Shotgun Metagenomics of 361 elderly women reveals gut microbiome change in bone mass loss

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Abstract:

Bone mass loss contributes to the risk of bone fracture in elderly. Many factors including age, obesity, estrogen and diet, are associated with bone mass loss. Some mice transplantation experiments suggest that the intestinal microbiome might influence the bone mass by regulating the immune system. However, there has been little evidence from human studies, not to mention the metagenome-wide association studies (MWAS). We have recruited 361 Chinese elderly women to explore the influence of gut microbiome on bone health by metagenomic shotgun sequencing data. Our results indicate that some lifestyle habits, like tea-drinking, have beneficial effects on bone mass loss. In addition, the gut microbiome diversity mildly increases with bone mass loss which might be contributed by the raise of pathogenic genera, such as *Escherichia*. Moreover, we have detected some microbial species and modules as markers for bone mineral density (BMD). Functionally, we observed positive correlation between bone mass loss and some modules which might influence the BMD, saying pectin degradation, trehalose degration and arginine degration.

Importance:

Our study firstly indicates that the gut microbiota might play an important role in bone mass loss. Our findings offer new insights on the bone mass loss process, and suggest better diagnosis as well as mechanistic understandings of this devastating disease.

Key words

Bone mass loss, Bone mineral density (BMD), Chinese elderly women,

Metagenome-Wide Association Study (MWAS)

Introduction

Bone mass loss is a process that reabsorb calcium and phosphate from the bones instead of keeping these minerals just make our bones weaker [1]. It is a severe and common disease in elderly population, also the most common reason for fracture, giving rise to ache, even death [2]. Many factors influence the illness including the age, obesity, estrogen level and diet [3]. More seriously, for elderly women the bone loss will increase after menopause due to lower levels of estrogen [4].The high-morbidity and serious drawbacks of elderly bone mass loss urge us to do more to its prevention and treatment. Recently a new concept, “osteoimmunology”, have revealed tight interaction between the immune system and bone metabolism [5]. The new term highlights the role of immune-related factors in modulating bone remodeling. Interestingly, it has been widely recognized that the gut microbiota could influence host health by interacting with the host immune system [6, 7].

The human gastrointestinal tract harbors trillions of microbial cells [8]. These microorganisms help us to digest food and also could access to many important complex functions including regulation of the host immune system [9]. Moreover, the transplantation of fecal or specific bacteria to specific pathogen free (SPF) mice or germ-free mice also showed that the gut microbiota could also modulate the bone homeostasis by immune system modulating and osteoclast formation [10]. But most of these experiments [7, 11] were carried on mice and 16S sequencing which is poor taxonomic resolution, low sensitivity and no functional related information [12].Notably, the metagenome-wide association studies (MWAS) based on the human shotgun

sequencing which could reveal the relationship between the bone mass loss and the microorganisms [13].

Here we carry out the MWAS on fecal samples from 361 Chinese elderly women in the city town. Species and functional profile are calculated by Metaphlan2 [33]and the GMMs [23]. Gut microbial species and modules that change along with T-score of BMD are identified and some species as well as modules which could serve as biomarkers for diagnosis of bone mass lose are suggested.

Results

To explore the gut microbiome in bone loss, feces from 361 Chinese elderly women in city town are collected and metagenomic shotgun sequencing is performed to obtain an average of 7.7 gigabase (Gb) host removing clean data per sample (sTable1C). For the result part, firstly, the life and clinical index (sTable1A-B) to the T-score in our cohort is assessed and the significant factors such as age and body mass index (BMI) to the microbiome are excluded, then the alteration of the gut microbiome along with the T-score was evaluated. Lastly, stable regression model is built at species and module level for the cohort. The T score of the BMD in the lumbar spine is used to represent the bone mass [14]. The specific details are showed following.

Result 1. Beneficial effects of tea drinking on BMD.

The influence of the life and clinical index to the T-score of BMD are accessed. We find that the age (p = 0.000279, adjusted R^2=0.034, linear regression, Fig1B), BMI (p = 8.08e-8, adjusted R^2=0.11, linear regression, Fig1C) and one interesting factor, tea drinking (p = 0.017, Wilcox test, Fig1A) have benign effect on the bone. But for other

life index including coffee drinking, alcohol and smoking show no significance (sFig1). And for the clinical index, N-amino terminal propeptide of type I collagen (P1NP) (p = 0.00224, adjusted R^2=0.0234, linear regression, Fig1D, sTab1C), β-Crosslaps (CROSSL) (p = 5.87e-5, adjusted R^2=0.0421, linear regression, Fig1E, sTab1C) and high-density lipoprotein (HDL) (p = 0.00168, adjusted R^2=0.0249, linear regression, Fig1F, sTab1C) are significant. Among these indexes, the age [15] and BMI [16] are reported to have key influence on gut microbiota. So, the two-stage least square [17] are used to exclude the error which caused by age and BMI. The description of this method is showed in the methods part.

Result 2. A mild gut microbiome dysbiosis seen for bone mass loss.

To show the alteration of the gut along with the change of the T-score, the change in different taxonomy levels are analyzed. Diversity at each level increase with the T-score probably caused by the flourish of pathogenic microcells in our gut. In detail, gene (p = 4.53e-9, adjusted R^2=0.0904, linear regression, sFig2B, sTab2), species (p = 1.17e-15, adjusted R^2=0.162, linear regression, sFig2D, sTab2) and genus (p = 7.98e-14, adjusted R^2=0.144, linear regression, Fig2B, sTab2) level are showed. In addition, the count data also shows increase with the T-score at gene (p = 0.0114, adjusted R2=0.0152, liner regression, sFig2A, sTab2), species (p = 2.33e-5, adjusted R2=0.0465, liner regression, sFig2C, sTab2), and genus (p = 7.73e-10, adjusted R2=0.0992, liner regression, Fig2A, sTab2) level. Then, the top 20 abundant species are chosen (Fig2C, sTab3A). The data show that the *B.stercoris, E.coli, B.uniformis, B.coprocola, B.fragilis , E.rectale* and *E.eligens* significantly negatively associated with T score. While for the *B.vulgatus,*

B.massiliensis, B. caccae and *Megamonas unclassified* display obvious positive correlation with T score (Fig2C, sTab3A). In addition, in the top 15 abundant genera, the *Eubacterium, Escherichia, Subdoligranulum, Klebsiella, Clostridium* and *Blautia* have significant negative correlation with the T-score (sFig3, sTab3B). Among these genera，the *Eubacterium, Escherichia* are normal microorganism of the intestinal tract and can cause infection under opportunistic conditions [5, 18]. For the positively correlated genera, the *Prevotella, Parabacteroides, Megamonas* and *Akkermansia* are inside of them (sFig3, sTab3B). For the top 10 enriched phyla, the *Bacteroidetes, Verrucomicrobia, Fusobacteria, Euryarchaeota* and *Ascomycota* are positive to BMD T-score (sFig4, sTab3C), while the *Proteobaccteria, Actinobacteria, Synergistetes* and *Chlorobi* are negative (sFig4, sTab3C).

Result 3. Species linked to BMD

To select the species which have strong connection with the T-score, we use the two-stage least square method [17] to regress the species to the T-score (details are showed in methods part). The model shows a high R square (more than 0.99, sTab3, Fig3A), and 18 species are selected. The importance of these species (Fig3B, sTab3A) are ranked in order. Spearman` rank correlation method is used to evaluate the relationship between the selected species and the clinical indexes (Fig3C). From the results, it is easy to find that some T-score negatively correlated species like the *Streptococcus parasanguinis* has enriched in atherosclerotic cardiovascular patients [19], *Clostridium perfringens, Haemophilus sputorum, Enterobacter aerogenes, Actinobacillus*

unclassified and *Chlorobium phaeobacteroides* are negatively connected with the triglyceride (TG), but positively to CROSSL and HDL. And some T-score positively correlated species, for example the *Roseburia intestinalis* which is a butyrate-producing bacterium that could influence human` immune system [20], *Enterobacter cloacae* that could promote obesity level in mice model [21] and *Sutterella wadsworthensis*. These species have positive correlation to TG, but negative to CROSSL and HDL.

Result 4. Modules suggesting for bone mass loss

The functional analysis is a critical advantage of the shotgun sequencing data. Traditional Kyoto Encyclopedia of Gene and Genomes (KEGG) annotation [22] methods hold redundant information and not suitable for the interaction between the host and microorganism. So, we use the Gut metabolic modules (GMMs) [23] to show the functional changes in bone mass loss cohort.

To find the higher correlation modules with the T-score, we also use the two-stage least square method [17] mentioned as before. 13 modules with more than 0.99 R square (sTab4, Fig4A) are obtained by the model and plotted in rank by their importance (Fig4B, sTab4). In addition, for the correlation with clinical indexes, the negatively correlated modules like the lactate consumption, sucrose degradation that would impart a significant impact on bone structural integrity [24] and tryptophan degradation which plays a complicated role in osteoblastic differentiation [25] are positively associated with HDL and CROSSL, but negatively with TG. By comparison, the BMD positive modules, for

example the pectin degradation that would inhibit bone resorption and strength the bone [26], trehalose degradation which could be effective on the prevention of bone mass loss [27], arginine degradation that can prevent bone mass loss and bone collagen breakdown in rats' model [28], mucin degradation and rhamnose degradation. These modules are positive to TG, but negative to HDL and CROSSL. And for the detail part (enzyme) of these modules, you could refer to the supplementary figure (sFig5).

Discussion:

We carry out the first study to explore the alteration of the gut microbiome along with bone mass loss in the 361 elderly Chinese urban women with MWAS. Firstly, the life and clinical indexes are evaluated and tea drinking is suggested as a factor which could reduce the bone mass loss. Then, taxonomy diversity is observed to increase at many levels which may be contributed by the growth of some opportunistic pathogens. In addition, some high correlated species and functional modules are also suggested which might offer us a new way for better diagnosis as well as mechanistic understandings of the bone mass loss.

First, the change of the life indexes with the T score shows us some ways to prevent the loss of the bone mass in our daily life. The life indexes show that the people who with high BMI [29] and tea drinking [30] will have higher bone mass, which is consistent with the previous study. BMI is not a percentage of body fat which should take age, gender, and occupation into consideration when using it to predict body fat percentage or obesity.

And higher BMI could recover by regular exercise. As tea is an important part of Chinese tradition and tea drinking is common in Chinese, especially green, black, white, and Oolong tea. High quality tea contains many nutrients, in particular flavonoids and vitamins, which might contribute to higher bone density [31].

Second, the gut is closely connected with the bone mass loss. With the loss of bone mass, some pathogenic microorganisms including *Escherichia* will mildly increase which attribute to the up of the species diversity. *F. prausnitzii* is reported as the 'probiotic of the future' microbe and short chain fatty acid (SCFAs) producer[32], its profile lightly declines with the loss of bone mass.

Third, the observed species and modules markers offer new insights about the microbial cells' role in the bone mass loss process. These bacteria, taking *Roseburia intestinalis* [20] as an example, may be involved in SCFAs synthesis related pathways to influence the immune system of the host. Some targeted modules, like the degradation of pectin [26], trehalose [27] and arginine [28], are related to bone protection. But for the sucrose degradation module, it would impart a significant impact on bone structural integrity [24], which suggest that we should reduce the intake of foods rich in sucrose in our daily diet.

In all, our study suggests that the gut microbiome is closely related to the process of bone mass loss in elder population. Although the mechanism of how do the gut microbes affect and modulate bone metabolism is not fully understood, our research indicates that gut

microbiota may be novel targets for the protection of bone mass loss and provide a new avenue for the future studies and treatment of this field.

Materials and Methods

Statement of IRB approval. This study was approved by the Institutional Review Board on Bioethics and Biosafety at Shenzhen people`s Hospital.

Study cohort and sample information. Fecal samples and clinical indexes were collected at Shenzhen people`s Hospital, transported frozen, and extracted at BGI-Shenzhen [12]. The BMD was calculated by Hologic dual energy X-ray machine at Shenzhen people`s Hospital. We use the T-score of BMD in the lumbar spine to represent the bone mass [14]. A sample`s T-score is a relative measure of the sample`s BMD compared to the reference population of young, healthy individuals with the same gender. The sequencing data of 361 sample were filtered low quality reads and the reads align to host genome (Hg19) with in-house scripts, finally we get the cleaned data.

Taxonomic abundance calculation

The cleaned data were used for the annotation and profile of taxon by MetaPhlan2 [33]. We remove species presented in less than 10% of the samples for later analysis.

Gut metabolic module analysis

Each GMM abundance was calculated as the median of KO abundance with 66% coverage just as showed in the former article [23].

Two-stage least square

Stage 1: In the first step we regress the taxonomic abundance or metabolic module abundance on the Age and BMI with linear regression and save the prediction value. The detail of the taxonomic abundance is in 'Taxonomic abundance calculation' part (sTab 3). The detail of the metabolic module abundance is in 'Gut metabolic modules analysis' part (sTab 4). This step is used to adjust the effects of Age and BMI to the contribution of BMD by the taxonomic abundance or metabolic module abundance.

Stage 2: Five-fold cross-validation is performed ten times on a random forest regression model (Y: the BMD T score; X: the prediction value from the stage 1). The error curves from ten trials of fivefold cross-validation are averaged. We chose the model which minimized the sum of the test error and its standard deviation in the averaged curve.

Alpha-diversity and count

The within-sample diversity is calculated by profile of samples with Shannon index, as described previously [14]. Genes were considered present with more than one read map to it.

Data Availability

The sequencing reads from each sequencing library have been deposited at EBI with the accession number: PRJNA530339.

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Authors' contributions

Design of the study: H.J. and S.P.; methodology: Q.W., Q.S., H.Z., J.C.; data analysis: Q.W., Q.S., H.Z., J.C., Y.J., R.G., Z.J.; sample collection: Q.W., J.C., Z.W., X.Y, X.S.; Clinical information collection: Q.W., F.W., J.C., Y.L., T.H.; writing of the first version of the manuscript: Q.W., J.C., H.Z.; restructuring and extensive revision of the manuscript: Q.S., X.S., T.Z., H.Y, X.X.; funding acquisition: H.J. and S.P.

Competing Interests

The authors declare no competing financial interests.

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Figure legends

Figure 1: The clinical index of the samples. Different distribution of the BMD T-score versus tea drinking or not. (A, two-tailed Wilcoxon-rank sum test); The correlation between Age (B), BMI (C), P1NP(D), CROSSL (E), HDL (F) with the BMD Tscore. (Liner regression)

Figure 2 |slightly increase gut microbial richness. (A-B) Richness and alpha-diversity (Shannon index) at the genus level of the two cohorts (liner regression). (C). The top 15 species. (The spearman`s correlation,'+' for $p<0.05$;'*' for $p<0.01$).

Figure 3 |. Fecal microbial species markers for BMD. (A) The R square during the Ten-time cross-validation process (the blue lines show the ten different process, the red line for the average of the ten-time cross validation, and the pink line show the best variables). (B). The lncMSE of the 18 chosen species markers. (C). The correlation between the marker with the clinical index. (Spearman` correlation,'+' for $p<0.05$;'*' for $p<0.01$).

Figure 4 |. Fecal microbial module markers for BMD. (A) The R square during the Ten-time cross-validation process (the blue lines show the ten different process, the red

line for the average of the ten-time cross validation, and the pink line show the best variables). (B). The lncMSE of the 13 chosen modules markers. (C). The correlation between the marker with the clinical index. (Spearman` correlation,'+' for $p<0.05$;'*' for $p<0.01$).

Supplementary materials description

sFigure1.The other clinical index. Different distribution of the BMD T-score in coffee, drink and smoking (A-C, two-tailed Wilcoxon-rank sum test).

sFigure2 |slightly increase gut microbial richness. (A-D) Richness and alpha-diversity (Shannon index) at the gene and species level of the two cohorts (liner regression).

sFigure3. The top 15 genera. (The spearman`s correlation,'+' for $p<0.05$;'*' for $p<0.01$).

sFigure4. The top 10 phyla. (The spearman`s correlation,'+' for $p<0.05$;'*' for $p<0.01$).

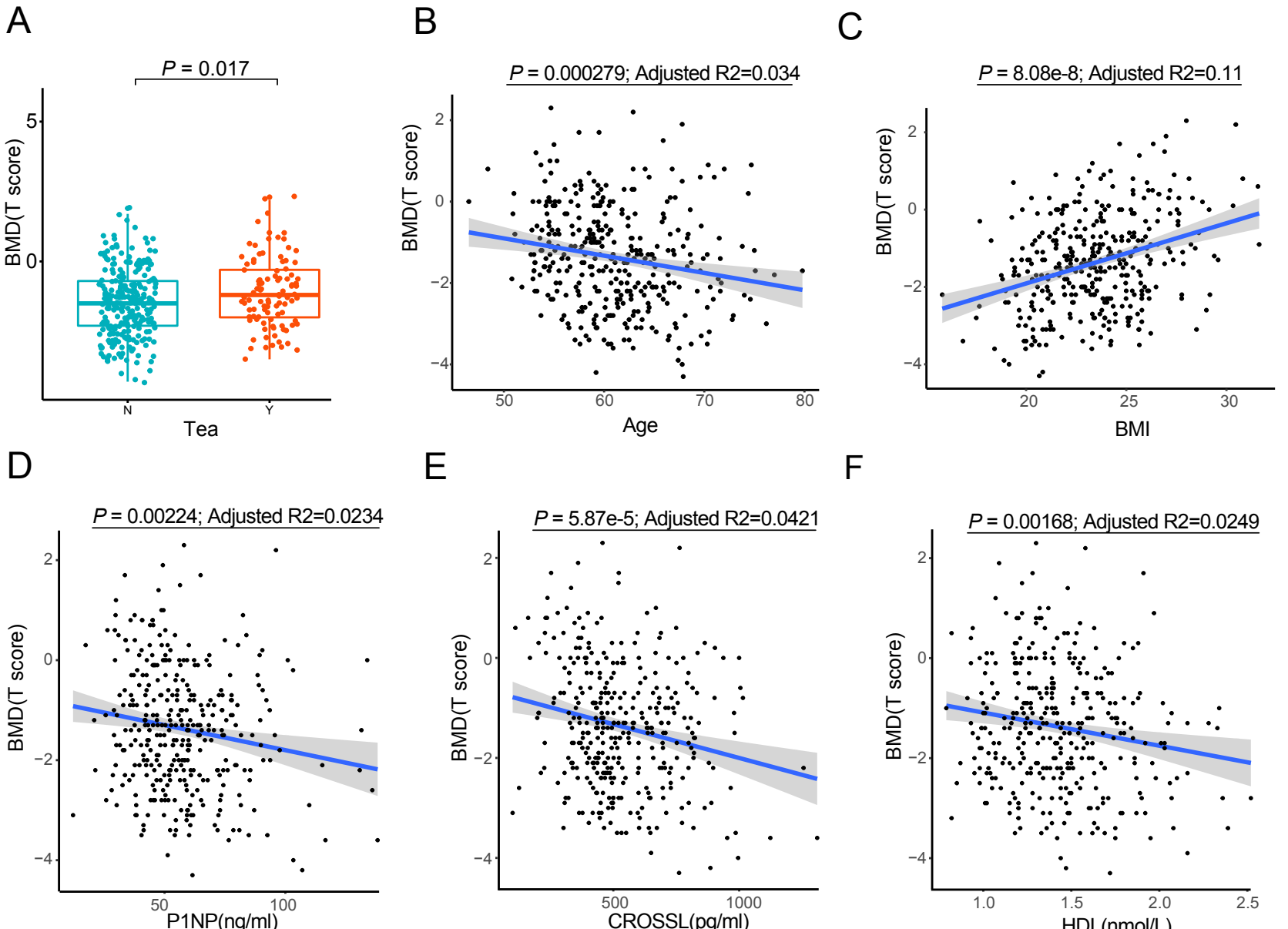
sFigure5. The detail information of the chosen modules with the clinical index. (The spearman`s correlation,'+' for $p<0.05$;'*' for $p<0.01$).

sTab1.The basic information of the samples.

sTab2. The diversity data.

sTab3.The taxon related data.

sTab4.The GMMs related data.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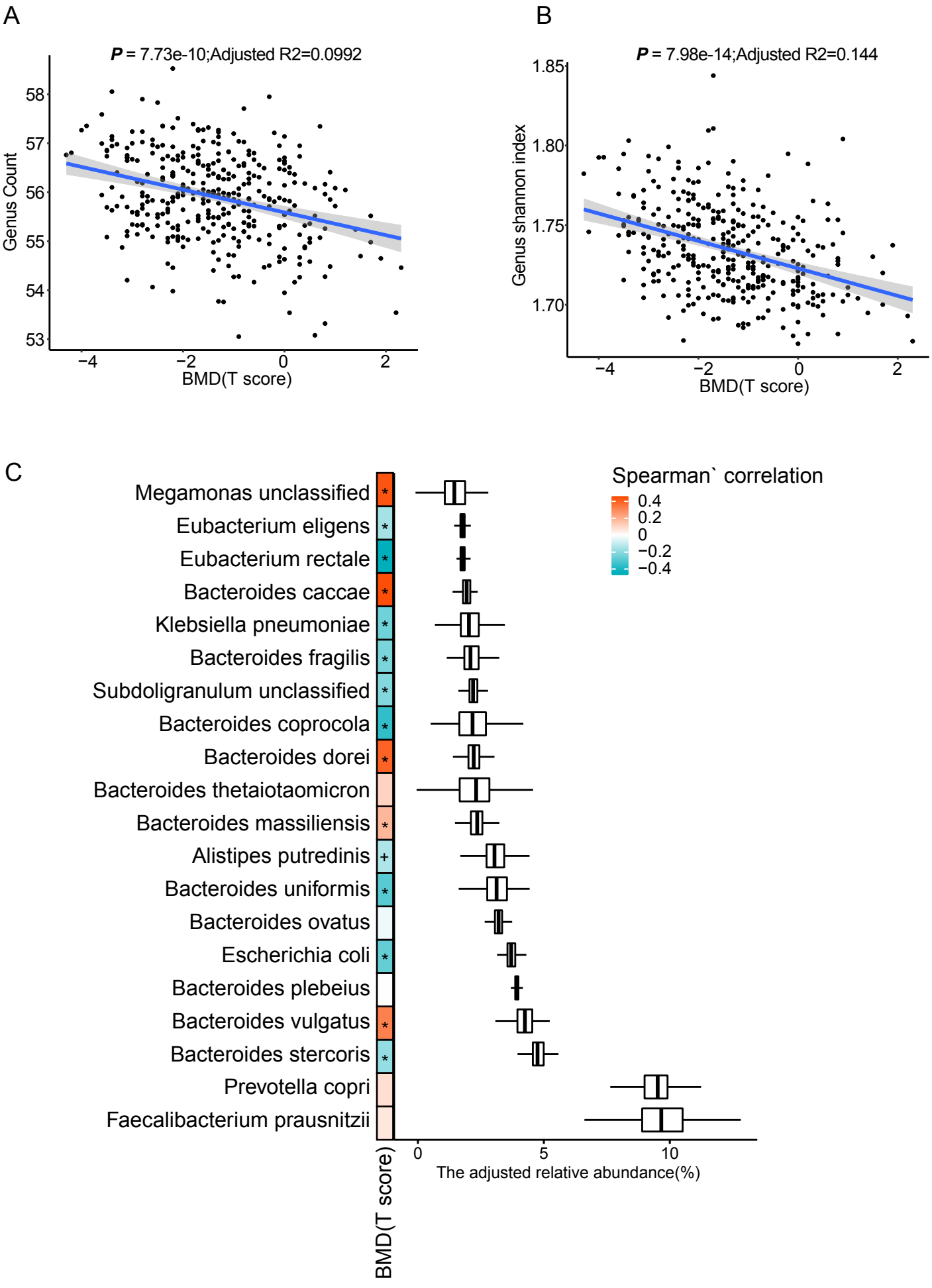

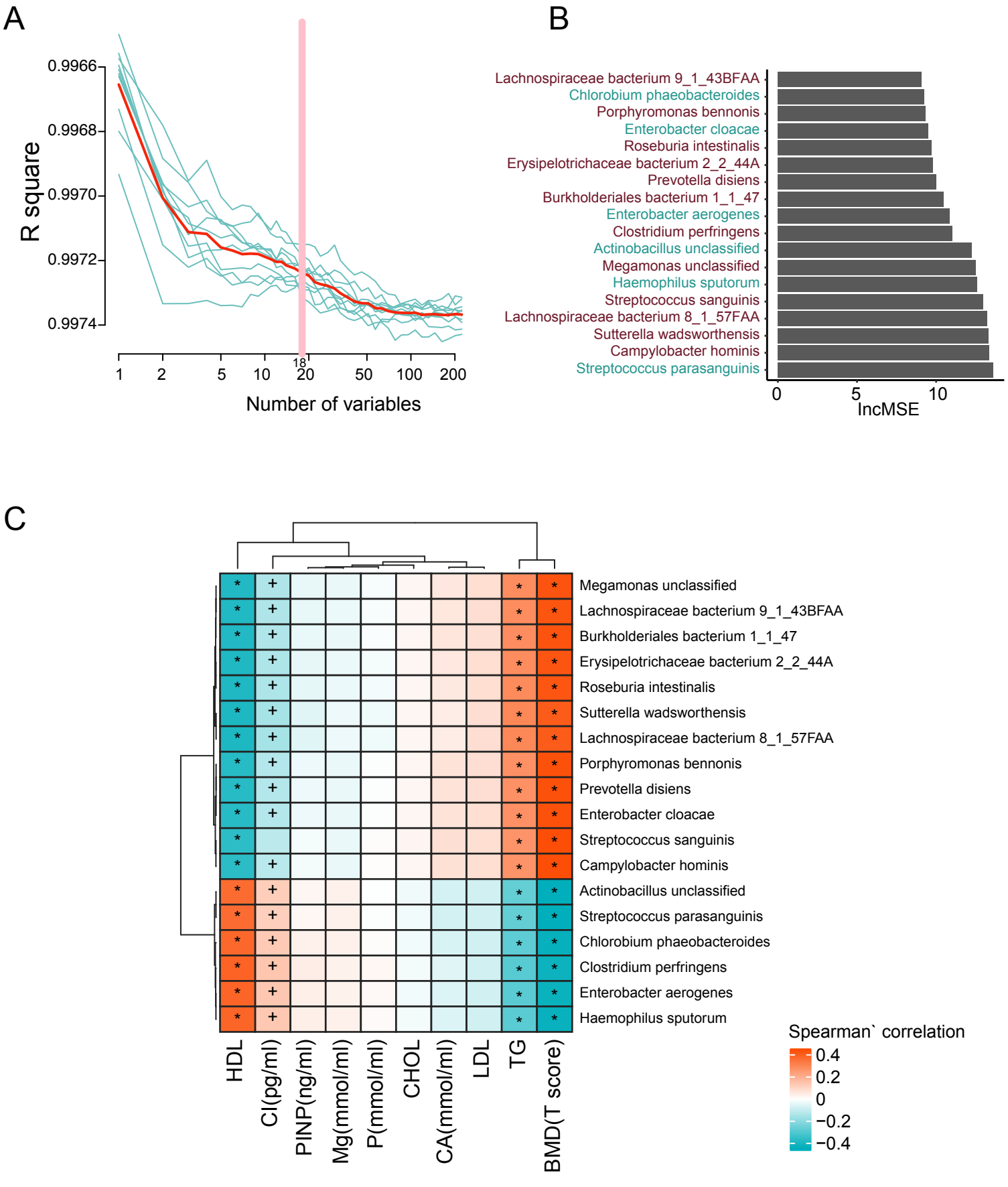
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Enterobacter cloacae
Roseburia intestinalis
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Prevotella disiens
Burkholderiales bacterium 1_1_47
Enterobacter aerogenes
Clostridium perfringens
Actinobacillus unclassified
Megamonas unclassified
Haemophilus sputorum
Streptococcus sanguinis
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Porphyromonas bennonis
Prevotella disiens
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Streptococcus sanguinis
Campylobacter hominis
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PINP(ng/ml)
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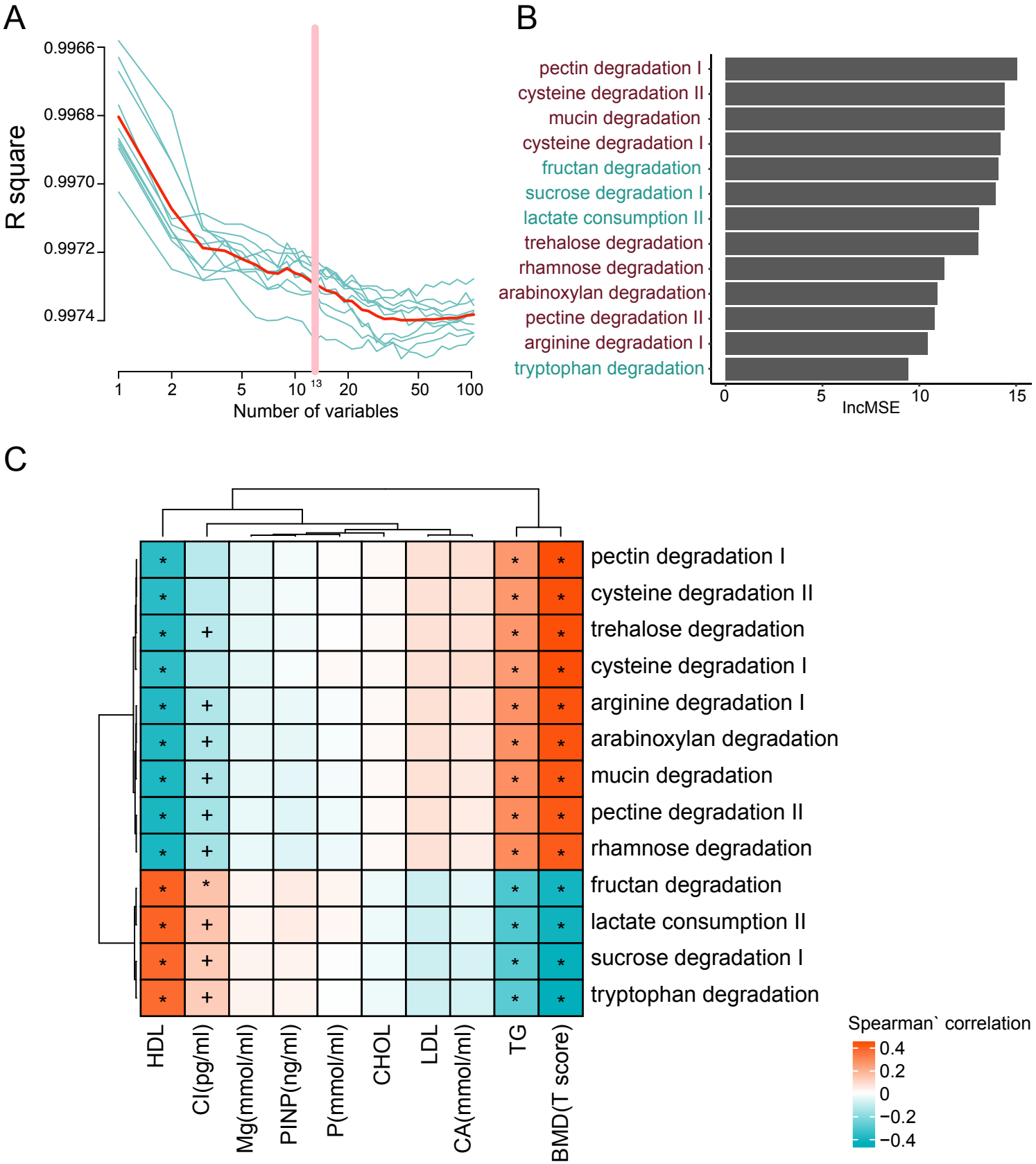
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13
20
50
100
Number of variables
B
pectin degradation I
cysteine degradation II
mucin degradation
cysteine degradation I
fructan degradation
sucrose degradation I
lactate consumption II
trehalose degradation
rhamnose degradation
arabinoxylan degradation
pectine degradation II
arginine degradation I
tryptophan degradation
0
5
10
15
IncMSE
C
pectin degradation I
cysteine degradation II
trehalose degradation
cysteine degradation I
arginine degradation I
arabinoxylan degradation
mucin degradation
pectine degradation II
rhamnose degradation
fructan degradation
lactate consumption II
sucrose degradation I
tryptophan degradation
HDL
CI(pg/ml)
Mg(mmol/ml)
PINP(ng/ml)
P(mmol/ml)
CHOL
LDL
CA(mmol/ml)
TG
BMD(T score)
Spearman` correlation
0.4
0.2
0
−0.2
−0.4