Vitamin D Deficiency: The Invisible Accomplice of Metabolic Endotoxemia?

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Abstract: The aetiology of obesity is multi-factorial. Recent research has identified a novel association between endotoxaemia (circulating lipopolysaccharide in the systemic circulation) and low-grade inflammation in the adipose organ, which may contribute to obesity. The mechanisms for the low-grade elevation of circulating lipopolysaccharide in obesity are poorly understood.

Vitamin D has been increasingly recognised for its pleiotropic actions beyond maintenance of musculoskeletal health. The parathyroid-vitamin D axis is altered in obesity. Circulating vitamin D levels are lower in obese individuals. The regulatory role of vitamin D in the immune system and colonic mucosa may explain the under-appreciated contribution of vitamin D deficiency in the obese to the pathogenesis of endotoxaemia and adipose inflammation.

We propose a hypothetical model linking metabolic endotoxaemia with vitamin D deficiency in obesity. A therapeutic approach involving the use of probiotics and vitamin D metabolites in the obese is described.

INTRODUCTION

While the aetiology of obesity is multifactorial, high-energy diet and physical inactivity are major obesogenic factors in the modern obesity epidemic. Recent research has identified a novel association between inflammation and obesity. Specifically inflammation in the adipose organ may be secondary to endotoxaemia, defined as the entry of altered gut flora into the systemic circulation, resulting in low-grade elevation of circulating endotoxin (lipopolysaccharide (LPS)), [1-3]. However the mechanisms linking endotoxaemia and systemic inflammation with the immune system and the strong human genetic predisposition to obesity are not clear [4]. Vitamin D, which is increasingly recognised for its pleiotropism beyond its known effects in bone and calcium metabolism [5], may represent an important link between inflammation and obesity.

The parathyroid-vitamin D axis is altered in obesity: circulating vitamin D levels are lower in obese individuals [6-9], possibly secondary to physical inactivity and lack of sunlight exposure [10,11], with sequestration of vitamin D in adipose tissue [12,13]. A hyperactive parathyroid-vitamin D axis, characterised by vitamin D deficiency and secondary hyperparathyrodism, has not only been associated with obesity [6,7] but also increased obesity-related cardiovascular mortality [14].

With recent unmasking of the regulatory role of vitamin D in the immune system [15,16] and colonic mucosa [17], we propose a hypothetical model of metabolic endotoxaemia with vitamin D deficiency in obesity.

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GUT MICROBIOTA AND METABOLIC ENDOTOXAEMIA

Gut microbiota exist in a symbiotic relationship with the host, performing important roles including stimulating epithelial growth, enhancing mucosal immunity and fermenting dietary fat to provide short-chain fatty acids [18,19]. With a probable evolutionary advantage, some microbiota potentiated host survival by increasing energy extraction from food [20]. In our modern environment, however, alteration of gut flora, with associated increased entry into the systemic circulation (endotoxaemia), may increase obesity. "Conventionalisation" of germ-free mice with usual intestinal organisms increased monosaccharide absorption and hepatic lipogenesis, while suppressing expression of fasting-induced adipocyte factor (FIAF), which inhibits lipoprotein lipase (LPL), thus promoting adipocyte triglyceride storage [20].

The nature of the bacterial flora in the gastrointestinal tract has been shown to influence the degree of endotoxemia and inflammation in adipose tissue [1-3]. In mice, antibiotic treatment significantly altered the bacterial flora, leading to reduction of LPS levels, inflammation, oxidative stress and diabetes [21,22]. The *CD14* receptor and its signalling co-receptor, toll-like receptor 4 (*TLR4*), are important in the innate immune response to microbes [23]. LPS receptor *CD14* knockout and germ free mice were resistant to weight gain during overfeeding [20,24]. High-fat feeding also increased intestinal permeability by reducing expression of genes coding for tight junction proteins [22].

The consequence of metabolic endotoxaemia from changes in gut flora induced by high-fat feeding in a "leaky" gut is an inflammatory microenvironment in visceral and omental fat and liver, produced by macrophage infiltration with increased local production of *PAI-1, IL-1* and *TNF-*α

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mRNA [22,25]. However it is unclear whether endotoxaemia alone causes an inflammatory microenvironment in adipose tissue *via* local *TLR4* receptors.

In obesity, there may be an altered immune response to gut flora, as the innate immune response is under genetic influence [26], hence compounding effects of endotoxaemia with subsequent chronic low-grade inflammation in the liver and adipose tissue. Specific obesogenic environmental regulators of host innate response to LPS have not yet been reported.

VITAMIN D AND OBESITY

The parathyroid-vitamin D axis is influenced by adiposity: circulating 25-hydroxy vitamin D (25-OH D) levels are lower in obese subjects [5-8] and may be associated with secondary hyperparathyroidism [27]. The negative correlation between vitamin D and obesity has been attributed to decreased physical activity and lack of sunlight exposure in obese individuals, decreased vitamin D synthesis in subcutaneous fat and sequestration of vitamin D in adipose tissue. Using liquid chromatography mass spectrometry, mean subcutaneous fat D3 was 102.8 nmol/kg compared to only 7.78 nmol/L in serum in 17 obese subjects [28].

The negative correlation between vitamin D and obesity could be interpreted differently. Could vitamin D deficiency contribute to obesity? Loss of body fat in women with higher serum 25-OH D (>50 nmol/L) was greater than those with lower levels during a hypocaloric diet [29]. In observational studies, ionised parathyroid hormone (PTH) levels are higher in obese than non-obese young adults [30,31], declining with weight loss. Similarly, hyperparathyroid postmenopausal women have a greater fat mass and more android fat distribution than do age-matched controls [32]. PTH may be involved in the postulated effect of dietary calcium on body composition [33]. Secondary hyperparathyroidism due to vitamin D deficiency can lead to compensatory increase in 1,25-$(OH)_2$ vitamin D (1,25-$(OH)_2$D) formation (Fig. **1**). Higher PTH levels have been shown to increase intra-adipocyte calcium concentration resulting in increased lipogenesis [33].

The relationship between 1,25-$(OH)_2$D and adiposity *in vivo* is less clear. Some [6,28,34,35] but not all [11,36] human studies reported a positive correlation between 1,25-$(OH)_2$D and BMI. Discrepancy in these studies may relate to assay variability and different degrees of PTH activation (Fig. **1**). Circulating 1,25-$(OH)_2$D level is dependent on severity of vitamin D deficiency. High 1,25-$(OH)_2$D levels may be found in obese individuals with a hyperactive PTH axis and 25-OH D deficiency. This is consistent with the *in vitro* stimulatory effects of 1,25-$(OH)_2$D on adipogenesis [37-39]. Also, there is recent evidence that VDR knock-out mice have reduced fat mass [40]. 1,25-$(OH)_2$D has been shown to inhibit uncoupling protein 1 expression in brown adipose tissue. Contrary to the traditional view that brown adipose tissue is scarce in adult humans, the advance in positron-emission tomography scanning has identified significant depots of brown adipose tissue in adult humans [41]. 1,25-$(OH)_2$D may therefore not only be adipogenic in white adipose tissue, but may contribute to obesity through blunting of thermogenesis in brown adipose tissue.

The vitamin D receptor (VDR) is present in most mammalian cells and is involved in immunity, epidermal differentiation and haemopoiesis. Whether vitamin D deficiency contributes to the low-grade inflammation reported in obesity has not been examined. However vitamin D deficiency is associated with type 2 diabetes, which is also associated with adipose tissue inflammation. There is evidence that vitamin D supplementation improves metabolic markers [42,43] and post-prandial insulin sensitivity [44]. Although inflammatory markers were not measured, there was a trend to lower C-reactive protein levels in subjects treated with vitamin D, suggesting vitamin D repletion could reduce inflammation.

HYPOTHESIS

We propose that low level endotoxaemia and vitamin D deficiency observed in obese subjects act synergistically, fuelling inflammation in the adipose organ. A high energy diet promotes a "pro-inflammatory" pattern of gut flora. The level of endotoxaemia is regulated by LPS entry from the gut as well as innate immune responsiveness, both of which are affected by vitamin D insufficiency.

Loss of Gastrointestinal Homeostasis

The relationship between circulating low-grade endotoxaemia and gut microbiota requires increased mucosal permeability. While an altered gut flora has been shown to control gut permeability [22], obesity-associated hypovitaminosis D may also contribute.

Inflammatory bowel disease (IBD) provides an extreme illustration of the role of vitamin D in maintaining mucosal integrity. VDR null mice are prone to development of IBD [45]. Vitamin D sufficient mice were resistant to dextran sulphate sodium induced colonic inflammation; VDR null mice developed mucosal ulceration and poor healing with severe disruption in epithelial junctions. Both local and systemic synthesis of 1,25-$(OH)_2$D are important as mice lacking 1-α hydroxylase are prone to colitis [46]. Mucosal disruption can be rescued by 1,25-$(OH)_2$D administration which enhanced junction protein expression. Epithelial migration was also improved by 1,25-$(OH)_2$D with resultant mucosal healing [47,48].

The importance of the VDR in colonic mucosa in the pathogenesis of IBD is demonstrated through the induction of severe IBD by deletion of VDR in *Il-10* KO mice [49]. VDR expression in *CD4* T cells may regulate bacterial antigen-dependent responses in the gut. Without appropriate signalling *via* the VDR, an inappropriate local inflammatory response is initiated to otherwise harmless gut flora. This animal model provides further evidence that vitamin D insufficiency may trigger local colonic inflammation to microbiota, which may increase endotoxaemia, a phenomenon associated with both obesity and IBD.

Loss of gastrointestinal homeostasis in the obese may therefore be a consequence of chronic low-grade mucosal inflammation due to poor healing and loss of mucosal integrity from vitamin D insufficiency leading to deficit of local 1,25-$(OH)_2$D synthesis. A high-energy diet with associated changes in gut flora could further exacerbate chronic mucosal micro-injury, exacerbating metabolic endotoxaemia.

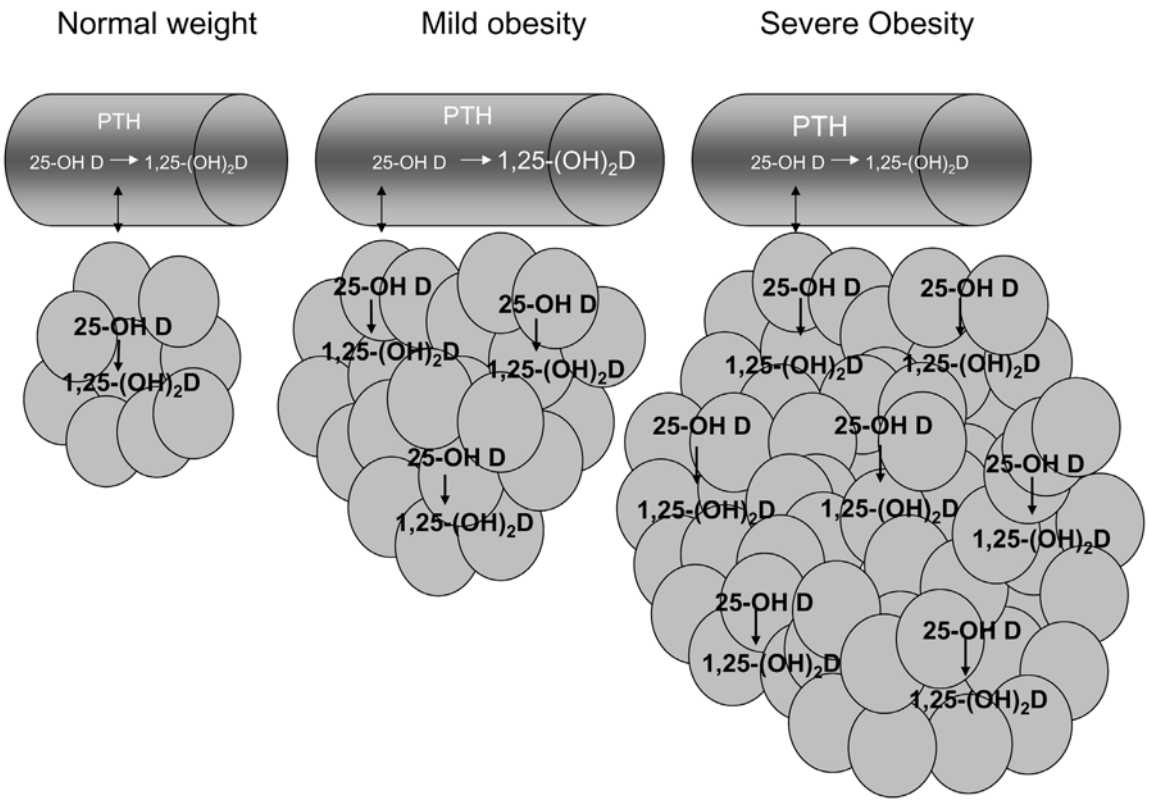

Fig. (1). Schematic diagram illustrating progressive dis-equilibrium between endocrine and paracrine PTH-vitamin D systems. Increased adiposity leads to progressive sequestration of 25-OH D, secondary hyperparathyroidism and increase local synthesis of 1,25-(OH)2D, which further fuels lipogenesis. Circulating 1,25-(OH)2D is variable depending on PTH and 1α-hydroxylase activity.

Altered Immune Response to LPS

Vitamin D and its metabolites play an intricate role in the immune system. The key activating enzyme, 1α-hydroxylase, encoded by *CYP27B1*, regulates both the adaptive and innate immune system through local synthesis of 1,25-$(OH)_2$D. Experimental evidence supports an inhibitory role of the active metabolite, 1,25-$(OH)_2$D, in the adaptive immune response, and a stimulatory role in the innate system [16,17].

LPS interacts specifically with *TLR-4* receptor on the surface of immune cells, which leads to nuclear activation and expression of antimicrobial peptides, such as LL-37 [50]. Proinflammatory cytokines, such as TNF-α, are produced from adipocytes, which contribute to low-grade systemic inflammation. It is uncertain whether the inflammatory response is systemic, or whether elevated circulating inflammatory markers "spill over" from local inflammatory foci, such as the adipose, hepatic or other organ. For circulating LPS to induce chronic low-grade systemic inflammation, there must be i) increased LPS entry into circulation and ii) incomplete eradication by the immune system and probably iii) local inflammatory foci such as adipose tissue, liver, endothelial plaque [51].

In addition to mucosal leakiness (see Section on *Loss of gastrointestinal homeostasis*), entry of LPS into systemic circulation may also be increased by alteration in mucosal immunity. Vitamin D deficiency impairs the expression of the human cathelicidin antimicrobial protein (CAMP) in macrophages [15,16,52]. Failure of mucosal CAMP up-regulation has been postulated as a cause of mucosal inflammation leading to endotoxaemia in patients with Crohn's disease [53]. As vitamin D deficiency is highly prevalent in adults with Crohn's disease [54], it supports a link between vitamin D insufficiency, impaired innate immunity and colonic mucosal inflammation, not dissimilar to the endotoxaemia and low grade inflammation in obesity.

However, the reasons for the discordant immune response resulting in impaired LPS clearance and exaggerated local tissue inflammation are not known. Indeed, while increased infiltration of adipose tissue by macrophages is observed in the more obese individuals, the pathophysiology of macrophage accumulation in adipose tissue is not clear [55]. Interestingly, 1,25-$(OH)_2$D has been shown to mediate macrophage-adipocyte cross-talk. Expression of macrophage inhibitory factor (MIF) and macrophage surface-specific protein CD14 were both increased in human adipocytes treated with 1,25-$(OH)_2$D *in vitro* [56]. Sequestered 25-OH D in adipose tissue may produce high local 1,25-$(OH)_2$D despite systemic vitamin D deficiency (Fig. **1**), leading to increased MIF and CD14-mediated macrophage survival.

Moreover, eradication of LPS from the systemic circulation may be impaired with low circulating 25-OH D, through impaired induction of CAMP expression. A recent study demonstrated CAMP levels to be significantly lower in dialysis patients, which correlated with 1,25-$(OH)_2$D levels [57].

We therefore suggest that the low grade inflammation in obese subjects is enhanced by migration of activated macro-

phages from vitamin D-insufficient systemic circulation into adipose tissue rich in locally derived lipogenic 1,25-$(OH)_2$D from D_3 sequestration (Fig. **1**).

Fig. (**2**) summarises the proposed pathogenesis of endotoxaemia and low-grade inflammation in vitamin D deficiency in a step-wise manner.

Post-Prandial Hypertriglyceridaemia, Vitamin D Absorption and Inflammation

Hypertriglyceridemia is a common dyslipidaemia associated with obesity, related to increased dense LDL cholesterol particles and lower HDL cholesterol levels [58-61]. Hypertriglyceridemia is an independent risk factor for cardiovascular complications, especially premature coronary artery disease, even after adjustment for traditional risk factors, but not LDL or HDL cholesterol sub-fractions [62]. Whether hypertriglyceridemia itself causes cardiovascular disease or is a "marker" of common metabolic abnormalities remains controversial [59,60]. Post-prandial hypertriglyceridaemia is associated with low-grade systemic inflammation in obesity. Although high fat diet promotes endotoxaemia and is associated with post-prandial hypertriglyceridaemia, the relationship between hypertriglyceridemia and endotoxaemia is uncertain.

A possible pathway is *via* the liver X receptors (LXRs) which are crucial in the regulation of lipid metabolism and

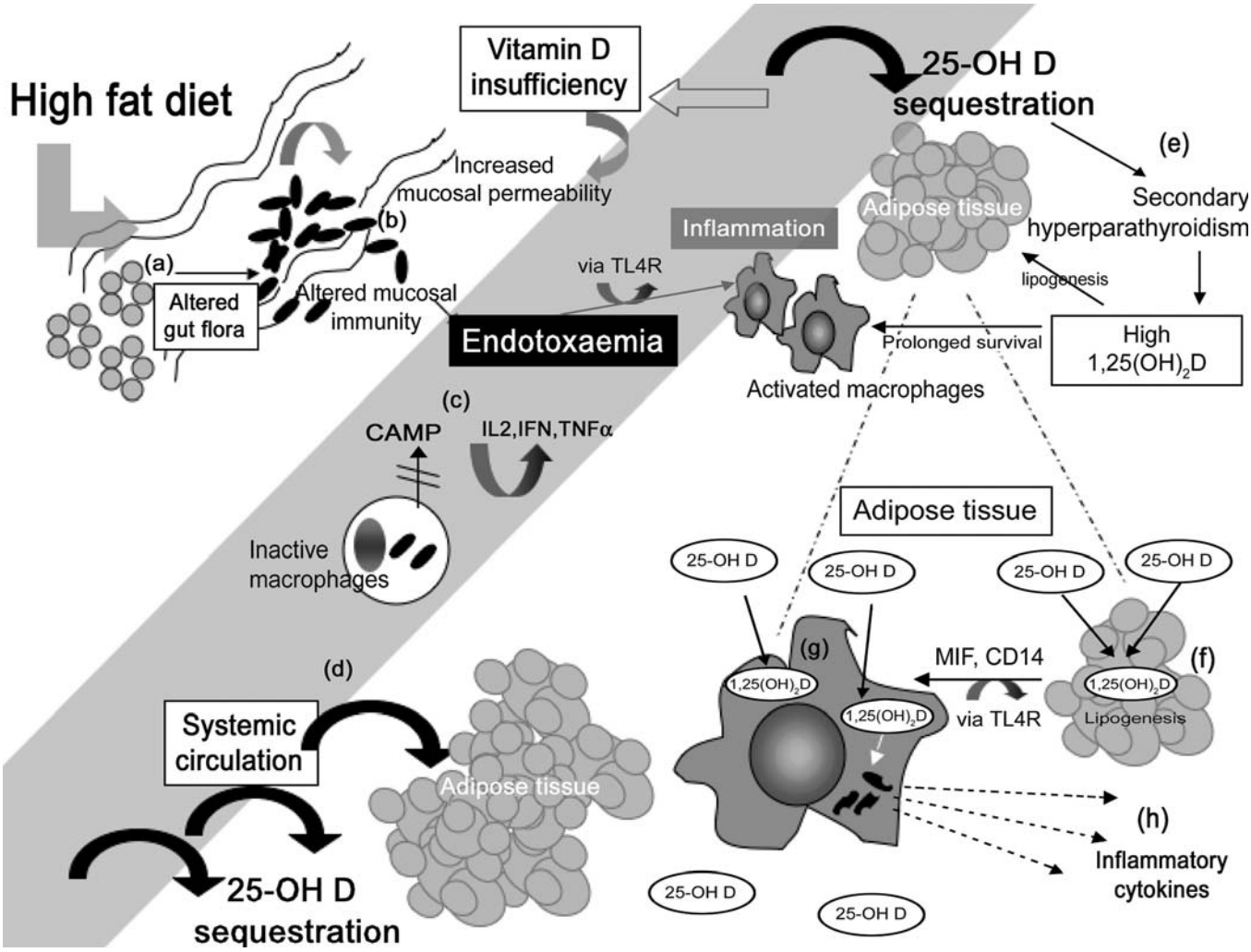

Fig. (2). Schematic diagram of the hypothetical model of endotoxaemia and vitamin D insufficiency. *Systemic circulation*: (**a**) High fat diet leads to an altered gut flora; (**b**) defective mucosal integrity and immunity due to vitamin D deficiency allows entry of LPS into systemic circulation; (**c**) systemic clearance of LPS is impaired due to blunting of innate immunity by low circulating vitamin D level, resulting in endotoxaemia; (**d**) 25-OH vitamin D is sequestered in adipose tissue. *Adipose tissue*: (**e**) secondary hyperparathyroidism and (**f**) abundant local 25-OH D leads to formation of high 1,25$(OH)_2$D; (**g**) Macrophage migration and activation is increased by high local 25-OH D with production of 1,25$(OH)_2$D; (**h**) LXR in human macrophages increases TLR4 responsiveness to LPS and increases TNFα and MCP1 release. The latter promote further migration from blood to inflamed tissues. The crosstalk results in the production of multiple cytokines and chemokines, including TNFα, MCP1 and CD14 which activate, propagate, and sustain local tissue inflammatory responses.

macrophage function. Treatment of macrophages by LXR agonists enhances LPS-induced inflammation [63], lipogenesis and hypertriglyceridemia [64]. The latter is mediated by the stimulation of angiopoietin-like protein 3, which similar to FIAF, inhibits LPL, causing hypertriglyceridaemia [65]. LXR agonists induce the expression of the LPS receptor *TLR-4* in human macrophages, as well as MCP-1 and TNF-α secretion in LPS-stimulated human macrophages. Moreover, LXR activation increases reactive oxygen species (ROS) production in LPS-activated human macrophages. Activation of *TLR-4* results in the induction of cytokine expression, which regulates cell migration and activation to sites of inflammation. In addition, LPS-activated macrophages produce ROS, which are bactericidal within the phagosome [63,64].

Importantly, vitamin D may also be an unrecognized immune regulator in the gut in the pathogenesis of endotoxaemia due to altered colonic exposure to vitamin D by hypertriglyceridaemia. Being fat soluble, absorption of vitamin D occurs mainly through the gut lymphatic system *via* chylomicrons [66,67]. High-fat diet and postprandial hypertriglyceridaemia enhance absorption of lipophilic medications, including vitamin D [68,69], which is hydroxylated by the hepatic 25-hydroxylase. However recently all 3 key D_3-hydroxylase gene transcripts (25-hydroxylase, 24-hydroxylase and 1α-hydroxylase) are found in human foetal small and large intestines [70], implying ability to activate vitamin D locally in the colon. Subsequently, colonic exposure to local vitamin D may be reduced in the obese due to high fat diet. Local vitamin D deficiency may therefore lead to colonic mucosal abnormalities, microbiota changes and endotoxaemia.

The concept of high fat diet and poor local vitamin D delivery resulting in systemic inflammation may explain the improvement in circulating inflammatory markers following gastric-bypass surgery [71]. Vitamin D deficiency is common following obesity surgery [72]. Reduction of fat absorption post-surgery simultaneously reduces local gut inflammation from nutrient load, and increases colonic exposure to vitamin D due to vitamin D malabsorption.

FOR SURVIVAL OR DESTRUCTION?

If metabolic endotoxaemia and vitamin D deficiency contribute synergistically in generation of modern obesity, the fundamental question is whether alteration in gut microbiota and vitamin D deficiency are the cause or consequence of obesity. Weight loss in obese individuals led to an increase in the proportion of Bacteroidetes and a decrease in the proportion of Firmicutes in faecal gut microbiota [1], leading to speculation that Firmicutes may promote more efficient energy extraction. This may have had survival benefit allowing such individuals to combat famine and more effectively.

Similarly seasonal storage of vitamin D in adipose tissue may also be a survival strategy. Increase in circulating vitamin D paralleled weight loss in mice [73], suggesting the adipose tissue was a seasonal buffer ensuring systemic vitamin D availability. During famine, while most endocrine function is down-regulated from weight loss, gradual release of stored vitamin D into systemic circulation may provide essential substrate for local 1,25-$(OH)_2$D synthesis, for maintenance of immunity and mucosal functions. Indeed the high mortality in lean people in famine may in part be due to impaired immune and epithelial functions from vitamin D deficiency contributing to fatal infective and diarrheal illnesses.

In summary, altered gut microbiota may precede obesity, pre-selected by the host gut by a cluster of "survival" genes, which may include genetic variations of 1α-hydoxylase activity. Tissue level 1,25-$(OH)_2$D in the gut may allows selectivity and immune tolerance for specific gut microbiota. It may be adaptive during the winter months when decreasing vitamin D allowed more obesogenic microbiota, allowing the host to gain fat, surviving the cold and hunger in winter.

Survival mechanisms become promoters of obesity with modern industrialisation. Vitamin D deficiency is increasingly prevalent as humans become less sun-exposed. Ozone, a common air pollutant, reduces cutaneous vitamin D synthesis by absorbing the essential ultraviolet radiation (290-315 nm) [74]. With increasing obesity, vitamin D is increasingly sequestered in fat stores, leading to systemic vitamin D deficiency, resulting in increased colonic permeability with enhanced immune response to LPS.

IMPLICATIONS TO THERAPEUTIC DESIGN

The intriguing relationship between vitamin D insufficiency and metabolic endotoxaemia may provide insight for novel therapeutic interventions to combat obesity. There are 2 fundamental approaches: i) manipulation of microbiota by probotics, antibiotics and/or prebiotics and ii) tissue vitamin D repletion. We advocate a combined use of probiotics to promote a "less obesogenic" microbiotic and vitamin D supplementation for immune reconstitution with maintenance of the new gut flora, therefore reducing endotoxaemia and low-grade systemic inflammation.

Manipulation of Gut Microbiota

There are over 100 trillion microbial organisms in the adult human gut, consisting of two main phyla, the Bacteroidetes and Firmicutes [1,75]. The unique composition of the adult gut microbiota is influenced by genotype and environmental exposure to micro-organisms, as early as the perinatal period [76]. Obese individuals have more Firmicutes compared to lean controls. Following a weight-loss programme in obese individuals for 12 months, the proportion of Bacteroidetes increased over Firmicutes in faecal gut microbiota [1]. Although probiotics have been used for the treatment of inflammatory bowel disease and irritable bowel syndrome, they have not been used in randomised trials for the "treatment" of obesity.

Theoretically, administration of probiotics to constitute a specific microbiota, less efficient in energy extraction and less pro-inflammatory, may reverse metabolic changes of endotoxaemia. This is supported by recent demonstration of improvement of high fat diet-induced hepatic steatosis and insulin resistance following oral probiotic treatment in mice [77]. However the reason behind the difference in microbiota between lean and obese individuals is not known. It is also uncertain how weight loss changes the microbiota. A plausible explanation is that obesity creates a gut microenvironment which pre-selects particular microbiota (ie Fir-

micutes) and weight loss alters microbial selectivity. However, therapeutic re-constitution of "less obesogenic" microbiota may not be sustainable in the obese, unless underlying factors, such as vitamin D deficiency, are corrected. Further research is required to understand how host genotype interacts with the environment, in particular local colonic and immune factors, in the constitution and maintenance of a specific microbiota.

Antibiotics are another possible therapeutic intervention for endotoxaemia, as demonstrated in animal models. However, long-term antibiotic treatment is associated with emergence of resistant organisms and development of Clostridium *difficle*-associated colitis. Until more specific antibiotics become available, it is unlikely to be a practical therapeutic option for endotoxaemia.

Tissue Vitamin D Repletion

Vitamin D repletion in the obese may appear logical, after earlier discussion of vitamin D deficiency fueling metabolic dysregulation through altered calcium balance, immune function and lipogenesis. However oral vitamin D administration has limitations: Conventional doses (1000-2000 units/day) are frequently inadequate to achieve sufficiency (>80 nmol/L) and circulating 25-OH D poorly reflects true vitamin D status, especially at the colonic epithelium. We recently showed that adequacy of vitamin D replacement in severely deficient individuals is dependent on body mass index (unpublished data) and obese individuals may require a larger dose of vitamin D supplement in deficient states.

Mega-dose vitamin D up to 300,000 units per dose may be more efficacious than conventional doses between 1000-2000 units. It has a safe therapeutic window and has been shown to be safe with minimal adverse effects [78]. Local delivery to colonic mucosa may be important: rectal administration of 1,25-(OH)2D improved DSS-colitis in mice more than oral [79].

Reduction of Post-Prandial Hypertriglyceridaemia

The extent of the pathogenic role of hypertriglyceridaemia in endotoxaemia remains uncertain. Hypertriglyceridemia-lowering medications, such as the fibrates, may modulate LPS-associated inflammation in addition to its triglyceride lowering effect. Fenofibrate therapy improves glycaemia and reduces hyperinsulinaemia and hyperleptinaemia in patients with type 2 diabetes [80]. A recent study demonstrates an increase in adiponectin levels in patients with hypertriglyceridemia and the metabolic syndrome following fenofibrate therapy, which correlate inversely with LPS-stimulated production of TNF-α, IL-1β, MCP-1 and MIP-1α [81]. Whether fibrate treatment by reducing hypertriglyceridaemia, results in better colonic vitamin D delivery and reduces LPS-associated inflammation merits further studies.

CONCLUSION

Up to 7% of total health care costs can be attributed to obesity, ranked fourth globally as a cause of morbidity and mortality. The possible contribution of microbiota to gut homeostasis and obesity is an exciting new area of research. Low vitamin D, known to be prevalent in the obese population, may be under-appreciated in the pathogenesis of endotoxaemia. The need to identify safe, simple and cost-effective means to fight obesity is urgent, but such methods currently seem distant. This hypothetical model provides hope, by combining novel research directions which allow for both testing the hypotheses and designing potential therapy.

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