nmol/L ($p<0.001$) from winter to summer and was accompanied by significant improvements in indices of IS,

McAuley's index and QUICKI and multiple markers of inflammation. PBMC Mitochondrial Stress Test parameters basal respiration, non-mitochondrial respiration, ATP production, proton leak, and maximal respiration decreased in summer compared to winter. Similarly, PBMC Glycolysis Stress Test parameters glycolytic activity, glucose response, and glycolytic capacity were all reduced in summer compared to winter. There was also a trend for RMR to decrease ($p=0.066$). Participants who entered winter with a low 25(OH)D (< 50 nmol/L), had the greatest alteration in bioenergetic parameters in summer, relative to those with winter 25(OH)D concentrations of 50-75 nmol/L or > 75 nmol/L. The absolute change in 25(OH)D was not associated with modulation of bioenergetics.

The concurrent observation of lowered bioenergetic parameters and reduced inflammation in summer as compared to winter supports the hypothesis that a lower 25(OH)D in winter may result in greater inflammation which has an energetic cost that could account for part of the higher RMR in winter. It is clear that those who had an initial low 25(OH)D (< 50 nmol/L) were associated with decreased bioenergetic parameters from winter to summer, while individuals with higher initial status (> 50 nmol/L) did not. In contrast, multiple systemic inflammatory markers decreased within each group suggesting higher levels of 25(OH)D may be required to dampen inflammation. Taken together, these results may suggest that the optimal 25(OH)D level depends on the target variable.

This is the first study to track bioenergetics in the same participants across seasons. Similar to the study of Chapter 4, the current study also suggests there may be an effect of circulating 25(OH)D on PBMC bioenergetics. Seasonal increases in 25(OH)D significantly reduced bioenergetic responses such that greater improvement was seen in those who normalized their status to ~ 50 nmol/L, while those who achieved values > 50 nmol/L showed no further improvements. Intervention trials are required to establish a potential cause-and-effect relationship between 25(OH)D and altered cellular bioenergetic parameters.

The content of this chapter is covered by Paper 5:
Calton, E. K., K. N. Keane, R. Raizel, J. Rowlands, M. J. Soares, and P. Newsholme. 2017. "Winter to summer change in vitamin D status reduces systemic inflammation

and bioenergetic activity of human peripheral blood mononuclear cells." *Redox Biol* 12:814-820. doi: http://doi.org/10.1016/j.redox.2017.04.009.

Chapter 6 - The impact of cholecalciferol supplementation on the systemic inflammatory profile: a systematic review and meta-analysis of high quality randomized controlled trials

Chapter 2 convincingly demonstrated an anti-inflammatory benefit following exposure to $1,25(OH)_2D$, and Chapter 5 showcased a reduction in levels of several inflammatory markers that coincided with increased 25(OH)D from winter to summer. Given these findings, the sixth publication of this thesis (Chapter 6) examined whether there was a causal link between 25(OH)D following cholecalciferol supplementation with the systemic inflammatory profile (n=14 studies). Nine studies (n=15 study arms) permitted extraction of data for interleukin (IL)-6 and C-reactive protein (CRP) and a meta-analysis was conducted. Whether baseline 25(OH)D, change in 25(OH)D or the final concentration achieved, modulated the effects of cholecalciferol supplementation on circulating inflammatory markers were also explored.

The data did not support a beneficial effect of cholecalciferol supplementation on the concentration of systemic inflammatory markers. However, sub-group analysis indicated a trend for lower CRP in studies that achieved ≥ 80 nmol/L (p=0.067). A higher daily dose of cholecalciferol supplementation showed a favourable effect on CRP. Meta-regression indicated that older age predicted a significant decrease in IL-6 and CRP, while a greater percentage of females and a longer study duration also independently predicted a higher CRP.

The content of this chapter is covered by Paper 6:
Calton, E. K., K. N. Keane, P. Newsholme, Y. Zhao, and M. J. Soares. 2017. "The impact of cholecalciferol supplementation on the systemic inflammatory profile: a systematic review and meta-analysis of high-quality randomized controlled trials." *Eur J Clin Nutr*. doi: 10.1038/ejcn.2017.67.

CHAPTER 1-EXEGESIS

Introduction

Obesity

The increasing prevalence of obesity worldwide over the last 30 years, represents a modern pandemic, with serious health consequences (Ogden et al. 2014; Twells et al. 2014). Obesity is not just a state of excess adiposity, having long been recognised as a chronic disease that threatens to reduce quality of life and life expectancy (Ng et al. 2014). Coinciding with this, are increases in chronic metabolic diseases, such as type 2 diabetes mellitus (T2DM) (Chen et al. 2012; Lin et al. 2013), dyslipidaemia (Choi et al. 2011), and non-alcoholic fatty liver disease (Welsh et al. 2013; Loomba and Sanyal 2013). Obesity adversely influences tissue function throughout the body; including the liver (Koteish and Diehl 2001), pancreas (Morioka and Kulkarni 2011), muscle (Akhmedov and Berdeaux 2013), heart (Poirier et al. 2006), and joints (King et al. 2013). In healthy lean males and females, adipose tissue (AT) represents ~20% and ~30% of tissue mass, respectively. In morbid obesity, AT can account for greater than 50% of total body mass (Morigny et al. 2016). AT is a heterogeneous tissue composed of adipocytes (mature adipocytes, pre-adipocytes), structural cells (fibroblasts, endothelial cells), and immune cells (mast cells, granulocytes, lymphocytes and macrophages) (Calder 2013).

A chronic low-grade inflammation underscores most, if not all, obesity-associated conditions including T2DM (Pickup 2004; Lontchi-Yimagou et al. 2013; Akash et al. 2013; Wellen and Hotamisligil 2005), cardiovascular disease (CVD) (Frostegard et al. 1999; Ikeoka et al. 2010; Zelkha et al. 2010; Abe et al. 2013), metabolic syndrome (MetS) (Esser et al. 2014) and some cancers (Ramos-Nino 2013). Systemic inflammation precedes chronic disease development (Luft et al. 2013; Duncan et al. 2003; Libby 2002) and can predict the occurrence of chronic disease (Koenig et al. 1999), while anti-inflammatory cytokines such as interleukin 13 (IL-13), supress diet-induced obesity and subsequent IR (Darkhal et al. 2015).

Inflammation

Inflammation is the defence mechanism that protects the host from infection or tumour development; provides tolerance to non-threatening organisms, food components and the human body; initiates pathogen killing; and promotes tissue repair processes with

the aim of restoring homeostasis at infected or damaged sites. The classical signs of inflammation include redness, swelling, heat, pain and loss of function (Calder 2013). The inflammatory response involves the production of, and responses to, a variety of chemical mediators. The inflammatory response is self-regulated; involving the activation of negative feedback mechanisms such as the secretion of anti-inflammatory cytokines, inhibition of pro-inflammatory signalling cascades, and activation of regulatory cells (Calder 2013).

Inflammation and adiposity relationship

While the existence of inflammatory disease and infectious conditions hallmarked by acute levels of inflammation have long been recognised, chronic low-grade inflammation has also received considerable attention, particularly in relation to obesity-related chronic disease. Chronic low-grade inflammation is characterised by a small (~2-fold) albeit clinically relevant increase in systemic indicators of inflammation such as leucocyte count, acute-phase proteins, pro-inflammatory cytokines, chemokines, soluble adhesion molecules and pro-thrombotic mediators (Calder et al. 2011). In contrast to acute inflammation, whereby the liver and the lymphoid organs are the major sites of production of inflammatory mediators, the AT (specifically immune cells such as macrophages localised in the AT) is the major source of inflammatory mediators in low-grade chronic inflammation (Calder et al. 2011).

The mechanistic link between obesity and low-grade inflammation was first proposed in 1993 (Hotamisligil et al. 1993) when it was shown that TNF-α was expressed in AT of mice, and expression was elevated in obese mice compared to their lean counterparts. Importantly, increased expression translated to increased TNF-α in the AT and in circulation. Investigations of TNF-α neutralisation revealed that TNF-α was also linked to IR, since increased peripheral glucose uptake and utilisation was evident on TNF-α neutralisation.

The discovery of the hormone leptin in 1994 (Zhang et al. 1994) refuted the view of AT as an inert energy store. Instead, AT was acknowledged as the largest endocrine gland. The concept of adipokines thereby emerged; with the recognition that proteins were produced by and secreted by AT into the circulation, thus exerting systemic

effects (Trayhurn and Wood 2004). The products of AT are diverse and include cytokines such as TNF-α, interleukin 1β (IL-1β), interleukin 1 receptor antagonist (IL-1RA), interleukin 6 (IL-6), interleukin 7 (IL-7), interleukin 8 (IL-8), interleukin 10 (IL-10) and interleukin 18 (IL-18) (Trayhurn and Wood 2004; Calder et al. 2011); chemokines which include MCP-1, and C–C chemokine motif ligand 5 (CCL5) (Calder et al. 2011; Trayhurn and Wood 2004); hormones such as leptin, adiponectin and resistin (Calder et al. 2011) and acute-phase proteins which are secreted in the acute phase of inflammation such as plasminogen activator inhibitor 1 (PAI-1) and haptoglobin (Calder et al. 2011).

At the onset of obesity, AT is infiltrated by bone-marrow-derived monocytes that mature to possess a classically activated M1-like phenotype (Weisberg et al. 2003; Xu et al. 2003) (Figure 1). T lymphocytes (Prietl et al. 2013) (specifically Th1 cells), natural killer T cells, mast cells, B cells (Anderson et al. 2010) and dendritic cells (DCs) also enter AT and promote the recruitment of additional monocytes and local proliferation of macrophages (Stefanovic-Racic et al. 2012). Inflammatory cytokines regulate macrophage phenotype (Johnson et al. 2012). Anti-inflammatory cytokines stimulate macrophages to acquire an anti-inflammatory phenotype. These alternatively activated (M2) macrophages secrete anti-inflammatory mediators, such as IL-10. The M2 macrophages utilise oxidative phosphorylation for energy generation (Kelly and O'Neill 2015) (Figure 1). In contrast, inflammatory cytokines such as TNF-α induce macrophages to adopt a pro-inflammatory M1 phenotype (Johnson et al. 2012) which rely on glycolysis, the conversion of glucose to pyruvate, for energy production (Kelly and O'Neill 2015). Almost all of this glucose is converted into lactate and very little is oxidized (Newsholme et al. 1987). Activated M1 macrophages produce large amounts of pro-inflammatory mediators, such as TNF-α and IL-1β, that act on adipocytes to induce an IR state (Johnson et al. 2012) (Figure 1). This establishes a positive feedback loop that further amplifies inflammation and IR (Olefsky and Glass 2010). The elevated levels of cytokines increase energy expenditure (EE), perhaps in an effort to offset weight gain (Wang and Ye 2015). The M1 macrophages continue to reside in the expanded AT mass unless weight loss is achieved or physical activity is increased. Following either intervention, they revert to an M2 phenotype (Fjeldborg et al. 2014)

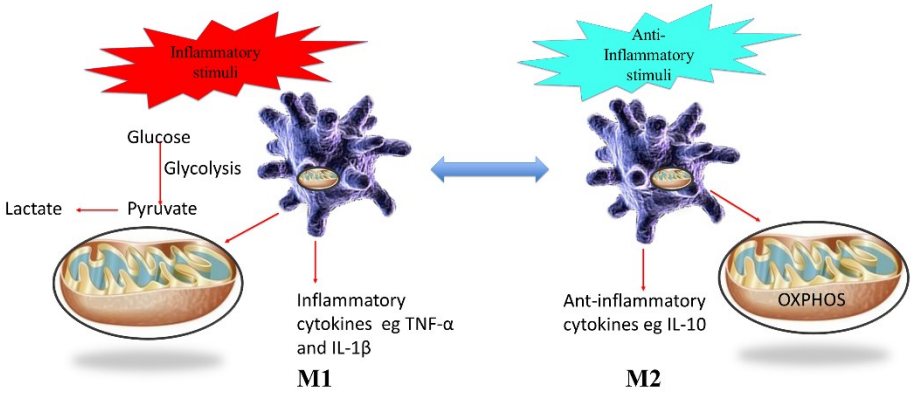

Figure 1. Inflammatory and metabolic profile according to macrophage phenotype

Mechanisms of obesity-induced inflammation

Monocytes infiltrate the AT and become AT macrophages. These macrophages then secrete their own chemical mediators, attracting additional monocytes and a feed-forward inflammatory process is established (Osborn and Olefsky 2012). The cause for infiltration and activation of macrophages in the AT is likely to be multifactorial (Surmi and Hasty 2008). Consequences of AT expansion include changes in fatty acid flux, hypoxia and adipocyte cell death. Increased lipolysis is associated with the IR state that accompanies obesity (Morigny et al. 2016). Endogenous free fatty acids (FFAs) released from adipocytes following lipolysis induce inflammatory changes through activation of the pro-inflammatory nuclear factor kappa B (NFκB) pathway (Suganami et al. 2007). AT undergoes hypertrophic enlargement in obesity, resulting in hypoxia (Trayhurn 2013). Hypoxia induces the expression of several inflammatory molecules in adipocytes (Trayhurn et al. 2008) and inhibits macrophage departure from the hypoxic region (Turner et al. 1999). Adipocyte death is a hallmark feature of obesity and macrophages may enter the AT to phagocytose dead or dying adipocytes present in the expanding AT (Surmi and Hasty 2008).

Over nutrition and dietary factors also promote monocyte recruitment and the inflammatory response. Triggers of the postprandial inflammatory response include fat type (Myhrstad et al. 2011; Cruz-Teno et al. 2012), glucose (Chu et al. 2014; Dickinson et al. 2008) and high glycaemic index foods (Dhindsa et al. 2004). A Westernized dietary pattern is characterised by excessive consumption of energy, salt, sugar, saturated fat and low intakes of healthy fats, fibre and antioxidants (Calton et al. 2014).

A diet rich in saturated fat promotes monocyte infiltration into AT and IR (Enos et al. 2013). Saturated fatty acids (SFA) are ligands for toll-like receptor 2 (TLR-2) and toll-like receptor 4 (TLR-4) located on the surface of macrophages and adipocytes. When the ligand-receptor complex is formed, activation of NFκB, the c-jun N-terminal Kinase and p38 mitogen activated protein kinase (MAPK) signalling cascades stimulate pro-inflammatory gene transcription pathways. Continuous stimulation of these pathways results in a chronic state of inflammation and IR follows (Enos et al. 2013). SFA also stimulate pro-inflammatory mechanisms through a TLR-independent pathway via the production of ROS which impacts on IR through the production of IL-1β (Teng et al. 2014). The consumption of a meal high in carbohydrate (>50%) increases oxidative stress and reduces antioxidant defences (Ceriello et al. 1998) and is related to the extent of meal-induced hyperglycaemia (Ceriello et al. 1999). Vitamin C (specifically ascorbic acid), is a potent antioxidant that scavenges ROS (Ottoboni and Ottoboni 2005). *In vitro* acute hyperglycaemia leads to a significant decrease in leucocyte vitamin C content (Chen et al. 1983). It has been hypothesised that acute hyperglycaemia may induce intracellular vitamin C deficiency through competitive inhibition because vitamin C and glucose share a common transport system, the glucose transporters (Price et al. 2001). Specifically, without transport of dehydroascorbic acid into cells, vitamin C is unable to be reduced to ascorbic acid.

In contrast, fatty acid docosahexaenoic acid (DHA), which is found in fish oil, seafood and sea weed (Singh 2005), exerts an anti-inflammatory effect. Although the precise pathways remain to be elucidated, activation of cytosolic phospholipase A2 and cyclooxygenase 2 (COX-2) have been suggested as potential candidate mechanisms (Liu et al. 2014). The antioxidant resveratrol, found in grapes, wine, peanuts, and soy (Burns et al. 2002), promotes anti-inflammatory effects in circulating macrophages through down regulation of the NFκB pathway and decreased cytokine release follows (Buttari et al. 2014). Vitamins C and E and energy restriction are additional dietary factors that have been identified with anti-inflammatory characteristics (Calder et al. 2011).

Inflammation and insulin resistance

Low-grade inflammation influences tissue metabolism locally and systemically via the circulation (Figure 2). Obesity-driven inflammation induces IR within the muscle,

liver, and AT. IR within the liver results in increased gluconeogenesis and elevated blood glucose in the circulation. In insulin-sensitive individuals, insulin acts on the insulin receptor leading to tyrosine-phosphorylation of insulin receptor substrate 1 (IRS-1) and subsequent intracellular signalling promotes glucose transporter 4 translocation and uptake of glucose into the cell (glucose disposal) (de Luca and Olefsky 2008; Marette 2002). TNF-α decreases insulin signalling by inducing IRS-1 serine phosphorylation instead (de Luca and Olefsky 2008; Marette 2002). TNF-α has been demonstrated to induce IR via a p38 MAPK-dependent pathway (Li et al. 2007). Activation of the Janus kinase (JAK)/ STAT pathway may be another signalling pathway through which inflammation induces IR (Gurzov et al. 2016). Suppressor of cytokine signalling (SOCS) proteins are expressed with activation of the JAK/STAT pathway. In mouse models of obesity, there is an increase in suppressor of cytokine signalling proteins, SOCS-1 and SOCS-3, in the liver, muscle, and AT, which supress tyrosine phosphorylation of IRS-1 and IRS-2 leading to a reduction in glycogen synthesis and glucose uptake (Ueki et al. 2004).

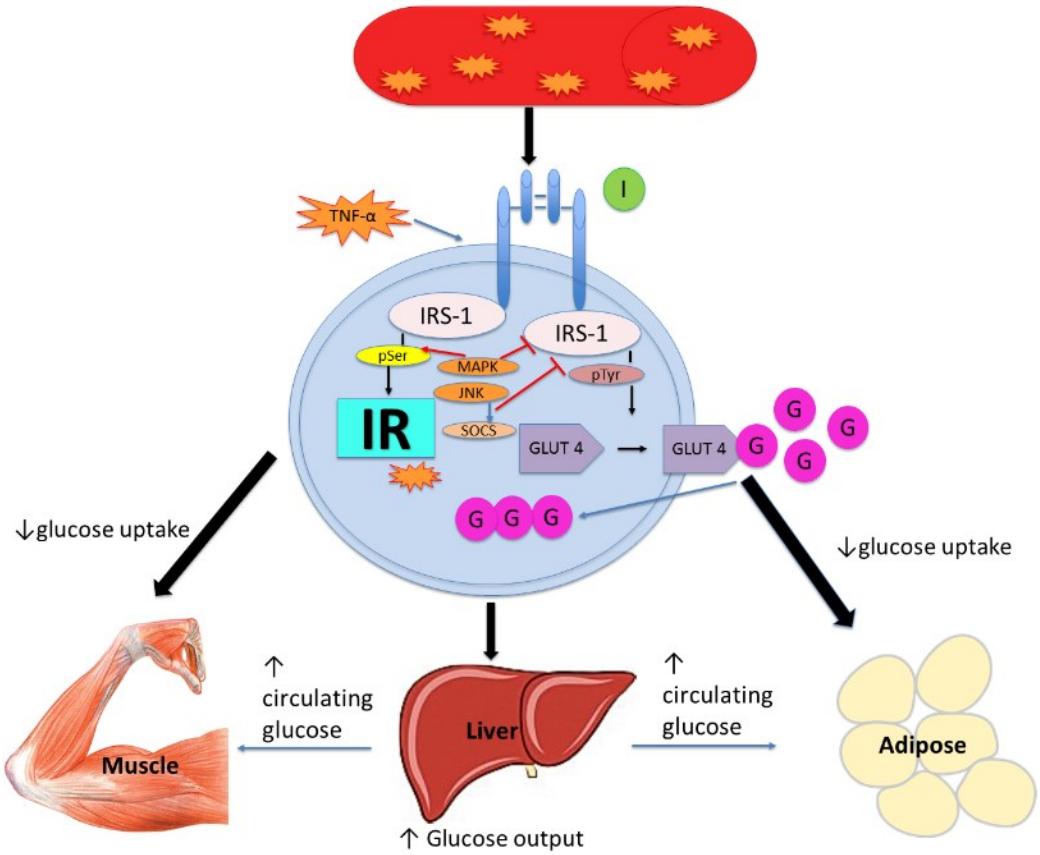

Figure 2. Inflammatory mediation of insulin function

In humans, the most direct approach to assess the contribution of low-grade inflammation to the development of IR is to analyse the consequences of anti-inflammatory pharmacotherapy. A double-blind randomized controlled trial (RCT) of salsalate treatment for 1 month in twenty non-diabetic obese individuals improved IS. This was gauged by decreased fasting blood glucose (FBG), fasting C-peptide, and changes in glucose and C-peptide following an oral glucose challenge. Level of inflammatory marker C-reactive protein (CRP) and anti-inflammatory marker adiponectin also improved following salsalate (Fleischman et al. 2008).

The metabolic effects of inflammatory cytokines can be neutralised by injections of recombinant monoclonal antibodies. In non-diabetic individuals, several studies have observed improvement of IS after treatment with TNF-α antibodies (Tam et al. 2007; Pina et al. 2015), although improvement in IS is not consistently observed despite dampening of systemic inflammation (Di Rocco et al. 2004). Reasons for disparity include different methods for determining IS and lengths of study design. A number of RCTs (Larsen et al. 2007; Cavelti-Weder et al. 2012; Sloan-Lancaster et al. 2013) in humans have suggested the involvement of IL-1β in impaired insulin secretion and in IR. An initial proof-of-concept study randomly assigned 70 patients with T2DM to receive either a recombinant human IL-1RA or placebo for 13 weeks. Improved glycaemia, β-cell secretory function and systemic inflammation were observed with the antagonist pharmacotherapy (Larsen et al. 2007). Gevokizumab, an IL-1β antagonist, was given to patients with T2DM for 13 weeks. CRP levels decreased as did HbA1c and insulin secretion improved (Cavelti-Weder et al. 2012). Together, these data support the hypothesis that low-grade chronic inflammation contributes to the pathogenesis of obesity-related IR and that targeting inflammation may provide a therapeutic route for diabetes prevention and/or treatment.

Bioenergetics

Bioenergetics is the field of study concerned with the flow of energy through living systems. In the cell cytosol, glycolysis produces pyruvate from glucose and adenosine triphosphate (ATP) is generated (2 molecules of ATP per molecule of glucose). However, the mitochondria produce over 90% of cellular ATP through the Kreb's cycle, also known as the tricarboxylic acid (TCA) cycle, and oxidative phosphorylation (38 molecules of ATP per molecule of glucose). The TCA cycle

converts carbohydrate, protein and fat into carbon dioxide (CO_2) and reduced coenzymes NADH and FADH are generated. These coenzymes then carry electrons into the electron transport chain (ETC). The electrons flow through the five complexes that make up the ETC and finally are passed on to molecular oxygen (O_2) with the generation of water (H_2O). Concurrently, protons (H^+) are moved from the inner mitochondrial membrane to the intermembrane space creating a proton motive force. This force is then used to phosphorylate adenosine diphosphate (ADP) via the mitochondrial ATP synthase complex and ATP is generated (Pieczenik and Neustadt 2007). It is in this fashion that the mitochondria consume ~85% of cellular O_2 during the production of ATP (Shigenaga et al. 1994), with the remaining oxygen being used by cellular enzymes including NADPH oxidase and cytochrome P450, during the generation of ROS and via proton leak. The uncoupling of electron transport to H^+ transfer leads to the generation of heat instead of generating ATP. Uncoupling proteins (UCPs), are a family of mitochondrial transport proteins, located in the inner mitochondrial membrane, which transport H^+ to the mitochondrial matrix dissipating the proton motive force as heat and uncoupling substrate oxidation from the production of ATP (Figure 3). Five UCPs (UCP1 to UCP5) are found in mammals and are named according to their tissue distribution through the body. UCP1 is expressed in brown adipose tissue in newborns, UCP2 is ubiquitously distributed throughout the body, UCP3 is mainly restricted to the skeletal muscle, and UCP4 and UCP5 are expressed in the brain. It is widely accepted that UCPs reduce oxidative damage by decreasing mitochondrial ROS formation (Liu et al. 2013). UCPs are believed to be implicated in the pathogenesis of obesity and T2DM. UCP 2 and 3 expression is induced by fasting, as well as hyperglycemia, which indicates there may be an important link between UCPs and the availability of fuel substrates (Dalgaard 2011).

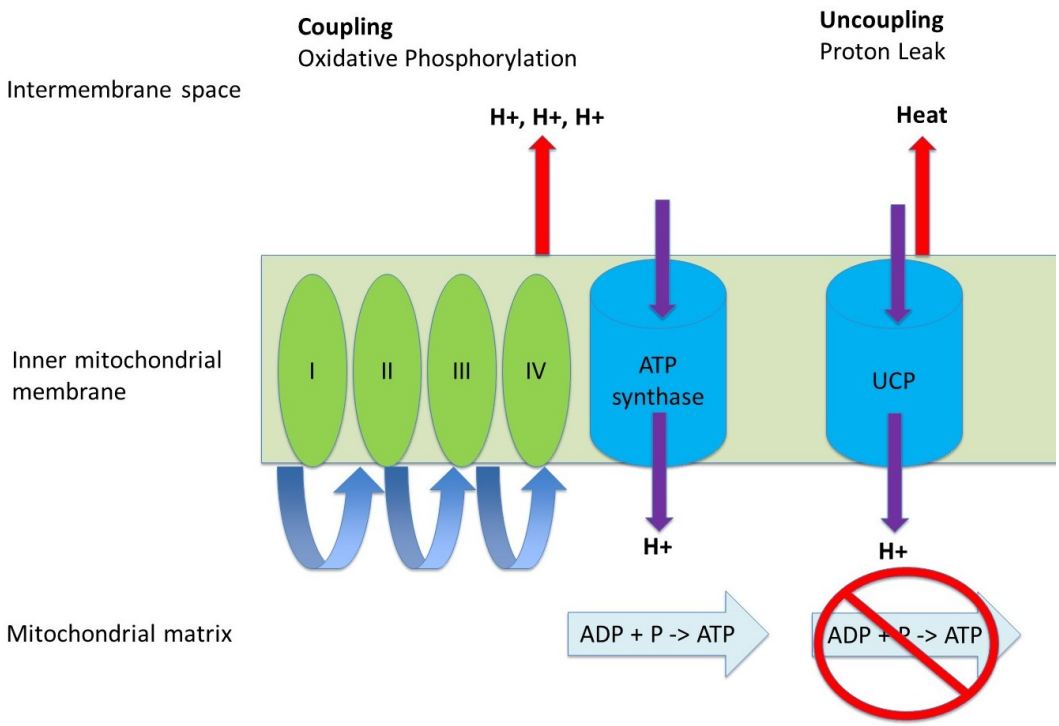

Figure 3. Uncoupling and coupling of oxidative phosphorylation to ATP production

Bioenergetics can be measured at the whole-body level through measurements of heat production via direct calorimetry or indirect calorimetry. The most common validated method to measure ATP production and substrate utilisation at the whole-body level in humans is via indirect calorimetry. Indirect calorimetry is a non-invasive method whereby whole body bioenergetics are estimated in vivo through respiratory gas measurements of O_2 consumption and CO_2 production (Ferrannini 1988). Substrate utilisation or respiratory quotient (RQ), provides an indication of average fuel utilisation across the whole body. Substrate utilization is determined through the measurement of the CO_2 production to O_2 consumption ratio. Lower values (eg 0.7), correspond to greater fat oxidation (FO), while higher values (eg 0.9) indicate carbohydrate oxidation. However true rates of carbohydrate or fat oxidation requires the additional measurement of nitrogen excretion, as a reflection of protein oxidation (Ferrannini 1988).

Indirect calorimetry measurements made at rest and in the fasted state give an indicator of the minimum EE that is compatible with life and this can be defined as the RMR. RMR is the major determinant of daily energy requirements in humans, accounting for 60–75% of total daily EE. FFM is the single greatest predictor of RMR accounting for

up to 60% of RMR variance (Johnstone et al. 2005; Arciero et al. 1993). A significant contribution of FM is also apparent in obesity (Johnstone et al. 2005). Additionally, small but important contributions to RMR are made by age (Johnstone et al. 2005), sex (Arciero et al. 1993) and ethnicity (Sharp et al. 2002). However, even after accounting for these factors, there still remains significant inter-individual variation in RMR which are not understood (Johnstone et al. 2005; Larsen et al. 2011) Addressing the underlying causes of this variation has long been recognised as essential (Scrimshaw et al. 1994), particularly if such factors are modifiable through therapeutic interventions.

Bioenergetics of individual tissues and cells can also be measured to gauge tissue specific energetic function and examine dysfunction in energy generating pathways. Many methods exist to measure bioenergetics at the cellular level. Extracellular flux analyzers, such as the Seahorse XF^{e}96 flux analyser, represent a novel and convenient method to measure O_2 consumption and proton production. This method requires a relatively small number of cells compared to alternative methods of O_2 consumption measurement such as the Clark electrode (Chacko et al. 2013; Fink et al. 2012). Importantly, the cell isolation procedure and attachment to the extracellular flux analyser cell culture plate does not result in detectable activation of the cells (Chacko et al. 2013). The Seahorse XF^{e}96 flux analyser measures the oxygen consumption rate (OCR), an index of mitochondrial respiration, and the change in pH (the extracellular acidification rate or proton production rate), an indicator of glycolytic flux (Dranka et al. 2011; Brand and Nicholls 2011; Maynard et al. 2013). Different reagents are preloaded into the four reagent delivery chambers for sequential injection into the media containing the cells or tissue. Injecting inhibitors and activators of mitochondrial respiratory chain allows determination of the defects in individual cellular respiration pathways (Chacko et al. 2013).

Tracking cellular bioenergetics may allow for a dynamic assessment of health status (Chacko et al. 2014). It has been suggested that the calculation of BHI, a composite of mitochondrial bioenergetic parameters, may represent a novel prognostic or diagnostic biomarker in disease states (Chacko et al. 2014). Chronic metabolic stress progressively decreases mitochondrial function and mitochondrial ETC damage occurs (Chacko et al. 2014). This manifests as a low BHI which is associated with

increased proton leak and greater non-mitochondrial respiration. However, whether individual factors such as age and sex influence the BHI requires investigation (Chacko et al. 2016).

The use of peripheral blood mononuclear cells as biomarkers

Peripheral blood mononuclear cells (PBMCs) are biosensors influenced by circulating molecules produced by cells, tissues and organs (Japiassu et al. 2011), as well as environmental factors, such as diet and exercise. Changes in gene expression in PBMCs have been shown in response to dietary interventions such as calorie restriction (Van Bussel et al. 2016). Recent studies in humans have demonstrated that changes in gene expression (Hulsmans et al. 2012), and surface protein markers (Poitou et al. 2011; van der Weerd et al. 2012; Bories et al. 2012) in PBMCs provide robust correlations with risk of MetS, CVD and obesity. Thus, PBMCs are postulated to be of potential use as biomarkers of health status (Stepien et al. 2014; Pecht et al. 2014).

Similar to classically studied organs such as AT, skeletal muscle, liver, and the pancreas, PBMCs have been utilised to determine bioenergetics status in a variety of different disease states, including sepsis (Japiassu et al. 2011; Belikova et al. 2007), rheumatic disease (Kuhnke et al. 2003), T2DM (Widlansky et al. 2010; Hartman et al. 2014), fibromyalgia (Cordero et al. 2010), and Alzheimer's disease (Maynard et al. 2013). PBMCs obtained from anorexia nervosa patients (Omodei et al. 2015) and Alzheimer's disease patients (Maynard et al. 2013) have a reduced bioenergetic profile compared to healthy controls, while PBMCs isolated from patients with T2DM have a higher basal respiration, maximal respiration, and coupling efficiency compared to controls (Hartman et al. 2014).

Vitamin D background

Vitamin D is a generic name given to two molecules, ergocalciferol (vitamin D_2) and cholecalciferol (vitamin D_3). The sources of vitamin D include consumption via diet and supplementation with either ergocalciferol or cholecalciferol (Liu 2012). The major source of vitamin D is endogenous production; commencing with solar UV-B irradiation of 7-dehydrocholesterol present in the skin to generate cholecalciferol (Pilz et al. 2013; Liu 2012). Subsequent hydroxylation in the liver produces 25-hydroxy

vitamin D [25(OH)D], the main circulating form of vitamin D. A final hydroxylation step in the kidney catalysed by the enzyme CYP27B1 produces the active metabolite 1,25-dihydroxy vitamin D [1,25$(OH)_2$D]. Extrarenal synthesis of 1,25$(OH)_2$D has been widely demonstrated including sites such as the colon, prostate, placenta and cells of the immune system (Bikle 2010). CYP27B1 expression and activity is well established in activated macrophages (Bikle 2010). 1,25$(OH)_2$D exerts biological function via binding to its nuclear vitamin D receptor (nVDR) (Pilz et al. 2013; Fleet 2004) and modulating gene expression (Haussler et al. 2008). These receptors are present in many non-skeletal tissues including the intestinal epithelial cells, pancreatic β cells and immune cells (Wang et al. 2012). Membrane-bound vitamin D receptors (mVDR) (Vuolo et al. 2012; Huhtakangas et al. 2004) and cytosolic VDRs (Trochoutsou et al. 2015) are also involved in vitamin D action (Huhtakangas et al. 2004; Trochoutsou et al. 2015) through induction of rapid, non-genomic effects (Haussler et al. 2011; Hii and Ferrante 2016). Non-genomic actions of 1,25$(OH)_2$D include the activation of signalling molecules (Vuolo et al. 2012), rapid generation of second messengers, activation of protein kinases and opening of ion channels (Hii and Ferrante 2016). Moreover, non-genomic pathways regulate a wide range of 1,25$(OH)_2$D-mediated genomic and cellular functions (Hii and Ferrante 2016).

Individual vitamin D status is evaluated by circulating serum levels of 25(OH)D. Inadequate 25(OH)D, defined as < 50 nmol/L, is commonly observed across the world (Daly et al. 2012), with the greatest levels of insufficiency occurring during winter months (Gill et al. 2014; Pittaway et al. 2013; Nowson et al. 2012). Despite the temperate Australian climate, 25(OH)D inadequacy is found in one third of adults (Daly et al. 2012; Nowson et al. 2012). The appropriate level of circulating 25(OH)D required for good health is highly disputed; while some research groups argue that 50 nmol/L is sufficient (Ross et al. 2011; Peterlik 2012), others have proposed that this value should be raised to 75 nmol/L (Heaney 2008; Vieth 2011) and recommendations for optimal status as high as 90-100 nmol/L have been made (White 2012; Tran et al. 2013; Bischoff-Ferrari et al. 2006). Geographical location, season, sex and adiposity are well-documented to influence status (Verdoia et al. 2015; Malvy et al. 2000; Palacios et al. 2012). Typical seasonal variations in Australia are 10-20 nmol/L (Gill et al. 2014; Nowson et al. 2012; Pittaway et al. 2013). In contrast, the circulating concentration of 1,25$(OH)_2$D is regulated in vivo through tight homeostatic

mechanisms (Holick 2007). Local generation of 1,25$(OH)_2$D from its less active circulating precursor, 25(OH)D, is believed to be important for regulatory function (Zhang et al. 2012b; White 2012; Hewison 2011).

Vitamin D in energy metabolism

A role for vitamin D in energy metabolism is plausible, but is not yet well explored. The VDR has been detected in the mitochondria of human platelets (obtained from fresh blood) and megakaryocytes (MEG-01 cell line) (Silvagno et al. 2010). In cell line studies, the translocation of nVDR to mitochondria in keratinocytes, has been demonstrated (Silvagno et al. 2013). Through genetic silencing of the VDR in a human keratinocyte cell line, the VDR was found to inhibit oxidative phosphorylation (Consiglio et al. 2014). Such effects may signal the potential to alter mitochondrial function and cellular bioenergetics. Mice lacking nVDR, known as VDR null mice, gained less FM on a high fat diet through increased EE, greater fatty acid oxidation and UCP expression (Wong et al. 2009). As to be expected, overexpression of the VDR, specifically in the AT, resulted in increased FM, reduced EE, and reduced fat oxidation (Wong et al. 2011). In contrast, mice supplemented with cholecalciferol for 10 weeks were protected against diet-induced obesity via enhanced EE and fat oxidation (Marcotorchino et al. 2014). Injection of 1,25$(OH)_2$D into the peritoneal cavity of rats on alternating days for 8 weeks reduced body weight gain following a high fat diet, inhibited lipogenesis and promoted fat oxidation (Yin et al. 2012). The origin of this discrepancy is presently not understood and requires further investigation.

Human supplementation studies examining the impact of cholecalciferol on EE are scarce (Soares et al. 2012). Cholecalciferol supplementation in 10 non-obese men for 1 week, increased 1,25$(OH)_2$D but did not impact substrate oxidation or EE (Boon et al. 2006). A meta-analysis consisting of 12 high quality RCTs examined the impact of cholecalciferol supplementation on body weight and multiple fat indices (FM kg, fat mass percentage, BMI). No differences between treatment and placebo groups were observed (Pathak et al. 2014). There is a need for future studies to investigate the impact of cholecalciferol supplementation and/or 25(OH)D on whole body EE and substrate utilisation.

Vitamin D in immunity and inflammation

The importance of sufficient 25(OH)D for resistance to infection in humans has long been appreciated. Seasonal oscillation of influenza is prominent, with explosive rates during winter coinciding with the seasonality of 25(OH)D. It has been hypothesized that the seasonal fluctuation of 25(OH)D concentrations may affect the seasonal pattern of influenza (Cannell et al. 2008). In a RCT, 104 post-menopausal African American women supplemented with cholecalciferol (800IU/d) were three times less likely to report cold and flu symptoms than 104 placebo controls (Aloia and Li-Ng 2007). Interestingly, this seasonal variation in 25(OH)D also coincides with seasonal variations in blood pressure (Rostand 1997), $HbA1_C$ (Tseng et al. 2005) and circulating lipids (Ockene et al. 2004), indicating a potential association between seasonality of 25(OH)D and chronic metabolic disease. However, there is a lack of reliable information to indicate whether seasonal variation in 25(OH)D positively or negatively influences health (Lucas and Neale 2014).

Cellular and animal models present many indications of a role for vitamin D in modulating inflammation. The presence of the VDR (Khoo et al. 2011; Mocanu et al. 2013) and the enzymatic machinery necessary to locally produce $1,25(OH)_2D$ within immune system cells, suggests a role for the vitamin in immune function (O'Brien and Jackson 2012). In activated macrophages, proinflammatory cytokines are major drivers for CYP27B1 expression and activity (Bikle 2010). $1,25(OH)_2D$ has been shown to enhance the antimicrobial actions, promote chemotaxis and phagocytic capabilities in macrophages (Baeke et al. 2010). PBMCs (Jirapongsananuruk et al. 2000; Sadeghi et al. 2006) and human-derived cell lines (Matilainen et al. 2010a; Matilainen et al. 2010b) have been used to study the impact of $1,25(OH)_2D$ on cytokine expression and production with anti-inflammatory effects found. In adipocytes, $1,25(OH)_2D$ inhibits inhibitor kappa-B alpha (IκBa) phosphorylation and subsequent activation of the NFκB signalling pathway. $1,25(OH)_2D$ also inhibits inflammation by preventing p38 MAPK phosphorylation. Gene transcription and protein production of proinflammatory factors IL-1β, IL-6, IL-8, MCP-1, and CCL5, were also suppressed (Ding et al. 2013).

In contrast, the link between 25(OH)D and low grade systemic inflammation in human studies is unclear. A population-based study of older individuals showed an inverse

relationship between 25(OH)D and pro-inflammatory makers, suggesting a potential anti-inflammatory role in older individuals (De Vita et al. 2014). Another population-based study, found the association between 25(OH)D and inflammatory biomarkers depends on the marker utilised and smoking status (Mellenthin et al. 2014). A U-shaped association between 25(OH)D and high-sensitivity C-reactive protein (hs-CRP) indicated that higher (> 62.5 nmol/L) 25(OH)D concentrations may be related to a pro-inflammatory state. Few RCTs utilising ergocalciferol or cholecalciferol supplementation have been conducted in humans and of those which have been conducted, methodology is vastly different between trials. These differences include study duration, vitamin D metabolite used for supplementation (ergocalciferol or cholecalciferol), dose, route of supplementation, presence of simultaneous interventions, inflammatory markers measured, study population demographics, assay technique used to measure 25(OH)D status, varying degree of change in 25(OH)D achieved, and different absolute levels achieved (Witham et al. 2010; Zittermann et al. 2009; Bischoff-Ferrari et al. 2012; Rahimi-Ardabili et al. 2013; Barker et al. 2015). Two meta-analyses have been conducted to examine the impact of 25(OH)D on systemic inflammation. The first meta-analysis to be conducted concluded that cholecalciferol supplementation reduced hs-CRP (Chen et al. 2014). However, a small number of RCTs were included and only the impact of supplementation on hs-CRP was investigated which is concerning as there is no consensus on which inflammatory marker best represents chronic low-grade inflammation (Calder et al. 2011). The second meta-analysis utilsied RCTs conducted in overweight/obese children and adults and found no benefit of cholecalciferol/ergocalciferol supplementation on CRP, IL-6 or TNF-α levels (Jamka et al. 2015). However, the review included trials that had implemented additional dietary interventions. The scarcity of good quality RCTs and reliance on poor quality studies limit these previously conducted systematic reviews.

Energy metabolism and inflammation

Inflammation has an energy cost, which is believed to be due to immune cell activation requiring energy for protein synthesis, inflammatory mediator release, and cell division (Keane et al. 2015; Straub 2011). A raised RMR is seen in metabolic conditions hallmarked by chronic inflammation such as T2DM and MetS (Weyer et al. 1999; Huang et al. 2004) and RMR has been shown to decline in those who recover

from MetS (Soares et al. 2011). Mechanistically there is a close positive relationship between mitochondrial activity and RMR in humans (Larsen et al. 2011).

Mitochondria have an important role in pro-inflammatory signaling; similarly, pro-inflammatory mediators may also alter mitochondrial function. Both of these processes increase mitochondrial oxidative stress, promoting a vicious inflammatory cycle (Brookes et al. 2004). Mitochondrial dysfunction is a recognised feature in IR, obesity and T2DM (Crescenzo et al. 2014), through a process which promotes low-grade inflammation via excessive ROS generation (Murphy and Siegel 2013) and damage to mitochondrial deoxyribonucleic acid (mtDNA) (Pieczenik and Neustadt 2007). Aside from the nucleus, the mitochondria are the only cell organelle to contain DNA. Unlike in the nucleus, the DNA is not protected by histones which physically protect DNA from free radical damage and can repair damaged DNA (Pieczenik and Neustadt 2007). Mitochondrial dysfunction can be due to either changes in the number and density of mitochondria (Abdul-Ghani and DeFronzo 2008) or reduced function, specifically reduced oxidative capacity (Ritov et al. 2005; Mogensen et al. 2007) and elevated ROS production. Under pathological conditions, an excess of superoxide (O_2^-) is produced, with superoxide dismutase unable to convert all of the anion to the more stable hydrogen peroxide (H_2O_2) within a rapid time frame (Pieczenik and Neustadt 2007). This accumulation causes the activation of redox-sensitive transcription factors, such as the key regulator of tissue inflammation, NFκB, and a subsequent increase in the expression of cytokines, chemokines, and adhesion molecules occurs (Chung et al. 2009), followed by increased production of these factors.

The first peer-reviewed publication forms the next part of the exegesis and is a narrative review summarising the literature at the commencement of the PhD program which informed the thesis objectives.

The content of this chapter is partially covered by Paper 1:
Calton, E. K., K. N. Keane, and M. J. Soares. 2015. "The potential regulatory role of vitamin D in the bioenergetics of inflammation." *Curr Opin Clin Nutr Metab Care* 18 (4):367-73

REVIEW

The potential regulatory role of vitamin D in the bioenergetics of inflammation

Emily K. Calton[a], *Kevin N. Keane*[b], *and Mario J. Soares*[a]

Purpose of review
The extraskeletal health benefits of vitamin D still need scientific endorsement. Obesity and related chronic diseases are pathogenically linked by inflammation, which carries a considerable energetic cost. Recent techniques for the determination of the bioenergetic demand of inflammation, offer an avenue to cement the regulatory role of vitamin D in this process.

Recent findings
Nuclear vitamin D receptors may be translocated into mitochondria of certain cell types, opening up a pathway for direct action on cellular bioenergetics. Classical M1 (inflammatory)/M2(anti-inflammatory) phenotypes can vary with the clinical context. M2 macrophages do not always depend on oxidative metabolism/fatty acid oxidation. Newer methodologies offer real-time bioenergetic measurements that can be used as an index of metabolic health.

Summary
Vitamin D may prove to be a therapeutic agent for inflammation of chronic disease and understanding its role in cellular bioenergetics may offer a diagnostic/prognostic indicator of its action.

Keywords
bioenergetics, energy metabolism, inflammation, macrophage, obesity, vitamin D

INTRODUCTION

Vitamin D insufficiency is a global issue and clusters with numerous chronic diseases including obesity, type 2 diabetes mellitus (T2DM) and even some cancers [1▪]. However, the current evidence base supports the micronutrient mainly for its effects in preventing osteoporosis and related conditions. The majority of biological functions associated with vitamin D are mediated through the regulation of gene expression. The active metabolite of vitamin D 1,25-dihydroxyvitamin D (1,25(OH)2D3) binds to its nuclear receptor (nVDR) with high affinity and specificity. These receptors are present in the majority of nonskeletal tissues including the brain, pancreas and macrophages [2▪,3▪]. The vitamin D-nVDR forms a heterodimer with the retinoid X receptor [4▪] and this complex amplifies or represses transcription of the target genes through its binding to vitamin D responsive elements. In this fashion, nVDR activation may primarily regulate the expression of between 200 and 600 genes. As most of these genes respond to vitamin D in a cell-specific fashion; the total number of vitamin D targets in the human genome is believed to be much higher [5▪].

Some immediate vitamin D action occurs in cells that possess the membrane vitamin D receptor (mVDR) and this modulates the synthesis of parathyroid hormone [6▪]. More recent developments include the translocation of nVDR to mitochondria in platelets, megakaryocytes and keratinocytes, through involvement of functional permeability transition pore activity [7▪▪]. Such effects may signal the potential to alter mitochondrial function and indeed, cellular bioenergetics. Not surprisingly, VDR has been likened to a 'gatekeeper' of mitochondrial respiratory chain activity [8▪▪]. Many cells of the body, including the immune cells, have the capacity to locally produce 1,25(OH)2D3 [9▪▪,10▪]. This production most likely depends on the availability of circulating 25(OH)D3 as a substrate. In deficiency

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KEY POINTS

- Vitamin D has regulatory roles in both immune function and energy metabolism.
- Nuclear vitamin D receptor is translocated into mitochondria of certain cell types, raising the possibility of a direct impact of vitamin D on cellular bioenergetics.
- Circulating peripheral blood mononuclear cells are a useful model for studying metabolic dysfunction in chronic noncommunicable disease states.
- Aberrant bioenergetics in obesity and T2DM may be dynamically reflected in a new parameter: the Bioenergetic Health Index.

and insufficiency states, 25(OH)D3 is unavailable to the macrophage CYP27B1-hydroxylase, therefore, insufficient 1,25(OH)2D3 is generated locally. This, in turn, limits binding of 1,25(OH)2D3 to the macrophage VDR, thereby limiting the activation of 1,25(OH)2D3-VDR-directed genes. Hence, determining the precise circulating level of 25(OH)D3 would be critical to extraskeletal health effects, such as the modulation of inflammation.

A chronic low-grade inflammation is an important underlying characteristic of obesity and related chronic diseases such as the metabolic syndrome (MetS), T2DM and cardiovascular disease [11▪▪]. Mitochondrial dysfunction has been implicated in these disorders [12▪], through a process that promotes low-grade inflammation via excessive reactive oxygen species (ROS) generation [13▪▪]. There is an energy cost associated with inflammation [14] that is ascribed to the increased energetic demand of many inflammatory cells [15▪]. This link between energy metabolism and the immune system is both physiological and biochemical. Tissues of the body such as adipose tissue and the liver have an architectural organisation in which metabolic and immune cells are in close proximity to one another. In addition, both have immediate access to a vast network of blood vessels allowing for cross-communication between the two systems, both locally and at other sites of the body.

It is, therefore, tempting to speculate that adequate vitamin D status would improve inflammatory cell bioenergetics and possibly lessen whole body energetic demand, as part of its immunomodulatory role. In this article, we provide an overview of the link between energy metabolism and inflammation, while targeting recent advances in the bioenergetics of peripheral blood mononuclear cells. These cells are expected to reflect metabolic dysfunction in much the same fashion as adipose tissue, skeletal muscle and liver; thereby, providing important insights into the clinical significance of adequate vitamin D status.

Diet, activated macrophages and adiposity

A Westernized Dietary Pattern is characterized by excessive consumption of energy, salt, sugar, saturated fat and low intakes of healthy fats and antioxidants [16▪]. This dietary pattern is found in most developed countries and is increasingly seen in Asian-Pacific countries [17▪]. A diet high in saturated fatty acids leads to macrophage infiltration into adipose tissue and insulin resistance [18▪] (See Fig. 1). It is believed that these fatty acids serve as ligands for Toll-like receptor (TLR) 2 and 4 on the surface of macrophages and adipocytes, resulting in proinflammatory gene transcription through activation of NF-κB, the c-jun N-terminal Kinase and p38 mitogen-activated protein kinase (MAPK) signaling cascades. Continuous stimulation of these pathways results in a chronic state of inflammation and insulin resistance [18▪]. In contrast, docosahexaenoic acid has an anti-inflammatory effect through several mechanisms. Recently, activation of cytosolic phospholipase A2 and cyclooxygenase 2 (COX-2) have been suggested as potential pathways [19▪]. Prostaglandin A2, the dehydrated product of prostaglandin E2, may inhibit NF-κB signaling by preventing phosphorylation and degradation of the NF-κB subunit inhibitor kappa-B alpha (IκBα), similar to prostaglandin A1 [20]. Antioxidants such as resveratrol, also have anti-inflammatory effects on the circulating macrophage through downregulation of NF-κB pathway and decreased cytokine release [21▪,22▪]. A conceptual scheme is presented in Fig. 1.

Adipose tissue undergoes hypertrophic enlargement in obesity, which results in an imbalanced blood flow leading to inflammation and macrophage infiltration [23▪]. The hypertrophied adipocytes are characterized by increased secretion of several proinflammatory cytokines such as tumor necrosis factor (TNF-α), interleukin (IL) 6, IL-8, IL-12, C-reactive protein, macrophage-chemoattractant protein-1 and resistin with a reduced secretion of the anti-inflammatory adiponectin and IL-10. In addition, proinflammatory cytokines are also released by macrophages residing within adipose tissue and infiltrating macrophages from circulation. If unchecked a feed forward loop is established that amplifies the release of cytokines into systemic circulation.

Vitamin D and inflammation

The biology underlying the effect of vitamin D on inflammatory processes has been investigated in

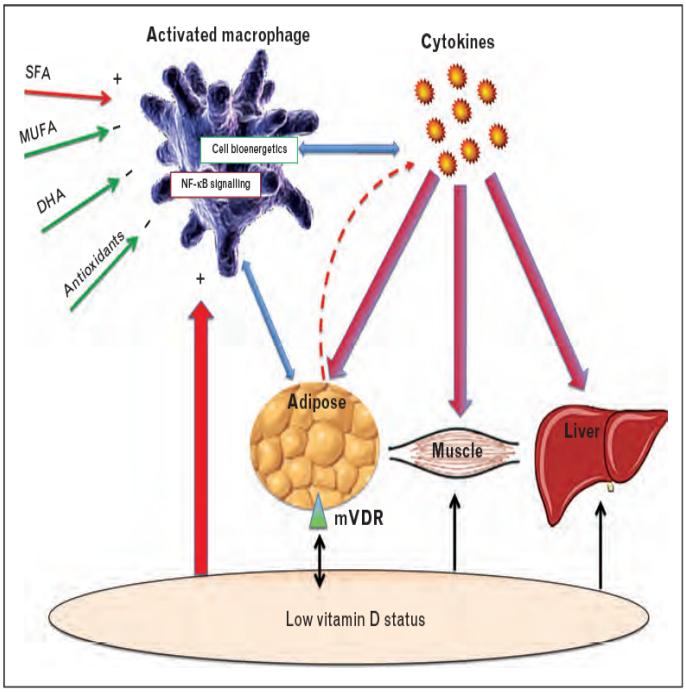

FIGURE 1. A conceptual scheme linking dietary components and low vitamin D status to inflammation. Chronic exposure to high nutrient load including saturated fatty acids (SFA) can activate circulating macrophages to release proinflammatory cytokines. This would lead to a low-grade inflammation and immune cell infiltration into peripheral tissues, which is perpetuated in individuals with low vitamin D status. Adipose tissue can also release adipokines that activate macrophages inducing a vicious proinflammatory cycle, whereas monounsaturated fatty acids (MUFA), docosahexaenoic acid (DHA) or high-levels of antioxidants induce anti-inflammatory conditions.

cell-culture models of adipose tissue and circulating macrophages [4▪,24▪▪]. Mechanisms established have included attenuating p38 activation, blocking NF-κB activation and downregulating concentration of matrix metalloproteinases among others [25▪]. 1,25(OH)2D3 inhibits IκBα phosphorylation and subsequent translocation of NF-κB and p38MAPK into the nucleus [26▪,27▪,28▪▪,29▪▪]. Thus, gene transcription of the proinflammatory factors are also suppressed. Vitamin D also has anti-inflammatory effects in other tissues of the body including the pancreas [30▪] and the liver [31▪].

Cross-sectional studies and the few human clinical trials investigating the links between vitamin D status and systemic inflammatory markers do not provide consistent outcomes [32▪–35▪]. In part, this arises from the different inflammatory markers measured, varying study populations and the variety of assay techniques used. Commensurate with these observations is the need to account for the significant interindividual variation of the change in serum vitamin D from the same dose of vitamin D. Hence, it is unclear whether a defined change in vitamin D status or the absolute level achieved, is important to vitamin D-related outcomes. In future, the protocol of vitamin D dosing may be central to clinical studies exploring extraskeletal effects of the vitamin.

The energetics of inflammation

The regulation of energy metabolism and inflammation share common pathways including peroxisome proliferator-activated receptors, TLRs, fatty acid-binding proteins [36] and signaling factors such as ROS. Furthermore, many products of metabolic and immune cells, such as hormones and cytokines, function in both metabolic and immune pathways [37▪,38]. Inflammatory cytokines regulate metabolic homeostasis [36]. For instance, TNF-α induce macrophages to adopt a proinflammatory M1 phenotype whereby ATP is generated primarily through anaerobic respiration in the cytoplasm and glucose is converted into lactate. In contrast, anti-inflammatory cytokines stimulate macrophages to acquire an anti-inflammatory M2 phenotype that depends on aerobic catabolism of glucose via the oxidative phosphorylation pathway [39]. This classical dichotomy is seen in murine models of inflammation. However there is good reason to

believe that depending on the clinical situation, M1 or M2 may reversibly assume an inflammatory or anti-inflammatory phenotype in humans [40▪]. Moreover, there is intriguing data to suggest that in human M2 macrophages, the reliance on oxidative metabolism and fatty acid oxidation is not absolute, instead gluconeogenesis may predominate [41▪▪]. Hence, the area of macrophage activation/polarization is an evolving one and will offer significant insights into the bioenergetics of these cells. Other white blood cell types also have a typical bioenergetic profile with each cell type utilizing glycolysis and oxidation phosphorylation to varying extents. For example, monocytes use high levels of both glycolysis and oxidative phosphorylation, whereas neutrophils use glycolysis with very little oxidative phosphorylation. Both lymphocytes and platelets rely more on oxidative phosphorylation and less on glycolysis, with lymphocytes having the lowest absolute value for glycolysis [42▪▪].

At the onset of weight gain macrophages convert to an M1 proinflammatory phenotype [36] and they are believed to be the primary source of diet-induced inflammation. The elevated levels of cytokines increase energy expenditure, perhaps in an effort to offset weight gain [15▪], and mitochondrial uncoupling may serve as a potential pathway in this process. The M1 macrophages continue to reside in the expanded adipose tissue mass unless weight loss is achieved or physical activity is increased. Following either intervention they revert to an M2 phenotype [43▪]. It was interesting, to note, that following weight loss, those who recovered from the MetS had a significantly lower adjusted basal metabolic rate compared with those who did not recover, or compared with those who had never suffered from MetS [44]. Clearly, there is a need for future clinical trials to integrate changes in whole body energy metabolism and the bioenergetics of cellular inflammation.

Chronic inflammation is an energetically expensive process. The early work of Newsholme *et al.* [45] validated the high use of glucose and glutamine in macrophages. Peripheral blood mononuclear cells are now readily used for assessing metabolic function as these cells are exposed to the systemic environment and are easily accessible through blood collection rather than invasive tissue biopsy [46▪▪,47▪▪]. Importantly, metabolic changes in these blood cells may mirror the altered mitochondrial respiratory capacity and function seen in classically studied tissues such as skeletal muscle, adipose, liver and the pancreas. There are many in-vitro and in-vivo techniques available to study the metabolism of body tissues as well as cells. Recent technological advances such as extracellular flux analysers (e.g. Seahorse Bioscience XF^{e}96 flux analyser), make the measurement of oxygen consumption and proton production in real-time, a convenient method to measure ATP turnover and mitochondrial function. The technique requires smaller numbers of cells compared with traditional methods such as the Clark electrode [48]. Overweight and obese participants with T2DM show impaired bioenergetics in platelets, as evidenced by higher basal and maximal oxygen consumption rate, consistent with a higher production of ROS [46▪▪].

Mechanisms for a vitamin D effect on bioenergetics

A link between vitamin D status and the mitochondrial oxidative phosphorylation pathway in skeletal muscle has recently been demonstrated using P-magnetic resonance spectroscopy [49▪▪,50▪▪]. Vitamin D supplementation resulted in improved vitamin D status and bioenergetics (specifically improved maximal respiratory capacity) in those who were vitamin D deficient [50▪▪]. However, the precise mechanism for vitamin D effect was not investigated. It was speculated that vitamin D was able to increase calcium uptake by the mitochondria or perhaps vitamin D acted through genomic pathways. Mitochondria are important organelles for metabolism and ATP supply, but they also hold a critical position in regulating other cellular processes including apoptosis, calcium homeostasis, steroid biosynthesis and immunity, with the latter three processes closely associated with Vitamin D status. Interestingly, the generation of mitochondrial-derived ROS (mtROS) is important for cytokine production and release in immune cells, as ROS is considered a signal transducer, and can consequently influence activation of immunological signaling pathways such as, NF-κB, Jun N-terminal Kinase (JNK) and MAPK (Fig. 2) [51]. mtROS levels are very much dependent on metabolic regulation and electron transport chain activity, but also calcium homeostasis. Studies have shown that TNF-α can promote inflammation by shedding of the TNF receptor from the immune cell membrane through a pathway that is dependent on mtROS generation and calcium influx into the mitochondria [52]. Inhibition of this calcium transport prevents TNF receptor shedding and alleviates the proinflammatory condition. Vitamin D is a known regulator of calcium influx via channels such as transient receptor potential family located in gut-epithelial cells, and these transporters function to increase calcium absorbance. However, we speculate that vitamin D, via its interaction with mitochondrial translocated VDR, may modulate calcium influx into the mitochondria through similar salt transporter channels, or possibly

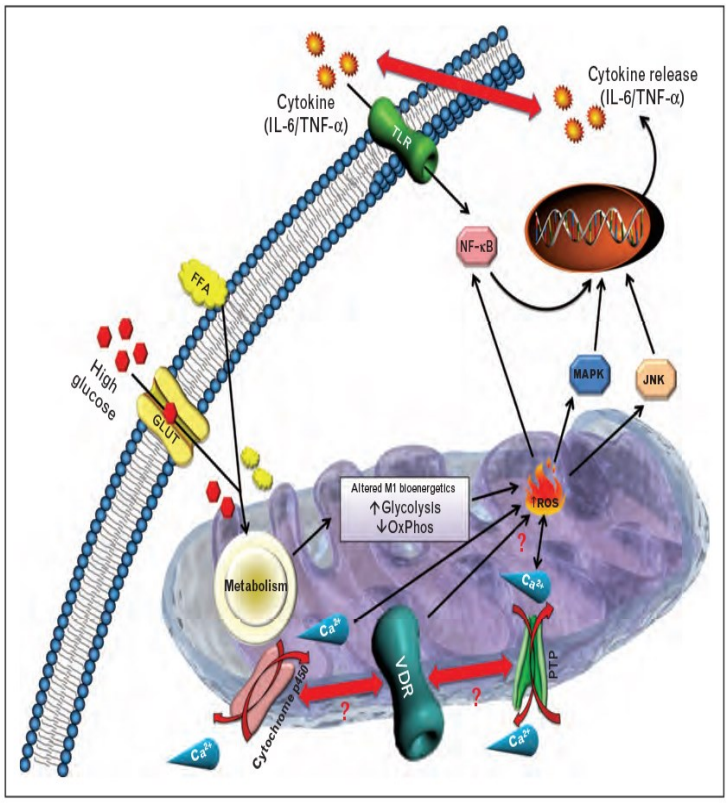

FIGURE 2. A mechanistic model of potential vitamin D actions in mitochondria of inflammatory cells. Metabolism of chronically high nutrient loads, including high glucose and free fatty acids (FFA), can promote proinflammatory conditions and cytokine release via generation of ROS and subsequent activation of NF-κB, MAPK or JNK signaling. The model proposes that vitamin D plays an immune-modulatory role through translocation of the vitamin D receptor (VDR) to the mitochondria, wherein it possibly alters cell bioenergetics through modulation of mitochondrial calcium influx.

through the cytochrome p450 systems (Fig. 2). Collectively, vitamin D may regulate immune cell cytokine production through a novel mechanism of mitochondrial metabolism and mtROS generation. These studies are currently underway in the authors' laboratory.

Future approaches to assessing metabolic health

It has recently been hypothesized that dysfunctional energetics associated with diabetes, cardiovascular disease, liver disease, cancer and environmental toxins can be dynamically assessed in patient populations through a new parameter: the Bioenergetic Health Index (BHI). This index incorporates various mitochondrial bioenergetic measurements and has the potential to act as a personalized measurement of health status, both as a diagnostic and prognostic marker [53▪▪]. Healthy patients would have a high BHI with low uncoupled respiration, low nonmitochondrial respiration (e.g. low ROS generation) or an enhanced ability to meet energy demand via increased ATP-coupled respiration or reserve capacity. [53▪▪].

Chronic metabolic stress, however, will induce damage in the mitochondrial respiratory machinery by progressively decreasing mitochondrial function. This would manifest as a low BHI, which is associated with a low reserve capacity, increased uncoupled respiration and/or nonmitochondrial respiration and elevated ROS. These bioenergetically inefficient damaged mitochondria require higher levels of ATP for maintaining organelle integrity, which increases the basal oxygen consumption. The persistence of unhealthy mitochondria damages the mtDNA, impairs the integrity of the biogenesis programme, leading to a progressive deterioration in bioenergetic function [53▪▪]. Our recent study has revealed that BHI measurements in circulating leukocytes are sensitive to cryopreservation [54]. When cells were cryogenically stored for more than 4 weeks, the BHI substantially decreased. This suggests that defects were present either in the electron transport chain machinery or that the cells

were adapting by enhancing their glycolytic activity [53▪▪]. Consequently, the way in which primary cells are handled and their duration of cryopreservation must be carefully planned. Nevertheless, the BHI as a concept for chronic disease diagnosis and/or prognosis is an exciting one and will progress this area.

CONCLUSION

Vitamin D has regulatory roles in both immune function and energy metabolism. Recent findings that VDR may be translocated to the mitochondria highlight the distinct possibility of an impact of vitamin D on cell bioenergetics, either directly or through its established influence on calcium metabolism. Future studies need to confirm the immune-modulatory action of vitamin D and its twinning with energy metabolism. The BHI is a novel and dynamic measure derived from circulating immune cells. It holds promise as a diagnostic indicator for chronic disease and in ascertaining the response to newer treatment modalities.

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Conflicts of interest

There are no conflicts of interest.

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Knowledge gaps

- After accounting for FFM, FM, age, sex and ethnicity, there still remains significant inter-individual variation in adult RMR which are not understood. Addressing the underlying causes of this variation is essential, particularly if such factors are modifiable through therapeutic interventions.
- A role for 25(OH)D in energy metabolism is plausible, but has not been well explored. Human studies investigating the link between 25(OH)D and whole-body bioenergetics are scarce. Circulating PBMCs are exposed to metabolic and immunological stimuli that influence their functionality. It is unknown whether *in vivo* 25(OH)D status modulates the resting bioenergetic profile of *ex vivo* PBMCs. Investigating this hypothesis will elucidate a role for the vitamin in cellular energy metabolism.
- Circulating 25(OH)D varies with season and this mimics the oscillation of a number of chronic disease risk markers. Seasonal variation in 25(OH)D and the seasonal changes in cellular bioenergetics, whole body energy metabolism and inflammation have not been previously described.
- Both human-derived PBMCs and cell lines have been used to investigate the immune-modulating effect of $1,25(OH)_2D$. To date, a systematic review that comprehensively assesses the evidence for an anti-inflammatory effect of $1,25(OH)_2D$ and/or 25(OH)D in PBMCs and immune cell lines has not been conducted.
- Results of previously conducted meta-analyses investigating the impact of cholecalciferol supplementation on inflammatory markers are inconsistent. RCTs investigating the impact of supplementation on systemic inflammatory markers are vastly different in methodology. These differences include study duration, vitamin D dose, vitamin D metabolite used for supplementation, route of supplementation, presence of simultaneous interventions, different inflammatory markers measured, varying study populations, variety of assay techniques used to measure 25(OH)D, varying degree of change in 25(OH)D achieved, and different absolute levels achieved. Therefore, there is a need to investigate the impact of the methodological factors identified above in order to comprehensively assesses the evidence for an anti-inflammatory effect of the vitamin in humans.

Research aim

The broad hypothesis on which this PhD thesis is based is that circulating vitamin D [25(OH)D or 1,25$(OH)_2$D] modulates bioenergetics (whole body and cellular) and inflammation both directly and through indirect cross-communication between energy metabolism and inflammatory pathways.

The overall aim of the thesis was to study explore the inter-relationships between vitamin D, energy metabolism and inflammation in adult humans at both the whole-body and cellular level. Specifically, the effects of cholecalciferol and/or 25(OH)D and/or 1,25$(OH)_2$D on whole body energy metabolism, the bioenergetic profile of isolated PBMCs, the inflammatory status of human-derived cell lines and PBMCs and the inflammatory marker concentration in circulation were investigated.

Thesis objectives

1. To carry out a comprehensive systematic review that examines the impact of vitamin D metabolites [cholecalciferol, 25(OH)D, 1,25$(OH)_2$D] on the inflammatory profile of human-derived immune cell lines and *ex vivo* PBMCs.
2. To investigate whether 25(OH)D contributes to whole-body bioenergetics (RMR) in adults.
3. To determine whether *in vivo* 25(OH)D is associated with *ex vivo* PBMC bioenergetics.
4. To investigate whether seasonal variations in 25(OH)D are associated with seasonal changes in whole body energy metabolism, circulating PBMC bioenergetic profiles and markers of systemic inflammation.
5. To examine the causal link between 25(OH)D and the systemic inflammatory profile in adults.

Discussion

Main findings

Through a collection of studies, the findings of this thesis demonstrate that 25(OH)D levels are associated with whole-body and cellular bioenergetics (Chapter 3, Chapter 4 and Chapter 5). Chapter 3 demonstrated that 25(OH)D was a significant positive predictor of whole-body bioenergetics in a Caucasian adult Australian population. In contrast, low 25(OH)D (< 50 nmol/L) was associated with increased PBMC bioenergetic responses in Chapter 4. Reasons for the seemingly conflicting findings may be the different study population used and varied methods for measuring bioenergetic parameters (cellular vs whole body and different equipment used for each approach). Although season of data collection varied, the mean (SD) 25(OH)D obtained in each study were similar [Chapter 3 study 58.89 (23.48) nmol/Lvs Chapter 4 study 65.61 (22.87) nmol/L. As Chapter 3 measured whole body bioenergetics and Chapter 4 measured PBMC bioenergetics, it cannot be ruled out that 25(OH)D has different bioenergetic effects in various tissues and cells of the body. In Chapter 4, it was demonstrated that *in vivo* circulating 25(OH)D was inversely associated with *ex vivo* PBMC cell bioenergetic responses. Furthermore, significantly greater bioenergetics responses in those with 25(OH)D < 50 nmol/L were observed compared to those with status > 50 nmol/L. It appeared that this effect may plateau above 50 nmol/L, since the group > 75 nmol/L showed no further change in these bioenergetic parameters. Similarly to Chapter 4, the findings of Chapter 5 also suggests there may be an effect of circulating 25(OH)D on PBMC bioenergetics. Chapter 5 demonstrated a concurrent observation of increased 25(OH)D and lowered bioenergetic parameters in summer as compared to winter. Seasonal increases in 25(OH)D significantly reduced bioenergetic responses such that greater improvement was seen in those who normalized their status to ~ 50 nmol/L in summer, while those who already had achieved values > 50 nmol/L in winter showed no further improvements in summer. With the findings of Chapters 4 and 5 interpreted together, reaching and maintaining 25(OH)D status > 50 nmol/L year-round may be important for bioenergetic function in PBMCs.

In Chapter 3, IS was reported to be a negative determinant of RMR and a direct positive relationship was found between IS and 25(OH)D. In Chapter 4, an association was

also found between IS and 25(OH)D, with greater IS in the highest 25(OH)D group. Furthermore, as the links between bioenergetics, inflammatory markers and whole-body bioenergetics disappeared on adjustment for QUICKI, whole body IS may be implicated in the derangement of bioenergetics associated with 25(OH)D. In Chapter 5, IS was reported to be greatest in summer compared to winter, which mirrored the seasonal pattern in 25(OH)D. However, despite adjusting for IS in this study, the relationship between bioenergetic responses and 25(OH)D remained. The relationship between 25(OH)D, IS and bioenergetics will require intervention studies to establish cause-and-effect.

The thesis clearly demonstrates that the active vitamin D metabolite, $1,25(OH)_2D$, exerts an anti-inflammatory effect on cytokine expression and production, in both human-derived immune cell lines and PBMCs originating from humans (Chapter 2). However, whether 25(OH)D modulates immune cell inflammation is unclear, as few studies used this form of vitamin D. Increasing circulating 25(OH)D levels can be achieved through sun exposure, diet or supplementation and this will increase the availability of substrate to immune cells for local production of the active metabolite. In Chapter 5, it was reported that all inflammatory markers significantly decreased with season from winter to summer, with the exception of CRP which did not change. Despite this, only the seasonal increase in 25(OH)D was positively associated with the anti-inflammatory marker IL-10. As multiple systemic inflammatory markers decreased within each tertile of 25(OH)D, this suggests that higher levels of 25(OH)D may be required to dampen inflammation than to modulate bioenergetic responses. If this is confirmed by future studies, optimal 25(OH)D will depend on the specific extra-skeletal effect and future recommendations for 25(OH)D will need to be directed at health across all systems of the body.

Given the clear anti-inflammatory effect of $1,25(OH)_2D$ observed in Chapter 2, it was surprising to find that modulating 25(OH)D through cholecalciferol supplementation did not influence the concentration of systemic inflammatory markers CRP or IL-6 in high-quality RCTs conducted in humans (Chapter 6). One possible explanation for this discrepancy is that cellular experiments allow greater control of the local environment while *in vivo*, the local response to supplementation by individual cells may be diluted out in the whole body, resulting in non-detectable changes. Interestingly, our meta-

analyses did suggest that a status ≥ 80 nmol/L was associated with a favourable reduction in CRP. This finding is in accordance with those of Chapter 5 suggesting that higher levels of 25(OH)D may be required to dampen inflammation than for bioenergetic function in PBMCs.

Significance and impact of publications

The body of research presented in this thesis presents several novel and interesting facets that further the understanding of immuno-metabolic processes modulated by vitamin D. It is acknowledged that while this research is still in its infancy, such studies may reveal therapeutically relevant targets for the treatment of the increasing pandemic of metabolic disease by manipulating the metabolic-immunological interface. Modulation of immune cell function/inflammation or targeting of bioenergetic function with vitamin D metabolites may present a valuable future strategy for therapeutic intervention.

No study had previously examined whole body and cellular bioenergetics within the same group of individuals (Chapter 4), nor tracked the same individuals over time (Chapter 5). This is a developing area of research in the field of whole body and cellular bioenergetics and may encourage further studies to link whole body and cellular bioenergetics of PBMCs or other cell types.

There is significant debate whether extra-skeletal benefits of vitamin D exist. The publication of Chapter 4 is the first to suggest there may be an effect of circulating 25(OH)D on PBMC bioenergetics. From the publications of Chapters 4 and 5, it appears that 25(OH)D should be kept above 50 nmol/L year-round. Intervention studies are warranted to confirm these findings in a larger group of individuals.

This thesis adds further evidence that BHI may be a prognostic or diagnostic indicator of health status. BHI, a proposed indicator of mitochondrial health status, was directly related to IS and inversely to RMR, and markers of systemic inflammation. This potentially validates a scenario where attenuation of systemic inflammation, improved IS, reduced whole body energetic demand and improved BHI are inter-linked. These novel findings will promote research in the area of immuno-metabolic health.

The systematic review described in Chapter 2 clearly demonstrates an anti-inflammatory effect of $1,25(OH)_2D$ on the cytokine expression and protein levels in human-derived immune cells. It was identified that few studies have investigated whether 25(OH)D modulates the inflammatory profile, and this may promote future research in the area.

Research limitations and future directions

Vitamin D is a broad term and is used throughout the literature to refer to a variety of metabolites. It is vital that the effects of cholecalciferol, ergocalciferol, 25(OH)D, $1,25(OH)_2D$ are clearly identified and differentiated.

Chapter 2 revealed there are very few studies which investigated whether 25(OH)D modulates parameters of inflammation, thus the impact of 25(OH)D on human-derived PBMCs and cell lines remains unclear. However, we and others (Zhang et al. 2012a) hypothesise that 25(OH)D levels are crucial since they influence local tissue concentrations of $1,25(OH)_2D$ (Hewison 2011). Therefore, future studies should investigate the potential role of various doses of 25(OH)D that reflect whole body circulating concentrations on inflammatory marker expression and production in human-derived immune cells.

As the impact of $1,25(OH)_2D$ on subsequent cell function was outside the scope of the systematic review of Chapter 2, whether the harmful effects of inflammation are able to be antagonised without affecting host defense functions remains to be investigated. Further studies are therefore required to determine the full effect of $1,25(OH)_2D$ on other parameters of immune and cellular function.

Chapters 3 and 4 both utilised a cross-sectional design and Chapter 5 an observational study design, therefore the work described in this thesis is unable to confirm a causal effect of 25(OH)D on bioenergetics. At most, the findings of these publications suggest a potential role for 25(OH)D in bioenergetic responses. While the results strongly support the further examination of studies aimed at correcting inadequate 25(OH)D and assessment of bioenergetic parameters. Well-designed high-quality RCTs are required to extend these findings and explore whether a causative relationship exists.

It is a limitation of the publications in Chapter 4 and Chapter 5 that a heterogenous population of immune cells was utilised, as each cell population has a unique bioenergetic profile. However, the use of a mixed population may provide a better index of the inflammatory status of the whole body than studying one specific type of immune cell. Moreover, cells *in vivo* do not exist in isolation, rather they circulate together and interact with one another. Never-the-less, future research should determine the causal influence of 25(OH)D on bioenergetics in isolated and purified populations of immune cells.

Both publications of Chapter 4 and Chapter 5 utilised relatively small sample sizes (n=38 and n=30, respectively). While this did not impact the ability to report significant findings in PBMC bioenergetic responses, the larger biological variability associated with the whole-body approach, relative to isolated cell systems, may have contributed to the non-significant decrease in RMR observed in the seasonal study described in Chapter 5. Although the hypotheses and findings of both of these studies are novel, it is crucial to assess whether similar results are found in other studies and larger sample sizes should be utilised when examining whole-body effects.

It must be acknowledged that measuring systemic cytokine concentrations as was described in Chapters 4-6, do not provide information on cytokine release at the local tissue level (Albers et al. 2005), thus local effects of 25(OH)D cannot be ruled out. Furthermore, we cannot eliminate the possibility that 25(OH)D modulates aspects of immune function other than cytokine release such as immune cell proliferation, differentiation and migration (Prietl et al. 2013), and future RCTs and cell studies should include such parameters in their design. Furthermore, there is a lack of studies which have investigated the impact of 25(OH)D on inflammatory markers other than IL-6 and CRP. This is important as there is currently no consensus at to which inflammatory marker best represents low grade inflammation.

The publications described in Chapter 4 and Chapter 5 did not explore the changes in protein or gene expression of inflammatory cytokines or energy metabolism components such as electron transport chain proteins, glucose transporters, or glycolytic enzymes, as the cellular material was limited. Further studies are therefore

required to determine the full effect of 25(OH)D on these parameters of immune and bioenergetic function.

Finally, future studies are required to investigate the potential for BHI to be a diagnostic indicator for chronic disease and ascertain the response to treatment modalities. Studies are required to develop the BHI calculation and ascertain whether the use of exponents to modify the relative weighting of the bioenergetic parameters should be utilised. Furthermore, an exploration of whether individual factors such as age and sex influence the BHI is also required.

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CHAPTER 2 - VITAMIN D PROMOTES AN ANTI-INFLAMMATORY CYTOKINE PROFILE IN HUMAN-DERIVED IMMUNE CELLS

The content of this chapter is covered by Paper 2:

Calton, E. K., K. N. Keane, P. Newsholme, and M. J. Soares. 2015. "The impact of vitamin D levels on inflammatory status: A systematic review of immune cell studies." *PLoS ONE* 10 (11):e0141770. doi: 10.1371/journal.pone.0141770.

Thesis objectives addressed in this chapter:

Objective 1: To carry out a comprehensive systematic review that examines the impact of vitamin D metabolites on the inflammatory profile of human-derived immune cell lines and *ex vivo* PBMCs.

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RESEARCH ARTICLE

The Impact of Vitamin D Levels on Inflammatory Status: A Systematic Review of Immune Cell Studies

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Abbreviations: (CRP), C-reactive protein; (IL-1β), interleukin-1β; (IL-2), interleukin-2; (IL-4), interleukin-4; (IL-6), interleukin-6; (IL-8), interleukin-8; (IL-10),

Abstract

Chronic low-grade inflammation accompanies obesity and its related chronic conditions. Both peripheral blood mononuclear cells (PBMCs) and cell lines have been used to study whether vitamin D has immune modulating effects; however, to date a detailed systematic review describing the published evidence has not been completed. We therefore conducted a systematic review on the effect of vitamin D on the protein expression and secretion of inflammatory markers by human-derived immune cells. The review was registered at the International Prospective Register for Systematic Reviews (PROSPERO, Registration number CRD42015023222). A literature search was conducted using Pubmed, Science Direct, Scopus, Web of Science and Medline. The search strategy used the following search terms: Vitamin D or cholecalciferol or 1,25-dihydroxyvitamin or 25-hydroxy-Vitamin D and Inflam* or cytokine* and supplement* or cell*. These terms were searched in the abstract, title and keywords. Inclusion criteria for study selection consisted of human-derived immune cell lines or cellular studies where PBMCs were obtained from humans, reported in the English language, and within the time period of 2000 to 2015. The selection protocol was mapped according to PRISMA guidelines. Twenty three studies (7 cell line and 16 PBMCs studies) met our criteria. All studies selected except one used the active metabolite $1,25(OH)_2$, with one study using cholecalciferol and two studies also using 25 (OH)D. Four out of seven cell line studies showed an anti-inflammatory effect where suppression of key markers such as macrophage chemotactic protein 1, interleukin 6 and interleukin 8 were observed. Fourteen of sixteen PBMC studies also showed a similar anti-inflammatory effect based on common inflammatory endpoints. Mechanisms for such effects included decreased protein expression of toll-like receptor-2 and toll-like receptor-4; lower levels of phosphorylated p38 and p42/42; reduced expression of phosphorylated signal transducer and activator of transcription 5 and decreased reactive oxygen species. This review demonstrates that an anti-inflammatory effect of vitamin D is a consistent observation in studies of cell lines and human derived PBMCs.

interleukin-10; (IL-12), interleukin-12; (IL-23), interleukin-23; (MCP-1), monocyte chemotactic protein 1; (nVDR), nuclear vitamin D receptor; (PBMCs), peripheral blood mononuclear cells; (PRISMA), preferred reporting items for systematic reviews and meta-analyses; (TNF-α), toll-like receptor (TLR), tumour necrosis factor α; (VDR), vitamin D receptor.

Introduction

Inflammation is recognised as the underlying characteristic of obesity and related chronic disease including type two diabetes [1–3] and cardiovascular disease [4–7]. In fact, inflammation may contribute to a multitude of chronic diseases [8]. Peripheral blood mononuclear cells (PBMCs) play a key role in the development and progression of obesity-related chronic diseases and have recently been suggested to be of potential use as biomarkers of health status [9–11]. Systemic inflammation is characterised by elevated levels of inflammatory biomarkers in the blood stream such as tumour necrosis factor α (TNF-α), interleukin-1β (IL-1β), interleukin-2 (IL-2), interleukin-6 (IL-6), interleukin-8 (IL-8) and interleukin-12 (IL-12).

Inadequate vitamin D status is common in many parts of the world [12] and is associated with obesity and related chronic disease [13–16]. The main source of vitamin D is through endogenous production, whereby solar UV-B irradiates 7-dehydrocholesterol present in the skin to generate cholecalciferol [17, 18], which is subsequently activated in the liver and kidney. The second source of vitamin D is dietary intake, which includes supplementation with either ergocalciferol (vitamin D_2) or cholecalciferol (vitamin D_3) [18]. Classifying vitamin D status is based upon the serum levels of 25(OH)D [17, 19]. However, the appropriate level of circulating 25(OH)D required for good health, is hotly debated [20, 21]. Vitamin D is argued by many to have potential extra-skeletal health effects, impacting energy balance and possibly reducing inflammation [21–25]. However, the findings are inconsistent from cross-sectional studies, and human clinical trials that have investigated the potential links between vitamin D status and systemic inflammatory markers [26–29].

Cellular studies indicate that vitamin D is a key modulator of immune function and inflammation [30, 31]. There is an increasing appreciation that vitamin D exerts broad regulatory effects on cells of the adaptive and innate immune system [32]. Current evidence suggests that the circulating level of 25(OH)D may be crucial for the optimal anti-inflammatory response of human monocytes [22]. The conversion of 25(OH)D to its active form $1,25(OH)_2$ occurs locally in immune system cells. The active metabolite of vitamin D has an anti-inflammatory effect on the inflammatory profile of monocytes [17, 33, 34], down-regulating the expression and production of several pro-inflammatory cytokines including TNF- α, IL-1β, IL-6, and IL-8 [33, 34]. Some immediate vitamin D action occurs in cells that possess the membrane vitamin D receptor (mVDR) [35]. However, the majority of vitamin D's biological functions are mediated through the regulation of gene expression. The active metabolite of vitamin D 1,25 dihydroxyvitamin D (1,25(OH)2D3) binds to its nuclear receptor (nVDR) with high affinity and specificity. The vitamin D-nVDR forms a heterodimer with the retinoid X receptor and this complex amplifies or represses transcription of the target genes through its binding to vitamin D responsive elements on DNA [17]. The nVDR is found in multiple cells of the immune system such as human Treg cells [36], neutrophils [37], dendritic cells, B cells [38] and macrophages [39].

To the best of our knowledge, there are no previously published systematic reviews that comprehensively assess the evidence for anti-inflammatory effects of vitamin D in human derived immune cells and human cell lines. We therefore embarked on this objective by targeting human-derived immune cell lines or PBMCs obtained from healthy participants or those with obesity-related chronic disease. In addition, we aimed to identify the pathways by which vitamin D modulated inflammation. We conclude that vitamin D has an anti-inflammatory effect with respect to cytokine expression and production, in both immune cell lines and PBMCs originating from humans. Furthermore, our review also highlights several mechanisms of action that may explain this anti-inflammatory effect of vitamin D.

Materials and Methods

This systematic review assessed the effect of vitamin D on the inflammatory profile of immune cells, using both human-derived immune cell lines and PBMCs obtained from adult humans. The primary outcomes were protein expression and secretion of common inflammatory markers such as pro-inflammatory cytokines MCP-1, IL-1β, IL-2, IL-6, IL-8, IL-12, TNF-α, CRP and anti-inflammatory markers such as IL-10 and IL-4 by immune cells. The protocol has been registered at the International Prospective Register for Systematic Reviews (PROSPERO) website (registration number CRD42015023222, S1 Table. Systematic review protocol).

A literature search was conducted independently by two reviewers (EKC and KNK) using Pubmed, Science Direct, Scopus, Wiley and Medline (search updated 19th June 2015). A third independent reviewer was consulted to resolve discrepancies (PN). The search strategy used the following search terms: Vitamin D or cholecalciferol or 1,25-dihydroxyvitamin or 25-hydroxy-Vitamin D and Inflam* or cytokine* and supplement* or cell*. These terms were searched in the abstract, title or keywords. Inclusion criteria for study selection included articles reported in the English language and within the time period of 2000 to 2015. The study selection process was mapped according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses guidelines (PRISMA) and can be seen in Fig 1. Study characteristics such as cell line, cell type, participant demographics, vitamin D form, dose, duration of exposure, presence of an inflammatory stimulus, direction of inflammatory marker change and pathway were extracted by two independent reviewers (EKC and KNK) and cross-checked as required (PN) (S2 Table. PRISMA Checklist).

Results

Cell line studies

Seven cell line studies (Table 1) were identified. Six out of the seven studies used the THP-1 cell line, while two studies used the U937 cell line and one study used Jurkat cells. All cell line studies administered vitamin D in the form of $1,25(OH)_2$ and one study also used 25(OH)D. All studies except one administered vitamin D in conjunction with an inflammatory stimulus. Overall, the majority of cell line studies (4 out of 7) reported that vitamin D had an anti-inflammatory effect, one study reported mixed effects and two studies reported a pro-inflammatory effect. The most common concentration of $1,25(OH)_2$ that indicated an anti-inflammatory effect was 10 nM (4 out of 7 studies). Mechanisms likely to mediate the anti-inflammatory effect of vitamin D included suppressed phosphorylated p38 (pp38) expression [40], reduced expression of p-STAT5 [41], and decreased reactive oxygen species levels due to increased cellular glutathione [42] (Fig 2).

PBMC studies

We identified sixteen studies that used PBMCs (Table 1). Of these, fifteen studies administered vitamin D in the form of $1,25(OH)_2$, one study used cholecalciferol and one study also used 25 (OH)D. All studies examined the effect of vitamin D in conjunction with an inflammatory stimulus. Of these, three studies also examined the effect of vitamin D alone without an inflammatory stimulus. The majority of PBMC studies showed that vitamin D had an anti-inflammatory effect (14 out of 16 studies), with two studies reporting mixed effects (Table 1). PBMCs were obtained from healthy participants in fourteen out of sixteen studies and the health status of participants in two studies was unknown. Six studies used PBMCs, four studies used monocytes, one study used macrophages, five studies used T-cells and one study used mixed

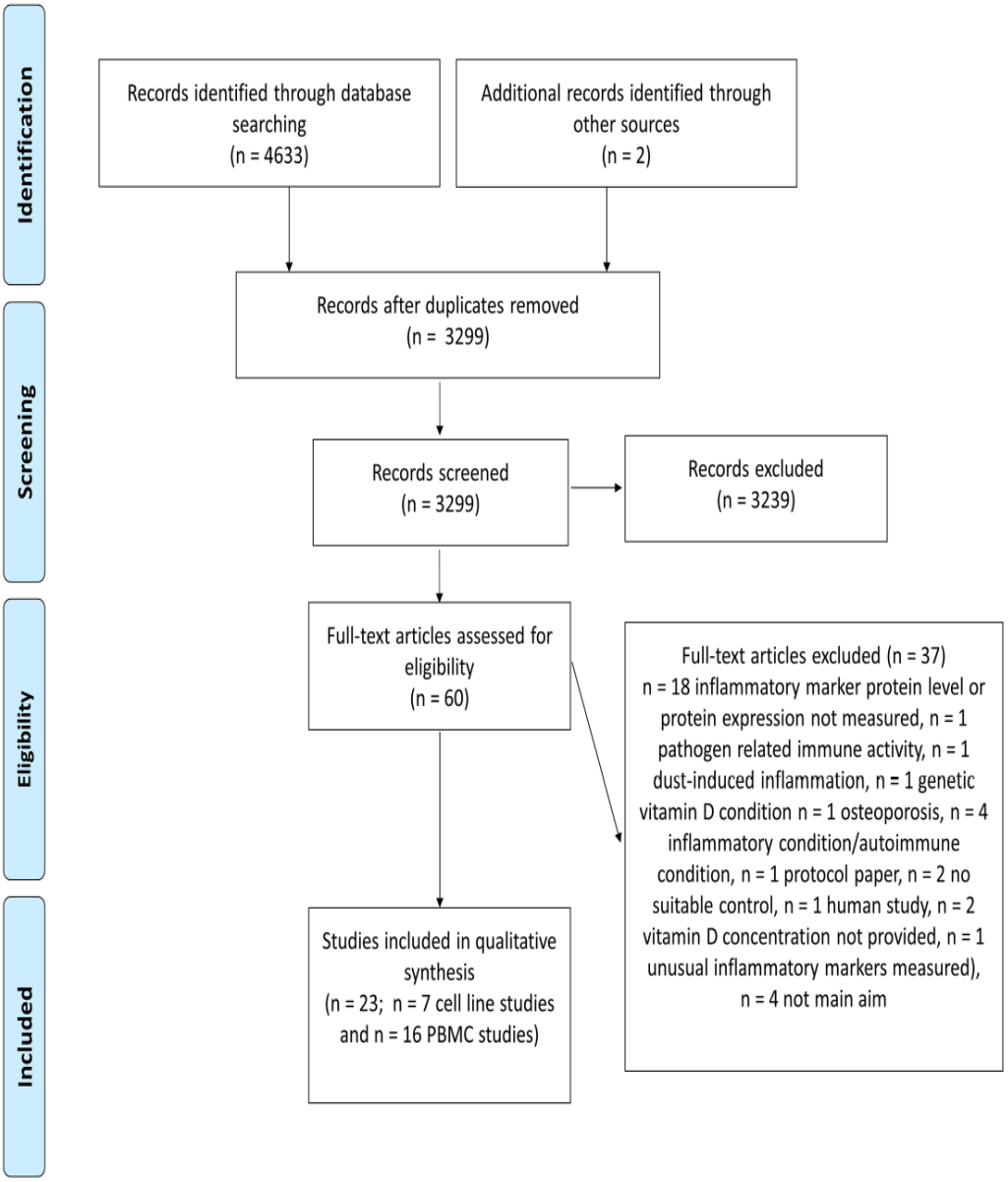

Fig 1. PRISMA flow diagram depicting the systematic study selection process. PBMC, peripheral blood mononuclear cells.

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lymphocytes. The two most common concentrations of $1,25(OH)_2$ that elicited an anti-inflammatory response was 10 nM (7 studies) and 100 nM (7 studies). Four studies demonstrated a dose-dependent response of vitamin D with respect to reducing inflammation, with 1 nM and 10 nM concentrations causing the greatest effects [22, 43–45]. Mechanisms likely to mediate the anti-inflammatory effect of vitamin D included decreased protein expression of toll-like receptor-2 (TLR-2) [43, 46] and toll-like receptor-4 (TLR-4) [43, 46], elevated trans-acting T-cell-specific transcription factor (GATA-3) mRNA through elevating upstream factor signal transducer and activator of transcription 6 (STAT6) [32], VDR [43], lower levels of pp38 and p42/42 (ERK1/2) [22, 43], and localization of p65 [43] (Fig 2).

Table 1. Cell studies examining the impact of vitamin D on inflammation.

Study	Cell line/cell type	Vitamin D form, dose and time	Stimulation method	Significant inflammatory marker change	Net effect
Cell line studies					
Tulk et al 2015 [47]	THP-1	$1,25(OH)_2$ (0, 0.1, 10, 100, 500 nM) 25(OH)D (0, 0.1, 10, 100, 500 nM)	PMA 100 nM overnight	IL-1β ↑	Pro-inflammatory (≥100 nM 25(OH)D and ≥1 nM 1,25 $(OH)_2D$)
Wang et al 2014 [40]	THP-1	$1,25(OH)_2$ (0, 100, 1000, 10000 nM) for 2 h	LPS 0.2 ug/ml for 6, 24 and 48 h	MCP-1 ↓	Anti-inflammatory
Yang et al 2012 [41]	THP-1	$1,25(OH)_2$ (0, 100 nM) for 48 h	LPS 1 ug/ml + IL-15 100 ng/ml for 4 h	IL-6, MCP-1 ↓	Anti-inflammatory
Matilainen et al 2010 [48]	THP-1	1,25(OH)2 (0, 10 nM) for 48 h	LPS 100 ng/ml for 24 h	IL-10 mRNA ↓ (8 h) then ↑ (48 h)	Anti-inflammatory
Matilainen et al 2010 [49]	THP1 + Jurkat lymphocyte cells	$1,25(OH)_2$ (0, 10 nM) for 24 h	LPS 100 ng/ml for 24 h or 2 ug/ml PHA and 50 ng/ml TPA	IL-2, IL-10 mRNA ↓ (3, 6 h) then ↑(24 h) IL-12 mRNA ↓ (6h)	Mixed
Lee et al 2011 [50]	U937 THP	$1,25(OH)_2$ (0, 10 nM) for 24 h	PMA	IL-1β protein expression and protein level ↑	Pro-inflammatory
Jain & Micinski 2013 [42]	U937 monocytes	$1,25(OH)_2$ (0, 10, 25 nM) for 24h	No inflammatory stimulant	IL-8, MCP-1 ↓	Anti-inflammatory
PBMC studies					
Cantorna 2015[a] [51]	PBMCs	$1,25(OH)_2$ (0, 10, 50 nM) for 72 hours	α-Galactoceramide for 72 hours	INF- γ ↓ IL-4 ↑	Anti-inflammatory
Ojaimi 2013[b] et al [52]	PBMCs	Cholecalciferol, 50,000 IU daily for 10 days, then 50000 monthly for 3 months	Pam3Cys 100 ng/ml Poly I: C 10 μg/ml LPS 100 ng/ml or unstimulated media for 24 h.	TNF-α, IL-6 ↓, then NC Unstimulated showed no effect as basal cytokine production was so low	Anti-inflammatory (when serum levels >100 nM)
Khoo 2011 et al [36]	PBMCs	$1,25(OH)_2$ 0 or 10^{-7} M (100 nM) for 30 min	Pam3Cys 10 mg/ml or LPS 10 ng/ml or RPMI control for 24 h	IL-6, TNF-α ↓	Anti-inflammatory
Rausch-Fan et al 2002 [44]	PBMCs	$1,25(OH)_2$ (0.01 to 100 nM) for 48 h	PMA 10 ng/ml and ionomycin 1.25 uM	INF- γ, IL-2, IL-10, TNF-α, IL-12, IL-1β ↓, IL-5, IL-10 ↑, IL-4 NC	Anti-inflammatory (10^{-8}, 10^{-7} M)
Takahashi 2002 [53]	PBMCs	$1,25(OH)_2$ (0, 0.1, 100nM) for 2 h, 4 h, 8 h and 24 h	LPS 1 ug/ml or IL-1β 10ng/ml	IL-8 ↓ (24 h)	Anti-inflammatory
Giovanni 2001 et al [45]	PBMCs	$1,25(OH)_2$ (25, 50, 100 ng) for 12 h	LPS 100 ng/ml	TNF-α, IL-1β, IL-6, IL-10 ↓, dose-dependent NE when PBMC incubated without LPS	Anti-inflammatory
Di Rosa 2012 et al [46]	Monocyte derived macrophages & monocytes	$1,25(OH)_2$ (0, 1000 nM) for 24 h	alone or in combination with TNF-α 100 U/ml or LPS 50 ng/ml for 2 h	Monocytes: IL-1β, IL-6, TNF-α mRNA NC Macrophages + LPS: IL-1β, IL-6 mNRA NC TNF-α mRNA ↑, Macrophages + TNF- α: IL-1β mNRA NC IL-6, TNF-α mRNA ↓, Macrophages without stimulation: IL-1β, IL-6, TNF-α ↓	Monocytes: No effect; Macrophages: Anti-inflammatory
Zhang 2012 et al [22]	Monocytes	$1,25(OH)_2$ (0, 1, 10 nM) for 24 h25(OH)D (0, 15 ng/ml, 30 ng/ml, 50 ng/ml and 70 ng/ml) for 24 h	10 ng/ml LPS for 24 h	IL-6 ↓ dose-response	Anti-inflammatory
Du 2009 [54]	Monocytes	$1,25(OH)_2$ (0, 100 nM) for 48 h	LPS 100 ng/ml and LTA 10 ug/ml for 3 h	TNF- α, IL1β ↓	Anti-inflammatory

(*Continued*)

Table 1. (*Continued*)

Study	Cell line/cell type	Vitamin D form, dose and time	Stimulation method	Significant inflammatory marker change	Net effect
Sadeghi 2006 et al [43]	Monocytes	$1,25(OH)_2$ (0.01 to 100 nM) for 48 h	10 ng LPS or 10 ug LTA for 4 h	TNF- α ↓, dose-response	Anti-inflammatory (10^{-9} to 10^{-7} M)
Sloka 2011 et al [32]	T cells	$1,25(OH)_2$ (0, 0.1 and 10 nM) of $1,25(OH)_2$	mouse anti-human CD3 10 or 1000 ng/mL for 3 days	IFN- γ, IL-17 ↓, IL-5 ↑	Anti-inflammatory
Thien 2005 et al [55]	T cells	$1,25(OH)_2$ (0, 10 nM) for 7–14 days	IL-4 500 U/mL or IL-12 200 U/mL	INF- γ, IL-4, IL-6, IL-13 ↑, IL-2 ↓	Mixed
Khoo 2011 et al [36]	Treg cells, T convential cells	$1,25(OH)_2$ (0, 100 nM) for 8 days	Treg and Tconv cells were stimulated with anti-CD3/ anti-CD28 monoclonal antibody-coated microbeads and PMA	IL-4, IL-10 ↑, TNF-α ↑, IL-2, IFN-γ, IL-17 NC	Mixed
Zhang, Leung & Goleva 2013 [56]	PBMCs-$CD14^+$ and $CD14^-$ T cells	$1,25(OH)_2$ (0, 10 nM) for 24 h	LPS 10ng/ml for 6 h	IL-6 ↓	Anti-inflammatory
Jeffery 2009 et al [a] [57]	T cells $CD4^+CD25^-$	$1,25(OH)_2$ (0, 100 nM) for 5 days	anti-CD3- and anti-CD28 Antibody-coated beads	IFN- γ, IL-2, IL-17, IL-21 ↓, IL-10 ↑	Anti-inflammatory
Jirapongsananuruk 2000 et al [58]	PBMCs-lymphocyte	$1,25(OH)_2$ (0, 1000 nM) for 72 h	anti-CD3	IL-5, IL-13 ↑ IFN- γ ↓	Anti-inflammatory

25-hydroxyvitamin D (25(OH)D), 1,25-dihydroxyvitamin D ($1,25(OH)_2$), interferon gamma IFN-γ, interleukin 1β (IL1β), interleukin 2 (IL-2), interleukin 4 (IL-4), interleukin 5 (IL-5), interleukin 6 (IL-6), interleukin 8 (IL-8), interleukin 10 (IL-10), interleukin 12 (IL-12), interleukin 13 (IL-13), interleukin 15 (IL-15), interleukin 17 (IL-17), interleukin 21 (IL-21), monocyte chemotatic protein-1 (MCP-1), no change NC, peripheral blood mononuclear cells (PBMCs), tumor necrosis factor alpha (TNF- α).

[a] Health status of participants unknown

[b] Study conducted in participants with inadequate vitamin D status (serum 25(OH)D < 50 nM

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Discussion

Inadequate vitamin D status is commonly observed in populations across the world [12]. This observation parallels the high prevalence of obesity-related chronic diseases that carry a heavy inflammatory burden. Our objective was to comprehensively review the cellular evidence linking vitamin D with the inflammatory profile of human-derived immune cells.

Our results demonstrated that the active form of vitamin D decreased the inflammatory status of cellular models. We found evidence that vitamin D was able to indirectly quench ROS, which are accepted as a major factor in the onset and development of chronic diseases including type 2 diabetes [59]. We also found evidence that vitamin D decreased TLR expression, which is increased in both immune cells and adipose tissue from overweight and obese subjects [60]. Furthermore, TLR activation has been implicated in mechanisms of obesity-related insulin resistance and metabolic dysfunction [61, 62]. TLRs are shown to be stimulated by both endogenous and exogenous factors such as dietary saturated fatty acids [63] and resistin [64], both of these factors induce inflammatory changes in circulating immune cells [65]. The TLR transmembrane proteins subsequently initiate classical signaling cascades leading to the activation of transcription factors, such as NFκB [66] and cytokine production [62]. TLR pathways also stimulate a variety of cellular responses including host defense in response to microbial products, and subsequently impact energy metabolism. Stimulated NFκB exerts its action through binding to DNA and inducing the transcription of many genes involved in various aspects of innate and adaptive immune responses, such as those coding for cytokines, growth factors, adhesion molecules [67], and multiple genes that regulate cellular differentiation, survival and proliferation [68]. Clearly, evidence suggests that $1,25(OH)_2$ acts to suppress NFκB

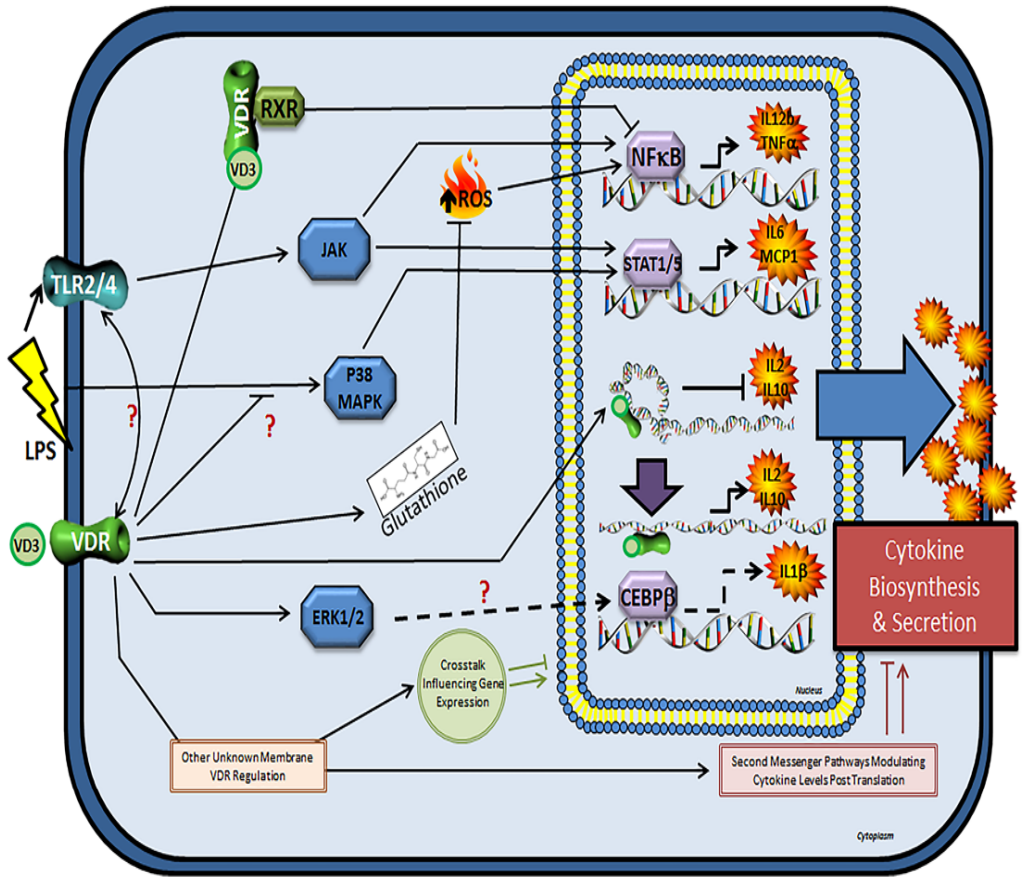

Fig 2. Overview of VDR-mediated regulation of cytokine transcription, production and secretion in immune cells. Interaction of VD3 and VDR leads to anti-inflammatory effects through negative regulation of NFκB and STAT1/5-mediated signalling. This results in decreased transcription of TNF-α, IL-6, MCP1 and IL-12β. VDR activation promotes increased intracellular glutathione levels that partially or fully attenuates excessive ROS production (ROS can activate pro-inflammatory NFκB signalling). Activated VDR regulates transcription of IL-2 and IL-10 through epigenetic and conformational changes in the promoter region of these genes. VDR association with the promoter region occurs in a cyclic fashion, which leads to initial gene suppression, followed by upregulation of IL-2 and IL-10 expression after 48 hours. Pro-inflammatory effects of VD3 were reported and suggested to be linked to increased IL-1β production possibly related to increased ERK1/2 phosphorylation and the transcription factor CEBPβ. The VDR is believed to modulate pro-inflammatory TLR expression both positively and negatively, but the mechanisms are unknown. Plasma membrane associated VDR may induce rapid effects through non-genomic pathways such as modulation of intracellular calcium levels, parathyroid hormone G-protein coupled or other second messenger systems. Non-genomic pathways may cooperate with genomic pathways to influence gene expression. CCAAT/enhancer binding protein beta (CEBPβ), extracellular signal-regulated kinase1/2 (ERK1/2), janus kinase (JAK), monocyte chemotatic protein1 (MCP-1), nuclear factor kappa light chain enhancer of activated B cells (NFκB), mitogen activated protein kinase (p38 MAPK), retinoid X receptor (RXR), reactive oxygen species (ROS), signal transducer and activator of transcription1/5 (STAT1/5), toll-like receptor-2/4 (TLR2/4), tumour necrosis factor alpha (TNF-α), vitamin D3 (VD3), vitamin D receptor (VDR).

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activity. It is possible that vitamin D acts through suppression of the NFκB transcriptional activity, or through regulation of cellular ROS levels, which subsequently alter NFκB transcriptional activity. However, the precise pathway(s) awaits confirmation. It is also possible, that inhibition of inflammatory signalling by vitamin D could happen upstream of modulation of transcriptional factor action.

It is also possible that vitamin D may be exerting anti-inflammatory effects through non-genomic pathways initiated at the plasma membrane VDR [35] (Fig 2). Binding of 1,25(OH) 2D at the plasma membrane VDR may result in the activation of one or more second messenger systems, such as phospholipase C (and subsequently protein kinase C, through generation of both diacylglycerol and a rise in intracellular Ca^{2+}), and G protein-coupled receptors.

Furthermore, non-genomic pathways could cooperate with the classical genomic pathway via cross-talk to influence gene expression. Perhaps application of a systems biology approach may reveal additional mechanisms of action.

We are unable to comment on whether 25(OH)D modulates inflammation, as few studies used this form of vitamin D. However, we and others [22] believe that prevailing 25(OH)D levels may be crucial since they influence local tissue concentrations of the active vitamin D metabolite [69]. Serum 25(OH)D levels as high as 120 nmol/L may be necessary for optimal immune function [52]. Indeed, it was reported [52] that the anti-inflammatory benefit of vitamin D was only seen in those individuals in whom 25(OH)D rose to >100 nmol/L. Beneficial effects disappeared when vitamin D status dropped to below 100 nmol/L. Since human recommendations for good health are based on appropriate serum levels of 25(OH)D, cellular studies could asses the effect of various doses of 25(OH)D that reflect/mimic whole body circulating concentrations of the hormone. In this systematic review, we did not investigate the impact of vitamin D on subsequent cell function. Potential therapeutic agents like vitamin D which target immune pathways such as NFκB, ROS quenching and JAK, must be able to antagonize the harmful effects of inflammation without affecting host defense functions. Further studies are therefore required to determine the full effect of vitamin D on other parameters of immune and cellular function.

Conclusion

Vitamin D consistently displayed anti-inflammatory effects in both human cell lines and PBMCs. Cellular studies which examine the impact of 25(OH)D on inflammatory status and responses are now required. Clinical studies are warranted to confirm whether supplementation and elevation in circulating vitamin D levels are able to modulate inflammation and improve outcomes or prevent chronic disease.

Supporting Information

S1 Table. Systematic review protocol.
(PDF)

S2 Table. PRISMA checklist.
(PDF)

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Author Contributions

Conceived and designed the experiments: EKC MJS KNK PN. Performed the experiments: EKC KNK MJS PN. Analyzed the data: EKC KNK MJS PN. Contributed reagents/materials/analysis tools: EKC KNK MJS PN. Wrote the paper: EKC KNK MJS PN.

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PROSPERO International prospective register of systematic reviews

Review title and timescale

1 Review title

Give the working title of the review. This must be in English. Ideally it should state succinctly the interventions or exposures being reviewed and the associated health or social problem being addressed in the review.

The effect of vitamin D manipulation on the inflammatory profile: a systematic review of immune cell studies

2 Original language title

For reviews in languages other than English, this field should be used to enter the title in the language of the review. This will be displayed together with the English language title.

3 Anticipated or actual start date

Give the date when the systematic review commenced, or is expected to commence.

02/02/2015

4 Anticipated completion date

Give the date by which the review is expected to be completed.

02/07/2015

5 Stage of review at time of this submission

Indicate the stage of progress of the review by ticking the relevant boxes. Reviews that have progressed beyond the point of completing data extraction at the time of initial registration are not eligible for inclusion in PROSPERO. This field should be updated when any amendments are made to a published record.

The review has not yet started ×

Review stage	Started	Completed
Preliminary searches	Yes	No
Piloting of the study selection process	Yes	No
Formal screening of search results against eligibility criteria	Yes	No
Data extraction	No	No
Risk of bias (quality) assessment	No	No
Data analysis	No	No

Provide any other relevant information about the stage of the review here.

Review team details

6 Named contact

The named contact acts as the guarantor for the accuracy of the information presented in the register record.

Mario Soares

7 Named contact email

Enter the electronic mail address of the named contact.

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8 Named contact address

Enter the full postal address for the named contact.

School of Public Health Curtin University Bentley Campus, Kent Street, Perth, Western Australia 6845

9 Named contact phone number

Enter the telephone number for the named contact, including international dialing code.

+ 61-8- 92663220

10 Organisational affiliation of the review

Full title of the organisational affiliations for this review, and website address if available. This field may be completed as 'None' if the review is not affiliated to any organisation.

Curtin University

Website address:

www.curtin.edu.au

11 Review team members and their organisational affiliations

Give the title, first name and last name of all members of the team working directly on the review. Give the organisational affiliations of each member of the review team.

Title	First name	Last name	Affiliation
Dr	Mario	Soares	Curtin University

Miss	Emily	Calton	Curtin University
Dr	Kevin	Keane	Curtin University
Professor	Philip	Newsholme	Curtin University

12 Funding sources/sponsors

Give details of the individuals, organizations, groups or other legal entities who take responsibility for initiating, managing, sponsoring and/or financing the review. Any unique identification numbers assigned to the review by the individuals or bodies listed should be included.

School of Public Health and School of Biomedical Sciences, Curtin University. EC is a recipient of an APA scholarship provided by the Australian Government.

13 Conflicts of interest

List any conditions that could lead to actual or perceived undue influence on judgements concerning the main topic investigated in the review.

Are there any actual or potential conflicts of interest?

None known

14 Collaborators

Give the name, affiliation and role of any individuals or organisations who are working on the review but who are not listed as review team members.

Title	First name	Last name	Organisation details

Review methods

15 Review question(s)

State the question(s) to be addressed / review objectives. Please complete a separate box for each question.

What is the impact of vitamin D manipulation on immune cell lines derived from humans and peripheral blood mononuclear cells obtained directly from humans on inflammatory markers/anti-inflammatory markers

16 Searches

Give details of the sources to be searched, and any restrictions (e.g. language or publication period). The full search strategy is not required, but may be supplied as a link or attachment.

PubMed, Scopus, Science Direct, MEDLINE, and Web of Science will be searched. Abstract, title and keywords to be searched Restricted to studies published within 2000-2015 and reported in the English language

17 URL to search strategy

If you have one, give the link to your search strategy here. Alternatively you can e-mail this to PROSPERO and we will store and link to it.

I give permission for this file to be made publicly available

Yes

18 Condition or domain being studied

Give a short description of the disease, condition or healthcare domain being studied. This could include health and wellbeing outcomes.

Change in inflammatory/anti-inflammatory markers

19 Participants/population

Give summary criteria for the participants or populations being studied by the review. The preferred format includes details of both inclusion and exclusion criteria.

Inclusion criteria: immune cells originating from humans (either cell lines or obtained from blood) The peripheral blood mononuclear cells must be from healthy participants or those with obesity-related chronic inflammatory disorders The peripheral blood mononuclear cells must be from adults Exclusion criteria: participants have a highly active inflammatory condition that is not associated with obesity or chronic disease peripheral blood mononuclear cells come from children or babies

20 Intervention(s), exposure(s)

Give full and clear descriptions of the nature of the interventions or the exposures to be reviewed

Inclusion criteria: cells are exposed to a form of vitamin D there is a control (no vitamin D) vitamin D is given alone or in conjunction with a stimulatory agent and not given in conjunction with a drug common inflammatory markers are measured (e.g. cytokines, adiponectin) protein level, protein expression or functional tests of cytokines are reported primary aim of the study is to examine the effect of vitamin D on inflammatory markers Exclusion cells are not exposed to vitamin d vitamin D is the control group vitamin D is given in combination with a drug neither protein level or protein expression or gene expression with functional/mechanistic results are measured the impact of vitamin D on inflammatory markers is not the main aim of the study

21 Comparator(s)/control

Where relevant, give details of the alternatives against which the main subject/topic of the review will be compared (e.g. another intervention or a non-exposed control group).
Non-exposed control group (no vitamin D) Inclusion: 0 vitamin D is the control group Exclusion: studies which use vitamin D as the control group or another form of vitamin D

22 Types of study to be included
Give details of the study designs to be included in the review. If there are no restrictions on the types of study design eligible for inclusion, this should be stated.
Inclusion criteria: cell studies which are directly exposed to a form of vitamin D there is a control (no vitamin D) Exclusion criteria study design is not a cellular study

23 Context
Give summary details of the setting and other relevant characteristics which help define the inclusion or exclusion criteria.

24 Primary outcome(s)
Give the most important outcomes.
The impact of vitamin D compared to no vitamin D on inflammatory markers in immune cell lines and immune cells obtained from peripheral blood mononuclear cells
Give information on timing and effect measures, as appropriate.

25 Secondary outcomes
List any additional outcomes that will be addressed. If there are no secondary outcomes enter None.
What are the mechanisms by which vitamin D exerts its effect on inflammatory markers?
Give information on timing and effect measures, as appropriate.

26 Data extraction (selection and coding)
Give the procedure for selecting studies for the review and extracting data, including the number of researchers involved and how discrepancies will be resolved. List the data to be extracted.
Literature search will be conducted using Pubmed, Science Direct, Scopus, Web of Science and MEDLINE. Restricted to studies in English and from 2000-2015. Duplicates removed. Study inclusion will be assessed by two independent reviewers and a third independent reviewer will be consulted if discrepancy exists. Data extraction will occur for cell type, vitamin D form, dose and duration of exposure, presence or absence of an inflammatory stimulus, inflammatory marker change and mechanisms of action by two independent reviewers and cross-checked by a third independent reviewer when discrepancy exists.

27 Risk of bias (quality) assessment
State whether and how risk of bias will be assessed, how the quality of individual studies will be assessed, and whether and how this will influence the planned synthesis.
The quality of each study will be assessed as good (and the study therefore included) if the study provides vital information including the cell type studied, vitamin D form, vitamin D concentration, inflammatory marker change and if the results and discussion are consistent

28 Strategy for data synthesis
Give the planned general approach to be used, for example whether the data to be used will be aggregate or at the level of individual participants, and whether a quantitative or narrative (descriptive) synthesis is planned. Where appropriate a brief outline of analytic approach should be given.
A semi-quantitative descriptive synthesis is planned whereby the number of studies which showed an anti-inflammatory or pro-inflammatory effect or no effect will be described.

29 Analysis of subgroups or subsets
Give any planned exploration of subgroups or subsets within the review. 'None planned' is a valid response if no subgroup analyses are planned.
If there are enough studies in more than 1 cell line type (at least 3 studies in each type), results will be divided according to type of cell line. Studies will be discussed based on the form of vitamin D administered. Studies will be divided based on whether they use vitamin D alone or stimulate the cells with an inflammatory agent if there are enough studies in each situation (at least 3 studies with and without an inflammatory stimulus).

Review general information

30 Type and method of review
Select the type of review and the review method from the drop down list.
Intervention, Systematic review

31 Language
Select the language(s) in which the review is being written and will be made available, from the drop down list. Use the control key to select more than one language.
English
Will a summary/abstract be made available in English?

Yes

32 Country

Select the country in which the review is being carried out from the drop down list. For multi-national collaborations select all the countries involved. Use the control key to select more than one country.

Australia

33 Other registration details

Give the name of any organisation where the systematic review title or protocol is registered together with any unique identification number assigned. If extracted data will be stored and made available through a repository such as the Systematic Review Data Repository (SRDR), details and a link should be included here.

34 Reference and/or URL for published protocol

Give the citation for the published protocol, if there is one.
Give the link to the published protocol, if there is one. This may be to an external site or to a protocol deposited with CRD in pdf format.

I give permission for this file to be made publicly available

Yes

35 Dissemination plans

Give brief details of plans for communicating essential messages from the review to the appropriate audiences.

publication and thesis

Do you intend to publish the review on completion?

Yes

36 Keywords

Give words or phrases that best describe the review. (One word per box, create a new box for each term)

systematic review
cell
inflammation
cytokine
vitamin D
immune

37 Details of any existing review of the same topic by the same authors

Give details of earlier versions of the systematic review if an update of an existing review is being registered, including full bibliographic reference if possible.

38 Current review status

Review status should be updated when the review is completed and when it is published.

Ongoing

39 Any additional information

Provide any further information the review team consider relevant to the registration of the review.

40 Details of final report/publication(s)

This field should be left empty until details of the completed review are available.
Give the full citation for the final report or publication of the systematic review.
Give the URL where available.

PRISMA 2009 Checklist

Section/topic	#	Checklist item	Reported on page #
TITLE			
Title	1	Identify the report as a systematic review, meta-analysis, or both.	1
ABSTRACT			
Structured summary	2	Provide a structured summary including, as applicable: background; objectives; data sources; study eligibility criteria, participants, and interventions; study appraisal and synthesis methods; results; limitations; conclusions and implications of key findings; systematic review registration number.	2
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of what is already known.	3-4
Objectives	4	Provide an explicit statement of questions being addressed with reference to participants, interventions, comparisons, outcomes, and study design (PICOS).	5
METHODS			
Protocol and registration	5	Indicate if a review protocol exists, if and where it can be accessed (e.g., Web address), and, if available, provide registration information including registration number.	5
Eligibility criteria	6	Specify study characteristics (e.g., PICOS, length of follow-up) and report characteristics (e.g., years considered, language, publication status) used as criteria for eligibility, giving rationale.	5-6
Information sources	7	Describe all information sources (e.g., databases with dates of coverage, contact with study authors to identify additional studies) in the search and date last searched.	6
Search	8	Present full electronic search strategy for at least one database, including any limits used, such that it could be repeated.	6
Study selection	9	State the process for selecting studies (i.e., screening, eligibility, included in systematic review, and, if applicable, included in the meta-analysis).	6
Data collection process	10	Describe method of data extraction from reports (e.g., piloted forms, independently, in duplicate) and any processes for obtaining and confirming data from investigators.	6
Data items	11	List and define all variables for which data were sought (e.g., PICOS, funding sources) and any assumptions and simplifications made.	6
Risk of bias in individual studies	12	Describe methods used for assessing risk of bias of individual studies (including specification of whether this was done at the study or outcome level), and how this information is to be used in any data synthesis.	6
Summary measures	13	State the principal summary measures (e.g., risk ratio, difference in means).	9-12
Synthesis of results	14	Describe the methods of handling data and combining results of studies, if done, including measures of consistency (e.g., I^2) for each meta-analysis.	n/a

PRISMA 2009 Checklist

Section/topic	#	Checklist item	Reported on page #
Risk of bias across studies	15	Specify any assessment of risk of bias that may affect the cumulative evidence (e.g., publication bias, selective reporting within studies).	n/a
Additional analyses	16	Describe methods of additional analyses (e.g., sensitivity or subgroup analyses, meta-regression), if done, indicating which were pre-specified.	n/a
RESULTS			
Study selection	17	Give numbers of studies screened, assessed for eligibility, and included in the review, with reasons for exclusions at each stage, ideally with a flow diagram.	Figure 1
Study characteristics	18	For each study, present characteristics for which data were extracted (e.g., study size, PICOS, follow-up period) and provide the citations.	9-12
Risk of bias within studies	19	Present data on risk of bias of each study and, if available, any outcome level assessment (see item 12).	n/a
Results of individual studies	20	For all outcomes considered (benefits or harms), present, for each study: (a) simple summary data for each intervention group (b) effect estimates and confidence intervals, ideally with a forest plot.	n/a
Synthesis of results	21	Present results of each meta-analysis done, including confidence intervals and measures of consistency.	n/a
Risk of bias across studies	22	Present results of any assessment of risk of bias across studies (see Item 15).	n/a
Additional analysis	23	Give results of additional analyses, if done (e.g., sensitivity or subgroup analyses, meta-regression [see Item 16]).	n/a
DISCUSSION			
Summary of evidence	24	Summarize the main findings including the strength of evidence for each main outcome; consider their relevance to key groups (e.g., healthcare providers, users, and policy makers).	6-8, 14-17
Limitations	25	Discuss limitations at study and outcome level (e.g., risk of bias), and at review-level (e.g., incomplete retrieval of identified research, reporting bias).	17
Conclusions	26	Provide a general interpretation of the results in the context of other evidence, and implications for future research.	17
FUNDING			
Funding	27	Describe sources of funding for the systematic review and other support (e.g., supply of data); role of funders for the systematic review.	Funding statement provided at submission, in accordance with Journal

CHAPTER 3 - VITAMIN D IS A NOVEL DETERMINANT OF RESTING METABOLIC RATE

The content of this chapter is covered by Paper 3:

Calton, E. K., K. Pathak, M. J. Soares, H. Alfonso, K. N. Keane, P. Newsholme, N. K. Cummings, W. Chan She Ping-Delfos, and A. Hamidi. 2016. "Vitamin D status and insulin sensitivity are novel predictors of resting metabolic rate: a cross-sectional analysis in Australian adults." *Eur J Nutr* 55 (6):2075-80. doi: 10.1007/s00394-015-1021-z.

Thesis objective addressed in this chapter:

Objective 2: To investigate whether 25(OH)D contributes to RMR in adults.

Eur J Nutr (2016) 55:2075–2080
DOI 10.1007/s00394-015-1021-z

ORIGINAL CONTRIBUTION

Vitamin D status and insulin sensitivity are novel predictors of resting metabolic rate: a cross-sectional analysis in Australian adults

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Abstract

Purpose Resting metabolic rate (RMR) accounts for two-thirds of the total energy expenditure in sedentary individuals. After accounting for traditional factors, there still remains a considerable unexplained variance in RMR. There is a pandemic of obesity and metabolic syndrome (MetS) which coexists with a high prevalence of vitamin D insufficiency. The aim of this study was to evaluate the potential effects of vitamin D status, insulin sensitivity (IS) and the metabolic syndrome (MetS) on RMR in Australian adults.

Methods RMR, respiratory quotient (RQ), McAuley's insulin sensitivity index, fat mass (FM), fat-free mass (FFM) and vitamin D status were assessed in Australian adults. The presence of MetS was evaluated by current standard criteria. Predictors of RMR were examined through multiple linear regression based on stepwise and backward regression approaches with attention to multicollinearity. All analyses were conducted on SPSS version 21.

Results One hundred and twenty-seven participants (45 men, 82 women), aged 53.4 ± 11.7 years and BMI 31.9 ± 5.2 kg/m^2, were included. Forty-one subjects were insufficient in vitamin D status (<50 nmol/L), and 75 participants had the MetS. A parsimonious regression model explained 85.8 % of RMR and was given by: RMR (kJ/d) = 1931 + 83.5 × FFM (kg) + 29.5 × FM (kg) + 5.65 × 25(OH)D (nmol/L) − 17.6 × age (years) − 57.51 × IS.

Conclusion Vitamin D status and IS are novel independent predictors of RMR in adults. Future studies could validate a causal role for these factors in human energy metabolism.

Keywords Resting metabolic rate · Metabolic syndrome · Vitamin D · Insulin sensitivity

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Introduction

Obesity, metabolic syndrome (MetS) and type 2 diabetes (T2DM) represent a global pandemic, which place a substantial burden on health care. All these conditions are associated with low-grade chronic inflammation and reduced insulin sensitivity (IS). Due to enhanced inflammation, there is an elevated energy cost, most probably due to activated immune cell responses [1]. It has recently been proposed that aberrant energy generation [2], possibly through altered mitochondrial activity [3], may also characterize

these diseases and manifest as a raised resting metabolic rate (RMR). An increase in RMR in T2DM and in MetS has been observed [4, 5], and RMR declined the most in those who recovered from MetS following weight loss [2]. Therefore, insulin resistance, obesity and MetS are potentially also linked through an increased energy expenditure secondary to the energetic cost of chronic inflammation.

Vitamin D insufficiency, as judged by circulating levels of 25(OH)D, is highly prevalent in Australia, with one-third of adults having inadequate status [6]. Vitamin D has been suggested to play a pivotal role in the amelioration of many chronic diseases [7], possibly through modulation of inflammation and insulin sensitivity (IS) [8]. The majority of biological functions regulated by vitamin D are mediated through the nuclear vitamin D receptor (nVDR) [9]. The active metabolite of vitamin D ($1.25(OH)_2D$) binds to nVDR with high affinity and specificity. These receptors are present in many non-skeletal tissues including the brain, pancreas and immune cells. The vitamin D-nVDR forms a heterodimer with the retinoid X receptor, and this complex amplifies or represses transcription of the target genes.

The link between vitamin D status and adiposity is increasingly being investigated. Cross-sectional studies report an inverse relationship between overweight and obesity and circulating levels of 25(OH)D [10], and prospective studies have reported that low 25(OH)D plasma levels may determine the incidence of obesity [11, 12]. However, a recent study based on a bi-directional Mendelian randomization approach concluded that while a high BMI was always associated with low vitamin D status, a low vitamin D status was unlikely to result in a high BMI [13]. It is hence probable that cross-sectional associations represent a volume dilution effect [14]. A role for vitamin D in energy metabolism is likely, but is not yet well defined or understood. Mice lacking nVDR gained less body fat mass on a high-fat diet through increased energy expenditure and greater fatty acid oxidation [15]. In contrast, cholecalciferol supplementation was shown to protect against a high-fat-diet-induced obesity in C57BL/6 J mice, by enhancing 24-h energy expenditure and fatty acid oxidation [16]. Hence extrapolation from animals to human obesity is not straight forward. Clinical studies examining the impact of vitamin D supplementation on human energy expenditure are scarce and show no effect, possible due to their short duration [10, 17].

RMR accounts for 60–75 % of total energy expenditure and thus forms the basis for human energy requirements. RMR reflects the energy production of the respiring tissue mass at rest. The major predictor of RMR is fat-free mass (FFM), which accounts for up to 60 % of the variance in RMR [18, 19]. A significant contribution of fat mass (FM) is also recognized [18]. Minor contributions to RMR are also made by age [18], gender [19] and ethnicity [20]. However, despite the contribution of these various factors, there still remain significant inter-individual variations in RMR which are not understood [18]. Addressing the underlying causes of this variation has long been recognized as essential [21]. Given the pandemic of obesity and MetS, which commonly coexists with inadequate vitamin D status, we hypothesized that vitamin D status, IS and/or the presence of MetS could contribute to RMR in an adult population group.

Methods

We used the data of 127 Caucasian adults with body mass index $\geq$18.5 kg/m^2 who were participants of three separate clinical trials conducted in our laboratory. All studies were approved by the Human Research Ethics Committee at Curtin University. Written informed consent was obtained from all participants. Energy metabolism was measured by indirect calorimetry (Deltatrac II, Datex Instrumentarium, Finland), using a standard protocol that emphasized an overnight fast, abstinence for 24 h from heavy physical activity and a mandatory 30 min rest in the supine position prior to measurement [22]. Minute–minute measurements of O_2 consumption and CO_2 production were then conducted over a minimum 30 min. Weir's equation was used to calculate RMR from the average of the last 25 min of data collection [23]. None of the participants in this study had a measured RQ < 0.7 or >1.0 [24]. Body composition was assessed using dual energy X-ray absorptiometry (DEXA, DPX-L ($n = 29$) or Prodigy Models ($n = 97$), Lunar Corporation, USA), and a validation study has demonstrated equivalence in their estimates [25]. All fasting blood clinical chemistry measurements were made at an accredited laboratory of the Department of Pathology, Royal Perth Hospital, Perth, WA. Vitamin D status [25(OH)D] was determined using the chemiluminescent immunoassay method (Liaison, DiaSorin and Architect, Abbott). MetS status was defined according to the most recent criteria advocated by an expert consultation [26]. IS was assessed using McAuley's insulin sensitivity index based on fasting insulin and triglycerides [27].

Statistical analysis

Continuous variables were checked for normality prior to analysis and were summarized by their means and standard deviations. The possible associations between RMR, vitamin D, IS, MetS and potential confounders were initially explored by Pearson's correlation coefficients, partial correlation and simple linear regression. Predictors of RMR were examined through multiple linear regression. Both stepwise and backward elimination approaches were tested to determine the best overall model. Stepwise regression used the SPSS default p value of 0.05. Variables with less than this p value enter the regression model one by one,

but the model may remove variables already in the equation if their p value increases above 0.05 during the process. Backward regression used the SPSS default p value of 0.1. All potential variables enter the model at the first step, and then each one is progressively removed if their p values are greater than 0.1. The final model by both methods was checked to make sure each variable made a significant contribution to RMR. Multi-collinearity analysis indicated that tolerance was >0.2 and variance inflation factor was <2.0 in the parsimonious model reported. Statistical analysis was performed using SPSS (version 21).

Results

All 127 participants (82 females, 45 males) were included in this analysis. Their general characteristics are shown in Table 1. Seventy-five participants had MetS, and 52 subjects were without MetS. Two participants were vitamin D deficient (<25 nmol/L), 39 were insufficient (25–50 nmol/L), and 86 had values considered sufficient by current guidelines (>50 nmol/L).

Table 1 General characteristics of the participants

Variable	Mean (SD)
Age (years)	53.42 (11.65)
Gender (M/F)	45/82
Weight (kg)	91.09 (18.48)
BMI (kg/m^2)	31.87(5.24)
FFM (kg)	54.24 (12.46)
FM (kg)	35.78 (34.96)
25(OH)D (nmol/L)	63.03 (22.69)
Systolic blood pressure (mm Hg)	127 (15)
Diastolic blood pressure (mm Hg)	76 (9)
MetS status (yes/no)	75/52
IS	7.13 (1.99)

Data are mean (SD), and $N = 127$

BMI body mass index, *FFM* fat-free mass, *FM* fat mass, *IS* McAuley's index, *MetS* metabolic syndrome, *25(OH)D* 25-hydroxyvitamin D

An inverse relationship between IS and RMR was observed ($r = -0.516$, $p < 0.001$), which remained significant after adjusting for age, gender, FFM, FM and vitamin D ($r = -0.184$, $p = 0.043$). There was a positive relationship between vitamin D and RMR after adjustment for age, gender, FFM and FM ($r = 0.185$, $p = 0.041$) and a similar relationship between adjusted vitamin D and IS ($r = 0.304$, $p = 0.001$). A positive relationship between MetS and RMR was found ($r = 0.312$, $p < 0.001$), but was not significant after adjustment for age, gender, FFM, FM, vitamin D and IS ($r = 0.122$, $p = 0.181$). An inverse relationship between MetS and IS ($r = -0.427$, $p < 0.001$) remained after adjustment of age, gender, FFM, FM and vitamin D ($r = -0.284$, $p = 0.001$).

Table 2 depicts the significant predictors of RMR based on a parsimonious model. The final equation was similar for both forward stepwise and backward regression approaches and was given by:

$$\begin{aligned}\text{RMR (kJ/days)} = {} & 1931.21 + 83.49 \times \text{FFM (kg)} + 29.48 \\ & \times \text{FM (kg)} + 5.65 \times 25\ \text{(OH)D (nmol/L)} \\ & - 17.56 \times \text{age (years)} - 57.51 \times \text{IS} \qquad (1)\end{aligned}$$

where $n = 127$, SEE = 486 and $r^2 = 0.86$. Independent predictors that did not enter the equation were: gender, study investigator, vitamin D analysis method and season of measurement.

The model predicted that for every unit increase in IS, RMR would be lower by 57.5 kJ/d. In addition, for every 10 nmol/L increase in 25OHD, RMR would increase by 56.5 kJ/d. When traditional predictors of RMR (age, gender, FM and FFM) were forced into the model, IS and

Table 2 Prediction model for RMR in Australian men and women

	Estimated regression coefficient			R^2 change		Collinearity statistics	
	Unstandardized coefficient	T value	p value	%	p value	Tolerance	VIF
Variable							
Constant	1931.21	4.193	<0.001				
FFM (kg)	83.49	20.805	<0.001	77.4	<0.001	0.748	1.336
FM (kg)	29.48	6.867	<0.001	5.8	<0.001	0.891	1.122
Age (years)	−17.56	−4.686	<0.001	2.1	<0.001	0.983	1.018
25(OH)D (nmol/L)	5.65	2.710	0.008	0.5	0.036	0.836	1.196
IS	−57.51	−2.118	0.036	0.5	0.034	0.644	1.553

Adjusted r^2 for model was 85.8 % using both backward elimination and stepwise linear regression

RMR resting metabolic rate, *FFM* fat-free mass, *FM* fat mass, *IS* McAuley's index, *25(OH)D* 25-hydroxycholecalciferol

vitamin D status were still retained as significant predictors (IS, β coefficient = −55.58, $p = 0.043$; vitamin D, β coefficient = 5.46, $p = 0.01$). To further explore the relationship between MetS and RMR, we removed IS from the list of independent predictors. MetS entered the model only when using the backward regression approach ($p = 0.051$). The resultant equation had an adjusted r^2 of 85.8 % and was given by:

$$\begin{aligned}\text{RMR (kJ/d)} = {} & 1472 + 85.37 \times \text{FFM (kg)} + 29.49 \\ & \times \text{FM (kg)} + 4.67 \times 25\text{ (OH)D (nmol/L)} \\ & + 197.84 \times \text{MetS} - 19.57 \times \text{age (years)} \quad (2)\end{aligned}$$

where MetS = 0 is the absence and 1 is the presence of the syndrome.

Discussion

The global health status has changed dramatically in the last 30 years, with an increasing number of countries reporting that the majority of their population are overweight or obese [28, 29]. Australia is no exception with increasing prevalence of obesity, MetS and T2DM. Additionally, a significant proportion of the Australian population is vitamin D inadequate, based on 50 nmol/L cut-off [30]. Like many other authors, we have been engaged in determining a possible causal role for vitamin D in chronic disease [31]. The aim of this study was to evaluate the effects of vitamin D status, IS and the MetS on RMR in Australian adults. To the best of our knowledge, this hypothesis has not been previously investigated.

In this analysis, we obtained an independent effect of both vitamin D status and IS on RMR. We found that IS had an inverse association with RMR. This is in agreement with studies which reported that obese individuals with impaired glucose tolerance have higher RMR compared to subjects with normal glucose tolerance [4]. Similarly, other studies have reported that higher fasting hyperglycaemia, closely related to insulin resistance, is associated with increased RMR [32]. When IS was not included in the model, MetS just entered the regression model. This was not surprising since IS and MetS are intimately and inversely linked [26]. One explanation for this linkage may involve the chronic low-grade inflammatory state that characterizes obesity, IR and MetS [33, 34]. Thus, the higher RMR seen with lesser IS or higher RMR seen with MetS (Eqs. 1, 2) may reflect the energy cost of inflammation [1] that is in part due to activated immune cells [35]. However, we did not measure inflammatory markers in our participants and are unable to confirm this at present.

Studies from VDR-null mice clearly show that vitamin D is involved in energy metabolism [15, 36]. However, human supplementation studies examining the impact of the vitamin on energy expenditure are scarce. One study with a very short duration of 1 week found no effect on energy or substrate utilization following vitamin D [17]. Nevertheless, a direct effect of vitamin D on human energy metabolism is intriguing since the regression indicates an independent effect of 25OHD after controlling for traditional factors (Eq. 1). The β coefficient for 25OHD suggests that changing vitamin D status from 40 to 75 nmol/L could have a sizeable and clinically relevant effect of ~200 kJ/d on RMR. A greater RMR would then act to attenuate weight gain. The role of vitamin D in energy metabolism has recently been reviewed [37]; however, the mechanisms responsible for a potential thermogenic action of vitamin D in humans are yet to be elucidated. There could be a direct thermogenic effect via nVDR, or vitamin D may impact skeletal muscle size and function [38, 39] and therefore increase metabolic rate. Vitamin D may also modulate mitochondrial function and thereby affect resting oxygen consumption. Mechanistically there is a close positive relationship between mitochondrial activity and RMR in humans [40]. Recent evidence indicates that vitamin D supplementation influences mitochondrial activity during exercise by improving ATP production [41] and has also been shown to have a similar effect at rest [42]. There was a small but significant direct relationship between 25(OH)D and IS in this study ($r = 0.304$, $p < 0.001$). This would support the findings from recent meta-analyses of both prospective studies [43] and randomized controlled trials [44], which indicate that at best, there is only a small positive effect of 25(OH)D on insulin sensitivity. It could therefore be argued that any increase in RMR through improvements in vitamin D status would only be partially offset by the decrease in RMR from an improvement in IS (Eq. 1). We acknowledge that our interpretation of the relationships described will require intervention studies to provide causal evidence.

Conclusion

In this cross-sectional study, vitamin D status and IS were independent predictors of RMR in adult Australians. Future human studies that investigate the causal associations between these variables and energy expenditure are therefore recommended.

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Author contributions EC, KP, MS, KK and PN conceived the idea. KP, NK, WCSPD conducted the measurements. KP and MS collated the data, EK cross-checked data entry and calculations, EK and MS

wrote the first draft. HA performed the statistical analysis. KK, PN and AH critically reviewed the manuscript. All authors contributed to the writing of the manuscript, and approved the submission.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical standards All human data collection was approved by the Curtin University Ethics Committee and was therefore performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments. All participants gave their informed written consent prior to their participation.

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CHAPTER 4 - *IN VIVO* 25(OH)D IS ASSOCIATED WITH THE BIOENERGETIC PROFILE OF *EX VIVO* PERIPHERAL BLOOD MONONUCLEAR CELLS

The content of this chapter is covered by Paper 4:

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Thesis objective addressed in this chapter:

Objective 3: To determine whether *in vivo* 25(OH)D is associated with *ex vivo* PBMC bioenergetics.

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Prevailing vitamin D status influences mitochondrial and glycolytic bioenergetics in peripheral blood mononuclear cells obtained from adults

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ABSTRACT

Background: Circulating peripheral blood mononuclear cells (PBMCs) are exposed to metabolic and immunological stimuli that influence their functionality. We hypothesized that prevailing vitamin D status [25(OH)D] would modulate the bioenergetic profile of PBMCs derived from humans.

Materials and methods: 38 participants (16 males, 22 females) ranging in body fat from 14–51% were studied. PBMCs were isolated from whole blood, counted and freshly seeded for bioenergetic analysis using the Seahorse XF^{e}96 flux analyser. Whole body energy metabolism via indirect calorimetry, body composition by *dual-energy X-ray* absorptiometry, and relevant clinical biochemistry were measured. Data was analysed based on 25(OH)D cut-offs of < 50 nmol/L (Group 1, n=12), 50–75 nmol/L (Group 2, n=15) and ≥75 nmol/L (Group 3, n=11). A multivariate general linear model adjusting for age, fat mass, fat-free mass, parathyroid hormone and insulin sensitivity was used.

Results: There were significant differences in cellular mitochondrial function between groups. Group 1 had significantly higher basal respiration (p=0.001), non-mitochondrial respiration (p=0.009), ATP production (p=0.001), proton leak (p=0.018), background glycolysis (p=0.023) and glycolytic reserve (p=0.039) relative to either Group 2 or Group 3; the latter two did not differ on any measures. There were no differences in bioenergetic health index (BHI), resting metabolic rates and systemic inflammatory markers between groups.

Conclusions: Inadequate vitamin D status adversely influenced bioenergetic parameters of PBMCs obtained from adults, in a pattern consistent with increased oxidative metabolism and activation of these cells.

1. Introduction

Insufficient vitamin D status [25(OH)D] is commonly observed worldwide. Several epidemiological studies in the United States, Canada, United Kingdom, and New Zealand have reported a high prevalence of inadequate levels of 25(OH)D [1]. Currently, the importance of achieving and maintaining adequacy is limited to the prevention of rickets, osteoporosis and fractures [2]. However, many have argued that a causal link between 25(OH) D insufficiency, obesity and other diseases may exist [2–5]. Consequently the concentration of circulating 25(OH)D that best represents sufficiency for extra-skeletal health, is currently debated. While some research groups argue that 50 nmol/L [6,7] is sufficient, others have proposed that the value should be raised to 75 nmol/L [8,9].

Cellular and animal models strongly argue for a role of vitamin D in immune function and energy metabolism. The vitamin D receptor (VDR), through which the majority of effects dependent on vitamin D are exerted, is found in most cells of the immune system, including activated lymphocytes, dendritic cells and macrophages [10]. The active metabolite, 1,25$(OH)_2$D, is a well-known regulator of immune function and in a recent systematic review we showcased that the active metabolite was strongly associated with an anti-inflammatory cytokine profile in peripheral blood mononuclear cells (PBMCs) [11]. 1,25$(OH)_2$D also enhanced the antimicrobial actions of macrophages, and promoted chemotaxis and phagocytic capabilities of innate immune cells [12]. The effect of 25(OH)D on cytokine release from immune cells is under-researched. One study has suggested that 25(OH)D had an anti-inflammatory effect as gauged from an increased IL-10 release with decreased concentrations of TNF-α levels [13], IL-6 and TNF-α mRNA, while another has observed a mixed profile [14]. The VDR has been identified in all key organs of energy metabolism including the pancreas, adipose, liver and skeletal muscle [15]. Low

Abbreviations: 2DG, 2-deoxyglucose; BHI, bioenergetic health index; FM, fat mass; FFM, fat free mass; PBMCs, peripheral blood mononuclear cells; PPR, proton production rate; OCR, oxygen consumption rate; RQ, respiratory quotient; RMR, resting metabolic rate; UCP, uncoupling proteins; VDR, vitamin D receptor

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25(OH)D, and the absence of vitamin D function in VDR knockout mice, resulted in an increase in energy expenditure [19,20]. Prevailing 25(OH)D levels are crucial since they influence local tissue concentrations of the active metabolite [16]. Hence inadequate 25(OH)D may indirectly affect whole body energetics through promoting activation of the immune system, which typically increases resting metabolic rate by 30–50% [17].

As PBMCs circulate in the vasculature, they are exposed to various metabolic and immunological stimuli such as glucose, amino acids, free fatty acids and vitamins. There is growing evidence that circulating factors can mediate bioenergetic function of PBMCs [18]. Numerous studies have harnessed PBMC's as a potential tool to determine the inflammatory and metabolic status in a variety of different disease states, including sepsis [19], neurodegeneration [20], rheumatic disease [21], obesity [22,23], cardiovascular disease [24], diabetes mellitus [25,26], and anorexia nervosa [27], all of which have been linked to mitochondrial dysfunction or altered bioenergetics. These studies in immune cells are consistent with a large body of work suggesting altered mitochondrial respiratory capacity and function in tissues classically studied such as skeletal muscle, liver, islet cells, and the myocardium [28–31]. Recently, the concept of a bioenergetic health index (BHI) has been proposed as a novel prognostic or diagnostic biomarker of disease [32].

The aim of the current study was to determine whether prevailing 25(OH)D influenced the bioenergetics of circulating leukocytes. To the best of our knowledge, the impact of the prevailing 25(OH)D status on bioenergetics and the BHI has not been previously described. A secondary objective was to determine whether PBMC bioenergetic parameters were associated with whole body energy metabolism in the same person. We have presented our hypothesis through a schematic that links low vitamin D status to a decreased BHI (Fig. 1). Intermediates in this pathway are higher levels of inflammation and greater insulin resistance (lesser insulin sensitivity) that independently, or in combination, would affect BHI. Since a heightened inflammatory status is energetically expensive, we have also proposed a link to whole body resting metabolic rate (RMR) and whole body fuel oxidation, i.e. respiratory quotient (RQ). Finally, we have included the possibility that a lower BHI per se could increase the demand and utilisation of vitamin D in order to restore cell functioning, and hence act as a driver of the low vitamin status in a feed forward loop (Fig. 1).

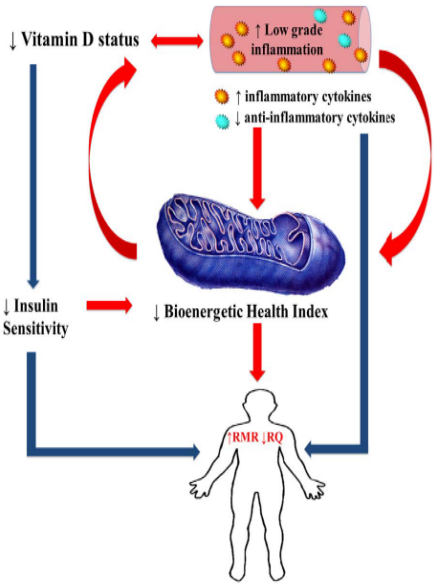

Fig. 1. Vitamin D status, inflammation and bioenergetic health. Legend: Inadequate vitamin D status promotes an inflammatory state. Together they reduce whole body insulin sensitivity and the bioenergetics of peripheral blood mononuclear cells. The resultant lower BHI, an indicator of mitochondrial health status, will possibly drive an increase in whole body resting metabolism (RMR). A heightened inflammatory state is also energetically expensive and account for a greater whole body energy requirement, while a lower insulin sensitivity will increase RMR and decrease respiratory quotient (RQ). Finally a low BHI per se has the potential to increase the demand for vitamin D in an attempt to maintain cellular function, hence leading to a further lowering of vitamin D status. Improvement of vitamin D status (increased sun exposure or supplementation) will act to reverse this dysmetabolism through lesser inflammation and greater BHI. ↑, increased; ↓, decreased; red arrows indicate pathways tested in this paper and blue arrows indicate what is known. BHI, bioenergetic health index; RMR, resting metabolic rate. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2. Materials and methods

2.1. Participant recruitment

Participants were recruited via flyer advertisement, radio advertisement, community newspapers and social media websites. Interested participants were assessed for eligibility through a short screening questionnaire. Inclusion criteria were as follows: Australians of European origin; aged between 20 and 70 years; body mass index (BMI) ≥18.5 kg/m^2, weight stable (±3 kg over the last six months); not suffering from any medical conditions involving the thyroid, liver, kidney or heart; absence of pregnancy; no history of cigarette smoking within a year prior to the study; not suffering from any current illness or infection requiring antibiotics; no gastrointestinal problems or history of gastrointestinal surgeries; no history of blood disorders; no history of mitochondrial disease; not on any medications that influence mitochondrial function (insulin, HMG-CoA reductase inhibitors, thiazolidinedione's), not on anti-convulsants (increased catabolism of vitamin D), not taking the following: parathyroid hormone (PTH) or its derivatives, calcitonin, HRT, corticosteroids, vitamin D supplements or any special or commercial diet programs that may affect the body's metabolism. This study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human volunteers were approved by the Human Research Ethics Committee of Curtin University, Perth, Western Australia (Ethics approval number RDHS-13-15). Written informed consent was obtained from all participants.

2.2. Intervention

Participants arrived at the laboratory after an overnight fast of 10–12 h, after refraining from strenuous physical activity 24 h prior to, and on the morning before the study day.

2.2.1. Physical characteristics and body composition

Weight was measured using an electronic platform balance (CW-11, Precision Balances Pty Ltd). Height was measured using a stadiometer fixed to a wall (Seca, Hamburg, Germany). Waist circumference was measured at the umbilicus. Body composition including fat mass (FM) and fat free mass (FFM) was assessed by dual energy x-ray absorptiometry (Prodigy, Lunar Corp USA).

2.2.2. Resting metabolic rate

Resting metabolic rate (RMR) was measured in the environmental chamber housed at the School of Public Health, Curtin University, Western Australia. The chamber is a purpose built structure within a large room of the building. It has a volume of 57.75 m^3, insulated walls and roof and is independently controlled for temperature range from 4 °C to 50 °C. Participants first rested in the supine position for 30 min to equilibrate with the temperature of the environmental chamber that was set at 25 °C the day before. A trial RMR measurement was made to accustom participants to the canopy of the TrueOne indirect calorimetry system (Parvo Medics, USA). RMR was then measured for 30 min, with the first 10 min excluded from the calculations. RMR in kJ/d was derived from CO_2 production and O_2 consumption according

to the Weir's formula, neglecting protein oxidation in the fasting state. Fasting respiratory quotient was calculated as volume of CO_2 produced/volume O_2 consumed. The TrueOne system was calibrated with gas mixtures of known composition before each measurement session and performance of the system was regularly checked through 30 min ethanol burn tests. A mean ± SD for six tests gave an RQ of 0.67 ± 0.01.

2.2.3. Blood collection and analysis

A fasting venous sample was obtained after 90 min exposure to the set temperature by experienced phlebotomists. Briefly, fasting blood glucose were determined using routine automated procedures on an Architect c16000 analyser that used specific enzyme-based colorimetric reagents (Abbott Diagnostics; CV < 2%). Fasting insulin was determined by PathWest Laboratories Perth Australia using an Architect i2000SR Analyser (Abbott Diagnostics; CV < 3%). PTH was determined using a Cobas e601 Analyser (Roche Diagnostics). Quantitative insulin sensitivity check index (QUICKI) was determined from fasting glucose and insulin [33]. 25(OH)D was measured by chemiluminescent micro particle immunoassay (Architect 25-OH Vitamin D assay, Abbott Diagnostics). Inflammatory cytokines IL-6, IL-8, IL-10, IL-12p70 and TNF-α were measured using Human High Sensitivity T Cell kits and run on a MAGPIX® system (Merck Millipore, Germany). All samples were measured in duplicate, and the average of the two values was used for data analyses. CRP was measured using QuickRead go CRP kits from Orian Diagnostica (Espoo, Finland).

2.2.4. Immune cell isolation and population determination

Eighteen millilitres of whole blood was drawn by venepuncture into commercially available EDTA-citrate vacutainers, from patients fasting overnight and diluted 1:1 in PBS-EDTA (2 mM). The diluted blood was transferred to a fresh tube containing an equivalent volume of Histopaque 1077 (Sigma-Aldrich, St Louis, USA) and centrifuged at 600×*g* for 20 min with minimum acceleration and no braking. Autologous plasma samples (3mls) were taken from the upper layer, while immune cells were isolated from the "buffy coat" and washed with EDTA-free PBS. Washing and centrifugation was repeated at 300, 200 and 100 g for 10 min each, to remove contaminating platelets. The cell pellet was re-suspended in 0.5 ml of warm RPMI-1640 (10% FBS, 2 mM glutamine, 100 U/ml penicillin & 0.1 mg/ml streptomycin), and an aliquot taken to determine the cell number and percentage proportion of immune cells (lymphocytes, monocytes and granulocytes) using the automatic Mindray BC2800 haematological analyser. The cell suspension was seeded into the Seahorse assay XF^{e}96 culture plate. All samples were processed within 5 h of blood collection.

2.2.5. Seahorse XF^{e}96 measurements

As per our previously established protocol [34], cells were seeded at a density of 3.5×10^5 cells/well into 96 well plates previously coated with poly-D-lysine (50 μg/mL) to maximise adherence and allowed to adhere overnight. After recording of basal measurements, the Mito Stress Test injection strategy consisted of oligomycin (5 μM), FCCP (1.5 μM), and rotenone/antimycin A in combination (5 μM). The Glycolytic Stress Test injection strategy consisted of glucose (25 mM), oligomycin (5 μM), followed by 200 mM 2-deoxyglucose (2DG). Oxygen consumption rate (OCR) and proton production rate (PPR) was measured using five 2 min cycles of mix and measurement following each injection.

2.2.6. Seahorse data analysis

Basal respiration was calculated by subtracting the minimum OCR following addition of rotenone/antimycin A (non-mitochondrial respiration) from the last OCR measurement recorded prior to addition of oligomycin. Proton leak was calculated by subtracting the minimum OCR following addition of rotenone/antimycin A (non-mitochondrial respiration) from the minimum OCR measurement recorded after addition of oligomycin. OCR related to ATP production (turnover) was calculated by the difference between the proton leak and basal respiration. Coupling efficiency percentage was calculated by dividing the ATP production dependent OCR by the basal respiration and multiplying by 100. Maximal respiration was determined by subtracting the non-mitochondrial respiration OCR from the maximum OCR in response to FCCP, while reserve capacity was the difference between the basal respiration and the calculated maximal respiration. Basal glycolysis in the presence of 0 mM glucose was determined by the last PPR measurement recorded prior to addition of 25 mM glucose. Glycolytic response to 25 mM glucose was determined by subtracting the maximum PPR following addition of glucose from the last PPR measurement prior to addition of glucose. Glycolytic capacity was measured by subtracting the minimum PPR following 2DG addition from the maximum PPR after injection of oligomycin. Finally, Glycolytic reserve was determined from the difference between the glycolytic capacity and the glycolytic response to 25 mM glucose. Each treatment was measured in at least triplicate wells.

2.2.7. Calculation of Bioenergetic Health Index (BHI)

The BHI of each sample was calculated as previously defined [35,36] and is presented below. No power function was applied to these parameters [35].

$$\text{Bioenergetic Health Index} = [\text{Reserve Capacity} \times \text{ATPproduction}] \div [\text{Non-mitochondrial respiration} \times \text{Proton Leak}] \quad (34)$$

2.3. Statistical analysis

We categorized our study sample into three groups based on cut offs for 25(OH)D of 50 nmol/L and 75 nmol/L. Participants with 25(OH)D < 50 nmol/L formed Group 1, 50–75 nmol/L were Group 2 and those with status ≥75 nmol/L were defined as Group 3. Normally distributed data are presented as mean (SD) and skewed data are presented as median (IQR). Skewed data were transformed and statistical analyses comparing the three independent groups were performed using multivariate GLM. Multivariate regression was used to adjust for effects of age, fat mass (kg), fat-free mass (kg), PTH (pmol/L), and QUICKI (quantitative insulin sensitivity check index) on all bioenergetics parameters. These covariates were selected by a parsimonious backward approach that tested for many potential variables. Pearson's partial correlation coefficients were used to determine correlations for BHI, RQ and RMR with inflammatory cytokines and QUICKI. All statistical calculations were performed using SPSS version 22 and graphics were generated using GraphPad Prism software v. 6.0.

3. Results

3.1. Demographics, body composition and inflammatory profile of participants

The participant cohort consisted of 16 males and 22 females, ranging in percent body fat from 14–51% and aged between 19 and 69 years. There were no differences between groups in gender distribution (M/F) (Group 1: 7/5, Group 2: 5/10, Group 3: 4/7, p=0.420), age [Group 1: 43.25 (16.65) years, Group 2: 42.00 (20.09) years, Group 3: 40.18 (18.78) years, p=0.925] and BMI [Group 1: 26.32 (3.77) kg/m^2, Group 2: 26.68 (4.04) kg/m^2, Group 3: 23.6418 (3.39) kg/m^2, p=0.115]. Further details of their body composition are provided in Table 1. We did not detect differences in CRP (p=0.174), TNF-α (p=0.952), IL-6 (p=0.883), IL-8 (p=0.986), IL-10 (p=0.499), or IL-12p70 (p=0.09) between the three groups whether unadjusted or adjusted for age, FM, FFM, PTH and QUICKI. Percentage of lymphocytes (p=0.217) and monocytes (p=0.424) also did not differ between

Table 1
Body composition, whole body energy metabolism and bioenergetic profiles of participants, according to vitamin D status group[†].

Characteristic	Whole group (n=38)	< 50 nmol/L (n=12)	50–75 nmol/L (n=15)	≥75 nmol/L (n=11)	P value
Body composition & whole body energy metabolism					
Fat mass (kg)	22.15 (12.84)	26.41 (19.89)	25.56 (16.07)	19.69 (5.01)	0.113
Fat free mass (kg)	52.14 (11.76)	54.93 (10.97)	51.67 (12.74)	49.75 (11.66)	0.574
PTH (pmol/L)	3.04 (1.38)	4.27 (2.52)[a]	2.8 (0.84)[b,c]	3.04 (0.86)[c]	0.065
QUICKI	0.37 (0.04)	0.37 (0.04)	0.37 (0.04)[a]	0.39 (0.02)[b]	0.078
RMR (kJ/d)	6136 (1415)	6535 (1444)	6333 (1513)	5433 (1058)	0.138
RQ	0.83 (0.03)	0.82 (0.04)	0.84 (0.03)	0.84 (0.03)	0.260
Mito stress test parameters					
Basal respiration (pmol O_2/min)	53.88 (21.84)	69.59 (22.22)[a]	43.69 (13.85)[b,c]	50.64 (22.13)[c]	0.005
Non mitochondrial respiration (pmol O_2/min)[#]	3.5667 (0.32)	3.7389 (0.33)	3.4729 (0.23)	3.507 (0.37)	0.074
ATP production (pmol O_2/min)[#]	6.16 (1.52)	7.27 (1.37)[a]	5.52 (1.05)[b,c]	5.82 (1.67)[c]	0.005
Proton leak (pmol O_2/min)	15.06 (4.58)	16.78 (5.04)	13.75 (4.67)	14.96 (3.62)	0.239
Maximal respiration (pmol O_2/min)	207.89 (106.95)	240.57 (106.58)	183.98 (76.31)	204.84 (139.74)	0.402
Coupling efficiency (%)	72.46 (7.20)	76.37 (6.05)[a]	69.68 (7.42)[b]	71.93 (6.57)	0.050
Reserve capacity (pmol O_2/min)	157.03 (88.54)	178.08 (83.67)	140.91 (72.66)	156.05 (113.98)	0.568
BHI[#]	2.29 (0.70)	2.45 (0.68)	2.14 (0.72)	2.31 (0.73)	0.534
Glycolysis stress test parameters					
Background glycolysis (pmol H^+/min)[#]	4.52 (1.05)	5.03 (0.92)	4.27 (1.03)	4.30 (1.10)	0.127
25 mM Glucose response (pmol H^+/min)[#]	0.03 (0.01)	0.03 (0.01)	0.04 (0.01)	0.04 (0.01)	0.321
Glycolytic reserve (pmol H^+/min)[#]	5.49 (1.40)	6.03 (1.38)	5.27 (1.40)	5.16 (1.36)	0.257
Glycolytic capacity (pmol H^+/min)[#]	7.83 (1.51)	8.55 (1.38)	7.64 (1.73)	7.29 (1.09)	0.110

Values not sharing the same superscript are significantly different from each other.
ATP, adenosine triphosphate; BHI, Bioenergetic Health Index; RMR, resting metabolic rate; RQ, respiratory quotient; QUICKI, quantitative insulin sensitivity check index.
[†] Data are mean (SD) following unadjusted multivariate ANOVA.
[#] Variables were transformed.

vitamin D status groups.

Thirty one participants had no contamination by platelets in their samples. Seven individuals showed marginal contamination ranging from 1000 platelets/μL to 8000 platelets/μL. Based on the total volume aliquoted into each well, the sample with the highest contamination would have had ~1.22 million platelets/well. The latter value is 1/20th the number of cells needed to detect a change in oxygen consumption of platelets [32].

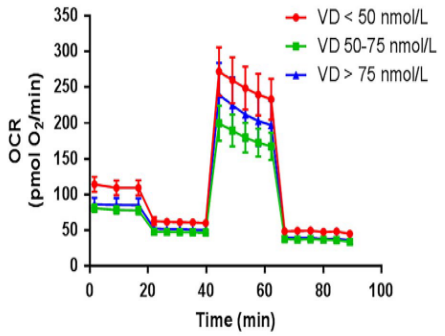

Fig. 2. Effect of vitamin D status on oxygen consumption rate during the Mito Stress Test. Legend: 25(OH)D, 25-hydroxy vitamin D; OCR, oxygen consumption rate.

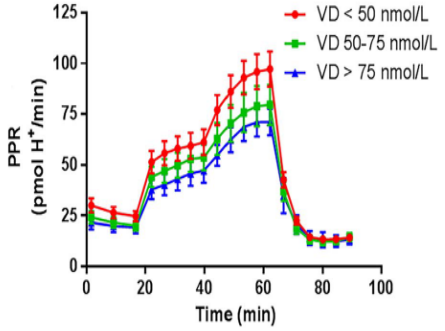

Fig. 3. Effect of vitamin D status on proton production rate during the glycolysis stress test. Legend: 25(OH)D, 25-hydroxy vitamin D; PPR, proton production rate.

3.2. Bioenergetic parameters

Insulin sensitivity was marginally different between vitamin D status groups, while RMR and RQ were similar among groups (Table 1). The Mito Stress Test trace and Glycolysis Stress Test bioenergetics responses are shown in Figs. 2 and 3, respectively. The unadjusted bioenergetic profile indicated significant group differences in basal respiration and ATP production. Basal respiration and ATP production were highest in the lowest vitamin D group (Group 1; < 50 nmol/L) with trends towards a greater non-mitochondrial respiration, and coupling efficiency (Table 1). After adjustment for age, FM, FFM, PTH and QUICKI, group differences were accentuated for basal respiration, non-mitochondrial respiration, ATP production, and proton leak (Table 2) with Group 1 having the highest values compared to the other two groups. Glycolytic stress test parameters also indicated a greater background glycolysis and glycolytic capacity (Table 2), with a trend for higher glycolytic reserve as well. There were no differences in adjusted parameters between Group 2 and Group 3 (Table 2). Analysis of the data without the 7 individuals where contamination by platelets was detected, is presented in Table S1. Those outcomes were similar in direction and statistical significance to outcomes of the complete dataset in Table 2.

3.3. Correlations between bioenergetics and whole body measurements

After adjustment for age, FM, FFM and PTH, BHI was positively related to QUICKI (r=0.527, p=0.002) and demonstrated inverse trends with RMR (r=−0.335, p=0.061), IL-6 (r=−0.312, p=0.082) and TNF-α (r=−0.325, p=0.07). A similar analysis demonstrated RMR to be positively associated with TNF-α (r=0.396, p=0.025), while RQ was inversely correlated with QUICKI (r=−0.433, p=0.013) and CRP (r=−0.477, p=0.006). QUICKI demonstrated a trend whereby values were inversely correlated with MCP-1 (r=−0.305, p=0.09). Upon

Table 2
Adjusted bioenergetic measurements compared across three groups varying in vitamin D status[†].

Characteristic	< 50 nmol/L (n=12)	50–75 nmol/L (n=15)	> 75 nmol/L (n=11)	P value
Whole body energy metabolism				
RMR (kJ/d)	6251 (785)	6294 (766)	5796 (755)	0.236
RQ	0.82 (0.03)[a]	0.84 (0.04)	0.85 (0.03)[b]	0.100
Mito stress test parameters				
Basal respiration (pmol O_2/min)	75.14 (19.94)[a]	40.74 (19.46)[b,c]	48.61 (19.18)[c]	0.001
Non mitochondrial respiration (pmol O_2/min)[#]	3.84 (0.33)[a]	3.41 (0.32)[b,c]	3.48 (0.32)[c]	0.009
ATP production (pmol O_2/min)[#]	7.59 (1.4)[a]	5.35 (1.37)[b,c]	5.70 (1.35)[c]	0.001
Proton leak (pmol O_2/min)	18.23 (4.61)[a]	12.56 (4.49)[b]	15.00 (4.43)	0.018
Maximal respiration (pmol O_2/min)	248.83 (109.58)	189.15 (106.94)	188.77 (105.43)	0.337
Coupling efficiency (%)	76.23 (7.76)	70.17(7.55)	71.36 (7.49)	0.156
Reserve capacity (pmol O_2/min)	182.78 (90.55)	146.81 (88.37)	142.87 (87.12)	0.522
BHI[#]	2.39 (0.69)	2.25 (0.67)	2.23 (0.66)	0.839
Glycolysis stress test parameters				
Background glycolysis (pmol H^+/min)[#]	5.27 (1.04)[a]	4.07 (1.01)[b,c]	4.31 (0.99)[c]	0.023
25 mM Glucose response (pmol H^+/min)[#]	0.03 (0.01)	0.04 (0.01)	0.04 (0.01)	0.253
Glycolytic reserve (pmol H^+/min)[#]	6.31 (1.39)	5.14 (1.46)	5.03 (1.43)	0.094
Glycolytic capacity (pmol H^+/min)[#]	8.79 (1.45)[a]	7.44 (1.43)[b,c]	7.30 (1.39)[c]	0.039

Values not sharing the same superscript are significantly different from each other.
ATP, adenosine triphosphate; BHI, Bioenergetic Health Index; RMR, resting metabolic rate; RQ, respiratory quotient; QUICKI, quantitative insulin sensitivity check index.
[†] Data are mean (SD) following multivariate ANCOVA with adjustment for fat mass (kg), fat-free mass (kg), PTH (pmol/L), and QUICKI.
[#] Transformed variables.

further adjustment for QUICKI, all associations with BHI disappeared, however RMR remained directly related to TNF-α levels (r=0.375, p=0.038), while RQ was inversely related to CRP (r=−0.40, p=0.024).

4. Discussion

Bioenergetic dysfunction has been demonstrated in various disease states including Alzheimer's disease [37], type 2 diabetes [38] and anorexia nervosa [39]. Each of these disorders has also been associated with low vitamin D status [40–42]. There are few studies investigating the impact of vitamin D status on energetic parameters of PBMCs, and whether prevailing 25(OH)D, whole body energy metabolism, inflammatory markers and cellular energetics are interrelated in the same individual was, up to now, unknown.

4.1. Main findings

Our results suggest that in vivo circulating 25(OH)D, a proxy for vitamin D status, was associated with ex-vivo PBMC cell bioenergetics resulting in significantly greater basal respiration, ATP production, proton leak, and non-mitochondrial respiration in those with 25(OH)D < 50 nmol/L (Table 2). Concomitantly, we also uncovered significantly greater background glycolysis and glycolytic capacity in the same vitamin D inadequate group (Table 2). These outcomes were consistent whether we used the entire dataset or restricted our analysis to those with no platelet contamination (Table S1). Overall, a lower 25(OH)D was associated with heightened PBMC bioenergetics, both oxidative phosphorylation and glycolysis. It appears from our data that this effect may plateau above 50 nmol/L, since the group > 75 nmol/L showed no further change in these bioenergetic parameters (Tables 2 and S1).

To the best of our knowledge, this is the first study to suggest there may be an effect of circulating 25(OH)D on PBMC bioenergetics. Other studies have investigated the effect of cholecalciferol ± calcium on skeletal muscle bioenergetics using P Magnetic resonance spectroscopy [43–45]. No overt abnormalities of skeletal muscle mitochondrial oxidative function in vitamin D-deficient subjects compared with healthy controls were found [43], which are in contrast to the present findings. Reasons for the discrepancy may include the different tissues/cells studied, and the use of small sample sizes that may not overcome the biological variability in measurement of mitochondrial function.

Previous animal and cellular studies suggested that vitamin D modulated energy metabolism. Mouse models demonstrate that vitamin D deficiency or impaired VDR signalling (through VDR knockout), results in increased energy expenditure. In global VDR null mice, increased energy expenditure measured by indirect calorimetry is seen [46]. These VDR null mice fed a high-fat diet displayed reduced lipid accumulation in the liver through greater fatty acid oxidation and increased expression of uncoupling proteins (UCPs), UCP-1, UCP-2, which increases energy expenditure [46]. Dietary induced vitamin D deficiency also alleviates hepatic lipid accumulation, upregulates key enzymes involved in fatty acid oxidation and uncoupling protein 3 (UCP-3) [47]. UCP-1 is an important regulator of proton flux, and can allow dissipation of the proton gradient across the mitochondrial inner membrane in specialised tissues such as brown adipose tissue [46]. While UCP-2 and UCP-3, will transport protons and increase the net proton conductance of mitochondria in the presence of specific activators. Such observations would support our findings of increased proton leak and ATP production with low 25(OH)D, which may reflect increased energy demand, increased energy expenditure, and/or dysfunctional energy utilisation. Interestingly, recent molecular studies have shown that the silencing of VDR signalling and impairment of VDR translocation to the mitochondria in cancer cells, promoted elevated mitochondrial respiration and electron transport chain activity through upregulation of cytochrome oxidase enzymes (COX II and IV) [48]. In another study, treatment with 1,25$(OH)_2$D reduced glycolytic and citric acid cycle metabolic flux by decreasing the concentration of key intermediates [49]. Collectively, these cellular studies demonstrate that VDR signalling is a regulator of glycolytic and oxidative metabolism, and appears to function by restraining metabolic flux under normal physiological conditions.

The increased bioenergetic profile associated with 25(OH)D < 50 nmol/L is consistent with enhanced oxidative stress and activation of PBMCs. Others have previously demonstrated that oxidative stress increases ATP-linked oxygen consumption and proton leak [50]. Chacko et al. hypothesized that oxidative stress induces increased non-mitochondrial respiration (e.g. ROS generation), which leads to increased proton leak and greater ATP demand; together this is reflected by an increased basal respiration [35]. In leukocytes, non-mitochondrial respiration is attributed to enzymes associated with inflammation, such as cyclooxygenases, lipoxygenases and NADPH oxidases and possibly intra-mitochondrial sources of ROS [35,36]. It is well-recognized that activation of leukocytes increases metabolic rate; with the increase depending on the condition that activates the immune system. RMR was positively associated with TNF-α, after accounting for several confounders including insulin sensitivity, while RQ was inversely related to CRP. Such observations mimic the increased energetic cost of an activated immune system [51]. However, immune cell fuel utilisation during low grade inflammation is an area which requires further investigation as no consensus exists regarding fuel choice by immune cells and the impact of circulating hormones and cytokines associated with stress responses.

We found that BHI, a proposed indicator of mitochondrial health status [32], was directly related to insulin sensitivity but inversely to

RMR, and some markers of systemic inflammation. This potentially validates a scenario where attenuation of systemic inflammation, improved insulin sensitivity, reduced whole body energetic demand and BHI are inter-linked (Fig. 1). Given that proton leak is accountable for ~25% of RMR [52] and proton leak is associated with inefficient ETC activity, this would further enhance the conceptual arguments presented. That these relationships were not obvious once the data were adjusted for QUICKI, could indicate the primacy of whole body insulin sensitivity in the derangement of bioenergetics associated with a poor vitamin D status.

Vitamin D is recognized as an anti-inflammatory agent [11], thus vitamin D may influence bioenergetics through inflammatory mechanisms. However, our study did not find any group differences in circulating inflammatory markers. Many randomized controlled trials report no difference in inflammatory markers following vitamin D supplementation [53–56], however cellular studies convincingly demonstrate an anti-inflammatory benefit following exposure to $1,25(OH)_2D(11)$. Although the majority of cellular studies have tested the effects of 1, $25(OH)_2D$, there is close relationship between circulating 25(OH)D and its active metabolite. This is especially crucial for tissues that can convert 25OHD to $1,25(OH)_2D$ such as the immune cells [16]. In support of this, observations that the concentration of serum vitamin D influences cytokine secretion from peripheral blood mononuclear immune cells has been reported [57]. We acknowledge that measuring systemic cytokine concentrations does not provide information on cytokine release at the local tissue level [58]. It is also likely that we could not detect group differences between study groups because we excluded participants with acute inflammatory conditions.

We also observed that insulin sensitivity tended to be highest in Group 3 (≥75 nmol/L) (Table 1). This positive association between vitamin D status and insulin sensitivity has been found in many cross-sectional studies [59–61]. Furthermore in this study QUICKI trended towards an inverse association with MCP-1, and MCP-1 has been repeatedly demonstrated to induce insulin resistance in obese mice models [62,63]. It is well-accepted that the insulin resistance that accompanies obesity is attributable, at least in part, to changes in the secretion of adipokines [64]. Overall, as the links between BHI, inflammatory markers and whole body energy expenditure disappeared on adjustment for QUICKI, insulin sensitivity may also be key to the relationship of bioenergetics and systemic inflammation.

4.2. Study limitations, strengths and future directions

As a cross-sectional design we cannot confirm a causal effect of 25(OH)D on PBMC bioenergetics but the results strongly support the examination of correcting inadequate vitamin D status on bioenergetics parameters. The samples used contained a heterogeneous population of immune cells, each of which have unique bioenergetic profiles, as eloquently discussed by Chacko et al. [32] and Kramer et al. [65]. Since immune cells in vivo interact with one another, it is likely that the metabolic state of the whole human system is better reflected through a heterogeneous sample, rather than just one immune cell type. We do however acknowledge the value in determining the influence of vitamin D on bioenergetic parameters in isolated and purified populations of immune cells. Future studies may investigate whether this explains the relationship between vitamin D status and bioenergetics that we have observed. Despite finding significantly increased ATP production and non-significant increased reserve capacity [numerator terms of the BHI equation], we also uncovered an increased proton leak and non-mitochondrial respiration [denominator terms]. It would appear such effects approximately cancelled out as we found no significant differences between vitamin D groups in BHI. While BHI was 7% marginally higher in those with < 50 nmol/L compared to those with status > 75 nmol/L, our study was not powered to detect such a difference. It is also possible that the BHI equation as originally proposed may need modification, however developing such an equation was outside the scope of this manuscript given its cross sectional design.

The major strength of our study is its holistic approach that combined metabolic profiling of PBMCs, whole body energy metabolism parameters, markers of whole body insulin sensitivity, body composition and systemic inflammatory profile. This allowed a broad overview of bioenergetics across the range of prevailing 25(OH)D seen in this study. We believe such studies are better equipped to allow translation of key cellular and molecular events to a clinical scenario of disease. However they necessarily have to overcome the complexity and inherent larger biological variability associated with the whole systems approach, relative to isolated cell systems. There is a requirement for well-designed randomized controlled trials to extend such findings towards a causal role for the vitamin in human energy metabolism and bioenergetics of leukocytes in chronic disease states.

In conclusion, this study documents for the first time the potential influence of vitamin D status on bioenergetics in freshly isolated peripheral blood mononuclear cells. Taken together, these data indicate a relationship between Vitamin D and immune cell bioenergetic responses. Specifically we propose that low vitamin D status engenders a pattern consistent with increased oxidative metabolism and inflammatory activation that is reflected in altered bioenergetics of PBMCs. Future studies need to validate whether vitamin D has a causal role in cellular function and bioenergetic health.

Author Contributorship

The present work was designed by KNK, MJS, PN and EKC. Initial manuscript preparation and draft was undertaken by EKC and revised by KNK, MJS, PN, and JR. Patients were recruited by EKC and body composition was assessed by EKC. Immune cell isolation was performed by JR, KNK and EKC. Bioenergetic parameters were measured by EKC. Data analysis and statistical analysis were made by EKC and KNK. Figure preparation was made by EKC, MJS and KNK. Supervision of the manuscript was made by PN and MJS. All authors approved the final version of the paper.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.redox.2016.10.007.

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Supplementary Table 1. Adjusted bioenergetic measurements according to vitamin D status groups in those participants with no platelet contamination[†]

Characteristic	<50 nmol/L (n=12)	50-75 nmol/L (n=15)	>75 nmol/L (n=11)	P value
Whole body energy metabolism				
RMR (kJ/d)	6295 (783)	6424 (789)	5901 (767)	0.337
RQ	0.82 (0.04)[a]	0.84 (0.04)	0.85 (0.04)[b]	0.097
Mito Stress Test parameters				
Basal respiration (pmol O_2/min)	75.14 (21.62)[a]	42.00 (21.80)[b,c]	46.13 (21.19)[c]	0.005
Non mitochondrial respiration (pmol O_2/min)[#]	3.82 (0.34)[a]	3.47 (0.35)[b,c]	3.43 (0.34)[c]	0.038
ATP production (pmol O_2/min)[#]	7.58 (1.54)[a]	5.48 (1.56)[b,c]	5.55 (1.51)[c]	0.009
Proton leak (pmol O_2/min)	18.01 (4.43)[a]	13.00 (4.46)[b]	13.87 (4.34)	0.045
Maximal respiration (pmol O_2/min)	253.25 (122.05)	197.31 (123.05)	189.72 (119.63)	0.467
Coupling efficiency (%)	76.38 (7.83)	69.85 (7.86)	72.30 (9.07)	0.201
Reserve capacity (pmol O_2/min)	187.38 (101.46)	152.09 (102.3)	143.85 (99.45)	0.610
BHI[#]	2.44 (0.76)	2.27 (0.77)	2.32 (0.75)	0.886
Glycolysis Stress Test parameters				
Background glycolysis (pmol H^+/min)[#]	5.32 (0.97)[a]	3.84 (0.98)[b,c]	4.28 (0.95)[c]	0.008
25mM Glucose response (pmol H^+/min)[#]	0.03 (0.01)	0.04 (0.01)	0.04 (0.01)	0.219
Glycolytic reserve (pmol H^+/min)[#]	6.35 (1.32)[a]	4.50 (1.26)[b,c]	4.79 (1.29)[c]	0.013
Glycolytic capacity (pmol H^+/min)[#]	8.85 (1.19)[a]	6.85 (1.20)[b,c]	7.12 (1.16)[c]	0.002

Data are mean (SD) following following multivariate ANCOVA with adjustment for age (years), fat mass (kg), fat-free mass (kg), PTH (pmol/L), and QUICKI.
[#]Transformed variables
Values not sharing the same superscripts are significantly different from each other. ATP, adenosine triphosphate; BHI, Bioenergetic Health Index; RMR, resting
metabolic rate; RQ, respiratory quotient; QUICKI, quantitative insulin sensitivity check index.

Supplementary Table 1. Adjusted bioenergetic measurements according to vitamin D status groups in those participants with no platelet contamination[†]

Characteristic	<50 nmol/L (n=12)	50-75 nmol/L (n=15)	>75 nmol/L (n=11)	P value
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Proton leak (pmol O_2/min)	18.01 (4.43)[a]	13.00 (4.46)[b]	13.87 (4.34)	0.045
Maximal respiration (pmol O_2/min)	253.25 (122.05)	197.31 (123.05)	189.72 (119.63)	0.467
Coupling efficiency (%)	76.38 (7.83)	69.85 (7.86)	72.30 (9.07)	0.201
Reserve capacity (pmol O_2/min)	187.38 (101.46)	152.09 (102.3)	143.85 (99.45)	0.610
BHI[#]	2.44 (0.76)	2.27 (0.77)	2.32 (0.75)	0.886
Glycolysis Stress Test parameters				
Background glycolysis (pmol H^+/min)[#]	5.32 (0.97)[a]	3.84 (0.98)[b,c]	4.28 (0.95)[c]	0.008
25mM Glucose response (pmol H^+/min)[#]	0.03 (0.01)	0.04 (0.01)	0.04 (0.01)	0.219
Glycolytic reserve (pmol H^+/min)[#]	6.35 (1.32)[a]	4.50 (1.26)[b,c]	4.79 (1.29)[c]	0.013
Glycolytic capacity (pmol H^+/min)[#]	8.85 (1.19)[a]	6.85 (1.20)[b,c]	7.12 (1.16)[c]	0.002

Data are mean (SD) following following multivariate ANCOVA with adjustment for age (years), fat mass (kg), fat-free mass (kg), PTH (pmol/L), and QUICKI.

[#]Transformed variables

Values not sharing the same superscripts are significantly different from each other.

ATP, adenosine triphosphate; BHI, Bioenergetic Health Index; RMR, resting metabolic rate; RQ, respiratory quotient; QUICKI, quantitative insulin sensitivity check index.

CHAPTER 5 - WINTER TO SUMMER CHANGE IN VITAMIN D STATUS REDUCES SYSTEMIC INFLAMMATION AND BIOENERGETIC ACTIVITY OF HUMAN PERIPHERAL BLOOD MONONUCLEAR CELLS

The content of this chapter is covered by Paper 5:

Calton, E. K., K. N. Keane, R. Raizel, J. Rowlands, M. J. Soares, and P. Newsholme. 2017. "Winter to summer change in vitamin D status reduces systemic inflammation and bioenergetic activity of human peripheral blood mononuclear cells." *Redox Biol* 12:814-820. doi: http://doi.org/10.1016/j.redox.2017.04.009.

Thesis objective addressed in this chapter:

Objective 4: To investigate whether seasonal variations in 25(OH)D are associated with seasonal changes in whole body energy metabolism, circulating PBMC bioenergetic profiles, and markers of systemic inflammation.

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Winter to summer change in vitamin D status reduces systemic inflammation and bioenergetic activity of human peripheral blood mononuclear cells

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ABSTRACT

Background: Vitamin D status [25(OH)D] has recently been reported to be associated with altered cellular bioenergetic profiles of peripheral blood mononuclear cells (PBMCs). No study has tracked the seasonal variation of 25(OH)D and its putative influence on whole body energy metabolism, cellular bioenergetic profiles, inflammatory markers and clinical chemistry.

Material and methods: Whole body energy metabolism and substrate utilisation were measured by indirect calorimetry. PBMCs obtained from the same subjects were isolated from whole blood, counted and freshly seeded. Bioenergetic analysis (mitochondrial stress test and glycolysis stress test) was performed using the Seahorse XF^{e}96 flux analyser. 25(OH)D was assessed using the Architect immunoassay method.

Results: 25(OH)D increased by a median (IQR) of 14.40 (20.13) nmol/L ($p < 0.001$) from winter to summer and was accompanied by significant improvements in indices of insulin sensitivity, McAuley's index ($p = 0.019$) and quantitative insulin sensitivity check index ($p = 0.028$). PBMC mitochondrial parameters basal respiration, non-mitochondrial respiration, ATP production, proton leak, and maximal respiration decreased in summer compared to winter. Similarly, PBMC glycolytic parameters glycolytic activity, glucose response, and glycolytic capacity were all reduced in summer compared to winter. There was also a trend for absolute resting metabolic rate (RMR) to decrease ($p = 0.066$). Markers of systemic inflammation MCP-1, IL-6, IL-8, IL-10, and IL-12p70 decreased significantly in summer compared to winter. Participants who entered winter with a low 25(OH)D (< 50 nmol/L), had the greatest alteration in bioenergetic parameters in summer, relative to those with winter 25(OH)D concentrations of 50–75 nmol/L or > 75 nmol/L. The absolute change in 25(OH)D was not associated with altered bioenergetics.

Conclusion: Seasonal improvements in 25(OH)D was associated with reduced systemic inflammation, PBMC bioenergetic profiles and whole body energy metabolism. These observational changes in PBMC bioenergetics were most pronounced in those who had insufficient 25(OH)D in winter. The data warrants confirmation through cause and effect study designs.

1. Introduction

Immunology and metabolism have, until recently, existed as two predominantly separate fields of research [1]. However, immune cell activation influences metabolic function in adipose and the liver, while the microenvironment within these tissues modulates immune cell function. Low-grade, systemic inflammation ('metaflammation') is linked to many types of chronic disease [2] including obesity and the metabolic syndrome [3]. The cytokines most widely studied in relation to obesity are IL-6 and TNF-α [3] and are consistently elevated in serum and adipose tissue derived from obese subjects [4].

Insufficient vitamin D status, determined by serum 25-hydroxyvitamin D [25(OH)D], is commonly observed worldwide, with the greatest levels of insufficiency occurring during winter months [5–7]. The level of 25(OH)D in humans and other animals is well known to rise in summer and decline in winter in response to seasonal variation in the

Abbreviations: 2DG, 2-deoxyglucose; BHI, bioenergetic health index; FBG, fasting blood glucose; FM, fat mass; FFM, fat free mass; IPAQ, international physical activity questionnaire; McA, McAuleys index of insulin sensitivity; PBMCs, peripheral blood mononuclear cells; PPR, proton production rate; OCR, oxygen consumption rate; RQ, respiratory quotient; RMR, resting metabolic rate

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intensity of solar UV light. In Australia, typical seasonal variations are 10–20 nmol/L [5–7]. However, there is a lack of reliable information to indicate whether seasonal variation in 25(OH)D positively or negatively influences health, or indeed has no significant impact [8]. Interestingly, this seasonal variation coincides with seasonal variations in blood pressure [9], $HbA1_C$ [10] and circulating lipids [11], indicating a potential association between seasonality of 25(OH)D and chronic metabolic disease.

Seasonal variation in energy demands and inflammation may co-exist. There is a seasonal variation in C-reactive protein (CRP) levels, with higher values observed during winter than in summer. Elevated CRP levels can be related to an increased risk of cardiovascular events, which are more prominent during the winter months [12]. Seasonal variations in the number of white blood cells and subtypes such as neutrophils, monocytes, lymphocytes, $CD4^+$ T cells, $CD8^+$ T cells, $CD25^+$ T cells, $CD20^+$ B cells, and serum IL-6 have also been reported [13]. Elevated inflammatory cytokines can damage cell function by promoting a reduction in mitochondrial DNA integrity and protein integrity, and can thus induce redox stress which may result in bioenergetic dysfunction [14]. We are aware of only one study that has examined whether the bioenergetic profile of peripheral blood mononuclear cells (PBMCs) was related to differences in inflammatory status; those data support an association of changes in interleukin-6 and bioenergetic profile [14]. Recently, we demonstrated that 25(OH)D below 50 nmol/L was associated with increased oxidative and glycolytic bioenergetic profile responses in PBMCs obtained from adults [15]. Whether change in 25(OH)D was associated with altered bioenergetic profiles remains to be elucidated. Other research groups have proposed that baseline 25(OH)D, change in vitamin status and the final concentration achieved, may well be crucial considerations in uncovering the extra-skeletal effects of vitamin D supplementation [16,17].

The aim of the present study was to track seasonal variations in 25(OH)D and investigate the influence on whole body energy metabolism, circulating PBMC bioenergetic profiles, and markers of systemic inflammation. To the best of our knowledge, the impact of seasonal variation in 25(OH)D on cellular energy demand and inflammation, and the potential flow through impact on whole body energy metabolism, has not been previously described. We examined the hypothesis that a lower 25(OH)D in winter may promote greater inflammation and insulin resistance; the attendant higher energetic cost would be reflected in higher resting metabolic rate (RMR) and increased PBMC bioenergetic parameters. Consequently, a seasonal increase in 25(OH)D would reverse these effects and be most evident in those who started with the lowest 25(OH)D (Fig. 1).

2. Materials and methods

2.1. Participant recruitment

Participant recruitment and eligibility criteria have previously been reported [15]. Briefly, participants were Australians of European origin, aged between 20 and 70 years, not suffering from any current illness or infection requiring antibiotics, not taking medications that influenced mitochondrial function (insulin, HMG-CoA reductase inhibitors, thiazolidinedione's), or any of the following: parathyroid hormone (PTH) or its derivatives, calcitonin, hormone replacement therapy, corticosteroids, or vitamin D supplements. This study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects were approved by the Human Research Ethics Committee of Curtin University, Perth, Western Australia (Ethics approval number RDHS-13-15]. Written informed consent was obtained from all participants.

2.2. Period of observation

The data collection described below was conducted in Australian winter 2015 (July-September) and the protocol was repeated in Australian summer 2016 (February-March). Participants were instructed not to perform any strenuous activity the day before the experiment to avoid effects of physical activity on RMR. Data were collected at the Curtin University laboratory with participants in the fasted state.

2.2.1. Physical characteristics, body composition and physical activity

Weight was measured using an electronic platform balance (CW-11, Precision Balances Pty Ltd). Height was measured using a stadiometer fixed to a wall to the nearest 0.1 cm (Seca, Hamburg, Germany). Body composition was assessed by dual energy x-ray absorptiometry (Prodigy, Lunar Corp USA). Physical activity was determined from the short version of international physical activity questionnaire (IPAQ) [18].

2.2.2. Resting metabolic rate and forearm-fingertip gradient

RMR was measured by indirect calorimetry in the environmental chamber housed at the School of Public Health, Curtin University, Western Australia as per our established protocol [15]. The TrueOne system (Parvo Medics, USA) was calibrated with gas mixtures of known composition before each measurement session and regularly checked by 30 min ethanol burn tests. Mean and SD for six tests during the winter phase of the study was substrate oxidation = 0.67 ± 0.01. Mean and SD for seven tests during the summer phase of the study was substrate oxidation = 0.68 ± 0.01. Forearm-fingertip gradient (FFG), an indicator of peripheral vasoconstriction, was measured using iButtons (iButton type DS1921H-F#, Maxim Integrated Products). One iButton was placed on the ventral side of the left middle fingertip and a second iButton was located along the dorsal left forearm, midway between the elbow and wrist [19].

2.2.3. Blood collection and analysis

A fasting venous sample was obtained by an experienced phlebotomist. Blood from fasted participants was collected, processed, and serum stored at −80 °C, until analysis. Triglycerides (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL) and fasting blood glucose (FBG) were determined using routine automated procedures on an Architect c16000 analyser that used specific enzyme-based colorimetric reagents (Abbott Diagnostics; CV < 2%). Low-density lipoprotein cholesterol (LDL) was estimated using the Friedewald equation [20]. Fasting insulin was measured using an Architect i2000SR Analyser (Abbott Diagnostics; CV < 3%). McAuley's index for insulin sensitivity (McA) was calculated from TG and insulin concentrations. Quantitative insulin sensitivity check index (QUICKI) was determined from FBG and insulin [21]. Parathyroid hormone (PTH) was determined using a Cobas e601 Analyser (Roche Diagnostics). 25(OH)D was measured by chemi-luminescent micro particle immunoassay (Architect 25-OH Vitamin D assay, Abbott Diagnostics). Inflammatory cytokines IL-6, IL-8, IL-10, IL-12p70 and TNF-α were measured using Human High Sensitivity T Cell kits and run on a MAGPIX® system (Merck Millipore, Germany). All samples were measured in duplicate, and the average of the two values was used for data analyses. CRP was measured using QuickRead Go CRP kits from Orian Diagnostica (Espoo, Finland). MCP-1 was measured by ELISA (elisakit.com, Australia).

2.2.4. Immune cell isolation, population determination, Seahorse XFe96 measurements and analysis

PBMCs were isolated and the population determined as previously described [22]. After recording the basal oxygen consumption rate, the Mito Stress Test inhibitor/activator injection strategy consisted of oligomycin (5 μM), FCCP (1.5 μM), and rotenone/antimycin A in combination (5 μM). Following the measurement of the basal proton production rate, the Glycolytic Stress Test injection strategy commenced with glucose (25 mM), then oligomycin (5 μM), followed by

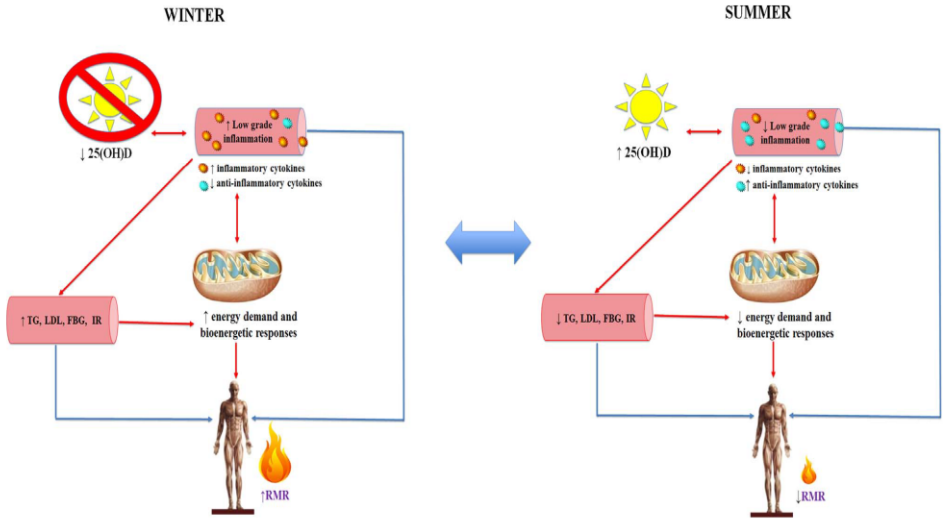

Fig. 1. Seasonal change in 25(OH)D influences changes in inflammation, PBMC bioenergetics, and whole body energy metabolism. A lower 25(OH)D in winter may promote greater inflammation and insulin resistance; the attendant higher energetic cost would be reflected in higher resting metabolic rate (RMR) and increased PBMC bioenergetic parameters. Consequently, a seasonal increase in 25(OH)D would reverse these effects. Legend: 25(OH)D, 25-hydroxy vitamin D; FBG, fasting blood glucose; IR, insulin resistance; LDL, low-density lipoprotein cholesterol; RMR, resting metabolic rate; TG, triglycerides. ↓, decrease; ↑, increase.

200 mM 2-deoxyglucose (2DG). The bioenergetic health index (BHI) of each sample was calculated as previously described and no power function was applied to these parameters [15,23].

2.3. Statistical analysis

Data are presented as median (IQR). Differences among seasons were compared using Wilcoxon non-parametric tests. Change variables were calculated as the summer value minus the winter value. To examine the influence of initial 25(OH)D on bioenergetics and inflammation change, we categorized our study sample into three groups based on cut offs for 25(OH)D of 50 nmol/L and 75 nmol/L. Participants with 25(OH)D < 50 nmol/L formed Group 1, 50–75 nmol/L Group 2 and those with status ≥ 75 nmol/L were defined as Group 3. To examine the influence of final 25(OH)D achieved and absolute change in 25(OH)D on bioenergetics and inflammation change, we categorized our study sample into three tertiles. The three independent groups were compared using multivariate GLM adjusting for effects of change in fat mass (FM) (kg), fat-free mass (FFM) (kg), PTH (pmol/L), and QUICKI on all bioenergetics and inflammatory parameters. All statistical calculations were performed using SPSS for Windows version 24.0 (SPSS, Chicago, IL). Statistical significance was accepted at $p < 0.05$.

3. Results

3.1. Demographic characteristics of participants

The participant cohort consisted of 30 adults aged between 20 and 69 years.

There were 15 males and 15 females, 14 of which were lean (BMI < 25 kg/m^2) and 16 were overweight or obese (BMI ≥ 25 kg/m^2).

3.2. Seasonal influence on 25(OH)D, body composition and physical activity

Median 25(OH)D significantly increased from winter 62.85 (30.35) nmol/L to summer 73.75 (34.05) nmol/L, $p < 0.001$. Initial winter 25(OH)D was strongly associated with final summer status unadjusted ($r = 0.711$, $p < 0.001$) and adjusted for PTH, QUICKI, FFM, FM ($r = 0.766$, $p < 0.001$). FFM (kg) did not change between seasons [winter 53.31 (18.81) kg vs summer 53.89 (20.25) kg, $p = 0.299$]. Similarly, FM (kg) was not different between seasons [winter 22.15 (11.20) kg vs summer 22.68 (9.10) kg, $p = 0.136$], nor was IPAQ [winter 1870 (2982) vs summer 1780 (4155), $p = 0.922$].

3.3. Seasonal influence on whole body and PBMC bioenergetics

There was a trend for absolute RMR to decrease ($p = 0.060$) and RQ to increase ($p = 0.066$) from winter to summer (Table 1). PBMC basal respiration, non-mitochondrial respiration, ATP production, proton leak, and maximal respiration were significantly lower in summer than winter. Background glycolytic activity, glucose response and glycolytic capacity were also reduced over the same period (Table 1).

3.4. Seasonal influence on inflammation and clinical biochemistry

All inflammatory markers significantly decreased from winter to summer, with the exception of CRP which did not change (Table 2). There were no significant changes in the percentages of lymphocytes (winter 66.32 (9.58)%; summer 65.91 (9.94)%; change −0.41 (12.51)%, $p = 0.860$) or monocytes (winter 11.00 (4.21)%; summer 9.59 (3.32)%; change −1.01 (5.28)%, $p = 0.305$). From winter to summer, FBG, TG, TC, LDL and HDL decreased, while indices of insulin sensitivity, QUICKI and McA significantly increased (Table 2). Forearm to fingertip skin temperature gradient (FFG) did not significantly change between seasons [winter −0.02 (4.84) °C vs summer −0.85 (3.92) °C, $p = 0.098$].

3.5. The influence of baseline (winter) 25(OH)D on change in seasonal bioenergetics and inflammation

When the patient data was analysed according to baseline (winter) 25(OH)D, with adjustment for change in FM, FFM, PTH and QUCKI, significant differences in bioenergetic parameters such as basal respiration ($p = 0.001$), non-mitochondrial respiration ($p = 0.021$), ATP production ($p = 0.001$), maximal respiration ($p = 0.022$), coupling efficiency ($p = 0.047$), reserve capacity ($p = 0.047$), background glycolysis ($p = 0.002$), glycolytic reserve ($p = 0.039$) and glycolytic capacity ($p = 0.026$) were observed across the 25(OH)D groups (Table 3). Group 1 (< 50 nmol/L) had the greatest reduction in bioenergetic parameters compared to the other two groups, and no differences were found

Table 1
Whole body and cellular bioenergetics in winter and summer.

Characteristic	Winter	Summer	P value	Direction & degree of change (%)
Whole body energy metabolism markers				
RMR (kJ/d)	6107 (1707)	5915 (2661)	0.060	↓ 3
RQ	0.83 (0.04)	0.85 (0.04)	0.066	↑ 2
Mito stress test parameters				
Basal respiration (pmol O_2/min/350,000 cells)	47.22 (25.17)	36.15 (16.82)	0.003	↓ 23
Non mitochondrial respiration (pmol O_2/min/350,000 cells)	36.10 (12.16)	28.86 (10.33)	0.005	↓ 20
ATP production (pmol O_2/min/350,000 cells)	33.79 (23.80)	29.52 (13.66)	0.009	↓ 14
Proton leak (pmol O_2/min/350,000 cells)	15.71 (5.59)	11.57 (4.85)	0.015	↓ 26
Maximal respiration (pmol O_2/min/350,000 cells)	212.67 (139.58)	164.71 (75.54)	0.027	↓ 24
Coupling efficiency (pmol O_2/min/350,000 cells)	73.22 (12.80)	73.57 (10.98)	0.992	0
Reserve capacity (pmol O_2/min/350,000 cells)	159.42 (119.35)	127.41 (65.52)	0.072	↓ 20
BHI	10.55 (9.31)	10.37 (6.04)	0.491	↓ 11
Glycolysis stress test parameters				
Background glycolysis (pmol H^+/min/350,000 cells)	19.65 (14.88)	15.88 (7.46)	0.011	↓ 19
Glucose response (pmol H^+/min/350,000 cells)	28.36 (13.41)	20.55 (11.43)	0.002	↓ 28
Glycolytic reserve (pmol H^+/min/350,000 cells)	30.79 (18.68)	28.00 (10.28)	0.399	↓ 9
Glycolytic capacity (pmol H^+/min/350,000 cells)	59.78 (25.48)	47.29 (19.81)	0.004	↓ 21

Data are presented as median (IQR).
P value determined by Wilcoxon non-parametric tests.
ATP, adenosine triphosphate; BHI, bioenergetics health index; RMR, resting metabolic rate; RQ, respiratory quotient.
↓, decrease in summer compared to winter; ↑, increase in summer compared to winter.

between Group 2 and Group 3 in any of the cellular bioenergetic parameters measured (Table 3). No differences in inflammatory marker change among the three 25(OH)D groups was found after adjustment for changes in FFM, FM, PTH and QUICKI (data not shown).

Within the low 25(OH)D group (Group 1), significant decreases in inflammatory markers MCP ($p=0.027$), IL-6 ($p < 0.001$), IL-8 ($p < 0.001$), IL-10 ($p < 0.001$), IL-12p70 ($p < 0.001$), and TNF-α ($p < 0.001$) were found in summer. In addition, oxidative phosphorylation parameters decreased in this group in summer including basal respiration ($p < 0.001$), non-mitochondrial respiration ($p=0.011$), ATP production ($p < 0.001$), proton leak ($p=0.024$), maximal respiration ($p=0.001$), reserve capacity ($p=0.002$), and BHI ($p=0.020$). Glycolytic parameters background glycolysis ($p=0.004$), glucose response ($p=0.013$), glycolytic reserve ($p=0.024$), and glycolytic capacity ($p=0.003$) also decreased. In those with 25(OH)D between 50 nmol/L and 75 nmol/L (Group 2), insulin sensitivity increased as determined by QUICKI ($p=0.014$) and McA ($p=0.011$), while bioenergetics parameter glucose response and glycolytic capacity and inflammatory markers MCP ($p=0.049$), IL-6 ($p < 0.001$), IL-8 ($p < 0.001$), IL-10 ($p=0.001$), and TNF-α ($p < 0.001$) decreased in summer. In those with 25(OH)D > 75 nmol/L (Group 3), inflammatory markers IL-6 ($p=0.007$), IL-8 ($p < 0.001$), IL-10 ($p=0.027$), IL-12p70 ($p=0.002$), and TNF-α ($p < 0.001$) decreased in summer but there were no significant alterations in bioenergetics.

3.6. The influence of final 25(OH)D achieved (summer) on change in seasonal bioenergetics and inflammation

After adjustment for change in FM, FFM, PTH and QUCKI, significant differences across tertiles of final 25(OH)D achieved were found in bioenergetic parameters basal respiration ($p=0.016$), ATP production ($p=0.019$), proton leak ($p=0.013$), and background glycolysis ($p=0.023$) (Table 4). Tertile 1, representing the lowest tertile of summer 25(OH)D, had the greatest reduction in bioenergetics compared to the other two groups; no differences were found between Tertile 2 and Tertile 3 in any of the cellular bioenergetic parameters (Table 4). There were no significant between group differences among

Table 2
Inflammatory markers and clinical biochemistry in winter and summer.

Characteristic	Winter	Summer	P value	Direction & degree of change (%)
Inflammatory markers				
CRP (mg/L)	1.00 (1.65)	0.75 (1.33)	0.565	↓ 25
MCP-1 (pg/mL)	190.48 (135.80)	133.78 (58.62)	< 0.001	↓ 30
TNF-α (pg/mL)	11.72 (4.12)	3.31 (2.13)	< 0.001	↓ 71
IL-6 (pg/mL)	2.39 (1.99)	0.66 (0.80)	< 0.001	↓ 72
IL-8 (pg/mL)	9.29 (4.47)	2.04 (1.70)	< 0.001	↓ 78
IL-10 (pg/mL)	21.96 (21.22)	3.73 (6.54)	< 0.001	↓ 83
IL-12 (pg/mL)	8.46 (5.26)	1.84 (2.17)	< 0.001	↓ 78
Clinical biochemistry				
FBG (mmol/L)	5.20 (0.63)	4.50 (1)	< 0.001	↓ 13
Insulin (uU/mL)	4.75 (3.65)	4.95 (3.5)	0.552	↑ 4
QUICKI	0.38 (0.04)	0.40 (0.06)	0.028	↑ 5
McA	9.17 (2.33)	9.83 (3.64)	0.019	↑ 7
TG (mmol/L)	0.88 (0.77)	0.80 (0.51)	0.015	↓ 9
TC (mmol/L)	4.96 (1.58)	4.20 (1.87)	< 0.001	↓ 15
LDL (mmol/L)	2.82 (1.58)	2.58 (1.44)	< 0.001	↓ 9
HDL (mmol/L)	1.49 (0.57)	1.26 (0.33)	< 0.001	↓ 15

Data are presented as median (IQR).
P value determined by Wilcoxon non-parametric tests.
CRP, C reactive protein; FBG, fasting blood glucose; IL, interleukin; HDL, high density lipoprotein cholesterol.; LDL, low density lipoprotein cholesterol; McA, McAuleys index of insulin sensitivity; MCP, macrophage chemoattractant protein; QUICKI, quantitative insulin sensitivity check index; TC, total cholesterol; TG, triglycerides; TNF-α, tumour necrosis factor alpha.
↓, decrease in summer compared to winter; ↑, increase in summer compared to winter

Table 3
Summer change in adjusted bioenergetic measurements according to winter vitamin D status at start.

Characteristic	< 50 nmol/L at start Group 1 n = 8	50–75 nmol/L at start Group 2 n = 13	≥75 nmol/L at start Group 3 n = 9	P value
25(OH)D (nmol/L)	42.76 (3.78)	62.18 (6.13)	86.39 (13.26)	0.416
Range of final 25(OH)D achieved (nmol/L)	37–48.3	51.5–72.4	76.8–116.5	–
Whole body energy metabolism markers				
ΔRMR (kJ/d)	−291 (797)	−342 (782)	−216 (742)	0.935
ΔRQ	+0.03 (0.04)*	−0.001 (0.03)	+0.02 (0.04)	0.161
Mito stress test				
ΔBasal respiration (pmol O_2/min/350,000 cells)	−42.91 (21.43)[a*]	−6.44 (21.03)[b,c]	−1.37 (19.95)[c]	0.001
ΔNon-mitochondrial respiration (pmol O_2/min/350,000 cells)	−20.63 (14.27)[a*]	−5.56 (14.01)	−1.15 (13.29)[b]	0.021
ΔATP production (pmol O_2/min/350,000 cells)	−36.67 (19.20)[a*]	−4.30 (18.85)[b,c]	+1.91 (17.87)[c]	0.001
ΔProton leak (pmol O_2/min/350,000 cells)	−6.42 (5.96)*	−2.41 (5.85)	−0.95 (5.55)	0.159
ΔMaximal respiration (pmol O_2/min/350,000 cells)	−139.25 (105.72)[a*]	−21.70 (103.76)	−1.67 (98.41)[b]	0.022
ΔCoupling efficiency (%)	−6.67 (12.79)[a]	−0.81 (12.55)	+9.13 (11.91)[b*]	0.047
ΔReserve capacity (pmol O_2/min/350,000 cells)	−102.99 (93.61)*	−10.92 (91.88)	+6.29 (87.14)	0.047
ΔBHI	−8.10 (11.26)*	+1.69 (11.05)	+1.62 (10.48)	0.129
Glycolysis stress test				
ΔBackground glycolysis (pmol H^+/min/350,000 cells)	−15.03 (9.33)[a*]	−3.94 (8.56)[b,c]	+2.00 (8.24)[b,c]	0.002
ΔGlucose response (pmol H^+/min/350,000 cells)	−14.28 (12.27)*	−14.74 (18.03)*	−4.45 (20.30)	0.355
ΔGlycolytic reserve (pmol H^+/min/350,000 cells)	−18.41 (18.62)[a*]	−1.74 (18.01)	+6.27 (17.08)[b]	0.039
ΔGlycolytic capacity (pmol H^+/min/350,000 cells)	−38.05 (29.56)[a*]	−16.44 (28.73)*	+3.78 (27.12)[b]	0.026

Data are presented as mean (SD) after adjustment for change in PTH, FFM, FM and QUICKI.
Identical superscripts denote no significant difference between groups. Different superscripts denote significant differences between groups.
25(OH)D, 25-hydroxy vitamin D; ATP, adenosine triphosphate; BHI, Bioenergetic Health Index; RMR, resting metabolic rate; RQ, respiratory quotient.
Δ, change.
* Denotes significant within group differences.

inflammatory markers (data not shown).

The lowest tertile of summer 25(OH)D achieved (Tertile 1), demonstrated significant decreases in basal respiration, non-mitochondrial respiration, ATP production, proton leak, maximal respiration, reserve capacity, background glycolysis, glucose response, glycolytic reserve and glycolytic capacity in comparison to winter bioenergetic parameters. Decreases in RMR, basal and glucose response occurred in the middle tertile (Tertile 2). In those who achieved the greatest summer 25(OH)D (Tertile 3), no changes in bioenergetics with season were observed (Table 4). All tertiles displayed significant reductions in TNF- α, IL-6, IL-8, IL-10 and IL-12p70 in summer, with Tertile 2 also demonstrating a reduction in MCP-1 (data not shown).

3.7. The influence of absolute change in 25(OH)D on change in seasonal bioenergetics and inflammation

After adjustment for change in FM, FFM, PTH and QUCKI, no significant differences across tertiles of change in 25(OH)D were found in any bioenergetic parameters (data not shown). A smaller reduction in IL-10 in those with the greatest change in 25(OH)D (Tertile 3) compared to the other two groups occurred [Tertile 3 + Δ25(OH)D (nmol/L) +30.89 (8.95), ΔIL-10 (pg/mL) −7.02 (12.95) vs Tertile 1

Table 4
Summer change in adjusted bioenergetic measurements according to tertiles of final vitamin D status achieved.

Characteristic	Final 25(OH)D Tertile 1 n = 10	Final 25(OH)D Tertile 2 n = 10	Final 25(OH)D Tertile 3 n = 10	P value
Final 25(OH)D (nmol/L)	54.31 (7.45)[a]	74.16 (8.06)[b]	101.36 (7.17)[c]	< 0.001
Range of final 25(OH)D achieved (nmol/L)	40.7–63	63.2–91.9	93.3–112.40	–
Whole body energy metabolism markers 3)				
ΔRMR (kJ/d)	−110 (738)	−654 (700)*	−116 (726)	0.166
ΔRQ	+0.02 (0.04)	+0.01 (0.04)	+0.01 (0.04)	0.575
Mito stress test				
ΔBasal respiration (pmol O_2/min/350,000 cells)	−33.63 (24.40)[a*]	−16.83 (23.14)*	+2.37 (23.98)[b]	0.016
ΔNon-mitochondrial respiration (pmol O_2/min/350,000 cells)	−13.96 (16.12)*	−9.65 (15.28)	−3.11 (15.84)	0.361
ΔATP production (pmol O_2/min/350,000 cells)	−27.60 (22.24)[a*]	−13.71 (21.09)	+4.24 (21.86)[b]	0.019
ΔProton leak (pmol O_2/min/350,000 cells)	−7.89 (5.33)[a*]	−1.48 (5.06)[b,c]	+0.43 (5.24)[c]	0.013
ΔMaximal respiration (pmol O_2/min/350,000 cells)	−102.28 (112.15)*	−65.63 (106.35)	+15.70 (110.24)	0.087
ΔCoupling efficiency (%)	−4.33 (13.56)	−0.66 (12.86)	+7.34 (13.33)	0.185
ΔReserve capacity (pmol O_2/min/350,000 cells)	−77.54 (98.02)*	−40.54 (92.95)	+16.21 (96.35)	0.139
ΔBHI	−4.08 (11.30)	−4.49 (10.72)	+4.74 (11.11)	0.145
Glycolysis stress test				
ΔBackground glycolysis (pmol H^+/min/350,000 cells)	−13.00 (9.67)[a*]	−3.04 (9.38)[b,c]	+0.19 (10.03)[c]	0.023
ΔGlucose response (pmol H^+/min/350,000 cells)	−14.46 (19.37)*	−14.64 (18.79)*	−6.51 (20.08)	0.628
ΔGlycolytic reserve (pmol H^+/min/350,000 cells)	−12.06 (20.28)	0.70 (19.40)	−0.29 (20.77)	0.347
ΔGlycolytic capacity (pmol H^+/min/350,000 cells)	−29.06 (31.86)*	−6.51 (30.90)	−5.53 (33.03)	0.323

Data are presented as mean (SD) after adjustment for change in PTH, FFM, FM and QUICKI.
Identical superscripts denote no significant difference between groups. Different superscripts denote significant differences between groups.
25(OH)D, 25-hydroxy vitamin D; ATP, adenosine triphosphate; BHI, Bioenergetic Health Index; RMR, resting metabolic rate; RQ, respiratory quotient.
Δ, change.
* Denotes significant within group differences.

Δ25(OH)D (nmol/L) −1.63 (8.06), ΔIL-10 (pg/mL) −19.25 (11.66) (p=0.049); Tertile 3+Δ25(OH)D (nmol/L) +30.89 (8.95), ΔIL-10 (pg/mL) −7.02 (12.95); (p=0.064 vs Tertile 2+Δ25(OH)D (nmol/L) +12.14 (8.23); ΔIL-10 (pg/mL) −21.35 (11.91 (p=0.026)]. All tertiles displayed significant reductions in TNF- α, IL-6, IL-8, IL-10, and IL-12p70, with the exception of Tertile 3 where no significant reduction in IL-10 was observed. Tertile 3 also demonstrated a reduction in MCP-1 (data not shown).

3.8. Correlations between inflammation, bioenergetics, insulin sensitivity and 25(OH)D

The seasonal increase in 25(OH)D was positively associated with IL-10 (r=0.389, p=0.033). As RQ decreased, McA increased (r=−0.367, p=0.046), as did IL-10 (r=−0.611, p < 0.001). RMR was positively associated with CRP (r=0.379, p=0.039). Non-mitochondrial respiration was positively associated with CRP (r=0.570, p=0.001) and TNF-α (r=0.411, p=0.024). After adjustment for FM, FFM, PTH, and QUICKI, RMR was positively associated with TNF-α (r=0.540, p=0.006) and CRP (r=0.462, p=0.023). Non-mitochondrial respiration was positively associated with CRP (r=0.668, p < 0.001) and TNF-α (r=0.452, p=0.027).

4. Discussion

Recently, we demonstrated that *in vivo* circulating 25(OH)D, a proxy for vitamin D status, was associated with *ex-vivo* PBMC cell bioenergetic capacity and activity. Specifically, 25(OH)D below 50 nmol/L was associated with increased oxidative and glycolytic bioenergetic profile responses in PBMCs obtained from adults and we speculated that this was due to inflammatory activation of these cells [15]. The present study investigated whether seasonal change in 25(OH)D was associated with altered bioenergetic and inflammatory profiles. We also investigated whether baseline (winter) 25(OH)D, the final 25(OH)D achieved in summer, and absolute change in vitamin status mediated the associations between 25(OH)D, bioenergetics and inflammation.

The winter to summer increase in 25(OH)D observed was typical of Australia [5–7] and we document that seasonal variation in circulating 25(OH)D, was associated with a change in *ex vivo* PBMC bioenergetic profiles. A reduction in non-mitochondrial respiration was associated with a reduction in CRP and TNF-α. This reduction in non-mitochondrial respiration may represent a reduction in reactive oxygen species generation or reduced demand for use in cytochrome P-450 monooxygenases, cyclooxygenases, and pro-inflammatory NADPH oxidases. The concurrent observation of lowered bioenergetic parameters and reduced inflammation in summer as compared to winter supports our hypothesis that a lower 25(OH)D in winter may result in greater inflammation which has an energetic cost that could account for part of the higher RMR in winter (Fig. 1). It is clear that those who had an initial low 25(OH)D (< 50 nmol/L) were associated with decreased bioenergetic parameters from winter to summer, while individuals with higher initial status (> 50 nmol/L) did not. In contrast, multiple systemic inflammatory markers decreased within each group suggesting higher levels of 25(OH)D may be required to dampen inflammation. Taken together, these results may suggest that the optimal 25(OH)D depends on the target variable.

Those who started with lower 25(OH)D in winter were likely to demonstrate a lower status in summer compared to those who started with greater 25(OH)D. We found that those who began the study with a low 25(OH)D in winter (< 50 nmol/L), were associated with reduced bioenergetic parameters (to a greater extent) in summer compared to the other two groups (50–75 nmol/L and > 75 nmol/L). We also found that in summer, those who were in the lowest tertile of final 25(OH)D achieved a reduced bioenergetic profile, while those in the middle quartile reduced only some bioenergetic parameters, and those in the upper quartile demonstrated no significant change in bioenergetics with season. Taken together, these results suggest that there is a 25(OH)D level which is associated with change in bioenergetic parameters and above this, bioenergetics do not change with season. To further these findings, we also investigated whether absolute change in 25(OH)D may be important in the association between bioenergetics, inflammation and 25(OH)D. Although we found no relationship between these variables, the results are none-the-less interesting and warrant further investigation through cause-and-effect study designs.

Although three of the four bioenergetic parameters used to calculate BHI were associated with significant reductions from winter to summer, BHI remained similar between seasons. Our observations of a significantly decreased ATP production and non-significant decreased reserve capacity [numerator term] were cancelled out by the decreased proton leak and non-mitochondrial respiration [denominator term], such that no significant effect across seasons was observed. It is unlikely that the lack of significant seasonal variation in BHI is due to sample size, since a very minimal 1.71% difference between winter and summer values was found. It is possible that a more refined BHI calculation which used exponents to modify the relative weighting of the respiratory parameters may be more appropriate to reflect seasonal difference [24]. We [19] and others [20] have not adopted the use of power functions, as there is insufficient data in the field to be confident that such power functions actually apply to the individuals under study. We also acknowledge that there may be several possible variants for a BHI calculation, however, developing such an equation was outside the scope of this manuscript.

As reductions in PBMC bioenergetic parameters were apparent with season, it is not surprising that a change in the same direction occurred in whole body energy metabolism. The trend towards a lower RMR in summer, reflected other longitudinal studies where a greater RMR was observed in winter compared to summer [4–7]. A seasonal change in RMR may be explained by several mechanisms including indoor temperature at measurement, outside ambient temperature, and level of thyroid hormones. RMR, is influenced by environmental temperature, specifically room temperature, wherein RMR is higher when measured in a cold environment [24]. Our results are not an artefact of indoor temperature during RMR measurement as the indoor temperature was set to 25 °C on both measurement occasions and patients were allowed to equilibrate prior to measurement. There was, however, an average difference in ambient temperatures between winter and summer in Perth, WA, of ~27 °C. The existence of other factors such as thyroid hormone levels may control modifications of RMR in the normal population throughout the year [25]. Serum levels of thyroid hormones T_3 and T_4 have been shown to decrease in summer compared to winter [26]. It is likely that a complex interplay of mechanisms are responsible for the change in RMR observed with season.

There are several limitations of study. We acknowledge that our study design does not allow demonstration of a causal effect of 25(OH)D on bioenergetics and inflammation. Also, we cannot rule out potential changes to energy intake and food intake that are known to influence systemic inflammation. However, given the lack of difference in body composition, we are confident that participants were in energy balance. The samples used contained a heterogeneous population of immune cells, with each subpopulation known to have a unique bioenergetic profile [27,28]. The use of a mixed population is likely to reflect the metabolic state of the human system as immune cells interact with one another *in vivo*. Moreover our method of isolation results in minimal platelet contamination with no measurable oxygen consumption [15]. However, we accept the value of future studies examining the influence of 25(OH)D on bioenergetic parameters in isolated and purified populations of immune cells. Furthermore, different cell types and the subsets within each immune cell type display a unique seasonal variation [29,30]. As it has been demonstrated, that CD4+ and CD8+ T cells reveal a reduced capacity to produce pro-inflammatory cytokines in summer [31], whether the seasonal variation in 25(OH)D in vivo throughout the year is associated with changes in bioenergetics

through changes in T cell compartment or other immune cell subsets remains to be elucidated. Knowledge of metabolic control of immunity is primarily derived from cultured cells exposed to potent stimuli, such as LPS, rather than stimulating them with physiologic ligands that might activate cells *in vivo*. Therefore, our understanding of immune cell metabolism is still in its infancy and merits further investigation. Future intervention trials should investigate our hypothesis that 25(OH)D should remain above 50 nmol/L throughout the year, and whether very high 25(OH)D has deleterious health effects as a U-shaped relationship is not uncommon between health indicators and dietary/nutritional components. We also recommend that future studies of larger sample size investigate whether changes in immune cell subsets explains the relationships between 25(OH)D, inflammation and bioenergetics that we have observed.

In conclusion, this study extends our previous cross sectional demonstration that PBMC bioenergetics varies with 25(OH)D (15). Seasonal increases in 25(OH)D significantly reduce bioenergetics such that greater improvement was seen in those who normalized their status to ~ 50 nmol/L, while those who achieved values > 50 nmol/L showed no further improvements. Future intervention trials are required to establish a potential cause-and-effect relationship between 25(OH)D and altered cellular bioenergetic parameters.

Author contributorship

The present work was designed by KNK, MJS, PN and EKC. Initial manuscript preparation and draft was undertaken by EKC and revised by KNK, MJS, PN, JR and RR. Patients were recruited by EKC and body composition was assessed by EKC. PBMC isolation was performed by JR, KNK, RR and EKC. Bioenergetics parameters were measured by EKC. Data analysis and statistical analysis were made by EKC, KNK, and MJS. Figure preparation was made by EKC. Supervision of the manuscript preparation was the responsibility of PN and MJS. All authors approved the final version of the paper.

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CHAPTER 6- THE IMPACT OF CHOLECALCIFEROL SUPPLEMENTATION ON THE SYSTEMIC INFLAMMATORY PROFILE: A SYSTEMATIC REVIEW AND META-ANALYSIS OF HIGH QUALITY RANDOMIZED CONTROLLED TRIALS

The content of this chapter is covered by Paper 6:

Calton, E. K., K. N. Keane, P. Newsholme, Y. Zhao, and M. J. Soares. 2017. "The impact of cholecalciferol supplementation on the systemic inflammatory profile: a systematic review and meta-analysis of high-quality randomized controlled trials." *Eur J Clin Nutr*. doi: 10.1038/ejcn.2017.67.

Thesis objectives addressed in this chapter:

Objective 5: To examine the causal link between 25(OH)D and the systemic inflammatory profile in adults.

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REVIEW

The impact of cholecalciferol supplementation on the systemic inflammatory profile: a systematic review and meta-analysis of high-quality randomized controlled trials

EK Calton[1], KN Keane[2], P Newsholme[2], Y Zhao[3] and MJ Soares[1]

Causal links between vitamin D status [25(OH)D] and systemic inflammation were examined through a systematic review of randomized controlled trials (RCTs). Selected RCTs were ⩾12 weeks, conducted in adults free of acute inflammatory disease, and of high-quality (Jadad score ⩾3). Of 14 studies that met our criteria, 9 studies (15 study arms) permitted extraction of data. There was no effect on the weighted mean difference (WMD) of IL-6 (WMD (95% confidence interval) = 0.1, (−0.166, 0.366) pg/ml, $P = 0.462$) or C-reactive protein (CRP) (WMD = − 0.324, (−1.007, 0.359) mg/l, $P = 0.352$). Subgroup analyses of trials achieving ⩾80 nmol/l indicated a trend for lower CRP (WMD = − 0.834, (−1.726, 0.058) mg/l, $P = 0.067$), however heterogeneity was significant ($I^2 = 66.7\%$, $P = 0.017$). Studies employing a low dose (< 1000 IU/d) showed increased CRP (WMD = 0.615, (0.132, 1.098), $P = 0.013$). In contrast, ⩾1000 IU/d had a favourable effect on CRP (WMD = − 0.939, (−1.805, − 0.073), $P = 0.034$) but heterogeneity was significant ($I^2 = 61.3\%$, $P = 0.017$). Meta-regression indicated that older age predicted a significant decrease in IL − 6 ($\beta = -0.02$, (−0.034, − 0.006) pg/ml, $P = 0.013$) and CRP ($\beta = -0.06$, (−0.103, − 0.017), $P = 0.01$), whereas a greater percentage of females ($\beta = 0.027$, (0.011, 0.044), $P = 0.004$) and longer study duration independently predicted a higher WMD for CRP ($\beta = 0.049$, (0.018, 0.079), $P = 0.005$). Available high-quality RCTs did not support a beneficial effect of cholecalciferol on systemic IL-6 and CRP. Future studies should consider the confounding effects of age, gender and study duration, while possibly targeting an achieved 25(OH)D ⩾80 nmol/l.

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INTRODUCTION

Low-grade inflammation is recognised as an underlying characteristic of obesity and other chronic disorders such as metabolic syndrome (including insulin resistance), type 2 diabetes, atherosclerosis and cardiovascular disease.[1–5] The presence of systemic inflammation is evaluated by the circulating concentrations of a panel of biomarkers, such as C-reactive protein (CRP), tumor necrosis factor α (TNF-α), interleukin-6 (IL-6), interleukin-10 (IL-10) and interleukin-12 (IL-12). Markers of inflammation predict the occurrence of chronic disease,[6] whereas anti-inflammatory cytokines such as interleukin-13 (IL-13) are closely associated with suppression of diet-induced obesity and subsequent insulin resistance in mouse models.[7] Thus, immunomodulation through diet and lifestyle may represent a worthy public health approach in the battle against obesity.

Over the past decade, vitamin D has received much interest as a potential modulator of inflammation. Accordingly, cellular studies consistently show that supplementation with $1,25(OH)_2D$ reduces inflammatory cytokine production and increases anti-inflammatory marker levels.[8–14] The effect of vitamin D supplementation on levels of plasma inflammatory markers is less clear from human studies. One recent systematic review and meta-analysis concluded that cholecalciferol supplementation reduced high-sensitivity C-reactive protein levels (hs-CRP).[15] However, only a small number of randomized controlled trials (RCTs) were included. Another systematic review and meta-analysis in overweight/obese children and adults found no benefit of cholecalciferol and ergocalciferol supplementation on inflammatory cytokine levels.[16] However, the review included trials that had implemented additional dietary interventions. The scarcity of good quality RCTs and reliance on poor quality studies limit these previously conducted systematic reviews.[17] Moreover, there is no consensus on which inflammatory mediator best represents chronic low-grade inflammation.[18]

There are close relationships between vitamin D status, determined by circulating 25-hydroxyvitamin D [25(OH)D] levels, the active hormone $1,25(OH)_2D$ and functional outcomes.[19] Adequate 25(OH)D allows production of sufficient $1,25(OH)_2D$ to optimise cellular functions, and this is particularly relevant to cells and tissues that have the enzymatic ability to convert 25(OH)D to its active form. There are now several studies that show a positive relationship between higher levels of 25(OH)D and its conversion to $1,25(OH)_2D$,[20–23] with some indications of a plateau to the relationship when 25(OH)D reached ~80 nmol/l.[20] The latter observations suggest that an 'optimal' 25(OH)D is required to best observe the beneficial extra-skeletal effects of $1,25(OH)_2D$. Others have suggested use of the concentration of 25(OH)D needed to maximally suppress intact serum parathyroid hormone, as a possible approach for determining optimal vitamin D status. Recent analyses suggest that ~70 nmol/l may represent such a threshold value for maximal intact serum parathyroid hormone

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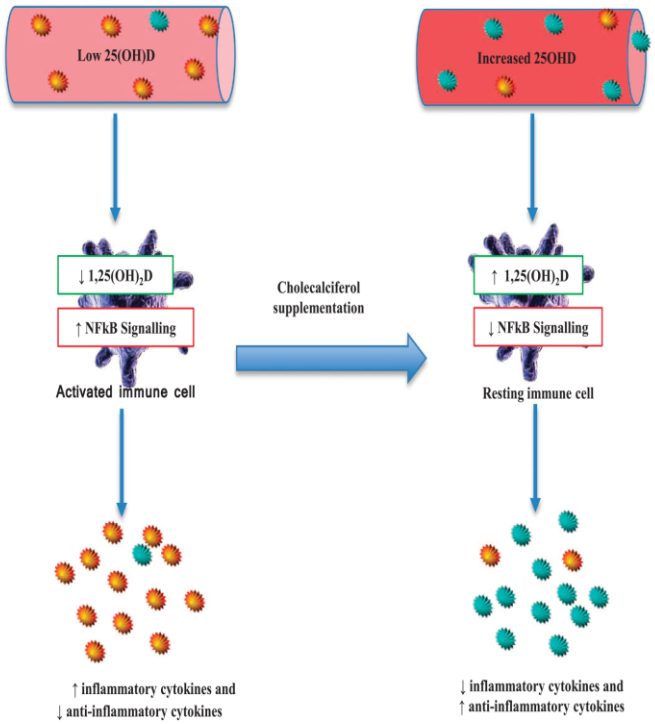

Figure 1. Schematic depicting the hypothesized role of cholecalciferol supplementation on systemic inflammation. We hypothesized that low 25(OH)D status would result in activated circulating immune cells. These immune cells would have increased signaling of cellular pathways that promote inflammation (for example, NFκB), resulting in a pro-inflammatory pattern of plasma cytokines (increased levels of pro-inflammatory markers, reduced anti-inflammatory cytokines). Improving 25(OH) status through cholecalciferol supplementation would be expected to raise 25(OH)D levels, suppress activation of circulating immune cells and restore the balance of circulating inflammatory markers in favor of a net anti-inflammatory profile.

suppression.[24] Still others have proposed that baseline 25(OH)D status, change in vitamin status and the final concentration achieved, may well be crucial considerations in uncovering the extra-skeletal effects of vitamin D supplementation.[18,25]

The present systematic review and meta-analysis has attempted to address these major concerns, and was based on a model of achieving a critical or threshold concentration of 25(OH)D in order to detect favourable effects on systemic inflammation Specifically, we hypothesized that achieving a plasma concentration of 70–80 nmol/l, through cholecalciferol supplementation, would promote an anti-inflammatory cytokine profile (low inflammatory cytokines and increased anti-inflammatory markers) in adults free of acute inflammation (Figure 1).

SUBJECTS AND METHODS

Review purpose

This systematic review assessed RCTs investigating the impact of cholecalciferol supplementation on systemic inflammatory markers in good health and low-grade chronic inflammatory conditions associated with obesity. Table 1 describes the research criteria using the PICOS (patients, intervention, comparator, outcomes and study design format).

The meta-analysis also aimed to explore the sources of heterogeneity and whether the relationship between cholecalciferol supplementation and changes in systemic inflammation markers differs by baseline 25(OH)D, change in 25(OH)D, final status achieved (70 nmol/l, 80 nmol/l), study duration, daily dose of cholecalciferol, assay method used to quantify 25(OH)D, whether D3 or total 25(OH)D was measured, and age of participants.

Study selection process

In an initial step, a limited search of Scopus and PubMed was undertaken in order to analyse the text words contained in the title, abstract and keywords.[26] A systematic literature search was then independently conducted by two reviewers (EKC and KNK) using PubMed, Medline, Science Direct, Scopus and Wiley Online Library. The search strategy used the following search terms: Vitamin D or cholecalciferol or 25-hydroxy-Vitamin D and Inflam* or cytokine* and supplement*. Articles were restricted to those conducted in humans, reported in the English language, and within the time period of 2000–2016. The search was updated in August of 2016. The systematic review included RCTs where cholecalciferol had been administered in at least one study arm. The primary outcomes were systemic inflammatory and anti-inflammatory markers. The study selection process was conducted in accordance with the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) guidelines and are described in Figure 2. Studies were restricted to those of at least 12 weeks duration as evidence indicated that following supplementation, 25(OH)D levels plateau at this time.[27] Studies in pregnant women, breastfeeding women, those diagnosed with cancer, acute infection, illness or were immunocompromised, were excluded from the review. RCTs were also required to be of high-quality, based on the Jadad score[28] (score $\geqslant 3$ is rated good quality), and this was independently assessed by two reviewers (EKC and KNK), and cross-checked by a third independent reviewer (MJS). Any discrepancy was resolved through discussion. The protocol was prospectively registered at PROSPERO (http://www.crd.york.ac.uk/PROSPERO).

Ethics

There was no ethical requirement for this review as primary data were not collected.

Table 1. PICOS criteria

Patient/population: adults with obesity and related chronic disease, free of acute inflammation
Intervention: cholecalciferol supplementation for ⩾12 weeks Comparison: placebo group Outcome: change in concentration of circulating inflammatory markers Study design: RCTs

Abbreviations: PICOS, Patient, Intervention, Comparison, Outcome, Study design; RCTS, randomized controlled trials.

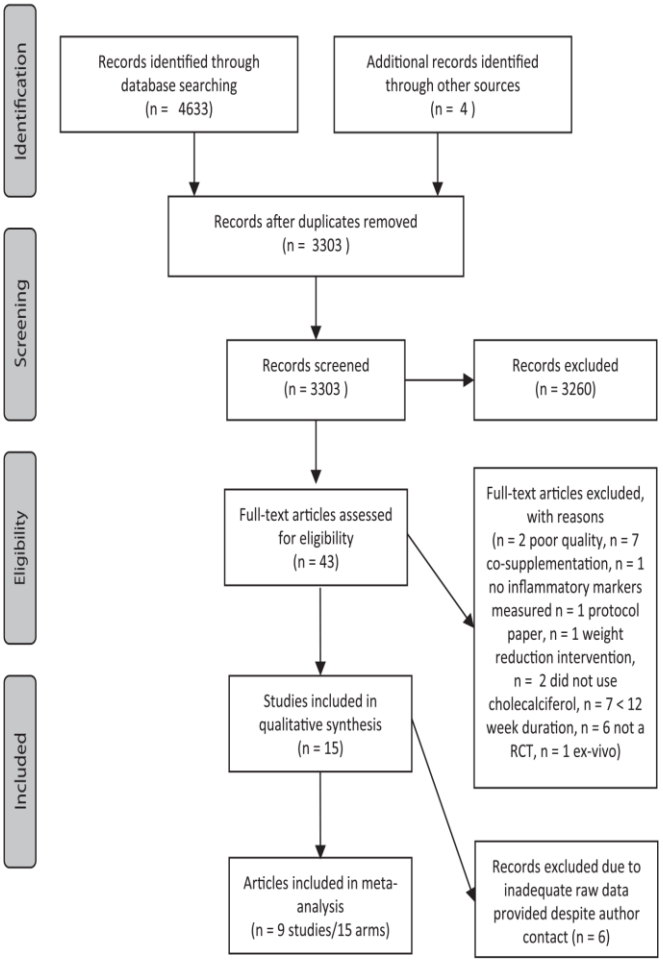

Figure 2. PRISMA flow diagram depicting study selection for systematic review and meta-analysis. PRISMA, Preferred Reporting Items for Systematic Reviews and Meta-Analyses; RCT, randomized controlled trial.

Data extraction

Data extracted included location of the study, participant demographics (number of participants, % female, mean age), health status, study duration (weeks), daily dose of cholecalciferol, assay method used to quantify 25(OH)D, whether D3 or total 25(OH)D was measured, study quality, placebo and treatment group 25(OH)D status (mean (s.d.) or median (interquartile range) for baseline, final and change), direction of inflammatory marker change, *P*-values for statistically significant systemic inflammatory marker changes and whether the impact of supplementation on inflammatory markers was a primary or secondary objective of the study. All cholecalciferol dosage data were converted to IU/d and values for 25(OH)D were expressed as nmol/l, where necessary. CRP and hs-CRP were converted to mg/l values. CRP values from both CRP and hs-CRP assays were used interchangeably because we were only interested in the CRP change and we were not predicting cardiovascular disease risk. Historically, CRP tests were not designed to quantitate CRP at low (normal) concentrations. Whereas, the hs-CRP assay design is technically optimized by the manufacturer to measure CRP at low levels, which are within the reference (normal) range.[29] Where information was missing, authors were contacted and the missing information was requested.

Statistical methods

Meta-analyses (both fixed and random effects models) were carried out to examine the weighted mean difference (WMD) of IL-6 and CRP

Table 2. Randomised controlled trials examining the impact of cholecalciferol supplementation on systemic inflammatory markers

Study	*Study details*	*Jadad score*	*25(OH)D status nmol/l mean ± s.d. or median (IQR)*	*Change in inflammatory markers between trial arms*	*Change in inflammatory markers within trial arms*
Forouhi *et al.*[39]	Country: England, United Kingdom Subject characteristics: $n = 221$, 30–75 years, elevated risk of diabetes Dose: 3300 IU/d Assay method: liquid chromatography–tandem mass spectrometry Duration: 16 weeks Secondary objective	5	Pre: 45.8 ± 22.6 Post: 83.8 ± 22.7 Change: 38.1 ± 23.8	hs-CRP, NS	hs-CRP, NS
Tuomainen *et al.*[33]	Country: Finland Subject characteristics: $n = 66$, ⩾60 years, overweight and obese, prediabetes, < 75 nmol/l 25(OH)D Dose: 1600 IU/d or 3200 IU/d Assay method: high-performance liquid chromatography Duration: 20 weeks Primary objective	5	Change 3200 IU/d: 45.0 (23.4) Change 1600 IU/d: 27.7 (17.2)	Trend for IL-1RA to ↓ as dose↑ ($P = 0.07$) Adiponectin, hs-CRP, sTNFrII, IL-6, NS	hs-CRP ↓ ($P < 0.05$) in the 3200 IU/d treatment group All others, NS
Sollid *et al.*[31]	Country: Norway Subject characteristics: $n = 484$, mean (s.d.) age 62.1(8.7) years, 21–80 years, prediabetes Dose: 2857 IU/d Assay method: liquid chromatography–tandem mass spectrometry Duration: 52 weeks Secondary objective	4	Pre: 59.9 ± 21.9 Post: 105.7 Change: 45.8 ± 24.2	hs-CRP, NS	hs-CRP, NS
Sharifi *et al.*[44]	Country: Ahvaz, Iran Subject characteristics: $n = 53$, 18–70 years, non-alcoholic fatty liver disease Dose: 3571 IU/d Assay method: radioimmunoassay Duration: 16 weeks Secondary objective	5	Pre: 28.75 (49) Post: 75 (52) Change: 40.5	Trend for hs-CRP to↑in placebo group and not the treatment group ($P = 0.06$) TNF- α, NS	Trend for hs-CRP to↑in the placebo group ($P = 0.05$) Both groups↑TNF- α (placebo group $P = 0.033$), treatment group $P = 0.002$)
Kampmann *et al.*[32]	Country: Aarhus, Denmark Subject characteristics: $n = 15$, T2DM ⩾18 years, < 50 nmol/l 25(OH)D Dose: 11200 IU/d 2 weeks, 5600 IU 10 weeks Assay method: ELISA Duration: 12 weeks Primary objective	5	Pre: 31.0 ± 1.85 Post: 73.9 ± 7.79 Change: 42.9	CRP, IL-6, IL-10, TNF-α, NS	CRP, IL-6, and IL-10 and TNF-α, NS
Yiu *et al.*[42]	Country: Hong Kong Subject characteristics: $n = 100$, mean (s.d.) age 65(8) years, 50 men, T2DM, < 75 nmol/l 25(OH)D Dose: 5000 IU/d Assay method: 25(OH)D enzyme immunoassay Duration: 12 weeks Secondary objective	5	Pre: 53.5 Post: 146.5 Change: 86.75	hs-CRP, NS	Trend for hs-CRP to ↑ in the treatment group ($P = 0.05$)

Table 2. (Continued)

Study	*Study details*	*Jadad score*	*25(OH)D status nmol/l mean ± s.d. or median (IQR)*	*Change in inflammatory markers between trial arms*	*Change in inflammatory markers within trial arms*
Wamberg *et al.*[34]	Country: Aarhus, Denmark Subject characteristics: $n = 43$, aged 18–50 years, BMI > 30 kg/m^2, < 50 nmol/l 25(OH)D Dose: 7000 IU/d Assay method: liquid chromatography–tandem mass spectrometry Duration: 26 weeks Primary objective	5	Pre: 33.0 ± 10.8 Post: 110.2 ± 21.2 Change: 77.2	hs-CRP, IL-6, MCP-1, adiponectin, leptin, MMP-9, PAI-1, NS	MCP-1↑in treatment group ($P = 0.04$) All others, NS
Witham *et al.* Study 1[37]	Country: Scotland, United Kingdom Subject characteristics: $n = 73$, mean age 66 years, myocardial infarction Dose: 1786 IU/d Assay method: radioimmunoassay Duration: 24 weeks Secondary objective	5	Pre: 49 ± 20 Post: 62 ± 22 Change: 12.9 ± 24.5	CRP ↓ in the treatment group ($P = 0.03$) compared to placebo TNF- α, NS	Significance not reported
Witham *et al.* Study 2[36]	Country: Scotland, United Kingdom Subject characteristics: $n = 142$, $\geqslant 70$ years, isolated systolic hypertension, < 75 nmol/l 25 (OH)D Dose: 1190 IU/d Assay method: not reported Duration: 52 weeks Secondary objective	5	Pre: 45 ± 15 Post: 67 ± 17 Change: 24.6 ± 17.6	CRP, NS	Significance not reported
Gepner *et al.*[40]	Country: Winconsin, America Subject characteristics: $n = 110$, mean (s.d.) 63.9 (3.0) years, postmenopausal women, 25 nmol/l-150 nmol/l 25(OH)D Dose: 2500 IU/d Assay method: reverse phase high-performance liquid chromatography Duration: 12 weeks Secondary objective	5	Pre: 75.75 ± 26.75 Post: 115 Change: 39.25 ± 23.25	CRP, NS	hs-CRP, NS
Wood *et al.*[35]	Country: Scotland, United Kingdom Subject characteristics: $n = 265$, 60–70 years, postmenopausal women Dose: 400 IU or 1000 IU/d Assay method: high-pressure liquid chromatography Duration: 52 weeks Primary objective	5	Pre: (32.27 ± 13.2) 400 IU, (33.35 ± 13.9) 1000 IU Post: (64.86 ± 19.8) 400 IU, (75.66 ± 19.1) 1000 IU Change: (33.04) 400 IU, (42.90) 1000 IU	hs-CRP, IL-6, sICAM-1, NS	hs-CRP, IL-6, sICAM-1, NS
Barnes *et al.*[38]	Country: Ireland, United Kingdom Subject characteristics: $n = 211$, 20–40 years, $n = 202$, 20–40 years and $\geqslant 64$ years Dose: 200 IU/d, 400 IU/d, 600 IU/d Assay method: enzyme linked immunosorbent assay Duration: 22 weeks Primary objective	4	Pre: 75.9 (34) 20–40 years old, 55.1 (31.4) $\geqslant 64$ years Post: 69 (25.3) 20–40 years, 73.9 (28.3) $\geqslant 64$ years Change:-6.9 20–40 years, 18.8 $\geqslant 64$ years	hs-CRP, IL-6, IL-10, sCD40L, TGF-β, TNF-α, fibrinogen, NS	hs-CRP, IL-6, IL-10, sCD40L, TGF-β, TNF-α and fibrinogen, NS

Table 2. (Continued)

Study	*Study details*	*Jadad score*	*25(OH)D status nmol/l mean ± s.d. or median (IQR)*	*Change in inflammatory markers between trial arms*	*Change in inflammatory markers within trial arms*
Von Hurst *et al.*[43]	Country: Auckland, New Zealand Subject characteristics: $n = 81$, 23–68 years, IR, <50 nmol/l 25(OH)D Dose: 4000 IU/d Assay method: radioimmunoassay Duration: 24 weeks Secondary objective	5	Pre: 21 (29) Post: 80 (27) Change: 49 (45)	Trend for hs-CRP to ↓ in the treatment group compared to placebo group ($P = 0.05$)	hs-CRP, NS
Yusupov *et al.*[41]	Country: New York, America Subject characteristics: $n = 120$, 18–80 years Dose: 2000 IU/d Assay method: radio-receptor assay Duration: 12 weeks Primary objective	4	Pre: 64.3 ± 25.4 Post: 88.5 ± 23.2 Change: 24.2	IL-2, IL-4, IL-5, IL-6, IL-8, IL-13, GM-CSF, IFN-γ, TNF-α, NS IL-10 decreased to a greater extent in the treatment group ($P = 0.02$)	A significant ↓ in levels of GM-CSF ($P < 0.0001$), IFN-γ ($P < 0.0001$), IL-4 ($P = 0.001$), IL-8 ($P < 0.0001$), IL-10 ($P < 0.0001$) and IL-13 ($P = 0.0104$) in the treatment group. In the placebo group, a significant↓in levels of GM-CSF ($P = 0.0007$), IFN-γ ($P = 0.0011$), IL-4 ($P = 0.006$), IL-8 ($P = 0.0210$) and IL-10 ($P = 0.0401$)

Abbreviations: 25(OH)D, 25-hydroxyvitamin D; BMI, body mass index; CDCL10, C-X-C motif chemokine ligand 10; CRP, C-reactive protein; d, day; GM-CSF, granulocyte macrophage colony-stimulating factor; hs-CRP, high-sensitivity C-reactive protein; IFN-γ, interferon gamma; IL-10, interleukin-10; IL-12, interleukin-12; IL-13, interleukin-13; IL-1RA, interleukin-1 receptor antagonist; IL-1δ½, interleukin-1 beta; IL-2, interleukin 2; IL-4, interleukin 4; IL-5, interleukin 5; IL-6, interleukin-6; IL-8, interleukin 8; IR, insulin resistance; MCP-1, macrophage chemotactic protein 1; MMP-9, matrix metallopeptidase 9; NS, non-significant; PAI-1, plasminogen activator inhibitor-1; sCD40L, Soluble CD40 ligand; sICAM-1, soluble intracellular adhesion molecule 1; sTNFRII, soluble tumor necrosis factor receptor type II; T2DM, type two diabetes; TGF-β, transforming growth factor beta; TNF-α, tumor necrosis factor alpha.

following cholecalciferol supplementation. No other inflammatory markers were included in the meta-analyses owing to insufficient number of studies retrieved with relevant raw data. Mean (s.d.) change in IL-6 and CRP were calculated as (post supplementation value minus baseline value), for the cholecalciferol and placebo arms, respectively. Some RCTs had multiple treatment arms and each arm was included as a separate study in the meta-analysis, matched to the placebo arm and is denoted by a letter following the study citation. If more than one time point for the follow up was reported, the data from the longest supplementation period were used. To test for heterogeneity between studies, I^2 statistics were used. Potential publication and small sample size bias were assessed by visual inspections of funnel plots and Egger test. Subgroup analyses to examine the influence of baseline 25(OH)D status (<50 nmol/l, $\geqslant 50$ nmol/l), final 25(OH)D status obtained (<70 nmol/l, $\geqslant 70$ nmol/l), final 25(OH)D status obtained (<80 nmol/l, $\geqslant 80$ nmol/l) and increase in 25(OH)D status following supplementation (<20 nmol/l, $\geqslant 20$ nmol/l) were conducted. Additional subgroup analyses were conducted for studies measuring 'D3 only' versus 'total 25(OH)D; studies with a 'short duration (defined as 3–6 months inclusive)' versus a 'long duration (defined as >6 months)', and studies using 'low daily dose (defined as $\leqslant 1000$ IU/d)' versus 'high daily dose (defined as >1000 IU/d)'. Sensitivity analyses were conducted to test the robustness of the results regarding whether use of gold standard LCMS (liquid chromatography mass spectrophotometry) impacted on WMD of IL-6 and CRP.

A meta-regression analysis was performed to assess the predictive effect of explanatory variables on WMD of IL-6 and CRP. Demographic factors age (years), % of female participants, study duration (weeks), daily cholecalciferol dose and overall 25(OH)D status ((post supplementation value-baseline value for the intervention group) − (post supplementation value minus the baseline value for the placebo group)) were investigated as potential confounders. These factors have previously been identified by us[30] and reported in the literature[25] and therefore were included initially in the development of the regression model. Backward elimination process was used to build a parsimonious regression model for identifying significant predictors of WMD of IL-6 and CRP. All statistical analyses were performed using Stata SE version14.0 (StataCorp LP, College Station, TX, USA). A *P*-value <0.05 was considered statistically significant.

RESULTS

Included studies

The PRISMA search strategy is depicted in Figure 2. Forty-three articles were retrieved for full-text assessment. After careful assessment, 14 high-quality articles met the inclusion criteria for qualitative analysis (systematic review). These high-quality studies are presented in Table 2. Relevant raw data could only be extracted from nine studies (15 arms), either through author contact or directly from the publication.

Characteristics of studies included in the systematic review

A total of 1984 subjects were included in the systematic review. The largest study had a population size of 484 individuals,[31] whereas the smallest study included 15 participants.[32] All studies were parallel randomised controlled trials. Studies were published recently, during the period of 2010–2016, and were conducted in Finland,[33] Denmark,[32,34] Norway,[31] Scotland,[35–37] Ireland,[38] England,[39] the United States,[40,41] Hong Kong,[42] New Zealand[43] and Iran.[44] A wide range of cholecalciferol dosages (200–7000 IU/d) were used in the included studies. Four studies reported 25(OH)D_2,[33,38,39,44] and the remaining studies reported total 25(OH)D.[31,32,34–37,40–43] Three studies included subjects with inadequate 25(OH)D status based on a cutoff value of <50 nmol/l[32,34,43] and three studies defined suboptimal D status using a cutoff of 75 nmol/l.[33,36,42] Eight studies started from a baseline <50 nmol/l[32,34–37,39,43,44] and six studies did not achieve a final 25(OH)D status above 80 nmol/l (Table 2).[32,35–38,44] CRP was

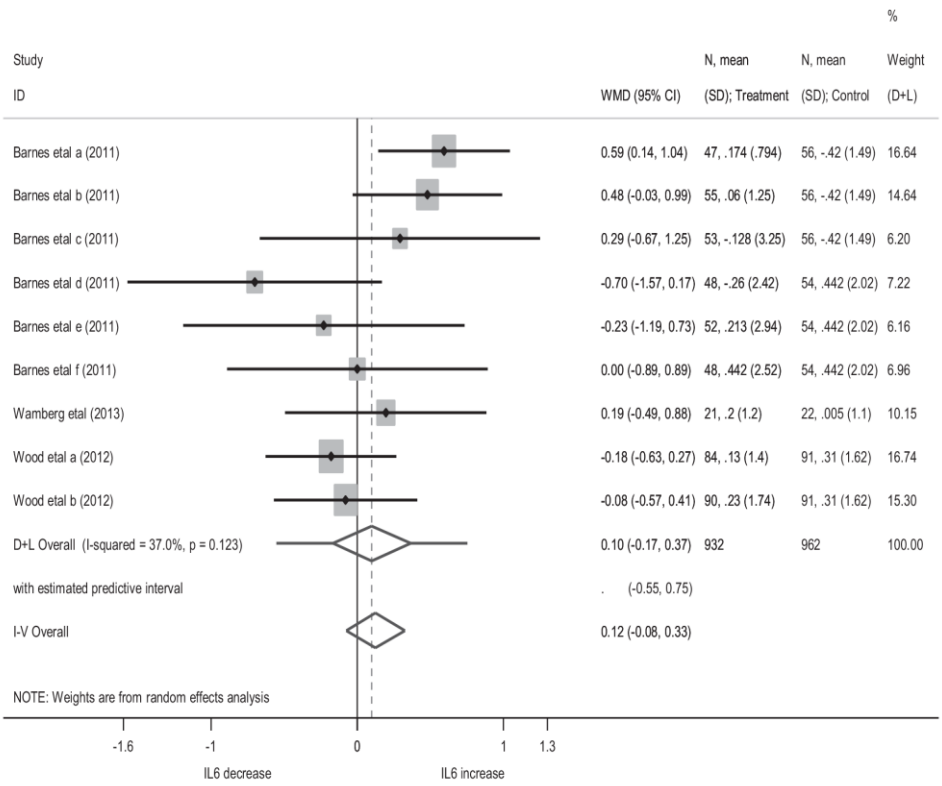

Figure 3. Forest plot of the effect of cholecalciferol supplementation on IL-6. Each square represents the unadjusted weighted mean difference (WMD) for each individual study with 95% confidence interval (CI) indicated by horizontal whiskers. Size of square is proportional to the precision of the estimate. Wood *et al.* (2012) is a multi-armed study and the letter 'a' denotes the comparison between the 400 IU/d arm and placebo and the letter 'b' denotes the comparison between the 1000 IU/d arm to placebo group. Barnes *et al.* (2011) is a multi-armed study conducted in both a younger and older age groups. In the younger age group, the letters 'a', 'b' and 'c' denote the comparison between the groups receiving 200 IU/d, 400 IU/d,1000 IU/d vs placebo, respectively. In the older age group, studies 'd', 'e' and 'f' denote the comparison between the groups receiving 200 IU/d, 400 IU/d,1000 IU/d vs placebo, respectively. IL-6, interleukin-6.

measured in all studies and IL-6 was the next commonly measured marker (measured in 6 of 14 studies). Most studies were unable to demonstrate an effect of cholecalciferol supplementation on systemic inflammatory markers. Four studies demonstrated significant improvements (or trends) in anti-inflammatory status with cholecalciferol supplementation compared with placebo.[33,37,43,44]

Quality assessment

Quality assessment as determined by the Jadad Score,[28] ranged from 4 to 5 among included trials. All of the selected studies provided sufficient details about randomization and double blinding, however description of withdrawals and dropouts was limited in the studies scoring 4.

Meta-analysis results for IL-6 and CRP

For the given nine study arms in which IL-6 mean (s.d.) change data were able to be extracted, the meta-analysis failed to find a significant overall change in IL-6 (WMD = 0.1, 95% confidence interval; CI = − 0.166–0.366, $P = 0.462$; Figure 3). No statistically significant heterogeneity was found in the effect sizes across the studies involved ($\chi^2 = 12.69$, $P = 0.123$, $I^2 = 37\%$). The meta-analysis also found no change in CRP (WMD = − 0.324, 95% CI = − 1.007–0.359, $P = 0.352$; Figure 4). However, there was significant heterogeneity in the effect sizes across the studies involved ($\chi^2 = 42.93$, $P < 0.001$, $I^2 = 67.4\%$).

Sensitivity analysis results for IL-6

Only one study used the gold standard LCMS assay to measure vitamin D status. On removing this study, sensitivity analysis revealed a negligible impact on WMD, however the I^2 statistic of heterogeneity increased from 37 to 44.6% with a trend towards significance ($P = 0.081$).

Subgroup analyses results for IL-6

Subgroup analysis to examine the impact of baseline 25(OH)D on IL-6 was conducted using a cutoff of 50 nmol/l. No significant effect of baseline 25(OH)D was found when status was < 50 nmol/l (WMD = 0.185, 95% CI = − 0.204–0.574, $P = 0.352$; heterogeneity test: $\chi^2 = 0.80$, $P = 0.670$, $I^2 = 0.0\%$) or ⩾ 50 nmol/l (WMD = − 0.073, 95% CI = − 0.371–0.225, $P = 0.632$; heterogeneity test: $\chi^2 = 8.79$, $P = 0.118$, $I^2 = 43.1\%$). Subgroup analysis to examine the impact of change in 25(OH)D on IL-6 was conducted using a modest increase of 20 nmol/l. No significant effect of 25(OH)D change was found when change was < 20 nmol/l (WMD = 0.195, 95% CI = − 0.254–0.645, $P = 0.394$; heterogeneity test: $\chi^2 = 8.34$, $P = 0.080$, $I^2 = 52.1\%$) or ⩾ 20 nmol/l (WMD = − 0.066, 95% CI = − 0.348–0.217, $P = 0.649$; heterogeneity test: $\chi^2 = 0.82$, $P = 0.844$, $I^2 = 0.0\%$). Subgroup analysis to examine the impact of final 25(OH)D achieved on IL-6 concentration was conducted using a cutoff of 70 nmol/l. No significant effect of final 25(OH)D was found when final status was < 70 nmol/l (WMD = 0.118, 95% CI = − 0.397–0.633, $P = 0.653$; heterogeneity test: $\chi^2 = 10.98$, $P = 0.012$, $I^2 = 72.7\%$) or ⩾ 70 nmol/l (WMD = 0.015, 95% CI = − 0.306–0.336, $P = 0.926$; heterogeneity test: $\chi^2 = 0.97$, $P = 0.914$, $I^2 = 0.0\%$). Subgroup analysis to examine

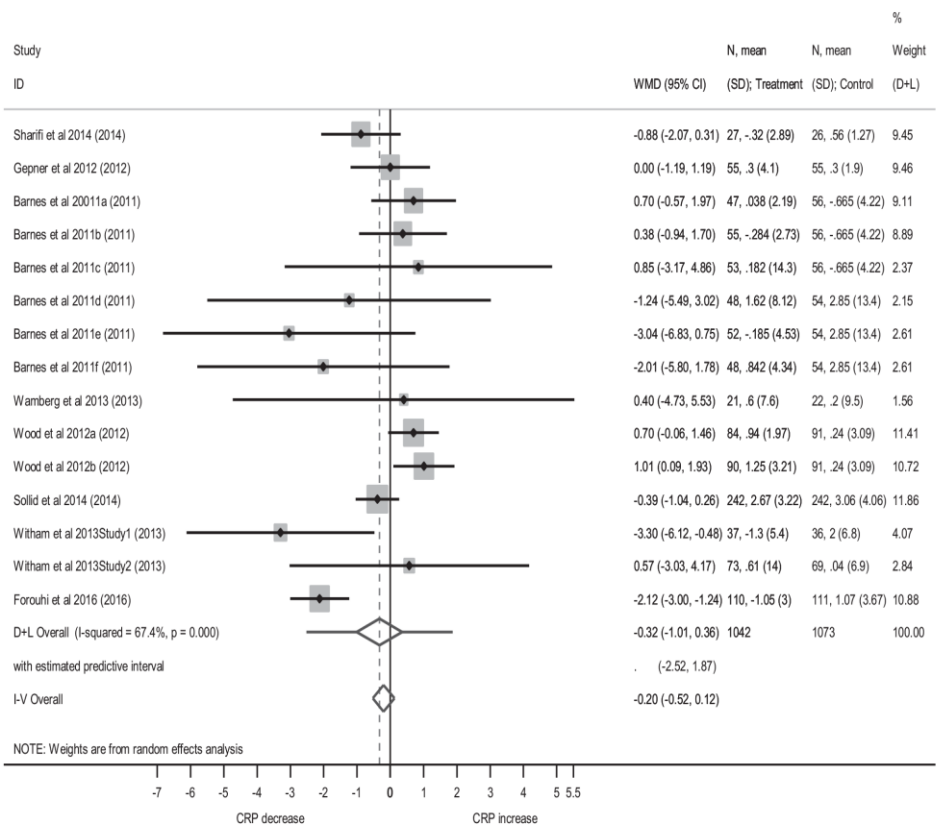

Figure 4. Forest plot of the effect of cholecalciferol supplementation on CRP. Each square represents the unadjusted weighted mean difference (WMD) for each individual study with 95% confidence interval (CI) indicated by horizontal whiskers. Size of square is proportional to the precision of the estimate. Wood *et al.* (2012) is a multi-armed study and the letter 'a' denotes the comparison between the 400 IU/d arm and placebo and the letter 'b' denotes the comparison between the 1000 IU/d arm to placebo group. Barnes *et al.* (2011) is a multi-armed study conducted in both a younger and older age groups. In the younger age group, the letters 'a', 'b' and 'c' denote the comparison between the groups receiving 200 IU/d, 400 IU/d, 1000 IU/d vs placebo, respectively. In the older age group, studies 'd', 'e' and 'f' denote the comparison between the groups receiving 200 IU/d, 400 IU/d, 1000 IU/d vs placebo, respectively. CRP, C-reactive protein.

the impact of final status achieved on IL-6 using a cutoff of 80 nmol/l was not conducted owing to inadequate studies with final status $\geqslant$ 80 nmol/l.

Subgroup analysis revealed that there was no impact of assay measurement (D3 only (WMD = 0.185, 95% CI −0.204, 0.574; $P = 0.352$; heterogeneity test: $\chi^2 = 8.89$, $P = 0.118$, $I^2 = 43.1\%$) versus total 25(OH)D (WMD = −0.073, 95% CI = −0.371, 0.225; $P = 0.632$); heterogeneity test: $\chi^2 = 0.80$, $P = 0.670$, $I^2 = 0\%$)). No impact of study duration on WMD was found for IL-6 (Shorter duration of 3–6 months (WMD = 0.208; 95% CI = −0.116–0.532, $P = 0.209$; heterogeneity test: $\chi^2 = 8.85$, $P = 0.182$, $I^2 = 32.2\%$ versus longer duration >6 months (WMD = −0.134, 95% CI = −0.465–0.196, $P = 0.425$; heterogeneity test: $\chi^2 = 9.09$, $P = 0.768$, $I^2 = 0.0\%$). Subgroup analysis to examine the impact of daily dose used on IL-6 was not conducted owing to inadequate number of studies which used a low dose of $\leqslant$1000 IU/d. There were not enough studies conducted in younger adults ($\leqslant$40 years) to examine the impact of age (younger adults versus older adults) on IL-6.

Sensitivity analyses results for CRP

Three studies used the gold standard LCMS assay to measure vitamin D status for CRP. Sensitivity analysis whereby each study was removed one at a time revealed negligible impact on WMD (data not shown). However, the removal of the study by Forouhi *et al.*[39] reduced the degree of heterogeneity for CRP (from $\chi^2 = 42.93$, $P < 0.001$, $I^2 = 67.4\%$ to $\chi^2 = 21.96$, $P = 0.056$, $I^2 = 40.8\%$).

Subgroup analyses results for CRP

Subgroup analyses to examine the impact of baseline 25(OH)D, change in 25(OH)D and final 25(OH)D achieved were conducted. No significant effect of baseline 25(OH)D was found when status was <50 nmol/l (WMD = −0.520, 95% CI = −1.812–0.772, $P = 0.430$; heterogeneity test: $\chi^2 = 36.24$, $P < 0.001$, $I^2 = 83.4\%$) or $\geqslant$50 nmol/l (WMD = −0.139, 95% CI = −0.611–0.332, $P = 0.562$; heterogeneity test: $\chi^2 = 6.57$, $P = 0.475$, $I^2 = 0.0\%$). No significant effect of 25(OH)D change was found when status change was <20 nmol/l (WMD = −0.487, 95% CI = −1.847–0.874, $P = 0.483$; heterogeneity test: $\chi^2 = 9.71$, $P = 0.084$, $I^2 = 48.5\%$) or $\geqslant$20 nmol/l (WMD = −0.286, 95% CI = −1.122–0.549, $P = 0.501$; heterogeneity test: $\chi^2 = 32.92$, $P < 0.001$, $I^2 = 75.7\%$). No significant effect of final 25(OH)D was found when final status was <70 nmol/l (WMD = 0.232, 95% CI = −0.630–1.094, $P = 0.597$; heterogeneity test: $\chi^2 = 7.97$, $P = 0.158$, $I^2 = 37.3\%$) or $\geqslant$70 nmol/l (WMD = −0.585, 95% CI = −1.488–0.318, $P = 0.204$; heterogeneity test: $\chi^2 = 27.35$, $P = 0.001$, $I^2 = 70.8\%$). No significant effect of final 25(OH)D was found when final status was <80 nmol/l (WMD = 0.238, 95% CI = −0.482–0.958, $P = 0.517$; heterogeneity test: $\chi^2 = 14.24$, $P = 0.114$, $I^2 = 36.8\%$; Figure 5), but a trend was observed when final 25(OH)D was $\geqslant$80 nmol/l (WMD = −0.834, 95% CI = −1.726–0.058, $P = 0.067$; heterogeneity test: $\chi^2 = 12.02$, $P = 0.017$, $I^2 = 66.7\%$; Figure 5).

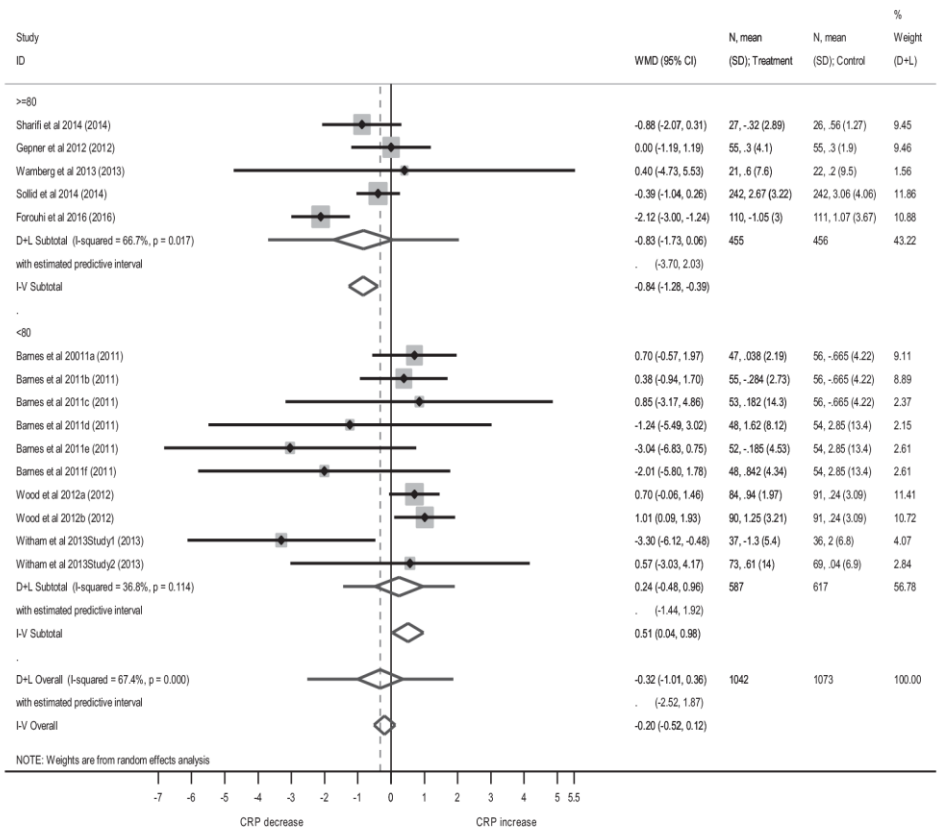

Figure 5. Forest plot of the effect of final 25(OH)D status on CRP by subgroup analysis. Wood *et al.* (2012) is a multi-armed study and the letter 'a' denotes the comparison between the 400 IU/d arm and placebo and the letter 'b' denotes the comparison between the 1000 IU/d arm to placebo group. Barnes *et al.* (2011) is a multi-armed study conducted in both a younger and older age groups. In the younger age group, the letters 'a', 'b' and 'c' denote the comparison between the groups receiving 200 IU/d, 400 IU/d, 1000 IU/d vs placebo, respectively. In the older age group, studies 'd', 'e' and 'f' denote the comparison between the groups receiving 200 IU/d, 400 IU/d,1000 IU/d vs placebo, respectively. CRP, C-reactive protein.

Subgroup analyses to examine the impact of assay measurement, study duration and daily dose of cholecalciferol on WMD of CRP were conducted. No significant impact of assay measurement on the WMD of CRP was found (D3 only (WMD = −0.746, 95% CI = −1.831–0.339; $P = 0.178$; heterogeneity test: $\chi^2 = 19.37$, $P = 0.007$, $I^2 = 63.9\%$) versus total 25(OH)D (WMD = 0.124, 95% CI −0.619 to 0.870, $P = 0.741$; heterogeneity test: $\chi^2 = 13.84$, $P = 0.031$, $I^2 = 56.6\%$)). Among the studies which measured D3 only, Galbraith plot (not shown) confirmed that Forouhi *et al.*[39] was a potential source of the heterogeneity. In contrast, studies which measured total 25(OH)D showed Wood *et al.*[35] and Witham *et al.* 2013 Study 1[37] as two potential sources of the heterogeneity. A trend for decreased CRP was found among studies with a short duration (WMD = −0.774, 95% CI = −1.675–0.128, $P = 0.092$; heterogeneity test: $\chi^2 = 24.42$, $P = 0.007$, $I^2 = 59.0\%$) while no significant WMD of CRP was found among studies with a longer duration (WMD = −0.324, 95% CI = −1.007–0.359, $P = 0.319$; heterogeneity test: $\chi^2 = 7.67$, $P = 0.053$, $I^2 = 60.9\%$; Figure 6). Galbraith plots (not shown) confirmed Forouhi *et al.*[39] and Wood *et al.*[35] were the potential source of the heterogeneity for the short and long duration group, respectively. When studies were grouped by daily dose (Supplementary Figure 1), significant WMD were found for both low (⩽1000 IU/d) and high (>1000 IU/d) daily dose groups. Among the studies using the low daily dose, the subgroup meta-analysis found a significant overall increase in CRP (WMD = 0.615, 95% CI 0.132 to 1.098, $P = 0.013$; heterogeneity test: $\chi^2 = 7.05$, $P = 0.424$, $I^2 = 0.6\%$). In contrast, among the studies with a high daily dose, an opposite effect with a significant overall decrease in CRP was found (WMD = −0.939, 95% CI = −1.805–−0.073, $P = 0.034$; heterogeneity test: $\chi^2 = 15.51$, $P = 0.017$, $I^2 = 61.3\%$). Examination of the Galbraith plot (not shown) revealed that the study by Forouhi *et al.*[39] was the potential source of the significant heterogeneity for the high-dose group. There were not enough studies conducted in younger adults (⩽40 years) to examine the impact of age (younger adults versus older adults) on CRP.

Meta-regression

Age was found to be a significant predictor of IL-6 change ($\beta = -0.02$, 95% CI = −0.034 to −0.006 pg/ml, $P = 0.013$; Table 3). No effect of gender (% female), study duration, total cholecalciferol dose or overall 25(OH)D change influenced IL-6 WMD. Age ($\beta = -0.06$, 95% CI −0.103 to −0.017, $P = 0.01$), gender ($\beta = 0.027$, 95% CI 0.011–0.044, $P = 0.004$) and supplementation duration ($\beta = 0.049$, 95% CI = 0.018–0.079, $P = 0.005$) were significant predictors of CRP change (Table 3).

Publication bias

There was reasonable symmetry to the funnel plot for IL-6 (Supplementary Figure 2a), with no publication bias or small-study effects found for studies included in the meta-analysis (Egger's test; $P = 0.379$). A slight trend of asymmetry was seen in the funnel plot for studies on the effect of cholecalciferol supplementation on CRP (Supplementary Figure 2b), no significant publication bias or small-study effect was found (Egger's test; $P = 0.538$).

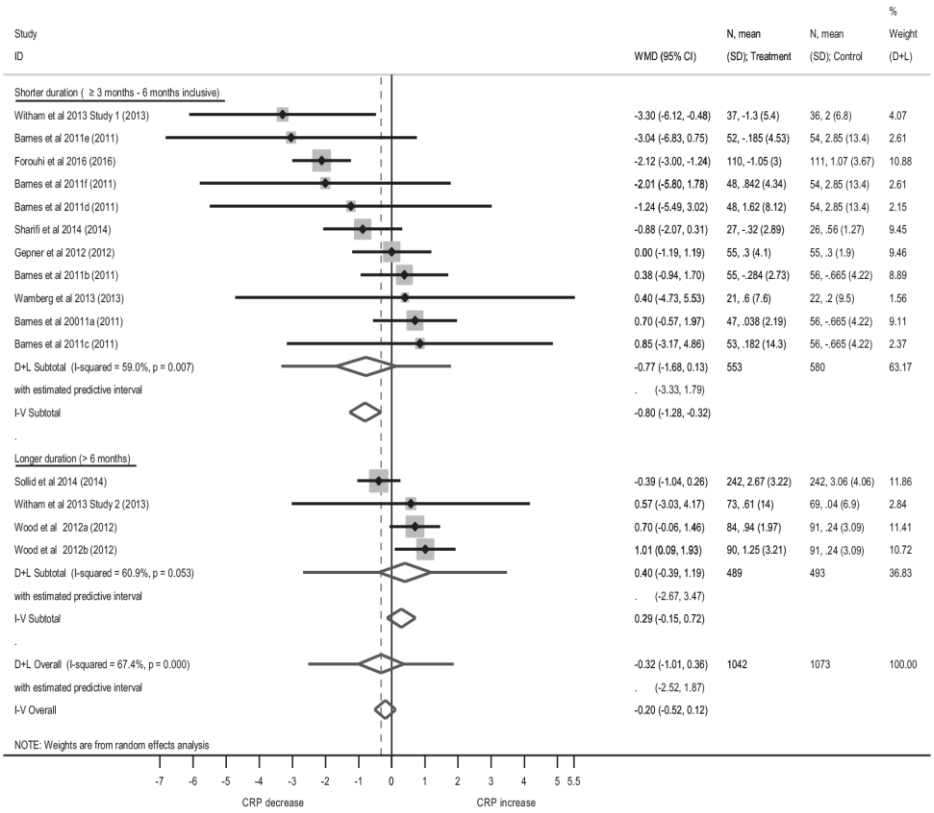

Figure 6. Forest plot of the effect of study duration on CRP by subgroup analysis. Wood *et al.* (2012) is a multi-armed study and the letter 'a' denotes the comparison between the 400 IU/d arm and placebo and the letter 'b' denotes the comparison between the 1000 IU/d arm to placebo group. Barnes *et al.* (2011) is a multi-armed study conducted in both a younger and older age groups. In the younger age group, the letters 'a', 'b' and 'c' denote the comparison between the groups receiving 200 IU/d, 400 IU/d,1000 IU/d vs placebo, respectively. In the older age group, studies 'd', 'e' and 'f' denote the comparison between the groups receiving 200 IU/d, 400 IU/d,1000 IU/d vs placebo, respectively. CRP, C-reactive protein.

Table 3. Meta-regression analyses[a] of potential factors influencing the WMD in IL-6 and CRP following cholecalciferol supplementation

Variable	*Estimated coefficient β*	*95% CI*	P-*value*
IL-6			
Age (years)	−0.02	−0.033, −0.006	0.013
Constant	1.1	0.357, 1.842	0.010
CRP			
Age (years)	−0.049	−0.091, −0.008	0.025
Gender (% female)	0.022	0.0008, 0.036	0.005
Study duration (weeks)	0.034	0.006, 0.068	0.024
Constant	−0.079	−1.878, 1.719	0.924

Abbreviations: CRP, C-reactive protein; IL-6, interleukin-6; RCTs, randomized controlled trials; WMD, weighted mean difference. [a]In both analyses, age (years), gender (%female), study duration (weeks), overall change in 25(OH)D (nmol/l), total cholecalciferol dose (daily dose IU/d × number of days of supplementation) were entered as a first step and non-significant factors were removed in a backward elimination procedure.

DISCUSSION

Vitamin D is widely accepted to have an anti-inflammatory role in human patho-physiology.[45,46] From a cellular perspective, this anti-inflammatory response is convincing.[9,45] In this review, we questioned whether increasing 25(OH)D levels in humans, through cholecalciferol supplementation, would result in an improvement in the systemic inflammatory profile of healthy adults and those with obesity-related chronic disease.

We did not find convincing evidence that cholecalciferol supplementation modified systemic inflammation as measured by a wide spectrum of cytokine profiles. It has previously been suggested that a high baseline 25(OH)D status, a small change in vitamin status and a relatively low final level achieved, may explain the lack of significant findings.[18,25] In support of this, participants in the study by Yusupov *et al.*[41] had adequate baseline 25(OH)D status (mean 64 nmol/l) and the change induced by supplementation was small. Similarly the study by Barnes *et al.*[38] also had high baseline 25(OH)D status (median 75.9 nmol/l) and resulted in a small change in status. Both of these studies employed doses of 200–2000 IU/d, which could have been too low to be effective in modulating inflammatory markers. Indeed other researchers have also suggested that much higher doses are needed.[47] However, two trials included in this review employed high doses ⩾5000 IU/d[32,34] and their participants also had a low initial 25(OH)D status (~33 nmol/l). Despite these subject characteristics, both studies failed to detect any effect on inflammatory markers, regardless of a large change in 25(OH)D following supplementation (mean (s.d.) values at end of study 73.9 ± 7.79 nmol/l[32] and 110.2 ± 21.2 nmol/l,[34] respectively). Furthermore, almost half of the RCTs that fulfilled our selection criteria started from a relatively low baseline (< 35 nmol/l) and achieved a final value between 65 and 110 nmol/l, following supplementation. In addition, we did not observe any clear

evidence in support of a link between baseline vitamin D status, change in status or final status following cholecalciferol supplementation on IL-6 or CRP inflammatory markers in our subgroup analyses. However, there was a trend that achieving a final status of ⩾80 nmol/l promoted a reduction in CRP following cholecalciferol supplementation. This needs to be interpreted cautiously since considerable heterogeneity was also found. However the result does warrant further investigation to explore whether such a cutoff (or higher) is needed to beneficially impact on levels of CRP. We do acknowledge that it is challenging to come to a come to a definite cutoff value from a worldwide collation of data on 25(OH)D. To an extent, the outcomes reported depend on the different 25(OH)D assays used, and even studies using the same gold standard LCMS technique can show a substantial (approximately twofold) variation.[48,49] The implication is that any proposed cutoff value is significantly dependant on the assay employed. Hence in this review, we appreciate that there may well be an underestimation or overestimation of the true status required to influence CRP levels. Furthermore, some studies report D3 only, whereas others report total 25(OH)D (sum of D2 and D3). This could potentially introduce a small but clinically significant error when a cutoff is utilised.

We found age to be a determinant of IL-6 change, with older adults displaying an effect of a reduced IL-6 following of cholecalciferol. A similar effect of age was evident for CRP as well (Table 3). In contrast, a greater percentage of females in these trials and a greater trial duration, both independently predicted an increase in CRP following supplementation. Whether studying older adults, only males or employing a short supplementation period favours a reduction in CRP by cholecalciferol, remains to be determined. In general, men have less body fat than women, so it is plausible that supplemented 25(OH)D was taken up by adipose tissue and cleared from the circulation to a greater extent in women.[50] Females also have greater circulating cytokine levels,[51] so overall they may need a much greater 25(OH)D levels to suppress inflammation. Well-conducted RCTs require good compliance, and it is possible that in longer term RCTs (>6 months), the compliance to the intervention decreases over time. Moreover CRP is an acute-phase protein, so a dampening effect of cholecalciferol may only be seen in the short term. These reasons would explain our meta-analysis outcomes where a greater study duration lessened the effect of cholecalciferol on reducing CRP.

There are two previous systematic reviews and meta-analyses in this area, but they are not strictly comparable. One review found no benefit of cholecalciferol supplementation on inflammatory cytokines CRP, IL-6 and TNF- α[16] in overweight and obese children and adults. This would be in agreement with our outcomes, but in that study there was additional confounding factors through the use of other dietary manipulations. Our results however are in contrast to another study that found cholecalciferol and ergocalciferol supplementation reduced CRP.[15] In the review of Chen *et al.*[52] participants had much higher levels of inflammation, with baseline circulating CRP levels varying from 1.71 to 22 mg/l (median of 5 mg/l). In contrast, baseline levels of CRP in our meta-analysis ranged from 0.18 to 4.55 mg/l and we only used high-quality trials of ⩾12 weeks. It is well-accepted that quality of studies selected is a key factor that can modify the conclusions of a systematic review.[53]

The lack of beneficial outcomes from high-quality RCTs, contrast with the strong cellular evidence in support of a role for vitamin D in regulating immune function. The active form of vitamin D, 1,25 $(OH)_2D$, demonstrates an anti-inflammatory effect on the production and release of cytokines from circulating immune cells.[54,55] Although cellular studies use 1,25$(OH)_2D$, we and others[56] strongly believe that maintaining a high plasma 25(OH)D level is crucial since 25(OH)D influences local tissue production of the active vitamin D.[57] Circulating immune cells come into contact with vitamin D levels and the level of serum vitamin D has been shown to influence cytokine secretion from peripheral blood mononuclear immune cells.[58] We acknowledge that measuring systemic cytokine concentrations does not provide information on cytokine release at the local tissue level.[59]

There are several possible explanations for the lack of significant effects of cholecalciferol supplementation on inflammatory markers. First, cellular experiments allow greater control of the local environment, however *in vivo*, local response to supplementation by individual cells may be diluted out in the whole body, resulting in non-detectable changes. It is possible that the absence of significant findings may be due to the low levels of baseline inflammation in relatively healthy participants without acute inflammatory conditions. We cannot rule out that cholecalciferol modulates other aspects of immune function other than cytokine release such as immune cell proliferation, differentiation and migration,[10] and future RCTs should include such measures in their design. Lastly, our results need to be treated with caution as considerable heterogeneity was detected, among studies which investigated the outcome of cholecalciferol on CRP. Heterogeneity was significantly reduced when our analyses were restricted to studies where baseline 25(OH)D was ⩾50 nmol/l, final status achieved was <70 nmol/l and <80 nmol/l. This suggests that these factors might contribute to inconsistency between studies for CRP outcomes. Future larger studies addressing these factors are warranted.

In conclusion, based on a systematic review of high-quality RCTs of medium to long duration, we did not find any evidence of a benefit of cholecalciferol supplementation on systemic inflammatory markers. Neither high baseline 25(OH)D, a small change in status nor a low final concentration achieved, accounted for our findings. Instead, future studies should consider the confounding effects of age, gender and study duration, while targeting a high daily dose (>1000 IU/d) and possibly an achieved 25(OH)D ⩾80 nmol/l.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

EKC, KNK, MJS and PN designed the research; EKC, KNK and MJS conducted the research; YZ designed the data extraction sheet, planned and performed all statistical analyses; EKC, MJS and KNK wrote the paper; EKC and MJS had primary responsibility for final content. All authors have approved and contributed to the final written manuscript.

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APPENDICES

APPENDIX A: COPYRIGHT PERMISSION

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PLoS One

The Impact of Vitamin D Levels on Inflammatory Status: A Systematic Review of Immune Cell Studies

Emily K. Calton,[1] Kevin N. Keane,[2] Philip Newsholme,[2,*] and Mario J. Soares[1]

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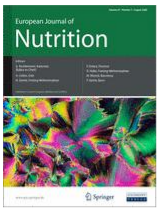

Title: Vitamin D status and insulin sensitivity are novel predictors of resting metabolic rate: a cross-sectional analysis in Australian adults
Author: E. K. Calton
Publication: European Journal of Nutrition
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Author: Emily K. Calton,Kevin N. Keane,Mario J. Soares,Jordan Rowlands,Philip Newsholme

Publication: Redox Biology

Publisher: Elsevier

Date: December 2016

Title: Winter to summer change in vitamin D status reduces systemic inflammation and bioenergetic activity of human peripheral blood mononuclear cells

Author: Emily K. Calton,Kevin N. Keane,Raquel Raizel,Jordan Rowlands,Mario J. Soares,Philip Newsholme

Publication: Redox Biology

Publisher: Elsevier

Date: August 2017

Title:	The impact of cholecalciferol supplementation on the systemic inflammatory profile: a systematic review and meta-analysis of high-quality randomized controlled trials
Author:	E K Calton, K N Keane, P Newsholme, Y Zhao and M J Soares
Publication:	European Journal of Clinical Nutrition
Publisher:	Nature Publishing Group
Date:	May 10, 2017

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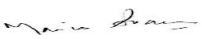
APPENDIX B: STATEMENTS FROM CO-AUTHORS

To Whom It May Concern

I, Emily Kathleen Calton, contributed (figure design, wrote the first draft, approved and contributed to the final written manuscript) to the paper/publication entitled (Calton, E. K., K. N. Keane, and M. J. Soares. 2015. "The potential regulatory role of vitamin D in the bioenergetics of inflammation." *Curr Opin Clin Nutr Metab Care* 18 (4):367-73).

I, as a Co-Author, endorse that this level of contribution by the candidate indicated above is appropriate.

Kevin Keane 

Mario Soares 

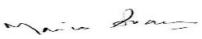
To Whom It May Concern

I, Emily Kathleen Calton, contributed (the idea for this review, the initial search, study selection, figure design, data extraction, wrote the first draft, approved and contributed to the final written manuscript) to the paper/publication entitled (Calton, E. K., K. N. Keane, P. Newsholme, and M. J. Soares. 2015. "The impact of vitamin D levels on inflammatory status: A systematic review of immune cell studies." *PLoS ONE* 10 (11):e0141770. doi: 10.1371/journal.pone.0141770).

I, as a Co-Author, endorse that this level of contribution by the candidate indicated above is appropriate.

Kevin Keane 

Philip Newsholme 

Mario Soares 

To Whom It May Concern

I, Emily Kathleen Calton, contributed (the idea for this paper, cross-checking of data entry, wrote the first draft, contributed to and approved the final written manuscript) to the paper/publication entitled (Calton, E. K., K. Pathak, M. J. Soares, H. Alfonso, K. N. Keane, P. Newsholme, N. K. Cummings, W. Chan She Ping-Delfos, and A. Hamidi. 2016. "Vitamin D status and insulin sensitivity are novel predictors of resting metabolic rate: a cross-sectional analysis in Australian adults." *Eur J Nutr* 55 (6):2075-80. doi: 10.1007/s00394-015-1021-z).

I, as a Co-Author, endorse that this level of contribution by the candidate indicated above is appropriate.

Kaveri Pathak 

Mario Soares 

Helman Alfonso 

Kevin Keane 

Philip Newsholme 

Nikki Cummings 

Wendy Chan She Ping-Delfos 

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To Whom It May Concern

I, Emily Kathleen Calton, contributed (the design, initial manuscript preparation, patient recruitment, body composition assessment, immune cell isolation, bioenergetic parameters measurement, data analysis, statistical analysis, figure preparation and approved the final version of the paper) to the paper/publication entitled (Calton, E. K., K. N. Keane, M.J. Soares, J. Rowlands, and P. Newsholme. 2016. "Prevailing vitamin D status influences mitochondrial and glycolytic bioenergetics in peripheral blood mononuclear cells obtained from adults." *Redox Biol* 10:243-250. doi: 10.1016/j.redox.2016.10.007).

I, as a Co-Author, endorse that this level of contribution by the candidate indicated above is appropriate.

Kevin Keane 

Mario Soares 

Jordan Rowlands 

Philip Newsholme 

To Whom It May Concern

I, Emily Kathleen Calton, contributed (the design, initial manuscript preparation, patient recruitment, body composition assessment, immune cell isolation, bioenergetic parameters measurement, data analysis, statistical analysis, figure preparation and approved the final version of the paper) to the paper/publication entitled (Calton, E. K., K. N. Keane, R. Raizel, J. Rowlands, M. J. Soares, and P. Newsholme. 2017. "Winter to summer change in vitamin D status reduces systemic inflammation and bioenergetic activity of human peripheral blood mononuclear cells." *Redox Biol* 12:814-820. doi:http://doi.org/10.1016/j.redox.2017.04.009).

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Kevin Keane 

Raquel Raizel 

Jordan Rowlands 

Mario Soares 

Philip Newsholme 

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I, as a Co-Author, endorse that this level of contribution by the candidate indicated above is appropriate.

Kevin Keane 

Philip Newsholme 

Yun Zhao 

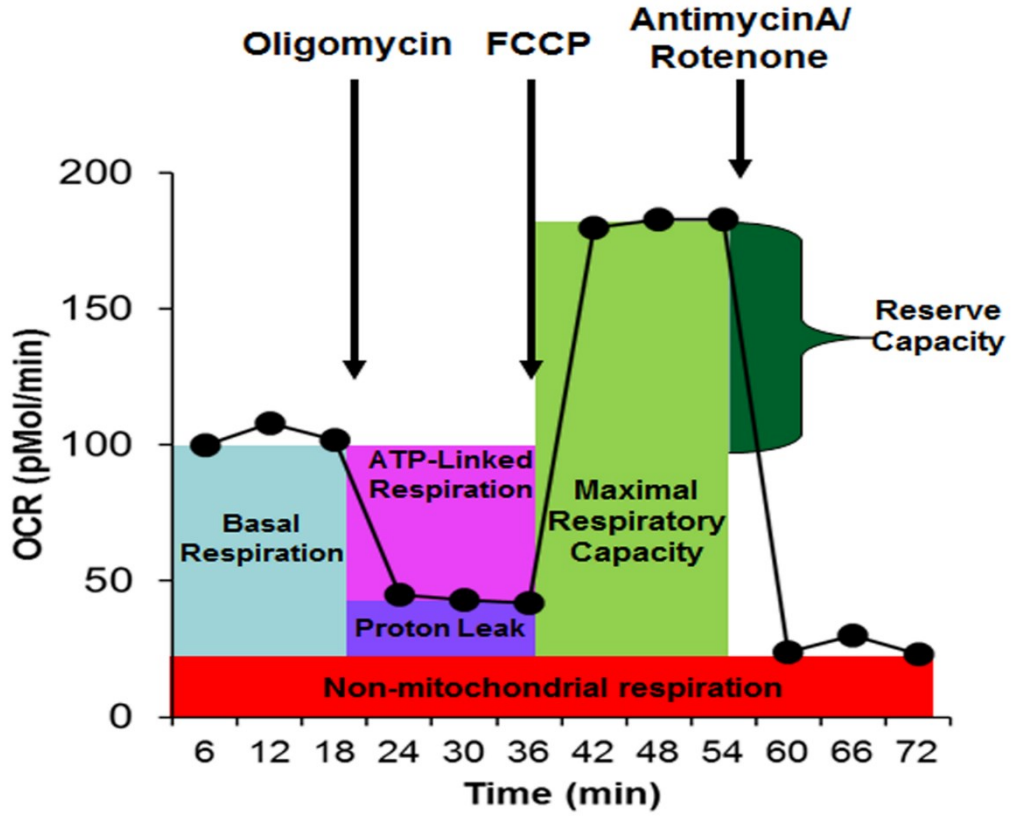
Mario Soares 

APPENDIX C: DETAILED METHODOLOGY

The Mitochondrial Stress Test Assay

Before any injections are made, the basal OCR is measured (See Figure 1). The basal OCR represents the net sum of all mitochondrial processes in the cell capable of consuming O_2 (Hill et al. 2012). That is, coupled mitochondrial respiration as well as uncoupled consumption of oxygen to ATP production (eg to form reactive oxygen species) (Hartman 2014). Energetically active cells have a high basal OCR. An increase in basal OCR could be due to an increase in ATP turnover, indicative of an increase in ATP demand. Secondly, an increase in basal OCR could be due to an increase in proton leak. Basal respiration represents a threshold below which the cell cannot sustain oxidative phosphorylation to meet energy demand. If this threshold activity cannot be met, glycolysis is then stimulated to meet the energetic needs of the cell (Chacko et al. 2013). Whereas a decrease in basal OCR could be due to decreased ATP demand, a lack of substrate availability (eg glucose, pyruvate), or severe damage to the ETC, which would impede the flow of electrons (Hill et al. 2012).

Figure 1. The Seahorse Mitochondrial Stress Test



Sourced from (Rose et al. 2014)

Basal OCR= last measurement prior to oligomycin addition-minimum non mitochondrial respiration value

The first injection is of oligomycin, an inhibitor of mitochondrial ATP synthase. By inhibiting ATP synthesis, oligomycin enables determination of oxygen consumption for ATP synthesis, and the coupling efficiency (Hill et al. 2012). The fall in OCR following oligomycin injection is the rate of oxygen consumption that is coupled to ATP synthesis, and the oligomycin-insensitive rate is considered as proton leak across the inner mitochondrial membrane.

Coupling efficiency (%)=100- (ATP production/basal respiration)

Proton leak= (minimum measurement after oligomycin injection through to the measurement prior to FCCP) – minimum non mitochondrial respiration

ATP production= basal – proton leak

Next, the theoretical maximum oxygen consumption that can take place at cytochrome c oxidase (complex IV) whether limited by availability of substrate or activity of the electron transport chain is measured by injecting uncoupling reagent, Carboynl cyanide-p trifluoromethoxyphenylhydrazone (FCCP). FCCP disrupts ATP synthesis by transporting hydrogen ions across the mitochondria membrane instead of through ATP synthase resulting in rapid consumption of energy and oxygen without the generation of ATP. The difference between the basal rate and this FCCP- stimulated rate is the reserve capacity of the mitochondria, which is a measure of the maximal potential respiratory capacity the cell can utilise under conditions of stress and/or increased energetic demands. The larger the value for reserve capacity the more effectively mitochondria can meet both the ATP needs of the cell and deal with increased energetic demand or metabolic stress.

Spare respiratory capacity = maximal respiration- basal

Maximal respiration= maximum OCR in response to FCCP - non mitochondrial respiration

The third injection is a combination of rotenone, a complex I inhibitor and antimycin A, a complex III inhibitor. Together, ETC activity is completely inhibited (Hill et al. 2012) allowing for non-mitochondrial derived OCR to be measured. Non-mitochondrial derived OCR measures cellular oxygen consumption occurring elsewhere in the cell such as by cellular enzymes including NADPH oxidase and cytochrome P450.

Non-mitochondrial derived OCR= first measurement after addition of rotenone and antimycin A through to the end of assay

The Glycolysis Stress Test Assay

First, cells are incubated in the glycolysis stress test medium containing no glucose but with pyruvate and glutamine and the basal PPR is measured. This measured PPR is not attributable to glucose metabolism. The first injection is a saturating concentration of glucose. The cells utilize the glucose injection and catabolize it through the glycolytic pathway to pyruvate, producing ATP, NADH, water, lactate and consequently protons. As the protons increase in the surrounding medium they are detected.

Glycolytic response to 25 mM glucose= maximum PPR following addition of glucose - last PPR measurement prior to addition of glucose.

The second injection is oligomycin, an ATP synthase inhibitor. Oligomycin inhibits mitochondrial ATP production and shifts the energy production to glycolysis, with the subsequent increase in PPR revealing the cellular maximum glycolytic capacity.

The last injection is 2-deoxy-glucose (2-DG), which inhibits glycolysis through competitive binding to glucose hexokinase, the first enzyme in the glycolytic pathway. As a result, the PPR decreases which acts to confirm that the PPR produced with the addition of glucose and oligomycin, is due to glycolysis.

Glycolytic capacity= maximum PPR after injection of oligomycin- the minimum PPR following 2-DG addition

Glycolytic reserve=glycolytic capacity-glycolytic response to 25 mM glucose.

Methods for bioenergetics analysis

Preparation of plate

Prior to the addition of PBMCs, XF-96 plates (Seahorse Bioscience, Billerica, MA) are treated with Poly-D-Lysine) to improve cell adherence. Poly-D-Lysine is diluted in sterile water to reach 50 µg/ml and the plate coated with 30µl per well and incubated for 1 hour at 37 °C. The solution is removed, the wells washed with sterile water, and the plate dried in a laminar flow hood overnight.

PBMC isolation and count

Venous blood is obtained from fasting donors and diluted 1:1 with PBS containing EDTA and added to an equal volume of histopaq solution to be centrifuged at 600xg for 20 min with minimum acceleration and no braking. A sample of plasma is taken from the upper layer, the buffy coat containing the leucocytes is removed and PBS (EDTA free) is added to the leucocytes for centrifugation at 300xg for 10 min. The pellet is resuspended in PBS (EDTA-free) and spun at 200xg for 10 min and then again at 100xg for 10 min. The pellet is resuspended in complete RPMI media. An aliquot is taken and a 1:20 dilution is made with the sample and PBS. This is then used to determine the cell number and proportion (%) of immune cells (lymphocytes, monocytes and granulocytes) using the automatic Mindray BC2800 haematological analyser. The cell

suspension is freshly seeded at a density of 3.5×10^5 cells/well into 96 well plates previously coated with poly-D-lysine (50 µg/mL) and allowed to adhere overnight in 5% CO_2.

Media change and calibration

Media change of the cell culture plate is performed manually. This involves 3 cycles to replace the initial serum- and bicarbonate-containing media, leaving a final volume of 175µl serum- and bicarbonate-free media per well. The cell culture plate is placed in the CO_2-free incubator for about 30 mins at 37°C. Cartridges are hydrated with 200µl of calibration solution added to each well and the cartridge and plate placed into a CO_2-free incubator for 24 h at 37°C. The cartridge with calibration solution is then inserted into the Seahorse instrument and the calibration protocol run.

APPENDIX D: STUDY FORMS

Participant information Sheet

Information to Volunteers

This document serves to assist you in understanding what is involved in this project. For this study to be a success, we request the volunteers to adhere to all parts of the study. This study is being conducted by a PhD student in partial fulfilment of a PhD degree. This study will be overseen by the supervisor. Please read this document carefully before you agree to join the study. Please be sure that you fully understand the nature of the study, and feel free to query us on any aspect, at any time.

Summary of program

It is well-known that immune function and energy metabolism (the way we use the food we eat) are connected to each other. Vitamin D is known to have effects on immune function and energy metabolism. This study seeks to explore relationships between change in vitamin D status with season and change in inflammation and energy metabolism.

Participation in this program is on a voluntary basis. Participants may opt out of this study at any time, without any fear of recrimination. Personal and medical information collected for this study by the research team will be kept confidential.

Please feel free to visit your GP to discuss your involvement in the research.

Background information

Vitamin D status is surprisingly poor in Australia, considering that Australia is well known for its abundant sunshine. The percentage of people with an overactive immune system is on the rise all over the world. An overactive immune system and impaired energy metabolism is more common among people who are overweight, have a sedentary life style or belong to certain ethnic groups.

Study Aim

The aim of this study is to understand how vitamin D status, inflammatory status and energy metabolism change in response to season change.

Study design

This study consists of a minimum of 2 visits

Visit 1 (start of the study) ~4 hour visit, Building 400.209 and 404 and Visit 2 (~4 hour visit)

The ***day prior to this visit,*** you will need to consume your last meal by at least 12 hours prior to your arrival time for the study. After this meal, you may drink as much as water you want; however **please do not eat or drink ANYTHING else including tea and coffee**. This will ensure that you are in a truly fasting state for our measurements. To ensure accuracy of our measurements, it is ***vital*** to fulfil the following conditions:

1. A minimum of 8 hours sleep.
2. No strenuous exercise for at least 1½days (36 hours) prior to the visit.
3. Try to avoid too much activity on the morning of the test.
4. Please take a shower the night beforehand and not in the morning.
5. No alcohol should be consumed for at least 36 hours prior to the study.

You will need to come to Curtin University anytime between 6.30am - 9 am as per your convenience. All measurements on this day will be conducted in Room 209 of Building 400 (Public Health).

During this visit, it is important that you come to the laboratory as relaxed as possible and remain relaxed until the end of the testing.

You will rest in bed for 30 minutes. We will then determine your metabolism. This is done using 'indirect calorimetry method' by placing a transparent canopy over the face and recording all the oxygen you breathe in and carbon dioxide you breath out over a 30 minute time period. We will then take 24 ml of venous blood (1 tablespoon) while you are in the fasting state to assess your inflammatory status, as well as your blood glucose levels, insulin levels and vitamin D status. We will use part of the blood sample

to look at the change in energy metabolism of your immune cells. We will provide you with a 75 g glucose drink to consume and 2 hours later we will then take a small 6 ml venous sample (1 teaspoon) to test your glucose, insulin and inflammatory response. We will also measure your blood endothelium function by placing the pulse trace clip over your index finger and we will also measure blood pressure using a cuff around your arm.

Your responsibility in this study

If you agree to participate in this study, you should follow the advice given to you by the study team. We will arrange study days to suit your availability, as far as possible but all visits will occur on a TUESDAY or a THURSDAY morning. If you decide to withdraw from the study, or change your mind, then we request you to inform us beforehand. If you do not follow the instructions provided by the study team, we will ask you to withdraw from the study.

Possible benefits from participating in the study

At the end of the study, we will provide a complete report on your vitamin D status, blood glucose and insulin levels, diabetes risk, body composition and metabolic rate. You will be informed of your personal results and when all the data have been analysed, a brief final outcome will be sent to your mailing address. If we detect any irregular results that need medical attention, we will inform you of these straight away.

Possible risks and side effects

There are very few, minimal risks and side effects that we foresee. The risk involved in the blood collection process includes the possibility of having minor bruises and temporary pain around the area where the blood will be taken. This usually subsides within 24-48 hours. Should you have any issues after the trial please contact the Principal Investigator (A/Prof Mario Soares) as soon as possible by emailing him on m.soares@curtin.edu.au or by calling him on +61-8-92663220.

Costs and payments for participating in the study

There will be no charge to you for participating in this study. At the end of the study, you will be given a small token of appreciation (equal to approximately AUD 20) for your attendance.

Voluntary participation

You may opt out of this study at any time, without any negative consequences. Your decision to quit will not affect your relationship with us or your existing relationship with Curtin University, nor will it impinge on the level of any medical care you may be receiving outside of Curtin University. If you choose to cease taking part in this study, please inform the Principal Investigator or research scientist, as a courtesy.

Your participation in this study may be stopped by the Principal Investigator for any of the following reasons:

- If you do not follow the study instructions; and/or
- If the study doctor decides it is in the best interest of your health and welfare to stop participating.

You will be informed of any new information that might affect your willingness to continue this study.

Confidentiality of study and medical records:

Personal and medical information collected for this study by the study doctor and the study team will be kept confidential. Data will be de-identified by assigning each participant a code. All data will be stored in a locked cabinet for a period of 7 years, in the supervisor's office. Any e-data will be password protected. Your records, to the extent of the applicable laws and regulations, will not be made publicly available. However, the Curtin University Institutional Review Board for the study will be granted direct access to your original personal and medical records if they need to check that the study was done properly, but still keeping such information confidential. By signing the Informed Consent Form attached, you are authorizing such access to your personal and medical records by concerned people. Data collected and entered into the Case Report Forms are the property of Curtin University. We will ensure that when we publish our results, your identity will always remain confidential.

In Summary

To participate in this study you will need to be able to:

✓ Have blood taken for glucose and other blood tests

✓ Undergo body composition assessment

✓ Receive an oral drink of glucose solution

✓ Attend Curtin University twice, on either a Tuesday or Thursday morning

As a participant, you will benefit by the following:

- An opportunity to understand whether you are at a higher risk of developing/progressing diabetes, as compared to other people
- On completion of the entire program, you will receive your results including vitamin D status, body composition, metabolic rate, blood glucose, insulin and triglyceride levels

Please take care that you do not:

- Wear any jewellery on the day of the study. If you do, it will need to be taken off for the duration of the study.
- Take a shower on the morning of the study.
- Wear any nail paints, such as fingernail polish.
- Do any vigorous exercise or consume alcohol on the day prior to the study.

Further information:

If you have any further queries, please do not hesitate to contact:

Emily Calton, Nutritionist, Dietitian, PhD Student
e-mail: emily.calton@postgrad.curtin.edu.au

Principal Investigator:
Dr Mario Soares, MBBS, PhD, RNutr
(08) 9266 3220 (Working hours)
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Associate Professor
Department of Nutrition, Dietetics & Food Science,
School of Public Health, Curtin University
GPO Box U1987, WA 6845

This study has been approved by the Curtin University Human Research Ethics Committee (Approval number: HR RDHS-13-15). The Committee is comprised of members of the public, academics, lawyers, doctors and pastoral carers. If needed, verification of approval can be obtained either in writing *to the Curtin University Human Research Ethics Committee c/- Office of Research Development, Curtin University of Technology, GPO Box U1987, Perth 6845, or by telephoning 9266 9223 or by emailing hrec@curtin.edu.au*

Screening Survey

January 2015

Thank you for your interest in this research program. This study has been approved by the Curtin University Human Research Ethics Committee (Approval number: RDHS-13-15). The Committee is comprised of members of the public, academics, lawyers, doctors and pastoral carers. If needed, verification of approval can be obtained either in writing to the Curtin University Human Research Ethics Committee c/- Office of Research Development, Curtin University of Technology, GPO Box U1987, Perth 6845, or by telephoning 9266 2784 or by emailing *hrec@curtin.edu.au*.

Purpose of study

You are invited to participate in a study, which aims to investigate whether seasonal vitamin D status is associated with impaired immune function and energy metabolism. At the end of the study, you will receive a report that indicates your body composition (fat % and muscle mass), metabolic rate, levels of blood glucose (sugar), insulin, diabetes risk and vitamin D status.

Screening Survey

In order for us to determine your suitability for our program, we invite you to kindly fill in this short survey which will take approximately 5 minutes. There are no known risks in filling this application.

Confidentiality

Any information that you provide us regarding your identity will be de-identified before being stored securely in a locked cabinet in a locked office to protect your privacy.

If you have any further questions or would like to have the information sheet/informed consent form prior to filling this survey please email Emily.calton@postgrad.curtin.edu.au

Proceed to the survey

Please complete the spaces as indicated below. Please place an (X) in the box which corresponds to your answer. If your response is YES to any question, you may provide further details in the space provided.

1. Demographics

- First name: _____ Last name: _____
- Address: _____

c) Suburb:_____

d) Postcode:_____

e) Telephone: _____ (home) _____ (mobile)

f) Email: _____

g) Date of Birth: _____ Age: _____ years

h) Country of Birth: _____

i) Duration lived in Australia: _____ years

j) Parents' country of birth:

Father: _____

Mother: _____

k) Menopause: pre ☐ peri ☐ post ☐

2. Are you currently a smoker? Y N

3. Do you have more than 2 alcoholic drinks per day? Y N

4. What is your current weight? _____

5. What is your height? _____

6. Has your weight fluctuated by 3(or more) kg in the last 6 months?

Y N

7. Do you plan on trying to lose weight in the next 3 months?

Y N

8. Are you pregnant or planning to be pregnant in the next 6 months?

Y N

9. Are you breastfeeding or plan to breastfeed in the next 6 months?

Y N

10. Do you suffer from any of the following chronic diseases/other health conditions:

Asthma	Y	N
Diabetes	Y	N
Kidney disease	Y	N
Kidney stones (more than 2)	Y	N
Thyroid disease	Y	N
Cancer	Y	N
Gastrointestinal disease/previous gastrointestinal surgery	Y	N

Other? Please specify here

Please write in the space below the medications and supplements/vitamins you are currently taking

Thanks. End of Screening Survey.

Consent form

Chief investigators:

Emily Calton

I understand that this study is being conducted by the above PhD student in partial fulfillment of the PhD degree. I understand that the data collected will be used to investigate relationships between vitamin D status, energy metabolism and immune function in adults. I consent for the data to be used in this manner and for the data to be collated with similar information collected from other studies. I understand that all personal details will be de-identified and my privacy respected.

Please tick the boxes if you agree to the following:

I agree to visiting Curtin University on a Tuesday or Thursday morning on two occasions.

☐

Having an oral glucose tolerance test where I consume a 75g drink of glucose (a sweet drink) twice (one during the winter visit and one drink during the summer visit).

☐

Having my body composition assessed by standing on scales(BIA) and laying on a bed (DEXA) at each visit to Curtin University.

☐

Maintaining my normal level of physical activity as recorded in the IPAQ questionnaire.

☐

Maintaining my normal diet as recorded in the FFQ questionnaire.

☐

Having my resting energy metabolism monitored during the winter and summer visit.

☐

Having two blood tests on each visit to test for vitamin D, insulin, glucose, inflammatory markers.

☐

I agree to all data from this study being collated with other similar data for purposes of research enquiry.

☐

I have read the participant information sheet and understand the risks and benefits of this research. I have been given the opportunity to ask questions and am satisfied with the responses.

I, ... (Participant's name) consent to participate in the above research conducted by Emily Calton under the supervision of Dr Mario Soares.

Participant name:

Signed ..... Date ...../...../.....

Witness name:

Signed ..... Date ...../...../.....

Physical activity checklist: International Physical Activity Questionnaire (IPAQ)

We are interested in finding out about the kinds of physical activities that people do as part of their everyday lives. The questions will ask you about the time you spent being physically active in the last 7 days. Please answer each question even if you do not consider yourself to be an active person. Please think about the activities you do at work, as part of your house and yard work, to get from place to place, and in your spare time for recreation, exercise or sport.

Think about all the vigorous activities that you did in the last 7 days. Vigorous physical activities refer to activities that take hard physical effort and make you breathe much harder than normal. Think only about those physical activities that you did for at least 10 minutes at a time.

1. During the **last 7 days**, on how many days did you do **vigorous** physical activities like heavy lifting, digging, aerobics, or fast bicycling?

_____ Days per week

☐ No vigorous physical activities → Skip to question 3

2. How much time did you usually spend doing **vigorous** physical activities on one of those days?

_____ Hours per day

_____ Minutes per day

☐ Don't know/Not sure

Think about all the moderate activities that you did in the last 7 days. Moderate activities refer to activities that take moderate physical effort and make you breathe somewhat harder than normal. Think only about those physical activities that you did for at least 10 minutes at a time.

3. During the **last 7 days**, on how many days did you do **moderate** physical activities like carrying light loads, bicycling at a regular pace, or doubles tennis? Do not include walking.

_____ Days per week

☐ No moderate physical activities → Skip to question 5

4. How much time did you usually spend doing **moderate** physical activities on one of those days?

_____ Hours per day

_____ Minutes per day

☐ Don't know/Not sure

Think about the time you spent walking in the last 7 days. This includes at work and at home, walking to travel from place to place, and any other walking that you might do solely for recreation, sport, exercise, or leisure.

5. During the last 7 days, on how many days did you walk for at least 10 minutes at a time?

_____ Days per week

☐ No walking → Skip to question 7

6. How much time did you usually spend **walking** on one of those days?

_____ Hours per day

_____ Minutes per day

☐ Don't know/Not sure

The last question is about the time you spent **sitting** on weekdays during the **last 7 days**. Include time spent at work, at home, while doing course work and during leisure time. This may include time spent sitting at a desk, visiting friends, reading, or sitting or lying down to watch television.

7. During the **last 7 days**, how much time did you spend **sitting** on a **week day**?

_____ Hours per day

_____ Minutes per day

☐ Don't know/Not sure

This is the end of the questionnaire. Thank you for participating.

Participant checklist and short answers

Please read the following statements and circle true of false

I am fasting, I have not had anything to eat or drink this morning except for plain water (this means no tea or coffee)

TRUE FALSE

I am not sick (I do not have a cold/flu and I have not had a recent allergic reaction)

TRUE FALSE

I have not been taking any vitamin D supplements or cod liver oil supplements within the last 3 months

TRUE FALSE

The supplements and medications I currently take are

_____

_____

_____

_____

_____

_____

_____

_____

Thinking back over the last 6 months, please write down when you holidayed outside of Perth, the city/country visited and length of time you were away for below. If this question is not applicable, please write NA below.

Please write below if there are any planned holidays outside of Perth within the next 6 months, the city/country to be visited and length of time you will be away. If this question is not applicable, please write NA below.

Last night I ate the following (please write as much detail as you can of your dinner meal and any food/drink consumed after dinner)