# Within-Day Baseline Variation in Salivary Biomarkers in Healthy Men

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Firman Prathama Idris[1,2], Yunxia Wan[1], Xi Zhang[1] and Chamindie Punyadeera[1]

[1]Institute of Health and Biomedical Innovation, School of Biomedical Science, Queensland University of Technology (QUT), 60 Musk Avenue, Kelvin Grove, Australia

[2]School of Chemistry and Molecular Biosciences, University of Queensland, Brisbane, Australia

**Short title:** *Baseline Variation in Salivary Biomarkers*

**Correspondence**
*Chamindie Punyadeera, PhD*
*Associate Professor*
*The School of Biomedical Sciences*
*Institute of Health and Biomedical Innovation*
*Queensland University of Technology*
*60 Musk Avenue, GPO Box 2434*
*Brisbane QLD 4001*
*Australia*
Email: chamindie.punyadeera@qut.edu.au

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## Abstract

Saliva is an easily accessible sample and offers practical and noninvasive biomarker solutions as an alternative to blood and urine based diagnostics. Saliva contains a plethora of biomolecules such as nucleic acids, hormones, proteins, and electrolytes. On the other hand, little is known on the extent to which the biomolecules in saliva vary over time within a given person. This baseline information is crucial for future development of robust saliva-based diagnostics. We have collected unstimulated whole mouth saliva from 20 healthy young men at four times during the day, including before and after a meal. We measured the salivary cortisol, testosterone, C-reactive protein (CRP), stability of genomic DNA and DNA methylation levels of *APC*, *P16*$^{INK4a}$ and *PCQAP* in these samples. We found that the salivary CRP, DNA methylation and *CD44* gDNA levels did not vary significantly across four time points ($P > 0.05$) while the salivary cortisol and testosterone levels varied significantly from the morning collection to the afternoon collection ($P < 0.05$). Furthermore, salivary cortisol levels were significantly affected by eating ($P < 0.05$). Our study offers a within-person baseline temporal assessment of several clinically relevant biomolecules and diagnostics, and suggests that salivary cortisol and testosterone levels vary over time in a given day whereas CRP and DNA methylation of tumour suppressor genes and CD44 amplification are stable throughout the day. Future research and clinical applications of salivary biomarkers and diagnostics should take into consideration their temporal variations.

## Introduction

The potential of using saliva as an alternative diagnostic medium has extensively been researched in the past (Miller et al., 2010; Punyadeera et al., 2011; Ovchinnikov et al., 2014; Salazar et al., 2014). Saliva contains a plethora of biomolecules such as nucleic acids, hormones, proteins, and electrolytes (Pfaffe et al., 2011). These biomolecules could either be directly produced from the salivary glands or derived from blood through ultrafiltration, diffusion or active transport (Pfaffe et al., 2011). Changes in salivary biomolecules could offer a snapshot of an organism's pathophysiological condition at the time of sample collection (Nunes et al., 2015).

Unlike blood, saliva collection is noninvasive, simple, and multiple samples can be drawn from an individual at different times (Mandel et al., 1976). Furthermore, it involves minimal risk to the person collecting these samples and it could easily be stored and transported (Hofman, 2001; Pfaffe et al., 2011). However, the analytes that originate from blood can only be detected in saliva at $100^{th}$ to $1000^{th}$ fold less than in blood and as such, sensitive technologies that enable the detection of these molecules are a prerequisite (Christodoulides et al., 2005). In addition, salivary biomarker composition could be influenced by the method of saliva collection, site of collection and the type of saliva, i.e., stimulated *versus* unstimulated (Hofman, 2001; Pfaffe et al., 2011; Mohamed et al., 2012; Ovchinnikov et al., 2012; Topkas et al., 2012; Ovchinnikov et al., 2014).

A key disadvantage of using saliva to diagnose systemic diseases is the temporal variations in biomolecule concentrations (Granger et al., 2003; Hardt et al., 2005; Harmon et al., 2008; Hayes LD, 2012; Smyth et al., 2013). Concentrations of salivary alpha-amylase, histatins, cortisol and testosterone have diurnal variations (Granger et al., 2003; Hardt et al., 2005;

Harmon et al., 2008; Hayes LD, 2012; Smyth et al., 2013). For example, salivary alpha-amylase concentrations display a non-linear pattern throughout the day; it is relatively low in the morning with a rapid rise from morning to noon (Harmon et al., 2008). Conversely, salivary cortisol levels are elevated early in the morning, a process known as cortisol awakening response (CAR) which causes peak cortisol levels 30 to 45 minutes after being awake. In CAR, the concentrations of salivary cortisol could increase by 50-100% (Smyth et al., 2013).

*(Granger et al., 2003)*has shown that salivary testosterone levels decrease by 3.04 pg/mL per hour across the day in adolescent men. In contrast, Hayes et al. (Hayes LD, 2012)) has reported that salivary testosterone levels in young university male students showed no significant variations throughout the day. This may be attributed to the type and time of saliva collection. In addition, it is important to identify both internal (e.g., age, gender) and external factors (diet) that could affect salivary biomarker profile changes (Mayeux, 2004). These variabilities are major limitations in advancing salivary diagnostics.

Understanding variation of biomarkers in healthy subjects is an essential step prior to implementation in a clinical setting (Wu, 2013). Furthermore, biomarkers with high intra-individual variations are clinically less valuable than those with less variations (Wu, 2013). The aim of this study was to evaluate the temporal daily changes in salivary biomolecules that are currently being used either in a research phase or commercially. The salivary biomarkers validated within this study are most commonly used salivary biomarkers to evaluate health and disease status. As such, we have chosen these biomarkers to investigate the changes within a day. Only healthy men were recruited in this study to avoid cyclic hormonal changes which could influence salivary biomolecule composition (Gröschl et al.,

2001; Nepomnaschy et al., 2011). We investigated the levels of salivary cortisol, testosterone, C-reactive protein (CRP), stability of genomic DNA (gDNA) and DNA methylation levels at four different time points, including before and after eating. We found that salivary cortisol and testosterone levels vary over time in a given day whereas CRP, *DNA* methylations of tumour suppressor genes and CD44 amplifications were stable throughout the day.

## Materials and methods

*Study Participants*

This study was approved by the University of Queensland's Medical Ethical Institutional Board (HREC number 2014000679) and the Queensland University of Technology Ethics Board (HREC number 1400000617). All participants gave informed written consent to donate saliva samples. In total, 20 healthy men were involved in this study and the age of the participants ranged from 20 to 32 years. All of the participants were of Caucasian ethnical origin and were in good general health. The exclusion criteria included having fever or signs and symptoms of suggesting active infection/illness on the day of saliva donation, having history of Hepatitis A or B, ear-nose-throat complaints, undergoing dental treatment or wearing dentures, having history of diabetes or hypoglycaemia, having recent history of alcohol or substance abuse, having history of any cancer, having irradiation to head and neck region and taking any medication during the sample collection process.

*Saliva sample collection and processing*

One hour before saliva collection, volunteers were asked to refrain from drinking and eating with the exception of drinking water as per previous work (Punyadeera et al., 2011; Ovchinnikov et al., 2014; Salazar et al., 2014). Before collection, volunteers were asked to rinse their mouths with water and to sit in a comfortable position, tilt their head down and

pool saliva in their mouths for 3-5 minutes. The pooled saliva was then expectorated into a 50 mL Falcon tube. Collected saliva was then frozen on dry ice and transported to the laboratory. Saliva samples were then aliquoted into multiple Eppendorf tubes and stored at -$80^0$C.

*Bisulfite conversion in DNA extracted from saliva samples*

Extraction of DNA from saliva was performed using Qick-gDNA™ MiniPrep Kit (Cat. No. D3025, Zymo Research, Irvine, CA, U.S.A) with slight modifications. In the DNA extraction method, we used 100 µL of saliva with 95 µL of DNA/RNA Shield (Cat. No R1100-50, Zymo, Irvine) and 5 µL of (20 mg/mL) proteinase K (Cat. No. 76225, Affymetrix, Santa Clara, California United States) to obtain high DNA yields from saliva samples.

The extraction and subsequent bisulfite conversion of DNA was performed by centrifuging 200 µL of collected whole unstimulated saliva samples at 1,500xg for 15 minutes at 4ºC. Supernatant was discarded and approximately 30 µL cellular pellet was used in subsequent DNA methylation analysis. The Epitect® Plus DNA Bisulfite Kit (Cat. No. 59124, Qiagen, Duesseldorf, Germany) was then used according to the manufacturer's protocol to simultaneously extract DNA and bisulfite convert. However, some adjustments were made to the protocol. The eluted volume of the sample was 17 µL instead of 15 µL and the bisulfite converted samples were incubated for an additional 10 minutes before the final elution. The concentration and purity of the bisulfite converted DNA was then measured using a Nano Drop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA).

*Real-time quantitative methylation specific PCR for PCQAP, APC and $p16^{INK4a}$*

A methylation specific quantitative PCR (qMSP) was performed in order to analyse the promoter methylation levels of *PCQAP*, *APC* and $p16^{INK4a}$ tumour suppressor genes. These genes have been selected as these have been widely reported to be methylated in cancers (Merlo et al., 1995; Kawamoto et al., 2006; Ovchinnikov et al., 2014). The primers used to amplify the methylated promoters of *APC* and $p16^{INK4a}$ genes are shown in Table 1. *MYOD* was used as a DNA normaliser.

The qMSP reaction was performed in a 10 µL reaction containing 5 µL iTaq™ Universal SYBR® Green Supermix (Cat. No. 1725120, Bio-Rad Laboratories, Inc., Hercules, California, United States), 200 nM of the forward and reverse primers for $p16^{INK4a}$ and *MYOD*, 400 nM of *APC* primers and 250 nM for *PCQAP* primers. For *PCQAP*, *APC* and $p16^{INK4a}$, 100 ng of bisulfite converted DNA was used in the qMSP and 25 ng of template were used for *MYOD*. For *APC* and $p16^{INK4a}$, the qMSP reaction was as follows: initial denaturing step at 95ºC for 3 minutes, followed by 50 cycles of denaturing step at 95ºC for 30 seconds and annealing stage at 56ºC (*APC* and $p16^{INK4a}$), 60ºC (*PCQAP)*, or 62.5ºC (*MYOD*) for 30 seconds (*MYOD, APC,* $p16^{INK4a}$) or 60 seconds (*PCQAP*). qMSP were performed in duplicate. Subsequent melt curve analysis was performed to determine the specificity of the PCR amplicons and found to be consistent. Methylation ratio was calculated by dividing CT value of the target genes with their corresponding CT value for *MYOD* ($Methylation\ ratio = \frac{Ct\ value\ of\ target\ genes}{Ct\ value\ of\ MYOD} x100$) as previously described (Arantes et al., 2015).

The PCR amplification of *CD44* was performed by using *CD44* primers (see Table 1) and *β-Globin* was used as a normaliser. PCR Reactions of 10 µL were made using 5 µL iTaq™

Universal SYBR® Green Supermix, 200 nM of the forward and reverse primers and 20 ng of gDNA template. The qMSP-PCR condition was as follows: initial denaturation at 95ºC for 10 minutes followed by 40 cycles of denaturing step at 95ºC for 15 seconds and 60ºC for 30 seconds. Melt curve analysis was then performed. Delta Ct of the samples were used to determine the amplification of *CD44* using the following calculation: $\Delta Ct = Ct_{CD44} - Ct_{B-Globin}$.

*C-Reactive protein concentrations using AlphaLISA® Immunoassay Kit*

Concentrations of CRP in saliva samples were measured using the human CRP Kit (Product-No: AL233C, Perkin Elmer®, MA, USA). Samples were analysed in triplicates as per manufacturer instructions using a 384 well ProxiPlate-384 Plus (Cat. No. 6008289, PerkinElmer, Inc., Waltham, Massachusetts, United States) as per our previous publication (Mohamed et al., 2012; Zhang et al., 2013; Zhang et al., 2014). The total reaction volume for each well was reduced to 10 µL containing 1 µL of undiluted saliva samples (analyte), 40 µg/mL acceptor beads, 50 µg/mL streptavidin donor beads, and 10 nM of biotinylated antibody. The mixture was incubated for 1.5 hours at room temperature in the dark and measured using an EnSpire® Multimode Plate Reader (Model No. 2300, PerkinElmer, Inc., Waltham, Massachusetts, United States). Twelve assay point standards were measured in order to generate a standard curve. The intra-assay variations for the triplicates were analysed using coefficient of variation that was calculated by: $\%CV = \frac{Mean\ of\ SD}{Mean} x100$

*Salivary testosterone measurements*

The measurement of testosterone levels was performed by using the Salivary Testosterone Enzyme Immunoassay Kit (Salimetrics, Inc., State College, PA) as per manufacturer instructions. In brief, saliva samples were centrifuged at 1,500xg for 15 minutes and 25 µL of

the supernatants were added into microtiter plates coated with rabbit anti-human testosterone. Samples and standards were then incubated with horseradish peroxidase and tetramethylbenzidine and reaction was stopped using 2M of sulfuric acid. Plates were then read on a plate reader at 450 nm. Six standard assay points were measured to generate a standard curve and measurements were done in duplicates to increase the reliability of measurement.

*Salivary cortisol measurement*

Measurement of cortisol concentrations in saliva samples was performed using the IPRO Lateral Flow Device and read using the IPRO Lateral Flow Device Reader (IPRO Interactive, Wallingford, UK). Saliva samples were diluted (1:1) with IPRO Oral Fluid Collector buffer and 66 µL of the mixture was then placed onto the Lateral Flow Device sample pad and incubated for 10 minutes before being read by the IPRO Lateral Flow Device reader.

*Statistical analysis*

The statistical analyses were performed using the GraphPad Prism software version 6.07 (GraphPad Software Inc., USA). Normal distributions were evaluated for all measurements and data using Shapiro-Wilk normality test and found that the data are not Gaussian distributed. ANOVA and Friedman test was used to analyse the measurement data across four different time points and Wilcoxon matched-pairs signed rank tests were used to analyse measurements between two time points. The non-parametric Spearman correlation analyses were carried out in order to determine the correlation between analytes and the salivary flow rates. The % coefficient of variation (CV) regarding the time-dependent variations in salivary biomolecule concentrations was calculated as (standard deviation/mean X 100) and by taking

the average of these values across the study subjects. Statistical significance was assessed as $p<0.05$.

## Results

*Participants characteristics and salivary flow rates*

The average age of the healthy volunteers was 25.6 years old with a Standard Deviation (SD) of 3.3 years. All participants were men and of Caucasian ethnicity. Additionally, all of the participants showed no periodontal disease, were non-smokers, and 90% of them were mild/social alcohol drinkers but no alcohol was permitted during the study. Salivary flow rates were measured and ranged from 0.10 g/mL to 2.46 g/mL with a median of 0.43 g/mL (interquartile range (IQR), 0.29 g/mL to 0.84 g /mL).

*Salivary C-reactive protein concentrations*

The intra-assay CV for salivary CRP measurement was 7.3% ± 0.8 and the lower limit of detection (LOD) was 30.0 pg/mL. **Figure 1a** demonstrates that the salivary CRP concentrations did not change significantly ($p=0.23$) throughout the day, confirming our previous findings (Punyadeera et al., 2011). Furthermore, there was no correlation between the salivary flow rates and CRP levels (Spearman correlation $r=0.10$, $p=0.37$). The average CVs of salivary CRP levels in subjects' samples across four time points was 116%.

*Within-day salivary cortisol and testosterone levels*

Salivary cortisol concentrations for 11 out of 20 participants (55%) peaked at the first collection time point (9:00 a.m.). The median levels and inter quartile ranges (IQR) values appear in Table 2. The Friedman test for cortisol levels revealed statistically significant differences across four different collection times ($p=0.0007$). Cortisol levels at 9:00 a.m.

showed significant differences to the cortisol levels at 12:00 p.m. (p=0.0007) and 16:00 (p=0.0008). Additionally, salivary cortisol levels were affected by eating (12:00 pm vs 13:00 pm; p=0.0250). However, there were no significant differences in cortisol levels at 9:00 and 13:00 (p=0.0638), 12:00 and 16:00 (p=0.330), 13:00 and 16:00 (p=0.105). The average CVs for salivary cortisol levels in the samples for 4 time points was 58%.

Intra-assay and inter-assay CVs for salivary testosterone measurements were 3.9% ±2.3 and 0.9% ± 0.01 respectively. Salivary testosterone levels had a trend to decrease from 9:00 a.m. until 13:00 p.m., and increased in the afternoon (16:00 p.m.). This trend is shown to have statistically significant differences throughout the day (p=0.0004) and this was especially true for salivary testosterone levels in the morning and after a meal consumption (9:00 vs 13:00, p=0.0001) and in the afternoon (9:00 vs 16:00, p=0.001) and before and after eating (12:00 vs 13:00, p=0.0042). No significant differences were found between other time points (9:00 vs 12:00, p=0.189; 12:00 vs 16:00, p=0.330; 13:00 vs 16:00, p=0.105). The average CVs of salivary testosterone level of the samples across four 4 time points was 52%.

There were no significant correlations between salivary testosterone and cortisol levels (p=0.18), cortisol levels and CRP levels (p=0.89) or salivary testosterone levels and CRP levels (p=0.12). In addition, there were significant correlations between salivary flow rates and testosterone levels (r=-0.24, p=0.03) and between salivary flow rates and cortisol concentrations (r=0.27, p=0.01). Furthermore, our results also demonstrated that the salivary testosterone concentrations were negatively correlated with salivary flow rates. In contrast, salivary cortisol concentrations were positively correlated with flow rates.

*Salivary DNA methylation levels*

Methylation ratio for all methylated genes (*APC, p16$^{INK4a}$*, and *PCQAP*) analysed in this study revealed no significant difference ($p>0.05$) across four collection time points (**Figure 2**). Similarly, methylation ratios between two different time points also showed the methylation stability of these gene promoter sequences. For *p16$^{INK4a}$* and *APC*, samples for all 20 individuals across four time points were analysed, however, due to the unavailability of bisulphite DNA from saliva, analysis of *PCQAP* methylation was done in 8 out of 20 individuals. *PCQAP* and *APC* promoters were shown to have significantly lower methylation ratio than *p16$^{INK4a}$* ($p<0.0001$). There were no correlations between flow rates and promoter methylation of *APC, p16$^{INK4a}$*, and *PCQAP* ($r=-0.116$, $p=0.306$; $r=0.084$ $p=0.457$ and $r=0.099$, $p=0.587$ respectively). The results of these analyses showed that promoter methylation for these genes are not affected by the time of the day and meal consumption. The average CVs for the methylation levels of *APC*, *p16$^{INK4a}$*, and *PCQAP* in the samples at four time points were 70%, 37% and 92%, respectively.

*CD44 DNA PCR amplifications using saliva samples*

The amplification of CD44 DNA from saliva samples collected at four time points revealed no significant differences ($p=0.577$) (Figure 3) across the four time points. The mean $\Delta$Ct for *CD44* at 9:00, 12:00, 13:00 and 16:00 are -1.033 ± 0.294, -1.483 ± 0.329, -1.605 ± 0.163, -1.126 ± 0.354. Furthermore, there was no correlation between flow rates and *CD44 PCR* amplifications ($r=-0.164$, $p=0.146$). The average CV of CD44 amplification of the samples across four time points was 195%

## Discussion

Disease biomarkers should meet several criteria before these can be implemented in clinical assays. Some of the criteria include easy access, clinical sensitivity, specificity, and can be reliably measured with minimal influence by both external and internal factors (Etheridge et al., 2011). We have measured biomarker profile changes in saliva samples across four different time points in a well-controlled group of men. Men were recruited in this study to minimise the influence of cyclic hormonal changes as found in women (Liu et al., 2010; Bocklandt et al., 2011). We found that salivary CRP, DNA methylation of three tumour suppressor genes *PCQAP, APC* and *p16* and CD 44 DNA levels did not significantly differ across the four time points whilst salivary cortisol and testosterone levels changed.

Salivary CRP, testosterone, and cortisol levels corroborated with previous findings (Goncharov et al., 2006; Mohamed et al., 2012). We found elevated levels of cortisol and testosterone in the morning saliva samples agreeing with previous findings (Granger et al., 2003; Smyth et al., 2013). Similarly, Granger *et al.*, (1999) found that 100% of salivary testosterone levels were detectable in the morning samples compared to 97% in the afternoon samples. The large SD in the morning cortisol levels may be as a result of variations in the CAR between individuals as reported before (Hansen et al., 2003). We have also observed lower salivary testosterone and cortisol levels postprandially agreeing with previous work (Mikulski et al., 2010). Decreased testosterone levels after eating is most likely due to splanchnic blood flow that caused hepatocytes to clear testosterone (Mikulski et al., 2010). Salivary CRP levels did not change significantly during the day. Similarly, Punyadeera *et al*., (Punyadeera et al., 2011) also found no significant changes in salivary CRP levels during the day. Similarly, DNA methylation and DNA CD44 levels were not affected during the day. In

contrast, Lim *et al.*, has analysed 400,000 genomic sites in human brain samples and discovered that global DNA methylation follows a 24-hour rhythm (Lim et al., 2014).

*APC* and $p16^{INK4a}$ tumour suppressor genes are responsible for cell cycle regulation and it was shown in previous studies that cell division is controlled by the circadian clock (Matsuo et al., 2003; Nagoshi et al., 2004). It has also been previously reported that the circadian PERIOD proteins and the nuclear protein, NONO, regulate the circadian expression of $p16^{INK4a}$ (Kowalska et al., 2013). Limitations to this study are as follows: relatively small sample size, sleep pattern and type of meals consumed were also not controlled or recorded. Our study revealed that salivary cortisol and testosterone levels follow a pattern throughout the day, whilst, CRP, *DNA* methylations of tumour suppressor genes and CD44 amplifications were stable throughout the day. Future studies involving these biomarkers should also take into consideration their subject to subject variations before applying these in a clinical context.

## Conclusions

We have shown that the salivary CRP, DNA methylation and CD44 DNA levels did not significantly vary across the four time points while the salivary cortisol and testosterone levels varied significantly from a morning saliva collection to an afternoon saliva collection. We have also observed that salivary cortisol levels were affected by eating. Our findings suggest that future studies involving cortisol and testosterone measurements should take temporal variations into account.

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**Abbreviations**

CAR: cortisol awakening response

CRP: C-reactive protein

gDNA: genomic DNA

IQR: interquartile range

qMSP: methylation specific quantitative PCR

LOD: Lower limit of detection

## References

Arantes LM, De Carvalho AC, Melendez ME, et al. (2015). Validation of methylation markers for diagnosis of oral cavity cancer. Eur J Cancer 51, 632-641.

Bocklandt S, Lin W, Sehl ME, et al. (2011). Epigenetic predictor of age. PLoS ONE 6, e14821.

Christodoulides N, Mohanty S, Miller CS, et al. (2005). Application of microchip assay system for the measurement of C-reactive protein in human saliva. Lab Chip 5, 261-269.

Etheridge A, Lee I, Hood L, Galas D and Wang K (2011). Extracellular microRNA: a new source of biomarkers. Mutat Res 717, 85-90.

Goncharov N, Katsya G, Dobracheva A, et al. (2006). Diagnostic significance of free salivary testosterone measurement using a direct luminescence immunoassay in healthy men and in patients with disorders of androgenic status. Aging Male 9, 111-122.

Granger DA, Shirtcliff EA, Zahn–Waxler C, Usher B, Klimes–Dougan B and Hastings P (2003). Salivary testosterone diurnal variation and psychopathology in adolescent males and females: Individual differences and developmental effects. Dev Psychopathol 15, 431-449.

Gröschl M, Rauh M, Schmid P and Dörr H-G (2001). Relationship between salivary progesterone, 17-hydroxyprogesterone, and cortisol levels throughout the normal menstrual cycle of healthy postmenarcheal girls. Fertil Steril 76, 615-617.

Hansen AM, Garde AH, Christensen JM, Eller NH and Netterstrom B (2003). Evaluation of a radioimmunoassay and establishment of a reference interval for salivary cortisol in healthy subjects in Denmark. Scand J Clin Lab Invest 63, 303-310.

Hardt M, Witkowska HE, Webb S, et al. (2005). Assessing the effects of diurnal variation on the composition of human parotid saliva: quantitative analysis of native peptides using iTRAQ reagents. Anal Chem 77, 4947-4954.

Harmon AG, Towe-Goodman NR, Fortunato CK and Granger DA (2008). Differences in saliva collection location and disparities in baseline and diurnal rhythms of alpha-amylase: A preliminary note of caution. Horm Behav 54, 592-596.

Hayes Ld GF, Kilgorejl Yjd, Baker Js. (2012). Interactions of cortisol, testosterone, and resistance training: influence of circadian rhythms. Sports SPA 9, 5-13.

Hofman LF (2001). Human saliva as a diagnostic specimen. J Nutr 131, 1621s-1625s.

Kawamoto K, Enokida H, Gotanda T, et al. (2006). p16INK4a and p14ARF methylation as a potential biomarker for human bladder cancer. Biochem Biophys Res Commun 339, 790-796.

Kowalska E, Ripperger JA, Hoegger DC, et al. (2013). NONO couples the circadian clock to the cell cycle. Proc Natl Acad Sci U S A 110, 1592-1599.

Lim AS, Srivastava GP, Yu L, et al. (2014). 24-hour rhythms of DNA methylation and their relation with rhythms of RNA expression in the human dorsolateral prefrontal cortex. PLoS Genet 10, e1004792.

Liu J, Morgan M, Hutchison K and Calhoun VD (2010). A study of the influence of sex on genome wide methylation. PLoS ONE 5, e10028.

Mandel ID and Wotman S (1976). The salivary secretions in health and disease. Oral Sci Rev, 25-47.

Matsuo T, Yamaguchi S, Mitsui S, Emi A, Shimoda F and Okamura H (2003). Control mechanism of the circadian clock for timing of cell division in vivo. Science 302, 255-259.

Mayeux R (2004). Biomarkers: Potential Uses and Limitations. Neurorx 1, 182-188.

Merlo A, Herman JG, Mao L, et al. (1995). 5' CpG island methylation is associated with transcriptional silencing of the tumour suppressor p16/CDKN2/MTS1 in human cancers. Nat Med 1, 686-692.

Mikulski T, Ziemba A and Nazar K (2010). Metabolic and hormonal responses to body carbohydrate store depletion followed by high or low carbohydrate meal in sedentary and physically active subjects. J Physiol Pharmacol 61, 193-200.

Miller CS, Foley JD, Bailey AL, et al. (2010). Current developments in salivary diagnostics. Biomark Med 4, 171-189.

Mohamed R, Campbell J-L, Cooper-White J, Dimeski G and Punyadeera C (2012). The impact of saliva collection and processing methods on CRP, IgE, and Myoglobin immunoassays. Clin Transl Med 1, 19-19.

Nagoshi E, Saini C, Bauer C, Laroche T, Naef F and Schibler U (2004). Circadian Gene Expression in Individual Fibroblasts: Cell-Autonomous and Self-Sustained Oscillators Pass Time to Daughter Cells. Cell 119, 693-705.

Nepomnaschy PA, Altman RM, Watterson R, Co C, Mcconnell DS and England BG (2011). Is Cortisol Excretion Independent of Menstrual Cycle Day? A Longitudinal Evaluation of First Morning Urinary Specimens. PLoS ONE 6, e18242.

Nunes LaS, Mussavira S and Bindhu OS (2015). Clinical and diagnostic utility of saliva as a non-invasive diagnostic fluid: a systematic review. Biochemia Medica 25, 177-192.

Ovchinnikov DA, Cooper MA, Pandit P, et al. (2012). Tumor-suppressor Gene Promoter Hypermethylation in Saliva of Head and Neck Cancer Patients. Transl Oncol 5, 321-326.

Ovchinnikov DA, Wan Y, Coman WB, et al. (2014). DNA Methylation at the Novel CpG Sites in the Promoter of MED15/PCQAP Gene as a Biomarker for Head and Neck Cancers. Biomark Insights 9, 53-60.

Pfaffe T, Cooper-White J, Beyerlein P, Kostner K and Punyadeera C (2011). Diagnostic potential of saliva: current state and future applications. Clin Chem 57, 675-687.

Punyadeera C, Dimeski G, Kostner K and Beyerlein P (2011). One-step homogeneous C-reactive protein assay for saliva. J Immunol Methods 373, 19-25.

Salazar C, Nagadia R, Pandit P, et al. (2014). A novel saliva-based microRNA biomarker panel to detect head and neck cancers. Cell Oncol (Dordr) 37, 331-338.

Smyth N, Hucklebridge F, Thorn L, Evans P and Clow A (2013). Salivary Cortisol as a Biomarker in Social Science Research. Social and Personality Psychology Compass 7, 605-625.

Topkas E, Keith P, Dimeski G, Cooper-White J and Punyadeera C (2012). Evaluation of saliva collection devices for the analysis of proteins. Clin Chim Acta 413, 1066-1070.

Wu AH (2013). Biological and analytical variation of clinical biomarker testing: implications for biomarker-guided therapy. Curr Heart Fail Rep 10, 434-440.

Zhang X, Dimeski G and Punyadeera C (2014). Validation of an immunoassay to measure plasminogen-activator inhibitor-1 concentrations in human saliva. Biochem Med (Zagreb) 24, 258-265.

Zhang X, Wan Y, Cooper-White J, Dimeski G, Atherton J and Punyadeera C (2013). Quantification of D-dimer levels in human saliva. Bioanalysis 5, 2249-2256.

**Table 1. qPCR primer sequences used in the study.**

| Target | | Sequence | Predicted size |
|---|---|---|---|
| p16 | Forward | 5' TCGGTTTTCGATCGTAATTATTC 3' | 133 bp |
| | Reverse | 5' ACGAAAAACAACATAAAACCTTCG 3' | |
| APC | Forward | 5' GAACCAAAACGCTCCCCAT 3' | 74 bp |
| | Reverse | 5' TTATATGTCGGTTACGTGCGTTTATAT 3' | |
| PCQAP/MED15 | Forward | 5' GGGAGTTGGGGAATAGGTATGGA 3' | 159 bp |
| | Reverse | 5' CGACCCTAACCTCGCCCG 3' | |
| CD44 | Forward | 5' CAGACAGGCTCACTCAAGCTCTTT 3' | 50 bp |
| | Reverse | 5' CTGGAGTGGCTTGTTGCTTTTCAGTT 3' | |
| MYOD | Forward | 5' TGATTAATTTAGATTGGGTTTAGAGAAGGA 3' | 162 bp |
| | Reverse | 5' CCAACTCCAAATCCCCTCTCTAT 3' | |
| β-Globin | Forward | 5' CAACTTCATCCACGTTCACC 3' | 268 bp |
| | Reverse | 5' GAAGAGCCAAGGACAGGTAC 3' | |

## Figure legends

**Figure 1.** Temporal variability of salivary concentrations of CRP (a), cortisol (b), and testosterone (c). There are no significant differences among time points (p=0.38) for salivary CRP levels. For salivary cortisol levels, there was a trend of increased levels early in the morning and a slight increase after meal consumption. Salivary testosterone levels peaked in the morning before gradually decreasing. Friedman's test was used to analyze statistical significance (* = $p > 0.05$, ** = $p < 0.05$, **** = $p \leq 0.001$).

**Figure 2.** The stability of APC (a), $p16^{INK4a}$ (b), and PCQAP (c) tumour suppressor gene methylations at four collection time points. There were no statistical differences among the methylation ratio throughout the day (p=0.32, p=0.85, p=0.61, respectively). Mean of the methylation ratio with standard deviations are shown.

**Figure 3.** Stability of CD44 gDNA amplification at four collection points. ΔCt with standard deviations is shown.

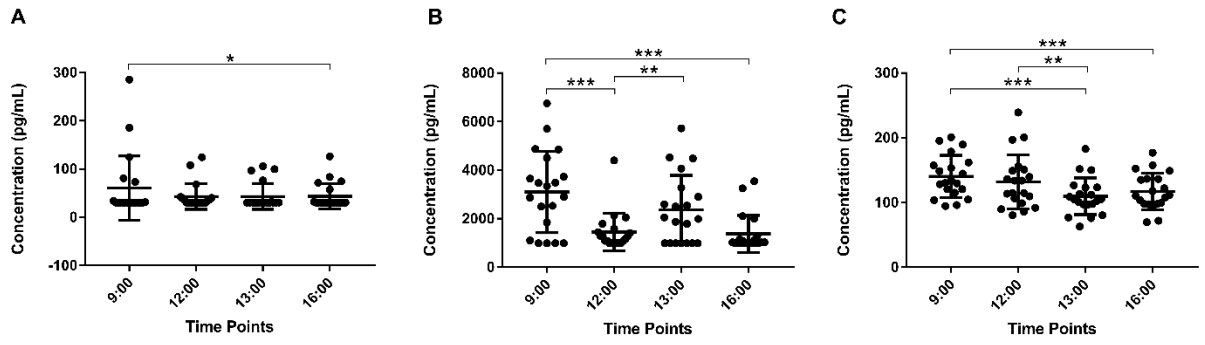


Figure 1

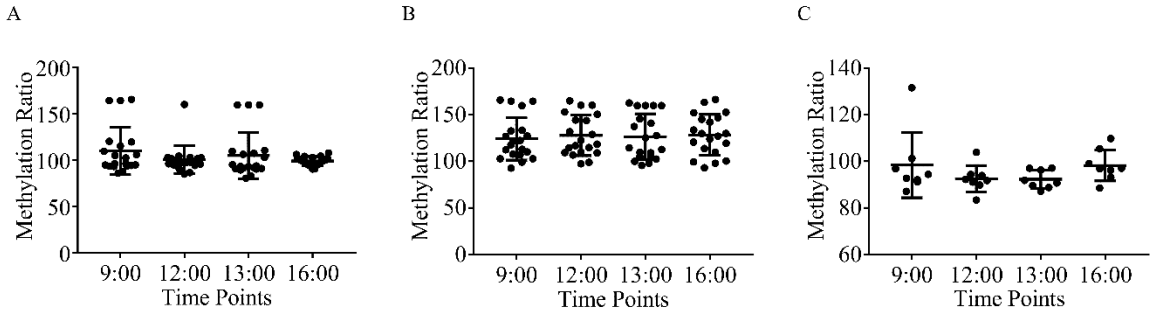


Figure 2

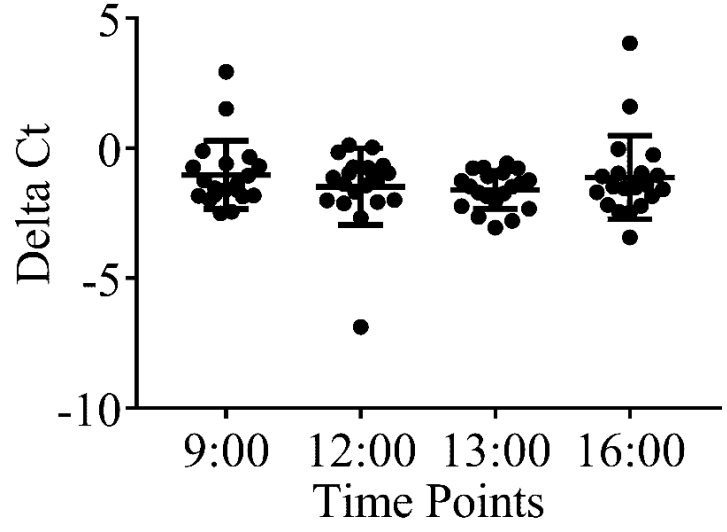


Figure 3