

# Can saliva testing replace blood measurements for health monitoring? Insights from a correlation study of salivary and whole blood glutathione in humans. †

Received 00th January 20xx,  
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

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The feasibility of using saliva samples as diagnostic for health status is assessed. Although blood is regularly used for this purpose, an alternative non-invasive route which yields equivalent clinical information is desirable. The non-invasive saliva testing is validated by comparing its result to that of blood examination. In this investigation, we used glutathione as a paradigmatic example of a biomarker and diagnostic auxiliary. Correlation between the levels of total unbound glutathione, reduced and oxidized, in saliva and whole blood samples from healthy individuals is evaluated. Both salivary and blood glutathione were measured using an enzymatic kinetic assay which was improved to eliminate measurement errors arising from the variation in the enzyme activity from different batches.

## 1 Introduction

Blood specimens are used extensively to monitor the general state of health and for analysis of many specific diagnostic analytes. The concentrations of metabolites in blood are taken as representative of tissues in major organs. Blood specimen collection, however, is invasive and has raised difficulties in clinical analysis. Drawing blood can be impractical for people with blood-injection-injury type phobia and those who require daily monitoring of biomarker levels. Non-invasive technology has thus become increasingly important and would be ideal for point-of-care diagnosis.

Among the non-invasive bodily fluids, saliva is one of the most preferable and practical specimens for health monitoring as it is readily available as well as easily collected and stored. Saliva contains various significant biomarkers including viral antibodies,<sup>1</sup> drugs,<sup>2</sup> steroid hormones such as cortisol,<sup>3</sup> progesterone<sup>4</sup> and estriol<sup>5</sup>, and glutathione.<sup>6, 7</sup> Recent successful development of non-invasive saliva technologies<sup>8, 9</sup> further leads to the urge for investigations concerning the relevance of saliva test results towards the actual health status.

Guilbault et al.<sup>10</sup> reported excellent correlations between salivary and blood serum concentrations of alcohol and lactate. On the other hand, it was reported that salivary glucose showed no correlation with blood glucose.<sup>11</sup> Discrepancy in the results regarding the correlations between salivary and blood levels of different biomarkers together with the relatively few reported works on saliva-blood correlations bring into question the reliability of saliva as a diagnostic tool.

In this paper, we use the most abundant and relevant thiol biomarker in human body, glutathione (reduced, GSH and oxidized, GSSG), as a prime example to assess the possibility of saliva as a blood-alternative for clinical analysis. Glutathione is an anti-oxidant which responds to both xenobiotic and endogenous compounds. Blood glutathione has been extensively proved to show significantly altered concentrations in patients with various major human diseases<sup>12-15</sup> and life styles.<sup>16, 17</sup>

The content of total unbound glutathione (GSH and GSSG) in saliva and whole blood samples collected from 15 healthy participants were analyzed using an enzymatic kinetic method developed by Tietze et al.<sup>18</sup> In addition, we present in this paper modifications made to the Tietze enzymatic assay to eliminate the inaccuracy arising from possible variation in the enzyme activity. The enzymatic assay specific to the analysis of saliva, which to the authors' best knowledge has not been reported before, is also included.

The results evidence a positive but weak correlation between salivary and whole blood glutathione ( $p$ -value = 0.112). Possible explanation for the lack of strong correlation is addressed later in the text. In this paper, we have further constructed a chart and a table to review the published relationships (correlation or no correlation) of glutathione

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† Electronic Supplementary Information (ESI) available. See

DOI: 10.1039/x0xx00000x

levels in specimens collected from different compartments of the body. As to date, there is little information on this topic despite the extensive uses of glutathione concentrations in various biological specimens in the examination of diseases.

From all the results and literature research gathered in this paper, we believe that saliva tests, although evidenced to not strongly correlate with blood measurements in the assessment of the general health status of healthy humans, are valuable biomarkers for certain diseases. Nonetheless, this work will serve to stimulate further studies regarding the value of information and consistency of results obtained from saliva, both of which are crucial steps before any of the saliva sensors can be used clinically and commercially as a sole diagnostic tool.

## 2 Materials and Methods

### 2.1 Participants

Healthy subjects were recruited through local and online advertising. Inclusion criteria included: participant willing and able to give informed consent, not currently taking any medications (except the contraceptive pill), non-smoker, alcohol consumption no more than 28 units weekly (men) or 21 units (women), no recent involvement (last month) in an interventional research study and female subjects reporting not being pregnant or breast feeding at the time of the study. Altogether 15 healthy subjects were studied (13 female, 2 male; mean age 33.8 years; range 22-50 years; BMI range 19-30). All gave written informed consent to the study which was approved by Oxford University Medical Sciences Interdivisional Research Ethics Committee.

### 2.2 Sample collection and storage

Subjects consumed no food or drink at least one hour prior to sampling. 5 mL venous blood samples were taken into ethylenediaminetetraacetic acid (EDTA) bottles and frozen whole. Saliva samples were taken directly after the blood sample using a salivette with the participant subjects seated. The salivette remained in the oral cavity for one minute. The saliva was then centrifuged for 15 minutes at 2400 rpm and frozen.

Blood samples were stored at  $-70^{\circ}\text{C}$  prior to analysis. Richie et al.<sup>19</sup> have reported that the concentration of glutathione in human blood sample is stable for up to 20 days when the blood is frozen at  $-70^{\circ}\text{C}$ . The level of glutathione in saliva samples has been reported by Emekli-Alturfan et al.<sup>20</sup> to be stable for up to 30 days when stored at  $-20^{\circ}\text{C}$ . The saliva samples were hence stored at the recommended temperature.

### 2.3 Sample preparation

For the determination of glutathione concentration, 10  $\mu\text{L}$  of whole blood was hemolyzed in 1.99 mL 0.01 M phosphate/5 mM EDTA buffer, pH 7.5. Saliva samples were used as received from the collection point.

### 2.4 Reagents and Instrumentation

All reagents were purchased from Sigma-Aldrich and were used as received without further purification; sodium phosphate dibasic ( $\geq 99.0\%$ ), sodium phosphate monobasic dihydrate ( $\geq 99.0\%$ ), ethylenediaminetetraacetic acid disodium salt dehydrate (EDTA, 99.0-101.0%), 5,5'-Dithiobis(2-nitrobenzoic acid) (DTNB,  $\geq 98\%$ ),  $\beta$ -nicotinamide adenine dinucleotide 2'-phosphate reduced tetrasodium salt hydrate (NADPH,  $\geq 97\%$ ), glutathione reductase from baker's yeast (*S. cerevisiae*) (GR, 100-300 units/mg protein (biuret)) and L-glutathione reduced (GSH,  $\geq 98.0\%$ ).

All glutathione measurements were performed with Hitachi U-2001 UV-Vis spectrophotometer in disposable cuvettes (Eppendorf UVette, Sigma-Aldrich) using a 10 mm optical path length.

### 2.5 Procedures

**Blind tests.** Blood and saliva were given to the testing laboratory without notifying which pair of blood and saliva samples was collected from the same subject.

**Assays.** Total glutathione (GSH and GSSG) concentrations were determined using the Tietze enzymatic kinetic assay<sup>18</sup> modified by Rahman et al.<sup>21</sup> The blood samples nevertheless were prepared according to the original preparation procedure by Tietze<sup>18</sup> as we chose to measure glutathione content in whole blood instead of erythrocytes to avoid the variability that may arise from sample preparation.

The solutions of 40  $\mu\text{L}$ /3.0 mL GR, 1.68 mM DTNB, 0.80 mM NADPH and 32.5  $\mu\text{M}$  GSH were prepared in 0.1 M phosphate buffer solution (pH 7.5) containing 10 mM EDTA salt. All solutions were prepared fresh and their containers were wrapped in aluminium foil.

For the measurement, 100  $\mu\text{L}$  of the standards (or samples), 60  $\mu\text{L}$  of the DTNB solution and 60  $\mu\text{L}$  of the GR solution were added respectively to a cuvette containing 700  $\mu\text{L}$  of the 0.1 M phosphate/10 mM EDTA/pH 7.5 buffer solution. 60  $\mu\text{L}$  of the NADPH solution was then added 30 seconds later. After the solution was mixed thoroughly, the cuvette was immediately transferred to the UV-Vis spectrophotometer. The absorbance at 412 nm was then recorded for 60 seconds by the UV-Vis spectrophotometer in kinetic mode.

**Modified quantitative analysis.** In contrast to the calibration curve technique usually employed in the Tietze enzymatic method, we used the standard addition technique, described next, to significantly improve the accuracy of the measurements. The principles underlying this change will be discussed later in the text.

For the standard addition procedure, GSH concentration increments of 332 nM were added to the assay by replacing 0  $\mu\text{L}$ , 10  $\mu\text{L}$ , 20  $\mu\text{L}$ , 30  $\mu\text{L}$  and 40  $\mu\text{L}$  of the 0.1 M phosphate/10 mM EDTA/pH 7.5 buffer solution by equal volumes of the 32.5  $\mu\text{M}$  GSH solution.

### 2.6 Statistical analysis

Two-tailed Pearson's product-moment correlation test was performed by R statistical software version 3.2.3.<sup>22</sup>

### 3 Results

#### 3.1 Assay validation

The Tietze enzymatic recycling assay is based on the reaction between reduced glutathione (GSH) and 5,5'-Dithiobis(2-nitrobenzoic acid) (DTNB) which produces a mixed disulfide (GS-TNB) and 2-nitro-5-thiobenzoate ( $\text{TNB}^{2-}$ ).  $\text{TNB}^{2-}$  has a strong absorption peak at 412 nm in the UV-Vis spectra. GS-TNB is then reduced by glutathione reductase (GR) in the presence of NADPH to form another  $\text{TNB}^{2-}$  and GSH which can undergo further reaction with DTNB. Any GSSG present in the solution is converted to GSH by GR at the beginning of the process; refer to Figure 1. The measurement of absorbance values at the wavelength of 412 nm as a function of time and the slope of the graph of the absorbance against time gives the rate of  $\text{TNB}^{2-}$  formation which is linearly proportional to the concentration of GSH equivalents present in the sample.<sup>18</sup> Throughout this paper, GSH equivalents refer to the total glutathione concentration ( $[\text{GSH}] + 2[\text{GSSG}]$ ).

The Tietze enzymatic measurements were first performed for a series of standard GSH solutions to obtain a calibration plot. The rate of  $\text{TNB}^{2-}$  formation was calculated using the time interval of 0 to 60 s. The results yielded a linear increase in the rate of  $\text{TNB}^{2-}$  formation as a function of GSH concentration ( $R^2 > 0.99$ ) in the concentration range of interest (0.1  $\mu\text{M}$  to 2.7  $\mu\text{M}$ ) with the sensitivity of  $2 \times 10^4 (\mu\text{M}_{\text{TNB}^{2-}}) (\mu\text{M}_{\text{GSH}})^{-1} \text{s}^{-1}$ ; refer to Figure 2.

The assay has also been studied for cysteine (Cys) and homocysteine (HCys), the two thiol species which are present in human body alongside glutathione. The results displayed in Figure 3 show significantly smaller rates of reactions for Cys and HCys compared with GSH. The assay is thus highly specific to glutathione. The high selectivity of the enzyme glutathione reductase towards glutathione minimizes translational matrix effects.<sup>23</sup> On the other hand, rotational matrix effects may be overcome by the method of standard addition. The need for standard addition is further enhanced by the variation of the glutathione reductase activity from different batches of the commercially available enzyme. The concentration of the enzyme is specified as 100-300 units/mg protein (biuret), suggesting a maximum of three times variation in activity. Such variability would lead to significant errors if not correctly accounted for. Note that an error in the measurement of only ca. 30% can give misleading information about diseases and health diagnosis.<sup>12, 13, 15, 24, 25</sup> In accordance with the Michaelis-Menten kinetics,<sup>26</sup> the rate of reaction is a function of enzyme concentration, and consequently standard additions were used as an internal calibration to avoid this problem.

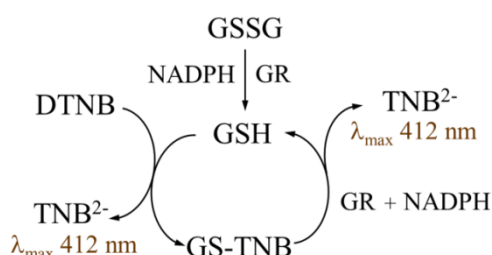


Figure 1: The Tietze enzymatic kinetic assay

At this stage, the validated and calibrated assay can be applied to the real biological samples where standard addition procedures will be carried out. The GSH concentration increments of 332 nM were used in the standard additions, the justification of which is provided in the Supplementary Information.

#### 3.2 Total glutathione concentration in saliva and whole blood

The amounts of total glutathione in saliva and whole blood samples collected from 15 healthy subjects were determined. The concentrations of glutathione equivalents in the saliva were low:  $4.0 \pm 2.0 \mu\text{M}$ . In contrast, glutathione equivalents in whole blood were  $1289 \pm 259 \mu\text{M}$ , approximately 320-fold higher than the salivary glutathione concentrations. Both levels of salivary and whole blood glutathione agree with the range of the values reported by Iwasaki et al.<sup>27</sup> and Richie et al.<sup>19</sup> respectively.

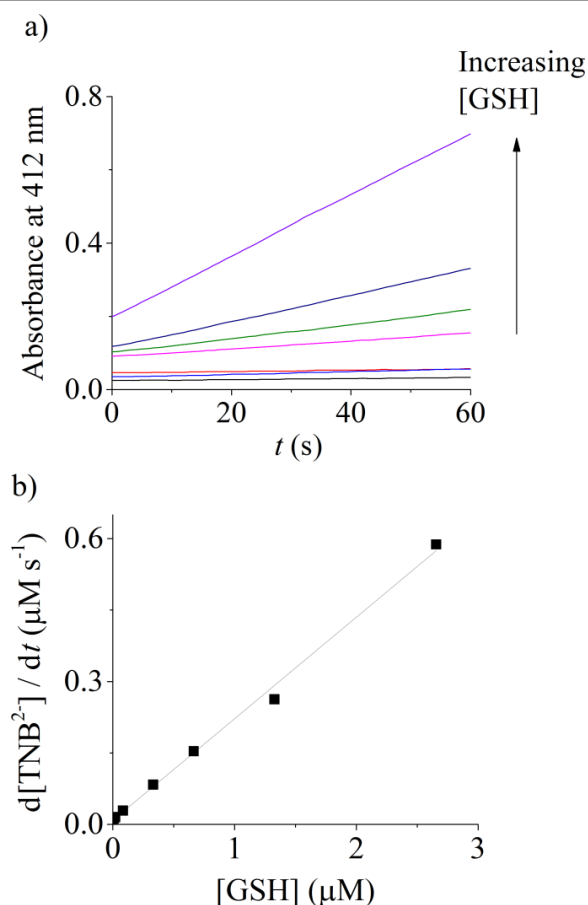
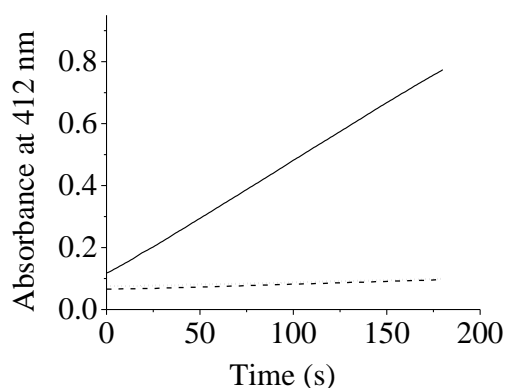


Figure 2: Results of Tietze enzymatic kinetic assay studies on GSH standards: 0, 21, 83, 332, 664, 1328 and 2656  $\mu\text{M}$ ; a) absorbance at 412 nm as a function of time; b) rate of  $\text{TNB}^{2-}$  formation ( $d[\text{TNB}^{2-}]/dt$ ) as a function of GSH concentration

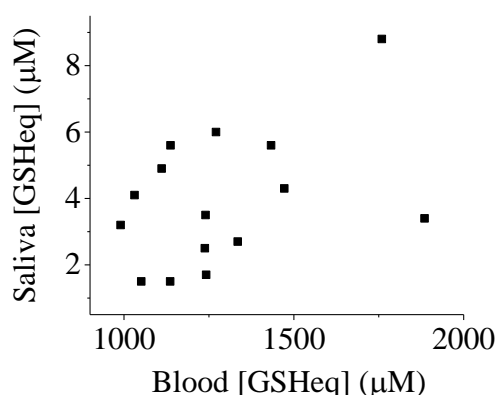


**Figure 3:** Tietze enzymatic kinetic assay studies: absorbance at 412 nm as a function of time for 2  $\mu\text{M}$  cysteine (dotted line), 2  $\mu\text{M}$  homocysteine (dash line) and 1  $\mu\text{M}$  reduced glutathione (solid line)

The comparison of glutathione equivalents concentrations in blood and saliva samples is shown as the scattered plot in Figure 4. Each point in the plot corresponds to salivary and whole blood glutathione concentrations from the same subject. The actual values and other relevant information can be found in the Supplementary Information. The statistical analysis yielded the Pearson's product-moment correlation ( $r$ ) of 0.427,  $p$ -value of 0.112 and the 95% confidence interval ranging from -0.109 to 0.771.

In addition, the analysis of the connection between age and saliva glutathione content showed negative correlation,  $r = -0.442$ ,  $p = 0.099$  and the 95% confidence interval ranges from -0.778 to 0.091. This is in agreement with previous studies by Nassar et al.<sup>28</sup> that there is some indication that the glutathione content is lower in the elderly group. However, both our results and that reported by Nassar et al. are not considered statistically significant.

The correlation between age and whole blood glutathione concentration was very weak;  $r = -0.249$ ,  $p = 0.371$  and the 95% confidence interval ranges from -0.675 to 0.302, in agreement with the non-correlation in a large-scale human study (>700 subjects) reported by Richie et al.<sup>19</sup>



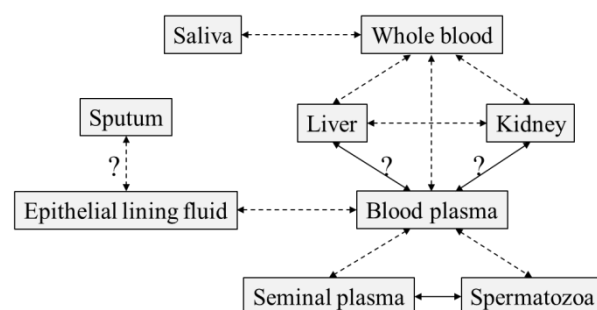
**Figure 4:** Correlation of whole blood and salivary total unbound glutathione concentrations expressed in terms of GSH equivalents ( $[\text{GSH}] + 2[\text{GSSG}]$ );  $n = 15$ ,  $r = 0.427$ ,  $p = 0.112$

## 4 Discussion

The results of our study show that there is no strong correlation between whole blood and salivary glutathione contents in healthy people. The use of saliva as a diagnostic specimen of oxidative status in general then is not likely, but its use with specific diseases remains a possibility. Apart from oral diseases where glutathione deficiency is most relevant in the oral cavity, saliva may act as a potential diagnostic auxiliary in the evaluation of systemic diseases where the levels of glutathione are affected throughout the entire human body. Unless the disease or disorder has been proved to be systemic, the use of saliva as an auxiliary diagnostic tool has to be studied and justified on a case by case basis.

In addition to saliva and whole blood, we would like to discuss in general the relationships between glutathione content in different specimens; summarized as a mapping chart in Figure 5 with more detail given in Table 1. Furthermore, the suspected correlations (represented by solid lines with question marks in Figure 5) are taken from the proof of inter-organ transport of glutathione in rat reported by Anderson et al.<sup>29</sup> No studies however have been done to study the direct correlations between the three specimens, viz., liver, kidney and blood plasma. The dashed line showing the non-correlation between epithelial lining fluid and sputum is marked by a question mark to declare that the non-correlation reported by Dauletbaev et al.<sup>30</sup> has not been demonstrated to be statistically significant.

There is little information on direct comparison in glutathione levels between different specimens collected from the general population. The correlations and non-correlations between different specimens summarized here are in most cases, due to the studies of the effects of different diseases, aging or lifestyles on the levels of glutathione in certain specimens. These papers report the correlation or non-correlation between the two or three specimens that they investigated. This does not necessary mean that there will be no correlation between those in other diseases. However, this brief review of literature further evidences, as expected, that glutathione levels will not help in the diagnosis of many diseases. Careful choices of specimens have to be made to gain physiologically meaningful information from the glutathione biomarkers.



**Figure 5:** Mapping chart displaying the correlation (solid line) and non-correlation (dashed line) between glutathione levels in different specimens. The question marks represent the results which have been stated but not proven.

**Table 1:** Summary of published results on the correlation and non-correlation between different specimens; the measurement methods are described by superscripts, where 'a' refers to HPLC with fluorescence detection and 'b' refers to enzymatic kinetic assay; unless specified otherwise, the subjects are human

Matrix 1	Matrix 2	Analytes	Subjects	Correlation	Ref.
whole blood	blood plasma	GSH, GSSG <sup>a</sup>	healthy	No	31
blood plasma	epithelial lining fluid	GSH+GSSG <sup>b</sup>	smokers / non-smokers	No	32
blood plasma	seminal plasma	GSH+GSSG <sup>b</sup>	healthy / andrological patients	No	33
blood plasma	spermatozoa	GSH+GSSG <sup>b</sup>		No	33
seminal plasma	spermatozoa	GSH+GSSG <sup>b</sup>		Yes	33
epithelial lining fluid	induced sputum	GSH:GSSG <sup>b</sup>	*	No	30, 32, 34, 35
spontaneous sputum	induced sputum	GSH+GSSG <sup>b</sup>	cystic fibrosis patients	Yes	36
mice whole blood	mice kidney	GSH+GSSG <sup>b</sup>	fasting and aging mice	No	37
mice whole blood	mice liver	GSH+GSSG <sup>b</sup>		No	37
mice liver	mice kidney	GSH+GSSG <sup>b</sup>		No	37

\* The subjects are asthma patients, smokers/non-smokers, cystic fibrosis patients and healthy individuals, in the order of references listed 30, 32, 34 and 35 respectively.

## Conclusions

We have found a weak positive correlation between salivary and whole blood glutathione content (GSH + 2GSSG) in 15 healthy participants. The result, which is not statistically significant ( $p$ -value = 0.112), suggests that salivary glutathione is not an appropriate marker for the general state of health. Instead, salivary glutathione may only likely be used as a biomarker in specific diseases. Caution is thus advised when choosing saliva as a diagnostic auxiliary.

Further investigations into the stability and factors affecting the stability of glutathione and other relevant biomarkers in biological fluids, especially in specimens of non-invasive nature are encouraged and will be highly valuable in the development of minimally invasive biosensors for health application.

## Acknowledgements

KN receives funding from the Royal Thai government (DPST scholarship). LMG wishes to acknowledge FCT for his post-doctoral grant (SFRH/BPD/76544/2011). KN, CBM and RGC are sponsored by the funding from the European Research Council under the European Union's Seventh Framework Programme (FP/2007-2013)/ERC Grant Agreement no. [320403].

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