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Determination of magnesium body concentration

Abstract

The presently-disclosed subject matter includes methods for detecting methylation biomarkers in a biological sample from a subject in need of assessment for magnesium deficiency status, methods of diagnosing magnesium deficiency, methods of diagnosing magnesium insufficiency, methods of treating magnesium deficiency, methods of diagnosing magnesium insufficiency, and methods of preventing or reducing a risk of developing a condition linked to magnesium deficiency.

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Background/Summary

RELATED APPLICATIONS (1) This application claims priority from U.S. Provisional Application Ser. No. 62/865,544 filed Jun. 24, 2019 and 63/042,878 filed Jun. 23, 2020, the entire disclosures of which are incorporated herein by this reference.

TECHNICAL FIELD

(1) The presently-disclosed subject matter generally relates to predicting and determining body magnesium status, treatment of magnesium deficiency, and treatment of conditions associated with magnesium deficiency.

INTRODUCTION

(2) According to the US National Health and Nutrition Examination Survey (NHANES), 79% of US adults do not meet their Recommended Dietary Allowance (RDA) of Mg.sup.3.

(3) In the US and other Western populations at high risk of Mg deficiency, epidemiologic studies have linked low Mg intake to risks of colorectal neoplasia.sup.4-6, insulin resistance.sup.7-12 systemic inflammation.sup.45;46 metabolic syndrome.sup.6-18, type 2 diabetes (T2D).sup.11;19-23 and cardiovascular disease (CVD).sup.19;24-28 although not entirely consistent.sup.26;27. Conversely, in populations not at high risk of Mg deficiency, the opposite results have been reported.sup.29. In two large-scale Chinese cohort studies, high Mg intake was associated with an increased risk of total mortality (e.g. mortality due to total cancer, colorectal cancer (CRC), and CVD) among both men and women, particularly when Ca intake was below the median.

(4) These findings are intriguing and suggest that the effects of high Mg intake may completely depend on the underlying Mg status. High-dose Mg supplementation or fortification may lead to problems in the subset of the US population who do not have Mg deficiency (i.e. at least 20%). Accordingly, it becomes critical to develop a personalized prevention strategy to minimize potential adverse effects (i.e. first identify individuals with Mg deficiency and target only those with Mg deficiency) for the prevention of CRC and other common diseases linked to Mg deficiency.

(5) Serum Mg has been used to clinically diagnose Mg deficiency.sup.1. However, only about 0.3% of the total body Mg is in serum. Further, serum Mg is frequently still in the normal range even when an individual has actual Mg deficiency because concentrations of serum Mg are tightly regulated.sup.31;47;48. This is very similar to serum Ca, which is also tightly regulated in a narrow range.sup.49-53 If serum Mg is under 0.7 mmol/l, a clinical diagnosis of Mg deficiency is made.sup.31. Serum Mg may be a good biomarker in the clinic for patients with severe symptomatic Mg deficiency.sup.1. Only 10% of all patients with Mg deficiency are likely to be diagnosed if the current critical value of 0.7 mmol/l is used.sup.31. Based on the data from a published study, if the critical value is elevated to 0.80 or 0.85 mmol/l, 34% or 40% of non-deficient subjects, respectively, were misclassified as Mg deficient.sup.54. Outside of serum, the remainder of the approximately 25 grams (g) of Mg in humans is in soft tissue (19%), bone (53%), and muscle (27%).sup.47;48. It is not practical to routinely measure Mg in bone or muscle through biopsy.

(6) A prior study found patients with Mg deficiency frequently had abnormal Mg tolerance tests in spite of a normal serum level of Mg.sup.55;56. Moreover, Lukaski et al..sup.57, among 10 postmenopausal women, conducted a feeding study using low dietary Mg levels based on ordinary Western foods, in amounts taken by some Americans. After administration of this low Mg diet for three months, serum Mg was slightly reduced by 6% ($p=0.07$), but still within the normal range. However, there was a significant reduction in skeletal muscle Mg ($p<0.05$).sup.57. Muscle Mg levels returned to normal after 49 days of a Mg repletion diet 57. Simsek et al. conducted a study among patients newly diagnosed with insulin dependent diabetes. They found that the plasma concentration of Mg was lower among diabetes patients than among normal controls, but, again, the level was still in the normal range. However, compared with normal controls, patients with diabetes had significantly higher Mg retention after intravenous (IV) infusion of Mg in the Mg tolerance test. A recent study found that the correlation between dietary intake of Mg and blood Mg was poor ($r=0.02$).sup.58. In the ongoing trial, $r=0.11$ ($p=0.66$) was found between serum Mg and the Mg tolerance test. Thus, serum Mg is a poor test for discriminating Mg deficiency.sup.1;32.

(7) Although the Mg tolerance test is the most accurate approach currently available for measuring Mg status.sup.1, it has several major limitations that prevent its adoption in both clinical and research practice. First, it cannot be used in patients with renal dysfunction or critical illness.sup.30. Secondly, it requires two 24-hour urine samples and a 4-hour IV Mg infusion,.sup.1 making it impractical to implement in the clinic. Even in research trials using Mg supplementation, the Mg tolerance test cannot be used to measure Mg status at baseline or to monitor treatment effects or compliance because it substantially changes Mg status due to the 4-hour IV infusion of Mg.sup.59.

(8) Accordingly, there is a need in the art for a sensitive, specific, and more implementable method to assess Mg status and to treat Mg deficiency and prevent conditions associated therewith.

SUMMARY

(9) The presently-disclosed subject matter meets some or all of the above-identified needs, as will become evident to those of ordinary skill in the art after a study of information provided in this document.

(10) This Summary describes several embodiments of the presently-disclosed subject matter, and in many cases lists variations and permutations of these embodiments. This Summary is merely exemplary of the numerous and varied embodiments. Mention of one or more representative features of a given embodiment is likewise exemplary. Such an embodiment can typically exist with or without the feature(s) mentioned; likewise, those features can be applied to other embodiments of the presently-disclosed subject matter, whether listed in this Summary or not. To avoid excessive repetition, this Summary does not list or suggest all possible combinations of such features.

(11) The presently-disclosed subject matter includes methods for detecting methylation biomarkers in a biological sample from a subject in need of assessment for magnesium deficiency status, methods of diagnosing magnesium deficiency, methods

of diagnosing magnesium insufficiency, methods of treating magnesium deficiency, methods of diagnosing magnesium insufficiency, and methods of preventing or reducing a risk of developing a condition linked to magnesium deficiency.
(12) In some embodiments, the presently-disclosed subject matter includes a method of detecting methylation biomarkers in a biological sample from a subject. In some embodiments, the method involves obtaining the biological sample from the subject, wherein the subject is in need of assessment for magnesium deficiency status; and detecting 5-hydroxymethylcytosine (5-hmC) and 5-methylcytosine (5-mC) methylation biomarkers and/or differentiating between 5-hydroxymethylcytosine (5-hmC) and 5-methylcytosine (5-mC) methylation biomarkers.
(13) In some embodiments, the presently-disclosed subject matter includes an improved method for determining magnesium deficiency status in a subject as compared to detecting serum magnesium levels. In some embodiments the method involves detecting 5-hydroxymethylcytosine (5-hmC) and 5-methylcytosine (5-mC) methylation biomarkers and/or differentiating between 5-hydroxymethylcytosine (5-hmC) and 5-methylcytosine (5-mC) methylation biomarkers in a biological sample from the subject.
(14) In some of the embodiments of the methods disclosed herein, bisulfite (BS) treatment of DNA is used to detect overall methylation biomarkers in the biological sample. In some of the embodiments of the methods disclosed herein, a TAB-Seq and TAB-Array protocol is used to differentiate between 5-hydroxymethylcytosine (5-hmC) and 5-methylcytosine (5-mC) methylation biomarkers in the biological sample.

Description

BRIEF DESCRIPTION OF THE DRAWINGS
(1) The novel features of the invention are set forth with particularity in the appended claims. A better understanding of the features and advantages of the present invention will be obtained by reference to the following detailed description that sets forth illustrative embodiments, in which the principles of the invention are used, and the accompanying drawings of which:
(2) FIG. **1** is a flow chart showing the design of an exemplary study used to identify and assess methylation modifications disclosed herein for use in assessing magnesium status.
(3) FIG. **2** illustrates the Mg and Cytosine Modification Pathway.
(4) FIG. **3** includes the results of a 5-hmC affinity enrichment sequencing analysis of rectal tissues.
(5) FIG. **4** depicts a TAB-array protocol to identify 5-hmC and 5-mC.
(6) FIGS. **5**A-**5**D include bar graphs depicting stratified distribution of exemplary markers cg00430271 and cg25731074.
DESCRIPTION OF EXEMPLARY EMBODIMENTS
(7) The details of one or more embodiments of the presently-disclosed subject matter are set forth in this document. Modifications to embodiments described in this document, and other embodiments, will be evident to those of ordinary skill in the art after a study of the information provided in this document. The information provided in this document, and particularly the specific details of the described exemplary embodiments, is provided primarily for clearness of understanding and no unnecessary limitations are to be understood therefrom. In case of conflict, the specification of this document, including definitions, will control.
(8) The presently-disclosed subject matter includes methods for detecting methylation biomarkers in a biological sample from a subject in need of assessment for magnesium deficiency status, methods of diagnosing magnesium deficiency, methods of diagnosing magnesium insufficiency, methods of treating magnesium deficiency, methods of diagnosing magnesium insufficiency, and methods of preventing or reducing a risk of developing a condition linked to magnesium deficiency.
(9) The presently-disclosed subject matter includes methods for detecting methylation biomarkers in a biological sample from a subject in need of assessment for magnesium deficiency status, methods of diagnosing magnesium deficiency, methods of diagnosing magnesium insufficiency, methods of treating magnesium deficiency, methods of diagnosing magnesium insufficiency, and methods of preventing or reducing a risk of developing a condition linked to magnesium deficiency.
(10) In some embodiments, the presently-disclosed subject matter includes a method of detecting methylation biomarkers in a biological sample from a subject. In some embodiments, the method involves obtaining the biological sample from the subject, wherein the subject is in need of assessment for magnesium deficiency status; and detecting 5-hydroxymethylcytosine (5-hmC) and 5-methylcytosine (5-mC) methylation biomarkers and/or differentiating between 5-hydroxymethylcytosine (5-hmC) and 5-methylcytosine (5-mC) methylation biomarkers.
(11) In some embodiments, the presently-disclosed subject matter includes an improved method for determining magnesium deficiency status in a subject as compared to detecting serum magnesium levels. In some embodiments the method involves detecting 5-hydroxymethylcytosine (5-hmC) and 5-methylcytosine (5-mC) methylation biomarkers and/or differentiating between 5-hydroxymethylcytosine (5-hmC) and 5-methylcytosine (5-mC) methylation biomarkers in a biological sample from the subject.
(12) In some of the embodiments of the methods disclosed herein, bisulfite (BS) treatment of DNA is used to detect overall methylation biomarkers in the biological sample. In some of the embodiments of the methods disclosed herein, a TAB-Seq and TAB-Array protocol is used to differentiate between 5-hydroxymethylcytosine (5-hmC) and 5-methylcytosine (5-mC) methylation biomarkers in the biological sample.
(13) In some of the embodiments of the methods disclosed herein, the methylation biomarkers include one or more of the methylation biomarkers set forth in Appendixes B-G.
(14) In some of the embodiments, the methods disclosed herein further involve identifying the subject has having an MTT score of greater than or equal to 50 (magnesium deficiency), an MTT score of less than 50 and greater than or equal to 25 (magnesium insufficiency), or an MTT score of less than 25 (magnesium sufficiency). In some embodiments, the MTT score

is determined using a method as described in Example 1.
(15) In some of the embodiments, the methods disclosed herein further involve identifying the subject as having a magnesium deficiency when one or more of the methylation biomarkers set forth in any one of Tables 3-42 are detected in the sample. In some embodiments, two, three, four, five, six, seven, eight, nine, ten or more biomarkers are detected.
(16) In some of the embodiments, the methods disclosed herein further involve administering an effective amount of magnesium to the subject identified as having a magnesium deficiency.
(17) Some embodiments of the presently-disclosed subject matter include a method of preventing or reducing a risk of developing a condition linked to magnesium deficiency. In some embodiments, the methods involve determining magnesium deficiency status in a subject using the methods as disclosed herein; identifying the subject as having a magnesium deficiency when one or more of the methylation biomarkers associated with magnesium deficiency are detected in the sample; or identifying the subject as having a magnesium insufficiency when one or more of the methylation biomarkers associated with magnesium insufficiency are detected in the sample; and administering an effective amount of magnesium to the identified subject.
(18) While the terms used herein are believed to be well understood by those of ordinary skill in the art, certain definitions are set forth to facilitate explanation of the presently-disclosed subject matter.
(19) Unless defined otherwise, all technical and scientific terms used herein have the same meaning as is commonly understood by one of skill in the art to which the invention(s) belong.
(20) All patents, patent applications, published applications and publications, GenBank sequences, databases, websites and other published materials referred to throughout the entire disclosure herein, unless noted otherwise, are incorporated by reference in their entirety.
(21) Where reference is made to a URL or other such identifier or address, it understood that such identifiers can change and particular information on the internet can come and go, but equivalent information can be found by searching the internet. Reference thereto evidences the availability and public dissemination of such information.
(22) As used herein, the abbreviations for any protective groups, amino acids and other compounds, are, unless indicated otherwise, in accord with their common usage, recognized abbreviations, or the IUPAC-IUB Commission on Biochemical Nomenclature (see, Biochem. (1972) 11(9):1726-1732).
(23) Although any methods, devices, and materials similar or equivalent to those described herein can be used in the practice or testing of the presently-disclosed subject matter, representative methods, devices, and materials are described herein.
(24) The present application can “comprise” (open ended) or “consist essentially of” the components of the present invention as well as other ingredients or elements described herein. As used herein, “comprising” is open ended and means the elements recited, or their equivalent in structure or function, plus any other element or elements which are not recited. The terms “having” and “including” are also to be construed as open ended unless the context suggests otherwise.
(25) Following long-standing patent law convention, the terms “a”, “an”, and “the” refer to “one or more” when used in this application, including the claims. Thus, for example, reference to “a cell” includes a plurality of such cells, and so forth.
(26) Unless otherwise indicated, all numbers expressing quantities of ingredients, properties such as reaction conditions, and so forth used in the specification and claims are to be understood as being modified in all instances by the term “about”. Accordingly, unless indicated to the contrary, the numerical parameters set forth in this specification and claims are approximations that can vary depending upon the desired properties sought to be obtained by the presently-disclosed subject matter.
(27) As used herein, the term “about,” when referring to a value or to an amount of mass, weight, time, volume, concentration or percentage is meant to encompass variations of in some embodiments ±20%, in some embodiments ±10%, in some embodiments ±5%, in some embodiments ±1%, in some embodiments ±0.5%, in some embodiments ±0.1%, in some embodiments ±0.01%, and in some embodiments ±0.001% from the specified amount, as such variations are appropriate to perform the disclosed method.
(28) As used herein, ranges can be expressed as from “about” one particular value, and/or to “about” another particular value. It is also understood that there are a number of values disclosed herein, and that each value is also herein disclosed as “about” that particular value in addition to the value itself. For example, if the value “10” is disclosed, then “about 10” is also disclosed. It is also understood that each unit between two particular units are also disclosed. For example, if 10 and 15 are disclosed, then 11, 12, 13, and 14 are also disclosed.
(29) The presently-disclosed subject matter is further illustrated by the following specific but non-limiting examples. The following examples may include compilations of data that are representative of data gathered at various times during the course of development and experimentation related to the present invention.

EXAMPLES

Example 1

(30) Magnesium Tolerance Test
(31) In a magnesium tolerance test, a health care professional, such as a doctor, gives a participant a shot of a known amount of magnesium and then later uses the participant's urine to determine how much magnesium the participant's body retained, and how much the body eliminated.
(32) If the participant's body retains most of the magnesium then it is assumed he or she needed it because of a deficiency, and if most of it is eliminated in the urine then it is assumed that the magnesium was not needed because he or she does not have a deficiency.
(33) In an exemplary study as described herein, participants who completed the study received a schedule of planned magnesium tolerance tests on two consecutive days. A 24-hour urine sample was collected for determining the basal urinary

magnesium excretion prior to the intravenous infusion of magnesium sulfate. Another 24-hour urine sample was obtained starting with the IV infusion.

(34) Procedure

(35) 1. Collect 24-hour urine the day before the load test for basal magnesium and creatinine ratio. 2. Infuse 0.2 mEq (2.4 mg) elemental magnesium per kilogram of body weight in 50 ml of 5% detrose over 4 hours, which was conducted by nurses. 3. Collect urine (starting with infusion) for magnesium and creatinine for 24 hours 4. Check the blood pressure and heart rate for the participant before initiating magnesium sulfate IV. Infusion, and monitor the same vital signs in the course of infusion and within 1 hour following infusion completed. 5. Deliver two 24-hour urine samples to the Molecular Epidemiology Lab at Vanderbilt Epidemiology Center. 6. Handle and process the urine samples followed by the protocols, and then stored at −80° C. until urine magnesium/creatinine assay were applied. 7. Measure 24-hour urine magnesium, creatinine concentration using an atomic absorption spectrophotometer, provided by the Vanderbilt Pathology Laboratory Services.

Body Magnesium Status 1. Percentage magnesium retained is calculated by the formula below (Mg: magnesium)

(36)

$$[1 - \frac{(\text{post infusion Mg excretion} - \text{basal Mg excretion}^*)}{\text{total Mg infused}}] \times 100 \quad * \text{basal Mg excretion} = \frac{\text{pre-infusion urine Mg}}{\text{pre-infused urine Creatinine}} \times \frac{\text{post-infusion 24 hour urine}}{\text{Creatinine}}$$

2. Criteria for magnesium status ≥50% retention at 24-hour urine: definite deficiency >25% to <50% retention at 24-hour urine: probable deficiency (insufficiency) ≤25% retention at 24-hour urine: normal (sufficiency)

Example 2

(37) Blood Leukocyte Methylation as a marker of Mg Deficiency The majority of Mg is stored in bone.sup.47;48. Although it is very similar for Ca, the Ca content in bone is 40 to 50 times higher than Mg.sup.60. Thus, bone density is conventionally used to measure body status of Ca, but not Mg. About 30% of bone Mg is exchangeable and serves to stabilize serum concentration of Mg.sup.61 because maintaining normal serum Mg is so critical that low serum Mg could be fatal.sup.62;63 Thus, the serum Mg concentration is unlikely to significantly drop until the exchangeable stores of Mg in bone are used up. Diet with moderate depletion of Mg led to reduction in bone Mg content, but no significant change in serum Mg.sup.64. Recent studies found that Mg concentrations are essential for the differentiation.sup.65, metabolism and activities.sup.66 of human osteoclasts. Notably, hematopoietic stem cells (HSC) and osteoclasts are linked.sup.67 and osteoclasts are important components of niches for HSCs in bone marrow, from which the majority of blood leukocytes (or white blood cells) develop.

(38) It is known that DNA methylation changes are inducible by environmental exposures, including nutrients.sup.34, and reversible when the exposure disappears.sup.35. Thus, methylation biomarkers have potential to be biomarkers of nutrient status. It is likely reduced Mg status in bone affects the differentiation (i.e. methylation) of HSC into blood leukocytes. Since the concentrations of Mg in bone drop long before serum Mg decreases, it is possible that methylation biomarkers in blood leukocytes affected by low Mg concentrations are promising biomarkers for Mg deficiency. Further, the rapid turn-over for the majority of blood leukocytes ranging from a few hours to a few days mean methylation biomarkers in leukocytes may readily reflect the changes in Mg status in bone. Thus, the present inventors contemplated sensitive methylomic markers in blood leukocytes for Mg deficiency.

(39) 5-hmC is a Newly Emerged Epigenetic Biomarker. DNA methylation at the cytosine in CpG dinucleotides, the most common epigenetic modification.sup.68;69, regulates gene function without changing primary DNA coding sequence.sup.70. Although DNA methylation research has increased substantially over the past three decades, previous studies were limited by the inability to distinguish between the two major cytosine modifications: 5-methylcytosine (5-mC) and 5-hydroxymethylcytosine (5-hmC). 5-hmC can be generated by the oxidation of 5-mC through the ten-eleven translocation (Tet) family of enzymes (See FIG. **2**).sup.70, and 5-hmC has distinct regulating functions in gene expression from 5-mC. In fact, it was not until very recently, in 2009 over 50 years after its discovery, that 5-hmC was “re-discovered” to be the 6.sup.th base in the human genome.sup.71-73. Although Tet proteins can further oxidize 5-hmC to 5-formylcytosine (5-fC) and 5-carboxylcytosine (5-caC), previous studies found the levels of 5-hmC were 100-fold higher than 5-fC and 5-caC.sup.74-79. Therefore, 5-mC and 5-hmC are the two major types of cytosine modifications in DNA.

(40) 5-mC is often associated with suppressed gene expression.sup.80;81 while 5-hmC is specifically enriched in expressed genes and plays a critical role in activating and/or maintaining gene expression.sup.36;82. Thus, without differentiating these two major cytosine modifications with distinct and opposite effects on gene expression, the findings from previous studies are difficult to interpret. In a recent study, the present inventors found 5-hmC may be a more sensitive marker than 5-mC for reflecting the changes in environment exposure. In fact, current epigenome-wide association study (EWAS) profiling platforms, including the widely-used Illumina Infinium HumanMethylation450 BeadChip.sup.83 (HM450K), cannot distinguish between 5-mC and 5-hmC. However, as described herein, there is a unique technique, Tet-assisted Bisulfite Sequencing (TAB-Seq) and TAB-Array protocol.sup.42-44 (See FIG. **4**), which now allows accurate distinguishing of 5-hmC from 5-mC in the whole human genome.

(41) Mg, 5-hmC and 5-mC and Methylation Capacity. The present inventors found Mg intake is significantly and positively linked to global methylation pattern (i.e. LINE-1) and methylation biomarkers in genes/pathways with Mg as a co-factor. As shown in FIG. **2**, two useful co-factors for the Tets to catalyze the oxidation of 5-mC to 5-hmC are Fe (iron).sup.2+ and α-ketoglutarate.sup.70. Previous studies found Mg affects metabolism of α-ketoglutarate.sup.84;85. Thus, it is very likely Mg may also affect the activity of Tets and, in turn, the oxidation of 5-mc to 5-hmC (See FIG. **2**). It was found among those at risk of Mg deficiency Mg treatment may lead to substantially more changes, primarily upregulation, in 5-hmC (e.g. 296 genes with ≥4-fold changes in 5-hmC peaks in treatment arm compared to 39 genes in placebo arm) in colorectal tissues compared to the placebo arm (See section D1.1.3 in detail). Although the mostly up-modified changes in 5-hmC caused by Mg treatment are consistent with mostly up-modified changes also found in both in vitro and in vivo studies of vitamin C.sup.86;87, the top differential CpG sites (either 5-mC or 5-hmC) linked to Mg treatment/intake do not overlap with those caused by vitamin

C.sup.86;87, indicating the changes in 5-mC or 5-hmC levels are unique to each nutrient. Thus, 5-mC and 5-hmC may be used as both specific and sensitive biomarkers for Mg status.
(42) S-adenosylmethionine (SAM), the second most widely used substrate molecule in the cell.sup.88, is the methyl donor for almost all methylation reactions including DNA methylation (FIG. **2**), which has profound physiologic consequences. S-adenosylhomocysteine (SAH) is a product of the transfer of a methyl group from SAM to 80 other molecules and is the metabolic precursor of all homocysteine (Hey) produced in the body in an irreversible reaction in which the equilibrium favors SAH. SAM has two products, a methylated molecule, such as DNA, and SAH. As SAH level increases, it prevents conversion of SAM to SAH.sup.89;90. This, in turn, results in decrease in DNA methylation.sup.91;92. Based on a strong biological basis, it is not surprising that positive associations between DNA methylation and circulating levels of SAM and SAH have been observed in previous studies. Thus, SAM and SAH levels are considered as the methylation capacity.sup.93-97. In a pilot study conducted in a randomized clinical trial, it was found that Mg treatment affected SAH level vs. placebo arm in those at risk of Mg deficiency.
(43) Methylation Markers Can be Altered by the Changes in Nutritional Status in a Dose-Response Manner. There is mounting evidence that nutritional factors differentially involved directly or indirectly in methylation and hydroxymethylation affect 5-mC or 5-hmC. In the following, folate and vitamin C will be used as examples to present further evidence that 5-hmC and 5-mC can serve as sensitive biomarkers of Mg status.
(44) Folate indirectly affects SAH (FIG. **2**) and has been widely studied for its potential effect in methylation. The findings from human, animal and in vitro studies indicate folate affects global as well as gene-specific and (gene) region-specific methylation biomarkers. An in vitro study using the sensitive Comet approach found folate depletion led to “a dose-dependent response” in hypomethylation while folate supplementation reversed the global and (gene) region specific (e.g. p53) hypomethylation caused by folate depletion.sup.98. Similar to in vitro findings, there are two human randomized trials of moderate folate depletion followed by moderate folate repletion.sup.99;100. Both trials consistently found that leukocyte genomic methylation.sup.99;100 decreased in response to moderate folate depletion. One trial.sup.99 found folate repletion reversed the hypomethylation caused by folate depletion while the other trial.sup.100 indicated it may take longer to reverse hypomethylation among elderly women. The authors concluded that “DNA methylation status may be used as a functional indicator of moderately depleted folate status”.sup.100. Consistent with this finding, other randomized trials found folate supplementation did not change leukocyte methylation in normal subjects.sup.101;102, but reversed methylation among high risk populations (e.g. those with hyperhomocysteinemia).sup.103;104 However, none of these previous studies were able to differentiate 5-hmC from 5-mC.
(45) Vitamin C is important in reducing F.sup.3+ to F.sup.2+, one of two key co-factors for the Tets to catalyze the oxidation of 5-mC to 5-hmC while Mg affects the other key cofactor (i.e. α-ketoglutarate).sup.70 (FIG. **2**). One study published in *Nature*.sup.86, found vitamin C supplementation enhanced Tet activity and, in turn, altered DNA methylation and gene expression pattern in embryonic stem cells. Similar to the observed Mg effect on 5-hmC, vitamin C supplementation led to a genome-wide increase in 5-hmC but a decrease in 5-mC. The change in 5-hmC in response to vitamin C supplementation was quicker than that on 5-mC. Both 5-hmC and 5-mC changes are correlated with over 2-fold changes, primarily up-modifications, in expressions of approximately 200 genes. Furthermore, the effect of vitamin C supplementation was reversible on both 5-hmC and 5-mC in promoter, and 5-hmC also more rapidly responded to vitamin C withdrawal than 5-mC. Further, the observed changes in 5-hmC are specific to vitamin C, but not other antioxidants (i.e. glutathione, selenite, vitamin E, vitamin B1, L-carnitine and α-lipoic acid). Another study.sup.87 found similar results in embryonic stem cells. In the mouse model deficient in vitamin C synthesis, although there were overlaps between 5-mC changes and 5-mC alterations caused by vitamin C treatment, specific genes with elevated 5-hmC in response to vitamin C treatment were identified. Further, vitamin C supplementation significantly elevated 5-hmC concentrations in all three tissues, but decreased 5-mC in two tissues; indicating 5-hmC may be a more sensitive biomarker than 5-mC to reflect changes in nutritional status.
(46) From the examples of folate and vitamin C it can be seen that 1) changes are specific to individual nutrients and these methylation changes locate in specific genes and gene regions, 2) changes are dependent on dose and duration, 3) changes likely have functional significance (i.e. linked to gene expression), 4) changes could differ by tissue or cell type, and 5) 5-hmC may more rapidly and sensitively respond than 5-mC to changes in nutritional status. These indicate 5-hmC and 5-mC biomarkers respond to nutritional status in a dose-response and nutrient-specific manner.
(47) Unlike folate, which may only indirectly affect SAH, or vitamin C, which solely influences the conversion from 5-mC to 5-hmC, Mg may affect leukocyte differentiation in bone and circulation SAH as well as the conversion from 5-mC to 5-hmC. As such, the combination of 5-hmC and 5-mC biomarkers in blood leukocytes are contemplated to serve as sensitive biomarkers unique for Mg deficiency. Further, these 5-hmC and 5-mC biomarkers have functional significance given its regulatory function of gene expression.
(48) During Mg depletion process, Mg concentration in bone drops long before a significant decrease in serum Mg.sup.64. Reduced Mg concentrations in bone are contemplated to affect the differentiation of bone HSC, the primary source of blood leukocytes and, thus, lead to modifications in blood leukocyte 5-mC and 5-hmC. Further, due to the rapid turn-over in blood leukocytes, the status of 5-mC and 5-hmC in blood leukocytes may readily reflect bone Mg status. This is supported by the four lines of novel pilot finding suggesting that Mg treatment affects both methylation capacity and the conversion from 5-mC to 5-hmC among those at high risk of Mg deficiency (D1.1). Thus, it is further contemplated that blood 5-mC and 5-hmC biomarkers, particularly when combined with serum Mg, are more sensitive and specific than serum Mg to differentiate Mg deficiency vs. non-deficiency. Thus, the proposed study uniquely identifies methylation biomarkers for Mg deficiency. Unlike serum Mg, the 5-mC and 5-hmC biomarkers have potential functional significance given their regulatory function of gene expression. The investigation includes studying the role of top modified CpG (5-hmC and 5-mC) loci in gene expression to

identify functional biomarkers for Mg deficiency.
(49) The current EWAS profiling platforms, including the widely-used Illumina HM450K.sup.83, cannot distinguish 5-hmC from 5-mC, two major types of modified cytosines with distinct regulating functions.sup.36;36;73;80-82;105. A state of the art technique.sup.42-44 has been developed, which now allows accurate distinguishing of 5-hmC from 5-mC methylation in the whole human genome. In addition, integrative analyses will be conducted to determine whether an index developed from combinations of biomarkers from multiple pathways achieve better prediction than a single biomarker. Finally, a personalized prevention application will be investigated by examining whether the newly identified biomarkers can be used to identify individuals who derive chemopreventive benefit for CRC from Mg supplementation.
(50) Importance of Methylomic Biomarkers in Clinics and Screening. In 2014, a screening multi-target tool which combines aberrantly methylated BMP3 and NDRG4 promoter regions with mutations in KRAS and β-actin has been approved by the FDA for colorectal neoplasia screening in asymptomatic individuals at average risk of CRC. Using this tool, a study published in *New England Journal of Medicine*.sup.106 found DNA tests (i.e. DNA methylation and mutations) have much higher sensitivity (Se) and specificity (Sp) to identify CRC or precancerous lesions than clinically used hemoglobin biomarkers in fecal samples with a Se of 92.3% for CRC. A qPCR-based approach was used to determine DNA 5-mC biomarkers and DNA mutations. The success of this screening tool indicates it is practical to use an index derived from combined methylation biomarkers in clinical or general populations. Similar to this study, a qPCR-based approach is used to quantify both 5-mC and 5-hmC biomarkers. A composite index combining 5-mC and 5-hmC biomarkers with serum Mg levels will be used to identify individuals with Mg deficiency. The clinical use of this composite index is tested for the prevention of CRC among those with Mg deficiency.
(51) Importance of a Sensitive Biomarker of Mg Status for Disease Prevention and DRI Revision. Previous studies indicate that the associations between Mg intake and risk of CRC and cardiometabolic disease completely differ by deficient or sufficient Mg status.sup.29. Even among US and Western populations at high risk of Mg deficiency, individual studies have generated inconsistent results on the association between Mg intake with colorectal neoplasia.sup.4-6, T2D.sup.19, coronary heart disease.sup.19;24-27 and stroke.sup.28. Similarly, the associations have also been inconsistent by using serum/plasma.sup.27;58;107-113. One possible explanation for the inconsistent findings is that Mg intake may not be a sensitive measure of Mg status. A recent study found that the correlation between dietary Mg and blood Mg was poor (r=0.02).sup.58. On the other hand, only about 0.3% of the total body Mg is in serum and concentrations of serum Mg are tightly regulated.sup.31;47;48.
(52) In addition to Mg intake, many other factors affect body overall Mg status. These factors include 1) other minerals (e.g. Ca).sup.49;51;114-119; 2) alcohol drinking.sup.120;121; 3) disease conditions (e.g. type 2 diabetes).sup.122; 4) medications, such as medications commonly used to treat reflux symptoms (i.e. proton pump inhibitors).sup.123-126, thiazide diuretics (i.e. one antihypertensive medication).sup.127, cisplatin-based chemotherapy medications.sup.128, cetuximab.sup.129 (i.e. a monoclonal antibody inhibitor of the epithelial growth factor receptor), lipid lowering medications (e.g. statins).sup.130; and 5) genetic polymorphisms.sup.131 (e.g. TRPM7.sup.49;132). Thus, an accurate measure of overall body Mg status which can reflect many known and unknown factors affecting Mg status is necessary.
(53) Although the Mg tolerance test is currently the most accurate approach to measure Mg status.sup.1, this approach has several major limitations which prevent its adoption in the clinic, prospective human studies, and randomized clinical trials (See B1.3 for details). Therefore, it is critical to develop simpler, more sensitive, and more specific biomarkers for Mg deficiency which reflect the aggregate factors affecting Mg status.
(54) In growing recognition of the importance of Mg in human health, very recently, Mg was selected for updating of the recommended intake level by the US and Canadian Federal Dietary Reference Intake (DRI) Committee. However, previous studies have used either Mg intake or serum Mg and generated inconsistent results using these metrics on which the new DRI review will also be based. Thus, the new DRI will still be controversial because, due to non-differential misclassification, it is likely that the true associations of low Mg status with CRC or cardiometabolic disease risk may be much stronger than those observed. As such, in April 2015, a workshop has been organized by the American Society for Nutrition (ASN) to discuss the challenges in accurately measuring Mg status. If the proposed study succeeds, a new composite index will be developed. The composite index will be used in randomized or cohort studies to accurately examine the associations and understand the etiology between Mg status and the above-mentioned common diseases, which will lay a solid foundation for future DRI revision.
(55) Importance of Sensitive and Functional Biomarkers for Precision Medicine. Using the Mg tolerance test.sup.1, studies conducted in 5 different US populations have found mean retention rates of Mg in excess of 50%, which is the criterion to define Mg deficiency.sup.2. In an ongoing trial, it was found, after 12-week Mg supplementation, that 42% of individuals still had Mg deficiency. These findings indicate a large proportion, but not all of the US population, is at risk of Mg deficiency. As mentioned, in contrast to the US population at high risk of Mg deficiency, in populations with lower intake of Ca and medications related to Mg status, high Mg intake has been related to an increased risk of total mortality (e.g. total cancer, CRC, and CVD), particularly when Ca intake is low.sup.29.
(56) Thus, a simpler biomarker or index will be useful for the success of personalized medicine for the prevention of Mg deficiency and other common diseases (i.e. CRC and CVD) related to Mg deficiency. In this application, a sensitive composite index is proposed to identify individuals who still have serum Mg within a normal range, but have a reduced body Mg status which already leads to 5-mC and 5-hmC modifications in DNA. Further, the functional significance of these biomarkers will be examined by profiling gene expression. Thus, the functional significance of the biomarkers included in the final composite index will be known. This new index can be used to identify individuals with Mg deficiency in the general population, clinics, or clinical trials for personalized prevention that maximizes efficacy and minimizes adverse effects. The effect of Mg

supplementation on biomarkers related to CRC carcinogenesis is examined.
(57) Mg treatment on plasma methylation capacity. Mg is essential in over 300 biological activities.sup.149, including multiple processes in genomic stability.sup.38;39. A study was conducted to evaluate whether Mg supplementation affects SAM and SAH (biomarkers for methylation capacity) among those at high risk of Mg deficiency. The finding suggests change in Mg status causes alteration in methylation capacity and, in turn, leads to methylation changes.
(58) 5-hmC alterations induced by Mg treatment. As mentioned, due to technological limitations, distinguishing 5-hmC from 5-mC has only recently been possible. Using the technology an evaluation was conducted of 5-hmC in normal rectal mucosa collected at baseline from 7 participants of the PPCCT. 5-hmC-specific affinity pull-down.sup.142 samples were sequenced using the Illumina next-generation sequencing (50 bp single-end). The raw sequencing reads were mapped to the human genome reference (hg19) using Bowtie2.sup.153. 5-hmC peaks (i.e. enriched 5-hmC regions) were then called using the MACS algorithm.sup.154. There are two important findings. 1) There are approximately 13,000-15,000 high quality 5-hmC peaks across the genomes at p<1e-5 calculated by MACS. 2) The distribution plot in FIG. **3** shows that there is apparent inter-individual variation in 5-hmC levels with a distinct genic distribution (e.g. much higher in the gene body).
(59) Genome-wide 5-hmC was also measured in pre- (i.e. baseline) and post-treatment rectal tissues (i.e. after 3-month Mg supplementation). In this analysis, one individual was included in the treatment arm and the other in the placebo arm. Two-hundred ninety-six (296) and 1271 genes were found with ≥4-fold or ≥3-fold changes in 5-hmC levels from baseline, respectively, in the treatment arm compared to 39 and 176 in the placebo arm (Table 1). Thus, the treatment arm has a much larger number of affected genes than the placebo arm.
(60) Furthermore, the majority of the changes found in 5-hmC caused by Mg treatment are up-modifications, very consistent with findings from in vitro and in vivo studies of vitamin C treatment.sup.86;87. Functional annotation analysis using the NIH/DAVID tool.sup.155 indicated that the 296 genes at ≥4-fold in the treatment arm were enriched with such Gene Ontology biological processes as “regulation of Ras protein signal transduction”, “regulation of small GTPase mediated signal transduction”, “regulation of ARF protein signal transduction”, and “Wnt receptor signaling pathway” relative to the genome reference at a FDR <10%, indicating that, although with a limited sample size, differential 5-hmC modifications in relevant pathways (e.g. Ras and GTPase as Mg plays a key role in energy metabolism.sup.37-41) could be detected with Mg treatment.
(61) TABLE-US-00001 TABLE 1 Genes with greatest changes in 5-hmC peaks Fold change Number of genes Number of genes in 5-hmC in the treatment arm in the placebo arm ≥4 296 39 ≥3 1271 176
(62) 5-hmC may be a sensitive biomarker of environmental exposure. In a recent study, global 5-mC and 5-hmC were measured in blood DNA by ELISA and evaluated the effects of ambient particulate matter (PM10) on blood global 5-mC and 5-hmC levels in two highly exposed groups of adults in Beijing, China from The Beijing Truck Driver Air Pollution Study.sup.156-158. The study found that exposure to ambient PM10 increased 5-hmC over time from 20% to 133% with p values ranging from 0.01 to <0.001), but not of 5-mC. This finding suggests 5-hmC is a sensitive biomarker in reflecting the changes in environmental exposure.
(63) The finding suggests 5-hmC is a sensitive biomarker in reflecting environmental change. Low Mg intake was linked to blood leukocyte global methylation and differentially methylated CpGs in genes with Mg as a co-factor. Although Mg intake is not an accurate measure of Mg status, it was found in a trial that 3-month Mg treatment significantly altered SAH, one biomarker of methylation capacity (D1.1.1) and may lead to changes in 5-hmC (D1.1.3) among individuals at risk of Mg deficiency. The change in SAH was about 20% while 296 genes had ≥4-fold change in 5-hmC levels. On the other hand, in a previous human study, 3-month Mg depletion reduced serum Mg from 0.85 to 0.81 mmol/L (p=0.07), and subsequent 49 days of Mg repletion increased the serum Mg by 6% from 0.81 to 0.86 (p=0.06).sup.57. Taken together, the findings, although preliminary, indicate that the combination of 5-hmC and 5-mC may serve as a more sensitive biomarker of Mg deficiency than serum Mg.
(64) When comparing the top differential CpGs (5-hmC or 5-mC) linked to Mg treatment/intake and those affected by vitamin C.sup.86;87, there was not any overlap found, indicating the 5-mC or 5-hmC can be specific biomarkers for Mg status. Further, the 5-mC and 5-hmC biomarkers have potential functional significance.
(65) High total Ca or Mg was reported to be related to a reduced risk of colorectal adenoma or hyperplastic polyps when the Ca/Mg ratio was below the median.sup.159. In a subsequent analysis in a large-scale randomized clinical trial in the US, it was found that Ca treatment significantly reduced colorectal adenoma recurrence risk only when the baseline dietary Ca/Mg ratio was under 2.6.sup.160. Furthermore, it was found that the Ca/Mg intake ratio significantly interacted with the common Thr1482Ile polymorphism (rs8042919, G.fwdarw.A) in TRPM7 (transient receptor potential melastatin 7), in relation to both adenomas and hyperplastic polyps. Based on these findings, a trial is being conducted among 240 colorectal polyp patients with Ca/Mg ratio ≥2.6. Results will determine 1) if reducing the Ca/Mg ratio through Mg supplementation affects biomarkers related to Mg homeostasis and CRC carcinogenesis (e.g. TRPM7, apoptosis, cell proliferation and COX expression in rectal biopsies); and 2) whether the effects differ by TRPM7 genotype.
(66) The study is a double-blind, placebo-controlled randomized trial conducted in Nashville, TN, comparing 12 weeks of Mg supplementation with placebo. Participants are generally healthy, have a history of colorectal polyps, are aged 40 to 85 years, do not have histories of cancer, IBD and colon resection, haveTRPM7 genotypic data, are not using anti-coagulant medications, and have a baseline Ca/Mg ratio ≥2.6, among other inclusion and exclusion criteria. 240 participants are expected to be recruited and randomized to either Mg treatment or placebo with permuted-block randomization.
(67) A Mg tolerance test, the most accurate approach to evaluate Mg nutriture, is conducted at the end of trial. Participants are collecting a 24-hour urine sample at home. The next day, at their clinic visit, after confirmation of adequate renal function, participants receive 0.2 mmol/kg body weight of Mg sulfate in 500 ml of 5% glucose by intravenous infusion over a four hour period. A second 24-hour urine sample begins at the time of the infusion and continues through the next day. For the Mg

tolerance test, the participant's percent retention is calculated by the following formula:
[1−(post infusion Mg excretion-pre infusion Mg excretion)/total Mg infused]×100.
(68) A retention of ≥50% indicates Mg deficiency.sup.161. 42% of the subjects are Mg deficient at the end of the trial (blinded analysis). The Pearson's correlation between serum Mg and the Mg tolerance test was found to be only 0.11 (p=0.66) among the 18 participants with both serum Mg and the Mg tolerance test. Based on current participation, it is estimated about 100 participants will complete the Mg tolerance test (before the proposed study starts).
(69) Magnesium supplementation. In the parent study, participants are using either daily Mg glycinate capsules or identical-appearing lactose placebo. Mg doses average 205 mg. Ca, Mg, and vitamin D intakes are determined through the administration of six days of dietary record-based 24-hour recalls (two prior to intervention and four during intervention) which include supplements. Pill counts and compliance calls are conducted throughout the study to monitor compliance.
(70) Data collection. An interviewer-administered questionnaire includes family history of cancer and lifestyle information, including smoking and alcohol histories. During the in-person visits to the clinic, information is also collected regarding recent, long-term, and current medication use, anthropometric measurements, and diet in addition to the six days of dietary recalls described above.
(71) Biological sample collection/storage. Fasting blood sample (separated into serum, plasma and buffy coat with leukocytes) and 8 rectal biopsies are collected at day 0, and again at the conclusion of the intervention (week 12), and are stored on ice until processed. All blood samples are processed within 4-6 hours. All samples are placed in long-term storage in −80° C. freezers.
(72) Overview of the Study Design. An initial study was completed with more than 200 participants. The study design is summarized in FIG. **1**. An unbiased EWAS study is conducted to identify 5-hmC/5-mC biomarkers of Mg deficiency by taking a four-phase approach.
(73) By using the TAB-Seq and TAB-Array protocol (See FIG. **4**).sup.42-44, both 5-hmC and 5-mC biomarkers are measured in half of the participants in the parent study. 5-mC and 5-hmC biomarkers with the greatest changes in blood leukocyte DNA are identified, comparing Mg treatment vs. placebo arm.
(74) These findings are replicated in the remaining half of participants using the TAB-Seq and TAB-Array protocol (See FIG. **4**).sup.42-44.
(75) Confirmed 5-hmC/5-mC biomarkers are tested alone or combined with serum Mg to differentiate Mg status with higher sensitivity and specificity than serum Mg, among 100 participants with the Mg tolerance test. Further, integrative analysis will be conducted to identify the combinational biomarker index from different pathways that may have even higher sensitivity and specificity. The final composite index will be generated from 5-mC and 5-hmC biomarkers from multiple pathways and serum Mg.sup.106.
(76) The top 20 modified CpG loci are selected from those identified and then their functional significance is examined by evaluating their correlation with their gene expression levels to functionally evaluate the findings.
(77) Biomarker Assays
(78) Bioassay quality control. Samples are organized in treatment-placebo (i.e. one treatment arm with one placebo arm) sets (4 samples in each set: 2 from pre-, and 2 from post-treatment) to minimize between-assay variation. Extracted DNA samples and plasma samples are shipped on dry ice via overnight to the labs in two locations. To control for batch-to-batch variability, samples from each set are analyzed in the same run. Quality control (QC) samples (5% of samples) will be added to each batch of samples to be assayed. Lab staff will be blinded to the sample status (in treatment or placebo arms, or QC).
(79) Measurement of DNA Methylation and Hydroxymethylation.
(80) Genomic DNA is extracted from buffy coat fractions collected in the PPCCT using a QIAamp DNA mini-kit (Qiagen Inc, Valencia, CA) according to the manufacturer's protocol.sup.159.
(81) Unbiased interrogation screen of DNA methylation and hydroxymethylation will be undertaken across a wide swath of the genome. The TAB-Array assay combines the Illumina HM450K array profiling and the TAB-Seq assay.sup.42. The TAB-Array protocol has been successfully used to profile 5-hmCs in human cells.sup.44 and has been incorporated into Illumina's protocol (See FIG. **3**). Briefly, each sample will be profiled twice using the HM450K array according to the Illumina recommended protocol at the Northwestern University Genomics Core. To distinguish 5-hmC from 5-mC, besides one regular bisulfite conversion-based profiling for 5-mC, the second HM450K profiling will be performed after the TAB assay, which employs the feature that genomic 5-hmC loci are protected by glucosylation from Tet conversion.sup.43. Specifically, in the TAB assay, genomic DNA is treated with β-glucosyltransferase (β-GT) to conjugate all 5-hmC residues to glucose. The DNA is then treated with Tet1 to convert 5-mC and 5-formylcytosine (5-fC) to 5-carboxylcytosine (5-caC), while cytosine and glycosylated 5-hmC remain unaffected. During subsequent bisulfite treatment, unmodified cytosines and 5-caC are converted to uracil or 5-carboxyuracil (5-caU), respectively, whereas 5-hmC remains protected by glycosylation. The location of 5-hmC is then indicated by a cytosine in the HM450K array profiling results since all other cytosine species (C, 5-mC, 5-fC, and 5-caC) have been converted to thymine. Data generated from these two array profiling assays in each sample will then allow for distinguishing these two modified cytosines.
(82) Bisulfite sequencing (for 5-mC) and the TAB-Seq (for 5-hmC) is conducted to confirm the TAB-Array results. The top 20 differential CpG loci are selected to be evaluated using both approaches in at least 30 random samples. Correlation with the TAB-Array data will be used to confirm the reliability of profiling.
(83) Measurement of Plasma Methionine Cycle Metabolites.
(84) LC/MS-MS. All protocols for SAM and SAH analysis are well-established and are used on a regular basis. Stable-isotope dilution liquid chromatography-electrospray tandem mass spectrometry (LC-ESI-MS/MS) will be used to determine methionine, SAM and SAH in plasmas.sup.95;162. A second LC-ESI-MS/MS method will be used to determine plasma total

homocysteine (tHcy).sup.163. Table 2 indicates the multiple reaction monitoring transitions (m/z) for each metabolite and respective stable isotope internal standard and retention time.
(85) QC samples will be included at the start and end of each batch of plasma samples to be analyzed. All QC data must be within two standard deviations of the preset limits (inter-assay variation) indicated in Table 2. Ten (10) paired samples collected before and the day following fasting were also compared, and a bowel cleansing preparation and found the correlation between the two collection times was high (r=0.89 for SAH, r=0.79 for SAM) consistent with a previous study with repeated measurements of SAM and SAH.sup.164.
(86) TABLE-US-00002 TABLE 2 LC-MS/MS MRM methods and assay performance for methionine cycle metabolites Labeled Inter-assay Precision MRM Labeled Isotope RT Level 1 Level 2 Analyte (m/z) Isotope (m/z) (min) (CV %) (CV %) Methionine 150 .fwdarw. 104 .sup.2H.sub.3- .sup.2H.sub.3- 5.0 7.0% 5.9% Methionine Methionine SAM 399 .fwdarw. 250 .sup.2H.sub.3-SAM .sup.2H.sub.3-SAM 5.7 7.6% 5.5% SAH 385 .fwdarw. 136 .sup.2H.sub.4-SAH .sup.2H.sub.4-SAH 5.4 8.1% 6.8% Hcy 136 .fwdarw. 90 .sup.2H.sub.4-Hcy .sup.2H.sub.4-Hcy 0.9 7.9% 6.9%
(87) Serum Mg is measured by standard method on the Beckman DXC 800 chemistry analyzer provided by the Vanderbilt Pathology Laboratory Services with an intra-assay coefficient of variation of 2.0.sup.165.
(88) Functional validation by gene expression. To examine the functional significance of the top differentially modified loci identified, real-time (RT)-PCR is performed to measure the expression levels of 20 selected genes in leukocytes in all 200+ participants.
(89) Expression of genes located locally (e.g. 10 kb) will be evaluated to the differentially modified CpGs. Briefly, RNA will be obtained from leukocytes collected in the PPCCT using the RNeasy Plus Mini Kits (Qiagen Inc, Valencia CA). mRNA will be reverse transcribed to cDNA using the Agilent Biosystems High Capacity Reverse Transcription Kit. qRT-PCR will be performed for each gene together with an endogenous control using TaqMan Gene Expression Assays following standard TaqMan protocols. Each sample will be run on a minimum of two plates in triplicates with standard deviation controlled to less than 15%.
Example 3
(90) In PPCCT study, 250 participants were enrolled, randomly allocated to treatment or placebo and had the 1st dose of intervention. Two hundred forty (240) completed week 12 data collection (the end of the study intervention and the primary data collection time point). Ten (10) participants withdrew from the study due to: moving (1), change of mind (4), side effects (5). Among the 240, 17 participants had compliance less than 90% based on pill counts.
(91) MTT was conducted on 78 subjects in PPCCT. After completion of the 12-week intervention, MTT was conducted among those who were willing to participate and 77 participants finished the test. In the test, two 24 hour urine samples were obtained, one prior to the intravenous infusion of magnesium sulfate and the other starting with the IV infusion. Urine magnesium level was measured using 7D70 Magnesium Reagent Kit from Abbot Laboratories (Abbott Park, TL). The method utilizes an arsenzo dye which binds preferentially with magnesium. The absorbance of the arsenazo-magnesium complex is measured at 572 nm and is proportional to the concentration of magnesium present in the sample.
(92) Their diagnosis was made by serum magnesium concentration. Serum magnesium concentration was determined using 7D70 Magnesium Reagent Kit from Abbot Laboratories (Abbott Park, TL) the assay was conducted at the Vanderbilt Pathology Laboratory Services. The method utilizes an arsenzo dye which binds preferentially with magnesium. The absorbance of the arsenazo-magnesium complex is measured at 572 nm and is proportional to the concentration of magnesium present in the sample. This diagnosis criterion is considered the gold standard. Unfortunately, this gold standard diagnosis sometimes cannot be obtained early to guide treatment decision.
(93) The diagnostic values of the identified methylation markers was determined to distinguish Mg-deficiency (>=50%) and Mg-insufficiency (>=25%) early
(94) Statistical Methods
(95) To discover the top picks in the bisulfite (BS) dataset, various approaches are contemplated.
(96) In one approach, a 3-phase study is carried out. The participants (N=240) are first divided in the randomized trial into two groups (n=120 in each group) by medium enrollment date.
(97) In Phase 1, methylation biomarkers with the greatest changes in blood leukocyte DNA comparing Mg treatment vs. placebo (n=114) were identified. For differential methylation analysis, the t-test was used for change score (change score=post âĂŞ pre) using limma software. The selected sites include 100,766 CpG sites out of 836,588 with a Type I error of 0.1.
(98) In Phase 2, the Phase 1 findings (i.e. 100,766 CpG sites) were replicated in an independent set of participants (n=110) from the PPCCT and 5,539 CpG sites were found out of 100,766 at a Type I error rate of 0.05. The analyses were limited to the CpG sites for which the changes caused by Mg treatment were in the same direction for both phases 1 and 2 and for which the difference between the treatment arm and the placebo arm were also in the same direction for both phases 1 and 2. Finally, 4,449 CpG sites that changed with Mg treatment vs. placebo were identified.
(99) For those selected biomarkers, logistic models were fit to describe the probability of the Mg status being deficient (or insufficient) (Y.sub.i=1 for MTT less than 50% for subject i, alternatively, Y.sub.i=1 for MTT less than 25% for subject i), otherwise Y.sub.i=0 (nondeficient/noninsufficient).
(100) There are two steps in the analyses. Step one is data reduction. LASSO and elasticnet regressions are conducted for variable selection. In step two, the selected variables are used to develop a classification rule for Mg status or a prediction model for MTT.
(101) The LASSO and elasticnet regressions are penalized logistic regressions or GLM conducted as:
(102)

$$\hat{} = \text{argmin}\{-[\log \text{ likelihood } ( )]/n + [(1 -)/2 \text{ .Math. } \text{.Math. } {}_2^2 + \text{ .Math. } \text{.Math. } {}_1]\} \quad (1)$$

where $\text{.Math. } \text{.Math. } {}_2 = (\text{.Math. } \sum_{j=1}^{p} {}_j^2)^{1/2}$, $\text{.Math. } \text{.Math. } {}_1 = \text{.Math. } \sum_{j=1}^{p} \text{.Math. } {}_j \text{.Math. }$,

and log likelihood (β) is the logistic likelihood function.

(103) In (1), α=1 gives the Least Absolute Shrinkage and Selection Operator (LASSO) regression (Tibshirani, 1996) and α<1 gives us elasticnet (Zou and Hastie, 2005) regression. Parameter λ is chosen through k-fold cross-validation.

(104) The accuracy of the classification rule is measured by calculating the rule's discrimination using area under receiver operating characteristic (ROC) curve or concordance (c-index). The rule's calibration demonstrated will be calculated with a smooth nonparametric calibration curve or scatter plot of estimated versus observed outcome. The calibration and discrimination of the rule will be internally validated using bootstrap resampling in order to estimate the likely performance of the rule on a new sample of patients from the same patient stream (Sections 10.8 and 10.9 of Harrell 2001xxx). Future prospective investigations will be planned to externally validate the classification rule.

(105) In Phase 3, the 4,449 CpG sites were used to predict Mg deficiency status at the end of the PPCCT trial. Mg deficiency status was measured by the Mg tolerance test results among 77 participants who completed the two 24-hour urine samples and a 4-hour IV Mg infusion71. Using logistic regression models by adjusting for serum Mg measured at the end of the trial and least absolute shrinkage and selection operator (LASSO) to reduce potential overfitting, 84 CpG sites are reported over minimum mean cross-validated error by LASSO.

(106) Finally, via LASSO composite indices of multiple methylation biomarkers in predicting Mg deficiency status were identified.

(107) As noted above, in the first approach, the original dataset was imputed, then biomarker sites were selected by phase1/phase2 and directional restriction from 836588 sites.

(108) The logistic model for Mg deficiency/insufficiency status was fist via penalized maximum likelihood (1). The regularization path was computed for LASSO with α=1 and for the elasticnet with α=0.25, 0.5, 0.75.

(109) There were 4449 sites selected from 836588 sites by phase1/phase2 and directional restriction. For Mg deficiency, LASSO/elasticnet regression fits pick 84 sites.

(110) Table 3 presents the methylation markers selected by LASSO or elasticnet at A value that minimizes the cross-validation missclassification rate.

(111) TABLE-US-00003 TABLE 3 Variables selected by LASSO/elasticnet for Mg deficiency in BS site Name cg05019905 cg25731074 cg06407417 cg11840205 cg06295308 cg06922496 cg10350957 cg00924527 cg12496307 cg26335127 cg00576263 cg07739604 cg11333566 cg00430271 cg07777270 cg10951786 cg12087941 cg18219712 cg18534872 cg23260330 cg21636911 cg26074539 cg01407874 cg22236894 cg02635020 cg18947305 cg10983873 cg02276825 cg00023056 cg18427968 cg16477259 cg12187394 cg19608680 cg24351671 cg04987122 cg17813946 cg01110440 cg26864174 cg15021089 cg18917736 cg15916745 cg03084350 cg21457401 cg01152729 cg12800781 cg14265145 cg10127610 cg04578283 cg12062489 cg01842741 cg11295178 cg20613400 cg18082638 cg01501912 cg24423359 cg14950072 cg18664866 cg10613701 cg16418754 cg18956714 cg15210809 cg26186954 cg02469161 cg23725734 cg12660093 cg26794993 cg17379666 cg17500902 cg08108029 cg21397480 cg13663211 cg06756499 cg10231801 cg26909602 cg04729491 cg17370417 cg13992360 cg04386563 cg02074074 cg06894687 cg03901836 cg26951706 cg17313709 cg23194229

(112) Pick 2, 3, or 4 from the union of 84 sites, run logistic regression model and some combination examples are as follows. Table 4 presents AUC for some combination of the methylation markers selected by LASSO or elasticnet at λ value that minimizes the cross-validation missclassification rate.

(113) TABLE-US-00004 TABLE 4 Logistic fit of some combination of selected sites for Mg deficiency in BS site 1 site 2 site 3 site 4 AUC 0.8230028 cg01842741 cg10051786 0.8195592 cg03084350 cg11333566 0.8140496 cg11840205 cg128000781 0.8092287 cg00430271 cg26335127 0.8057851 cg06922496 cg26864174 0.8019972 cg00430271 cg10951786 0.8016529 cg12187394 cg25731074 0.8016529 cg00924527 cg11840205 0.7982094 cg01842741 cg25731074 0.7975207 cg06022406 cg11333566 cg18056714 0.8801653 cg00430271 cg11840205 cg26335127 0.8794766 cg00430271 cg11840205 cg25731074 0.8753444 cg00430271 cg15916745 cg25731074 0.8746556 cg00430271 cg01842741 cg25731074 0.8698347 cg04578283 cg11840205 cg14265145 0.8670799 cg00430271 cg04578283 cg11840205 0.8050138 cg00430271 cg18917736 cg25731074 0.8643251 cg00430271 cg03084350 cg25731074 0.8626033 cg00430271 cg04987122 cg25731074 0.8626033 cg00430271 cg04386563 cg11840205 cg25731074 0.9256198 cg00430271 cg02074074 cg11840205 cg26335127 0.9214876 cg00430271 cg04386563 cg11840205 cg26335127 0.9187328 cg00430271 cg15916745 cg18956714 cg25731074 0.9173554 cg00430271 cg01110440 cg11840205 cg26335127 0.9139118 cg00430271 cg01110440 cg01842741 cg10951786 0.9097796 cg00430271 cg11840205 cg15916745 cg25731074 0.9084022 cg00430271 cg11840205 cg26335127 cg26864174 0.9077135 cg00430271 cg01842741 cg23260330 cg25731074 0.9070248 cg00430271 cg10127610 cg12187394 cg25731074 0.9056474

(114) Detailed logistic regression results of some interesting combination of markers in Table 5 were double checked. The stratified distribution of marker cg00430271 and cg25731074 A listed in FIG. **5**.

(115) TABLE-US-00005 TABLE 5 some logistic regression models for Mg deficiency in BS Logistic Model AUC Mg deficiency - cg00430271 0.6646 cg25731074 0.7094 cg00430271 + cg25731074 0.8230 cg00430271 + cg11840205 + 0.8753 cg25731074 cg00430271 + cg04386563 + 0.9256 cg11840205 + cg25731074

(116) For Mg insufficiency, LASSO/elasticnet regression fits pick 117 sites. Table 6 presents the methylation markers selected by LASSO or elasticnet at λ value that minimizes the cross-validation missclassification rate.

(117) TABLE-US-00006 TABLE 6 Variables selected by LASSO/elasticnet for Mg insufficiency in BS site Name cg17102582 cg12600069 cg24216889 cg06407417 cg10270306 cg13914600 cg22997415 cg05031696 cg09858749 cg18956714 cg25033719 cg00823602 cg19422947 cg16722435 cg05382973 cg17965622 cg02795691 cg16244786 cg16731240 cg10231801 cg02117656 cg03955314 cg13233725 cg25557858 cg23812215 cg11567608 cg01336268 cg01100448 cg14584422 cg15645605 cg04416635 cg01382875 cg26331945 cg10855773 cg20658450 cg05019905 cg09486166 cg23060646 cg12513738 cg07908870 cg02506875 cg06108900 cg15652683 cg11078674 cg18233497 cg24624572 cg19594772 cg16619071 cg16418754 cg12477050 cg27198485 cg07425005 cg15182613 cg22315933 cg26335127 cg01850352 cg05630957 cg11921952 cg16393730 cg13823415 cg02469161 cg17441998 cg26794993 cg14901226 cg08539620 cg16464483 cg23358699 cg27632704 cg02117713 cg02276825 cg01135781 cg18790771 cg23260330 cg11513221 cg12502223 cg21178653 cg10581837 cg20931474 cg10848640 cg17399385 cg16477259 cg07273980 cg01076051 cg04589248 ch.16.50217098R cg12259593 ch.2.54406426R cg02192472 cg00843631 cg10242496 cg01657422 cg16589555 cg19446777 cg13431373 cg12284971 cg22333471 cg06719651 cg02461690 cg25392154 cg24985235 cg09223811 cg21107235 cg00946712 cg15721020 cg16790645 cg05264252 cg04165824 cg00145955 cg00884973 cg18670278 cg03307560 cg10159922 cg21882593 cg16577509 cg04321753 cg04645444 cg01588826

(118) Pick 2, 3, or 4 from the union of 117 sites, run logistic regression model and some combination examples are as follows. Table 7 presents AUC for some combination of the methylation markers selected by LASSO or elasticnet at λ value that minimizes the cross-validation missclassification rate.

(119) TABLE-US-00007 TABLE 7 Logistic fit of some combination of selected sites for Mg insufficiency in BS site 1 site 2 site 3 site 4 AUC cg10231801 cg16722435 0.9048964 cg10231801 cg25033719 0.8935970 cg04165824 cg10231801 0.8907721 cg12259593 cg16790645 0.8841808 cg10231801 cg12259593 0.8822976 cg10231801 cg16619071 0.8794727 cg06843631 cg10231801 0.8771186 cg05264252 cg10231801 0.8766478 cg04321753 cg10231801 0.8747646 cg10231801 cg16577509 0.8747646 cg10231801 cg16722435 cg25033719 0.9576271 cg10231801 cg23358699 cg25033719 0.9529190 cg12259593 cg16790645 cg19422947 0.9500942 cg10231801 cg13914600 cg25033719 0.9463277 cg00823602 cg02795691 cg10231801 0.9458569 cg10231801 cg10270306 cg16722435 0.9444444 cg04165824 cg10231801 cg25557858 0.9435028 cg10231801 cg12259593 cg16790645 0.9435028 cg02795691 cg10231801 cg16722435 0.9425612 cg07908870 cg10231801 cg16619071 0.9387947 cg00823602 cg02795691 cg10231801 cg25033719 0.9783427 cg00823602 cg02795691 cg10231801 cg16722435 0.9764595 cg02795691 cg10231801 cg13914600 cg25033719 0.9726930 cg02795691 cg10231801 cg16722435 cg24216889 0.9717514 cg02795691 cg10231801 cg16722435 cg18956714 0.9708098 cg00823602 cg02795691 cg10231801 cg15645605 0.9680266 cg02795691 cg10231801 cg16722435 cg25033719 0.9689266 cg00823602 cg10231801 cg16731240 cg25033719 0.9679849 cg02795691 cg10231801 cg17965622 cg25033719 0.9679849 cg02795691 cg10231801 cg10270306 cg16722435 0.9670433

(120) In approach 2, all 836,588 CpG sites are used to predict Mg deficiency status at the end of the PPCCT trial. Using logistic regression models and LASSO, 118 sites are reported over minimum mean cross-validated error by LASSO.

(121) The original dataset of 836588 sites was inputted without further selection. The logistic model for Mg deficiency/insufficiency status was fit via penalized maximum likelihood (1). The regularization path was computed for LASSO with α=1 and for the elasticnet with α=0.25, 0.5, 0.75 in (1).

(122) For Mg deficiency, LASSO/elasticnet regression fits pick 118 sites.

(123) Table 8 presents the methylation markers selected by LASSO or elasticnet at A value that minimizes the cross-validation missclassification rate.

(124) TABLE-US-00008 TABLE 8 Variables selected by LASSO/elasticnet for Mg deficiency in BS site Name cg17102582 cg12600069 cg24216889 cg06407417 cg10270306 cg13914600 cg22997415 cg05031696 cg09858749 cg18956714 cg25033719 cg00823602 cg19422947 cg16722435 cg05382973 cg17965622 cg02795691 cg16244786 cg16731240 cg10231801 cg02117656 cg03955314 cg13233725 cg25557858 cg23812215 cg11567608 cg01336268 cg01100448 cg14584422 cg15645605 cg04416635 cg01382875 cg26331945 cg10855773 cg20658450 cg05019905 cg09486166 cg23060646 cg12513738 cg07908870 cg02506875 cg06108900 cg15652683 cg11078674 cg18233497 cg24624572 cg19594772 cg16619071 cg16418754 cg12477050 cg27198485 cg07425005 cg15182613 cg22315933 cg26335127 cg01850352 cg05630957 cg11921952 cg16393730 cg13823415 cg02469161 cg17441998 cg26794993 cg14901226 cg08539620 cg16464483 cg23358699 cg27632704 cg02117713 cg02276825 cg01135781 cg18790771 cg23260330 cg11513221 cg12502223 cg21178653 cg10581837 cg20931474 cg10848640 cg17399385 cg16477259 cg07273980 cg01076051 cg04589248 ch.16.50217098R cg12259593 ch.2.54406426R cg02192472 cg00843631 cg10242496 cg01657422 cg16589555 cg19446777 cg13431373 cg12284971 cg22333471 cg06719651 cg02461690 cg25392154 cg24985235 cg09223811 cg21107235 cg00946712 cg15721020 cg16790645 cg05264252 cg04165824 cg00145955 cg00884973 cg18670278 cg03307560 cg10159922 cg21882593 cg16577509 cg04321753 cg04645444 cg01588826

(125) Pick 2, 3, or 4 from the union of 118 sites, run logistic regression model and some combination examples are as follows.

(126) Table 9 presents AUC for some combination of the methylation markers selected by LASSO or elasticnet at λ value that minimizes the cross-validation missclassification rate.

(127) TABLE-US-00009 TABLE 9 Logistic fit of some combination of selected Mg deficiency in BS cg18910313 cg26286077 0.9049587 cg05095647 cg11368923 0.9039256 cg11368923 cg14844977 0.9028926 cg11368923 cg17431860 0.9001377 cg16106313 cg18910313 0.8980716 cg03597723 cg11368923 0.8973829 cg06045761 cg21494343 0.8973829 cg02692511 cg22798756 0.8929063 cg06213635 cg14844977 0.8925620 cg05863683 cg25498045 0.8911846 cg02692511 cg08878802 cg16106313 0.9662534 cg03597723 cg05095647 cg11368923 0.9648760 cg05095647 cg11613875 cg18746826 0.9600551 cg06213635 cg14450620 cg14844977 0.9593664 cg00327506 cg06840298 cg17162453 0.9586777 cg03043243 cg06292683 cg18746826 0.9566116 cg01331062 cg03941824 cg04285064 0.9559229 cg03597723 cg11368923 cg17338208

0.9552342 cg03597723 cg11368923 cg17431860 0.9552342 cg06213635 cg20384683 cg21369695 0.9552342 cg01331062 cg03043243 cg06292683 cg23862011 0.9931129 cg05095647 cg11368923 cg11613875 cg18746826 0.9917355 cg16106313 cg16484042 cg18746826 cg25739288 0.9910468 cg01331062 cg03597723 cg05095647 cg11368923 0.9903581 cg01331062 cg03043243 cg06292683 cg25498045 0.9889807 cg06045761 cg11613875 cg15613012 cg16106313 0.9889807 cg01331062 cg05095647 cg06045761 cg11613875 0.9862259 cg01331062 cg03043243 cg06292683 cg12064372 0.9855372 cg05095647 cg11613875 cg14131256 cg18746826 0.9855372 cg01331062 cg03043243 cg06292683 cg18746826 0.9848485

(128) For Mg insufficiency, LASSO/elasticnet regression fits pick 252 sites.

(129) Table 10 presents the methylation markers selected by LASSO or elasticnet at A value that minimizes the cross-validation missclassification rate.

(130) TABLE-US-00010 TABLE 10 Variables selected by LASSO/elasticnet for Mg insufficiency in BS site Name cg10055621 cg02563636 cg21057880 cg08520660 cg12269394 cg21710569 cg15482690 cg16643840 cg07442759 cg06809055 cg10054262 cg11018337 cg18867708 cg19051504 cg02074478 cg03906697 cg16103996 cg13133961 cg06756169 cg26744387 cg22511432 cg05542957 cg24613479 cg16762072 cg12038684 cg12307823 cg25623271 cg17545218 cg18522266 cg10575376 cg19851029 cg17102089 cg19300474 cg22740700 cg23186116 cg10191772 cg05995260 cg19354851 cg01654446 cg10308906 cg27145495 cg18469778 cg15848792 cg09232499 cg02550027 cg10044839 cg21258377 cg15367487 cg11058730 cg05798223 cg26874164 cg01520858 cg13792460 cg22736872 cg13751775 cg11166759 cg18763656 cg10732215 cg10672567 cg23792658 cg04822405 cg23823879 cg09950256 cg21923861 cg07525971 cg02919116 cg23806084 cg24659093 cg23686983 cg10091335 cg11953383 cg17265279 cg03826594 cg27148952 cg10261952 cg15724296 cg14416747 cg23377495 cg26608883 cg16879197 cg09142260 cg21062661 cg09603594 cg07584331 cg20832125 cg12278099 cg10072995 cg17089444 cg08960045 cg26142604 cg17164520 cg21565543 cg20568108 cg04536393 cg06217399 cg15269754 cg02941697 cg01785473 cg12573318 cg12566078 cg11713008 cg18467358 cg12259892 cg12988231 cg19560579 cg03021910 cg09030187 cg04127455 cg27005373 cg23471617 cg16458834 cg08452613 cg01516591 cg13075456 cg12925904 cg27413396 cg12614789 cg04379126 cg15711208 cg02845345 cg06203009 cg12943441 cg25498045 cg14999518 cg08198851 cg05314679 cg05888518 cg00826536 cg17094127 cg07604566 cg26722858 cg03389215 cg24343524 cg24001601 cg27266060 cg02235760 cg19667913 cg26990667 cg22776504 cg02795691 cg25670545 cg05836790 cg08159120 cg23251282 cg13861802 cg00415057 cg00088575 cg07410597 cg21997109 cg23175573 cg04344303 cg19859445 cg05682319 cg19021236 cg26903218 cg20772106 cg10498390 cg19301992 cg00170421 cg25976440 cg21150271 cg24210818 cg22173794 cg17630144 cg17692879 cg16753420 cg04175109 cg27457631 cg03428945 cg06045761 cg17547883 cg04559604 cg00017630 cg25997661 cg20926461 cg07189587 cg08143605 cg00999950 cg04763519 cg17678928 cg15002115 cg00908117 cg11620475 cg02658969 cg02341645 cg20100675 cg27661212 cg07360304 cg01705888 cg02805354 cg26291276 cg12568536 cg01480545 cg02916173 cg15727032 cg12319618 cg16896144 cg23564460 cg00401721 cg14764085 cg16372825 cg05101463 cg16258229 cg24613906 cg24073042 cg20533553 cg22617044 cg19602694 cg14526241 cg22699815 cg25654768 cg16664915 cg07973125 cg18515510 cg18964630 cg15595495 cg25843873 cg00181497 cg10406482 cg21198880 cg14451730 cg02742551 cg11498908 cg12080079 cg16181135 cg18436123 cg13988329 cg06626140 cg11440629 cg20659752 cg05083647 cg21545071 cg07015412 cg00482488 cg15133564 cg22914616 cg01797652 cg13649960 cg19701205 cg03408135 cg17239714 cg15131187 cg26655697* cg06509153 cg12875665 cg11304573 cg10544554 cg29886858 cg27115113 cg27055732 cg17585910 cg17747199

(131) Pick 2, 3, or 4 from the union of 252 sites, run logistic regression model and some combination examples are as follows.

(132) Table 11 presents AUC for some combination of the methylation markers selected by LASSO or elasticnet at λ value that minimizes the cross-validation missclassification rate.

(133) TABLE-US-00011 TABLE 11 Logistic fit of some combination of selected sites for Mg insufficiency in BS site 1 site 2 site 3 site 4 AUC cg10055621 cg11166759 0.9538606 cg07360304 cg12038684 0.9463277 cg10055621 cg19021236 0.9425612 cg12269394 cg15367487 0.9425612 cg12269394 cg14416747 0.9416195 cg01785473 cg20832125 0.9406780 cg00482488 cg08520660 0.9392655 cg12269394 cg18763656 0.9387947 cg19560579 cg24343524 0.9387947 cg05995260 cg25843873 0.9378531 cg08520660 cg16643840 cg21710569 0.9698082 cg10055621 cg16643840 cg21710569 0.9698082 cg10055621 cg12269394 cg16643840 0.9623352 cg08520660 cg12269394 cg16643840 0.9613936 cg02563636 cg08520660 cg16643840 0.9604520 cg08520660 cg10055621 cg16643840 0.9557439 cg08520660 cg16643840 cg21057880 0.9557439 cg10055621 cg16643840 cg21057880 0.9548023 cg02563636 cg08520660 cg12269394 0.9519774 cg02563636 cg10055621 cg12269394 0.9519774 cg02563636 cg10055621 cg16643840 cg21710569 0.9934087 cg02563636 cg08520660 cg16643840 cg21710569 0.9868173 cg02563636 cg08520660 cg10055621 cg16643840 0.9839925 cg02563636 cg10055621 cg12269394 cg16643840 0.9821092 cg02563636 cg08520660 cg12269394 cg16643840 0.9802260 cg08520660 cg12269394 cg16643840 cg21710569 0.9802260 cg10055621 cg12269394 cb16643840 cg21710569 0.9802260 cg08520660 cg10055621 cg16643840 cg21710569 0.9792844 cg08520660 cg12269394 cg16643840 cg21057880 0.9774011 cg08520660 cg16643840 cg21057880 cg21710569 0.9774011

(134) For Mg deficiency, LASSO/elasticnet regression fits pick 72 sites using approach 1 (i.e., two-phase study).

(135) TABLE-US-00012 TABLE 12 Variables selected by LASSO/elasticnet for Mg deficiency in BS “cg07121807” “cg08001123” “cg15863924” “cg20435284” “cg18315623” “cg25428389” “cg15152945” “cg20858033” “cg10350957” “cg04780086” “cg10983873” “cg09182447” “cg24687894” “cg25368651” “cg04671914” “cg16052920” “cg00430271” “cg18477635” “cg18947305” “cg19103546” “cg10432364” “cg03889299” “cg23691220” “cg25040679” “cg17105557” “cg01037314” “cg23997664” “cg14282531” “cg11441416” “cg07731404” “cg24202000” “cg11049018” “cg01716499” “cg26196087” “cg27123859” “cg13207790” “cg20805104” “cg16737267” “cg24687806” “cg07582923” “cg06108900” “cg14227325” “cg14883993” “cg20653128” “cg15347131” “cg26864174” “cg18956714” “cg24833731” “cg03240920”

“cg12660093” “cg13001274” “cg05382973” “cg10451502” “cg26592319” “cg12502223” “cg16821345” “cg14863124” “cg00492691” “cg06976222” “cg01334432” “cg04891094” “cg24396686” “cg13992360” “cg11343713” “cg02074074” “cg17446661” “cg01793068” “cg06432889” “cg17313709” “cg14185463” “cg09272948” “cg07070882”

(136) For Mg insufficiency, LASSO/elasticnet regression fits pick 160 5-mC sites using approach 1.

(137) TABLE-US-00013 TABLE 13 Variables selected by LASSO/elasticnet for Mg deficiency in BS “cg09936824” “cg05660803” “cg12600069” “cg27649897” “cg10503473” “cg24216889” “cg17105557” “cg02460812” “cg07554408” “cg24135151” “cg18233497” “cg25600049” “cg02027123” “cg19490001” “cg18956714” “cg24576206” “cg21875096” “cg14503881” “cg22328746” “cg16621176” “cg16602850” “cg19405842” “cg16722435” “cg05382973” “cg13503928” “cg04426842” “cg10581837” “cg09182447” “cg10231801” “cg24025119” “cg07522508” “cg01152729” “cg06917858” “cg03301025” “cg24396686” “cg18096764” “cg12463089” “cg01660034” “cg11466504” “cg09307977” “cg00946712” “cg03007338” “cg05241538” “cg06131046” “cg19980575” “cg07686023” “cg10909141” “cg08759026” “cg11368392” “cg10855773” “cg21593628” “cg20360416” “cg04896013” “cg18326365” “cg12475128” “cg16398761” “cg17420619” “cg09949366” “cg25789861” “cg10062141” “cg14759277” “cg05417607” “cg07908870” “cg27483474” “cg19206146” “cg19987296” “cg16055294” “cg00134295” “cg06108900” “cg10420854” “cg07081465” “cg05351998” “cg02928110” “cg27188491” “cg19594772” “cg25117895” “cg12477050” “cg13431573” “cg14880655” “cg07425005” “cg25276640” “cg10491546” “cg11485283” “cg21178711” “cg02779870” “cg09962477” “cg23997664” “cg04094346” “cg21130113” “cg11534242” “cg15486846” “cg15715690” “cg10983873” “cg16152741” “cg26592319” “cg03538548” “cg01758805” “cg27156529” “cg22721434” “cg22673476” “cg16821345” “cg05315240” “cg23027329” “cg25089903” “cg10848640” “cg21956258” “cg18777236” “cg07009570” “cg07070882” “cg24611214” “cg24354380” “cg25794707” “cg16477259” “cg21656199” “cg14681115” “cg00492691” “cg07399846” “cg01076051” “cg25826576” “cg03374632” “cg01043567” “cg13233725” “cg08440418” “cg24687894” “cg20002846” “cg16503559” “cg24013810” “cg11495399” “cg16484858” “cg08937612” “cg23902439” “cg19926480” “cg23664783” “cg05071577” “cg20051696” “cg09223811” “cg02013841” “cg05064673” “cg18477635” “cg18049571” “cg02074074” “cg27617214” “cg06868100” “cg18848965” “cg24202485” “cg22287711” “cg27658811” “cg08822689” “cg03901836” “cg01095103” “cg15185001” “cg02456675” “cg19440992” “cg18670278” “cg03234777” “cg24351671” “cg09445162” “cg04645444” “cg21244116” “cg25225632”

(138) For Mg deficiency, LASSO/elasticnet regression fits pick 43 sites using approach 2.

(139) TABLE-US-00014 TABLE 14 Variables selected by LASSO/elasticnet for Mg deficiency in BS “cg18910313” “cg01892689” “cg18016565” “cg24375627” “cg21494343” “cg16484042” “cg15916246” “cg16978871” “cg03170318” “cg08690634” “cg19066691” “cg01016169” “cg14436032” “cg19406106” “cg23255151” “cg15740518” “cg13867683” “cg08878802” “cg01278712” “cg10660498” “cg16085056” “cg15711208” “cg08198851” “cg25962755” “cg06844749” “cg13865595” “cg14595922” “cg19109677” “cg19615147” “cg20808227” “cg13192508” “cg16106313” “cg16395700” “cg14480619” “cg26510597” “cg26553263” “cg19591710” “cg25041035” “cg00444883” “cg04464650” “cg05347567” “cg15522298” “cg13877657”

(140) For Mg insufficiency, LASSO/elasticnet regression fits pick 253 sites using approach 2.

(141) TABLE-US-00015 TABLE 15 Variables selected by LASSO/elasticnet for Mg deficiency in BS “cg01654446” “cg13323097” “cg12461252” “cg10055621” “cg25034625” “cg21480996” “cg07912402” “cg26608883” “cg13272280” “cg09603594” “cg08570492” “cg02563636” “cg04353660” “cg15269754” “cg11962515” “cg00514268” “cg18308359” “cg12566078” “cg15916628” “cg10712573” “cg00264384” “cg07537978” “cg11705931” “cg26722858” “cg22662556” “cg08520660” “cg08159120” “cg19301992” “cg12269394” “cg05285687” “cg25545088” “cg26291276” “cg22524174” “cg16350010” “cg06981948” “cg02847344” “cg05083647” “cg02942142” “cg16495809” “cg23012185” “cg09886858” “cg00873704” “cg24999679” “cg07146974” “cg13626842” “cg19504661” “cg10061496” “cg16655404” “cg26744387” “cg06005396” “cg03859915” “cg19752094” “cg11909137” “cg10308906” “cg22120017” “cg08170757” “cg27145495” “cg25732732” “cg21622381” “cg04231467” “cg17431746” “cg21041792” “cg00997174” “cg07817409” “cg05988267” “cg10732215” “cg25178784” “cg01157169” “cg24659093” “cg11967835” “cg23377495” “cg10679147” “cg10498926” “cg24287362” “cg17492717” “cg00700455” “cg22455392” “cg09142260” “cg22492435” “cg01884445” “cg26539873” “cg07763398” “cg10273666” “cg26142604” “cg09044656” “cg17753476” “cg18048027” “cg06217399” “cg21809040” “cg16136290” “cg08506990” “cg02941697” “cg06482534” “cg01280589” “cg01851573” “cg05362892” “cg26125864” “cg01748805” “cg21057880” “cg07283630” “cg19560579” “cg10680854” “cg06246435” “cg18315249” “cg20393707” “cg04379126” “cg15711208” “cg05347173” “cg01640958” “cg25498045” “cg08934843” “cg26045331” “cg07604566” “cg23352145” “cg01458041” “cg19592898” “cg27506098” “cg10130703” “cg08191469” “cg25912173” “cg19667913” “cg07708947” “cg26369996” “cg26352440” “cg23175573” “cg04344303” “cg19021236” “cg26903218” “cg24406391” “cg12406651” “cg08379517” “cg27488807” “cg00640253” “cg18998321” “cg06045761” “cg17547883” “cg07581775” “cg08706575” “cg03826480” “cg00908117” “cg18304936” “cg02341645” “cg20963002” “cg07698266” “cg01705888” “cg01456285” “cg04861494” “cg20984904” “cg21710569” “cg23564460” “cg09055507” “cg02339032” “cg04088074” “cg24073042” “cg19260922” “cg13259063” “cg07508452” “cg18964630” “cg23262555” “cg00181497” “cg21198880” “cg09450352” “cg16181135” “cg13988329” “cg06626140” “cg20659752” “cg15328328” “cg07015412” “cg22715072” “cg22914616” “cg03408135” “cg17239714” “cg23757365” “cg11304573” “cg11231279” “cg27115113” “cg06647930” “cg22775776” “cg18981248” “cg03080336” “cg11775846” “cg14132288” “cg12319618” “cg16896144” “cg07973125” “cg14451730” “cg22920609” “cg17760895” “cg18867708” “cg19051504” “cg22511432” “cg25623271” “cg20911168” “cg05141695” “cg05356308” “cg18930100” “cg26589591” “cg17304878” “cg09041485” “cg20252903” “cg22736872” “cg24184350” “cg22869660” “cg27326823” “cg23321841” “cg11918124” “cg13831860” “cg26832999” “cg17601661” “cg27296963” “cg01443408” “cg05980922” “cg27413396” “cg27383534” “cg26127187” “cg14999518” “cg05314679” “cg10713715” “cg13865595” “cg18523042” “cg02992951” “cg27266060” “cg26990667” “cg19916364” “cg19335381”

“cg00594167” “cg04260065” “cg06788751” “cg19486702” “cg06773584” “cg11633280” “cg02222170” “cg02658969” “cg21947394” “cg21085679” “cg12144374” “cg17887537” “cg25687573” “cg16258229” “cg20405742” “cg14526241” “cg22699815” “cg26912485” “cg06190612” “cg22547226” “cg26667659” “cg10673318” “cg04272994” “cg19701205” “cg22536351” “cg21293943” “cg03356877” “cg26954056”

(142) For Mg deficiency, LASSO/elasticnet regression fits pick 71 5-hmC sites using approach 1.

(143) TABLE-US-00016 TABLE 16 Variables selected by LASSO/elasticnet for Mg deficiency in BS “cg19942593” “cg25883327” “cg25779653” “cg25506514” “cg08688393” “cg10129485” “cg16880176” “cg17980786” “cg01783579” “cg15768413” “cg02468643” “cg00162806” “cg02272814” “cg10997203” “cg09443697” “cg19277119” “cg26916871” “cg24705717” “cg07461432” “cg18414025” “cg19836423” “cg08752155” “cg17235953” “cg01211097” “cg04124858” “cg13306164” “cg06600135” “cg03383975” “cg19451311” “cg16075006” “cg05559978” “cg06007434” “cg17787108” “cg19404184” “cg10940515” “cg11514839” “cg16735465” “cg03451760” “cg19188464” “cg26517176” “cg05852416” “cg24613956” “cg16646600” “cg23703711” “cg14725164” “cg09313831” “cg16371860” “cg07565441” “cg22979531” “cg10641986” “cg07785314” “cg19872463” “cg08316054” “cg03345059” “cg07513561” “cg07814712” “cg00685863” “cg07734259” “cg03092609” “cg08288894” “cg11824316” “cg21627706” “cg15961993” “cg09600529” “cg17066470” “cg22571271” “cg04202957” “cg26390078” “cg25763709” “cg06331446” “cg00552805”

(144) For Mg insufficiency, LASSO/elasticnet regression fits pick 54 sites using approach 1.

(145) TABLE-US-00017 TABLE 17 Variables selected by LASSO/elasticnet for Mg deficiency in BS “cg20678128” “cg05595943” “cg01783579” “cg11514839” “cg19702274” “cg02272814” “cg09078754” “cg07461432” “cg05138062” “cg11528570” “cg06858541” “cg08752155” “cg14725164” “cg00446065” “cg09718640” “cg06830769” “cg27649897” “cg03381216” “cg17819702” “cg08790584” “cg19993845” “cg13432294” “cg03345059” “cg05351998” “cg00968310” “cg02804655” “cg19404184” “cg00666845” “cg24987590” “cg17934130” “cg18936620” “cg25257051” “cg19783306” “cg02858997” “cg22689016” “cg18610423” “cg01211109” “cg02484886” “cg02017534” “cg04144515” “cg05365607” “cg16195970” “cg04426842” “cg15732149” “cg19277119” “cg23657865” “cg07439208” “cg26278666” “cg18634848” “cg13753657” “cg08456112” “cg06894069” “cg21099767” “cg21884589”

(146) For Mg deficiency, LASSO/elasticnet regression fits pick 54 sites using approach 2.

(147) TABLE-US-00018 TABLE 18 Variables selected by LASSO/elasticnet for Mg deficiency in BS “cg01672172” “cg15965578” “cg02812510” “cg24197051” “cg07869659” “cg24330553” “cg08984686” “cg11997359” “cg03924111” “cg14160449” “cg03539876” “cg17572903” “cg02968715” “cg10342304” “cg25921358” “cg03235871” “cg09664812” “cg00574206” “cg24970361” “cg14810004” “cg20153590” “cg02383399” “cg07119028” “cg08411738” “cg20149871” “cg07574385” “cg17251609” “cg12670862” “cg05962733” “cg00577164” “cg24022829” “cg01628181” “cg02722511” “cg08216099” “cg23785114” “cg22095253” “cg17528662” “cg25130134” “cg07613047” “cg05135521” “cg07976328” “cg16385758” “cg14859088” “cg04275362” “cg07230522” “cg16642721” “cg14991358” “cg08558495” “cg20299810” “cg05244236” “cg17767224” “cg13877657” “cg02423930” “cg25005374”

(148) For Mg insufficiency, LASSO/elasticnet regression fits pick 130 sites using approach 2.

(149) TABLE-US-00019 TABLE 19 Variables selected by LASSO/elasticnet for Mg deficiency in BS “cg12546785” “cg20558790” “cg23050436” “cg23262555” “cg27070952” “cg23043438” “cg24955156” “cg27141863” “cg07139928” “cg21057271” “cg24034568” “cg13323097” “cg05780543” “cg25349350” “cg26747293” “cg02085507” “cg26644049” “cg18833720” “cg01400516” “cg23361930” “cg23115387” “cg05372730” “cg08463929” “cg13673137” “cg16616370” “cg08327708” “cg04762756” “cg18581781” “cg10273666” “cg18372013” “cg05168062” “cg03065601” “cg01612232” “cg25965355” “cg11727826” “cg27646850” “cg00036272” “cg14322298” “cg19495079” “cg01814149” “cg06839650” “cg17137671” “cg19504736” “cg26753307” “cg24702286” “cg26196162” “cg19703425” “cg01808547” “cg00203160” “cg14375890” “cg14983108” “cg16829297” “cg09312590” “cg04276750” “cg23169762” “cg03521625” “cg08841098” “cg02128882” “cg17178489” “cg11008674” “cg22832808” “cg02961620” “cg10720040” “cg25151806” “cg18156003” “cg27607805” “cg05285687” “cg14796889” “cg12579764” “cg03071553” “cg10739136” “cg17409731” “cg02339369” “cg21852208” “cg25182665” “cg18270378” “cg13164309” “cg26596734” “cg05847038” “cg11369564” “cg10142237” “cg07442244” “cg12031217” “cg04548032” “cg25025983” “cg23651872” “cg07943346” “cg23012185” “cg14626660” “cg23757365” “cg27371264” “cg09150559” “cg11333968” “cg23695209” “cg16923826” “cg20537611” “cg27241190” “cg00597107” “cg06177599” “cg25362525” “cg04396185” “cg07912402” “cg11621211” “cg02235659” “cg27030854” “cg16554615” “cg10538654” “cg15548346” “cg06483661” “cg12638844” “cg14309111” “cg23894443” “cg02362409” “cg24141156” “cg17382986” “cg01972688” “cg05324991” “cg12317021” “cg23281384” “cg13776199” “cg17796982” “cg02646091” “cg07502730” “cg00851782” “cg16018314” “cg13168333” “cg03940024” “cg04754212” “cg17602884” “cg18122443”

(150) Table 20 presents the BS methylation markers selected by LASSO at lambda value that minimizes the mean squared error rate for continuous magnesium status.

(151) TABLE-US-00020 TABLE 20 Variables selected by LASSO Name Beta OR cg05019905 167.198677 4.106416e+72 cg00924527 −10.087378 4.160000e−05 cg15021089 −140.149493 0.000000e+00 cg12800781 −4.044777 1.751360e−02

(152) Table 21 presents the BS methylation markers selected by Elasticnet with alpha=0.25 at lambda value that minimizes the mean squared error rate for continuous magnesium status.

(153) TABLE-US-00021 TABLE 21 Variables selected by Elasticnet fit alpha = 0.25 Name Beta OR cg05019905 122.144402 1.113371e+53 cg11840205 2.564487 1.299399e+01 cg15210809 −9.464701 7.750000e−05 cg00924527 −15.353084 2.000000e−07 cg15021089 −85.633748 0.000000e+00 cg12800781 −11.881832 6.900000e−06

(154) Table 22 presents the BS methylation markers selected by Elasticnet with alpha=0.5 at lambda value that minimizes the mean squared error rate for continuous magnesium status.

(155) TABLE-US-00022 TABLE 22 Variables selected by Elasticnet fit alpha = 0.5 Name Beta OR cg05019905 129.343026 1.489232e+56 cg15210809 −1.401056 2.463368e−01 cg00924527 −10.158863 3.870000e−05 cg15021089 −104.449342 0.000000e+00 cg12800781 −7.871308 3.815000e−04
(156) Table 23 presents the BS methylation markers selected by Elasticnet with alpha=0.75 at lambda value that minimizes the mean squared error rate for continuous magnesium status.
(157) TABLE-US-00023 TABLE 23 Variables selected by Elasticnet fit alpha = 0.75 Name Beta OR cg05019905 151.731653 7.874437e+65 cg00924527 −10.338433 3.240000e−05 cg15021089 −125.496642 0.000000e+00 cg12800781 −6.063569 2.326100e−03
(158) Table 24 presents the BS methylation markers selected by LASSO at lambda value that minimizes the mean square error rate for continuous magnesium status.
(159) TABLE-US-00024 TABLE 23 Variables selected by LASSO fit at lambda.min Name Beta OR cg08828389 138.865506 2.034806e+60 cg05995260 −135.501610 0.000000e+00 cg04778331 1183.296218 Inf cg17431860 641.914211 6.022818e+278 cg09823095 12.929707 4.123829e+05 cg06510234 −7.448462 5.823000e−04 cg12418357 121.079716 3.839303e+52 cg04530860 351.014430 2.777347e+152 cg02073796 −8.946129 1.302000e−04 cg07937803 −24.130111 0.000000e+00 cg08202165 50.496900 8.521679e+21 cg22798756 48.704348 1.419155e+21 cg24210818 −26.845604 0.000000e+00 cg06045761 32.657780 1.524381e+14 cg02916173 −29.488199 0.000000e+00 cg19434718 16.827155 2.032080e+07 cg03043243 3.446144 3.137916e+01
(160) Table 25 presents the BS methylation markers selected by Elasticnet with alpha=0.25 at lambda value that minimizes the mean squared error rate for continuous magnesium status.
(161) TABLE-US-00025 TABLE 24 Variables selected by Elasticnet fit alpha = 0.25 Name Beta OR cg08828389 115.1918321 1.064577e+50 cg05995260 −100.9931990 0.000000e+00 cg04778331 707.2274189 1.396257e+307 cg17431860 385.7032569 3.226979e+167 cg09823095 68.5944187 6.168471e+29 cg06510234 −5.7014958 3.341000e−03 cg12418357 333.4379260 6.460280e+144 cg04530860 240.3691265 2.460284e+104 cg02073796 −3.6532932 2.590570e−02 cg19400873 0.3081523 1.360908e+00 cg07937803 −8.7811010 1.536000e−04 cg15711208 −0.1705206 8.432257e−01 cg08202165 22.0573539 3.796532e+09 cg08520660 −4.0741794 1.700620e−02 cg22798756 18.4489354 1.028658e+08 cg24210818 −15.8078627 1.000000e−07 cg19615147 7.2197536 1.366152e+03 cg06045761 18.9400187 1.680914e+08 cg25767906 5.8479146 3.465110e+02 cg02916173 −31.9175271 0.000000e+00 cg17373759 0.1197168 1.127177e+00 cg20405742 −0.9694672 3.792851e−01 cg19531475 −8.1667245 2.839000e−04 cg19434718 10.5741498 3.911064e+04 cg03043243 2.3987769 1.100970e+01 cg13175850 −0.1669210 8.437315e−01
(162) Table 26 presents the BS methylation markers selected by Elasticnet with alpha=0.5 at lambda value that minimizes the mean squared error rate for continuous magnesium status.
(163) TABLE-US-00026 TABLE 25 Variables selected by Elasticnet fit alpha = 0.5 Name Beta OR cg05995260 −98.671875 0.000000e+00 cg04778331 743.208587 Inf cg17431860 314.640730 4.433358e+136 cg09823095 3.468884 3.210090e+01 cg12418357 60.600049 2.080975e+26 cg04530860 186.419395 9.139333e+80 cg02073796 −3.932358 1.959740e−02 cg07937803 −6.139188 2.156700e−03 cg08202165 32.846534 1.841063e+14 cg22798756 14.758575 2.567838e+06 cg24210818 −13.940851 9.000000e−07 cg19615147 3.267453 2.624441e+01 cg06045761 22.445195 5.595311e+09 cg25767906 2.907708 1.831477e+01 cg02916173 −28.512119 0.000000e+00 cg19434718 9.221180 1.010899e+04 cg03043243 2.337433 1.035462e+01
(164) Table 27 presents the BS methylation markers selected by Elasticnet with alpha=0.75 at lambda value that minimizes the mean squared error rate for continuous magnesium status.
(165) TABLE-US-00027 TABLE 26 Variables selected by Elasticnet fit alpha = 0.75 Name Beta OR cg08828389 210.851913 3.730974e+91 cg05995260 −141.564206 0.000000e+00 cg04778331 1143.339735 Inf cg17431860 608.753224 2.388724e+264 cg09823095 38.430211 4.898108e+16 cg06510234 −9.172619 1.038000e−04 cg12418357 262.019506 6.217640e+113 cg04530860 347.116610 5.634148e+150 cg02073796 −8.035824 3.237000e−04 cg07937803 −21.915744 0.000000e+00 cg08202165 43.975895 1.254551e+19 cg22798756 42.012923 1.761897e+18 cg24210818 −25.701806 0.000000e+00 cg06045761 30.178299 1.277229e+13 cg25767906 1.962401 7.116395e+00 cg02916173 −35.188482 0.000000e+00 cg19434718 17.253811 3.113401e+07 cg03043243 3.472041 3.220240e+01
(166) Table 28 presents the 5-mC methylation markers selected by LASSO at lambda value that minimizes the mean squared error rate for continuous magnesium.
(167) TABLE-US-00028 TABLE 27 Variables selected by LASSO Name Beta OR cg07121807 6.028887 415.25252 cg08001123 −32.979351 0.00000 cg04426842 −17.210661 0.00000 cg24687894 7.094252 1205.02029 cg06392664 4.201440 66.78244
(168) Table 29 presents the 5-mC methylation markers selected by Elasticnet with alpha=0.25 at lambda value that minimizes the mean squared error rate for continuous
(169) TABLE-US-00029 TABLE 28 Variables selected by Elasticnet fit alpha = 0.25 Name Beta OR cg07121807 3.731745 41.7519114 cg08001123 −20.288714 0.0000000 cg03889299 1.924902 6.8544797 cg10983873 −3.046954 0.0475034 cg04426842 −9.041472 0.0001184 cg24687894 3.353182 28.5935612 cg06382664 3.937142 51.2718752
(170) Table 30 presents the 5-mC methylation markers selected by Elasticnet with alpha=0.5 at lambda value that minimizes the mean squared error rate for continuous magnesium.
(171) TABLE-US-00030 TABLE 29 Variables selected by Elasticnet fit alpha = 0.5 Name Beta OR cg07121807 1.710080 5.5294042 cg08001123 −17.752705 0.0000000 cg04426842 −10.386653 0.0000308 cg24687894 2.477333 11.9094608 cg06382664 2.580519 13.2039880
(172) Table 31 presents the 5-mC methylation markers selected by Elasticnet with alpha=0.75 at lambda value that minimizes

the mean squared error rate for continuous magnesium.
(173) TABLE-US-00031 TABLE 30 Variables selected by Elasticnet fit alpha = 0.75 Name Beta OR cg07121807 5.839709 343.6791269 cg08001123 −31.113077 0.0000000 cg04426842 −15.634694 0.0000002 cg24687894 6.301561 545.4228447 cg06382664 4.475123 87.8053585
(174) Table 32 presents the 5-mC methylation markers selected by LASSO at lambda value that minimizes the mean squared error rate for continuous magnesium.
(175) TABLE-US-00032 TABLE 31 Variables selected by LASSO Name Beta OR cg16978871 168.8655581 2.174603e+73 cg26744387 −454.0816703 0.000000e+00 cg15577559 16.0920146 9.742561e+06 cg22120017 425.3692651 5.439070e+184 cg03529803 −193.6133078 0.000000e+00 cg16436867 −323.2394483 0.000000e+00 cg14258935 −308.6214036 0.000000e+00 cg22492435 −32.5284342 0.000000e+00 cg02073796 −2.3424258 9.609420e−02 cg15989720 3.2959008 2.700173e+01 cg13226135 1.7299539 5.640394e+00 cg15740518 97.0888704 1.462718e+42 cg23172059 6.2167359 5.010650e+02 cg15711208 −34.0070716 0.000000e+00 cg08202165 32.2456398 1.009492e+14 cg08520660 −18.5568863 0.000000e+00 cg03238677 6.0886201 4.408127e+02 cg19615147 53.7861012 2.285635e+23 cg06045761 17.9116196 6.010597e+07 cg13192508 4.4040006 8.177737e+01 cg22043275 −5.8273224 2.946000e+03 cg12026858 10.7278595 4.560896e+04 cg24520234 −14.0790374 8.000000e−07 cg25767906 1.1096137 3.033186e+00 cg20405742 −15.8009896 1.000000e+07 cg12544293 0.0563505 1.057968e+00 cg21801549 10.1838319 2.647171e+04 cg12165250 −42.4279469 0.000000e+00 cg10413151 9.4485487 1.268974e+04 cg05031435 −53.2325187 0.000000e+00 cg25143609 3.4701042 3.214009e+01 cg17542650 2.1587528 8.660330e+00 cg05525594 4.4426723 8.500179e+01 cg15522298 0.9449790 2.572759e+00 cg21302538 −21.4355254 0.000000e+00 cg11757337 −0.8649319 4.210802e−01 cg05444524 −6.1084300 2.224000e−03
(176) Table 33 presents the 5-mC methylation markers selected by Elasticnet with alpha=0.25 at lambda value that minimizes the mean squared error rate for continuous magnesium.
(177) TABLE-US-00033 TABLE 32 Variables selected by Elasticnet fit alpha = 0.25 Name Beta OR cg16978871 153.6391738 5.304512e+66 cg06026520 2.3928664 1.094482e+01 cg26744387 −214.8360175 0.000000e+00 cg05995260 −24.5952038 0.000000e+00 cg22120017 274.0051372 9.975142e+118 cg13323097 −142.0225249 0.000000e+00 cg23029526 116.4674538 3.812172e+50 cg03529803 −138.3640631 0.000000e+00 cg16436867 −143.7549508 0.000000e+00 cg22280238 67.1420760 1.443554e+29 cg19066691 7.7304470 2.276620e+03 cg14258935 −77.7791875 0.000000e+00 cg22492435 −15.2793723 2.000000e−07 cg02073796 −1.9894817 1.367663e−01 cg15989720 1.4248432 4.157206e+00 cg13226135 3.8225455 4.572044e+01 cg15740518 43.0302025 4.872810e+18 cg23172059 8.6536099 5.730797e+03 cg00781388 −3.1187924 4.421050e−02 cg15711208 −30.5642598 0.000000e+00 cg04312620 −0.3283286 7.201264e−01 cg08202165 17.8940211 5.905744e+07 cg08520660 −10.4024631 3.040000e−05 cg03238677 2.1111501 8.257733e+00 cg06528228 −3.0062450 4.947710e−02 cg19615147 29.8630053 9.318337e+12 cg06045761 14.7964860 2.667056e+06 cg22676923 −5.7299470 3.247200e−03 cg13192508 6.5129613 6.738188e+02 cg12026858 6.1264297 4.577988e+02 cg24520234 −11.9423197 6.500000e−06 cg25767906 5.4487252 2.324616e+02 cg20405742 −7.3223499 6.606000e−04 cg09383456 −14.2588785 6.000000e−07 cg21801549 9.4633042 1.287837e+04 cg17753427 −0.4919550 6.114299e−01 cg12165250 −21.2878781 0.000000e+00 cg16642721 0.0199829 1.020184e+00 cg11635325 −1.6269720 1.965237e−01 cg10413151 3.1638279 2.366100e+01 cg19531475 −16.2727070 1.000000e−07 cg05031435 −26.7068285 0.000000e+00 cg15355341 6.3353840 5.641860e+02 cg03043243 0.3733803 1.452637e+00 cg19247001 81.9360640 3.840436e+35 cg17542650 2.4427495 1.150463e+01 cg05525594 1.8610654 6.430584e+00 cg15522298 4.5193889 9.177950e+01 cg13410153 0.7554803 2.128634e+00 cg21302538 −24.6333983 0.000000e+00 cg11757337 −11.3735808 1.150000e−05 cg05444524 −5.7626209 3.142900e−03
(178) Table 34 presents the 5-mC methylation markers selected by Elasticnet with alpha=0.5 at lambda value that minimizes the mean squared error rate for continuous
(179) TABLE-US-00034 TABLE 34 Variables selected by Elasticnet fit alpha = 0.75 Name Beta OR cg16978871 176.5659273 4.804055e+76 cg06026520 0.8235186 2.278503e+00 cg26744387 −310.9720561 0.000000e+00 cg22120017 357.4438279 1.720024e+155 cg13323897 −97.6926325 0.000000e+00 cg23029526 00.9472404 2.944782e+28 cg03529803 −164.1334361 0.000000e+00 cg16436867 −216.0442581 0.000000e+00 cg22280238 54.4268508 4.337914e+23 cg14258935 −147.4915193 0.000000e+00 cg22492435 −19.7679952 0.000000e+00 cg02073796 −1.8387406 1.590176e−01 cg15989720 1.9796313 7.241521e+00 cg13226135 4.0350752 5.654717e+01 cg15740518 66.6554394 8.873389e+28 cg23172059 9.9175384 2.028300e+04 cg00781388 −0.4737788 6.226450e−01 cg15711208 −35.7591629 0.000000e+00 cg19850463 −0.0079288 9.921025e−01 cg08202165 24.3994422 3.949509e+10 cg08520660 −13.6994832 1.100000e−06 cg03238677 4.7777196 1.188381e+02 cg10615147 41.7501915 1.354808e+18 cg06045761 17.5943937 4.376713e+07 cg22676923 −1.5919824 2.035218e−01 cg13192508 5.7547783 3.156955e+02 cg12026858 7.4808056 1.087166e+03 cg24520234 −14.9258319 3.000000e−07 cg25767906 4.5692175 9.646859e+01 cg20405742 −10.2489541 3.540000e−05 cg21801549 10.5525170 3.827365e+04 cg12165250 −30.5918328 0.000000e+00 cg10413151 4.6902916 1.088849e+02 cg19531475 −4.4139937 1.210670e−02 cg05031435 −36.8859424 0.000000e+00 cg15355341 0.3642230 1.439395e+00 cg19247001 15.9408548 8.375780e+06 cg17542850 2.5037163 1.222785e+01 cg05523594 0.7784029 2.177991e+00 cg15522298 4.3593168 7.820369e+01 cg21302538 −24.8053154 0.000000e+00 cg11757337 −7.8961447 3.722000e−04 cg05444524 −7.3638780 6.337000e−04
(180) Table 35 presents the 5-mC methylation markers selected by Elasticnet with alpha=0.75 at lambda value that minimizes the mean squared error rate for continuous
(181) TABLE-US-00035 TABLE 33 Variables selected by Elasticnet fit alpha = 0.5 Name Beta OR cg16978871 168.185669 1.101813e+73 cg26744387 −376.932712 0.000000e+00 cg22120017 400.377142 7.613484e+173 cg13323097 −29.580911

0.000000e+00 cg03529803 −181.745902 0.000000e+00 cg16436867 −280.279288 0.000000e+00 cg22280238 34.581507 1.043657e+15 cg14258935 −229.972560 0.000000e+00 cg22492435 −24.887309 0.000000e+00 cg02073796 −1.933014 1.447114e−01 cg15989720 2.756013 1.573697e+01 cg13226135 3.177646 2.399021e+01 cg15740518 83.192548 1.349163e+36 cg23172059 8.937008 7.608397e+03 cg15711208 −34.847537 0.000000e+00 cg08202165 29.008663 3.965540e+12 cg08520660 −15.968992 1.000000e−07 cg03238677 6.656079 7.774965e+02 cg19615147 49.138812 2.191367e+21 cg06045761 19.015199 1.812158e+08 cg13192508 4.957573 1.422482e+02 cg12026858 9.316269 1.111742e+04 cg24520234 −15.270334 2.000000e−07 cg25767906 3.395298 2.982355e+01 cg20405742 −12.773773 2.800000e−06 cg21801549 10.492268 3.603581e+04 cg12165250 −36.461723 0.000000e+00 cg10413151 7.077818 1.185379e+03 cg05031435 −45.442638 0.000000e+00 cg17542650 2.752226 1.567749e+01 cg05525594 1.273569 3.573583e+00 cg15522298 2.917090 1.848741e+01 cg21302538 −24.213658 0.000000e+00 cg11757337 −4.138331 1.594940e−02 cg05444324 −7.768477 4.229000e−04

(182) Table 36 presents the 5-hmC methylation markers selected by LASSO at lambda value that minimizes the mean squared error rate for continuous magnesium.

(183) TABLE-US-00036 TABLE 35 Variables selected by LASSO Name Beta OR cg18999855 −6.512791 1.484300e−03 cg17980786 −45.581059 0.000000e+00 cg01783579 19.329694 2.481872e+08 cg02272814 3.055827 2.123874e+01 cg19277119 −84.958210 0.000000e+00 cg18414025 −38.861833 0.000000e+00 cg08752155 158.457317 6.563542e+68 cg17235953 378.222471 1.819417e+164 cg21884589 13.257369 5.722716e+05

(184) Table 37 presents the 5-hmC methylation markers selected by Elasticnet with alpha=0.25 at lambda value that minimizes the mean squared error rate for continuous

(185) TABLE-US-00037 TABLE 36 Variables selected by Elasticnet fit alpha = 0.25 Name Beta OR cg19942593 59.9506481 1.087015e+26 cg25779653 75.4684777 5.964080e+32 cg18999855 −49.4331796 0.000000e+00 cg08688393 −10.6285860 2.420000e−05 cg16752029 −5.3622066 4.698500e−03 cg16880176 2.9051535 1.826805e+01 cg05559978 −17.343271 0.000000e+00 cg17980786 −64.8489291 0.000000e+00 cg01783579 76.8154912 2.293739e+33 cg11514839 32.2543656 1.018340e+14 cg12252979 −1.9493353 1.423687e−01 cg12814059 −1.5352711 2.153973e−01 cg16193970 −9.1923593 1.018000e−04 cg04426842 4.9648411 1.432858e+02 cg02272814 10.0907890 2.411981e+04 cg11944024 −27.8805872 0.000000e+00 cg10997203 −20.4650335 0.000000e+00 cg19277119 −76.5182092 0.000000e+00 cg15795515 0.5030806 1.653808e+00 cg26916871 0.3109956 1.364783e+00 cg24613956 −3.6990990 2.474580e−02 cg22571271 64.9045160 1.540542e+28 cg19870668 107.0485910 3.094658e+46 cg18414025 −63.0026625 0.000000e+00 cg08752155 126.7241156 1.085386e+55 cg17235953 498.7505991 4.023769e+216 cg03030098 19.4807725 2.886636e+08 cg21884589 60.4170626 1.732993e+26

(186) Table 38 presents the 5-hmC methylation markers selected by Elasticnet with alpha=0.5 at lambda value that minimizes the mean squared error rate for continuous

(187) TABLE-US-00038 TABLE 38 Variables selected by Elasticnet fit alpha = 0.5 Name Beta OR cg18999855 −50.274410 0.000000e+00 cg08688393 −4.679491 9.283700e−03 cg17980786 −64.452640 0.000000e+00 cg01783579 66.118950 5.189140e+28 cg04426842 2.797767 1.640797e+01 cg02272814 11.764888 1.286548e+05 cg11944024 −5.336252 4.813900e−03 cg19277119 −91.884163 0.000000e+00 cg22571271 42.372980 2.525523e+18 cg19870668 57.928091 1.438302e+25 cg18414025 −67.252074 0.000000e+00 cg08752155 153.334947 3.913104e+66 cg17235953 557.915188 1.992910e+242 cg03030098 2.002318 7.406203e+00 cg21884589 48.266700 9.161387e+20

(188) Table 39 presents the 5-hmC methylation markers selected by Elasticnet with alpha=0.75 at lambda value that minimizes the mean squared error rate for continuous magnesium.

(189) TABLE-US-00039 TABLE 37 Variables selected by Elasticnet fit alpha = 0.5 Name Beta OR cg18999855 −61.708361 0.000000e+00 cg08688393 −2.056172 1.279429e−01 cg17980786 −65.464170 0.000000e+00 cg01783579 65.683167 3.356115e+28 cg04426842 2.258579 9.569480e+00 cg02272814 14.307720 1.635927e+06 cg19277119 −102.434209 0.000000e+00 cg22571271 47.623753 4.816517e+20 cg19870668 34.276798 7.695279e+14 cg18414025 −75.293465 0.000000e+00 cg08752155 171.928844 4.653161e+74 cg17235953 637.287627 5.895216e+276 cg21884589 41.600807 1.166812e+18

(190) Table 40 presents the 5-hmC methylation markers selected by LASSO at lambda value that minimizes the mean squared error rate for continuous magnesium.

(191) TABLE-US-00040 TABLE 39 Variables selected by LASSO Name Beta OR cg24374538 −26.5206555 0.000000e+00 cg03924111 384.6988050 1.181866e+167 cg06219103 −72.9059171 0.000000e+00 cg01331064 −95.4872762 0.000000e+00 cg17572983 −29.7135139 0.000000e+00 cg15494524 134.5388524 2.688322e+58 cg01672172 −387.4245715 0.000000e+00 cg15740518 −6.1086325 2.223606e−03 cg27639644 149.5627707 9.000001e+64 cg04509481 −61.8892859 0.000000e+00 cg15252243 −8.3300984 2.411000e−04 cg00175823 0.1041116 1.109724e+00 cg16586442 96.8739100 1.179790e+42 cg05397609 106.5209582 1.825847e+46 cg25182665 −39.8583041 0.000000e+00 cg09728629 −75.2506493 0.000000e+00 cg18270174 255.8941701 1.359647e+111 cg10480824 −46.1886444 0.000000e+00 cg23651872 −501.0590248 0.000000e+00 cg20399983 6.3128010 5.515878e+02

(192) Table 41 presents the 5-hmC methylation markers selected by Elasticnet with alpha=0.25 at lambda value that minimizes the mean squared error rate for continuous

(193) TABLE-US-00041 TABLE 40 Variables selected by Elasticnet fit alpha = 0.25 Name Beta OR cg24374538 −28.509247 0.000000e+00 cg03924111 247.688897 3.714679e+107 cg06219103 −25.912247 0.000000e+00 cg01331064 −35.788300 0.000000e+00 cg20894341 21.888360 3.206225e+09 cg17572903 −13.655918 1.200000e−06 cg15494524 25.583430 1.290454e+11 cg01672172 −140.738371 0.000000e+00 cg15740518 −8.601643 1.838000e−04 cg27639644 45.528992 5.929118e+19 cg13565702 −1.071689 3.424295e−01 cg16044562 −14.266913 6.000000e−07 cg04509481 −31.326172

0.000000e+00 cg00175823 7.532584 1.867927e+03 cg24022829 27.108228 5.928621e+11 cg16586442 37.373413 1.702421e+16 cg05397609 49.189377 2.305022e+21 cg25182665 −21.935949 0.000000e+00 cg09728629 −27.799970 0.000000e+00 cg18270174 112.349530 0.205556e+48 cg10480824 −20.210551 0.000000e+00 cg01093429 34.128650 6.635662e+14 cg16642721 −3.264202 3.827740e−02 cg05244236 1.387170 4.003504e+00 cg23651872 −163.050291 0.000000e+00 cg04562755 3.059624 2.131954e+01 cg20399983 12.021346 1.662663e+05

(194) Table 42 presents the 5-hmC methylation markers selected by Elasticnet with alpha=0.5 at lambda value that minimizes the mean squared error rate for continuous magnesium.

(195) TABLE-US-00042 TABLE 41 Variables selected by Elasticnet fit alpha = 0.5 Name Beta OR cg24374538 −33.655254 0.000000e+00 cg03924111 332.534362 2.617210e+144 cg06219103 −33.745629 0.000000e+00 cg01331064 −54.299997 0.000000e+00 cg20894341 18.187072 7.916719e+07 cg17572903 −20.249879 0.000000e+00 cg15494524 55.977350 2.044815e+24 cg01672172 −236.721324 0.000000e+00 cg15740518 −9.812386 5.480000e−05 cg27639644 74.936304 3.502865e+32 cg16044562 −2.734779 6.490830e−02 cg04509481 −47.209553 0.000000e+00 cg00175823 7.043800 1.145802e+03 cg24022829 14.340020 1.689630e+06 cg16586442 49.683857 3.779417e+21 cg05397609 73.347851 7.154298e+31 cg25182665 −31.950541 0.000000e+00 cg09728629 −44.607688 0.000000e+00 cg18270174 174.079862 3.998742e+75 cg10480824 −31.126525 0.000000e+00 cg23651872 −275.875194 0.000000e+00 cg20399983 13.478978 7.142428e+05

(196) Table 43 presents the 5-hmC methylation markers selected by Elasticnet with alpha=0.75 at lambda value that minimizes the mean squared error rate for continuous magnesium.

(197) TABLE-US-00043 TABLE 42 Variables selected by Elasticnet fit alpha = 0.75 Name Beta OR cg24374538 −32.171798 0.000000e+00 cg03924111 370.545965 8.434164e+160 cg06219103 −48.565503 0.000000e+00 cg01331064 −73.913877 0.000000e+00 cg20894341 2.453452 1.162842e+01 cg17572903 −25.138338 0.000000e+00 cg15494524 95.403975 2.712814e+41 cg01672172 −317.082391 0.000000e+00 cg15740518 −8.204276 2.735000e−04 cg27639644 111.607470 2.954665e+48 cg04509481 −56.164089 0.000000e+00 cg00175823 5.079676 1.607221e+02 cg16586442 71.292907 9.164651e+30 cg05397609 94.473546 1.069891e+41 cg25182665 −36.736160 0.000000e+00 cg09728629 −60.992877 0.000000e+00 cg18270174 221.199825 1.163759e+96 cg10480824 −39.192160 0.000000e+00 cg23651872 −389.318823 0.000000e+00 cg20399983 10.780226 4.806096e+04

(198) All publications, patents, and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication, patent, or patent application was specifically and individually indicated to be incorporated by reference, including the references set forth in the following list:

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(200) It will be understood that various details of the presently disclosed subject matter can be changed without departing from the scope of the subject matter disclosed herein. Furthermore, the foregoing description is for the purpose of illustration only, and not for the purpose of limitation.

Claims

1. A method for predicting and treating magnesium deficiency in a subject, said method comprising: a) detecting methylation at CpG sites in DNA obtained from a biological sample from the subject, wherein the subject is in need of assessment for magnesium deficiency status; and b) identifying the subject as being in need of magnesium supplementation when methylation is found at the CpG site: cg00430271 or cg25731074; and c) administering an effective amount of magnesium to

the identified subject.
2. The method of claim 1, and further comprising detecting 5-hydroxymethylcytosine (5-hmC) and 5-methylcytosine (5-mC) methylation in the DNA obtained from the biological sample from the subject and differentiating between 5-hmC and 5-mC methylation in the sample.
3. The method of claim 1, further comprising detecting 5-hmC and 5-mC methylation, 5-hmC methylation, or 5-mC methylation by performing bisulfite (BS) treatment of the DNA in the biological sample.
4. The method of claim 2, further comprising differentiating between 5-hmC and 5-mC methylation in the biological sample using a TAB-Seq and TAB-Array protocol.
5. The method of claim 1, and further comprising determining for the subject a magnesium tolerance test (MTT) score, where an MTT score of greater than or equal to 50% is further indicative of a magnesium deficiency, an MTT score of less than 50% and greater than or equal to 25% is further indicative of a magnesium insufficiency, and an MTT score of less than 25% is further indicative of a magnesium sufficiency.
6. The method of claim 1, and further comprising identifying the subject as being in need of magnesium supplementation when methylation is found at the CpG sites comprising: cg00430271 and cg25731074.
7. The method of claim 6, and further comprising administering an effective amount of magnesium to the identified subject.
8. The method of claim 1, wherein the biological sample includes blood leukocyte DNA.
9. The method of claim 1, and further comprising identifying the subject as being in need of magnesium supplementation when methylation is found at the CpG sites comprising: cg00430271, cg11840205, and cg25731074.
10. The method of claim 9, and further comprising administering an effective amount of magnesium to the identified subject.
11. The method of claim 1, and further comprising identifying the subject as being in need of magnesium supplementation when methylation is found at the CpG sites comprising: cg00430271, cg04386563, cg11840205, and cg25731074.
12. The method of claim 11, and further comprising administering an effective amount of magnesium to the identified subject.
13. The method of claim 1, and further comprising determining Ca:Mg ratio of the diet of the subject, and selecting the effective amount of magnesium to decrease the Ca:Mg ratio.