Characterization of salivary microbiome of Japanese IgA nephropathy patients in comparison with chronic tonsillitis and ulcerative colitis

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1 General Introduction

General Introduction

Chronic kidney disease (CKD) is defined as gradual loss of kidney function over a period of 3 months to years (Al Khodor and Shatat 2017; Levey et al. 2005). With comorbidities such as cardiovascular disease and infectious diseases (Al Khodor and Shatat 2017; Levey et al. 2005), CKD is a worldwide health issue affecting millions of individuals every year. According to a report in 2005 by the Japanese Society of Nephrology, approximately 13.3 million people in Japan have CKD (Imai et al. 2009).

In Japan, one of the leading causes of CKD is immunoglobulin A nephropathy (IgAN) (Tomino 2016). IgAN is an idiopathic disease which means despite years of research and advancement in medical science, the definite cause of the disease is unknown. To address this gap in the existing research and thus, advance the development of medical science in curing IgAN, our current work explores the salivary microbiome of IgAN patients in a Japanese cohort. For a better understanding of our research motivation, objectives and hypotheses, in this chapter we provide you with a brief background of IgAN disease, followed by an introduction to human salivary microbiome and how these two topics might be linked to each other. The last section of this chapter describes the motivation behind the research, and the objectives of this study.

1.1 Overview of IgA Nephropathy

a. *Clinical description*

IgAN is the most common and prevalent form of glomerulonephritis worldwide and was first classified in 1968 by Jean Berger and his co-author Nicole Hinglais (Berger and Hinglais 1968; D'Amico 1987; Gharavi et al. 2000; Schena 1990). This is why IgAN is also addressed as Berger's disease. IgAN is clinically characterized by the mesangial deposition of immunoglobulin A (IgA) and immunoglobulin G (IgG) complexes in the

kidney glomeruli (Moriyama et al. 2014; Tashakkorinia et al. 2021). Upon classification, IgAN was thought to be a benign disease involving the kidneys and the immune system. However, several decade-long survival analytical studies revealed that around 40% of the patients diagnosed with IgAN progressed towards end-stage renal disease (ESRD) within 20 years of their diagnosis (Alamartine et al. 1991; D'Amico 1988; Koyama et al. 1997; Moriyama 2019). Another retrospective study conducted from 1974 to 2011 at a single center in Japan concluded that with about 50% of the IgAN patients progressing towards ESRD within 30 years of their diagnosis, IgAN can no longer be classified as a benign disease (Moriyama et al. 2014).

b. *Types of IgA Nephropathy*

Broadly there are two types of IgAN – primary IgAN and secondary IgAN.

The primary IgAN, which is often addressed as only IgAN, is the idiopathic form of disease. Simply put this translates to absence of any underlying ailment or disease as a starting point for IgAN in the patient.

The secondary IgAN is when IgAN is diagnosed upon biopsy of patients suffering from other diseases (Obrișcă et al. 2019). However, despite such observations in clinical settings, there is no concordant definition of secondary IgAN in the scientific literature (Saha et al. 2018).

- *Secondary IgAN* – when IgAN and the underlying ailment is presumed to share common pathogenesis (Obrișcă et al. 2019). Underlying ailments for secondary IgAN reported till date are liver diseases (cirrhosis, hepatitis C virus, non-alcoholic steatohepatitis), hepatitis B virus (HBV) infection, human immunodeficiency virus (HIV) infection, inflammatory bowel diseases (IBD), psoriasis (Ahuja et al. 1998; Beaufils et al. 1995; Filiopoulos et al. 2010; Koçak et al. 2012; Lai et al. 1989; McGuire et al. 2006; Obrișcă et al. 2019; Saha et al. 2018; Trimarchi et al. 2001).

- *Associated IgAN* – when the relationship between the pathogenesis of IgAN and the underlying disease is unclear or presumed to be coincidental (Obrișcă et al. 2019). Associated IgAN is often discussed in relation to non-Hodgkin and Hodgkin lymphoma (Obrișcă et al. 2019; Saha et al. 2018).

In literature, secondary IgAN and associated IgAN are often clubbed together as secondary IgAN.

c. *Symptoms and clinical manifestations in IgA Nephropathy*

Symptoms in IgAN patients vary from asymptomatic microscopic hematuria or proteinuria to severe hypertension due to kidney damage (Lai et al. 2016; Rodrigues et al. 2017). Two most common phenotypes of IgAN are asymptomatic hematuria and progressive CKD (Rodrigues et al. 2017). Most of the IgAN patients in the early stages exhibit asymptomatic hematuria with minimal proteinuria (*i.e.,* <0.5g/d) and this is usually diagnosed by routine screening programs like urinalysis (Rodrigues et al. 2017; Yamagata et al. 2008). In IgAN patients with progressive CKD, symptoms usually occur in the order of moderate to severe proteinuria, followed by hypertension and a decline in kidney function (Yuzawa et al. 2015). Gross macroscopic hematuria paired with non-specific mucosal infection (synpharyngitic macrohematuria) is a classic clinical syndrome associated with first presentation of IgAN (Lai et al. 2016; Rodrigues et al. 2017). This type of clinical manifestation is less frequent, occurring in about 10%-15% of the patients and is more common in patients aged under 40 (Ibels and Györy 1994; Rodrigues et al. 2017). In these patients, microscopic hematuria is associated with fluctuating proteinuria and in less than 30% of these patients, the proteinuria levels are more than 1g per day (Lai et al. 2016). Nephrotic syndrome, primarily characterized by edema and proteinuria, is a rare clinical manifestation observed in about 5% of the IgAN patients (Lai et al. 2016; Rodrigues et al. 2017).

According to Mayo Clinic repository (MayoClinic 2019), the signs and symptoms for IgAN are as follows:

- Tea or Cola-colored urine caused by red blood cells discharged in the urine
- Recurrent episodes of tea or Cola-colored urine during or after an infection in the body.
- Swelling (edema) in limbs
- Proteinuria or foamy urine due to protein discharge into the urine
- Severe pain in one or both sides at the back below the ribs
- High blood pressure
- Compromised immune system

d. *Pathogenesis of the IgAN patients*

The pathogenesis of IgAN is described by multi-hit hypothesis. Briefly, the multi-hit hypothesis model can be broken down into four steps or “hits” (Lai et al. 2016; Rodrigues et al. 2017; Suzuki et al. 2011; Wyatt et al. 1995). The mechanism of IgAN pathogenesis according to multi-hit model is shown in **Fig. 1**.

(1) Hit 1 is the production of galactose-deficient IgA1 or Gd-IgA1.

(2) Recognition of these antibodies by circulating anti-Gd-IgA1 antibodies is the Hit 2.

(3) The antigen-antibody complex or immune complex formation is the Hit 3. This step is considered to be vital for nephritogenicity of the Gd-IgA1.

(4) Deposition of these immune complexes in the kidney glomeruli and activation of inflammatory responses in the mesangial cells is the Hit 4.

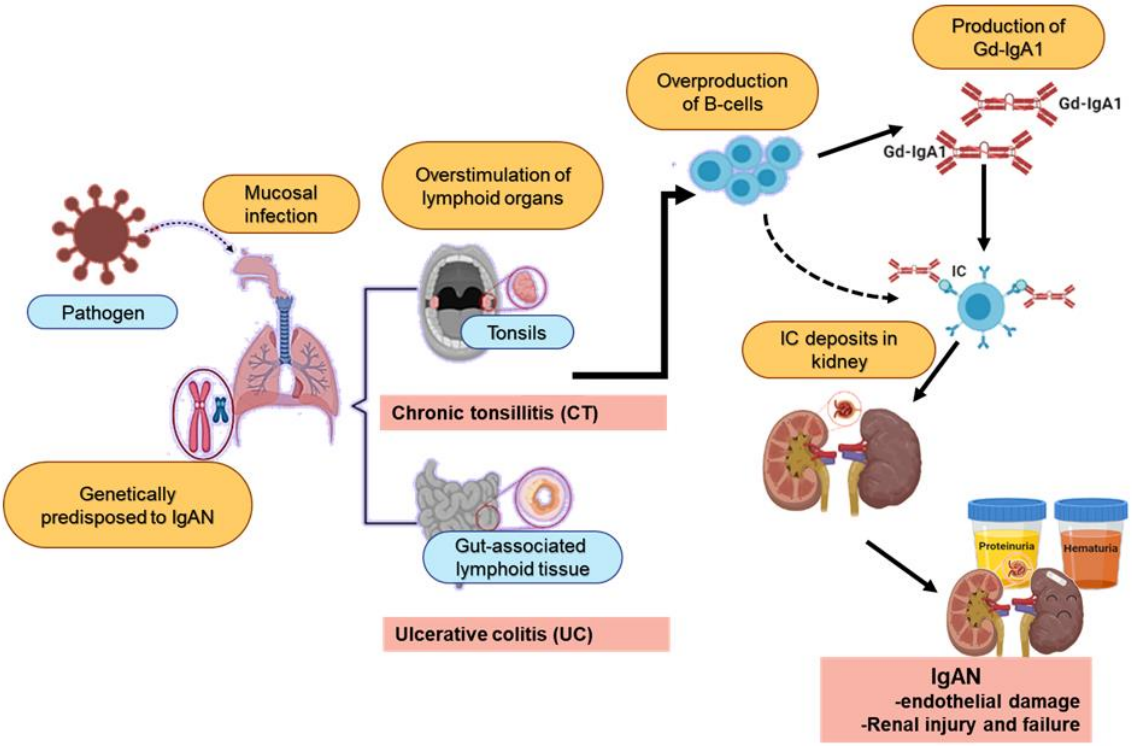

Fig. 1. Main mechanisms involved in the pathogenesis of IgAN. IgAN, Immunoglobulin A Nephropathy. IC, Immune complex. Gd-IgA1, galactose-deficient Immunoglobulin A 1. *(Adapted by permission from Oxford University Press. Clinical Kidney Journal. Rojas-Rivera, J et al. Rapidly progressive IgA nephropathy: a form of vasculitis or a complement-mediated disease? Copyright © 2021 Oxford University Press. 2015)*

Activated mesangial cells overproduce and release components of extracellular matrix, angiotensin II, aldosterone, proinflammatory and profibrotic cytokines and several growth factors which in turn mediate renal injury and tubulointerstitial damage (Amore et al. 2000; Lai 2012; Lai et al. 2016; Novak et al. 2012; Wyatt et al. 1995). The renal injury characterized by complement activation, expansion of mesangial matrix, podocyte injury, and increased glomerular permeability is depicted by clinical manifestations such as hypertension, proteinuria, hematuria and reduced kidney functioning (Amore et al. 2000; Lai 2012; Lai et al. 2016; Novak et al. 2012; Wyatt et al. 1995). Each of these steps in the

multi-hit model for IgAN pathogenesis is affected by a varied number of environmental and genetic factors, which in turn affect disease progression and susceptibility of IgAN in humans (Rodrigues et al. 2017).

e. *Diagnosis*

There is no specific laboratory test available for screening IgAN patients. While clinical assessments are used to determine the need for kidney biopsy, kidney biopsy or histopathological assessment is the confirmatory test for IgAN diagnosis.

Urinalysis: Asymptomatic hematuria or proteinuria is the most common clinical manifestation in the IgAN patients (Yuzawa et al. 2015). The best way to detect this is by urinalysis. A first voided (first morning) urine sample is examined using unstained bright-field microscope or phase contrast microscope (Lai et al. 2016). Renal injury and glomerular bleeding are detected by the presence of red blood cell casts and dysmorphic red blood cells in the urine sample under microscope (Lai et al. 2016). According to the Clinical Guidelines for IgAN version 3 by Japan nephrology society, persistent microscopic hematuria is an essential observation and intermittent or persistent proteinuria is a frequently associated observation in the urinalysis of IgAN patients (Yuzawa et al. 2015). A timed urine collection and a spot urine protein to creatinine ratio measurement are different ways to quantify proteinuria (Lai et al. 2016). The Clinical Practice Guidelines for IgAN by Japan Nephrology Society recommends conducting at least 3 urinalyses of the subject before concluding the urinary abnormality (Yuzawa et al. 2015). The guidelines also instruct that at least 2 of these urinalyses should include analysis of the urinary sediment in addition to qualitative dipstick test (Yuzawa et al. 2015).

Blood biochemical analysis: Despite the absence of IgAN specific blood based diagnostic test, elevated serum total IgA levels suggests IgAN when paired with other

findings such as microscopic or macroscopic hematuria and proteinuria (Lai et al. 2016; Yuzawa et al. 2015). Additionally, an elevated levels of serum IgAN/C3 ratio is often used as a differential diagnostic tool for IgAN detection (Yuzawa et al. 2015). Large cohort study of white patients in the United States reported serum levels of Gd-IgA1 as useful blood biomarker for diagnosing sporadic IgAN (Lai et al. 2016; Yuzawa et al. 2015).

Secondary markers: According to the IgAN pathogenesis, high levels of IgA immune complexes are common in IgAN; however, this finding has limited diagnostic use on its own. Previous studies identified urinary cytokines and chemokines such as monocyte chemoattractant protein 1 (MCP1; also known as CCL2), IL-6, IL-8 and epidermal growth factor as predictors of renal function outcome in IgAN (Lai et al. 2016; Stangou et al. 2009). GWAS of renal miRNA expression has identified a number of novel miRNAs related to IgAN pathogenesis (Lai et al. 2016; Szeto and Li 2014). For example, miR-148b is connected with production of Gd-IgA1 and is thus a potential biomarker of IgAN in the early stages of the disease(Szeto and Li 2014). Similarly, an antifibrotic miRNA, miR-29c can be a biomarker for diagnosing later stages of IgAN progression (Szeto and Li 2014). However, most of these secondary biomarkers are still at the research stage, which explains the lack of specific blood or urine test for IgAN diagnosis.

Kidney function assessment: More than initial diagnosis, renal function assessment every 6 months is essential for monitoring the stage of disease progression. Endogenous creatinine clearance and/or estimated glomerular filtration rate (eGFR) are common parameters in the renal function assessment. Renal function at the baseline is considered to have prognostic implications (Lai et al. 2016). Clinical outcome of patients with an eGFR less than 60 ml/min/1.73 m^2 at the time of renal

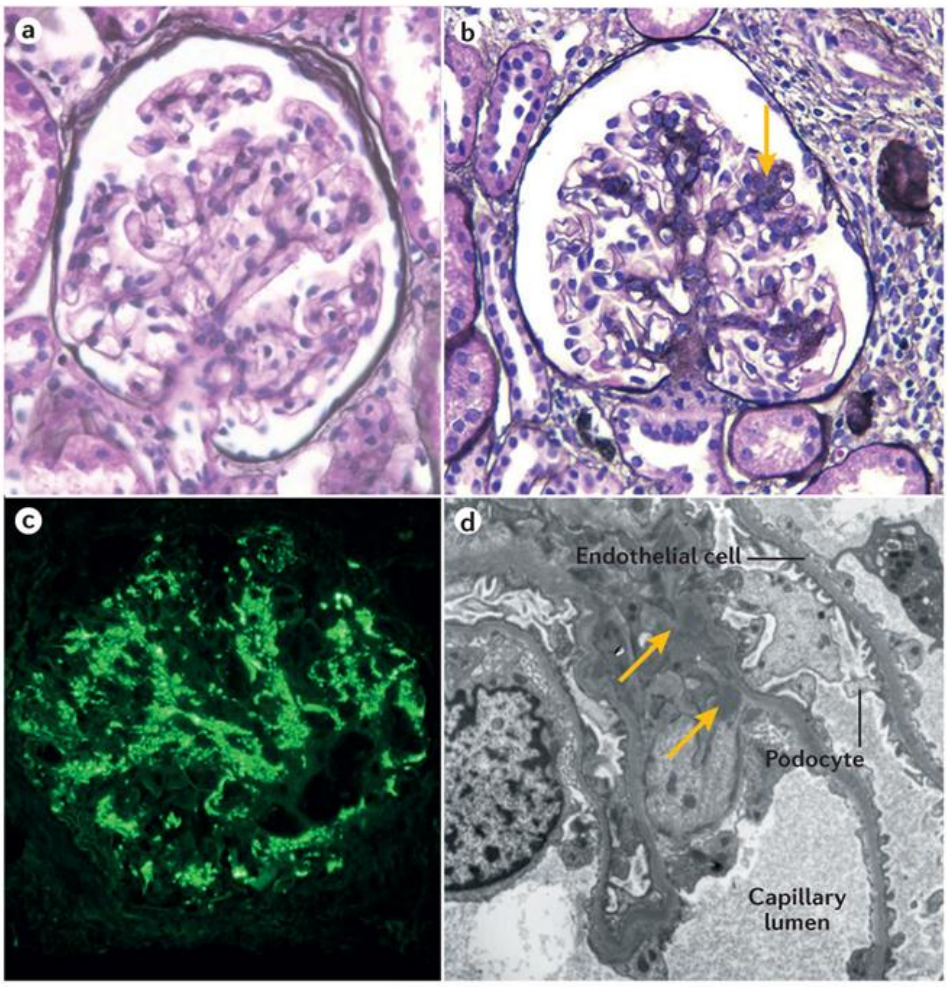

Fig. 2. Renal biopsy findings in a patient with IgA nephropathy. a | Light microscopy image of a normal glomerulus (Jones' silver stain; 200× magnification). **b** | Light microscopy image of a glomerulus showing mild increase in the mesangial matrix and cellularity (arrow; Jones' silver stain; 200× magnification). **c** | Immunofluorescence image of a glomerulus showing heavy granular staining of IgA in the mesangium (200× magnification). **d** | Transmission electron micrograph of the mesangium demonstrating electron-dense mesangial deposits (arrows; 5,000× magnification). *(Adapted by permission from Springer Nature. Nature Reviews Disease Primers. Lai, K., Tang, S., Schena, F. et al. IgA nephropathy. Copyright © 2021 Springer Nature Limited. 2016)*

biopsy is much worse than those with normal eGFR (90 -120 ml/min/1.73m^2) at the baseline (Lai et al. 2016). In addition to the renal function, the degree of histological

injury detected by pathological tests is of prognostic value in IgAN diagnosis.

Kidney biopsy and pathological features: High serum IgA/C3 ratio, persistent microscopic hematuria paired with proteinuria, elevated levels of serum IgA, and macroscopic hematuria with mucosal infection are strong indicators of IgAN in clinical settings (Yuzawa et al. 2015). However, kidney biopsy is the gold standard for definitive diagnosis of IgAN (Lai et al. 2016; Suzuki 2019). Renal biopsy is also essential for assessment of disease progression and prognosis in IgAN patients and thus helps in long term patient management (Suzuki 2019; Yuzawa et al. 2015).

The features of IgAN presented as seen by light microscopy of kidney biopsy specimens varies greatly among patients and even within individual samples (Wyatt and Julian 2013). The clinical presentation of IgAN in biopsy specimen ranges from mesangial cell proliferation (***Fig. 2a,b***) to crescentic lesions or advanced sclerosing appearance (Lai et al. 2016). The diagnostic hallmark of IgAN is presence of predominant IgA deposits (***Fig. 2c***) in the glomerular mesangium, with prevalent λ-light chains (Lai et al. 2016; Wyatt and Julian 2013). The IgA deposits are rarely monoclonal and often found in congregation with IgG, IgM or both (Lai et al. 2016; Wyatt and Julian 2013). In addition to IgA deposits, some other histopathological features are noted in the biopsy specimens from IgAN patients using light microscopy. Increase in the mesangial matrix along with hypercellularity are common findings. Glomerular lesions including focal necrosis, segmental scarring and crescents in the Bowman's capsule are also reported from IgAN renal biopsy specimens. Electron-dense material corresponding to the immune deposits are specifically located by immunofluorescence electron microscopy (Lai et al. 2016; Wyatt and Julian 2013). The deposits are commonly observed in the mesangium, adjacent to activated mesangial cells and occasionally in subendothelial areas of glomerular basement membranes associated

with proliferative lesions (***Fig. 2d***) (Lai et al. 2016; Wyatt and Julian 2013). In rare occasions, subepithelial deposits are also observed (Lai et al. 2016). Immune deposits are observed in other glomerular diseases such as lupus nephritis and Henoch-Schönlein purpura (Lai et al. 2016). Lupus nephritis and IgAN can be distinguished from each other using staining for the immunoglobulins (IgA, IgG and IgM) and staining for classic complement pathway proteins (C1q and C3) present in deposits, tubule reticular aggregates (interferon fingerprints) and the patient's clinical history (Lai et al. 2016). Renal pathology of Henoch–Schönlein purpura cannot be distinguished from IgAN except for a greater propensity to produce necrotizing crescentic glomerulonephritis (Lai et al. 2016).

Despite the effectiveness in IgAN diagnosis, renal biopsy is not recommended by many clinical guidelines for patients with mild proteinuria or isolated hematuria which may in turn lead to poor prognosis of IgAN in the long run. Other reasons of infrequent renal biopsies may stem from procedural risk of this invasive tool or limitations of health insurance coverage (Suzuki 2019). The histopathological findings from renal biopsy is time point dependent and may be inconclusive when performed during the early stages of IgAN (Chauveau and Droz 1993; D'Amico 2004; Suzuki 2019). This could lead to poor prognosis in chronic IgAN patients (Suzuki 2019). 30%-40% of the IgAN patients with mild proteinuria and mild histological lesions at the time of kidney biopsy exhibit disease progression marked by rapidly progressing proteinuria (Donadio and Grande 2002; Imai and Miura 2012; Suzuki 2019; Szeto et al. 2001). Therefore, noninvasive disease biomarkers for diagnosis and assessment of real-time disease progression is the need of the hour (Suzuki 2019).

f. *Classification*

There was a need for a global scoring system made up of limited number of pathologic parameters which is highly reproducible among pathologists, predictive of renal outcomes and which can be used to analyze and grade the kidney biopsy specimens for IgAN diagnosis. The Oxford classification is one of the first scoring system to receive a global consensus for it usage in IgAN diagnosis (Moriyama 2019). The Oxford Classification of IgAN consist of four pathologic parameters (MEST), which were independently associated with clinical outcome: mesangial proliferation (M), endocapillary proliferation (E), segmental sclerosis (S), and tubular atrophy and interstitial fibrosis (T)

Table 1. Updated Oxford Classification of IgAN and description of MEST-C criteria. M, Mesangial hypercellularity; C, crescents; E, endocapillary hypercellularity; s, segmental glomerulosclerosis; T, tubular, atrophy/interstitial fibrosis. (*Reprinted with permission from Springer Nature. Nature Reviews Nephrology. Markowitz, G. Updated Oxford Classification of IgA nephropathy: a new MEST-C score. Copyright © 2021 Springer Nature Limited. 2017)*

Histological variable	Definition	Score
Mesangial hypercellularity	More than four mesangial cells in any mesangial area of a glomerulus	• M0: <50% of glomeruli showing mesangial hypercellularity • M1: >50% of glomeruli showing mesangial hypercellularity
Endocapillary hypercellularity	Hypercellularity due to an increased number of cells within glomerular capillary lumina	• E0: no endocapillary hypercellularity • E1: any glomeruli showing endocapillary hypercellularity
Segmental glomerulosclerosis	Adhesion or sclerosis (obliteration of capillary lumina by matrix) in part but not the whole glomerular tuft	• S0: absent • S1: present in any glomeruli
Tubular atrophy/ interstitial fibrosis	Estimated percentage of cortical area showing tubular atrophy or interstitial fibrosis, whichever is greater	• T0: 0–25% of cortical area • T1: 26–50% of cortical area • T2: >50% of cortical area
Cellular or fibrocellular crescents	Percentage of glomeruli with cellular or fibrocellular crescents	• C0: absent • C1: 0–25% of glomeruli • C2: ≥25% of glomeruli

(Markowitz 2017; Rodrigues et al. 2017). Several studies have reported that pairing up

the MEST score with clinical data collected at the time of biopsy (proteinuria, creatinine, mean arterial pressure) significantly improved the predictive accuracy in identifying patients at higher risk of poor prognosis (Barbour et al. 2016; Rodrigues et al. 2017). The study that developed the Oxford Classification of IgAN system recruited patients with proteinuria ≥0.5 g/d, eGFR ≥ 30 ml/min/1.73 m^2 at renal biopsy, and at least 1 year of follow-up (Rodrigues et al. 2017). These criteria excluded patients with most rapidly progressing form of IgAN, which was characterized as crescentic forms of IgAN (Markowitz 2017). Crescents represent a severe form of renal injury in which cellular proliferation and infiltration of white blood cells lead to rupture of the glomerular basement membrane and successive extracapillary proliferation of parietal and visceral epithelial cells and intermixed mononuclear white blood cells within the Bowman's capsule (Markowitz 2017). This proliferation confined by the rounded contour of the Bowman capsule looks like a crescent moon (Markowitz 2017). Various studies emphasized on the importance of including crescent in the MEST score system. Following the results from a 2017 study, crescent (C) score was included in the Oxford Classification of IgAN and the updated classification system called MEST-C score system is presented with

Table 2. Japanese Histological Grading Classification. Active lesion (A) indicates cellular crescent and fibrocellular crescent; chronic lesion (C) indicates global sclerosis, segmental sclerosis, fibrous crescent. *(Reprinted from Tomino, Y. Diagnosis and treatment of patients with IgA nephropathy in Japan. Kidney Res Clin Pract. 2016;35(4):197-203. doi: 10.1016/j.krcp.2016.09.001 Published under the terms of CC BY-NC-ND 4.0.)*

Histological grade	No of lesions*/total no. of glomeruli	Active lesions only	Active lesion + chronic lesion	Chronic lesion only
H-grade I	0–24.9%	A	A/C	C
H-grade II	25–49.9%	A	A/C	C
H-grade III	50–74.9%	A	A/C	C
H-grade IV	75%	A	A/C	C

corresponding definitions of the parameters in ***Table 1***. In Japan, the third edition of the *Clinical Practice Guidelines for IgA Nephropathy* is also used for histological classification in addition to the Oxford Classification (***Table 2***) (Matsuo et al. 2011; Moriyama 2019; Tomino 2016; Yuzawa et al. 2015).

g. *Risk factors for IgA Nephropathy*

There are several risk factors associated with IgAN. Broadly these can be grouped into clinical findings and histological findings (Moriyama 2019). Risk factors associated with IgAN and ESRD are as follows (Moriyama 2019):

- Clinical findings
    - Deteriorated renal function
    - Higher amount of urinary protein excretion
    - Hypertension
    - Sex (male)
    - Age (older, younger)
    - Hematuria (mild, severe, without macrohematuria)
    - Higher uric acid
    - Lower serum albumin or total protein
    - Dyslipidemia (higher total cholesterol, LDL-cholesterol, and triglyceride)
    - Obesity
    - Higher serum IgA or IgA/C3
    - Anemia
- Histological findings
    - Active lesions
        - Cellular and fibrocellular crescent, endothelial hypercellularity, mesangial hypercellularity, tuft necrosis

- Chronic lesions
  - Global sclerosis, segmental sclerosis, fibrous crescent, glomerular tuft adhesion to Bowman's capsule, mesangial matrix increase, interstitial fibrosis and tubular atrophy
- Depositions
  - IgG, IgA, IgM, C3, C4d

h. *Epidemiology of IgA Nephropathy*

Prevalence of IgAN varies with geography. Biopsy registry and dialysis registries are two key sources used in the epidemiological studies of IgAN. According to biopsy and organ replacement registries, Pacific Asian regions have higher incidence of IgAN(Rodrigues et al. 2017). Compared to only 1.3% autopsies of the deceased in Finland, lanthanic mesangial IgA deposits were observed in 15.6% of deceased donors in Japan (Rodrigues et al. 2017; Suzuki et al. 2003; Varis et al. 1993). Using biopsy registry data to understand the disease distribution according to geography and ethnicity will not always paint the real picture due to differences in the clinical practices like routine screening program vs clinical referrals (Rodrigues et al. 2017). Australia is the country with highest incidence rate of IgAN (105 cases pmp/y) followed by Japan (39-45 cases pmp/y) (Schena and Nistor 2018). The prevalence of IgAN is often underestimated in low-income regions owing to differences in guidelines for performing kidney biopsies and access to quality health care (Lai et al. 2016). Efficiency of the health care system depends on the socioeconomic status. In developing countries, individuals with asymptomatic urinary abnormalities do not benefit from early referral to nephrologists since the public health care resources are largely dedicated to more severe clinical presentations such as nephrotic syndrome (Lai et al. 2016). On the other hand, in many developed countries in Asia, routine urine screening tests are conducted in schools which explains higher frequency of IgAN cases

in these countries (Cho et al. 2013; Imai et al. 2007; Lai et al. 2016). Additionally, in a country like Japan with rapidly decreasing number of children and young adults, IgAN, which more commonly affects these age groups, is a severe burden socially as well as economically(Schena and Nistor 2018).

Ethnicity is one of the factors affecting the prevalence of IgAN across the globe. Several GWAS on IgAN identified genetic susceptibility loci associated with the disease and the prevalence of these risk alleles varied with ethnicity, with highest frequency in people with East Asian origin (Lai et al. 2016; Sallustio et al. 2019). Furthermore, meta-analysis of GWAS reported a higher frequency of protective alleles in Europeans than in Asians, both in controls as well as IgAN patients (Yeo et al. 2019; Yeo et al. 2018b).

Prevalence of IgAN also varies with age. Studies have reported a higher incidence rate in children and young adults (less than 30 years old) (Lai et al. 2016; Pontier and Patel 1994; Yokoyama et al. 2012). However, this frequency may also be influenced by routine urinalysis which is more common for children and young adults as compared to the elderly (more than 65 years of age) (Lai et al. 2016). Earlier studies indicated a male predominance in IgAN patients of Caucasian origin (Schena 1990). Additionally, according to the 2005 nationwide survey of IgAN in Japan by Ministry of Health, Labor and Welfare of Japan, male gender is one of the predictive factors associated with IgAN prognosis (Tomino 2016).

i. *Treatment and therapeutic strategies*

Japan has an established system of annual health check-ups, especially for children and young adults, which include urinalysis screening test and therefore, a varied range of stages of IgAN is observed and are managed using wide variety of treatments in Japanese population (Matsuzaki et al. 2013).However, there are no specific treatment regime for IgAN owed to its complex nature of pathogenesis varying across geographical regions

(Selvaskandan et al. 2019). There is a marked heterogeneity in the IgAN management practices adopted internationally but they can be typically grouped into supportive management, immunosuppression and tonsillectomy (Selvaskandan et al. 2019). The Kidney Disease Improving Global Outcomes (KDIGO) guidelines list out treatment regimens for IgAN across the globe (KDIGO Clinical Practice Guideline for Glomerulonephritis Chapter 10: Immunoglobulin A nephropathy 2012) whereas clinical guidelines for IgAN (Version 3) are followed for IgAN management in Japan (Matsuo et al. 2011; Yuzawa et al. 2015). According to regional guidelines of Japan, corticosteroid therapy, steroid pulse therapy paired with tonsillectomy (TSP), non-steroidal immunosuppressants, antiplatelet agents, n-3 fatty acids (fish) oil are various types of therapeutic strategies recommended at different stages of IgAN progression (Matsuo et al. 2011; Yuzawa et al. 2015).

Conventional therapies and supportive management: Both the guidelines (KDIGO and Japanese guidelines) recommend the use of renin-angiotensin system (RAS) inhibitors, such as an angiotensin-converting enzyme inhibitor (ACE-I) or an angiotensin II-receptor blocker (ARB), for treating IgAN patients with proteinuria ≥ 1 g/day (Lai et al. 2016; Selvaskandan et al. 2019). A meta-analysis of 11 randomized control trial (RCT) reported that ACE-I/ARBs succeeded in reducing proteinuria, lowering blood pressure and subsequently reducing the renal function decline rate (Selvaskandan et al. 2019). Interestingly, this observation still held true when 6 out of the 11 studies were removed from meta-analysis due to low Jadad score (Cheng et al. 2009; Selvaskandan et al. 2019). Studies suggest the combination of ACE-I and ARB for greater benefits (greater reduction in urinary protein levels, blood pressure levels and thus, better kidney outcome) in adult as well pediatric IgAN patients (KDIGO Clinical Practice Guideline for Glomerulonephritis Chapter 10: Immunoglobulin A nephropathy 2012; Russo et al. 1999; Yang et al. 2005).

It has also been noted that sodium restriction enhances the antiproteinuric effects of RAS-I in IgAN management (Selvaskandan et al. 2019; Suzuki et al. 2009).A small cross-over study reported that low-sodium diets reduced levels of urinary proteins even in IgAN patients with normal blood pressure (Konishi et al. 2001; Selvaskandan et al. 2019). Japanese guidelines recommend limiting salt intake to 3-6g/day in order to lower risk of cardiovascular disease and progression to ESRD in IgAN patients (Yuzawa et al. 2015). A RCT noted that weight loss led to decrease in proteinuria in overweight primary IgAN patients (Kittiskulnam et al. 2014). Another study noted that overweight/obese patients were more likely to have high blood pressure, worse eGFR, proteinuria and greater prevalence of CKD stage 3 or higher (Rodrigues et al. 2017). Therefore, Japanese guidelines strongly recommends IgAN patients with BMI ≥ 25 to lose weight (Yuzawa et al. 2015). Studies have identified smoking as one of the risk factors in IgAN disease progression (Selvaskandan et al. 2019; Yamamoto et al. 2010). Additionally, in study cohorts across the globe, current and past smoking history is associated with renal dysfunction, increased proteinuria and albuminuria in IgAN patients (Yuzawa et al. 2015). Furthermore, smoking is also a consistent risk factor for upper respiratory tract diseases such as chronic obstructive disease (COPD), lung cancer, and cardiovascular ailments (Yuzawa et al. 2015). Given these evidences, Japanese guidelines strongly recommends IgAN patients to stop smoking (Yuzawa et al. 2015). Fish oil is rich in omega 3 fatty acids which have anti-inflammatory properties. It is hypothesized that fish oil may reduce inflammations in the kidney and thus provide a supportive care when used in combination of other therapies like RAS-I (Lai et al. 2016). Despite weak scientific evidences in support of beneficiary effects of fish oil in IgAN patients, both KDIGO and Japanese guidelines suggests its use in treating IgAN patients, especially those with persistent proteinuria even after optimized supportive management of the disease (KDIGO Clinical Practice Guideline

for Glomerulonephritis Chapter 10: Immunoglobulin A nephropathy 2012; Selvaskandan et al. 2019; Yuzawa et al. 2015).

Immunosuppressive therapy: Various types of immunosuppressive agents are often prescribed to IgAN patients such as corticosteroids, cyclophosphamide, azathioprine and mycophenolate mofetil (Selvaskandan et al. 2019). Both KDIGO and Japanese guidelines suggest a treatment course of corticosteroids however the patients' criteria is different for both guidelines. KDIGO suggests a 6-month long steroid therapy course for IgAN patients with persistent proteinuria of more than 1g/day and eGFR of more than 50 ml/min/1.73m^2 despite adequate ACEI or ARB usage and control for hypertension (KDIGO Clinical Practice Guideline for Glomerulonephritis Chapter 10: Immunoglobulin A nephropathy 2012; Lai et al. 2016). Japanese guidelines recommend steroid therapy to IgAN patients with urinary protein more than 1g/day and eGFR more than 60ml min/1.73m^2 despite supportive care (Lai et al. 2016). However, the use of corticosteroids for IgAN treatment often comes at the expense of more adverse events such as infections, weight gain, and disturbances in the glucose metabolism (Feehally 2017; Rauen et al. 2018; Selvaskandan et al. 2019). This was noted by STOP-IgAN study which also reported that higher rate of clinical remission is associated with corticosteroid therapy compared to supportive therapy (Feehally 2017; Rauen et al. 2018; Selvaskandan et al. 2019). Findings of RCTs suggest that corticosteroid intervention for a 6 month period may have a "legacy effect" with sustained benefits (like decline in the risk of progressive renal dysfunction) even after cessation of the treatment in the IgAN patients (Pozzi et al. 2004; Pozzi et al. 1999; Rodrigues et al. 2017).

Tonsillectomy or Tonsillectomy with steroid-pulse therapy (TSP): Tonsillectomy is more commonly recommended by nephrologists in Asia as compared to Europe (Selvaskandan et al. 2019) Studies from Asian cohorts suggests an association between

reduction in urinary protein levels and tonsillectomy and highest benefits were noted when tonsillectomy was paired with corticosteroids (Kawamura et al. 2014; Komatsu et al. 2008; Matsumoto et al. 2018; Selvaskandan et al. 2019; Xie et al. 2003b). Retrospective studies on European cohorts found no benefit associated with tonsillectomy in IgAN patients (Selvaskandan et al. 2019). Given the lack of randomized trials about tonsillectomy of IgAN patients , KDIGO does not recommend tonsillectomy or TSP for IgAN management (KDIGO Clinical Practice Guideline for Glomerulonephritis Chapter 10: Immunoglobulin A nephropathy 2012). On the other hand, Japanese guidelines suggest tonsillectomy in the early stages of the disease irrespective of medical history of tonsilitis in the IgAN patient (Selvaskandan et al. 2019; Yuzawa et al. 2015). According to immunological studies, production of Gd-IgA1 is induced by mucosal infections and microbial antigen present in the tonsillar crypts are often associated with remission of IgAN after kidney transplantation (Lai et al. 2016). Thus by affecting the Hit 1 (production of Gd-IgA1) in IgAN pathogenesis model, tonsillectomy derails renal dysfunction which can be noted by improvement in urinary findings (Yuzawa et al. 2015).

1.2 Overview of human salivary microbiome

a. Definition

Human body is home to trillions of microorganisms and genetic pool of all these microbes put together is known as human microbiome (Turnbaugh et al. 2007). The oral microbiome is defined as a collective of complex and diverse microbial communities living in the human oral cavity (Acharya et al. 2017a). According to human oral microbiome database (HOMD), the oral microbiome is one of the most diverse community of the human body and contains about 500-700 bacterial species (Acharya et al. 2017a; Chen et al. 2010; Huttenhower et al. 2012; Shaw et al. 2017; Wade 2013).

Saliva is sterile at the time of secretion from salivary glands into the oral cavity (Belstrom 2020). Salivary microbiome is the conglomeration of genetic material of the microbes that shed into the saliva from adherent epithelial cells of oral mucosa, dental plaque, tongue, throat and other distinct ecological niches of the oral cavity (Dawes 2003). Therefore, characterizing salivary microbiome will provide a deeper understanding of the workings of oral microbiome (Belstrom 2020; Shaw et al. 2017; Takeshita et al. 2016).

b. *Role of salivary microbiome in human health and disease*

Unlike gut microbiome, salivary microbiome composition is largely consistent with little variations across the globe (Nasidze et al. 2009; Shaw et al. 2017). The salivary microbiome structure established early in life is able to persist for several years (Shaw et al. 2017). Environmental factors such as lifestyle practices and living space also impacts the composition of salivary microbiome. For example, salivary microbiome of twins became increasingly less similar as they grew from babies to adults and lived in separate households (Stahringer et al. 2012). Instead of genetic makeup, cohabiting is one of the dominant factors that affect both salivary as well as gut microbiome composition (Shaw et al. 2017). This may help unravel the familial aggregation of diseases such as IBD (Nunes et al. 2011; Shaw et al. 2017) or CKD (Carlassara et al. 2021) that have an environmental component. Climate and diet are other environmental factors that affect the salivary microbiome composition (Belstrom 2020; De Filippis et al. 2014; Lassalle et al. 2018; Li et al. 2014a; Shaw et al. 2017). The diet patterns also affects the functionality of salivary microbiome (Lu et al. 2019). Salivary microbiota of tobacco smokers shifted from before drinking green tea to after drinking green tea and was marked by shifts in abundance of *Streptococcus* and *Staphylococcus.* The study inferred from these findings that salivary microbiota is involved in processing food extracts and affect carcinogenesis (Adami et al. 2018; Lu et al. 2019). Oral cavity is subject to frequent internal and external

perturbations as compared the gut mucosa (Marsh 2018). These ecological perturbations have made the salivary microbiome resilient to stress such as antibiotic therapy (Rosier et al. 2018; Shaw et al. 2017; Zaura et al. 2015).

Early life development of salivary microbiome is influenced by ecological factors such as mode of delivery, duration of breastfeeding and antibiotic therapy and changes in the colonization pattern during this developmental stage (1st year of a baby's life) may have long-term consequences for oral and systemic health (Belstrom 2020; Dzidic et al. 2018). Salivary microbiome composition is also affected by age, with the most active window for increase in species richness being from 6 to 18 months (Lif Holgerson et al. 2020). Interestingly, individuality in the salivary microbiota transformation materializes within the first few months of life(Lif Holgerson et al. 2020). Maturation and final homeostasis of salivary microbiome was influenced by determinants such as socio-economic conditions, environmental and cultural factors, host biology and genetics(Lif Holgerson et al. 2020). The salivary microbiome attains diversity within 2 days after birth and remains in a continuous variable form up until 5 years (Lif Holgerson et al. 2020), and these can be paralleled with the rapid developmental changes as a baby grows to become a child. The salivary microbiome structure may also be affected by individual's body-size and gender (Raju et al. 2019). The successive organization of the salivary microbiome is niche dependent and distinct in health and disease (Zenobia et al. 2021). Mucins and proline rich proteins in the saliva helps in biofilm formation as well as clearance of bacteria from oral surfaces (Freire et al. 2021; Zenobia et al. 2021). Initially the microbes interact with each other using quorum sensing pathways and adhere to the hard and soft tissue surfaces in the oral cavity. However host factors like cytokines regulate the biofilm formation to establish immune homeostasis(Zenobia et al. 2021).

1.3 Salivary microbiome and systemic diseases

Salivary microbiota is closely associated with and sensitive to host physiological state. For example, circadian oscillations are observed in salivary microbiome which is correlated with the host circadian rhythm (Takayasu et al. 2017). Dysbiosis of salivary microbiome are linked with various oral and systemic diseases. The nature of dysbiosis in salivary microbiome is different in diseases involving the immune system, owing to the connection between immune homeostasis and microbiome (Acharya et al. 2017a). Chronic inflammatory disease states are correlated with burst of salivary opportunistic Grammaproteobacteria (Acharya et al. 2017a; Acharya et al. 2017b; Diaz et al. 2013; Schuurhuis et al. 2016). In cirrhosis patients with hepatic encephalopathy, salivary *Enterobacteriaceae* are negatively associated with anti-inflammatory cytokine IL-10 (Bajaj et al. 2015), whereas it is positively associated with proinflammatory IL-1β in healthy individuals (Acharya et al. 2017b). HIV disease status is associated with *Haemophilus, Porphyromonas, Treponema*, and *Eubacterium* (Kistler et al. 2015). Furthermore, there was a shift in salivary microbiome in HIV patients who underwent retroviral therapy, presented by increase in *Prevotella, Fusobacterium, Capnocytophaga, Campylobacter* and simultaneous decrease in *Aggregatibacter* (Acharya et al. 2017a; Li et al. 2014b). Increase in abundance of *Prevotella* is associated with pro-inflammatory Th-17 responses in chronic inflammatory disease cohort (Larsen 2017), IL-1β activity in healthy individuals (Acharya et al. 2017b) as well as in IBD patients (Said et al. 2014).

On other end of immunological disease spectrum, similar patterns of dysbiosis in salivary microbiome is noted in autoimmune diseases (Acharya et al. 2017a). *Lactobacillus salivarius* is enriched in the rheumatoid arthritis patients (Zhang et al. 2015); similarly, higher abundance of *Lactobacillus* was noted in celiac disease (Tian et al. 2017).

From these findings we can infer that there is an intricate balance between host immune system and salivary microbiome. Therefore, characterizing the salivary microbiome specific to diseases may help in deeper understanding of disease pathogenesis and progression.

1.4 Motivation, objectives and hypotheses

a. *Motivation*

With rapidly aging population and ever rising numbers of chronic dialysis patients (Masakane et al. 2018), IgAN is a major health burden upon the Japanese population. A confirmed diagnosis of the disease is obtained only after a highly-invasive procedure of renal biopsy (Tomino 2016). Keeping in mind the risk of performing a renal biopsy in the population such as the elderly, we understood the need to expedite the development of a non-invasive and efficient tool for IgAN diagnosis.

Several genome-wide association studies (GWAS) on large cohorts reported that genetics only account for about 5% of the disease risk in IgAN (Sallustio et al. 2019). Thus, as we mentioned in section 1.1, various other factors are associated with the pathogenesis and maintenance of immune reactions in IgAN (Sallustio et al. 2019). IgAN patients often present gross hematuria in association with upper respiratory tract infections or gastrointestinal infections (Haas 2007; Park et al. 2020; Wyatt and Julian 2013; Wyatt et al. 1995). Additionally, GWAS studies of IgAN patients identified several significant loci associated with the maintenance and functioning of the mucosal barrier (Kiryluk et al. 2014; Park et al. 2020). These findings indicate that mucosal immune system plays an important role in the IgAN pathogenesis (Sallustio et al. 2019).

Given the co-evolution of the immune system and commensal microbiota, especially at the mucosal surfaces, microbiota is linked with maintenance of immune homeostasis and defense against pathogens (Bain and Cerovic 2020). The mucus layer

provides nutrients and a suitable platform for growth and sustenance of the commensal microbes, which in turn is the foundation of host-microbiota relationships (Bäckhed et al. 2005; Li et al. 2015; Martin et al. 2012; Nakajima et al. 2018). This symbiotic relationship is clearly understood if we focus on the interactions between commensal microbiota and mucosal-associated lymphoid tissue (MALT). The microbiota is essential for the development of the MALT and in turn, MALT regulates the microbiota composition of mucosal surfaces like gut or oral cavity (Nakajima et al. 2018; Sallustio et al. 2019). IgA and more specifically, secretory IgA that originates from MALT plays an important role in maintaining the homeostasis at the mucosal surfaces(Li et al. 2020; Sallustio et al. 2019) . Studies have also reported that IgA secretion is induced and regulated by the commensal microbes (Macpherson and Harris 2004; Pabst et al. 2016). In humans, mucosal layer starts at the oral cavity and the inhabitants of oral mucosa, the salivary microbiome consisting of more than 200 species (Krishnan et al. 2017), plays indispensable roles in regulating and maintaining the mucosal immunity (Moutsopoulos and Konkel 2018). Changes in salivary microbiome in terms of composition and diversity reflects inflammatory responses and microbial dysbiosis in the gut (Bajaj et al. 2015; Abe et al. 2018). Several studies have also noted that salivary microbiome is a potential source of diagnostic biomarkers for several immunological diseases such as rheumatoid arthritis (Chen et al. 2018), celiac disease (Francavilla et al. 2014), primary sclerosing cholangitis (Iwasawa et al. 2018), colorectal cancer (Flemer et al. 2018), pancreatic cancer (Coit et al. 2016; Torres et al. 2015) and CKD (Hu et al. 2018). Additionally, the simplicity and inexpensiveness of the sample collection procedure paired with its low risk factor are advantageous for profiling salivary microbiome of IgAN patients to discover microbial biomarkers for IgAN diagnosis (Hemadi et al. 2017).

According to current treatment guidelines for the early-stage primary IgAN patients in Japan, tonsillectomy monotherapy or tonsillectomy paired with steroid pulse therapy are the recommended therapeutic regimens (Nihei et al. 2017; Tomino 2016; Xie et al. 2003a). The efficacy of these treatments can be attributed to the underlying immune reactions connecting tonsils with IgAN pathogenesis. As mentioned earlier, macroscopic hematuria in IgAN patients often coincides with upper respiratory tract infections, especially, tonsilitis (Park et al. 2020; Wang et al. 2012; Wyatt and Julian 2013). It was also noted that in the tonsils of the IgAN patients, there was a marked increase in polymeric IgA (pIgA) producing B-cells (Harper et al. 1995; Kodama et al. 2001; Meng et al. 2012) which are the flag bearers of IgAN pathogenesis. Additionally, pathogenic bacteria associated with chronic tonsilitis (CT) and periodontitis were also found at the tonsillar crypts of IgAN patients (Jensen et al. 2013; Nagasawa et al. 2014; Watanabe et al. 2017). However, despite these evidences indicating an underlying connection between CT and IgAN pathogenesis, there has been no studies exploring the salivary microbiome associated with these diseases in comparison with each other.

A study using transgenic mouse model of IgAN (designed to overexpress BAFF, a B-cell activation factor of the tumor necrosis factor (TNF) super family) revealed that IgA deposition in the kidney glomeruli was regulated by commensal microbiota (McCarthy et al. 2011). Inflammation in the gut and dysbiosis of gut microbiota is often considered as a source of inflammation in CKD (Han et al. 2016; Lau et al. 2015). In case of IgAN, the GWAS-identified risk loci for IgAN overlapped with those of IBD (Han et al. 2016; Sallustio et al. 2019). Swedish cohort-based study indicates that IgAN patients are at greater risk to incur IBD (Rehnberg et al. 2021). Based on these observation and hypotheses, several studies across Caucasian, Chinese and Korean populations attempted at exploring microbiome associated with IgAN. As expected, these studies highlighted a dysbiosis in

the gut(De Angelis et al. 2014; Dong et al. 2020; Hu et al. 2020), salivary(He et al. 2021b; Luan et al. 2019; Piccolo et al. 2015), periodontal (Cao et al. 2018) and tonsillar (Park et al. 2020) microbial communities. However, there has been no study till date that has compared the microbiome dysbiosis in IgAN with that in the IBD.

b. *Objectives and hypotheses*

Based on these motivations and identified gap in the literature, in this research project we used 16S rRNA gene sequence-based analysis to

I. Compare the salivary microbiome composition of Japanese IgAN patients and age-, gender-matched Japanese healthy controls (HC).

II. Compare the salivary microbiome composition of the Japanese IgAN patients and Japanese chronic tonsilitis (CT) patients.

III. Compare the salivary microbiome composition of the Japanese IgAN patients and Japanese ulcerative colitis (UC) patients.

Depending on these objectives, we hypothesized that the salivary microbiome of IgAN differed from the HC in terms of the composition and diversity. Similarly, we hypothesized that the salivary microbiome of IgAN also differed from the CT and UC patients in terms of the above-mentioned criteria. And lastly, we hypothesized that the salivary microbiome of the IgAN patients is a source of potential microbial biomarkers that can differentiate the IgAN from the HC as well as the CT and UC samples. In this thesis, Chapter 2 addresses with first objective and Chapter 3 addresses all the three objectives together.

2 Comparison of Salivary Microbiome of Japanese IgA Nephropathy Patients and Healthy Controls

Comparison of Salivary Microbiome of Japanese IgA Nephropathy Patients and Healthy Controls

Abstract

Background: With an incidence rate of 39-45 immunoglobulin A nephropathy (IgAN) cases per million population per year, Japan is ranked second in the world for IgAN prevalence. Being an idiopathic disease, IgAN is poorly understood and the development of diagnostic methods independent of renal biopsy had been slow. Genome wide association studies have identified several risk loci in IgAN pathogenesis to be associated with regulation of mucosal immunity. Salivary microbiome is a key player in the maintenance of immune homeostasis. In recent years, dysbiosis of salivary microbiome has been associated with oral as well as systemic diseases like arthritis, autism spectrum disorder, cancer, inflammatory bowel disease, and chronic kidney disease. This study aimed to investigate the salivary microbiome profile of IgAN patients in comparison with age-, gender-matched healthy controls (HC) in Japanese population.

Methods: Saliva was collected from 43 IgAN patients and 50 age- and gender matched HC. The hypervariable V1–V2 regions of 16S rRNA gene were purified from all the samples and amplified using 27Fmod and 338R primers. The salivary microbial profile of IgAN and HC samples were investigated by using high-throughput 454-pyrosequencing technology and bioinformatic analyses pipelines. Statistical analyses were conducted on R software. We used Linear discriminant analysis effect size (LEfSe) tool and R-based Area under curve-random forest (AUC-RF) package to identify microbial biomarkers for differentiating IgAN from HC.

Results: Alpha diversity parameters for richness and diversity (Observed Operational Taxonomic Units, Chao1, Shannon) were significantly lower in the IgAN than HC

samples ($p<0.05$, wilcoxon test). At the phylum level, Bacteroidetes significantly decreased whereas Proteobacteria significantly increased in abundance in IgAN samples compared with HC samples. Using LEfSe followed by AUC-RF, we were able to identify a group of 7 genera (*Staphylococcus, Prevotella, Neisseria, Peptostreptococcus, Corynebacterium, Stomatobaculum* and *Veillonella*) to differentiate IgAN from HC, with area under curve of 0.90. Additionally, there was a significant difference between IgAN male and HC male samples in terms of the microbiome structure and composition. A similar difference was also observed between IgAN female and HC female samples.

Conclusion:

There is a significant dysbiosis in salivary microbiome of IgAN patients. Salivary microbiome is potential source of biomarkers for development of effective and non-invasive diagnostic tool for IgAN diagnosis and association of microbiome and gender in the context of IgAN patients should be explored in future studies. To our best knowledge, this is the first report of salivary microbiome profile of Japanese IgAN patients.

Keywords: salivary microbiome; IgA nephropathy; oral microbiota; kidney disease; random forest algorithm.

2.1 Introduction

Oral mucosal barrier is the site of first encounters with a cornucopia of immunological triggers such as transient or commensal microbiota, dietary or airborne antigens and food (Moutsopoulos and Konkel 2018). Therefore, homeostasis of the oral mucosa is vital for maintenance of human health (Wilharm et al. 2019). At the barrier sites, one of the vital tasks of the local immune system is to balance between effective immune surveillance without spiking inflammatory response and tolerate local resident

microbiota (Moutsopoulos and Konkel 2018). Therefore, the oral epithelial cells and oral microbiota are in a continuous flux with each other to regulate the local and systemic homeostasis (Wilharm et al. 2019). Oral microbiome consists of diverse community of microbiota, compared to other barrier sites (Huttenhower et al. 2012; Moutsopoulos and Konkel 2018). The human microbiome is closely associated with mucosal immunity including Th-17 response and mucosal IgA-regulated immunity (Zenobia et al. 2021). Oral cavity is often overlooked in terms of mucosal immunity; however, it houses the oral microbiome which interacts with the immune landscape and regulates local and systemic homeostasis (Wilharm et al. 2019; Zenobia et al. 2021). The salivary microbiome is considered as the representative of the oral microbiome as the microbiota adhering to niches on various intraoral surfaces sheds into the saliva (Duan et al. 2020). Changes in salivary microbiome in terms of composition and diversity reflects inflammatory responses and microbial dysbiosis in the gut (Bajaj et al. 2015; Abe et al. 2018). Several studies have also noted that salivary microbiome is a potential source of diagnostic biomarkers for several immunological diseases such as rheumatoid arthritis (Chen et al. 2018), celiac disease (Francavilla et al. 2014), primary sclerosing cholangitis (Iwasawa et al. 2018), colorectal cancer (Flemer et al. 2018), pancreatic cancer (Coit et al. 2016; Torres et al. 2015) and CKD (Hu et al. 2018). Additionally, the simplicity and inexpensiveness of the sample collection procedure paired with its low risk factor are advantageous for profiling salivary microbiome(Hemadi et al. 2017).

IgAN patients often present gross hematuria in association with upper respiratory tract infections or gastrointestinal infections (Haas 2007; Park et al. 2020; Wyatt and Julian 2013; Wyatt et al. 1995). Additionally, GWAS studies of IgAN patients identified several significant loci associated with the maintenance and functioning of the mucosal barrier (Kiryluk et al. 2014; Park et al. 2020). These findings indicate that mucosal

immune system plays an important role in the IgAN pathogenesis (Sallustio et al. 2019). A study using transgenic mouse model of IgAN (designed to overexpress BAFF, a B-cell activation factor of the tumor necrosis factor (TNF) super family) revealed that IgA deposition in the kidney glomeruli was regulated by commensal microbiota (McCarthy et al. 2011). Inflammation in the gut and dysbiosis of gut microbiota is often considered as a source of inflammation in CKD (Han et al. 2016; Lau et al. 2015). Recent studies reported dysbiosis in salivary microbiota in CKD patients(Hu et al. 2018). Studies also identified a rise in number of periodontal pathogens in ESRD patients(Araújo et al. 2015; Schmalz et al. 2016) and a decline in oral health along with salivary microbiome dysbiosis in ESRD patients undergoing hemodialysis (Duan et al. 2020). With this background, several studies across the world attempted at exploring microbiome associated with IgAN. As expected, these studies highlighted a dysbiosis in the gut(De Angelis et al. 2014; Dong et al. 2020; Hu et al. 2020), salivary(He et al. 2021b; Luan et al. 2019; Piccolo et al. 2015), periodontal (Cao et al. 2018) and tonsillar (Park et al. 2020) microbial communities. Two Japanese cohort studies focused on exploring tonsil microbiome of IgAN patients in comparison with recurrent tonsilitis, tonsillar hyperplasia (Watanabe et al. 2017) and chronic tonsilitis(Nagasawa et al. 2014). However, there has been no study till date that explored the salivary microbiome profile of IgAN patients in comparison with healthy individuals in Japanese population.

In the current study, we performed 16S rRNA gene sequence-based analysis of the salivary microbiome of IgAN and healthy subjects in a Japanese cohort. Using random forest models, we identified a set of potential microbial biomarkers that can differentiate IgAN from other diseases and healthy individuals. According to the Ministry of Health, Labor, and Welfare 2005 survey in Japan, the male gender is one of the predictive factors of IgAN (Tomino 2016). Thus, we also explored the gender-specific

association of the salivary microbiome for IgAN patients by grouping the dataset based on genders prior to analysis. We found a significant difference in the salivary microbiome structure between IgAN and healthy subjects based on gender-grouping.

2.2 Methods

a. Sample collection and DNA extraction

The study was approved by the ethics committees of Azabu University (029, 14 March 2013) and RIKEN (H30-4, 29 August 2019). Saliva samples were collected from 43

Table 3. Study subject demographics of IgAN and HC group

Demography	**IgAN (n=43)**	**HC (n=50)**
Age, years, median (IQR)	39(20.5)	37.5(8)
Male	20	36
Female	23	14

Median age in terms of years is shown for each group along with IQRin parentheses. Small IQR values represent data points that are spreadcloser to the median. IgAN, Immunoglobulin A nephropathy; CT, chronic tonsillitis; UC, ulcerative colitis; HC, healthy control; IQR, interquartile range.

IgAN patients (median age 39) and 50 age and sex-matched healthy controls (the HC , median age 37.5) were recruited for this study (**Table *3***). IgAN patients collected their saliva prior to their tonsillectomy surgery. Any subject with history of oral disease, gastrointestinal or hepatobiliary surgery and antimicrobial usage within the past three months of the recruitment date were excluded from the study. Informed consents were obtained from the subjects before sampling and the study participants were instructed

to restrain from eating or drinking for 2 hours prior to sampling. The saliva samples were transported to the laboratory at 4°C within 24 hours of collection. Upon reaching the laboratory, the samples were immediately frozen using liquid nitrogen and stored at -80°C until further downstream analysis.

The DNA extraction from these salivary samples was performed as described previously (Morita et al. 2007; Said et al. 2014). 1ml of saliva was centrifuged at 3,300g for 10 mins at 4°C. The resulting bacterial cell pellets were then suspended in 10mM Tris–HCl/10mM EDTA buffer and incubated with 15mg/ml lysozyme (Sigma-Aldrich Co. LLC) for 1 h at 37°C. Purified achromopeptidase (Wako Pure Chemical Industries, Ltd.) was added to the samples at a final concentration of 2000 units/ml before incubating the samples for an additional 30 mins at 37°C. The suspension was treated with 1% (wt/vol) sodium dodecyl sulphate (SDS) and 1 mg/ml proteinase K (Merck Japan) and incubated for 1h at 55°C. The resultant lysate was treated with phenol: chloroform: isoamyl alcohol (25:24:1) (Life Technologies Japan, Ltd.) and centrifuged at 3,300g for 10mins at 4°C. To precipitate the microbial DNA, 1/10 volume of 3M sodium acetate (pH= 4.5) and 2 volumes ethanol (Wako Pure Chemical Industries, Ltd.) was added to the supernatant. The suspension was centrifuged at 3,300g for 15mins at 4°C and the resulting DNA pellets were rinsed with 75% ethanol, dried and dissolved in 10mM Tris–HCl/1mM EDTA (TE) buffer. For purification, the DNA samples were treated with 1 mg/ml RNase A (Wako Pure Chemical Industries, Ltd.) at 37°C for 30 min and precipitated by adding equal volumes of 20% polyethylene glycol solution (PEG6000-2.5MNaCl). These were centrifuged at 8,060g at 4°C and double-rinsed with 75% ethanol before drying the pellets. The dried pellets were then dissolved in TE buffer and stored at -20°C until polymerase chain reaction (PCR) steps followed by sequencing.

b. 16S rRNA amplicon library preparation and sequencing

The salivary microbial DNA was sequenced using the 454 GS FLX Titanium or 454 GS Junior system (Roche Applied Science, Indianapolis, IN) according to the manufacturer's instructions (Said et al. 2014; Tsuda et al. 2015). Prior to the sequencing the 16S rRNA amplicon libraries were prepared by PCR amplification of the V1-V2 region of the 16S rRNA gene. For the PCR amplification, barcoded 27Fmod (5′-AGRGTTTGATYMTGGCTCAG-3′) and 338R (5′-TGCTGCCTCCCGTAGGAGT-3′) primers were used(Kim et al. 2013).

The protocol for PCR amplification was same as previously described (Tsuda et al. 2015). Briefly, 50 µl of PCR mix is composed of 10mM Tris-HCl (pH 8.3), 50mM KCl, and 1.5mM MgCl2 in the presence of 250 µM dNTP, 1 U Ex Taq polymerase (TakaraBio, Kyoto, Japan), forward and reverse primers (0.2 µM) and ~ 20 ng template DNA. At the thermal cycler, the PCR protocol consisted of initial denaturation at 96 °C for 2 min, followed by 25 cycles of denaturation at 96 °C for 30 s, annealing at 55 °C for 45 s, and extension at 72 °C for 1 min, a final extension at 72 °C and hold at 4 °C on a 9700 PCR system (Life Technologies Japan, Tokyo, Japan).

The resultant PCR amplicons were then purified by AMPure XP magnetic purification beads (Beckman Coulter, Brea, CA, USA) and quantification using the Quant-iT PicoGreen dsDNA Assay Kit (Life Technologies Japan). Equal amounts of the PCR amplicons were pooled and used for the 16S rRNA sequencing.

c. Data analysis

The raw sequences were analyzed using a predesigned pipeline for analysis of 454 pyrosequencing data of the 16S rRNA gene V1-V2 region, as reported previously(Kim et al. 2013). Reads lacking both universal primers, low-quality reads (with an average quality score ≤ 25) and possible chimeric reads together accounted for 44-46% of total

reads and were excluded from the downstream analysis (***Table 4***). After filtering, 2300 reads per sample were chosen randomly from the remaining high-quality reads for further analysis. The selected reads were sorted on the basis of their average quality score and grouped into OTUs using the UCLUST algorithm with 96% identity as threshold (Said et al. 2014). We conducted taxonomic assignments for each OTU by

Table 4. Statistics of 16S V1-V2 pyrosequencing of salivary microbiome samples of IgAN and HC groups

Total Reads	Filter-Passed Reads	Reads Removed		
		Reads lacking primer sequences	Reads with average Qv<25	Possible chimeric reads
1124798(100)	619041(55)	499955(44.4)	2258(0.2)	7864(0.7)

Note: The number in the parentheses indicate the ratio in percentage to the total read number.

similarity searching against RDP, CORE and NCBI genome databases using the GLSEARCH program. For taxonomic assignments at the phylum, genus and species levels, 70%, 94% and 96% sequence similarity thresholds were used, respectively.

We used Chi-squared test on the gender data across groups for gender-matching the samples. Similarly, for age-matching, we used the one-way ANOVA test on the demographic data of the samples. Beta-diversity was assessed by using the UniFrac distance metric (Lozupone et al. 2011) followed by the principal coordinate analysis (PCoA) to visualize the overall microbiome structure of the saliva samples. We used The permutational multivariate analysis of variance (PERMANOVA) to find the significance of beta-diversity between the groups, and the corresponding p-values

were adjusted for multiple testing using Benjamin-Hochberg (BH) correction method. We assessed within-sample diversity or alpha diversity in terms of microbial richness and microbial diversity. In this assessment, the observed OTU number and Chao1 index were the measures to quantify microbial richness whereas Shannon's index quantified the microbial diversity of the samples. To check the statistical significance of the relative abundance of individual taxa, we used the Wilcoxon-test with BH correction for multiple comparisons for the data at different taxonomic levels, namely, OTU, phylum, and genus.

This protocol was also used for revealing gender-specific microbiome association in the study groups. Briefly, we first grouped the complete dataset into male and female sample. UniFrac weighted and unweighted metrics corresponding to this dataset was assessed using PERMANOVA in order to evaluate existence of gender-effect. Statistically significant groups (p-value < 0.05, Wilcoxon test) were further investigated in terms of microbial diversity as well as taxonomic profile of microbiome, specific to male and female samples in the groups.

To identify differentially abundant taxa between IgAN and HC groups at the genus and OTU levels, we used Linear Discriminate Analysis (LDA) effect size (LEfSe) tool (http://huttenhower.sph.harvard.edu/lefse/) on the galaxy server (https://huttenhower.sph.harvard.edu/galaxy/). LEfSe identifies the features (in our case, taxa) which are most likely to explain differences between the groups in a dataset. For this LEfSe uses the non-parametric Kruskal-Wallis test and unpaired Wilcoxon rank sum test to evaluate the statistical significance of the features and pairs this with LDA method to estimate the effect relevance of each feature/taxa among the groups (Segata et al. 2011). For this study, LEfSe analysis was carried out with default parameters, i.e., alpha value was set to 0.05 for statistical analyses, non-negative

threshold for the logarithmic LDA score was set to 2 and strategy for multi-class analysis was set to all-against-all (more stringent).

To identify a group of taxa among the selected biomarkers from the previous step (LEfSe analysis) which can act as potential biomarkers and classify IgAN from HC group, we used Area Under Curve-Random Forest (AUC-RF) (version 1.1) (Calle et al. 2011) package. To generate random forest (RF) models using AUC-RF we used the protocol previously described (Iwasawa et al. 2018). Brielfy, RF was built, using all the variables selected by LEfSe tool and here the variables are ranked in terms of their importance (which is depicted by the mean decrease Gini score). Subsequently, 5% of the least important features were eliminated and RF was built with the remaining variables. The area under curve (AUC) was calculated for each of the RF models and was based on the out-of-the-bag (OOB) predictions. The best model was the RF model with the best AUC value. The performance of the best model was confirmed by evaluating the mean AUC of a 10-fold cross-validation repeated 20 times using the AUC-RF (Calle et al. 2011) package. The AUC-RF based analyses were performed using the R (v3.6.1) within RStudio environment (v1.2.5019).

d. Data availability

The high-quality 16S V1-V2 sequences used in this study for downstream analysis were deposited on the DDBJ/GenBank/EMBL database (accession no. DRA002611, DRA002617, and DRA002618(Tsuda et al. 2015) and DRA011285).

2.3 Results

a. *Summary of 454 reads*

We obtained a total of 1,124,798 high-quality 16S reads from the two groups by using 454 GS FLX Titanium platform (***Table 4***) (Roche Applied Science, Indianapolis, IN). After removing low quality and possibly chimera reads, we obtained a total of 619,041

reads from 93 samples. Of them, we randomly selected 2300 reads per sample (213,900 reads from 93 samples), and further analyzed them using the pipeline for 454 barcoded pyrosequencing of 16S amplicons in order to minimize overestimation of species richness in the clustering due to intrinsic sequencing error (Kim et al. 2013). The average Good's coverage index(Good 1953; Singleton et al. 2001) of the 2300 reads per sample was 0.97 (values ranging from 0.94 to 0.99), indicating a high degree of coverage. Hence, the sequence data was sufficient for analysis in the current study.

b. *Differences in alpha and beta diversity of salivary microbiota of HC and IgAN groups*

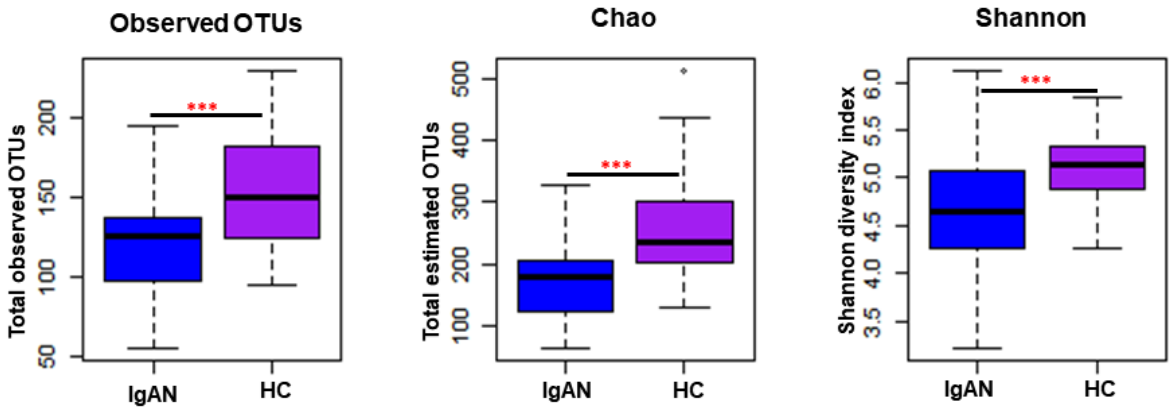

Fig. 3. Alpha diversity in IgAN and HC subjects. Samples from 43 IgAN (blue), and 50 HC (purple) subjects are shown. (A) The observed and Chao1-estimated OTU numbers, and the Shannon index of salivary microbiome from the two groups. * p-value <0.05; ** p-value < 0.01; *** p-value <0.001 based on Wilcox test. OTU, operational taxonomic unit.

The salivary microbiome of IgAN and HC samples were compared based on their alpha (***Fig. 3***) and beta diversity (***Fig. 4***). Alpha diversity estimates the variation of microbiome within a sample and is often denoted in terms of richness and diversity. In this study microbiome richness was estimated in terms of number of observed OTUs and Chao1 index. The number of observed OTUs as well as the Chao1 index in the

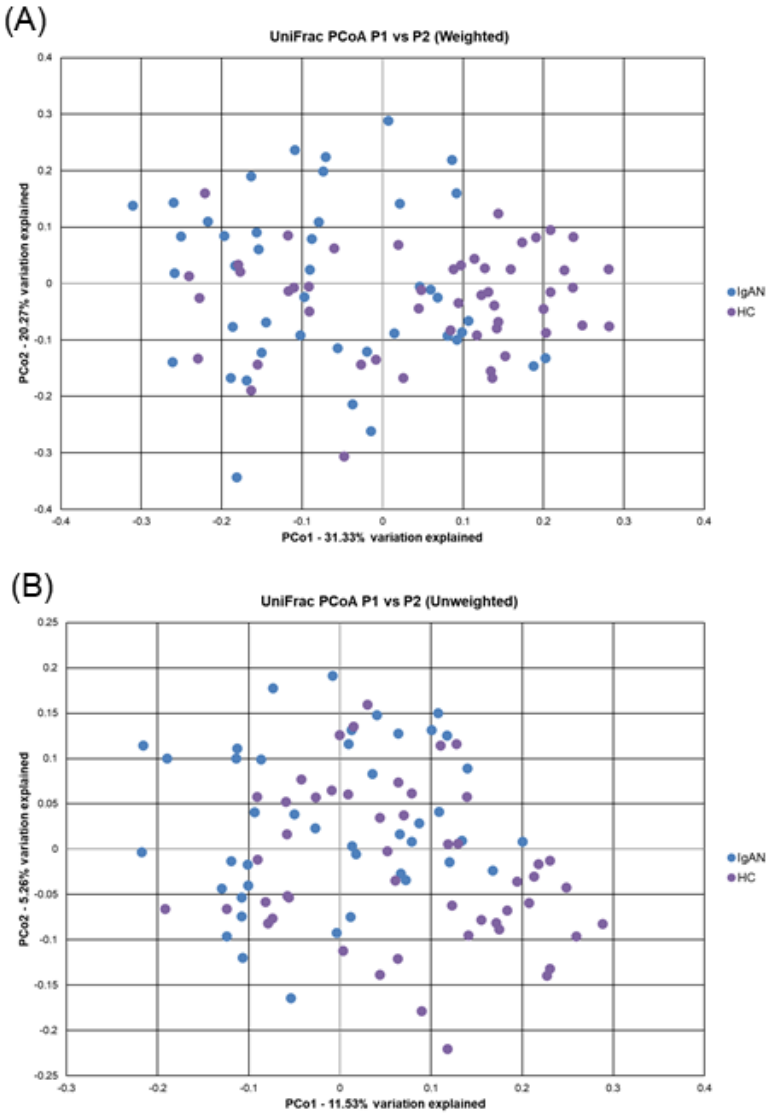

Fig. 4. Beta diversity in IgAN and HC subjects. Each dot represents a scaled measure of the composition of a given sample color coded by groups with blue dots code for IgAN and purple dots code for HC samples. (A) Weighted UniFrac –PCoA and (B) Unweighted UniFrac – PCoA of the salivary microbiome from the two groups. PCoA, principal coordinate analysis.

IgAN samples were significantly lower than the HC samples (p= 1.06E-05 and 2.81E-07, respectively; ***Fig. 3***). Shannon index, which takes into account both the richness and evenness of the microbiome and thus indicates within-sample diversity, was significantly decreased in the IgAN patients as compared to the HC (p= 9.66E-05, ***Fig. 3***).

Between-samples diversity is depicted as the beta diversity which explains the variations of microbial communities between the samples. In this study, sequence-based distances or UniFrac distance metrics are used to estimate the beta-diversity of the dataset. Unweighted UniFrac metric is entirely based on the sequence distances only whereas the weighted UniFrac metric includes both the sequence distance and abundance information(Lozupone and Knight 2005). PCoA of the unweighted and weighted UniFrac distance metrics of our dataset is shown in the ***Fig. 4***. In the unweighted plot, the largest principal coordinates were 11.53% and 5.26% of total variation, respectively, while they were 31.33% and 20.27%, respectively, in the weighted plot. The PCoA based on the unweighted UniFrac distance metric showed that many of the IgAN samples segregated from the HC sample ***Fig. 4B***. PERMANOVA shows that the IgAN samples significantly differed from the HC samples in terms of both weighted as well as unweighted UniFrac metrics (***Table 5***).

Table 5. Permutational multivariate analysis of variance (PERMANOVA) in salivary microbiome samples among two groups—IgAN and HC.

Category	No. of Subjects	Weighted UniFrac		Unweighted UniFrac	
		R^2	p-value	R^2	p-value
IgAN vs HC	IgAN = 43 HC = 50	0.07	**0.001**	0.03	**0.001**

p-values <0.05 are in bold.

c. *Variations in the salivary microbiome taxonomic profiles between the two groups*

We taxonomically assigned the 213,900 reads to 2,784 OTUs according to the phylotypes in the public microbial 16S rRNA gene databases. The phyla with relative mean abundances of more than 0.1% across both the groups (IgAN and HC) and thus accounting for 99.8% of total abundances were Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, Fusobacteria, Candidatus Saccharibacteria (TM7), and Streptophyta. The relative mean abundances of Bacteroidetes and Fusobacteria were significantly lower in the IgAN compared to the HC samples (p= 0.0002 and

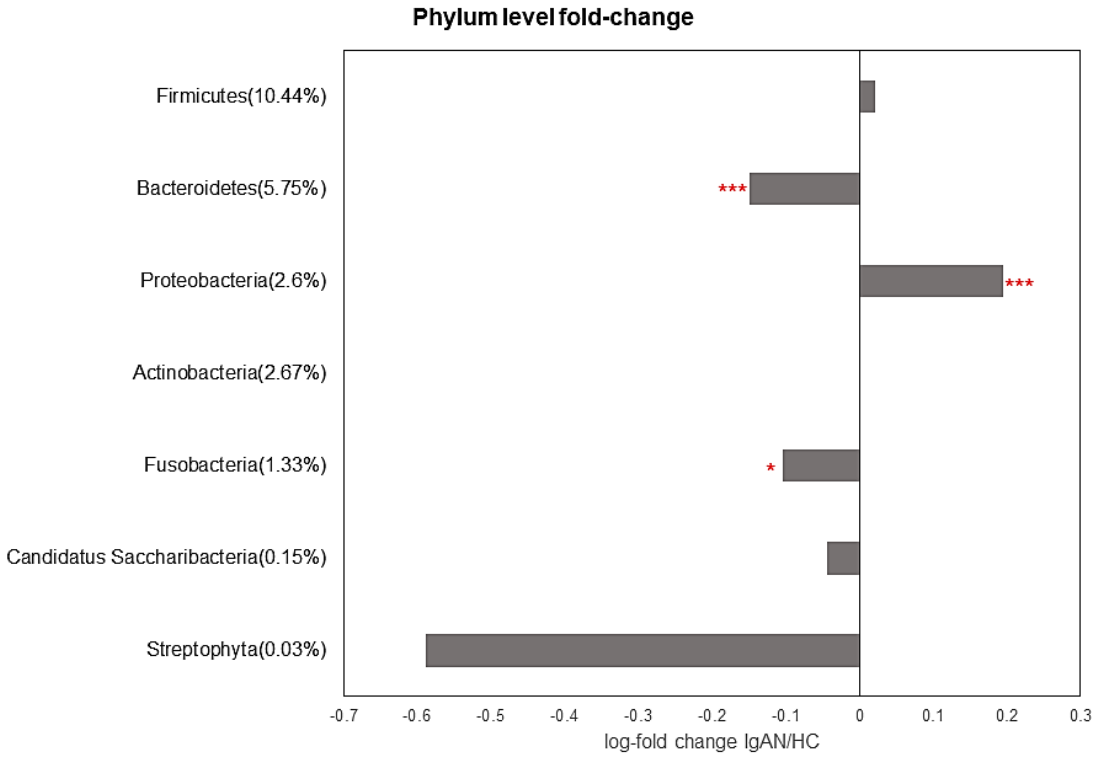

Fig. 5. Phylum level fold-change between IgAN and HC samples. For each phylum, fold-change is calculated by dividing (mean relative abundance) % in IgAN by (mean relative abundance) % in HC. The (mean relative abundance) % in HC is given in parentheses besides corresponding phyla. * p-value <0.05; ** p-value < 0.01; *** p-value <0.001 based on Wilcox test.

0.0361, respectively). On the other hand, the relative mean abundance of

Proteobacteria was significantly higher (p = 0.0004) in the IgAN patients than the HC

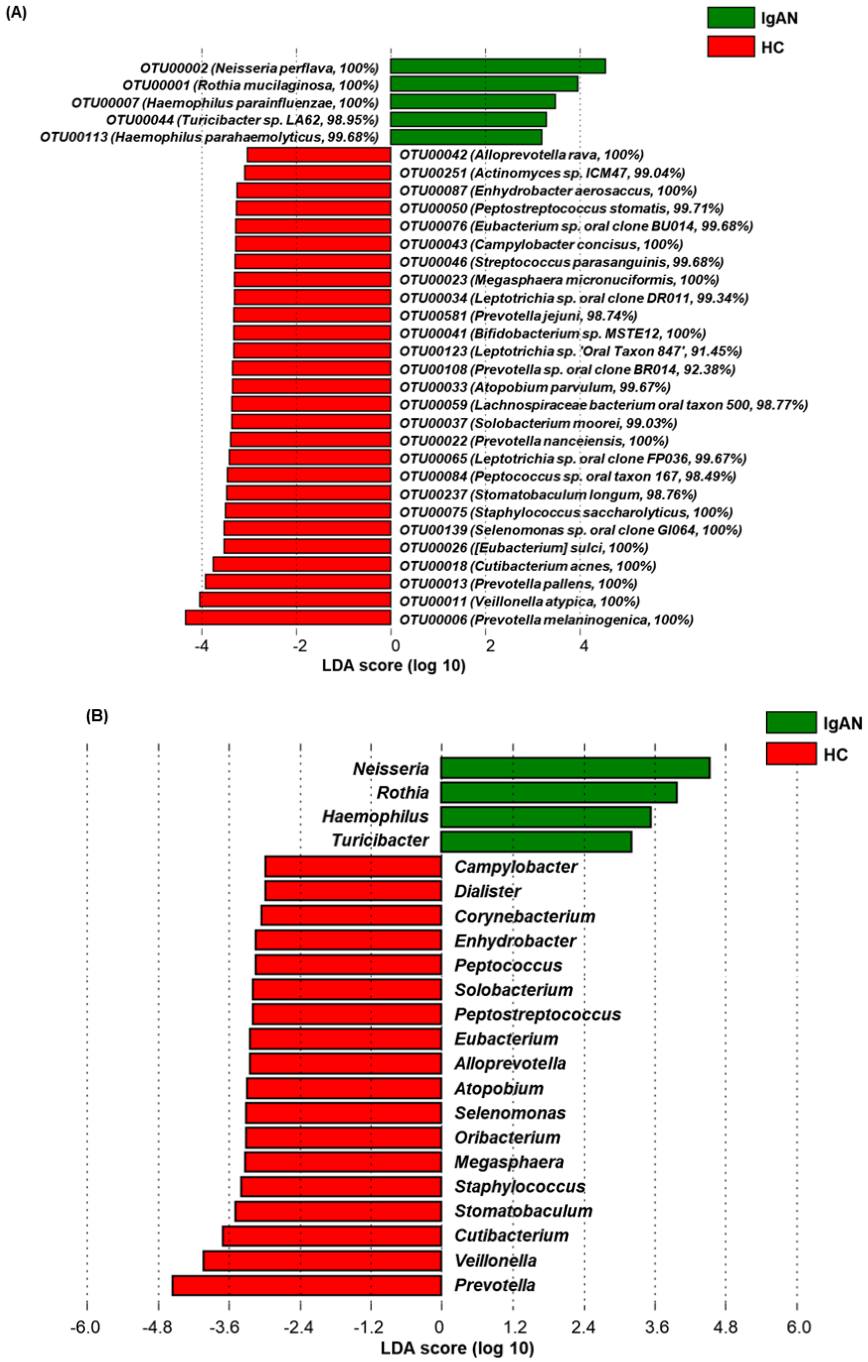

Fig. 6. Differential taxa between IgAN and HC derived using LEfSe. Histogram of LDA score indicating the effective size and ranking of each differentially abundant (A) OTU and (B) genus is shown here. LDA>2 threshold was used for LEfSe analysis. LEfSe, Linear discriminant analysis (LDA) Effect Size; IgAN, Immunoglobulin A Nephropathy; HC, Healthy Control; OTU, operational taxonomic unit.

(***Fig. 5***). The ratio of relative mean abundances of Firmicutes to Bacteroidetes was higher in the IgAN as compared to the HC. On the other hand, the Firmicutes to Proteobacteria ratio was lower in the IgAN as compared to the HC samples.

The taxonomic assignment at the genus level identified 270 bacterial genera, of which 48 genera had a relative mean abundance of more than 0.1%, accounting for 97.8% of the total abundance. Among these 48 genera, 21 genera varied significantly in terms of relative mean abundance between the two groups. *Streptococcus, Neisseria, Rothia, Haemophilus* and *Turicibacter* were significantly more abundance in the IgAN patients than the HC ($p = 0.0382, 0.0001, 0.0169, 0.0431$, and 0.0285, respectively). On the other hand, *Prevotella, Veillonella, Alloprevotella, Cutibacterium, Megasphaera, Atopobium, Campylobacter, Eubacterium, Corynebacterium, Solobacterium, Peptostreptococcus, Staphylococcus, Enhydrobacter, Peptococcus,* and *Dialister* were significantly depleted in abundance in the IgAN samples compared to the HC samples ($p = 0.0003, 0.0004, 0.0277, 0.0002, 0.0040, 0.0380, 0.0178, 0.0256, 0.0096, 0.0083, 0.0011, 2.3295E-07, 0.0002, 0.0004, 0.0325$, and 0.0342, respectively).

To further evaluate salivary microbiome differences among patients with IgAN and healthy subjects (HC), the LEfSe method was employed to identify significant discriminative features between the two groups (with logarithmic LDA score threshold >2). From LEfSe analysis, we were able to identify 32 differential taxa at the OTU level and 22 differential taxa at the genus level between IgAN and HC samples (***Fig. 6***).

To determine the potential value of the identified microbial biomarkers to

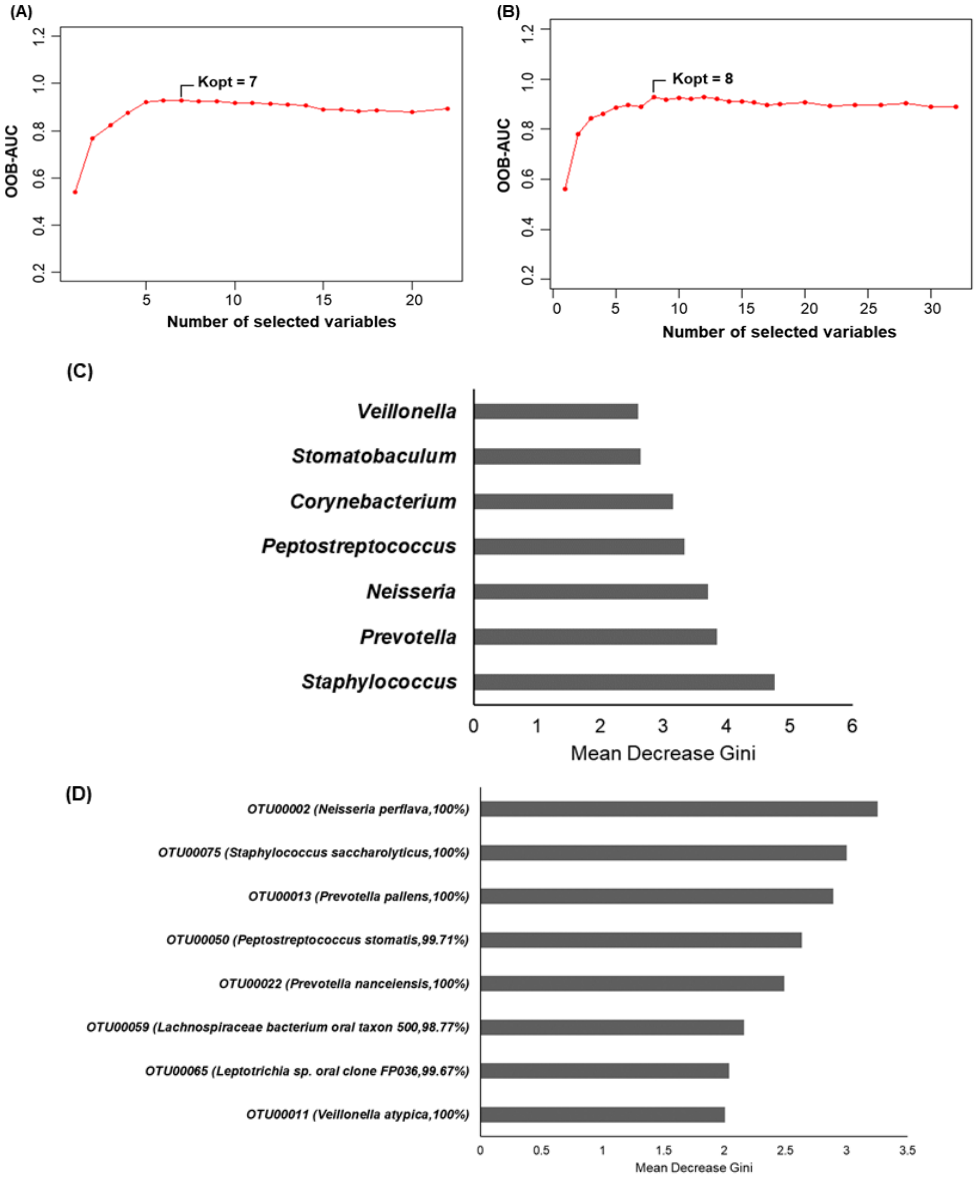

Fig. 7. Random Forest (RF) analysis of the salivary microbiota of IgAN and HC groups using AUC-RF package at OTU and genus level. Best RF models (comparison based on combination of best mean area under curve (AUC) value) was obtained. The corresponding AUC curve with Kopt is shown for (A)Genus and (B)OTU levels. (C), (D) The selected features of best RF models to differentiate IgAN from HC are shown; the features are ranked as highest Mean Decrease Gini score (top-most taxa on each plot) to lowest Mean decrease Gini score (last taxa on each plot). Kopt, optimal number of features to distinguish between the two groups under comparison. OTU, operational taxonomic unit.

clinically differentiate the IgAN from the HC samples, we generated RF models using the AUC-RF package. We generated models for two taxa levels (genus and OTU).

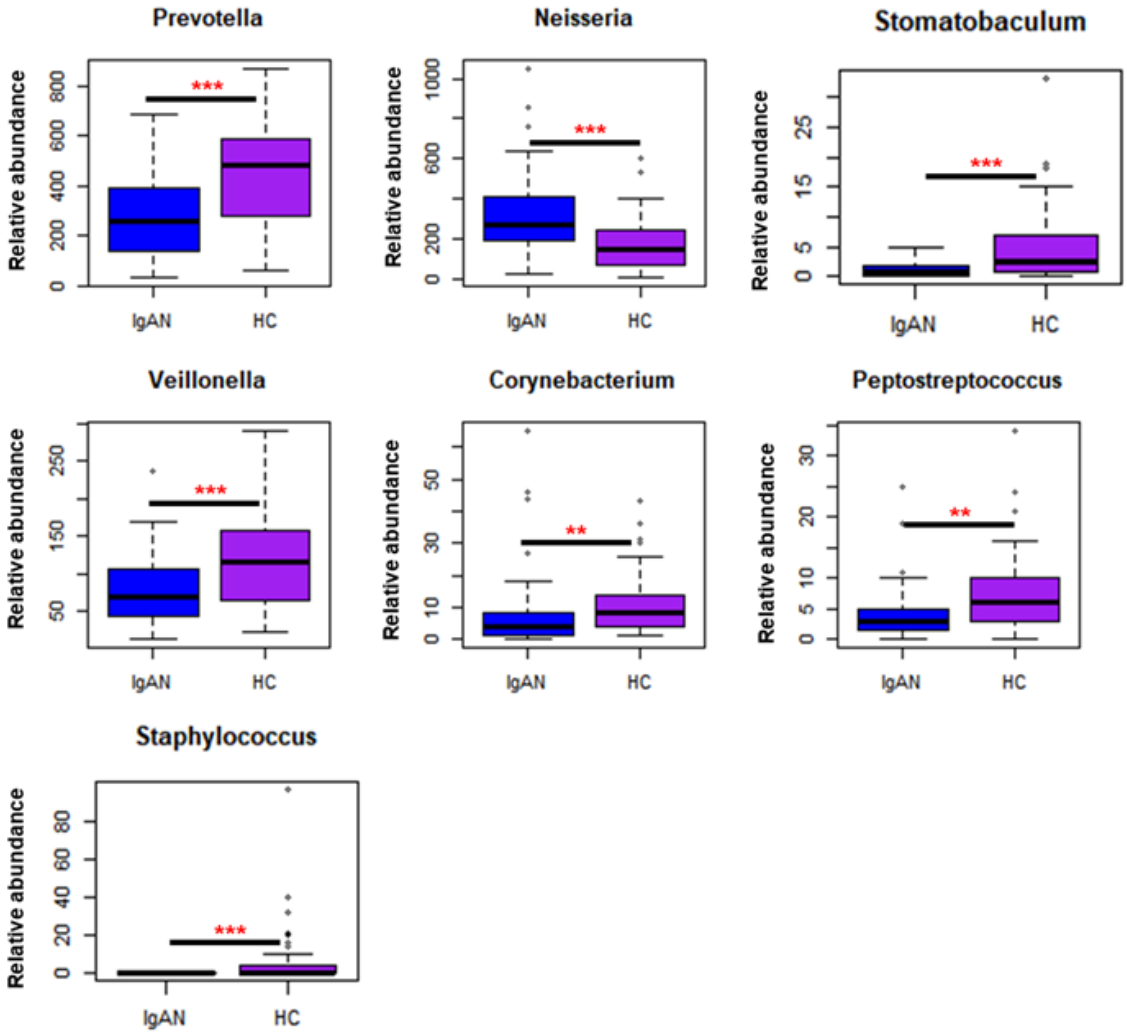

Fig. 8. Genus level variables from Random Forest (RF) analysis of the salivary microbiota using AUC-RF package. The relative abundance of taxa selected by AUC-RF are shown here. Seven genera selected for IgAN vs HC model* p-value <0.05; ** p-value < 0.01; *** p-value <0.001 based on Wilcox test with Benjamin-Hochberg correction. IgAN=blue, and HC=purple. IgAN, Immunoglobulin A Nephropathy; HC, Healthy Control; OTU, operational taxonomic We used the AUC of the receiver operative curve (ROC), that was in turn based upon the out-of-bag (OOB) error rate, to find the combination of multiple taxa from the 22 differential genera selected by the LefSe tool which can contribute in

discriminating IgAN group from the HC group. The model with best AUC value at the genus level was observed for 7 genera between IgAN and HC samples (***Fig. 7B***). These 7 genera (*Staphylococcus, Prevotella, Neisseria, Peptostreptococcus,*

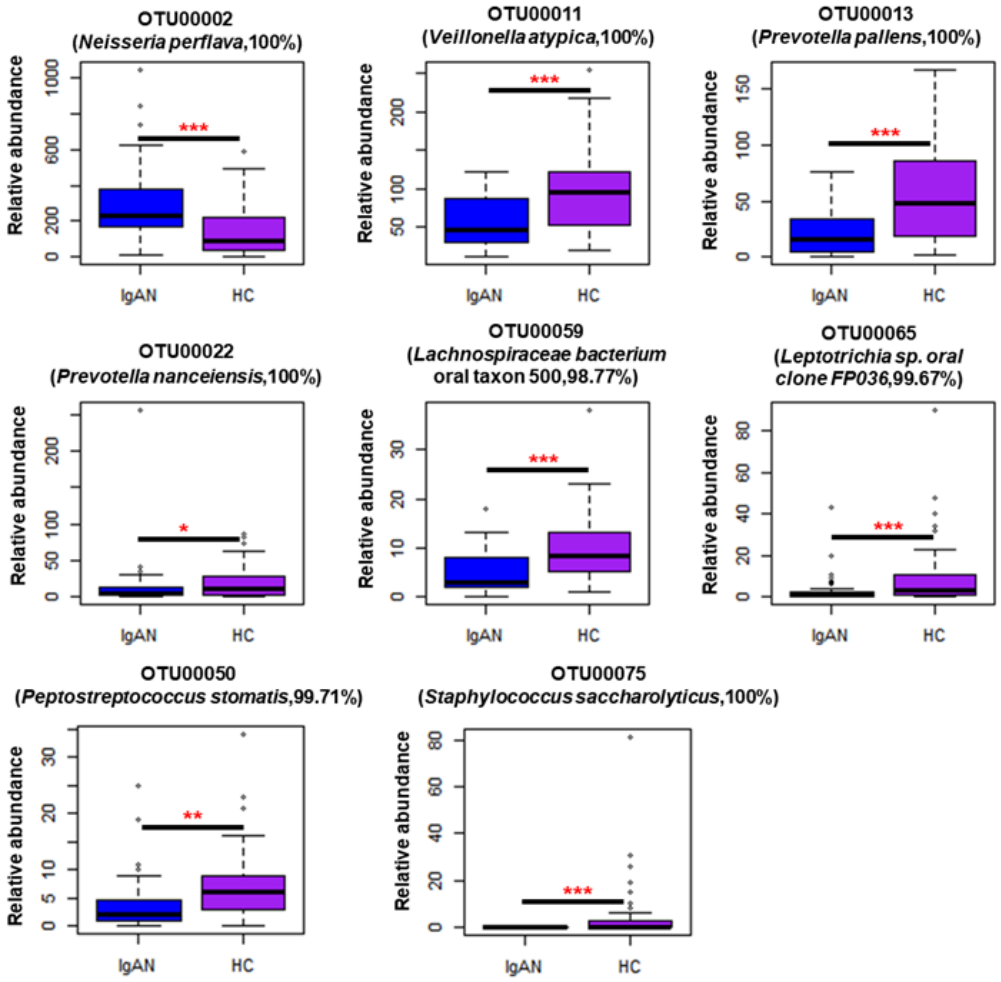

Fig. 9. OTU level variables from Random Forest (RF) analysis of the salivary microbiota using AUC-RF package. The relative abundance of taxa selected by AUC-RF are shown here. Eight OTUs were selected for IgAN vs HC model. The corresponding species and percentage of identity for the OTUs are given in the parentheses. * p-value <0.05; ** p-value < 0.01; *** p-value <0.001 based on Wilcox test with Benjamin-Hochberg correction. IgAN=blue, and HC=purple. IgAN, Immunoglobulin A Nephropathy; HC, Healthy Control; OTU, operational taxonomic unit.

Corynebacterium, Veillonella and *Stomatobaculum*), thus, contributed in

distinguishing the IgAN from the HC samples. Out of 7 genera, only *Neisseria* was significantly more abundant in the IgAN patients than the HC (p = 8.77E-05) (***Fig. 8.*** **Genus level variables from Random Forest (RF) analysis of the salivary microbiota using AUC-RF package.** The relative abundance of taxa selected by AUC-RF are shown here. Seven genera selected for IgAN vs HC model* p-value <0.05; ** p-value < 0.01; *** p-value <0.001 based on Wilcox test with Benjamin-Hochberg correction. IgAN=blue, and HC=purple. IgAN, Immunoglobulin A Nephropathy; HC, Healthy Control; OTU, operational taxonomic unit.). On the other hand, *Staphylococcus, Prevotella, Peptostreptococcus, Corynebacterium, Veillonella* and *Stomatobaculum* were significantly less abundant in the IgAN samples than the HC samples (p = 2.33E-07, 0.0003, 0.0011, 0.0096, 0.0002, and 0.0004, respectively) (**Fig. 8**). We confirmed the results by 10-fold cross-validation of AUC-RF model repeated 20 times to obtain the mean AUC of 0.903 for IgAN vs HC.

At the OTU level, we used the 32 differential OTUs selected by the LefSe tool to build the RF model. The models with best AUC values had 8 OTUs between the IgAN and HC samples (***Fig. 7A***). Out of 8 OTUs, only OTU00002 (100% identity with *Neisseria perflava*) was significantly more abundant in IgAN patients than the HC . On the contrary, remaining 7 OTUs, namely, OTU00011 (100% identity with *Veillonella atypica*), OTU00065 (99.67% identity with *Leptotrichia sp*. oral clone FP036), OTU00059 (98.77% identity with *Lachnospiraceae bacterium* oral taxon 500), OTU00022 (100% identity with *Prevotella nanceiensis*), OTU00050 (99.71% identity with *Peptostreptococcus stomatis*), OTU00013 (100% identity with *Prevotella pallens*) and OTU00075 (100% identity with *Staphylococcus saccharolyticus*), were significantly less abundant in the IgAN samples than the HC samples (p = 0.000240349, 8.17E-05, 9.46E-05, 0.0117, 0.0014, 4.26E-05 and 9.90E-07,

respectively) (***Fig. 9***). The mean AUC of the 10-fold cross-validation repeated 20 times was 0.86 between the IgAN and HC.

d. *Gender-specific microbiome association*

Table 7. Permutational multivariate analysis of variance (PERMANOVA) in salivary microbiome samples in the two groups to check gender effect. p-value <0.05 are bold and underlined.

HC(male vs female)	female:14	0.01	0.84	0.03	**0.04**
IgAN(male vs female)	male:20 female:23	0.05	**0.04**	0.03	0.17
All(male vs female)	male:56 female:37	0.03	**0.01**	0.02	**0.02**

To check whether there is any underlying gender-specific association of the salivary microbiome, we performed a PERMANOVA test where the samples were grouped

Table 6. Permutational multivariate analysis of variance (PERMANOVA) in salivary microbiome samples in the two groups, namely IgAN and HC, grouped by gender. The adjusted p-values were adjusted for multiple testing by Benjamin-Hochberg method. p-value <0.05 are bold and underlined.

IgAN female vs HC female	HC:14 IgAN:23	0.09	**0.02**	**0.02**	0.05	**0.002**	**0.002**
IgAN male vs HC male	HC:36 IgAN:20	0.06	**0.005**	**0.01**	0.04	**0.001**	**0.002**

according to their genders. PERMANOVA test showed a significant difference between male and female samples in the IgAN group for weighted UniFrac metric and

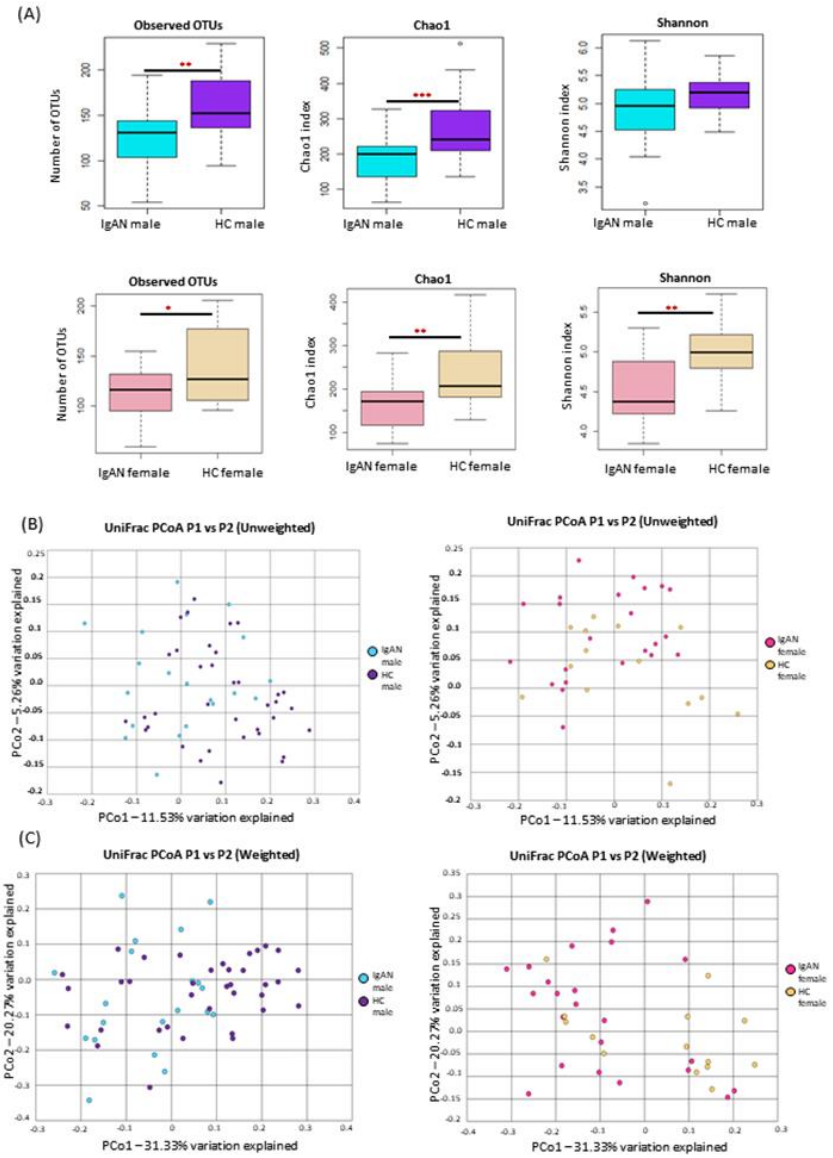

Fig. 10. Alpha and beta diversity in IgAN male, HC male, IgAN female and HC female subjects. Samples from 20 IgAN male, 36 HC male, 23 IgAN female and 14 HC female subjects are shown. (A) The observed and Chao1-estimated OTU numbers, and the Shannon index of salivary microbiome from the four groups. * p-value <0.05; ** p-value < 0.01; *** p-value <0.001 based on Wilcox test. (B) Unweighted UniFrac –PCoA and (C) weighted UniFrac –PCoA of the salivary microbiome from the four groups (IgAN=blue, and HC=purple). IgAN, Immunoglobulin A Nephropathy; CT, Chronic Tonsillitis; UC, Ulcerative Colitis; HC, Healthy Control; OTU, operational taxonomic unit; PCoA, principal coordinate analysis.

in the HC group for unweighted UniFrac metric only (***Table 6***). The observed OTU

numbers of the IgAN male and IgAN female samples were significantly lower than HC

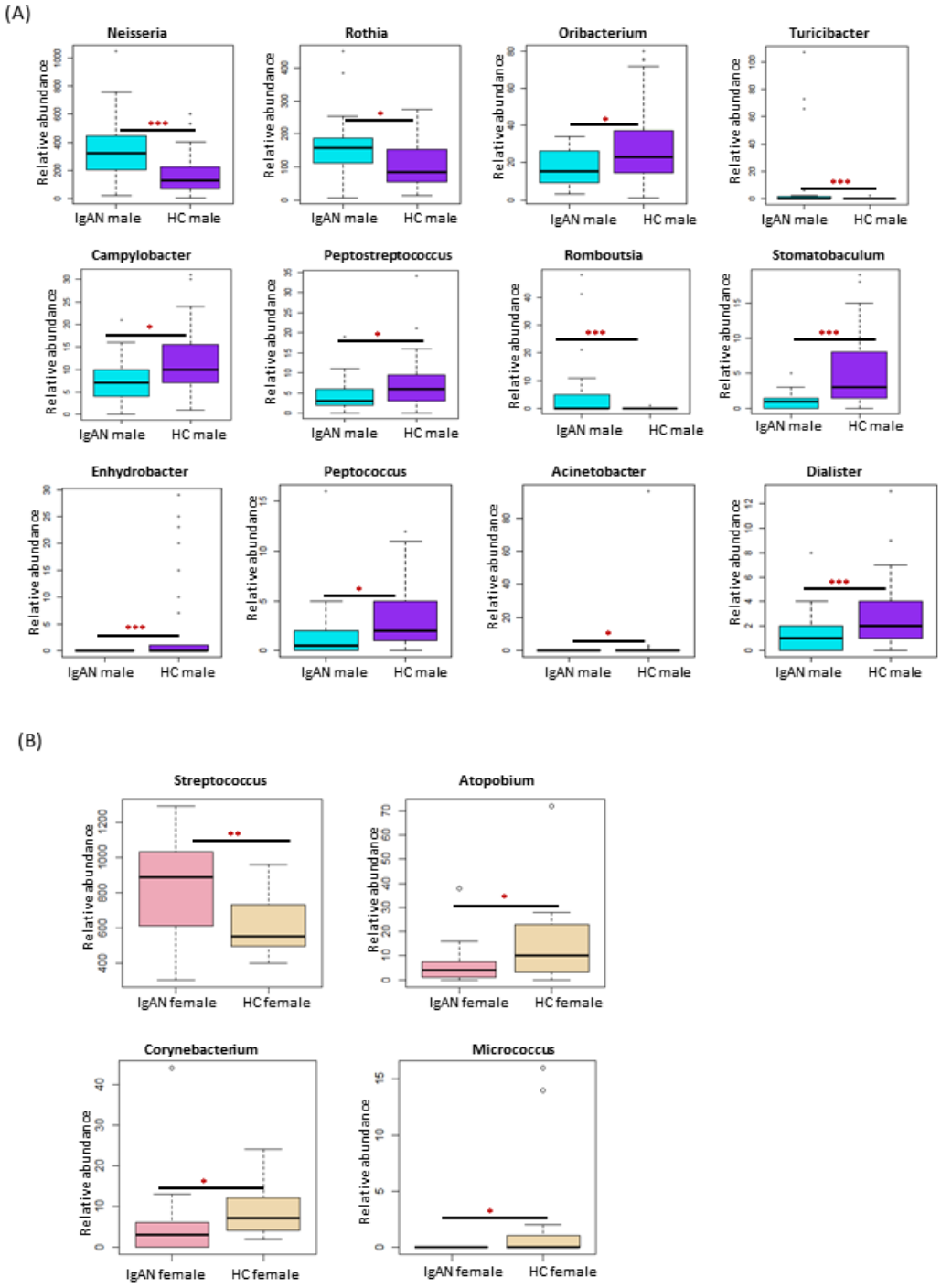

Fig. 11. Unique Gender-associated genera with significant difference between the IgAN and HC groups. (A) 12 genera with more than 0.1% mean relative abundance and p-value < 0.05 between IgAN male and HC male groups are shown. (B) 5 genera with more than 0.1% mean relative abundance and p-value <0.05 between IgAN Female and HC male are shown.

male and HC female samples, respectively (p = 0.0010, and 0.04, respectively) (***Fig. 10A***). Similarly, the chao1-estimated OTU numbers of the IgAN male and IgAN female samples were significantly lower than HC male and HC female samples, respectively (p= 0.0002 and 0.003, respectively) (***Fig. 10B***). Alpha diversity, indicated by the Shannon index, showed a significant difference between IgAN female and HC female samples (p = 0.005) but not between IgAN male and HC male samples (***Fig. 10A***). The PCoA based on unweighted UniFrac distance metric segregated IgAN male and IgAN female samples from HC male and HC female samples, respectively (***Fig. 10B***). There was a similar sample segregation trend between IgAN male and HC male, and IgAN female and HC female samples in PCoA plots based on weighted UniFrac distance metrics (***Fig. 10B***). Additionally, there was a significant difference between IgAN male and HC male samples for both weighted and unweighted UniFrac metrics (***Table 7***). We observed a similar scenario in PERMANOVA between IgAN female and HC female samples (***Table 7***).

At the taxonomic composition, there was no phylogenetic demarcation between taxa that were differentially abundant in two gender-based analysis. At the phylum level, Bacteroidetes was significantly less abundant in IgAN male group than the HC male group, and Candidatus Saccharibacteria (TM7) was significantly less abundant in IgAN female group than HC female group. However, phylum Proteobacteria is significantly higher in IgAN male and IgAN female samples than the HC male and HC female samples, respectively. At the genus level, 12 genera (*Neisseria, Rothia, Oribacterium, Turicibacter, Campylobacter, Peptostreptococcus, Romboutsia, Stomatobaculum, Enhydrobacter, Peptococcus, Acinetobacter, and Dialister*) with more than 0.1% mean relative abundance were significantly higher in IgAN male samples than HC male samples. Three genera (*Atopobium, Corynebacterium and*

Micrococcus), with more than 0.1% mean relative abundance, were significantly decreased while *Streptococcus* genus significantly increased in IgAN female samples as compared with HC female samples (***Fig. 11***).

2.4 Discussion

Mucosal immunity has a symbiotic relationship with the microbiome. GWAS studies have indicated that several risk loci involved in IgAN pathogenesis is associated with mucosal immunity (Sallustio et al. 2019). Additionally, a transgenic murine model for IgAN didn't exhibit any IgA deposits in kidney glomeruli in the absence of commensal microbiota, thus highlighting a connection between microbiome and IgAN pathogenesis (McCarthy et al. 2011; Sallustio et al. 2019). There have been reports about the gut, tonsil, periodontal and salivary microbiota associated with IgAN (Cao et al. 2018; De Angelis et al. 2014; Dong et al. 2020; Hu et al. 2020; Luan et al. 2019; Nagasawa et al. 2014; Park et al. 2020; Piccolo et al. 2015; Watanabe et al. 2017). However, despite Japan being the second highest in the world in terms of incidence and frequency of IgAN (Schena and Nistor 2018), the IgAN associated microbiota studies for the Japanese cohort are limited. Our present study represents the characterization of salivary microbiome of patients with IgAN in comparison with healthy subjects. To the best of our knowledge, this is the first study to characterize salivary microbiome profile of Japanese IgAN patients.

In this study, we observed altered salivary microbial composition in the IgAN patients as compared to the healthy subjects, indicated by the lower species richness and microbial diversity in the IgAN patients' salivary microbiota. Similarly, salivary microbial richness and diversity were lower but not significant ($P>0.05$) in IgAN than HC in the Caucasian population(Piccolo et al. 2015) whereas a Chinese population based study of IgAN salivary microbiota did not report any significant difference(Luan et al.

2019). Another oral microbiome of IgAN patients from Han Chinese ancestry reported a decrease in Chao1 diversity index in IgAN group compared to health group; however, the difference was not statistically significant ($p>0.05$)(He et al. 2021a). In fecal microbiome studies associated with IgAN, few studies reported similar findings of lower microbial richness (De Angelis et al. 2014; Hu et al. 2020) and diversity(De Angelis et al. 2014) in IgAN compared to HC, whereas another study did not find any significant difference(Dong et al. 2020). The observed differences might be rooted in the variability of study design, like using different hypervariable regions for 16S rRNA sequencing, using different sequencing platforms, sample size, or ethnicity differences. In our study, statistical analysis of the phylogeny-based weighted and unweighted UniFrac metrics shows that the observed dysbiosis is associated with differences in the presence or absence of the microbial taxa as well as their abundance in the population. Therefore, as seen in Caucasian(De Angelis et al. 2014; Piccolo et al. 2015) and Chinese(Dong et al. 2020; He et al. 2021a; Luan et al. 2019) population studies, our study demonstrates microbial dysbiosis in IgAN patients as compared to the healthy subjects. A detailed and larger population-based study with collaborative research across globe using same protocol from subject recruitment to sample processing and analysis is required for more concrete understanding of IgAN microbiome.

There were differences at the taxonomic level between the IgAN and HC samples. The phylum Firmicutes dominated all the samples and was most abundant in the IgAN, following previous studies on fecal and salivary microbiota studies of IgAN patients (De Angelis et al. 2014; Hu et al. 2020; Luan et al. 2019). However, the difference between the mean relative abundance of Firmicutes in IgAN and HC group was not statistically significant ($p>0.05$) and this observation also matches with previous IgAN salivary microbiome studies (Luan et al. 2019; Piccolo et al. 2015). The

Firmicutes/Proteobacteria ratio was lower in the IgAN group as compared to the HC groups. This ratio agrees with the previously reported salivary microbiota study on the Caucasian population (Piccolo et al. 2015) and periodontal microbiome study on the Asian population (Cao et al. 2018) but is in contrast with the salivary microbiota study in Chinese cohort (Luan et al. 2019).

The significant differences between IgAN and HC samples in terms of microbial taxa prompted us to investigate the selected biomarkers for discriminating IgAN group from the healthy controls. In our study, a combination of salivary taxa separated IgAN from healthy individuals with AUC of 0.86 and 0.90 at OTU and genus levels, respectively. A Chinese cohort study reported separation of IgAN from HC with predictive accuracy up to 80% upon using salivary microbial OTUs in combination with biochemical characteristics (Luan et al. 2019). These results indicate that salivary microbiome derived biomarkers can be used for predictive diagnosis of IgAN from healthy population.

Staphylococcus is one of the genera that differentiates IgAN from HC samples. This genus significantly decreased in abundance in IgAN samples compared to the HC. *Staphylococcus* genera is often represented by pathogenic species such as *Staphylococcus aureus* which are known to cause postoperative wound infection, food poisoning, septicemia, endocarditis, and toxic shock syndrome (Kluytmans et al. 1997; Koyama et al. 2004). Studies have indicated that *S. aureus* cell antigen is associated with IgAN pathogenesis(Koyama et al. 2004). However, *Staphylococcus* is also a commensal microbiota in the oral cavity (Lamm 1997). Further research is needed to reveal members of *Staphylococcus* genera which contribute in IgAN pathogenesis.

Neisseria was also one of the contributors in distinguishing IgAN from healthy individuals. This genus is enriched in the IgAN group than healthy groups, similar to the

findings of prior studies (He et al. 2021a; Piccolo et al. 2015). Also, at the OTU level, some OTUs belonging to the *Neisseria* genus were among the selected taxa for differentiating IgAN from the HC samples and they were significantly enriched in IgAN patients. Previous salivary microbiome studies report the co-occurrence of *Neisseria* and *Haemophilus* in salivary ecosystem (De Angelis et al. 2014; Takeshita et al. 2016). Salivary community type comprising of *Neisseria*, *Haemophilus, Gemella, Porphyromonas,* and *Streptococcus mitis* along with reduced phylogenetic diversity was associated with better periodontal health (Takeshita et al. 2016). A salivary microbiome study of IBD patients reported that elevated salivary IgA levels were correlated with lower abundance of *Neisseria*, *Haemophilus, Gemella* and *Streptococcus (Said et al. 2014)*. On the other hand, salivary microbiome study of IgAN patients reported that *Haemophilus* was positively correlated with levels of serum IgA(He et al. 2021a). According to scientific literature, salivary and serum IgA levels are elevated in IgAN patients and they are positively correlated (Yamabe et al. 1987). In our study, abundance of *Neisseria*, *Haemophilus, Gemella* and *Streptococcus* genera increased in the IgAN patients. The difference in the relationship between abundance of these genera and salivary IgA levels can be attributed to difference in the disease pathogenesis of IgAN and IBD. However, given the lack of data corresponding to salivary IgA levels of the IgAN patients in our study, the underlying relationship of salivary IgA and microbiome in light of these two diseases has a scope of future research.

Prevotella also contributed in differentiating IgAN patients from HC. A study that used Projection to Latent Structure (PLS) models for discriminant analysis reported that *Prevotella* was among the top 5 genera that differentiated between IgAN and HC samples (He et al. 2021a). In line with previous reports(Cao et al. 2018; Luan et al.

2019; Piccolo et al. 2015), there was significant enrichment of *Prevotella* in HC than IgAN samples. Though OTU clustering methods in our study is not sufficient for species level assignment, we found OTUs assigned to *Prevotella pallens* with 100% identity which were among the selected taxa for distinguishing IgAN patients from HC and similar to Italian cohort study, these were significantly enriched in HC than IgAN patients (Piccolo et al. 2015). The Chinese fecal microbiome study comparing the IgAN patients with membranous nephropathy patients, and healthy controls noted a positive correlation between *Prevotella* and a higher serum albumin level (Dong et al. 2020). Serum albumin plays an essential role in reducing oxidative stress in the mesangial cells by attenuating the reactive oxygen species like hydrogen peroxide, thereby lowering the risk of IgAN progression towards ESRD (Kawai et al. 2018). From this, we can infer that *Prevotella* genera has a protective role in the salivary and gut ecosystem, and a decline in its abundance may facilitate systemic disease progression like IgAN.

Under Firmicutes phyla, *Streptococcus* is the predominant genera, and is more abundant in the IgAN group (p= 0.0382). In previous reports, *Streptococcus* was enriched in the subgingival microbiome of chronic periodontitis patients with IgAN (Cao et al. 2018) and is also one of the core members of tonsillar crypt microbiome (Jensen et al. 2013). 16S rDNA reads of *Streptococcaeae* family increased in the fecal microbiome of IgAN patients with persistent proteinuria (De Angelis et al. 2014). Another study reported an increase in the cell surface collagen-binding Cnm protein of *Streptococcus mutans* in tonsillar specimens of IgAN patients compared to chronic tonsilitis patients (Ito et al. 2019). This study also reported an association between the presence of Cnm-positive *S. mutans* strain with greater severity of symptoms in IgAN patients, marked by higher proteinuria and lower serum albumin levels (Ito et al. 2019). In our study, we found the mean relative abundance of *S. mutans* was higher in IgAN

than HC samples. These evidences along with findings such as presence streptococcal proteins in the renal tissues from IgAN patients (Schmitt et al. 2010) indicate that bacterial causal agents of focal tonsillar infection, like *Streptococcus*, can be associated with IgAN pathogenesis.

Several OTUs belonging to *Haemophilus* genus varied significantly between the IgAN and HC groups. Some members of *Hemophilus* genus, like *H. parahaemolyticus*, are commensal microflora in the upper respiratory tract, and literature suggests these are to be considered as opportunistic pathogens capable of causing invasive and severe diseases (Le Floch et al. 2013). *H. parahaemolyticus* belongs to the *H. parainfluenzae* group and is one of the few species in *Haemophilus* genera like *H. influenzae,* which produces IgA1 protease that attributes to its pathogenicity in human host (Norskov-Lauritsen 2014). Piccolo *et al.* reported an increase of *H. parainfluenzae* in salivary microbiota of IgAN patients with the lowest proteinuria (Piccolo et al. 2015). In our study, *H. parahaemolyticus* and *H. parainfluenzae* are more abundant in the salivary microbiome of the IgAN patients. Authors recommending the use of *H. influenzae* derived IgA protease for IgAN treatment state that the lack of mesangial IgA1 specificity of the *H. influenzae* derived IgA protease limits its immediate application for IgAN therapy (Eitner and Floege 2008). These observations suggest that some members of *Hemophilus* genera contribute to the early stages of IgAN pathogenesis. Further research based on these associative members might contribute towards a better understanding of the progression of IgAN and thus expedite the therapeutic applications.

While some epidemiological studies observed a male predominance in North American and Western European populations(Barratt and Feehally 2014; Sukcharoen et al. 2020), others show an equal distribution of IgAN incidence between genders in

the Asian population(Cheng et al. 2013; Feehally and Barratt 2015; Lee et al. 2012). A single-center study in Japan showed that primary glomerulonephritis showed higher frequency in men as compared to women(Moriyama et al. 2010). In microbiome studies gender may act as a confounding factor as some show a significant difference between male and female microbiome(Minty et al. 2020; Raju et al. 2019). Thus, upon exploring existence of gender-associated microbiota in our cohort we found that unlike previous study on a cohort of Hans Chinese origin(He et al. 2021a), microbial richness of the IgAN male and IgAN female groups was significantly lower than corresponding HC male and HC female groups (***Fig. 10***). We also found that some genera had significant difference of abundance only between HC and IgAN males (***Fig. 11A***), whereas some others differed only between HC and IgAN females (***Fig. 11B***). Similarly, oral microbiome study of IgAN patients from Chinese Han ancestry reported several genera with potential to discriminate between IgAN and HC samples in terms of gender(He et al. 2021a). These observations suggest that microbiota may play a role in the gender-associated risk of IgAN disease progression and, further research in this field is essential.

The current study has some limitations. Firstly, our study included non-uniform sample size across the groups. The imbalance in dataset was not statistically significant and did not affect the microbiome analysis. Disease predictive modeling is affected by an imbalanced dataset; however, the AUC-RF package uses a random forest classifier for modeling and the random forest classifier is relatively robust when dealing with an imbalanced dataset(Dittman et al. 2015). Secondly, data associated with stage of disease for IgAN patients is not available for clinical metadata correlation analysis. However, since in Japan, tonsillectomy is recommended and effective in the early stages IgAN (Hotta et al. 2001) and the IgAN patients in the current study collected the

saliva sample before their tonsillectomy surgery, it implies that patients were in the early stage of the disease. Lastly, our study design is limited by the lack of clinical data reporting the serum or salivary IgA levels in the IgAN patients. As secretory IgA plays important role in IgAN pathogenesis, there is a scope for future microbiome study exploring the relationship between the secretory IgA levels and predominant microbiota associated with IgAN.

In conclusion, our findings from the current study, where we explored the variations in salivary microbiome profiles of IgAN patients, and HC, indicate that the salivary microbiome is a potential biomarker source to develop a non-invasive diagnostic tool for IgAN. However, the biological role of the microbial biomarkers identified in this study in the pathogenesis and disease progression of IgAN is a scope of further research.

3 Comparison of Salivary Microbiome of Japanese IgA Nephropathy Patients versus Chronic Tonsillitis Patients, Ulcerative Colitis Patients and Healthy Controls

Comparison of Salivary Microbiome of Japanese IgA Nephropathy Patients versus Chronic Tonsillitis Patients, Ulcerative Colitis Patients and Healthy Controls

Abstract

Background: In Japan, IgA nephropathy (IgAN) is one of the leading causes of chronic kidney disease, with 50% of the cases progressing to end stage renal disease. Understanding about the origin and maintenance of IgAN has limited the development of a diagnostic tool independent of renal biopsy. The salivary microbiome is a rich source of biomarkers for systemic diseases. In this study, we primarily aimed to investigate the salivary microbiome as a tool for the non-invasive diagnosis of IgAN. IgAN has been reported to be triggered by upper-respiratory tract and gastrointestinal infections. Recent studies reported that patients with IgAN are more likely to incur inflammatory bowel diseases. Researchers have also established that chronic inflammation of tonsils triggered the recurrence of IgAN. Therefore, this study also aimed at investigating salivary microbiome profile of IgAN patients in comparison with mucosal diseases like chronic tonsillitis and ulcerative colitis.

Methods: Saliva was collected from 43 IgAN patients (IgAN), 50 healthy controls (HC), 20 chronic tonsilitis (CT) patients and 22 ulcerative colitis (UC) patients. The hypervariable V1–V2 regions of 16S rRNA gene were purified from all the samples and amplified using 27Fmod and 338R primers. The salivary microbial profile of all the samples were investigated by using high-throughput 454-pyrosequencing technology and bioinformatic analyses pipelines. Statistical analyses were conducted on R software. We used Linear discriminant analysis effect size (LEfSe) tool and R-based Area Under

Curve-Random Forest (AUC-RF) package to identify microbial biomarkers for differentiating IgAN from mucosal diseases (CT and UC) and HC.

Results: We noted a significant difference in microbial diversity and richness between IgAN patients and HC. IgAN and CT differed significantly in terms of both microbial diversity and richness whereas there was no significant difference in terms of alpha diversity between IgAN and UC. PERMANOVA of UniFrac distance metrics to quantify the beta-diversity, we found that when compared with the HC samples, the IgAN samples presented lower degree of dysbiosis than the other two disease groups (CT and UC). Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, Fusobacteria, Candidatus Saccharibacteria (TM7), and Streptophyta were the major phyla with relative mean abundances of more than 0.1% across all the groups (IgAN, CT, UC and HC) and accounted for 99.8% of the total abundances. Combining the genera selected by the random forest algorithm, we were able to distinguish the IgAN from the HC samples with the area under the curve (AUC) of 0.90, from the UC samples with the AUC of 0.88, and from the CT samples with AUC of 0.70. Additionally, genus *Neisseria* was common among the selected genera that contributed in distinguishing the IgAN samples from HC, CT and UC samples.

Conclusion:

There is a significant dysbiosis in salivary microbiome of IgAN patients. IgAN can be differentiated from related mucosal disease using biomarkers derived from the salivary microbiome. Therefore, salivary microbiome is a potential source of biomarkers for development of effective and non-invasive diagnostic tool for IgAN diagnosis.

Keywords: salivary microbiome; IgA nephropathy; oral microbiota; kidney disease; random forest algorithm.

3.1 Introduction

Chronic kidney disease (CKD) is a worldwide health issue affecting millions of individuals every year. According to the Japanese Society of Nephrology report in 2005, approximately 13.3 million people in Japan have CKD (Imai et al. 2009). In Japan, Immunoglobulin A nephropathy (IgAN) has been identified as one of the leading causes of CKD (Tomino 2016). IgAN also carries the reputation of being the most common form of primary glomerulonephritis reported globally (D'Amico 1987; Schena 1990). With 50% of IgAN patients progressing to end-stage renal disease (ESRD)(Moriyama et al. 2014), IgAN is a substantial health burden, especially in Japan. Japan is the home to the world's most rapidly aging population (Chen et al. 2019) and has witnessed an annual rise in the number of patients undergoing dialysis (Masakane et al. 2018). Even after more than 50 years since its first classification, the confirmed diagnosis of IgAN is only by kidney biopsy (Tomino 2016), which is an invasive diagnostic tool and possesses greater risk, especially for the elderly. Thus, research studies aiming to develop a non-invasive but effective diagnostic tool for IgAN is the need of the hour.

IgAN is an idiopathic disease, as indicated by the multi-hit hypothesis (Suzuki et al. 2011). Clinical diagnosis of IgAN includes detection of the differently glycosylated polymeric IgA1 (pIgA1) immune complex deposits in the kidney glomeruli by histopathology, along with microscopic or macroscopic hematuria and proteinuria by urinalysis (Sallustio et al. 2019). However, the trigger or maintenance of these immune reactions is still mostly unexplored. Genome-wide association studies (GWAS) on large patient cohorts have discovered loci that only account for about 5% of the disease risk in IgAN (Sallustio et al. 2019). Thus, apart from genetics, various other environmental factors (Sallustio et al. 2019) are thought to be associated with IgAN progression and pathogenesis. Among these, mucosal immunity is an essential factor in IgAN

pathogenesis as upper respiratory tract microflora is associated with recurrent macroscopic hematuria (Sallustio et al. 2019). The microbiota plays a vital role in the development of mucosal-associated lymphoid tissue (MALT), and in turn, MALT regulates the microbiota composition (Nakajima et al. 2018; Sallustio et al. 2019). IgA primarily originates from MALT, and GWAS of IgAN identified several risk loci involved in maintaining mucosal immunity (Sallustio et al. 2019). Oral mucosa is the gateway to the human body, and the salivary microbiome plays a crucial role in oral mucosal immunity (Moutsopoulos and Konkel 2018). Salivary microbiome dysbiosis often reflects inflammatory responses and microbiome changes in the gut (Abe et al. 2018; Bajaj et al. 2015). Members of the salivary microbiome are potential diagnostic biomarkers for immunological diseases such as rheumatoid arthritis (Chen et al. 2018), primary sclerosing cholangitis (Iwasawa et al. 2018), and pancreatic cancer (Coit et al. 2016; Torres et al. 2015). Also, saliva collection and storage are non-invasive, inexpensive, and pertain to lower risk (Hemadi et al. 2017). These factors can be advantageous in conducting salivary microbial profiling in IgAN patients to find non-invasive microbial biomarkers for effective low-risk diagnostics of IgAN.

The GWAS also revealed common risk loci between IgAN and IBD like Crohn's disease(CD) or ulcerative colitis(UC) (Sallustio et al. 2019). A Swedish population-based study reported that patients with IgA nephropathy were more likely to incur IBD (Rehnberg et al. 2021). The same study revealed that IgAN patients have 2.6 times higher risk of UC than those without IgAN (Rehnberg et al. 2021). A transgenic murine model of IgAN highlighted an essential dependence of signals from commensal microbiota for kidney IgA deposition in IgAN pathogenesis (McCarthy et al. 2011). Few studies have reported a change in the gut (De Angelis et al. 2014; Dong et al. 2020; Hu et al. 2020), salivary (He et al. 2021a; Luan et al. 2019; Piccolo et al. 2015),

periodontal(Cao et al. 2018) and tonsillar (Park et al. 2020) microbiota composition in IgAN patients from Caucasian and Asian origin. However, none of these studies have explored the underlying microbiome changes in IgAN versus IBD. To better understand the common underlying mechanisms in IgAN and IBD pathogenesis and to find out the reason behind co-occurrence of IgAN and IBD, we aimed to explore the microbiome profile associated with IgAN and UC in comparison to each other.

In Japan, tonsillectomy monotherapy or tonsillectomy paired with steroid pulse therapy is one of the most effective therapeutic regimens for early-stage primary IgAN patients (Nihei et al. 2017; Xie et al. 2003a). The reason behind the efficacy of this treatment is rooted in the association of focal tonsillar infection and a rise in pIgA secreting plasma cells in tonsils of IgAN patients (Meng et al. 2012). At the molecular level, galactose deficient IgA1 (Gd-IgA1) is one of the key players in the IgAN pathogenesis and its production is linked with overexpression of toll-like receptor 9 (TLR9) in the tonsils, especially after upper respiratory tract infections like tonsilitis (Moriyama et al. 2020). Activated TLR-9 leads to production of APRIL (a proliferation-inducing ligand) and BAFF (B cell activating factors from the TNF super family), which in turn activates B lymphocytes to generate Gd-IgA1 producing plasma cells (Moriyama et al. 2020; Suzuki 2019; Suzuki et al. 2011). Therefore, tonsillectomy cuts off the primary step in the Gd-IgA1 production and prevents disease progression and severity in IgAN(Moriyama et al. 2020). IgAN is also called synpharyngitic glomerulonephritis as often the onset of the disease is triggered by upper respiratory tract infection (Rodrigues et al. 2017). Some studies reported pathogenic bacteria, commonly associated with chronic tonsillitis (CT) and periodontitis, to be present in tonsillar crypts of IgAN patients (Jensen et al. 2013; Nagasawa et al. 2014; Watanabe et al. 2017). Despite the previous reports indicating periodontal pathogens involved in IgAN pathogenesis, there have

been no studies exploring the salivary microbiome profile of IgAN in comparison with CT patients.

In the current study, we performed 16S rRNA gene sequence-based analysis of the salivary microbiome of IgAN, UC, and CT patients and healthy subjects in a Japanese cohort. In our attempt to distinguish IgAN from other mucosal diseases (UC and CT), we identified a set of potential microbial biomarkers that can differentiate IgAN from other diseases and healthy individuals. According to the Ministry of Health, Labor, and Welfare 2005 survey in Japan, the male gender is one of the predictive factors of IgAN (Tomino 2016). Also, according to previous reports, gender may incur an effect on microbiome composition (He et al. 2021a). Thus, we also explored the gender-specific association of the salivary microbiome for IgAN patients.

3.2 Methods

a. *Sample collection and DNA extraction*

The study was approved by the ethics committees of Azabu University (029, 14 March 2013), Osaka university (2413, 29 September 2014), and RIKEN (H30-4, 29 August 2019). Any subject with history of oral disease, gastrointestinal or hepatobiliary surgery and antimicrobial usage within the past three months of the recruitment date were excluded from the study. Informed consents were obtained from the subjects before sampling and the study participants were instructed to restrain from eating or drinking for 2 hours prior to sampling. Depending on the inclusion and exclusion criteria of the study, saliva samples were collected from 43 IgAN patients (median age 39), 20 CT patients (median age 34.5), 33 UC patients (median age 47) and 65 HC (median age 37). After age and gender matching, 11 UC samples and 15 HC samples were removed from the current study which led to the median age for the UC samples to be 44.5 and for the HC samples to be 37.5 (**Table *8***). Please note that the subjects in the

IgAN and HC cohorts are the same as the ones included in the first part of the study presented in Chapter 2. IgAN and CT patients collected their saliva prior to their tonsillectomy surgery (**Table *8***).

Table 8. Demography of the subjects included in four different groups – IgAN, CT, UC and HC. Median age in terms of years is shown for each group along with IQR enclosed in parentheses. Small IQR value represents data points to be spread closer to median. IgAN, Immunoglobulin A Nephropathy; CT, Chronic Tonsillitis; UC, Ulcerative Colitis; HC, Healthy Control; IQR, interquartile range.

Demography	**IgAN (n=43)**	**CT (n=20)**	**UC (n=22)**	**HC (n=50)**
Age, years, median(IQR)	39(20.5)	34.5(14.5)	44.5(7.75)	37.5(8)
Male	20	13	11	36
Female	23	7	11	14

IQR:Inter-quartile range.

The saliva samples were transported to the laboratory at 4°C within 24 hours of collection. Upon reaching the laboratory the samples were immediately frozen using liquid nitrogen and stored at -80°C until further downstream analysis. The DNA extraction from these salivary samples was performed as described previously (Morita et al. 2007; Said et al. 2014). 1ml of saliva was centrifuged at 3,300g for 10 mins at 4°C. The resulting bacterial cell pellets were then suspended in 10mM Tris–HCl/10mM EDTA buffer and incubated with 15mg/ml lysozyme (Sigma-Aldrich Co. LLC) for 1 h at 37°C. Purified achromopeptidase (Wako Pure Chemical Industries, Ltd.) was added to the samples at a final concentration of 2000 units/ml before incubating the samples for an additional 30 mins at 37°C. The suspension was treated with 1% (wt/vol) sodium dodecyl sulphate (SDS) and 1 mg/ml proteinase K (Merck Japan) and incubated for 1h at 55°C. The resultant lysate was treated with phenol: chloroform: isoamyl alcohol

(25:24:1) (Life Technologies Japan, Ltd.) and centrifuged at 3,300g for 10mins at 4°C. To precipitate the microbial DNA, 1/10 volume of 3M sodium acetate (pH= 4.5) and 2 volumes ethanol (Wako Pure Chemical Industries, Ltd.) was added to the supernatant. The suspension was centrifuged at 3,300g for 15mins at 4°C and the resulting DNA pellets were rinsed with 75% ethanol, dried and dissolved in 10mM Tris–HCl/1mM EDTA (TE) buffer. For purification, the DNA samples were treated with 1 mg/ml RNase A (Wako Pure Chemical Industries, Ltd.) at 37°C for 30 min and precipitated by adding equal volumes of 20% polyethylene glycol solution (PEG6000-2.5MNaCl). These were centrifuged at 8,060g at 4°C and double-rinsed with 75% ethanol before drying the pellets. The dried pellets were then dissolved in TE buffer and stored at -20°C until polymerase chain reaction (PCR) steps followed by sequencing.

b. *16S rRNA amplicon library preparation and sequencing*

The salivary microbial DNA was sequenced using the 454 GS FLX Titanium or 454 GS Junior system (Roche Applied Science, Indianapolis, IN) according to the manufacturer's instructions (Said et al. 2014; Tsuda et al. 2015). Prior to the sequencing the 16S rRNA amplicon libraries were prepared by PCR amplification of the V1-V2 region of the 16S rRNA gene. For the PCR amplification, barcoded 27Fmod (5′-AGRGTTTGATYMTGGCTCAG-3′) and 338R (5′-TGCTGCCTCCCGTAGGAGT-3′) primers were used(Kim et al. 2013).

The protocol for PCR amplification was same as previously described (Tsuda et al. 2015). Briefly, 50 µl of PCR mix is composed of 10mM Tris-HCl (pH 8.3), 50mM KCl, and 1.5mM MgCl2 in the presence of 250 µM dNTP, 1 U Ex Taq polymerase (TakaraBio, Kyoto, Japan), forward and reverse primers (0.2 µM) and ~ 20 ng template DNA. At the thermal cycler, the PCR protocol consisted of initial denaturation at 96 °C for 2 min, followed by 25 cycles of denaturation at 96 °C for 30 s, annealing at 55 °C

for 45 s, and extension at 72 °C for 1 min, a final extension at 72 °C and hold at 4 °C on a 9700 PCR system (Life Technologies Japan, Tokyo, Japan).

Table 9. Statistics of 16S V1-V2 pyrosequencing of salivary microbiome samples

Total Reads	Filter-Passed Reads	Reads Removed		
		Reads lacking primer sequences	Reads with average Qv<25	Possible chimeric reads
1576683 (100)	809607 (51.3)	758604(48.1)	3683(0.2)	10478(0.01)

Note: The number in the parentheses indicate the ratio in percentage to the total read number.

The resultant PCR amplicons were then purified by AMPure XP magnetic purification beads (Beckman Coulter, Brea, CA, USA) and quantification using the Quant-iT PicoGreen dsDNA Assay Kit (Life Technologies Japan). Equal amounts of the PCR amplicons were pooled and used for the 16S rRNA sequencing.

c. *Data analysis*

The raw sequences were analyzed using a predesigned pipeline for analysis of 454 pyrosequencing data of the 16S rRNA gene V1-V2 region, as reported previously(Kim et al. 2013). Reads lacking both universal primers, low-quality reads (with an average quality score ≤ 25) and possible chimeric reads together accounted for 48-49% of total reads and were excluded from the downstream analysis (**Table *9***). After filtering, 2300 reads per sample were chosen randomly from the remaining high-quality reads for further analysis. The selected reads were sorted on the basis of their average quality score and grouped into OTUs using the UCLUST algorithm with 96% identity as threshold (Said et al. 2014). We conducted taxonomic assignments for each OTU by similarity searching against RDP, CORE and NCBI genome databases using the

GLSEARCH program. For taxonomic assignments at the phylum, genus and species levels, 70%, 94% and 96% sequence similarity thresholds were used, respectively.

We used Chi-squared test on the gender data across groups for gender-matching the samples. Similarly, for age-matching, we used the one-way ANOVA test on the demographic data of the samples. Beta-diversity was assessed by using the UniFrac distance metric (Lozupone et al. 2011) followed by PCoA to visualize the overall microbiome structure of the saliva samples. We used PERMANOVA to find the significance of beta-diversity between the groups, and the corresponding p-values were adjusted for multiple testing using Benjamin-Hochberg (BH) correction method. We assessed within-sample diversity or alpha diversity in terms of microbial richness and microbial diversity. In this assessment, the observed OTU number and Chao1 index were the measures to quantify microbial richness whereas Shannon's index quantified the microbial diversity of the samples. To check the statistical significance of the relative abundance of individual taxa, we used the Wilcoxon-test with BH correction for multiple comparisons for the data at different taxonomic levels, namely, OTU, phylum, and genus.

This protocol was also used for revealing gender-specific microbiome association in the study groups. Briefly, we first grouped the complete dataset into male and female sample. UniFrac weighted and unweighted metrics corresponding to this dataset was assessed using PERMANOVA in order to evaluate existence of gender-effect. Statistically significant groups (p-value < 0.05, Wilcoxon test) were further investigated in terms of microbial diversity as well as taxonomic profile of microbiome, specific to male and female samples in the groups.

To identify differentially abundant taxa between IgAN, CT, UC and HC groups at the genus and OTU levels, we used LEfSe tool

(http://huttenhower.sph.harvard.edu/lefse/) on the galaxy server (https://huttenhower.sph.harvard.edu/galaxy/). LEfSe identifies the features (in our case, taxa) which are most likely to explain differences between the groups in a dataset. For this LEfSe uses the non-parametric Kruskal-Wallis test and unpaired Wilcoxon rank sum test to evaluate the statistical significance of the features and pairs this with LDA method to estimate the effect relevance of each feature/taxa among the groups (Segata et al. 2011). For this study, LEfSe analysis was carried out with default parameters, i.e., alpha value was set to 0.05 for statistical analyses, non-negative threshold for the logarithmic LDA score was set to 2 and strategy for multi-class analysis was set to all-against-all (more stringent).

To identify a group of taxa among the selected biomarkers from the previous step (LEfSe analysis) which can act as potential biomarkers and classify IgAN from HC samples as well as other patients' samples (UC and CT), we used Area Under Cuver-Random Forest (AUC-RF) (version 1.1) (Calle et al. 2011) package. To generate RF models using AUC-RF we used the protocol previously described (Iwasawa et al. 2018). Brielfy, RF was built, using all the variables selected by LEfSe tool and here the variables are ranked in terms of their importance (which is depicted by the mean decrease Gini score). Subsequently, 5% of the least important features were eliminated and RF was built with the remaining variables. The AUC was calculated for each of the RF models and was based on the OOB predictions. The best model was the RF model with the best AUC value. The performance of the best model was confirmed by evaluating the mean AUC of a 10-fold cross-validation repeated 20 times using the AUC-RF (Calle et al. 2011) package. The AUC-RF based analyses were performed using the R (v3.6.1) within RStudio environment (v1.2.5019).

d. *Data availability*

The high-quality 16S V1-V2 sequences used in this study for downstream analysis were deposited on the DDBJ/GenBank/EMBL database (accession no. DRA002611, DRA002617, and DRA002618 (Tsuda et al. 2015), DRA011285 and DRA011286). Additionally, this chapter of the thesis is published as a research paper titled "Dysbiosis in the Salivary Microbiome Associated with IgA Nephropathy —A Japanese Cohort Study" (Khasnobish et al. 2021).

3.3 Results

a. *Summary of 454 reads*

We obtained a total of 1,576,683 high-quality 16S reads from the four groups by using 454 GS FLX Titanium platform (Roche Applied Science, Indianapolis, IN) (**Table *9***). After removing low quality and possibly chimera reads, we obtained a total of 809,607 reads from 135 samples. Of them, we randomly selected 2300 reads per sample (310,500 reads from 135 samples), and further analyzed them using the pipeline for 454 barcoded pyrosequencing of 16S amplicons in order to minimize overestimation of species richness in the clustering due to intrinsic sequencing error (Kim et al. 2013). The average Good's coverage index (Good 1953; Singleton et al. 2001) of the 2300 reads per sample was 0.97 (values ranging from 0.94 to 0.99), indicating a high degree of coverage. Hence, the sequence data was sufficient for analysis in the current study.

b. *Differences in alpha and beta diversity of salivary microbiota of IgAN, CT, UC and HC groups*

The salivary microbiome of the IgAN samples was compared with the CT, UC and HC samples based on their alpha and beta diversities. Richness and diversity are the two

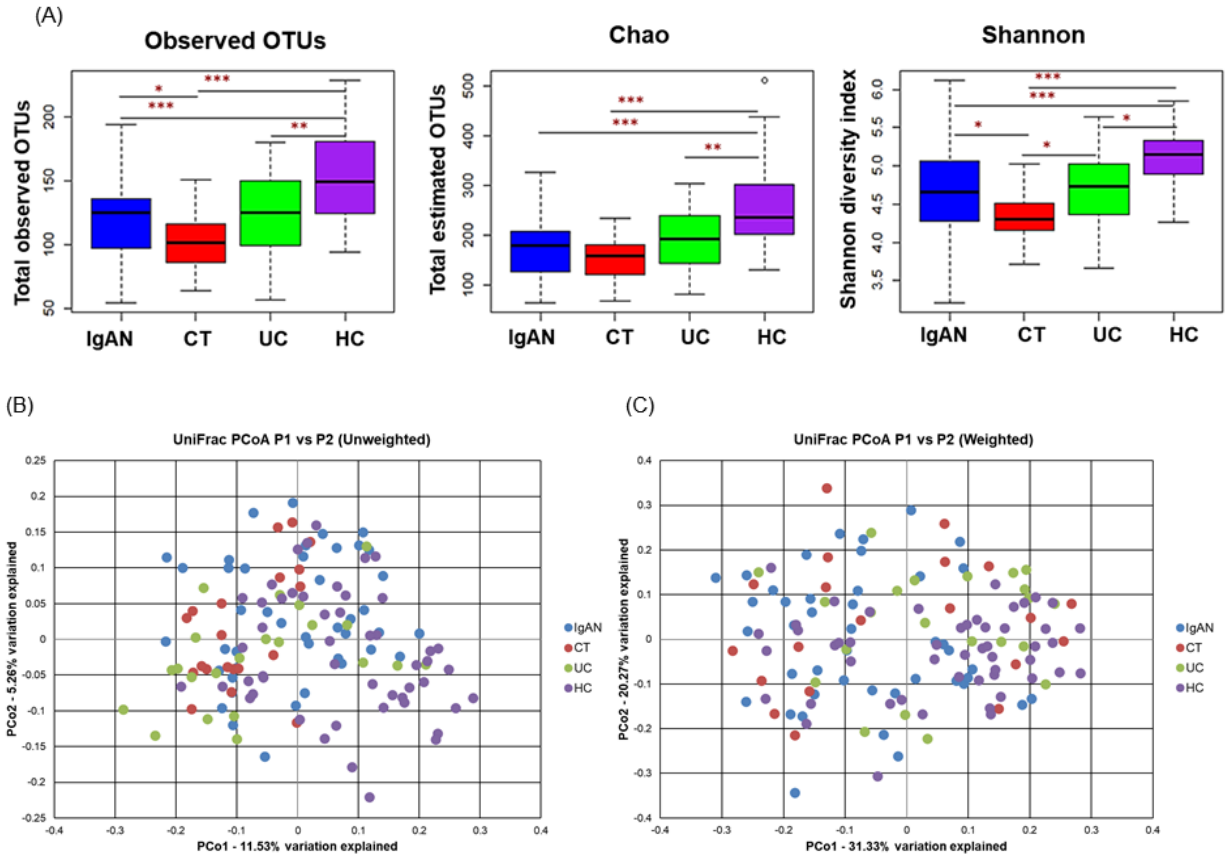

Fig. 12. Alpha and beta diversity in IgAN, CT, UC and HC subjects. Samples from 43 IgAN (blue), 20 CT (red), 22 UC (green) and 50 HC (purple) subjects are shown. (A) The observed and Chao1-estimated OTU numbers, and the Shannon index of salivary microbiome from the four groups. * p-value <0.05; ** p-value < 0.01; *** p-value <0.001 based on Wilcox test with Benjamin-Hochberg correction. (B) Weighted UniFrac–PCoA and (C) Unweighted UniFrac–PCoA of the salivary microbiome from the four groups. OTU, operational taxonomic unit; PCoA, principal coordinate analysis; IgAN, Immunoglobulin A Nephropathy; CT, Chronic Tonsillitis; UC, Ulcerative Colitis; HC, Healthy Controls.

most commonly used indices under alpha diversity. For this study, richness was estimated in terms of number of observed OTUs and Chao1 index whereas diversity

was estimated in terms of Shannon index. Similar to the findings in the last chapter, we observed a significant decrease in the number of observed OTUs as well as the Chao1 index in the IgAN samples than the HC samples (p = 5.31E-05 and 1.40E-06, respectively). The number of observed OTUs in the IgAN samples was significantly higher than the CT samples (p = 0.04407) (**Fig. 12*A***). There was no significant difference between the IgAN and the UC samples in terms of the observed OTU numbers (**Fig. 12*A***). The Chao1-estimated OTU numbers of the IgAN, CT, and UC samples were significantly lower than that of the HC samples (p= 1.40E-06, 6.79E-07, and 0.00459, respectively) (**Fig. 12*A***). There was no significant difference between the IgAN samples and the UC samples in terms of the Chao1-estimated OTU numbers (p>0.05). Similarly, the Chao1-estimated OTU numbers of the CT was lower than the

Table 10. Permutational multivariate analysis of variance (PERMANOVA) in salivary microbiome samples among the four groups – IgAN, CT, UC and HC.

Category	No. of Subjects	Weighted UniFrac		Unweighted UniFrac	
		R^2	Adjusted p-value	R^2	Adjusted p-value
CT vs HC	CT: 20 HC: 50	0.06	**0.006**	0.06	**0.001**
IgAN vs CT	IgAN: 43 CT: 20	0.02	0.217	0.03	**0.003**
IgAN vs HC	IgAN: 43 HC: 50	0.07	**0.003**	0.03	**0.001**
CT vs UC	CT: 20 UC: 22	0.04	0.179	0.04	**0.002**
UC vs HC	UC: 22 HC: 50	0.04	**0.043**	0.05	**0.001**
IgAN vs UC	IgAN: 43 UC: 22	0.06	**0.003**	0.04	**0.001**

The adjusted p-values were adjusted for multiple testing by Benjamin-Hochberg method. p-value <0.05 are in bold.

IgAN samples but the difference was not statistically significant (p>0.05). Shannon index, representing the within-sample microbial diversity, was significantly different among all the groups except between IgAN and UC samples (**Fig. 12*A***). The Shannon

index was significantly lower in the IgAN, CT, and UC samples than the HC samples (p= 0.00048, 4.34E-08, and 0.01055, respectively). Among the patient samples, the Shannon index of the CT was significantly lower than the IgAN and UC samples (p= 0.02099 and 0.01763, respectively) (**Fig. 12*A***).

Beta diversity, also known as between-sample diversity, is used to explain the variations in the microbiome structure between the samples. In our study we used weighted and unweighted UniFrac metrics to determine the beta diversity between IgAN, CT, UC and HC samples. PCoA of the unweighted and weighted UniFrac distance metrics of our dataset is shown in the (**Fig. 12*B, C***). In the unweighted plot, the largest principal coordinates were 11.53% and 5.26% of total variation, respectively, while they were 30.33% and 20.27%, respectively, in the weighted plot. PCoA based on the unweighted UniFrac distance metric showed that many of the IgAN, CT, and UC samples were segregated from HC samples (**Fig. 12*B***). According to the PERMANOVA, the IgAN and CT samples exhibited a significant difference based on the unweighted UniFrac metric ($p<0.01$) but there was no significant difference between the groups depending on the weighted UniFrac metric (**Table *10***). Additionally, the PERMANOVA results also indicate that the IgAN samples differed significantly from both HC and UC samples based on unweighted ($p <0.01$) as well as weighted ($p< 0.01$) UniFrac metrics, respectively (**Table *10***). When compared with the HC, the IgAN samples presented lower degree of dysbiosis than the CT and UC samples. This was evident from the R^2 values from the PERMANOVA on to unweighted UniFrac metrics (R^2 value= 0.03 (IgAN vs HC); 0.06 (CT vs HC); 0.05 (UC vs HC); **Table *10***).

c. *Variations in the salivary microbiome taxonomic profiles between the two groups*

Next, 310,500 reads from 135 samples were taxonomically assigned to 2782 OTUs using the phylotypes in the public microbial 16S rRNA gene databases. The major phyla with relative mean abundances of more than 0.1% across all the groups (IgAN, CT, UC and HC) accounted for 99.8% of the total abundances and consisted of Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, Fusobacteria, Candidatus Saccharibacteria (TM7), and Streptophyta. Firmicutes was the major phyla across all groups, with highest mean relative abundance in the IgAN samples but there was no significant difference in terms of mean relative abundance of Firmicutes amongst the groups ($p>0.05$). Bacteroidetes was significantly less abundant ($p = 0.0014$), whereas Proteobacteria was significantly more abundant ($p = 0.0021$) in the IgAN samples than the HC samples. The relative mean abundance of Actinobacteria was significantly higher in the UC samples than the IgAN and HC samples ($p = 0.0256$ and 0.0085, respectively). Candidatus Sachharibacteria (TM7) was significantly less abundant in the CT samples than the IgAN and HC samples ($p = 0.0074$ and 3.75909E-05, respectively). Given the higher relative mean abundances of Firmicutes corresponding with lower relative mean abundances of Bacteroidetes in the disease groups (IgAN, CT and UC), the Firmicutes/Bacteroidetes ratio was higher in the disease samples than the HC samples. However, the Firmicutes/Proteobacteria ratio was lower in the disease samples than the HC samples.

The taxonomic assignment at the genus level identified 270 bacterial genera. 51 out of these 270 genera had a relative mean abundance of more than 0.1% and accounted for 97.8% of the total abundance. *Streptococcus* was the highest in abundance in the IgAN samples followed by CT, UC and HC samples, but there was no significant difference amongst the groups in terms of its mean relative abundance. *Neisseria* was significantly more abundant in the IgAN samples than the HC and UC

samples (p = 0.0005 and 0.0032, respectively), whereas *Prevotella, Megasphaera*, and *Solobacterium* were significantly less abundant in the IgAN samples compared to the HC and UC samples (p = 0.0019, 0.0335, 0.0241, 0.0261, 0.0417 and 0.0391, respectively). *Stomatobaculum* was significantly more abundant in the IgAN samples than the CT and UC samples (p= 0.0385 and 0.005, respectively). However, *Stomatobaculum* significantly depleted in abundance in the IgAN samples as compared to the HC samples (p = 0.0008). Similarly, the abundance of *Peptostreptococcus* in the IgAN samples was significantly lower when compared to the HC samples (p = 0.0044) but significantly higher when compared to the CT samples (p = 0.0431). *Peptococcus* was significantly more abundant whereas *Schaalia* was significantly less abundant in the IgAN samples than the CT samples (p= 0.0361 and 0.0147, respectively). *Schaalia* was also lower in abundance in the IgAN samples than the UC samples (p = 0.0017). *Actinomyces* and *Selenomonas* were significantly less abundant, whereas *Gemella* was significantly more abundant in IgAN samples than the UC samples (p = 0.0020, 0.0006, and 0.0098, respectively).

Additionally, there were some similar patterns regarding genus abundance in the patient samples (IgAN, CT and UC) compared to the HC samples. *Stomatobaculum, Staphylococcus, Cutibacterium*, and *Peptostreptococcus* significantly decreased in abundance in all the patient samples (IgAN, UC, and CT) as compared with the HC samples (respective p-values < 0.05). Similarly, the genera, namely, *Veillonella, Solobacterium*, and *Corynebacterium*, showed a significant decrease in abundance in the IgAN and the CT samples than the HC samples (respective p-values < 0.05). The mean relative abundance of *Enhydrobacter* genera was significantly lower in the IgAN and the UC samples than the HC samples (p = 0.0021, and 0.0457, respectively).

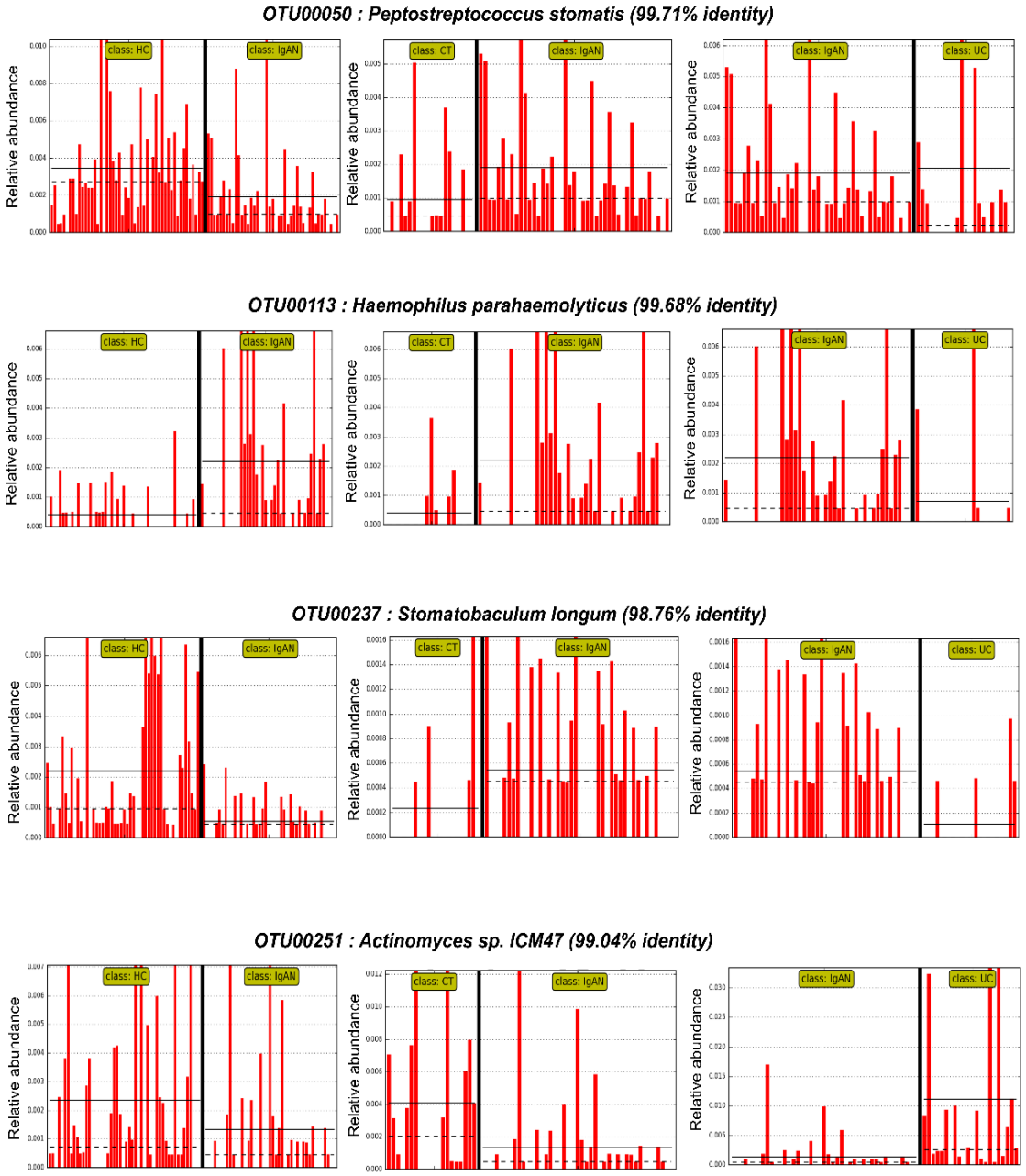

Fig. 13. Common differential features between IgAN and remaining three groups (CT, UC and HC) from LefSe analysis. With threshold LDA score >2, differentially abundant taxa were identified between IgAN vs CT, IgAN vs UC and IgAN vs HC groups. The four common taxa at the OTU level are shown in here. Individual red bars represent relative abundance of the taxa in a sample. LefSe, Linear discriminant analysis (LDA) effect size; IgAN, Immunoglobulin A Nephropathy; CT, Chronic Tonsillitis; UC, Ulcerative Colitis; HC, Healthy Controls.

To further evaluate the salivary microbiome differences among patients with IgAN, CT,

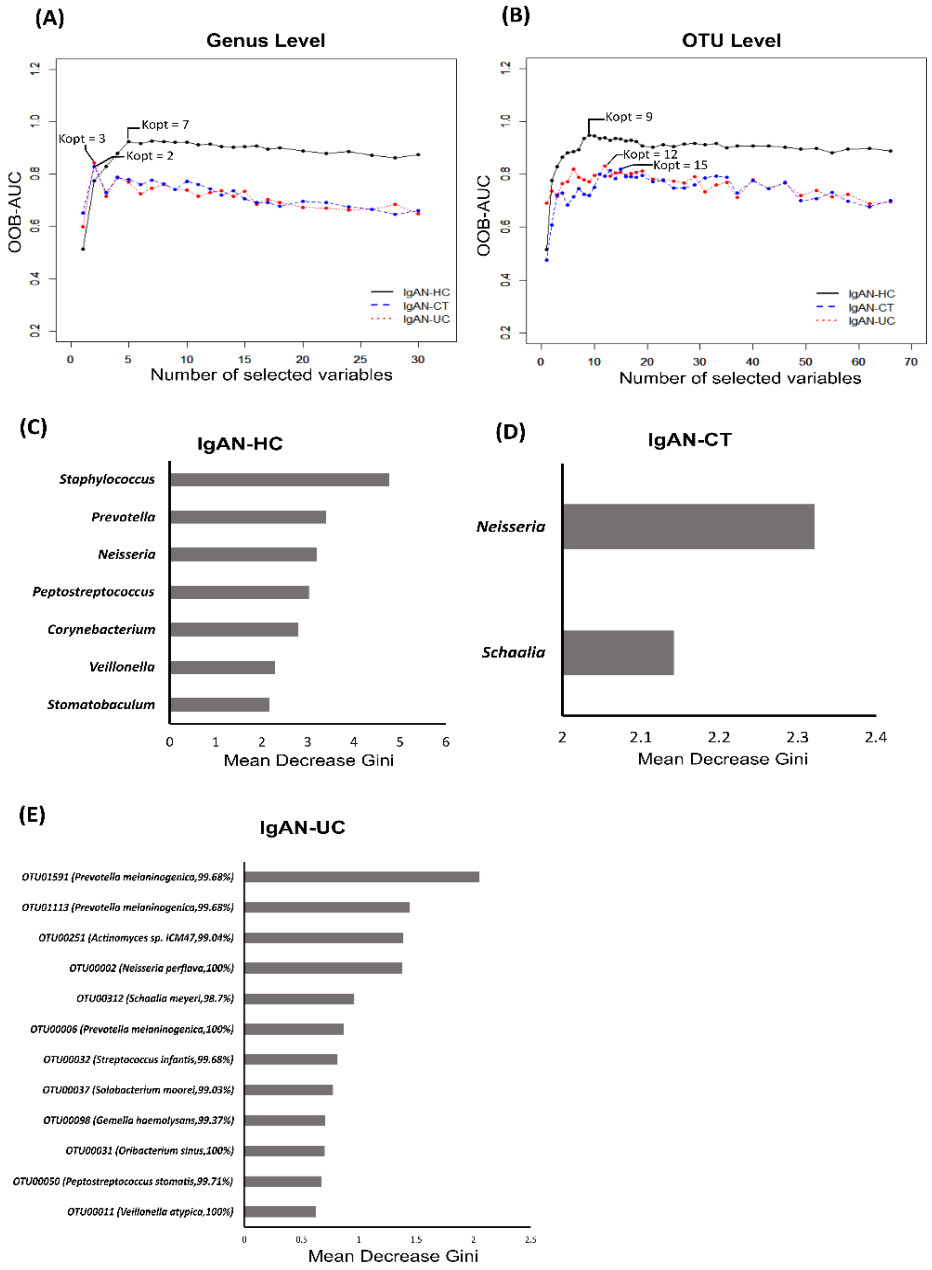

Fig. 14. Random Forest (RF) analysis of the salivary microbiota of the four groups using AUC-RF package at OTU level. Best RF models (comparison based on combination of best mean area under curve (AUC) value) was obtained at (a) Genus and (b) OTU level. The selected features of best RF models for (c) IgAN-HC, (d)IgAN-CT and (e)IgAN-UC comparisons are shown. Kopt = optimal number of features to distinguish between the two groups under comparison; IgAN, Immunoglobulin A Nephropathy; CT, Chronic Tonsillitis; UC, Ulcerative Colitis; HC, Healthy Controls.

UC, and the healthy subjects (HC), the LEfSe method was used to identify significant

discriminative features between the groups. With the logarithmic LDA score threshold set at 2, at the OTU level, we identified 19, 33, and 36 differential taxa between IgAN and CT, IgAN and UC, and IgAN and HC samples, respectively (Fig. S1). Among these, four taxa, namely, OTU00113 (99.68% similarity with *Haemophilus parahaemolyticus*), OTU00237 (98.76% similarity with *Stomatobaculum longum*), OTU00251 (99.04% similarity with *Actinomyces sp. ICM47*) and OTU00050 (99.71% similarity with *Peptostreptococcus stomatis*) were common differential features between IgAN and remaining three groups (CT, UC and HC) (**Fig. 13**).

To determine the potential value of the identified microbial biomarkers to clinically differentiate the IgAN group from disease (CT and UC) and the HC samples,

Table 11. The mean area under curve for salivary microbiome samples among the IgAN, CT, UC and HC groups.

Category	cvAUC	
	OTU Level	**Genus Level**
IgAN vs HC	0.88	0.90
IgAN vs CT	0.62	0.707
IgAN vs UC	0.88	0.851

The mean AUCs (cvAUC) of the 10-fold cross validation process repeated 20 times using the best RF model in the AUC-RF package are shown here. cvAUC, mean area under curve from 20 repetitive 10-fold cross validation of random forest model; RF, Random Forest.

we generated random forest (RF) models using the AUC-RF package. We generated models for two taxa levels (genus and OTU) (**Table *11***). We used the AUC of the receiver operative curve (ROC), that was in turn based upon the OOB error rate, to

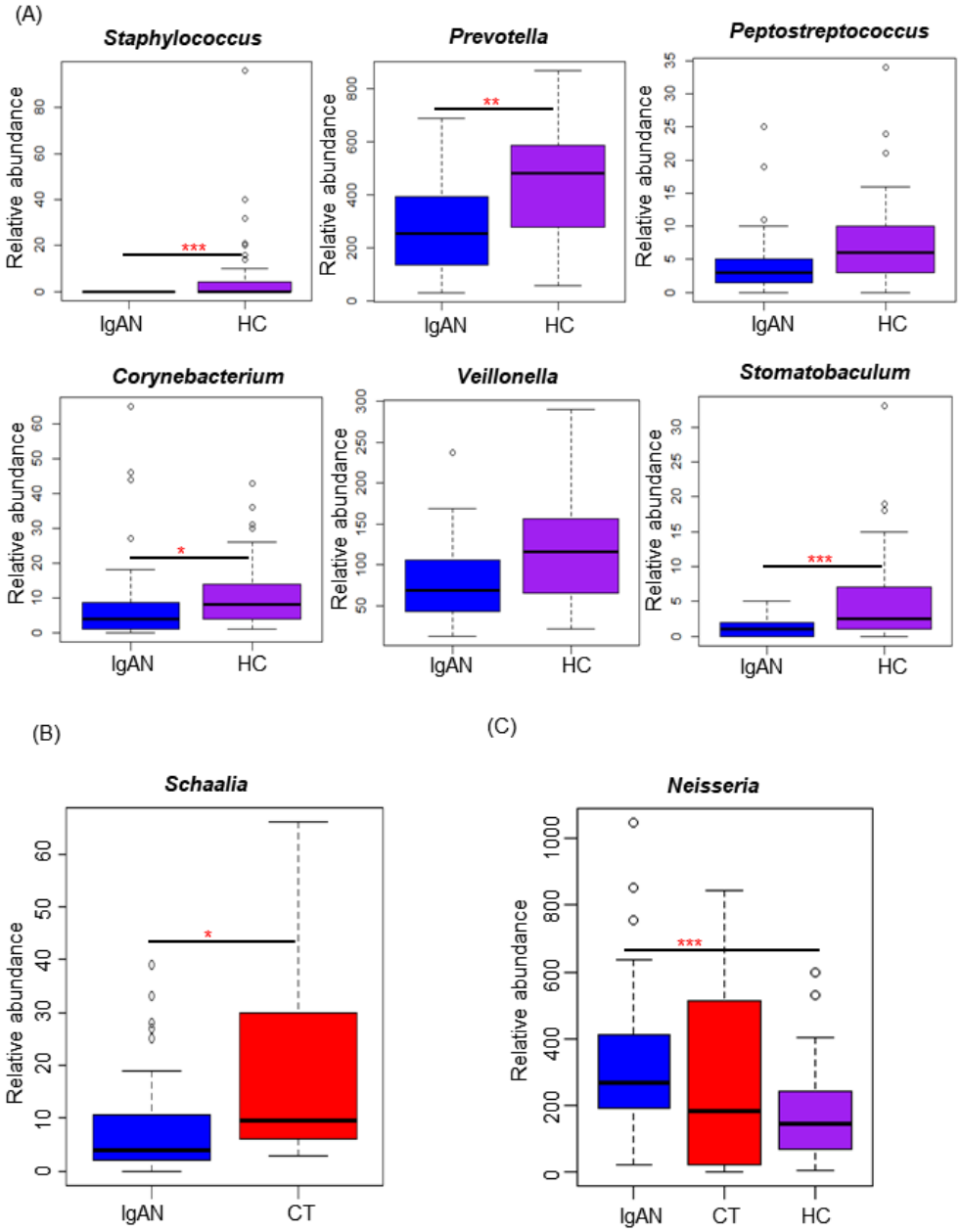

Fig. 15. Genus level variables from Random Forest (RF) analysis of the salivary microbiota using AUC-RF package. The mean relative abundance of taxa selected by AUC-RF are shown here.(A)Six unique genera selected for IgAN vs HC model; (B) One unique genera selected for IgAN vs CT model; and (C)Common genera selected for IgAN vs HC and IgAN vs CT model . * p-value <0.05; ** p-value < 0.01; *** p-value <0.001 based on Wilcox test with Benjamin-Hochberg correction. IgAN=blue, CT=red, and HC=purple. IgAN, Immunoglobulin A Nephropathy; CT, Chronic Tonsillitis; HC, Healthy Control; OTU, operational taxonomic unit.

find the combination of multiple taxa from the 30 genera selected by the LefSe tool as

potential features which can contribute in differentiating IgAN group from the rest. The models with best AUC values at the genus level were observed for 7, 2, and 3 genera between the IgAN and HC, the IgAN and CT, and the IgAN and UC samples, respectively (**Fig. 14*A***). Among these selected genera, *Neisseria* was a common contributor for distinguishing the IgAN samples from HC, CT, and UC samples, and *Schaalia* was a common contributor for distinguishing the IgAN samples from CT and UC samples (***Fig. 15C***). *Neisseria* was significantly higher in abundance in the IgAN than the HC and the UC samples (p= 0.0005, and 0.0032 respectively), whereas there was no significant difference in terms of its abundance between IgAN and CT samples. *Schaalia* was significantly less abundant in the IgAN samples than the CT and UC samples (p = 0.01 and 0.002, respectively) (**Fig. 15*B***). Out of 7 genera, 6 (*Staphylococcus, Prevotella, Peptostreptococcus, Corynebacterium, Veillonella* and *Stomatobaculum*) solely contributed to distinguishing the IgAN samples from the HC samples (**Fig. 15**). Out of the 3 genera, genus *Selenomonas* only contributed to distinguishing the IgAN samples from the UC samples. We confirmed the results by 10-fold cross-validation of the AUC-RF models repeated 20 times and obtained the mean AUCs, which were 0.90 between the IgAN and HC samples, 0.707 between the IgAN and CT samples, and 0.851 between the IgAN and UC samples (**Table *11***).

At the OTU level, we used 66 differential OTUs selected by the LefSe tool to build the RF model. The models with best AUC values had 9, 15, and 12 OTUs between the IgAN and HC samples, the IgAN and CT, and the IgAN and UC samples, respectively (**Fig. 14*B***). Out of these OTUs, OTU00002 (100% similarity with *Neisseria perflava*) and OTU00050 (99.71% identity with *Peptostreptococcus stomatis*) were common contributors in distinguishing the IgAN samples from the healthy (HC) as well as patient samples (CT, and UC). OTU00002 (100% similarity with *Neisseria perflava*)

was significantly higher in abundance in the IgAN samples than the HC and UC samples (p= 0.0002 and 0.0060, respectively), but the difference between its abundance in the IgAN and CT samples was not significant (p > 0.05). UC and HC samples had significantly higher whereas the CT samples had a significantly lower abundance of the OTU, OTU00050 (99.71% identity with *Peptostreptococcus stomatis*), than the IgAN samples (p = 0.02, 0.01, and 0.03, respectively). Four OTUS, OTU00312 (98.7% similarity with *Schaalia meyeri*), OTU00251 (99.04% similarity with *Actinomyces sp. ICM47*), OTU00011 (100% similarity with *Veillonella atypica*), and OTU00031 (100% similarity with *Oribacterium sinus*) were the common contributors in distinguishing the IgAN group from the other two disease (CT and UC) samples. OTU00312 (98.7% similarity with *Schaalia meyeri*) was significantly lower in abundance in the IgAN samples as compared to CT and UC samples (p = 0.005 and 0.005, respectively). While OTU00251 (99.04% similarity with *Actinomyces sp. ICM47*) had significantly lower abundance, OTU00031 (100% similarity with *Oribacterium sinus*) had significantly higher abundance in the IgAN samples than UC group (p = 0.0005 and 0.04, respectively). There was no significant difference between IgAN and the other two disease groups in terms of the abundance of OTU00011 (100% similarity with *Veillonella atypica*). The mean AUC of the 10-fold cross-validation repeated 20 times for the best AUC-RF models was 0.88 between the IgAN and HC samples, 0.62 between the IgAN and CT samples, and 0.88 between the IgAN and UC samples (**Table *11***).

d. *Gender-specific microbiome association*

As we observed in the previous chapter there was an underlying gender-specific microbiome structure in IgAN and HC samples. Therefore, we aimed to check whether any such structure presents itself when we are comparing the four samples – IgAN, CT, UC and HC in terms of the gender distribution amongst the samples. To check whether there is any underlying gender-specific association of the salivary microbiome, we performed a PERMANOVA test where the samples were grouped according to their genders. PERMANOVA test showed a significant difference between male and female groups with respect to weighted and unweighted UniFrac metrics irrespective of the disease-state grouping (**Table *12***). Upon further analyzing the dataset group-wise, there was a significant difference between male and female samples in the IgAN samples for weighted UniFrac metric and in the HC samples for unweighted UniFrac metric only (**Table *12***). However, the CT and UC samples didn't exhibit any gender-specific microbiome structure. These findings match our findings in the previous

Table 12. Permutational multivariate analysis of variance (PERMANOVA) in salivary microbiome samples in the four groups to check gender effect. The adjusted p-values were adjusted for multiple testing by Benjamin-Hochberg method. p-value <0.05 are in bold.

Category	No. of subjects	Weighted UniFrac		Unweighted UniFrac	
		R^2	p-value	R^2	p-value
CT(male vs female)	male:13 female:7	0.12155	0.06194	0.05878	0.2198
HC(male vs female)	male:36 female:14	0.01044	0.8362	0.03011	**0.04196**
UC(male vs female)	male:11 female:11	0.05306	0.3087	0.04002	0.7413
IgAN(male vs female)	male:20 female:23	0.05431	**0.03696**	0.02829	0.1748
All(male vs female)	male:80 female:55	0.02927	**0.003996**	0.01096	**0.02697**

chapter which accounts for detailed gender-specific microbiome structure in the IgAN and HC samples.

3.4 Discussion

The human microbiome is closely associated with mucosal immunity, and there have been reports about the gut, tonsil, periodontal and salivary microbiota associated with IgAN (Cao et al. 2018; De Angelis et al. 2014; Dong et al. 2020; Hu et al. 2020; Luan et al. 2019; Nagasawa et al. 2014; Park et al. 2020; Piccolo et al. 2015; Watanabe et al. 2017). However, despite Japan being the second highest in the world in terms of incidence and frequency of IgAN (Schena and Nistor 2018), the IgAN associated microbiota studies for the Japanese cohort are limited. Our present study represents the characterization of salivary microbiome of patients with IgAN in comparison with patients with CT and UC, and healthy subjects. To the best of our knowledge, this is the first study to characterize salivary microbiome of Japanese IgAN patients.

In this study, we observed altered salivary microbial composition in the IgAN patients as compared to the healthy subjects, indicated by the lower species richness and microbial diversity in the IgAN patients' salivary microbiota. Similar trend was observed in salivary microbiome composition of CT and UC patients. Similarly, salivary microbial richness and diversity were lower but not significant ($P>0.05$) in IgAN than HC in the Caucasian population(Piccolo et al. 2015) whereas a Chinese population based study of IgAN salivary microbiota did not report any significant difference(Luan et al. 2019). In fecal microbiome studies associated with IgAN, few studies reported similar findings of lower microbial richness(De Angelis et al. 2014; Hu et al. 2020) and diversity(De Angelis et al. 2014) in IgAN compared to HC, whereas another study did not find any significant difference(Dong et al. 2020). The observed differences might be rooted in the variability of study design, like using different hypervariable regions for

16S rRNA sequencing, using different sequencing platforms, sample size, or ethnicity differences. Statistical analysis of the phylogeny-based weighted and unweighted UniFrac metrics shows that the observed dysbiosis is associated with differences in the presence or absence of the microbial taxa as well as their abundance in the population. Therefore, as seen in Caucasian(De Angelis et al. 2014; Piccolo et al. 2015) and Chinese(Dong et al. 2020; Luan et al. 2019) population studies, our study demonstrates microbial dysbiosis in IgAN patients. From PERMANOVA analysis of unweighted UniFrac distance metrics, IgAN and CT samples differ significantly only in terms of microbial taxa composition. In contrast, both the membership and abundance of the microbial taxa contribute to a significant difference between IgAN and UC samples. Thus, we can infer that the CT and IgAN samples's overall microbial population is similar. However, as this is the first instance of comparing salivary microbiome of IgAN, CT and UC patients, a detailed and larger population-based study exploring relationships among these diseases in terms of microbiome is required for more concrete understanding.

There were differences at the taxonomic level between the IgAN and the rest of the samples. The phylum Firmicutes dominated all the samples and was most abundant in the IgAN, following previous studies on fecal and salivary microbiota studies of IgAN patients(De Angelis et al. 2014; Hu et al. 2020; Luan et al. 2019). The Firmicutes/Proteobacteria ratio was lower in the IgAN group as compared to the HC samples. This ratio agrees with the previously reported salivary microbiota study on the Caucasian population (Piccolo et al. 2015) and periodontal microbiome study on the Asian population(Cao et al. 2018) but is in contrast with the salivary microbiota study in Chinese cohort(Luan et al. 2019).

Our study reported an increase in Firmicutes/Bacteroides ratio in the UC group compared to the HC group, which is similar to the findings of a few gut microbiome studies (Kabeerdoss et al. 2015; Xun et al. 2018). Also, being in line with a meta-analysis study of the gut microbiota composition in IBD (Walters et al. 2014) and salivary microbiome ecotypes associated with UC (Xun et al. 2018), we observed a significant enrichment of Phyla Actinobacteria in the UC group as compared to IgAN and HC. This confirms that abnormal physiological state is associated with microbial dysbiosis in gut inflammatory diseases (Xun et al. 2018).

The significant differences between IgAN group and the remaining three groups (CT, UC and HC) in terms of microbial taxa prompted us to investigate the selected biomarkers for discriminating IgAN samples from other diseases as well as healthy controls. In our study, a combination of salivary taxa separated IgAN from healthy individuals with AUC of 0.88 and 0.90 at OTU and genus levels, respectively. A Chinese cohort study reported separation of IgAN from HC with predictive accuracy up to 80% upon using salivary microbial OTUs in combination with biochemical characteristics (Luan et al. 2019). These results indicate that salivary microbiome derived biomarkers can be used for predictive diagnosis of IgAN from healthy population.

Neisseria was a common contributor in distinguishing IgAN from healthy individuals and CT patients. This genus is enriched in the IgAN samples when compared with the disease as well as healthy samples, similar to the findings of a previous study(Piccolo et al. 2015). Also, at the OTU level, some OTUs belonging to the *Neisseria* genus were among the selected taxa for differentiating IgAN from the UC samples and they were significantly enriched in IgAN patients. Previous salivary microbiome studies report the co-occurrence of *Neisseria* and *Haemophilus* in salivary ecosystem(De Angelis et al. 2014; Takeshita et al. 2016). Salivary community type

comprising of *Neisseria*, *Haemophilus, Gemella, Porphyromonas,* and *Streptococcus mitis* along with reduced phylogenetic diversity was associated with better periodontal health(Takeshita et al. 2016). A salivary microbiome study of IBD patients reported that elevated salivary IgA levels were correlated with lower abundance of *Neisseria*, *Haemophilus, Gemella* and *Streptococcus (Said et al. 2014)*. Salivary IgA levels are elevated in IgAN patients according to scientific literature (Yamabe et al. 1987). In our study, abundance of *Neisseria*, *Haemophilus, Gemella* and *Streptococcus* genera increased in the IgAN patients. Therefore, we can infer that abundance of *Neisseria*, *Haemophilus, Gemella* and *Streptococcus* genera are not directly associated with salivary IgA response in IgAN pathogenesis. This can be attributed to difference in the disease pathogenesis of IgAN and IBD. However, as this is the first instance of comparison between IgAN and UC associated salivary microbiome and given the lack of data corresponding to salivary IgA levels of the IgAN patients in our study, the underlying relationship of salivary IgA and microbiome in light of these two diseases has a scope of future research.

Some of the OTUs belonging to the genus *Gemella* differentiated IgAN from UC samples (Fig. S1(B)). Unlike previous study results (Piccolo et al. 2015), this taxa was relatively more abundant in IgAN patients than UC and HC samples. Such differences in IgAN salivary microbiome across population supports the heterogeneity of IgAN pathology across different ethnicities (Yeo et al. 2018a).

Prevotella is one of the selected genera that contributes to differentiating IgAN patients from healthy controls. In line with previous reports(Cao et al. 2018; Luan et al. 2019; Piccolo et al. 2015), there was significant enrichment of *Prevotella* in HC than IgAN samples. The Chinese fecal microbiome study comparing the IgAN patients, membranous nephropathy patients, and healthy controls noted a positive correlation

between *Prevotella* and a higher serum albumin level. Serum albumin plays an essential role in reducing oxidative stress in the mesangial cells by attenuating the reactive oxygen species like hydrogen peroxide, thereby lowering the risk of IgAN progression towards ESRD (Kawai et al. 2018). From this, we can infer that *Prevotella* genera has a protective role in the salivary and gut ecosystem, and a decline in its abundance may facilitate systemic disease progression like IgAN.

Though OTU clustering methods in our study is not sufficient for species level assignment, we found several OTUs assigned to *Prevotella melaninogenica* species, with more than 99% identity, which were among the selected taxa for distinguishing IgAN patients from UC patients and these were significantly enriched in UC patients than IgAN patients. Even though *P. melaninogenica* is identified as a member of the Japanese salivary core microbiome (Takeshita et al. 2016), various studies have associated *P. melaninogenica* with oral and systemic diseases (Brook 1995; Kondo et al. 2018; Sibley et al. 2011). In blood mononuclear cells of IgAN patients (Chang and Li 2020; Saito et al. 2016) and in dendritic cells of IBD patients (Crohn's disease and UC)(Hug et al. 2018), the TLR2 expression increases. Since *P. melaninogenica* reportedly stimulates cytokine response via TLR2 pathways (Kondo et al. 2018), it has the potential to contribute to the progression of inflammatory disorders like UC and IgAN. On the other hand, the depletion of CD4+ T cells correlated with an increase of *P. melaninogenica* in salivary microbiota of HIV patients (Lewy et al. 2019). Thus, lower abundance of *P. melaninogenica* in IgAN patients in our study implied the autoimmune nature of IgAN, and further investigation is needed to clarify the role of this genera in T helper cell imbalance in IgAN.

Under Firmicutes phyla, *Streptococcus* is the predominant genera, with highest abundance in the CT samples, followed by the IgAN samples. In previous reports,

Streptococcus was enriched in the subgingival microbiome of chronic periodontitis patients with IgAN (Cao et al. 2018) and is also one of the core members of tonsillar crypt microbiome (Jensen et al. 2013). 16S rDNA reads of *Streptococcaeae* family increased in the fecal microbiome of IgAN patients with persistent proteinuria(De Angelis et al. 2014). Another study reported an increase in the cell surface collagen-binding Cnm protein of *Streptococcus mutans* in tonsillar specimens of IgAN patients compared to CT patients (Ito et al. 2019). This study also reported an association between the presence of Cnm-positive *S. mutans* strain with greater severity of symptoms in IgAN patients, marked by higher proteinuria and lower serum albumin levels(Ito et al. 2019). In our study, we found the mean relative abundance of *S. mutans* was higher in IgAN than CT samples (though $p>0.05$). These evidences along with findings such as presence streptococcal proteins in the renal tissues from IgAN patients (Schmitt et al. 2010) indicate that bacterial causal agents of focal tonsillar infection, like *Streptococcus*, can be associated with IgAN pathogenesis.

Several OTUs belonging to *Haemophilus* genus vary significantly only between the IgAN and UC samples. Some members of *Hemophilus* genus, like *H. parahaemolyticus*, are commensal microflora in the upper respiratory tract, and literature suggests these are to be considered as opportunistic pathogens capable of causing invasive and severe diseases (Le Floch et al. 2013). *H. parahaemolyticus* belongs to the *H. parainfluenzae* samples and is one of the few species in *Haemophilus* genera like *H. influenzae,* producing IgA1 protease that attributes to its pathogenicity in its human host (Norskov-Lauritsen 2014). Piccolo *et al.* reported an increase of *H. parainfluenzae* in salivary microbiota of IgAN patients with the lowest proteinuria(Piccolo et al. 2015). Authors recommending the use of *H. influenzae* derived IgA protease for IgAN treatment state that lack of mesangial IgA1 specificity of the *H.*

influenzae derived IgA protease limits its immediate application for IgAN therapy (Eitner and Floege 2008). These observations suggest that some members of *Hemophilus* genera contribute to the early stages of IgAN pathogenesis. Further research based on these associative members might contribute towards a better understanding of the progression of IgAN and thus expedite the therapeutic applications.

In this study the microbial diversity and richness of salivary microbiome decreased significantly in UC patients compared to HC. This was in contrast to previous studies that reported no significant difference between these two groups in terms of richness or diversity(Said et al. 2014; Xun et al. 2018). Also, unlike the previous reports(Xun et al. 2018), Firmicutes/Bacteroidetes ratio decreased in UC samples compared to HC samples. Like previous reports(Said et al. 2014; Xun et al. 2018), *Oribacterium* decreased significantly in UC than HC. While our study and another study(Xun et al. 2018) reported enrichment of *Streptococcus* in UC patient's salivary microbiome, Said et al. (2014) reported a depletion of this genus in UC patients. Moreover, unlike the previous report (Said et al. 2014), in our study *Veillonella* was less abundant in UC samples than HC samples. We also noted a decrease in abundance of *Prevotella* in UC samples (though $P>0.05$). This was in line with the Chinese cohort study (Xun et al. 2018) where they reported a significant depletion in *Prevotella* in UC samples. However, Said et al. (2014) reported an increase in abundance of this genus in UC samples. The contrast in the observations between our study and previous study can be attributed to multiple factors like study design, sample size, and genetic history of the study subjects.

While some epidemiological studies observed a male predominance in North American and Western European populations(Barratt and Feehally 2014; Sukcharoen et al. 2020), others show an equal distribution of IgAN incidence between genders in

the Asian population(Cheng et al. 2013; Feehally and Barratt 2015; Lee et al. 2012). A single-center study in Japan showed that primary glomerulonephritis showed higher frequency in men as compared to women(Moriyama et al. 2010). In microbiome studies gender may act as a confounding factor as some show a significant difference between male and female microbiome(Minty et al. 2020; Raju et al. 2019). Thus, upon exploring existence of gender-associated microbiota in our cohort we found that unlike previous study on a cohort of Hans Chinese origin(He et al. 2021a), microbial richness of the IgAN male and IgAN female groups was significantly lower than corresponding HC male and HC female groups (**Fig. 10**). We also found that some genera had significant difference of abundance only between HC and IgAN males (***Fig. 11A***), whereas some others differed only between HC and IgAN females (***Fig. 11B)***. These observations suggest that microbiota may play a role in the gender-associated risk of IgAN disease progression and, further research in this field is essential.

The current study has some limitations. Firstly, our study included small and non-uniform sample size across the groups. The imbalance in dataset was not statistically significant and did not affect the microbiome analysis. Disease predictive modeling is affected by an imbalanced dataset; however, the random forest classifier is relatively robust when dealing with an imbalanced dataset(Dittman et al. 2015) and the AUC-RF package uses a random forest classifier for modeling. Secondly, data associated with stage of disease for IgAN patients is not available for clinical metadata correlation analysis. However, since tonsillectomy is recommended and effective in the early stages IgAN(Hotta et al. 2001) and the IgAN patients in the current study collected the saliva sample before their tonsillectomy surgery, it implies that patients were in the early stage of the disease. Lastly, our study design is limited by the lack of clinical data reporting the serum or salivary IgA levels in the IgAN patients. As secretory IgA plays

important role in IgAN pathogenesis, there is a scope for future microbiome study exploring the relationship between the secretory IgA levels and predominant microbiota associated with IgAN.

In conclusion, our findings from the current study, where we explored the variations in salivary microbiome profiles of IgAN patients, UC patients, CT patients and HC, indicate that the salivary microbiome is a potential biomarker source to develop a non-invasive diagnostic tool for IgAN. However, the biological role of the microbial biomarkers identified in this study in the pathogenesis and disease progression of IgAN is a scope of further research.

4 Summary

Summary

CKD is a worldwide health issue affecting millions of individuals every year. According to the Japanese Society of Nephrology report in 2005, approximately 13.3 million people in Japan have CKD(Imai et al. 2009). IgAN has been identified as one of the leading causes of CKD in Japanese population (Tomino 2016). With an incidence rate of 39-45 IgAN cases per million population per year and 50% of these patients progressing to ESRD (Moriyama et al. 2014), Japan is second highest in the world in terms of IgAN prevalence. Japan is the home to world's most rapidly aging population (Chen et al. 2019) and with annually rising numbers of dialysis patients (Masakane et al. 2018), IgAN is a significant health burden on the Japanese population. Even after more than 50 years since its first classification, underlying mechanisms of IgAN pathogenesis is poorly understood and the development of diagnostic methods independent of renal biopsy has been slow. IgA primarily originates from MALT, and GWAS of IgAN identified several risk loci involved in maintaining mucosal immunity (Sallustio et al. 2019). The salivary microbiome plays crucial roles in regulating mucosal immunity for local and systemic homeostasis (Moutsopoulos and Konkel 2018). Members of the salivary microbiome are potential diagnostic biomarkers for immunological diseases such as rheumatoid arthritis (Chen et al. 2018), primary sclerosing cholangitis (Iwasawa et al. 2018), pancreatic cancer (Coit et al. 2016; Torres et al. 2015) and CKD (Hu et al. 2018). Interestingly, the GWAS revealed common risk loci between IgAN and IBD (UC or CD) (Sallustio et al. 2019). IgAN patients have 2.6 times higher risk of UC than those without IgAN (Rehnberg et al. 2021). Some studies detected pathogenic bacteria, commonly associated with CT and periodontitis, in tonsillar crypts of IgAN patients (Jensen et al. 2013; Nagasawa et al. 2014; Watanabe et al. 2017). According to the Ministry of Health,

Labor, and Welfare 2005 survey in Japan, the male gender is one of the predictive factors of IgAN (Tomino 2016). Also, according to previous reports, gender may incur an effect on microbiome composition (He et al. 2021a). Thus, we also explored the gender-specific association of the salivary microbiome for IgAN patients.

To address the gaps in IgAN research, this study aimed to investigate the salivary microbiome profile of IgAN patients in comparison with that of age-, gender-matched healthy individuals in Japanese population. Second objective of this research study was to compare the salivary microbiome profile of IgAN patients with related mucosal diseases like CT and UC. Lastly, we aimed to investigate the salivary microbiome as a tool for the non-invasive diagnosis of IgAN.

The first study aimed to compare the salivary microbiome profile of IgAN patients with that of HC in a Japanese cohort. Saliva was collected from 43 IgAN patients and 50 age- and gender matched HC. The hypervariable V1–V2 regions of 16S rRNA gene were purified from all the samples and amplified using 27Fmod and 338R primers. The salivary microbial profile of IgAN and HC samples were investigated by using high-throughput 454-pyrosequencing technology and bioinformatic analyses pipelines. Statistical analyses were conducted on R software. We used Linear discriminant analysis effect size (LEfSe) tool and R-based AUC-RF package to identify microbial biomarkers for differentiating IgAN from HC. Alpha diversity parameters for richness and diversity (Observed Operational Taxonomic Units, Chao1, Shannon) were significantly lower in than HC samples ($p<0.05$, wilcoxon test). At the phylum level, Bacteroidetes significantly decreased whereas Proteobacteria significantly increased in abundance in IgAN samples compared with HC samples. Using LEfSe followed by AUC-RF, we were able to identify a group of 7 genera (*Staphylococcus, Prevotella, Neisseria, Peptostreptococcus, Corynebacterium, Stomatobaculum* and *Veillonella*) to

differentiate IgAN from HC samples, with area under curve of 0.90. Additionally, there was a significant difference between IgAN male and HC male samples in terms of the microbiome structure and composition. A similar difference was also observed between IgAN female and HC female samples. There is a significant dysbiosis in salivary microbiome of IgAN patients. Salivary microbiome is potential source of biomarkers for development of effective and non-invasive diagnostic tool for IgAN diagnosis and association of microbiome and gender in the context of IgAN patients should be explored in future studies. To our best knowledge, this is the first report of salivary microbiome profile of Japanese IgAN patients.

The second study aimed to explore the salivary microbiome profile of IgAN patients in comparison with salivary microbiome profiles of chronic tonsilitis patients, ulcerative colitis patients and healthy controls. Saliva was collected from 43 IgAN patients (IgAN), 50 healthy controls (HC), 20 chronic tonsilitis patients (CT) and 22 ulcerative colitis patients (UC). The hypervariable V1–V2 regions of 16S rRNA gene were purified from all the samples and amplified using 27Fmod and 338R primers. The salivary microbial profile of all the samples were investigated by using high-throughput 454-pyrosequencing technology and bioinformatic analyses pipelines. Statistical analyses were conducted on R software. We used LEfSe tool and R-based AUC-RF package to identify microbial biomarkers for differentiating IgAN from mucosal diseases (CT and UC) and HC. We noted a significant difference in microbial diversity and richness between IgAN patients and HC. IgAN and CT differed significantly in terms of both microbial diversity and richness whereas there was no significant difference in terms of alpha diversity between IgAN and UC. PERMANOVA of UniFrac distance metrics to quantify the beta-diversity, we found that when compared with the HC samples, the IgAN samples presented lower degree of dysbiosis than the other two

disease groups (CT and UC). Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, Fusobacteria, Candidatus Saccharibacteria (TM7), and Streptophyta were the major phyla with relative mean abundances of more than 0.1% across all the groups (IgAN, CT, UC and HC) and accounted for 99.8% of the total abundances. Combining the genera selected by the random forest algorithm, we were able to distinguish the IgAN from the HC samples with the area under the curve (AUC) of 0.90, from the UC samples with the AUC of 0.88, and from the CT samples with AUC of 0.70. Additionally, genus *Neisseria* was common among the selected genera that contributed in distinguishing the IgAN samples from HC, CT and UC samples. IgAN can be differentiated from related mucosal disease using biomarkers derived from the salivary microbiome. Therefore, salivary microbiome is a potential source of biomarkers for development of effective and non-invasive diagnostic tool for IgAN diagnosis.

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Acknowledgement

‘*Gratitude can transform common days into thanksgivings, turn routine jobs into joy and change ordinary opportunity into blessings.*’

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