PROTOCOL

AGE-RELATED ABILITY TO SYNTHESISE VITAMIN D IN THE SKIN ON EXPOSURE TO SUNLIGHT

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1. BACKGROUND

Sunlight exposure of skin is the major source of vitamin D, which is essential for musculoskeletal health. Vitamin D status may be compromised in older adults through assumed reduced capacity of skin to produce vitamin D. The levels of precursor 7-Dehydrocholesterol (7DHC) in skin are understood to decline with age (MacLaughlin and Holick, 1985), but this older work has been queried through its design limitations, and it is not clear whether vitamin D synthesis in the elderly is limited through amount of 7DHC available, or UV limited through less sun exposure as opposed to younger adults.

Importance of vitamin D status in healthy ageing

Vitamin D is critical for calcium homeostasis and healthy bones through the lifespan, particularly in the older-aged, where osteoporosis, osteomalacia, muscle weakness and bone fractures have major negative health impact (Lips, 2001). Low vitamin D status, i.e. low circulating 25-hydroxyvitamin D (25(OH)D), causes the deficiency disorder osteomalacia and is associated with low bone mineral density (Lips, 2001; Bischoff-Ferrari *et al.*, 2004; Gerdhem *et al.*, 2005; Vanderschueren *et al.*, 2013), falls (Bischoff-Ferrari *et al.*, 2009), and poor physical performance ≥65y (Houston *et al.*, 2007; Hill *et al.*, 2013). Impact of vitamin D supplements is varied, with compliance a limitation of some studies (Hill *et al.*, 2013). Low vitamin D status is linked with increased markers of bone turnover (Gerdhem *et al.*, 2005), implicated in enhanced bone loss and fracture risk (Bischoff-Ferrari *et al.*, 2009). Effect of season on such markers is inconsistent (Wheater *et al.*, 2013), with no impact in Aberdeen post-menopausal women (Mavroeidi *et al.*, 2013). In addition to its role in bone health, there is a large volume of evidence associating vitamin D with protective effects against immune-based conditions and malignancies, notably colon cancer (IARC, 2008). All-cause mortality increases with lower 25(OH)D levels in ≥65y free-living adults (IARC, 2008; Pilz *et al.*, 2009; Gröber *et al.*, 2015; Sun *et al.*, 2017), as does cancer mortality in older males (Michaëlsson *et al.*, 2010), while reverse causality may operate. Low vitamin D status may be under-recognised in the aged (Kuchuk *et al.*, 2009). Due to concerns about the potential adverse health effects of low vitamin D status, the Department of Health (DH) recommends all ≥65y take 400 IU vitamin D daily (Department of Health, 1991, 1998; SACN, 2016), while Institute of Medicine guidance for USA/Canada (Institute of Medicine, 2011) advises a higher dose of 800 IU/day >70y. Only a minority of older people, however, take supplements and a significant proportion remain at risk.

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UK demographic changes and the knowledge gap regarding vitamin D in ≥65 year-olds

There was a 10.6% increase in those aged ≥65y at the 2011 Census versus 2001, this group now representing 16% (10.4M) of the UK population (Office for National Statistics, 2011). Changing age-profile, leisure activity and longer retirement period require adjustment of our concept of older adults and their health issues. There is current public health concern regarding low vitamin D levels in the UK, and it is imperative to address the knowledge gap regarding vitamin D acquisition through sunlight in those aged ≥65y, particularly with report that older adults have reduced skin capacity to produce vitamin D (MacLaughlin and Holick, 1985). Understanding of vitamin D status in the UK's older people has been based particularly on the institutionalised (Corless *et al.*, 1975; Lester *et al.*, 1977) and cross-sectional data including the NDNS and HSE surveys (Smithers *et al.*, 1998; Hirani and Primatesta, 2005). More recently, longitudinal vitamin D status was reported for 55-70y women in Aberdeen and Surrey, highlighting seasonal and latitudinal differences (MacDonald *et al.*, 2011; Mavroeidi *et al.*, 2013). We have determined relationships between vitamin D status and ambient and simulated UK sunlight exposure in a wide range of population groups at mid-UK latitude, including younger adults (Stafford *et al.*, 2010; Webb *et al.*, 2010; Farrar *et al.*, 2013; Kift *et al.*, 2013; Rhodes *et al.*, 2014; Gould *et al.*, 2015) and have the expertise to model our data according to climatic conditions across the UK (Kazantzidis *et al.*, 2015).Our data are crucially informing current reviews of vitamin D by Public Health England's Advisory Group on Non-Ionising Radiation (AGNIR) and the Scientific Advisory Committee on Nutrition (SACN). Such data are urgently required for a key at-risk population, the present-day ≥65y, to provide underpinning evidence for their appropriate public health guidance.

What influences vitamin D status in the UK young and aged population?

Vitamin D status is influenced by extrinsic and intrinsic factors and modified by lifestyle. The major source of vitamin D is cutaneous synthesis following exposure to ultraviolet (UV)B in sunlight, with usually only low amounts provided by diet (Ashwell *et al.*, 2010).Thus, minimal ambient UVB in sunlight across the UK's winter months causes a seasonal fall in status.

In older people the risk of vitamin D deficiency may be augmented through reduced capacity of skin to make vitamin D (National Radiological Protection Board, 2002),potentially aggravated by less sunlight exposure. Low dietary vitamin D or impaired conversion to the active hormone may contribute. Older studies suggested the UK aged

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population had lower 25OHD levels than younger adults (Corless *et al.*, 1975; Lester *et al.*, 1977) with less seasonal variation (Lester *et al.*, 1977), while national cross-sectional surveys indicate a dichotomy, with lower levels in institutionalised than free-living (Smithers *et al.*, 1998; Hirani and Primatesta, 2005) Information is required from longitudinal studies regarding seasonal 25OHD change in ambulant older people in relation to contributors, particularly their sunlight exposure levels. Levels of 25(OH)D are assessed against risk of vitamin D deficiency (25OHD <10ng/ml or 25nmol/l; where the bone deficiency disease osteomalacia occurs); several authorities including Institute of Medicine for USA/Canada also propose a level for insufficiency (25OHD <20ng/ml or 50nmol/l; associated with risk of poor musculoskeletal health) (Department of Health, 1991; German Nutrition Society, 2012; NORDEN, 2013). In a Norwegian study where >50y was regarded as the older group, higher 25OHD levels were found than in those <50y, with less seasonal variation (Moan *et al.*, 2009), but few recent studies worldwide report on comparative research with the post-retirement age group (Arabi *et al.*, 2010; Lippi *et al.*, 2012). Our modelling work and ambient UVR measurements show Manchester is representative of mid-UK for UVB availability, and using our simulated and natural sunlight exposure data, the collated UVR-25OHD responses of other population sectors in Manchester are extrapolated to the wider UK. However, the relative efficiency of skin synthesis of vitamin D of ambulant older adults following sunlight exposure, and how this relates to their actual vitamin D status, is still unknown.

Sunlight exposure, vitamin D synthesis and skin ageing

Cutaneous synthesis of vitamin D is tightly regulated; unlike the oral route, vitamin D toxicity including hypercalcaemia is not seen. The skin uniquely synthesises vitamin D from 7-dehydrocholesterol (7-DHC), which shows highest concentration in the lower epidermis, and is converted to pre-vitamin D_3 on UVB exposure (MacLaughlin *et al.*, 1982). Pre-vitamin D_3 undergoes slower thermochemical isomerisation to vitamin D_3, which then classically undergoes hepatic hydroxylation to 25OHD (the major circulating form and accepted best indicator of vitamin D status) followed by renal hydroxylation to 1,25$(OH)_2$D (active hormonal form). *Ex vivo* studies indicated only 10-20% of 7-DHC can be converted to pre-vitamin D_3 during one sunlight exposure, continued exposure converting photo-labile pre-vitamin D_3 to inert isomers (Webb *et al.*, 1988), and degrading formed vitamin D_3 (Webb *et al.*, 1989). Some of these reactions are attributable to UVA rather than UVB, and limit amount of vitamin D formed with one sun-exposure (Webb *et al.*, 1989). Hence it is important in human experimental research to use UVR with a

spectrum simulating sunlight's UVA and UVB content at ground-level, as in our studies (Rhodes *et al.*, 2010; Farrar *et al.*, 2011, 2013). Skin undergoes intrinsic and extrinsic ageing, the latter occurring at exposed sites and largely attributable to UVR (photoageing). Both may reduce vitamin D synthesis, as skin shows an inverse correlation for 7-DHC content with age (MacLaughlin and Holick, 1985) while in repeatedly photoexposed skin, thickening of upper epidermis may impact on UVR transmission to lower levels. Vitamin D synthesis can be initiated by artificial UVR in the older-aged (Corless *et al.*, 1978), but impact of skin ageing on its level of production is poorly understood.

Sunlight exposure recommendations and vitamin D

National sunlight exposure guidance advises the public, particularly those with light skin, to minimise summer sunlight exposure to protect against skin cancer, the majority being attributable to UVR exposure (IARC, 2008). Indeed, over-exposure to UVR is also unhelpful for vitamin D synthesis in view of pre-vitamin D_3 isomerisation and vitamin D_3 degradation. Recently, the robust datasets of our intervention (simulated sunlight) and seasonal (sunlight) studies enhanced understanding of the relationships between UK sunlight exposure level and vitamin D outcome in younger light-skin adults (Rhodes *et al.*, 2010; Webb *et al.*, 2011); and provided evidence for national guidance advising ~15 minutes sun exposures several times per week (National Radiological Protection Board, 2002; Cancer Research UK, 2017).Going outdoors is acknowledged to give additional social and physical (exercise) benefits, and possibly further health benefits of sunlight exposure (Wright and Weller, 2015). Public Health England's Advisory Group on Non-Ionising Radiation has updated its scientific report on sunlight exposure/vitamin D relationship (PHE, 2017).However, the impact of ageing is still unclear, and there is a need to examine UVR exposure-vitamin D outcomes in younger and older adults under dosimetry-controlled conditions utilising safe, low dose solar simulating UVR and known skin exposure, and to relate this to sunlight exposure occurring naturally in daily life where behavioural factors are at play.

Research Question:

Is skin 7-DHC level lower in skin of older vs younger people when assessed under carefully controlled protocols, and is skin ability to synthesise Vitamin D following ultraviolet radiation exposure under conditions similar to natural sunlight different in young and aged adults?

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2. STUDY OBJECTIVES

Overall Aim: Evaluate 7-DHC levels and vitamin D production in young vs aged adults in an intervention study of cutaneous exposure to known amounts of low-level UVR simulating national recommendations on UK summer sunlight exposure, and compare those two data sets, through skin and blood assays.

Detailed Aims:

1. Measure concentration of 7-DHC (and pre-vitamin D/vitamin D) in the skin of aged vs young adults
2. Examine the loss of 7-DHC (and gain of pre-vitamin D/vitamin D) in the skin following UV irradiation (photoconversion and heat-induced isomerisation)
3. Measure concentration of vitamin D related molecules in blood of aged vs young adults pre- and post- UV irradiation
4. Compare the dietary vitamin D intake of the two groups
5. Compare the lifestyle questionnaires of the two groups

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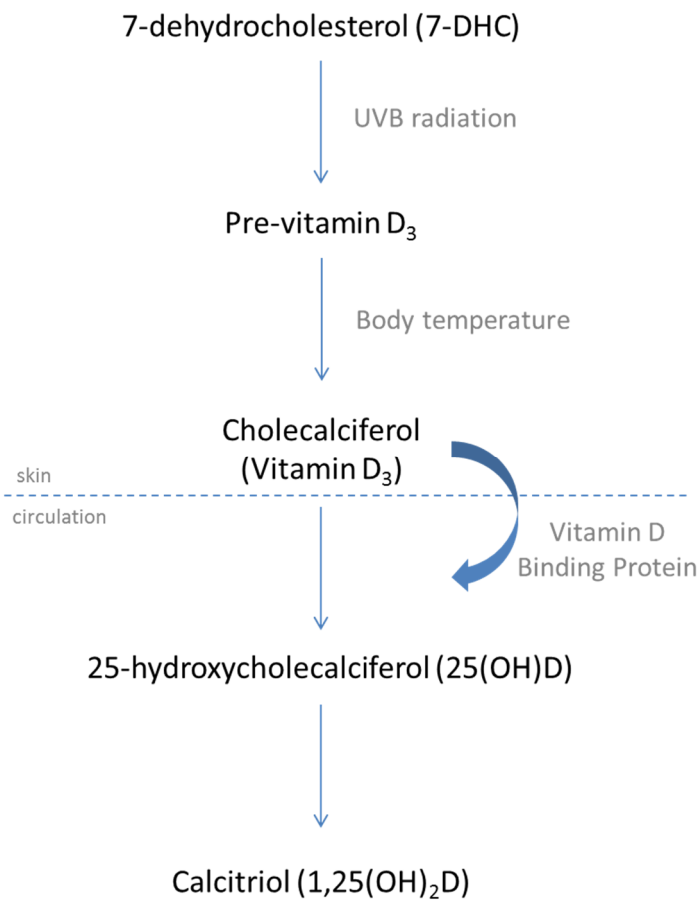

Figure 1. The Vitamin D_3 production pathway in human skin. 7-DHC undergoes photoconversion to pre-vitamin D3. Pre-vitamin D3 undergoes heat-induced isomerisation to form Vitamin D_3 which then binds to Vitamin D binding Protein and enters circulation. Firstly, in liver, it is hydroxylated to 25(OH)D, then in kidney it is converted to form biologically active Calcitriol.

3. STUDY DESIGN

To address the objectives we propose a trial of low-level UVR exposures in a comparative study of human volunteers in 2 age groups, young (18-40 years old) and aged adults (65-89 years old).
Volunteers will be matched for other attributes including skin type, and UV dose administered, biopsy and blood sampling procedures will be identical.

Exposures will be equivalent to 15 mins June noon-time sun-exposure in Manchester (53.5°N). Skin area exposure will reflect casual summer clothing style with lower legs,

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arms, hands, neck and face exposed (~35% surface area). This work will be done during November-February (when there is negligible ambient UVB and subjects are at their trough 25(OH)D level).

Blood samples pre-, 24 hours post-irradiation and 1 week after will allow analysis for vitamin D and 25(OH)D as used in previously published studies carried out in Salford Royal Foundation Trust's Photobiology Unit.
Skin biopsies from previously protected skin sites of the upper buttock, will be taken before and immediately after pre-determined doses of simulated summer sunlight, and at 24 hours post-irradiation, for measurement of 7-DHC, pre-vitamin D and vitamin D.

Biopsy samples will be analysed by extraction, chromatographic separation, and quantification by Mass Spectrometry (Tandem MS) for epidermal and dermal content of 7DHC and (after irradiation) pre-vitamin D and related molecules; similar techniques will provide for 25(OH)D analysis.

Volunteers will also complete diet diaries and lifestyle exposure questionnaires, as used in previous work at Salford Royal Foundation Trust's Photobiology Unit, to assess their background sources of vitamin D.

Outcomes can be set in context against data from existing studies of large numbers of young and elderly adults showing annual cycles in vitamin D status. Outcomes will address a significant knowledge gap - whether ability to synthesise Vitamin D in human skin changes with age.

4. PARTICIPANTS AND RECRUITMENT

We will recruit up to 12 (in each age group) of white Caucasian (skin type I-III) 18-40 year olds and 65-89 year olds using current Salford Royal Foundation Trust's Photobiology Unit contacts.
Potential subjects will be invited to participate and sent the Participant Information Sheet if they request more information. They will then be invited for informed consent and baseline assessments with a research team member.

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Potential participants will also be identified and approached through departmental participant databases and via advertisements placed in Salford Royal NHS Foundation Trust and other Trusts of the Manchester Academic Health Science Centre, The University of Manchester, local media (e.g. newspapers, radio), community locations (e.g. libraries, shops), and online (University and Trust websites, Citizen Scientist including social media sites e.g. Twitter, Facebook).

The text used to advertise on Social Media (some of which impose word count limitations) will be as follows: “Are you healthy and aged 18-40 or 65-89? You are invited to take part in a Vitamin D study. More info: <link to advert>”

Volunteers will be invited to contact the Photobiology Unit, Salford Royal NHS Foundation Trust to obtain more details of the study prior to recruitment and will only be enrolled onto the study following their written informed consent.

5. INCLUSION / EXCLUSION CRITERIA

Inclusion	Exclusion
Healthy, ambulant human volunteers Male and female Aged 18-40 or 65-89 years White Caucasian (sun-reactive skin types I-III)	History of photosensitivity disorder History of skin cancer Taking photoactive or bone active therapies Sunbathing/sunny holiday/sunbed use in past 3 months Taking vitamin D doses >200 IU (5 µg) Taking anticoagulation medicines including Aspirin, Clopidogrel and Warfarin or Propranolol Unable to comply with the requirements of the study

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6. STUDY PROCEDURES

Baseline assessments

Volunteers' height and weight will be assessed.

Controlled UV exposures

A whole body irradiation cabinet will be used to expose volunteers to low, sub-sunburn threshold doses of solar simulated ultraviolet radiation, specifically 1.3 SED (equivalent to 1.1 SED in sunlight) per dose. Exposure will be identical to those in our previous studies. Lamps emitting low dose solar UVR as close as possible to summer sunlight (5% UVB, 95% UVA) will be used which produce a rise in serum 25(OH)D levels at doses insufficient to cause skin redness. Subjects will wear standardised clothing (shorts and T-shirt) to expose hands, forearms, face, and lower legs. There will be a 10 cm x 10 cm cut out area in the shorts to expose an upper buttock area from where the skin biopsies will be taken.

Blood sampling and analysis

Blood samples will be taken by our research team experienced and competent in phlebotomy. Each participant will provide blood samples on 3 occasions during the study i.e. during 1st visit (baseline assessments), 24h post irradiation and 1 week after the exposure.

Peripheral blood (approximately 20ml) will be collected and separated by centrifugation and serum samples will be kept.

Serum samples will be stored at -80^{o}C prior to analysis. Samples will be analysed locally at Salford Royal Foundation Trust for routine biochemistry including renal and hepatic function, and parathyroid hormone (PTH).

Serum analysis of 25(OH)D and other circulating Vitamin D molecules will be performed by LC-MS/MS in the Runcorn facility of the University of East Anglia (UEA) with supervision of Professor William Fraser and John Dutton (senior research fellow). However, they will not have access to personal data, only anonymised samples will be transported at UEA facilities for analysis.

Skin biopsies

2 skin biopsies will be taken on three occasions pre-, immediately post- and 24h post radiation (6 biopsies overall). The biopsies will be circular (punch biopsies) with a diameter of 5 mm. Biopsies will be taken from the upper buttock area. The procedures will be done with accordance to Salford Royal NHS Foundation Trust policies.

Skin colour assessment

Skin colour will be assessed by spectrophotometry using a hand-held spectrophotometer (Konica Minolta CM600d) on exposed and unexposed sites including upper buttock area.

Dietary vitamin D intake

Volunteers will keep a dietary log of daily consumption of vitamin D containing foods for one week, as described (see Diet Log) (Rhodes *et al.*, 2010, 2014; Webb *et al.*, 2010; Farrar *et al.*, 2011, 2013; Kift *et al.*, 2013).

Lifestyle questionnaire

This questionnaire will give an overview of holidays, activities and the time spent outdoors by volunteers throughout the year. It gives a picture if a person's lifestyle as related to sun-exposure.

Sample size and data analysis

Up to 12 subjects will be recruited for the assessment in each age group.

The data gathered will be then analysed using suitable parametric test.

There are very limited data on 7DHC levels in human skin. Based on data from Moody *et al.*, 1990 (mean 7DHC=44.4 ug/g dry weight; SD=14.8, n=15) and MacLaughlin and Holick, 1985 we calculated that a sample size of n=10 is sufficient to detect an approximate 2 fold difference in 7DHC content between old and young subjects (80% power, alpha=0.05).

We will recruit n=12 to allow for drop out.

7. PATIENT AND PUBLIC INVOLVEMENT AND ENGAGEMENT (PPIE)

2 people in each of the two age groups have been involved into derivation of this protocol, and approved the questionnaires and diaries.

8. DATA MONITORING AND QUALITY ASSURANCE

The study will be subject to the audit and monitoring regime of The University of Manchester and Salford Royal NHS Foundation Trust.

9. DATA MANAGEMENT PLAN

All electronic data will be stored and backed up daily on secure University of Manchester servers. Hardcopy documents will be stored securely by the Principal Investigator (Prof Lesley Rhodes) who will be the data custodian. Personal data will be stored for 5 years after the last publication of the study or for 10 years, whichever is the greater, and will only be accessed by the research team and members of the University of Manchester, Trust and regulatory authorities for auditing and monitoring purposes. Consent forms will be retained for 5 years following the end of study. The project will use the University Research Data Management Service (RDMS) which will allow researchers to store, manage and curate their data, as well as preserve data after project completion. The RDMS will also cater for the publishing and sharing of research data.

10. ETHICAL CONSIDERATIONS

University of Manchester Research Ethics Committee (REC) and HRA approvals will be obtained before commencing this research. The study will be conducted according to the principles of the Declaration of Helsinki and Good Clinical Practice, and within the laws and regulations of the country in which it is conducted.

11. STATEMENT OF INDEMNITY

The University has insurance available in respect of research involving human subjects that provides cover for legal liabilities arising from its actions or those of its staff or supervised students. The University also has insurance available that provides compensation for non-negligent harm to research subjects occasioned in circumstances that are under the control of the University.

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