Impact of lifestyle on carotid vascular pathogenesis is mediated by the gut microbiome: A population-based analysis of gut microbiome in Chinese elderly

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Research

Keywords: Gut microbiome, metagenomics, vascular physiology, carotid atherosclerosis, mediation analysis

Posted Date: November 14th, 2019

DOI: https://doi.org/10.21203/rs.2.17233/v1

Abstract

Background The altered microbiota, considered as quantitative traits has also been identified to play pivotal roles in the host vascular physiology and might contribute to diseases. To understand the role of fecal microbiota on the vascular physiology in the healthy elderly population and how lifestyle such as diet and income shape the composition of host gut microbiota to further impact pathogenesis of carotid vascular diseases.

Results We performed a population-based fecal metagenomic study over 569 elderly asymptomatic healthy individuals in rural China. An association network was built based on clinical measurements and detailed epidemiologic questionnaires including blood chemistry, arterial stiffness, carotid ultrasonography, and metagenomic datasets. Carotid arterial atherosclerosis indices including intima-media thickness (IMT) were shown essential in the network and were significantly associated with lifestyles and diet. Fresh fruit, fresh vegetables, and fresh aquatic food can significantly reduce carotid artery IMT, especially in the mediation of Alistipes and Parabacteroides genus. Higher income, and exercise which are shown to improve carotid arteries mediated by Prevotella and Oligella genus.

Conclusions Our study provided a Chinese population-wide phenotype-metagenomic network, revealing association and mediation effect of gut microbiota on carotid atherosclerosis by lifestyle, hinting at a therapeutic and preventive potential of microbiota in cardiovascular diseases.

Background

There is increasing evidence showing that the dynamic changes in the gut microbiota can influence host physiology [1]. The altered microbiota, considered as quantitative traits has also been identified to play pivotal roles in the host vascular physiology and diseases [2]. Vascular diseases are a variety of circulatory diseases that normally affect carotid, cerebral, coronary circulation and thus cause systematic damage. Subclinical atherosclerosis has been linked to higher risk of coronary vascular disease and stroke [3, 4]. Behavioral Risk Factor Surveillance data also showed that physical activity, fruit and vegetable consumption are key heart-healthy standards for vascular diseases [5]. However, microbiota related mechanisms of how lifestyle and diet alter the vascular structure and pre-symptomic atherosclerosis remain to be elucidated.

Population-based metagenomic analyses revealed that the composition of the microbiome can be explained by a variety of exposures [6, 7]. On the other hand, the lifestyle and diet influence the composition of gut microbiota [8]. Moreover, most of these studies were well implemented in western populations and little is known about the association between gut metagenomics and exposures or vascular phenotypes in Chinese populations, with indigenous dietary habit and living style. To understand the association and mediation role of fecal microbiota in host vascular physiology, herein we performed a comprehensive population-based fecal metagenomic study over 569 participants aged 55–65 years in the Taizhou Imaging Study (TIS) [9]. (Figure 1a)

Results

Phenotypic and metagenomic data description

A total 600 samples were included. After sequencing, we performed quality control to excluded 3 samples with low quality and 28 samples with incomplete exposure and phenotype records. Finally 569 samples passed QC. The fecal metagenomes were sequenced using a paired-end whole metagenomic shotgun sequencing (WMS) on a NovaSeq sequencer. Each sample was sequenced at an average depth of 3.6 Gigabytes of data (~24.5 million reads per sample). (Figure S1)

Composition of gut microbiome in TIS individuals

The dataset is composed of a total richness of 26 phyla and 699 genera which is comparable with Falony et al.'s studies [6]. After excluding taxa present in <5% of individuals and reads from Homo Sapien, we collectively detected 9 and 221 highly confident commensal phyla and genera for further tests [10]. The average abundances of Firmicutes (~46%), Proteobacteria (~27%) and Bacteroidetes (~24%) account for more than 96% of the microbiota as core phyla, while >70% of genera were shared by Bacteroides, Prevotella, Faecalibacterium, Klebsiella, Escherichia, and Clostridium. (Figure S2) In terms of functional annotation, the main SEED and EGGNOG functions were enriched in carbohydrates and amino acid metabolism. (Figure S3)

Association analysis of variables in TIS based metagenome

Exposome, clinical phenotype, diseases and metabolomics variables are strongly associated with metagenomic taxonomy. (Figure S4) Diet intake frequency including staple, fresh vegetable, egg, fresh fruit, pickled food, meat and aquatic food are also linked to the composition of gut microbiome. Clinical examinations including blood chemistry indices, ECG measurements, and carotid ultrasonography were also shown correlated to abundance of genus taxonomy.Table S2)

Alpha diversities, i.e. Simpson's index and Shannon index were calculated to estimated species diversity per individual. Functional principal component analysis (FPCA) was applied to summarize the 14 categorical factors from 142 pre-categorized variables, and Pearson's correlation was tested between the categorical factors and individual alpha-diversity measurements. Blood chemistry, arterial stiffness, bone mass density and carotid ultrasonographic measurements significantly explained variation of phylum-leveled alpha-diversity. (Figure S5)

Mantel matrix correlation test was further calculated of overall variables and categorical factors between Gower distances and microbiome Bray-Curtis dissimilarity [6]. Categorical phenotypes such as heart function, disease status, demographic data, social economic status and arterial stiffness were found strongly linked to individual gut distances. (Figure S6)

Network analysis

We further calculated the edge counts, eccentricity and their product to obtain the breadth, depth and impact of each node respectively using association network. (Figure 2a) We found demography, carotid ultrasonography, arterial stiffness, blood biochemistry and physical examination measurements were among the most influential or influenced categories in the network with respect to the in- and out-bound connections. (Figure 2b, S7) The carotid ultrasonography variables were also among the top categories in terms of degree, neighborhood connectivity and stress network.

Mediation analysis of carotid ultrasonography

We further carried out analyses over the role of gut microbiota in the mediation of exposures on carotid artery physiology, performed as per MacKinnon *et al.*'s method [11]. Results showed that the effect of the life styles on carotid stiffness was mediated by microbiome, including negative regulation of IMT in common carotid arteries (CCAs), intracranial carotid arteries (ICAs), and extracranial carotid arteries (ECAs) by fresh fruit and fresh aquatic food through Alistipes, Bacteroides and Oligella genus. (Figure 3a) It is worth noting that, Alistipes contributed more than 10% of mediation effect in the reduction of PSV and IMTs by fresh fruit, while similar effect was achieved by Oligella through intake of Fresh Aquatic Food.Table S3) Fresh fruit is responsible for reduction of IMT indices through mediation of Alistepes. (Figure 3b) Education and total income are also negatively correlated with IMT in carotid arteries through promoting Prevotella genus and decreasing Oligella genus. (Figure 3c) The staple food intake frequency showed strong positively association with for the thickness of IMT in ECAs and RICA, and resistive index in LECA through Acinetobacter, however the results are marginally significant. Interestingly, total income are also negatively correlated with IMT in carotid arteries through promoting Prevotella, Lachnoclostridium genus or lactate producing Ruthenibacterium, and decreasing Acinetobacter and Oligella. To find deeper mechanism of the gut microbiota's effect on carotid IMT, we also calculated the mediation effect of metabolomics. The results showed that food-derived amino acids, choline and phosphocholine were significantly enriched as mediators. (Figure S8)

Discussion

In the current study we built an association network based on multiple phenotypes related to cardiovascular features and metagenomic datasets in an elderly Chinese population, finding the mediation effect of microbiome on carotid atherosclerosis by lifestyle and diet. Carotid intima-media thickness, as a good indicator of the severity of atherosclerotic disease well reflects carotid and systemic inflammation [12, 13]. In our mediation analysis, IMT indices in CCAs, ICAs, and ECAs were negatively associated with, fresh fruit, vegetables, and fresh aquatic food through promoting *Alistipes, Oligella,* and *Bacteroides* genus. It is suggested that greater intake of fruit was associated with lower carotid IMT, especially in high cardiovascular risk individuals [14–16]. This can be explained by a higher frequency of vegetable and fruit intake promoted SCFA metabolism by *Alistipes* to alemiorate system inflammation.

Alistipes relative abundance is reported to be highly correlated with butyrate concentration which pacifies inflammation through generating anti-inflammation butyrate [17, 18]. *Alistipes* and *Lachnoclostridium* have also been proved to be negatively correlated with chemical and pathogen-induced inflammation [19]. Interestingly, in our study the staple food intake frequency is positively associated with for the IMTs in ECAs and RICA, and RI in LECA through *Acinetobacter.* It is worth noted that *Acinetobacter* is able to promote degradation of carnitine to trimethylamine (TMA) which contribute to cardio-vascular diseases or stroke [20].

Higher income, education and exercise improve carotid vessel through modulation of a variety of bacterial genus. This is partly proved by Wildman et al.'s study that dietary and exercise intervention slowed progression of subclinical atherosclerosis [21]. In previous population-wide analyses, lower socio-economic status, including low education and low income, or employed in a manual occupation were associated with increased IMT [22–25]. These factors are negatively associated with IMT through promoting butyrate producing Lachnoclostridium genus and lactate producing Ruthenibacterium genus. On the other hand the socio-economic factors decrease the abundance of Acinetobacter and Oligella genus, implying the anti-co-occurrence of the two groups. A similar co-linearity of the microbes was observed in a rodent colitis model [26]. We have observed that total income and education are positively correlated to fruit intake frequency and education levels[27], which explained the protective roles of socio-economy.

In the TIS cohort the composition of microbiota was mainly contributed by Firmicutes, Proteobacteria and Bacteroidetes. There are less Firmicutes and more Probacteria phyla present in our datasets as compared to FGFP cohort [6]. The main composition of genera was not fully in concordance with Western cohort and FGFP cohort, implying different diet pattern, life style and other extrinsic factors [7, 10].

It is an obstacle in current microbiota-based translational medicine that the mild and long-term of effects of microbiota may not be significantly standing out in the short-term assessment. For example lifestyle and diet changes including the fruit, vegetables, and lean fish meal were not responsible for the change of carotid artery in a 20-week short [28].The limited number of exposure and diet variables we currently apply can only partly explain the outcomes. More features employed will characterize a more comprehensive role of gut microbiota in the network.

Although additional work is still needed, to our knowledge it is the first work to provide a population-wide phenotype-metagenomic network and revealed association and mediation effect of lifestyles on carotid atherosclerosis through microbiome in Chinese population. This work hopefully pushes the frontiers of modifying the gut microbiome composition via diet and change of living habit to pave the way for improving vascular physiology targeting the gut microbiome.

Conclusions

Here, we provided a population-wide phenotype-metagenomic network, revealing association and mediation effect of gut microbiota on carotid atherosclerosis by lifestyle, hinting at a therapeutic and

preventive potential of microbiota in cardiovascular diseases.

Methods

Cohort Description and Participants

The TIS (Taizhou image study) is an ongoing longitudinal study that aiming to explore the risk factors and etiology of cerebrovascular disease and dementia, which derives from the Taizhou Longitudinal Study (TZL) [29]. The TIS has been detailed in our previous studies [30]. Briefly, a total of 1049 Han Chinese individuals aged 55–65 years without stroke, cancer, cardiovascular disease, psychiatric disorders, or other serious illness in three villages that previously exhibited high response rates in the TZL from Taixing (a county level city of Taizhou), were invited to participate the baseline survey of TIS from March 2013 to October 2018. Eventually, 904 participants met the inclusion criteria and finised the baseline examination. For the present study, 600 individuals with adequate fecal samples at baseline were included, among them 31 were excluded for low sequencing quality and phenotype missing, and the final analysis consisted of 569 participants. A total 142 variables from 14 variable categories were collected and classified into exposome, clinical phenotype, diseases and metabolomics which were further classified into 142 factors including 20 sociodemographic and exposures *(1 demographics, 4 lifestyle, 3 physical measurement results, 2 socio-economic, 10 diet habits),* 16 diseases' status, 68 physiological phenotypes *(2 demographics, 4 arterial stiffness, 12 blood biochemistry, 6 blood pressure, 6 electrocardiography features, 30 carotid ultrasonographic, 2 cognitive function, 6 bone mass density),* and 38 metabolite features. (Table S1, S4)

Cohort Description and Selection

TIS, Taizhou image study is a longitudinal investigation of cerebrovascular disease risk factors in three natural villages from Taizhou city in China. A total 936 participants are recruited ranging from 55~65 years old and has been described in detail from our previous article [29]. The cohort is composed of 42.7% and 57.3% male and female adults, respectively. Institutional review boards at each site approved the protocol and all participants provided informed consent.

Fecal sample collection, DNA extraction and WMS library preparation

Stool specimens were transferred to a −80°C freezer immediately after collection. The metagenomic DNA extraction kit (Tiangen, China) and Lysozyme (Sigma-Aldrich, Canada) were employed to purify the DNA specimens to generate WMS libraries. Informed consent was obtained from all four donors. Microbial DNA was used for fragmentation (Nextera XT kit, Illumina, USA). DNA fragments were then amplified by 12 cycles of PCR using NPM Polymerase (Illumina, USA) and Illumina N5 and N7 index-specific primers. Libraries were purified with 0.8× AMPure XP beads (Beckmann Coulter, USA). After library profile analysis by 2100QC and Qubit quantification, the libraries were pooled 1:1 in molarity at a 2 nM final

concentration. Samples were sequenced in a NovaSeq sequencer (Illumina, USA) in a 150 Pair-end mode. Each sample was sequenced at an average depth of 3.6 Gigabytes of data (~24.5 million reads per sample).

Metagenomic data processing

All raw FASTQ files were first analysed with FASTQC to trim filter low-quality bases. Paired-end sequences of WMS were combined using the zcat command line to obtain long reads. WMS files were calculated using DIAMOND software to generate DAA files. As soon as the long reads were generated, the DIAMOND tool was employed for mapping reads to the reference NCBI nr metagenomic database [31]. The taxonomic assignments including phylum, class, order, family, genus, and species levels of assigned reads were determined using MEGAN 6 to generate the taxonomic count tables for each of the samples [32]. Taxa present in <5% of individuals and reads from *Homo Sapien* were excluded. Alpha diversity analyses were subjected to richness, Simpson's and Shannon indices. To determine the population-wide taxonomic differences, Bray-Curtis dissimilarity beta-diversity was applied for each species with non-zero abundance. SEED and EGGNOG were applied as databases for functional annotations using MEGAN 6 [33, 34]. The Shannon Weaver index, Simpson's reciprocal index, PCoA, and the Bray-Curtis matrix were calculated using *vegan* package in R (v3.5.2) and MEGAN6 (version 6.10.3). Mantel test, Gower distance matrix, Pearson's correlation, and Whiskers plot were calculated using R. The overall gut microbiome in subjects are composed of a total richness of 26 phyla and 699 genera, which is comparable with Falony *et al.*'s studies [6]. After excluding taxa present in <5% of individuals [10] and reads from Homo Sapien, we collectively detected 9 and 221 highly confident commensal phyla and genera for further tests. The average abundances of Firmicutes (~46%), Proteobacteria (~27%) and Bacteroidetes (~24%) account for more than 96% of the microbiota as core phyla, while >70% of genera were shared by Bacteroides, Prevotella, Faecalibacterium, Klebsiella, Escherichia, and Clostridium. (Figure S2)

Association studies

Shapiro's test was applied to determine the distribution of each clinical variable. Univariate association analysis was done using ordinary linear regression, logistic regression, beta regression and multinomial regression respectively, according to the distribution of the response variables (Table S4). All association effects were tested considering age and gender of individuals. Medicine intake information was also considered in the test, but no significant difference had been found. To account for multiple testing, we applied a stepwise Benjamini & Hochberg correction to the resulting P values [35]. The significant level was selected at 0.05. We applied functional principal component analysis to extract the information of the predefined categories, and test the association between the predefined groups and alpha diversities using Pearson correlation [36].

Alpha diversities, i.e. Simpson's index and Shannon index were calculated to estimated species diversity per individual. Functional principal component analysis (FPCA) was applied to summarize the 14

categorical factors from 142 pre-categorized variables, and Pearson correlation was tested between the categorical factors and individual alpha-diversity measurements. Socio-demographic data such as marriage, year of education and total income were significantly correlated with gut microbiota. Notably, previously less known factors such as ECG indicator RV5, cognitive assessment including MoCA, and socio-demographic factors like total income were especially responsible for the gut beta-diversity. (Figure S6)

Network and Mediation analysis

We first calculated the edge attributes of all variable nodes using network analysis in Cytoscape 3.4 [37]. The edge counts and eccentricity of each node were calculated to obtain the breadth and depth respectively [38]. We define the product of edge count and eccentricity as impact of a certain category. We further studied the effect of metagenome on carotid ultrasound considering of factors related to living habits (diet, lifestyle, social economics). Firstly, we tested if the effect of metagenome on carotid ultrasound would be masked by living habits factors. The living habits variables that significant associated with carotid ultrasound had been filtered according to their correct P values (after Benjamini & Hochberg correction). Variables related to diet had been added to the model using their first three principal component factors, in order to remove colinearity. Besides, we used mediation analysis to test the mediation effect of metagenome (M) in the regulation of carotid ultrasonography (CU) [11]. We use a three-step mediation analysis to assess if there is a mediation effect of metagenome between living habits (L) and carotid ultrasound. Firstly, we selected living habits variables that have significant association with any carotid ultrasound variables. That is,

[Due to technical limitations, the diagram and formula could not be displayed here. Please see the supplementary files to access the diagrams and formulas.]

Secondly, we tested if there were any significant associations between the living habits variables selected in step 1 and genera (metagenome).

[See supp. files.]

Thirdly, we tested if the effect of living habits variables on carotid ultrasound variables would disappear when metagenome variables were added in the regression.

[See supp. files.]

The mediation effects were tested using Sobel test [39]. Mediation edge analysis and network graphic was plotted with Cytoscape (3.4.0) Percentage of mediation effect by each significant microbe was shown in table S3.

Hypertension status is associated with carotid pathogenesis and was applied in the stratified mediation analysis. However, results showed no significant mediation effect of microbiome in population without

hypertension.Mediation effect with significance and coefficient of microbiota and metabolome are listed in detail in supplementary files. The results showed that food-derived amino acids, choline and phosphocholine were significantly enriched as mediators. (Figure S8)

List Of Abbreviations

TIS: Taizhou Imaging Study

CCAs: common carotid arteries

ICAs: intracranial carotid arteries

ECAs: extracranial carotid arteries

TMA: Trimethylamine

ABI: ankle brachial index

baPWV: brachial-ankle pulse wave velocity

BMI: body mass index

DBP: diastolic blood pressure

HDL-C: high-density lipoprotein cholesterol

IMT: carotid intima-media thickness

IQR: interquartile range

LDL-C: low-density lipoprotein cholesterol

MMSE: Mini-Mental State Examination

MoCA: Montreal Cognitive Assessment

SBP: systolic blood pressure

SD: standard deviation

TC: total cholesterol

TG: triglycerides

Declarations

Ethics approval and consent to participate

Ethics approval was granted by the Ethics Committee of the School of Life Sciences, Fudan University.

Consent for publication

Not applicable.

Availability of data and material

The datasets used and analysed during the current study are available from the corresponding author on reasonable request

Competing interests

The authors declare that they have no competing interests.

Funding

This work was supported by the National Key Research and Development program of China (grant number: 2017YFC0907002, 2017YFC0907501), National Nature Science Foundation of China (grant number: 81502870, 31521003), the International Science and Technology Cooperation Program of China (grant number: 2014DFA32830), the Key Basic Research Grants from Science and Technology Commission of Shanghai Municipality, China (grant number: 16JC1400500) and Shanghai Municipal Science and Technology Major Project (grant number: 2017SHZDZX01).

Authors' contributions

XDC, LJ, SBZ, KLX, and YFJ designed the work. SBZ, CS, MC, YZW, ZY, JLX, TTH, JCW, TJZ joined the experiment. SBZ, KLX, YFJ, WMY analyzed datasets. SBZ, XDC, KLX, YFJ wrote this paper.

Acknowledgements

Not applicable.

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Table

Table 1. Characteristics of key measurements in TIS metagenomic study.

Characteristics	*N*	Overall
Age, years, mean (SD)	569	59.8 (2.9)
Male, *N* (%)	569	243 (42.7)
Years of education, median (IQR)	564	6 (9)
BMI, mean (SD)	569	24.4 (3.2)
Current smoker, *N* (%)	564	164 (29.1)
Current alcohol drinker, *N* (%)	565	161 (28.5)
SBP, mmHg, mean (SD)	567	138.1 (19.9)
DBP, mmHg, mean (SD)	567	82.7 (11.6)
Heart rate, beats/min, mean (SD)	569	70.0 (10.9)
Fasting glucose, mmol/L, mean (SD)	514	5.6 (1.5)
Total cholesterol, mmol/L, mean (SD)	520	5.2 (1.0)
Triglycerides, mmol/L, mean (SD)	520	1.5 (1.2)
HDL-C, mmol/L, mean (SD)	512	1.6 (0.4)
LDL-C, mmol/L, mean (SD)	513	2.7 (0.8)
MMSE, median (IQR)	569	27 (6)
MoCA, median (IQR)	505	19 (9)
Cerebral small vessel disease, *N* (%)	566	272 (48.1)
Hypertension, *N* (%)	569	324 (56.9)
Diabetes mellitus, *N* (%)	569	66 (11.6)
Hyperlipidemia, *N* (%)	569	301 (52.9)
Metabolic Syndrome, *N* (%)	569	177 (31.1)
Medication of antihypertensive, *N* (%)	569	376 (0.66)
Medication of antidiabetic, *N* (%)	569	519 (0.91)
Plaque, *N* (%)	564	148 (26.2)
LCA_IMT, mm, mean (SD)	534	0.81 (0.22)
RCA_IMT, mm, mean (SD)	533	0.81 (0.21)
LCA_DIA, mm, mean (SD)	545	6.7 (0.83)
RCA_DIA, mm, mean (SD)	545	6.8 (0.81)
baPWV, cm/s, mean (SD)	538	1544.1 (277.7)

ABI, mean (SD)	530	1.1 (0.1)

Abbreviations: ABI, ankle brachial index; baPWV, brachial-ankle pulse wave velocity; BMI, body mass index; DBP, diastolic blood pressure; HDL-C, high-density lipoprotein cholesterol; IMT, carotid intima-media thickness; IQR, interquartile range; LDL-C, low-density lipoprotein cholesterol; MMSE, Mini-Mental State Examination; MoCA, Montreal Cognitive Assessment; SBP, systolic blood pressure; SD, standard deviation; TC, total cholesterol; TG, triglycerides.

Figures

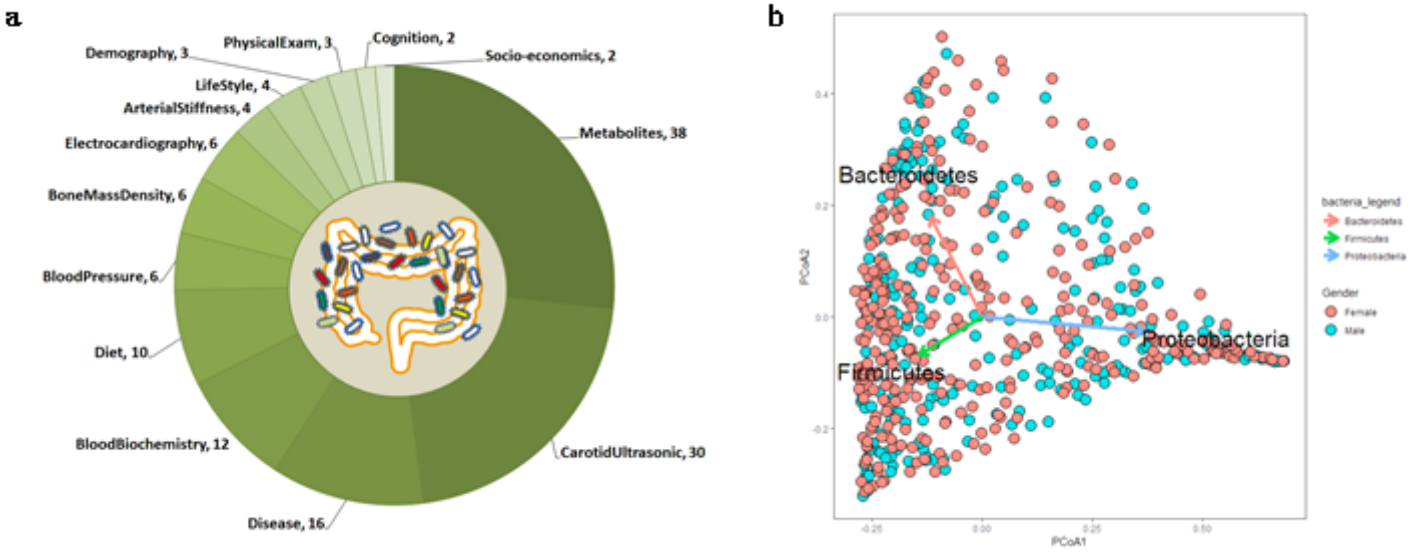

Figure 1

Metagenomic data and cohort characteristic summary. (a) The ring chart arc sizes reflected the number of collected covariates in each category. (b) Bray-Curtis distance based PCoA biplot showed that Firmicutes, Bacteroidetes, and Proteobacteria were dominant phyla.

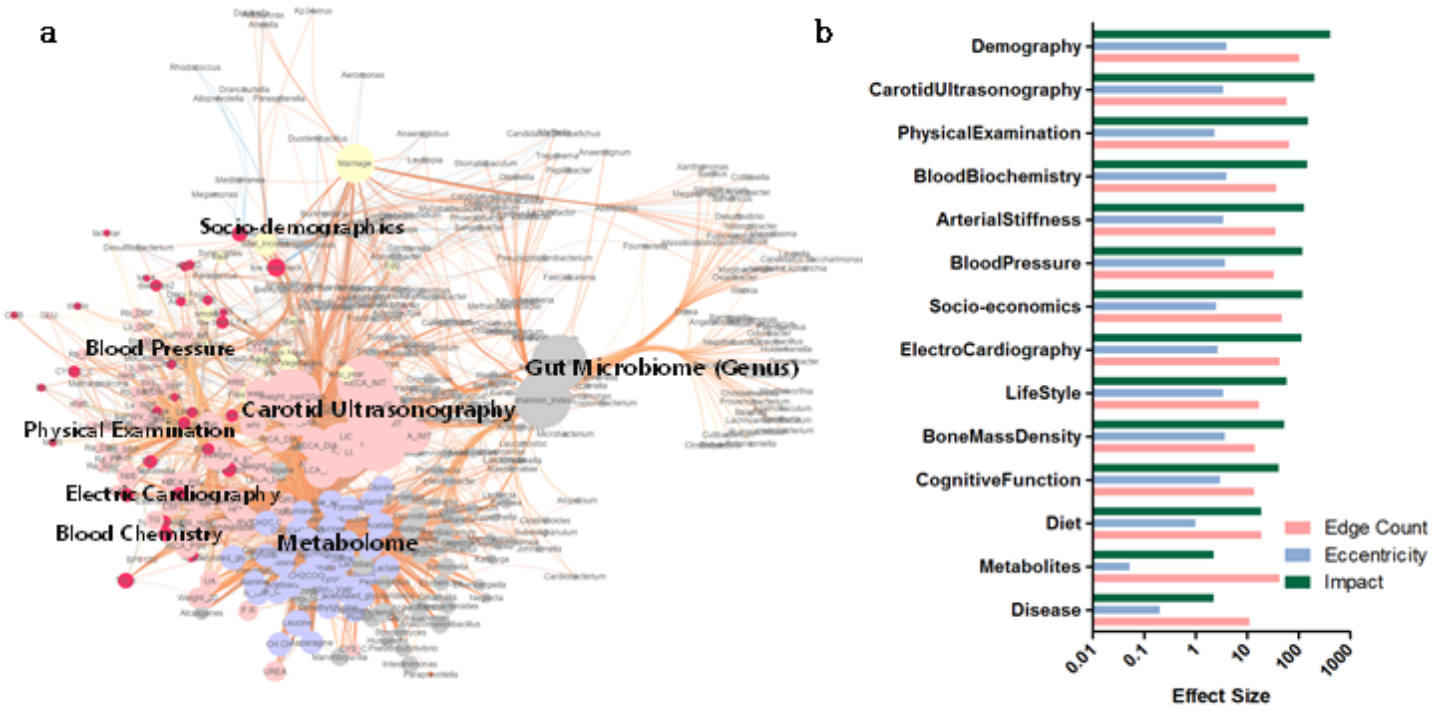

Figure 2

Network analysis and influence analysis of variables. (a) We first calculated the edge attributes of all variable nodes using association network analysis. (b) Demography, carotid ultrasonography, arterial stiffness, blood biochemistry and physical examination measurements were among the most influential categories in the network.

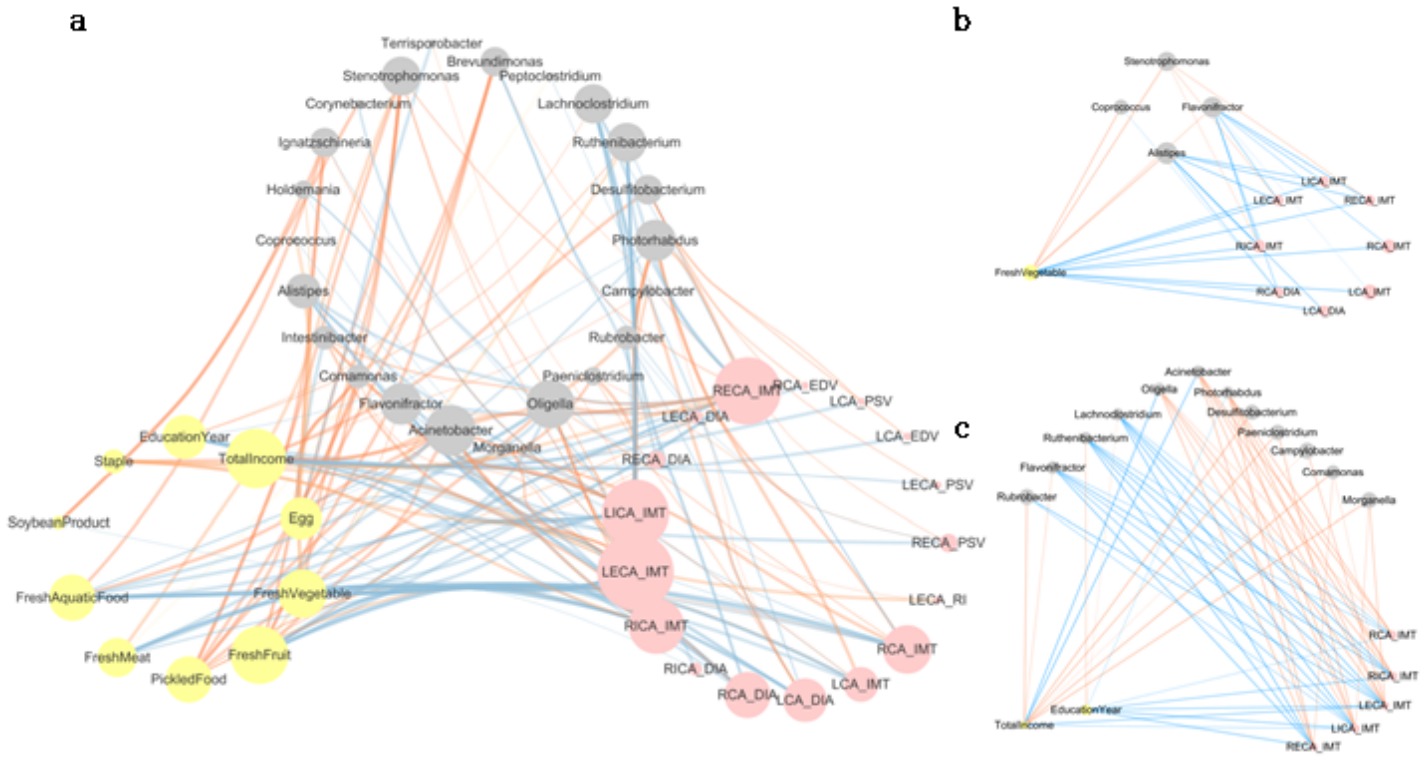

Figure 3

Mediation analysis of microbiota. (a) The effect of the life styles on carotid artery physiology was mediated by microbiome, including negative regulation of IMT in CCAs, ICAs, and ECAs by fresh fruit, fresh aquatic food and fresh vegetables through genus leveled microbiota, especially Alistipes, Parabacteroides and Oligella. (b) Fresh fruit is responsible for reduction of IMT indices through mediation of Alistepes. (c) Education and total income are also negatively correlated with IMT in carotid arteries through promoting Prevotella genus and decreasing Oligella genus.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- TableS1.csv
- TableS2.csv
- Supplementary.docx
- TableS3.csv
- TableS4.csv
- Methodsformulasanddiagrams.docx