Revisiting the cortisol reference ranges in humans: the role of demographics

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Abstract

The current study explores the effect of demographics on serum cortisol expression in a study group of 52 individuals to improve the current serum reference ranges to produce personalized expression profiles consequently increasing clinical confidence in the diagnosis. The serum cortisol concentration was inspected against demographical data like age, sex, and body mass index and showed an association with age and sex. The serum cortisol values also indicated a positive association with chronic illnesses however this finding requires a more focused study for establishment. Additionally, saliva samples are also collected from the same study group at the same time through drool and an absorbent sponge and correlated with serum values to draw an alternative route of serological testing. Salivary cortisol from drool showed a linear correlation of 0.51 and 0.52 with serum cortisol and with saliva samples collected using a porous sponge respectively. Overall, the study shows the role of demographics in shaping the reference ranges for cortisol, suggesting a path for developing personalized diagnostics. The study also highlights the efficacy of saliva as an alternative to serum cortisol to facilitate cortisol measurement for efficient stress management.

1. Introduction

Cortisol is the main molecule associated with stress, released by the adrenal glands in response to the HPA-axis stimulation in a state of threat.[1–3] Besides its special role as the *stress hormone*, cortisol is involved in a general alertness routine, following a circadian cycle, being highest in the morning to *wake* the body, a phenomenon known as *Cortisol Awakening Response* (CAR).[4] However, individuals suffering from acute or chronic stress may display either an abnormal cortisol cycle or consistently elevated levels.[5] Therefore, the measurement of cortisol is the pivotal step in assessing an individual's stress level. Cortisol is released in the blood, from where it finds its way to various other bodily fluids like sweat, saliva, urine, and cerebrospinal fluid.[6–9] The estimation of any cortisol-associated pathology involves comparing the serum cortisol expression to a set of reference ranges. However, variations in the serum cortisol concentrations in *normal* population are well documented based on an individual's demographics among other variables.[10–13] Consequently, an individual predisposed to have higher cortisol levels may be misdiagnosed as having physiological/psychological stress whereas individuals having a lower physiological cortisol may have to suffer through unnecessary medication resulting from the faulty diagnosis due to underlying biases. It is evident that the imminent paradigm shift of personalized medicine, where the treatment is tailored specifically for the diseased individual is entwined with personalized diagnosis where the biomarker analysis is driven from the individual's normal reference ranges. While getting the individual reference ranges would require estimation of the analyte under physiological conditions, a more efficient route is to draw out the influencers for the analyte and adjusting the reference range in accordance to the found derivatives.

Furthermore, due to the diurnal fluctuations of cortisol, a random estimation is less informative and a series of measurements are needed to establish the trend of cortisol expression to confidently assess the overall cortisol profile and eliminate erroneous diagnoses due to any transient spikes in its concentration.

This repeated sampling makes serological cortisol measurement tedious and unfeasible for an individual, and the process is further cumbersome in infants, or individuals with pre-existing conditions.[14] Alike blood, cortisol is also dynamically expressed in the saliva that follows any changes in the serological level of cortisol closely. Further, salivary cortisol represents the free, active form of cortisol due to the absence of cortisol-binding globulin (CBG) thereby being a more precise tool for cortisol assessment.[15–17] Saliva also presents itself as a non-invasive biological alternative to blood and can dynamically reflect the serum analyte concentration effectively.[9, 18] However, the method of salivary sample collection is not unanimous and saliva can be collected through a variety of methods like spitting, drooling, aspiration, external absorbent materials, etc., each having its advantages in terms of ease of collection, collection time, and collected volume.[19, 20] Saliva production can also be stimulated through an olfactory or gustatory stimulus or through mastication, which in turn varies the effective concentration of the analytes in the saliva. Therefore, the method of saliva collection and its standardization is of paramount importance for salivary diagnosis of cortisol. In this study, we conducted a cross-verification of cortisol expression in saliva and serum to understand the linearity of the relationship while also testing the feasibility of using a porous sponge as an alternative method of saliva collection. Further, the diversity of the study group was maintained to elucidate the role of demographics as influencers in the expression of cortisol in individuals to help attain a personalized reference range based on the characteristics of an individual.

2. Materials And Methods

The study was designed and conducted in alignment with the *Code of Ethics of the World Medical Association*.[21] The study was conducted at DA Pandu Memorial RV Dental College, Bangalore between 03 June 2022 to 01 July 2022, and the ethical clearance for the study was given by the Institutional Review Board (Approval Number 452/VOL-2/2022).

The study group size was calculated through performing a power analysis using the G*power software. A Statistical test on means for a two-tails t-test using α of 0.05 and power of 0.95 with an effect size of 0.5 gave a study size of 54 individuals.[22–24] The study group was divided into 3 age groups of 18–33, 34–48, 49–62 years with 20 participants in each group (total 60 individuals) attempting near-equal number of both sexes in each group. A written informed consent was signed by the participants after thoroughly understanding the purpose, procedure, and risks of the study. The participants provided saliva samples with 2 different methods *viz.* drooling, and a porous sponge made of PVA (**P**oly**v**inyl **a**lcohol). PVA sponge was procured from Cologenesis Healthcare Private Limited, India commercially sold as *Bsorb PVA sponges*, compressed PVA sheets of A4 sizes and an average thickness of 8 mm. The PVA sheet was cut down to small sponge pieces of 1 cm × 2 cm and used for saliva collection in its compressed form without any further processing. Simultaneously, blood samples were drawn during the same time to correlate the two methods. Samples were collected and analyzed on the Roche Cobas instrument using an ECLIA (**E**lectro**c**hemi**l**uminescence **I**mmuno**a**ssay) cortisol assay kit (Cortisol II) at a

NABL-certified diagnostics lab. All the samples were collected between 9:30 am to 11:30 am to avoid any discrepancies.

2.1. Population distribution

The study group for the survey was divided into three age groups 18–33 years, 34–48 years, and 49–62 years old, with 20, 21, and 11 individuals in each category. The age groups were maintained with a nearly equal sex distribution with age groups containing 9 (45%), 14 (66.7%), and 5 (45.5%) females respectively. The volunteers were selected on the exclusion criteria as follows,

- Visible bleeding in the mouth
- Active prescription of a steroidal medication
- Dental procedure in the past week
- Presence of oral or dental conditions

2.2. Sample collection

Keeping in purview the diurnal variation in physiological concentration of cortisol, the sample collection time was kept and maintained between 9:30 am to 11:30 am to mitigate the effect of sampling time on the expressed concentrations of cortisol. 5 mL of blood samples were collected by a resident phlebotomist aseptically from each participant into anticoagulant-coated tubes. The saliva samples were collected by a standard drool method.[25] For this, the participants were asked to sit in a relaxed position with their heads tilted slightly forward. The saliva collected in the mouth was then channelled directly into sterile sample containers (50 mL capacity) to gather 5 mL of saliva sample. For the collection of saliva samples through a sponge, each participant was given a sterile PVA sponge to keep on the top of their tongues for 2:30 minutes undisturbed. Following the incubation times, the sponges were taken out of the mouth and the absorbed saliva was squeezed into sterile sample containers. The saliva samples were frozen overnight, thawed on the next day, and centrifuged to separate mucus or any other particulate material. The processed samples were then analysed in the Roche COBAS system operating an ECLIA cortisol assay for the quantification of cortisol from the samples.

3. Results And Discussion

A total of 52 serum and saliva samples were collected from the participants with their consent. In the last age group of 49–62, the sample collection was terminated at 11 individuals as multiple saliva samples failed due to undetectable cortisol. Further, in the individual data sets where one or more data points were lost due to technical failures or remained undetected due to the limitations of the testing method, the whole data set was excluded from the correlation study to reduce bias. Following such exclusion, 5 data sets were discarded and the rest 47 data sets were used for interpretation of serum-saliva correlation. The average levels of serum cortisol across all individuals were recorded to be 82.61 ng/mL (42.2–172.1 ng/mL). The corresponding average for salivary cortisol for the same was 2.04 ng/mL (0.56–5.56

ng/mL). The data implies the serum levels of total cortisol to be approximately 40.49 times higher than salivary cortisol.

The serum and salivary cortisol values were plotted using a scatterplot and the correlation between the two was checked. The data presented in Fig. 1 for a sample size of 47, demonstrates the correlation of serum and salivary cortisol levels from random samples. The correlation coefficient between the two was calculated to be 0.51, and the p-value for the same was found to be < 0.05, suggesting saliva as a reliable serum alternative for cortisol measurement.

Further, to test the applicability of a porous sponge as the medium for saliva collection was also tested in the same study group to mitigate the variations arising from the saliva collection methods. PVA sponge was chosen for this purpose owing to its remarkable absorbent nature along with non-toxic nature and the absence of interaction with cortisol, a vital property for analysis.[26–28] The average cortisol values in the saliva samples extracted from the PVA sponge were found to be 0.93 ng/mL (0.54–3.31 ng/mL). The correlation coefficient for the same with cortisol expressed in drool samples was significant with an r^2 of 0.52, and a p-value of < 0.05 (Fig. 2). The cortisol recovery from the PVA sponges was calculated by comparing it with the cortisol expression in the drool samples, and was found to be 45.6% on average. Based on the findings along with the convenience of sample collection as reported by the participants, the sponge method was declared to be a better route of saliva collection requiring minimal instructions and steps.

To understand the effect of age on the expression of cortisol, age related cortisol values were assessed. The study group was divided into subsequent age groups to understand and validate age as a variable to cortisol expression to build toward the personalization of the cortisol reference ranges. For this purpose, the samples were sorted in groups of 5 years to improve the resolution of the data and to limit the bias of having individuals concentrated in a small age band across the wide age groups. The graph in Fig. 3 shows that the youngest age group of 18–22 years had the highest levels of serum cortisol averaging 95.50 ng/mL (47.7-172.1 ng/mL). The cortisol levels dropped gradually with the progression of age up to the age group of 49–53 years with average serum cortisol levels of 59.18 ng/mL. However, in the last age group, an elevation in the serum cortisol expression was observed, rising to 89.33 ng/mL (46.1–121.3 ng/mL) almost matching the highest age-related average seen in 18–22 years age group. This reversal in trend is suspected to be the outcome of persistent physiological stress arising from chronic ailments and/or administration of regular medications as 83.33% of the participants in this age group presented with diabetes, hypertension, or both. The validation of this hypothesis was not possible due to the unavailability of a *normal* population group in that age group and remains beyond the scope of the current study. The salivary cortisol values followed a similar trend closely, being highest in the youngest age group with an average value of 2.91 ng/mL (1.66–5.56 ng/mL), falling to the lowest levels of 0.75 ng/mL (0.64–0.85 ng/mL) in the 49–53 years age group before rising again to 1.20 ng/mL (0.654–2.18 ng/mL) in the age group of 54–58 years. Although the increase in the salivary cortisol expression was not as profound as in serum levels, suggesting an underlying role of the decreased diffusion rates of cortisol from blood to saliva in the elderly participants.

The overall serum cortisol levels, sorted for sex showed a clear demarcation in expression, with an average of 94.4 ng/mL, and 72.52 ng/mL for males, and females respectively. The observation was found to be maintained in age-correlated cortisol levels where in age groups of 18–22, 44–48 and 54–58 years the serum cortisol levels in males were 9.15%, 21.91% and 10.21% higher than females. The cortisol expression in the participants was found to be independent of the body mass indices (Data not shown).

Based on the observed influence of demographics on the expression of cortisol, it was logical to find the reference ranges for the individuals based on their demographics. As the influence was found only with sex and age of an individual, the various reference ranges found are tabulated in the Table 1. The grouped ranges are evidently showing stark differences from the average for the same study group and are a crucial step for generating the personalized reference ranges based on the personal data of an individual.

Table 1: Personalised reference ranges for serum cortisol encompassing the variation caused by age and sex.

Sex / Age	Male	Female
18-33	104.08 ± 29.71 ng/mL	82.65 ± 29.5 ng/mL
34-48	78.13 ± 19.60 ng/mL	67.32 ± 27.29 ng/mL
49-62	93.98 ± 32.37 ng/mL	71.13 ± 20.1 ng/mL

4. Conclusion

Stress monitoring is of paramount importance in the physiological and psychological well-being of individuals, and its assessment is fundamentally dependent on the quantification of cortisol. The current study on a population group of 47 individuals demonstrates the applicability of saliva as a substitute to serum-based cortisol quantification, expressing cortisol at ~ 2.5% of the serum concentration with a linear correlation having an r^2 value of 0.51 and a p-value of < 0.05. Alternatively, a porous PVA sponge presented itself as an improvement in the sample collection method for saliva-based cortisol measurement with an r^2 of 0.52 and a p-value of < 0.05. The study further demonstrates the role of age and sex as variables to cortisol quantification while suggesting medication and chronic illnesses as potential effectors. The dependence of cortisol expression from body mass index is inconclusive from the data obtained in this study. Finally, the current study peeks in the development of personalized reference ranges for cortisol based on the individual personal data, and paves the way for the personalization of

analyte references based on the studied variables, a finding that can be critical in improving the efficacy of clinical diagnosis.

Declarations

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Figures

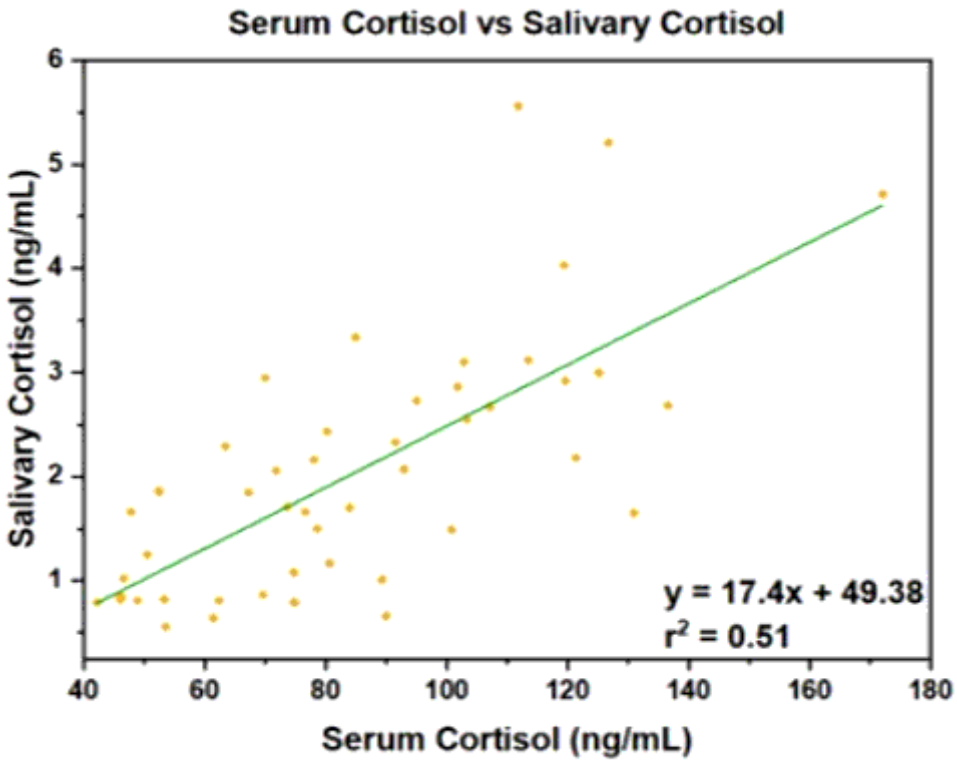

Figure 1

Correlation between serum and salivary cortisol levels

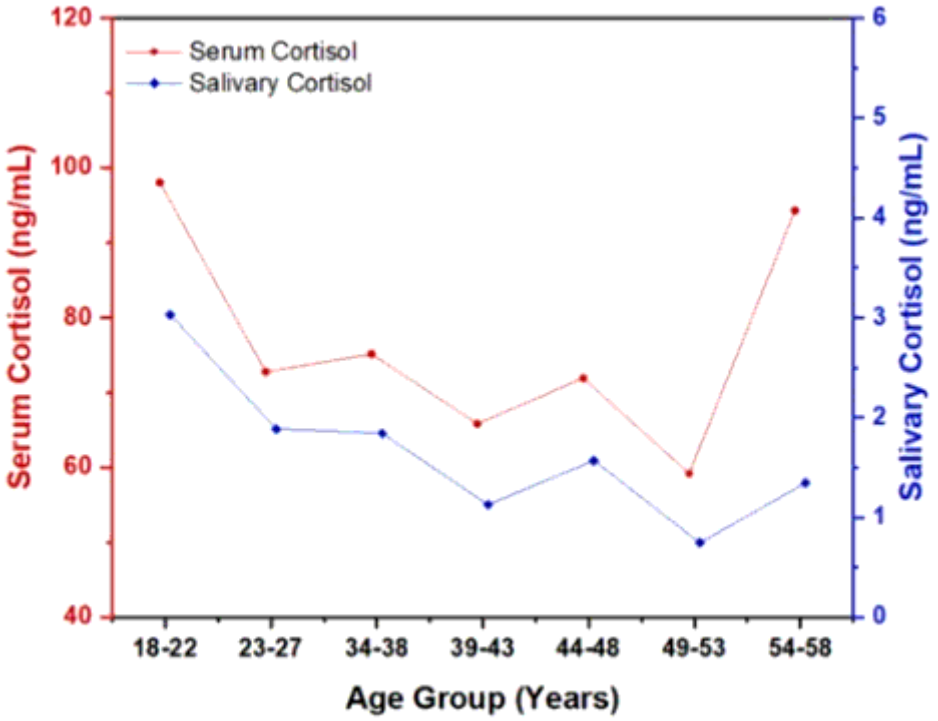

Figure 2

Correlation between drool and PVA Sponge

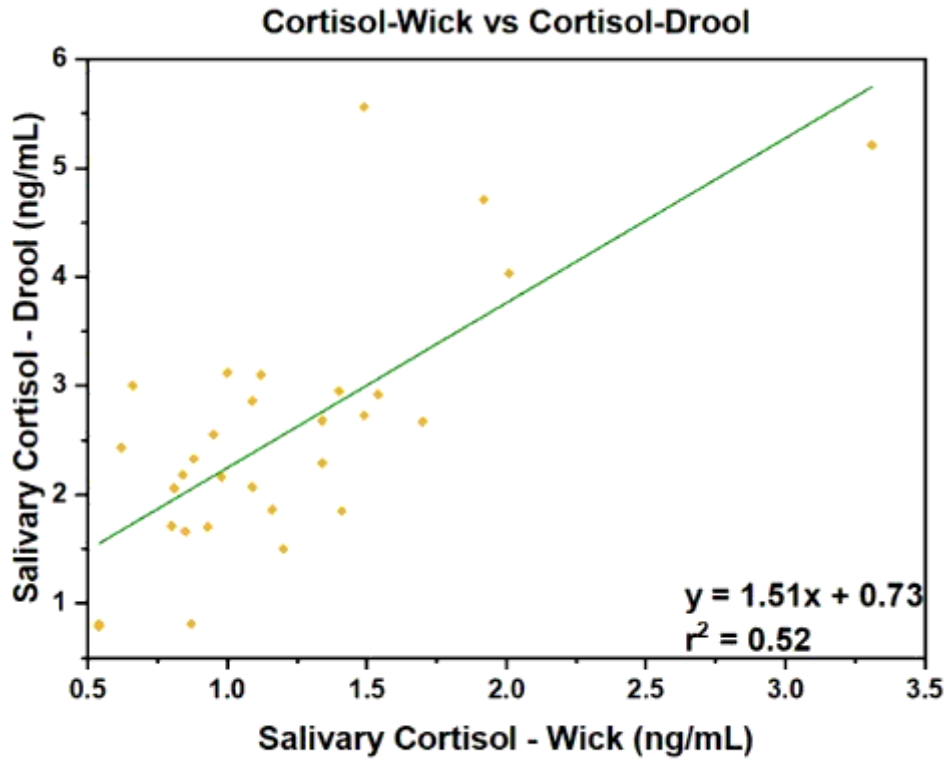

Figure 3

Trend as seen in cortisol levels across different age groups in both serum and saliva samples