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Does promoter methylation of the *SLC30A5* (ZnT5) zinc transporter gene contribute to the ageing-related decline in zinc status?

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A decline in Zn status with ageing may contribute to the development of frailty, including impaired immune function, and increased incidence of age-related degenerative diseases. This decline may be a result of reduced dietary Zn intake and/or impaired Zn absorption in the gut. The Zn transporter ZnT5 may play a key role in the absorption of dietary Zn. The corresponding gene (*SLC30A5*) has a CpG island in its promoter region, so could be regulated by epigenetic mechanisms. It is hypothesised that methylation of the *SLC30A5* promoter region is increased with age and that a resulting reduction in ZnT5 expression contributes to the decline in Zn status observed with ageing. This hypothesis has been addressed through (1) studies of effects of *SLC30A5* promoter methylation on gene expression *in vitro* and (2) *in vivo* measurements of the DNA methylation status of this gene domain. It has been established *in vitro* that methylation of the human *SLC30A5* promoter region results in reduced expression of an associated reporter gene. Second, this gene region shows variable levels of methylation *in vivo*. Correlation between the level of methylation at this locus and age would support the hypothesis that age-related hypermethylation of this region has the potential to modulate dietary Zn absorption. This premise is being investigated by analysis of additional samples from a human adult cohort to test the hypothesis that methylation of the *SLC30A5* promoter region contributes to the age-related decline in Zn status.

Zinc status and ageing: Zinc transporter gene: Age-related promoter hypermethylation

Nutritional and biological importance of Zn

Adequate dietary Zn intake is essential for the maintenance of optimal health (reference nutrient intake is 10·2 and 7·4 mg/d for men and women respectively)[1]. Zn is not stored in the body but is present in all organs and tissues, with greatest concentrations found in bone, liver, kidney, muscle and skin[2]. The lack of a Zn store within the body invokes the requirement for a constant adequate supply of dietary Zn. Primary food sources of Zn are the protein-rich foods; seafood, lean beef and chicken are therefore key sources of readily-absorbed Zn (Table 1). Zn is an essential component in the structure or function of hundreds of enzymes, spanning all six enzyme classifications[3,4]. Thus, Zn is central to a number of biological functions within the human body (Table 2) and deficiency is potentially detrimental to human health in many ways (Table 3).

Zinc deficiency

Zn deficiency is a global problem; the WHO estimates that annually >700 000 deaths in children aged 0–4 years are a result of the consequences of Zn deficiency[5,6]. Based on individual requirements for Zn and the absorbable content of each nation's food supply, it has been estimated that approximately 20·5 % of the world's population are at increased risk of inadequate dietary Zn intake[7]. Zn

Abbreviation: DNMT, DNA methyltransferase.

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Table 1. Dietary zinc food sources (data from US Department of Agriculture[53])

Food	Measure	Zn (mg per measure)
Oyster, wild, raw	Six medium	76·3
Crustaceans, crab, cooked	85 g	6·48
Baked beans, canned	One cup	5·79
Beef, top sirloin	85 g	4·64
Roasted turkey	One cup	4·34
Kidney beans, canned	One cup	4·20
Plain yoghurt, low-fat	227 g	2·01
Pine nuts, dried	28·3 g	1·83
Swordfish, cooked	One piece	1·56
Salted cashew nuts, with salt	28·3 g	1·52
Boiled spinach	One cup	1·37
Cooked mushrooms	One cup	1·36
Whole-wheat spaghetti	One cup	1·13

deficiency may not be a result of inadequate intake *per se* but may arise through elevated Zn requirements, decreased absorption efficiency or even genetic predisposition. Acrodermatitis enteropathica, a genetic condition first described in 1943, predisposes an individual to Zn deficiency as a result of a defective intestinal Zn transporter, ZIP4 (*SLC39A4* gene), which is functionally involved in dietary Zn absorption within the duodenum and jejunum[8,9].

Deficiency is not easily diagnosed because of the tight homeostatic control of blood plasma Zn concentration. For example, 6 months on a Zn-deficient diet (2·6 mg Zn/d compared with the reference nutrient intake of 7·4 mg Zn/d for women) did not induce significant changes in either plasma Zn levels or in the activity of Zn-containing enzymes, such as carbonic anhydrase[10]. Natural daily fluctuations in plasma Zn status, which accompany ever changing inflammatory status or infection levels within the body, also contribute to complications in using plasma Zn levels as a marker of Zn status (for review, see Fairweather-Tait *et al.*[11]).

The risk of Zn deficiency is increased in regions of the world where diets are typically low in animal protein and high in plant-based foods containing higher concentrations of Zn-chelating phytates, which reduce dietary Zn absorption[5]. Furthermore, in countries with foods containing readily available and absorbable Zn some population subgroups may be at increased risk of Zn deficiency. For example, pregnant women and growing infants have an increased demand for Zn and this requirement must be met via dietary intake. Individuals with malabsorption conditions such as Crohn's disease may need to supplement their Zn intake to maintain adequate Zn status[12]. It is recognised that dietary Zn intake is negatively associated with increased age, resulting in increased risk of compromised Zn status in the elderly. The third US National Health and Nutrition Examination Survey has shown that median Zn intakes are lower in the elderly compared with younger individuals in white, Hispanic and black populations (reported intakes for 30–39-year-old males of 13·88, 13·19, 10·77 mg/d *v.* reported intakes for males >80 years of 9·06, 7·74 and 7·04 mg/d respectively)[13]. Median Zn intakes for females in the same populations show similar reductions in the older age-groups. In addition, the UK National Diet and Nutrition Survey reported a decrease in mean daily dietary Zn intakes from 10·2 and 7·4 mg/d for males and females respectively at age 19–64 years to 8·4 and 7·1 mg for men and women respectively at age $\geqslant$65 years[1,14]. This decreased Zn intake may reflect a lower demand for, and intake of, total dietary energy among older individuals and it may reflect a move towards lower animal-protein consumption as older individuals become increasingly concerned with cholesterol levels[15].

In contrast with studies of Zn intake during ageing, there have been few studies of the effect of ageing on Zn absorption. Studies carried out in the 1980s have noted that Zn absorption is reduced in elderly subject groups compared with younger groups. For example, mean Zn absorption from a purified formula diet (15 mg Zn/d for 12 weeks) for subjects aged 22–30 years and 65–74 years was reported to be 31% and 17% respectively[16]. Similarly, mean Zn absorption from an adequate-Zn diet (12·8–15 mg Zn/d for 2 weeks) was found to be 39% and 21% for subjects aged 18–22 years and 67–83 years respectively[17].

It is becoming increasingly apparent that many characteristics of ageing, such as increased inflammatory status and decreased immune function, are also characteristic of Zn deficiency[18]. Dramatic increases in inflammatory status amongst the elderly[15] may be linked to metallothionein function. Metallothioneins are a family of cysteine-rich metal-binding proteins involved in Zn homeostasis[19]. During chronic inflammation metallothioneins release Zn ions to stimulate the activity of numerous antioxidants, subsequently reducing oxidative damage[18,20]. During ageing metallothioneins have been found to sequester Zn rather than releasing Zn in response to inflammatory stimuli, so that the inflammatory response may be compromised in elderly populations[21,22]. In addition, osteoporosis, a disease characterised by reduced bone mass, becomes more common with age and has been linked with compromised Zn status. A significant increase in renal Zn excretion has been reported in post-menopausal women with osteoporosis compared with post-menopausal healthy controls ($P = 0{\cdot}002$)[23]. This observation was suggested to be a compensatory mechanism to maintain plasma Zn levels following the release of Zn during increased bone resorption in patients with osteoporosis.

The concept that ageing may have a direct effect on dietary Zn absorption has only begun to be explored. A potential mechanism responsible for altered Zn absorption is differential expression of the Zn transporters involved in the absorption of Zn from the intestinal lumen and this aspect is explored in a recent review[11].

Zinc absorption

Zn is absorbed primarily in the proximal small intestine[24], involving specific Zn-transport proteins. Mammalian Zn transporters play a key role in the tight regulation of Zn

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Table 2. Examples of the biological functions of zinc

Biological process	Function of Zn	References
Inflammation	Zn is released from metallothioneins in response to inflammatory exposure: Stimulates the activity of numerous antioxidants Acts to reduce oxidative damage	Frazzini *et al.*[20], Vasto *et al.*[22]
DNA and protein synthesis	Zn is a cofactor for many enzymes: DNA polymerase, RNA polymerase and reverse transcriptase DNA-binding Zn finger proteins enhance protein–DNA interactions promoting gene transcription	Truong-Tran *et al.*[54], Vallee *et al.*[55]
Bone metabolism	Zn has been shown to: Stimulate osteoblast activity Inhibit osteoclast formation Zn deficiency retards bone growth Women with osteoporosis excrete higher concentrations of urinary Zn	Yamaguchi & Hashizume[56], Kishi & Yamaguchi[57], Herzberg *et al.*[58], Oner *et al.*[59]
Immunity	Zn deficiency is associated with impaired immune response: Reduced T lymphocyte proliferation and function	Vallee & Falchuk[4], Prasad[60]
Skin	Patients with acrodermatitis enteropathica (a disease characterised by Zn deficiency) manifest scaly skin patches that become vesicular, pustular and if untreated may develop into alopecia	Maverakis *et al.*[61]
Taste	Zn supplements have been shown to prevent and treat taste disorders and enhance taste acuity, which may be a result of the involvement of Zn as a component of gustin, a protein component of saliva	Stewart-Knox *et al.*[62], Yamagata *et al.*[63], Stoll & Oepen[64], Takeda *et al.*[65]

Table 3. Symptoms associated with zinc deficiency (from Hambridge *et al.*[66] and World Health Organization[5])

Severe Zn deficiency symptoms
Growth retardation
Delayed sexual and bone maturation
Skin lesions
Diarrhoea
Alopecia
Impaired taste
Marginal Zn deficiency symptoms
Reduced growth rate
Impairment of immune defence
Impaired taste
Impaired wound healing

homeostasis[18] and are classified into two metal-transporter families, ZRT or IRT-like proteins (ZIP transporters; classified as solute carrier family SLC39) and the cation diffusion facilitators (ZnT transporters; classified as solute carrier family SLC30). Members of the SLC39 family have been found to promote influx of Zn into the cytosol of the cell, whereas those of the SLC30 family reduce cytosolic Zn concentration by either Zn efflux from the cell or the sequestration of Zn into intracellular compartments[25]. To date, a number of human Zn transporters have been characterised. Some of these transporters are expressed in the intestine, the site of Zn absorption, including ZIP4[26], ZnT1[27] and ZnT5[28,29]. ZnT5 (*SLC30A5* gene), a member of the CDF family, is expressed in the intestine, localised to the apical membrane of the enterocyte, and is down regulated in response to increased Zn supply[30]. The human splice variant, ZnT5 variant B, acts as a bidirectional Zn transporter when expressed in *Xenopus laevis* oocytes, i.e. it is active in both the uptake and efflux of Zn[25]. The bidirectional function of ZnT5 suggests a possible role in dietary Zn absorption.

The functions of ZnT5 do not appear to be restricted to its proposed role in dietary Zn absorption. ZnT5-knock-out mice display poor growth with decreased bone density and hunched backs[31]. These observations have linked ZnT5 to the maturation of osteoblasts into osteocytes. In addition, >60% of male ZnT5-deficient mice were reported to have died aged 15–40 weeks as a consequence of sudden cardiac failure[31], suggesting an additional role of ZnT5 in the cardiac conduction system.

It seems probable that intestinal Zn absorption does not rely on a single Zn transporter. As mentioned earlier, the Zn-deficiency condition acrodermatitis enteropathica results from defective function of the ZIP4 transporter, which is localised to the apical membrane of the enterocyte and is involved in dietary Zn absorption[9,26]. The fact that appropriate dietary Zn supplementation alleviates Zn deficiency symptoms in patients with acrodermatitis enteropathica suggests another transporter, such as ZnT5, may compensate for the lack of transport by ZIP4.

Epigenetic regulation of gene expression

Epigenetics describes a number of genomic modifications, principally DNA methylation and histone modifications (phosphorylation, methylation and acetylation), which result in a heritable change in gene expression with no corresponding change in DNA sequence[32]. DNA methylation involves the addition of a methyl group to deoxycytosine to form deoxymethylcytosine within a CG dinucleotide (CpG site)[33]. CpG sites may be clustered in regions of higher frequency termed CpG islands, which are defined as regions of DNA >200 bp in length with >50% content being C and G residues[34]. DNA methylation is mediated by the DNA methyltransferase (DNMT) enzyme family, which includes DNMT1, 3a and 3b. DNMT1 is responsible for the maintenance of DNA methylation patterns and has a preference for hemi-methylated DNA,

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whereas DNMT3a and 3b are associated with the *de novo* methylation of previously unmethylated CpG sites[35,36].

These DNMT are essential for normal embryonic development[37,38]. During early mammalian embryogenesis most methylation marks on the human genome are removed, with the main exceptions being imprinted genes[39]. DNA methylation patterns are subsequently reprogrammed during early development[39]. In addition to maintenance of specific methylation patterns throughout life, methylation patterns may also be maintained from one generation to another. It has been demonstrated that feeding pregnant rats (F0) a protein-restricted diet results in altered methylation patterns of specific promoter regions including PPARα and the glucocorticoid receptor. These diet-induced changes in methylation patterns are subsequently maintained in both the F1 and F2 generations[40], demonstrating trans-generational inheritance of methylation patterns. Inter-individual variation in health outcomes and in responses to nutritional factors at different stages of the life course may be influenced by the patterns of DNA methylation, which are unique to each individual[41].

The progression of ageing and the development of cancer are associated with aberrant DNA methylation patterns, typically global hypomethylation and gene-specific hypermethylation[33]. Specific cancer types demonstrate differing patterns of hypermethylation of tumour-suppressor-gene promoters, e.g. *MLH1* in colon cancer[42] or *BRAC1* in breast cancer[43]. It has been assumed that aberrant methylation of gene promoter regions, such as the hypermethylation of the promoter for *IGF2* (encoding insulin-like growth factor 2) observed with age[44], may result in substantial modifications of gene expression.

Specific dietary components are known to affect mammalian DNA methylation status[45]; for example, through affecting the supply of methyl groups as substrates for the DNA methylation reaction or through affecting the activity of specific DNMT[46]. Rats fed Zn-deficient diets have markedly decreased liver DNA methylation status[47]. The methionine cycle, key in the production of the universal methyl donor *S*-adenosylmethionine, is sensitive to a number of micronutrient deficiencies including Zn[48]. The depletion in DNA methylation following exposure to Zn-deficient diets may be the result of a reduction in the function of Zn-dependent enzymes in the methionine cycle, such as betaine-homocysteine methyltransferase[48,49].

The potential influence of DNA methylation on Zn transporter gene expression

The effects of ageing on the expression and/or function of Zn transporters such as ZnT5 are unknown. To date there has been little investigation of the effects of typical age-related epigenetic events on methylation of the promoter regions of the genes encoding such transporters and how their expression, and ultimately function, may be compromised in ageing. It is hypothesised that epigenetic modifications of specific Zn transporters may influence Zn absorption and therefore Zn status in the elderly.

Since there is a CpG island in the *SLC30A5* gene promoter region the expression of ZnT5 may potentially be regulated by its promoter methylation status. Data based on expression of a reporter gene downstream of the *SLC30A5* promoter expressed in the human intestinal Caco-2 cell line from a plasmid construct demonstrate that *in vitro* methylation of the *SLC30A5* gene promoter region represses reporter gene expression (LJ Coneyworth, KA Jackson, JC Mathers and D Ford, unpublished results), indicating that *SLC30A5* promoter activity is modulated by methylation status. If this effect is confirmed *in vivo* then methylation of the *SLC30A5* promoter region may result in decreased Zn absorption from the intestinal lumen. Pyrosequencing (Biotage, Uppsala, Sweden) is currently being used to measure methylation at individual CpG dinucleotides in the region 946 bp upstream of the *SLC30A5* coding region in three adult cohorts from northern England. Preliminary data from these studies show correlations between methylation of specific CpG sites in the *SLC30A5* promoter and age (LJ Coneyworth, KA Jackson, JC Mathers and D Ford, unpublished results), which support the hypothesis that age-related reduction in ZnT5 expression in the intestine may contribute to the decline in Zn status observed with ageing. Differential methylation of individual CpG sites within a promoter region, acquired with ageing, may be critical in determining the expression of the associated gene if the site is important for binding of an essential transcription factor and binding is sensitive to the methylation status of the CpG[50]. The effects of methylation of individual CpG dinucleotides, alone and in combination, on transcription factor binding to a key regulatory region of the *SLC30A5* promoter are currently being studied. The functional consequences of altered promoter methylation will be examined by establishing whether ZnT5 expression at the RNA level changes in parallel with age-related changes in promoter methylation. Detailed molecular studies of this nature are required to demonstrate links between observed ageing-related changes in gene methylation and functional effects.

Changes in promoter methylation patterns in the elderly may therefore influence the absorption of Zn, providing a possible mechanism for the reduction of Zn status with age. Expression of other Zn transporters involved in dietary Zn absorption may also be potentially regulated by DNA methylation. ZnT1 is expressed in the intestinal mucosa, localised to the basolateral membrane of the enterocyte, and is involved in the transport of Zn from the enterocyte into the portal blood[27,51]. Since there is a CpG island within the promoter region of this gene[52] it is reasonable to speculate that ZnT1 expression, and subsequently transport of Zn into the portal blood, may be modified as a consequence of age-related changes in DNA methylation status.

Concluding remarks

Zn deficiency may contribute to compromised immune response, systemic inflammation and physical frailty amongst the elderly. Whilst many studies have investigated the decline in dietary Zn intake with increased age,

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currently the effects of age on dietary Zn absorption, specifically the expression of intestinal Zn transporters, are poorly understood. DNA methylation is an epigenetic event, known to be modified by age, which results in altered gene expression. Age-related modifications in the promoter methylation status of specific Zn transporters such as ZnT5 (potentially involved in dietary Zn absorption) may contribute to diminished Zn absorption with age and, ultimately, reduced Zn status. Further research is required to investigate the effects of ageing on the methylation status of genes involved in Zn absorption and to probe underlying molecular mechanisms through which observed ageing-related changes in methylation exert their functional effects.

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