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UV-associated decline in systemic folate: Implications for human nutrigenetics, health, and evolutionary processes

Mark Lucock[1]*, Emma Beckett[1], Charlotte Martin[1], Patrice Jones[1], John Furst[2], Zoe Yates[3], Nina G. Jablonski[4], George Chaplin[4], Martin Veysey[5]

[1]School of Environmental & Life Sciences, [2]Maths & Physical Sciences and [3]Biomedical Sciences & Pharmacy, University of Newcastle, PO Box 127, Brush Rd, Ourimbah, NSW 2258, Australia.

[4]The Pennsylvania State University, Anthropology Department, 409 Carpenter Building, University Park, PA 16802, USA.

[5]Teaching & Research Unit, Central Coast Local Health District, PO Box 361, Gosford, NSW 2250, Australia.

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Ph: +61 2 4348 4109

Fax: +61 2 4348 4145

***Corresponding author:** Mark.Lucock@newcastle.edu.au

Abstract

Objectives: To examine whether UV exposure alters folate status according to C677T-MTHFR genotype, and to consider the relevance of this to human health and the evolutionary model of skin pigmentation.

Methods: Total Ozone Mapping Spectrometer (TOMS) satellite data was used to examine surface UV-irradiance in a large (n=649) Australian cross-sectional study population. PCR/RFLP analysis was used to genotype C677T-MTHFR.

Results: Significant negative correlations between all examined wavelengths of UV-irradiance (including erythemal UV dose-rate) and red cell folate (RCF) levels were found (cumulative irradiance for 42 and 120 days pre-clinic). This relationship was maintained when analyses were stratified by C677T-MTHFR CC, CT and TT genotype, with the inverse association between folate and UV-irradiance ($mW/m^2/nm$) being most pronounced in 677TT-MTHFR subjects (cumulative exposure over 42 days pre-clinic). Inclusion of an interaction term (p-interaction) indicates that the influence of UV-exposure on RCF status is dependent on genotype, but does not interact with dietary intake of folate. This was supported by z-score analysis, which indicated 677TT-MTHFR subjects have a significantly different correlation coefficient for the UV-RCF relationship to those who are wildtype 677CC-MTHFR.

Conclusions: Data provides strong evidence that surface UV-irradiance reduces long-term systemic folate levels, and that this is influenced by the C677T-MTHFR gene variant. We speculate this effect may be due to 677TT-MTHFR individuals containing more 5,10CH_2-H_4PteGlu, and that this folate form may be particularly UV labile. Since UV-irradiance lowers RCF in an MTHFR genotype-specific way, there are likely implications for human health and the evolution of skin pigmentation; both are discussed.

Key Words

Folate; Pigmentation; UV; MTHFR; Evolution; Nutrigenetics

Introduction

Folate is a generic term for a large group of vitamers that transfer one-carbon units into important biosynthetic pathways, of which DNA-thymidylate (dTMP) and methionine are of particular relevance. Methionine is produced from homocysteine (Hcy) using both 5-methyltetrahydrofolate (5CH_3-H_4PteGlu) and vitamin B_{12} as cofactors. Methyl groups derived from methionine can be utilised for both genomic [CpG] and non-genomic methylation reactions. In addition, folyl-vitamers are required for purine synthesis and serine-glycine interconversion as well as histidine catabolism (Lucock, 2000).

This means that anything that perturbs folate metabolism such as genetic or environmental factors, or a restricted dietary intake can promote uracil misincorporation into the primary DNA base sequence in place of thymine, a phenomenon associated with DNA fragility. Additionally, since half

our methionine is generated *de novo* from Hcy, a folate shortage can also adversely influence the methylome and alter/disrupt epigenetic control (Lucock et al., 2015). Although cause and effect are not always proven, correlations of various evidentiary strengths suggest that dysregulated folate nutrition, genetics and hence metabolism are associated with neural tube defects (NTD) and several other developmental and degenerative disorders, including many cancers, vascular disease, and neuro-psychiatric conditions (Selhub, 2008; Lucock et al., 2015). As a result, there is now considerable interest in folate-related nutrigenetics and how genotype might modify the level and character of the body's folate pool (Smulders et al., 2007; Sohn et al., 2009).

With these factors in mind, many countries have opted to introduce mandatory folic acid fortification of the diet, ostensibly to reduce the incidence of NTDs such as spina bifida (MRC Vitamin Study Group, 1991). However, the form of folate used as a fortificant and in supplements is pteroylmonoglutamic acid (PteGlu), a synthetic analogue that does not occur naturally and has different physico-chemical properties to natural vitamers such as $5CH_3$-H_4PteGlu. PteGlu is fully oxidised and hence is very stable (relative to other folyl vitamers) under normal circumstances, and therefore until recently was the reference folate for studies of the 'relative absorption' of reduced folates in human subjects. This is now considered invalid as the initial site of folic acid biotransformation in humans is the liver, and not the upper small intestine as once thought (Wright et al., 2007). Because PteGlu must first be converted to tetrahydrofolate (H_4PteGlu) by dihydrofolate reductase (DHFR) before it can enter human metabolism, and given limited human liver DHFR activity, this hepatic enzyme is easily saturated leading to unmetabolised PteGlu in the plasma (Bailey and Ayling, 2009). Saturation of PteGlu would typically occur at around a 400µg dose (Lucock et al., 1989). So while both vitamers are bioavailable, historically PteGlu had been considered to have the greatest bioavailability (Choumenkovitch et al., 2002), and this led to the concept of "dietary folate equivalence" (DFE) in order to express a mixed intake of PteGlu and natural folate where 1µg of natural folate equals 1µg of DFE, but 1µg of PteGlu equates to 1.7µg of DFE (Suitor and Bailey, 2000). Given recent discoveries, views on folate bioavailability may therefore change in the future (Wright et al., 2007; Bailey and Ayling, 2009).

Studies have looked at the light sensitivity of both synthetic and natural folyl-vitamers, however, no large definitive population study has yet been conducted to examine whether ultraviolet light can degrade systemic folate stores taking account of key genetic factors. *In vitro* studies carried out by the present authors have demonstrated UV-B light at 312nm can degrade plasma/cellular $5CH_3$-H_4PteGlu, leading to the formation of labile, oxidised $5CH_3$-H_2PteGlu, with the eventual, irreversible loss of all vitamin activity via C9-N10 bond scission (Lucock et al., 2003). However, longer wavelengths in the UV-A spectrum (315-400nm) are more likely to penetrate the skin and reach the dermal circulation, and have been attributed to photolytic degradation of synthetic PteGlu that remains unmetabolised in the circulation, and which in this unmodified form is increasingly being linked to negative health correlates (Wright et al., 2007), including the potential production of 6-formylpterin (6-FP), which eventually oxidizes to form pterin-6-carboxylic acid (PCA) (Offer et al., 2007). These photolytic scission products can lead to oxidation of 2'-deoxyguanosine 5'-monophosphate (Serrano et al., 2012) and sequence-specific DNA cleavage, a major risk for carcinogenesis (Hirakawa et al., 2003; Ito and Kawanishi, 1997). Indeed, 6-FP has actually been examined as an agent for use in photodynamic chemotherapy (Wada et al., 2007; Arai et al., 2001; Yamada et al., 2005). Nevertheless, a 2015 *ex vivo* study shows that like PteGlu, epidermal $5CH_3$-H_4PteGlu is also degraded by longer UV-A wavelengths, in addition to UV-B light (Lucock et al., 2003;

Hasoun et al., 2015). Although few population studies have studied the effect of daily UV exposure on folate degradation, one paper is notable. Borradale et al. (2014) have shown that solar UV radiation exposure over three weeks reduces the efficacy of PteGlu supplements. Although this was a relatively small study with only 45 participants completing it, and was limited to serum folate (SF) measurements that do not reflect overall folate status as well as red cell folate (RCF) values do, it did focus on a young female population of reproductive age who live in a region with the potential for extreme UV exposure. Others have shown a similar photolytic effect, with the most profound influence being on systemic PteGlu, although the study did not adequately demonstrate chromatographic resolution of natural $5CH_3$-H_4PteGlu from its photolytic scission products to categorically define the UV effect on this blood vitamer (Fukuwatari et al., 2009).

Irrespective of the differential effect of UV exposure on photolytic degradation of natural and synthetic folyl-vitamers, the wider health implications of a loss of folate stores due to sun exposure are considerable given the vitamin's role in so many developmental and degenerative disorders as alluded to above. However, another important implication of any UV -associated folate degradation relates to whether this might have influenced the evolution of skin melanisation as a response to a clinal variation in UV levels between equatorial and temperate latitudes. Contemporary findings that support UV-related folate loss in human population studies would provide the strongest evidential validation of the folate–vitamin D-sunlight hypothesis of skin pigmentation first put forward by Jablonski and Chaplin (Jablonski and Chaplin, 2000). It would also help explain observational studies and hypotheses that explore novel evolutionary and developmental biological ideas linking light-sensitive vitamins to human health and phenotype, both within and across the lifecycle (Lowell and Davis, 2008; Marzullo and Fraser, 2005 and 2009; Torrey et al., 1997; Templer et al., 1992; Bayes et al., 2010; Disanto et al., 2012; Skjaervo et al., 2015; Gavrilov and Gavrilova, 1999; Juckett and Rosenberg, 1993; Doblhammer and Vaupel, 2001; Foster and Roenneberg T, 2008; Lucock and Leeming, 2013a; Lucock, 2011; Lucock et al., 2010, 2012 and 2014). These ideas have been developed into a unifying theory suggesting environmental (UV) and nutritional agents (folate and vitamin D) interact to modify gene-phenotype relationships across the lifespan (Lucock et al., 2010 and 2014). For example, a lack of systemic folate due to UV loss before and during embryogenesis might enhance the viability of embryos that have a specific genetic signature. In particular, the MTHFR 677TT variant is thought to maintain the fidelity of DNA-dTMP synthesis when folate levels are low (Blount et al., 1997). It is important to recognise that only the naturally occurring compound ($5CH_3$-H_4PteGlu) is likely to have been assimilated in the human circulation during evolutionary history, and so it is the effect of UV irradiance on this vitamer, not synthetic PteGlu that would be central to the folate–vitamin D-sunlight hypothesis of skin pigmentation.

With these thoughts in mind, it seems reasonable to explore not just how UV might alter systemic folate levels, but how any such effect is influenced by genetic variants considered to be particularly important in determining human health outcomes and which may be potentially relevant in linking environmental factors with evolutionary processes such as skin pigmentation. We therefore used Total Ozone Mapping Spectrometer (TOMS) satellite data to examine the effect of local noon time surface UV irradiance (305, 310, 324, 380nm, and the erythemal UV dose-rate, a measure of the potential for biological damage due to solar UV irradiance) on a large Australian cross-sectional study population. Our aim was to examine how accumulated UV exposure might influence RCF and SF, and to see if there was any nutrigenetic effect evident via the common C677T-MTHFR variant.

Methods

Subjects and sample collection: Six hundred and forty nine volunteers from the cross-sectional Retirement Health and Lifestyle Study (RHLS), Central Coast, New South Wales (65–95 years, 287 males and 362 females) were assessed for the prevalence of the C677T-MTHFR polymorphism, RCF and SF. They were also assessed according to the surface UV-irradiance on the day of blood collection, accumulated exposure over 42 days (6 weeks) before blood collection, and accumulated exposure over 120 days (17 weeks) before blood collection. This equates to the time taken for vitamin D photosynthesised in the skin to appear in the blood (Ca. 6 weeks), and the lifespan of the red blood cell (Ca. 17 weeks). Six hundred and thirty two subjects had complete data for genetics, RCF and UV exposure, while 639 had complete data for RCF and UV exposure.

Total Ozone Mapping Spectrometer (TOMS satellite data collection: UV data expressed as $mW/m^2/nm$ was obtained for wavelengths of 305nm, 310nm, 324nm and 380nm as well as for the erythemal dose-rate. This was gathered from the National Aeronautics and Space Administration's (NASA) Total Ozone Mapping Program via NASA's Aura OMI level 3 Atmospheric portal (http://gdata1.sci.gsfc.nasa.gov/daac-bin/G3/gui.cgi?instance_id=omi). See ESM for further information.

Haematology/folate analysis: Venous blood was collected by a trained phlebotomist following at least a 10 hour overnight fast. Blood samples were analysed by Hunter Area Pathology Service and RCF and SF were analysed by chemiluminescent immunoassay.

Food Frequency Questionnaire: The estimated daily intake of folate from all sources including supplements (synthetic PteGlu and natural $5CH_3$-H_4PteGlu) was assessed by a self-administered FFQ that covered 225 food items and all food groups. The FFQs were analysed using FoodworksTM 2.10.146 (Xyris Software, Brisbane, QLD, Australia) (Lucock et al., 2013b).

DNA analysis: The folate C677T-MTHFR gene polymorphism was analysed according to van der Put *et al.* (Van der Put et al., 1995).

Statistics: Statistical testing was performed using JMP (version 11; SAS Institute Inc., Cary, NC, USA). The associations between key variables and related parameters assessed on an *a priori* basis were examined using either standard least squares, or, where categoric data were examined, logistic regression analysis that fits the cumulative response probabilities to the logistic distribution function of a linear model using maximum likelihood. The *p*-value ($p < 0.05$) for the Wald Chi-square test/effect likelihood ratio test (use as indicated), provided a significance marker for screening effects. All statistical analyses have been performed adjusting for both age and gender. Only adjusted data is presented in the Tables. The p (interaction) for influence of UV exposure with a) total dietary folate, b) synthetic dietary folate, c) natural dietary folate and d) genotype with respect to RCF level has been calculated and is presented after adjustment for age and gender. All parameters were approximately normally distributed.

Descriptive statistics were calculated and presented as appropriate using mean, standard error of mean (SE) and number of observations. These helped define the basic levels of dietary folate, RCF and SF, age and erythemal UV dose-rate for all subjects, by gender and for genotype, as well as the overall genetic characteristics of the population. A one-way ANOVA/unpaired T-test was performed

as appropriate to establish whether significant variation between means occurs for variables (age, folate levels/intake, erythemal UV dose-rate) with respect to the population criteria - gender or genotype.

Where data was explored for potential relationships, analysis using stepwise regression was performed in a mixed direction with significant probability [0.250] that a parameter be considered as a forward step and entered into the model or considered as a backward step and removed from the model. Mallow's Cp criterion was used for selecting the model where Cp first approaches *p* variables.

In order to establish whether a significant difference exists between two age and gender adjusted correlation coefficients, a Fisher r-to-z transformation was used to calculate a value for z (http://vassarstats.net/rdiff.html). In all cases, the z score and two-tailed *p* value are given, and the reference genotype in the Fisher r-to-z transformation is the one with the least number of T alleles and/or the least accumulated UV exposure.

Results

In order to provide overall context to the data, Figure S1 (Supporting Information) shows how surface UV-irradiance on the day of each clinic alters over the course of the study. Although irradiance relates to the actual day of the clinic, in the supplementary figure it is only categorised by month, with several clinics potentially occurring in any one month and the entire study occurring over a total period that spanned January 2010 through March 2012. Over this time course, 85 separate clinics were held. Measurements are given as $mW/m^2/nm$ and relate to the irradiance at 305nm, 310nm, 324nm, 380nm and the erythemal UV dose-rate. The figure shows what might be predicted for this sub-tropical southern hemisphere region with UV-irradiance maxima in February and minima in July.

Table S1a (Supporting Information) provides descriptive data and statistical analyses for the key measurements central to this population study. No significant gender differences were observed for any of the key variables. However, when C677T-MTHFR genotype was examined to see if a significant variation between means occurs for the variables; age, RCF and SF, folate intake, and erythemal UV dose-rate, only one variable was affected: C677T-MTHFR genotype led to a significant variation in the red blood cell folate mean values (ANOVA; $p = 0.0039$). The effect was to show a progressively higher RCF with increasing carriage of the T allele (1303, 1331, 1530 nmol/L for CC, CT, TT respectively). The potential explanation for this may have significance in considering the implications of the overall UV-folate data analysis, and is discussed fully later.

Although ANOVA did not show any similar effect of C677T-MTHFR genotype on variation in mean SF level, stepwise regression was used to generate the best model for exploring the relationship between surface UV-irradiance (305nm, 310nm, 324nm, 380nm and erythemal UV dose-rate) and both a) SF and b) RCF levels independent of genotype. Using this approach, surface irradiance at 310nm was negatively associated with SF (whole model $r^2=0.0124$; $p=0.0049$; slope estimate=-0.0005), while 310nm was again negatively associated with RCF (whole model $r^2=0.036$; $p<0.0001$; slope estimate = - 0.0274). These models were performed for the accumulated UV exposure over

120 days. Although this study is limited primarily to RCF, the best overall measure of long-term systemic levels, it is important to note that a significant but lesser effect is also observed on SF – an index that is far more dynamic, with diurnal levels strongly influenced by dietary intake. The authors believe the most valuable data relates to RCF levels, and this paper therefore focuses on this, but the authors acknowledge that a signature of the effects presented for RCF is present, but to a lesser extent in SF as has been described previously (Borradale et al., 2014). Using this modelling for SF and RCF, only around 1 and 4% variation respectively can be explained by the regression model. This effect may be small due to other interactive factors not examined, such as antioxidant status (i.e. vitamin C) and photosensitisers (i.e. riboflavin), as well as undefined genetic and dietary factors. In previous genotype-by-environment studies, we have typically found around 2-6% of the response variable can be explained by the regression model, and so the present findings, that additionally reflect low p values, are not unexpected (Lucock et al., 2013b). A summary of the effect of UV on SF is described below, following the statistical interpretation of the effect on RCF values.

There appears to be little difference in diet across genotype, with mean values all above an acceptable level of 400µg/day. Similarly, erythemal UV dose-rate does not vary significantly across genotypes.

Table S1b (Supporting Information) provides basic genetic information, including genotype prevalence, allele frequency and carriage of the polymorphic T allele.

The major outcomes from this research are presented in Table 1, S2a (Supporting Information) and S2b (Supporting Information), and in Figures 1 and S2 (Supporting Information). Tables 1 and S2a demonstrate significant negative correlations between all wavelengths of UV light, including erythemal UV dose-rate, which have accumulated over 42 and 120 days pre-clinic respectively, and RCF levels. This correlation has been performed for all subjects, and C677T-MTHFR CC, CT and TT subjects. In all cases, the correlation is negative and is significant in most cases. Only 677CC-MTHFR subjects after 42 days accumulated UV did not show significance (and 677CT-MTHFR at 380nm). Similarly, only 677TT-MTHFR subjects after 120 days accumulated UV did not show significance. However, the majority of correlations conducted showed a clear and significant negative correlation between UV exposure and RCF level. Clearly, this effect is partially down to nearby wavelengths having similar intensity and is therefore perhaps predictable; nonetheless, the generality of the effect is worth noting. Figures 1 and S2 show this graphically for all 40 correlations, many of which are statistically robust (Tables 1 and S2a show r^2, and p values, along with the corrected slope [standardised β value] and SE of the regression).

It is evident from Figures 1 and S2 that the slope indicative of folate loss with increasing surface UV-irradiance ($mW/m^2/nm$) is more acutely negative in the 677TT-MTHFR subjects, and that this effect is most obvious when the accumulated UV dose over 42 days pre-clinic is used. Table 1 demonstrates that this effect is significant at all wavelengths of UV. In order to establish that this was a real genotype-related effect, a z-score for significance of difference between correlation coefficients was conducted (z score and two-tailed p value are given in Tables 1, S2a and S2b). By using the genotype with the least number of T alleles as the reference genotype in the Fisher r-to-z transformation, it has been possible to demonstrate that the 677TT-MTHFR subjects have a significantly different age and gender adjusted correlation coefficient to the wildtype 677CC-MTHFR subjects. This only occurs for accumulated UV dose over 42 days, and does not occur between other

genotypes at either 42 or 120 days UV exposure (with one exception). Furthermore, Table S2b shows virtually no significant z-score for significance of difference between correlation coefficients between UV data obtained at 42 days vs 120 days when comparing CC vs CC, CT vs CT and TT vs TT (again with one exception).

A close examination of Figure 1 in particular, extends this finding that TT individuals exhibit a greater decline in RCF per unit UV-irradiance than do CC individuals. In fact, it points to the possibility that increasing carriage of the polymorphic T allele leads to a progressive increase in folate loss per unit UV-irradiance across the entire spectrum of wavelengths examined: Loss in TT>CT>CC. This effect is most evident after measuring 42 days of accumulated UV where exposure to this environmental factor explains as much as 6% of the RCF variance. It becomes less clear when using the data for the 120 day exposure. Despite this, the strength of the associations are generally greater at 120 days compared to 42 days. The explanation for this can be inferred from Figure S1. This figure shows that over 4 months (120 days) or one third of the Earths annual cyclic period, the variation in UV-irradiance will be time-averaged out. This would not be the case over 42 days. A more detailed biochemical explanation for this is suggested in the discussion in relation to the physico-chemical properties of specific folyl-vitamers.

The following brief summary of how the C677T-MTHFR polymorphism modified the influence of surface UV-irradiance on SF levels supports the overall findings: 42 days of accumulated UV-irradiance showed a significant reciprocal association at all wavelengths for CT individuals only ($p<0.05$ but >0.01) . Similarly, at 120 days, UV-irradiance again showed a significant reciprocal association at all wavelengths for CT individuals only ($p<0.05$ but >0.005). No effect was detected for either CC or TT individuals at any of the wavelengths examined after either 42 or 120 days exposure.

The FFQ for folate intake is based not only on the amount of food eaten, but also frequency – i.e. daily, weekly, monthly, yearly, never. It is therefore not ideal for accurate assessment of intake over a temporal profile such as has been used here, particularly when linked to environmental phenomena over lengthy specified time periods. However, it does provide an estimation of nutrient consumption, and is therefore included for completion. Since folate intake is a determinant of RCF, an ANOVA was conducted to see whether any variation in mean folate intake (as determined by FFQ) occurred over the year (by month of clinic). No significant variation in mean folate intake was found to occur by month of clinic ($p=0.983$; $n=647$). However, variation in mean RCF did occur over the year (by month of clinic); ANOVA $p=0.0002$; $n=640$ (minima RCF in March, maxima in September). Although causation cannot be proven, this latter point is consistent with the effect of a southern hemisphere seasonal flux in UV-irradiance, and taken collectively, the two ANOVAs lend support to the idea that variation in dietary folate intake is not a critical factor in this observed seasonal variation in RCF. It is worth noting that this is likely consistent with the increased use of both discretionary and mandatory sources of synthetic folate throughout the year. In the present study, subjects were found to have a mean daily intake of synthetic PteGlu of 191.7µg/day (SE=10.1 µg/day) and a mean daily intake of natural $5CH_3$-H_4PteGlu of 339.7µg/day (SE=5.9 µg/day). Ninety nine subjects (15.2%) took PteGlu containing supplements, while only 1.2% received no dietary PteGlu. These figures are reflective of what can be considered a well-nourished population.

Table 1 also shows a significant p-interaction between UV exposure at all wavelengths and MTHFR genotype with respect to RCF. Therefore, it is not simply an additive effect of both UV and genotype

influencing RCF, rather, the response of RCF to UV varies by genotype. This effect was observed after 42 days UV exposure (Table 1), but not 120 days exposure (Table S2a). No significant interaction was found between either duration of UV exposure and a) total dietary folate, b) synthetic dietary folate, or c) natural dietary folate with respect to RCF level, indicating that none of these dietary relationships are interactive, although any two may influence the outcome variable independently.

Although not presented in the tables, when adjusted for dietary folate intake, UV exposure remains a significant negative predictor of RCF, such that increased UV (all wavelengths) leads to a decreased RCF. This was true following 42 and 120 days of accumulated UV (only the adjusted 42days correlation for 380nm did not achieve significance, although it was borderline significant [p=0.0595]).

In general, results for all correlations remained significant when adjusted for age and gender. Furthermore, neither age nor gender were significant independent predictors of RCF.

Discussion

The elderly study population examined was relatively homogeneous, being primarily white and of northern and western European descent. This makes the present population ideal to carry out a study of the effects of UV-irradiance on folate status because, as we age, constitutive pigmentation declines and the ability to tan is diminished due to lower numbers of active melanocytes (Quevedo et al., 1969; Jablonski and Chaplin, 2010). This is at variance with needs during the reproductive phase of the lifecycle where maximal pigmentation is paramount – possibly to help protect/preserve UV labile folate from photolytic loss so it can be used for reproductive advantage enhancing the fidelity of cell division and differentiation (Jablonski and Chaplin, 2010). With this in mind, and given the obvious discord between a lightly pigmented elderly phenotype and an exceptionally high UV, subtropical environment, this study population is well positioned to detect any environment-nutrient-gene interaction.

The results presented here strongly support the assertion that exposure to UV light reduces systemic folic acid levels using the best overall long-term marker of folate status–RCF. This is the first study to demonstrate this, and has significant implications for health and evolutionary biology. Despite this, it also raises some other interesting and very important questions.

The first of these questions relates to the nature of dietary folate. Following recent increased use of synthetic PteGlu on a mandatory and discretionary basis, dietary folate is now a combination of both synthetic and natural vitamers. Given that these have very different physico-chemical properties and that above 400µg doses, PteGlu appears in the blood in unmodified form (Choi et al., 2014), many concerns relating to negative health correlates of PteGlu have been raised (Choi et al., 2014). The one that is most relevant here, given that on average the intake of the synthetic PteGlu vitamer in the RHLS group is 36% of all dietary folate ingested, is the potential production of 6-FP and its oxidation product, PCA (Offer et al., 2007). These UV scission products can lead to oxidation and sequence-specific DNA cleavage, both considered a major risk for carcinogenesis (Serrano et al., 2012; Hirakawa et al., 2003; Ito and Kawanishi, 1997). Natural $5CH_3$-H_4PteGlu is even less UV-stable, particularly in the presence of photosensitisers, but does not form potentially toxic degradation

products like PteGlu. Therefore, the interesting effects depicted in Figures 1 and S2 raise the question as to whether PteGlu generates meaningful levels of these UV scission products, and if so, are there any toxic consequences?

The second question relates to the nature of RCF coenzymes, and whether this varies according to C677T-MTHFR genotype. Data from the present study shows that individuals who are homozygous TT have the highest RCF, followed by CT and then CC (wild type) individuals (see Table S1a). This may suggest that the TT folate pool contains a greater proportion of a more labile folyl-vitamer that responds differentially to the non-specific folate assay used in this analysis. This point is worth further consideration, with Figure 2 showing a speculative view on how the expressed MTHFR enzyme protein might affect this area of folate-dependent one-carbon metabolism according to the C677T-MTHFR variant. It is evident from Figure 1b, that the reduction in RCF with increasing UV-irradiance (at all wavelengths assessed and erythemal UV dose-rate) increases from CC to CT to TT MTHFR genotype. There is some evidence to support the idea that the amount of non-methylfolate vitamers increases with carriage of the mutant T allele, and that this might be in the form of either formyl-H_4 or methylene-H_4 -folate vitamers (mostly polyglutamates) (Smulders et al., 2007; Bagley and Selhub, 1998; Lucock and Yates, 2005). There are no routine assays for folyl-coenzyme speciation as the low levels, extreme instability and propensity for coenzyme interconvertability makes this one of the most difficult of all micronutrients for bioanalysts to measure with any degree of confidence. Assays are nearly always non-specific chemiluminescence or microbiological methods run through routine pathology laboratories. These give a single measurement for what is often a complex mixture of folyl-vitamers. Nevertheless, even with these non-specific assays, others have found that folate concentrations are not being measured accurately in individuals who are TT homozygous for the MTHFR variant (15% of people); typically these individuals exhibit higher RCF values than CC or CT individuals (Molloy et al., 1998), as presented here (Table S1). However, it has also been shown that this finding is variable and depends on the assay and laboratory used, with some studies showing lower overall RCF in TT individuals (Bailey and Gregory, 1999). We have previously shown that a routine hospital folate assay responds differentially to a variety of folylmonoglutamates (Lucock et al., 1999). What was interesting here was that although most folylmonoglutamates gave around a 100% response to the routine non-specific folate assay, one vitamer, 5,10methylenetetrahydrofolate (5,10CH_2-H_4PteGlu) gave an unprecedented 299% response (Lucock et al., 1999). This surprising assay response to 5,10CH_2-H_4PteGlu needs to be considered in concert with the fact that 5,10CH_2-H_4PteGlu is an extremely labile vitamer (Wilson and Horne, 1983; Lucock et al., 1996). Taken together, these observations and previous work raise the possibility that the RCF in our TT subjects with low UV-irradiance exposure, and which seem to reflect higher RCF than CT and CC subjects (Figure 1b) could simply be reflecting a differential assay response to two or more different vitamers. The levels may not actually be higher; there may simply be more 5,10CH_2-H_4PteGlu present in TT individuals (this idea fits with what is already known about folate metabolism (Blount et al., 1997)). Furthermore, the rapid decline in TT RCF with respect to UV-irradiance when compared to CT and CC individuals could reflect greater UV destruction of 5,10CH_2-H_4PteGlu compared to 5CH_3-H_4PteGlu, the latter likely predominating in CT and CC subjects. The possible implications of this in the context of a role for folate in the evolutionary biology of skin pigmentation are discussed later.

The third question that is raised by these findings is whether the loss of RCF with increasing UV-irradiance reflects a greater need for folate to repair UV-induced DNA damage. That is to say, is all

the folate loss attributable to photolytic scission or is there a component involved that directs folate towards DNA repair? 5,10CH_2-H_4PteGlu is the immediate precursor for the conversion of dUMP into dTMP, and taken as whole, this family of coenzymes is crucial for several other biosynthetic pathways of central importance to the cell under conditions of stress. However, in either event (cell repair/UV scission), folate is clearly being lost. RCF is considered a surrogate marker of systemic levels, but mature red cells contain no DNA, so a key issue is whether or not these data have any bearing on DNA maintenance and repair in other tissues.

The final, immediate question raised is whether these effects would have a more critical importance in individuals with a particularly low folate status. The present population have a high folate status, but at some point, this environmental stress would likely have serious impact – it would be interesting to know at what point this cuts in.

So if we assume UV lowers RCF in an MTHFR genotype specific way, a process that involves effects at a metabolic locus with considerable inherent complexity (Lucock 2000; Mathews and Haywood, 1979; Selhub, 1999; Mathews, 2002), and therefore alters the cellular equilibrium between 5,10CH_2-H_4PteGlu and 5CH_3-H_4PteGlu, what are the implications for human health and evolutionary biology? The metabolic balance between these two cellular vitamers is a determinant of both folate-dependent DNA-dTMP synthesis and maintenance of the epigenome via CpG methylation patterns. 5,10CH_2-H_4PteGlu is also the major entry point for one-carbon units into one carbon metabolism, a process by which serine donates a carbon for the CH_2 folate moiety with production of glycine, a reversible reaction involving serinehydroxymethyl transferase, and one important for amino acid synthesis. Both dTMP and CpG methylation are considered critical during early development/embryogenesis and in the development of many cancers. These metabolic loci would be very sensitive to a low folate status, which would undoubtedly enhance DNA and methylomic damage that is recognised as being critical in the development of a variety of patho-aetiologies. Fortunately, the study population has an overall healthy folate status (estimated mean folate intake 527µg/d, mean RCF 1339 nmol/L), despite a clear lowering of folate following UV exposure. The study does raise the possibility that populations with high UV exposure and low folate status might be compromised to the extent that this environment-nutrient interaction could lead to pathological changes at the molecular level, and further that this might be exacerbated by C677T-MTHFR genotype, with TT individuals more sensitive to UV than CT or CC individuals. Although the negative effect of UV-irradiance occurs at all wavelengths examined, there is an indication that the more energetic 305nm and 310nm wavelengths may exhibit a marginally greater loss of folate. It is clear from a previous *in vitro* kinetic study that UV exposure at 312nm leads to a rapid decay in 5CH_3-H_4PteGlu with C9-N10 bond scission clearly detected (Lucock et al., 2003), and so the wavelengths examined in the present study at 305nm and 310nm are also likely to degrade 5CH_3-H_4PteGlu, although no information is available on the effect of UV on 5,10CH_2-H_4PteGlu, and no UV-5CH_3-H_4PteGlu kinetic studies have been carried out directly in red cells. However, this reduced methylene vitamer form of folate has proven even more labile than 5CH_3-H_4PteGlu in terms of its stability for analysis (Lucock et al., 1996).

This environment-nutrient-gene interaction may therefore have relevance in a plethora of clinical phenotypes in which low folate levels or folate-related gene variants are aetiologically important factors, for example: in birth defects (MRC Vitamin Study Group, 1991), vasculo-, embryo- and neurotoxicicity associated with elevated Hcy (Lucock, 2006; Boushey et al., 1995), cognitive decline

(Tangney et al., 2009), Alzheimer's disease (Clarke et al., 1998), mood disorders (Bottiglieri, 2005) and many cancers (Smith et al., 2008), although in the latter case it is more complex since too little or too much as well as the C677T-MTHFR are relevant in cancer development and progression (Sohn et al., 2009; Ulrich and Potter, 2007).

One of the most interesting questions relates to whether this UV loss of folate, and the possible modulation of this process by the C677T-MTHFR variant has any relevance in the development of human skin pigmentation. We believe the current work provides the strongest evidence to date in support of the folate–vitamin D-sunlight hypothesis of skin pigmentation first put forward by Jablonski and Chaplin (Jablonski and Chaplin, 2000). UV-labile folate and the light-dependent steroid vitamin D, have both been implicated in the evolution of human skin pigmentation and depigmentation, respectively (Jablonski and Chaplin, 2000, and 2010; Jablonski 2004). However, any such role needs to bear in mind the evidence for multifactorial involvement of several key genes in this process such as MC1R, MATP (SLC45A2), OCA2, TYRP1, DCT, KITLG, PPARD, DRD, and EGFR (Lao et al., 2007; Rees, 2004; Graf et al., 2005).

Maintaining an adequate folate status is critical for reproductive success; skin melanisation is an adaptation that is maintained via natural selection as part of a polygenic effect, and is required, amongst other things, to protect UV-labile folate and hence maintain fecundity in high UV regimes at lower latitudes. Jablonski's work (Jablonski and Chaplin, 2000, and 2010; Jablonski 2004) also contends that at higher latitudes, depigmentation was driven by selection pressures to enhance cholecalciferol biosynthesis from 7-dehydrocholesterol in the skin because of the lower and more seasonal ambient UV levels – again, this is a vitamin critical early in the lifecycle via both direct and calcium-related effects. In the context of recent human evolution, this fits a paradigm where depigmentation evolved as early humans radiated out from equatorial latitudes where dark, melanin-rich skin provided a barrier against UV-induced cellular damage, including protection against UV loss of $5CH_3$-H_4PteGlu via C9-N10 bond scission. This vitamin is critical for cell division and embryogenesis (Lucock et al., 2003 and 2014; Jablonski 2004), and is also part of intrinsic folate-dependent DNA repair processes that occur following UV-induced DNA damage (Jablonski and Chaplin, 2010). Taking account of the obligate need for maintaining folate and vitamin D status, it is possible to integrate these aspects into an evolutionary model that has gained popular acceptance over the past few years: Natural selection maintains two opposing phenotypic clines of skin pigmentation. The first cline (and evolutionarily the original one) emphasises photoprotection of folate, from darkest pigmentation under high UV-R at the equator to lesser pigmentation near the poles. The second one, in the opposite direction, relates to promotion of vitamin D photosynthesis through the evolution of lesser pigmentation under low UV-B conditions nearer the poles. Between clinal extremes in UV exposure, humans evolved facultative melanisation so as to adapt to the significant annual flux in UV exposure that can occur at intermediate latitudes ((Jablonski and Chaplin, 2010; Lucock 2011).

The present findings strongly support the dual cline hypothesis, providing the best evidence yet that this idea may be correct. However, it goes one step further, raising the question as to whether individuals who are homozygous TT for the C677T-MTHFR might have higher levels of a form of folate ($5,10CH_2$-H_4PteGlu) that is less stable (Wilson and Horne, 1983; Lucock et al., 1996) than in CT and CC genotypes. Figures 1 and S2 tend to support this notion, and the evidence in comparing regression coefficients between CC and TT subjects again supports such a proposition (Table 1). The

implications of such an effect might mean that under lower levels of folate intake, TT subjects are more susceptible to serious UV induced folate loss (5,10CH_2-H_4PteGlu being more UV-labile than 5CH_3-H_4PteGlu) and that natural selection would operate to favour a more highly pigmented phenotype. Some evidence may already exist to support this idea, with large studies suggesting the presence of selective pressures leading to marked geographical and ethnic variation in the frequency of the C677T-MTHFR T allele (Wilcken et al., 2003; Gueant-Rodriguez et al., 2006). However, such MTHFR genotype variation has largely been attributed to the availability of dietary folate with geographic gradients tending to reflect this; to the authors' best knowledge, no one has yet directly linked the MTHFR T allele to the pigmentation phenotype. In the light of the present findings, it seems plausible to consider a possible interaction between dietary folate, systemic folate (particularly 5,10CH_2-H_4PteGlu), the C677T-MTHFR genotype and UV exposure in the natural selection of skin pigmentation. Future studies that span latitude should carefully examine this possibility, and should do so over a range of pigmentation phenotypes, and in concert with other gene variants that can be linked to the utilisation or production of 5,10CH_2-H_4PteGlu.

Although no direct link between the MTHFR T allele and the pigmentation phenotype has yet been detected, one study that aggregated data by location merged 154 population groups, and has linked prevalence of the C677T-MTHFR variant to UV radiation in Eurasia (Yafei et al., 2012). Linear modelling suggests that latitude (directly) and UV-A (inversely) are associated with the T allele. The authors suggest this clinal effect results from natural selection via environmental factors, especially UV radiation, but note that quadratic regression indicates an inverse U-shape relationship. In the context of folate photolysis, they hypothesise that at low latitudes and in the presence of dark skin, strong UV predicts the C allele; at intermediate latitudes, medium dark skin and medium UV predicts the T allele; and at higher latitudes, weak UV and light skin predicts the C allele. This model seems reasonable and considers both genetic and stochastic factors that drive genotypic shifts in a population, with likely important consequences for natural selection (Soloway, 2006). Unlike our study, it does not directly link UV to folate status nor does it demonstrate the clear influence of genotype on this relationship. However, taken together, these studies provide compelling evidence for the "folate-vitamin D-UV hypothesis of skin pigmentation", a model that should now include MTHFR genotype as an important component.

Linkage disequilibrium (LD) is influenced by many factors including selection, recombination, mutation, and genetic drift and linkage. The CEPH (Centre d'Etude du Polymorphisme Humain) human diversity panel for variants in folate pathway genes (Shi et al., 2003) indicates a LD for the MTHFR gene that is consistent with reported early origins of African populations, where LD is lower due to historical recombination events being large. Globally, the report indicates that the T allele is at relatively low frequency in Africa and S Asia compared to Europe, Asia, and the Americas. This tends to support the above hypothesised model linking latitude/UV, folate and MTHFR genotype (Yafei et al., 2012) The precise molecular mechanisms involved in this evolutionary model remain unclear, but one area of future study should focus on whether the potentially greater amount of labile folate in TT individuals is being routed to repair UV-damage, and whether such a mechanism could fit this evolutionary hypothesis, an area where opinion is likely to be divided (Osborne and Hames, 2014). Figure 3 integrates these ideas, including the present observations, into a simplified model.

The health implications are also of interest given the critical role of folate in so many disorders, with future work needing to acknowledge the degrading potential of sun exposure in assessing the role of the vitamin in human biology – particularly in developmental biology and in neoplastic disorders. Although it seems fortuitous that mandatory fortification has enhanced population folate status in many countries, questions exist regarding the possible toxicity of the photolytic degradation product of synthetic PteGlu. Populations without mandatory fortification in place, low pigmentation, and high UV exposure are at greatest risk of folate loss. The C677T-MTHFR TT genotype will exacerbate this process.

Although the present study limits itself to the interaction between UV, folate and MTHFR, other nutrients might also be relevant: photo-sensitisers like vitamin B_2 may enhance UV-dependent folate degradation (Steindal et al., 2008), while vitamin C and glutathione likely enhance folate stability (Lucock et al., 2003 and 2013b). Within the skin itself, carotenoids are important anti-oxidants and UV-protective pigments (Alaluf et al., 2002), and could be determinants of UV-related biological outcomes. Future studies should therefore also take nutrient-nutrient interactions into account in fully addressing this environment-related nutrigenetic phenomenon and its possible health sequelae. Such future studies should also take account of other related genetic polymorphisms as well as clothing and outdoor lifestyle, the latter being a significant limitation of the present observational study.

Conclusions

UV exposure at all wavelengths studied (305-380nm) appears to degrade RCF, a process that seems enhanced in individuals that are homozygous for the TT C677T-MTHFR variant (42 days UV) or carry one T allele (120 days UV). If this translates into higher levels of labile 5,10CH_2-H_4PteGlu, it would have relevance in considering the role of UV-labile folate in driving the natural selection of skin pigmentation. These findings may also have health implications, particularly in the absence of mandatory fortification, in populations with low pigmentation and high UV exposure – a combination that likely facilitates the greatest risk of folate loss. Future studies would benefit from greater clarity on the nature of red cell folate as defined by MTHFR genotype.

Ethics

Informed consent was obtained prior to participation under University of Newcastle Human Research Ethics Committee approval number H-2008-0431.

Data Accessibility

Access to the ARC RHLS database is via the RHLS Steering Committee (Chair – A/Prof Martin Veysey)

Competing Interests

We have no competing interests

Author Contributions

Idea researched and developed by ML; Genetics examined by CM and EB; Statistical input ML and EB; Physical science aspects carried out by JF and PJ; Overall chief investigator and clinician with ultimate oversight MV; Evolutionary aspects led by NJ, GC and ML; Article crafted in final form by ML, EB, MV, NJ, GC and ZY; all authors contributed to final manuscript.

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Table 1: Correlation between 42 days of accumulated UV exposure prior to blood sampling ($mW/m^2/nm$) and red cell folate level. Spectral irradiance is given for 305nm, 310nm, 324nm, 380nm and erythemal UV dose-rate. The z-scores for significance of difference between correlation coefficients using a two-tailed p value are given. In all cases, the reference genotype in the Fisher r-to-z transformation is the one with the least number of T alleles. The p (interaction) for influence of UV exposure with a) total dietary folate, b) synthetic dietary folate, c) natural dietary folate and d) genotype in respect to the red cell folate level has been calculated. All the data presented in this table has been adjusted for age and gender.

Figure 1: a) Association of red cell folate level with accumulated 42 day surface UV-irradiance ($mW/m^2/nm$ at 305nm, 310nm, 324nm, 380nm) for whole population and b) according to C677T-MTHFR genotype. Shading shows 95% confidence bands.

Figure 2: UV lowers red cell folate in an MTHFR genotype specific way. The figure presents a speculative model, in which it is suggested that an alteration in the cellular equilibrium between $5,10CH_2\text{-}H_4PteGlu$ and $5CH_3\text{-}H_4PteGlu$ occurs with implications for dTMP and methionine biosynthesis. This may influence human health and have been important in the evolution of pigmentation phenotypes.

Figure 3: Schematic of the evolutionary model for the “folate-vitamin D-UV hypothesis of skin pigmentation”, with MTHFR genotype as an important component.

Table 1

Correlation between 42 days of accumulated UV exposure prior to blood sampling (mW/m^2/nm) and red cell folate level; data adjusted for age and gender; slope=standardised beta value					
	305nm	**310nm**	**324nm**	**380nm**	**Erythemal UV dose rate**
All subjects	R^2=0.0147; p=0.0038; slope= -0.1144; SE=0.019; n=639	R^2=0.0133; p=0.0065; slope= -0.1078; SE=0.015; n=639	R^2=0.0094; p=0.0268; slope= -0.0877; SE=0.006; n=639	R^2=0.0079; p=0.0459; slope= -0.0079; SE=0.004; n=639	R^2=0.0137; p=0.0055; slope= -0.1099; SE=0.007; n=639
677CC-MTHFR	*R^2=0.0055; p=0.4468; slope= -0.0466; SE=0.030; n=272*	*R^2=0.0044; p=0.5899; slope= -0.0330; SE=0.024; n=272*	*R^2=0.0033; p=0.9395; slope= -0.0047; SE=0.010; n=272*	*R^2=0.0033; p=0.9430; slope= 0.0044; SE=0.006; n=272*	*R^2=0.0050; p=0.5055; slope= -0.0408; SE=0.010; n=272*
677CT-MTHFR	R^2=0.0231; p=0.0148; slope= -0.1406; SE=0.026; n=306	R^2=0.0221; p=0.0179; slope= -0.1366; SE=0.020; n=306	R^2=0.0172; p=0.0420; slope= -0.1174; SE=0.008; n=306	*R^2=0.0151; p=0.0629; slope= -0.1074; SE=0.005; n=306*	R^2=0.0212; p=0.0187; slope= -0.1356; SE=0.009; n=306
677TT-MTHFR	R^2=0.1373; p=0.0395; slope= -0.2919; SE=0.087; n=54	R^2=0.1420; p=0.0336; slope= -0.3002; SE=0.067; n=54	R^2=0.1421; p=0.0336; slope= -0.2992; SE=0.027; n=54	R^2=0.1400; p=0.0361; slope= -0.2951; SE=0.017; n=54	R^2=0.1369; p=0.0400; slope= -0.2911; SE=0.029; n=54
z-score for significance of difference between correlation coefficients (z score and two-tailed p value are given). In all cases, the reference genotype in the Fisher r-to-z transformation is the one with the least number of T alleles, and results have been adjusted for age and gender					
TT vs CC	z=2.06; p=0.0394	z=2.16; p=0.0308	z=2.22; p=0.0264	z=2.95; p=0.003	z=2.08; p=0.038
TT vs CT	*z=-1.56; p=0.119*	*z=1.63; p=0.103*	*z=1.75; p=0.080*	*z=1.78; p=0.075*	*z=1.58; p=0.114*
CT vs CC	*z=-0.70; p=0.484*	*z=0.99; p=0.322*	*z=0.89; p=0.374*	z=2.16; p=0.031	*z=0.93; p=0.352*
P (interaction) for influence on red cell folate level adjusted for age and gender					
UV exposure x total dietary folate	NS	NS	NS	NS	NS
UV exposure x synthetic dietary folate	NS	NS	NS	NS	NS
UV exposure x natural dietary folate	NS	NS	NS	NS	NS
UV exposure x genotype	p=0.0191	p=0.0152	p=0.0120	p=0.0118	p=0.0181

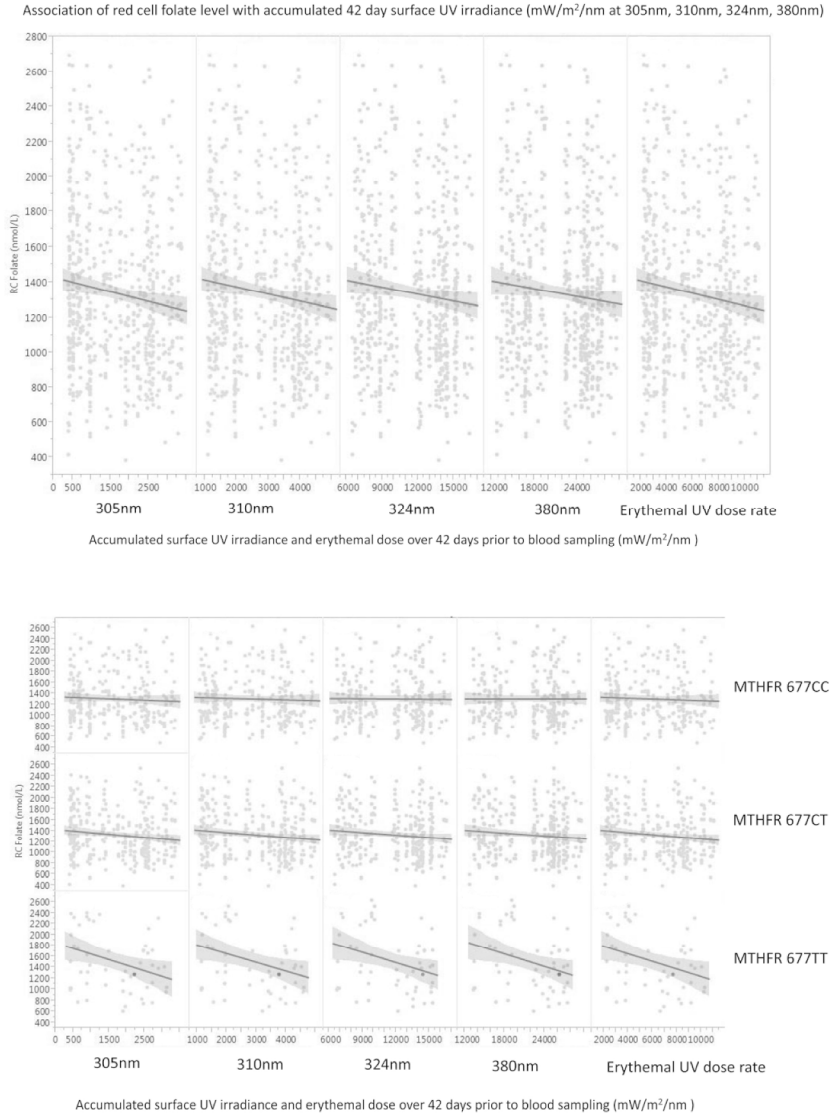
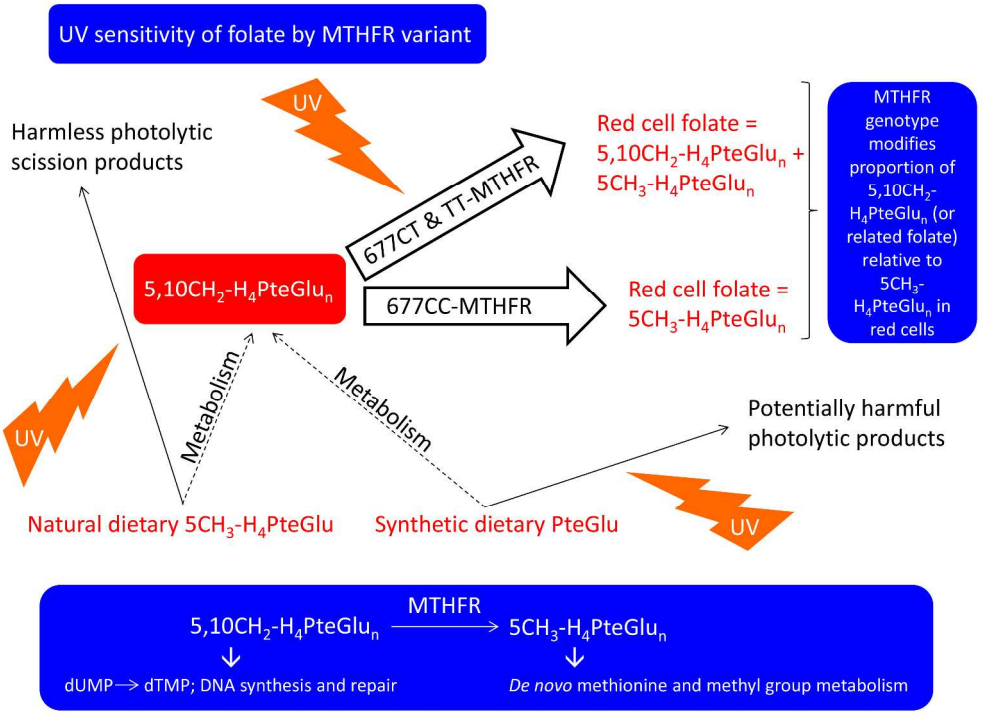


Figure 1: a) Association of red cell folate level with accumulated 42 day surface UV-irradiance (mW/m2/nm at 305nm, 310nm, 324nm, 380nm) for whole population and b) according to C677T-MTHFR genotype. Shading shows 95% confidence bands.
228x304mm (300 x 300 DPI)



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304x227mm (300 x 300 DPI)

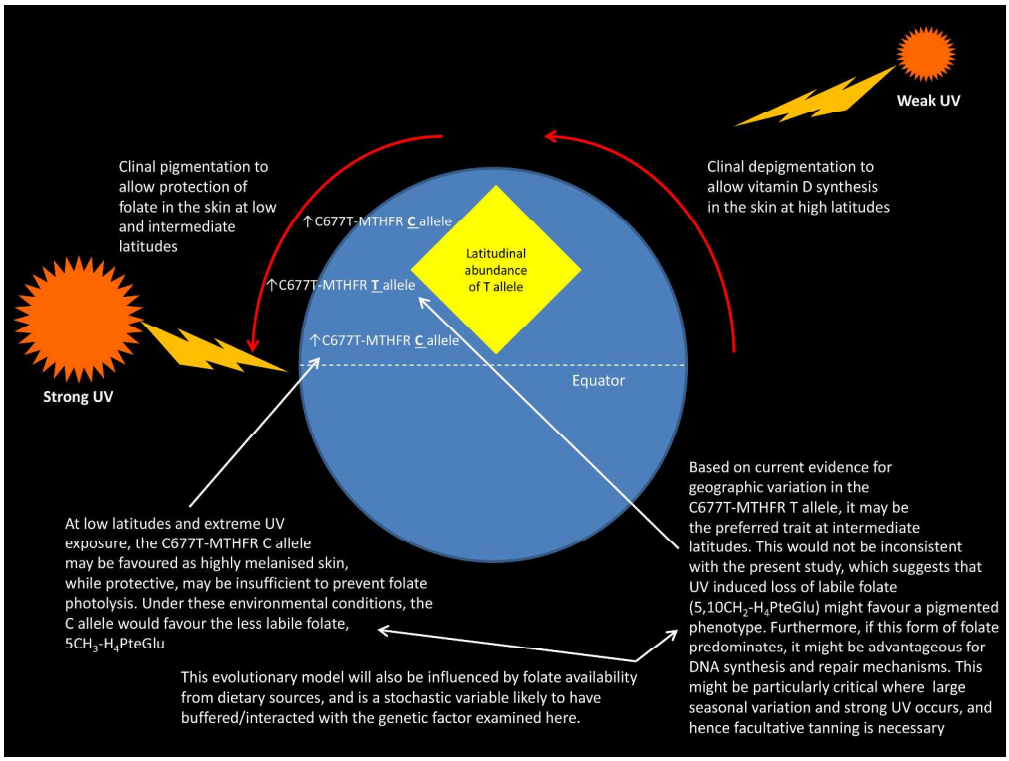

Figure 3: Schematic of the evolutionary model for the "folate-vitamin D-UV hypothesis of skin pigmentation", with MTHFR genotype as an important component.
299x224mm (300 x 300 DPI)