Original Paper

Vitamin D Receptor Genotype Modulates the Correlation between Vitamin D and Circulating Levels of let-7a/b and Vitamin D Intake in an Elderly Cohort

Emma Louise Beckett^{a, d} Charlotte Martin^a Konsta Duesing^d
Patrice Jones^a John Furst^b Zoe Yates^c Martin Veysey^e Mark Luccock^a

^aSchool of Environmental and Life Sciences, ^bSchool of Mathematical and Physical Sciences, and ^cSchool of Biomedical Sciences and Pharmacy, University of Newcastle, Ourimbah, N.S.W.,

^dFood and Nutrition Flagship, CSIRO, North Ryde, N.S.W., and ^eTeaching and Research Unit, Central Coast Local Health District, Gosford, N.S.W., Australia

Key Words

let-7 microRNA · Vitamin D · Vitamin D receptor · *BsmI* restriction site · *ApaI* restriction site · Nutrigenomics

Abstract

Background and Aims: Circulating microRNAs (miRNAs) are linked to disease and are potential biomarkers. Vitamin D may modulate miRNA profiles, and vitamin D status has been linked to risk of disease, including cardiovascular disease and cancers. We hypothesise that genotypic variance influences these relationships. We examined the correlations between vitamin D intake and circulating levels of the miRNAs let-7a/b, and the involvement of two common vitamin D receptor (*VDR*) polymorphisms, *BsmI* and *ApaI*. **Methods:** Two hundred participants completed food frequency and supplement questionnaires, and were assayed for circulating let-7b expression by qPCR. Polymorphisms were detected using restriction fragment length polymorphism-PCR. **Results:** let-7b expression negatively correlated with vitamin D intake ($r_s = -0.20$, $p = 0.005$). The magnitude and direction of correlation were maintained in the presence of the *BsmI* restriction site ($r_s = -0.27$, $p = 0.0005$). However, in the absence of *BsmI* restriction site, the direction of the correlation was reversed ($r_s = +0.319$, $p = 0.0497$). These correlations were significantly different (z -score = 2.64, $p = 0.0085$). The correlation between

This paper was presented at the 8th Congress of the International Society of Nutrigenetics/Nutrigenomics (ISNN), Gold Coast, Qld., Australia, May 2–3, 2014.

Emma Louise Beckett
School of Environmental and Life Sciences
University of Newcastle
Brush Rd, Ourimbah, NSW 2258 (Australia)
E-Mail emma.beckett@uon.edu.au

vitamin D intake and let-7a was only significant in those without the *ApaI* restriction site. **Conclusions:** The correlation between vitamin D intake and let-7a/b expression in this cohort varies with *VDR* genotype. This study highlights the importance of considering underlying genotypic variance in miRNA expression studies and in nutritional epigenetics generally.

© 2015 S. Karger AG, Basel

Introduction

MicroRNAs (miRNAs) are a class of short non-coding RNAs that can regulate gene expression at the post-transcriptional level, normally by blocking mRNA translation or triggering degradation [1–5]. miRNAs are estimated to participate in nearly all cellular processes [6]. To date, over 2,500 mature miRNAs have been identified (miRBase, <http://www.mirbase.org>, release June 21, 2014). Disease-specific miRNA profiles have been identified, including for those diseases with known dietary risk factors [7–9]. Evidence is mounting that miRNA profiles can be modulated by nutrients and bioactive compounds [10]. Traditionally, the focus has been on tissue expression of miRNAs, but circulating miRNAs have recently been identified in serum and plasma, which have the advantage of being easily accessible for use as biomarkers. These circulating miRNAs are now being linked to particular diseases [11].

let-7a and let-7b are well-characterised members of the let-7 family of tumour suppressor miRNAs. They are highly expressed in the cardiovascular system [12] and are robustly detected in plasma samples [13–15]. Altered expression of let-7 has been implicated in a number of later-life diseases; circulating let-7b has been suggested as a potential biomarker for cardiovascular diseases, such as atherosclerosis and myocardial infarction [12], and the let-7 family may be involved in regulation of glucose homeostasis in diabetes [16]. Furthermore, expression of let-7a/b is often reduced in malignant tissue, relative to healthy tissue [17], and in some cases these altered profiles are reflected in blood circulation [14, 18]. For circulating miRNAs to be valuable as biomarkers for disease, it is important to understand how other environmental factors influence their expression levels.

Vitamin D is well known for the influence it has on gene expression. The active vitamin D metabolite 1,25-dihydroxyvitamin D₃ (calcitriol) directly regulates gene transcription via the vitamin D receptor (VDR), which acts as a nuclear receptor transcription factor [19]. The potential for vitamin D to modulate gene expression via additional indirect pathways is now being investigated. Vitamin D is synthesised in the skin in response to sunlight, consumed in the diet as cholecalciferol (vitamin D₃) or ergocalciferol (vitamin D₂), or taken as a supplement. Although current recommended intakes are based only on its role in bone health [20], epidemiological evidence is now mounting for a potential role for suboptimal vitamin D levels in multiple conditions, including diabetes [21], cardiovascular disease [22], autoimmune disease [23] and cancers [24]; however, results to date remain inconsistent [20]. Vitamin D supplementation is now being investigated in the treatment and prevention of a number of these conditions [25–27], in particular its potential as a cancer chemopreventative, due to the identified role of the VDR in cell cycle regulation and differentiation [28].

Multiple in vitro models using malignant cell lines have demonstrated the anti-proliferative effect of vitamin D [29–32], which correlates with the altered expression of a number of miRNAs, including the let-7 family [33–36], suggesting that aberrant expression of these miRNAs may contribute to the malignant phenotype and that this can be moderated by vitamin D treatment [37]. In a model of serum starvation using a breast epithelial cell line (MCF12F), calcitriol treatment had a protective effect. Serum starvation led to a significant increase in the expression of multiple miRNAs, including the let-7 family; however, this was reversed in the presence of calcitriol, suggesting miRNA expression may be a mechanism

protecting against cellular stress via a vitamin D-related process [38], which may have implications in multiple disease states including diabetes, cardiovascular disease, and cancers [39].

Limited studies investigating the correlation between serum levels of vitamin D and miRNA expression in humans have been conducted. In a study of 13 pregnant women, categorised by low (≤ 25.5 ng/ml) or high (≥ 31.7 ng/ml) plasma calcitriol level, 11 miRNAs were differentially regulated [40]. In a small study, 2 groups of 5 subjects were given high doses of vitamin D for 12 months, and their miRNA levels were assessed relative to baseline. Several miRNAs were differentially expressed after 12 months, including let-7a [41], an established target of VDR, with a vitamin D response element (VDRE) in the regulatory region of the gene controlling its expression [42].

To further investigate the role of vitamin D in modulating levels of circulating miRNAs, we studied the relationship between vitamin D intake and the expression of circulating let-7a/b, 2 well-characterised tumour-suppressor miRNAs, in a large human cohort. To further investigate the role of the VDR in this relationship, two common polymorphisms in the VDR gene, *BsmI* (rs1544410) and *Apal* (rs7975232), were assayed in participants.

Methods

Subjects and Sample Collection

This study consisted of 200 participants, aged 65 years or more, living on the Central Coast of New South Wales, Australia, who completed a food frequency and supplement questionnaire, and donated blood for analysis. Blood was collected in heparin-lined tubes (Greiner Bio-one, Frickenhausen, Germany), and plasma was isolated by centrifugation. Informed consent was obtained prior to participation under University of Newcastle Human Research Ethics Committee approval number H-2008-0431.

Food Frequency and Supplement Questionnaires

Daily intake of vitamin D was estimated using a validated food frequency questionnaire, covering 225 food items and all food groups. Subjects also provided a list of all supplements and their frequency of intake. The food frequency questionnaires were analysed using Foodworks™ (version 2.10.146; Xyris Software, Brisbane, Qld., Australia) [43, 44]. The correlation between estimated intake and plasma concentrations of 25(OH) vitamin D has been validated in a subset ($n = 80$; adjusted $r^2 = 0.46$, $p < 0.001$) of this cohort (see online suppl. methods and fig. S1; see www.karger.com/doi/10.1159/000381676 for all online suppl. material).

Genotyping

Genomic DNA was isolated from whole blood and amplified using PCR. Restriction fragment length polymorphism assays and gel electrophoresis were used to genotype two diallelic polymorphisms of the VDR gene, *BsmI* G/A (rs1544410) and *Apal* A/C (rs7975232) [43, 45], both located on the last intron [46]. The presence of restriction sites for the *BsmI* and *Apal* enzymes were coded as 'b' and 'a' and the absence of restriction sites as 'B' and 'A', respectively (see online suppl. methods for additional details).

Circulating let-7a/b Expression by qPCR

miRNAs were isolated from plasma using TRIzol LS and GlycoBlue [47]. Three synthetic *Caenorhabditis elegans* miRNAs (cel-238, 54, 38) were added to plasma samples as exogenous spike in controls to normalise data as no appropriate endogenous housekeeping gene exists in plasma [48]. cDNA was synthesised using universal primers, and qPCR primer design was conducted as per the rules described in Balcells et al. [49].

Statistics

Statistical analyses were performed using JMP (version 11; SAS Institute Inc., Cary, N.C., USA). Correlations between continuous variables were assessed using Spearman's correlation coefficient due to the non-normal distribution of vitamin D intake in this cohort; z-scores were used to assess significant differences

Table 1. Allelic and genotypic frequencies of *VDR* SNPs *BsmI* (rs1544410) and *ApaI* (rs7975232)

rs1544410	
B allele	38.7%
b allele	61.2%
BB genotype	12.0%
Bb genotype	53.5%
bb genotype	34.5%
rs7975232	
A allele	51.0%
a allele	49.0%
AA genotype	27.0%
Aa genotype	48.0%
aa genotype	25.0%

Upper case signifies restriction site absent, lower case signifies restriction site present.

between correlations (<http://vassarstats.net/rdiff.html>). Analyses were performed correcting for age and sex; however, this had no notable significant impact on results.

Categorical data were assessed using the Mann-Whitney test when comparing two categories, and the Kruskal-Wallis test was used when comparing multiple categories. Differences between groups were considered to be statistically significant at $p \leq 0.05$.

Results

Participant Characteristics and Vitamin D Intake

The mean age of participants was 75.0 ± 0.5 years (range: 65–94 years), and 57.5% were female. The median daily reported vitamin D intake was $6.8 \mu\text{g}/\text{day}$ (range: 0–65.6 $\mu\text{g}/\text{day}$). Fifty-seven (28.5%) participants reported taking a supplement containing vitamin D. The recommended adequate daily intake for vitamin D in Australia is $10 \mu\text{g}/\text{day}$ for 51- to 70-year-olds and $15 \mu\text{g}/\text{day}$ for those aged over 70. The recommended upper limit of consumption is $80 \mu\text{g}/\text{day}$ [50]. Based on these criteria, 177 participants had inadequate intake for their age group, and none had excess intake above the upper limit.

VDR Genetic Variants

The allelic and genotypic frequencies for both polymorphisms tested are given in table 1. The genotype frequency did not deviate from Hardy-Weinberg equilibrium expectations for either the *BsmI* or the *ApaI* polymorphism ($p = 0.19$ and 0.85 , respectively). These two polymorphisms exhibit strong linkage disequilibrium [51]. In this population, 91.7% of those with the BB genotype (*BsmI* restriction site absent) were also AA (*ApaI* restriction site absent); however, the AA genotype was more than twice as frequent as the BB genotype.

Association between Vitamin D Intake and let-7a/b Expression

A weak but significant negative association was found between let-7b expression and vitamin D intake (table 2; $r_s = -0.20$, $p = 0.005$). The same trend was seen for let-7a, but this did not reach statistical significance (table 2; $r_s = -0.14$, $p = 0.057$). Adjustment for age and sex did not significantly alter these relationships.

Categorical analysis was conducted on the data, based on adequate daily intake (greater than or adequate daily intake vs. less than adequate daily intake), as these are clinically relevant categories. There was a significant difference between the mean let-7b expression

Fig. 1. let-7b (a) and let-7a (b) expression in those who reported consuming adequate versus inadequate levels of vitamin D (n = 200). Data are presented as means and standard error of means. ns = Not significant. * p < 0.05.

Table 2. Correlation between vitamin D intake and let-7a/b expression, stratified by absence/presence of *BsmI* (rs1544410) and *ApaI* (rs7975232) restriction polymorphism

	Total (n = 200)	<i>BsmI</i>		z-score	<i>ApaI</i>		z-score
		restriction site present (n = 176)	restriction site absent (n = 24)		restriction site present (n = 146)	restriction site absent (n = 54)	
let-7b	-0.20 (0.005)	-0.27 (0.0005)	0.319 (0.049)	2.64 (0.0085)	-0.2899 (0.0006)	0.0614 (0.6621)	2.21 (0.027)
let-7a	-0.1367 (0.0566)	-0.0655 (0.3958)	0.0254 (0.9106)	1.14 (0.12)	-0.0209 (0.086)	0.3132 (0.0224)	2.12 (0.034)

Figures indicate r_s (p) values. Italic type highlights those relationships with a p value < 0.05.

levels between those who reported adequate vitamin D intake or above, and those with below adequate intake (fig. 1a; relative expression = -0.49 vs. 0.14, p = 0.013). Results for let-7a were not significant (fig. 1b; relative expression = -0.19 vs. 0.10, p = 0.1132). Comparison of let-7a/b levels between those who reported taking a supplement containing vitamin D and those who relied on diet alone for vitamin D intake found lower levels of relative expression of both let-7b (-0.49 vs. 0.14, p = 0.0014) and let-7a (-0.09 vs. 0.15, p = 0.039) in those consuming supplements.

Association by Genotype

To determine the effect of *BsmI* and *ApaI* genotype on the relationship between let7a/b expression and vitamin D intake, analyses were repeated, stratified by genotype. Genotypes were stratified as 'restriction site absent' (AA, BB genotypes) or 'restriction site present' (Aa/aa, Bb/bb genotypes). This revealed that the magnitude and direction of correlation between let-7b relative expression and vitamin D intake was maintained in carrying an allele with a restriction site (table 2; n = 176, r_s = -0.27, p = 0.0005). However, the direction of the correlation was reversed (n = 24, r_s = +0.319, p = 0.0497) in those where the restriction site was absent. These correlations were significantly different (z-score = 2.5, p = 0.0124). Similar results for let-7b were obtained for the *ApaI* variant (table 2). The only significant correlation observed between let-7a and vitamin D intake was in the subpopulation without *ApaI* restriction site (AA; table 2). Neither vitamin D intake nor miRNA expression levels alone differed significantly between genotype groups.

Fig. 2. let-7b expression in those who reported consuming adequate versus inadequate levels of vitamin D, stratified by presence/absence of *BsmI* (a) or *ApaI* (b) restriction site polymorphisms. *BsmI*[−] (restriction site absent, BB), n = 24; *BsmI*⁺ (restriction site present, Bb/bb), n = 176; *ApaI*[−] (restriction site absent, AA), n = 54; *ApaI*⁺ (restriction site present, Aa/aa), n = 146. Data are presented as means and standard error of means. ns = Not significant. * p < 0.05, ** p < 0.01, *** p < 0.001.

Fig. 3. let-7a expression in those who reported consuming adequate versus inadequate levels of vitamin D, stratified by the absence or presence of *BsmI* (a) or *ApaI* (b) restriction site polymorphisms. *BsmI*[−] (restriction site absent, BB), n = 24; *BsmI*⁺ (restriction site present, Bb/bb), n = 176; *ApaI*[−] (restriction site absent, AA), n = 54; *ApaI*⁺ (restriction site present, Aa/aa), n = 146. Data are presented as means and standard error of means. ns = Not significant. * p < 0.05.

While no significant difference was found when let7a/b expression data were analysed by genotype alone (online suppl. fig. S3), analysis by genotype and category of intake did reveal some interesting patterns. Reflective of the continuous analysis presented in table 2 and figure 2a shows that inverse relationships of expression to intake exist between participants in which the *BsmI* restriction site polymorphism is absent and those in which it is present. A similar pattern is seen in the analysis stratified by presence or absence of the *ApaI* restriction site polymorphism; however, not all relationships were statistically significant. For let-7a, the only statistically significant relationships found were for those possessing the *BsmI* restriction site and those possessing the *ApaI* restriction site (fig. 3).

Discussion

This study is the largest study to date investigating the relationship between vitamin D intake and circulating miRNA levels. We have demonstrated firstly that there is a significant correlation between reported vitamin D intake and let-7b expression in circulation. Secondly, we have demonstrated that this relationship varies depending on the underlying *VDR* genotype. The relationship between let-7a expression and vitamin D was only significant in participants without the *ApaI* restriction site in the *VDR* gene.

While studies such as this can only demonstrate correlation, and not causation, the modulation of the relationship by genotype suggests the *VDR* may play a causative role. However,

evidence of this interaction is greater for let-7b than let-7a, despite the presence of a VDRE in the let-7a-2 gene [42]. While vitamin D is able to modulate gene expression via the interaction with VDR and the VDRE, modulation of miRNA expression may also occur via indirect mechanisms, such as vitamin D modulation of epigenetic mechanisms. Regulation of mRNA levels via miRNA signalling is now becoming recognised as a potential additional mechanism for the action of vitamin D, and consequences of these interactions are now being investigated [52–54]. These additional pathways need to be considered when investigating the potential therapeutic and preventative use of vitamin D for chronic diseases. Additionally, understanding the molecular influence of current recommended daily intakes and supplementation regimens may shed light on the proposed links between vitamin D intake and later-life diseases.

Evidence as to the links between vitamin D status and diseases such as cancer and cardiovascular disease has so far been inconsistent [20]. This study demonstrates that underlying genotype can influence molecular markers, and this may contribute to explaining some of these inconsistencies. *BsmI* and *Apal* are both intronic polymorphisms, and as such they do not influence the coding amino acids in the VDR protein. However, several studies have linked these polymorphisms to altered outcomes, including bone density, fracture risk and cancer risk [55–58]. This may be linked to these polymorphisms influencing mRNA levels or mRNA stability [59, 60]; however, results have been mixed [61, 62]. Furthermore, both *BsmI* and *Apal* are in high linkage disequilibrium with a number of other *VDR* polymorphisms, including *TaqI* in the 3'UTR and the polyA variable number tandem repeat (VNTR) [51], which may alter expression levels, stability or transcriptional activity of the *VDR* [60, 63, 64]. The VNTR has at least 12 different alleles, but essentially follows bimodal distribution, with 'b' found with long repeats (n = 18–24) and 'B' found with short repeats (n = 13–17) [51]. Resolution of haplotypes with additional polymorphisms in this area of the *VDR* gene may provide additional information on the results observed here. Strong linkage disequilibrium may exist with additional polymorphisms in the regulatory or coding regions that may explain the observed associations.

An obvious shortcoming of this work is that vitamin D status was only estimated by reported dietary and supplementary intake. However, a significant correlation between plasma 25(OH) vitamin D and reported dietary and supplementary intake has been demonstrated in a subset of this cohort (online suppl. fig. S1). Several other studies have shown reasonable correlations between reported intake using food frequency questionnaires and plasma levels [65–68]. Additionally, as this is an elderly cohort, relative contribution of endogenously synthesised and intake of exogenous vitamin D is altered compared to the general population. Cutaneous production of vitamin D reduces with age due to decreased levels of provitamin D₃ (7-dehydrocholesterol) [69] the precursor for vitamin D synthesis in the skin. Additionally, changes in lifestyle habits that reduce sun exposure occur, therefore making dietary intake increasingly important [70–72]. In this cohort, plasma 25(OH) vitamin D levels did not appear to be influenced by solar activity in the 6 weeks prior to collection (online suppl. fig. S2), suggesting intake is the major contributor to plasma 25(OH) status in this elderly cohort. Unfortunately, no information is available on skin pigmentation or estimated time spent outside for this cohort, which may explain the remaining variance between intake and blood levels of vitamin D. However, due to the health risks associated with excess sun exposure, any intervention looking to modulate vitamin levels in an 'at risk' population is likely to attempt to do so using dietary and supplementary means [71].

In conclusion, the correlation between let-7a/b expression and vitamin D intake in this cohort varies with *VDR* genotype. This is the first time such a relationship has been described between vitamin D status, *VDR* genotype and the level of circulating miRNAs. While this is yet to be demonstrated for additional miRNAs, and the mechanisms behind these correlations are yet to be elucidated, this study highlights the importance of considering underlying genotypic variance in miRNA expression studies and in nutritional epigenetics generally.

Acknowledgements

The authors would like to acknowledge Drs. Paul Roach, Katrina King, Suzanne Niblett, Peter Lewis and David Kennedy for their contribution to sample collection, database maintenance and design of the larger study. UV analyses used in this paper were produced with the Giovanni online data system, developed and maintained by the NASA GES DISC.

Disclosure Statement

The authors have no conflicts of interest to disclose.

References

- Yekta S, Shih I, Bartel D: MicroRNA-directed cleavage of HOXB8 mRNA. *Science* 2004;304:594–596.
- Davis E, Caiment F, Tordoir X, Cavaillé J, Ferguson-Smith A, Cockett N, Georges M, Charlier C: RNAi-mediated allelic trans-interaction at the imprinted Rtl1/Peg11 locus. *Curr Biol* 2005;15:743–749.
- Chendrimada T, Finn K, Ji X, Baillat D, Gregory R, Liebhaber S, Pasquinelli A, Shiekhattar R: MicroRNA silencing through RISC recruitment of eIF6. *Nature* 2007;447:823–828.
- Petersen C, Bordeleau M, Pelletier J, Sharp P: Short rNAS repress translation after initiation in mammalian cells. *Mol Cell* 2006;21:533–542.
- Wu L, Fan J, Belasco J: MicroRNAs direct rapid deadenylation of mRNA. *Proc Natl Acad Sci USA* 2006;103:4034–4039.
- Friedman R, Farh K, Burge C, Bartel D: Most mammalian mRNAs are conserved targets of microRNAs. *Genome Res* 2009;19:92–105.
- Papageorgiou N, Tousoulis D, Androulakis E, Siasos G, Briassoulis A, Vogiatzi G, Kampoli A, Tsiamis E, Tentolouris C, Stefanadis C: The role of microRNAs in cardiovascular disease. *Curr Med Chem* 2012;19:2605–2610.
- Lorenzen J, Kumarswamy R, Dangwal S, Thum T: MicroRNAs in diabetes and diabetes-associated complications. *RNA Biol* 2012;9:820–827.
- Calin G, Croce C: MicroRNA signatures in human cancers. *Nat Rev Cancer* 2006;6:857–866.
- Beckett EL, Yates Z, Veysey M, Duesing K, Lucock M: The role of vitamins and minerals in modulating the expression of microRNA. *Nutr Res Rev* 2014;27:94–106.
- Weiland M, Gao X, Zhou L, Mi Q: Small RNAs have a large impact: circulating microRNAs as biomarkers for human diseases. *RNA Biol* 2012;9:850–859.
- Bao MH, Feng X, Zhang YW, Lou XY, Cheng Y, Zhou HH: let-7 in cardiovascular diseases, heart development and cardiovascular differentiation from stem cells. *Int J Mol Sci* 2013;14:23086–23102.
- Long G, Wang F, Li H, Yin Z, Sandip C, Lou Y, Wang Y, Chen C, Wang DW: Circulating miR-30a, miR-126 and let-7b as biomarker for ischemic stroke in humans. *BMC Neurol* 2013;13:178.
- Tsujiura M, Ichikawa D, Komatsu S, Shiozaki A, Takeshita H, Kosuga T, Konishi H, Morimura R, Deguchi K, Fujiwara H, Okamoto K, Otsuji E: Circulating microRNAs in plasma of patients with gastric cancers. *Br J Cancer* 2010;102:1174–1179.
- Zuo Z, Calin GA, de Paula HM, Medeiros LJ, Fernandez MH, Shimizu M, Garcia-Manero G, Bueso-Ramos CE: Circulating microRNAs let-7a and miR-16 predict progression-free survival and overall survival in patients with myelodysplastic syndrome. *Blood* 2011;118:413–415.
- Frost RJ, Olson EN: Control of glucose homeostasis and insulin sensitivity by the let-7 family of microRNAs. *Proc Natl Acad Sci USA* 2011;108:21075–21080.
- Rutnam ZJ, Yang BB: The involvement of microRNAs in malignant transformation. *Histol Histopathol* 2012;27:1263–1270.
- Brase JC, Wuttig D, Kuner R, Sultmann H: Serum microRNAs as non-invasive biomarkers for cancer. *Mol Cancer* 2010;9:306.
- Carlberg C: Current understanding of the function of the nuclear vitamin D receptor in response to its natural and synthetic ligands. *Recent Results Cancer Res* 2003;164:29–42.
- Ross AC, Manson JE, Abrams SA, Aloia JF, Brannon PM, Clinton SK, Durazo-Arvizu RA, Gallagher JC, Gallo RL, Jones G, Kovacs CS, Mayne ST, Rosen CJ, Shapses SA: The 2011 report on dietary reference intakes for calcium and vitamin D from the Institute of Medicine: what clinicians need to know. *J Clin Endocrinol Metab* 2011;96:53–58.
- Pittas A, Lau J, Hu F, Dawson-Hughes B: The role of vitamin D and calcium in type 2 diabetes. A systematic review and meta-analysis. *J Clin Endocrinol Metab* 2007;92:2017–2029.
- Wang T, Pencina M, Booth S, Jacques P, Ingelsson E, Lanier K, Benjamin E, D'Agostino R, Wolf M, Vasan R: Vitamin D deficiency and risk of cardiovascular disease. *Circulation* 2008;117:503–511.
- Adorini L, Penna G: Control of autoimmune diseases by the vitamin D endocrine system. *Nat Clin Pract Rheumatol* 2008;4:404–412.

- 24 Lappe J, D Travers-Gustafson, Davies K, Recker R, Heaney R: Vitamin D and calcium supplementation reduces cancer risk: results of a randomized trial 1, 2. *Am J Clin Nutr* 2007;85:1586–1591.
- 25 Stein MS, Liu Y, Gray OM, Baker JE, Kolbe SC, Ditchfield MR, Egan GF, Mitchell PJ, Harrison LC, Butzkueven H, Kilpatrick TJ: A randomized trial of high-dose vitamin D₂ in relapsing-remitting multiple sclerosis. *Neurology* 2011;77:1611–1618.
- 26 Wactawski-Wende J, Kotchen JM, Anderson GL, Assaf AR, et al: Calcium plus vitamin D supplementation and the risk of colorectal cancer. *N Engl J Med* 2006;354:684–696.
- 27 Zittermann A, Frisch S, Berthold HK, Gotting C, Kuhn J, Kleesiek K, Stehle P, Koertke H, Koerfer R: Vitamin D supplementation enhances the beneficial effects of weight loss on cardiovascular disease risk markers. *Am J Clin Nutr* 2009;89:1321–1327.
- 28 Lamprecht S, Lipkin M: Chemoprevention of colon cancer by calcium, vitamin D and folate: molecular mechanisms. *Nat Rev Cancer* 2003;3:601–614.
- 29 Essa S, Reichrath S, Mählknecht U, Montenarh M, Vogt T, Reichrath J: Signature of VDR miRNAs and epigenetic modulation of vitamin D signaling in melanoma cell lines. *Anticancer Res* 2012;32:383–389.
- 30 Essa S, Denzer N, Mählknecht U, Klein R, Collnot EM, Tilgen W, Reichrath J: VDR microRNA expression and epigenetic silencing of vitamin D signaling in melanoma cells. *J Steroid Biochem Mol Biol* 2010;121:110–113.
- 31 Alvarez-Díaz S, Valle N, Ferrer-Mayorga G, Lombardía L, Herrera M, Domínguez O, Segura M, Bonilla F, Hernando E, Muñoz A: MicroRNA-22 is induced by vitamin D and contributes to its antiproliferative, antimigratory and gene regulatory effects in colon cancer cells. *Hum Mol Genet* 2012;21:2157–2165.
- 32 Mohri T, Nakajima M, Takagi S, Komagata S, Yokoi T: MicroRNA regulates human vitamin D receptor. *Int J Cancer* 2009;125:1328–1333.
- 33 Sonkoly E, Lovén J, Xu N, Meisgen F, Wei T, Brodin P, Jaks V, Kasper M, Shimokawa T, Harada M, Heilborn J, Hedblad M, Hippe A, Grandér D, Homey B, Zaphiropoulos P, Arsenian-Henriksson M, Ståhle M, Pivarcsi A: MicroRNA-203 functions as a tumor suppressor in basal cell carcinoma. *Oncogenesis* 2012;1:e3.
- 34 Giangreco A, Vaishnav A, Wagner D, Finelli A, Fleschner N, Van der Kwast T, Vieth R, Nonn L: Tumor suppressor microRNAs, miR-100 and -125b, are regulated by 1,25-dihydroxyvitamin D in primary prostate cells and in patient tissue. *Cancer Prev Res (Phila)* 2013;6:483–494.
- 35 Ting H, Messing J, Yasmin-Karim S, Lee Y: Identification of microRNA-98 as a therapeutic target inhibiting prostate cancer growth and a biomarker induced by vitamin D. *J Biol Chem* 2013;288:1–9.
- 36 Ma Y, Hu Q, Luo W, Pratt RN, Glenn ST, Liu S, Trump DL, Johnson CS: 1 α ,25(OH)₂D₃ differentially regulates miRNA expression in human bladder cancer cells. *J Steroid Biochem Mol Biol* 2015;148:166–171.
- 37 Wang X, Gocek E, Liu C, Studzinski G: MicroRNAs181 regulate the expression of p27Kip1 in human myeloid leukemia cells induced to differentiate by 1,25-dihydroxyvitamin D₃. *Cell Cycle* 2009;8:736–741.
- 38 Peng X, Vaishnav A, Murillo G, Alimirah F, Torres K, Mehta R: Protection against cellular stress by 25-hydroxyvitamin D₃ in breast epithelial cells. *J Cell Biochem* 2010;110:1324–1333.
- 39 Fulda S, Gorman A, Hori O, Samali A: Cellular stress responses: cell survival and cell death. *Int J Cell Biol* 2010;2010:214074.
- 40 Enquobahrie D, Williams M, Qiu C, Siscovick D, Sorensen T: Global maternal early pregnancy peripheral blood mRNA and miRNA expression profiles according to plasma 25-hydroxyvitamin D concentrations. *J Matern Fetal Neonatal Med* 2011;24:1002–1012.
- 41 Jorde R, Svartberg J, Joakimsen R, Coucheron D: Plasma profile of microRNA after supplementation with high doses of vitamin D₃ for 12 months. *BMC Res Notes* 2012;5:245.
- 42 Guan H, Liu C, Chen Z, Wang L, Li C, Zhao J, Yu Y, Zhang P, Chen W, Jiang A: 1,25-dihydroxyvitamin D₃ up-regulates expression of hsa-let-7a-2 through the interaction of VDR/VDRE in human lung cancer A549 cells. *Gene* 2013;522:142–146.
- 43 Luccock M, Yates Z, Martin C, Choi JH, Boyd L, Tang S, Naumovski N, Furst J, Roach P, Jablonski N, Chaplin G, Veysey M: Vitamin D, folate, and potential early lifecycle environmental origin of significant adult phenotypes. *Evol Med Public Health* 2014;2014:69–91.
- 44 Luccock M, Yates Z, Boyd L, Naylor C, Choi JH, Ng X, Skinner V, Wai R, Kho J, Tang S, Roach P, Veysey M: Vitamin C-related nutrient-nutrient and nutrient-gene interactions that modify folate status. *Eur J Nutr* 2013;52:569–582.
- 45 Zmuda JM, Cauley JA, Danielson ME, Wolf RL, Ferrell RE: Vitamin D receptor gene polymorphisms, bone turnover, and rates of bone loss in older African-American women. *J Bone Miner Res* 1997;12:1446–1452.
- 46 Falletti E, Bitetto D, Fabris C, Cussigh A, Fontanini E, Fornasiere E, Fumolo E, Bignulin S, Cmet S, Minisini R, Pirisi M, Toniutto P: Vitamin D receptor gene polymorphisms and hepatocellular carcinoma in alcoholic cirrhosis. *World J Gastroenterol* 2010;16:3016–3024.
- 47 Hastings ML, Palma J, Duelli DM: Sensitive PCR-based quantitation of cell-free circulating microRNAs. *Methods* 2012;58:144–150.
- 48 Mitchell PS, Parkin RK, Kroh EM, Fritz BR, Wyman SK, Pogosova-Agadjanyan EL, Peterson A, Noteboom J, O'Briant KC, Allen A, Lin DW, Urban N, Drescher CW, Knudsen BS, Stirewalt DL, Gentleman R, Vessella RL, Nelson PS, Martin DB, Tewari M: Circulating microRNAs as stable blood-based markers for cancer detection. *Proc Natl Acad Sci USA* 2008;105:10513–10518.
- 49 Balcells I, Cirera S, Busk PK: Specific and sensitive quantitative RT-PCR of miRNAs with DNA primers. *BMC Biotechnol* 2011;11:70.

- 50 Australian Government, National Health and Medical Research Council: Nutrient Reference Values for Australia and New Zealand Including Recommended Dietary Intakes. Canberra, Commonwealth of Australia, 2006.
- 51 Uitterlinden AG, Fang Y, Van Meurs JB, Pols HA, Van Leeuwen JP: Genetics and biology of vitamin D receptor polymorphisms. *Gene* 2004;338:143–156.
- 52 Witwer K: XenomiRs and miRNA homeostasis in health and disease. *RNA Biol* 2012;9:1147–1154.
- 53 Lundstrom K: MicroRNA and diet in disease prevention and treatment. *J Med Res Sci* 2012;2:11–18.
- 54 McKay J, Mathers J: Diet induced epigenetic changes and their implications for health. *Acta Physiol (Oxf)* 2011;202:103–118.
- 55 Gandini S, Gnagnarella P, Serrano D, Pasquali E, Raimondi S: Vitamin D receptor polymorphisms and cancer. *Adv Exp Med Biol* 2014;810:69–105.
- 56 Kostner K, Denzer N, Muller CS, Klein R, Tilgen W, Reichrath J: The relevance of vitamin D receptor (VDR) gene polymorphisms for cancer: a review of the literature. *Anticancer Res* 2009;29:3511–3536.
- 57 McClung JP, Karl JP: Vitamin D and stress fracture: the contribution of vitamin D receptor gene polymorphisms. *Nutr Rev* 2010;68:365–369.
- 58 Nelson DA, Vande Vord PJ, Wooley PH: Polymorphism in the vitamin D receptor gene and bone mass in African-American and white mothers and children: a preliminary report. *Ann Rheum Dis* 2000;59:626–630.
- 59 Jurutka PW, Whitfield GK, Hsieh JC, Thompson PD, Haussler CA, Haussler MR: Molecular nature of the vitamin D receptor and its role in regulation of gene expression. *Rev Endocr Metab Disord* 2001;2:203–216.
- 60 Morrison NA, Qi JC, Tokita A, Kelly PJ, Crofts L, Nguyen TV, Sambrook PN, Eisman JA: Prediction of bone density from vitamin D receptor alleles. *Nature* 1994;367:284–287.
- 61 Durrin LK, Haile RW, Ingles SA, Coetzee GA: Vitamin D receptor 3'-untranslated region polymorphisms: lack of effect on mRNA stability. *Biochim Biophys Acta* 1999;1453:311–320.
- 62 Verbeek W, Gombart AF, Shiohara M, Campbell M, Koeffler HP: Vitamin D receptor: no evidence for allele-specific mRNA stability in cells which are heterozygous for the Taq I restriction enzyme polymorphism. *Biochem Biophys Res Commun* 1997;238:77–80.
- 63 Wang GJ, Yang P, Xie HG: Gene variants in noncoding regions and their possible consequences. *Pharmacogenomics* 2006;7:203–209.
- 64 Whitfield GK, Remus LS, Jurutka PW, Zitzer H, Oza AK, Dang HT, Haussler CA, Galligan MA, Thatcher ML, Encinas Dominguez C, Haussler MR: Functionally relevant polymorphisms in the human nuclear vitamin D receptor gene. *Mol Cell Endocrinol* 2001;177:145–159.
- 65 Lehtonen-Veromaa M, Mottonen T, Irjala K, Karkkainen M, Lamberg-Allardt C, Hakola P, Viikari J: Vitamin D intake is low and hypovitaminosis D common in healthy 9- to 15-year-old Finnish girls. *Eur J Clin Nutr* 1999;53:746–751.
- 66 Pritchard JM, Seechurn T, Atkinson SA: A food frequency questionnaire for the assessment of calcium, vitamin D and vitamin K: a pilot validation study. *Nutrients* 2010;2:805–819.
- 67 Sauvageot N, Alkerwi A, Albert A, Guillaume M: Use of food frequency questionnaire to assess relationships between dietary habits and cardiovascular risk factors in NESCAV study: validation with biomarkers. *Nutr J* 2013;12:143.
- 68 Wu H, Gozdzik A, Barta JL, Wagner D, Cole DE, Vieth R, Parra EJ, Whiting SJ: The development and evaluation of a food frequency questionnaire used in assessing vitamin D intake in a sample of healthy young Canadian adults of diverse ancestry. *Nutr Res* 2009;29:255–261.
- 69 MacLaughlin J, Holick MF: Aging decreases the capacity of human skin to produce vitamin D₃. *J Clin Invest* 1985;76:1536–1538.
- 70 Holick MF: Sunlight and vitamin D for bone health and prevention of autoimmune diseases, cancers, and cardiovascular disease. *Am J Clin Nutr* 2004;80:1678S–1688S.
- 71 Holick MF: Sunlight, ultraviolet radiation, vitamin D and skin cancer: how much sunlight do we need? *Adv Exp Med Biol* 2014;810:1–16.
- 72 Holick MF, Matsuoka LY, Wortsman J: Age, vitamin D, and solar ultraviolet. *Lancet* 1989;2:1104–1105.