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Gene expression levels of salivary inflammatory markers and their association with obesity, glycemic status, and beta cell function

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ABSTRACT

Introduction: Chronic low-grade inflammation is central to the pathogenesis of obesity-related metabolic disorders, and identifying circulating inflammatory biomarkers may facilitate early risk stratification and targeted preventive strategies. This study investigated whether salivary mRNA expression levels of the pro-inflammatory cytokines interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α) differ between obese and non-obese individuals and examined their associations with glycemic status and β-cell function.

Methods: Salivary mRNA levels of IL-6 and TNF-α were quantified using real-time polymerase chain reaction. Bivariate analyses explored fold-change differences across glycemic categories, obesity status, and C-peptide levels, and adjusted logistic regression models were employed to assess independent associations with metabolic outcomes, controlling for age, sex, smoking, periodontal disease, and co-variables.

Results: Multinomial logistic regression showed that IL-6 mRNA fold-change was independently associated with obesity (aOR = 1.49, 95% CI: 1.09–2.08) and overweight (aOR = 1.56, 95% CI: 1.11–2.16), while TNF-α was independently associated with overweight (aOR = 1.74, 95% CI: 1.03–2.92); however, no significant associations were found between cytokine expression and glycemic status or C-peptide to glucose ratio.

Conclusion: Salivary mRNA expression levels of IL-6 and TNF-α may serve as non-invasive biomarkers for obesity-associated inflammation, supporting their potential utility in metabolic risk stratification, although their role in early glycemic dysregulation warrants further investigation.

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1. Introduction

Estimates from the 2021 Global Burden of Disease study indicate a strengthening association between obesity and Type 2 Diabetes, with the proportion of global type 2 diabetes DALYs attributable to high BMI growing by nearly 25% between 1990 and 2021 (Ong et al., 2023). In low-income and middle-income countries, the transition from underweight dominance to obesity dominance has led to a higher obesity prevalence than industrialised high-income countries (Phelps et al., 2024). Obesity represents a state of persistent low-grade inflammation that disrupts metabolic homeostasis, contributing to the development of insulin resistance and predisposing individuals to Type 2 Diabetes Mellitus (Hotamisligil, 2017). The involvement of obesity-induced inflammation in the pathogenesis of Type 2 Diabetes and its related metabolic complications has sparked increasing interest in targeting specific inflammatory mediators or pathways as potential strategies for preventing the onset of diabetes.

Currently, there is a growing scientific curiosity in the application of less invasive diagnostic techniques using saliva which contains biomolecules with clinical relevance which can serve as biomarkers for many chronic health conditions (Pfaffe et al., 2011). Saliva provides benefits compared to serum as it can be gathered noninvasively by people with minimal training and has the potential to become a first-line diagnostic sample of choice owing to advancements in detection technologies (Pfaffe et al., 2011). Recent studies have shown a strong positive correlation between the concentration of inflammatory markers like CRP and interleukins in serum and saliva (La Fratta et al., 2018; Tvarijonaviciute et al., 2020).

In obesity, white adipose tissue undergoes dynamic remodeling and is classified into two primary compartments: subcutaneous adipose tissue (SAT) and visceral adipose tissue (VAT), each with distinct metabolic functions and characteristics. These compartments exhibit differential gene expression patterns, including genes associated with adipocyte function (Hildebrandt et al., 2023). Visceral adipose tissue (VAT) expands mainly through adipocyte hypertrophy which triggers chronic low-grade inflammation, characterized by the activation of immune cells, particularly monocytes, which infiltrate expanding adipose tissue and differentiate into resident macrophages. Adipose tissue secretes a range of proinflammatory and acute-phase mediators in proportion to fat mass, with adipose-resident macrophages releasing cytokines that disrupt insulin signaling and promote systemic inflammation (Caër et al., 2017; Michaud et al., 2013; Weyer et al., 2000). Among these molecules, TNF-α and IL-6have been implicated in the development of adverse pathophysiological phenotypes associated with obesity (Weisberg et al., 2003).

Epidemiological evidence from the Whitehall II cohort demonstrates that low-grade systemic inflammation impairs β-cell function by disrupting calcium handling, inducing secretory dysfunction, and activating apoptotic pathways (Herder et al., 2016). This provides a strong rationale for investigating whether salivary cytokine mRNA, obtained non-invasively, mirrors these pathological processes. Examining the relationship between salivary inflammatory transcripts and β-cell function, evaluated through the C-peptide to glucose ratio, may facilitate the development of a novel, practical biomarker approach for monitoring inflammation-associated β-cell dysfunction in metabolic disorders (Herder et al., 2016; Saisho, 2016).

Chronic low-grade inflammation is a well-established contributor to the pathogenesis of obesity and its associated metabolic complications. Circulating inflammatory biomarkers have shown promise for future use in risk stratification and early identification of individuals at heightened risk for obesity-related disorders. The identification and validation of such biomarkers may enable the prediction of individuals who are more likely to develop metabolic sequelae, thereby facilitating timely preventive or therapeutic interventions. Given this context, we aimed to determine whether salivary mRNA expression levels of the pro-inflammatory cytokines interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α) differ between obese and non-obese individuals and to evaluate their associations with glycemic status and β-cell function, assessed using the C-peptide to glucose ratio as a surrogate measure. We hypothesized that salivary mRNA expression of IL-6 and TNF-α would be upregulated in obesity and independently associated with metabolic dysregulation, including altered glycemic status and β-cell function.

2. Materials & methods

2.1. Study design and study population

This was a cross-sectional observational study that included adult men and women aged 18–70 years. Participants were recruited between January 2023 and November 2024 from five healthcare facilities across the south Indian states of Kerala and Karnataka. Inclusion criteria comprised adults within the specified age range who were willing to provide written informed consent and complete study assessments. Individuals were excluded if they had a self-reported diagnosis of malignancy, autoimmune disorders, chronic kidney disease, chronic liver disease, or endocrine disorders other than diabetes. Participants with self-reported acute illness at the time of recruitment, including recent febrile or infectious conditions, or recent use of anti-inflammatory medications were also excluded to minimize the influence of acute inflammatory states. In total, 276 participants were enrolled. The study was approved by the Institutional Ethical Review Committee of Mar Baselios Dental College, Kerala, and written informed consent was obtained from all participants prior to enrollment.

2.2. Study procedure

For the evaluation of salivary IL-6 and TNF-α mRNA expression, unstimulated saliva samples were collected in the morning using a sterile swab placed in the sublingual region of the lingual vestibule for 20 s to ensure adequate absorption. The swabs were then immediately transferred into universal transport medium and stored at 4 °C until RNA extraction and subsequent PCR analysis. Venepuncture was done to collect blood sample from participants and stored in EDTA bottle for Hb1A1c (glycated hemoglobin)

determination. The plasma glucose and C-peptide levels were also measured. A clinical assessment by a dental surgeon was carried to determine presence of active tooth and periodontal disease. An analysis of microbial markers using another multiplex RT-PCR test kit, for the detection of three prominent bacteria associated with periodontal disease, specifically *P. gingivalis*, *T. forsythia*, and *T. denticola*, collectively referred to as the Red Complex bacteria was also undertaken on the saliva sample.

The information about the participants' socio-demographic and behavioural factors were obtained through a self-reported questionnaire. Anthropometric measurements were collected from the participants as per the standardized procedures. Body weight was evaluated to nearest 0.1 kg using Omron digital Scale without shoes and with light clothing. Height was measured with a stadiometer attached to the scale. The participants were classified based on glycemic status as normoglycemia (A1C <5.7%), impaired fasting glucose (A1C 5.7–6.4%), and diabetes (A1C ≥ 6.5%). The diagnosis of IFG and diabetes was based on the criteria of the American Diabetes Association (American Diabetes Association Professional Practice Committee et al., 2024). The Asian BMI thresholds of BMI ≥23 kg/m^2 for overweight and ≥27.5 kg/m^2 for obesity were used (Appropriate Body-Mass Index for Asian Populations and Its Implications for Policy and Intervention Strategies," 2004).

2.3. Quantitative assessment of gene expression by qRT-PCR

IL-6 and TNF-α mRNA expression was measured using real-time quantitative PCR (qRT-PCR) with actin as the housekeeping gene. RNA isolation was performed using Magnetic Bead-based RNA Isolation Kit. The isolated RNA samples were stored at −80 °C for long-term preservation. Subsequently, these RNA samples were amplified using the Multiplex Real-Time PCR Kit to identify inflammatory markers. The inflammatory markers—IL-6 and TNF-α were analyzed, along with Actin as an internal control. This was accomplished using gene-specific primers and probes through Reverse Transcriptase PCR. Amplification of all samples took place in a 96-well PCR plate on the Bio-Rad CFX96™ Real-Time System. Each well contained a 22 μL PCR mixture, which included 10 μL of Master Mix, 1 μL each of forward and reverse primers for IL-6, TNF-α, and Actin, 1 μL each of probes for IL-6, TNF-α and Actin, and 4 μL of the extracted RNA. Standard curves were generated by plotting the number of amplification cycles against relative fluorescence units (RFU). The cycle threshold (Ct) values for each marker were subsequently recorded. Fold change was calculated using the 2ˆ(-ΔΔCt) method, comparing each marker to actin as the reference.

2.4. Statistical analysis

Baseline patient characteristics were presented as frequency and proportions for categorical variables and mean and standard deviation [SD]) for continuous variables with a normal distribution. We initially conducted unadjusted analyses using the Mann–Whitney *U* test and the Kruskal–Wallis test to assess differences in the mRNA fold-change expression levels of IL-6 and TNF-α between the two categories of C-peptide levels and across the three categories of glycemic status and obesity classification. To investigate the associations between IL-6 and TNF-α expression and the three outcomes—obesity, glycaemic status, and β-cell function—we fitted distinct logistic regression models for each endpoint. Each model was mutually adjusted for the other two metabolic variables and additionally controlled for age, sex, smoking status, and periodontal disease. To compare the goodness of fit of the models, we used the Akaike information criterion (AIC). All statistical analyses were performed using the R software (R Core Team −2024) and R packages 'VGAM', 'ggplot2' and 'dplyr'.

3. Results

The demographic and clinical profiles of the 276 participants included in the study are summarized in Table 1. Median age of the study population was 41.5 years (IQR 36-50) with a predominance of male participants. The bivariate analysis revealed a statistically significant difference in foldchange across obese, overweight and normal BMI groups for IL-6 (p-value = 0.006) and TNF-α (p-value =

Table 1
Sociodemographic and clinical characteristics of the study population.

Characteristic		n(%), N = 276
Age	<30 years	53 (19.2)
	31 to 60 years	210 (76.1)
	61 and above	13 (4.7)
Sex	Male	172 (62.3)
	Female	104 (37.7)
Periodontitis	Present	117 (42.4)
	Absent	159 (57.6)
Glycemic status	Normal	151 (54.7)
	Prediabetes	62 (22.5)
	Diabetes	63 (22.8)
BMI	Normal BMI	41 (14.8)
	Overweight	130 (47.1)
	Obese	105 (38.1)
Smoking	Smokers	24 (8.7)
	Non smokers	252 (91.3)

0.036). However, the observed differences in fold change of IL-6 ($p = 0.65$) and TNF-α ($p = 0.73$) across the normal, prediabetic, and diabetic groups did not reach statistical significance. Fig. 1 illustrates the mRNA fold change of IL-6 and TNF-α across categories of BMI and glycemic status revealing higher expression levels among individuals with obesity compared to those with normal BMI, with no consistent trends observed across glycemic categories. To further explore these associations, three logistic regression models were fitted for each outcome (Table 2). In the multinomial logistic regression analysis, IL-6 mRNA fold change was independently associated with both obesity (aOR = 1.49, 95% CI: 1.09–2.08) and overweight (aOR = 1.56, 95% CI: 1.11–2.16) whereas TNF-α fold change emerged as an independent predictor of overweight (aOR = 1.74, 95% CI: 1.03–2.92) alone. No statistically significant associations were observed between the mRNA fold changes of IL-6 or TNF-α and glycemic status, including prediabetes and diabetes. Additionally, the C-peptide to glucose ratio, a surrogate marker of β-cell function, showed no significant correlation with the expression levels of either cytokine. Sensitivity analyses using continuous outcome variables demonstrated similar directions of association between salivary IL-6 mRNA expression and obesity, although effect sizes were attenuated and did not materially alter the overall interpretation of the findings.

4. Discussion

Obesity is characterized by chronic, sustained systemic inflammation, as evidenced by increased adipose tissue inflammation occurring alongside elevated systemic inflammatory markers (Kunz et al., 2021). In the present study, we have chosen to measure the mRNA levels of inflammatory markers, which are more likely to provide a better reflection of the pathophysiological process in individual patients. Salivary mRNA quantification offers enhanced sensitivity, specificity, and analytical robustness compared to protein-based measurements, as demonstrated by a study on IL-6 mRNA in oral squamous cell carcinoma patients (Márton et al., 2019).

The present study demonstrated significantly higher gene expression levels of IL-6 in the saliva of subjects who are overweight or obese compared to individuals with normal body weight. This finding appears to be consistent with previous research, including a study that examined adipose tissue expression of IL-6R and IL-6 in obese, overweight, and lean non-diabetic adults, which reported a significant upregulation of IL-6R mRNA expression in obese individuals compared to their lean and overweight counterparts (Sindhu et al., 2015). Another related study assessed salivary concentrations of IL-6 and leptin in pediatric populations, comparing obese or overweight children to those with normal weight. The findings revealed that salivary IL-6 levels were significantly elevated in the obese/overweight group relative to their normal-weight counterparts. Furthermore, a strong positive correlation was observed between salivary IL-6 levels and Body Mass Index (BMI), indicating that IL-6 concentrations in saliva increase proportionally with rising BMI(Pîrsean et al., 2019). Similarly, peripheral administration of IL-6 at concentrations comparable to those observed in obesity has been shown to induce hyperlipidemia, hyperglycemia, and insulin resistance in both human and rodent models (Tsigos et al., 1997).

In our study, a significant association was observed between TNF-α expression levels and individuals classified as overweight (Table 2); however, this association was not evident among those categorized as obese. This result may be explained by the fact that serum TNF-α levels increase significantly during early abdominal fat gain (Olszanecka-Glinianowicz et al., 2011). In accordance with the present results, a previous study assessing visceral and subcutaneous adipose tissue volumes investigated their correlation with concentrations of inflammatory markers, revealed a positive association between visceral adipose tissue (VAT) and salivary TNF-α levels (Ostrowska et al., 2020). Katsuki et al. conducted a study comparing obese individuals with diabetes to lean controls found that serum TNF-α concentrations were significantly elevated in obese diabetic patients, correlating positively with visceral fat area and negatively with insulin sensitivity (Katsuki et al., 1998). The finding also corroborates the results of a systematic review and meta-analysis of 34 studies where obese/overweight individuals presented higher levels of TNF-α in saliva than those without obesity (Duffles et al., 2019).

On examining the relationship between inflammatory gene expression and metabolic status, we also sought to determine whether expression levels of IL-6 and TNF-α were associated with glycemic status and β-cell function. Although individuals with prediabetes

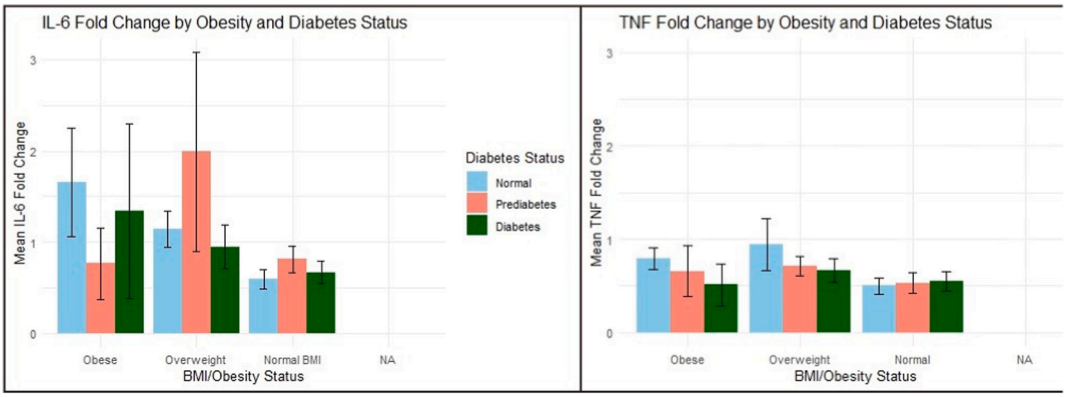

Fig. 1. Salivary cytokine mRNA fold change across BMI and glycemic status.

Table 2
Multinomial Logistic Regression models and Cytokine expression.

Outcome variable		IL-6 Odds Ratio (95% CI)	TNF α Odds Ratio (95% CI)
Model 1-Obesity	Obese	**1.49 (1.09–2.08)**	1.87(0.98-3.01)
	Overweight	**1.56 (1.11–2.16)**	**1.74(1.03–2.92)**
	Normal BMI	Ref	Ref
Model 2-Glycemic status	Diabetes	1.07(0.87- 1.31)	1.03(0.72-1.48)
	IFG	1.03(0.85-1.26)	0.89(0.53-1.51)
	Normal	Ref	Ref
Model 3- Beta cell function (C-Peptide to Glucose Ratio)	Elevated(>2)	1.18(0.96-1.73)	1.39(0.91-2.63)
	Normal(<2)	Ref	Ref

Notes: All three models were adjusted for age, sex, periodontal status and smoking.

exhibited higher expression levels of the markers compared to those with diabetes and normoglycemic individuals, the differences did not achieve statistical significance. Findings across studies have been heterogeneous with studies both supporting and refuting the association. A Study to examine the possible direct relationship of interleukin-6 and TNF-α with insulin sensitivity in humans by a series of euglycaemic-hyperinsulinaemic clamp experiments demonstrated that the increased circulating IL-6 concentrations seen in patients with Type 2 diabetes are strongly related to fat mass and not insulin responsiveness, and suggest that neither IL-6 nor TNF-α are indicative of insulin resistance (Carey et al., 2004). The present findings appear to align with those of a case-control study conducted among individuals with type 2 diabetes attending a diabetes clinic in Nigeria, which reported no statistically significant differences in IL-6 and TNF-α levels between diabetic patients and non-diabetic controls (Agho et al., 2021). Our findings contrast with numerous published studies that have reported a positive association between inflammatory cytokines and insulin resistance or diabetes (Aguirre et al., 2000; Liu et al., 2016; Lucas et al., 2013; Nisoli et al., 2000). A comparative analysis by Tigno et al. demonstrated that inflammatory markers were most pronounced during the prediabetic stage (Tigno et al., 2006). In addition, an interventional study examining the effects of a 10-week lifestyle intervention in obese children found a 9% reduction in serum TNF-α levels, along with decreases in other inflammation-related molecules, and reported potential benefits in glucose metabolism (Marti et al., 2018).

While previous studies have reported both inverse and positive associations between circulating IL-6 and TNF-α levels and β-cell function, our study did not observe a significant relationship between the mRNA expression of these cytokines and C-peptide levels (Dai et al., 2021; Dauriz et al., 2016). This lack of association may be attributable to differences in the biological matrix analyzed, as prior studies predominantly measured serum cytokine concentrations, whereas we assessed gene expression levels in peripheral blood. It is also possible that post-transcriptional regulation or local tissue-specific cytokine signaling, not captured by peripheral mRNA expression, plays a more critical role in modulating β-cell function. However, the findings observed in this study mirror those of a dose-response study of recombinant human IL-6 in normal volunteers, the levels of plasma insulin and C-peptide were not affected by any IL-6 dose (Tsigos et al., 1997).

The present study is limited by certain caveats. Firstly, comorbidity status was self-reported, which may introduce recall bias and potential misclassification. Additionally, although IL-6 and TNF-α are key markers of metabolic inflammation, they are not specific to obesity and may be elevated in a range of inflammatory or infectious conditions, thereby reducing their diagnostic specificity. Secondly, the quantification of salivary mRNA is inherently variable due to fluctuations in RNA yield and stability, which may be influenced by factors such as hydration status, dietary intake, and oral hygiene. Although we adjusted for periodontal status and potential microbiome-related interference, residual pre-analytical variability may still affect the accuracy and reproducibility of salivary cytokine mRNA measurements. In addition, saliva samples were collected during morning clinic hours; however, time since waking was not recorded, and potential diurnal variation in salivary cytokine mRNA expression cannot be fully excluded. Finally, salivary IL-6 and TNF-α mRNA expression cannot be assumed to directly reflect systemic circulating cytokine levels, representing an important limitation, although these markers may still have utility as non-invasive biomarkers for metabolic risk stratification.

5. Conclusion

Salivary mRNA expression levels of IL-6 and TNF-α were significantly associated with overweight and obesity, with IL-6 demonstrating a specific correlation with obesity, underscoring their potential utility as non-invasive biomarkers for obesity-related inflammatory states. Although elevated salivary cytokine mRNA levels were also noted in individuals with prediabetes, the absence of statistical significance indicates the need for further studies with larger cohorts to validate their diagnostic performance in early glycemic dysregulation. Overall, the analysis of salivary cytokine transcripts offers a promising, non-invasive modality for assessing low-grade inflammation in metabolic disorders, and may serve as a valuable tool for risk stratification and early intervention in clinical practice.

CRediT authorship contribution statement

Ronnie Thomas: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Methodology, Investigation, Formal analysis, Conceptualization. **Prashanth Varkey Ambooken:** Writing – review & editing, Visualization, Validation, Supervision, Resources, Project administration, Investigation, Funding acquisition, Data curation,

Conceptualization. **Asina Palakuzhy Abdurahman:** Writing – review & editing, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Formal analysis, Data curation. **Mathew John:** Writing – review & editing, Supervision. **Rahul Thampi:** Writing – review & editing, Supervision, Methodology. **Muni Rubens:** Writing – review & editing, Methodology, Conceptualization.

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Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Dr. Prashanth Varkey Ambooken is the founder and director of Zum Heilen Healthcare Private Limited and he received research funding from this company to support the current study. The other authors declare no competing interests.

Abbreviations

mRNA	Messenger Ribonucleic Acid
TNF-α	Tumor Necrosis Factor Alpha
IL-6	Interleukin-6
aOR	Adjusted Odds Ratio
95% CI	95% Confidence Interval
RNA	Ribonucleic Acid
DALY	Disability-Adjusted Life Year
HbA1C	Hemoglobin A1C
A1C	Glycated Hemoglobin
EDTA	Ethylenediaminetetraacetic Acid
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SAT	Subcutaneous Adipose Tissue
VAT	Visceral Adipose Tissue
Ct	Cycle Threshold
SD	Standard Deviation
IQR	Interquartile Range
AIC	Akaike Information Criterion

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