Progress in Microbes and Molecular Biology

HH PUBLISHER

Original Research Article

Association Between Salivary C-Reactive Protein Levels and Covariates in an Older Adult Population in Malaysia – A Cross-Sectional Study of The MyAgeWell Cohort

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Article History

Received: 10 September 2024;

Received in Revised Form: 28 December 2024;

Accepted: 14 January 2025;

Available Online: 24 February 2025

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Abstract: Malaysia's aging population faces heightened risks of cardiovascular diseases, cancers, metabolic disorders, and physical and cognitive decline. Inflammatory biomarkers, such as C-reactive protein (CRP), play a key role in age-related ailments. For instance, elevated CRP levels are linked to an increased risk of cardiovascular diseases and cognitive decline. It is of particular interest to investigate CRP levels and their correlation with various factors among older adults to develop potential predictive markers and interventions for age-related diseases. In recent years, salivary samples have become a viable non-invasive alternative approach to studying CRP levels. Hence, this study aims to examine salivary CRP levels and explore the influences of age, ethnicity, gender, body mass index (BMI) and economic status on salivary CRP levels among older adults. The study was registered at ClinicalTrials.gov (NCT06376656) and ethical approval was obtained from the Sunway University Research Ethics Committee (SUREC 2020/039). A total of 382 saliva samples were collected from the older adults (≥ 60 years) through the MyAgeWell cohort for analysis. Various covariates including age, gender, ethnicity, BMI, and economic status were recorded. Salivary CRP levels were measured using ELISA. We observed a median CRP concentration of 0.39 ng/mL (IQR = 0 – 1.36 ng/mL). Correlation analysis shows salivary CRP levels were associated with gender, ethnicity, and BMI ($p < 0.05$), but not age and economic status. These findings suggest diverse influences on inflammation among Malaysian older adults. In conclusion, this study advances the understanding of salivary CRP levels in healthy older adults, highlighting the complex nature of inflammation in this

population and the importance of addressing modifiable risk factors to reduce health disparities.

Keywords: Saliva, C-reactive protein (CRP), inflammation, Malaysia, aging, older adults

1. Introduction

Malaysia is a multiethnic country with a total population of 33.7 million [1]. The multiethnic population comprises 57.9% Malays, 22.7% Chinese, 6.60% Indians, and the remaining 12.8% which includes Malaysia aborigines and foreigners [1]. The total number of older adults is anticipated to increase from 7% to 14% from 2020 – 2043 [2]. Malaysia, like many other developing countries, is experiencing a demographic shift as the number of older adults increased to a total of 2.4 million in 2023 [1]. This raises a huge concern in the healthcare system as the aging population is highly susceptible to various diseases such as cardiovascular diseases, hypertension, cancer, osteoarthritis, diabetes mellitus, osteoporosis, and multiple complex health conditions [3].

Inflammatory biomarkers play an important role in age-related diseases among older adults, reflecting the intricate interplay between aging, chronic inflammation, and various health conditions [4]. C-reactive protein (CRP) is one of the several inflammatory biomarkers that has been implicated in the development and progression of age-related diseases [4,5]. Studies have shown an elevation of CRP is linked to various age-related diseases including atherosclerosis [6], cardiovascular diseases [7,8], and cognitive decline [9,10] in older adults. CRP is a class of acute-phase proteins produced by the liver in response to inflammation [11]. CRP plays a key role in host defence, clearance of cellular debris, and regulation of the complement system [12]. CRP is also known to be tightly regulated by pro-inflammatory cytokine such as interleukin-6 (IL-6) [13]. In clinical settings, serum CRP levels are extensively used as a reliable biomarker for systemic inflammation and tissue damage [14]. A broad spectrum of acute and chronic conditions, including infections, autoimmune diseases, cardiovascular disorders, and malignancies can trigger the rapid elevation of serum CRP levels within 24 to 72 hours. Hence, serum CRP has become a valuable biomarker for the diagnosis and monitoring of various inflammatory diseases [13,14].

Clinically, serum is the common specimen for the measurement of CRP levels. However, the blood collection procedure is invasive and poses some challenges in its collection and storage. Alternative, non-invasive, and cost-effective methods have received increased interest in response to the limitations of traditional methods. One such approach

involves using salivary biomarkers for disease detection. In recent years, the concept of using saliva specimens in disease screening and diagnosis has garnered significant attention due to its potential to provide an alternative, less invasive means of evaluating health conditions [15,16,17]. Various biomarkers that are typically found in serum, such as cytokines, and inflammatory markers, have also been detected in saliva. This indicates that saliva could be a valuable alternative to reflect our body's physiological and pathological state [15,18].

Hence, understanding the average range of salivary CRP levels among Malaysian older adults, and examining the influences of various covariates on salivary CRP levels, are essential for the development of salivary CRP levels as a biomarker to diagnose and monitor age-related diseases. The objectives of this study are: (1) To establish the average range of salivary CRP levels of older adults, and (2) To establish the relationship between salivary CRP levels and multiple covariates among Malaysian older adults.

2. Materials and Methods

2.1. Study population and sample collection

The MyAgeWell cohort is a community-based longitudinal cohort study of older adults aged 60 years or older, residing in Selangor and Federal Territory Kuala Lumpur. This cohort consists of a diverse multiethnic group with an equal distribution of gender and economic status based on the average household income group in Malaysia, classified as B40 (representing the bottom 40% of household-income individuals) and non-B40 (representing the moderate household-income and high household-income individuals). All participants in this cohort were recruited based on the following criteria: (1) healthy and this includes those seeking regular medical attention, (2) have some form of mobility (ability to walk short distances at least 3 m, based on participant self-reported), and (3) able to communicate in at least one of the following languages: English, Malay, Mandarin, or Tamil.

Individuals with confirmed diagnoses of neurodegenerative diseases, such as Alzheimer’s, Parkinson’s disease or with cognitive impairment defined as a Montreal Cognitive Assessment (MoCA) score <13, psychiatric disorders, immobility, and/or any form of uncorrected auditory or visual impairment were excluded from the cohort. All procedures and methods used in the study were informed to all participants. Upon agreement, all participants provided the informed consent form as required by the Declaration of Helsinki guidelines. The study was approved by the Sunway University Research Ethics Committee (SUREC 2020/036) and registered at the ClinicalTrials.gov website (NCT06376656).

2.2. Cognitive assessment and questionnaire data

MoCA was performed on all participants to determine the cognitive status of the participants. The MoCA assessment was conducted physically through an interview session at Sunway University with a cut-off point of 13 as it has been suggested to be suitable to be implemented on populations with low education levels and minor ethnic groups [19]. Participants who scored below MoCA score of 13 were excluded from this study.

Additional health, biological and demographic data including age, height, weight, sleep intake, measured as sleep duration and sleep quality, comorbidities, ethnicity, and gender were self-reported in an hour-long questionnaire interview conducted at our institute. BMI was calculated based on the formula: weight (kilograms) / height (meters²).

2.3. Collection of saliva

An unstimulated passive drooling method was used to collect salivary samples with a minimum volume of 5 mL in a centrifuge tube as adapted from Bhattarai et al., 2018 [20]. Participants were told to rinse their mouths five times with normal drinking water to remove any food or beverage residues, prior to collecting saliva samples. Food and beverage intake was prohibited for at least 30 minutes before saliva samples were collected. All saliva samples were then stored on ice until the end of the day. 1 mL of the sampled saliva was aliquoted into a 1.5 mL centrifuge tube and stored at -20°C for the experimental assays, while the unused saliva samples were stored at -80°C freezer until further analysis.

2.4. Salivary CRP assay

Salivary CRP levels were determined using the Human C-reactive Protein (PTX1) ELISA kit (Abcam, ab108826) as adapted from Dillon et al. (2010) [21]. The salivary samples were first thawed and centrifuged at 800xg for 10 minutes to remove any solid residue. The CRP standards (16 ng/mL – 0.25 ng/mL) were prepared according to the manufacturer's protocol. 50 µl of the CRP standard or samples were added into the individual wells, followed by incubation for 2 hr on a shaker at 400 rpm under room temperature. A series of treatments with 1X biotinylated CRP antibody and 1X Streptavidin conjugate was applied according to the manufacturer's protocol. The optical density was read immediately at a wavelength of 450 nm using a Tecan Infinite M Plex plate reader after the stop solution was added. All samples were analysed in duplicates and plotted against the standard curve for the quantification of salivary CRP according to the manufacturer's protocols.

2.5. Statistical analysis

All normally distributed variables are reported as mean and standard deviation (SD), while median and interquartile range (IQR) are for non-normal distribution. Spearman correlation analysis was performed between individual salivary CRP levels with age and BMI scores. T-test analysis, Kruskal-Wallis test and Mann-Whitney U test were performed to determine the statistical significance of the differences observed with a *p*-value of less than 0.05 is considered statistically significant. The Kruskal-Wallis test is used to compare three or more independent groups of numbers, in this case, ethnic groups, while a t-test is used to compare the means of two groups, ie., age using the grouping approach, and the Mann-Whitney U test is used to compare two independent groups with non-parametric data, ie., gender and economic status. All statistical analyses were conducted using GraphPad Prism version 9.

3. Results

3.1. Demographic characteristics of the MyAgeWell cohort

The demographic characteristics of the MyAgeWell cohort are presented in Table 1. This cohort consists of 400 older Malaysian adults but only 382 subjects were included in this study (suboptimal saliva sample quality for 18 subjects). The recruited subjects were presented with a mean age of 67.7 ± 5.4 (range 60 – 98 years), an almost equal distribution of gender (49.5% male; 50.5% female), and an ethnicity ratio of approximately 40 Malays:40 Chinese:20 Indians. 193 (50.5%) participants were of the lower socioeconomic status, B40 community. The median BMI of our study cohort was 24.80 kg/m² (IQR: 15.11 – 51.42 kg/m²).

3.2. Salivary CRP levels across the MyAgeWell cohort

The salivary CRP levels of the MyAgeWell cohort were analyzed using the ELISA assay. The median salivary CRP concentration was 0.39 ng/mL (IQR: 0 – 1.36 ng/mL) (Table 1, Figure 1) with a lower detection limit of 0.2 ng/mL, and intra-assay coefficient of variation (% CVs) of 3.9%.

Table 1. Demographics of the MyAgeWell cohort.

Parameters	Number of participants, n (%)
Gender (n = 382)	
Female	193 (50.5)
Male	189 (49.5)
Ethnicity (n = 382)	
Malay	151 (39.5)
Chinese	148 (38.7)
Indian	78 (20.4)
Others	5 (1.3)
Economic status (n = 382)	
B40	193 (50.5)
Non-B40	189 (49.5)
Mean age (n = 382)	67.6 ± 5.4 (ranging 60 - 98)
Median BMI (kg/m^2) (n = 357)	24.80 (IQR: 15.11 – 51.42)
Median salivary CRP conc. (ng/mL) (n = 382)	0.39 (IQR: 0 – 1.36)

Data is expressed as mean ± standard deviation (SD) or median and interquartile range (IQR).

Abbreviations: BMI – Body Mass Index, CRP – C-reactive protein, kg/m² - kilogram per square meter, ng/mL – nanograms per milliliter.

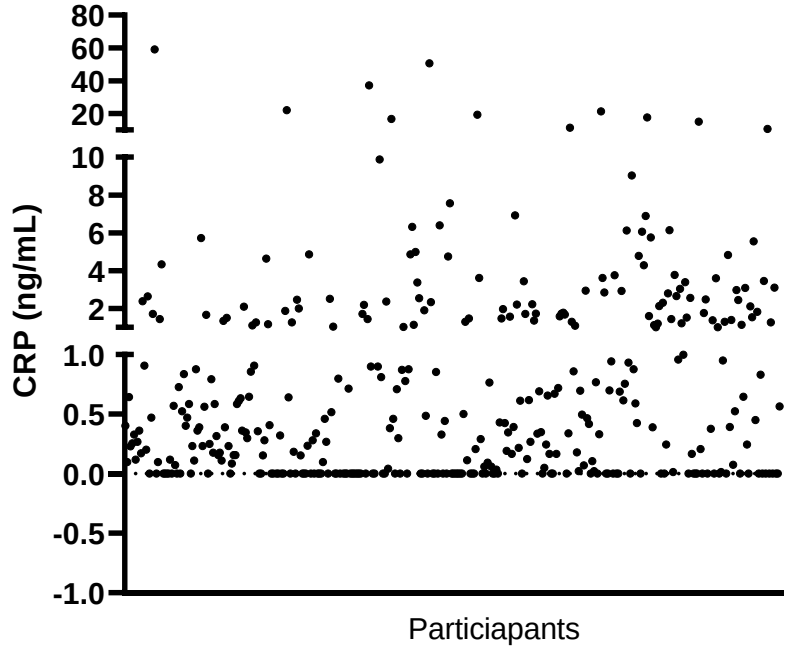

Figure 1. Scatter plot of salivary CRP (ng/mL) level. The y-axis scales were divided into distinct sections to clearly represent the wide range of CRP levels detected in this study.

3.3. Association between the salivary CRP levels (ng/mL) and age

Spearman coefficient correlation was performed to determine the correlation between the salivary CRP levels and age. There was no correlation between salivary CRP levels with age (r = -0.07 (95% CI: 0.25 to 0.44), p = 0.09) (Figure 2 a). T-test was also performed to compare the salivary CRP levels by age groups stratified as < 65 years and ≥ 65 years in age, and the results showed no significant differences in the salivary CRP levels between the two age groups (r = -0.01, (95% CI = -0.14 to 0.06), p = 0.23) (Figure 2b).

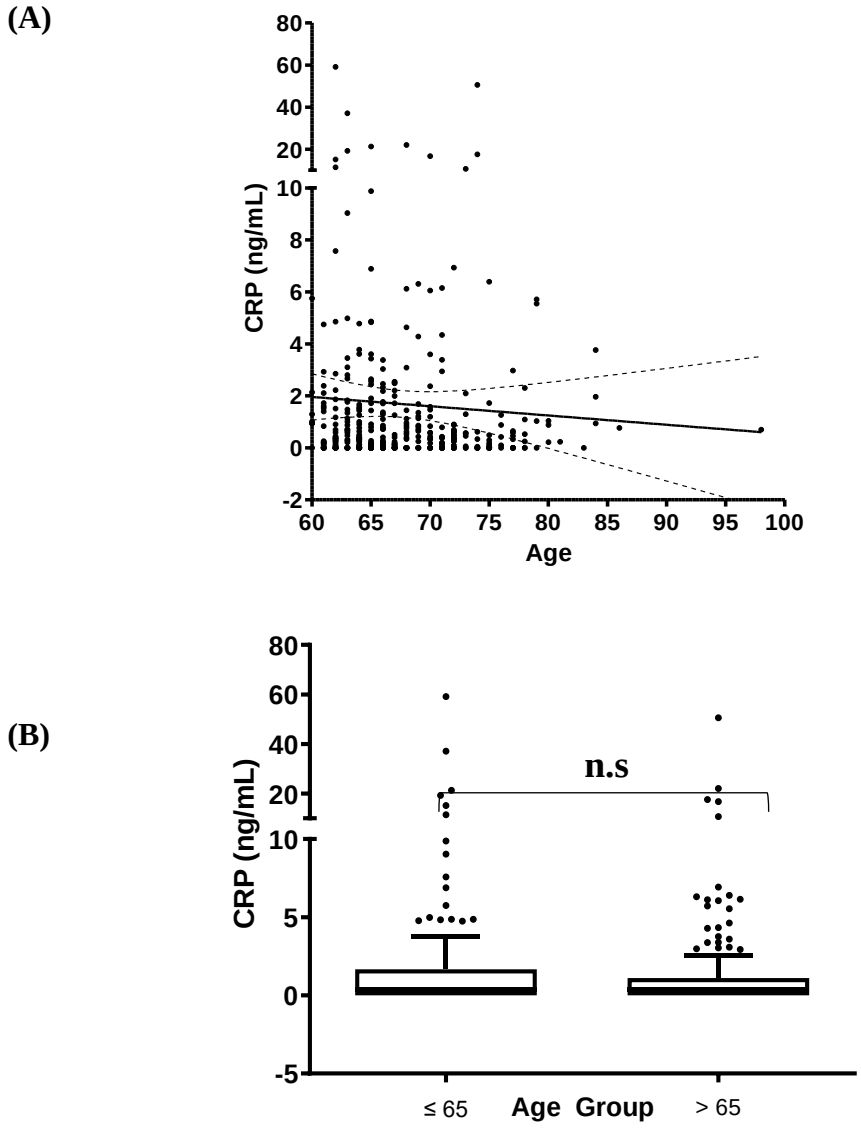

Figure 2. (A) Correlation coefficient of salivary CRP levels across age range of 60 – 100. (r = -0.01, (95% CI: -0.17 to 0.15), p = 0.193); (B) Distribution of salivary CRP levels in <65 years of age and ≥ 65 years. (r = -0.01, (95% CI: -0.17 to 0.15), p = 0.198). *The y-axis scales were divided into distinct sections to clearly represent the wide range of CRP levels detected in this study.

3.4. Association between the salivary CRP levels and gender

We observed a significant difference in the salivary CRP levels between both genders ($p < 0.0001$) when a Mann-Whitney U test was conducted. Females have a higher median CRP level (0.59 ng/ml; IQR: 0.15 - 156) compared to males (0.21 ng/ml; IQ: 0 – 1.01) (Figure 3).

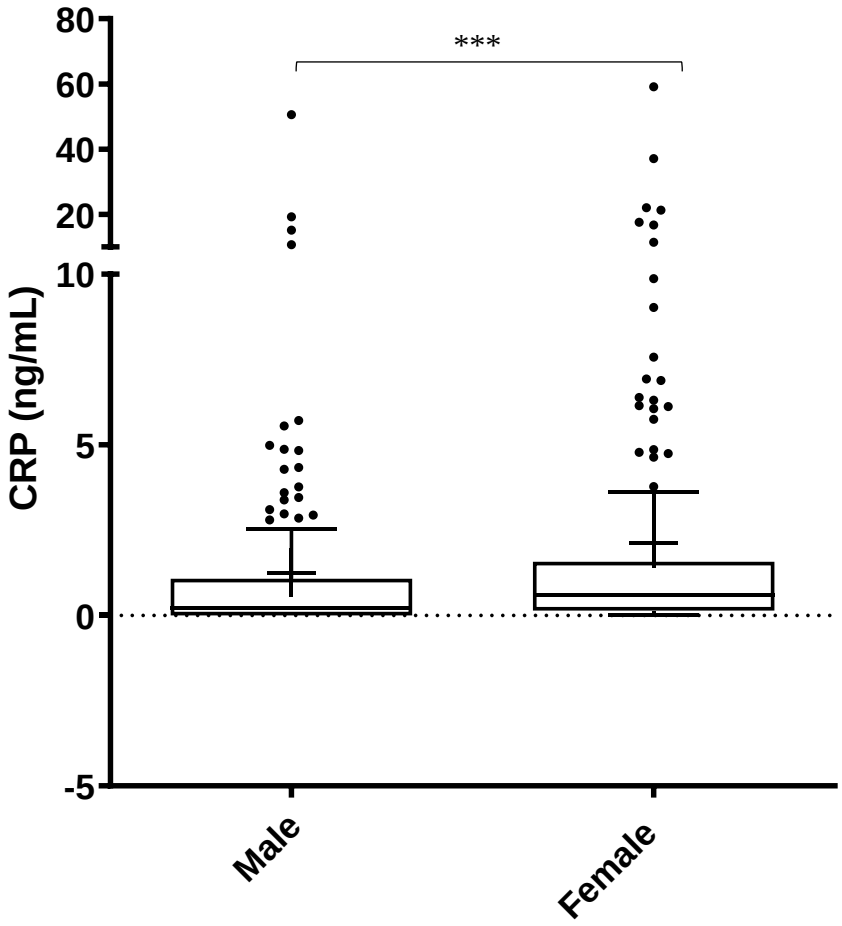

Figure 3. Distribution of the salivary CRP levels by gender. *The y- axis scales were divided into distinct sections to clearly represent the wide range of CRP levels detected in this study.

3.5. Association between the salivary CRP levels and different ethnicities

A significant difference in salivary CRP levels was observed among the ethnic groups ($p < 0.0001$) using the Kruskal-Wallis test, with Indians showing overall higher salivary CRP levels (median: 0.71 ng/mL; IQR: 0.24 – 1.65 ng/mL) compared to Chinese (median: 0.16 ng/mL; IQR: 0 – 0.59 ng/mL) and Malays (median: 0.61 ng/mL; IQR: 0 – 1.80 ng/mL) (Figure 4).

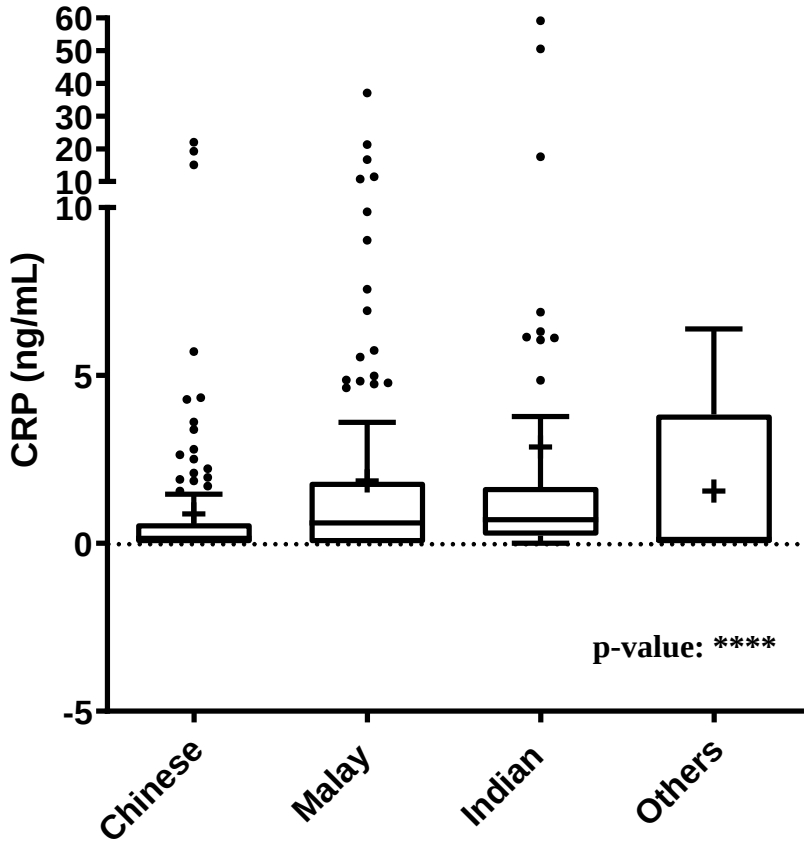

Figure 4. Distribution of the salivary CRP levels by ethnicity. *p*-values were calculated using Kruskal-Wallis test. $p < 0.0001$(****) by comparing across all ethnic groups. *The y- axis scales were divided into distinct sections to clearly represent the wide range of CRP levels detected in this study.

3.6. Association between salivary CRP levels and economic status

No significant difference was observed when the Mann-Whitney U test was performed by comparing the two economic groups (p = 0.2575) (Figure 5).

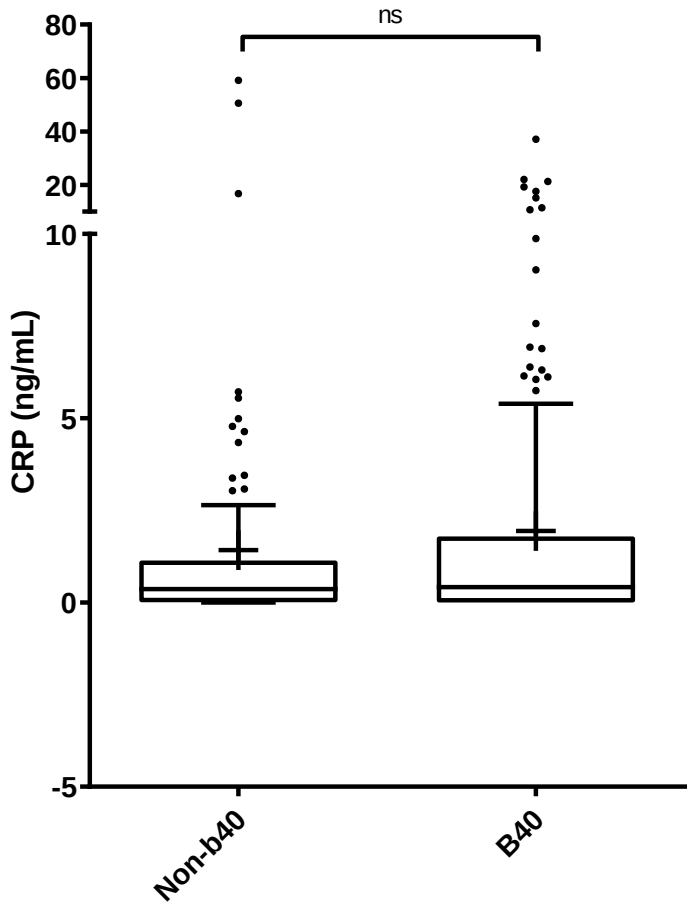

Figure 5. Distribution of salivary CRP levels within the B40 group (low household-income individuals) and non-B40 group (moderate- and high-household income individuals). *The y-axis scales were divided into distinct sections to clearly represent the wide range of CRP levels detected in this study.

3.7. Association between salivary CRP levels and BMI

A weak positive correlation was observed between salivary CRP levels and BMI scoring among the older adults (rho = 0.3491, p = < 0.0001) using Spearman correlation analysis. Participants with higher BMI tended to have higher salivary CRP levels (Figure 6).

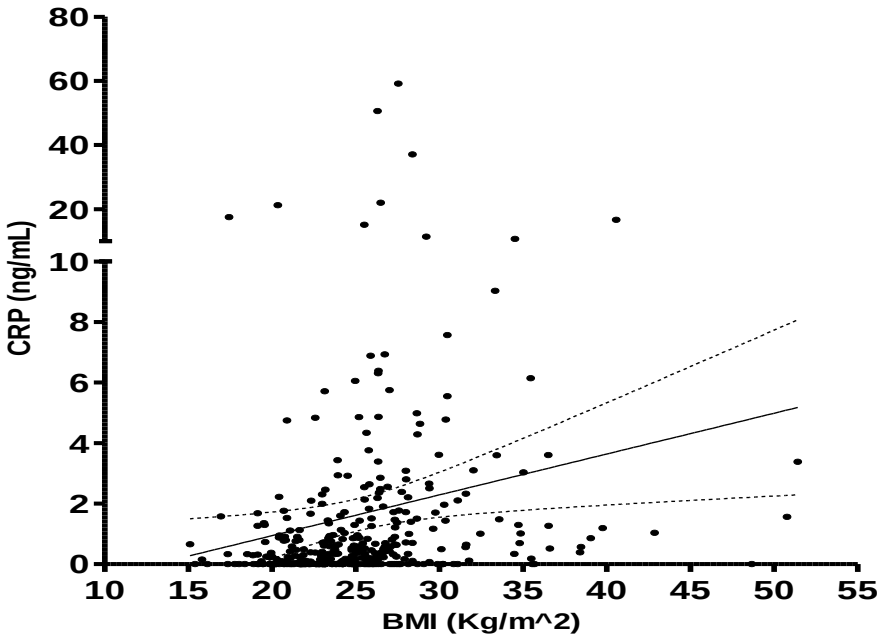

Figure 6. Distribution of salivary CRP levels across different BMI scores. *The y-axis scales were divided into distinct sections to clearly represent the wide range of CRP levels detected in this study.

4. Discussion

In most studies, the CRP levels in young and old adults were determined using serum samples [26,27,28,29]. In recent years, a shift from serum biomarkers to salivary biomarkers has been observed in detecting various human diseases [15,17]. CRP levels in saliva are one of the many inflammatory biomarkers that have been gaining attention. Given the CRP levels from saliva and serum vary depending on demographic distribution [26, 27, 30, 31], it is crucial to determine the reference range of the salivary CRP levels in the Malaysian population, especially in older adults, for future diagnostic and screening test development. Based on our cross-sectional study, we proposed that the salivary CRP levels of these healthy Malaysian adults aged 60 and above ranged between 0 and 59.13 ng/mL, with a median of 0.39 ng/mL. This proposed reference range falls within the range reported by Wettero et al. (2021) [31].

Our study delves into salivary CRP levels in healthy older adults in Malaysia and examines several factors that may influence salivary CRP levels in these individuals. Our study revealed that salivary CRP levels of healthy older adults in Malaysia were not significantly correlated with age. This observation contrasts with previous studies that reported a positive association between CRP levels and age in older adult populations aged 45 to 69 with a reported serum CRP level of 0.03 – 30.4 mg/L [27,28, 31]. Other studies also reported that the average salivary CRP levels and serum CRP levels in the population aged

60 and above are significantly higher compared to the younger age group [28,31]. The lack of correlation between CRP levels and age in our study cohort potentially indicates that other covariates such as BMI, ethnicity, gender, and economic status may play a significant role in influencing CRP levels among Malaysian older adults. Thus, correlation analysis and simple T-test were further performed to examine the relationship between salivary CRP levels and various covariates among the Malaysian older adult population.

The average BMI of the Malaysian population aged 18-59 years was reported to be lower (24.37 kg/m^2) when compared to the older adults aged 60 and above (25.54 kg/m^2) [24,25]. From our cross-sectional study, a similar observation was obtained, where the average BMI of older adults aged 60 and above is approximately 25.22 kg/m^2. Our cohort demonstrated a significant association between salivary CRP levels and BMI in older Malaysian, where the CRP levels increase as the BMI score increases. A similar positive correlation was observed in another study conducted by Norshafawati et al., (2014) [22]. A different observation was reported by another group of researchers where the salivary CRP levels were not correlated with BMI among older adults aged 45 – 69 in Sweden [31]. On the other hand, our findings are consistent with two other research studies, highlighting the plausible link between obesity and cardiovascular diseases and elevated salivary CRP levels [27,32]. The observed relationship between BMI and salivary CRP levels underscores the importance of addressing inflammation as a modifiable risk factor for obesity, cardiovascular diseases, and other related health outcomes among older adults in Malaysia.

Overall, the salivary CRP level in Malaysian older adults is significantly different across different ethnic groups with a higher CRP level among Indians, followed by the Malays and Chinese, which is consistent with the results observed by Mitra et al., (2018) [23]. Our findings coincide with various research studies demonstrating the ethnic disparities in serum or salivary CRP levels. These studies showed that the Black populations including African Americans (serum CRP = 0.05 – 0.29 mg/mL) and Indians (serum CRP = 0.2 – 9.8 mg/L) exhibit the highest average salivary and serum CRP levels compared to Caucasians, and other Southeast Asians [27, 29, 30, 32]. These further suggest the influence of genetic, cultural, and environmental factors on inflammatory responses in our body.

Similarly, our cohort also demonstrated a significant difference in salivary CRP levels based on gender where the females exhibited an average higher salivary CRP levels compared to the males. This gender disparity in CRP levels was also presented in other studies across various populations [27, 32, 33]. The observed variation in salivary CRP levels by gender may be plausibly explained by the differences in body composition, including fat

distribution and muscle mass, between females and males, as proposed by Khera et al., (2009) [34]. On the other hand, no significant difference in the salivary CRP levels based on economic status was demonstrated in our older adults' cohort. However, higher salivary CRP levels were documented in the low household-income group, which is in contrast with the observations reported by two other groups of researchers from the US and England [26, 35]. A different observation was documented in the older adult population of Indonesia where a higher CRP level was recorded in the moderate to high household-income group [35].

Although our research offers valuable insights into the connections between salivary CRP levels and several factors among older adults in Malaysia, it is important to acknowledge several limitations in our study. Firstly, due to the cross-sectional nature of our study, we are unable to establish the causal relationships between salivary CRP levels and the covariates under investigation. In future, a longitudinal study will be useful to clarify the temporal associations and other potential confounding variables such as physical activity, medication use, and comorbidities could be included in the analysis. These will allow a more comprehensive understanding of the determinants of CRP levels among the older adult population.

5. Conclusions

In conclusion, our study proposed the reference range of salivary CRP levels of 0 – 59.13 ng/mL among older adults, which can serve as the baseline for related research areas in the Malaysian population. The significant associations observed between salivary CRP levels and BMI, ethnicity, and gender, underscore the multifaceted nature of inflammation and its determinants among older adults in Malaysia. Overall, the salivary CRP results observed in our study show a similar observational pattern as the serum CRP results observed in other studies, further supporting the plausible alternative method of using saliva samples.

Author Contributions: Conceptualization, WLL, JC; methodology, WLL, WLL, JC; formal analysis, WGL, WLL, JC; investigation, WGL.; resources, WLL, JC, YCC.; data curation, WG, JC, WLL; writing—original draft preparation, WGL; writing—review and editing, WGL, WWL, YCC, TPL, JC.

Funding: This work was supported by a research grant provided by the Malaysian Department of Higher Education (MOHE), the Long-Term Research Grant Scheme (LRGS/1/2019/SYUC/02/1/5), and Sunway University.

Acknowledgments: We are grateful for the help of all postdocs, graduate research assistants, research assistants and interns in this project. We would also like to thank the participants who provided the information for the project.

Conflicts of Interest: The authors declare no conflict of interest.

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