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PROGNOSIS OF NASOPHARYNGEAL CARCINOMA: BODY MASS INDEX, PLASMA EPSTEIN-BARR VIRUS DNA AND ORAL MICROBIOME

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Prognosis of Nasopharyngeal Carcinoma: Body Mass Index, Plasma Epstein-Barr Virus DNA and Oral Microbiome

Thesis for Doctoral Degree (Ph.D.)

By

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To time.

Abstract

Nasopharyngeal carcinoma (NPC) has a geographically skewed distribution worldwide, with high incidence rates in East and Southeast Asia. Although hospital-based studies suggest that the development of new radiotherapy techniques has contributed to improved NPC prognosis, population-based research on NPC patient survival is lacking. In addition, potential environmental prognostic factors for NPC, including body mass index (BMI) and body shape, pretreatment plasma Epstein-Barr virus (EBV) DNA, and oral microbiome, are not yet well understood. Therefore, this thesis aims to characterize population-based NPC survival patterns and identify prognostic factors for NPC in a population-based context in southern China.

In **Paper I**, we aimed to estimate population-based NPC survival in an unbiased manner by implementing a tracing strategy to ensure a high follow-up rate in a representative cohort of NPC patients. We also aimed to compare this estimate with survival results from other studies in endemic areas, and to calculate the number of avoidable deaths from earlier detection or more widespread access to advanced medical care. Based on patients with incident NPC enrolled in the population-based NPC Genes, Environment, and EBV (NPCGEE) project, we developed a passive-active-passive circle follow-up strategy that achieved a high rate (98.3%) of complete follow-up for vital status through 2018. We estimated that 5-year overall survival for NPC diagnosed at stages I, II, III, IVa, IVb, and IVc was 91.1%, 88.1%, 79.8%, 63.8%, 57.7%, and 34.4%, respectively. In general, we found that population-based NPC survival lags by approximately 10 years behind survival reported in large hospital-based cohorts. We estimated that 174 NPC deaths per 1000 patients could be avoided within five years of diagnosis if all advanced-stage cases were instead diagnosed at early stages.

In **Paper II**, we examined whether pretreatment BMI and body shape were associated with the prognosis of NPC, using the NPC patient cohort from the NPCGEE project. We found that being overweight at diagnosis, was associated with a 25% lower all-cause mortality rate whereas those with a thinner body shape had a higher all-cause mortality rate, than those with a normal weight/body shape. When we examined associations with BMI and body shape 10 years before diagnosis, similar but weaker associations existed, but for BMI and body shape at age 20 years, the associations with NPC prognosis disappeared. The lack of effect modification by stage at diagnosis, along with the detection of similar associations with BMI and body shape 10 years before diagnosis, suggests that the results were not primarily due to reverse causation.

In **Paper III,** we assessed the relationship between pretreatment plasma EBV DNA and NPC survival, using the NPC patient cohort from the NPCGEE project. We found that higher pretreatment plasma EBV DNA load was associated with increased risks of all-cause and NPC-specific mortalities, particularly in the first five years after diagnosis; cases with detectable plasma EBV DNA (compared with undetectable) had more than double the risk of all-cause and NPC-specific death. Higher pretreatment EBV DNA levels may reflect a greater tumor burden, and may signal a need for more intensive chemotherapy and/or heightened clinical surveillance.

In **Paper IV,** we estimated associations of oral microbiome with NPC prognosis, using a subcohort of patients with saliva specimens from the NPCGEE project. We showed that lower within-community diversity was associated with higher all-cause and NPC-specific mortalities, and some (albeit not all) measures of between-community diversity were also associated with all-cause and NPC-specific mortalities. None of candidate bacteria were found to be significant prognostic biomarkers, suggesting

that the observed associations resulted from global patterns rather than specific microbiota. These results indicate that microbiota may affect host immune function or contribute to the development of adverse treatment effects that in turn influence NPC prognosis.

In conclusion, using a population-based patient cohort of NPCGEE project in southern China, we estimated generalizable 5-year survival rates and investigated potential environmental prognostic factors. We found that population-based NPC survival lags behind large-hospital-based survival; overweight at diagnosis indicated a favorable long-term prognosis, whereas a thinner body shape at diagnosis is associated with worse prognosis; pretreatment plasma EBV DNA is a strong prognostic factor for NPC; and decreased within-community diversity in oral microbiome is related to increased mortality. Taken together, these findings constitute some of the first population-based evidence on NPC prognosis in southern China, and point to potential routes to improving NPC management and long-term outcomes in this NPC-endemic region.

List of scientific papers

* Equal contribution

I. **Du Y***, Feng R*, Chang E T, Yin L, Huang T, Li Y, Zhou X, Huang Y, Zhou F, Su C, Xiao X, Jia W, Zheng Y, Adami H-O, Zeng Y, Cai Y, Zhang Z, Xu M, Ye W. Population-based nasopharyngeal carcinoma survival study in southern China. (Manuscript)

II. **Du Y***, Feng R*, Chang E T, Yin L, Huang T, Li Y, Zhou X, Huang Y, Zhou F, Su C, Xiao X, Jia W, Zheng Y, Adami H-O, Zeng Y, Cai Y, Zhang Z, Xu M, Ye W. Body mass index and body shape before treatment and nasopharyngeal carcinoma prognosis: a population-based patient cohort study in southern China. International Journal of Cancer, 2023 March; 1153(2):290-301

III. **Du Y***, Feng R*, Chen Y, Chang E T, Yin L, Huang T, Huang Y, Li Y, Zhou X, Zhou F, Su C, Xiao X, Jia W, Zheng Y, Adami H-O, Zeng Y, Cai Y, Xu M, Zhang Z, Ye W. Pre-treatment plasma EBV DNA and nasopharyngeal carcinoma prognosis: a prospective population-based cohort study in southern China. (Manuscript)

IV. **Du Y***, Feng R*, Chang E. T*, Debelius J. W, Yin L, Xu M, Huang T, Zhou X, Xiao X, Li Y, Liao J, Zheng Y, Huang G, Adami H-O, Zhang Z, Cai Y, Ye W. Influence of pre-treatment saliva microbial diversity and composition on nasopharyngeal carcinoma Prognosis. Frontiers in Cellular and Infection Microbiology, 2022 March; 12:831409

Related work

(Not included in the thesis)

Du Y, Yu X, Chang ET, Lian S, Wu B, Li F, Chu B, Wei K, Zhan J, Liang X, Ye W, Ji M. Pre-diagnostic anti-EBV antibodies and primary liver cancer risk: a population-based nested case-control study in southern China. BMC Cancer. 2023; 23:250.

Du Y, Zhang W, Lei F, Yu X, Li Z, Liu X, Ni Y, Deng L, Ji M. Long-term survival after nasopharyngeal carcinoma treatment in a local prefecture-level hospital in southern China. Cancer Manag Res. 2020;12:1329-1338

Yu X, Ji M, Cheng W, Wu B, Lian S, **Du Y**, Cao S. A retrospective cohort study of nasopharyngeal carcinoma screening and hepatocellular carcinoma screening in Zhongshang city. J Cancer 2019; 10(8):1909-1914.

Contents

List of abbreviations

ACT	Adjuvant chemotherapy
ASV	Amplicon sequences variants
BMI	Body mass index
CCRT	Concurrent chemoradiotherapy
NPC	Nasopharyngeal carcinoma
EBNA1-IgA	Immunoglobulin A of EBV nuclear antigen 1
EBV	Epstein-Barr virus
Faith's PD	Faith's phylogenetic diversity,
2DRT	Two-dimensional radiotherapy
3DRT	Three-dimensional radiotherapy
FDR	False discovery rate
HR	Hazard ratio
ICT	Induction chemotherapy
IMRT	Intensity-modulated radiotherapy
KPS	Karnofsky Performance Scale
NPCGEE	NPC Genes, Environment, and EBV project
PCoA	Principal coordinates analysis
qPCR	Quantitative polymerase chain reaction
PCs	Principal coordinates
RPCA	Robust Aitchison principal-component analysis
SEER	Surveillance, Epidemiology, and End Results Program
VCA-IgA	Immunoglobulin A of EBV viral capsid antigen

1 Introduction

Nasopharyngeal carcinoma (NPC) is an uncommon cancer worldwide but is prevalent in populations in Southeast Asia, Middle East and North Africa [1]. Due to the histology of undifferentiated type and hidden anatomical location, radiotherapy together with chemotherapy, rather than surgery plays an essential role in treatment [2]. The tumor location in the deep nasopharynx and asymptomatic feature brings difficulties in early diagnosis, thus, most patients were diagnosed at advanced stages [3]. The population-based 5-year survival probabilities of NPC were 79.3% and 65.2% in Hong Kong and Taiwan, respectively [4,5]. However, data on population-based survival probabilities remain largely unknown in China. Further, the investigation of potential environmental prognostic factors in a population-based setting has been limited.

This thesis outlines the long-term population-based survival probabilities of NPC, compares the estimates with those from other studies in endemic areas, and calculates the avoidable deaths from earlier detection or more widespread access to advanced medical care. Further, this thesis investigates the associations of pretreatment body mass index (BMI) and body shape, pretreatment plasma Epstein-Barr virus (EBV) DNA and saliva microbiota, with the prognosis of NPC in a population-based context.

2 Literature review

2.1 General background on nasopharyngeal carcinoma

2.1.1 Descriptive epidemiology

Nasopharyngeal carcinoma (NPC) is a highly malignant cancer that originates in the nasopharynx (**Figure 2.1**). NPC is relatively rare worldwide, with an age-standardized incidence of less than one per 100,000 person-years [1]. Age-standardized incidences in southern China, however, showed as high as 25 per 100,000 person-years (**Figure 2.2**). Based on global cancer statistics, the estimated number of incident NPC cases in 2020 was 133,354 globally and ranked 23rd among all cancers. By region, 113,659 (85.2%) were diagnosed in Asia, 10,041 (8.8%) in Africa and 5,204 (4.6%) in Europe in 2020. In China, the number of estimated incident cases was 62,444 in 2020 [6] and the distribution of NPC cases varied geographically. In northern China, the age-standardized incidence is lower than 2/100,000 person-years whereas in southern China, it may exceed 25/100,000 person-years [7]. In southeast Asia (i.e., Singapore and Malaysia), high NPC incidence seems to be associated with social and racial mixture with populations from southern China. However, in Japanese populations, which primarily interact with populations from northern China, NPC incidence is low [7]. The incidence in males is twice or triple, that in females [8]. It has declined gradually worldwide since 1970 [7,9], probably due to economic growth and its implications.

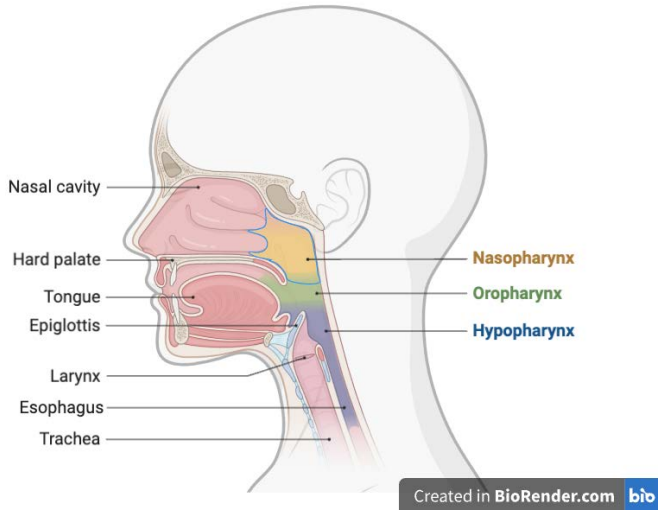


Figure 2.1 The location of the nasopharynx (Created by BioRender: https://www.biorender.com/)

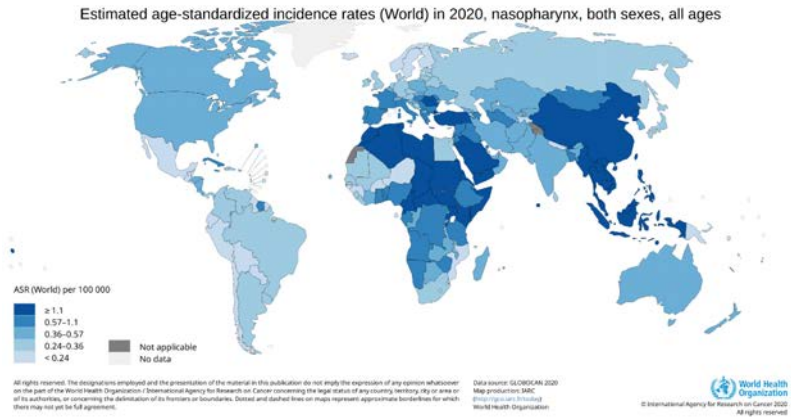


Figure 2.2 Estimated age-standardized incidence rate (World) of nasopharyngeal carcinoma in 2020 worldwide.

2.1.2 Risk factors

NPC occurrence concerns the interplay between genetic and environmental factors [1]. Epidemiological studies on migration patterns offer some hints of this interplay. The incidence of NPC among immigrants from high-risk regions to low-risk regions has declined, albeit has remained higher than that among local natives [10,11] and it is higher among their offspring [12,13]. The risk of NPC among populations emigrating from low-risk regions to high-risk areas has increased [14,15]. The phenomenon of family clustering of NPC also strengthens the importance of gene-environment interaction, as a segregation study of familial NPC explained [16].

2.1.2.1 Genetic risk factors

Numerous genetic risk factors have been identified and linked to the pathogenesis of NPC (**Table 2.1**). The most investigated genes include human leukocyte antigen (HLA)-A/B/C, which are involved in the immune system's ability to recognize and eliminate abnormal cells, based on large-scale genome-wide association studies [17–20]. The macrophage-stimulating 1 receptor (MST1R) gene plays an essential role in defense against viral infection according to a whole-exosome sequencing study [21]; integrin-α 9 (ITGA9) and RAD51L1 were reported to be associated with increased NPC risk [22,23], related to cell-cell mediation and DNA damage repair. In addition, many statistical associations have been observed but further validation studies across diverse populations are needed.

Table 2.1 Genetic alterations associated with NPC risk.

Studies	Genes	Function	Location	Direction of associations
Bei et al. [17]	Human leukocyte antigen (HLA)-A/B/C	Immune response	6p21.3	Increased/decreased risk
Dai et al. [21]	macrophage-stimulating 1 receptor (MST1R)	Host defense against viral infection	3p21.3	Decreased risk
Ng et al. [22]	Integrin-α 9 (ITGA9)	Mediate cell–cell and cell–matrix adhesion	3p21	Increased risk
Qin et al. [23]	RAD51L1	DNA damage repair		Increased risk

Abbreviations: NPC, nasopharyngeal carcinoma.

2.1.2.2 Environmental risk and preventive factors

The causes of NPC remain inconclusive, although certain genetic risk factors have been identified. There is no single causal risk factor responsible for the occurrence of NPC. A comprehensive gene-environment interaction has been postulated. In addition to the genetic factors, some environmental risk factors are well-confirmed, including older age, Cantonese background, EBV infection, family history of NPC, tobacco use, male sex and consumption of Chinese-style salted fish in early life (**Table 2.2**). In high-incidence areas, NPC incidence peaks at approximately 45-60 years of age. About 83% of NPC cases are associated with EBV infection [24]. Of note, specific EBV strains may be related to NPC. For instance, based on a large-scale genome sequencing study, two EBV variants within BALF2 genes, encoding a major binding protein, were found to be strongly correlated with NPC risk with an odds ratio of 8.69 [25]. Anti-EBV antibodies (i.e., immunoglobulin A of EBV nuclear antigen 1, EBNA1-IgA and immunoglobulin A of EBV viral capsid antigen, VCA-IgA) have been used for NPC screening due to their strong associations [26]. Tobacco is one confirmed cause of NPC according to the International Agency for Research on Cancer [27]. Traditional Asian pickled vegetables are defined as possible carcinogens. Our population-based cohort study found a modest association between hard salted fish (directly salted and afterwards dried) and NPC risk [28].

Table 2.2 Environmental risk and preventive factors for NPC[a].

Factors	Odds ratio/Relative risk	Direction of associations
Well-confirmed		
Older age (up to ~60 years in high-incidence areas)		↑↑
Cantonese		↑↑
EBV infection (anti-EBV IgA serology)		↑↑
First degree family history of NPC [29]	4-20	↑↑
Tobacco [30]	1.3-1.6	↑
Male	2-3	↑
Chinese-style salted fish in early life	1.1-1.5	↑
Possible		
EBV variation (BALF2 gene) [25]	8.69	↑↑
Indoor air pollution	1.1-3.5	↑
Chronic respiratory tract infection		↑
Occupational wood dust/smoke		↑
Animal-based diet [31]	2.2	↑
Plant-based diet [31]	0.5	↓
Inconclusive		
Traditional herbal medicine [32,33]		↓/↑/null
Alcohol [34]		↑/null
Tea [34]		↓/null
Occupational formaldehyde		↑/null

[a] Adapted from the article by Chang et al. [1] and other studies cited in the table. Abbreviations: NPC, nasopharyngeal carcinoma; EBV, Epstein-Barr virus.

2.1.3 Treatment

The histological subtypes of NPC include type I (keratinizing squamous cell carcinoma), type II (nonkeratinizing carcinoma) and type III (basaloid squamous cell carcinoma) [35]. The majority of NPC cases are nonkeratinizing undifferentiated carcinoma, which are sensitive to radiation. Radiotherapy plays a key role in NPC treatment, combined with/without chemotherapy because of its deep and hidden location (**Figure 2.1**) and histology. For patients with cancer stage I (according to the 8th version of the American Joint Committee on Cancer, AJCC) who are plasma EBV DNA negative, definitive radiotherapy is recommended. For patients with cancer stages T0-2 (EBV DNA positive),

N1 and M0 or T3, N0 and M0, concurrent radio-chemotherapy with/without induction/adjuvant chemotherapy is suggested. For patients with cancer stages T3-4, N1-3 and M0 or any T, N2-3 and M0, clinical trials or concurrent systematic therapy with induction/adjuvant chemotherapy is recommended. For patients with metastatic disease, the treatment principal is dependent on the conditions of the individuals, although clinical trials are strongly recommended (**Table 2.3**) [36].

Table 2.3 NPC treatment guideline based on national comprehensive cancer network[a]

Clinical stage[b]		Treatment
T1, N0, M0		Definitive RT.
T2, N0, M0		Definitive RT ± concurrent therapy if it is bulky tumor size or EBV+.
T0 (EBV+)-2, N1, M0 Or T3, N0, M0		Concurrent therapy ± induction/adjuvant chemotherapy if it is bulky tumor size or EBV+.
T3-4, N1-3, M0 Or any T, N2-3, M0		Clinical trials, or concurrent therapy + induction/adjuvant.
Any T, any N, M1	Oligometastatic	Induction chemotherapy + radiotherapy/concurrent radiotherapy
	Widely metastatic and good performance status	Systematic therapy
	Widely metastatic and poor performance status	Supportive care

[a] Adapted from national comprehensive cancer network [36].
[b] Clinical stage is the 8th Version of TNM clinical stage by American Joint Committee on Cancer.
Abbreviations: NPC: nasopharyngeal carcinoma; T, primary tumor stage; N, regional lymph nodes stage; M, metastasis stage; EBV, Epstein-Barr virus; RT, radiotherapy.

2.2 Prognosis

In general, the mortality rate for NPC is relatively low compared to other types of cancer. It accounted for approximately 1% of all cancer deaths, and was ranked as the 21st most frequent cause of cancer death worldwide in 2020 [6]. The age-standardized mortality rate declined from 3.9/100,000 in 1990 to 2.2/100,000 person-years in 2019 for Chinese males, and for Chinese females and populations worldwide, it followed a similar pattern [37–40].

According to the American Cancer Society, the 5-year overall survival probability of NPC is approximately 64%. However, the mortality rate for advanced stages of NPC is much higher, with a 5-year survival rate of less than 50% [41]. The well-established prognostic factors, which encompass the tumor characteristics, host factors, and treatment modalities, have been compiled and presented in **Table 2.4.**

Table 2.4 Well-established prognostic factors of NPC

Prognostic factors	Survival probability
Tumor	
Histology	Non-keratinizing squamous cell carcinoma better than (>) keratinizing squamous cell carcinoma [42]
Clinical cancer stage	Early stage better than (>) late stage
Host	
Age	Younger age better than (>) elder age
Sex	Female better than (>) male [43]
Karnofsky Performance Score (KPS)	Higher KPS better than (>) lower KPS
Smoking	Never smokers better than (>) smokers
Nutrition	Malnutrition with worse survival [44]
Treatment	
Radiotherapy	IMRT better than (>) 2DRT/3DRT [45]

Abbreviations: NPC, nasopharyngeal carcinoma; EBV, Epstein-Barr virus; IMRT, intensity-modulated radiation therapy; 2DRT, two-dimensional conformal radiotherapy; 3DRT, three-dimensional conformal radiotherapy.

In addition to the well-established prognostic factors described above, numerous unclear and novel factors are emerging. This thesis concentrates on three inconclusive prognostic factors within a population-based cohort. Specifically, the latest updates on associations between pretreatment body mass index (BMI) and plasma EBV DNA, as well as the microbiome, are highlighted in the following sections.

2.2.1 Population-based NPC survival probabilities worldwide.

Population-based studies investigate specific populations to gain insight into health, disease or social issues. Given the data collected from a representative sample of individuals within a particular region or demographic group, the findings are generalizable to the target population according to the study hypothesis, not only to individuals included in the study. Population-based cancer survival probabilities can be a good indicator for public health organizations and guide research and financial support for decision makers, as well as reflect treatment outcomes of the disease.

Generally, NPC survival probabilities have been increasing with the development of radiotherapy techniques, from two-dimensional radiotherapy (2DRT) era to intensity-modulated radiotherapy (IMRT) era [45]. One large-scale register-based study calculated dynamic age-standardized 5-year relative survival rates in China and observed a slight increase from 43.8% during the period of 2003-2005 to 45.4% during the period of 2012-2015 [46] , despite incomplete information on tumor morphology and cancer stage. A population-based survival study in Taiwan, China, retrospectively identified 13,407 cases of NPC between 2002 and 2010, using population-based registries and all cases recorded in the medical library, and reported an overall 5-year survival probability of 65.2%. However, the study did not include cancer stage information[4].

Beyond China, stable increases in 5-year survival rates were observed in the Americas and Europe. In Ontario Canada, it was 52.9% between 1984 and 1986 and increased to 60.2% between 1999 and 2001, whereas in Surveillance, Epidemiology, and End Results Program (SEER), it was 44.7% between 1984 and 1986, and 56% between 1999 and 2001 [47]. The 5-year overall survival rates in Europe rose from 39.0% between 1990 and 1994 to 46.0% between 2000 and 2007 [48].

These studies showed that the 5-year overall survival probabilities of NPC patients range from 40.0% to 78.2%, depending on the region and period, yet there are several limitations. First, few studies presented cancer stage distribution, one of the most important prognostic factors. One population-based NPC screening cohort identified 153 NPC cases with a 5-year survival of 77.6%, however the proportion of early-stage cancer was 45.9%, which was much higher than that in other population-based studies [49]. Second, limited studies have considered loss to follow-up, which would bias the estimates of 5-year survival rates [50]. Third, these studies encountered coverage issues, which may have impacted the generalizability and validity of the findings. For instance, the SEER program covers 50% of the American population, and most participants live in metropolitan areas [51].

2.2.2 BMI and body shape and NPC prognosis.

Overweight and obesity refer to an atypical or excessive buildup of body fat that poses a health hazard. BMI, which is determined by dividing the weight in kilograms (kg) by square of their height in meters, provides a useful approximation for determining overall body fat levels. For adults, overweight is characterized by a BMI ranging from 25.0 to 29.9, while obesity is indicated by a BMI

of 30 or higher based on the World Health Organization (WHO) [52]. Four more cutoff points specific to Asians at risk have also been established (23.0, 27.5, 32.5 and 37.5) [53]. According to estimates from 2014, the age-standardized prevalence of obesity among men and women worldwide, was 10.4% and 14.4%, respectively [54]. Between 1975 and 2016, there was a notable rise in the prevalence of obesity worldwide, with the number of cases almost tripling during this period. In 2016, 39% of individuals aged 18 years and over were classified as overweight, and 13% were categorized as obese [54,55].

In addition to BMI, other anthropometric measures are employed to access body fatness, such as weight, body shape, waist circumference, and waist-to-hip ratio. These measurements provide additional information, allowing for a more thorough evaluation of an individual's body composition and health status [56]. By considering multiple anthropometric measures, healthcare professionals can obtain a more comprehensive understanding of an individual's overall health. For instance, body shape, reflects how fat is distributed in the body.

The impact of obesity on cancer patient survival remains inconclusive [57] , although there is convincing evidence linking excess body fat, i.e., as measured by BMI, to an increased risk of various types of cancer [58,59]. Obesity paradox is used to describe the condition that a higher BMI is related to better survival outcomes for certain cancers, even though obesity is a recognized risk factor for multiple cancer types [55]. This paradox has been observed in several cancers, including breast, prostate, and lung cancer, and has stimulated considerable debate in the scientific community [60–63]. Proposed explanations for this paradox include the possibility that individuals with a higher BMI may have greater energy reserves to draw upon during cancer treatment, or that the inflammatory response associated with obesity may have a protective effect against certain cancers [64]. The obesity paradox does not represent a true causal relationship. Confounding factors such as age, sex, comorbidities and treatment pattern may influence the observed associations [65].

The risk of NPC is lower among individuals who were underweight or had a normal weight at age 20 years and 10 years before diagnosis, than those who were overweight or obese [66]. However, there is inconsistent evidence regarding the association between BMI and mortality after NPC diagnosis [67–69]. Some studies have reported that patients with a higher BMI showed a lower risk of NPC mortality compared to those who were underweight or normal weight [68,70,71]. However, concerns have been raised regarding the validity of these findings due to potential reverse causality and residual confounding [72]. Reverse causality may exist when low BMI is the result of cancer progression and cachexia, especially among patients diagnosed at advanced stages [73]. Additionally, residual confounding may occur when studies only control for certain factors such as sex, age, alcohol consumption, smoking, clinical cancer stage, and treatment modality, but no other relevant confounders such as education and occupation.

There is evidence suggesting that body weight in early life can impact health outcomes later in life [74]. However, there is a lack of research on the prognostic implications of BMI and body shape measured prior to the diagnosis of NPC. To address these gaps, we prospectively analyzed a population-based NPC patient cohort in southern China [75]. We examined the impact of BMI and body shape at age 20 years, as well as 10 years prior to diagnosis and at the time of diagnosis, on overall and NPC-specific survival rates to reduce reverse causation and residual confounding.

2.2.3 Plasma EBV DNA and NPC prognosis

Plasma EBV DNA has been considered marker for guiding treatment, predicting prognosis, and evaluating the response to therapy [36]. Circulating EBV DNA may originate from either apoptotic host cells or destroyed virus particles. Polymerase chain reaction (PCR) techniques targeting BamH1-W, LMP, EBNA1, and BZLF1 have been used to detect EBV DNA [76,77]. The BamH1-W sequence showed the highest sensitivity in quantitative PCR (qPCR). Assays using plasma had higher sensitivity (69%-99%) and specificity (87%-100%) compared to serum detection (sensitivity: 31%-87%, specificity: 83%-100%) [78,79].

The release of EBV DNA into the bloodstream, which likely originates from NPC tumor cells, may be correlated with tumor burden. The immune function of NPC cases is compromised, with decreased CD4+ and CD8+ T cells, disturbing surveilling and controlling EBV-host cells that harbor and replicate the virus [80]. Lo et al. [81,82] demonstrated that the median plasma EBV DNA copy number was significantly higher in advanced NPC cases than in early-stage NPC cases. Two meta-analyses reported that high pretreatment plasma EBV DNA levels, detectable midtreatment plasma EBV DNA, detectable posttreatment plasma EBV DNA, and slow clearance rates of plasma EBV DNA half-life were significantly linked to worse overall survival [83,84].

Pretreatment plasma EBV DNA concentration has been investigated as a predictor for both short-term and long-term survival in early- and late-stage of NPC [85–87]. Leung et al. [88] studied 90 early stages (I-II) NPC cases and found that higher pretreatment plasma EBV DNA concentration was associated with higher distant failure. Lo et al. [89] investigated the role of pretreatment serum/plasma EBV DNA in predicting early clinical events after treatment completion. Of 91 NPC patients with complete radiotherapy, those with local recurrence or distant metastasis had a median plasma EBV DNA concentration of 41,756 copies/ml, much higher than those without the corresponding events (median concentration, 5,807 copies/ml). The risk ratio of each tenfold increase in plasma EBV DNA was 3.8. Jin et al. [90] performed a retrospective study of 1036 NPC cases of stage III-IVb without distant metastasis and suggested an independent prognostic value of pretreatment plasma EBV DNA on long-term survival outcomes.

However, plasma EBV DNA for monitoring NPC prognosis remains uncertain in population-based validation studies [36]. To address the knowledge gaps, we utilized a large population-based cohort of NPC patients with collected plasma before treatment [75] to examine the associations between pretreatment plasma EBV DNA and NPC prognosis, with a focus on time-dependent and dose-response associations.

2.2.4 Microbiome and NPC

Human microbiota consists of bacteria, viruses and fungi, that colonize in different locations. Commensal bacteria are most frequently studied. The composition is influenced by age, diet, antibiotic use, environmental exposure, and geographic location [91–93]. New evidence has emerged indicating that these microbes could potentially increase the likelihood of developing specific types of cancer and affect the response to treatments.

Inadequate oral hygiene and related diseases have been associated with NPC risk [94,95]. Studies have shown that the microbiota in NPC patients was less abundant or rich in community diversity than in

healthy controls [96,97]. Additionally, the microbiota might interact with the known risk factor, EBV. Liao et al. [98] conducted a cross-sectional study of 186 NPC patients and 153 healthy controls from a physical examination center, tested saliva microbiota by 16S rRNA, and found that *Streptococcus sanguinis* was more abundant in NPC patients and was positively correlated with anti-VCA IgA, an established biomarker of NPC risk and EBV reactivation.

During radiation therapy, the composition of bacterial community undergoes gradual changes, accompanied by a significant rise in the proportion of certain gram-negative bacteria [99]. One longitudinal study collected nasopharyngeal swabs biweekly during radiotherapy. Significant beta diversity changes were observed between early and late responders. The study suggests that microbiome changes during radiotherapy could potentially serve as a short-term predictor of therapeutic response [100]. Another longitudinal study showed that the abundance of *Streptococcus* and *Actinobacillus* was higher among those with severe mucositis than those without severe mucositis during radiotherapy [101]. In a cohort study of 802 NPC patients [102], intratumoral bacterial load was found to be negatively associated with T-lymphocyte infiltration and linked to poor survival outcomes. Nevertheless, limited research has investigated the relationship between the long-term survival of NPC patients and the oral microbiota.

3 Research aims

The primary aim was to employ an efficient follow-up strategy to achieve a low loss to follow-up rate, measure the population-based NPC survival in southern China and investigate the potential prognostic factors regarding lifestyle, an EBV-related biomarker, and oral microbiota.

The specific research aims are illustrated in **Figure 3.1**:

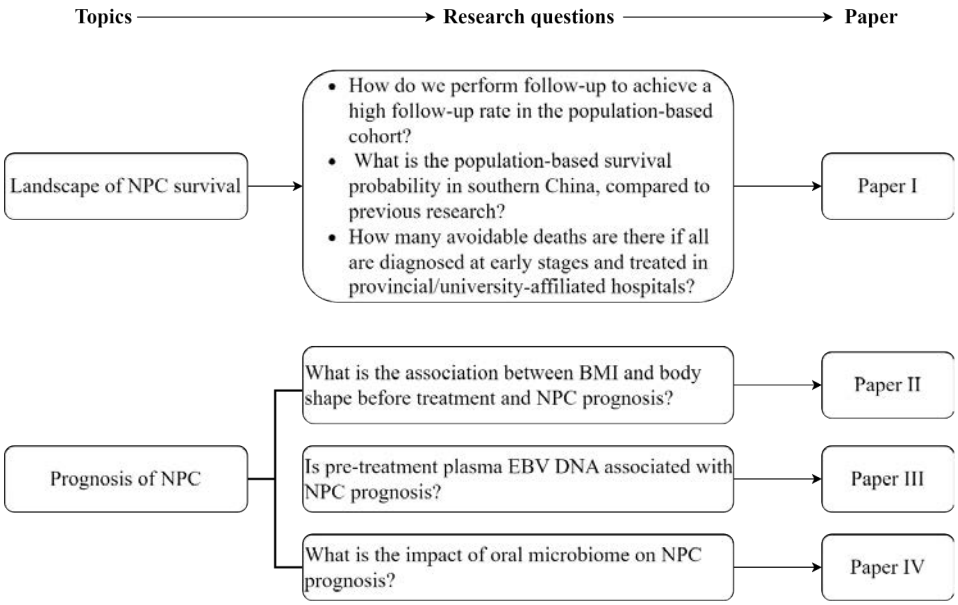


Figure 3.1 Illustration of research questions for each paper.
Abbreviations: NPC, Nasopharyngeal carcinoma; EBV, Epstein-Barr virus; BMI, body mass index.

4 Study design and methodology

4.1 Study design and study population

4.1.1 Population-based cohort study design

The population-based cohort in this thesis comprised the patient cohort of a project entitled 'NPC Genes, Environment, and EBV (NPCGEE)'. The case participants were newly diagnosed between 2010 and 2013 from the study base of 13 cities/counties with 8 million people in the Zhaoqing area of Guangdong Province and the Wuzhou and Guiping/Pingnan areas of Guangxi Autonomous Region in southern China [75]. The participants were eligible for the study if they currently resided in the study areas and were aged between 20 and 74 years, fluent in Cantonese, and able to participate in the study interview without prevalent malignancies or inherited or acquired immune deficiency. In total, we identified 3047 newly diagnosed NPC patients within the study areas, out of which 2553 (83.8%) were included based on eligibility criteria. All the participants completed the questionnaire through face-to-face interviews and 2350 patients donated saliva samples at diagnosis. Between 2018 and 2023, we performed an additional medical record review and found 24 cases that were not incident NPC cases, therefore, we excluded them, leaving 2529 participants in the patient cohort (**Figure 4.1**).

4.1.2 Study I

We aimed to explore a follow-up strategy to obtain a low rate of loss to follow-up, calculate the survival probabilities for this population-based patient cohort, compare our study with other studies in NPC-endemic areas, and compute avoidable deaths.

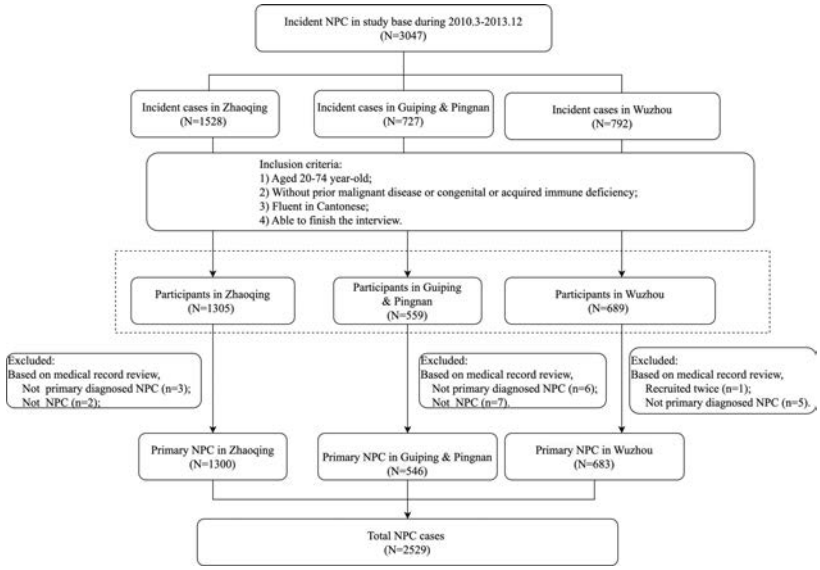


Figure 4.1 Study population for Study I. NPC, nasopharyngeal carcinoma.

4.1.3 Study II

We aimed to assess the associations between BMI and body shape, measured at age 20, 10 years before diagnosis, and at diagnosis, and all-cause and NPC-specific mortality among NPC patients,

accounting for reverse causation and residual confounding. After excluding three cases that lacked information on prediagnosis BMI and/or body shape, the final analysis included 2526 patients (**Figure 4.2**).

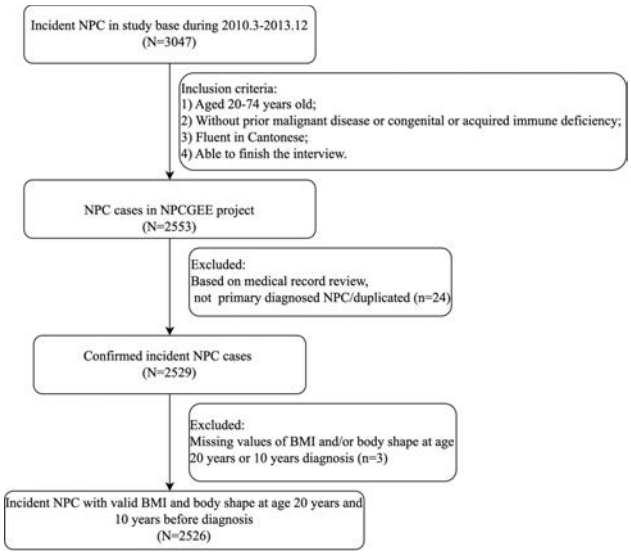


Figure 4.2 Study population for Study II. NPC, nasopharyngeal carcinoma.

4.1.4 Study III

A total of 1854 cases were included in the final analysis after excluding 386 cases who lacked measurements for the plasma EBV DNA load and 529 cases with samples collected after therapy (**Figure 4.3**).

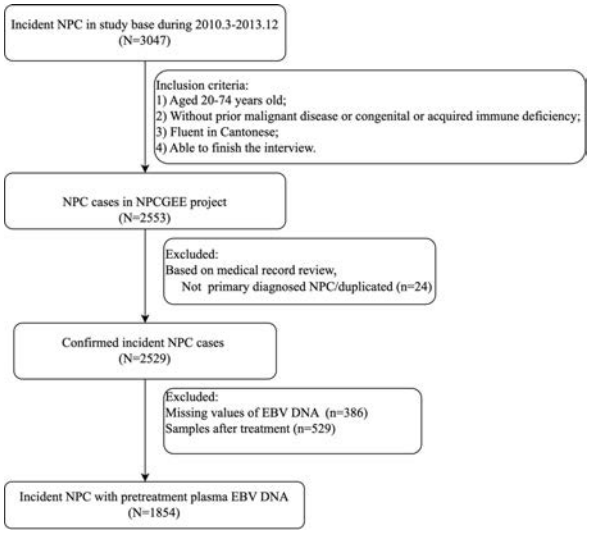


Figure 4.3 Study population for Study III. NPC, nasopharyngeal carcinoma.

4.1.5 Study IV

We aimed to detect potential associations between oral microbiota patterns and mortality of NPC patients, utilizing saliva bacterial profiles obtained through 16S rRNA sequencing. The study population comprised NPC cases in the Wuzhou area, a subcohort of NPCGEE. Eighty-nine were excluded because they refused to provide saliva specimens, and 58 had their saliva DNA extracted using varied procedures. Fifteen cases were excluded due to failed library preparation, low sequence counts, ambiguous sequencing identifiers, duplicated enrollment, or not having primary NPC. Thirty-two former smokers and 13 cases with missing values of covariates were further excluded. In total, 482 cases remained for the final analysis (**Figure 4.4**).

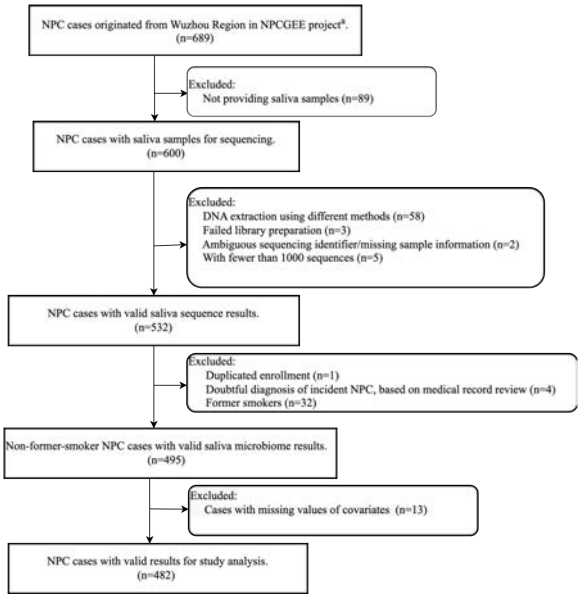


Figure 4.4 Study population for Study IV. NPC, nasopharyngeal carcinoma.

4.2 Main measurements

In the NPCGEE project, the electronic lifestyle questionnaires were composed of the following variables: basic personal characteristics, residential history, occupational history, medical history (oral hygiene, family medical history etc), smoking history, alcohol, and tea consumption history, dietary history (10 years ago and teenage), reproductive history and use of Chinese herbal medicine [75]. Biospecimens were collected, including blood, saliva, hair, fingernail, and toenail. Blood samples were refrigerated at 4°C within three days, delivered to the laboratory, processed to isolate plasma, serum, red blood cells, and buffy coat, and frozen at -80°C [75].

Between 2018 and 2023, an additional medical record review of NPC cases was launched. We made a user-customized follow-up sheet including information on histology, clinical cancer stage, and treatment. The medical records were reviewed by five clinical medical students and a junior oncologist. To ensure accuracy, a senior oncologist randomly checked 10% of the documents. The information from the follow-up sheets was entered into the *Epidata* (3.1 version) database by one person and then verified by another to ensure accuracy.

4.2.1 Exposures

*4.2.1.1 Pretreatment BMI (**Study II**)*

BMI and body shape at age 20 years, 10 years prior to diagnosis, and at diagnosis were obtained through lifestyle questionnaires or medical record reviews.

BMI was classified into four groups following WHO guidelines for Asian populations: underweight (<18.5 kg/m2), normal weight (18.5-22.9 kg/m2), overweight (23.0-27.4 kg/m2), and obese (≥ 27.5 kg/m2) [53]. Body shape was assessed using the revised Stunkard's Figure Rating Scale, which had seven male and nine female figures [103]. The reference groups were body shape 3 and normal weight due to their larger sample sizes, with some adjacent groups merged for subgroup analyses due to limited sample size.

*4.2.1.2 Pretreatment plasma EBV DNA (**Study III**)*

Pretreatment plasma EBV DNA load was quantified using real-time qPCR [81]. Initially, blood samples were centrifuged at 1500 rpm for 15 minutes, aliquoted into microtubes, and stored at -80 °C. Total nucleic acid was extracted from 400 µl plasma using a QIAamp DNA Blood Mini Kit, and eluted from the column using a final elution volume of 50 µL. Then qPCR was performed using primers and probe specific to the BamHI-W region (HONG KONG TECH DRAGON LTD). Five microliters of plasma EBV DNA was distributed and amplified in an ABI 7300 Real-Time PCR system together with a negative control (purified nuclease-free water) and positive controls of the clonal synthesis of the BamHI-W region diluted into six different concentrations. To calculate the target concentration of EBV DNA in plasma (copies/mL), the standard curve and the equation below were used.

$$C = Q \times \frac{V_{DNA}}{V_{PCR}} \times \frac{1}{V_{ext}}$$

C represents for the concentration in targeted plasma (copies/ml), Q for the targeted quantity (copies) based on the PCR detector, V_{DNA} for the total volume of DNA yielded after extraction (50 µL in our case), V_{PCR} for the volume of DNA used for PCR (5 µL in our case), and V_{ext} for the volume of plasma used for extraction (400 µL in our case). For analysis, we classified detectable plasma EBV DNA load into tertiles (low, medium, and high).

*4.2.1.3 Oral microbiota profiling (**Study IV**)*

16S rRNA sequencing

For DNA extraction, twenty-three saliva samples and one autoclaved blank Eppendorf tube with nuclease-free water as a negative control were used each round. The process involved mixing 1 mL of saliva sample with 100 µL lysozyme lysis buffer and incubating it at 37°C for 60 minutes. After adding 0.5-mm-diameter and 0.1-mm-diameter beads (BioSpec, Bartlesville, OK) to each sample, they were blended at top speed for 10 minutes to physically disrupt the microbial cells. The resulting clean liquid was then transferred to a new 2 mL Eppendorf tube for each sample.

The isolation and purification of total DNA was performed using the TIANGEN TIANamp Blood DNA Kit. The DNA concentration was measured using a Qubit 3.0 Fluorometer, Thermo Fisher Scientific. The 16S rRNA amplicon library was generated using 341F/805R primers

(CTACGGGNGGCWGCAG,GACTACHVGGGTATCTAATCC), amplified for 20 cycles (30 seconds at 98°C for melting, 30 seconds at 60°C, and 30 seconds at 72°C), and then barcoded and cleaned up in a second PCR step. The DNA purity and volume were evaluated using an Agilent 2100 Bioanalyzer system and real-time PCR. Finally, sequencing was performed at Beijing Genome Institute on an Illumina MiSeq using a 2 × 300 bp paired-end strategy. In every batch, two blank controls containing nuclease-free water and one control containing *E. coli*-positive single organisms were incorporated.

Data preprocessing

Sample sequencing data underwent demultiplexing, adaptor trimming, and joining paired-end sequences using *VSEARCH* (v.2.7) [104]. Then, it was uploaded to QIIME2 software (November 2018 release) to preprocessing [105]. The *deblur* (v. 1.0.4; *q2-dublur*) was then used for quality filtering (*q2-quality-filter*) and denoising to produce an amplicon sequences variant (ASV) table with default parameters [106,107]. Additionally, a phylogenetic tree was constructed by inserting fragments into the Greengenes 99% identity tree backbone using *q2-fragment-insertion* [108]. Finally, ASVs were assigned a taxonomy using a pretrained reference with a naive Bayesian classifier (*q2-feature-classifier*). ASVs were referred using the first letter of their lowest clearly assigned taxonomic level, the first five letters of their lowest taxonomic assignment, and the first six characters of an MD5 hash of the sequences [109].

Diversity analyses were conducted on rarefied samples of 6,500 sequences using *q2-diversity* in QIIME 2, which calculated alpha diversity measures including Faith's phylogenetic diversity (Faith's PD), observed ASVs and Shannon diversity [110,111] and beta diversity including unweighted UniFrac, weighted UniFrac, and Bray-Curtis metrics [112–114].

Exposure metrics

The exposures of **Study IV** were alpha diversity and beta diversity.

Alpha diversity describes the microbiota diversity within a sample [115]. Faith's PD provides a measure of richness (i.e., the count of different sequence variants in one sample) weighted by the phylogeny (i.e., shared evolutionary history between organisms) [111] , while observed ASVs indicate richness in the sample. The Shannon diversity index is a measure of both richness and abundance (i.e., the count of each sequence variant in one sample) [110]. Alpha diversity was treated as a continuous variable or categorized into tertiles (low, medium, and high diversity).

Beta diversity emphasizes the dissimilarity between samples [115,116]. The Bray-Curtis distance measures dissimilarities in relative abundance. The weighted UniFrac distance considers relative abundance and phylogeny, with emphasis on dominant organisms, and the unweighted UniFrac distance focuses on presence/absence and phylogeny, highlighting the uncommon microbiota.

4.2.2 Outcomes

The outcome was survival probability rate, all-cause mortality and/or NPC-specific mortality. NPC patients were monitored for their vital status, causes of death, and migration out of the study areas using a passive-active-passive circle strategy, which was adapted according to the practical situations of the regions (**Figure 4.5**). The circle strategy began with passive follow-up, which involved linking to databases such as the Cancer Registry, Medical Records, Causes of Death Registry, Medical

insurance system, and Population registry. If patients were lost to follow-up or had passed away without a known cause, we utilized active follow-up methods, such as contacting patients' relatives or the patients themselves, or conducting home visits by village doctors. Finally, we performed passive and active follow-ups repeatedly to complete the information of remaining cases (**Figure 4.5**).

The follow-up period started from diagnosis until either the 31st of December 2018, migration, or death, whichever occurred first. For two cases of death without a death date, we used the median survival time of cases of death with corresponding areas, age and cancer stage. The follow-up time for seven lost-to-follow-up patients (i.e., follow-up time=0) was set to half a day.

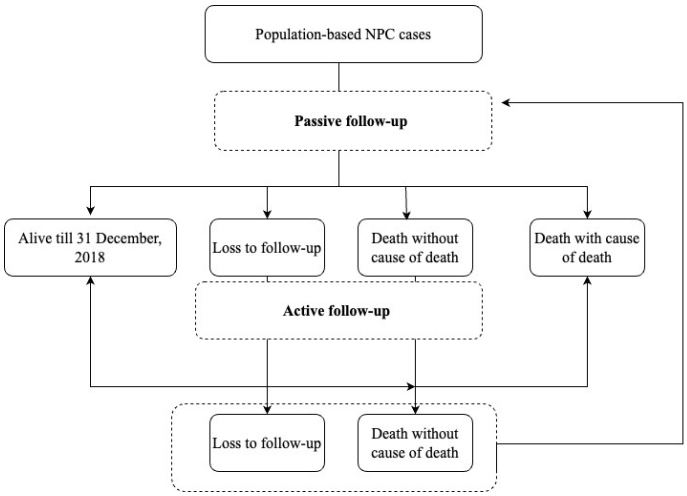


Figure 4.5 Strategy of NPCGEE follow-up. NPC, nasopharyngeal carcinoma.

4.2.3 Covariates

Covariate information was collected through questionnaires or medical records.

Education level at diagnosis was classified into four groups: illiterate/primary school, middle school, high school, and vocational or technical college/university and above [117]. Tobacco use and alcohol consumption were categorized as never, former, or current. Occupation at diagnosis was classified as farmer, blue collar, white collar, unemployed, or unknown/others.

NPC cases were treated in more than 20 hospitals in southern China. We grouped the hospitals into two categories: medical university-affiliated or province-level hospitals and prefecture-level hospitals. Karnofsky Performance Scale (KPS) was categorized into two levels (<90 and ⩾90), which determined the patient's ability to carry out regular activities [118]. The clinical TNM cancer stage was defined based on the 7th version of the AJCC staging system [119], while the pathological classification included non-keratinizing carcinoma and others according to the 2005 WHO tumour classification [35]. NPC therapy is radiotherapy-based, combined with or without chemotherapy; therefore, we categorized the treatment pattern into six groups according to clinical routine: concurrent chemoradiotherapy (CCRT), CCRT with adjuvant chemotherapy (ACT) and/or induction chemotherapy (ICT), only radiotherapy, only chemotherapy, neither radiotherapy nor chemotherapy and missing. Radiotherapy techniques included 2DRT, 3DRT and IMRT.

The selection of covariates to be included in the models followed common principles. First, we selected covariates with prior knowledge and identified covariates by examining their statistically significant univariate associations with mortality. To ensure there was no multicollinearity, we calculated a variance inflation factor and excluded variables that had a variance inflation factor greater than 10 [120].

4.3 Statistical analysis

4.3.1 Cox regression

Cox regression is a semi-parametric model for analyzing survival data without assuming any specific distribution of survival times. The basic assumption is proportional hazards over time, which is testable using the cumulative hazards plots or Schoenfeld's residuals[121,122]. In **Studies II, III, and IV**, we employed Cox regression models to estimate hazards ratios (HRs) for all-cause and NPC-specific mortality in relation to various exposures. To assess potential nonlinear dose-response associations, we used restricted cubic spline functions to visualize the HRs along the continuous exposure (**Studies II and III**) [123].

4.3.2 Flexible parametric model

Flexible parametric survival models utilize a smooth function to represent the transformation of survival. The Royston-Parmar model was used in this thesis [124,125]. In **Study I**, we performed flexible parametric models to calculate the avoidable deaths. In **Study III**, we utilized flexible parametric models to evaluate the time-dependent associations between plasma EBV DNA and mortality.

4.3.3 Permutational multivariate analysis of variance

Permutational multivariate analysis of variance (PERMANOVA) is a statistical method that uses distance matrices to fit linear models and partition distance matrix among sources of variation. PERMANOVA does not assume distributions of the variables or the dissimilarities in the matrix [126]. In **Study IV**, beta diversity was compared by PERMANOVA between groups, controlling for sex, age and sequencing plates, with 999 permutations.

4.3.4 Principal coordinates analysis

Principal coordinates analysis (PCoA) is an ordination technique used for analyzing and visualizing the similarities and differences among a set of objects. It involves transforming complex data, i.e., high-dimensional data, into a simpler format by creating a set of new variables, called principal coordinates (PCs), which represent the most important sources of variation in the data [127]. In **Study IV**, PCoA was employed to visualize the subjects and the top three PCs corresponding to microbiome pattern were included as covariates in adjusted Cox regression model.

4.3.5 Robust Aichison principal component analysis

Robust Aitchison principal component analysis (RPCA) is a statistical method used in microbiome studies to analyze and compare the composition of microbial communities across different samples. It accounts for the sparse composition and zero-flat of microbiota data. It contains two procedures, i.e.,

robust centered log-ratio transformation of absolute abundance of features and matrix completion of treating all zeros as missing values and imputation [128]. In **Study IV**, we used RPCA to depict and illustrate beta diversity. The top three PCs were incorporated into Cox regression models to evaluate the association between beta diversity and mortality.

4.4 Ethical considerations

This thesis relied on the NPCGEE project, approved by institutional review boards from various organizations, including Harvard T.H. Chan School of Public Health, Institute for Viral Disease Control and Prevention of the Chinese Center for Disease Control and Prevention, Sun Yat-sen University Cancer Center, Guangxi Medical University, and the Regional Ethical Review Board in Stockholm, Sweden. The ethical application for the follow-up of NPC patients enrolled in the NPCGEE study was further approved by the Regional Ethical Review Board in Stockholm, Sweden, Guangxi Medical University, Wuzhou Red Cross Hospital and Sun Yat-sen University Cancer Center, China. All participants provided written or oral informed consent during the interview.

5 Results

5.1 Population-based survival in southern China

Study I comprised 2529 newly diagnosed patients with NPC, with 1300 from Zhaoqing, 546 cases from Guiping & Pingnan, and 683 cases from Wuzhou. The average duration of follow-up was 5.50 years, and only 1.7% of the patients were lost to follow-up. A total of 11.3% of the patients in this cohort had early-stage (I-II) NPC. Among the patients, the majority (72.1%) were treated at prefecture-level hospitals (**Table 5.1**).

Table 5.1 Characteristics of primary NPC cases by geographic areas during 2010-2013 in Southern China

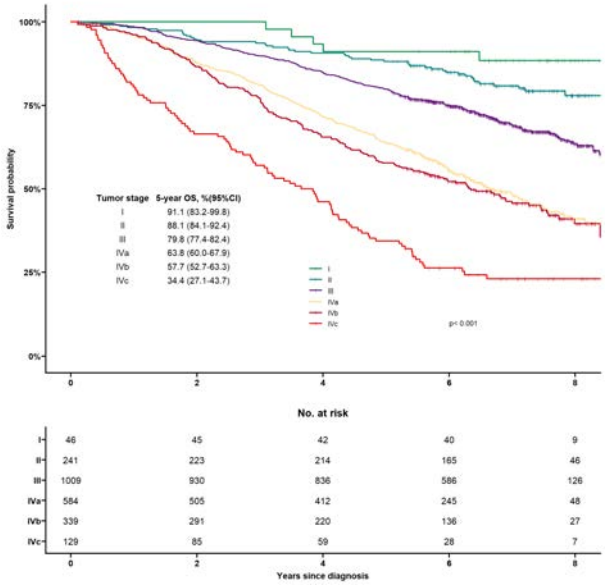
Characteristics	Zhaoqing (N=1300)	Guiping&Pingnan (N=546)	Wuzhou (N=683)	Total (N=2529)
Follow-up years				
Mean (SD)	5.67 (2.32)	5.19 (2.20)	5.42 (2.23)	5.50 (2.27)
Age at cancer diagnosis				
Mean (SD)	47.9 (10.7)	49.7 (10.9)	48.8 (10.4)	48.5 (10.7)
Vital status at end of follow-up, n (%)				
Alive	786 (60.5%)	309 (56.6%)	371 (54.3%)	1466 (58.0%)
Deceased	481 (37.0%)	228 (41.8%)	310 (45.4%)	1019 (40.3%)
Lost to follow-up	33 (2.5%)	9 (1.6%)	2 (0.3%)	44 (1.7%)
Sex, n (%)				
Female	349 (26.8%)	142 (26.0%)	182 (26.6%)	673 (26.6%)
Male	951 (73.2%)	404 (74.0%)	501 (73.4%)	1856 (73.4%)
Marital status at diagnosis, n (%)				
Married	1221 (93.9%)	514 (94.1%)	649 (95.0%)	2384 (94.3%)
Not married	79 (6.1%)	32 (5.9%)	34 (5.0%)	145 (5.7%)
Educational attainment, n (%)				
Illiterate/Primary school	478 (36.8%)	241 (44.1%)	286 (41.9%)	1005 (39.7%)
Middle school	558 (42.9%)	210 (38.5%)	243 (35.6%)	1011 (40.0%)
High school	204 (15.7%)	76 (13.9%)	127 (18.6%)	407 (16.1%)
Vocational or technical college/University and above	60 (4.6%)	19 (3.5%)	27 (4.0%)	106 (4.2%)
Occupation at diagnosis, n (%)				
Farmer	388 (29.8%)	246 (45.1%)	218 (31.9%)	852 (33.7%)
Blue collar	570 (43.8%)	179 (32.8%)	273 (40.0%)	1022 (40.4%)
White collar	195 (15.0%)	52 (9.5%)	103 (15.1%)	350 (13.8%)
Unemployed	43 (3.3%)	16 (2.9%)	18 (2.6%)	77 (3.0%)
Unknown/other	104 (8.0%)	53 (9.7%)	71 (10.4%)	228 (9.0%)
Smoking history at diagnosis, n (%)				
Never	545 (41.9%)	254 (46.5%)	317 (46.4%)	1116 (44.1%)
Former	111 (8.5%)	30 (5.5%)	38 (5.6%)	179 (7.1%)
Current	638 (49.1%)	262 (48.0%)	328 (48.0%)	1228 (48.6%)
Missing	6 (0.5%)	0 (0%)	0 (0%)	6 (0.2%)
Treatment hospitals, n (%)				
Medical university-affiliated/province-level	183 (14.1%)	315 (57.7%)	35 (5.1%)	533 (21.1%)
Prefecture-level	1026 (78.9%)	168 (30.8%)	629 (92.1%)	1823 (72.1%)
Missing	91 (7.0%)	63 (11.5%)	19 (2.8%)	173 (6.8%)
BMI before treatment, n (%)				
Normal weight	616 (47.4%)	233 (42.7%)	352 (51.5%)	1201 (47.5%)
Underweight	175 (13.5%)	45 (8.2%)	98 (14.3%)	318 (12.6%)
Overweight	335 (25.8%)	138 (25.3%)	181 (26.5%)	654 (25.9%)
Obese	63 (4.8%)	27 (4.9%)	30 (4.4%)	120 (4.7%)
Missing	111 (8.5%)	103 (18.9%)	22 (3.2%)	236 (9.3%)
KPS before treatment, n (%)				
< 90	170 (13.1%)	46 (8.4%)	96 (14.1%)	312 (12.3%)
≥ 90	1029 (79.2%)	392 (71.8%)	560 (82.0%)	1981 (78.3%)
Missing	101 (7.8%)	108 (19.8%)	27 (4.0%)	236 (9.3%)
Histological type, n (%)				
Others	39 (3.0%)	30 (5.5%)	22 (3.2%)	91 (3.6%)
Non-keratinizing carcinoma	1171 (90.1%)	457 (83.7%)	635 (93.0%)	2263 (89.5%)
Missing	90 (6.9%)	59 (10.8%)	26 (3.8%)	175 (6.9%)
Cancer stage, n (%)				

Characteristics	Zhaoqing (N=1300)	Guiping&Pingnan (N=546)	Wuzhou (N=683)	Total (N=2529)
I	19 (1.5%)	14 (2.6%)	13 (1.9%)	46 (1.8%)
II	154 (11.8%)	44 (8.1%)	43 (6.3%)	241 (9.5%)
III	530 (40.8%)	205 (37.5%)	274 (40.1%)	1009 (39.9%)
IVa	288 (22.2%)	113 (20.7%)	183 (26.8%)	584 (23.1%)
IVb	154 (11.8%)	70 (12.8%)	115 (16.8%)	339 (13.4%)
IVc	62 (4.8%)	33 (6.0%)	34 (5.0%)	129 (5.1%)
Missing	93 (7.2%)	67 (12.3%)	21 (3.1%)	181 (7.2%)
Treatment pattern, n (%)				
CCRT	484 (37.2%)	204 (37.4%)	351 (51.4%)	1039 (41.1%)
CCRT+ICT/ACT	561 (43.2%)	181 (33.2%)	215 (31.5%)	957 (37.8%)
RT only	96 (7.4%)	47 (8.6%)	68 (10.0%)	211 (8.3%)
Chemo only	45 (3.5%)	13 (2.4%)	19 (2.8%)	77 (3.0%)
Neither RT nor CT	26 (2.0%)	15 (2.7%)	11 (1.6%)	52 (2.1%)
RT+ICT/ACT	0 (0%)	2 (0.4%)	0 (0%)	2 (0.1%)
Missing	88 (6.8%)	84 (15.4%)	19 (2.8%)	191 (7.6%)
RT technique, n (%)				
2DRT	729 (56.1%)	159 (29.1%)	396 (58.0%)	1284 (50.8%)
3DRT	107 (8.2%)	32 (5.9%)	1 (0.1%)	140 (5.5%)
IMRT	300 (23.1%)	248 (45.4%)	236 (34.6%)	784 (31.0%)
No RT	71 (5.5%)	28 (5.1%)	30 (4.4%)	129 (5.1%)
Unknown technique	5 (0.4%)	18 (3.3%)	1 (0.1%)	24 (0.9%)
Missing	88 (6.8%)	61 (11.2%)	19 (2.8%)	168 (6.6%)
Nasopharyngeal radiation dose, n (%)				
< 70 Gy	211 (16.2%)	55 (10.1%)	175 (25.6%)	441 (17.4%)
≥ 70 Gy	909 (69.9%)	372 (68.1%)	453 (66.3%)	1734 (68.6%)
No radiotherapy	71 (5.5%)	28 (5.1%)	30 (4.4%)	129 (5.1%)
Missing	109 (8.4%)	91 (16.7%)	25 (3.7%)	225 (8.9%)

Abbreviations: EBV, Epstein-Barr virus; SD, standard deviation; BMI, body mass index; KPS, Karnofsky performance scale; CCRT, concurrent chemoradiotherapy; ICT, induction chemotherapy; ACT, adjuvant chemotherapy; RT, radiotherapy; Chemo, chemotherapy; 2DRT, two-dimensional radiotherapy; 3DRT, three-dimensional radiotherapy; IMRT, intensity-modulated radiotherapy.

The 5-year overall survival probability was 70.1% (95% CI, 68.4%-72.0%). The 5-year overall survival was 74.1% for patients from Zhaoqing, 67.0% from Guiping & Pingnan, and 65.2% from Wuzhou. By stage at diagnosis, it was 91.1% of stage I, 88.1% of stage II, 79.8% of stage III, 63.8% of stage IVa, 57.7% of stage IVb, and 34.4% of stage IVc (**Figure 5.1A**). Patients treated at medical university-affiliated/province-level hospitals had a 5-year overall survival probability of 77.2%, while for those treated at prefecture-level hospitals, it was 69.4% (p<0.001) (**Figure 5.1B**).

A



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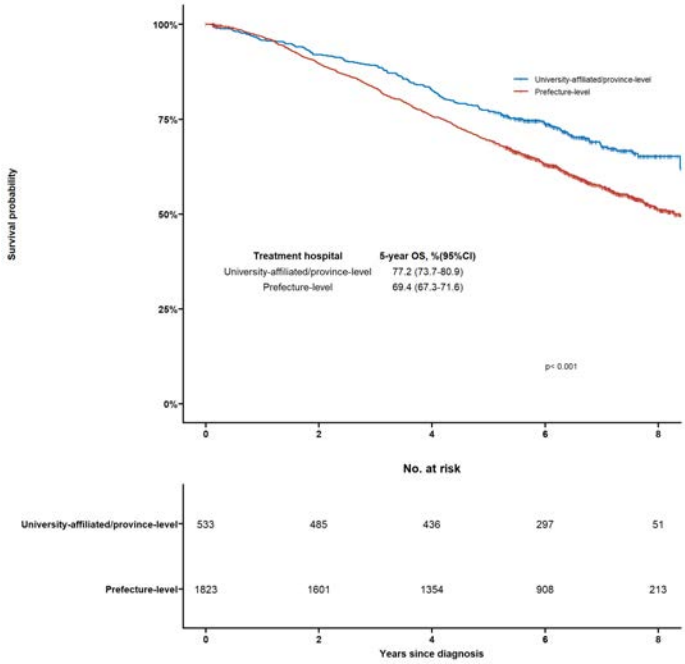


Figure 5.1 Overall survival curves of NPC cases estimated by Kaplan-Meier methods. Panel A, survival by clinical stage at diagnosis; Panel B, survival by treatment hospital type.

The most directly comparable study was performed on a hospital-based NPC cohort diagnosed between 1990 and 2012 at Sun Yat-Sen University Cancer Center, a medical university-affiliated hospital in Guangdong Province (the same province as Zhaoqing, one of our study regions) and with the largest sample size of NPC cases (20,305) ever reported in China [3]. There was a lag of approximately 10 years in the survival of our population-based NPC patients compared to those hospital-based patients. (**Figure 5.2**).

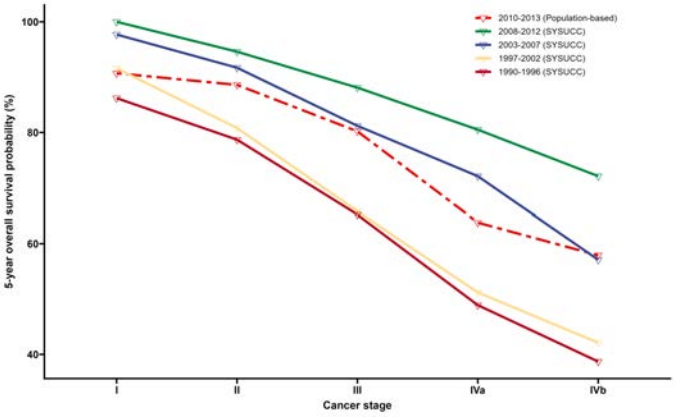


Figure 5.2 Comparison of 5-year overall survival by stage across calendar period of a large hospital-based NPC cohort in Sun Yet-Sen University Cancer Center (SYSUCC), with the results of NPCGEE.

If early diagnosis (stage I-II) was possible for all advanced-stage NPC cases (III-IVc), the standardized avoidable deaths within five years of diagnosis would be 174 (95% CI: 128-221) per

1000 patients (**Figure 5.3A**). Similarly, if all patients received treatment at medical university affiliated or province-level hospitals instead of prefecture-level hospitals, the standardized avoidable deaths within five years would be 58 (95% CI: 8-108) per 1000 patients (**Figure 5.3B**).

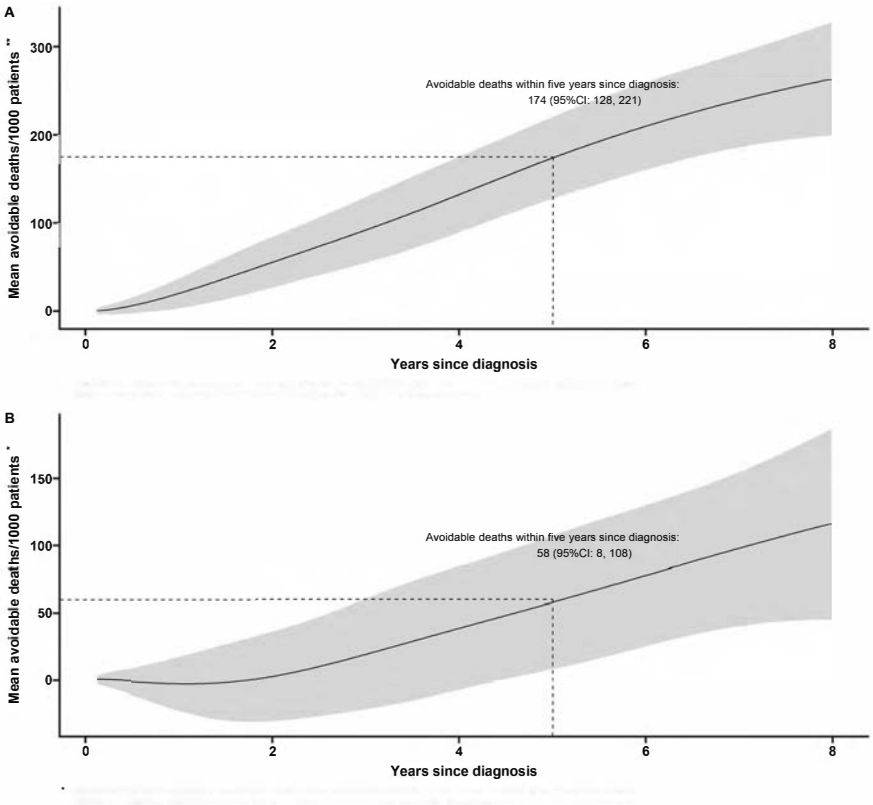


Figure 5.3 Mean avoidable deaths along years since diagnosis under different scenarios. Panel A: Mean avoidable deaths by year since diagnosis, comparing scenarios if 1000 patients were all diagnosed at early stages (I-II) vs. if all were diagnosed at advanced stages (III-IV). Panel B: Mean avoidable deaths by year since diagnosis, comparing scenarios if 1000 patients were all treated in university-affiliated or province-level hospitals vs. if all patients were treated in prefecture-level hospital.

5.2 BMI and body shape before treatment and NPC prognosis

In **Study II**, we included 2526 NPC patients, of which 316 (12.5%) were underweight, 1180 (46.7%) were normal weight, 638 (25.3%) were overweight, 115 (4.6%) were obesity and 277 (11.0%) had missing values at diagnosis.

At diagnosis, overweight and obese patients with NPC had a 25% (adjusted HR 0.75, 95% CI: 0.64-0.89) and a 28% (adjusted HR 0.72, 95% CI: 0.51-1.03) lower all-cause mortality than normal weight patients, respectively. Patients with thinner body shapes 1 or 2 at diagnosis had higher mortality rates than those with body shape 3. Both BMI and body shape at diagnosis showed a monotonic trend with all-cause mortality (**Table 5.2**).

To investigate presumable reverse causality, we conducted a stratified analysis by clinical cancer stage and observed no significant interactions. Of note, the effect size of lower BMI and thinner body shape

with higher mortality was stronger for early-stage NPC than for locally advanced or metastatic NPC. This argued against reverse causality. Patterns of associations with NPC-specific mortality were comparable to those for all-cause mortality (**Tables 5.2 and 5.3**).

Table 5.2 Hazard ratios for body mass index and body shape in association with mortality among 2526 NPC cases

	All-cause mortality				NPC-specific mortality			
	Events	Person-years	Crude HR (95%CI)	Adjusted HR (95%CI)†	Events	Person-years	Crude HR	Adjusted HR (95%CI)†
BMI at age 20 years								
Underweight	161	2234	0.96(0.81-1.14)	0.95(0.80-1.12)	111	2234	0.99(0.80-1.21)	0.97(0.79-1.19)
Normal	745	9953	ref	ref	502	9953	ref	ref
Overweight/obese	112	1701	0.87(0.72-1.07)	0.87(0.71-1.06)	88	1701	1.02(0.81-1.28)	1.02(0.81-1.28)
P for trend			0.523	0.627			0.806	0.733
BMI 10 years before diagnosis								
Underweight	102	1397	0.94(0.76-1.16)	0.93(0.76-1.15)	74	1397	1.01(0.79-1.30)	1.01(0.79-1.30)
Normal	672	8685	ref	ref	452	8685	ref	ref
Overweight	218	3287	**0.85(0.73-0.99)**	0.87(0.75-1.02)	160	3287	0.93(0.78-1.11)	0.97(0.81-1.17)
Obese	26	519	**0.63(0.43-0.94)**	0.75(0.50-1.11)	15	519	**0.55(0.33-0.91)**	0.64(0.38-1.07)
P for trend			**0.022**	0.131			0.060	0.256
BMI at diagnosis								
Underweight	152	1623	**1.23(1.03-1.48)**	1.07(0.89-1.29)	104	1623	1.21(0.97-1.51)	1.03(0.83-1.29)
Normal	490	6411	ref	ref	340	6411	ref	ref
Overweight	208	3737	**0.72(0.61-0.85)**	**0.75(0.64-0.89)**	142	3737	**0.71(0.58-0.86)**	**0.75(0.61-0.91)**
Obese	34	706	**0.62(0.44-0.88)**	0.72(0.51-1.03)	24	706	**0.63(0.42-0.96)**	0.75(0.49-1.14)
Missing	134	1412	**1.25(1.03-1.52)**	0.78(0.47-1.31)	91	1412	1.22(0.97-1.54)	0.81(0.44-1.49)
P for trend††			**< 0.001**	**< 0.001**			**< 0.001**	**0.002**
Body shape at age 20 years								
Shape 1 (thinnest)	35	462	1.06(0.75-1.49)	1.16(0.82-1.66)	22	462	1.00(0.65-1.55)	0.97(0.62-1.51)
Shape 2	379	5112	1.04(0.91-1.19)	1.08(0.94-1.24)	265	5112	1.09(0.93-1.29)	1.12(0.95-1.32)
Shape 3	448	6233	ref	ref	297	6233	ref	ref
Shape 4	125	1738	1.00(0.82-1.22)	0.93(0.76-1.14)	94	1738	1.14(0.90-1.43)	1.01(0.79-1.27)
Shape 5-9	31	343	1.26(0.87-1.81)	1.22(0.85-1.77)	23	343	1.41(0.92-2.16)	1.23(0.80-1.89)
P for trend			0.980	0.275			0.565	0.641
Body shape 10 years before diagnosis								
Shape 1 (thinnest)	27	342	1.15(0.78-1.70)	1.38(0.93-2.06)	21	342	1.30(0.84-2.03)	1.40(0.89-2.20)
Shape 2	282	3355	**1.24(1.06-1.43)**	**1.18(1.02-1.37)**	190	3355	**1.21(1.01-1.45)**	1.15(0.96-1.39)
Shape 3	447	6502	ref	ref	307	6502	ref	ref
Shape 4	181	2677	0.98(0.83-1.17)	0.92(0.78-1.10)	127	2677	1.01(0.82-1.24)	0.92(0.75-1.14)
Shape 5-9	81	1012	1.16(0.92-1.47)	1.06(0.84-1.36)	56	1012	1.17(0.88-1.55)	1.00(0.75-1.34)
P for trend			0.121	**0.025**			0.238	**0.048**
Body shape at diagnosis								
Shape 1 (thinnest)	59	476	**1.87(1.42-2.47)**	**1.68(1.26-2.23)**	45	476	**2.01(1.47-2.76)**	**1.65(1.19-2.29)**
Shape 2	314	3634	**1.29(1.11-1.51)**	**1.23(1.06-1.44)**	206	3634	**1.20(1.00-1.44)**	1.12(0.93-1.35)
Shape 3	363	5388	ref	ref	257	5388	ref	ref
Shape 4	203	3162	0.95(0.80-1.13)	0.94(0.79-1.12)	136	3162	0.90(0.73-1.11)	0.88(0.71-1.08)
Shape 5-9	79	1227	0.95(0.75-1.21)	0.96(0.75-1.24)	57	1227	0.97(0.73-1.29)	0.93(0.69-1.24)
P for trend			**< 0.001**	**< 0.001**			**< 0.001**	**0.002**

This table is adapted from Du et al [129].

Abbreviations: HR, hazard ratio; BMI, body mass index; CI, confidence interval; NPC, nasopharyngeal carcinoma. †HRs were adjusted by age, residential areas, sex, educational attainment, current occupation, smoking history, alcohol consumption, treatment hospital and KPS before treatment, and baseline hazards were stratified by cancer stage. ††Except for missing group.

Table 5.3 Hazard ratios for body mass index and body shape at diagnosis in association with all-cause and NPC-specific mortality stratified by cancer stage, among nasopharyngeal carcinoma cases during 2010-2013 in southern China

	Early				Locally advanced				Metastatic				
	Events	Person-years	Crude HR (95%CI)	Adjusted HR (95%CI)†	Events	Person-years	Crude HR (95%CI)	Adjusted HR (95%CI)†	Events	Person-years	Crude HR (95%CI)	Adjusted HR (95%CI)†	*P**
All-cause mortality													
BMI													0.466
Underweight	5	145	1.18 (0.45-3.08)	1.61 (0.56-4.59)	124	1364	1.18 (0.96-1.44)	1.09 (0.88-1.34)	23	101	1.00 (0.61-1.64)	1.16 (0.66-2.04)	
Normal	25	858	ref	ref	413	5319	ref	ref	52	222	ref	ref	
Overweight/obese	18	768	0.80 (0.44-1.47)	1.05 (0.54-2.05)	204	3546	**0.73 (0.62-0.86)**	**0.75 (0.63-0.89)**	20	123	0.70 (0.42-1.17)	0.60 (0.34-1.06)	
Body shape													0.398
Shapes 1-2 (thinnest)	16	455	1.41 (0.71-2.79)	1.45 (0.69-3.07)	253	3024	**1.25 (1.05-1.48)**	**1.28 (1.07-1.52)**	47	184	1.31 (0.83-2.06)	1.36 (0.80-2.28)	
Shape 3	17	671	ref	ref	282	4170	ref	ref	32	157	ref	ref	
Shape 4-9	15	662	0.89 (0.44-1.78)	0.98 (0.46-2.08)	218	3208	1.00 (0.84-1.19)	1.00 (0.84-1.20)	18	131	0.68 (0.38-1.21)	**0.46 (0.24-0.89)**	
NPC-specific mortality													
BMI													0.408
Underweight	2	145	0.66 (0.15-2.83)	0.78 (0.17-3.60)	83	1364	1.13 (0.88-1.44)	1.04 (0.81-1.33)	19	101	1.28 (0.73-2.25)	1.51 (0.80-2.87)	
Normal	18	858	ref	ref	288	5319	ref	ref	34	222	ref	ref	
Overweight/obese	13	768	0.81 (0.39-1.65)	1.00 (0.45-2.21)	135	3546	**0.69 (0.57-0.85)**	**0.70 (0.57-0.87)**	18	123	0.97 (0.55-1.73)	0.93 (0.50-1.74)	
Body shape													0.821
Shapes 1-2 (thinnest)	10	455	1.32 (0.56-3.12)	1.37 (0.53-3.53)	166	3024	1.13 (0.92-1.39)	1.15 (0.93-1.42)	35	184	1.35 (0.80-2.29)	1.46 (0.80-2.66)	
Shape 3	11	671	ref	ref	204	4170	ref	ref	23	157	ref	ref	
Shape 4-9	12	662	1.10 (0.49-2.50)	1.12 (0.46-2.74)	145	3208	0.92 (0.74-1.14)	0.89 (0.72-1.11)	14	131	0.74 (0.38-1.44)	0.57 (0.27-1.21)	

This table is adapted from Du et al [129].

Abbreviations: HR, hazard ratio; BMI, body mass index; CI, confidence interval; KPS, Karnofsky performance scale.

†HR was adjusted by age at cancer diagnosis, residential areas, sex, educational attainment, current occupation, smoking history, alcohol consumption, treatment hospital and KPS before treatment.

**P* for interaction

5.3 Pretreatment plasma EBV DNA and NPC prognosis

Of the 1854 NPC cases in **Study III**, 1673 (90.2%) had detectable plasma EBV DNA at diagnosis. Those individuals with detectable plasma EBV DNA were more likely to have lower education levels, be underweight, and have been diagnosed with advanced cancer stages.

Comparing those with detectable plasma EBV DNA to those with undetectable EBV DNA, the multivariate adjusted HRs for all-cause mortality and NPC-specific mortality were 2.16 (95% CI: 1.54-3.02) and 2.03 (95% CI: 1.37-3.02), respectively (**Table 5.4**).

Table 5.4 Hazard ratios of pretreatment plasma EBV DNA on mortality among 1854 nasopharyngeal carcinoma cases during 2010-2013 in southern China

Pretreatment Plasma EBV DNA	Person-years	All-cause mortality			NPC-specific mortality		
		Events	Crude HR (95%CI)	Adjusted HR (95%CI)†	Events	Crude HR (95%CI)	Adjusted HR (95%CI)†
Undetectable	1119	37	ref	ref	27	ref	ref
Detectable	8851	744	**2.59(1.86-3.61)**	**2.16(1.54-3.02)**	506	**2.41(1.63-3.55)**	**2.03(1.37-3.02)**
Low	3317	167	**1.53(1.07-2.19)**	**1.45(1.01-2.09)**	114	1.43(0.94-2.18)	1.40(0.91-2.14)
Medium	2987	242	**2.50(1.77-3.54)**	**2.22(1.56-3.17)**	158	**2.23(1.48-3.35)**	**1.99(1.31-3.03)**
High	2547	335	**4.16(2.96-5.84)**	**3.04(2.14-4.32)**	234	**3.95(2.65-5.88)**	**2.88(1.91-4.35)**

Abbreviations: NPC, nasopharyngeal carcinoma; HR, hazard ratio; BMI, body mass index; CI, confidence interval; EBV, Epstein-Barr virus.
†HR was adjusted by continuous age at cancer diagnosis, residential areas, sex, educational attainment, occupation at recruitment, smoking history, alcohol consumption, KPS before treatment, treatment hospital, pathological type and cancer stage, and baseline hazards were stratified by treatment pattern.

Treating plasma EBV DNA as a log-transformed continuous variable, the adjusted HRs per unit of increase for all-cause and NPC-specific mortality were 1.14 (95% CI: 1.11-1.17) and 1.14 (95% CI: 1.10-1.18), respectively. Furthermore, the restricted cubic spline models demonstrated that there were strong monotonic dose-response associations between pretreatment plasma EBV DNA levels and mortality (**Figure 5.4**).

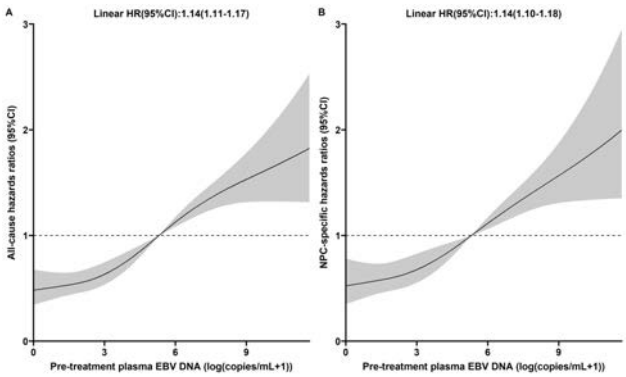


Figure 5.4 Dose-response associations between pretreatment plasma EBV DNA load with logarithm-transformation and all-cause mortality (Panel A) and NPC-specific mortality (Panel B).
Hazard ratios were adjusted for continuous age at cancer diagnosis, residential area, sex, educational attainment, current occupation, smoking history, alcohol consumption, BMI at diagnosis, KPS before treatment, pathological type and cancer stage at diagnosis, and baseline hazards were stratified by treatment pattern.
Abbreviations: BMI, body mass index; NPC, nasopharyngeal carcinoma; HR, hazard ratio; CI, confidence interval.

Five years after diagnosis, the relationships between plasma EBV DNA and mortality became weaker (**Figure 5.5**).

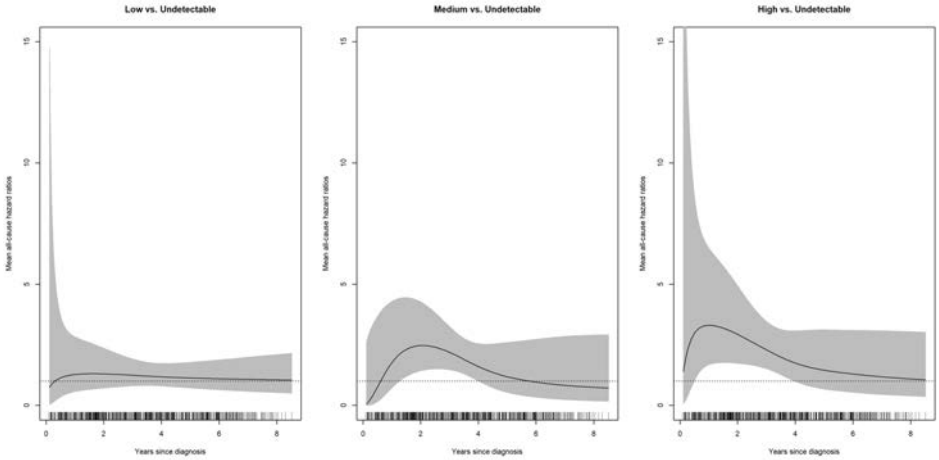


Figure 5.5 Time-dependent associations of plasma EBV DNA on mean all-cause mortality.
Hazard ratios were adjusted for continuous age at cancer diagnosis, residential area, sex, educational attainment, current occupation, smoking history, alcohol consumption, BMI at diagnosis, KPS before treatment, and cancer stage at diagnosis, considering effect modification of treatment pattern, with four degrees of freedoms.
Abbreviation: NPC, nasopharyngeal carcinoma.

5.4 Saliva microbiota and NPC prognosis

5.4.1 Characteristics of the study population

Study IV involved 482 patients with NPC. Among them, 71% were male, and 93% were diagnosed at stage III/IV. The average follow-up time was 5.29 years. With respect to the risk factors for NPC, 78% had less than 9 years of education, 66% brushed their teeth once or less per day, and 52% were current smokers at the time of the interview (**Table 5.5**).

Table 5.5 Characteristics of NPC cases and univariate associations between covariates and mortality

Characteristics	Total, n(%)[a]	Deaths, n(%)[a]	All-cause HRs, (95%CI)	Deaths from NPC, n(%)[a]	NPC-specific HRs, (95%CI)
Number of cases	482 (100.0)	210 (43.6)		181 (37.6)	
Mean follow-up yeas (SD)	5.29 (2.07)				
Mean age at diagnosis (SD)	48.45 (10.55)		**1.03 (1.01,1.04)**		**1.02 (1.00,1.03)**
Sex					
Male	342 (71.0)	165 (78.6)	ref	144 (79.6)	ref
Female	140 (29.0)	45 (21.4)	**0.58 (0.42,0.81)**	37 (20.4)	**0.55 (0.38,0.79)**
Residential community					
Wuzhou	108 (22.4)	41 (19.5)	ref	36 (19.9)	ref
Cangwu	115 (23.9)	51 (24.3)	1.26 (0.83,1.89)	46 (25.4)	1.29 (0.83,1.99)
Cenxi	165 (34.2)	79 (37.6)	1.45 (0.99,2.11)	63 (34.8)	1.31 (0.87,1.97)
Tengxian	94 (19.5)	39 (18.6)	1.17 (0.76,1.82)	36 (19.9)	1.23 (0.78,1.96)
Educational attainment					
≤ 6 years	204 (42.3)	95 (45.2)	ref	75 (41.4)	ref
7-9 years	170 (35.3)	77 (36.7)	0.96 (0.71,1.30)	70 (38.7)	1.11 (0.80,1.54)
≥ 10 years	108 (22.4)	38 (18.1)	0.72 (0.49,1.04)	36 (19.9)	0.86 (0.58,1.28)
Tobacco use					
Never	230 (47.7)	83 (39.5)	ref	67 (37.0)	ref
Current	252 (52.3)	127 (60.5)	**1.56 (1.18,2.06)**	114 (63.0)	**1.73 (1.28,2.34)**
Diagnosis calendar year					
2011	224 (46.5)	115 (54.8)	ref	96 (53.0)	ref
2012	149 (30.9)	55 (26.2)	0.75 (0.54,1.04)	53 (29.3)	0.87 (0.62,1.23)
2013	109 (22.6)	40 (19.0)	0.88 (0.61,1.26)	32 (17.7)	0.83 (0.55,1.25)
Season of saliva sampling					
Winter	114 (23.7)	51 (24.3)	ref	43 (23.8)	ref
Spring	141 (29.3)	60 (28.6)	0.98 (0.67,1.42)	50 (27.6)	0.97 (0.64,1.46)
Summer	101 (21.0)	45 (21.4)	1.03 (0.69,1.54)	40 (22.1)	1.09 (0.71,1.68)
Autumn	126 (26.1)	54 (25.7)	0.99 (0.67,1.45)	48 (26.5)	1.04 (0.69,1.58)
Tooth brushing frequency					
≤ 1/day	316 (65.6)	140 (66.7)	ref	119 (65.7)	ref
≥ 2/day	166 (34.4)	70 (33.3)	0.89 (0.67,1.18)	62 (34.3)	0.93 (0.68,1.26)
Missing or filled teeth					
0	209 (43.4)	77 (36.7)	ref	70 (38.7)	ref
1	61 (12.7)	25 (11.9)	1.15 (0.74,1.81)	22 (12.2)	1.12 (0.69,1.80)
2	52 (10.8)	21 (10.0)	1.13 (0.70,1.83)	16 (8.8)	0.95 (0.55,1.63)
3-5	79 (16.4)	39 (18.6)	**1.48 (1.00,2.17)**	36 (19.9)	**1.49 (1.00,2.23)**
6+	81 (16.8)	48 (22.9)	**1.87 (1.31,2.69)**	37 (20.4)	**1.58 (1.06,2.35)**
Cancer stage					
I-II	35 (7.3)	5 (2.4)	ref	4 (2.2)	ref
III	209 (43.4)	65 (31.0)	**2.51 (1.01,6.23)**	54 (29.8)	2.60 (0.94,7.19)
IV	238 (49.4)	140 (66.7)	**6.06 (2.48,14.81)**	123 (68.0)	**6.61 (2.44,17.92)**
Treatment regimen					
CCRT	250 (51.9)	103 (49.0)	ref	88 (48.6)	ref
CCRT+ICT/ACT	150 (31.1)	66 (31.4)	1.14 (0.84,1.56)	60 (33.1)	1.21 (0.87,1.69)
RT only	60 (12.4)	28 (13.3)	1.22 (0.80,1.85)	20 (11.0)	1.02 (0.63,1.66)
No RT	22 (4.6)	13 (6.2)	**2.01 (1.12,3.57)**	13 (7.2)	**2.33 (1.30,4.17)**
BMI before treatment					
Normal weight	257 (53.3)	130 (61.9)	ref	114 (63.0)	ref
Underweight	62 (12.9)	26 (12.4)	0.78 (0.51,1.19)	23 (12.7)	0.79 (0.50,1.24)
Overweight	93 (19.3)	36 (17.1)	**0.65 (0.45,0.95)**	29 (16.0)	**0.60 (0.40,0.90)**
Obese	70 (14.5)	18 (8.6)	**0.43 (0.26,0.70)**	15 (8.3)	**0.41 (0.24,0.70)**
History of alcohol use					
Never	330 (68.5)	134 (63.8)	ref	114 (63.0)	ref
Former	20 (4.1)	13 (6.2)	**1.97 (1.11,3.48)**	10 (5.5)	1.77 (0.93,3.39)
Current	132 (27.4)	63 (30.0)	1.24 (0.92,1.68)	57 (31.5)	1.32 (0.96,1.82)
Radiotherapy technique					
No radiotherapy	22 (4.6)	13 (6.2)	ref	13 (7.2)	ref
2DRT/3DRT	266 (55.2)	141 (67.1)	0.67 (0.38,1.19)	119 (65.7)	0.57 (0.32,1.02)
IMRT	194 (40.2)	56 (26.7)	**0.36 (0.19,0.65)**	49 (27.1)	**0.31 (0.17,0.58)**
NP radiation dose					
< 70 Gy	144 (30.1)	41 (19.8)	ref	38 (21.2)	ref
≥ 70 Gy	312 (65.3)	153 (73.9)	**1.73 (1.22,2.44)**	128 (71.5)	**1.56 (1.09,2.24)**
No radiotherapy	22 (4.6)	13 (6.3)	**2.83 (1.52,5.29)**	13 (7.3)	**3.03 (1.61,5.69)**

This table is adapted from Du et al [130].

Abbreviations: HRs, hazard ratios; SD, standard deviation; BMI, body mass index; CCRT, concurrent chemoradiotherapy; ICT, induction chemotherapy; ACT, adjuvant chemotherapy; RT, radiotherapy; IMRT, intensity-modulated radiation therapy; 2DRT, conventional 2D radiotherapy; 3DRT, conventional 3D radiotherapy, NP, Nasopharyngeal.

[a]Percentages may not be 100 because of rounding.

5.4.2 Alpha diversity and NPC prognosis

Patients with a lower Faith's PD, had significantly higher all-cause and NPC-specific mortality in unadjusted and adjusted models, compared to the medium diversity group. The adjusted hazard ratios were 1.52 (95% CI, 1.06–2.17) and 1.57 (95% CI, 1.07–2.29) for all-cause and NPC-specific mortality, respectively. The observed ASVs demonstrated a comparable relationship with mortality as compared to Faith's PD, although the associations were not statistically significant in adjusted models. There was no correlation found between Shannon diversity and either outcome (**Table 5.6**).

Table 5.6 Hazard ratios (HRs) for mortality of NPC cases in relation to alpha diversity, Cox regression models

			All-cause HRs (95%CI)			NPC-specific HRs (95%CI)	
Alpha diversity	Cases (n=482)	Deaths (n=210)	Crude	Adjusted[a]	Deaths from NPC (n=181)	Crude	Adjusted[a]
Faith's PD							
Low diversity	161	83	**1.62 (1.16,2.27)**	**1.52 (1.06,2.17)**	74	**1.64 (1.15,2.33)**	**1.57 (1.07,2.29)**
Medium diversity	161	59	ref	ref	52	ref	ref
High diversity	160	68	1.24 (0.88,1.76)	1.18 (0.82,1.72)	55	1.14 (0.78,1.66)	1.10 (0.73,1.64)
Observed ASVs							
Low diversity	161	77	**1.44 (1.03,2.02)**	**1.45 (1.01,2.10)**	68	**1.47 (1.02,2.10)**	1.44 (0.97,2.12)
Medium diversity	161	61	ref	ref	53	ref	ref
High diversity	160	72	1.30 (0.92,1.83)	1.27 (0.88,1.84)	60	1.25 (0.86,1.80)	1.24 (0.83,1.83)
Shannon							
Low diversity	161	73	1.05 (0.75,1.45)	1.07 (0.75,1.52)	65	1.08 (0.76,1.53)	1.14 (0.78,1.66)
Medium diversity	161	71	ref	ref	61	ref	ref
High diversity	160	66	0.94 (0.67,1.31)	0.96 (0.68,1.36)	55	0.91 (0.63,1.31)	0.94 (0.64,1.37)

This table is adapted from Du et al [130].
Abbreviations: Faith's PD, Faith's phylogenetic diversity.
aHRs were adjusted for age, sex, smoking history, BMI before treatment, cancer stage, treatment pattern, alcohol consumption, the number of missing or filled teeth, sequence running number, residential community and season of saliva sampling.

5.4.3 Beta diversity and NPC prognosis

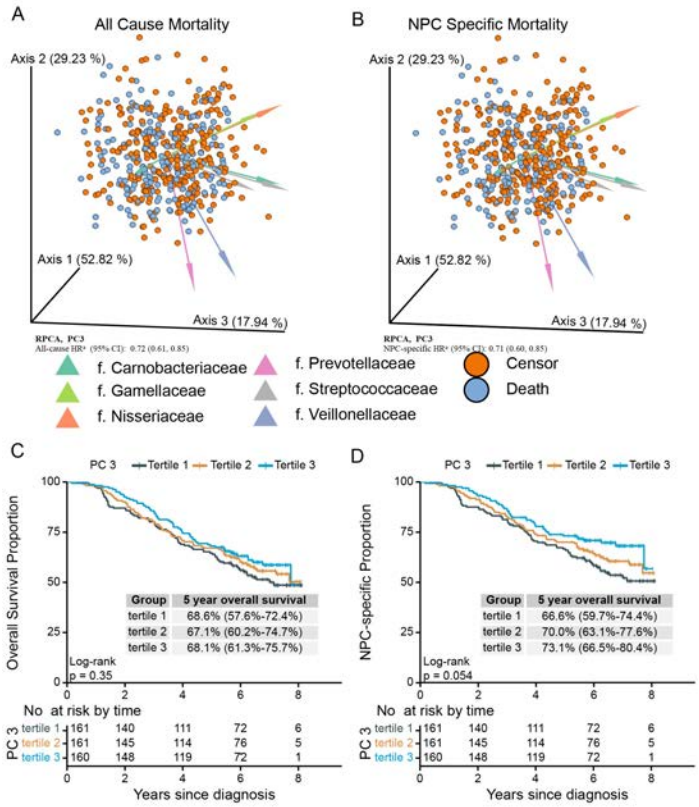
In the context of comparing nested models with and without PCs derived from PCoA, it was observed that nested models utilizing PC3 based on Bray-Curtis distance and PC1 based on weighted UniFrac distance demonstrated marginal significant associations with all-cause mortality (**Table 5.7**).

Table 5.7 Analysis of deviance table for nested with versus without top three PCoA coordinates separately

			All-cause mortality		NPC-specific mortality	
Beta diversity	PCs	Proportion of variation %	p^a	p (alpha)[b]	p^a	p (alpha)[b]
Bray curtis	PC1	10.85	0.456	0.468	0.681	0.562
	PC2	8.15	0.544	0.698	0.427	0.531
	PC3	5.17	0.061	**0.041**	0.132	0.178
Unweighted UniFrac	PC1	20.98	0.104	0.442	0.049	0.545
	PC2	5.99	0.681	0.683	0.984	0.586
	PC3	3.99	0.382	0.430	0.416	0.492
Weighted UniFrac	PC1	32.83	0.102	**0.053**	0.171	0.186
	PC2	24.89	0.434	0.407	0.940	0.580
	PC3	6.97	0.294	0.530	0.205	0.486

This table is adapted from Du et al [130].
p values were generated using likelihood ratio tests to compare nested models.
Abbreviations: PCs, principal coordinates; PCoA coordinates; Faith's PD, Faith's phylogenetic diversity.
[a]Adjusted for age, sex, sequencing plates, tobacco use, the number of missing or repaired tooth, cancer stage, treatment pattern, saliva sampling season, BMI before treatments, alcohol use, diagnosis calendar year and residential community.
[b] additionally adjusted for adjusted for Faith's PD.

As Bray-Curtis and weighted UniFrac mainly indicate relative abundance, we employed RPCA, which considers the abundance of taxa, to generate alternative beta diversity metrics. Normalized PC3 obtained through RPCA exhibited a significant correlation with both all-cause mortality (adjusted HR, 0.72, 95% CI: 0.61–0.85) and mortality specific to NPC (adjusted HR, 0.71, 95% CI: 0.60-0.85). However, we did not observe a significant correlation between PC1 or PC2 derived from RPCA and either of the outcomes. To strengthen the reliability of the associations between these PCs and the outcomes, we classified them into tertiles, plotted Kaplan-Meier curves, and developed multivariate Cox models. The analysis indicated that tertile 3 of PC3 had a 47% lower all-cause mortality and 51% lower NPC-specific mortality than tertile 1. Conversely, the associations with PC1 and PC2 remained statistically nonsignificant after adjusting for confounding factors (**Figure 5.6 and Table 5.8**).



This figure is adapted from Du et al [130].

Figure 5.6 Biplots of RPCA by survival status (A) and NPC-specific survival status (B) and Kaplan–Meier curves of overall (C) and NPC-specific survival (D) proportion between tertiled PC3 groups generated from RPCA. Arrows in (A) and (B) were top 8 taxa influencing the principal component axis. Axis1, axis2, and axis3 were equal to PC1, PC2, and PC3. The axes were labeled with the variation proportion that PCs explain. Sample loadings PC3 were z-normalized in Cox models. HRs were adjusted for age at diagnosis, sex, sequencing plates, tobacco use, the number of missing or filled tooth, cancer stage, treatment pattern, saliva sampling season, BMI before treatments, alcohol use, diagnosis calendar year, residential community and Faith's PD. RPCA, robust Aitchison principal-component analysis; NPC, nasopharyngeal carcinoma; Faith's PD, Faith's phylogenetic diversity.

Table 5.8 Hazard ratios (HRs) for mortality of NPC cases in relation to tertiled PCs from RPCA, Cox regression models

			All-cause HRs			NPC-specific HRs	
PCs[a]	Cases (n=482)	Deaths (n=210)	Crude	Adjusted[a]	Deaths of NPC (n=181)	Crude	Adjusted[b]
PC1							
tertile 1	161	63	ref	ref	51	ref	ref
tertile 2	161	81	**1.44 (1.04,2.00)**	1.21 (0.84,1.74)	73	**1.60 (1.12,2.29)**	1.31 (0.89,1.94)
tertile 3	160	66	1.13 (0.80,1.59)	1.30 (0.88,1.93)	57	1.20 (0.82,1.75)	1.32 (0.86,2.02)
PC2							
tertile 1	161	69	ref	ref	64	ref	ref
tertile 2	161	75	1.15 (0.83,1.60)	1.07 (0.75,1.54)	61	1.01 (0.71,1.43)	1.01 (0.68,1.48)
tertile 3	160	66	0.99 (0.71,1.39)	1.14 (0.78,1.67)	56	0.90 (0.63,1.29)	1.02 (0.68,1.52)
PC3							
tertile 1	161	77	ref	ref	72	ref	ref
tertile 2	161	70	0.88 (0.64,1.22)	**0.66 (0.46,0.96)**	61	0.82 (0.59,1.16)	**0.68 (0.46,1.00)**
tertile 3	160	63	0.78 (0.56,1.09)	**0.53 (0.36,0.80)**	48	**0.64 (0.44,0.92)**	**0.49 (0.32,0.76)**

This table is adapted from Du et al [130].

Abbreviation: RPCA, robust Aitchison principal-component analysis. PC, principle component.

[a] Sample loading of PCs were grouped into three tertiles.

[b]Adjusted for age at diagnosis, sex, sequencing running number, tobacco use, the number of missing or filled tooth, cancer stage, BMI before treatments, alcohol use, diagnosis calendar year, treatment pattern, saliva sampling season, residential community and Faith's phylogenetic diversity.

6 Discussion

6.1 Interpretation of findings and implications

6.1.1 What is population-based NPC survival in China?

Study I aimed to establish an effective follow-up strategy in a population-based setting in southern China. It would enable the accurate estimation of NPC survival probabilities. We also aimed to compare the results with those from other NPC-endemic areas, and to calculate avoidable deaths through early detection or treatment in advanced hospitals. We achieved a high follow-up rate of 98.3% using a passive-active-passive circle strategy that combined data linkages with direct outreach to ascertain vital status. Our population-based cohort consisted mostly of advanced-stage patients treated in prefecture-level hospitals between 2010 and 2013, and we found a 5-year overall survival rate of 70.1%, similar to that reported a decade earlier in a large cancer center in southern China. We estimated that early diagnosis could prevent 174 deaths per 1000 patients within five years, while treatment at university-affiliated or province-level hospitals could save 58 lives per 1000 patients.

During the late 20th and early 21st centuries, the development of linear accelerator technology in China enabled the use of 3DRT and IMRT, providing significant therapeutic advantages for NPC patients [131]. Our study found that 36.5% of the population received treatment with 3DRT and IMRT. Due to insufficient information on radiotherapy techniques provided in prior population-based studies [4,132], it is not possible to make direct comparisons with our findings. However, we hypothesize that the observed improvement in survival rates over time in those studies was likely influenced by the development of radiation treatment facilities, particularly around the early 21st century. Additionally, within our study population, the survival rate in Zhaoqing was significantly higher than those in the other two regions, likely attributable to its more prosperous economy [133].

Our study presents a successful approach to achieve complete follow-up for cancer patients in southern China, offering a reliable depiction of NPC survival over the past decade. Our findings reveal that NPC survival rates are lower in the general population than in hospital-based cohorts, which was an expected result but had not been previously established. These results are likely to be applicable to other regions or countries where NPC is endemic, and suggest that the actual benefits of newer therapies may be less than what is observed in clinical trials. To reduce disparities in NPC mortality rates nationally and globally, it is necessary to allocate more medical resources to enable widespread cancer screening and improve access to advanced radiation therapy facilities in remote areas.

6.1.2 Pretreatment BMI and body shape and NPC prognosis

Study II, a prospective cohort study on NPC in southern China examined the associations of BMI and body shape at different time points with all-cause and NPC-specific mortality, taking measures to address concerns of reverse causation. The study found that overweight individuals at diagnosis had a lower mortality rate of approximately 25% compared to those with normal weight. Additionally, individuals with the thinnest body shape at diagnosis had a 68% higher mortality rate than those with a normal body shape. These associations were not modified by cancer stage. BMI and body shape at age 20 did not have any correlation with overall or NPC-specific mortality. The study's lack of heterogeneity by tumor stage at diagnosis and the detection of similar associations with BMI and body shape 10 years before diagnosis suggest that the results were not primarily due to reverse causation.

Our study has implications for both biology and clinical practice. NPC is typically treated with radiation, with or without chemotherapy. However, radiation therapy can cause painful side effects on the oral and pharyngeal mucosa, which can negatively impact a patient's nutritional balance [134]. This can lead to patients' being reluctant to eat, which can have a significant impact on their overall health. The average radiation course for NPC patients lasts for about 45 days. Individuals with a lower BMI and thinner body shape, indicating poor nutrition at baseline, may be less tolerant of the side effects of radiation therapy, leading to discontinuation of treatment and poorer survival outcome [135,136]. Given our hypothesis that the observed associations between BMI and body shape, and mortality are related to adherence to radiotherapy, our findings suggest that greater clinical attention should be directed toward improving the nutrition and treatment tolerance to achieve better survival outcomes of this patient group.

6.1.3 Pretreatment plasma EBV DNA and NPC prognosis

Study III, a prospective population-based cohort study in southern China, revealed that individuals with detectable plasma EBV DNA (observed in over 90% of cases) had more than twice the risk of all-cause and NPC-specific mortality compared to those with undetectable EBV DNA in plasma. The study also demonstrated strong positive dose-response associations between log-transformed plasma EBV DNA and all-cause and NPC-specific mortality, as evidenced by both linear and spline models.

Higher pretreatment EBV DNA levels may reflect a more extensive tumor burden, which could contribute to a higher risk of mortality. Reportedly, EBV can facilitate metastasis, invasion, and recurrence of malignant cells. EBV can target cell adhesion molecules such as cadherin and integrin, which can trigger a mesenchymal-like phenotype in host malignant cells [137]. This phenomenon is an essential developmental program that can promote metastasis, drug resistance, and tumor recurrence. There are novel treatment trials targeting on EBV. For instance, EBV-specific tumor-infiltrating lymphocytes were taken from the patient, expanded in vitro, and then transferred back to the same patient [138,139]. One phase II clinical trial showed long-term benefits of adoptive transfer of EBV-specific T cells among 23 nonmetastatic NPC patients [140].

Our findings reveal that the correlation between plasma EBV DNA and mortality exhibited attenuation beyond the 5-year mark following diagnosis. Notably, prior investigations have not undertaken an exploration of whether this correlation undergoes temporal variation. In a broader context, patients with NPC who surpass the five-year survival threshold may be regarded as being statistically "cured", denoting a mortality pattern similar to that observed in the general population [141].

The present study's results suggest that NPC patients with high pretreatment plasma EBV DNA loads may benefit from heightened clinical management and surveillance for up to five years postdiagnosis. This is particularly relevant given that NPC recurrence is often the result of tumor cells invading the circulation, and pretreatment EBV DNA levels are indicative of tumor burden. Therefore, a high EBV DNA load may signal the need for more intensive chemotherapy to reduce the risk of recurrence. Conducting randomized controlled trials that stratify patients based on pretreatment plasma EBV DNA levels may be a useful avenue for further exploring this issue.

6.1.4 Oral microbiota and NPC prognosis

Study IV indicated that certain measures of reduced enrichment and phylogenetic within-community diversity were linked to increased disease-specific and overall mortality, and some measures of

between-community diversity and composition were associated with mortality. However, the results were not entirely conclusive since certain measures of alpha and beta diversities did not exhibit any association with either overall or NPC-specific mortality.

The concept that diverse and well-balanced microbiome plays a crucial role in the immune function of human oral mucosa is widely accepted [142]. The commensal microbiota has the potential to safeguard hosts from colonization by exogenous pathogens and overgrowth by indigenous pathobionts. The mechanism underlying the observed relationship between the oral microbiota and NPC prognosis remains unclear. A potential explanation is that the microbiota may impact host immune function or contribute to the development of severe mucositis, which is a common side effect of radiotherapy in NPC patients. A study conducted by Hou et al. [143], revealed that two specific microbial taxa in the retropharyngeal wall were significantly associated with the progression of mucositis. This complication can lead to discontinuation of chemotherapy or radiotherapy, as well as malnutrition, which may contribute to poorer prognosis among NPC patients. Another longitudinal study showed that the abundance of *Streptococcus* and *Actinobacillus* was higher among patients with severe mucositis than those without severe mucositis during radiotherapy [101]. In a cohort study of 802 NPC cases [102], intratumoral bacterial load was found to be negatively associated with T-lymphocyte infiltration and linked to poor survival outcomes.

Mechanisms underlying the microbiota and survival of patients with other cancers revealed that commensal microbiota was associated with the efficacy of immunotherapy [144,145]. A study demonstrated that an abnormal gut microbiome composition in mouse models can cause primary resistance to immune checkpoint inhibitors for those with advanced melanoma, non-small cell lung cancer, or renal cell carcinoma [146]. Antibiotics use could inhibit the clinical benefit of checkpoint inhibitors in patients with advanced cancer. Additionally, metagenomics analysis of patient stool samples at diagnosis revealed associations between clinical responses and fecal microbiota transplantation [146].

Our research provides insight into the connection between microbiota and NPC survival. As the relationship between gut microbiota and cancer immunotherapy response has been explored, we are inspired to investigate the potential impact of microbiota on NPC treatment response.

6.2 Methodological considerations

6.2.1 Strengths

To our knowledge, our study represents the only population-based investigation of NPC survival within an NPC-endemic region, featuring a notably high enrollment rate and an exceptional degree of follow-up completeness. Our research also encompasses comprehensive data collection regarding unified clinical stage, detailed radiation therapy modalities, and demographic and environmental risk factors, enabling us to adjust for numerous potential confounders. Consequently, our study yielded valid, representative, and generalizable insights regarding NPC survival in southern China, which is burdened with the greatest incidence of NPC.

6.2.2 Selection bias

Selection bias refers to a potential source of distortion in estimates of disease occurrence or of effects arising from procedures that influence initial, ongoing study participation or loss to follow-up. In the

NPCGEE study, we achieved relatively high participation rates among cases (84%), therefore, the selection bias that comes from initial participation should be limited. However, time-to-event studies can be prone to another form of selection bias that arises from incomplete follow-up correlated with both the exposure and outcome variables. Survival analysis typically assumes that individuals lost to follow-up are comparable to those who remain in the study. It is important to consider the possibility that certain factors may affect an individual's likelihood of dropping out, such as worsening symptoms or a belief that staying in touch with medical professionals will result in better care. As a result, the assumption of similarity between those who are lost to follow-up and those who remain in the study may not always hold true [147]. The high participation rates and near-complete follow-up suggest that selection bias may be minimal.

6.2.3 Information bias

Systematic error in a study can arise because the information on exposures, covariates and outcome collected about the study subjects is erroneous, for example, measurement error for continuous variables and misclassification for discrete variables. Recall bias, a common type of information bias, is one concern for **Study II**. Body size and shape at age 20 years and 10 years before diagnosis were assessed by self-report, and are therefore prone to error, although this error is likely to be non-differential due to the prospective data collection. Another potential misclassification is the cause of deaths in **Studies I, II, III and IV**, which are not always easily classified even in hospitals. However, due to the nondifferential misclassification of binary causes of death, it will not bias the direction of rate ratio estimate [148].

6.2.4 Confounding

Confounding is one systematic error and a common concern in nearly all non-randomized studies. Critics may argue that a third variable could potentially negate the relationship between the exposure and outcome. There are methods to control for confounding at study design stage, i.e., randomization, restriction, and matching, and at analytic stage, i.e., standardization, stratification, regression adjustment, and weighting [148].

To alleviate the concern regarding confounding, we adjusted for numerous potential confounders, including age, sex, education, occupation, sampling season, or cancer stage in **Studies I, II, III and IV**. However, there is always residual confounding unknown or unmeasured. In **Study IV**, we lacked information on antibiotic use and anti-inflammatory therapy prior to saliva collection, which could affect both microbiome diversity and survival outcomes and cause bias, although previous work has reported that oral microbiome is more stable to antibiotics than fecal microbiome [149].

6.2.5 External validity

External validity, also referred as generalizability, is a crucial aspect of research design that pertains to the extent to which the findings of a study can be applied to other situations, populations, and period. This thesis was conducted in a population-based NPC-endemic area where 84% of the cases were enrolled during the study period. Hence, the findings of our study have high external validity and may be applied to a broader population in southern China. The survival patterns of NPC patients from **Study I** can be generalized to NPC cases diagnosed with NPC between 2010 and 2013 in southern China. Similarly, the conclusions drawn from **Studies II and III** have high external validity in southern China and could potentially extend to other high-incidence NPC areas such as Hong Kong,

Taiwan, and southeast Asia, although the size of the association may be different due to the sensitivity and specificity of the qPCR test by amplified fragments and methods [79,150] and the different cut-off values of categorical BMI as well as population differences in the distribution of plasma EBV DNA load [83] and overweight. The result of **Study IV** was strictly generalizable to NPC in endemic Guangxi Autonomous Region in southern China, as the composition of the oral microbiome is highly influenced by dietary patterns and geographic location [151,152].

7 Conclusions

Based on the papers included in this thesis, several conclusions can be drawn.

- Using a passive-active-passive circle follow-up strategy, high complete follow-up in a population-based setting in southern China was achievable. Population-based NPC survival lags behind large-hospital-based survival. Earlier diagnosis could contribute to substantial reductions in NPC mortality (**Study I**).

- Being overweight or obese at NPC diagnosis was linked to lower all-cause and NPC-specific mortality, while having a leaner body shape was associated with higher mortality compared to a normal weight/body shape (**Study II**).

- Robust evidence existed indicating a correlation between pretreatment plasma EBV DNA levels and NPC prognosis, with a positive dose-response relationship. However, the associations were weakened beyond five-year postdiagnosis mark (**Study III**).

- Certain measures of oral microbiome diversity and NPC prognosis were correlated. Reduced within-community diversity was associated with increased mortality risk. The observed associations may result from global patterns, rather than specific microbiota (**Study IV**).

8 Points of perspective

This thesis not only addressed original research questions but also sparked further inquiry and inspired us to tackle unresolved NPC issues related to clinical management, as discussed in the following section.

In **Study I,** in addition to overall survival for NPC, we calculated net survival, i.e., NPC-specific survival, in southern China. This metric could be used to compare disease-specific survival outcomes between different populations or time periods, or provide insights into the effectiveness of healthcare interventions or treatment protocols. However, due to the incomplete data on cause of death, as well as variability in methods of classifying causes of death for those with available data, analyses of net survival are prone to bias. As an alternative, relative survival can be used as a proxy for net survival when the disease of interest is the primary cause of death in the population under study. To estimate relative survival in the NPCGEE patient cohort, we will try to obtain life tables based on the general population's age- and sex-specific mortality rates, thereby enabling us to perform relative survival analysis. Second, as we observed from the Kaplan-Meier curves, the overall survival probability declined slightly beyond five years of diagnosis. This observation prompts further inquiry to examine differences in survival rates and causes of death after the five-year mark, to provide potentially valuable evidence for patient monitoring. Third, our findings indicated that approximately one-fifth of deaths could have been avoided if all cases had been diagnosed at an early stage. These results motivate us to further explore early detection methods for NPC. Both serum EBV antibody and plasma EBV DNA levels have demonstrated favorable performance characteristics and cost-effectiveness as biomarkers for early NPC detection in high-risk populations, yet their predictive positive value (PPV) ranged from only 4% to 11% [153]. Our previous work showed that combining epidemiological characteristics, serum EBV antibody levels and human and EBV single-nucleotide polymorphisms (SNPs) increased the PPV to 32% [154]. Yet, few randomized controlled trials of NPC screening have been conducted, and more such studies are warranted to determine whether these tools, perhaps in combination with other biomarkers, are valid basis for effective NPC screening to reduce NPC mortality.

In **Study II,** we found associations of BMI and body shape, including 10 years before diagnosis with NPC prognosis. Yet, potential misclassification of BMI and body shape based on memory is a concern. We could eliminate this bias in a natural longitudinal cohort by capturing objective measurements of anthropometric index, whether through medical records or in-person measurements. Such a study design would also enable us to evaluate associations between post-diagnosis changes in BMI and treatment response, providing insight into how we can perform nutrition management in clinical practice. Although any associations between post-diagnosis BMI and NPC outcomes would be susceptible to reverse causation (i.e., an effect of disease severity on BMI, as opposed to an effect of BMI on NPC prognosis), it would be worthwhile to understand these associations both to improve NPC prognosis and to maintain a favorable BMI, given the importance of BMI as a mediator of other health outcomes and health-related quality of life.

In **Study III,** we observed a dose-response and time-dependent association between pre-treatment plasma EBV DNA and mortality among NPC patients in population-based setting. Our results suggest that a higher EBV DNA load may indicate higher tumour burden, which may in turn denote a requirement for more intensive chemotherapy. To further investigate this issue, randomized controlled

trials of chemotherapy regimens stratified by pre-treatment plasma EBV DNA levels could provide valuable insights. Other questions prompted by our findings include, how pre-treatment plasma EBV DNA could be incorporated into the current staging framework, and whether longitudinal monitoring of plasma EBV DNA could help with risk stratification and adaptation of therapeutic protocols. For example, there are some ongoing clinical trials (i.e., NCT02135042, NCT05772208) of applying individualized treatment based on consideration of both cancer stage and EBV DNA load. In addition, due to a lack of quantitative agreements among laboratories internationally, clinically relevant thresholds have not been standardized [150]. One possibility has been raised by Miller et al., who developed laboratory BamHI-W droplet digital PCR (dPCR) to define prognostic thresholds [155,156]. Further studies are needed to integrate dPCR or other EBV-DNA-based metrics with cancer stage to guide individualized treatment.

In **Study IV,** we uncovered associations between oral microbiota as a community and NPC prognosis. In light of our findings that oral microbiome diversity is associated with NPC mortality, the next step is to explore underlying mechanism. First, since diet impacts the gut microbiome and in turn, gut microbiota take part in the process of digestion, we could explore potential triangular associations among diet, microbiota and NPC prognosis. Second, alteration of the immune response (reflecting either an effect of the gut microbiome on the host immune system, or an impact of immunological changes on gut microbiome composition) is another potential explanation for the observed associations [102]. To explore this possibility, we plan to collect data on host immune status (e.g., as measured by C-reactive protein, immunoglobulins G, M and A, complete blood count, peripheral blood lymphocytes using epigenetic cell count measures [157] and tumor infiltrating lymphocytes phenotyping). Third, gut microbiota dysbiosis might indicate resistance to radiotherapy, chemotherapy and immunotherapy [158,159], thereby indirectly affecting NPC prognosis. To further investigate this potential mechanism, it may be worth considering incorporation of bacteria into NPC treatment protocols. Fourth, microbiota may have effects on toxicity of chemoradiotherapy, as some observational studies and animal experiments reported [160,161]. It is worth exploring associations of the interactions or modifications.

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