Optical Sensing of Salivary Cortisol for Stress Monitoring

Tashfia Ahmed

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Supervisor:
Prof. Panicos Kyriacou

School of Science and Technology
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DEDICATED IN LOVING MEMORY OF MY GRANDAD,

DADA

Your love for science and knowledge was an endless source of inspiration for me.

Thank you for being my guiding light throughout this journey.

I, Tashfia Nastaran Ahmed confirm that the work presented in this thesis is my own work. Where information has been derived from other sources, it has been indicated in the thesis.

Abstract

Clinical depression and major depressive disorder are considered one of the primary global burdens of disease, impacting society and health economies with monumental prevalence, as of recent years. A primary factor which facilitates the development of such mental illnesses is the prevalence of psychological stress, which has been rising globally, notably since the Covid-19 pandemic. The trajectory of psychological stress towards the development of depressions has been vastly studied from a predominantly qualitative perspective, which has given rise to a plethora of subjective measures for psychological stress evaluation, including interview-based tools. Alternatively, research within physiological stress has principally focused on the physiological biomarkers of stress, including heart rate variability (HRV), electrodermal activity (EDA) and electroencephalography (EEG), enabling the collection of quantitative data regarding the manifestations of stress in the human body. The measurement of the stress hormones in the body, such as cortisol, facilitates the understanding of the biochemical pathways and bio-signals which arise during stress elicitation, that hold vastly informative cues regarding the stress response and how it is managed and controlled via the central and peripheral nervous systems. Although previous research has shown great promise for the measurement and analysis of stress hormones with regards to psychological and physiological stress, this has seldom led to the development of point-of-care technologies for the betterment of the public. One of the leading concerns throughout the Covid-19 pandemic was the practicalities of face-to-face therapeutic interventions, which are commonly practiced for mental health management. Due to the nature of the pandemic, such interventions soon became impractical and led to the rise in virtual therapy sessions, further isolating patients suffering from mental illnesses and perpetuating the stigmatisation that surrounds mental health management. Therefore, the development of point-of-care technologies for the management of markers of mental health, such as psychological stress levels, is a vital necessity to facilitate patient empowerment and assist in creating impactful strategies that consider the objective and quantitative measures of stress in the human body.

The research outlined in this thesis showcases the novel development of a colorimetric method for the determination of cortisol in human saliva. Notably, cortisol governs the stress response through release in a ‘hormonal cascade’ that leads to mobilisation of energy,

increased awareness, and alertness, as reactions to restore the body to its homeostatic nature. Thereby, cortisol is a key logical indicator that can be used to determine stress levels, which can be monitored to facilitate stress management for mental health wellbeing. The research question underpinning this body of work is the prospect of fingerprinting cortisol in human saliva through optical techniques, such as infrared, ultraviolet, and visible light (UV-Vis) spectroscopy, towards a non-invasive, point-of-care application. Firstly, the existing state-of-the-art technologies for stress monitoring were evaluated, from which it was determined that cortisol is the fundamental biomarker to enable stress monitoring. Experimental studies were designed to critically evaluate the optical characteristics of cortisol using infrared and UV-Vis spectroscopy, towards fingerprinting the stress hormone in salivary samples. Further experimentation involved the development of a protocol for the fingerprinting of cortisol in the UV-Vis spectral range, with the aid of chromogenic reagents (tetrazolium blue and tetramethylammonium hydroxide), as well as comparison with the gold-standard salivary cortisol measurement technique, enzyme-linked immunoassays (ELISAs), showcasing promising results. After development of calibration curves through regression modelling, the novel method for colorimetric determination of cortisol was utilised for cortisol measurement in an in-vivo pilot study of 20 participants undergoing a standardised stress elicitation protocol. The Maastricht Acute Stress Test (MAST) protocol was implemented for stress elicitation in 20 healthy individuals from whom saliva samples were collected for rapid cortisol determination via the novel method and ELISA validation. Results from the in-vivo study demonstrated a coefficient of determination of 0.997, suggesting that the novel colorimetric method could determine cortisol levels from human saliva samples with great accuracy, comparable to the gold-standard technique. Finally, a preliminary sensor development stage was conducted in which a prototype colorimeter sensor was developed for the evaluation of cortisol in human saliva, towards point-of-care applications. The novel technological advancements presented in this research contribute towards the development of point-of-care devices which take a new and innovative approach to stress monitoring. Thus, deviating from dated practices that are limited by subjective and qualitative evaluations, towards transformative technology for the optical determination of stress hormones for improved mental health wellbeing. The work encompassed in this project showcases the development of a novel colorimetric method for the determination of salivary cortisol for the first time with the use of the blue tetrazolium dye and

tetramethylammonium hydroxide catalyst, towards the development of a simple prototype colorimetric sensor to evaluate salivary cortisol levels in line with the current gold standard, ELISAs, within in vitro and in vivo scenarios with great accuracy, showing great promise for the future of stress monitoring.

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Abbreviations

Abbreviation	Definition
HAM-D	Hamilton Depression Rating Scale
ELISA	Enzyme-linked Immunoassays
UV-Vis	Ultraviolet and Visible Light Spectroscopy
WHO	World Health Organisation
MDD	Major Depressive Disorder
CNS	Central Nervous System
ANS	Autonomic Nervous System
NA	Noradrenaline/Norepinephrine
SAM	Sympathetic-adreno-medullar
HPA	hypothalamus-pituitary-adrenal
CRF	Corticotropin-releasing Factor
AVP	Arginine Vasopressin
ACTH	Adrenocorticotropic Hormone
5-HT	Serotonin
DA	Dopamine
SSRIs	Selective Serotonin Reuptake Inhibitors
SNRIs	Serotonin-Norepinephrine Reuptake Inhibitors
mPFC	Medial Prefrontal Cortex
REM	Rapid Eye Movement
SWS	Short Wave Sleep
ECT	Electroconvulsive Therapy
BDI	Beck Depression Inventory
DST	Dexamethasone Suppression Test
CBT	Cognitive Behavioural Therapy
IPT	Interpersonal Therapy
TCAs	Tricyclic Antidepressants
HRV	Heart Rate Variability

EEG	Electroencephalography
EDA	Electrodermal Activity
GSR	Galvanic Skin Response or Galvanic Skin Resistance
SCL	Skin Conductance Levels
SCR	Skin Conductance Responses
SC	Skin Conductance
MDD	Major Depressive Disorder
HAM-D	Hamilton Depression Rating Scale
BDI	Beck Depression Inventory
ICD-10	International Classification of Diseases -10th Revision
STAI	State-Trait Anxiety Inventory
DSM-IV/DSM-III-R	Diagnostic and Statistical Manual on Mental Disorders
SDS	Self-rating Depression Scale
SRI	Stress Response Inventory
TSST	Trier Social Stress Test
ICG	Inventory of Complicated Grief
SNS	Sympathetic Nervous System
PNS	Parasympathetic Nervous System
ANS	Autonomic Nervous System
ECG	Electrocardiography
RSA	Respiratory Sinus Arrhythmia
HR	Heart Rate
LF	Low Frequency
VLF	Very Low Frequency
LF_{norm}	Normalised Low Frequency
LFv	LF/ (LF+HF)
HF	High Frequency
HF_{norm}	Normalised High Frequency

HFv	HF/ (LF+HF)
LF/HF ratio	Low frequency power to high frequency power ratio
NN	Normal-to-normal heartbeat intervals
SDNN	Standard deviation of NN intervals
SDANN	Standard deviation of average NN intervals in 5-minute segments over 24-hour recording
RMSSD	Root mean square of successive differences between normal heartbeats
NN50	Number of pairs of adjacent NN intervals differing by more than 50ms in the complete recording
pNN50	Proportion of NN50 divided by total number of NN intervals
NN20	Number of pairs of adjacent NN intervals differing by more than 20ms in the complete recording.
pNN20	Proportion of NN20 divided by total number of NN intervals
SD	Standard Deviation
RR	Time between two successive R-waves of the QRS complex on an ECG
SDRR	Standard deviation of RR intervals
SDSD	Standard deviation of successive RR interval differences
SDHR	Standard deviation of Heart Rate
BP	Blood Pressure
PEP	Pre-ejection Period
ACTH	Adrenocorticotropic hormone
FFT	Fast Fourier Transformation

AR Modelling	Auto-Regressive Modelling
DHEA	Dehydroepiandrosterone
MMST	Mannheim Multicomponent Stress Test
TSST	Trier Social Stress Test
CBG	Cortisol Binding Globulin
GRE	Glucocorticoid Response Elements
OXT	Oxytocin
ECF	Extracellular Fluid
ADHD	Attention Deficit/Hyperactivity Disorder
POC	Point-of-Care
LC	Liquid Chromatography
GC	Gas Chromatography
MS	Mass Spectrometry
PCR	Polymerase Chain Reaction
NIR	Near-infrared Spectroscopy
MIR	Mid-infrared Spectroscopy
FIR	Far-infrared Spectroscopy
FTIR	Fast-Fourier Infrared Spectroscopy
ATR	Attenuated Total Reflection
UV	Ultraviolet
PCA	Principal Component Analysis
PLS	Partial-Least Squares Regression
BT	Blue Tetrazolium
LED	Light Emitting Diode
MAST	Maastricht Acute Stress Test
HI	Hand Immersion
MA	Mental Arithmetic
PHQ-9	Personal Health Questionnaire
GAD-7	Generalised Anxiety Disorder Scale

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1. Introduction

Clinical depression or major depressive disorder is one of the most prevalent mood disorders affecting over 3.8% of the global population[1]. It is considered by the World Health Organisation (WHO) as one of the main contributors to the global health and economic burden. With over one in five adults (19.7%) experiencing a form of depression in the UK since the start of the COVID-19 pandemic, the correct diagnosis and treatment of the disorder is of utmost significance[2]. This has doubled from 9.7% since before the COVID-19 pandemic (July 2019)[2]. Moreover, financial difficulties during the COVID-19 pandemic and the anticipation of unexpected expenses, has further accelerated the rate of development of depressive symptoms with over 21.2% of adults developing moderate to severe depressive symptoms by June 2020[2].

Currently, the diagnostic procedures surrounding mental health focus on a qualitative approach, through the utilisation of mood-evaluation and subjective questionnaires. Questionnaires and surveys such as the Hamilton Depression Rating Scale (HAM-D) have proven to be effective in the assessment of depression, however cases of misdiagnosis still exist[3]–[6]. Several factors can contribute to the misdiagnosis of human depression, such as the miscoding of mental illnesses as secondary disorders to more socially accepted illnesses like brain tumours and cardiovascular disease[6]. The stigmatisation of mental illnesses, as well as the economic burden associated with such disorders further deteriorate the diagnostic processes involved. It has been reported that often physicians will deliberately treat mental illnesses as secondary illnesses to ensure that patients are receiving rightful imbursement for treatment from insurance companies[7]. However, by doing so, physicians are also contributing to the stigmatisation of mental illnesses and preventing the advancements and acceptance of mental illnesses as disorders of great severity[8]. This destructive societal process is leading to the further burdening of global economy and health, as the population affected by mental illnesses is increasing[2], [6].

To eradicate the issues of misdiagnosis and the subjective diagnoses of mental illnesses, the implementation of a quantitative system is necessary. Not only will this lead to greater confidence in making judgments regarding the depression diagnoses, but it will also

encourage the de-stigmatisation of mental illnesses through the association of the illnesses with tangible and comprehensible information from the human body. Otherwise, the existing systems will continue to cause disadvantages for the patients who are misdiagnosed, as well as those who are correctly diagnosed due to the economic implications.

Cortisol is considered as the main biomarker of stress, showing a strong relationship with the manifestation of clinical depression in the human body[9]. However, the regular monitoring of this stress hormone has not yet been considered as an objective measure of clinical depression, which could facilitate the early identification of the mental illness in patients from home settings. This is primarily due to the current measurement techniques involved with the monitoring of cortisol, such as enzyme-linked immunoassays (ELISA) which can be time consuming, costly and require complex processing in the laboratory[10]. Therefore, regular, and on-demand testing of cortisol levels under these conditions can be greatly troublesome for patients, especially for those in high-risk groups.

This project proposes the development of a point-of-care device which is capable of measuring cortisol levels from saliva samples, through optical techniques[9], [11]. The utilisation of ultraviolet, visible, and infrared, spectroscopy and colorimetric techniques will facilitate the rapid quantification of cortisol and stress levels from different media. As there have been successful attempts in the measurement of different analytes in blood and saliva through these techniques, it is considered that the measurement of cortisol could be feasible[12]. Moreover, in this research the current state of the art physiological monitoring techniques that are used for psychophysiological stress evaluation will be highlighted and critically investigated[13], [14]. This will highlight the necessity for the development of a point-of-care device which measures cortisol concentrations from saliva, for the complete evaluation of acute and chronic stress and its inevitable manifestation of clinical depression[13]–[15]. Evidently, the design and development of a prototype towards a patient-held device will lead to improvements in the understanding of the pathophysiology of the course of depression and its relationship with psychological stress. Additionally, this device could empower patients through enabling greater patient involvement in the management of mental illness.

1.1 Chapter Overview

In this thesis, the various aspects of psychological stress and clinical depression will be considered. Firstly, the relationship between psychological stress and the manifestation of clinical depression will be discussed. It will also highlight socioeconomic impact of the mental illness, as well as the current management techniques for treating clinical depression, or major depressive disorder of varying severity. Secondly, the thesis will discuss the current state-of-the art monitoring techniques used to measure stress, which primarily consist of physiological monitoring modalities. This will lead to deliberation of the various existing methods of physiological stress evaluation such as heart rate variability, electroencephalography and electrodermal activity. Then, the focus will shift towards the biochemical aspect of stress and on the different biomarkers that have been identified in relation to stress and depression. Cortisol and other stress hormones such as adrenaline and noradrenaline will be highlighted. Additionally, anti-stress neurotransmitters such as DHEA, dopamine and serotonin will be mentioned to further evaluate the biochemical reactions which occur during the development of the mental illness.

Furthermore, the main medium of interest, whole human saliva will be discussed. The chemical composition of whole human saliva will be considered, as well as the changes in chemical composition of saliva in response to variations in stress levels will be studied. Moreover, the primary optical techniques of interest for this project will be discussed. Chapter 6 will focus of the fundamental principles of infrared spectroscopy and the differences between the different infrared regions and the information conveyed within them. It will also highlight the mathematical techniques employed within spectral analysis for further evaluation of chemical compounds, such as cortisol.

This will lead to a discussion of the methodologies and protocols conducted within the scope of this project, such as ultraviolet and visible light (UV-Vis) spectroscopy-based protocols on artificial and real human saliva samples for the evaluation of stress via salivary cortisol measurement. The main methodology which will be focused on is the use of colorimetric approaches towards the determination of salivary cortisol. This will be conducted using the use of colorimetric reagents, including the blue tetrazolium dye and the novel use of the tetramethylammonium hydroxide catalyst. A rigorous comparison of the proposed method against the gold-standard enzyme-linked immunosorbent assay (ELISA) will be conducted to

strengthen and validate the use of the novel method towards mental health monitoring applications. The development of a novel colorimetric sensor will be discussed towards artificial salivary cortisol and human salivary cortisol analysis, towards stress monitoring in healthy populations. Finally, the thesis will conclude on the major findings and contributions made within the project, as well as propose future concepts for continuation of the work.

2. Mental Health, Stress and Depression

Mental health has become one of the primary concerns requiring substantial improvement in the clinical field, as it is ranked as the fourth medical condition with the greatest disease burden[16]. Mental wellbeing and its relationship with illnesses such as depression and schizophrenia are often categorised in subjective manners which prevent true quantification of the progression and developments of the illnesses in populations[17]. Due to the nature of mental illnesses and the vast array of symptomatic and asymptomatic cases, it is often difficult to characterise the diagnosis and treatment of a mental illness such as clinical depression[17], [18].

Reported to be the second condition with greatest disease burden globally by 2020, the need for a clearer comprehension between depression and its relationship with physiological and psychological stress has increased unequivocally[16]. Through physiological and biochemical monitoring of the developments of major depressive disorder, with regards to the chronic impact of physiological and psychological stressors on the human body, distinguished and educations solutions can be established for the mental illness[18]. Mental health encompasses mental wellbeing and can include a plethora of conditions ranging from clinical and major depression to schizophrenia and bipolar disorder. Whereas, stress primarily focuses on the interruption to homeostasis and has several trajectories towards the development of clinical depression, especially in the case of chronic stress.

2.1 Socioeconomic Impacts of Depression

According to the World Health Organisation (WHO), over 264 million people suffer from clinical depression globally, the socioeconomic impacts of the debilitating psychiatric disorder are immense[1]. As one of the leading causes of disability, major depressive disorder is a critical contributor to the global burden of disease. Strongly associated with high societal costs, the economic burden of clinical depression is exceedingly significant due to its attributions to medical expenses, costs related to comorbidities, workplace costs and suicide-related costs[19], [20]. Therefore, effective mental health care has become a necessity globally i.e. the service use of psychiatrists and psychologists as well as pharmaceutical-led

therapy, involving antidepressants or herbal medicines. Occurrence of the mental illness is highest in young individuals, aged 18-25, and it disproportionately affects women[21]. Suggesting that the development of future generations is vastly impacted by the prevalence of major depressive disorder (MDD) in the global population, especially as the burden is expected to increase due to longer life expectancy[22].

The burden of mental disorders, such as clinical depression and bipolar disorder, is often accountable beyond direct medical costs i.e., for diagnosis and treatment. The indirect costs associated with mental illnesses include income losses due to mortality, or loss of productivity due to work absences, early retirement, or imprisonment[23]. Unfortunately, the treatment gap for mental illnesses is higher than any other health sector, often due to lack of trained personnel and infrastructure for effective treatment of the illnesses[24]. The common misconception of psychiatric illnesses as minor disorders compared to somatic conditions has led to assumptions regarding the lack of societal significance of the diseases and the effectivity of treatments and preventive interventions. Stigmatisation and lack of comprehensiveness in diagnosis of MDD has led to the imbalance between the socioeconomic burden, presented by the disease, and the funding of mental health care persists[20], [25].

2.2 Endocrinology of Stress

The endocrinology of stress embodies the various afferent pathways involved with the highly specific dynamic and adaptive changes that occur in the body in response to a stressor[26], [27]. The catecholamines and the glucocorticoids are known as the two central components of the endocrinal response to stressors[28]. Catecholamines are monoamine hormones and neurotransmitters such as adrenaline and noradrenaline (also known as epinephrine and norepinephrine) and, dopamine[27].

Adrenaline is primarily produced in the adrenal gland medulla, as well as some neurons within the central nervous system (CNS) i.e., it acts as a hormone and a neurotransmitter, depending on the location of release in the body[9], [28]. Adrenaline and noradrenaline (NA) have very significant roles in maintenance of visceral (involuntary) functions in the autonomic nervous system (ANS), such as heart rate, respiratory rate and sexual arousal[9], [29]. Both are identified as sympathetic neurotransmitters and stress hormones which are released into the

bloodstream upon triggering of the 'fight or flight' response[27], [30]. NA can induce behavioural changes in the brain such as increased arousal and alertness, or enhancement of retrieval of memory[9], [29], [31]. Whereas in the body, through release from adrenal glands, it induces physiological changes i.e., increased blood flow to skeletal muscles and release of glucose from glycogen stores[28].

As the catecholamines are released immediately after perception of a stressor, there is rapid activation of cellular processes in targeted tissues to lead to large-scale responses for allostasis. Allostasis is the process of restoration of homeostasis through dynamic mechanisms in the body[9], [28], [32], [33]. These mechanisms via the sympathetic-adreno-medullar (SAM) axis can resolve situations of acute stress[28], [33]. Furthermore, the endocrinal response which leads to the secretion of glucocorticoids via the hypothalamus-pituitary-adrenal (HPA) axis is also highly significant in the resolution of acute stress[31], [34]. Glucocorticoids are steroid hormones and the final effectors of the HPA axis, some of which (cortisol and corticosterone) are secreted as stress hormones upon detection of a stressor[9], [28], [31]. Cortisol is the primary glucocorticoid for fish and mammals whereas, corticosterone is more significant for bird and amphibian species, with some rodent species relying on both[28].

In the human body upon detection of a stressor, corticotropin-releasing factor (CRF), or corticotrophin releasing hormone (CRH), is released into the bloodstream along with arginine vasopressin (AVP) to initiate the hormonal cascade (the HPA axis)[9], [11], [28], [31], [34]. After these hormones bind to receptors at the anterior pituitary, adrenocorticotropic hormone (ACTH) is secreted into the general circulation towards the adrenal cortex[9], [11], [28], [30], [31], [34]. Once ACTH reaches the adrenal cortex receptors, it stimulates glucocorticoid synthesis and the subsequent release of the steroid hormones into peripheral circulation, as they cannot be stored[9], [11], [28], [31], [34]. Through its release, the glucocorticoids can act on extrahypothalamic centres, the hypothalamus, and the pituitary gland to eradicate the stress response within a limited duration, because it follows a negative feedback loop. Thus, cell exposure to glucocorticoids is strictly controlled to prevent overexposure. Chronic stress is one of the main causes for overexposure of glucocorticoids and hyperactivation of the SAM and HPA axes, which can lead to overcompensation of the biological systems in place to counterbalance long-term stressful events. Persistent stressors

can lead to a chronic state of dyshomeostasis, often as a consequence of prolonged stress responses or disproportionate dynamic changes to the equilibriums existing in the body[9]. Such instances are known as allostatic overload and can often lead to the acceleration of stress-related diseases such as atherosclerosis and increased susceptibility to stroke, obesity and cardiovascular disease[32], [35].

Memory consolidation is one of the psychological effects caused by the secretion of stress hormones such as cortisol, NA and adrenaline into the bloodstream[9], [28], [31]–[33], [35]. The responses to acute stress are therefore learned through neuronal growth and enhancement, such that organisms can resolve stressful events prospectively. This learning mechanism (adaptive plasticity) coupled with consistent stressors i.e., stressors which present themselves for longer durations (over weeks), the memory can also be impaired through neurons becoming atrophied[29], [32], [35]. Adaptive plasticity often causes the suppression of neurogenesis and dendritic remodelling. Instead of positive evolutionary changes to increase chances of survival, allostatic overload mechanisms can exploit existing mediators to exacerbate the cumulative effects involved with stress responses[32], [33], [35]. For example, acute stress supports immune function through mobilisation of immune cells to targeted regions for increased pathogen defence whereas, chronic stress responses utilise similar mediators to promote immunosuppression[9], [11], [28], [31]–[33], [35], [36].

In individuals with chronic mental illnesses and anxiety disorders, such conditions are prevalent[32], [33], [35]. The imbalance of the stress mediators is reflected as ‘allostatic states’, i.e., blunted cortisol levels or chronic insomnia and chronic fatigue syndrome, in individuals suffering from severe depression[11], [32], [35]. Consequently, this signifies the importance of the relationship between chronic stress and mental illnesses, highlighting the implicated physiological, and often deteriorative changes they pose on the human body[17], [18].

2.3 The Pathophysiology of Clinical Depression and its Relationship with Stress

The pathophysiological mechanisms associated with MDD comprise of altered neurotransmission, HPA axis abnormalities and reduced neuroplasticity[37]. Furthermore, stress pathways have been historically interlinked with MDD, thus several models and

systems have been formed to allow for greater comprehension of the mental illness, as well as the associated pathophysiological mechanisms. The mechanisms can be categorised according to molecular, cellular, neurocircuitry, and behavioural levels, due to the high complexity and overlaps that exist between diagnostic entities.

The monoamine hypothesis for the pathophysiology of depression focuses on the alteration of monoamines such as serotonin (5-HT), noradrenaline (NA) and dopamine (DA)[37]. It is stated that the functional deficiency of monoamines, specifically catecholamines i.e. NA, are responsible for the psychiatric disorder. Furthermore, mania (periods of euphoria and heightened arousal) is caused by the functional excess of catecholamines and cellular actions of psychotropic agents. The neurotransmitter systems are interconnected such that the concentrations of each monoamine in the brain is affected by the release of the other monoamines. For example, dopamine has an inhibitory effect on the functional release of NA, whereas NA can have an excitatory or inhibitory effect on the release of dopamine. Moreover, both dopamine and NA have a positive excitatory effect on serotonin release[37], [38]. Through analysis of the origin of neurons associated with these monoamines (noradrenergic, serotonergic, and dopaminergic neurons), it has become evident that their projections in areas of the brain account for behavioural symptoms of MDD e.g., mood, motivation, fatigue etc.[39]. This hypothesis has contributed to the forefront of antidepressant therapy for MDD such as, selective serotonin reuptake inhibitors (SSRIs) and serotonin-norepinephrine reuptake inhibitors (SNRIs)[37]. However, monoamine depletion is perceived as an over-simplified view of the pathophysiology of MDD[17].

The pathophysiology of depression also interlinks with the aforementioned endocrinal processes and mechanisms that are frequently related with stress. Overactivity of the HPA axis is commonly associated with major depression as well as schizophrenia[40]. Altered levels of cortisol are indicative of endocrinal disturbances i.e., dysfunctionality of the HPA axis, which is a consistent observation made in the assessment of depressed individuals[39], [41]. Dysfunctionality of the HPA axis involves hypersecretion of CRH and hypercortisolism, as well as inadequate glucocorticoid feedback and enlarged adrenal glands[37]. There are several neurobiological ramifications of HPA dysfunctionality in the brain, specifically the depressive effects of hypercortisolism[41]. Increased glucocorticoid release affects the medial prefrontal cortex (mPFC), the hippocampus and the amygdala[37]. These brain areas

are responsible for executive function, memory and learning, and emotion processing[18], [42]–[44]. Decreased activity in the amygdala and mPFC, caused by the chronic stress decreasing dendritic complexity of neurons, leads to inadequate cognitive processing of emotions[45]. Alterations to the functional connections in the brain between the amygdala and the mPFC are triggered by elevated levels of cortisol. This increases the amygdala's excitability in response to stress i.e., increased stress reactivity and decreased cognitive processing[37], [42]. Moreover, the adaptability of the hippocampus is impaired by increased cortisol levels, thus chronic stress induces reduced neuronal plasticity and consequently, impaired adaptation and learning.

These mechanisms of behavioural adaptation include the hippocampus' and amygdala's high involvement in contextual fear conditioning[18]. The activity of both the hippocampus and the amygdala are strictly regulated by the moderate release of glucocorticoids. Glucocorticoids are involved in the formation of context in the hippocampus and limiting the flow of information within the amygdala through inhibitory mechanisms, affecting the processing of excitatory sensory inputs. In individuals suffering from chronic stress, functional memory is shifted from hippocampal-based learning to habitual striatum-based learning[37]. This means that mineralocorticoid receptor-mediated glucocorticoids uncouple the amygdala from the hippocampus and increase its connectivity to the striatum. These synaptic changes in the emotional-cognitive circuitry changes the individual's learning techniques from adaptive exploration and contextual learning to habitual learning[46]. Thus, exploration is avoided when certain stressors arise, and the individual relies heavily on the habit of autonomously reacting to that specific stressor i.e., cognitive processing is decreased substantially. Subsequently, this leads to misinterpretation and assumption of such stressors as dangerous threats which increases disinterest, a common depressive symptom[47]. Such effects in depressed individuals often lead to hippocampal loss and amygdala enlargement[18]. Reduced neurogenesis in the hippocampus and synaptic reorganisation has been proven to occur rapidly because of stress [18], [35], [37]. Therefore, the structural changes i.e., structural plasticity of the brain is responsible for the decreased cognitive processing and expands comprehension of the pathophysiology of major depressive disorder and its strong relations with chronic stress.

2.4 Symptoms of Clinical Depression

Clinical depression is considered one of the most common mental illnesses, often associated with anxiety or generalised anxiety disorder[17], [48]. Depression can be perceived as a mood state experienced by most individuals during periods of stress and sadness. However, it is also classified as a severe clinical and behavioural syndrome which can significantly affect moods, neurovegetative functions, cognition, and psychomotor activity[17]. There exists a strong correlation between anxiety and depression, often leading to difficulties in the meaningful separation between the clinical and behavioural syndromes[48]. Anxiety is characterised as the habitual perception of threat to stability in an individual's life e.g. through self-esteem, happiness and perception of normal activities, it is considered a stage of unfocused arousal[48], [49]. This concept also loosely correlates to ideas of fear, which is known as the 'awareness and appraisal of danger', instead of feelings of uncertainty commonly associated with general anxiety[50]. Behavioural characteristics of an individual with anxiety include apprehensiveness, nervousness, increased perspiration and rapid respiratory and heart rates[3], [50]. Thus, allowing for simple detection of the clinical and behavioural syndrome for diagnosis.

Comparatively, depression is exceedingly difficult to diagnose as it is often masqueraded as other illnesses such as diabetic neuropathy or chronic pain and fatigue[51]–[53]. The core symptoms of MDD are 'depressed mood' and 'loss of interest and pleasure in nearly all activities (anhedonia)', they are considered the essential requirements for diagnosis of MDD, yielding 83% sensitivity and 92% specificity in screenings for MDD[54]. Other prominent symptoms of clinical depression include insomnia (lack of sleep), hypersomnia (excessive daytime sleepiness), chronic fatigue, headaches and chronic pain[52]–[54]. Chronic pain is one of the predominant symptoms of individuals suffering from endogenous depression (melancholia) and it has been identified that most individuals suffering from a type of chronic pain indeed suffer from a form of depression[52], [53]. Furthermore, in the presence of more depressive symptoms, the intensity and frequency of painful conditions are seen as significantly more severe[53].

There exists a strong relationship between depression and increased intensity of pain perception, which can be elucidated through a cognitive neurobiological model[53]. There are three components to the model of pain perception: sensory, affective and evaluative[52],

[53]. During the affective stage, if there are negative or 'noxious' inputs regarding the source of the pain, this can cause escalation of the evaluative stage and the symptom states, thus causing deterioration of the pain intensity[53]. Subsequently, the presence of a mood disorder, such as depression can critically affect how an individual perceives pain from somatic stimulus[53].

Moreover, sleep disturbances and its relationship with depression involves several complexities[54]. Insomnia is one of the most frequent symptoms of depression, and the most unresponsive to antidepressants, with over 75% of depressed patients suffering from insomnia symptoms[54], [55]. Several antidepressants essentially worsen this symptom, thus overtreatment in some cases lead to excessive daytime sleepiness. The most prevalent marker in terms of sleep disturbance for MDD is characterised by the ratio of rapid eye movement (REM) sleep to non-REM sleep. Sleep continuity in depressed individuals is regularly compromised, with reduced sleep efficiency and longer and more frequent periods of wakefulness[54]. Thus, REM duration is increased, and slow wave sleep (SWS) is often significantly lower in depressed patients, compared to healthy individuals[54], [55].

The two processes that regulate the sleep-wake cycle are the circadian process and the homeostatic process[55]. The circadian process is responsible for the 24-hour patterns of activity of the body and brain, controlled by the cells of the suprachiasmatic nucleus (SCN) in the hypothalamus[55], [56]. The oscillatory activity pattern of the SCN cells drives the circadian rhythms which are accounted for the regulation of the sleep-wake cycle and hormone release etc. [55], [56]. The circadian process is independent of tiredness and amount of sleep; however, it is affected by light i.e., bright light in the evening delays the circadian clock, whereas bright light in the morning sustains the daily rhythm[55], [56]. Comparatively, the homeostatic process is wake-dependent, therefore dependent on tiredness and amount of sleep[55]. In scenarios where there is lack of sleep ('sleep debt') there is an increased drive, or pressure, by the homeostatic process which results in acceleration of time to sleep and increased sleep depth and duration. Lack of the homeostatic drive for sleep interlinked with interruptions to the circadian rhythm are attributed effectors of sleep disturbances in depressed individuals[55]. Furthermore, resetting of these processes through total sleep deprivation has proven to improve mood in MDD, however such treatment cannot be maintained due to rapid relapse once uninterrupted sleep is permitted[54], [55]. Through

consideration of some of the symptoms of clinical depression, it is evident that the relationship between the mental illness and chronic stress remains exceedingly complex.

2.5 Current Management and Diagnosis of Depression

The effective management of MDD and other psychiatric illnesses is extremely significant due to its major contribution to the global burden of disease[1]. The current management of clinical depression relies heavily on the effectivity of a doctor-patient relationship; however, the futility of single component interventions has been recognised e.g., mental health screening[25]. Thus, the efficacy of 'collaborative care' has been highlighted in recent studies, which focuses on multifaced interventions, systematic identification of patients and patients' preferred choices of treatment setting.

Furthermore, the diagnostic techniques used to screen for clinical depression and MDD are a large contributing factor to the management of the mental illness. Interview-based criteria is considered the 'gold standard' diagnostic practice, with individuals who suffer from serious recognised symptoms labelled as 'false positives' if they fail to meet the criteria of psychological intervention-based settings[57]. Such techniques, which involve interview-based instruments, are often heavily reliant on subjective interpretations[58]. Treatment responses and laboratory-based methods are also utilised for the diagnosis of the mental illness[58]. These techniques are uncommon due to the difficulties in administration of the involved tests, and the inconvenience caused to individuals being screened.

The current management and treatment of depression is dependent on the severity of the mental illness in an individual. For example, cases of mild depression are often treated with structured psychological interventions i.e., cognitive behavioural therapy[25]. Whereas the treatment for moderate to severe depression frequently involves the use of pharmacological treatment i.e., tricyclic antidepressants and selective serotonin reuptake inhibitors (SSRIs)[37]. As the severity of the mental illness increases, the focuses of the interventions utilised to manage the illness also vary. In high severity cases i.e., severe dysfunction and significant risk of life, combinational therapies are used e.g., a combination of medication and psychotherapeutic treatments. In extremely severe cases, electroconvulsive therapy (ECT) may be used[59]. Hence, focuses on the current management and treatment of clinical

depression is exceptionally significant to recognise the current benefits and drawbacks, facilitating the enhancement and the methods used to manage the psychiatric illness.

2.6.1 Diagnosis of Clinical Depression

Symptom-oriented questionnaires and psychometric tests such as the Beck Depression Inventory (BDI) and the Symptom Checklist, are standardised screening methods for the mental illness[60]. Most screening tools of this nature involve self-assessment buy the patients, which are then scored by the physicians, with high scores leading to further comprehensive evaluation of the disorder[61]–[63]. Although these diagnostic instruments have high sensitivity, they lack specificity thus, clinical history, such as prior substance abuse, must be assessed vigilantly by clinicians to correctly diagnose depression and reduce false-positive rates[60], [61]. An existing limitation of these symptom-based self-assessments is the risk of exaggeration or minimisation of the severity of the mental disorder, by the screened individual, as well as the subjectivity of the depression inventory criteria[58], [64]. Therefore, it is essential to assess symptom severity and suicidal ideation with great diligence, to ensure effective management of MDD[60].

Depressive symptoms often mimic other disorders such as generalised anxiety, which can lead to inaccuracies in diagnosis, thus treatment response techniques are also used regularly for the diagnosis of depression[58]. Additionally, laboratory-based techniques can also be used to diagnose MDD, in the form of a dexamethasone suppression test (DST) which assesses the negative feedback of dexamethasone on pituitary gland release of corticotropin[58]. In this test, dexamethasone is administered to the individual, which should reduce corticotropin levels and decrease cortisol levels in healthy, normal conditions. If cortisol levels remain elevated, the individual can be clinically diagnosed with MDD, however the efficacy of this technique differentiates between different types of depression e.g., melancholic depression and chronic psychosis[65]. Therefore, standardised self-assessments and subsequent interview-based techniques are primarily employed to diagnose MDD and facilitate the effective management and treatment of the mental illness.

2.6.2 Psychotherapy

Psychotherapeutic treatments for clinical depression are universally recognised by practitioners for the management of depression[25]. Cognitive behavioural therapy (CBT) is considered the gold standard of psychotherapy due to its efficacy which has been incomparable with other forms of psychotherapy[68]. Furthermore, as the most researched form of psychotherapy, the models and mechanisms employed in CBT are most aligned with the current mainstream theories of the human mind and behaviour [66]. CBT involves the development of a therapeutic relationship, seen as the most significant aspect of the treatment[67]–[69]. The treatment is often conducted by the psychotherapist through employment of an empathic and respectful attitude, to assure the depressed individual is not made to feel inferior to the therapist during the structured psychological interventions. The collaborative partnership between the therapist and the patient is highly emphasised in CBT, thus psychoeducation is another important aspect of treatment. Psychoeducation focuses on the active involvement of the patient in the establishment of the treatment plan; therefore, the patient is empowered, and their concerns are valued during sessions[25]. With various delivery techniques, chosen at the discretion of the therapist, the patient is informed of the role of thinking in depression and the process-based approach to the management of acute clinical depression.

At the beginning of a CBT session, the therapist often proceeds with a depression inventory to assess the current functioning of the patient, as well as any dramatic changes that may have occurred since the previous session[67]. The depression inventory is frequently implemented as a questionnaire, which is further supported by a 'mood check' assessment in which the therapist uses a point rating scale to evaluate the patient's emotional and psychological state prior to treatment. After a treatment session is complete, homework may be assigned by the therapist. Homework is considered to be one of the most significant aspects of CBT, due to the understanding that each session is fundamental for problem definition and teaching strategies to overcome such problems. Whereas the implementation of these strategies and translation of the insights from the session occurs beyond the treatment setting.

Another effective psychological treatment for acute clinical depression is interpersonal therapy (IPT). IPT focuses on the principle of relationships being the fundamental core of

psychological balance[70]. Therefore, a disturbance to an individual's interpersonal environment e.g. death of a loved one, could trigger the cascade of depressive illness. Thus, IPT focuses on developing a link between the life events of the patient and the onset of the mental illness, to reassure the patient is not at fault. Although IPT has shown great promise and effectivity in the psychological treatment of clinical depression, it is still considered to be in the development stages, whereas CBT has been recognised as a progressive and evolving process with several validating constructs[71].

While these techniques have been historically utilised for the evaluation of patients during a psychological intervention, the effectivity of such practices are not consistent with cases of moderate and severe depression[25]. CBT has rendered ineffective in cases of depression relapse and recurrence, due to its lack of enduring effects on depressed individuals[72]. Thus, with more severe cases of the mental illness, other practices such as ECT and pharmacological therapy is frequently employed.

2.6.3 Electrical Therapy

In cases of severe clinical depression or MDD where other treatments have been ineffective or resisted by depressed individuals, electroconvulsive therapy (ECT) may be employed[25]. Due to the serious implications of the use of ECT on patients suffering from MDD i.e. memory impairment and heart rhythm disturbances, the highly effective treatment is considered as a 'last resort' for intervention of the mental illness[73]. For instances of resistance to tricyclic antidepressants and behavioural therapy techniques, the use of ECT can be highly desirable. Furthermore, in depressed individuals where there is a risk of life or suicidal intent, there is a necessity for a fast-acting treatment which surpasses the effectivity of long-term courses of antidepressants and structured psychological interventions.

ECT involves the induction of electrical currents for a maximum of 6 seconds between electrodes which are placed on either side of the patient's head (bi-temporal or unilateral placement)[74]. The application of the electrical current to the scalp provokes a generalised epileptic seizure with intent to alleviate psychotic symptoms of the depressed individual. Although the efficacy of ECT for treatment of psychotic depression has been presented in several trials, the severity of the side effects has been recognised as great concerns[59].

Beyond the acute side effects of confusion, headaches and short-term memory disturbances, the long term adverse cognitive effects are yet to be thoroughly distinguished. For example, the risk of incidental dementia in patients' post-treatment has only recently been debunked[75].

Additionally, the efficacy of ECT for severe MDD is high only for repeated sessions, as single occurrences remain ineffective to facilitate chronic alterations of brain function, to alleviate the psychotic and depressive symptoms of the debilitating mental illness[76]. Hence, the use of ECT for the management of clinical depression is applied only in patients with history of failure of at least three medication trials i.e. ECT is mostly considered in cases of MDD with pharmacological resistance[73].

2.6.4 Pharmacological Therapy

There is compelling evidence to suggest the efficacy of tricyclic antidepressants, SSRIs and selective serotonin and noradrenaline reuptake inhibitors (SNRIs) in the management of clinical depression. Pharmacological treatment is often the most usual form of intervention for cases of moderate to severe depression, where sole psychotherapeutic treatment is redundant[25]. At least 50% of moderate to severe episodes are improved with the use of antidepressants[77].

Antidepressants are classified through their mechanism of action. SSRIs inhibit the reuptake of serotonin into the pre-synaptic terminal of the serotonin uptake site[78]. This increases the synaptic concentration of serotonin, subsequently increasing the activation of presynaptic inhibitory receptors and decreasing the firing of the serotonergic neuron. This negative feedback mechanism is applied to accelerate the attainment of higher levels of serotonin, such that a therapeutic effect is relayed to the depressed individual. Similarly, SNIRs work to amplify levels of noradrenaline through inhibition of reuptake at the synaptic cleft[77]. Alternatively, tricyclic antidepressants (TCAs) inhibit the reuptake of some neurotransmitters such as dopamine, noradrenaline and serotonin, into the presynaptic cleft in a nonselective manner[79]. Although they were once considered the gold standard of pharmacological treatment for MDD, there are several anticholinergic side effects associated with the use of

TCAs such as sedation, hypotension and issues with cardiac conduction[17]. Therefore, the use of TCAs is no longer a first-line treatment.

SSRIs are considered the primary pharmacological intervention after initial screening reveals cases of moderate depression due to its efficacy and high patient acceptance rates[17], [25]. However, long term courses of treatment involving SSRIs is associated with a plethora of troublesome side effects such as sexual dysfunction, weight gain, insomnia and apathy[79]. This can, in some cases, lead to relapse and recurrence of the mental illness, leading to cautious practice of pharmacological therapy by clinicians.

3. Physiological Signal Monitoring for Mental Health and Depression

The findings reported in this chapter have been published in:

***Ahmed T,** Qassem M, Kyriacou PA. Physiological monitoring of stress and major depression: A review of the current monitoring techniques and considerations for the future, Biomedical Signal Processing and Control, Volume 75,2022*[80].

The use of physiological signals monitoring is the leading approach to comprehending the progression of psychiatric disorders, such as clinical depression[81]. The physiological, behavioural, and psychological changes that transpire as a result of such illnesses are often indicative of the severity of the disorder[82]. These changes can be detected through heart rate variability, biofluid analysis, electroencephalography, electrodermal activity, as well as standardised questionnaires[14], [81]–[83]. Physiological monitoring facilitates a feasible and objective approach to evaluation and diagnosis of neuropsychiatric disorders, without the uncertainty of questionnaire-based psychoanalysis. The purpose of this chapter is to highlight the current state of the art monitoring techniques used to measure stress in the human body.

Neuropsychiatric evaluation through physiological monitoring primarily involves multimodal sensing approaches, with a focus on stress monitoring[14], [81]–[83]. Chronic and excessive stress are main causes of deterioration of mental illnesses, frequently leading to the progression of physiological and psychological concerns, such as hypertension and major depressive disorder respectively[18], [35], [37]. Major depressive disorder and other mood disorders, for instance schizophrenia can cause considerable degeneration of the brain, a severe consequence which is a predominant cause of disabilities[84]. Due to the debilitating nature of several psychiatric disorders, the necessity of employing a multisensory approach to investigate the underlying physiological signals that are indicative of the disorder is essential for the evaluation and treatment of suffering patients.

3.1 Introduction

Currently, the procedure for diagnosing depression involves self-reported questionnaires, interviews by trained professionals i.e., psychologists and psychiatrists; and the use of

standardised qualitative surveys and scoring systems, such as the Hamilton Depression Rating Scale (HAM-D) or the Beck Depression Inventory (BDI-II) for evaluation of depression severity[3]–[5]. The absence of physical tests is cause for alarming concern, as the common mental illness takes its toll on global productivity, economy and social health[1]. The concept of monitoring the physiological signals associated with stress has been established for many years, primarily focusing on the effects that different stresses can have on the sympathetic and parasympathetic nervous systems[85]. Physiological monitoring has provided essential information regarding mental illness sufferers that cannot be obtained through the existing diagnostic means. Monitoring techniques such as electrocardiography, electroencephalography and electrodermal responses are pioneering the realisation of the quantified form of stress and major depression. Such technologies have proven to be significantly effective in the classification of mental illnesses, despite its lack of presence in clinical psychology diagnostic applications[86].

The primary aims of this review chapter are to comprehensively review the common physiological monitoring techniques and their applications within stress and depression monitoring. Furthermore, the fundamental principles of such technologies will be highlighted, as well as results from notable physiological measurement studies. This will lead to a discussion focusing on the significance of standardised stress testing, the strengths, and shortcomings of physiological monitoring techniques and, the technical aspects that should be considered as psychological diagnosis approaches the concept of physical testing.

3.2 Methods of Literature Search

The purpose of this chapter to encapsulate studies of certain physiological measurement techniques and its utilisation in the monitoring of psychological stress, as it leads to the manifestation of mental illnesses, particularly clinical depression. English-written articles were obtained from SCOPUS and PubMed databases and selected based on the search criteria of inclusion of specific words in their title, abstract or keywords. The search criteria comprised of two stationary terms: ((‘Psychological Stress’) AND (‘Depression’)). Also, 3 independent terms were used to obtain articles relating to each of the physiological monitoring techniques of interest: (‘HRV’ OR ‘Heart Rate Variability’); (‘GSR’ OR ‘EDA’ OR ‘Galvanic Skin Response’ OR ‘Electrodermal Activity’); (‘EEG’ or ‘Electroencephalography’). Additionally, related articles were selected through reference lists and the ‘related articles’ feature on SCOPUS and PUBMED. After removal of duplicates, a total of 725 papers were obtained. Following the reading of the abstracts, 237 papers were selected for further evaluation and classified

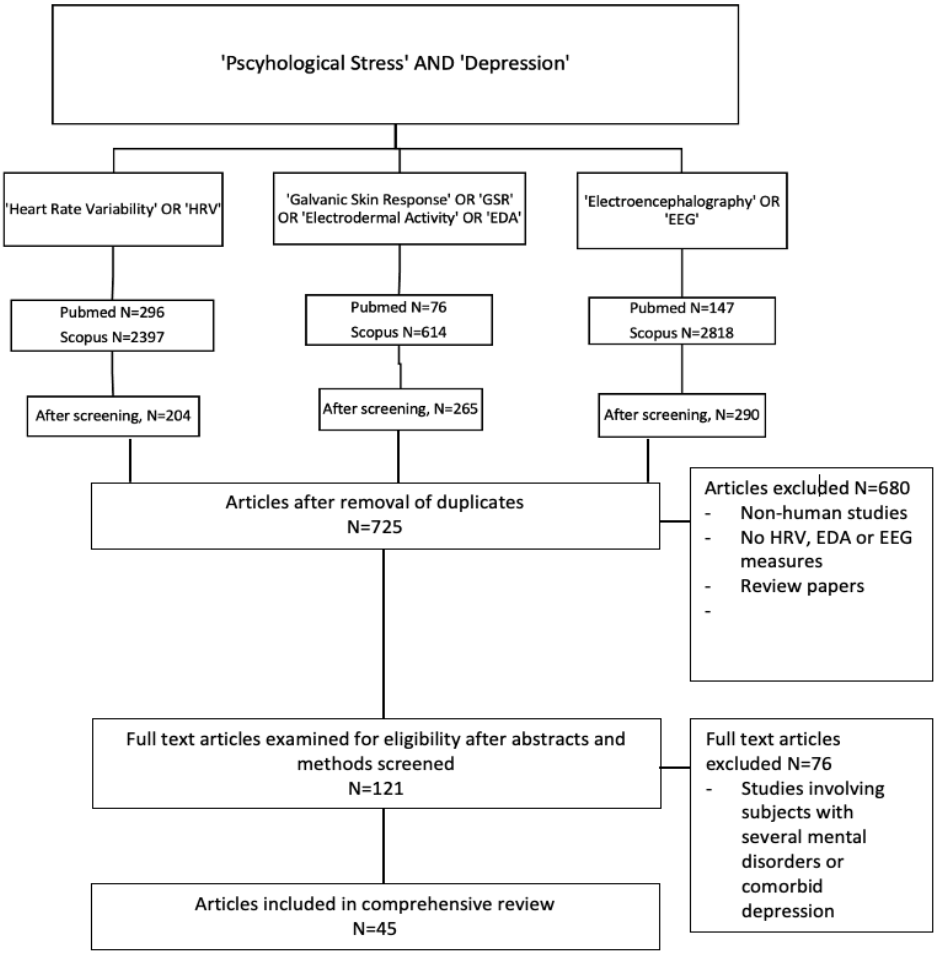

Figure 1 - Flow chart of article selection for comprehensive review of physiological monitoring of stress and depression.

according to the monitoring/diagnostic focus, i.e., psychological stress, physical stress, major depressive disorder, schizophrenia, bipolar disorder, co-morbid depression, and post-traumatic stress disorder. The review focuses primarily on the physiological monitoring of psychological stress and the development of major depression therefore, the most relevant papers that matched these criteria were selected. Eventually, 42 papers were chosen for complete evaluation and inclusion in this review. Figure 1 depicts a flow chart of the database search procedure and selection process for the review.

Prolonged or chronic stress commonly leads to the imbalance of stress mediators and hormones within the body. Such imbalances are often referred to as 'allostatic states', such as blunted cortisol responses to a stressor or chronic insomnia[11], [32], [35]. Several articles have highlighted the significance of chronic stress and the manifestation of depressive symptoms from the allostatic overloads, caused by psychological stressors and extreme environmental conditions. The relationship that exists between recurrent stress and depression is reflected within the human body in several forms; whether it is through biochemical reactivity and allostatic states, or the physiological changes that they pose on the body[17], [18], [39], [41]. These physiological changes have been a primary focus for several studies, as they can contribute greatly to the quantification of stress and depression. These are pioneering techniques that are shifting efforts from the subjective manner of psychological diagnoses, towards objective classifications of depression.

3.3 Physiological Monitoring Techniques for Stress and Depression

As previously mentioned, the comprehension of stress and its effects on the body is significant for the quantitative evaluation of depression, and major depressive disorder. Agelink et al. presented a study concerning the relationship between heart rate variability and major depression in 2002[13]. Through comparing time and frequency domain HRV indices between 32 patients suffering from Major Depressive Disorder (MDD), classified through the Hamilton Depression Scale (HAM-D), Agelink concluded that a negative correlative relationship existed between the HAM-D scores and the vagal HRV indices. Altered vagal tone suggests changes within the parasympathetic nervous system, which is illustrated in the low-frequency to high-frequency ratio (LF/HF) HRV index. Reduced vagal modulation translates to a higher LF/HF ratio, and reduced activation of the parasympathetic nervous system[87], [88]. This could

indicate that MDD patients take a longer period of time to reach a resting state in post-stress situations, when compared to healthy controls.

Additionally, Branković reported on the skin conductance responses of 57 depressed individuals, compared to 52 healthy controls in a 2008 stress study[89]. Branković developed a mathematical model to showcase the regulation of Skin Conductance Responses (SCRs) in the body during emotional arousal, resulting in the realisation of two feedback loops.

Both feedback loops exhibited significantly stronger signals from depressed individuals when compared to healthy subjects. Thus, facilitating the comprehension of the neurochemical characteristics of depression and its expression in SCR. Furthermore, several studies have involved the development of an electroencephalography-based diagnostic tool for major depression. Notably, Cai's study with a 3-electrode EEG system for depression diagnosis compared the successes and shortcomings of four feature selection algorithms for the selection of the optimum feature selection technique for the best classification performance[90]. This study revealed that in the case of distinguishing between 152 depressed patients and 113 healthy subjects, the decision tree classifier exhibited the highest accuracy of 76.4%. Several studies carried out a multimodal approach to stress monitoring which included heart rate variability, electroencephalography, galvanic skin response, etc. Such studies present the robust power in the utilisation of physiological signals as alternative, or complimentary tools to the current diagnostic and monitoring practices for clinical depression, its progression and treatment efficacy in modern societies.

3.3.1 Fundamental Principles and Applications of Heart Rate Variability

The significance of heart rate variability (HRV) in the field of psychophysiology was introduced by Wolf in 1967, which described HRV as an indicator of brain and vagal-heart communication[91]. HRV is variation in the time intervals that exist between successive heartbeats. This interval is referred to as 'inter-beat intervals' or IBIs[85]. Heart rate variability is known to be influenced by heart-brain interactions and the non-linear dynamics of the autonomic nervous system (ANS)[92]. The ANS can be separated into the sympathetic (SNS) and parasympathetic nervous system (PNS) branches. The balancing mechanisms between the two branches influence heart rate and are moderated by unique mediators. The

parasympathetic branch is mediated by acetylcholine, released from the vagal nerve. Whereas the sympathetic branch is facilitated by the release of epinephrine and norepinephrine from the adrenal medulla[9], [29]. Interactions between acetylcholine and, epinephrine and norepinephrine and their corresponding receptors leads to modulations in parasympathetic and sympathetic activity, respectively. Balance changes between the SNS and PNS lead to cardiovascular variations[92]. Wherein, a ratio of increased SNS activity to decreased PNS activity leads to cardio acceleration, whereas the opposite ratio (High PNS and low SNS) leads to cardio deceleration. The constant interactions and balancing mechanisms between the vagal and sympathetic activity encapsulates heart rate variability. Through electrocardiography (ECG), the rhythmic contributions of sympathetic and parasympathetic activity on the variations between heartbeats can be observed[85], [92]. Due to the natural irregularity of successive heartbeats, variations in IBIs are expected in healthy humans, whereas regularity and decreased variation can be indicative of homeostatic changes within the body, changes in the environment, or physical and mental disorders[93].

For the comprehension of psychophysiological stress, there are several HRV indices of interest within the time and frequency domains. Time domain analysis of HRV involves the calculations of mean normal-to-normal (NN) intervals and the variance between these intervals[93]. NN intervals are defined as the distance, in milliseconds (ms) that exist between successive normal heartbeats i.e., between the R peak of the QRS complex in the ECG[85]. Comparatively, frequency domain HRV indices are obtained through utilisation of Fast Fourier Transformation (FFT) or Auto-Regressive modelling (AR). HRV measures within the time and frequency domain are summarised in Table 1.

Table 1 - Heart Rate Variability (HRV) measures within the time and frequency domains.

VARIABLE	UNITS	DESCRIPTION	DOMAIN	FREQUENCY RANGE
SDNN	ms	Standard deviation of all NN intervals	Time	
SDANN	ms	Standard deviation of average NN intervals for each 5 min segment in 24-hour recording	Time	
SDNN INDEX	ms	Mean of standard deviation of all NN intervals for each C75 min segments in 24-hour recording	Time	
RMSSD	ms	Square root of the mean of the sum of squares of differences between successive NN intervals	Time	
SDSD	ms	Standard deviation of differences between successive NN intervals	Time	
NN50		Number of pairs of adjacent NN intervals differing by more than 50ms in the complete recording.	Time	
PNN50	%	NN50 divided by total number of all NN intervals	Time	
VLF	ms^2	Power in the very low frequency range	Frequency	<0.04Hz
LF	ms^2	Power in the low frequency range	Frequency	0.04-0.15Hz
LF $_{norm}$	v	LF power normalised units LF/ (total power - VLF) x100	Frequency	
HF	ms^2	Power in the high frequency range	Frequency	0.15-0.4Hz

HF $_{norm}$	v	HF power normalised units: HF/ (total power-VLF) x100	Frequency	
TOTAL POWER		Variance of all NN intervals	Frequency	<0.4Hz

Notably, the low-frequency/high-frequency (LF/HF) ratio has great emphasis in several HRV stress studies[94]. Delaney et.al. conducted a HRV stress study in 2000, which involved the HRV measurement of 30 healthy volunteers within a competitive setting whereby the Stroop Colour Word Conflict Test was conducted[94], [95]. Heart rate variability monitoring revealed that during stages of psychological stress, there was a significant reduction in the high frequency component, and a significant increase in the low frequency component. Therefore, it was evident that psychological stress from the stress test led to a significant increase in the LF/HF ratio. Similarly, in 2007 Udupa et.al. led a study amongst 40 patients suffering from major depression disorder and age- and gender-matched controls using heart rate variability measures. The LF/HF ratio in MDD patients were significantly higher than those in healthy controls. This would suggest that both psychological stress and the presence of depressive symptoms have a similar effect on the sympathovagal balance within the body. The sympathovagal balance, reflected by the LF/HF ratio was increased in both cases, signifying increased sympathetic activity, which is modulated by stress hormones, epinephrine and norepinephrine[9], [29]. Although, it should be noted that the former study involved healthy volunteers undergoing a standardised stress test whereas, the latter comprised of MDD patients that had undergone a deep breathing test, Valsalva manoeuvre and an orthostatic test. Therefore, direct comparisons cannot be made between the two studies as one focuses primarily on the effects of the stress inflicted on HRV measures whilst the other focuses on the HRV indices in MDD patients, without the presence of a stress test. However, Agelink et.al. 2002 study of the relationship between major depression and HRV examines the sympathovagal balance in MDD patients with a similar set of tests to Udupa's study and has shown findings which resonate with that of Udupa[13]. Psychological stress-based studies that have demonstrated the effects on HRV measures are summarised in Table 2.

Table 2 - Studies of heart rate variability (HRV) monitoring for psychological stress evaluation in depressed and non-depressed groups.

Authors	**Year**	**N (Number of participants)**	**Age in years (mean ± SD)**	**Stress Evaluation**	**Test**	**HRV measures**	**Major Findings**
Agelink, M.W.[13]	2002	32 MDD patients (16 mildly depressed i.e. M-HAMD; 16 severely depressed i.e. S-HAMD) and 64 non-depressed controls.	44.3±12.6 (M-HAMD); 53.5±15.8 (S-HAMD) 46.6±11.9 (controls)	HRV, Blood pressure, DSM-III-R and Hamilton Depression Scale (HAM-D)	Standardised 5 min resting study, deep breathing test, Valsalva manoeuvre	HR, log CV, log RMSSD, LF power, log HF power, HF power, LF/HF ratio, Valsalva ratio	S-HAMD patients had lowest HRV indices. Mean CV and RMSSD (during deep respiration test) for S-HAMD group was significantly lower in comparison to healthy controls, as well as M-HAMD group. Higher LF/HF ratio and resting HR in S-HAMD group compared to healthy controls. Negative correlation

Authors	Year	N (Number of participants)	Age in years (mean ± SD)	Stress Evaluation	Test	HRV measures	Major Findings
							between depression severity and vagal HRV indices.
Bosch, J.A.[96]	2009	61 healthy participants	20.3±1.09	HRV via ECG, salivary cortisol, ICG, Test Anxiety Scale for affective responses	2 back-to-back speeches, with 2 mins preparation and 4 mins of speech delivery.	HR, RMSSD	Affective response, salivary cortisol, HR, HRV and pre-ejection period all differentiated in different task conditions. Physiological reactivity increased with increasing audience size. Cortisol increase predicted by

Authors	Year	N (Number of participants)	Age in years (mean ± SD)	Stress Evaluation	Test	HRV measures	Major Findings
							sympathetic activation (pre-ejection) but not by affective responses. RMSSD responses were larger in 1 and 4 audience member settings than control, difference did not reach statistical significance.
Brugnera, A. [97]	2019	65 healthy participants	24.7±3.9	Beck Depression Inventory III (BD), State and Trait Anxiety Inventory, Cook-Medley Hostility Scale, Stress Rating	Montreal Imaging Stress Task (MIST) - involves mental arithmetic task	HR, SDNN, RMSSD, total power, HF power, LF power, HFv, LFv, LF/HF	Significant changes in HF power and RMSSD, with the lowest value reached during stress task. Only depressive symptoms positively correlated to higher resting HRV and to

Authors	Year	N (Number of participants)	Age in years (mean ± SD)	Stress Evaluation	Test	HRV measures	Major Findings
				Questionnaire (SRQ), Type D Scale-14, HRV via ECG			blunted reactivity in stress task. LF/HF variations were insignificant.
Castaldo, R. [15]	2016	42 healthy participants	18.7±28.7	HRV via ECG	Oral academic examination	RR, SDNN, RMSSD, pNN50, LF, HF, LF/HF	Higher values of all-time domain features were observed during stress phase except RMSSD. During stress phase, LF and HF also increased. Nonlinear HRV features were also assessed and

Authors	Year	N (Number of participants)	Age in years (mean ± SD)	Stress Evaluation	Test	HRV measures	Major Findings
							showcased better discrimination ability.
Chang, H.-A. [98]	2012	498 unmedicated MDD participants; 662 healthy participants	39.13 ± 14.12 (MDD); 40.66 ± 14.89 (controls)	DSM-IV, HAM-D, BDI, HRV via ECG, blood pressure		RMSSD, variance, LF, HF, LF/HF	MD patients exhibit reduced cardiac vagal control compared to healthy subjects. Reduced RR, variance, LF, HF in MDD patients compared with controls.
Delaney, J.P.A.[94]	2000	30 healthy participants	30.9 ± 3.9 (Women); 34.4 ± 8.7(Men)	Visual Analog Scales, HRV via ECG	Stroop Word Colour Conflict Test	HR, SD, RMSSD, pNN50, total power, VLF, LF, HF, LF_{norm}, HF_{norm}, LF/HF	Stress task caused significant increase in HR and overall reduction in autonomic system activity (decrease in SD of

Authors	Year	N (Number of participants)	Age in years (mean ± SD)	Stress Evaluation	Test	HRV measures	Major Findings
							normal inter-beat intervals). RMSSD was also significantly reduced. In frequency domain, there was significant decrease in total power. High frequency component also showed decrease and LF component showed significant increase.

Authors	Year	N (Number of participants)	Age in years (mean ± SD)	Stress Evaluation	Test	HRV measures	Major Findings
Hughes, J.W. W[99]	2000	53 healthy participants	18.7±1.53	HRV via ECG, BDI, STAI, blood pressure, respiratory rate, respiration amplitude	Videotaped speech task, 3-minute forehead cold pressor task	R-wave peak detection, IBIs, HF via Fast Fourier Transform (FFT)	Significant main effects of depressed mood on BP. High depressed mood participants had significantly higher BP than low depressed mood patients. No other significant main changes on interaction terms involving depressed mood. Participants with higher BDI had significantly different patterns of HF response to stressors than those with low BDI i.e. greater decreases in

Authors	Year	N (Number of participants)	Age in years (mean ± SD)	Stress Evaluation	Test	HRV measures	Major Findings
							HF to speech task and smaller increases to cold pressor task.
Light, K.C.[100]	1998	60 healthy women; 15 with highest BDI score formed depressed group vs 15 with lowest BDI score formed	32.5±9.9 (Depressed); 30.1±9.0 (Controls)	BDI, HAM-D, Interpersonal Support Evaluation List (ISEL), blood pressure, PEP, HRV via ECG, plasma epinephrine and norepinephrine	Postural challenge, speech task	HR, RMSSD	Depressive group had higher BP at rest and during stressors compared to healthy controls. Both groups showed equivalent increase in BP to stressors. Depressed group had shorter PEP and reduced HRV. Baseline levels of E and

Authors	Year	N (Number of participants)	Age in years (mean ± SD)	Stress Evaluation	Test	HRV measures	Major Findings
		healthy control group					NE didn't differ between the two groups. Depressed group showed greater increase in plasma NE to speech task and posture challenge. Plasma E increased from baseline in depressed group and decreased from baseline in healthy group for the speech task.
Moser, M.[101]	1998	26 unmedicated MDD patients and	33.7 (MDD); 32.1 (Controls)	BDI, STAI, HRV via ECG, blood pressure		HR, HF	Heart rate was significantly higher in patients and diastolic BP was significantly

Authors	Year	N (Number of participants)	Age in years (mean ± SD)	Stress Evaluation	Test	HRV measures	Major Findings
		26 healthy controls					higher in controls. Depressed patients showed slightly lower vagal tone but not significantly different from healthy control group.
Pereira, T.[87]	2017	14 healthy participants		STAI, HRV via ECG	Trier Social Stress Test (TSST)	AVNN (average value of NN intervals), SDNN, RMSSD, pNN20, pNN50, LF, HF, LF/HF and	STAI scores validated subjects had higher stress levels during tasks compared to baseline. All HRV metrics negatively correlated with STAI scores except LF/HF, LF (v) and (alpha1) which showed positive

Authors	Year	N (Number of participants)	Age in years (mean ± SD)	Stress Evaluation	Test	HRV measures	Major Findings
						nonlinear measures	correlation. AVNN, RMSSD, SDNN and pNN20 showed consistent differences between stress and non-stress phases of the TSST. AVNN, SDNN and pNN50 significantly reduced during stressor activation periods which was confirmed by results.

Authors	Year	N (Number of participants)	Age in years (mean ± SD)	Stress Evaluation	Test	HRV measures	Major Findings
Pulopulos, M.M.[102]	2020	52 female participants	21.06±2.58	BDI-II, Ruminative Responses Scale, Rosenberg Self-Esteem Scale, Perceived Stress Scale, Generalized Self-Efficacy Scale, Visual Analog Scales, salivary cortisol, HRV via telemetric HR monitor	modified TSST	RMSSD, pNN50	RMSSD (HRV) showed significant decrease from habituation phase to anticipation of stress phase. RMSSD was also lower during stress phase. There were no significant differences between groups in anticipatory HRV responses, HRV responses to stress and cortisol indexes. More negative anticipatory cognitive stress appraisal was associated with larger

Authors	Year	N (Number of participants)	Age in years (mean ± SD)	Stress Evaluation	Test	HRV measures	Major Findings
							decreases in HRV during stress anticipation and higher cortisol reactivity.
Schulz, S.[88]	2010	57 unmedicated MDD patients; 57 healthy controls	30±9 (MDD); 29±8 (Controls)	DSM-IV, HAM-D, BDI, HRV via ECG, blood pressure,		AVNN, SDNN, RMSSD, pNN50, LF $_{norm}$, HF $_{norm}$, LF/HF and nonlinear measures	All parameters from time and frequency domain of HRV showed no significant differences between MDD patients and controls. Time domain parameters tended to be reduced in MDD

Authors	Year	N (Number of participants)	Age in years (mean ± SD)	Stress Evaluation	Test	HRV measures	Major Findings
							compared to controls, frequency domain parameters were unaffected. Time domain parameters of BVP tended to be higher in MDD compared to controls.
Ahrens, T.[103]	2008	22 female MDD patients; 20 healthy female controls	51.0±1.7 (MDD); 54.2±1.6 (Controls)	DSM-IV, HAM-D, HRV via ECG, salivary cortisol, blood pressure, Visual Analog Scales, serum cortisol, ACTH, epinephrine and	Speech task, mental arithmetic, cognitive challenge	RR variance, LF, HF, LF/HF ratio, total power	Morning saliva cortisol lower in MDD than healthy. No differences in afternoon or night-time cortisol. Baseline after resting showed lower serum cortisol and NE in MDD than healthy, serum ACTH

Authors	Year	N (Number of participants)	Age in years (mean ± SD)	Stress Evaluation	Test	HRV measures	Major Findings
				norepinephrine, urinary cortisol			didn't differ. E levels below range of detection. No group differences in HRV. Blunted cortisol levels in MDD patients.
Vaccarino, V.[104]	2008	288 male twins of varying depression severity		DSM-IV, BDI, HRV via 24-hour ambulatory ECG, blood pressure		RR interval, ultra-low frequency (ULF), very low frequency (VLF), LF, HF, total power, LF/HF	Current depressive symptoms were associated with lower HRV. Increasingly higher BDI scores were associated with progressively lower HRV indices. Power in each HRV frequency band was 19-36% lower in the highest compared with

Authors	Year	N (Number of participants)	Age in years (mean ± SD)	Stress Evaluation	Test	HRV measures	Major Findings
							the lowest BDI scoring categories. HRV was 12-21% lower in twins with MDD history than those without. None of the HRV spectra remained significantly associated with a lifetime history of MDD in multivariate analysis.

3.4.2 Fundamental Principles and Applications of Galvanic Skin Resistance

Electrodermal activity (EDA) describes the active and passive electrical properties of the skin and is known to be modulated exclusively by the sympathetic nervous system[105]. Variations in sweat gland activity give rise to the tonic and phasic dynamics of EDA[106], [107]. Sympathetic innervations of sweat glands contribute to EDA dynamics, which are known to be affected by arousal of emotional and cognitive states[107]. Thus, clinical research within psychophysiology often highlights the significance of EDA monitoring. The inclusion of EDA monitoring in stress studies is further reinforced by the characteristic increase in EDA responses. The signals mirror other well-known physiological changes that occur in response to stress i.e., 'the fight or flight response', such as increases in heart rate and blood pressure[30]. Several studies have noted the significance of EDA as an objective measure for emotional behaviour and arousal[106], [108], [109]. There are distinct regions of the brain that are involved with the homeostatic control of sympathetic arousal which leads to the manifestation of EDA responses[107]. EDA responses can be classified based on the characteristics of the signal. Slow changes in the basal components are referred to as skin conductance levels (SCLs), whereas rapid transient peaks are known as skin conductance responses or galvanic skin responses (SCRs or GSRs, respectively)[106], [107].

A prominent study in the field of psychophysiological monitoring via EDA was the development of a system for continuous EDA measurement for the detection of MDD in 2018 by Kim et.al.[14]. The study involved a machine learning approach to the classification of major depressive disorder in 30 MDD patients and 37 healthy controls. Continuous EDA measurements were recorded during 5 experimental phases including baseline, stress and recovery stages to evaluate the alterations in autonomic activity for feature selection via support vector machines (SVMs). Selected features were used in a decision tree classifier, generating an accuracy of 74%. Kim et.al demonstrated the robust power of EDA responses in preliminary studies through the decomposition of the tonic and phasic components of obtained EDA signals, to facilitate feature extraction for time and frequency analysis[14]. Through feature extraction in preliminary studies, it was evident that all SCRs features were significantly lower in MDD patients than healthy controls, between the experimental phases, suggesting its efficacy in distinguishing between depressed and non-depressed subjects[110].

There are several studies which highlight the use of electrodermal activity measurements for the comprehension of psychophysiological stress, these are summarised in Table 3.

Table 3 - Studies of electrodermal activity (EEG) monitoring for psychological stress evaluation in depressed and non-depressed groups.

Authors	**Year**	**N (Number of participants)**	**Age in years (mean ± SD)**	**Stress Evaluation**	**Test**	**Major Findings**
Branković, S.B.[89]	2008	57 MDD patients; 52 healthy controls	43.1±10.9 (MDD); 39.8±8.1(Control)	DSM-IV, HAM-D, Skin conductance (SC), HR, respiration	11 short stories to elicit arousal	Stronger SCR signals in depression compared to healthy controls
Kim, A.Y.[14]	2018	30 MDD patients; 37 healthy controls	42.5±16.96(MDD); 41.3±15.97 (Control)	HAM-D, Hamilton Anxiety Rating Score (HAM-A), Stress Response Inventory (SRI), Skin conductance levels (SCL), skin conductance response (SCR)	Mental arithmetic task	MSCL (mean amplitude of SCL), SDSCL (standard deviation of SCL), NSSCR (non-specific SCR) were significantly affected by group and task. SKSCL (skewness of SCL) was significantly affected by arithmetic task.
Kim, A.Y.[110]	2019	30 MDD patients; 31 healthy controls	42.5±19.70 (MDD); 43.7±20.77 (Control)	SRI, PSS (Perceived Stress Scale), HAM-D, SC	Mental arithmetic task	All 6 SC features were lower in MDD patients compared to control groups during all phases of study.

3.4.3 Fundamental Principles and Applications of Electroencephalography

Electroencephalography (EEG) is the measurement of electrical impulses from the surface of the scalp to record spontaneous rhythmic brain activity[111]. The relationship between EEG signals and emotional states has been studied for several decades. Numerous studies have shown how certain components of EEG signals can be reflective of specific behavioural and emotional tendencies in humans[112], [113]. The EEG signal can be classified into 5 frequency-based bands, each wave contributing to specific functionality in the brain. The delta wave (<4Hz) characterises adult slow-wave sleep, whilst the theta wave (4-8Hz) is prevalent in adults reaching the stage of sleep[111], [114]. Additionally, the alpha band (8-14Hz) illustrates resting, relaxed states, whereas the beta band (14-30Hz) signifies active thinking phases[111], [114]. The gamma band (14-30Hz) indicates working memory and attention, often amplified by neurostimulation or meditation[115].

EEG monitoring for depression has been highlighted in numerous studies[116]–[118]. Predominantly, the findings of these studies presented the increase in absolute power in the theta and beta bands during eyes open and eyes closed conditions, in depressed individuals[116], [119]. Furthermore, Kan et.al. showcased the significance of alpha band frequencies in the discrimination between depressed and normal groups in a 2015 electroencephalogram study[120]. Kan highlighted the low alpha frequency signals originating from the parietal, occipital and temporal lobes, suggesting the lack of attentiveness in depressed individuals, when compared to healthy controls[120]. Depending on the frequency bands that are analysed, there are distinct characteristics that may be indicative of depressive symptoms within individuals under examination. For example, in a study by Debener et.al. on physiological markers of depression, the EEG frequency that was analysed was alpha wave asymmetry[118]. The findings from this study illustrated the increase in anterior alpha wave asymmetry in the depressed patients when compared to the healthy controls[117]. These studies have emphasised the differences in brain activity amongst depressed individuals, in comparison to healthy controls, as well as noting the influences of antidepressant treatments on the electroencephalographic signals of depressed patients[117]. A comprehensive summary of these studies can be found in Table 4.

Table 4 - Studies of electroencephalography (EEG) monitoring for psychological stress evaluation in depressed and non-depressed groups.

Authors	**Year**	**N (Number of participants)**	**Age in years (mean ± SD)**	**Stress Evaluation**	**Test**	**EEG measures**	**Major Findings**
Billones, R.K.C.[121]	2019	8 healthy participants		EEG, HR via ECG	2-minute jog, watching thriller clip with jump-scare sequence	alpha, beta, delta, theta band measurements from 3 electrode EEG	Increase in HR and alpha, beta, delta and theta waves upon stimulus activation. Dominance of delta activity after jog and theta waves after watching video.
Al-Shargie, Fares[122]	2018	18 healthy male participants		EEG, self-reporting about task load (NASA-TLX rating scale)	Mental arithmetic task of 3 levels of difficulty	alpha rhythm power, EEG from prefrontal cortex (PFC) with 7 active electrodes	no significant differences in EEG when compared to self-reporting questionnaire. Mean EEG alpha rhythm power significantly

Authors	Year	N (Number of participants)	Age in years (mean ± SD)	Stress Evaluation	Test	EEG measures	Major Findings
							reduced with increasing difficulty.
Bachmann, M.[123]	2015	17 female MDD patients; 17 healthy female controls	39±12	ICD-10, HAM-D, EEG		Lempel-Zig complexity (LZC) from 18 channel EEG in 10-20-electrode position classification system	Increased complexity in depressive subjects compared to normal controls, even in single channel EEG.
Baehr, E[124]	1998	13 MDD patients; 11 healthy controls	43.5±6.99 (MDD); 44.2±13.3 (Control)	DSM-IV, BDI, EEG		alpha asymmetry from 3 electrode commercial EEG system	Percent index (percentage of time in which asymmetry was >0) was better discriminator between depressed and control groups

Authors	Year	N (Number of participants)	Age in years (mean ± SD)	Stress Evaluation	Test	EEG measures	Major Findings
							compared to asymmetry score.
Hinrikus, H[125]	2010	18 female MDD patients; 18 healthy female controls	36±10 (MDD); 35±10.5 (Control)	ICD-10, HAM-D, EEG		Spectral asymmetry (SA) in alpha band from 19 electrode commercial EEG system	SA values were positive for depressive subjects, negative for healthy subjects. SA differences between 2 groups were significant in all EEG channels.

Authors	Year	N (Number of participants)	Age in years (mean ± SD)	Stress Evaluation	Test	EEG measures	Major Findings
Kan, D.P.X.[120]	2015	8 participants	23.38	DSM-IV, Patient Health Questionnaire (PHQ-9), Depression, Anxiety and Stress Scale (DASS-21), EEG		delta, theta, beta and alpha band analysis from 32 channel commercial EEG system	Alpha waves in depressed subjects were decreased compared to normal subjects significantly. Low alpha frequency in parietal lobe, occipital lobe and temporal lobe.

Table 5 – Studies of multimodal monitoring for psychological stress evaluation in depressed and non-depressed groups.

Authors	**Year**	**N (Number of participants)**	**Age in years (mean ± SD)**	**Stress Evaluation**	**Test**	**Measurements**	**Major Findings**
Cipresso, P.[126]	2019	60 healthy participants	21.2±2.25	Respiration, Blood volume pulse (BVP), GSR, HRV via BVP	Stroop Colour Word Task, mental arithmetic task	moment-to-moment HRV i.e., PRV (pulse rate variability), SDRR, SDHR, VLF, LF, HF, LF/HF, respiration rate, SC	Increase in sympathetic activity with significantly higher LF during acute mental stress. However, HF also increased suggesting increased parasympathetic activity. RSA shows HRV in synchrony with respiration, reduced vagal tone during acute mental stress.

Authors	Year	N (Number of participants)	Age in years (mean ± SD)	Stress Evaluation	Test	Measurements	Major Findings
Guinjoan, S.M.[127]	1995	18 MDD patients; 18 healthy controls	44±12 (MDD); 40±13 (Control)	Minnesota multiphasic personality inventory (MMPI), DSM-III-R, blood pressure, HR and HRV, GSR	Lying to standing manoeuvre, hand grip manoeuvre, mental arithmetic task, explosive sound, cold pressor task, hyperventilation	Systolic and diastolic BP, HR, GSR	Depressed patients had significantly lower indices than control subjects for parasympathetic activity as seen by HRV. Sympathetic skin responses were significantly larger in depressed individuals.
Papousek, I.[128]	2002	111 healthy participants	21	FBL-R (Freiburg Complaint Checklist), 17-point bipolar rating scale (KUSTA),	Public speech task	EDA, LF, HF	All participants showed increase in EDA and decreases in HRV-HF from rest to stress condition. Changes were larger

Authors	Year	N (Number of participants)	Age in years (mean ± SD)	Stress Evaluation	Test	Measurements	Major Findings
				Eysenck Personality Questionnaire (EPQ), EDA, HRV via ECG			in subjects that reported greater stress.
Reinhardt, T.[129]	2012	Study 1: 80 female participants. Study 2: 30 healthy participants	25.4±4.5 (Study 1); 24.7±4.6 (Study 2)	DSM-IV, SCID II Personality Questionnaire, International Personality Disorder Examination (IPDE), salivary cortisol (study 2), SCL (study 1), HR	Mannheim Multicomponent Stress Test (MMST)	HR via HR sensors in study 1 along with GSR. HR via ECG in study 2 along with salivary cortisol.	Significant increase in subjective stress ratings in response to MMST. HR significantly increased in response to MMST. Significant increases of SCL found in response to stress induction. In second study subjective

Authors	Year	N (Number of participants)	Age in years (mean ± SD)	Stress Evaluation	Test	Measurements	Major Findings
							stress ratings were significantly increased along with HR and salivary free cortisol. Mean peak of cortisol level observed 20 mins after stress cessation.
Ding, X.[130]	2019	144 MDD patients; 204 healthy controls	27.65±9.50 (MDD); 27.46±9.61 (Control)	ICD-10, SDS, eye tracking, EEG, GSR	Open Affective Standardised Image Set (OASIS) for eye tracking task. Watching 8 short videos	Eye tracking data, EEG, GSR	MDD patients had higher SDS scores than control. MDD showed significantly lower absolute EEG power in theta, alpha, beta and gamma bands than

Authors	Year	N (Number of participants)	Age in years (mean ± SD)	Stress Evaluation	Test	Measurements	Major Findings
							HC. MDD also showed attentional bias towards negative images during eye tracking.

3.5 Discussion

HRV reactivity is profoundly dependent on variations in ANS activity, which may be caused by stress induction or emotional arousal[85]. Several studies have presented the relationship that exists between psychological stress and the manifestation of changes in physiological signals within the body[13], [94], [131]. Evidently, the monitoring of heart rate variability in response to elicitation of psychological stress has revealed the significance of the balance between sympathetic and parasympathetic activity i.e., the sympathovagal balance. The LF/HF ratio in HRV indices reflects the sympathovagal balance and indicates vagal activity dominance in scenarios where the ratio is low, and sympathetic activity dominance when the ratio is high[85]. As it is known that the 'fight or flight' or stress response is activated by high sympathetic activity, a high LF/HF ratio can suggest increased stress in individuals[9]. Furthermore, in cases of persistent stress or mental illnesses, such as major depressive disorder, the LF/HF ratio is expected to increase. This is potentially due to the exhaustive stage of the Generalised Adaptation Syndrome which depicts the depletion of efforts to challenge persistent stressors[33], [35]. Exhaustion of the ANS can be signified by decreased vagal tone, increased LF values and decreased HF values i.e., overall increase in LF/HF ratio. This concept is in line with the findings from Delaney et.al's study of HRV monitoring in participants undergoing the Stroop Word Colour Conflict Test, Agelink et.al.'s study in the examination of HRV reactivity in MDD patients, and partially with Hughes' study of HRV during a stress task, which reported HF power decline in the presence of stressors[13], [94], [99].

However, several studies have suggested that the LF/HF ratio may not accurately reflect ANS activity, especially during stress tasks. Brugnera et.al's protocol involved HRV monitoring of 65 healthy volunteers during the Montreal Imaging Stress Task (MIST)[97]. Findings from this study revealed that although statistically significant decreases in HF power were presented during the stress task; the LF/HF ratio variations were insignificant. Similarly, Castaldo's 2016 study comprised of HRV monitoring of 42 healthy volunteers during oral academic examinations, which disclosed increases in both LF power, as well as HF power during the stress phase[15]. Discrepancies in LF/HF ratio in some HRV stress studies suggests that the complexity of psychophysiological stress cannot be illustrated through one HRV index, implying the necessity for the inclusion of other physiological signal monitoring methods.

Numerous articles have presented the utilisation of electrodermal activity responses to stressors for the monitoring of psychophysiological stress, such as those by Branković et. al[89]. Similarly, Kim et.al. led two studies in the application of electrodermal responses for the discrimination between MDD patients and healthy volunteers undergoing stress tasks[108], [110]. Kim et. al. studies involved mental arithmetic tasks as the stressor and the measurement of skin conductance levels (SCLs) and skin conductance responses (SCRs) for stress evaluation. Feature extraction from the acquired physiological signals demonstrated the reduction in skin conductance as a response to stress in MDD patients, compared to their healthy control counterparts[132].

Comparatively, electroencephalographic studies in the field of psychological stress analysis have proven to be successful in the discrimination between depressed and non-depressed individuals. Notable EEG studies include Al-Shargie et al. 2018 EEG study involving electroencephalographic monitoring from the prefrontal cortex (PFC) on 18 healthy male participants undergoing a mental arithmetic task of 3 levels of difficulty[133]. The EEG frequency band of interest in this protocol was the alpha wave (8-14Hz), which demonstrated reduced power with increasing arithmetic difficulty levels[114], [133]. Furthermore, this phenomenon was also reported by Kan et.al. in 2015 whereby it was found that alpha waves in depressed and stressed subjects were significantly reduced, when compared to their healthy control counterparts [120]. In Kan's study, low alpha frequency was found in EEG measurements from the parietal, occipital and temporal lobes.

Evidently, the mentioned studies have primarily focused on the alpha EEG band which is one of the most common EEG signals of interest, in its relation to stress. Chandra's study on the neurophysiology of mental stress reported similar decreases in alpha band power with accumulating mental stress, as well as reductions in frontal brain asymmetry as stress tolerance increased[134]. This feature has given rise to the utilisation of power ratios in EEG stress studies to reflect the brain activity during stress assessment. Wen et.al. describes the use of alpha/beta ratio and theta/beta ratio in a protocol of 40 subject undergoing a virtual reality (VR) session for stress elicitation[135]. EEG monitoring and signal processing of the subjects' physiological signals led to the acquisition of the alpha, beta and theta frequency bands. Subsequent computation of power spectral density (PSD) of each frequency facilitated the calculation of absolute power ratios. The alpha/beta ratio was used to determine the

differences in absolute power between the baseline session and the stress task, whereas the beta/theta ratio signified the differences between the relaxed state and stress state[135]. These correspond with the known associations between the brain activity and frequency bands in the existing literature[111], [114].

Although, it should be noted that respective studies have narrated increases in all EEG frequency band powers upon stress activation[121]. However, it should also be noted that such studies often involved non-standardised stress tests and lacked affective stress evaluations, which may have led to discrepancies in stress elicitation and subsequent inconsistencies in the results.

3.5.1 Stress Studies and Considerations

The significance of standardised stress tests is mentioned throughout this comprehensive review. Numerous studies have shown promising findings and results in accordance with the existing literature, as well as some studies which have given contradictory results[103], [121], [136]. However, in both cases, the legitimacy of the findings of numerous studies is diminished by the lack of standardised stress testing. Standardised stress tests such as the Trier Social Stress Test (TSST) or the Mannheim Multicomponent Stress Test (MMST) are vastly known to reliably elicit stress responses. Therefore, the utilisation of such tests certifies that the subjects of the protocol experienced stress, which can be monitored through physiological signals. In the case of non-standardised stress testing, such as those by Avdeeva et.al., Billones et.al, Castaldo et.al and Ahrens et.al, the major findings of the protocols found inconsistencies with the existing literature[15], [121], [136]. This could have been caused by the elicitation of other responses through the supposed stress tasks, due to lack of standardisation and previous utilisation.

3.5.2 Multimodal Approaches to Stress Monitoring

A multimodal approach to physiological monitoring of stress and depressive symptoms may be the future of psychophysiological stress evaluation. The multimodal approaches to stress evaluation are highlighted in Table 5. Numerous preliminary studies have shown promising results in the use of multiple monitoring techniques for the evaluation of stress, or

discrimination between depressed and non-depressed individuals[126], [128]–[130], [137]. Notably, Reinhardt et.al's 2012 study on the effects of the Mannheim Multicomponent Stress Test (MMST) on salivary cortisol, electrodermal activity and heart rate demonstrated the synchrony in these physiological signals in the event of stress elicitation[129]. However, the article clarifies that two separate studies were conducted in conjunction to evaluate the stress response in the physiological signals of interest. Therefore, future elaborations within this field may involve the development of a multimodal structure to fuse the noteworthy features of interest from the physiological signals for a complete system, which evaluates psychological stress responses in the human body.

3.6 Conclusion

In conclusion, the prominence of physiological monitoring for the evaluation of psychological stress and major depressive disorder is inevitable. Further elaborations within multimodal systems could direct efforts away from the existing diagnostic and monitoring practices, towards a rejuvenated complete stress evaluation process which considers both the physiological elements of the stress response, as well as the psychological considerations. As point-of-care physiological monitoring techniques focus on the indirect measurements of the effects of stress on the sympathetic and parasympathetic nervous system, it can lead to the misinterpretation of conditions which may give rise to increased stress levels e.g. changes in the environment which do not correlate to acute psychological stress. Therefore, the investigation of biochemical biomarkers of stress is essential to understanding the dynamic mechanisms which take place in the human body upon activation of the stress response due to acute psychological stress.

4. Biomarkers for Depression

The findings reported in this chapter have been published in:

***Ahmed T,** Qassem M, Kyriacou PA**.** Measuring stress: a review of the current cortisol and dehydroepiandrosterone (DHEA) measurement techniques and considerations for the future of mental health monitoring, Stress, 26:1, 29-42. 2023*[80]

Mental health and specifically, clinical depression can be monitored using various techniques, which can be categorised into physiological monitoring and biochemical signal analysis. Biochemical biomarkers monitoring can facilitate the understanding of underlying neurobiological processes involved in several mental illnesses[138], such as clinical depression and bipolar disorder, as well as neurological diseases, for example, Alzheimer's disease and dementia[139]. The monitoring of specific biomarkers aids in the early detection and diagnosis of mental illnesses as well as simplifying the observation of illness progression [139].

Clinical depression and major depressive disorder (MDD) are both highly associated with endocrine and metabolic dynamics[34]. Intervention and deliberate influences on these factors often contribute to the treatment of this mental illness e.g., through use of antidepressants[34]. Therefore, the observation of the endocrinology and metabolic markers is essential for the comprehension of psychological stress, its relationship to depression and the progression and treatment of the debilitating psychiatric disorder[28].

Stress and clinical depression are strongly associated with endocrine and metabolic dynamics therefore, studying the endocrinology of stress is essential to understand psychological stress and its relationship with depression. Cortisol is the key logical indicator of stress and can be monitored for the neuroendocrinal analysis of mental health [34]. Cortisol is a glucocorticoid which is responsible for increasing glucose levels in the blood through the inhibition of insulin, which favours immediate use of glucose in response to stressors. Regular levels of salivary cortisol range from 0.7 to 27.3ng/ml, with highest levels present during mornings, as cortisol follows a diurnal rhythm [153]. Other stress hormones included adrenaline and noradrenaline. Adrenaline is strongly linked to HPA axis activity [28]. The release of adrenaline

is regulated by the sympathetic nervous system. It is associated with the manifestation of emotional distress and anxiety. Noradrenaline is a hormone and neurotransmitter which is responsible for the sleep-wake cycle as well as the fight or flight response alongside cortisol and adrenaline [201]. Noradrenaline is associated with behavioural changes as well as memory formation.

Interestingly, DHEA is a steroid hormone which possesses anti-glucocorticoid properties and is known to have an antagonistic relationship with cortisol. The cortisol-to-DHEA ratio is becoming a recognised metric for stress evaluation, with studies showcasing that the cortisol to DHEA ratio can be used as an objective measure of stress in depressed and healthy populations [141].

4.1 Cortisol

Cortisol is often considered the key logical indicator of stress, and, in many cases, depression is characterised by the stable and sustained elevation of cortisol levels[34], [140]. Hypercortisolaemia and reduction of the cortisol awakening response are characteristics of depression. These qualities are often monitored in the assessment of biomarkers of HPA axis activity and for neuroendocrinal analysis of depression and major depressive disorder.

Additionally, in studies where cortisol levels are monitored during treatment of first-episode psychosis, it was found that a decline in cortisol and cortisol/dehydroepiandrosterone sulphate (DHEAS) ratio directly correlated to an improvement in depressive and psychotic symptoms[141]. As the most common HPA biomarker associated with depression, cortisol and its relationship with dexamethasone suppression is considered a promising neuroendocrine marker for analysis of treatment response, albeit not robust for clinical applications[142].

The implication of cortisol level dynamics on the depressive symptoms of psychosis and other mental illnesses showcases that cortisol is a critical factor in the development of several mental illnesses, especially those involving depressive and negative symptoms[143]. Thus, cortisol can be considered as the ideal biomarker for analysis and clinical staging in psychiatry[138]. The dexamethasone suppression test is categorised as the most promising neuroendocrine marker for treatment response in depression, although is it not considered

robust for clinical applications[142]. In the dexamethasone test, post administration of dexamethasone resulting in non-suppression of cortisol translates to a lower likelihood of remission of the illness, post-treatment. Dysfunction in neuroendocrine hormone function is regularly associated with depression, specifically the dynamics of the hypothalamic pituitary adrenal (HPA) axis, is considered the primary contributing factor to the development of depression via endocrinal means[144]. Thus, the necessitation of further analysis of cortisol and a plethora of other potential biomarkers can facilitate greater comprehension of major depressive disorder and associated psychiatric illnesses[142].

4.1.1 Fundamental Metabolic Activity of Cortisol

Cortisol is the main endogenous glucocorticoid in humans and secreted primarily due to the hormonal cascade involving adrenocorticotropic hormone (ACTH)[34]. Other than involvement in the biological stress response to physical and emotional stressors, cortisol is also associated with several homeostatic maintenance actions such as blood pressure; immune responses and; protein, carbohydrate and, adiendopose metabolism[145]. By definition, a glucocorticoid is responsible for the increase in glucose levels in the blood[146]. In the case of cortisol, insulin production is inhibited to prevent glucose storage, favouring immediate use in response to stressors. Furthermore, cortisol and other glucocorticoids stimulate gluconeogenesis (the formation of glucose)[146]. Due to this metabolic action, repeated cortisol elevation can lead to weight gain and obesity, as triglycerides are mobilised from storage and relocated to visceral fat cells[147]. Furthermore, cortisol promotes the development of adipocytes into mature fat cells. Moreover, as cortisol increases glucose levels and supresses insulin, this can lead to overeating and eventual storage of unused glucose as body fat. Moreover, within the central nervous system, glucocorticoids increase cerebral excitability, predisposition to seizures and psychoses[146]. As previously mentioned, sleep disturbances are a common accompaniment to elevated glucocorticoid levels, as many patients with increased cortisol commonly suffer from insomnia[148].

HPA activity leads to the release of neurohormones, such as CRH into general circulation[34]. This triggers a hormonal cascade in which ACTH is released, which induces glucocorticoid synthesis and secretion of glucocorticoids into circulation. The central nervous system (CNS)

and the endocrine system are tightly interconnected to coordinate glucocorticoid activity[144]. After a stressful event activates the HPA axis, the increase of cortisol and other glucocorticoids facilitate the body's recovery from the stressor[149]. Cortisol regulates its secretion through a negative feedback mechanism involving the activation of the glucocorticoid receptor in the anterior pituitary gland. This mechanism is necessary to eliminate the HPA axis response to stress i.e., to aid the body's recovery from the stressor, as well as the maintenance of optimal levels of cortisol secretion in basal conditions[149].

Cortisol increases blood pressure as well as blood glucose levels due to induction of insulin resistance[150]. Furthermore, excessively high cortisol levels in the body often result in suppression of the immune system[36]. HPA axis dysfunctionality and dysregulation of the biological stress response system has been linked with risk of depression[151]. As the primary coordinator of the stress response, cortisol secretion patterns can indicate HPA axis dysfunction, in response to laboratory stressors and other interventions. Cortisol reactivity studies have shown that depressed individuals often have higher levels of cortisol during the recovery period post-stressor[152].

Commonly known as the 'hormonal endpoint' of the HPA axis, cortisol is primarily responsible for the body's reactions to stressors[11]. Regular levels of salivary cortisol range from 0.7-11ng/mL [153]. Salivary cortisol levels are known to follow a circadian rhythm, with morning cortisol levels reaching as high as 27.3ng/mL before declining throughout the day into the evening [153]. This phenomenon is known as the cortisol awakening response, comprising of an increase in cortisol peak values by at least 50% within 30 minutes post-awakening[154]. The rate of its secretion is dependent on the level of circulating corticotrophin, under extreme stimulating conditions, the level of cortisol in the human body can exceed 250mg a day, approximately an 125% of its typical level of 20mg [146]. In healthy and normal individuals, there are exceptionally low/undetectable levels of cortisol at midnight. Comparatively, disturbances in this circadian rhythm are highly prevalent in individuals with depression[148]. In normal individuals, there is a decline in mood in the evening, compared with the morning whereas in depressed individuals, there are mood improvements in the evening, which is associated with increased dorsal neural network activity. Sustained activity in the brainstem and hypothalamus involved with the sleep/wake cycle and, increased brain glucose metabolism is also observed throughout the day, which is reversed in healthy

individuals[148]. Due to these disturbances in the cortisol circadian rhythm in depressed individuals, irregularities in sleeping patterns and the sleep/wake cycle are commonly observed. 90% of depressed patients have complained about difficulties with sleeping, staying asleep and early morning awakening, compared to only 6% with complaints about hypersomnia[155], [156]. Thus, cortisol monitoring can simplify the comprehension of the circadian rhythms and chemical balances of depressed individuals compared to healthy subjects.

4.1.2 The Chemical and Structural Properties of Cortisol

Originally discovered in the 1930s by Edward Kendall as Compound F, cortisol and its synthetic form, hydrocortisone have similar actions within the body[146]. Hydrocortisone has an absorption rate of 95% per dose, leading to a peak 1-2 hours post-ingestion, through blood tests[157]. The substance remains in the blood for approximately 6 hours, with a half-life of 80 minutes. However, the duration of action of cortisol is considerably longer (8-12 hours) and the absorption rate and half-life can vary amongst individuals.

Cortisol exists within the blood in two forms, bound and free. Bound cortisol is attached to cortisol binding globulin (CBG) or albumin, whereas free cortisol is not attached to proteins[158]. Through blood tests, both levels are measured to calculate the total plasma cortisol concentration. Free unbound cortisol is considered the active form and can be transported between cells through the cortisol receptor. Upon attachment to the receptor, the complex travels to the cell nucleus for attachment to the glucocorticoid response elements, which are responsible for gene expression and protein synthesis. Hydrocortisone is often used as a steroid medicine to treat several health issues including contact dermatitis, mouth ulcers, painful joints and for hormone replacement for natural cortisol i.e., for treatment of Addison's disease[159]. Hydrocortisone effectively suppresses the body's immune response to reduce pain, swelling and itching. With the molecular formula $C_{21}H_{30}O_5$, hydrocortisone or cortisol is the primary glucocorticoid of the adrenal gland with minor mineralocorticoid effects[160]. Hydrocortisone is applied via injection or topically to treat for inflammation and allergies, collagen diseases, asthma and shock[159].

When cortisol enters circulation, it binds to cytosolic glucocorticoid receptors to form receptor-ligand complexes which translocate to the cell nucleus to bind to glucocorticoid response elements (GRE)[149], [160]. The DNA-bound receptor interacts with transcription factors to increase targeted gene expression to suppress the products of inflammation (prostaglandins and leukotrienes). Furthermore, cortisol stimulates the release of lipocortin-1 into the extracellular space (ECS) for binding to leukocyte membrane receptors for the inhibition of several inflammatory events e.g., chemotaxis, phagocytosis etc. Furthermore, the decrease in functionality of the lymphatic system and interferences with antigen-antibody binding are other effects of glucocorticoids for the suppression of the immune system. Cortisol and its synthetic counterpart hydrocortisone have a molecular weight of 362.5g/mol and has a solid physical state[160]. Hydrocortisone is an odourless, white crystalline powder with a bitter taste and a melting point of 220 °C. It is sensitive to light and very stable at room temperature except in the present of alkalis or strong acids. The chemical structure of cortisol can be seen in figure 2, and the IR spectrum in figure 2[160].

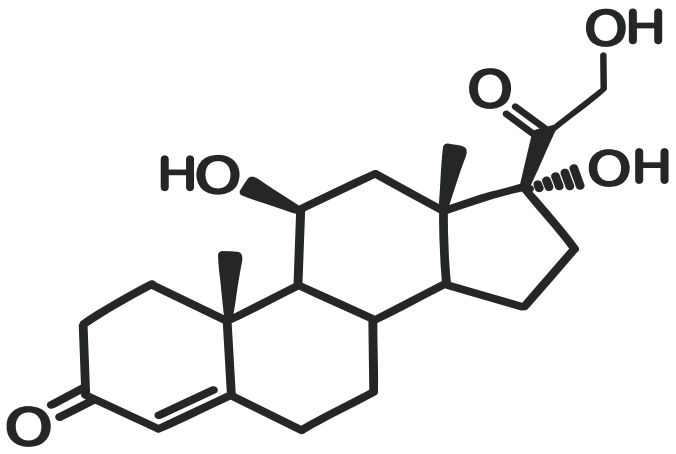

Figure 2 – 2D Chemical Structure of Cortisol.

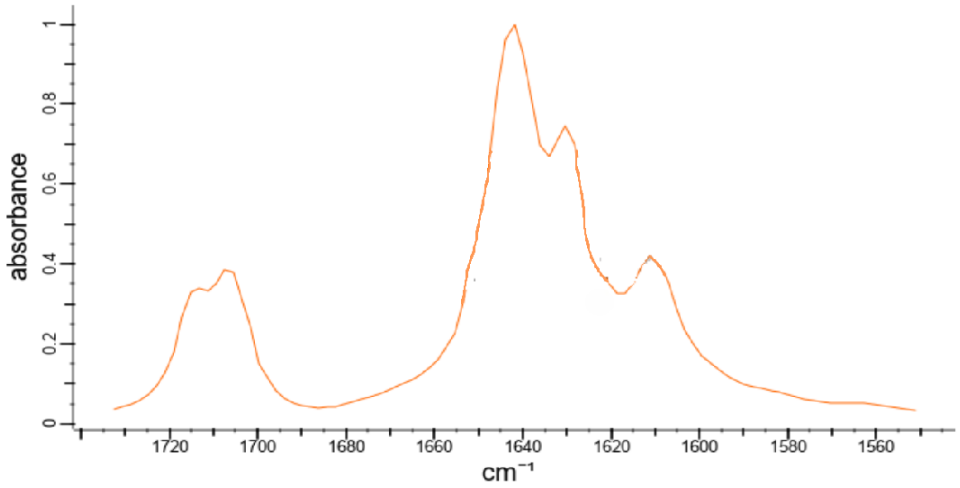

Figure 3 - ATR-IR Spectrum of Cortisol.

4.2 Oxytocin

Oxytocin (OXT) is a cyclic nonapeptide hormone and neurotransmitter which is responsible for social bonding, sexual reproduction and childbirth[161]. Additionally, OXT is the principal uterine contracting and milk-ejecting hormone of the posterior pituitary gland. Synthesised primarily in the hypothalamus alongside vasopressin, the nonapeptide hormones influence social cognition and behaviour[162]. OXT is considered an oxytocic, whereby it indirectly stimulates uterine smooth-muscle contraction and facilitates childbirth. Furthermore, OXT stimulates smooth muscles in mammary glands to induce lactation in mothers, whilst promoting strong social bonding and attachment between mothers and their babies[163].

OXT is recognised for its stimulation of anti-stress effects such as blood pressure reduction or lowering of cortisol levels, as well as increasing of pain thresholds[162]. Due to its association and long-lasting effects on the activity of transmitter systems, it is considered a clinically significant biomarker for the biochemical analysis of clinical depression. As several psychological mechanisms trigger the release of oxytocin, the analysis of endogenous oxytocin in individuals may elucidate to the positive effects of psychotherapy e.g., cognitive behavioural therapy (CBT) and social interventions. Thereby, facilitating the comprehension of the relationship between oxytocin and psychological stressors, and supporting the

conception of the underlying neurobiological processes which often progress into clinical depression.

4.2.1 Fundamental Metabolic Activity of Oxytocin

Oxytocin is primarily synthesised in the parvocellular neurones of the paraventricular nucleus (PVN) and the magnocellular neurons of the PVN and supraoptic nucleus, within the hypothalamus[31]. In humans, OXT can be considered anorectic, whereby an increased level of OXT leads to appetite suppression and decreased food intake. Due to this, weight loss is often observed in response to OXT. Labour and infant suckling are well-established stimuli for secretion of OXT however, other effective stimuli include exercise, sexual stimulation and stress[163], [164]. Due to its primary functions in parturition and lactation, OXT receptors are in the uterus and mammary glands. Oxytocin is considered a system activator due to its influence of the release of other signalling substances e.g., serotonin, dopamine and noradrenaline, which consequently leads to different behavioural and psychological effects.

Systemic oxytocin release impacts several psychological and physiological adaptations, often observed in breastfeeding mothers[163]. These adaptations include increased social interaction; couple bonding; decreased anxiety, cortisol levels and blood pressure; and, increased gastrointestinal tract functions[165].Due to these observations, it has been strongly theorised that systemic oxytocin release into circulatory blood is accompanied by OXT release into the brain in parallel. The relationship that exists between oxytocin and cortisol is highly significant, with regards to mental health studies. The inhibitory effects of OXT on the HPA axis occur through counteraction in the noradrenergic pathways[166]. This is observed as decreased functionality at each level of the cascade involved in cortisol release, which defines the role of oxytocin in decreasing stress levels. Oxytocin-linked stress diminution is frequently facilitated by several interventions e.g., skin exposure to touch, warmth and light pressure[163]. In rats, noradrenergic neurons which originate from the locus coeruleus (LC) and the nucleus solitarius (NTS) of the brainstem strongly influence HPA axis activity, specifically through stimulation of the CRF and activity of the sympathetic nervous system[166]. Once oxytocin is administered or released within these pathways, it is terminated in the LC and NTS, which consequently increases functionality of inhibitory alpha-2 adrenoreceptors on noradrenergic neurons[166]. Thus, oxytocin administration on

noradrenergic neurons suppresses LC responses to environmental and internal stimuli, which can account for the anti-stress-like effects of the neurotransmitter and hormone.

Additionally, oxytocin's stimulation of significant pathway within the parasympathetic nervous system e.g., the vagal nerve can lead to several physiological adaptations such as increased digestive, anabolic and restorative actions[167]. Furthermore, through promoting wound healing and increased plasma levels of growth factors, oxytocin has been perceived as the growth and relaxation hormone. Through repeated administration of oxytocin, several sustained or long-term effects can be observed. These include decreased blood pressure; pulse rate and corticosterone in rats (equivalent to cortisol). Chronic effects also include lowered basal levels of gastrointestinal hormones such as gastrin and insulin, as well as behavioural changes e.g., hostility in mothers when their offspring is threatened[168]. In humans, oxytocin administration facilitates labour initiation and reduces bleeding after birth[167]. Also, intranasal oxytocin may be administered to promote social bonding and decrease stress and anxiety, with potential opportunities for treatment of autism, schizophrenia and clinical depression[169].

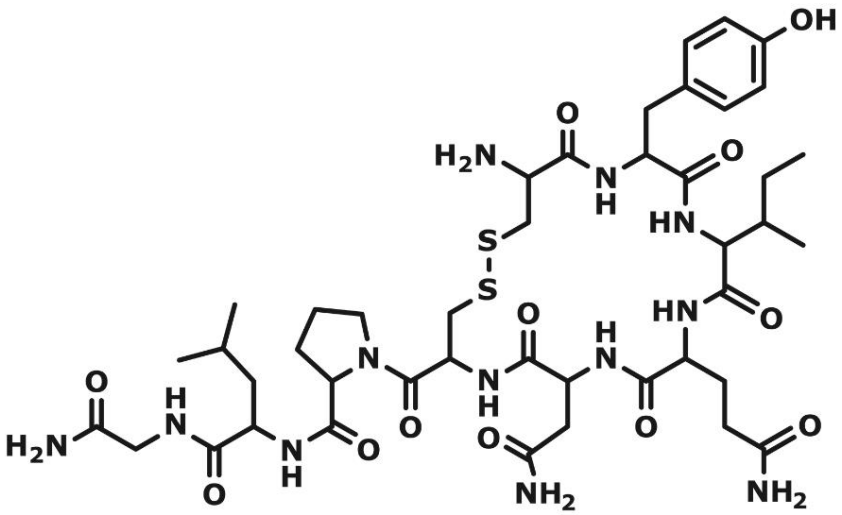

Figure 4 - 2D Chemical Structure of Oxytocin.

4.2.2 The Chemical and Structural Properties of Oxytocin

Notably, oxytocin secretion or administration can have various effects on the human body, dependent on the structural formation of the nonapeptide[167]. In its intact cyclic molecule state, oxytocin has been observed in initiation of social interaction and the associated psychophysiological effects. Alternatively, linear OXT and its fragments induce relaxation and anti-stress-like effects. Linear fragments with exposed cysteine-residue have suggested involvement in anti-inflammatory and antioxidant effects[167]. Oxytocin consists of a cyclic part with a disulphide bond and a chain of three amino acids. The release of oxytocin is under complex control, such that a multitude of factors influence its release into the brain or systemic circulation e.g., other neurotransmitters, hormones, and sensory stimuli. The OXT receptor is a G-protein coupled receptor which is present in uterine muscle tissue, the brain as well as other peripheral tissues. In humans, the oxytocin receptors are connected to different G-proteins which have different secondary messengers, thus leading to different effects[170].

Originally discovered in 1906 by Henry Dale, oxytocin was first noted for its stimulation of uterine muscle and myoepithelial cells of the mammary gland, leading to its establishment as a hormone involved in labour and lactation[167]. The cyclic structure and amino acid chain of oxytocin was determined in the 1950s by Du Vignaud. Once oxytocin binds to receptors, the G-protein coupled receptor signal transduction cascade is triggered such that intracellular calcium concentrations are increased[161]. The increase in calcium concentrations activate myosin light chain kinase which induces the formation of actomyosin, a contractile protein. This mechanism is responsible for uterine smooth muscle contractions, as well as mammary gland muscle stimulation which is crucial for lactation and milk let-down[161], [163]. With a molecular formula of $C_{43}H_{66}N_{12}O_{12}S_2$, molecular weight of 1007.2g/mol and, an amino acid sequence of CYIQNCPLG, the nonapeptide and neurotransmitter has several roles within the human body, often accompanied by vasopressin. The chemical structure of oxytocin can be seen in figure 4. Experimentally, the cyclic synthetic form of oxytocin, recombinant oxytocin is a white powder, which is soluble in water and thermally decomposes into toxic fumes of sulphur oxide and nitric oxide. Naturally, alongside vasopressin, oxytocin is distributed throughout the extracellular fluid (ECF) and is destroyed by chymotrypsin in the gastrointestinal tract. Oxytocin administration triggers uterine contractions almost

immediately, which subside within an hour[161]. Whereas intranasal application leads to contraction of myoepithelial tissues surround the breasts within 3-5 minutes, which subside after 20 minutes. Oxytocin has a plasma half-life of 3-5 minutes, although it can have several sustained and long-term effects on the body upon repeated administration[167].

4.3 Serotonin

Serotonin or 5-hydroxytryptamine (5-HT) is a monoamine neurotransmitter with a multitude of functions and roles within the body including modulation of mood, learning, memory, cognition and, physiological processes such as vasoconstriction[171]. Neurotransmitters are chemical messengers responsible for signal transmission and modulation of the central and sympathetic nervous systems[172]. Serotonin is found in the nervous system of humans and animals, as well as non-neuronal tissues, such as the GI tract, in blood platelets, and the cardiovascular system. As a derivative of L-tryptophan, serotonin possesses seven serotonin-receptor families with various subtypes, facilitating its responsibilities in control and regulation of several physiological and behavioural pathways. 15 serotonin receptors exist, which are involved in several processes within the brain as well as the body[173]. Consequently, serotonin is involved in the regulation of several biological processes including bowel motility, ejaculatory latency, and bladder control. Although, its relevance in neuropsychological processes is unprecedented. The regulation of serotonin levels and maintenance of the serotonergic system is essential, as the dysregulation of this system has implications in the pathogenesis of several psychiatric disorders, such as major depressive disorder, psychosis, bipolar disorder, and schizophrenia.

As a key component in the modulation of emotional states, the presence of serotonin in human saliva can be a significant factor in analysis of mental health stability in individuals[174]. Studies have shown the association between salivary serotonin and the recovery rate of depressive symptoms in patients suffering from clinical depression post-treatment, suggesting the importance of salivary serotonin analysis for the evaluation of serotonergic functionality[175]. Additionally, the circadian rhythm in salivary serotonin levels can be indicative of psychological well-being and the severity of clinical depression in patients,

further supporting the significance of serotonin as a biomarker in mental health studies to analyse the progression of major depressive disorder or clinical depression.

4.3.1 Fundamental Metabolic Activity of Serotonin

In the body, serotonin is involved in several regulatory processes for body temperature, blood pressure and smooth muscle functions in cardiovascular and gastrointestinal tissues. Furthermore, serotonin has significant roles in modulation of sleep, moods, appetite, and behavioural states such as aggression, attention, anger, and sexuality. Abnormalities in serotonergic brainstems or dysfunction of serotonin receptors can lead to aggression, depression, obsessive-compulsive disorder (OCD), migraines, anxiety, and borderline personality disorder. As serotonin is a derivative of tryptophan, fluctuations in tryptophan levels within the brain can implicate variations within serotonin synthesis[176]. Studies have demonstrated that manipulation of tryptophan levels can lead to low serotonin levels which can predispose subjects to mood and impulse control disorders. Behavioural effects associated with lower serotonin levels are often indicative of acute tryptophan depletion (ATD) including mood-lowering effects, increased irritability and, self-reported boredom[176].

For each behavioural process stated, there have been cases suggesting the presence of at least one specific brain region or nucleus which is critically responsible[173]. As the expression pattern of each serotonin receptor in the human brain is recognised, it insinuates that each behaviour is regulated by several serotonin receptors, which are expressed in multiple brain regions. For example, anxiety is regulated by 5-HT(1A) and 5-HT(2C) receptors, the latter is also involved in regulation of other behavioural processes such as reward processing, appetite, and energy balance. This further supports the concept of one-to-many mapping between serotonin receptors and behavioural processes[173]. Additionally, serotonin acts as a neuromodulator for nociceptive processing within the central and peripheral nervous systems, with varying effects dependent on the 5-HT receptor and anatomical location of action[173]. Receptors in the CNS have been associated with pain inhibition, whereas studies on peripheral receptors have shown that 5-HT release can increase inflammatory response and facilitate nociceptive effects[177]. Studies have shown that individuals suffering from

serotonergic abnormalities and its associated mood disorders often have heightened pain perception, due to altered serotonin modulation in pain processing[173], [178]. This concept is further supported by the efficacy of serotoninergic drugs in the treatment of chronic pain disorders[179].

Furthermore, hypothalamic 5-HT(2C) receptors are involved in energy balance regulation and modulation of glucose homeostasis, suggesting serotonin's impactful role in regulating metabolic rate and temperature control in the body[173]. Moreover, the location of serotonin synthesis implicates its involvement in several metabolic and endocrinologic processes. For example, serotonin that has been synthesised in the mammary gland is essential for gland development, epithelial tight junctions and milk release[180]. Whereas liver serotonin is involved in promoting liver regeneration following volume loss[181]. Evidently, 95% of total body serotonin is released into the gut by intestinal enterochromaffin cells, signifying its integral role in regulation of digestion at multiple levels of the GI system[173]. Upon entering the GI tract, food is propelled via peristaltic waves to promote digestion. These waves, along with intestinal motility and pancreatic enzyme secretion are modulated by serotonin. Due to serotonin's importance within the GI system, alterations within serotonin levels can lead to an array of functional bowel disorders, such as irritable bowel syndrome (IBS). Moreover, excessive release of serotonin within the GI tract activates 5-HT (3) receptors on afferent vagal nerves, which supply brainstem vomiting centres, resulting in emetic effects.

4.3.2 The Chemical and Structural Properties of Serotonin

Serotonin was first discovered by Vittorio Erspamer in 1935 as enteramine, through studies regarding its constricting and contracting effects in the intestinal tracts of rabbits, molluscs and frogs[182]. Since its discovery, serotonin has been found in most human tissues as well as faeces, urine, blood, saliva and cerebrospinal fluid. Serotonin is the 5-hydroxy derivative of tryptamine, and is considered a human and mouse metabolite, as well as a neurotransmitter[183]. In humans, several action pathways including methadone, ethylmorphine, oxycodone and ropivacaine, are regulated by serotonin, which has a half-life of 11 hours. The biochemical messenger is found primarily in the central nervous system, gastrointestinal tract, and blood platelets, to conduct several physiological functions such as

neurotransmission, gastrointestinal motility and haemostasis respectively. As previously mentioned, serotonin is associated with several psychiatric diseases including schizophrenia and major depressive disorder, as well as neurodegenerative disorders such as Parkinson's disease[173]. Serotonin receptors are essential to conduct the multitude of functions of the primary amino compound. The 15 5-HT receptors can be classified into 7 families of G protein-coupled receptors, excluding the 5-HT(3) receptor, which is a ligand-gated ion channel[172], [173].

The molecular formula for serotonin is $C_{10}H_{12}N_2O$, and its chemical structure can be seen in figure 5. With a molecular weight of 176.21g/mol, a hydroxyl group at position 5 and an indole with an aminoethyl group at position 2[183]. It is found in a solid state, with a melting point of 167.5 °C, with slight solubility in water. Cellular locations of serotonin include the cytoplasm and extracellular fluid, whereas the tissue locations include:

- Adipose tissue
- Adrenal cortex
- Adrenal gland
- Adrenal medulla
- Bladder
- Brain
- CNS
- Epidermis
- Fibroblasts
- GI Tract
- Hypothalamus
- Intestine
- Kidney
- Muscle
- Neuron
- Pancreas
- Placenta
- Platelets

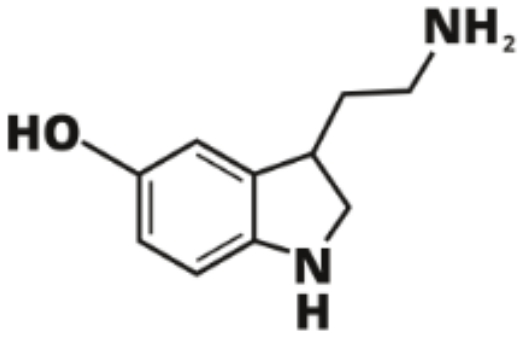

Figure 5 - 2D Chemical Structure of Serotonin.

4.4 Dopamine

Dopamine or 3,4-dihydroxyphenethylamine is a monoamine catecholamine neurotransmitter and hormone which controls several physiological functions within the brain and the body, through action with dopamine receptors[184]. There are 5 types of dopamine receptors, which are G protein-coupled receptors that are associated with the regulation of motor activity and neurological disorders, such as schizophrenia, bipolar disorder and attention-deficit/hyperactivity disorder (ADHD)[184]. Dopamine is produced in the substantia nigra, ventral tegmental area and the hypothalamus, and is involved in numerous pathways in the brain[185]. The main dopaminergic pathways include the mesolimbic and nigrostriatal pathways, which are both involved in reward-related cognition[186]. Dopamine plays a crucial role in the regulation of the reward pathway, including incentive salience (desire and wanting), positive reinforcement, aversion-related cognition, pleasure and other positive-valence emotions.

Evidently, cortisol is associated with attenuation of reward sensitivity, due to acute stress episodes causing disruptions to dopaminergic pathways related with reward cognition[187]. Furthermore, reward anticipation is an effective measure in activating reward pathways,

which leads to spikes in dopaminergic activity. Consequently, resilience against the effects of cortisol can be increased through anticipation of rewards e.g., monetary rewards or verbal positive reinforcement[187]. Therefore, the investigation of the relationship that exists between dopamine and cortisol, with regards to stress response and positive-valence emotions, is crucial for further comprehension of the connection between stress and psychiatric disorders, such as clinical depression. Deficits of dopaminergic systems have been demonstrated in cases of depression, however the relevance of dopaminergic systems and the modulatory effects they may have in individuals suffering from major depressive disorder have only recently been highlighted[188]. It is essential to derive the relationship between clinical depression and the role of dopaminergic systems within the psychiatric disorder, as well as its association with cortisol, the main stress hormone which has been observed in the attenuation of dopamine-controlled activities[187].

4.4.1 Fundamental Metabolic Activity of Dopamine

Dopamine is an adrenergic neurotransmitter which affects brain processes that regulate motor functions and emotional response[189]. Furthermore, within the vesicles of the adrenal medulla, dopamine acts as a hormone responsible for regulation of heart rate and blood pressure. Lower levels of dopamine in the brain can often be an implicative cause of Parkinson's disease, whereas high dopamine levels are strongly associated with schizophrenia[189]. As mentioned previously, dopamine is strongly associated with reward cognition and addiction, such that dopamine transmission within the brain increases significantly as a response to rewards or highly addictive drugs, such as cocaine. Roles of dopamine within the human body include modulation of behaviour and cognition; voluntary motor functions; motivation; punishment and reward; sleep and dreaming; attention; memory; and learning[185], [186]. Additionally, dopamine is a precursor for biosynthesis of other catecholamines, including adrenaline and noradrenaline[185]. Catalytic action of DA B-hydrolase in the presence of L-ascorbic acid and oxygen facilitates noradrenaline synthesis. Upon action of the enzyme phenylethanolamine *N*-methyltransferase and the cofactor *S*-adenosyl-L-methionine (SAMe), adrenaline is synthesised[185].

The physiological functions of dopamine within the brain and body are conducted through dopamine receptors[184], [190]. There exist two main types of dopamine receptors: D1-type (includes D1 and D5 receptors), and D2-type (includes D2, D3 and D4 receptors); these groupings are based on structural and functional similarities between receptors. D1-type receptors are involved in post-synaptic inhibition and ultimately open and close sodium and potassium channels respectively. D2-type receptors are involved in both pre- and post-synaptic inhibition, where the ultimate effect involves the inhibition of target neurons. Specifically, D2 receptors are involved with the regulation of mood, emotional stability in the limbic system and, movement control in the basal ganglia[190].

The dopaminergic system in the brain is regulated through four main pathways which include the nigro-striatal, mesolimbic, mesocortical and tuberoinfundibular pathways, which are involved with the regulation of motor controls, reward cognition, emotional behaviour and, prolactin release inhibition respectively[190]. Dopamine in the nigro-striatal pathway controls voluntary movement and the learning of new motor skills, the degeneration of this pathway can lead to Parkinson's disease[184], [190]. Alternatively, mesolimbic dopamine mediates pleasure and other positive-valence emotions in the brain, through release during pleasurable situations. Due to this, the mesolimbic pathway is highly associated with addictions. Also, antipsychotic drugs for the treatment of schizophrenia target the dopamine receptors involved within this pathway[190]. Significantly, dysfunctionality within the mesolimbic pathway and D1 receptor may be indicative of mood disorders such as clinical depression[190], [191]. Moreover, mesocortical dopamine controls cognitive and emotional behaviour, which includes fluctuating dopamine levels to assist in improving memory or attention[192]. Consequently, dysfunctionality within this pathway can contribute to issues with memory and neurological disorders such as ADHD[184], [190]. The use of antipsychotic drug treatment for schizophrenia on mesocortical dopamine receptors can induce worsened negative symptoms[190]. Dopamine in the tuberoinfundibular pathway inhibits prolactin release, which is pituitary gland hormone that facilitates lactation in mothers after childbirth[185].

4.4.2 The Chemical and Structural Properties of Dopamine

Discovered in 1957 by Avid Carlsson as a neurotransmitter, dopamine is a monoamine with positive inotropic activity[193]. Dopamine, or 3-hydroxytyramine, is a catecholamine formed from the decarboxylation of dehydroxyphenylalanine (Figure 6) [194]. The compound binds to alpha-1 and beta-1 adrenergic receptors, thus facilitating the increase of heart rate and force, and consequent cardiac output. Dopamine is found throughout most human tissues, and can be detected in faeces, urine, blood and cerebrospinal fluid (CSF). Cellularly, dopamine is found in the cytoplasm and myelin sheath of neurons. Dopamine is naturally found in solid state; it is slightly soluble in water and weakly acidic[194].

Additionally, with regards to drug treatments, dopamine is administered for the correction of haemodynamic imbalances as a result of myocardial infarction, trauma, open-heart surgery, renal failure and chronic cardiac decompensation[194]. Dopamine has a plasma half-life of approximately 2 minutes. As previously mentioned, most physiological and biochemical actions of dopamine require involvement of dopamine receptors. Dopamine receptors were first discovered in the CNS in 1972, in studies associating dopamine to the stimulation of adenylyl cyclase (AC)[195]. The two types of dopamine receptors that were classified were D1 and D2, based on their ability to stimulate AC or remain ineffective. Subsequent studies led to the confirmation that D1-type and D2-type receptors differed completely with regards to pharmacological activity, biochemical composition and physiological action, although they shared similar structural characteristics[195].

Dopamine is a catechol, wherein structurally the compound comprises a benzene ring with two hydroxyl side groups. Additionally, dopamine has a 2-aminoethyl group which substitutes hydrogen at position 4 of the benzene ring[194]. Dopamine is the simplest catecholamine and is a precursor in the biosynthesis of more complex neurotransmitters, such as adrenaline and noradrenaline[185]. The molecular formula of dopamine is $C_8H_{11}NO_2$, and the structural depiction can be seen in figure 5. The IUPAC name for dopamine is 4-(2-aminoethyl) benzene-1,2-diol. Moreover, dopamine has a molecular weight of 153.18g/mol, with a boiling point of 227 °C, and a melting point of 128.0 °C[194]. Dopamine is freely soluble in water, methanol and 95% ethanol. It is sensitive to oxygen and discolours rapidly upon oxidisation. Thermal decomposition of dopamine emits toxic nitrous oxide fumes, also the substance is highly unstable in alkaline media.

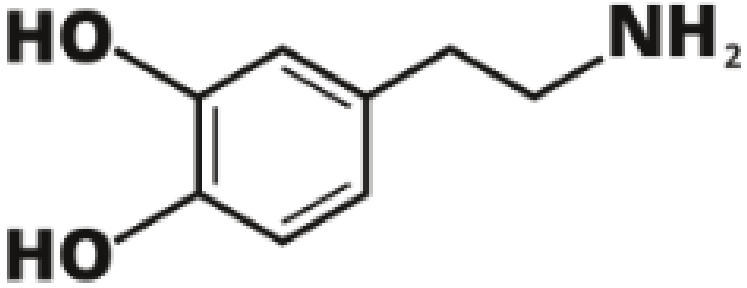

Figure 6 - 2D Chemical Structure of Dopamine.

4.5 Adrenaline

Adrenaline is a hormone and neurotransmitter which is primarily produced in the adrenal glands, as well as the neurons of the medulla oblongata[196]. Adrenaline is a catecholamine which is secreted via the adrenal medulla; it is a major determinant to metabolic or global disruptions to homeostasis. Adrenaline, alongside noradrenaline, is responsible for several visceral functions within the autonomic nervous system, such as regulation of heart rate and respiratory rate. Catecholamines, like adrenaline, and glucocorticoids (e.g., cortisol) are the two main constituents of the endocrinological response to stressors[28]. Adrenaline and noradrenaline are primarily responsible for the orchestration of the 'fight or flight' response upon detection of a stressor/disruption to homeostasis[33].

4.5.1 Fundamental Metabolic Activity of Adrenaline

Evidently, adrenaline responses to stressors are more strongly linked to HPA axis activity, instead of sympathetic nervous system responses to stressors[196]. Adrenaline's roles during the 'fight or flight' response is conducted through its action with alpha- and beta-adrenoceptors[196]. Adrenoceptors, or adrenaline receptors are present in effector cell membranes and can modulate physiological and metabolic effects of the catecholamines.

Upon stimulation of alpha-adrenoreceptors, vasoconstriction occurs in the arteries, whereby increasing blood pressure. Alternatively, beta-adrenoreceptors are stimulated in skeletal muscle to induce vasodilation. Additionally, stimulation of beta receptors affects the rate and force of the heartbeat.

Furthermore, adrenaline is a key component in the manifestation of emotional distress, alongside behavioural and autonomic inputs[197]. The release of adrenaline from the adrenal medulla is considered the adrenomedullary response to stress, which is regulated by the sympathetic nervous system. Studies have shown that the presence of adrenaline facilitates the encoding of emotionally arousing situations, contributing to higher levels of arousal due to fear[197]. Fear is the most associated emotion to adrenaline, due to its definitive result with the hormone in comparison to other emotions, which have not shown distinctive results. Historically, the relationship between anger and fear, and the action of adrenaline have been highlighted significantly, as well as the connection between the effects of adrenaline on the anxiety state[198]. The emotion response upon adrenaline administration is commonly observed and the sensitivity to adrenaline is characteristically higher in individuals suffering from anxiety, chronic stress or generalised anxiety disorder[198]. Once administered, adrenaline causes an initial rise and secondary prolonged drop in blood glucose levels, the latter of which is considered to be the primary cause of nervous symptoms of anxiety[198]. Due to its influence on anxiety symptoms and the stress response in humans, the study of adrenaline with regards to psychiatric disorders such as clinical depression is highly significant for the comprehension of the biochemical processes involved within the disorder[48].

4.5.2 The Chemical and Structural Properties of Adrenaline

Discovered in 1895 by Napoleon Cybulski, adrenaline or epinephrine is a naturally occurring sympathomimetic amine and catechol with vasoconstriction and bronchodilation properties, which are conducted through stimulation of alpha- and beta-adrenoreceptors respectively[196], [199]. As a therapeutic drug, adrenaline is used in treatment for anaphylaxis, cardiac arrest and septic shock. Predominantly, adrenaline is found in most human tissues as well as biofluids, such as urine, blood, and cerebrospinal fluid. Cellularly, adrenaline exists within the cell cytoplasm or myelin sheaths of neurons.

The biosynthesis of adrenaline occurs through enzymatic reactions with noradrenaline, involving the enzyme phenyl ethanolamine *N*-methyltransferase and the cofactor *S*-adenosyl-L-methionine (SAMe)[185]. Structurally, adrenaline is a catechol i.e., the compound contains a 1,2-benzenediol moiety. The molecular formula for adrenaline is $C_9H_{13}NO_3$, the structural depiction of the catecholamine can be seen in Figure 7[200]. Moreover, adrenaline exists in the solid state as an off-white, odourless, microcrystalline powder which is a weakly acidic compound and, soluble in water. IR spectrum of adrenaline can be seen in Figure 8. The IUPAC name for adrenaline is 4-[(1R)-1-hydroxy-2-(methylamino)ethyl]benzene-1,2-diol and it has a molecular weight of 183.2g/mol. Experimentally, adrenaline has a melting point of 211 °C and a boiling point of 215 °C[200]. Upon thermal decomposition, toxic fumes of nitrous oxide are emitted. The plasma half-life of the hormone and neurotransmitter is approximately 2-3 minutes. However, due to its vasoconstrictive effects, the complete absorption of adrenaline can be slightly delayed, resulting in prolonged effects.

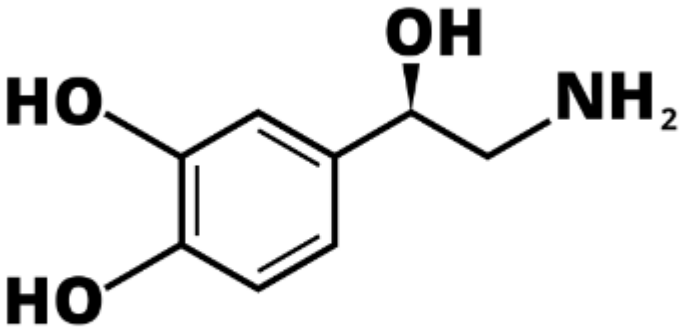

Figure 7 - 2D Chemical Structure of Adrenaline.

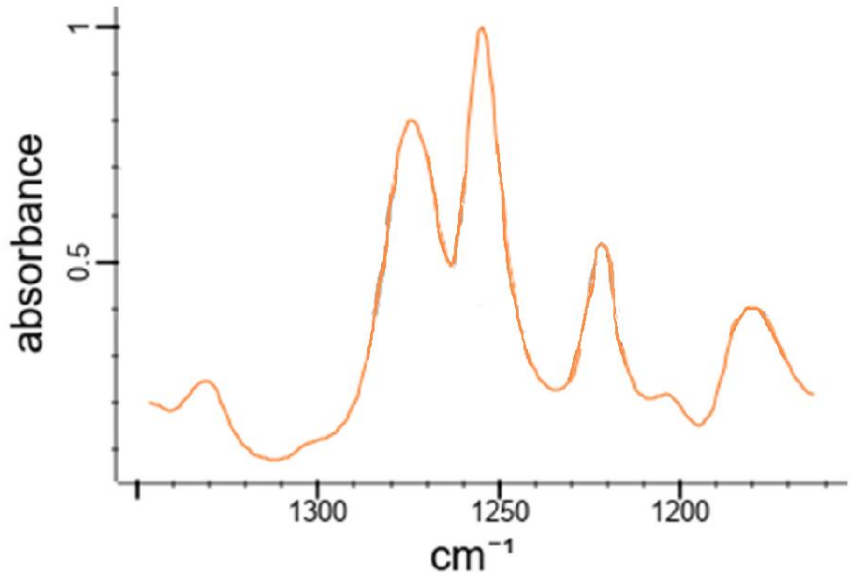

Figure 8 - ATR-IR Spectrum of Adrenaline.

4.6 Noradrenaline

Similarly, noradrenaline (NA) is also a hormone and neurotransmitter which is synthesised in the adrenal medulla[196]. As a member of the catecholamine family alongside adrenaline and dopamine, NA has several functions and responsibilities within the brain and body. NA is responsible for the regulation of the sleep-wake cycle and to orchestrate several physiological processes in conjunction with adrenaline and cortisol for the 'fight or flight' response to stressors[201]. Additionally, noradrenaline is involved in the maintenance of visceral functions in the body, as well as influence of several behavioural changes within the brain. As a sympathetic neurotransmitter, NA is able to induce changes such as increased arousal and alertness, as well as promote memory formation and retrieval[9]. Furthermore, within the body NA maintains the tonic level of cardiovascular performance and reflexive changes in cardiovascular tone, at rest[196].

4.6.1 Fundamental Metabolic Activity of Noradrenaline

As previously mentioned in chapter 2, NA has several physiological and neurological functions within the body and brain as a hormone and neurotransmitter respectively. Comparably alongside adrenaline, the functionality of NA is conducted through action with noradrenaline receptors[196]. NA receptors are classified as alpha- and beta-adrenergic receptors which function as G-protein coupled receptors. As G-protein coupled receptors, alpha and beta receptors exert their effects via a complex second messenger system, which triggers physiological changes at a cellular level[202]. Specifically, through stimulation of alpha-adrenergic receptors, vasoconstrictive effects are induced in several anatomical locations such as the radial smooth muscles of the iris, arteries, veins, urinary bladder and gastrointestinal tract sphincter[203]. Alternatively, stimulation of beta-1-adrenergic receptors leads to increased myocardial contractility, heart rate and atrioventricular (AV) conduction. Moreover beta-2-adrenergic receptor stimulation results in bronchodilation[203].

Furthermore, the role of noradrenaline and alpha-adrenergic receptors in the pathophysiology of major depressive disorder is highly significant[204], [205]. Studies have shown that NA activity and subsequent alpha-adrenergic receptor modulation can potentially influence symptoms of major depressive disorder (MDD), such as depressed mood, diminished ability to concentrate, disruptions to sleep and circadian rhythms and, immune responses[17], [204], [205]. As NA is strongly associated with the regulation and modulation of the systems responsible for these systems within the brain, the relationship between NA and MDD is evident. Additionally, the use of selective NA reuptake inhibitors and other pharmacological drugs which act on NA levels for the treatment of MDD suggests the importance of functional levels of NA for mental and biochemical stability in depressed individuals[204]. Many pharmacological treatments involve the blocking of alpha-adrenergic receptors to improve negative effects of MDD. Additionally, withdrawal from NA antidepressant treatment and experimental depletion of NA can result in the return of depressive symptoms in individuals suffering from clinical depression[205].

4.6.2 The Chemical and Structural Properties of Noradrenaline

Noradrenaline, or norepinephrine, was discovered in 1947 by Ulf von Euler as a neurotransmitter in the sympathetic nervous system, as well as a precursor in the biosynthesis of adrenaline[206]. Since its discovery, the functionality and effects of NA have been investigated to showcase the plethora of roles that the neurotransmitter and hormone holds in the brain and body. NA belongs to the catechol family, due to the presence of a 1,2-benzenediol moiety. NA can be found in most human tissues and biofluids, including urine, blood, saliva and cerebrospinal fluid; it has a half-life of approximately 2.5 minutes[203], [207]. Noradrenaline can be synthesised from its precursor dopamine through catalytic action of DA beta-hydroxylase.

The molecular formula for noradrenaline is $C_8H_{11}NO_3$, with a molecular weight of 169.18g/mol[203]. The IUPAC name for noradrenaline is 4-[(1*R*)-2-amino-1-hydroxyethyl] benzene-1,2-diol and the structural depiction of the catecholamine can be seen in figure 9. NA exists in solid state as colourless microcrystals with a melting point of 217 °C. It is slightly soluble in water, ethanol, and very soluble in alkali and dilute hydrochloric acid. Upon thermal decomposition, it emits nitrous oxide fumes, which is similar to dopamine and adrenaline.

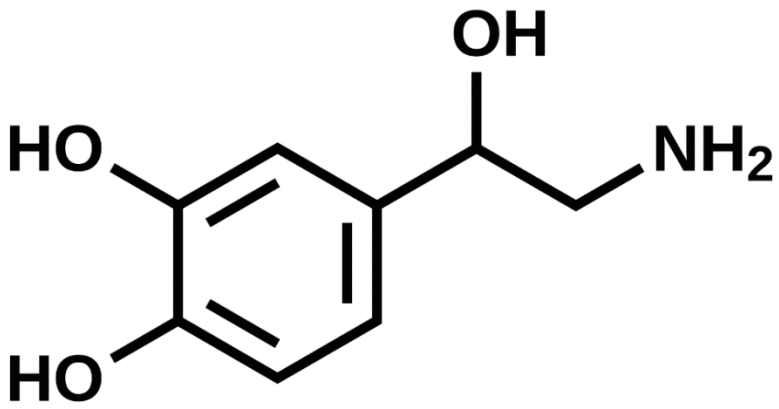

Figure 9 - 2D Chemical Structure of Noradrenaline.

4.7 Dehydroepiandrosterone (DHEA)

Dehydroepiandrosterone (DHEA) and dehydroepiandrosterone sulphate (DHEAS) are steroid hormones that are regulated by the ACTH and possess anti-glucocorticoid properties[208]. DHEA is produced by the zona reticularis area in response to the ACTH. It has a regenerative role in the body, often associated with ageing[209], [210]. The primary function of DHEA is its involvement as a metabolic intermediate in sex hormone biosynthesis i.e., to produce androgen and oestrogen[211]. DHEA is known to improve physical well-being through reduction of total cholesterol, and prevention of bone mineral density. The steroid has an antagonistic relationship with cortisol, the primary stress hormone in humans[208]. This relationship can translate to reduced stress and improved psychological well-being. The cortisol-to-DHEA ratio has been considered as a precise method of assessing HPA axis functionality[208]. Several studies have shown an association between DHEA levels and stress intensity, as well as focusing on the cortisol/DHEA ratio. Although the magnitude of fluctuations in DHEA levels caused by stress is known to decrease with age[209]. As a well-established biomarker of acute stress, the metabolism of DHEA and its release patterns in the human body are of great interest in stress studies. Several studies have noted that DHEA levels often appear as a peak at the end of a stressful period, and progressively return to baseline levels after recovery from stress[209]. However, these factors are sex and age dependent therefore, it is imperative to delve deeper into the functionality of DHEA in the human body and the roles it plays within the stress response.

There are clear gender differences in circulating DHEAS levels, higher levels are found in men than in women, with peak levels around ages 25-30[209]. After this, there is an age-dependent decline in levels, which can also be influenced by drastic developmental changes. The reactivity to developmental changes in unique to DHEA secretion and not commonly observed for other steroid hormones. DHEA is a naturally occurring C-19 adrenal steroid synthesised by the adrenal cortex from cholesterol. The adrenal cortex secretes 75-90% if the body's DHEA with the remainder produced by the sex organs i.e., testes and ovaries[212]. Clinical studies have shown that DHEA secretion has several effects on the human body such as, reduced inflammation, improved sexual function, cognitive function and memory enhancement[213]. Furthermore, studies have shown that low DHEA and DHEAS levels are

associated ischemic heart disease, endothelial dysfunction, atherosclerosis as well as psychological distress[209], [213], [214].

4.7.1 Fundamental Metabolic Activity of DHEA

DHEA has several functions in the human body, often posing as precursors to more potent androgens and oestrogens. With regards to the endocrinological pathway of DHEA, it is released on a negative feedback cycle upon characterisation of a stress response. DHEA secretion is controlled by the ACTH and pituitary factors and synthesised by the adrenal cortex from cholesterol. 75-90% of DHEA biosynthesis and release occurs at the adrenal cortex, with the remainder being produced and released via the testes or ovaries[215].

Once produced by the adrenal glands it is taken up by tissues including the brain, kidney, liver and gonads where it is metabolised into 5-androstene-3β,17β-diol, 4-androsten-3,17-dione, oestrogen and testosterone[215]. DHEA has been proven to have antagonistic effects on glucocorticoids such as cortisol, deeming it a biomarker of interest for stress evaluation. Evidently, the anti-aging properties of DHEA are also highlighted in prominent studies through anti-dementia and anti-osteoporosis effects[216].

4.7.2 The Chemical and Structural Properties of DHEA

DHEA is a naturally occurring anabolic steroid produced primarily in the adrenal cortex as well as the testes and ovaries. Most secreted DHEA is released in its sulphated form as DHEA-S. The molecular formula for DHEA is $C_{19}H_{28}O_2$, with a molecular weight of 288.4m/mol[217]. The IUPAC name for DHEA is (3S,8R,9S,10R,13S,14S)-3-hydroxy-10,13-dimethyl-1,2,3,4,7,8,9,11,12,14,15,16-dodecahydrocyclopenta[a]phenanthren-17-one and the structural depiction of DHEA can be seen in Figure 10. DHEA exists in the solid state with a melting point of 140°C. The FTIR for DHEA, using the KBr wafer technique can be seen in Figure 11.

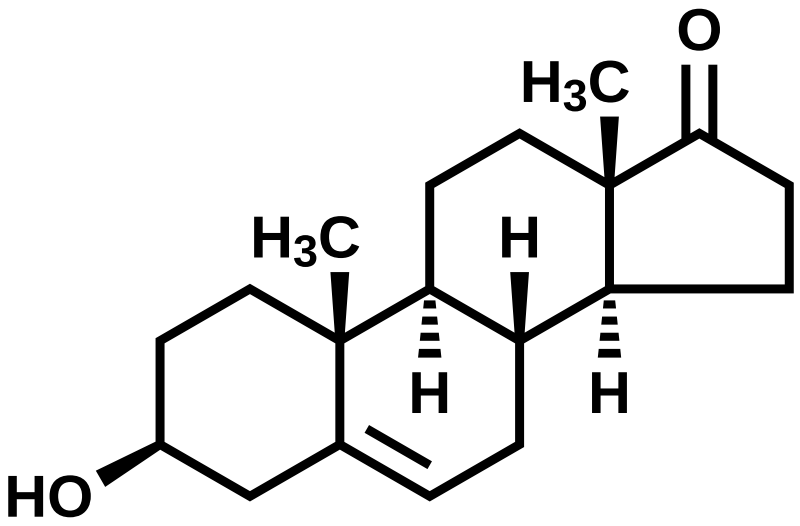

Figure 10 - 2D Chemical Structure of DHEA

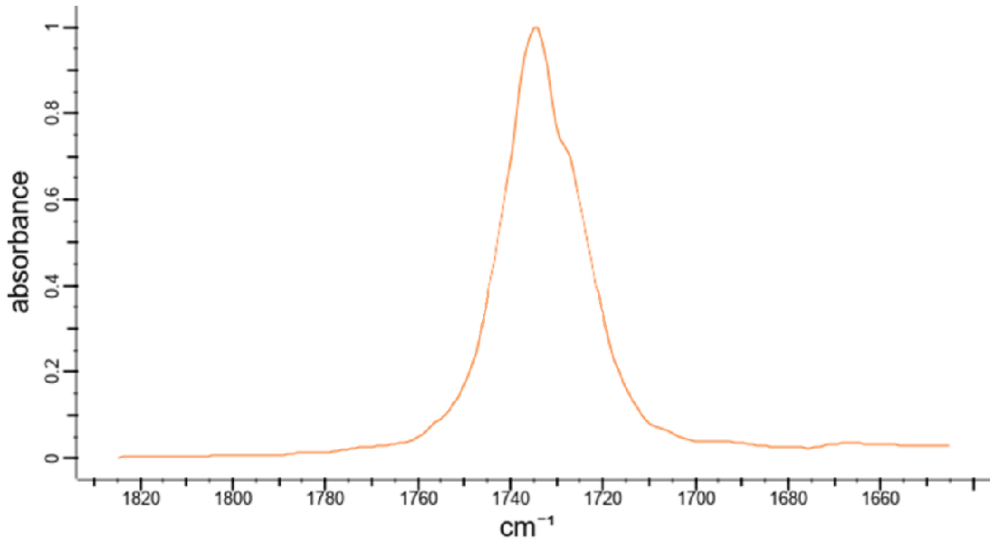

Figure 11 - FTIR Spectrum for DHEA, KBr Method

5. Salivary Hormone Analysis

The findings reported in this chapter have been published in:

***Ahmed T,** Qassem M, Kyriacou PA**. Measuring stress: a review of the current cortisol and dehydroepiandrosterone (DHEA) measurement techniques and considerations for the future of mental health monitoring, Stress, 26:1, 29-42. 2023*[80]

This chapter focuses on two key biomarkers which were previously mentioned in Chapter 4, cortisol and DHEA due to its significance in the characterisation of HPA axis functionality. The chapter highlights the antagonistic relationship between the two biomarkers and critically reviews the current state-of-the-art monitoring principles involved in measuring cortisol and DHEA for evaluation of stress and mental health.

5. 1 Introduction

Saliva is a mucoserous exocrine fluid that is clear and slightly acidic, with a pH range of 6.2-7.6[218]. The primary component of human saliva is water, which makes up 99% of the biofluid that is found abundantly in the oral cavity[219], [220]. Additionally, electrolytes, immunoglobins, proteins, enzymes and hormones can be found in saliva, albeit in small varying amounts[221]. The complex composition of saliva facilitates the multitude of functions of the biofluid, such as lubrication, buffering and remineralisation, as well as digestion and speech[222]. However, it should be noted that the chemical composition of saliva is known to change drastically in response to various physiological states and stimuli[220].

Analysis of salivary composition offers a non-invasive and straightforward approach for comprehensive studies within several fields of interest, especially psychiatry and stress research[223]. As whole human saliva is of a less varying composition to serum and requires non-invasive collection, it is considered far more desirable than other diagnostic fluids such as blood and urine[224]. The stress-induced increase in cortisol activity associated with blood sampling is avoided completely due to the non-invasive nature of saliva collection, reducing external factors that may affect investigations[225]. Furthermore, extensive research into salivary analysis has led to an abundance of developments in clinical diagnosis and monitoring, as it is widely accepted as a sample source for steroid and amine analysis, further

suggesting its significance in psychological stress studies[223], [224]. Both the primary glucocorticoid and catecholamines involved in the induction of the stress response are present in whole human saliva[224], [226]. Thus, salivary analysis is imperative for the understanding of the activity of cortisol, adrenaline and NA, and the interactions which are perceived as psychological stress in the human body.

Psychological stress is often associated with fluctuations of stress hormones, such as cortisol and adrenaline[11]. Cortisol is the primary stress hormone that is involved in governing the stress response from the moment of stress elicitation to recovery from stressful events. Alternatively, DHEA is a steroid hormone which has proven to express anti-glucocorticoid properties[208]. As two steroid hormones with inverse actions, the relationship between cortisol and DHEA is of great interest. The antagonistic relationship between cortisol and DHEA has been discussed at length in regard to their opposing actions on immune function, however, the relationship between cortisol and DHEA in stress management is seldom discussed[227]. DHEA is primarily implicated in aging research, whereby an increase in cortisol/DHEA ratio can be a contributing factor of age-related declination in immune function[227]. Evidently the biochemical interactions between cortisol and DHEA have proven to be of great importance in the determination of declining functions of biological systems. Therefore, observing the changes in these biomarkers with respect to stress and mental health monitoring could unveil vital details regarding stress management and mental health.

The key aims are to review the existing state-of-the-art technology comprehensively in the field of cortisol and dehydroepiandrosterone (DHEA) measurement and their applications within stress monitoring, whether it is in settings involving healthy participants or clinically depressed patients. Previous reviews within this field have focused primarily on the applications of cortisol monitoring for stress, albeit the importance of DHEA is seldom elaborated upon. Thereby, this review will consider the magnitude of DHEA in stress studies and discuss the strengths and shortcomings of measuring such stress biomarkers and whether it can impact the current perspective of mental health monitoring. The review will involve the evaluation of stress studies which focus on cortisol and DHEA measurements in various mediums, as well as its relationship with mental illnesses, primarily major depression. Stress studies include a broad set of studies involving either stress reactivity or chronic stress observations within adult populations. A broad set of stress studies were included to further

comprehension regarding the cortisol/DHEA ratio and its implications in different modalities of stress.

5.2 Enzyme-Linked Immunoassays (ELISAs)

Enzyme-linked immunoassays or ELISAs are labelled immunoassays, considered the gold-standard of immunoassays, as well as the gold-standard technique for salivary cortisol analysis[228]. ELISAs are commonly used for detection of analytes in saliva such as SARS-CoV-2 antibodies as they offer a highly specific and sensitive modality for the quantification of key analytes present in saliva, which can be collected non-invasively for further ease [229]. The purpose of the assay is to detect and quantify a variety of analytes, including hormones, antigens, antibodies, and proteins. ELISAs are commonly performed on 96-well plates. The four general steps to conduct an ELISA involves:

- Coating (with antigen or antibody)
- Blocking
- Detection
- Readout

There are 4 key types of ELISAs which include:

- Direct ELISA
- Indirect ELISA
- Sandwich ELISA
- Competitive ELISA

The direct and indirect ELISAs both involve the coating of the plates with an antigen of choice [230]. However, in a direct ELISA, the primary detection antibody directly binds to the analyte of interest whereas, in the indirect ELISA, two antibodies are involved i.e., a primary antibody for protein binding and a secondary enzyme-linked antibody which is complementary to the primary antibody. The indirect ELISA has higher sensitivity than the direct ELISA, although it is often more time-consuming and more expensive.

The sandwich ELISA begins with a capture antibody which is used to coat the plate. Once coating is complete, the plates are washed and blocked. The antigen of interest is

subsequently added to the plates to bind to the capture antibody, the antigen is 'sandwiched' between two layers of antibodies alluding to the name of the ELISA. After washing, the primary detection antibody is added to the plate, followed by the secondary enzyme-conjugated antibody. After incubation and washing steps, the substrate is added, and the colour change is observed. Although sandwich ELISAs offer the highest sensitivity amongst the immunoassays, they are often the most time consuming.

Finally, the competitive ELISA tests for the presence of an antibody which is specific for antigens in the test sample[231]. 2 antibodies (one enzyme conjugated and another present in the test sample) are added to the wells of the plate to allow for competition for antigen-binding. Upon processing of the ELISA, the presence of a colour change demonstrates a negative test i.e., the enzyme-conjugated antibody has bound to the antigens instead of the antibodies present in the test sample. Competitive ELISAs have relatively low specificity but can be used to measure for smaller antigens in a given sample. In studies that involve salivary sampling for stress biomarker determination, enzyme-linked immunosorbent assays (ELISA) are frequently used[234]. The technique used for ELISA involves the immobilisation of antigens (the target molecule) onto a microplate, which is then complexed with an antibody that is linked to an enzyme. Activity of the enzyme with a substrate leads to the creation of a product that is measured for accurate detection and quantification of macromolecules. Due to its ease of use, sensitivity and short analysis times, ELISA techniques have become the foundation for the development of pioneering point-of-care (POC) devices[234].

5.3 Diagnostic Applications of Saliva

Saliva has played a key role in molecular diagnostics, especially for the diagnosis of oral and systemic diseases, such as HIV and Alzheimer's disease[232], [233]. Primarily, oral sampling in the form of saliva testing is used for genomic profiling in commercial DNA analysis kits. Furthermore, oral sampling for quantification of steroid hormone levels in saliva is highly common[232]. Several studies have demonstrated the utilisation of saliva as a significant investigative tool within stress research, primarily focusing on the presence of salivary cortisol in periods of normal behavioural conditions versus stressed conditions. Predominantly, studies involving cortisol and catecholamine measurements employ immunoassay and

chromatographic methods. As saliva collection is considered non-invasive, saliva sampling studies are considered highly attractive in comparison to existing detection methods, such as blood sampling.

Furthermore, several studies have highlighted the use of liquid chromatography (LC) or gas chromatography (GC) in conjunction with mass spectrometry (MS) for salivary analysis. LC-MS and GC-MS both enable multi-analyte assessment, which is not possible with immunoassay techniques, due to the necessity of highly specific antibody-antigen interaction in ELISA. Additionally, DNA based salivary tests are commonly used for several analyses, such as clinical genetic testing, population studies, prenatal studies etc. Due to the sufficient availability of DNA in saliva and the biofluid's stability at high temperatures, polymerase chain reaction (PCR) results are highly reliable and have given rise to highly informative and discriminatory salivary assessment[224].

5.4 Stress and Salivary Analysis

A multitude of studies have reported the effects of stress on salivary characteristics. The composition of saliva is known to change distinctively following acute psychological stress exposure. Variations in saliva composition often involve the presence of cortisol and other stress hormones such as adrenaline and noradrenaline. However, studies have shown deviations in calcium and albumin concentrations in post-stress evaluations. Al-Nuaimy et.al. study focusing on the colorimetric assessment of saliva demonstrated a 78.50% decrease in calcium concentration during significant stress conditions compared to non-stressful conditions, as well as rising total protein concentrations by 10.60%[235]. The most common stress biomarker in salivary stress studies is cortisol. This is because the relationship between stress and cortisol is well-established and within biofluids, cortisol concentration varies in acute and chronic stress scenarios[234], [236]. As cortisol is the primary stress hormone, several studies have investigated the effects of stress on salivary cortisol levels. Additionally, adrenaline and noradrenaline concentrations have been examined to determine the relationships that exist between the onset of a stressor and the alterations in salivary compositions that it leads to. As salivary protein secretion is heavily influenced by

sympathetic nervous system activity, alterations in salivary characteristics can be indicative of stress within the body[237].

However, it should be noted that studies involving the catecholamine stress hormones (adrenaline and NA) have led to major discrepancies in results. This is prominent in Schwab et.al's study on the concentration changes in salivary adrenaline, noradrenaline and dopamine compared to serum concentrations during a bicycle ergometry-based stress scenario[238]. The investigation showcased that although serum concentrations for the catecholamines significantly increased during the stress phase, the salivary concentrations for the biomarkers of interest were insignificant and were not related to heart rate nor blood pressure variations. Further studies highlighted in Malamud et.al's review have determined that salivary levels of these stress hormones along with testosterone and oestrogen do not correlate strongly with serum levels[232]. Therefore, studies should be conducted cautiously with regards to the comprehension of the relationship between acute stressors and its effects on salivary stress hormone concentration variations.

Remarkably, dehydroepiandrosterone (DHEA) has been reliably measured in saliva, with its concentrations reflecting serum concentration levels[232]. Furthermore, the sulphated derivative of DHEA, DHEA-S also has great serum-saliva correlation, as highlighted by Yamaguchi et.al's review[239]. Salivary DHEA and DHEA-S are both known to increase during acute stress although detection of the biomarkers have not been deeply assessed in recent stress studies[234]. Thereby, the analysis of cortisol as the primary stress hormone, in conjunction with complimentary stress biomarkers such as DHEA and adrenaline is anticipated. Such investigations may lead to further developments in the understanding of the stress response and the detection of acute and chronic stress phases through saliva sampling.

5.5 Methods of Literature Search

The purpose of this review is to evaluate studies which have focused on the measurement of cortisol and DHEA, in different mediums e.g., saliva and plasma, simultaneously to comprehend the biochemical characteristics of psychological stress, and its trajectory to the manifestation of major depression. English-language articles were obtained from SCOPUS and

PubMed databases. They were selected based on the search criteria of inclusion of specific words in their title, abstract or keywords. The searching criteria comprised of two fixed terms: ((‘Cortisol’) AND (‘DHEA’)), alongside two independent terms to obtain articles focusing on healthy subjects as well as depressed patients: (‘Stress’ OR ‘Depression’). Additional articles that were related were selected through reference lists and the ‘related articles’ feature on SCOPUS and PubMed. After removal of duplicates, a total of 437 papers were obtained. Following the reading of paper abstracts, 98 papers were selected for further reading. The review focuses on the biochemical monitoring of psychological stress, therefore the most relevant papers that matched these criteria were selected. Ultimately, 31 papers were chosen for complete evaluation in this review. Figure 12 depicts a flow chart of the searching and selection process that was conducted for this review. Non-human studies were excluded from this review as the human stress response and stress hormones differ from other animals therefore the evaluation of animal studies would not be beneficial in the evaluation of stress biomarkers and the existing measurement techniques. Due to the implications of DHEA in aging function, the studies that were chosen for this review focus primarily on adults. Furthermore, papers involving participants with comorbid depression and other mental illnesses were excluded as these mental illnesses often have different and far more complex characteristics in stress hormone regulation which do not match the processes involved in acute or chronic psychological stress, nor major depression. The selection process was conducted by the primary author, upon shortlisting of articles a dual review was conducted with defined exclusion criteria to ensure minimisation of bias and subjective errors. Exclusion criteria is noted in Figure 12. Assorted studies were selected for review, including cross-sectional case control studies, randomised controlled trials and pilot studies. To ensure quality of the review was maintained, studies with lower sample sizes are explicitly mentioned in Table 6.

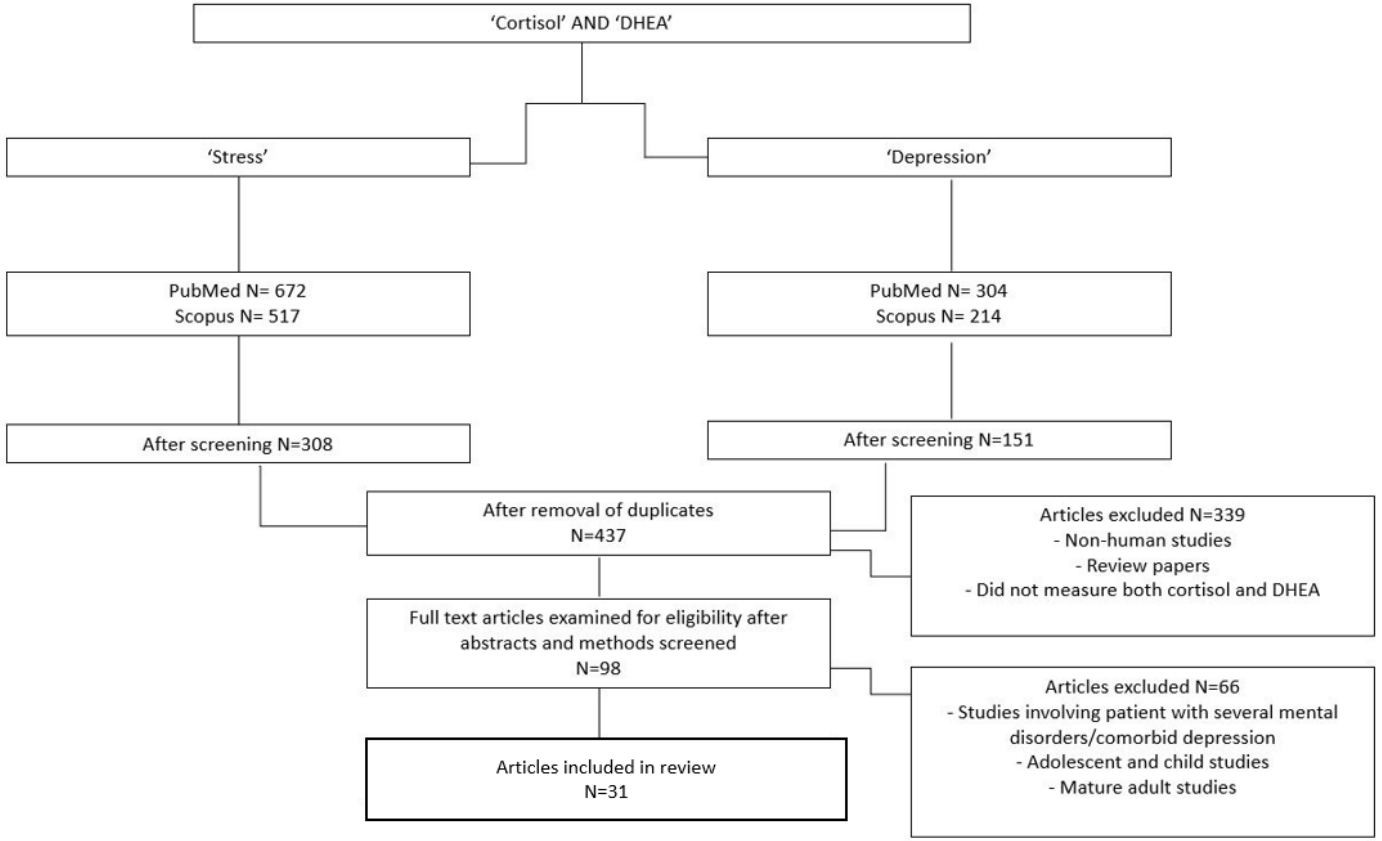

Figure 12- Flow chart of article selection process for comprehensive review of cortisol and DHEA monitoring for stress and depression.

5.7 Current State of the Art Monitoring Techniques for Cortisol and DHEA

The manifestation of stress and its effects on the human body are considerable for the comprehensive and quantitative evaluation of depression, and severe mental illnesses such as major depressive disorder. The relationship between stress hormones and clinical depression have been highlighted in a plethora of studies. Such studies have showcased the importance of cortisol monitoring for a better understanding of the neurobiological processes and their dysfunctionalities, which often lead to the development of depression. Evidently, Ter Horst et al. demonstrated the differences which exist between patients suffering from recurrent depression and healthy participants in their 2019 study[240]. This study highlighted HPA axis irregularities and subsequent hormonal imbalances which exist in depressed patients. Higher cortisol awakening responses were observed alongside lower cortisol/DHEA

Figure 12- Flow chart of article selection process for comprehensive review of cortisol and DHEA monitoring for stress and depression.

ratios, which have been noted as key characteristics commonly found in depressed patients.

The monitoring of stress-related biomarkers is well established in the field of stress studies, in cases of healthy participants as well as patients suffering from major depressive disorder. It is evident that the techniques used to measure cortisol, DHEA and other stress-related biomarkers often require the utilisation of highly complex and expensive equipment, and highly skilled specialists to comprehend and evaluate the quantification of the biomarkers and its translation into psychological and neurobiological changes in the human body [241]. Common techniques for the measurement of cortisol and DHEA include enzyme-linked immunoassays (ELISA) and liquid chromatography tandem mass spectrometry[242]–[244].

The cortisol/DHEA ratio was first coined in 2001 by Goodyer et al. in a selective review which related the three aspects of behavioural endocrinology i.e., the developmental changes of cortisol and DHEA and their roles in psychopathology and neurobiological mechanisms[245]. Goodyer observed that there exist medial changes in brain sensitivity following an excess in cortisol exposure, which often leads to dysfunctionalities and impairments in mental and behavioural function. Furthermore, it was noted that steroid hormones, therefore, contribute drastically to the shaping of behavioural and mental functions during early development and

such dysfunctionalities act as risk factors for psychopathology. The cortisol/DHEA ratio is a measure of the relative activity of both steroids and can be indicative of psychopathological issues[246]. Decreased ratios are often associated with dysfunctionality of the HPA axis. The two hormonal profiles that are common in depressed patients are either higher cortisol levels with normal levels of DHEA: or normal cortisol levels with lower levels of DHEA. Both of which lead to a lower cortisol/DHEA ratio. The cortisol/DHEA ratio has been a successful indicator of predicting recurrent major depressive disorder in adolescents[247]. Such studies have shown the clinical relevance of the cortisol/DHEA ratio and its impact in the prediction and evaluation of major depression. Several studies have demonstrated the efficacy of monitoring cortisol and DHEA for the assessment of stress and major depressive disorder. Comparatively, some studies have showcased the shortcomings in monitoring the stress and gonadal hormones and its correlations with psychological changes in the body, therefore it is imperative to critically discuss the benefits and limitations of studies highlighting such methodologies for more robust application of biomarker monitoring technologies for stress evaluation. The complete evaluation of these studies can be seen in Table 6.

Table 6 – Studies involving the measurement of cortisol and DHEA for stress and psychological evaluation in depressed and non-depressed groups.

Author	**Year**	**N (Number of Participants)**	**Age in years (mean ± SD)**	**Aim**	**Methods**	**Major Findings/Limitations**
Asadikaram, G.[248]	2019	79 MDD patients, 71 healthy controls	36.1±16.6 (MDD); 34.5± 19.8 (Controls)	Analyse differences in hormonal alterations between healthy participants and MDD patients.	Cortisol, ACTH, Testosterone, TSH, DHEA-S, T4, T3, FT4I, T3RU were evaluated via blood samples under fasting conditions between 7-8am using ELISAs. MDD confirmed through clinical interview.	Patients with other mental illnesses e.g., bipolar disorder were excluded. Depressed patients had substantially decrease in TSH, increase in FT4I compared to matched controls. ACTH levels were also higher in MDD patients. DHEA-S differences between healthy men and women were found but there were no differences between MDD and HC. MDD patients had considerably higher cortisol/DHEA-S ratio compared to HC. MDD testosterone was lower than HC. Small sample size, sex differences, blood sampling.

Author	Year	N (Number of Participants)	Age in years (mean ± SD)	Aim	Methods	Major Findings/Limitations
Assies, Johanna[241]	2004	13 MDD, 13 healthy controls	39.8±11.3 (MDD) 40.7±10.1 (Controls)	Measurement of salivary DHEAS and cortisol in MDD patients vs healthy controls	Salivary morning and evening DHEA-S and cortisol levels measured via ELISA. DSM-IV for MDD evaluation.	All depressed patients were medicated. No differences in cortisol levels between patients and controls. DHEA-S levels were elevated in MDD medicated patients vs healthy controls. It is possible that treatment may have normalised HPA axis dysfunction. DHEA-S levels may more adequately reflect 'state-related' HPA axis dysregulation than cortisol. Small sample size and only one day study.

Author	Year	N (Number of Participants)	Age in years (mean ± SD)	Aim	Methods	Major Findings/Limitations
Bae, Yoon Ju[249]	2019	33 TSST, 34 placebo (control)	18-35	Determine which stress biomarker had highest discriminatory power amongst groups of healthy males undergoing a stress test vs a placebo/control	67 healthy male participants, 33 of which completed TSST, 34 completed placebo TSST. Blood and saliva collected at 14 time points along with STAI and HR. Serum steroids, salivary cortisol and alpha amylase were analysed using LC-MS/MS, chemiluminescent immunoassays, intraassay and enzymatic colorimetric testing.	Salivary cortisone had highest discriminatory power 10 mins after peak salivary cortisol and meaningful correlations with subjective and autonomic stress measures. Salivary cortisone is superior surrogate marker for serum free cortisol compared to salivary cortisol due to irreversible conversion from cortisol to cortisone in saliva. Only tested stress response in healthy males.

Author	Year	N (Number of Participants)	Age in years (mean ± SD)	Aim	Methods	Major Findings/Limitations
Boudarene, M[250]	2002	40 subjects	42±12 years	define relationships between biological and physiological aspects of stress response.	stressed out participants but not mentally ill were asked to complete cognitive tasks with audio-visual cues. Amiel Lebrige questionnaire was filled as well as STAI. Serum cortisol and DHEA measured using radioimmunology and radio-immunoassays.	Most subjects had high STAI scores and life events impact. 25 subjects exhibited high level of STAI. 11 subjects had increase in cortisol plasmatic concentrations. Close correlation between DHEA and cortisol concentrations suggesting antagonistic relationship between the two hormones. High level of anxiety associated with increase in cortisol, low anxiety correlates to exclusive rise of DHEAs. Low sample size.

Author	Year	N (Number of Participants)	Age in years (mean ± SD)	Aim	Methods	Major Findings/Limitations
Cutshall, S.M.[251]	2016	21 female subjects	30-55	Assess the efficacy of functional medicine approach to improving stress, energy, fatigue, digestive issues and QoL in women	Measured salivary DHEA and cortisol via commercial salivary test kits.	Cortisol/DHEA ratio increased considerably from the beginning to the end of the study. Shows possible reversal of HPA dysregulation. Mean salivary DHEA levels also increased and SF pain subscale scores decreased. Small sample size, multiple intervention methods were applied.

Author	Year	N (Number of Participants)	Age in years (mean ± SD)	Aim	Methods	Major Findings/Limitations
Du, C.-L. Chung-Li C.-L. Chung-Li[252]	2011	63 city bus drivers and 54 staff		Validation of physiological stress biomarkers.	24-hour urine cortisol testing and blood draws for DHEAs.	Elevated cortisol level was associated with worker's relationship with supervisor and life changes in recent 3 months. DHEAS levels were higher in drivers of young age and in drivers with more concerns relating to their salary and bonuses. Non-drivers showed no association between urine cortisol and blood DHEA levels. Serum cortisol should have been taken. A standardised stress test was not used, subjective understanding of stress.

Author	Year	N (Number of Participants)	Age in years (mean ± SD)	Aim	Methods	Major Findings/Limitations
Ebrahimpour, Z.[253]	2011	10 female volleyball players	21.44±1.13	determine levels of salivary cortisol and DHEA in response to competition.	Salivary cortisol and DHEAs samples were collected 5 and 30 minutes before the match, between the sets and immediately and 30 minutes after the match, for 2 matches. ELISA used for concentration determinations	No substantial differences between DHEA concentrations and salivary cortisol. Slight increase in salivary cortisol during middle of the match but it was not statistically significant. Cortisol concentration increases more drastically during loss versus win in amateur players. Small sample size.
Ge, Fiona[254]	2016	218 couples	28.4	investigated the extent to which individual differences in HPA axis activity are associated with depressive symptoms among	5 saliva samples provided before and after discussion of major areas of disagreement in relationship. Samples assayed for cortisol and DHEA-S concentrations. Depressive symptoms assessed initially and 19,	Concordant levels of cortisol and DHEAS were concurrently and prospectively associated with higher depression scores. This effect was observed for wives only. Study was correlational so cannot determine whether differences in HPA axis functioning led to depressive symptoms.

Author	Year	N (Number of Participants)	Age in years (mean ± SD)	Aim	Methods	Major Findings/Limitations
				newlywed couples	37 months later with IDS-SR	
Ghiciuc, Cristina[255]	2011	102 healthy males		investigate presence of awakening response for various salivary biomarkers of adrenocortical activity including DHEAs, alpha amylase and cortisol	saliva was collected upon waking and 15,30,45,60 min afterwards and then at 8pm.	Salivary alpha amylase and DHEAS also produce awakening responses, similar to cortisol. Salivary cortisol and alpha amylase have opposite diurnal fluctuation patterns, Salivary cortisol and DHEAS represent HPA whereas alpha amylase represents sympathetic activity. Cortisol and DHEAS concentrations are inversely correlated to alpha amylase levels.

Author	Year	N (Number of Participants)	Age in years (mean ± SD)	Aim	Methods	Major Findings/Limitations
Grillon, C.[256]	2006	30 healthy male and female participants		Investigate relationship between cortisol/DHEAS ratio and fear-potentiated startle	30 subjects participated in differential aversive conditioning experiment with one of two stimuli (shock or no shock). Conditioned Responses were assessed with startle reflex. DHEAS and cortisol levels assayed from blood samples collected at baseline and after aversive conditioning session. State anxiety also assessed throughout testing.	Fear potentiated startle was higher in individuals with high/low cortisol/DHEA ratio. Multiple regression analysis showed that fear potentiated startle was positively associated with cortisol and negatively associated with DHEAS. No meaningful correlation between DHEAS and cortisol levels.

Author	Year	N (Number of Participants)	Age in years (mean ± SD)	Aim	Methods	Major Findings/Limitations
Heuser, I.[257]	1998	15 male MDD patients, 11 female MDD patients, 22 healthy males, 11 healthy female volunteers	47.7±14.8 (MDD males); 48.2+- 18.1 (MDD females); 53.1+-18.2 (healthy males); 47.9+-21.6 (healthy females)	Studied differences in diurnal plasma concentrations of DHEA in depressed patients vs healthy controls	Studied 24h DHEA plasma concentration in severely depressed patients and controls. Depressed patients were included after assessment using DSM-3-R and Hamilton Depression Scale.	Depression increases diurnal minimal and mean DHEA plasma concentrations but has no effect on diurnal maximal plasma concentrations or amplitude of DHEA. Novel finding of parallel increases in diurnal DHEA and cortisol plasma concentrations in depressed patients.
Irshad, Lylah[258]	2020	58 healthy adults	18-35	Investigate impact of exam period stress on salivary free light chains alongside stress biomarkers	saliva samples and questionnaires were filled during periods without exams to analyse baseline vs start of exam period. Saliva samples were	Cortisol concentration substantially increased during exams. DHEA did not change, leading to increase in cortisol/DHEA ratio.

Author	Year	N (Number of Participants)	Age in years (mean ± SD)	Aim	Methods	Major Findings/Limitations
					assessed for FLCs, IgA, cortisol and DHEA.	
Izawa, S.[259]	2008	33 male students	22.6+-3.6	Investigate DHEA secretion in response to acute psychosocial stress and relations of DHEA and cortisol, cardiovascular activity and negative mood changes.	33 participants were subjected to TSST, collections of saliva, BP measurements, HR and mood assessments were taken via visual analog scales. These were conducted before, during and immediately after the TSST.	Peak DHEA concentration preceded cortisol concentration by 10 min. Lower DHEA and elevated cortisol/DHEA ratio during TSST significantly correlated with increased negative mood during and after TSST. Indication that acute increase in DHEA concentration under stressful situations might be partly mediated by the activity of the HPA axis - could have some significance in improvement of negative mood.

Author	Year	N (Number of Participants)	Age in years (mean ± SD)	Aim	Methods	Major Findings/Limitations
Izawa, S.[260]	2012	33 women	19.5+-3.3	Investigates variation in salivary levels of cortisol and DHEA in prolonged stressful situation (2-week teaching practice).	Saliva samples taken at awakening, 30 min after, and bedtime at 2 weeks before practice, first week of practice, second week of practice and few days after practice. Completed questionnaires for perceived stress and subjective moods on each day. Cortisol and DHEA analysed via enzyme immunoassays.	Linear mixed model indicated that cortisol levels considerably increase during the first and second week of the practice compared with those before and after the practice period. DHEA levels decreased after the practice period compared with those at the other time points. Cortisol awakening response reduced substantially compared to other time points of saliva collection. Perceived stress and mood scores were higher during practice period. Negative feedback of the HPA axis may cause diminished cortisol awakening response and lower DHEA levels after the stress period. Limitations:

Author	Year	N (Number of Participants)	Age in years (mean ± SD)	Aim	Methods	Major Findings/Limitations
						not enough saliva samples taken, non-standardised stress test.

Author	Year	N (Number of Participants)	Age in years (mean ± SD)	Aim	Methods	Major Findings/Limitations
Jeckel, Cristina M Moriguchi[261]	2010	41 caregivers, 33 non-caregivers	60.56+-16.56 (caregivers); 60.27+-14.11 (non-caregivers)	Assessed neuroendocrine and immunological correlates of realistic chronic stress experience by strictly healthy caregivers of Alzheimer's disease and age-matched controls.	Salivary cortisol and DHEAS were assessed at multiple points by radioimmunoassay. Peripheral T cell proliferation and cellular sensitivity to glucocorticoids were evaluated by colorimetric assays.	Caregivers were drastically more stressed, anxious and depressed than controls. Similar cortisol levels between cohorts but caregivers had reduced DHEAS levels thus, increased cortisol/DHEAS ratio as well as impaired HPA axis response to DEX intake. Caregivers had higher T proliferation compared to controls.

Author	Year	N (Number of Participants)	Age in years (mean ± SD)	Aim	Methods	Major Findings/Limitations
Jozuka, H.[262]	2003	17 MDD patients, 10 controls	40.3+-15.1	Compared NK cell activity and blood levels of IL-2 to DHEA, DHEAS and cortisol in MDD patients vs healthy controls	Depression severity measured with Zung Self-rating depression scale. NK cell activity and IL-2 levels measured with chromium-51 release test and ELISA. Radioimmunoassay used to measure serum cortisol, DHEA and DHEAS.	NK cell activity and cortisol and DHEA levels were reduced in MDD patients compared with controls. IL-2 levels were increased. No differences in DHEAS levels. Reduction in NK levels and DHEA with increase in IL-2 is indicative of MDD.

Author	Year	N (Number of Participants)	Age in years (mean ± SD)	Aim	Methods	Major Findings/Limitations
Kim, M.-S.[263]	2010	74 participants	41.39+-10.22	Determine day-to-day differences in cortisol levels and molar cortisol/DHEA ratio in working subjects	Cortisol and DHEA levels measured from saliva samples via radioimmunoassay 30 mins after awakening for 7 consecutive days.	Cortisol levels from samples collected after awakening on workdays were radically different from cortisol level on Sunday. DHEA levels were not much different between the days of the week. DHEA levels on Monday and Tuesday were relatively lower than the levels on the other weekdays with levels on Thursday and Friday being the highest. Cortisol/DHEA ratios on Sunday were lower than those on workdays. Limitations: Short testing phase of only one week. No subjective measures of state anxiety taken.

Author	Year	N (Number of Participants)	Age in years (mean ± SD)	Aim	Methods	Major Findings/Limitations
Lac, G.[264]	2012	41 subjects suffering from bullying vs 28 healthy controls	46.3+-8.45 (Bullied); 46.0+-10.4 (Controls)	Measure salivary DHEAS and cortisol in individuals suffering from bullying at work	Bullied subjects screened for mental distress and institute of occupational health. Conditions causing bullying were recorded. Hospital anxiety and depression scale, Beech questionnaire and Visual analog scale of stress used to determine psychological state. Saliva samples taken at awakening, 30 min and 60 min after awakening and every 2 hours until bedtime. Cortisol and DHEA measured using commercial ELISA kits.	Bullied subjects had higher HAD scale scores, higher stress on the VAS and Beech questionnaire. Substantially higher salivary DHEA in bullied subjects but no meaningful differences between groups in cortisol levels at any time. Discrepancy probably arises from the stability and longer half-life of DHEA vs cortisol.

Author	Year	N (Number of Participants)	Age in years (mean ± SD)	Aim	Methods	Major Findings/Limitations
Laudenslager, Mark L M.L.[242]	2013	31 healthy subjects	43.5+-12.4	Constructed convenient and novel collection device for collecting saliva for determination of cortisol and DHEA	Saliva collected four times a day on three consecutive days using filter paper collection device. Subjects were asked to provide saliva at awakening, 30 min after, before lunch and 600 min after awakening. Cortisol and DHEA were measured from filter paper using EIA kits	Cortisol and DHEA revealed diurnal declines in similar patterns. Subjects did not adhere to collection times so caused disparities in the results.
Lennartsson, A.-K.[243]	2022	81 healthy subjects divided into low stress and high stress	20-50	Investigate the DHEA and DHEAS production capacity in relation to prolonged stress.	Participants underwent TSST. blood samples drawn before, during and after the stress test. Concentrations of cortisol, DHEA and DHEAS measured via LC-MS/MS,	High stress group had appreciably higher pre-test cortisol to DHEAs ratio than low stress group. Higher perceived stress in previous month related to attenuated DHEAS response during acute psychosocial stress. Limitation - only categorised

Author	Year	N (Number of Participants)	Age in years (mean ± SD)	Aim	Methods	Major Findings/Limitations
					radioimmunoassay and immuno-chemiluminescence assays.	using one question regarding perceived stress.

Author	Year	N (Number of Participants)	Age in years (mean ± SD)	Aim	Methods	Major Findings/Limitations
Lennartsson, A.-K.[265]	2012	20 men, 19 women	30-50	Study investigates effect of psychosocial stress on serum concentrations of DHEA and DHEAS in healthy subjects	Physiological measurements were performed before, directly after test and 30 mins after recovery. Blood samples were analysed via electrochemiluminescence assays, LC-MS/MS and radioimmunoassay for cortisol, DHEA and DHEAS respectively. Participants underwent TSST.	In both men and women, there was notably elevated DHEA and DHEAS in response to stressor. Large inter-individual variation in the magnitude of the response, especially for DHEA - no statistical difference between men and women. Magnitude of change in DHEA levels positively associated with magnitude of change in ACTH, cortisol and heart rate. Suggests capacity to secrete DHEA and DHEAS during acute psychosocial stress declines with age. Limitations - blood samples taken but there were only four time points available to measure DHEA and DHEAS levels.

Author	Year	N (Number of Participants)	Age in years (mean ± SD)	Aim	Methods	Major Findings/Limitations
Lennartsson, A.-K.[266]	2013	36 healthy subjects	37+-5	Investigate whether prolonged psychosocial stress is related to capacity to produce DHEA and DHEAS during acute psychosocial stress	Perceived stress at work measured using Stress-Energy Questionnaire. Participants divided into 3 groups based on their means scores (low stress, medium stress, high stress). Participants underwent TSST and blood samples were collected before, directly after test and after 30 min recovery. Same methods of concentration analysis as above	Higher perceived stress at work associated with attenuated DHEAS response during acute psychosocial stress. Cortisol/DHEAS ratio during acute stress test were higher in individuals reporting higher perceived stress at work vs individuals reporting lower perceived stress. No statistical difference in DHEA response between groups. All participants had relatively low perceived stress level as no individuals suffering from severe stress issues were included in the study - biased sample.

Author	Year	N (Number of Participants)	Age in years (mean ± SD)	Aim	Methods	Major Findings/Limitations
Mazgelyte, E.[267]	2021	40 subjects	21-53	Evaluate activity of the HPA axis by measuring salivary cortisol, cortisone, DHEA levels and their ratios to examine association with HRV measures in healthy adults.	Participants completed self-reported questionnaire on sociodemographic and lifestyle characteristics as well as the perceived stress scale and state trait anxiety inventory. Saliva samples were collected and resting HR and HRV were recorded during 3 data collection sessions. Salivary samples were analysed using ultra-high-performance liquid chromatography. HR measures - high-frequency IR earlobe sensor.	Statistically significant associations between HRV measures and higher salivary cortisol and lower DHEA levels. Decreased DHEA/cortisol ratio.

Author	Year	N (Number of Participants)	Age in years (mean ± SD)	Aim	Methods	Major Findings/Limitations
Michael, A.[268]	2000	44 MDD, 35 partially depressed and 41 healthy controls	20-64	Examine whether levels of DHEA are abnormal in depressed patients vs healthy controls	Salivary cortisol and DHEA samples were taken at 8am and 8pm for 4 days. Assayed with immunoassays	DHEA was lower at 8am and 8pm in depressed patients compared to controls. DHEA levels at 8am correlated negatively to severity of depression, were not related to drug treatment but decreased with age as expected. Cortisol was elevated in depression in the evening. Molar cortisol/DHEA ratio differentiated between those with depression vs the control group. Lower DHEA levels are additional state of abnormality in adult depression. DHEA may antagonise some effects of cortisol. Results could be affected by cortisol and DHEA awakening responses.

Author	Year	N (Number of Participants)	Age in years (mean ± SD)	Aim	Methods	Major Findings/Limitations
Mocking, R.J.T.[269]	2015	187 remitted recurrent MDD patients, 72 matched controls	18-65	Tested whether low DHEAS and high cortisol/DHEAS ratio in MDD reflects a trait or depressive state and its association with previous MDD episodes/effects of cognitive therapy	Salivary samples taken morning and evening for 8 weeks, repeated after 3 months and 2 years. Measured clinical symptoms during 10 year follow up. MDD reviewed with DSM-IV and HDRS. Saliva samples assayed via radioimmunoassay analysis.	Steeper diurnal DHEAS decline and flatter profile of cortisol/DHEAS ratio throughout follow up in remitted patients. Higher morning cortisol/DHEAS ratio predicted shorter time till recurrence over 10 year follow up. Cognitive therapy did not influence DHEAS or ratio.
Noser, E.[270]	2018	121 male participants	40-75	Investigate whether men with different degrees of vital exhaustion differ	Salivary cortisol, DHEAS, waist to hip ratio, systolic and diastolic BP were measured. Long-term cortisol and DHEAS were	Men who reported mild-severe levels of exhaustion had highest scores of cumulative indices of biological stress. Hair cortisol was unrelated to vital exhaustion. Hair

Author	Year	N (Number of Participants)	Age in years (mean ± SD)	Aim	Methods	Major Findings/Limitations
				in terms of allostatic load, chronic stress and social support	measured in hair. Chronic stress and social support assessed via questionnaires.	DHEA was highest in men with substantial levels of exhaustion.
Osran, H[271]	1993	9 MDD patients, 9 healthy matched controls		Observe abnormalities in adrenal androgen and cortisol metabolism in depressed patients	Serum levels of cortisol and DHEA measured at 8am and 4pm	Hypercortisolaemia and loss of diurnal DHEA variation but not cortisol variation in depressed patients. Suggests that in depression the adrenal androgens are partially regulated by mechanisms independent of ACTH. Very small sample size.

Author	Year	N (Number of Participants)	Age in years (mean ± SD)	Aim	Methods	Major Findings/Limitations
Ota, A.[272]	2015	115 healthy female nursery schoolteachers	30.8+-8.5	Examine associations of job strain and social support with daytime secretion amounts of DHEA and cortisol and daytime variations of cortisol/DHEA ratio.	Salivary DHEA and cortisol were measured with LC-MS/MS. Samples taken at 9am, 12pm and 3pm. Job strain measured via Job Content Questionnaire.	Social support scores were negatively associated with daytime DHEA secretion, not associated with cortisol/DHEA ratio. There were no major associations between job strain and the salivary measures.
Pérez-Valdecantos, Daniel[273]	2021	97 healthcare professionals	20.6+-+79.4	Assessed stress response in emergency health workers through measurement of cortisol, DHEA	Saliva samples obtained at 8am, 12pm, 3pm and midnight. Samples assayed by ELISA immunoassays.	Cortisol levels decreased throughout working day, similar pattern as DHEA. Alpha amylase values increased throughout the working day. Baselines not

Author	Year	N (Number of Participants)	Age in years (mean ± SD)	Aim	Methods	Major Findings/Limitations
				and salivary alpha amylase		calculated, only measured for single working day.
Persson, Roger[274]	2006	50 with 84 hour working week; 25 workers with 40 hour working week	21-65	Examines degree to which long workhours influenced stress biomarkers, metabolic processes and diurnal rhythm.	Blood samples obtained in the morning immediately prior to start of work on days 1, 5 and 7. Psychosocial circumstances assessed with questionnaire.	84-hour group had higher melatonin concentrations and reported higher job control scores than 40-hour group. Both groups had lower melatonin, cortisol and cholesterol concentrations on workday 5 than workday 1. DHEA and uric acid concentrations remained stable across all days.

Author	Year	N (Number of Participants)	Age in years (mean ± SD)	Aim	Methods	Major Findings/Limitations
Ter Horst, D M[240]	2019	73 unmedicated recurrent MDD patients; 46 matched controls	35-65	Determine whether alterations in HPA axis activity and fatty acids in recurrent MDD remain during remission.	Measurements of salivary cortisol and DHEAS were taken at awakening, evening and after sad-mood induction. Assayed via radioimmunoassay. Depressive symptoms measured using HDRS.	Patients had higher cortisol awakening responses and lower evening cortisol/DHEA ratios compared to healthy controls. Fatty acids did not differ between patients and controls.

5.7 Discussion

Several studies have demonstrated the benefits of utilising the cortisol/DHEA ratio as an objective and quantitative measure of stress in depressed and healthy participants[35], [39], [44]– [47], [50]– [57], [59], [61]. Moreover, some studies have noted the decline in cortisol levels i.e., hypercortisolaemia and in DHEA levels were characteristic in patients suffering from depression, compared to controls[240], [271]. Michael et al.'s 2000 study observed that a negative correlation relationship existed between DHEA awakening response levels and the severity of depression in a study consisting of 44 MDD patients and 35 partially depressed participants[268]. They went on to suggest that lower DHEA levels are an additional state of abnormality in adult depression, alongside hypercortisolaemia i.e., blunted cortisol response[271]. Additionally, the results of Jozuka et al.'s 2003 study further justify this argument[275]. In this study the cortisol and DHEA levels were observed to be radically lower in MDD patients compared to healthy controls, albeit in a smaller sample size of 17 MDD patients and 10 healthy participants. These studies suggest that the abnormalities found in secretion patterns of cortisol and DHEA may be indicative of the dysfunctionalities of the HPA axis, as well as irregularities in the antagonistic relationship between cortisol and DHEA, which would evidently be reflected in the cortisol/DHEA ratio.

In studies that compared the hormonal differences between depressed patients and healthy participants, such as Assies et al.'s 2004 study on salivary cortisol and DHEAS, it was observed that DHEAS levels were elevated in MDD patients, whereas no noteworthy differences existed in salivary cortisol levels amongst MDD and healthy cohorts[241]. Although this led to indications that MDD patients had greater cortisol/DHEA ratios, which correlate with existing literature, the behaviour of salivary cortisol and DHEA and its fluctuations did not corroborate with the hormonal patterns expected from MDD patients. Comparatively, Boudarene et al.'s 2002 study on the roles of cortisol and DHEA during the stress response showcased high levels of anxiety and stress were linked with higher cortisol levels and close correlations between DHEA and cortisol[250]. As this study was conducted on healthy participants, it brings forward the question of whether the cortisol fluctuations in the Assies et al.'s study were a result of blunted cortisol responses, commonly observed in MDD patients[11], [32], [35], [241]. Moreover, parallel increases in plasma cortisol and DHEA levels of depressed patients were

found in a 1998 study conducted by Heuser et al. whereby it was found that the mental disorder led to large increases in diurnal minimal and mean plasma DHEA concentrations in a comparative study between depressed patients and healthy control participants[257].

Evidently, stress studies which have been conducted on healthy participants have demonstrated results which further support this theory. In healthy human studies, such as those conducted by Izawa et al. and Irshad et al. the analysis of salivary biomarkers revealed the increase in salivary cortisol levels after stressful events[258], [259]. In both cases the presence of a stress-inducing event led to the increase in cortisol levels whereas, in Irshad et al.'s study there were no substantial changes in DHEA levels[258]. However, in Izawa et al.'s study, a peak in DHEA concentration was observed 10 minutes prior to peak cortisol concentration[259]. Both studies showcased increases in cortisol/DHEA ratios as a result of stress induction, albeit with different hormonal profiles. Although Izawa et al.'s study reveals the antagonistic nature of the relationship between salivary cortisol and DHEA, the results of Irshad et al.'s study suggests that further evaluation is required to fully assess the manner in which the fluctuations of cortisol and DHEA influence each other within the human body[258], [259].

Thus, an argument can be derived that is dependent on the behaviour of the stress-related biomarkers in the body and its roles in mental/psychological changes in humans. The cortisol/DHEA ratio can be considered as an objective indicator of mental stress in healthy humans. This is because the fluctuations of cortisol and DHEA are expected to follow a known pattern in response to stressors i.e., stressor leads to an increase in cortisol levels, which results in a higher cortisol/DHEA ratio, as seen in several studies. Whereas, in studies involving depressed patients, a lower cortisol/DHEA ratio can be expected due to declines in cortisol or DHEA, which coincides with existing literature regarding blunted cortisol responses in patients suffering from MDD due to reaching the 'exhaustive' stage of the stress or 'fight or flight' response[11], [32], [35].

It is therefore imperative to consider the methods in which cortisol and DHEA are measured from serum and saliva samples in stress studies. As previously mentioned, the primary methods of monitoring cortisol and DHEA involve immunoassay-based techniques or high-performance chromatography-based methods[261], [265]. Although such methods yield

highly accurate results regarding cortisol and DHEA concentrations, they require laboratory-based protocols and expensive equipment that are not easily accessible. Furthermore, in cases with high-risk individuals, such as those at risk of suicide, the time taken to obtain results is of utmost importance. The standard time taken to generate results from the gold standard salivary cortisol evaluation technique i.e., ELISA testing is minimally 24-48 hours. Ideally, in high-risk cases, the existence of a rapid and continuous cortisol and DHEA monitor is compulsory for the betterment and improvement to quality of life for depressed patients.

Additionally, only one study considered the physiological changes which arise during stress, as well as the hormonal fluctuations which are implicated by the cortisol/DHEA ratio. Mazgelyte et al. investigated the associations between salivary steroid hormone fluctuations and time domain heart rate variability (HRV) indices in healthy individuals[267]. Participants were asked to provide saliva samples during 3 collection sessions, which also involved a sociodemographic and lifestyle questionnaire, state trait anxiety inventory and the perceived stress scale. Salivary samples were analysed for cortisol and DHEA concentrations via high performance liquid chromatography whilst HRV measures were taken issuing a high frequency infrared earlobe sensor. The results of this study demonstrated statistically significant associations between HRV measures and salivary cortisol and DHEA levels[267]. The results coincided with previously mentioned studies whereby an increase in stress on the perceived stress scale correlated with an increased cortisol/DHEA ratio. Other studies involving multimodal measurements of stress include Ahrens et al.'s study on stress responses in recurrent MDD patients versus healthy participants through HRV, as well as serum and, salivary cortisol measurements[276]. However, such studies did not consider the presence of DHEA in response to stress elicitation, therefore it was not considered within the scope of this review. The importance of physiological biomarkers of psychological stress in tandem with the evaluation of biochemical measurements is evidently essential for the comprehension of the stress response, as well as the relationship between steroid hormones and mental health deterioration in humans.

5.7.1 Considerations

The implication of monitoring stress-related biomarkers such as cortisol and DHEA has been highlighted throughout this comprehensive review. Evidently, the quantification of the

relationship that exists between cortisol and DHEA is an important characteristic for the objective measurement of stress, and its manifestations in the human body. Various studies have shown promising findings which corroborate with the existing literature[267]. Whereby, spikes in cortisol concentration leads to increased cortisol/DHEA ratios in healthy subjects, whilst blunted cortisol responses and irregularities in DHEA secretion led to subsequent declines in cortisol/DHEA ratios in depressed individuals[240], [271]. Alternatively, several studies have demonstrated the insufficiencies in measuring only two steroid biomarkers for the understanding of the stress response. Evidently, the lack of standardised stress testing in stress studies involving healthy participants has led to diminished legitimacy in the results as there is no clear boundaries of stress elicitation in these individuals. The utilisation of standardised stress tests such as the TSST validifies that individuals were subjected to stress and genuine stress response were elicited. For example, in Bae et al.'s. 2019 study, the TSST was used to investigate the stress biomarkers which had the highest discriminatory power between healthy cohorts undergoing stress tests versus controls[249]. It was observed that salivary cortisone and salivary cortisol had substantial correlations with the subjective and autonomic stress measures, which were monitored via questionnaires and heart rate, respectively. Therefore, it is imperative to consider standardised stress tests such as the TSST when conducting stress studies as it is a reliable method for stress response elicitation.

5.7.2 Multimodal Approaches

Undertaking a multimodal approach for monitoring of stress and depressive symptoms may lead to great advances for the complete quantification of psychophysiological stress evaluation[80]. Particularly, Mazgelyte et al.'s study showcases promising results in the use of chemical biomarker monitoring alongside physiological monitoring techniques such as HRV measures in the discrimination between stress responses versus resting state responses in healthy adults[267]. Notably, the application of HRV measures in parallel with the cortisol/DHEA ratio established statistically significant associations between the physiological stress measures and biochemical stress biomarkers, which could be a promising characteristic to incite further investigations in stress measurement. In a recent review of studies involving the measurement of cortisol in tandem with physiological measurements of stress, promising results were obtained which further bridges the gap in knowledge in the quantification of psychological stress[80]. Therefore, efforts in the investigation of DHEA and the

cortisol/DHEA ratio in similar studies would ideally lead to a greater understanding of the fluctuations of stress hormones during the stress response, and the physiological markers that are expressed during an episode.

5.8 Conclusion

To conclude, the prominence of cortisol and DHEA monitoring for the evaluation of psychological stress and its discriminatory power between patients of major depressive disorder and healthy individuals is inevitable. With this understanding and the aide of standardised stress testing, it could direct efforts away from existing subjective stress monitoring practices and drive research towards the complete quantification of psychological stress in the human body, through optical methods, for the improvement of quality of life for stressed individuals as well as those suffering from chronic stress or, clinical depression.

6. Spectroscopy

Spectroscopy is the observation of interactions between matter and electromagnetic (EM) radiation through absorption, emission, and reflection. Specifically, infrared spectroscopy focuses on the application of infrared radiation to a wide variety of sample types for simple classification of functional groups, as well as in-depth qualitative and quantitative analysis of their chemical compositions[277]. This chapter introduces the primary optical method which was used to characterise cortisol for point-of-care applications.

Near-infrared spectroscopy (NIR) utilises the near-infrared region of the EM spectrum (12500-4000cm^{-1}) for the rapid analysis of molecular overtone and combination bands of fundamental vibrations in higher frequency modes[278]. This spectroscopic method is used in a multitude of medical diagnostic applications, including blood sugar, pulse oximetry and functional neuroimaging. The mid-infrared spectroscopic (MIR) method operates within the mid-IR region of the EM spectrum (4000-400cm^{-1}) for measurement of fundamental vibrational bands which coincide with critical functional groups within a sample[277]. Thus, MIR spectroscopy facilitates the determination of the chemical composition of a sample for the purpose of identifying its fingerprint. Furthermore, the far-infrared region (FIR) consists of frequencies between 400 and 20cm^{-1}[279]. The low energies characteristic of the far-IR region can be applied for rotational spectroscopy and identification of low-frequency vibrations[280].

6.1 Basic Principles of Infrared Spectroscopy

Infrared spectroscopy is characterised by the molecular vibrational activity within chemical compounds at differing absorption bands[279]. Through application of infrared radiation to chemical compounds, the composition of the sample can be quantitatively analysed. This is done through the analysis of peaks in specific absorption bands[281]. Vibrational frequencies are highly sensitive to bond structure, thus facilitating the determination of which molecular bonds, and therefore which molecules, exist within the sample being investigated. The Lambert-Beer Law can be used to elaborate the relationship that exists between intensities of the incident and transmitted IR radiation and analyte concentration[282]. IR spectra are obtained through plotting the intensity of the of the signal (in absorbance or transmittance

mode) against the wavenumber[282]. The wavenumber is proportional to the energy difference between ground and excited vibrational states.

Energy is transmitted from the IR photon to the molecule through absorption[282]. Subsequently the molecular vibration must cause a change in dipole moment of the molecule. The dipole moment is a function of the magnitude of the atomic charges and their positions, within a molecule. It can be signified by (1) [282]. The change in dipole moment for the molecule leads to a change in the vibrational energy level, which can be utilised for compound determination. The IR band intensity that is measured is proportional to the square of the change in the dipole moment, whereby, μ=dipole moment; e_i =atomic charges; r_i=molecular positions.

$$\mu = \sum e_i r_i \tag{1}$$

Fourier Transform-IR (FTIR) is the preferred method for infrared spectroscopy due to its speed and ease of use for infrared spectroscopy[283]. FTIR spectrophotometers offer a disperse method of measurement across a broad spectrum instead of a narrow band of frequencies[284]. The IR beam passes through an interferometer, consisting of a beam splitter, fixed mirror and moving mirror[284]. The interferometer separates the spectral components of the beam which then passes through the sample before reaching the detector. The absorbance or transmittance spectrum is then collected from a Fourier Transform of the interferogram. Due to the utilisation of a single beam spectrum, all radiation frequencies reach the detector at the same time, facilitating simultaneous calculation of wavelength intensities, as well as a large signal-to-noise ratio[282], [283]. Sequential measurements of the background spectrum are required, alongside the sample spectra, as the background provides information regarding wavelength-dependent elements of the spectrometer[282]. Furthermore, the background spectrum is associated to the source emission, detector response, beam splitter properties and residual atmospheric absorptions, which may affect the measurement of final IR spectra[282].

Larkin et.al. book concerning infrared and Raman spectroscopy describes the principles of spectroscopy, as well as the various sampling methods within IR spectroscopy in immense depth[282]. Larkin et.al. explains the need for IR transmitting materials for the ease in

sampling of a wide variety of sample types, such as samples at different temperatures or varying physical states[282]. Commonly, NaCl (sodium chloride) and KBr (potassium bromide) windows are used for FTIR spectroscopy, as well as ZnSe (zinc selenide) for aqueous/wet samples. Furthermore, the various methods of sampling are highlighted such as the utilisation of KBr discs and nujol molls for the collection of transmission spectra from solid-powdered samples. Additionally, reflection techniques are described, including attenuated total reflectance (ATR) mode. The ATR sampling method involves the utilisation of a crystal with a high refractive index for contact sampling without the requirement of sample preparation[285]. The phenomenon of total internal reflection facilitates the collection of spectral information in a quick and non-destructive manner. Total internal reflection relies on the angle of incidence being greater than the critical angle[282]. At frequencies within an absorption band, the reflection is attenuated, whilst at a frequency away from an absorption band, all light if reflected. The reflected light contains spectral information regarding the sample thus giving rise to spectral analysis of the sample under investigation.

The near-infrared region is considered the optimal technique for quantitative analysis of chemical compounds of interest[286]. Whereas the mid-IR region is known as the molecular fingerprinting region. This is because most molecules have intense fundamental vibrational bands within the mid-IR, making it the ideal technique for qualitative analysis of chemicals[287]. The sensitivity in molecular determination within the mid-IR region is unmatched due to the intensity of the transitions being more than a thousand-fold stronger than the spectral band transitions in the NIR region[287]. The utilisation of dual-band frequency combs has led to major developments in spectral analysis within the mid-IR[288]. Frequency combs offer ultra-high resolutions and high sensitivities in broad bandwidth spectroscopy. A dual frequency comb tool facilitates the measurement of a sample's spectral response on a tooth-by-tooth basis, where one comb interrogates the sample and interferes on a photodetector with the second comb, which acts as a local oscillator. The interference pattern is recorded as a function of time and its Fourier transform gives the spectrum for further high precision molecular spectral analysis[287]–[289]. Adjacent to the mid-IR region lies the NIR region, which contains absorption bands that coincide with combinations of fundamental vibrations[278].

6.2 UV-Vis Spectroscopy

Similar to other modalities of spectroscopy, UV-Vis spectroscopy focuses on the amount of wavelengths of light that are absorbed by or transmitted through a sample in comparison to a reference, showcased in figure 12a [290]. The wavelengths of interest for UV-Vis spectroscopy include the ultraviolet (UV) and visible light range which is 100nm (UV) to 780nm (red light). UV-Vis spectroscopy analysis often utilises absorbance spectra to examine analytes of interest. The absorbance is equal to the log of the fraction of the intensity of light passing through the sample, divided by the intensity of light after passing through the sample. This information alongside the molar absorptivity and path length can be used to obtain the concentration of the sample, in accordance with Beer-Lambert law[291].

Beer Lambert law is highly utilisable in situations where the substance concentration showcases a linear relationship with absorbance, when measured using standard solutions[292]. The wavelength corresponding to the maximum absorbance of the substance is often chosen for this type of analysis, this is referred to as peak absorbance values. The use of the peak absorbance value ensures maximum sensitivity to analyte the sample concentration.

To determine the concentration of a sample with the analyte of interest, often a standard curve is calculated with known concentration values which coincide with absorbance values. The linearity of the standard curve determines the feasibility of using the Beer-Lambert law to predict concentration values in samples with unknown levels of the target analyte[291].

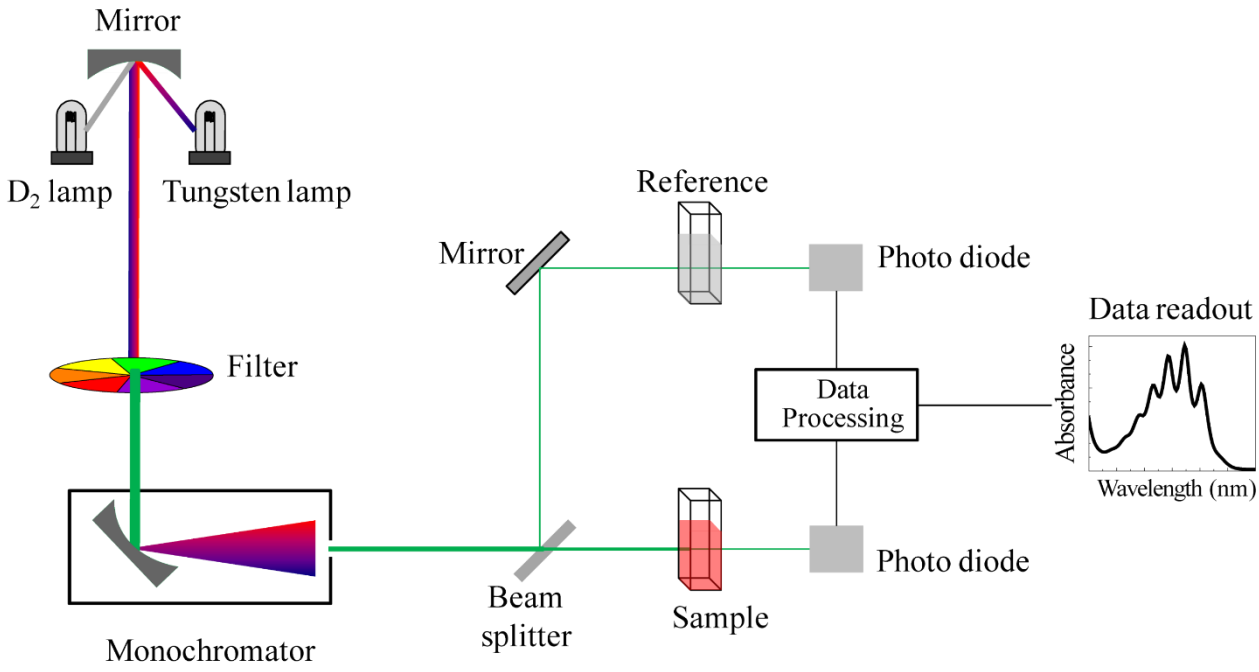

Figure 12a - Standard Dual-Beam Spectrophotometer Setup

6.3 Chemometrics and Spectral Analysis

Chemometrics and spectroscopy combine to form a powerful tool for spectral analysis of chemicals, especially in the pharmaceutical industry[286]. As spectroscopy is suitable for a wide range of physical state i.e., liquid, gaseous and solid powder forms, it is often implemented in pharmaceutical development industries e.g., in quality control laboratories[286]. Spectroscopy allows for non-destructive and effective analysis of chemical compounds at considerably low costs, which suggests its desirability in several investigative applications within chemistry and biotechnology industries.

Chemometric analysis eases the interpretation of spectra through three main technique groups. Mathematical pre-treatments involve the enhancement of spectral information through pre-processing techniques such as normalisation, derivative transformations, and smoothing filters. Furthermore, classification methods are used to categorise samples based on spectral characteristics, such as principal component analysis (PCA). Such methods are used to visualise the data and reduce variables or features in multivariate datasets for ease

of interpretation. Finally, the third technique consists of regression methods which connects spectral characteristics or information to quantifiable measures, such as multi-linear regression. Multi-linear regression establishes a correlation between studied specific wavelengths and the samples' properties to mathematically define the characteristics of the sample. This relationship can then be used to develop a predictive model, calibrated on the reference values of the studied sample properties[286].

Moreover, after classification techniques are applied to the sample dataset, quantitative modelling can enable the accurate and precise determination or prediction of more samples under investigation. Chemometrics alongside NIR spectroscopy have been utilised in several studies to develop highly precise models for the successful determination of chemical samples. Some methods within this field include multi-linear regression as previously mentioned, as well as partial least squares regression (PLS). PLS establishes a linear relationship between two matrices i.e., the spectral data and the reference values to find out how the spectral data best describes the studied properties. These techniques can be used to derive information regarding the physical properties of the samples under investigation, such as hardness and particle size[286].

However, it is worth noting that although chemometric utilisation can lead to powerful models for the chemical composition determination, good practices are of utmost significance[293]. With misuse of such methods, the model calibration may remain successful for a known dataset and fail to operate as intended with data outside of the calibration set. Therefore, calibration drift and the necessity of periodic re-calibration should be noted[293]. Otherwise, within mental health monitoring applications, this could lead to incorrect analysis of the chemical biomarkers of interest. Consequently, this may lead to severe misdiagnoses which could have unsafe consequences in a clinical environment involving high-risk patients.

7. Characterisation and Determination of Artificial Salivary Cortisol via FTIR

To comprehend the relationship between acute psychological stress and salivary cortisol levels, the following in vitro protocol was designed. The protocol involves the investigation of general trends in spectral behaviour as the concentration of cortisol increases in a known volume of saliva. To ensure cortisol can be dissolved in aqueous solutions, it is recommended to first dissolve the cortisol in an organic solvent such as ethanol or dimethyl formamide (DMF). The solubility of hydrocortisone in DMF is 30mg/ml. This is because hydrocortisone is sparingly soluble in aqueous buffers such as saliva or artificial saliva. The shelf-life of the aqueous solution including cortisol is a maximum of 24 hours. Therefore, random batch sampling was conducted to test the spectral properties of salivary cortisol in varying concentrations.

The PerkinElmer Frontier spectroscopy system was utilised which offers spectra in the near, mid, and far infrared regions. The HATR (Horizontal Attenuated Total Reflectance) accessory module was utilised as liquid samples were being investigated. Characteristic spectral peaks of interest for cortisol exist in the mid-IR region thus, MIR mode was applied for this protocol. The instrument settings can be found in Table 7. The scan settings can be found in Table 8.

Table 7 - PerkinElmer Frontier FTIR Spectrometer Instrument Settings

Mode	MIR
Range	8000-30cm^{-1}
Optimum Scan Range	7800-450cm^{-1}
J-Stop Image Size	8.94
J-Stop Wavenumber	4000

Table 8 - FTIR Spectroscopy Scan Settings

Units	**Nanometres (nm)**
Scan Range	2500-20000nm
Resolution	$4cm^{-1}$
Data Interval	$8cm^{-1}$
Number of Scans	20

7.1 Methods

Salivary Cortisol Clinical Range: 0.4nmol/L – 32nmol/L

Salivary Cortisol Testing Range: 1-24mmol/L

This testing range was chosen due to the availability of resources within the laboratory. It was not essential to investigate the clinical range of salivary cortisol at this preliminary stage as the aim of this protocol is to detect the general trends and molecular fingerprint of cortisol. Artificial saliva was used for this preliminary protocol due to risks in obtaining whole human saliva during the COVID-19 pandemic. Firstly, stock solutions of 100mmol/L and 200mmol/L of cortisol dissolved in DMF were made, using the following calculations:

$$\frac{362.47g}{mol} \times \frac{0.1mol}{L} = \frac{36.46g}{L} \qquad (2)$$
$$\frac{36.47}{L} \div 2 = \frac{18.125g}{500ml}$$

$$\frac{362.47g}{mol} \times \frac{0.2mol}{L} = \frac{72.494g}{L} \qquad (3)$$
$$\frac{72.494}{L} \div 10 = \frac{7.2494g}{500ml}$$

Subsequently, the samples were prepared in accordance with (4). The two-step dilution process is shown in Table 9.

$$C_1V_1 = C_2V_2 \quad (4)$$
$$V_2 = \frac{C_1V_1}{C_2}$$

Whereby: C1= concentration of cortisol in DMF

V1 = vial volume

C2 = concentration of stock solution

V2= amount of stock to be mixed with DMF top-up for sample

Table 9 - Two-step Dilution Process for Cortisol in Artificial Saliva Sample Preparation

C1 (mmol/L)	V1 (ml)	C2 (mmol/L)	V2(ml)	DMF top up (ml)	Final Conc. (mmol/L)	cortisol in DMF solution (ml of C1)	saliva top-up (ml)
5	5	100	0.25	4.75	1	1ml of 5mmol	4ml saliva
10	5	100	0.5	4.5	2	1ml of 10mmmol	4ml saliva
15	5	100	0.75	4.25	3	1ml of 15mmol	4ml saliva
20	5	100	1	4	4	1ml of 20mmol	4ml saliva
25	5	100	1.25	3.75	5	1ml of 25mmol	4ml saliva
30	5	100	1.5	3.5	6	1ml of 30mmol	4ml saliva
35	5	100	1.75	3.25	7	1ml of 35mmol	4ml saliva
40	5	100	2	3	8	1ml of 40mmol	4ml saliva
45	5	100	2.25	2.75	9	1ml of 45mmol	4ml saliva
50	5	100	2.5	2.5	10	1ml of 50mmol	4ml saliva
55	5	100	2.75	2.25	11	1ml of 55mmol	4ml saliva
60	5	100	3	2	12	1ml of 60mmol	4ml saliva
65	5	100	3.25	1.75	13	1ml of 65mmol	4ml saliva
70	5	100	3.5	1.5	14	1ml of 70mmol	4ml saliva
75	5	100	3.75	1.25	15	1ml of 75mmol	4ml saliva
80	5	100	4	1	16	1ml of 80mmol	4ml saliva
85	5	100	4.25	0.75	17	1ml of 85mmol	4ml saliva

C1 (mmol/L)	V1 (ml)	C2 (mmol/L)	V2(ml)	DMF top up (ml)	Final Conc. (mmol/L)	cortisol in DMF solution (ml of C1)	saliva top-up (ml)
90	5	100	4.5	0.5	18	1ml of 90mmol	4ml saliva
95	5	100	4.75	0.25	19	1ml of95mmol	4ml saliva
100	5	100	5	0	20	1ml of 100mmol	4ml saliva
105	5	200	2.625	2.375	21	1ml of 105mmol	4ml saliva
110	5	200	2.75	2.25	22	1ml of 110mmol	4ml saliva
115	5	200	2.875	2.125	23	1ml of 115mmol	4ml saliva
120	5	200	3	2	24	1ml of 120mmol	4ml saliva

7.2 Results

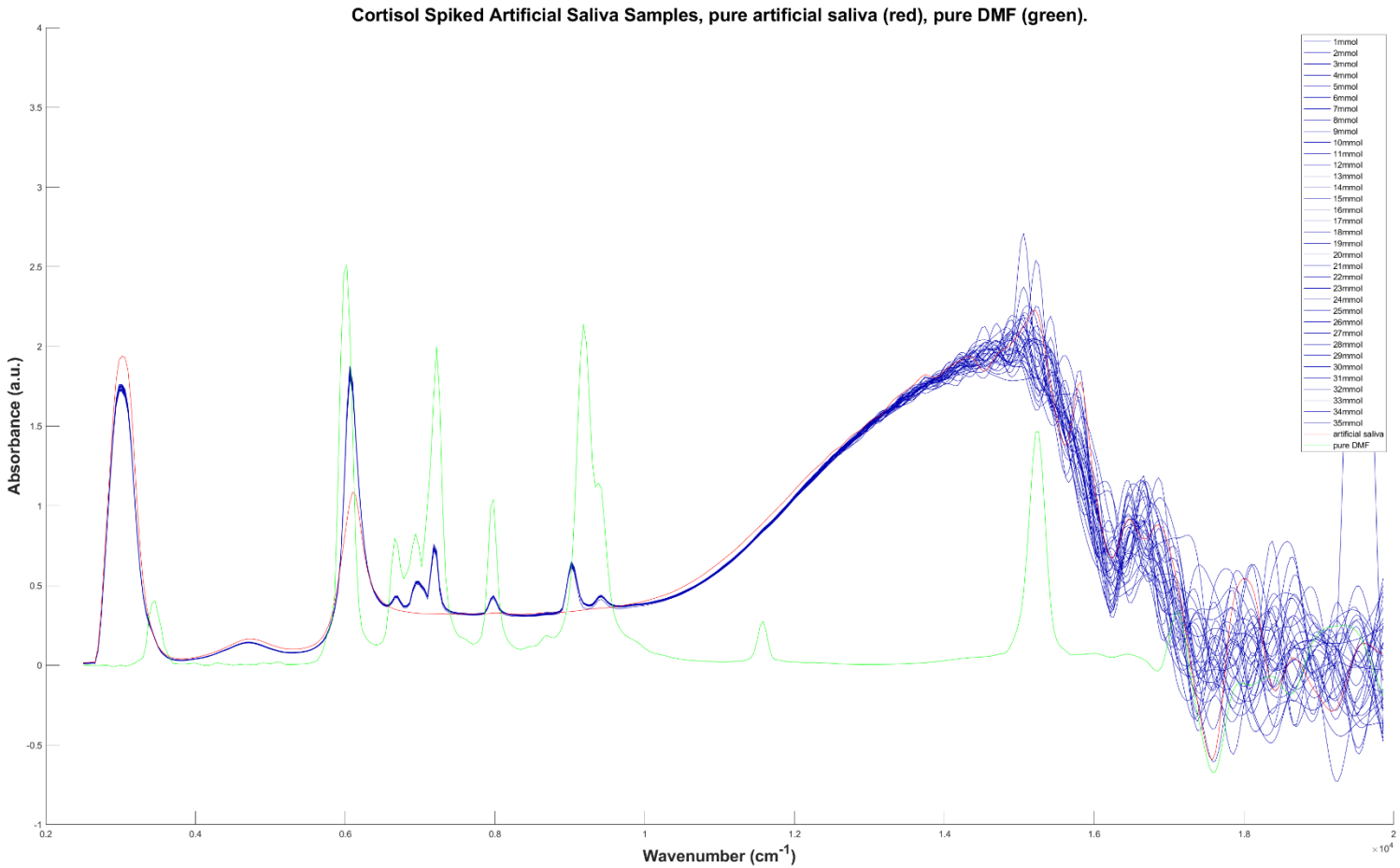

Figure 13 - Spectrogram of Cortisol-spiked saliva samples, pure artificial saliva and pure DMF. Window size – 2500-20,000nm.

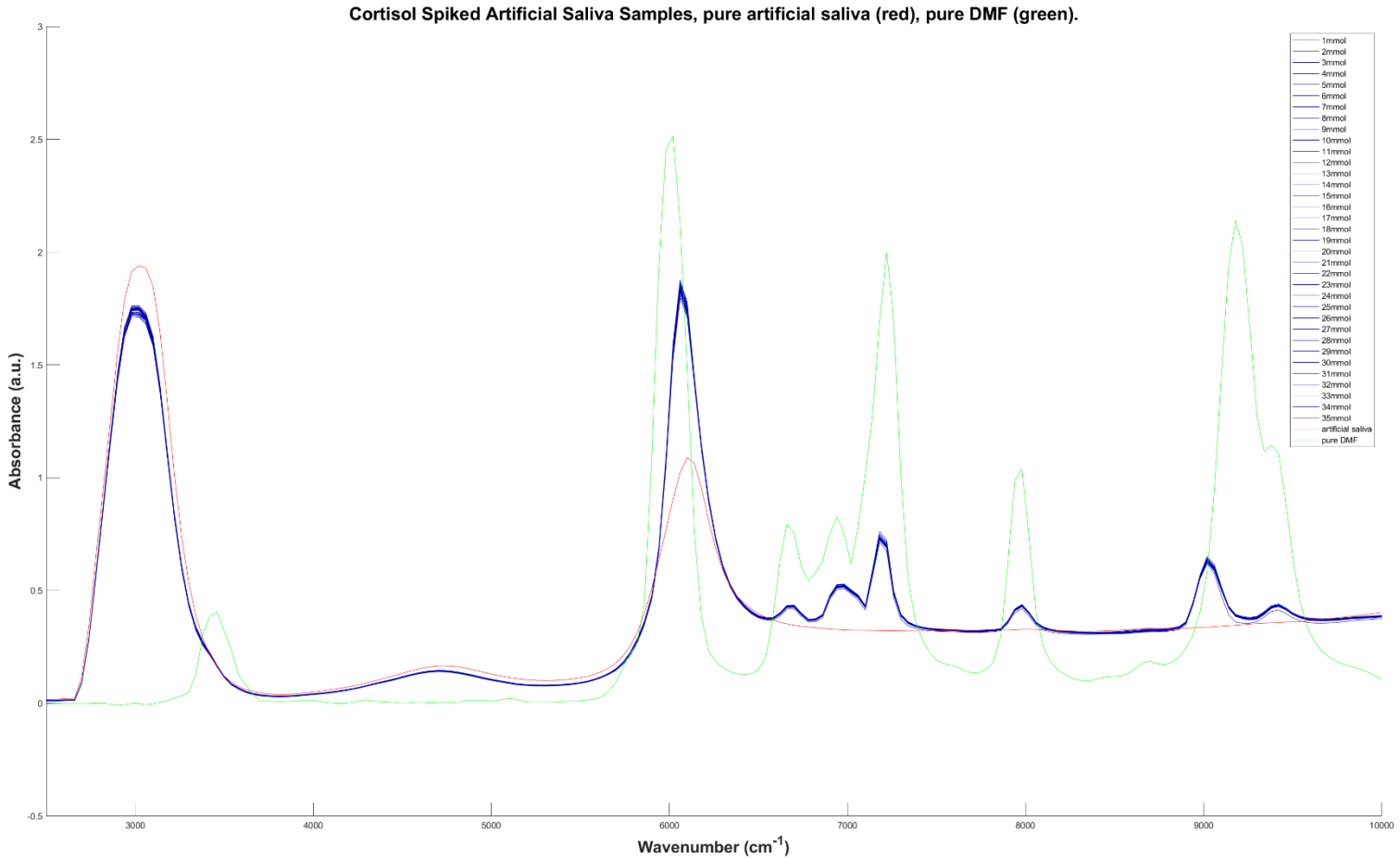

Figure 14 - Spectrogram of Cortisol-spiked saliva samples, pure artificial saliva and pure DMF. Window size-2500-10,000nm.

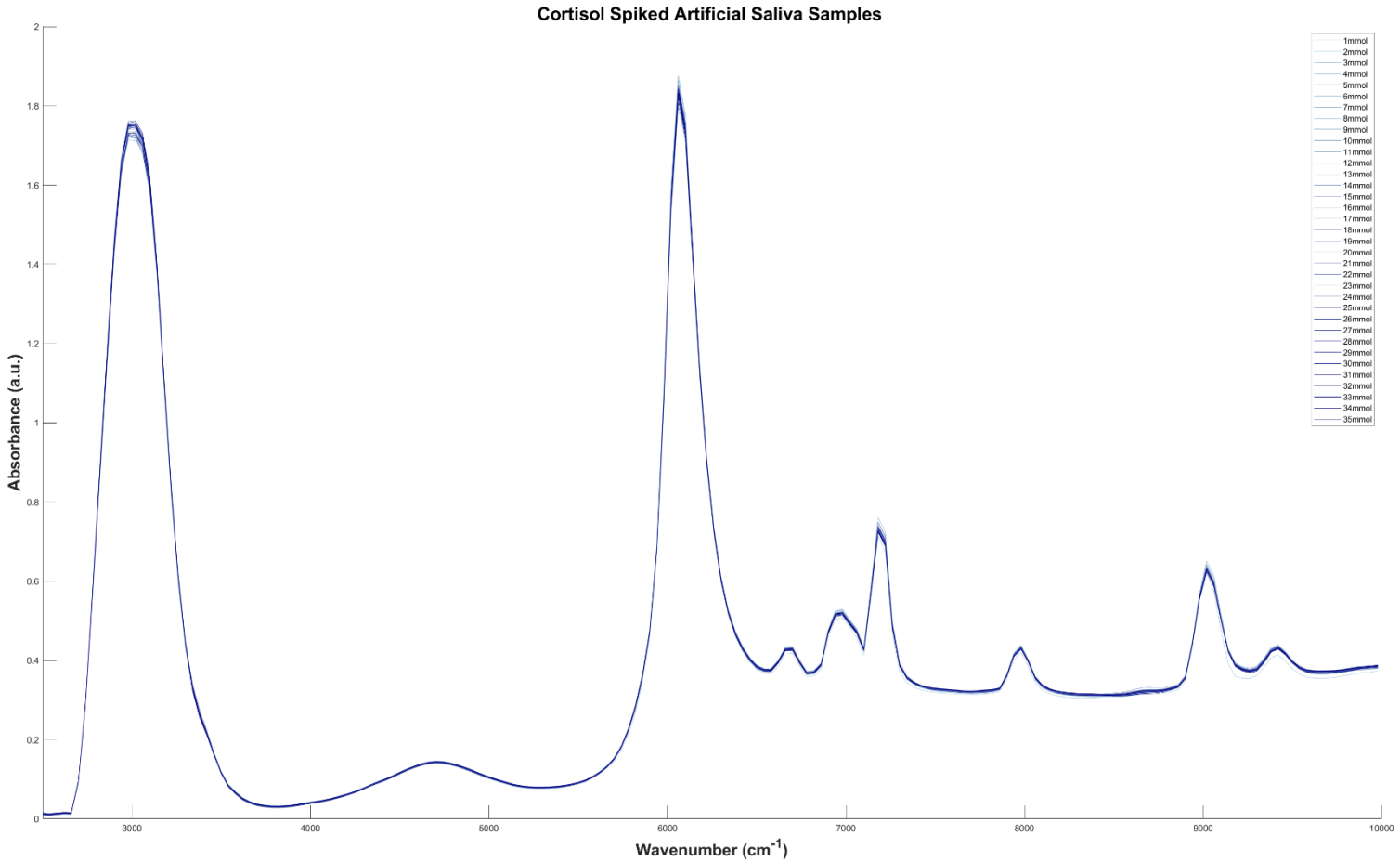

Figure 15 - Spectrogram of Cortisol-spiked saliva samples with increasing shades of blue representing increasing cortisol concentrations in artificial saliva samples. Window size - 2500-10,000nm.

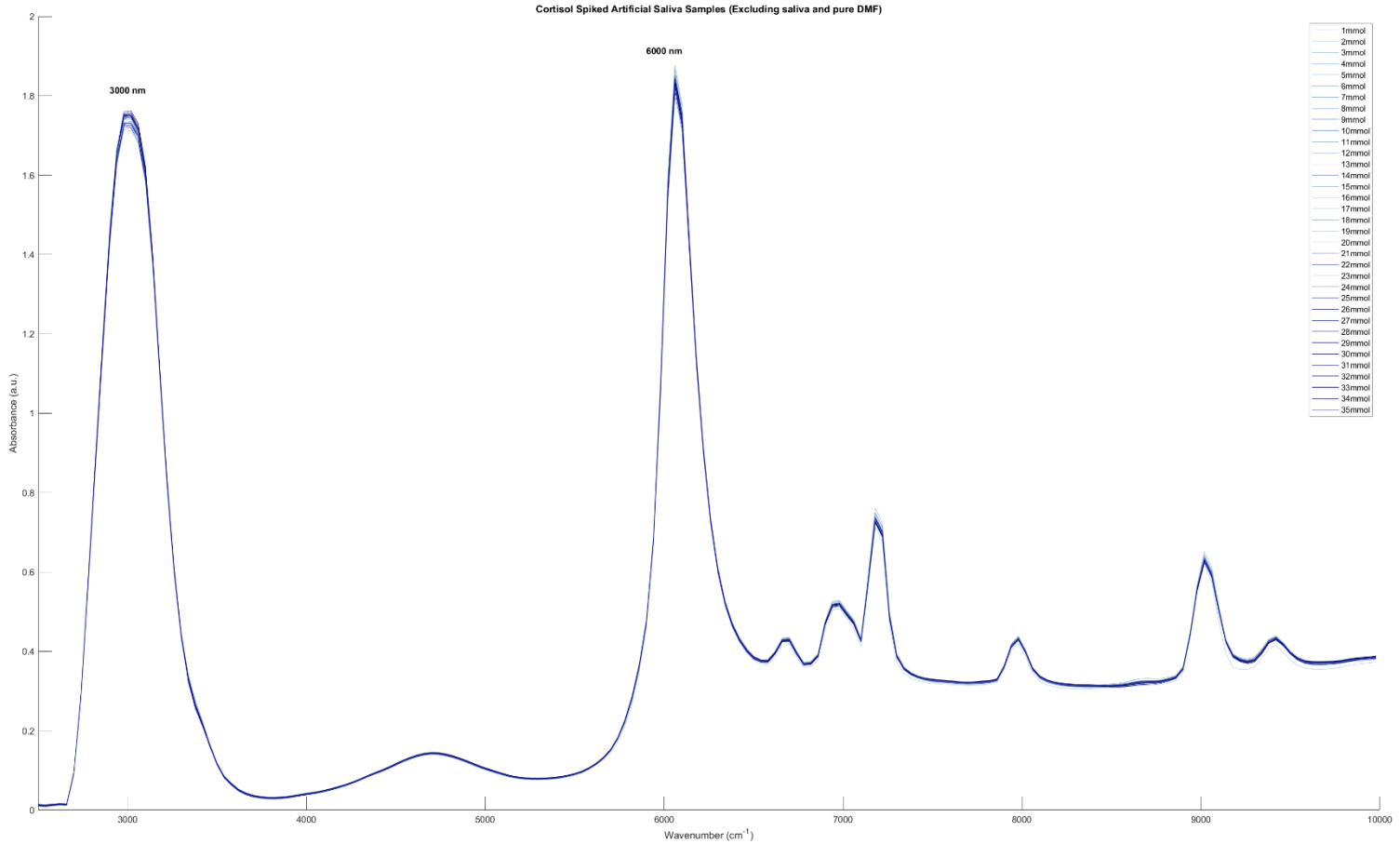

Figure 16 - Spectrogram of Cortisol-spiked saliva samples. Window size - 2500 -10,000nm. Including peak labels.

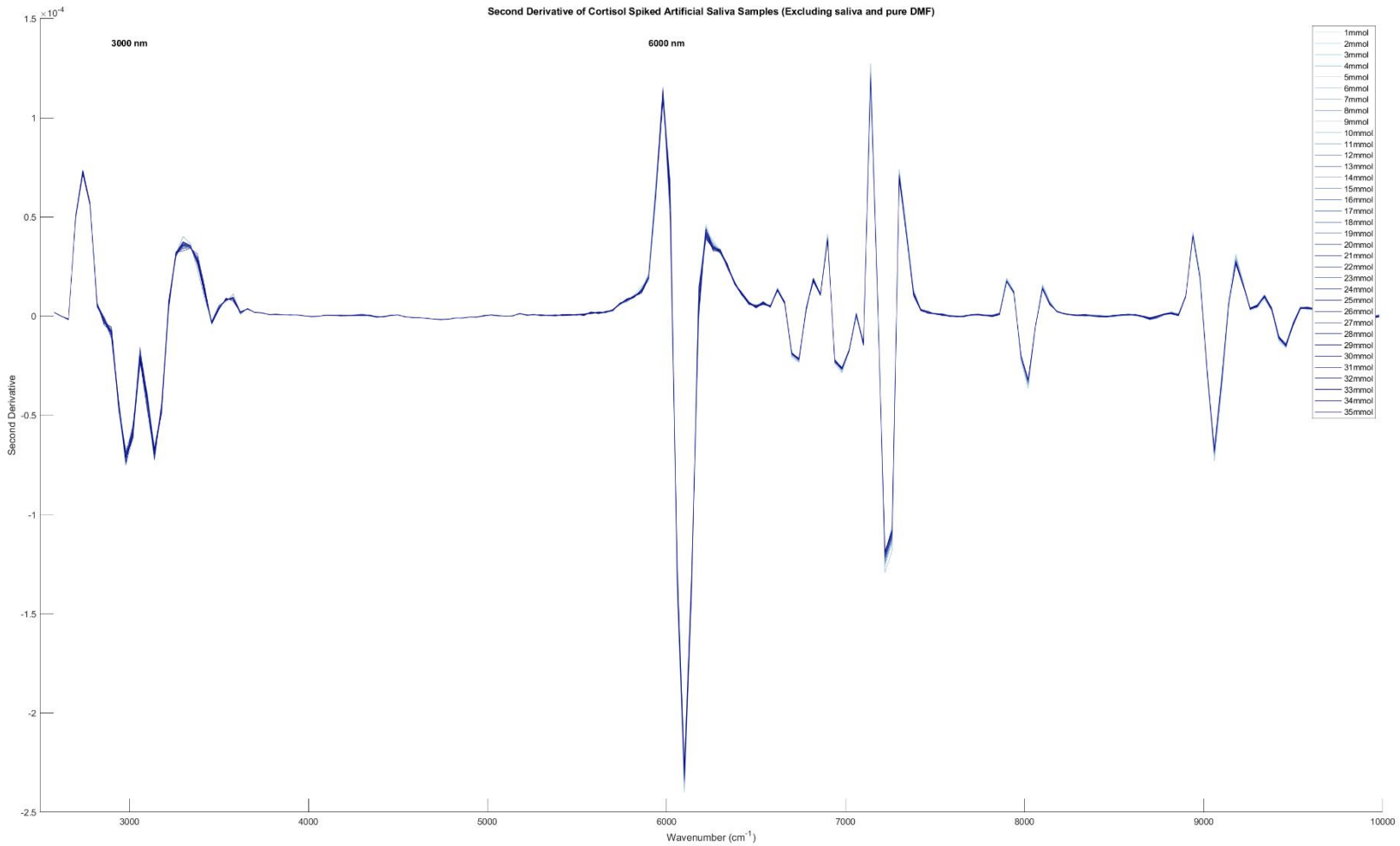

Figure 17 - 2nd Order Derivative Transformation of Cortisol-spiked saliva samples. Window size - 2500-10,000nm

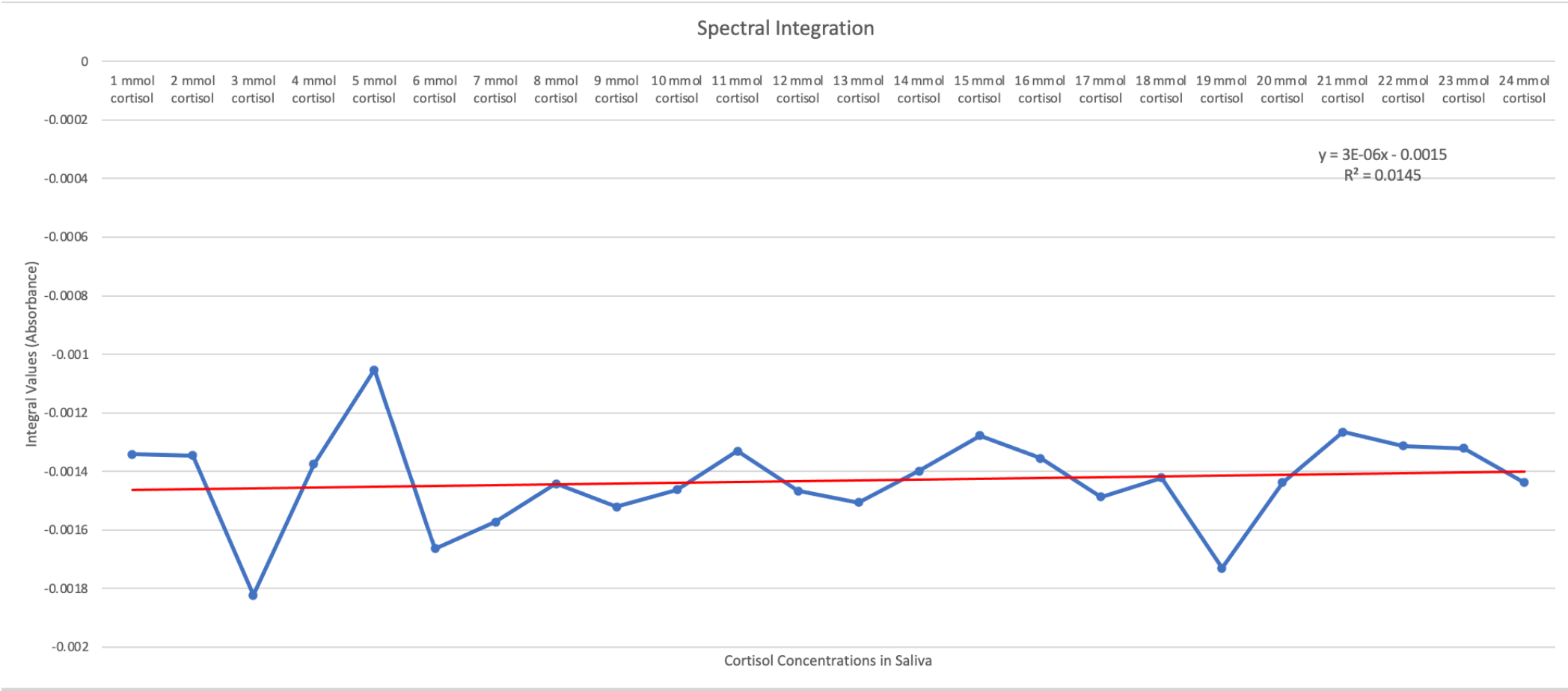

Figure 18 - Line Graph of integral values of cortisol-spiked saliva samples of increasing concentration (blue). Linear regression of integrals (red).

7.3 Results

Figure 13 depicts the spectra of cortisol-spiked saliva samples, along with the spectra of pure artificial saliva and pure DMF, as reference spectra. In accordance with Small et.al.'s review of good practices in chemometric spectral analysis, periodic background scans were taken every 3 samples or 20 minutes[293]. This ensures that spectral analysis of this data would yield legitimate results, whereby judgements can be made with greater confidence. From Figure 13, it is apparent that after 10,000nm, there is a lot of noise which masks a substantial portion of spectral data. This may have been due to the presence of water absorption bands throughout the spectra originating from the artificial saliva[295]. Fundamental vibrations of the water molecule result in intense absorption in broad spectral bands[295]. Therefore, for qualitative analysis the window size was reduced to a limit of 10,000nm (Figure 14).

Further qualitative analysis of figure 14 shows a clear similarity between the cortisol spiked saliva spectra and the pure saliva spectra at 3000nm. This could be a characteristic of symmetric O-H bond stretching at 2734nm as the chemical composition of the artificial saliva consists predominantly of water[296]. Furthermore, the secondary peak of artificial saliva at 6269nm is reflected in the cortisol-spiked spectra. This peak signifies the fundamental vibrations of the water molecule, specifically defined as H-O-H bending vibrations[296]. The peak at 3400nm for the pure DMF spectrum is one of the fingerprinting peaks of DMF[297]. Moreover, spectral peak activity from 6600nm to 7200nm is visible in the pure DMF spectrum, as well as the cortisol-spiked samples. This could signify fundamental DMF characteristics which are commonly found around 1500cm^{-1} or 6600nm[297]. Notably, the peak at 5900nm is not present in neither of the reference spectra and is a fundamental characteristic of alkene functional groups, which is present in cortisol[160], [298]. Moreover, the peak at 2700nm could be an indication of vibrational energy of the O-H bond in alcohol functional groups which ranges from 2700nm to 3500nm, this functional group is present in cortisol[160]. Although there are very distinct peaks in the spectra collected, as seen in figures 15 and 16, judgements regarding the chemical data should consider the high prevalence of water in the sample. As water has very intense absorption bands in the mid-infrared and near-infrared regions, several elements of key spectral data could be masked by the presence of fundamental vibrations of the water molecule[299].

7.4 Discussion

Spectral information can facilitate the investigation of chemical compounds in various means[286]. Primarily, fundamental chemical peaks can be used to derive the bond structure of a sample, as well as the specific chemical composition. Manipulation of spectral data can also lead to the comprehension of the relationships between peak placements and the concentrations of the molecules of interest within a sample[294]. For example, in some cases the increase in concentration of a chemical may lead to spectral shifts that can be determined through applied chemometrics[286].

Moreover, further qualitative analysis suggested that within the mid-IR region, the relationship between the cortisol concentration and peak intensity is considerably weak. From visual analysis, it was evident that as the cortisol concentration increased, variable results in the absorbance were found. Therefore, to comprehend this relationship, a 2nd order derivative transformation was applied to the cortisol-spiked sample spectra (figure 17)[300]. As is characteristic of calculating the 2nd order derivative, the spectra have a negative band with a minimum at the same wavelength as the maximum of the zero-order spectra i.e., the spectra are visually inversed[301]. The first order derivative showcases the rate of change of absorbance with respect to wavelength, whilst the second-order derivative depicts the instantaneous rate of change of the first derivative[301]. Thus, containing information regarding whether the rate of change of absorbance is increasing or decreasing. Ordinarily, second-order derivatives are used in spectroscopy to increase the molecular specificity by enhancing the separations of overlapping peaks[300]. Therefore, facilitating more efficient and successful determination of the samples' chemical compositions, as well as outliers[286]. For example, the spectrograph in figure 17 shows separation in bands at 2500-3500nm which is one broad peak in figure 13. This separation can be used to quantify the narrow peaks with greater determinability and confidence. Although it can be resolved with confidence that the peaks at 2500-3500nm are fundamental water absorption bands, other regions of the spectrogram can be used to analyse cortisol peaks[295], [296], [299].

Characteristic cortisol peaks are found around 5800nm and 6100-6250nm, which is present in the 2nd derivate spectrum at very high intensities, suggesting the successful determination of cortisol through FTIR spectroscopy[160], [298]. Furthermore, the integrity of 2nd derivative spectroscopy is discussed in Baldassarre et.al.'s article regarding absorption spectra and their

second derivatives[302]. Baldassarre et.al. considers the fitting of second derivative curves with zero-order absorption spectra to improve the description of complex structures of investigative compounds[302]. However, Baldassarre et.al. stresses the deterioration of the absorption spectra during simultaneous fitting with the second order transformation[302]. This can lead to the detection of weaker absorption bands of little significance that do not accurately represent the analysed samples. Therefore, the addition of new bands may lead to misrepresentation of the spectral data, which is the cortisol spiked samples in this case.

Evidently as mentioned in chapter 6, chemometric techniques have taken precedence in the quantitative analysis of spectra[286]. However, most chemometric techniques utilise FTIR within the near-infrared region[286]. Therefore, for the dataset from the conducted protocol the techniques had to be altered prior to application. The area under the curve was calculated for the cortisol-spiked samples to determine the amount of energy absorbed. The spectral integration values were then plotted in figure 18. Although there are varying levels of absorbance with increasing cortisol concentration, the general trend of the graph showcases positive correlation. Further quantitative analysis through linear regression justifies this positive correlation, also showcased in figure 18. The magnitude of correlation between the cortisol concentration and the absorbance of the samples is miniscule, justifying the utilisation of chemometrics techniques in the NIR region instead of MIR region as the NIR region is known to contain vast amounts of quantitative information, whilst the MIR region is optimal for fingerprinting and qualitative analysis[278], [282], [286]. The R2 value for the linear regression model is 0.0145, which suggests that the model cannot robustly predict data points, due to the lack of linearity between individual cortisol spiked samples[303].

In conclusion, the use of FTIR for the determination of cortisol in artificial saliva samples was deemed unsuccessful within the non-clinical range. As the clinical range of salivary cortisol would involve further dilution of the saliva samples, it is evident that other optical techniques such as UV-Vis spectroscopy and label based methods are necessary for the determination of salivary cortisol within the clinically significant range.

7.4 Further Work

Moving forward, other spectroscopic techniques will be utilised, such as ultraviolet-visible (UV-Vis) spectroscopy and colorimetric methods for the quantitative analysis of cortisol in saliva samples. Such techniques offer greater quantifiability at lower concentrations, which is necessary for testing within the clinical range of salivary cortisol in humans. Thereby facilitating psychological stress measurement with greater confidence and certainty.

8. Colorimetric determination of artificial salivary cortisol (non-clinical range) using UV-Vis spectroscopy (1-40mmol/L).

The detection and determination of salivary cortisol is of utmost significance towards the development of a point-of-care stress monitoring device. Previous studies using infrared spectroscopy have deemed the method inapplicable for salivary hormone analysis, specifically due to the presence of several water bands present in the spectra. This is unavoidable, due to the chemical composition of saliva consisting primarily of water. Therefore, the utilisation of other optical and spectroscopic techniques is essential for the characterisation and determination of cortisol in a simple and rapid manner. This chapter introduces the use of the blue tetrazolium method (BT method) for the optical determination of cortisol in artificial saliva.

8.1 Introduction

The use of colorimetric methods has been used vastly within the field of biosensing as they offer a simple and straightforward alternative to complex and expensive assay-based techniques, such as enzyme-linked immunoassays (ELISAs) and radioimmunoassay (RIAs)[304]. Salivary cortisol is regularly measurement with the use of ELISAs, deeming it the gold-standard method for salivary cortisol determination. However, the method can prove to be expensive and involve long waiting times, which suggests the need for a rapid and inexpensive method for cortisol detection.

Tu et.al. proposed the use of the blue tetrazolium (BT) dye and tetramethylammonium hydroxide reagent for the determination of cortisol concentration in sweat, utilising a colorimetric-based scale to analyse cortisol concentration[305]. As a proof-of-concept study, the following protocol aims to observe the feasibility of utilising the BT dye and aforementioned reagents to determine salivary cortisol concentration variations in a non-clinical testing range of 1mmol/L to 40mmol/L, with the aid of a PerkinElmer UV-Vis spectrophotometer. The clinical range for salivary cortisol analysis lies between 0.7ng/mL to 27.3ng/mL or 0.4nmol/L to 32nmol/L.

8.2 Methods

The aim of this protocol was to characterise the reaction between cortisol and the BT dye, and tetramethylammonium hydroxide reagent, via optical techniques i.e., UV-Vis spectroscopy. The testing range was defined as 1-40mmol/L. A stock solution of 200mmol/L of cortisol in methanol was prepared by adding 36.247g of hydrocortisone (>98% purity) powder to analytical grade methanol (>99.7%). Furthermore, the BT dye was prepared by adding 400mg of blue tetrazolium (3,3'-3,3'-dimethoxy-4,4'-biphenylene bis 2,5-diphenyl-2h-tetrazolium chloride) powder to 200ml of methanol for a 200ml volume solution. Moreover, the 1% v/v tetramethylammonium hydroxide solution was prepared by combining 20ml of tetramethylammonium hydroxide to 180ml of methanol for a 200ml volume reagent solution. These materials were acquired from Fisher Scientific (Fisher Scientific, Waltham, MA, USA). Artificial saliva was acquired from Sigma Aldrich (Sigma Aldrich, St Louis, MO, USA).

Pre-samples of diluted cortisol in methanol were created of 5ml volume. For each of the 40 samples, 0.1ml of each of these pre-samples were added to 2.8ml of artificial saliva and equal volumes (0.05ml) of the BT dye and tetramethylammonium hydroxide reagent. The sample preparation of the cortisol samples in artificial saliva can be seen in Table 10. Each sample was mixed and 1ml of the sample was pipetted into an Eppendorf disposable plastic cuvette. Spectra was collected after a 10 min colour development time. The Lambda 1050 dual-beam spectrophotometer equipped with the 3-detector module was used for optical measurements of the samples. The visible colour change from translucent pale yellow to magenta can be used to determine cortisol concentration levels within the samples. The colour intensity increases as a function of cortisol concentration, whereby higher concentrations of cortisol yield deeper and darker magenta samples. In each case a 3-cycle

configuration was used to obtain 3 consecutive absorbance spectra from each sample between 400-650nm at a step interval of 1nm. The reference cuvette remained blank for the entire protocol and routine baseline measurements at 100%/0% absorbance was taken to reduce the effects of background and ambient noise. Each sample was tested 3 times to yield average absorbance values for each concentration of cortisol that was tested. Spectragryph software was used for spectral visualisation.

Spectral analysis of the samples involved using linear regression-based modelling to return a coefficient of determination value R^2. This was used to determine the linearity of the relationship between the cortisol concentration and the absorbance obtained via the spectroscopy protocol.

8.3 Results

There is a characteristic absorption peak (seen in figure 19) observed at 510nmm which corresponds with existing literature [305]. The visual colour change from pale yellow to magenta which was observed in samples after the colour development time showed a distinct linear dependency on cortisol concentration, whereby higher concentrations of artificial salivary cortisol led to darker and more intense shades of magenta within the sample. Moreover, the second derivative spectra (see figure 20) reiterate this point as the band spacing in the second derivative spectra suggests linearity in the samples. Furthermore, regression analysis of the spectra confirmed an R^2 value of 0.83 which further reiterates the linear relationship between cortisol concentration and absorbance (Figure 21). The relationship was to be expected, in accordance with the Beer Lambert Law which facilitates target analyte concentration determination through accounting for analyte absorbance and the extinction coefficient of the analyte. Furthermore, the spectra showcase further linearity between the colour intensity and concentration of the sample as samples of greater cortisol concentration led to greater absorbance levels.

Table 10 - Two-step Dilution Process for Cortisol in Artificial Saliva Sample Preparation for the BT method.

Desired Cortisol Concentration **in Saliva (mmol/L)**	Cortisol Methanol Conc **C1 (mmol/L)**	Vial Volume (ml) **V1**	Stock Conc (mmol/L) **C2**	Cortisol Stock to be mixed **V2 (ml)**	Methanol **Top Up (ml)**	Sample Vial Volume **ml**	Cortisol in Methanol **µl**	Saliva **Top Up µl**	TB Dye **µl**	T Hydroxide Agent **µl**
1	5	5	200	0.125	4.875	4	100	2800	50	50
2	10	5	200	0.25	4.75	4	100	2800	50	50
3	15	5	200	0.375	4.625	4	100	2800	50	50
4	20	5	200	0.5	4.5	4	100	2800	50	50
5	25	5	200	0.625	4.375	4	100	2800	50	50
6	30	5	200	0.75	4.25	4	100	2800	50	50
7	35	5	200	0.875	4.125	4	100	2800	50	50
8	40	5	200	1	4	4	100	2800	50	50
9	45	5	200	1.125	3.875	4	100	2800	50	50
10	50	5	200	1.25	3.75	4	100	2800	50	50
11	55	5	200	1.375	3.625	4	100	2800	50	50
12	60	5	200	1.5	3.5	4	100	2800	50	50
13	65	5	200	1.625	3.375	4	100	2800	50	50
14	70	5	200	1.75	3.25	4	100	2800	50	50
15	75	5	200	1.875	3.125	4	100	2800	50	50
16	80	5	200	2	3	4	100	2800	50	50
17	85	5	200	2.125	2.875	4	100	2800	50	50
18	90	5	200	2.25	2.75	4	100	2800	50	50
19	95	5	200	2.375	2.625	4	100	2800	50	50
20	100	5	200	2.5	2.5	4	100	2800	50	50

Desired Cortisol Concentration **in Saliva (mmol/L)**	Cortisol Methanol Conc **C1 (mmol/L)**	Vial Volume (ml) **V1**	Stock Conc (mmol/L) **C2**	Cortisol Stock to be mixed **V2 (ml)**	Methanol **Top Up (ml)**	Sample Vial Volume **ml**	Cortisol in Methanol **µl**	Saliva **Top Up µl**	TB Dye **µl**	T Hydroxide Agent **µl**
21	105	5	200	2.625	2.375	4	100	2800	50	50
22	110	5	200	2.75	2.25	4	100	2800	50	50
23	115	5	200	2.875	2.125	4	100	2800	50	50
24	120	5	200	3	2	4	100	2800	50	50
25	125	5	200	3.125	1.875	4	100	2800	50	50
26	130	5	200	3.25	1.75	4	100	2800	50	50
27	135	5	200	3.375	1.625	4	100	2800	50	50
28	140	5	200	3.5	1.5	4	100	2800	50	50
29	145	5	200	3.625	1.375	4	100	2800	50	50
30	150	5	200	3.75	1.25	4	100	2800	50	50
31	155	5	200	3.875	1.125	4	100	2800	50	50
32	160	5	200	4	1	4	100	2800	50	50
33	165	5	200	4.125	0.875	4	100	2800	50	50
34	170	5	200	4.25	0.75	4	100	2800	50	50
35	175	5	200	4.375	0.625	4	100	2800	50	50
36	180	5	200	4.5	0.5	4	100	2800	50	50
37	185	5	200	4.625	0.375	4	100	2800	50	50
38	190	5	200	4.75	0.25	4	100	2800	50	50
39	195	5	200	4.875	0.125	4	100	2800	50	50
40	200	5	200	5	0	4	100	2800	50	50

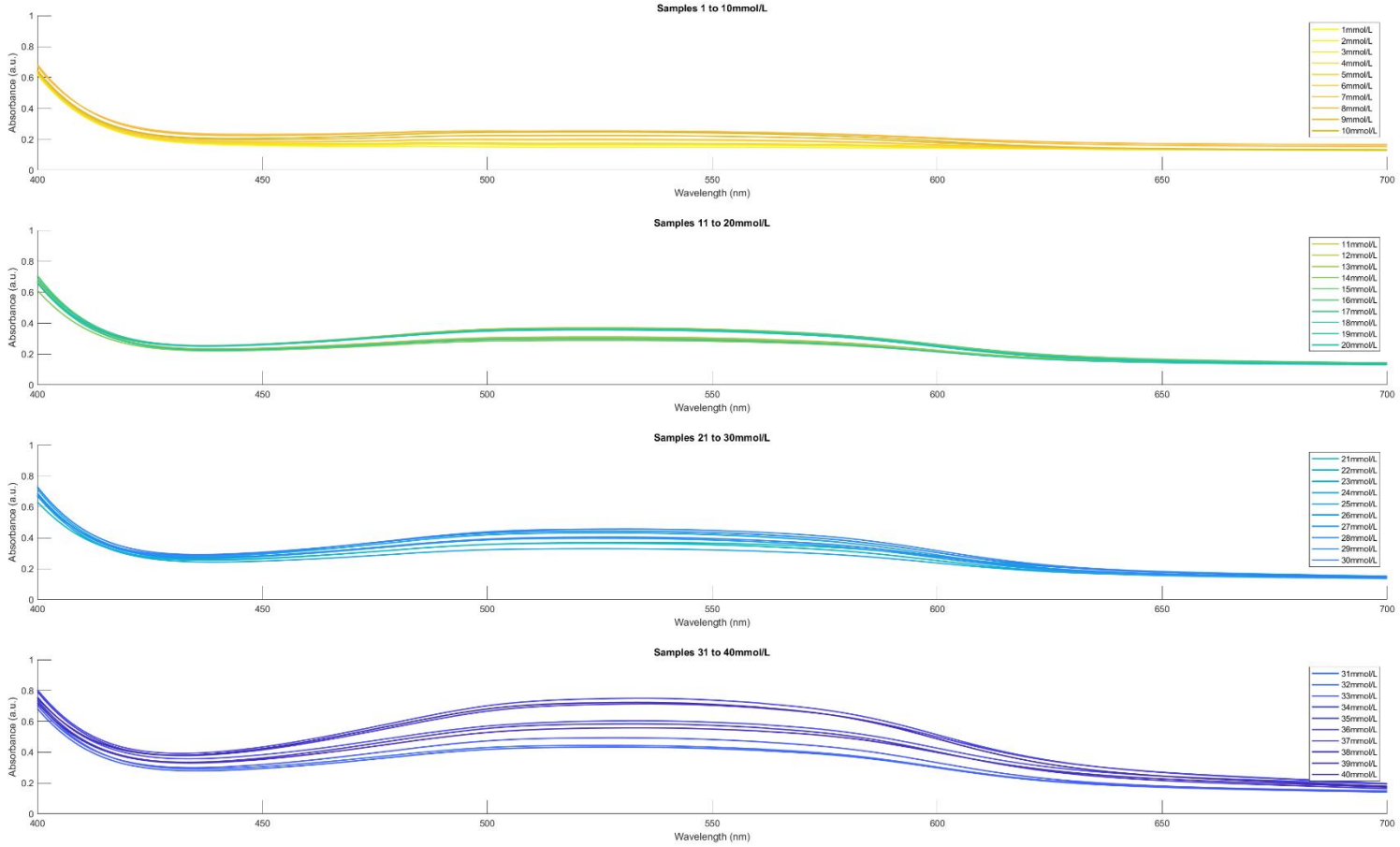

Figure 19 - Spectral visualisation of cortisol-spiked artificial saliva samples ranging from 1-40mmol/L in the spectral range 400-650nm.

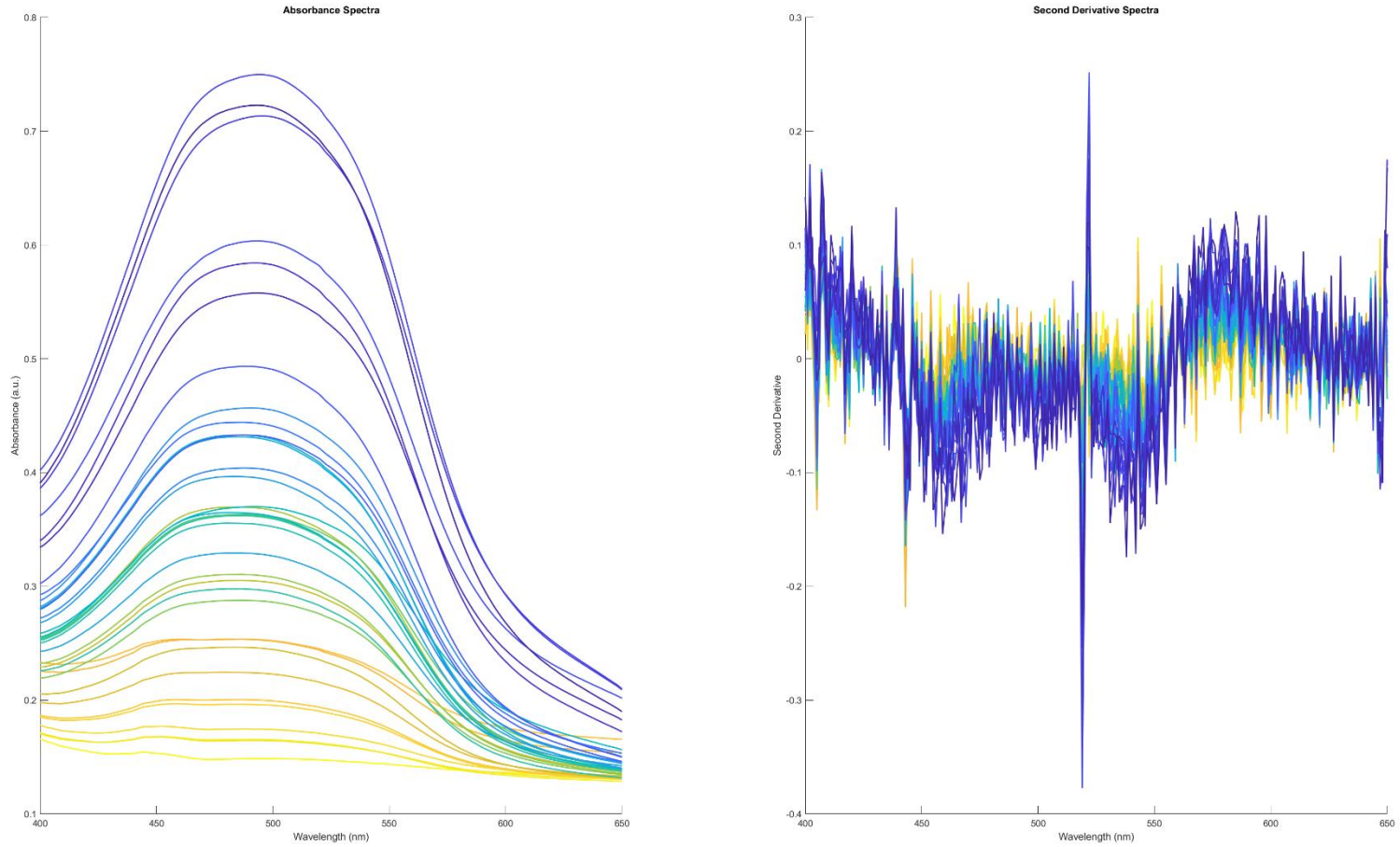

Figure 20 – Absorbance spectra of cortisol spiked saliva samples (left) with increasing colour intensity from yellow to blue showcasing increasing cortisol concentrations (left), 2nd order derivative transformation of cortisol-spiked artificial saliva samples(right) in the spectral range 400-650nm.

8.4 Discussion

The results showcase the potential feasibility of utilising colorimetric techniques for the spectroscopic evaluation of salivary cortisol from human participants. The linearity between the concentration and the colour intensity of the developed sample showcases the potential of employing this method towards a straightforward point-of-care device and system which can be introduced in clinical and home settings for patients suffering from mental illnesses or general public who are keen to use self-management tools for stress monitoring. Further studies will consider experimentation within a smaller range of salivary cortisol concentrations using cortisol spiked artificial saliva samples towards the development of a model which can be used within the actual clinical range of salivary cortisol. The development of these regression-based models will facilitate the development of a prototype sensor for real-time assessment of salivary cortisol for psychological stress monitoring.

The initial coefficient of determination for this series of experiments yielded an R^2 value of 0.83 (Figure 19) which shows great promise in further optimisation of the model towards a clinical testing range of salivary cortisol i.e., within the 0.7-27.3ng/mL range for cortisol [305]. Testing within this range would lead to greater quantification of one of the key logical indicators of psychological and physiological stress in a simple and inexpensive manner, which diverges from existing practices of subject-based interviews and time-consuming assay processing.

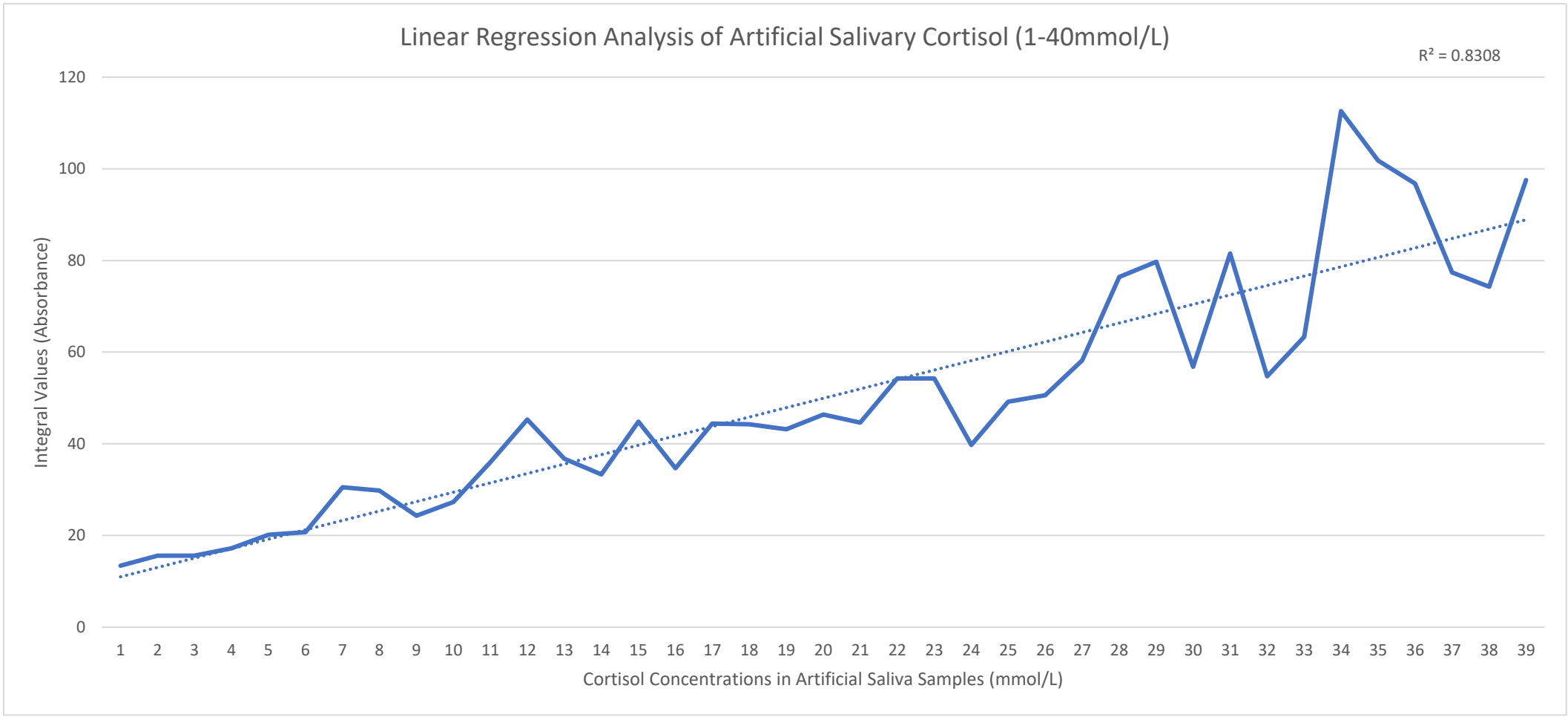

Figure 21 - Linear regression analysis of cortisol-spiked artificial saliva samples (1-40mmol/L).

9. Colorimetric determination of artificial salivary cortisol (non-clinical range) using UV-Vis spectroscopy (0.1 - 4mmol/L).

The findings reported in this chapter have been presented in the conference:

***Ahmed T,** Qassem M, Kyriacou PA. Colorimetric determination of cortisol concentrations in spiked samples of artificial saliva. BiomedEng 2022. University College London Institute of Education, London, September 2022.*

Following the initial colorimetric and UV-VIS spectroscopy-based protocol for optical determination of cortisol concentration in samples of spiked artificial saliva, the aim is to continue to develop models which can be utilised towards the determination of salivary cortisol in human saliva samples, within the clinically accepted salivary cortisol range i.e., 0.7ng/mL to 27.3ng/mL. Therefore, the current protocol aims to determine artificial salivary cortisol concentrations in a testing range from 0.1 to 4.0mmol/L with a resolution of 0.1mmol/L.

9.1 Introduction

As continuation from the previous study, the current protocol was designed to determine cortisol concentration levels from spiked artificial saliva samples in a range from 0.1 to 4.0mmol/L. The experimental design of the current protocol follows closely with the previous UV-Vis spectroscopy protocol, with the main alterations being the testing range. The significance of reducing the testing range by 10-fold dilution is due to the clinical range for salivary cortisol in humans being in the nanomolar region with respect to concentrations. Therefore, for the development of a point-of-care device for salivary cortisol determination, it is of utmost significance that the models developed for analysing cortisol concentrations via absorbance and colorimetric methods can be employed in practice, towards real-world applications. Future applications of this technology could involve utilisation towards a point-of-care sensor and mobile application for stress profiling in individuals seeking self-

management tools for psychological and physiological stress, as well as other avenues of mental health management such as anxiety and clinical depression.

9.2 Methods

As a repeated experiment from the previous protocol, the methodology of the current protocol follows closely in line. The same materials were utilised in this study which includes hydrocortisone (>98%), analytical grade methanol, tetrazolium blue chloride and tetramethylammonium hydroxide, all of which were acquired from Fisher Scientific (Fisher Scientific, Waltham, MA, USA). Artificial saliva was acquired from Sigma Aldrich (Sigma Aldrich, St Louis, MO, USA). With regards to sample preparation, the same principle of pre-samples (5ml) of cortisol in methanol were prepared, of which 0.1ml was pipetted into a mixture of 2.8ml artificial saliva and equal parts of 0.05ml volume of BT dye and tetramethylammonium hydroxide solution (Table 11). The 3ml final volume of the sample was then kept at room temperature conditions for 10 min to allow for the colour development process. Samples were pipetted into the Eppendorf UV-Vis cuvettes before being analysed spectroscopically via the PerkinElmer UV-Vis Spectrophotometer (Lambda 1050 dual-beam spectrophotometer with the 3-detector module. Each sample was spectroscopically analysed using a 3-cycle configuration to yield 3 absorbance spectra within the 400-650nm spectral range. The spectra were then processed through baseline corrections, Savitsky-Golay smoothing and averaging. All 40 samples of spiked artificial saliva were examined in triplicate.

Spectral analysis of the cortisol spiked artificial saliva samples involved linear regression modelling as well as performance metrics such as the coefficient of determination to analyse the model's determinability of cortisol concentration through absorbance readings at 510nm. MATLAB R2020b software was used for spectral visualisation and regression modelling.

A 5-point linear regression curve was developed using a small sub-set of the samples (0.1mmol/L, 1.0mmol/L, 2.0mmol/L, 3.0mmol/L and 4mmol/L) to analyse the concentration of the cortisol-spiked artificial saliva samples in the 0.1-4.0mmol/L testing range. The regression model was used as a calibration curve and can be seen in Figure 23. Furthermore, the absorbance data from all of the samples were implemented into the regression model to determine the concentration of cortisol found in each sample. Samples were also analysed

qualitatively to form a colorimetric scale depending on colour intensity, which coincides with increasing cortisol concentration, as expected (Figure 22).

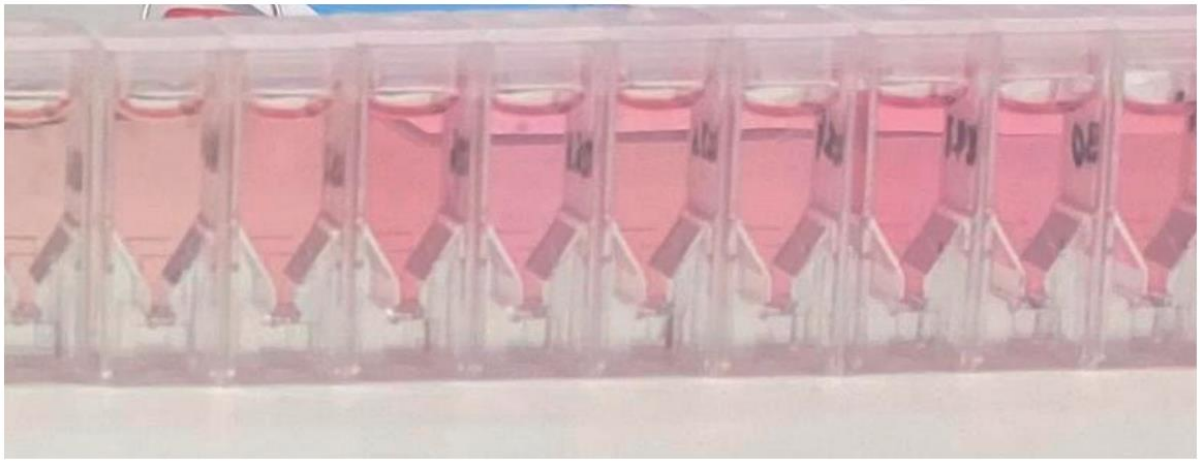

Figure 22 - Image of visual colorimetric scale depending on colour intensity, coinciding with increasing cortisol concentrations.

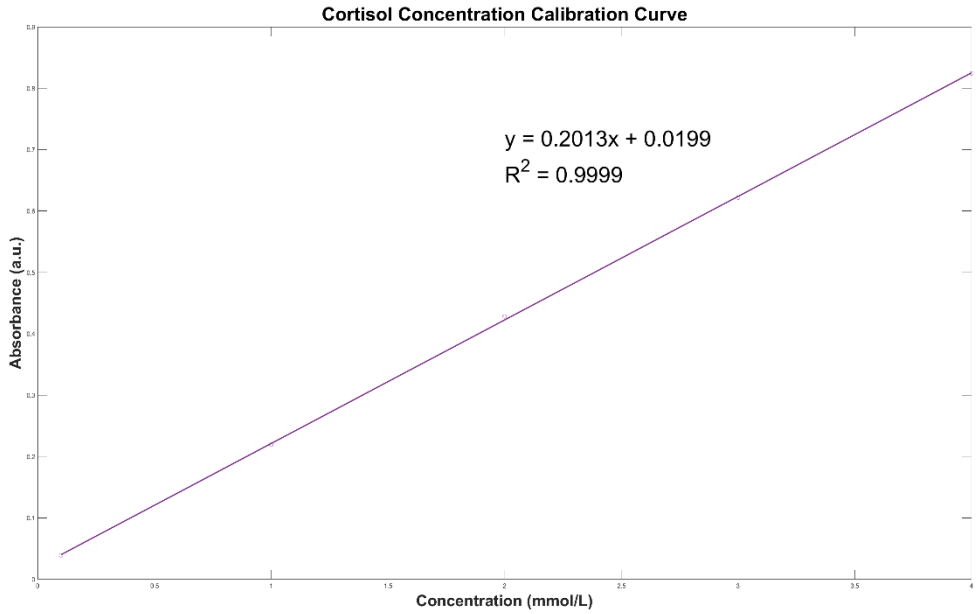

Figure 23 - Linear regression modelling based on a 5-point calibration curve for cortisol concentration analysis from artificial saliva samples.

Table 11 - Two-step Dilution Process for Cortisol in Artificial Saliva Sample Preparation for the BT method.

Desired Cortisol Concentration **in Saliva**	Cortisol Methanol Conc **C1**	Vial Volume **V1**	Stock Conc **C2**	Stock to be mixed **V2**	Methanol **Top Up**	Sample Vial Volume **ml**	Cortisol in Methanol **µl A1**	Saliva **Top Up µl**	TB Dye **µl**	T Hydroxide Agent **µl**
0.1	3	5	200	0.075	4.925	3	100	2800	50	50
0.2	6	5	200	0.15	4.85	3	100	2800	50	50
0.3	9	5	200	0.225	4.775	3	100	2800	50	50
0.4	12	5	200	0.3	4.7	3	100	2800	50	50
0.5	15	5	200	0.375	4.625	3	100	2800	50	50
0.6	18	5	200	0.45	4.55	3	100	2800	50	50
0.7	21	5	200	0.525	4.475	3	100	2800	50	50
0.8	24	5	200	0.6	4.4	3	100	2800	50	50
0.9	27	5	200	0.675	4.325	3	100	2800	50	50
1	30	5	200	0.75	4.25	3	100	2800	50	50
1.1	33	5	200	0.825	4.175	3	100	2800	50	50
1.2	36	5	200	0.9	4.1	3	100	2800	50	50
1.3	39	5	200	0.975	4.025	3	100	2800	50	50
1.4	42	5	200	1.05	3.95	3	100	2800	50	50
1.5	45	5	200	1.125	3.875	3	100	2800	50	50
1.6	48	5	200	1.2	3.8	3	100	2800	50	50
1.7	51	5	200	1.275	3.725	3	100	2800	50	50
1.8	54	5	200	1.35	3.65	3	100	2800	50	50
1.9	57	5	200	1.425	3.575	3	100	2800	50	50
2	60	5	200	1.5	3.5	3	100	2800	50	50

Desired Cortisol Concentration **in Saliva**	Cortisol Methanol Conc **C1**	Vial Volume **V1**	Stock Conc **C2**	Stock to be mixed **V2**	Methanol **Top Up**	Sample Vial Volume **ml**	Cortisol in Methanol **µl A1**	Saliva **Top Up µl**	TB Dye **µl**	T Hydroxide Agent **µl**
2.1	63	5	200	1.575	3.425	3	100	2800	50	50
2.2	66	5	200	1.65	3.35	3	100	2800	50	50
2.3	69	5	200	1.725	3.275	3	100	2800	50	50
2.4	72	5	200	1.8	3.2	3	100	2800	50	50
2.5	75	5	200	1.875	3.125	3	100	2800	50	50
2.6	78	5	200	1.95	3.05	3	100	2800	50	50
2.7	81	5	200	2.025	2.975	3	100	2800	50	50
2.8	84	5	200	2.1	2.9	3	100	2800	50	50
2.9	87	5	200	2.175	2.825	3	100	2800	50	50
3	90	5	200	2.25	2.75	3	100	2800	50	50
3.1	93	5	200	2.325	2.675	3	100	2800	50	50
3.2	96	5	200	2.4	2.6	3	100	2800	50	50
3.3	99	5	200	2.475	2.525	3	100	2800	50	50
3.4	102	5	200	2.55	2.45	3	100	2800	50	50
3.5	105	5	200	2.625	2.375	3	100	2800	50	50
3.6	108	5	200	2.7	2.3	3	100	2800	50	50
3.7	111	5	200	2.775	2.225	3	100	2800	50	50
3.8	114	5	200	2.85	2.15	3	100	2800	50	50
3.9	117	5	200	2.925	2.075	3	100	2800	50	50
4	120	5	200	3	2	3	100	2800	50	0

9.3 Results

As seen in the previous study, the presence of the characteristic absorption peak at 510nm remains prominent in the spectral visualisation of the samples in the 0.1mmol/L to 4.0mmol/L testing range. This correlates with the visual change in colour seen in samples from pale translucent yellow to magenta, of increasing intensity, as a function of cortisol concentration. The results of this study support the theory that blue tetrazolium chloride can be used as a colorimetric indicator of cortisol concentration with distinctive determinability between samples of 0.1mmol/L resolution. With a coefficient of determination (R^2) value of 0.99, it is evident that linear dependency between cortisol concentration and absorbance can be fully utilised to determine concentration of samples with unknown cortisol quantities.

Moreover, the spectral visualisation of all samples in Figure 24, further showcases the prominent linearity between absorbance and concentration. Moreover, the determinability at low concentrations i.e., below 1.0mmol/L shows great promise towards the utilisation of the blue tetrazolium (BT) method for cortisol determinability in further diluted samples, such as those in the clinical range of human salivary cortisol i.e. 0.4nmol/L to 32nmol/L.

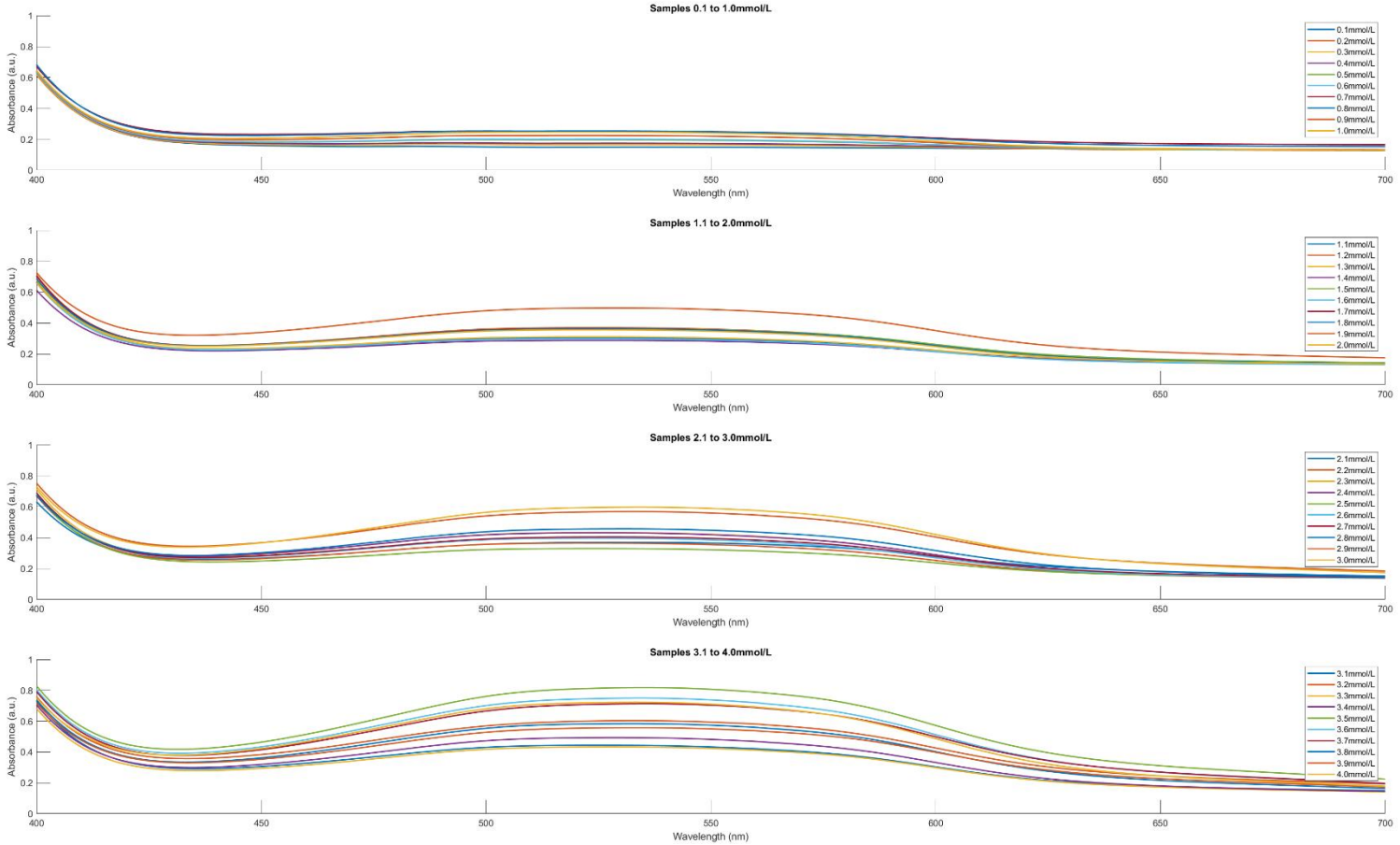

Figure 24 - Spectral visualisation of cortisol-spiked artificial saliva samples from 0.1-4mmol/L range, in spectral range 400-650nm.

9.4 Discussion

The coefficient of determination (R^2) for the salivary cortisol calibration curve was 0.99, suggesting successful determinability between salivary samples of varying cortisol concentration, including those in the lower region of the testing range i.e., 0.1mmol/L. This is an improvement from the previous study which had an R^2 value of 0.89. This may be due to the presence of a higher concentration of the BT dye and the tetramethylammonium hydroxide solution, leading to higher discernibility between results due to increased colour intensity across samples.

Moreover, visually the difference between samples of lower concentration and higher concentration of cortisol are discernible to the naked eye, as depicted in Figure 22. As the difference between samples are visible to the naked eye, this could be employed into a traffic light-based system in a point-of-care application for a user-friendly approach to stress management and monitoring. The traffic light system could focus on creating 3 levels of stress for the user:

- Low stress – pale yellow to pale pink in colour.
- Medium stress (stress response triggered) – light pink to dark pink.
- High stress (sustained stress response) – dark pink to intense dark magenta.

This system could be used in clinical and home-based settings to facilitate users in quantifying their stress levels and further destigmatising the conversation surrounding mental health [6].

10. Colorimetric determination of salivary cortisol levels in artificial saliva for the development of a portable colorimetric sensor (Salitrack).

The findings reported in this chapter have been published in:

***Ahmed T,** Powner MB, Qassem M, Kyriacou PA**. Colorimetric determination of salivary cortisol levels in artificial saliva for the development of a portable colorimetric sensor (Salitrack), Manuscript under review.*

As preliminary studies conducted for measurement of cortisol outside of the clinical range of significance have shown remarkable success, it was essential to test the feasibility of the BT method for the evaluation of cortisol levels in the ng/mL range. This chapter details the methodology followed to test the BT method on artificial saliva samples spiked with cortisol within the clinical range of 0.7-27.3ng/mL. The results of the BT method were then cross-validated against using reference ELISA measurements which are the gold-standard method for salivary cortisol analysis.

10.1 Introduction

The present study proposed the exploration of the use of blue tetrazolium dye for the measurement of artificial salivary cortisol through spectrophotometric analysis, with comparison and validation using commercial cortisol ELISA kits. This led to the establishment of a simple method for cortisol monitoring and the development of a prototype point-of-care device in the form of a pocket colorimeter for routine evaluation. Furthermore, the colorimetric method was utilised for the development of a colorimetric sensor which was tested on a small sub-set (n=17) of the artificial salivary cortisol range for sensor validation. The aim of this research is to provide accessible means for non-invasive stress hormone monitoring for regular usage, in non-laboratory-based settings by reducing the need for outsourced commercial laboratory testing kits and face-to-face interventions, which continues to remain inaccessible for the public, especially post-pandemic i.e., COVID-19.

10.2 Materials and Methods

10.2.1 Materials and Reagents

3,3'-3,3'-dimethoxy-4,4'-biphenylene bis 2,5-diphenyl-2h-tetrazolium chloride (blue tetrazolium), Tetramethylammonium hydroxide, 25 wt.% in methanol, hydrocortisone (>98%) and analytical grade methanol (>99.7%) were purchased from Fisher Scientific (Fisher Scientific, Waltham, MA, USA). Artificial saliva was obtained from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO, USA). For the development of the pocket colorimeter sensor, 1cm path length plastic disposable cuvettes were acquired from Fisher Scientific (Fisher Scientific, Waltham, MA, USA). An Arduino Uno microcontroller was used alongside blue green through-hole 20mA 510nm LEDs acquired from Farnell Electronics (Farnell Electronics, Leeds, UK). Furthermore, 30nA dark current 940nm 2-pin photodiodes were used from Farnell Electronics. A dual operational amplifier IC chip (TL082) was used (RS Components, Corby, UK). A 3D printed housing unit as developed with an Arduino Uno compartment, for the final sensor prototype.

10.2.2 Sample Preparation

A cortisol stock solution of 24umol/L was prepared by dissolving 0.008g of hydrocortisone powder into 1L of methanol. For the preparation of the blue tetrazolium dye, 1.2g of blue tetrazolium chloride was dissolved in 200ml of methanol. Additionally, a 1% v/v tetramethylammonium hydroxide solution was prepared by combining 5ml of tetramethylammonium hydroxide in 45ml of methanol. For sample preparation, diluted solutions of cortisol in methanol were made as ‘pre-samples’ of 5ml volume, of which 1ml was combined with 1.4ml of artificial saliva and 0.3ml each of blue tetrazolium solution and tetramethylammonium hydroxide solution to give a final sample volume of 3ml. The clinical range for salivary cortisol concentration is 0.7-27.3ng/mL, therefore, a calibration set (BT-Cal) was created which comprised of a series of 7 diluted solutions of cortisol in methanol (Table 1). Furthermore, a series of 34 samples of unknown concentration of cortisol within this clinically significant range were produced for spectral analysis with the BT method, Salitrack sensor and reference ELISA measurements.

10.2.3 Optical Spectroscopy Measures

For optical determination of salivary cortisol concentration from the artificial saliva samples, 2.4ml of the salivary cortisol sample was combined with equal volumes (0.3ml) of blue tetrazolium dye solution and tetramethylammonium hydroxide solution. 1ml of the prepared sample was pipetted into plastic Eppendorf disposable UV-Vis cuvettes with a spectral range between 220-1600nm and path length of 1cm. These samples were then optically analysed after 10 min using the Lambda 1050 dual-beam spectrophotometer equipped with the 3-detector module (PerkinElmer Corp, Waltham, MA, USA).

The utilisation of a chromogenic reagent such as blue tetrazolium chloride allows for the indirect measurement of salivary cortisol. Blue tetrazolium is a water-soluble nitro-substituted aromatic tetrazolium compound which oxidises the C-17 side chain of cortisol. This is subsequently reduced by free oxygen radicals in tetramethylammonium hydroxide to from nitro blue formazan. The BT method for colorimetric determination of cortisol was reported by Tu et al., for its notable colour change, visible to the naked eye, which is dependent on cortisol concentration. After the development period, the sample colour changes from translucent yellow to magenta. The colour intensity showcases a positive correlation with increasing cortisol concentrations. The observable colour change occurs due to the hydrolysis of cyclic diacetyl in the blue tetrazolium dye, with colour intensity depending on the cortisol concentration within the sample. This visible colour change can be used for the determination of cortisol levels through spectrophotometric and colorimetric methods. The characteristic absorption peak for the colorimetric reaction between the dye and cortisol is present at 510nm. A 3-cycle configuration was used to collect 3 consecutive absorbance spectra for each sample within a specified spectral region (450-650nm) at a step interval of 1nm. The reference cuvette was kept blank for the entire duration of the protocol and baseline corrections at 100% transmittance/0% absorbance was considered to reduce the effects of instrumental and ambient noise. The experiment was conducted in triplicate for each sample of artificial saliva with spiked cortisol, whereby each sample was measured three times.

10.2.4 Sensor Development

10.2.4.1 Circuit Design and Assembly

A simple electronic and portable colorimeter was developed for the measurement of spectral absorption of salivary cortisol samples at 510nm. 510nm was chosen and subsequently validated through the in-vitro measurements of artificial salivary cortisol concentrations using the benchtop spectrophotometer and blue tetrazolium (BT) method, due to the discernible characteristic peak of the nitro blue formazan product at this wavelength. A 510nm light emitting diode (LED) was used as the light source. A 30nA dark current photodiode was used at the photodetector. The components (seen in Table 11a) were connected to a TL082 dual-input operational amplifier chip as portrayed by the circuit diagram (figure 25). The circuit was developed on a miniature breadboard/Arduino shield, which was then connected to the Arduino Uno microcontroller for digitisation and further processing. The ArduinoUno and breadboard were placed into the customised 3D-printed housing unit and connected to a PC via USB.

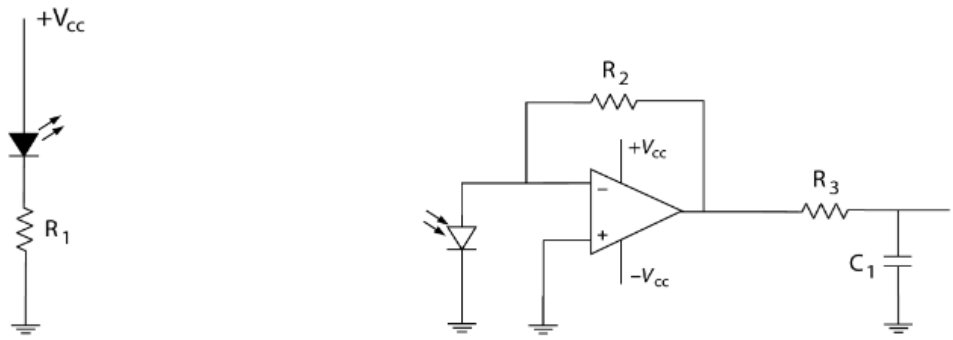

Figure 25 - Sensor circuit including LED and photodiode components for use as colorimeter.

Table 11a- Components selected for Sensor Development (Salitrack).

Component	Component Value
LED	510nm blue-green
Capacitor	220nF
Resistor	36MΩ
Operational Amplifier	TL082
Microcontroller	ArduinoUno

10.2.4.2 Testing and Calibration

The calibration of the colorimeter sensor (Salitrack-Cal) involved the use of the same samples as those used in the BT-Cal set of artificial saliva with known cortisol concentrations, which were validated by ELISAs and the benchtop spectrophotometric BT method. The sensor was then tested on the smaller sub-set (n=17) of the prediction set (SalitrackPred1) for the measurement of cortisol concentrations in a series of 17 samples with unknown salivary cortisol levels, which was then compared with the BT method, and validated with the gold-standard ELISA protocol. Voltage readings taken from the Salitrack sensor were used to calculate the absorbance of the tested samples. Testing of the Salitrack sensor was conducted within the 3D-printed housing unit, which was painted black to mimic a dark chamber for prevention of voltage fluctuations caused by ambient light. The final Salitrack sensor prototype is shown in figure 26.

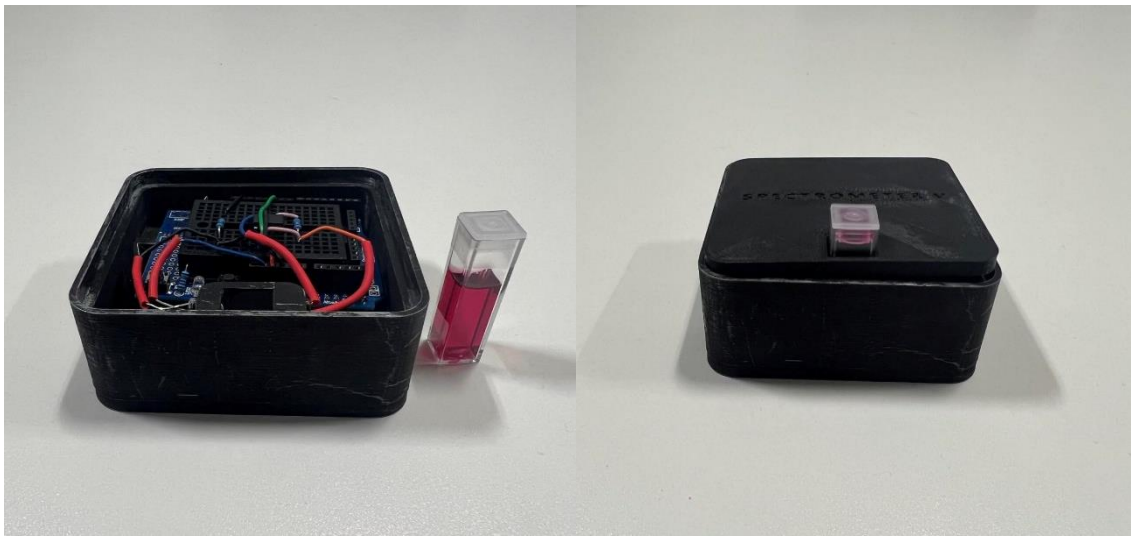

Figure 26 - Salitrack Sensor uncovered with sample (left), complete Salitrack sensor with sample inserted into collection point (right).

10.2.4.3 Sensor Optimisation and Software Development

The optimisation procedure for the sensor involved testing of the sensor with a range of feedback resistors to determine the optimal range for detection within the optical range of interest, and for further data analysis and post-processing. Software was developed for use alongside the ArduinoUno in Sketch, utilising C++. The Sketch program was run with a baud rate at 9600, to prevent complete CPU usage through the data transmission state, the delay

was set to be 20ms, to determine whether the signal was being successfully read by the Arduino board and system without lag. Furthermore, post-processing of the signal and subsequent data analysis was conducted in MATLAB, using the MATLAB support package for Arduino.

With decreased light absorbed by the sample, the intensity of the light reaching the photodiode increases, which leads to a lower resistance and smaller V_{out} reading, post-amplification. The Arduino software was developed to read the input voltage and the output voltage from the operational amplifier and calculate the absorbance of the sample using Beer Lambert's law, which dictates a linear relationship exists between absorbance and concentration, with a constant path length. The path length was maintained at 1cm through the use of 1cm plastic disposable cuvettes. Light intensity and output voltage follow an inverse relationship, which was then used to determine the absorbance, and subsequent concentration of each sample. The values were printed to the serial monitor, which in this case was a laptop. The input voltage was set at 5V. The output absorbance value can be applied to the standard curve to estimate the concentration of the artificial salivary cortisol sample, for point-of-care usage.

10.2.5 Reference ELISA Measurements

Reference measurements were made using a cortisol ELISA kit (Parameter Cortisol Assay, R&D Systems, Minneapolis, MN, USA). The BT-calibration (BT-Cal) and prediction set (BTPred) samples were measured via ELISA. The assay was prepared and used according to the recommended protocol as stated by the manufacturer to determine artificial salivary cortisol levels. 50ul of each sample was combined with 200ul of diluent (5-fold sample dilution). All samples were tested in duplicate, and a cortisol calibration standard curve was included on the assay plate. Cortisol concentrations were reported in ng/mL after considering the dilution factor (5-fold).

10.2.6 Data Analysis

10.2.6.1 Spectroscopy and Linear Regression Modelling

UVWinLab was utilised for spectral collection from the Lambda 1050 spectrophotometer, and pre-processing and analysis was carried out in MATLAB R20220b (MathWorksTM, Natick, MA, USA) which included the use of the Statistics and Machine Learning Toolbox for statistical testing of the results from the BT method, the sensor, and the cortisol ELISA protocol. Three spectra were obtained from each sample, which were then processed through baseline subtractions, averaging and Savitsky-Golay smoothing (interval 5, polynomial order 2). Subsequently, second derivative analysis was conducted to improve prominence of the characteristic peak and enhance spectral features for further evaluation. The sample calibration dataset (BT-Cal) was used to develop a linear regression model, which was then used as a calibration curve for prediction of the unknown cortisol concentrations. The peak absorbance value at 510nm were used to predict concentration levels by utilisation of the Beer-Lambert Law, which states that a linear relationship exists between target analyte concentration and absorbance of the sample.

10.2.6.2 Salitrack Sensor Performance Validation

The performance of the colorimetric sensor was validated against the BT benchtop spectrophotometric method as well as the ELISAs. A calibration curve was generated using the BT-Cal sample set of known cortisol concentrations and absorbances, which was subsequently validated with the BT method. The absorbance of each sample of known cortisol level was determined using (5), whereby A is absorbance and V_{out} and V_{in} represent the voltage outputs and input, respectively. The coefficient of determination (R^2) values from the Salitrack-Cal was compared with the BT-Cal dataset and the ELISA curve to determine the accuracy of both models against the gold standard. The performance of the sensor was tested in the determination of cortisol concentration from the SalitrackPred1 set, the absorbance data collected from the sensor was classified as SalitrackPred1. The concentration values yielded from the Salitrack method, and the BT method were individually compared with the results obtained from the cortisol ELISA protocol. Results from both the Salitrack and BT methods were compared with the gold standard ELISA results using Bland-Altman analysis.

$$A = \log\left(\frac{V_{out}}{V_{in}}\right) \quad (5)$$

10.2.6.3 Reference ELISA Analysis

Optical density values for each sample from the ELISA protocol were processed in Excel before analysis with a 4-PL (Parametric Logistic) regression model, which was conducted using the Quest Graph Four Parameter Logistic (4PL) Curve Calculator (AAT Bioquest Inc., Pleasanton, CA, USA) and MATLAB. The calibration curve was developed using the standard solutions from the cortisol ELISA kit, which was then implemented to analyse cortisol concentration levels from the artificial saliva samples. Concentration values were reported in ng/mL, after consideration of the dilution factor (5-fold dilution).

10.3. Results

10.3.1 Analytical performance of BT protocol and sensor for artificial salivary cortisol concentration determination.

The analytical performance of the BT protocol and the Salitrack sensor were evaluated through qualitative and quantitative comparison of the calibration curves derived from both methods, against the cortisol ELISA standard curve. The calibration curve (Table 12) for the BT method was determined using the calibration set of known artificial salivary cortisol concentrations (BT-Cal), this set was also used for the development of the Salitrack calibration curve. The calibration curves used for salivary cortisol determination in each of the methods, including the reference ELISA protocol, can be seen in figure 27. The BT protocol provides the basis of colorimetric determination of cortisol using the tetrazolium blue dye and tetramethylammonium hydroxide reagent. The characteristic peak of the nitro blue formazan, the product of the redox reaction between cortisol and the reagents, is seen at 510nm in the UV-Vis spectra of the samples.

The intensity/absorbance of this signal increases with increasing concentrations of cortisol, which translates visually to increasing intensity in the magenta colour, detectable by the naked eye within 10 min of the reaction taking place. There exists a linear relationship between cortisol concentration and the colour intensity/absorbance of the sample at 510nm which was further exploited to achieve colorimetric determination of salivary cortisol using

the developed Salitrack sensor. This method is supported by the Beer-Lambert Law which dictates that there is linear correlation between absorbance and concentration of an analyte.

For further enhancement and better visualisation of the linearity of the BT method, the second derivative spectra were obtained for the BT-Cal set of samples. The peak separation between the samples demonstrates the linear relationship between the cortisol concentration and absorbance. The collected spectra from the BTPred set of unknown cortisol concentration saliva samples can be seen in figure 28, after baseline subtractions, averaging and Savitsky-Golay smoothing. For better visualisation of the complete range of samples, the spectra were separated into three subsets. The observed peak is characteristic of the reaction between the cortisol and the tetrazolium blue and tetramethylammonium hydroxide reagents, showing distinctive prominence around 510-530nm across all samples. From the visualisation of the BTPred samples, it is evident that the increases in peak absorbance are proportional to the concentration of cortisol within the sample, which follows the Beer-Lambert law. The spectral relationship between the cortisol concentration of the sample and the peak absorbance is visualised for the BT-Cal sample set in figure 28.

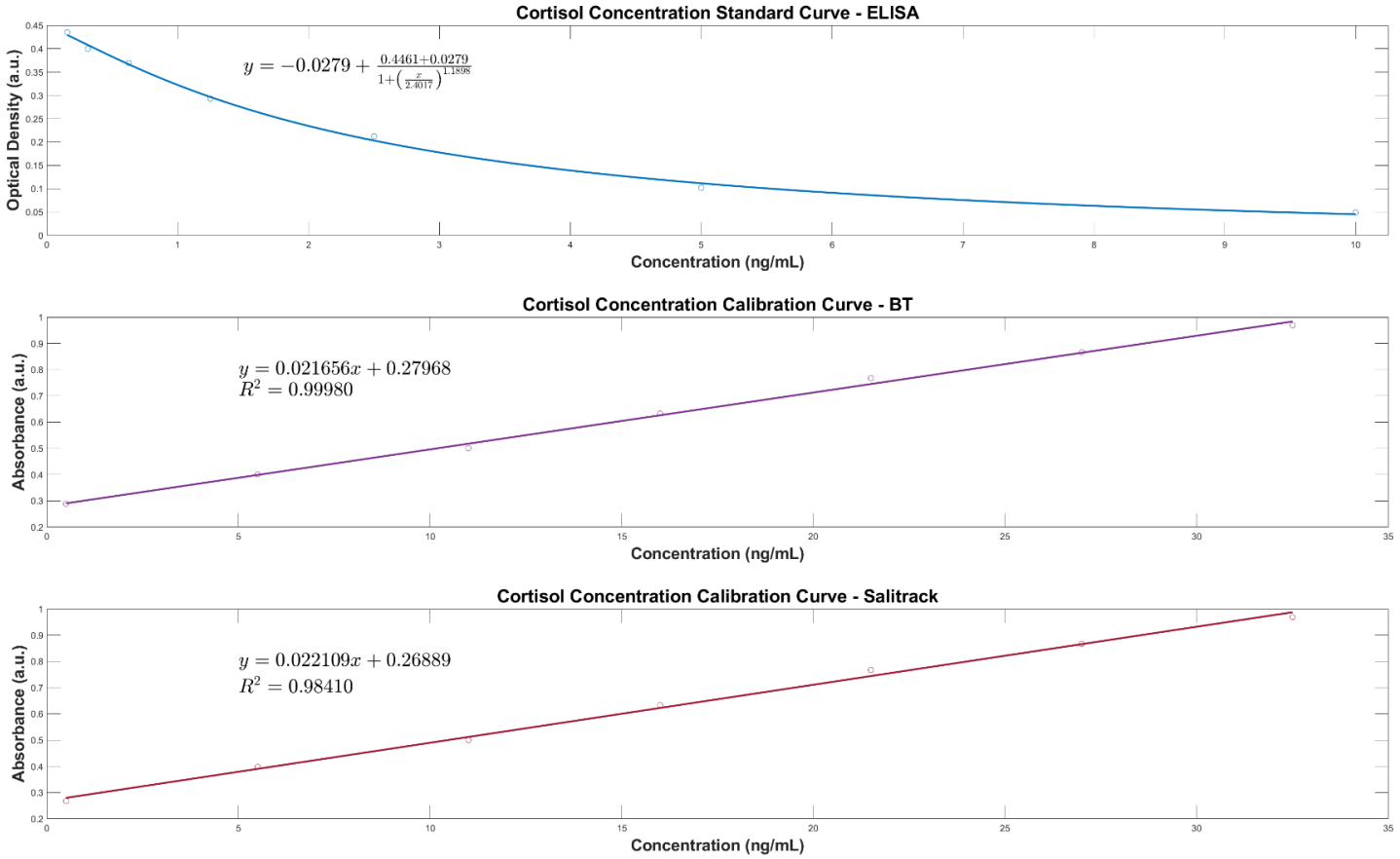

Figure 27 - Regression models for the analytical evaluation of artificial salivary cortisol in the clinical range (ng/mL) showing comparison between the gold standard ELISA protocol (Top), the Blue Tetrazolium (BT) method (Middle) and the prototype Salitrack colorimetric sensor (Bottom). The proposed BT method and prototype sensor show distinctive ability to determine cortisol in spiked artificial saliva samples.

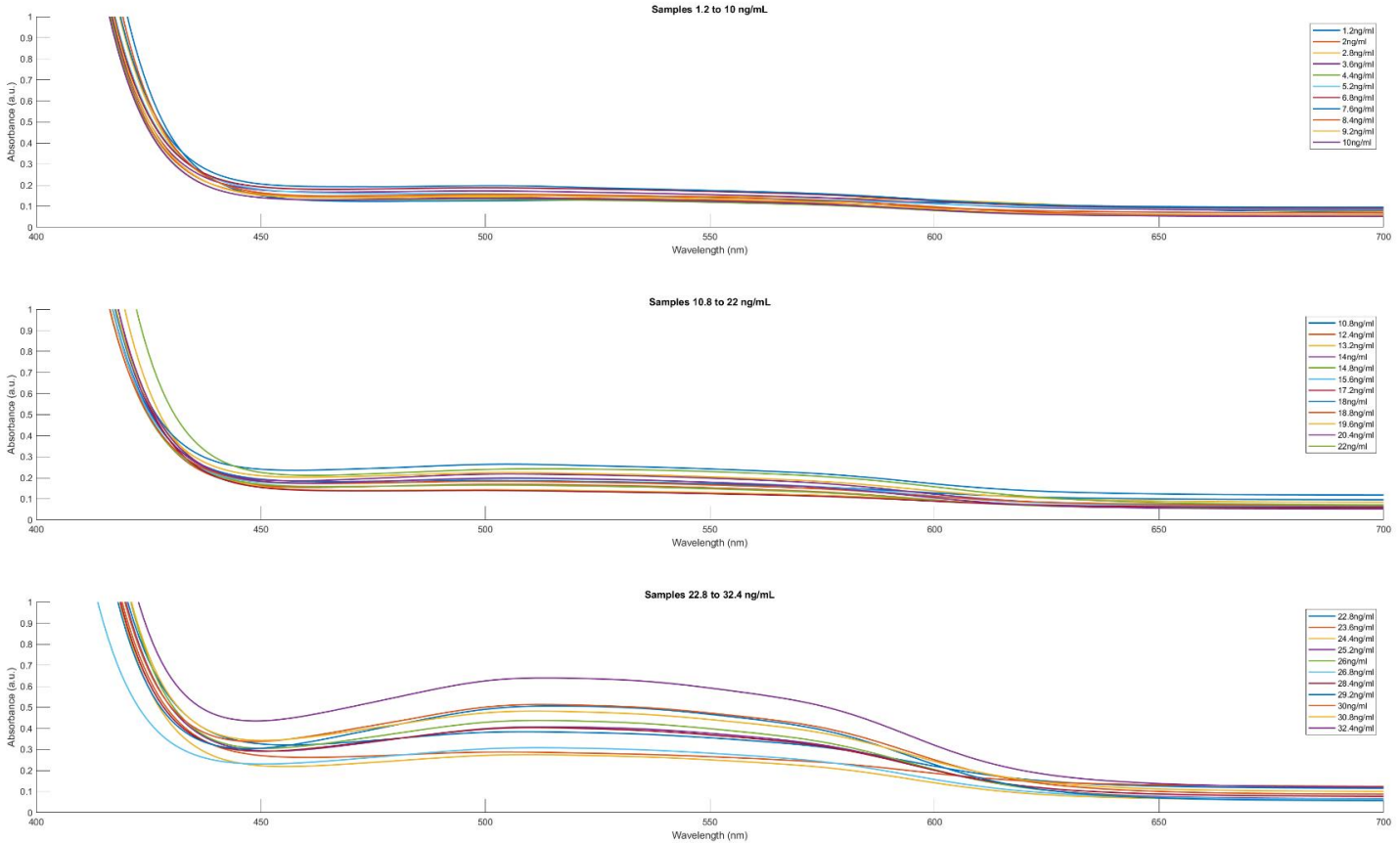

Figure 28 - Absorbance spectra of cortisol in spiked artificial saliva samples, with addition of blue tetrazolium and tetramethylammonium hydroxide reagents. Characteristic peak is observed at 510nm. Spectra shown in 3 plots ranging from 1.2ng/mL to 10ng/mL (Top), 10.8ng/mL to 22ng/mL (Middle) and 22.8ng/mL to 32.4ng/mL (Bottom), including post processing in the form of averaging, baseline corrections and Savitsky-Golay smoothing. Peak separation seen distinctly in increasing cortisol concentrations, showing clear linearity.

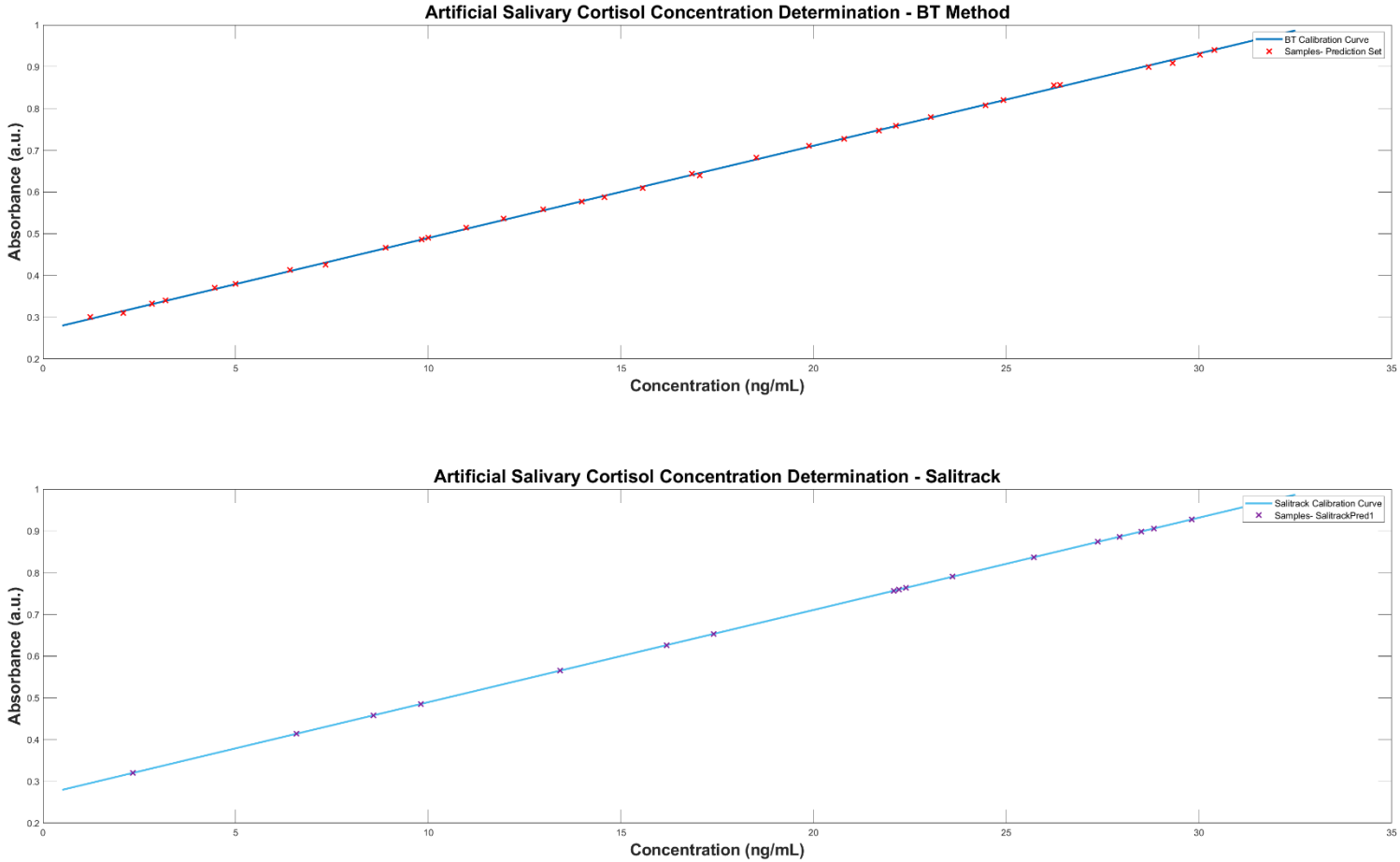

Figure 29 - Cortisol concentration determination using the BT calibration curve on the BTPred dataset (Top) and the SalitrackPred1 dataset (Bottom).

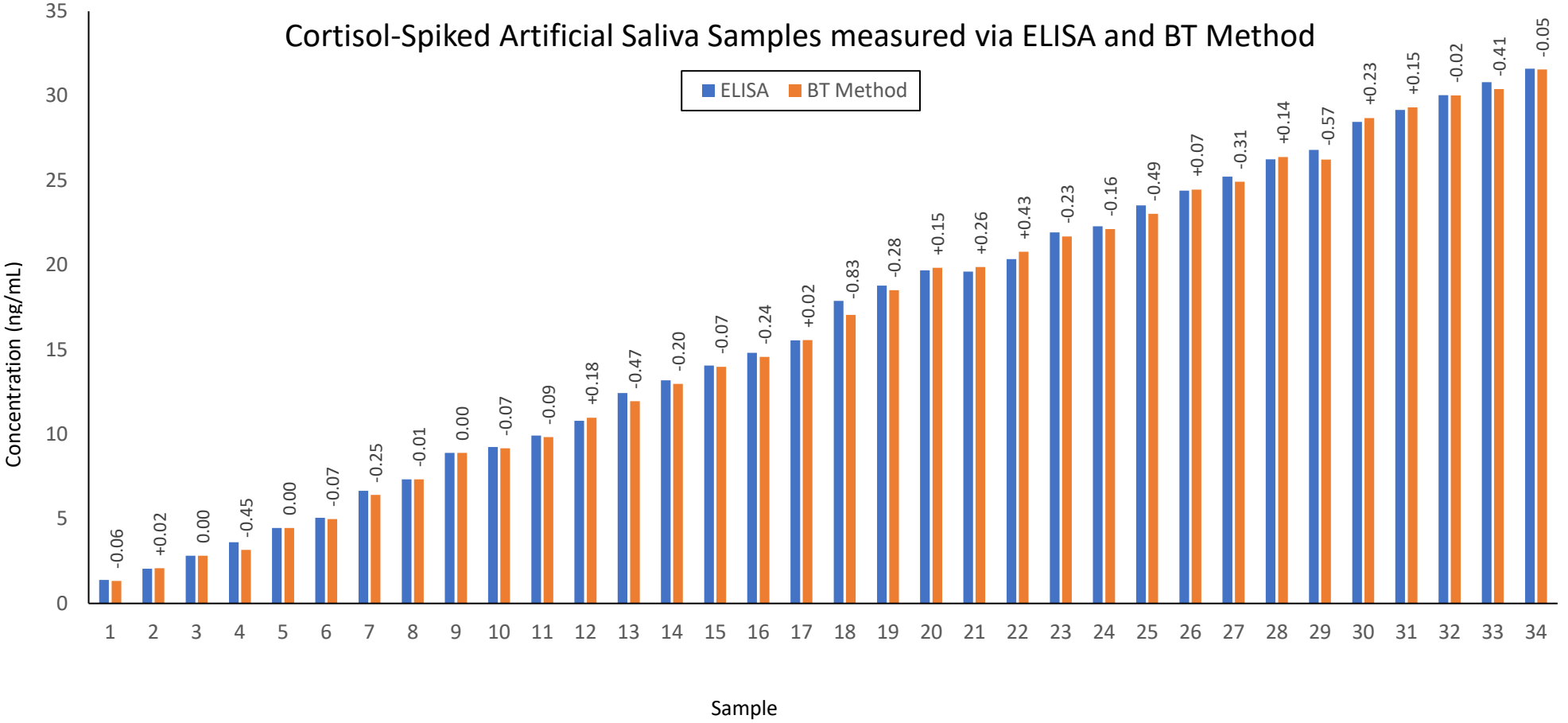

Figure 30 - Comparison between the gold-standard ELISA protocol (blue) and the BT method (orange) for cortisol determination in artificial saliva samples. Difference between concentration of cortisol between the two methods for each sample is denoted above the BT method bars, in ng/mL.

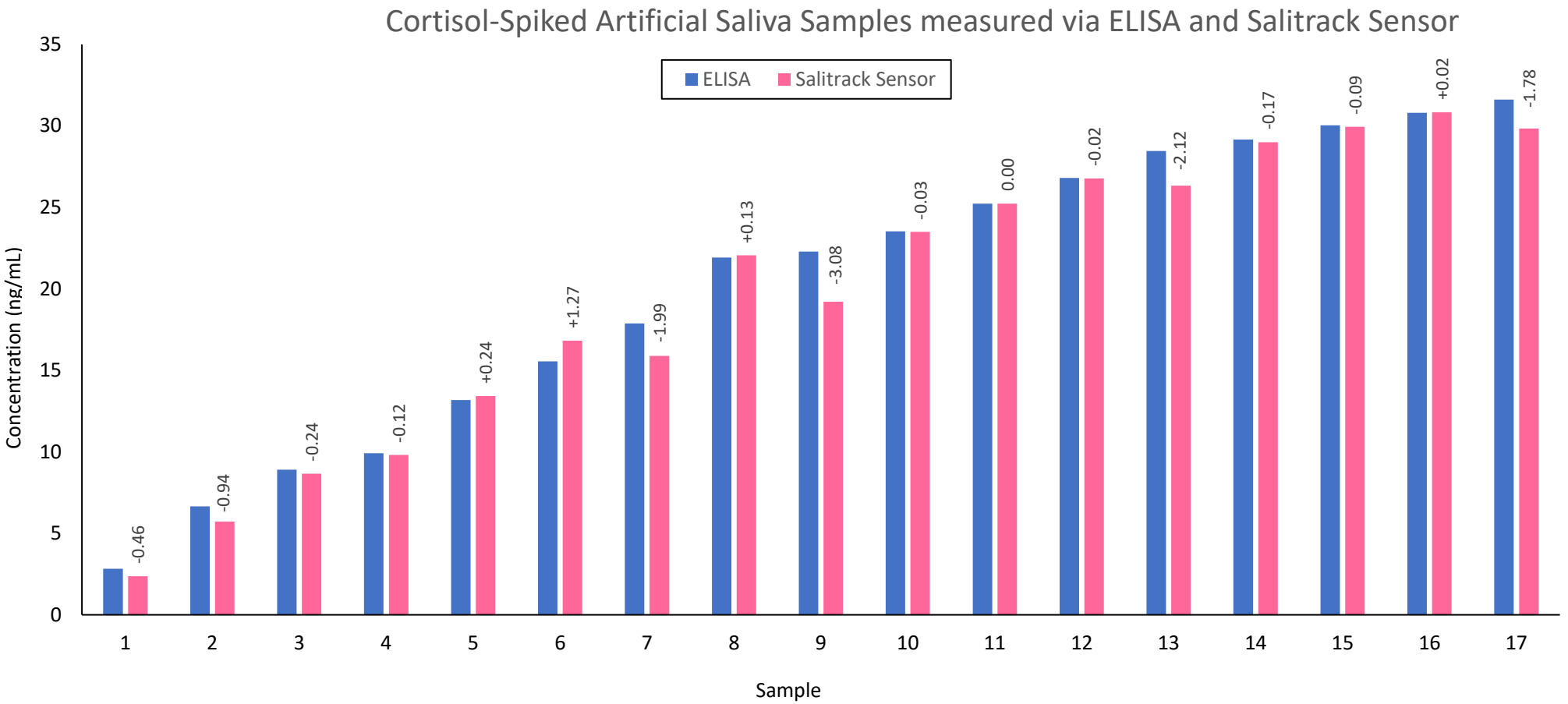

Figure 31 - Comparison between the gold-standard ELISA protocol (blue) and the Salitrack sensor (pink) for cortisol determination in artificial saliva samples. Difference between concentration of cortisol between the two methods for each sample is denoted above the BT method bars, in ng/mL.

Table 12 – Calibration set (BT-Cal) of known concentrations of cortisol-spiked artificial saliva and coinciding absorbance values at 510nm, for use in regression modelling for cortisol determination.

Artificial Salivary Cortisol Concentrations (ng/mL)	**Absorbance Value**
0.5	0.268
5.5	0.398
11	0.500
16	0.633
21.5	0.767
27	0.866
32.5	0.969

Table 13 – Performance metrics of the BT method, Salitrack sensor and the ELISA protocols.

BT vs ELISA	**Salitrack vs ELISA**
R^2= 0.99	R^2= 0.98
RMSE=3.31e-12	RMSE=1.48e-12
RMSECV=0.093	RMSECV=0.121

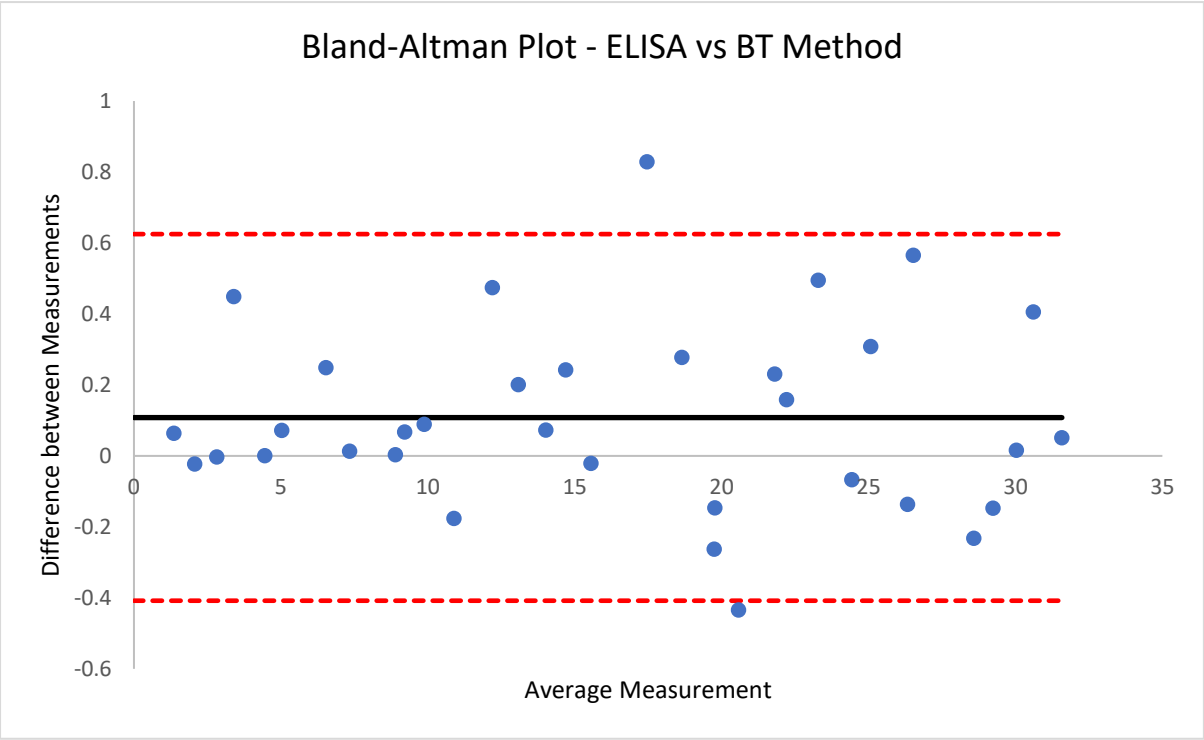

Figure 31a - Bland-Altman Plot for ELISA vs BT method. Showcasing average difference of 0.108ng/mL between concentration results obtained using the two methods.

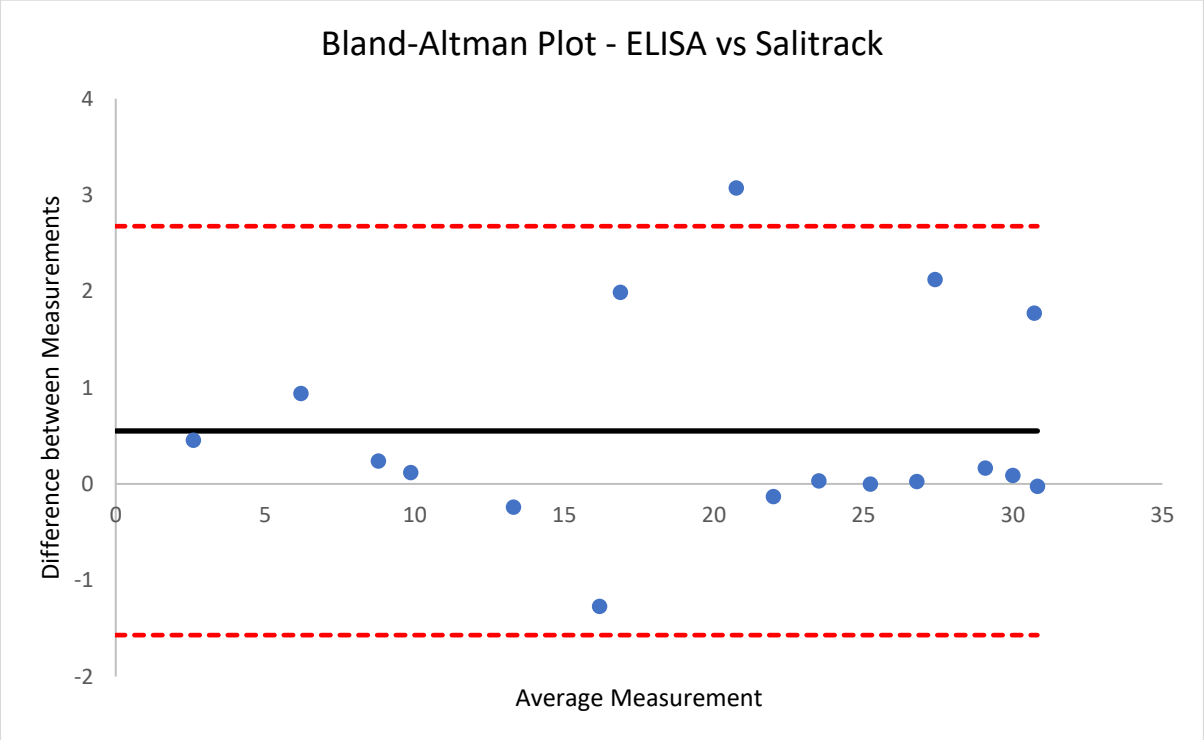

Figure 31b - Bland-Altman Plot for ELISA vs Salitrack Sensor. Showcasing average difference of 0.551ng/mL between concentration results obtained using the two methods.

10.3.2 Linear regression modelling for BT and Salitrack method at 510nm

Furthermore, the optical results obtained via the BT and Salitrack method were implemented into linear regression models to predict cortisol concentration in artificial saliva samples, with great accuracy in both cases (R^2=0.99 and R^2=0.98, respectively). A single wavelength (510nm) was chosen as this facilitated the development of a simple colorimetric point of care cortisol monitoring device and eliminated the necessity for broad-range spectral analysis. For the Salitrack sensor, the calibration curve was dependent on the saturation of implemented photodiode, therefore the relationship between cortisol concentration and absorbance shows linear correlation. Whereby, the higher the intensity of the pigment in solution (caused by increased sample concentration), the greater the absorption of light, which coincides with less light passing through the sample chamber i.e., a smaller signal is produced at the device's output. The regression analyses for the BT and Salitrack methods are shown in figure 27. The coefficient of determination of each of the models i.e., the BT method and Salitrack sensor versus the cortisol ELISA reference method showcases the successful determination of salivary cortisol concentration through utilisation of the linear relationship between cortisol concentration and peak absorbance and colour intensity, respectively.

Thereafter, the BT linear regression model was applied to the full range of spectral data collected from the BTPred samples, to investigate the feasibility of determining cortisol concentrations of unknown values in artificial saliva-based samples. As demonstrated in figure 29, via the characteristic peak at 510nm, the cortisol was detected in all samples within the prediction sets with great determinability. Further processing of the spectral data showcased the increased concentrations in samples with greater absorbance values, which follows the trend established by the BT calibration curve. The results show that the utilisation of the BT method for the determination of concentration of cortisol yields quantification of the target analyte with very high levels of accuracy. This reiterates the feasibility of the utilisation of this colorimetric method for the detection of salivary cortisol from human samples in in-vivo applications, such as cortisol monitoring during psychological stress-based interventions. This is supported by the implementation of the Salitrack regression model on a smaller sub-set of samples from the prediction set (SalitrackPred1) which demonstrated great discernibility between artificial saliva samples of varying cortisol concentrations.

10.3.3 Statistical comparison between BT protocol, sensor, and gold-standard ELISA protocol.

To analyse the accuracy of the regression models in predicting cortisol concentrations, the root mean square of errors (RMSE) was calculated. The RMSE is an absolute measure of fit of a model and can be used to determine model accuracy, with lower values indicating a better fit. To further investigate the accuracy of the BT method, which provides the basis for the Salitrack sensor, the root mean standard error of cross validation (RMSECV) was used, whereby the training set was predetermined as the ELISA data and the BT method results were used as the test set. The RMSECV is a measure of the difference between the predicted values and the actual values for the test set and can indicate the success of a model in determining the concentration of the target analyte, compared to the standard method i.e., ELISA. Lower values of RMSECV can be inferred as highly accurate modelling. The performance metrics for the regression models are stated in Table 13.

The coefficient of determination (R^2) for the BT method was 0.99, suggesting great accuracy in the spectroscopic determination of cortisol concentration with the use of the blue tetrazolium chromogenic agent. Moreover, the R^2 value for the Salitrack sensor was 0.98 which demonstrates the feasibility of using the BT method in point-of-care applications. The high values for R^2 in both cases showcase that the interrelationship between cortisol concentration and optical absorbance follows a distinctively linear trend and linear regression modelling can successfully predict cortisol concentrations in samples with unknown cortisol levels, which is ideal for feasibility of use in real-world applications. Moreover, results from both the BT method and the Salitrack sensor showcase very minor differences (0.11ng/mL and 0.55ng/mL) compared to the concentrations obtained from the reference ELISA, this is visualised in figures 30 and 31. Furthermore, Bland-Altman analysis of the results obtained from the BT method and the Salitrack versus the ELISA gold standard kits showcases average differences of 0.108ng/mL and 0.551ng/mL, respectively. This demonstrates the accuracy of both methods in the determination of cortisol concentration in artificial saliva samples, within line with the gold standard ELISA protocol.

10.4 Discussion

Commonly known as the 'fight or flight' response, the stress response is segmented into 3 key stages which regulate pathways and mechanisms to restore homeostasis within the body, upon stress elicitation[28], [32], [33]. Cortisol measurement is of utmost significance for the evaluation of the HPA axis as it is a vital regulator of the stress response in the human body. Furthermore, the non-invasive measurement of cortisol via saliva collection offers a straightforward method for evaluating stress levels within clinical and home settings. Thus, the proposed method of assessing artificial salivary cortisol through use of chromogenic agents offers a rapid and simple alternative to extensive laboratory-based analytical procedures such as liquid chromatography-mass spectrometry (LC-MS) and ELISAs[234].

Evidently, the observations from the current study define the feasibility of utilising the BT method for the spectroscopic and colorimetric determination of cortisol in artificial saliva, with great potential in applications towards measurement of human salivary cortisol levels. The proof-of-concept colorimetric sensor that was developed further reiterates that the BT method provides the basis for further technological advancements, specifically within the field of mental health monitoring and management, towards point-of-care devices for the evaluation of psychological and physiological stress. Existing technologies within this field comprise of the physiological monitoring techniques such as measurement of heart rate variability (HRV) and electrodermal activity (EDA), which provides substantial evaluation of physiological stresses, such as those experienced during periods of exercise and high physical activity[80]. However, such techniques neglect the measurement of the biochemical processes which occur during the stress response. Stress hormones, such as cortisol which govern the human stress response are key biomarkers of interest for the evaluation and quantification of physiological and psychological stress. The relationship between cortisol and stress response activity is prominent in stress studies which investigate the functionality of the HPA axis, therefore the measurement of cortisol in the body can aid in the quantification of stress and its progression into mental health disorders such as clinical depression[28]. Although cortisol is regarded as the stress hormone, several point-of-care technologies focus entirely on the physiological biomarkers of stress such as increased heart rate and irregular alpha-wave activity measured via electroencephalography (EEG)[118]. Evidently, the recognised methods for measurement of salivary cortisol involve the use of highly specialised

lab equipment and biochemical assays such as ELISAs[234]. ELISAs are considered the gold-standard method for measurement of salivary cortisol concentration but involves several time-consuming processes and remains inaccessible for routine use by the public. Hence, the employment of the BT method for the evaluation of cortisol and therefore, stress, can aid in the progression of point-of-care technologies which diverge away from benchtop applications and challenge traditional methods of stress assessment, such as subjective interviews and face-to-face interventions.

The results from the current study showcase the successful performance of the regression models for the prediction of salivary cortisol with accuracy levels that are strongly comparable to the ELISA method. It is evident that blue tetrazolium proposes an effective method of cortisol concentration measurement, which is in line with the gold-standard measurement technique. This facilitates the use of optical sensors for the mapping of psychological stress profiles, as the sensor offers readily available and highly accurate results within 10 minutes of sample collection. Further development and optimisation of the sensor can enable simple point-of-care testing alongside a mobile-based application for data collection and processing. Such advancements propose alternative means for screening therapy and mental health management, which diverge from current face-to-face based practices. Through the quantification of cortisol, which is a key logical indicator of stress in the human body, the proposed sensor facilitates the advancement of technologies towards the complete comprehension of stress, via biochemical monitoring. This challenges the existing screening methods for mental health related issues which primarily rely on interview-based tools that have led to gross misdiagnosis and poor adherence to treatment plans [29]. As mental health disorders are becoming an unprecedented global burden, the quantification and comprehension of the biomarkers of the leading disorders, such as chronic stress, offer an objective support tool for management in both the clinical environment and for point-of-care applications. Undoubtedly, using the blue tetrazolium dye exhibits great sensitivity for detecting variations in artificial salivary cortisol levels, which gives a greater understanding into the chemical interactions between the dye and the target analyte that led to the qualitative evaluation of cortisol. Notably, formation of the nitro blue formazan is the key indicator of presence of cortisol within a sample, which can be examined in the visible region of the optical spectrum for further quantitative analysis of cortisol levels for sensitive

monitoring of the clinical range of cortisol in saliva[305]. The present study sought to investigate the feasibility of developing a sensor using the BT method as the foundation for optical determination of cortisol, which yielded exceptional results and highlighted the ease of use of a visual tool for biomarker detection. The visual assessment of cortisol concentration offers a qualitative evaluation of stress with increasing cortisol levels correlating with prominent colour development ranging from translucent yellow to deep magenta. The sensor acts as an extension of this established method by measuring the absorbance of the sample to provide a quantitative result for the salivary cortisol evaluation, which can be monitored routinely in settings for stress management or for clinical evaluation of therapy adherence and efficacy.

Further elaboration of this technology will involve the optimisation of the sensor alongside the development of a smartphone application to allow for optical data analysis and storage. Mathematical modelling of collected data for a user can then be used towards the creation of personalised hormonal profiles which can predict stress levels for individual users, based on their lifestyles and hormone trends. Moreover, the introduction of other stress-related biomarkers would lead to robust modelling of user stress profiles, such as the inclusion of other stress hormones like adrenaline and noradrenaline, or antagonistic biomarkers like dehydroepiandrosterone sulphate (DHEA-S). The antagonistic relationship between cortisol and DHEA-S should be considered for the complete comprehension of stress regulation in the human body[306]. The current study has proposed a method for the optical determination of cortisol in samples of artificial saliva with great sensitivity and accuracy, as well as the development of a proof-of-concept sensor which quantifies the colorimetric reaction to ensure precise results are obtained as a readout signal within 10 min. Therefore, the proposed methodology offers a time-efficient and straightforward approach for cortisol measurement which eradicates the need for lengthy preparatory steps which are required from existing methods for cortisol analysis. Furthermore, the colorimetric sensor offers a rapid and cost-effective solution for accurate at-home cortisol testing which eradicates the need for sending off saliva samples for lab-based testing, which involves a plethora of costs such as sample transportation and processing, as well as general establishment costs.

10.5 Conclusion

In conclusion, the potential impact of the introduction of a colorimetric method for the measurement of cortisol levels, and in turn the evaluation of psychological stress is immense. With a growing population of mental health disorders, the necessity for simple and straightforward point-of-care technologies for mental health management is vastly prevalent. The current study has demonstrated the success of the BT method and the Salitrack sensor for the rapid and precise determination of cortisol levels in artificial saliva, with R^2 values of 0.99 and 0.98, respectively. Therefore, the employment of such methods proposes a feasible alternative to existing technologies which are less efficient with respect to costs and time. Further advancements of this method should involve the development of a mobile-based application for the complete development of a point-of-care device for stress monitoring, which can be used routinely for psychological stress evaluation and management.

11. Rapid optical determination of salivary cortisol responses in individuals undergoing physiological and psychological stress.

The findings reported in this chapter have been published in:

***Ahmed T,** Powner, MB. Qassem M, Kyriacou PA**. Rapid optical determination of salivary cortisol responses in individuals undergoing physiological and psychological stress. Manuscript under review.*

The previous chapter concluded the success of the BT method for determination of cortisol levels from artificial saliva samples, therefore for progression towards the development of a point-of-care application, it is essential to consider the use of the BT method for determination of cortisol from human saliva samples. The chapter outlines the present study which utilised one of the noted chromogenic agents (blue tetrazolium) for the determination of cortisol in human saliva samples, taken from participants who underwent a modified version of the Maastricht Acute Stress Test (MAST)[307].

11.1 Introduction

The MAST has been found to yield superior salivary cortisol responses in a rapid and non-invasive manner for effective stress elicitation [307]. Evidently, Tu et al. concluded that the BT method is comparable to the gold-standard ELISA technique due to the rapid reaction rate, low limit of detection (LoD) and greater range of detection [308]. Therefore, the measurement of salivary cortisol is an established method for determining psychological stress noninvasively, albeit through enzymatic methods such as ELISA. Tu et al. reported the use of various chromogenic agents for the determination of cortisol concentration through a series of in vitro experiments, and a pilot study using human sweat samples [[308]. Presently, the colorimetric determination of salivary cortisol concentration was observed through optical spectroscopy in the visible region (400-800nm). This method facilitates the rapid and accurate detection of salivary cortisol concentrations, which strongly correlated with cortisol ELISAs i.e., the gold standard. This study is fundamental step towards the development of a point-of-care device for rapid measurement of salivary cortisol levels in stressed individuals.

11.2 Materials and Methods

11.2.1 Participants

A total of 22 healthy adults with a mean age of 28.18 ± 9.00 years participated in the current study, including 12 males and 10 females. 3 participants from this cohort were excluded from this study due to insufficient saliva samples. The participant information sheet was provided to interested volunteers and eligibility was assessed using a screening questionnaire. Exclusion criteria was adopted from Smeets et al., which included diagnosed mental illnesses, endocrine disorders, cardiovascular diseases, pregnancy, oral infections, heavy smoking (>10 cigarettes/day), recreational drug use and excessive alcohol consumption (>14 units/week)[307], [309]. Written informed consent was provided by all participants. This study was carried out under approval by the City, University of London, School of Science and Technology Senate Research Ethics Committee. Participants were informed to refrain from caffeinated drinks at least 3 h prior to the MAST and abstain from food or drink consumption 1 h prior to the test. This was implemented to ensure that the results of the salivary analysis were not influenced by recent food or drink intake. Furthermore, the protocol was conducted at approximately similar times (between 12pm-4pm) for all participants to ensure the effects of the circadian rhythm of cortisol responses did not interfere with the study's findings.

11.2.2 Maastricht Acute Stress Test Protocol

Upon informed consent, participants were informed that the stress test would involve alternating trials between hand immersion (HI) and mental arithmetic (MA) tasks of randomly determined durations. Participants were unaware of the number and duration of trials. The HI task involved immersion of the participant's non-dominant hand into ice-cold water (2°C). The MA task involved counting aloud backwards from 2043 in steps of 17. Participants were informed that mistakes with accuracy or slow response (>5s) would lead to negative feedback from the experimenter and restarting of the MA task. The duration of all trials was pre-determined, and the same pattern was used for all participants. After the MAST was completed, the participant was debriefed and given instructions for the intervention period. The intervention period consisted of a 20 min relaxation phase where the participant was advised to relax in the monitoring room, unmonitored by the experimenter.

11.2.3 Physiological Measures

Salivary cortisol was measured in response to the MAST as a measure of stress activity. For each participant, a total of four saliva samples were collected via the Cortisol Salivette (Cortisol Salivette®, SARSTEDT, Numbrecht, Germany). For sample collection, the Salivette® swab is placed in the mouth for 2 minutes without chewing. A baseline saliva measurement was taken prior to the MAST (T-Base), immediately after MAST completion (T-0), 20 min after completion (T-20) and post-relaxation (T-Relax). After collection, the swab is placed into the Salivette Cortisol tube for centrifugation at 1000g for 2 min at room temperature, using the Biofuge Primo R (Sorvall, Waltham, MA, USA). The average saliva volume that was recovered was 1.8 ± 0.3ml. Centrifuged samples were stored at -80°C before subsequent analysis. Cortisol levels were determined spectroscopically as well as with commercially available ELISA kits (Parameter Cortisol Assay, R&D Systems, Minneapolis, MN, USA).

11.2.4 Subjective Measures

The NHS depression and anxiety self-assessment quiz was administered as a baseline measurement of participant mental health state, prior to starting the MAST[310]. The quiz consists of 18 questions based on the Personal Health Questionnaire (PHQ-9) and the Generalised Anxiety Disorder Scale (GAD-7) developed by Kroenke and Spitzer et al. as methods to assess and monitor depression and anxiety severities, respectively [[311], [312]. Based on the responses from the assessment, participants were given a depression score from 1-21 and an anxiety score from 1-21, with increasing scores correlating with increased severity of depression and anxiety symptoms. To ensure the safety of all participants, those scoring above 12 were advised to withdraw from the study, as approved by the Senate Research Ethics Committee, City, University of London. Immediately after the MAST protocol, participants were asked to rate their experiences of stress on a 5-point Likert scale, ranging from 1 to 5 (1=not stressful at all, 5=extremely stressful). The subjective measures are seen in Table 15.

11.2.5 Reagents

3,3'-3,3'-dimethoxy-4,4'-biphenylene bis 2,5-diphenyl-2h-tetrazolium chloride (blue tetrazolium), Tetramethylammonium hydroxide, 25 wt.% in methanol, and methanol

(>99.7%) were acquired from Fisher Scientific (Fisher Scientific, Waltham, MA, USA). For the preparation of the dye, 1.2g of blue tetrazolium was dissolved in 200ml of methanol. Additionally, a 1% v/v tetramethylammonium hydroxide solution was prepared by diluting 5ml of tetramethylammonium hydroxide in 45ml of methanol. As stated by Tu et al., methanol decreases the reaction time by 25%, with no changes in sensitivity[308]. Therefore, analytical grade methanol (>99.7%) was used as the primary solvent for reagent preparation.

11.2.6 Optical Spectroscopy Measures

To determine cortisol concentration in saliva samples through optical spectroscopy, 50µl of the centrifuged saliva was combined with equal volumes (200µl) of blue tetrazolium dye solution and tetramethylammonium hydroxide solution (4-fold sample dilution). The BT method was experimentally validated for the visual inspection of cortisol concentration by Tu et al. where the characteristic absorption peak of the colorimetric reaction was 510nm[308]. The visual colour change, from translucent yellow to magenta, is stable for up to 12 h, and therefore, measurements were taken after the 10 min development period. The intensity of the magenta colour increases with rising concentrations of cortisol; therefore, the determination of salivary cortisol at high levels is detectable by the naked eye. The peak absorption rises with respect to increasing salivary cortisol concentrations, showcasing a linear relationship. The colorimetric reaction is dependent on the hydrolysis rate of cyclic diacetyl in the blue tetrazolium dye, whereby the cortisol concentration determines the rate of full colour development in the sample. The cortisol concentration from each sample was reported in ng/mL after accounting for the dilution factor (4-fold sample dilution).

Notably, the colorimetric reaction is stabilised in solvents with a large dielectric constant e.g., water, which indicated the suitability of measuring cortisol levels in saliva via the BT method, as the composition of saliva is 99% water. Hence, the redox reaction which leads to the colour development as a function of cortisol concentration was decelerated to a discernible level, which could be detected by the spectrometer. 450µl of the prepared sample was pipetted into plastic Eppendorf disposable UV-Vis transparent cuvettes, with a spectral range between 220-1600nm and path length of 10mm. Three consecutive absorbance spectra were acquired for each prepared sample with the use of a dual beam spectrophotometer (Lambda 1050) in

the spectral region between 450-650nm (PerkinElmer Corp, Waltham, MA). The spectrophotometer was configured in a 3-cycle format, to yield three spectra from each sample, within the specified spectral region, at a step interval of 1nm. The Lambda 1050 was equipped with the 3-detector module. Reference and sample attenuators were set to 100% and the reference cuvette was kept blank for the entire duration of the protocol. Baseline correction of 100% transmittance/0% absorption was taken to account for the effects of instrument and ambient environmental noise.

11.2.7 ELISA

Cortisol ELISA kits (Parameter Cortisol Assay, R&D Systems, Minneapolis, MN, USA) were used according to manufacturer's recommended protocol with a 5-fold saliva sample dilution, to determine salivary cortisol levels. 50µl of saliva sample was mixed with 200µl of diluent. Cortisol concentrations were reported in ng/mL after considering the sample dilution. All samples were tested in duplicate, and a cortisol calibration standard curve was included on each assay plate.

11.2.8 Data Analysis

11.2.8a Spectroscopy and Linear Regression

Spectra collection was carried out using UVWinLab for the Lambda 1050 spectrophotometer. Spectral pre-processing and analysis were performed in MATLAB R2020b, (MathWorksTM, Natick, MA, USA), with use of the Statistics and Machine Learning Toolbox, on two datasets of samples to determine salivary cortisol concentrations in participants undergoing the MAST protocol. The datasets were divided according to sample placement on each ELISA plate, for simpler comparison and validation. 3 spectra were acquired from each sample, which were pre-processed through baseline subtractions, averaging, and smoothing. Savitsky-Golay smoothing was implemented (interval:5, polynomial order:2), as well as 2^{nd} derivative calculations, to reduce noise and improve discernibility between samples through enhanced spectral features. One dataset comprising of samples of known cortisol concentrations in artificial saliva was used to develop a calibration curve, which was later implemented in a linear regression curve to create a prediction model. The range of cortisol concentrations

used as a calibration set can be found in Table 14. Peak absorbance values at 510nm from each spectrum were used to predict concentration values against the prediction model. This was compared against the results obtained from the cortisol ELISA protocol using the Pearson's correlation coefficient.

Table 14 – Calibration set of known concentration cortisol-spiked artificial saliva samples and representative absorbance values at 510nm, for use in predictive regression model for cortisol determination in human saliva samples.

Artificial Salivary Cortisol Concentrations (ng/mL)	**Absorbance Value**
0.5	0.268
5.5	0.398
11	0.500
16	0.633
21.5	0.767
27	0.866
32.5	0.969

11.2.8b ELISA

Optical density results were obtained from each sample through the cortisol ELISA protocol. Pre-processing of this data was conducted in Excel, which involved averaging duplicates and subtracting the B0 value from each plate respectively. The results were then analysed as recommended in the manufacturer's instructions, using a 4-PL (Parameter Logistic) regression model, Quest Graph™ Four Parameter Logistic (4PL) Curve Calculator (AAT Bioquest Inc., Pleasanton, CA, USA). A calibration curve was developed using the non-linear regression model which was then implemented to determine the cortisol concentrations of all samples within the two datasets with a total of 19 participants i.e., 76 samples. The cortisol concentrations for each sample were reported in ng/mL, after accounting for the dilution factor (5-fold sample dilution).

11.3 Results

11.3.1 Analytical performance of BT protocol for salivary cortisol concentration determination.

The samples were separated into two datasets, to replicate the same order that was used in the cortisol ELISA protocol. A BT calibration curve was determined with a calibration set of known cortisol concentrations in artificial saliva samples which was then compared with the cortisol ELISA standard curves, shown in figure 32. The characteristic peak around 510nm from the UV-VIS spectra of the calibration set demonstrates the linear correlation between increasing cortisol concentration and absorbance. This linear relationship was used to predict salivary cortisol concentrations in the human saliva samples acquired from participants undergoing the MAST protocol. The Beer-Lambert Law was employed to determine the cortisol concentration from the samples. The law states that a linear relationship exists between the analyte concentration and the absorbance of the sample. A linear regression model (R^2=0.997) was utilised to facilitate in the quantification of the target analyte concentration (cortisol) in the collected saliva samples. The pre-processed spectra from the in-vivo trial are visualised in figures 33 and 34. The results demonstrate the prominence of the 510nm characteristic peak, with fluctuating absorbance values in varying samples collected from each participant (n=19). The characteristic peak is associated with the reaction between the blue tetrazolium dye-reagent mixture and the cortisol present in the saliva sample. A peak at 510nm corresponds with the absorbance of the colour green, which is complementary to the magenta colour that is observed within 10 minutes of the colorimetric reaction taking place (figure 35). The absorbance at 510nm changes as a function of cortisol concentration, which is further reinforced by the increased intensity of the magenta colour seen in samples of higher cortisol concentration, validated by the cortisol ELISA protocol. The peak absorbance values for each spectrum were implemented into the linear regression model to yield predicted cortisol concentration values for the collected saliva samples, presented in figure 36. The second derivative spectra were obtained for all samples, to further enhance the spectral features at 510nm. The second derivative spectrum is often used to quantify the absorption of sample for subsequent determination of the concentration of the target analyte, which is cortisol in this case. The peak separation in the second derivate spectra of all samples further emphasises the linearity of the relationship between blue

tetrazolium and cortisol. Thus, reiterating the feasibility of utilising this method towards point-of-care applications in cortisol monitoring. Through the spectral analysis of the collected data, the salivary cortisol concentration for each participant undergoing the MAST protocol was mapped in figures 37 and 38 for simpler observation of the trajectory of cortisol levels from baseline measurements (T-Base, point of stress elicitation (T-0) to stress recovery (T-20) and intervention (T-Relax).

11.3.2 Analysis of salivary cortisol concentrations during MAST protocol via BT and ELISA methods

Fluctuations in salivary cortisol concentrations throughout the duration of the MAST protocol amongst all participants' samples are presented in figures 37 and 38. Evidently, the salivary cortisol concentration values obtained from the prediction model devised from the BT method shows clear likeness to the known cortisol concentration values obtained from the ELISAS. Thus, there is confidence in the feasibility of employing the BT method for salivary cortisol determination within line of the existing gold standard protocols that are utilised by commercial laboratories. Distinctly, the peak cortisol level for each participant differs vastly throughout the MAST protocol, for example, participant 1 showcasing very high baseline cortisol levels (22ng/ml) with cortisol levels diminishing upon stress elicitation to 4ng/ml. Comparatively, the observations from samples collected from participant 10 presents low baseline cortisol concentration (1.6ng/ml) to a spike in cortisol upon stress elicitation and recovery in stages (T-0, T-20) with concentrations reaching 6 ng/ml. The disparity amongst cortisol concentrations in participants unveils the necessity for further stress/cortisol profiling analysis.

11.3.3 Statistical comparison and validation of performance of BT protocol versus gold-standard ELISA protocol.

As mentioned previously, ELISAs are the gold-standard technique for gold-standard for salivary cortisol measurement. Therefore, all samples were investigated using cortisol ELISA kits to yield optical density results which were analysed to determine cortisol concentration using standard curves for calibration, shown in figure 1 for each plate. Predicted cortisol concentration values from the BT method were compared the results from the cortisol ELISA

protocol to analyse the validity of the proposed optical method against the clinical standard. To analyse the correlation between the predictive models for cortisol concentration, the Pearson's correlation coefficient was determined (Table 16). There is strong correlation between the BT method and the ELISA method for cortisol concentration as shown in Table 1 for both plate 1, *r(38) = .99, p<.0001*, and plate 2*, r(34) =.99, p<.0001*.

Table 15 - NHS Self-Assessment Depression and Anxiety Inventory Scores, alongside subjective stress rating for each participant.

Participant	**Depression Score**	**Anxiety Score**	**Stress Rating**
P1	4	5	4.5
P2	0	0	4
P3	8	4	2.5
P4	0	0	3
P5	0	1	4
P6	9	8	2.5
P7	4	1	4
P8	2	4	4
P9	8	7	3.5
P10	11	11	3.5
P11	4	3	2.5
P12	5	3	4
P13	1	1	1.5
P14	7	9	3.5
P15	0	1	3
P16	2	0	1
P17	5	3	3
P18	1	2	3
P19	0	0	2

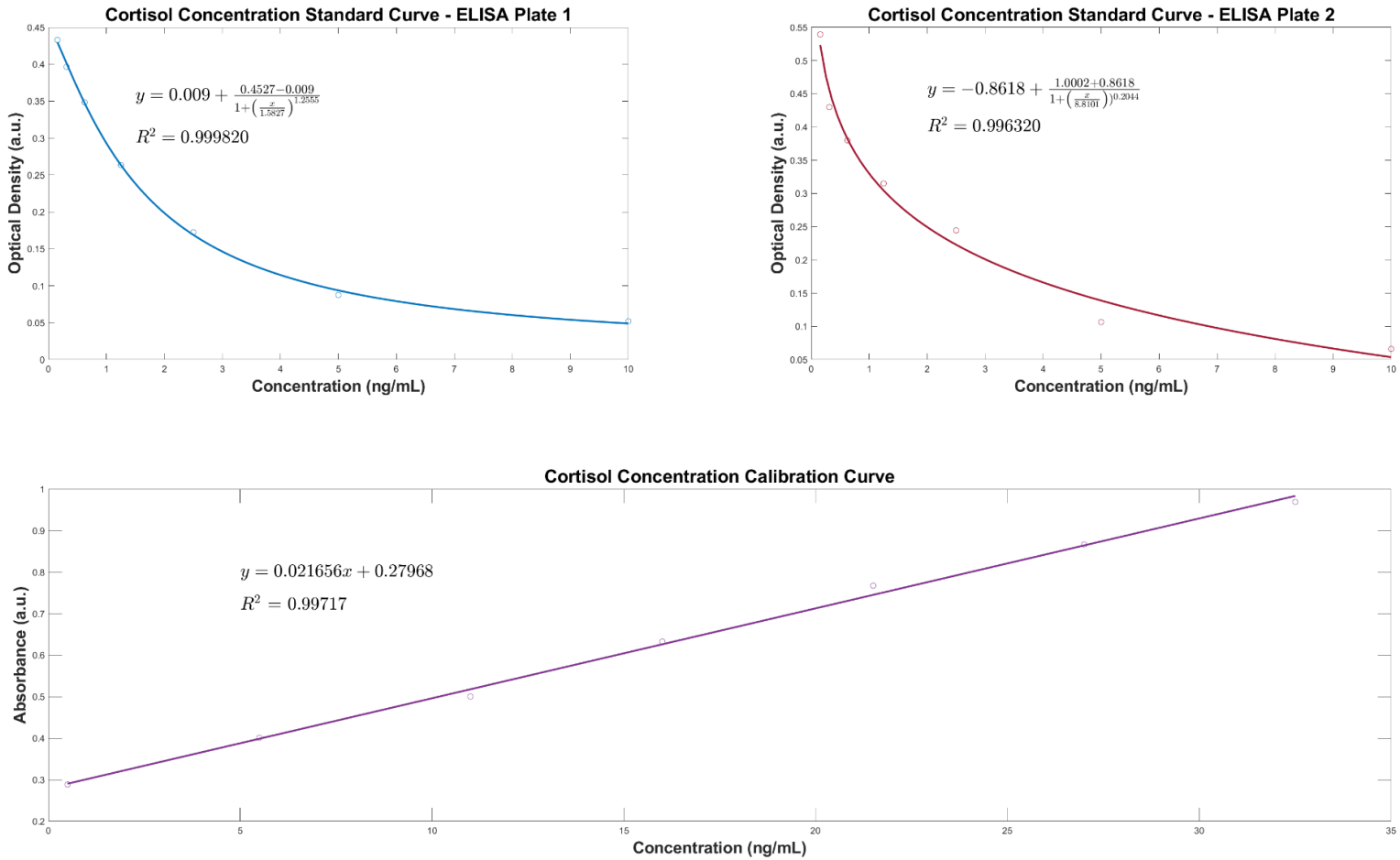

Figure 32 - Analytical performance comparison of the ELISA protocol (top left and right) and Blue Tetrazolium (BT) method (bottom) for monitoring salivary cortisol in clinical range (ng/mL) using linear regression analysis. Both methods show high sensitivity for cortisol determination.

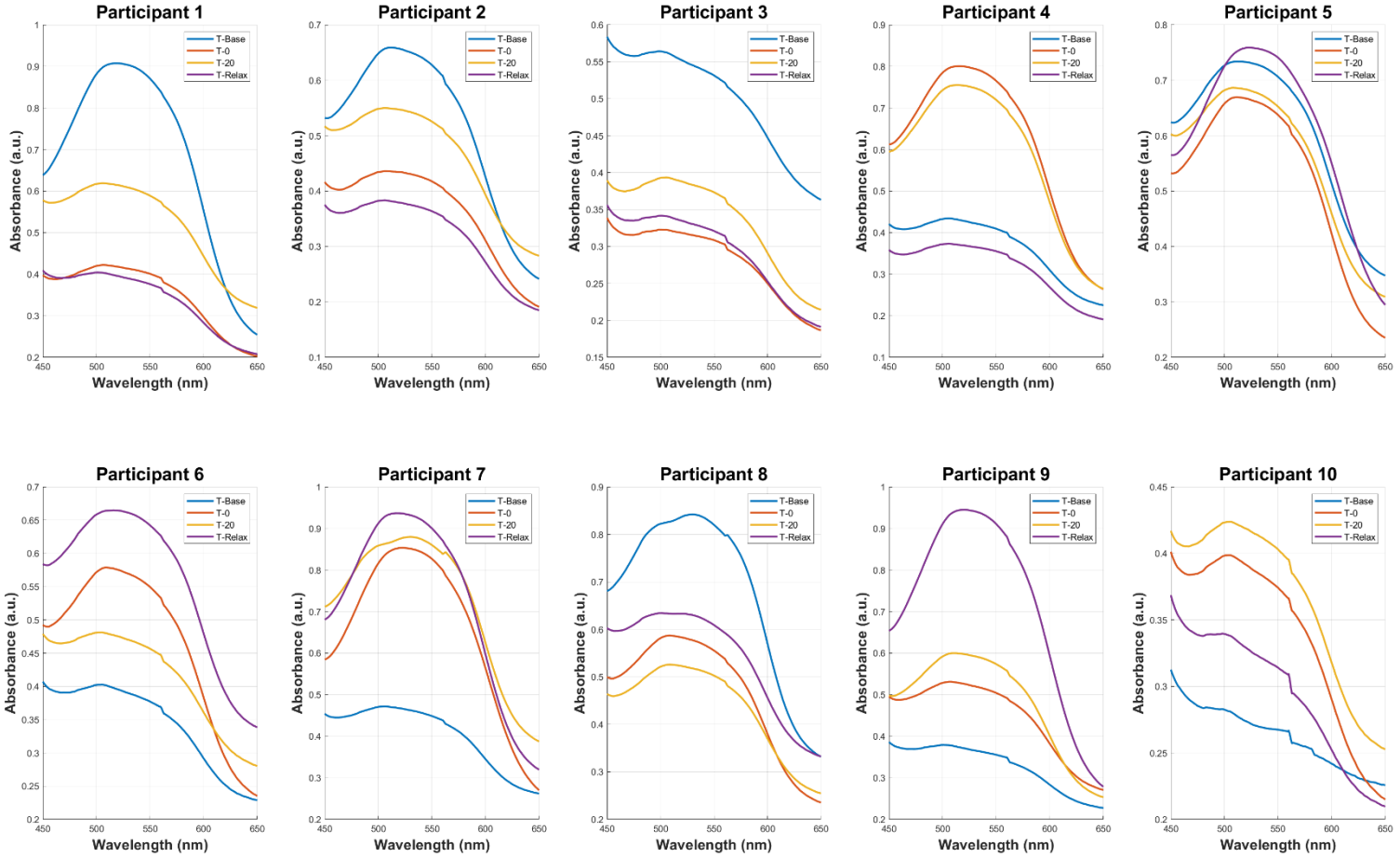

Figure 33– Absorbance spectra of cortisol in blue tetrazolium and tetramethylammonium hydroxide reagents with characteristic peak at 510nm, dataset 1 (Participants 1-10), including post-processing (averaging, baseline subtractions and Savitsky-Golay smoothing). Peak separation between varying concentrations shows clear distinction between detected cortisol concentrations.

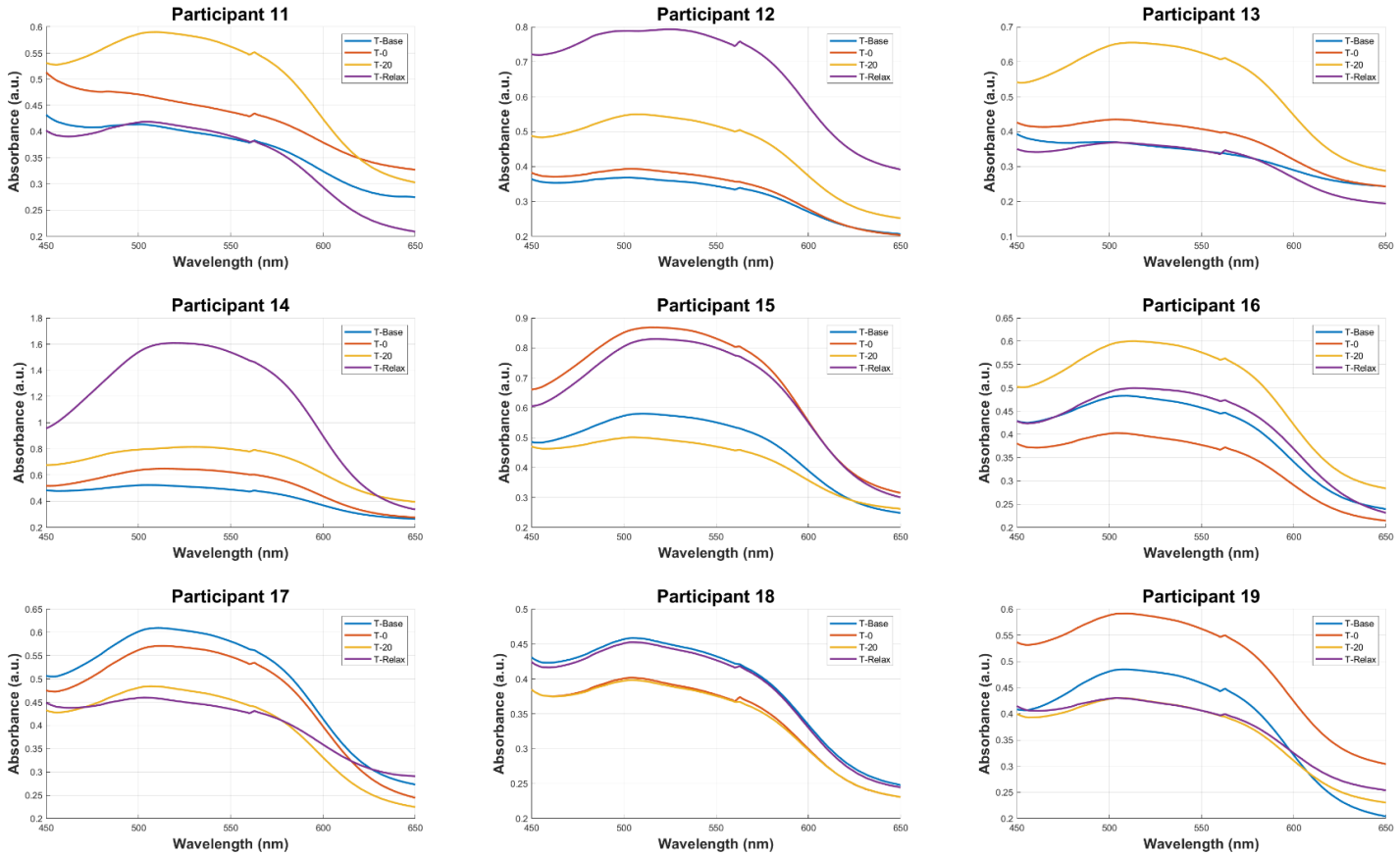

Figure 34 - Absorbance spectra of cortisol in blue tetrazolium and tetramethylammonium hydroxide reagents with characteristic peak at 510nm, dataset 2 (Participants 11-19), including post-processing (averaging, baseline subtractions and Savitsky-Golay smoothing. Peak separation between varying concentrations show clear distinction between detected cortisol concentrations.

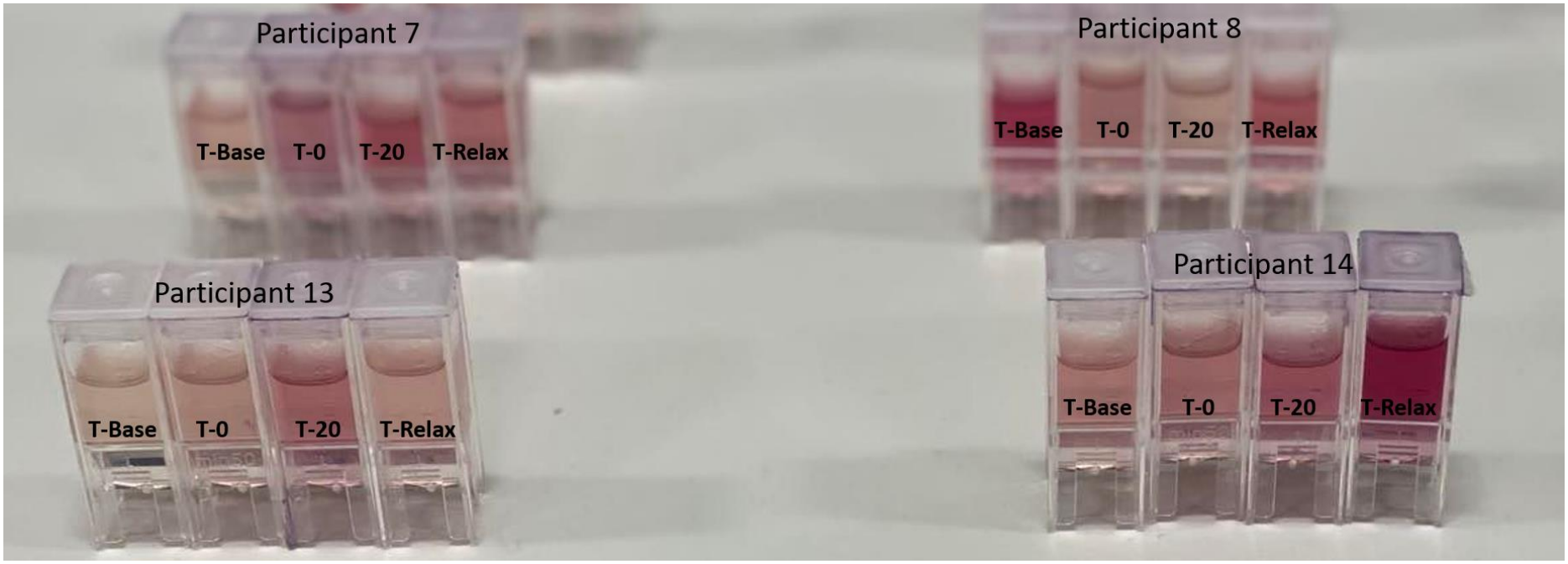

Figure 35 - Optical images of colour change with varying concentrations of salivary cortisol in participants 7 (top left), participant 8 (top right), participant 13 (bottom left) and participant 14 (bottom right) via BT method.

Table 16 - Statistical analysis of dataset 1 (participants 1-10) and dataset 2 (participants 11-19) via Pearson's correlation coefficient for analysis of BT method results versus cortisol ELISA protocol results.

Dataset 1	**Dataset 2**
P-value<.0001	P-value<.0001
R=0.9998	R=0.9985
R2=0.99717	R2=0.99717

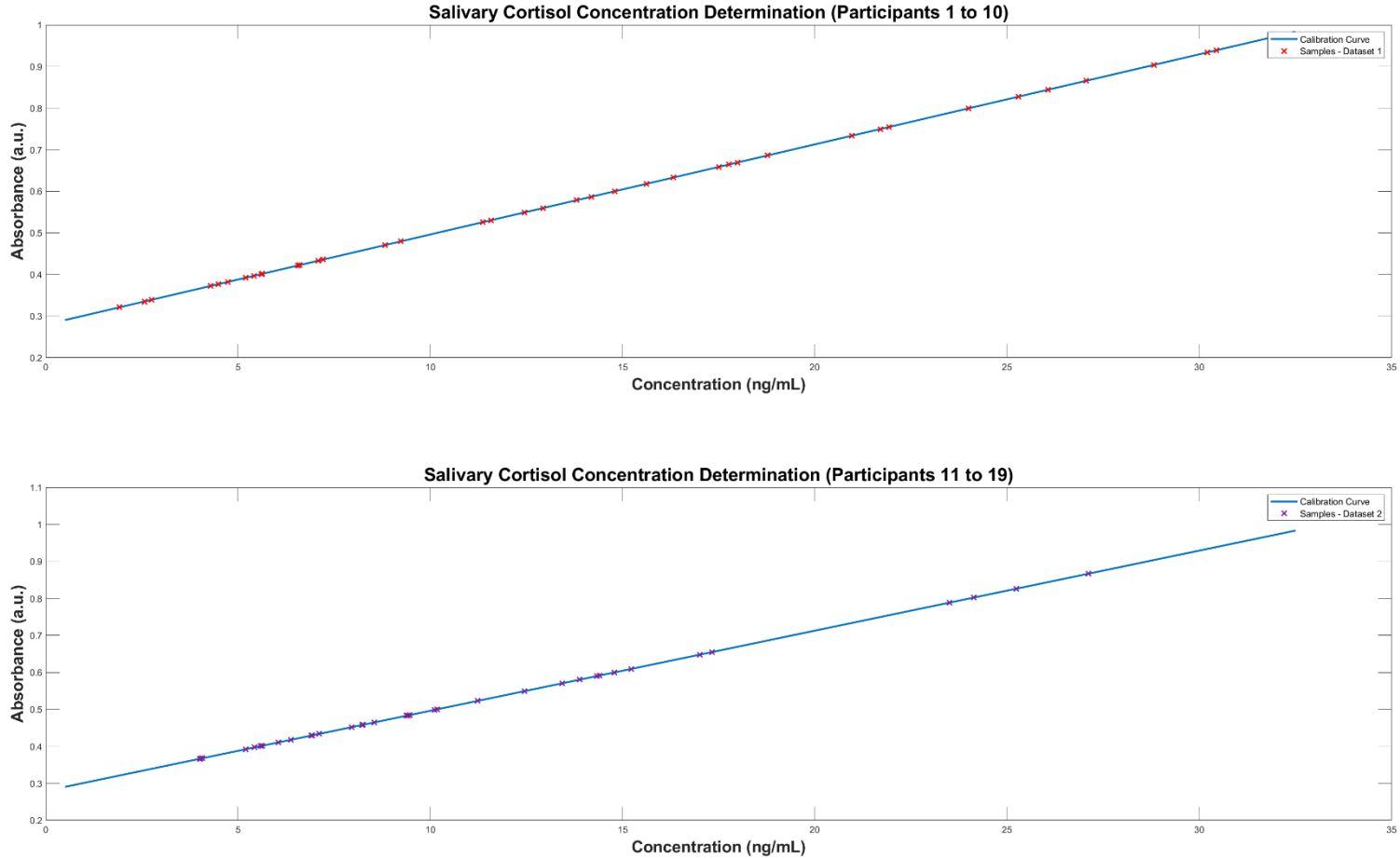

Figure 36 - Cortisol determination using the salivary cortisol concentration calibration curve at 510nm from BT method on dataset 1 – Participants 1-10 (top) and dataset 2 – Participants 11 to 19 (bottom).

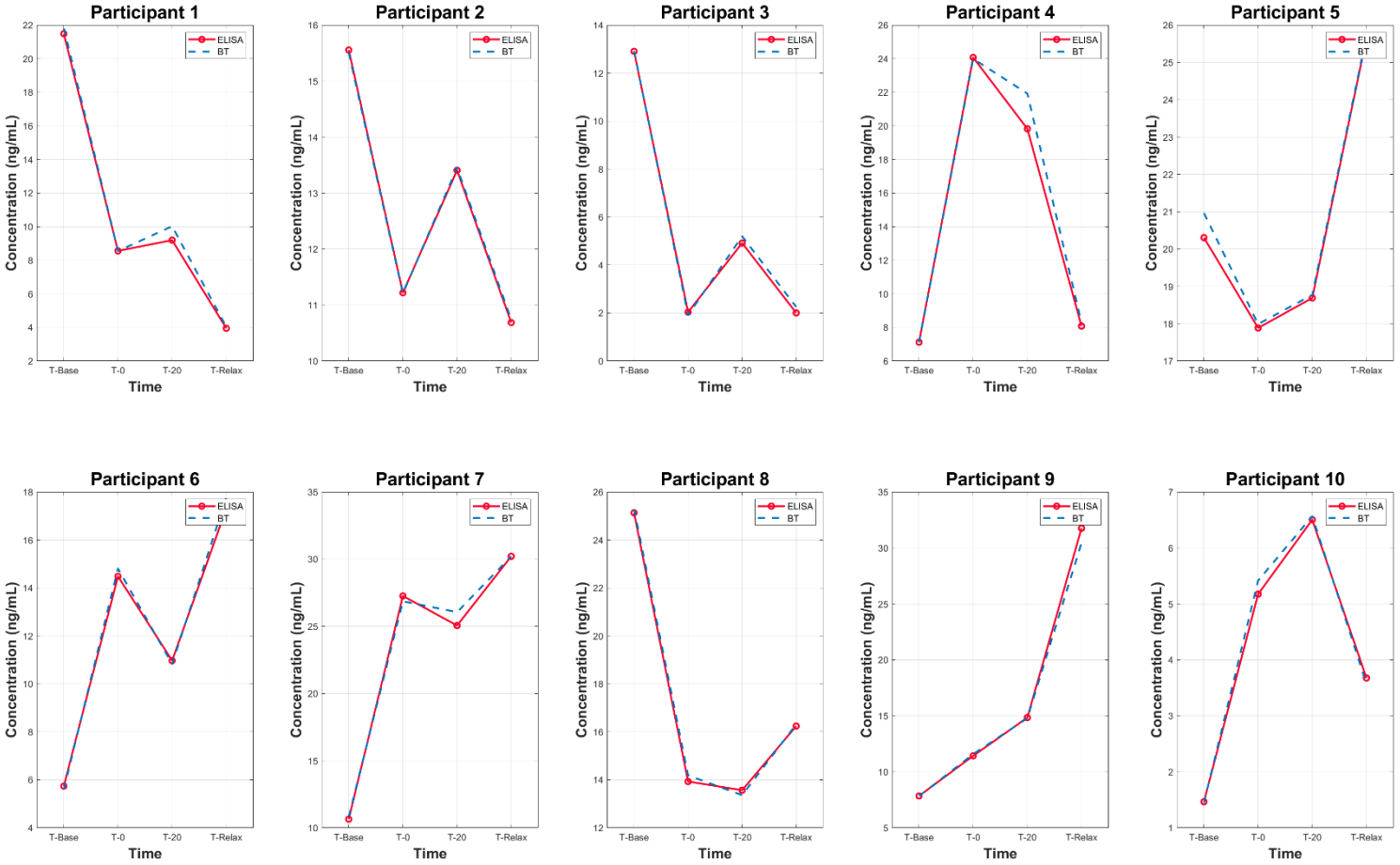

Figure 37 -Salivary cortisol monitoring via BT method (blue) and ELISA method (red) for dataset 1 (participants 1-10) undergoing the MAST protocol. Significant correlation between the proposed experimental (BT) method and the gold standard measured (ELISA).

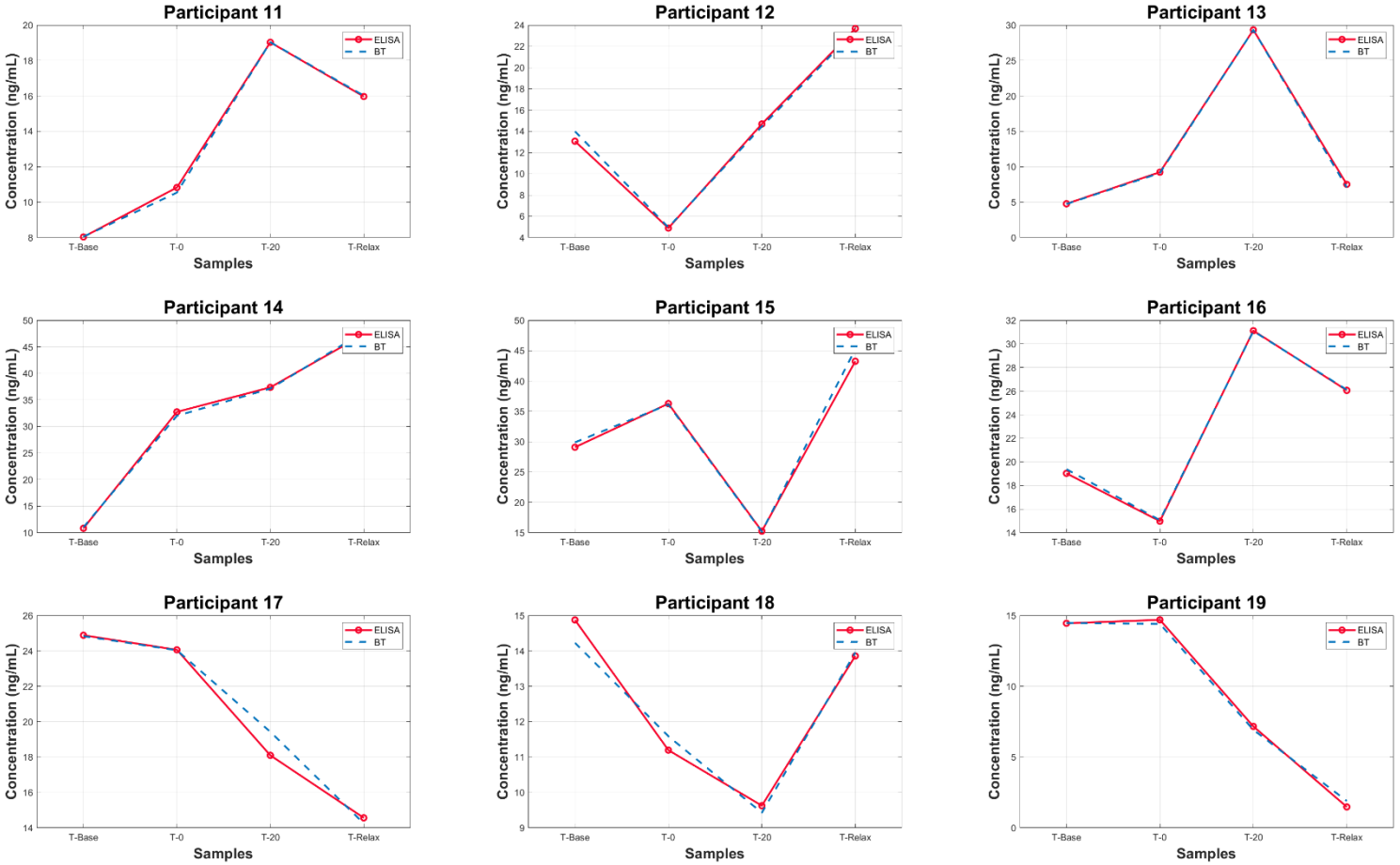

Figure 38 -Salivary cortisol monitoring via BT method (blue) and ELISA method (red) for dataset 2 (participants 11-19) undergoing the MAST protocol. Significant correlation between the proposed experimental method (BT) and the gold standard measurement (ELISA).

11.4 Discussion

Several conclusions can be drawn from the findings of this study, such as the application of the proposed method for potential technological advancements in the management of mental health. The current state-of-the-art technology for the use of mental health involves the applications of physiological monitoring for the evaluation of psychological stress e.g., smart watches. Physiological measures of stress can include heart rate variability, electroencephalography (EEG) data, and electrodermal activity. However, critical analysis of these existing monitoring techniques has shown several shortcomings in the true quantification of stress, due to the lack of consideration for the measurement of stress hormones[80]. Cortisol and other stress hormones, such as adrenaline and noradrenaline, are the key logical indicators of stress because of their involvement in the stress response. The stress response, often referred to as the 'fight or flight' response is the chain of events that take place in the human body upon stress elicitation i.e., a sudden change which interferes with homeostasis. Previous studies have reported on the direct relationship between cortisol concentration and HPA activity, with increases in cortisol in the body translating to resistance stage of the stress response. Hence, the utilisation of the cortisol response towards the quantification of stress and its relationship with mental health is quintessential for the progression towards innovative solutions for the support and treatment of mental disorders beyond traditional interventions such as interview-based techniques and face-to-face therapy[306].

The presence of a stressor activates the HPA axis and subsequently leads to the release of cortisol into the bloodstream, with previous findings reporting a peak in saliva is observed approximately 20-30 minutes after stress elicitation[313], [314]. The results from this protocol evidently reflect this trend, with salivary cortisol levels reaching maximum levels in the 3rd measurement (T3). Thus, demonstrating the success of the MAST protocol and the BT method in determination of salivary cortisol levels. The simplicity and cost-effectiveness of colorimetric analysis of cortisol holds immense potential towards point-of-care applications. The ability to monitor salivary cortisol levels towards psychological stress evaluation would be a major technological advancement for the field of mental health monitoring, which remains antiquated to this day by subjective interview-based tools. The aim of this study was to investigate the feasibility of using colorimetric and optical spectroscopy-based techniques

towards the determination of salivary cortisol levels with precision and high accuracy that matches the existing gold-standard methodology (ELISA). The results of this current study demonstrate the robustness of the BT method in participants undergoing the MAST protocol and serves as a foundation for the development of spectrophotometric sensor-based technology which exploits the reaction of the blue tetrazolium dye and tetramethylammonium reagents for the detection of precise levels of cortisol in saliva samples.

Findings from the current study showcase the successful performance of the spectrophotometric determination of salivary cortisol with remarkable accuracy of 99.7%, compared to ELISA methods. The proposed optical method showcases linearly correlated absorbance values with cortisol concentration present in the human saliva samples, which can be employed towards optimal stress monitoring. With t-values of 0.07 (dataset1, $P<.05$) and 0.08 (dataset2, $P<.05$), it is evident that the proposed method of utilising blue tetrazolium for cortisol concentration determination is in line with measurements from the current gold standard ELISA method. The high accuracy of the linear prediction model further reiterates the feasibility of employing this technology for accurate psychological stress monitoring. Following comparison and validation of the proposed methodology against the cortisol ELISA protocol, it is apparent that the chromogenic-based optical spectroscopy technique offers lucrative mapping of the participants' stress activity in response to the MAST, which is reinforced by the subjective stress scores provided by participants post-MAST.

Evidently, existing technologies towards salivary cortisol determination have proven to be highly accurate, albeit with several limitations such as reaction time, the need for skilled personnel and highly specialised instrumentation. Therefore, the accessibility of such technologies e.g., ELISAs and LC-MS/MS for routine use is vastly uncommon, hindered further by the cost of tests. Previous studies have noted the success of the tetrazolium protocol for cortisol determination in plasma and sweat [[308], [315]. However, salivary cortisol has been found to be directly proportional to serum unbound cortisol and a better measure of adrenal cortical function than serum cortisol [[316]. Consequently, the invasiveness of serum cortisol determination towards psychological evaluation can be an unnecessary measure which could potentially lead to further stresses for participants. Although, the use of sweat cortisol has emerged as a desirable measure in recent studies involving stress monitoring, the complexity

of HPA activity and its relationship with sweat production can lead to divisive results, especially if participant fitness levels and nutrition are considered. Thereby, salivary cortisol measurements are regarded as the gold standard biomarker of stress, due to its valuable non-invasive mapping of HPA activity and direct proportionality to blood cortisol levels [153], [317], [318].

Notably, the complexities between mental health complications and psychological stress processing are evident in this investigation. From the observed post-MAST cortisol concentrations (T-0, T-20), it can be hypothesised that participants that had higher depression inventory scores experienced the stressors of the MAST contrastingly when compared to those who scored lower in the NHS self-assessment (Table 15). Participants with higher depression inventory scores had lower cortisol concentrations post-MAST, which could suggest inactivity of the HPA axis. Previous investigations which observed cortisol concentrations in response to stressors showcase that blunted cortisol responses can be indicative of mental illnesses such as clinical depression [152]. The prevalence of this phenomenon in the current study suggests the necessity for future studies which focus on the optimisation of the investigated methodology towards personalised mental health monitoring, aided by adaptive cortisol profiling. Further investigations involving larger volunteer trials that include varied stress-elicitation methods should be considered towards the development of a complete cortisol monitoring system. Whereby, data from long-term routine measurement of salivary cortisol can be utilised in the development of a predictive model for individualised psychological stress evaluation.

Future work in this field will also focus on the optimisation of the spectroscopic technique towards the development of a point-of-care portable monitoring device which can readily analyse salivary cortisol levels for personal mental health monitoring. Most existing mental health monitoring devices utilise physiological signal processing towards stress detection. However, it has been reported that such methods may lead to several inaccuracies in psychological stress evaluation. Primarily due to lack of consideration of the implications of cortisol and other stress hormones on the HPA axis. Evidently, the significance of cortisol is unprecedented in stress evaluation, although the introduction of complimentary stress hormones such as adrenaline, or antagonistic hormones like dehydroepiandrosterone (DHEA) would further consolidate the hormonal determination of stress [306]. Thus, the

development of healthcare devices that account for both, physiological and biochemical attributes of psychological stress, would lead to more robust applications of stress monitoring.

11.5 Conclusion

In conclusion, the current study has demonstrated the successful implementation of the BT method for rapid and accurate determination of cortisol levels in saliva samples collected from participants undergoing the MAST protocol. With an R^2 value of 0.997, when compared to ELISAs, the proposed method can be considered as a viable alternative to existing time-consuming and expensive methods for cortisol analysis. This will provide significant advancement in the development of a miniaturised point-of-care device for stress monitoring. The utilisation of such a device on a routine basis will empower individuals to effectively monitor their psychological stress profiles and seek appropriate therapeutic intervention, which is of utmost significance in a growing population of mental illness prevalence.

12. Rapid colorimetric determination of salivary cortisol levels in individuals undergoing physiological and psychological stress using the Salitrack sensor (small n-study)

The findings reported in this chapter have been published in:

***Ahmed T,** Powner MB, Qassem M, Kyriacou PA**. Rapid colorimetric determination of salivary cortisol levels in individuals undergoing physiological and psychological stress using the Salitrack sensor (small n-study). Manuscript in submission.*

The success of the previous study (Chapter 11) showcases the feasibility of using the BT method for the optical determination of human salivary cortisol in participants undergoing the MAST stress protocol. Furthermore, the development of the prototype colorimetric sensor in Chapter 10 led to testing on a range of artificial salivary cortisol samples, with hopes of further testing on human saliva samples. The current study details the use of the colorimetric sensor (Salitrack) for the colorimetric determination of cortisol concentration from a small range (n=8) of participants who undertook the MAST stress protocol.

12.1 Introduction

For the development of a point-of-care device for psychological and physiological monitoring of stress, it is essential to consider the role of cortisol, and therefore cortisol measurement. Cortisol is the primary stress hormone in the human body which governs the stress response, commonly referred to as the 'fight or flight' response[11]. The stress response is triggered once a threat is perceived which translates physiologically to disruptions in homeostasis within the body. Cortisol, along with other stress hormones, such as adrenaline and noradrenaline, are involved in orchestrating the body's response to stressors which can be observed through a series of physiological changes in the body such as elevated heart rate, increased blood glucose levels and increased perspiration[9]. Evidently, the long-term effects of cortisol on the human body can lead to deterioration of physical and mental health. Therefore, the management of cortisol levels is of utmost significance for the management of stress and to promote mental wellbeing[35]. The current study proposes the utilisation of

a previously developed colorimetric sensor (Salitrack) for the determination of salivary cortisol levels in a small cohort (n=8) of participants undergoing the Maastricht Acute Stress Test (MAST) protocol[309].

12.2 Methods

12.2.1 Participants

8 participants with a mean age 28.50 ± 10.49 years participated in the current study, which included 4 males and 4 females. Written informed consent was provided by all participants and the study was carried out under approval by the City, University of London, School of Science and Technology Senate Research Ethics Committee. The exclusion and inclusion criteria followed those established in the previous study (Ahmed, T et.al). The study was conducted between 12pm-4pm for all participants to ensure the effects of cortisol's circadian rhythm did not interfere with the observations made.

12.2.2 Maastricht Acute Stress Test Protocol

Participants took part in the MAST protocol which involves alternating trials between cold (2°C) hand-immersion (H) and mental arithmetic (MA) tasks of predetermined durations, unknown to the participant. After the MAST was completed, participants were debriefed and given instructions for an intervention/relaxation period.

12.2.3 Physiological and Subjective Measurements

Salivary cortisol was measured throughout the MAST protocol, with a total of four saliva samples collected using the Cortisol Salivette (Cortisol Salivette, SARSTEDT, Numbrecht, Germany). A baseline measurement was taken prior to the MAST (T-Base), immediately after completion of the stress test (T-0), after the 20 min debriefing period (T-20), and post relaxation/intervention (T-Relax). Salivette swabs were centrifuged at 1000g for 2 min at room temperature using the Biofuge Primo R (Sorvall, Waltham, MA, USA). Samples were then stored at -80°C for subsequent ELISA and optical analysis using a benchtop spectrophotometer (Ahmed T. et al.) and the Salitrack sensor. Subjective measures involved

the use of the NHS depression and anxiety self-assessment for a baseline reading of participants' mental health status, and a rating of their experience of the MAST protocol on a 5-point Likert scale ranging from 1 to 5 (1= not stressed at all, 5=extremely stressed).

12.2.4 Salitrack and ELISA Measurements

The Salitrack sensor was developed (Ahmed T. et al 2) for the analysis of salivary cortisol levels from artificial and human saliva samples. A previous study conducted by (Ahmed T et al. 2) highlights its success in determining cortisol concentrations in the ng/mL region from samples of artificial saliva spiked with cortisol. The sensor was used to determine cortisol levels from the saliva samples of each of the 8 participants, which were then compared with the gold-standard ELISA measurements conducted using a commercially available ELISA kit (Parameter Cortisol Assay, R&D Systems, Minneapolis, MN, USA). Blue tetrazolium chloride and tetramethylammonium hydroxide reagents were used as chromogenic agents for the colorimetric determination of cortisol in the saliva samples, this methodology is highlighted in previous studies (Ahmed T et al, 2).

50ul of the saliva was combined with 200ul of blue tetrazolium dye and 200ul of tetramethylammonium hydroxide reagent (4-fold sample dilution). After a 10 min development period, the sample was analysed using the Salitrack sensor. The voltage change detected upon examining each sample was used to calculate the absorbance value for each sample. This absorbance value was then used to predict concentration of cortisol using the Salitrack calibration curve which was modelled in a previous study (Ahmed T. et al. 2).

Reference measurements for each sample were conducted using the cortisol ELISA kit, samples were tested in duplicate. The results from the Salitrack sensor were compared with the gold-standard measurements through Pearson's correlation coefficient.

12.3 Results

12.3.1 Analytical performance of the Salitrack Sensor for salivary cortisol determination from human saliva samples.

The analytical performance of the Salitrack sensor was evaluated through qualitative and quantitative comparison between the Salitrack calibration curve and the cortisol ELISA standard curve. The coefficient of determination (R^2) value for the Salitrack curve (Figure 39) and the ELISA curve were compared to analyse the models' determinability of unknown cortisol concentrations using absorbance values derived from a calibration set for cortisol.

The calculated absorbance values returned from the Salitrack sensor for each sample were used to determine the concentration of cortisol within the samples, by using the calibration curve. Therefore, the R^2 value of the calibration curve reflects the accuracy of the sensor in detecting cortisol concentrations in human samples, which is essential for progression towards complete stress monitoring. The evaluation of each participant's salivary cortisol levels via Salitrack and ELISA methods is presented in Figure 40.

12.3.2 Statistical comparison between Salitrack sensor performance and gold-standard ELISA protocol.

As with previous studies, the comparison between the gold-standard methodology for salivary cortisol analysis and the proposed Salitrack sensor is necessary to validate the performance of the sensor and its use in point-of-care applications. A paired t-test was conducted to evaluate the differences between the paired observations i.e., the measurements from the sensor vs. the reference measurements collected from the ELISA kit. The purpose of this analysis is to determine the success of the prototype sensor. Results from

the Pearson's correlation coefficient showcase strong positive correlation between the sensor results and the ELISA concentrations, *r(30) = .99, p<.001.*

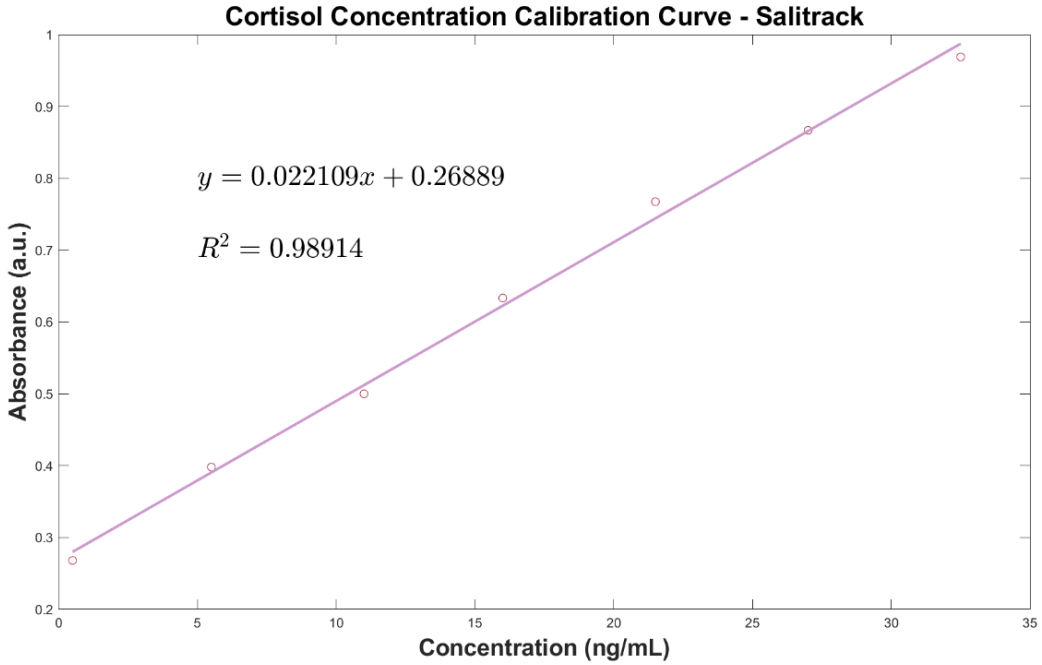

Figure 39 – Linear regression analysis-based calibration curve for cortisol concentration determination using the Salitrack sensor.

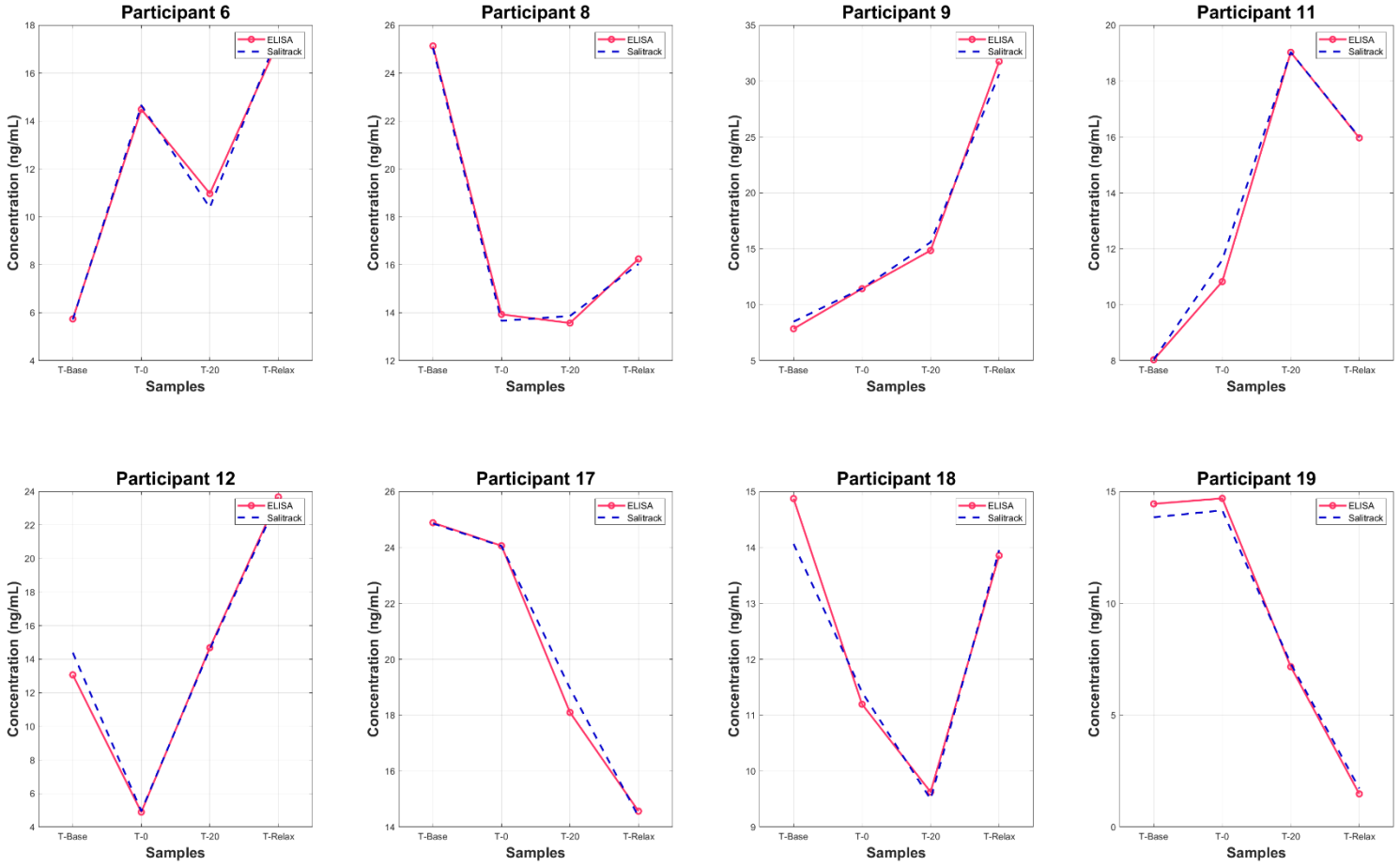

Figure 40 - Salivary cortisol monitoring via Salitrack method (blue) and ELISA method (red) for participants (n=8) undergoing the MAST protocol.

12.4 Discussion

Undoubtedly, the observations from the current study demonstrate the ability of the Salitrack sensor in the accurate determination of salivary cortisol levels from participants undergoing the MAST protocol, in line with the gold-standard ELISA measurements. The visual representation of cortisol concentration using the blue tetrazolium chloride and the tetramethylammonium hydroxide chromogenic reagents offer an alternative to expensive and time-consuming assay-based technologies such as ELISAs which are the current gold-standard method for analysing cortisol levels. With the accessibility and ease of use of the established colorimetric method, the future costs of the development of a complete point-of-care stress monitoring device can be significantly reduced.

The R^2 value for the Salitrack sensor based on prior calibration testing was 0.989, which demonstrates the accuracy of the model in predicting cortisol levels from saliva samples of varying cortisol concentration. Moreover, from the statistical testing conducted alongside the results from the ELISA protocol, the Pearson's correlation coefficient yielded, R = 0.9995 and the P <.001, suggesting that there is strong positive correlation between the proposed prototype sensor and the gold-standard methodology (ELISA kit). This establishes that the Salitrack sensor can be used for the successful and precise determination of cortisol concentrations from human saliva samples, towards point-of-care stress monitoring applications.

Moreover, by defining baseline cortisol measurements as conducted in the current and previous studies surrounding the BT method and Salitrack sensor, it is possible to develop mathematical models which consider a user's baseline and regular cortisol levels towards adaptive stress profiling. Adaptive stress profiling would enable users to monitor their physiological and psychological stress levels and how they fluctuate on a day-to-day basis to allow for personalised stress management tools, such as those offered by mindfulness and wellbeing mobile applications e.g., Headspace. Mindfulness applications have already shown vast improvement to multiple aspects of psychosocial well-being, as an alternative to face-to-face interventions and therapy[319]. Integration with such applications could lead to more robust and low-cost intervention regimes for the general public. The introduction of personalised stress profiles can be used to monitor an individual's stress levels, which could

lead to greater situational context to aid in a wide array of aspects e.g., occupational stress and financial stress.

Evidently, the development of the Salitrack sensor offers accessibility to stress and mental health management to a larger population. The introduction of tools and point-of-care devices for self-management of mental health facilitates a low-cost and highly engaging alternative to existing intervention methods, which are often associated with low patient adherence, mainly due to costs and practicality of face-to-face sessions in a post-pandemic setting[320], [321].

13. Concluding Discussion and Future Work

13.1 Discussion

As an overarching global burden, mental illnesses have created substantial detriment to society, especially in recent years due to the Covid-19 pandemic[322]. Dated methods of mental health assessment such as interview-based tools and trial-and-error medication regimes have led to gross deficiencies in patient adherence, further increasing the costs surrounding diagnosis and treatment of a plethora of mental illnesses[23]. The thesis focuses on the trajectories of chronic psychological stress towards the development of clinical depression, and specifically the biomarkers which can be characterised to lead to quantitative approaches in diagnostic and management tools. Through evaluation and analysis of existing methods of stress monitoring such as physiological measurements (Chapter 3) and biochemical measurements (Chapter 4), the clinical significance of monitoring cortisol as a key biomarker of physiological and psychological stress is proposed[80], [306].

The experimental work in this thesis demonstrates the novel colorimetric determination of cortisol concentrations in human and artificial saliva samples, within the clinical range (0.7-27.3ng/mL)[153]. The detection and monitoring of cortisol remains clinically significant for the characterisation of physiological and psychological stress, as detailed in chapters 3 and 5. Previous studies have noted the strong correlation between salivary cortisol and serum cortisol, deeming it an ideal non-invasive alternative for biochemical stress determination[323]. Historically, salivary cortisol analysis has depended primarily on assay-based technologies such as ELISAs and aptamer-based biosensors which can often be costly and time-consuming for result development[324].

The thesis proposes an alternative optical method (BT Method) which utilises chromogenic agents (tetrazolium blue chloride and tetramethylammonium hydroxide) to aid in the colorimetric determination of cortisol, facilitated by UV-Vis spectroscopy[308]. Through several experimental protocols, the BT method has been used to evaluate cortisol concentration within in-vitro and in-vivo environments. The BT method has proven to be successful in determining cortisol concentrations in samples of cortisol-spiked artificial saliva, as well as in samples of human saliva taken from participants undergoing a standardised stress test to induce physiological and psychological stress (MAST protocol). In both instances,

the concentration values obtained via the BT method remained in line with the reference measurements taken using the gold-standard salivary cortisol methodology, ELISAs. Statistical testing through calculation of Pearson's correlation coefficients demonstrated minor statistical differences between the data obtained from the proposed method and the gold-standard, further justifying the use of the BT method for cortisol monitoring.

Moreover, a simple prototype point-of-care colorimetric sensor was designed and developed which utilises the BT method for cortisol analysis without the need for benchtop spectroscopy. Testing of this sensor was conducted on a range of samples of artificial saliva spiked with cortisol, as well as on a small cohort of participants who undertook the MAST protocol to evaluate sensor performance. As showcased in chapter 12, the Salitrack sensor was able to successfully predict cortisol levels in human saliva samples, with minor statistical differences from the reference measurements (ELISA). Thus, the Salitrack sensor shows great promise for the future of stress monitoring, and justifiably considers the key logical indicator of stress, cortisol.

For the first time, the concentration of cortisol in saliva from human participants has been determined optically with the use of the tetrazolium blue dye and the novel use of the tetramethylammonium hydroxide catalyst within the clinically significant range of salivary cortisol. This led to the development of the novel colorimetric Salitrack sensor for salivary cortisol analysis which was validated with the current gold-standard ELISA method. The results from this work have shown great accuracy in the determination of cortisol well in line with the results obtained from the gold standard, showcasing the potential of the colorimetric approach for mental health monitoring.

13.2 Future Work

Primarily, future work beyond this thesis should consider the significance of secondary biomarkers of stress such as adrenaline and noradrenaline. Characterisation and detection of these biomarkers in human saliva through colorimetric methods could lead to a complete multiplexed system that can be translated to a point-of-care device for robust stress monitoring. Moreover, the significance of DHEA is highlighted in this thesis as having an antagonistic relationship with cortisol[306]. Therefore, exploring this relationship further with regards to stress could be used to identify key biochemical patterns for stress profiling.

The key limitation of the work presented in this thesis is the differences in baselines of stress across different participants. Evidently, the human study showcased participants experienced the standardised stress test (Chapter 11) with variable responses. For example, some participants with higher baseline levels did not respond as significantly to the stress test as those with loser baseline levels, suggesting blunted cortisol responses. Thus, the concept of personalised and adaptive stress profiling could harness significant data regarding how each individual responds to and intervenes in stressful situations. Through establishing baseline measurements, the change in salivary cortisol levels in response to stressors (temperature-induced and mental arithmetic) are apparent and allows for evaluation of the individual's stress levels in comparison to the reference measurements, as well as their subjective rating of the experience. Notably mentioned in the human study, participants who reported a higher/more stressful rating of the experience had lower NHS depression/anxiety scores than their counterparts and had lower baseline readings of cortisol. This further reiterates blunted cortisol responses, which are a key indicator of chronic stress, which can often lead to severe mental health deterioration[325], [326]. Evaluation of these behaviours, with the use of the Salitrack sensor and the BT method would facilitate a definitive system for identifying patients at risk of depression or comorbid depression in clinical settings.

Moreover, for the application of the Salitrack sensor towards depressed populations, further work needs to be undertaken to understand the degree of severity of depression which is associated with the blunting of cortisol responses. Information from such studies would facilitate the development of an adapted Salitrack sensor for use in depressed cohorts, who often present blunted cortisol responses to stress, compared to peak salivary cortisol levels when undertaking a stress test.

Furthermore, evaluation of baseline readings and cortisol levels in response to stressful situations could aid in the understanding of personalised stress profiling towards mental health management. This concept could be coupled with mindfulness and wellbeing mobile phone applications such as Headspace and BetterHelp to offer personalised intervention regimes in a more accessible manner, compared to the alternative which heavily relies on face-to-face therapy sessions and pharmaceuticals. As patient adherence declines, especially in a post-pandemic setting, the necessity for digital alternatives to existing therapeutic interventions in essential to ensure that more individuals have access to the treatment and

management techniques that they require to manage their stress levels, and in turn mental health. Therefore, connecting point-of-care devices such as the Salitrack to a mindfulness application could propose a compelling alternative to face-to-face therapy, as studies have already shown individuals report significant improvement to their psychosocial status upon using wellbeing applications for intervention, like Headspace[319].

It is also essential to note the broader significance of characterising cortisol through colorimetric and optical methods such as those highlighted in this thesis. Although cortisol is primarily associated with stress, the excessive production of cortisol in the body or hypercortisolism is known as Cushing's syndrome. In some cases, Cushing's syndrome is present in patients who have taken steroid medications, such as steroid tables for a prolonged period of time. Untreated Cushing's syndrome is associated with extremely poor prognosis, with an estimated 5-year survival rate of 50%[327]. Therefore, in patients suffering from or at risk of this disease due to steroid treatment, the monitoring of cortisol levels is absolutely essential. One of the commonly administered tests for Cushing's syndrome is a late-night/midnight salivary cortisol test to examine cortisol levels using ELISAs[328]. The introduction of the Salitrack sensor for this test could allow for ease of examination and improve patient adherence as tests could be conducted at home, without the need for sending off samples to an external laboratory facility, significantly reducing the costs associated with the procedure. Therefore, the use of the Salitrack sensor in patients undergoing steroidal treatment could be a pivotal in management of treatment and diagnosis of Cushing's syndrome.

Further development of the Salitrack sensor needs to be undertaken which involves optimisation of the biosensor, as well as consideration of more biomarkers, as mentioned previously. Further optimisation of the sensor would involve the implementation of a saliva collection device to be used alongside the Salitrack sensor. Currently, Cortisol Salivettes are used for sample collection, which involves a centrifugation step that is inaccessible for patient-held settings. An alternative method which has been tested is the use of cotton balls which can be placed in the mouth to collect saliva. The saliva can then be extruded from the cotton ball using a 5ml BD Plastipak Luer Slip Syringe with a MF-Millipore Membrane Filter (0.45µm pore size). The saliva sample can then be added to a pre-filled and sealed cuvette

containing a pre-determined volume of tetrazolium blue chloride and tetramethylammonium hydroxide reagents to facilitate easier sample collection and preparation.

Alternatively, the tetrazolium blue chloride and tetramethylammonium hydroxide reagents could be used to develop a coating on cellulose-based paper which could allow for a paper-based colorimetric technique. The coated strip could be dipped into a sample of saliva collected either via passive drool technique or swab technique. Preliminary studies within this field have shown great promise towards the development of point-of-care sensors[329].

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Appendix

Ethics ETH2122-0653: Miss Tashfia Ahmed (High risk)

Date Created	17 Nov 2021
Date Submitted	03 Jan 2022
Date of last resubmission	24 Mar 2022
Date forwarded to 2022 committee.	20 Jan
Academic Staff	Miss Tashfia Ahmed
	Prof Panicos Kyriacou
	Miss Meha Qassem
Student ID	150055889
Category	Doctoral Researcher
Academic Staff	Tashfia Ahmed
Supervisor	Prof Panicos Kyriacou
Project	Optical Sensing of Salivary Cortisol for Stress Monitoring
School	School of Science & Technology
Department	Department of Engineering
Current status	Approved after amendments made

Ethics application

Risks

R1) Does the project have funding? No

R2) Does the project involve human participants?
Yes

R3) Will the researcher be located outside of the UK during the conduct of the research?
No

R4) Will any part of the project be carried out under the auspices of an external organisation, involve collaboration between institutions, or involve data collection at an external organisation? No

R5) Does your project involve access to, or use of, terrorist or extremist material that could be classified as security sensitive?
No

R6) Does the project involve the use of live animals?
No

R7) Does the project involve the use of animal tissue?
No

R8) Does the project involve accessing obscene materials?
No

R9) Does the project involve access to confidential business data (e.g. commercially sensitive data, trade secrets, minutes of internal meetings)?
No

R10) Does the project involve access to personal data (e.g. personnel or student records) not in the public domain? No

R11) Does the project involve deviation from standard or routine clinical practice, outside of current guidelines? No

R12) Will the project involve the potential for adverse impact on employment, social or financial standing?
No

R13) Will the project involve the potential for psychological distress, anxiety, humiliation or pain greater than that of normal life for the participant?
No

R15) Will the project involve research into illegal or criminal activity where there is a risk that the researcher will be placed in physical danger or in legal jeopardy?
No

R16) Will the project specifically recruit individuals who may be involved in illegal or criminal activity?
No

R17) Will the project involve engaging individuals who may be involved in terrorism, radicalisation, extremism or violent activity and other activity that falls within the CounterTerrorism and Security Act (2015)? No

Applicant & research team

T1) Principal Applicant

Name
Miss Tashfia Ahmed

Provide a summary of the researcher's training and experience that is relevant to this research project.

Tashfia Ahmed received her Bachelor of Engineering Degree in Biomedical Engineering at City, University of London in 2018. She then went on to continue her studies at Imperial College London where she obtained her Master of Science Degree in Biomedical Engineering, focusing primarily on Neurotechnology, in 2019. Since October 2019, she has been enrolled as a full-time PhD student at the Research Centre for Biomedical Engineering at City, University of London. Her research focuses on the spectroscopic studies for the monitoring of stress biomarkers, such as cortisol, in human saliva. This will eventually lead to the development of a psychological stress monitor for use with sufferers of psychological stress and clinical depression.

T2) Co-Applicant(s) at City

Name

Prof Panicos Kyriacou

Provide a summary of the researcher's training and experience that is relevant to this research project.

Prof Kyriacou received a BESc degree in Electrical Engineering from the Engineering Department of the University of Western Ontario, Canada in 1994 and an MSc degree in Medical Electronics and

Physics from St. Bartholomew's Medical College, University of London in 1995. During 1995 -1996 Prof Kyriacou worked as a Senior Biomedical Engineer and application specialist in the medical devices industry. He received his PhD from St. Bartholomew's Medical College, University of London in 2001 where he engaged in research in the field of medical instrumentation and electro-optical sensors for monitoring critically ill patients. He is currently a Professor of Biomedical Engineering and

Associate Dean for Postgraduate studies at the School of Engineering and Mathematical Sciences at

City University London. He is also the Director of the undergraduate programme in Biomedical

Engineering and Director of the Biomedical Engineering Research Group. Prof Kyriacou is an

Honorary Professor in the Department of Anaesthesia at St. Andrews Centre for Plastic Surgery and

Burns, Broomfield Hospital, Mid Essex Hospital Services NHS Trust. He is also an Honorary Senior

Research Fellow at Great Ormond Street Hospital for Children and St. Bartholomew's Hospital and a Visiting Research Fellow at Yale Medical School, Yale University. Professor Kyriacou' s main research activities are primarily focused upon the understanding, development and applications of instrumentation, sensors and physiological measurement to facilitate the

prognosis, diagnosis and treatment of disease or the rehabilitation of patients.

His research pushes the frontiers of current optical and electronic technologies and demonstrates how such technologies can be used as medical "tools". His research is nationally and internationally recognised and this is evident by the number of organisations he is collaborating with and also from the wide spread of high impact factor scientific and clinical journals that he has published. He has authored and co-authored over 100 publications in peer reviewed journals, invited chapters in books and conference proceedings. He is also the holder of five patents with inventions in the area of Biomedical Instrumentation and Optical Biomedical Sensors. He is currently the Principle Investigator (PI) of more than nine research projects and has attracted funding from a variety of sources such as research councils, charities, NHS, and industry. Prof Kyriacou is the Chairman of the Instrument Science and Technology Group at the Institute of Physics and the Chair of the Engineering Advisory Group at the Institute of Physics and Engineering in Medicine. He is also a member of the Healthcare Science Advisory Group at NHS London.

Name

Miss Meha Qassem

Provide a summary of the researcher's training and experience that is relevant to this research project.

Dr Meha Qassem graduated with a BEng degree in Biomedical Engineering from City University in 2008, then went on to complete her PhD 2010 at City University, after working as a medical engineer for two years. During her PhD, she worked extensively in the areas of optical spectroscopy, spectral analysis and optical sensing. She had successfully completed multiple studies on animal and human skin, looking at the properties of skin and the effect of certain applicants on skin parameters using NIR spectroscopy. Her work has been published in several conferences and internationally recognised peer-reviewed journals. She is currently a lecturer in Biomedical Engineering at City, University of London.

T3) External Co-Applicant(s)

T4) Supervisor(s)

Prof Panicos Kyriacou

Miss Meha Qassem

T5) Do any of the investigators have direct personal involvement in the organisations sponsoring or funding the research that may give rise to a possible conflict of interest?

No

T6) Will any of the investigators receive any personal benefits or incentives, including payment above normal salary, from undertaking the research or from the results of the research above those normally associated with scholarly activity?

No

T7) List anyone else involved in the project.

Project details

P1) Project title

Stress Monitoring Through Cortisol Measurements from Saliva Using Spectroscopic Methods

P1.1) Short project title

Stress hormone measurements using saliva during a stress test

P2) Provide a lay summary of the background and aims of the research, including the research questions (max 400 words).

Clinical depression is one of the most common mood disorders, with over 264 million cases globally. This severity of this mental illness has only been accelerated by the covid-19 pandemic. Currently mental health evaluation focuses on the qualitative and subjective evaluation of patients through interviews and questionnaires, this has been an established method for several decades, although the process is considered lengthy and expensive, and can still lead to misdiagnoses. There remains a need for the quantification of psychological stress and its manifestation in the human body. Stress evaluation in the body primarily focuses on the analysis of stress biomarkers such as cortisol and adrenaline in the body, in response to stressors. The state-of-the-art technology in this field utilises enzyme linked immunoassays or ELISA sensors to monitor these biomarkers in the human body. ELISA sensors have proven to be highly effective, however they require the need for external lab testing which can be expensive, time-consuming, and highly inconvenient for patients during their day-to-day lives. Furthermore, commercial stress monitoring technologies often utilise PPG sensors with smart watch applications, which are cost effective, albeit ineffective in monitoring psychological stress when compared to physical stress i.e., during exercise.

The project proposes the utilisation of spectroscopic techniques to overcome the current limitations of psychological stress monitoring. With UV-visible spectroscopy and colorimetric analysis, cortisol concentrations within human saliva can be determined rapidly and accurately. This will provide a novel technique for the measurement of stress biomarkers in salivary applications, eradicating the need for use of blood sampling and ELISA sensors. As the RCBE has expertise in optical spectroscopy and the development of optical sensors, this project will be enhanced by the support of the research centre and expand the centre’s expertise into the field of mental health and stress monitoring. The project will involve the study of

interactions between various dyes and their aide in the spectroscopic evaluation of known concentrations of stress hormones in saliva, before application of this knowledge in an in vivo protocol involving human participants. Participants will be asked to complete a stress test whilst supplying the investigator with 4 saliva swabs to evaluate the change in cortisol levels in saliva as the participant undergoes a standardised stress test. This research will provide new knowledge regarding the spectroscopic 'fingerprint' of cortisol in saliva and aid the conception and development of a sensor which can detect cortisol fluctuates rapidly and with high sensitivity and accuracy.

P3) BRIEFLY explain how this project will further existing knowledge.

Cortisol is the main stress hormone in humans, and it has been studied by various groups, within a plethora of scenarios. It has been determined that cortisol fluctuations within the body, specifically within saliva and serum are a major indicator of psychological stress. This project will facilitate the comprehension of the optical properties of cortisol and aid in the development of a sensor which can monitor the concentration of cortisol in real-time without the need for clinical intervention, as a point of care device.

P4) Provide a summary and brief explanation of the research design, method, and data analysis.

- Participant Recruitment and General Health Screening

Once the participant has enrolled onto the study, they will be asked to fill out a consent form and general health questionnaire including COVID-19 Screening. Participants that fully meet the inclusion criteria as listed below will then be asked to take part in the study.

Inclusion Criteria

In order to be eligible for inclusion in this study, participants must meet the following criteria:

- Adult volunteers must be aged between 18-65 years who can give informed consent.
- Adult volunteers who are not taking any time of medication.
- Adult volunteers who do not suffer from any existing medical condition, including any heart conditions, peripheral vascular or arterial diseases, oral infections or mental health disorders.
- Adult volunteers who have not used recreational drugs during the last month.
- Adult volunteers who do not excessively consume alcohol (more than 14 units per week).
- Adult volunteers who have not used stimulating supplements, either natural or synthetic, at least 12 hrs prior to taking part in the study i.e. caffeinated drinks including coffee.
- Potential participants will only be enrolled if they have a good understanding of spoken English.

Exclusion Criteria

The following are a set of criteria which determines a participant's ineligibility to take part in the study:

Any inclusion criteria not met.

• Persons who decline or cannot give consent.

• Pregnant women.

• Persons with acute illness/cold or COVID-19 on the day of the study.

• Participation in drug trials/studies in the last 6 months.

• Participant declaring a Mental Illness in their Participant Information Sheet.

• Participants with cold sensitivity.

- COVID-19 Screening

Upon arrival to the testing facility, the participant's temperature will be taken to ensure they do not have a fever. Furthermore, they will be asked a series of questions to ensure the correct COVID-19 precautions have been taken:

- Do you have any signs or symptoms associated with COVID-19 infection, such as a high temperature, a new, continuous cough or loss or change to your sense of smell or taste?

- Have you or any member of your household had a confirmed case of COVID-19 in the last 10 days?

If the participant answers yes to these questions, they will be asked to reschedule their session and leave the facility.

- Mental Health Screening

Participants will be asked to fill out the NHS depression self-assessment quiz on a laptop in the laboratory for mental health screening purposes. Participants who score highly on the depression severity rating scale will not be included in this study. They will be advised to seek help from an experienced health professional through contact with their GP or the City, University of London Student Counselling and Mental Health Service at +44 (0)20 7040 8094 or coun@city.ac.uk.

Protocol Design

This research will adopt a range of analytical techniques on human saliva samples in the laboratory.

These analytical methods comprise of the following:

a. Commercial ELISA analysis from R&D Systems

b. Commercial Dye analysis from Thermo Fisher Scientific

c. Commercial UV-Vis Spectrophotometer from PerkinElmer

1. The Standardised Stress Test and Sample Collection

This experiment will be conducted at 2pm for any given day to ensure that cortisol measurements are not affected by the cortisol awakening response or the diurnal variations of cortisol.

Participants are introduced to experimenter and the lab environment in which the experiment will be conducted i.e. the Physiological Monitoring Room (CLG13). After COVID-19 screening, the participant is asked to make

themselves comfortable in the patient area of the Physiological Monitoring Room.

Upon participant informed consent, the participant is asked to complete a questionnaire to assess whether they meet inclusion criteria, as well as the NHS depression self-assessment quiz.

The participant is asked to provide 2 saliva swabs which will evaluate baseline cortisol levels. One sample will be used for spectroscopic evaluation and the other for ELISA analysis. The participant will be reminded of the stress test (MAST), which was be previously outlined in the Patient Information Sheet, involving alternation between hand immersion cold pressor task and a mental arithmetic task of randomly varying durations – unknown to the participant.

Hand Immersion Task (HI): Immerse non-dominant hand into ice-cold water (maintained at 2°C with water bath). Mental Arithmetic Task (MA): Count backwards from 2043 in steps of 17. If a mistake is made or a response is not given in 5 seconds, negative feedback is given by experimenter and the participant is asked to start again.

Trial Duration and Order: HI (90s) -> MA (45s) -> HI (60s) -> MA (60s) -> HI (60s) -> MA (90s) -> HI (90s) -> MA (45s) -> HI (60s).

Immediately after the stress test, participant is asked to provide 2 saliva swabs.

The participant is debriefed in which they are informed that the purpose of the test was to create stress and that the results do not reflect the participant's abilities. They are asked to provide 2 saliva swabs within 10 minutes of the debriefing.

The participant is asked to make themselves comfortable by laying on the patient bed in the physiological monitoring room whilst classical music or nature sounds are played, based on the participant's choice. This relaxation phase will last 20 minutes and will be unmonitored by the experimenter, to ensure that the participant is comfortable.

The participant is asked to provide the final 2 saliva swabs. The participant is informed that the study is complete and that they are free to leave. They are reminded that they can remain in contact if they have any queries.

2. Acquisition of Saliva

Human saliva samples will be acquired from volunteers following informed, written consent. Two forms will be signed so that the participant is provided with a signed copy to keep for their personal records. The saliva will be acquired through a self-swabbing technique. Unstimulated whole human saliva will be acquired by placing a synthetic swab into the mouth of the participant. The participant will be asked to chew on the swab for 60 seconds before removal. The swab will then be placed into a sealable blue cap plastic vial for subsequent laboratory-based analysis. The swab type that will be utilised will be commercially purchased from Salivette, as it is specific for salivary cortisol collection. Prior to saliva collection, the participant will be supplied with an instructional booklet to ensure they understand the procedure of saliva acquisition. The action of saliva

collection will be conducted entirely by the participant throughout the duration of the study. This will ensure that covid-19 guidelines regarding social distancing are maintained throughout the investigation. No more than 1.1ml of saliva will be acquired from the participant during each saliva collection. Saliva collection will be required twice during 4 phases of the investigation, for a total of 8 saliva samples per participant. One batch of saliva samples will be used for ELISA analysis and the other will be used for UV-Vis spectroscopic analysis.

3. Saliva Handling and Preparation

Following acquisition of the saliva samples they will be centrifuged for 2 minutes at 1000 x g to yield clear saliva samples. Particles and mucus strands will be collected in the extended tip of the Salivette vial and disposed of. The recovered saliva will be frozen in a -80 °C freezer for subsequent analysis. Saliva will only be handled in a suitable, well-prepared area within the Biomedical Engineering Research Laboratory. The area will be equipped will the analysing equipment and tools needed for handling the samples. The samples will not be removed from the dedicated space for the saliva analysis. The area will have smooth, easily sterilisable surfaces, a sink, a first aid kit sterilising agents and kit, sharps disposal bin, biological fluids disposal bin, and suitable storage for saliva samples. The investigator handling saliva will wear suitable attire, such as a disposable gown, white lab coat, disposable nitrile gloves, n95 mask and safety goggles. Saliva samples will be analysed within a maximum time of 3 months, depending on the recruitment of participants for the study as ELISA analysis will be conducted on all samples after they have been collected from all participants recruited throughout the duration of the study. After analysis, the samples will be disposed of in a suitable biological fluids' disposal bin. Waste from the ELISA method will be disposed of as clinical waste. Within this time, the saliva samples will be stored in a dedicated, padlocked freezer awaiting analysis.

4. Analytical Procedures on Saliva Samples

• ELISA method:

Following preparation and thawing of samples, the saliva samples will be pipetted into a clear microtiter plate coated with an antibody. A cortisol-peroxidase conjugate is added to the wells. After 1-hour of incubation, the plate will be washed, and the substrate will be added. After a 20-minute incubation, the reaction is stopped, and the intensity of the colour generated from the reaction is detected an analysed with a microtiter plate reader at 450nm.

• Spectroscopic method: Following preparation and thawing of samples, the saliva samples will be pipetted into individual vials containing a solution of tetrazolium blue dye and methanol. After 10 minutes, the solution will be pipetted into a disposable cuvette. The cuvette will be placed into the spectrophotometer where measurements will be taken. Optical absorption spectra of saliva samples will be taken from 400nm to 720nm. The concentration of cortisol in saliva will be measured by optical absorption spectrophotometry.

5. Disposal and Sterilisation

- All waste will be collected by a registered carrier on the day of sample analysis.
- Analysed saliva samples will be disposed of in appropriate biological fluids waste disposal bins (yellow biohazard-marked bin).
- All items that come into contact with biological fluids, such as used cuvettes and vials will either be disposed in the same manner or cleaned with sterilising solution.

6. Analysis of data

All acquired data will be de-identified and analysed. Signal processing techniques will be applied to the obtained optical spectra to acquire further information regarding salivary cortisol concentrations and its fluctuations in response to the undergone stress test. The specificity and sensitivity of the optical technique will be compared with the ELISA method through a cortisol calibration curve. Analysis of variance (e.g. t-test) will be conducted to compare measured variables using the different measurement techniques. The measurements will be saved on a computed in an encrypted and password protected folder. Artificial neural networks and machine learning based techniques will be applied to the results to determine data trends, for the development of a mathematical model.

P4.1) If relevant, please upload your research protocol.

P5) What do you consider are the ethical issues associated with conducting this research and how do you propose to address them?

The ethical issues associated with this research investigation include:

1. Risk to the volunteer acquiring saliva from their mouth

a. Discomfort: Placing the synthetic swab in the mouth of the volunteer may cause minimal discomfort.

Eradication: The volunteer will be reminded that they can cease the procedure at any time throughout the course of the study.

2. Risk to the volunteer during the Maastricht Stress Test

a. Discomfort: Placing their hand in the container of cold water (2 degrees Celsius) can cause minimal discomfort.

b. Mental Distress: The mental arithmetic stage of the stress test may cause minor mental distress.

Eradication: The volunteer will be given a hand towel to place their hand on throughout the phases of the stress test. The volunteer will be reminded that they can stop participation in the stress test during any time throughout the course of the stress test.

3. Risk to the principal investigator during preparation and analysis of saliva samples

a. Exposure: Risk of contact with hazardous chemicals and dyes.

b. Infection: Risk of contact with participants infected with Covid-19.

Eradication: The principal investigator will wear protective clothing, disposable gloves, safety goggles and a face mask. The area will be

maintained in a safe and sterile manner. Access to the laboratory area will not be permitted to any other person while samples or unsterile containers are exposed.

Participants will be asked to take a covid-19 PCR test or lateral flow test 48 hours prior to their entrance to the laboratory. This will be declared on the patient information sheet. Participants will be advised to not attend the study if they are feeling ill or develop any covid-19 symptoms on the day of the study.

P6) Project start date
The start date will be the date of approval.

P7) Anticipated project end date
28 Oct 2022

P8) Where will the research take place?
The research will take place in the physiological monitoring room (CLG13) and the Biomedical Laboratory (CLG11), at City, University of London.

P10) Is this application or any part of this research project being submitted to another ethics committee, or has it previously been submitted to an ethics committee? No

Human participants: information and participation

The options for the following question are one or more of:
'Under 18'; 'Adults at risk'; 'Individuals aged 16 and over potentially without the capacity to consent'; 'None of the above'.

H1) Will persons from any of the following groups be participating in the project? None of the above

H2) How many participants will be recruited?
40

H3) Explain how the sample size has been determined.
No formal power study has been performed to calculate the sample size as this is considered to be a preliminary study that will serve to inform the design of future studies involving sensor development. The number of participants has been selected to allow for sufficient measurements by the optical techniques and enable the measurement of accuracy to be determined.

H4) What is the age group of the participants?

Lower	Upper
18	65

H5) Please specify inclusion and exclusion criteria.
Inclusion Criteria

To be eligible for inclusion in this study, participants must meet the following criteria:

- Adult volunteers must be aged between 18-65 years who can give informed consent.
- Adult volunteers who are not taking any time of medication.
- Adult volunteers who do not suffer from any existing medical condition, including any heart conditions, peripheral vascular or arterial diseases, oral infections, or mental health disorders.
- Adult volunteers who have not used recreational drugs during the last month.
- Adult volunteers who do not excessively consume alcohol (more than 14 units per week).
- Adult volunteers who have not used stimulating supplements, either natural or synthetic, at least 12 hrs prior to taking part in the study i.e., caffeinated drinks including coffee.
- Potential participants will only be enrolled if they have a good understanding of spoken English.

Exclusion Criteria

The following are a set of criteria which determines a participant's ineligibility to take part in the study:

Any inclusion criteria not met.

- Persons who decline or cannot give consent.
- Pregnant women.
- Persons with acute illness/cold or COVID-19 on the day of the study.
- Participation in drug trials/studies in the last 6 months.
- Participant declaring a Mental Illness in their Participant Information Sheet.
- Participants with cold sensitivity.

Exclusion Criteria

The following are a set of criteria which determines a participant's ineligibility to take part in the study:

Any inclusion criteria not met.

- Persons who decline or cannot give consent.
- Pregnant women.
- Persons with acute illness/cold or COVID-19 on the day of the study.
- Participation in drug trials/studies in the last 6 months.
- Participant declaring a Mental Illness in their Participant Information Sheet.
- Participants with cold sensitivity.

H6) What are the potential risks and burdens for research participants and how will you minimise them?

The ethical issues associated with this research investigation include:

1. Risk to the volunteer acquiring saliva from their mouth

a. Discomfort: Placing the synthetic swab in the mouth of the volunteer may cause minimal discomfort.

Eradication: The volunteer will be reminded that they can cease the procedure at any time throughout the course of the study.

2. Risk to the volunteer during the Maastricht Stress Test

a. Discomfort: Placing their hand in the container of cold water (2 °C) can cause minimal discomfort.

b. Mental Distress: The mental arithmetic stage of the stress test may cause minor mental distress.

Eradication: The volunteer will be given a hand towel to place their hand on throughout the phases of the stress test. The volunteer will be reminded that they can stop participation in the stress test during any time throughout the course of the stress test. Participants with peripheral vascular diseases and cold sensitivity will be excluded from the study.

3. Risk to the principal investigator during preparation and analysis of saliva samples

a. Exposure: Risk of contact with hazardous chemicals and dyes.

b. Infection: Risk of contact with participants infected with Covid-19.

Eradication: The principal investigator will wear protective clothing, disposable gloves and gown, safety goggles and an n95 face mask. The area will be maintained in a safe and sterile manner. Access to the laboratory wet bench will not be permitted to any other person while samples or unsterile containers are exposed.

4. Covid-19 Risk Assessment

Upon arrival to the testing facility, the participant's temperature will be taken to ensure they do not have a fever. Furthermore, they will be asked a series of questions to ensure the correct COVID-19 precautions have been taken:

- Do you have any signs or symptoms associated with COVID-19 infection, such as a high temperature, a new, continuous cough or loss or change to your sense of smell or taste?
- Have you or any member of your household had a confirmed case of COVID-19 in the last 10 days?

If the participant answers yes to these questions, they will be asked to reschedule their session and leave the facility. Requirements such as the wearing of face masks, social distancing, ensuring adequate ventilation and the cleaning of surfaces will be maintained. All participants will be encouraged to regularly sanitise or wash their hands. The researchers will also sanitise or wash their hands regularly and will wipe down all surfaces after use. • The study will be conducted on participants one by one to allow enough space and avoid overcrowding.

H7) Will you specifically recruit pregnant women, women in labour, or women who have had a recent stillbirth or miscarriage (within the last 12 months)?

No

H8) Will you directly recruit any staff and/or students at City?
Staff
Students

H8.1) If you intend to contact staff/students directly for recruitment purpose, please upload a letter of approval from the respective School(s)/Department(s).

H8.2) Will you recruit students by virtue of their attendance on specific programmes or modules? No

H9) How are participants to be identified, approached and recruited, and by whom?
Advertisement in the form of a recruitment flyer which will be affixed around the University premises and emails, with the advertisement attached, will be sent to groups inviting interested persons to contact the investigator(s) about participating in the studies.

H10) Please upload your participant information sheets and consent form, or if they are online (e.g. on Qualtrics) paste the link below.
H11) If appropriate, please upload a copy of the advertisement, including recruitment emails, flyers or letter.

H12) Describe the procedure that will be used when seeking and obtaining consent, including when consent will be obtained.
Volunteers will be identified following an expression of interest on seeing a poster/email regarding the study. A participant information sheet will be provided, explaining exactly the aims and objectives of the study, and, if they agree to take part, exactly what will happen to them. The potential participants will be given enough time (min 24 hours) to think whether to take part or to consult relatives, etc. Volunteers will be clearly informed that participation in the study is voluntary and that refusal to participate will not affect their role, study etc within the University.
Participants will be provided with a full explanation of the nature, purpose and requirements of the study including Participant Information Sheet and Informed Consent Form. The consent form will be countersigned by the investigators only when satisfied that the participant has understood the information sheet and is willing to give written consent to participate in the study, and that the they understand that it is their right to withdraw from the study at any time without the need to explain their reason for doing so and without any prejudice for future treatment.
No participants will be recruited if they lack capacity and if it is deemed that capacity is lost during the trial period then the participant will be removed from the trial and all information relating to them will be destroyed.

If the volunteer agrees to take part and signs a consent form, they will be recruited to the study.

H13) Are there any pressures that may make it difficult for participants to refuse to take part in the project?

No

H14) Is any part of the research being conducted with participants outside the UK?

No

Human participants: method

The options for the following question are one or more of:
'Invasive procedures (for example medical or surgical)'; 'Intrusive procedures (for example psychological or social)'; 'Potentially harmful procedures of any kind'; 'Drugs, placebos, or other substances administered to participants'; 'None of the above'.

M1) Will any of the following methods be involved in the project:

Intrusive procedures (for example psychological or social)

M1.1) If you are using any of the procedures above, what precautions will you take to minimise any potential harm?

The acquisition of saliva from the volunteer may cause minimal discomfort. To eradicate any potential harm, the volunteer will be asked to provide the saliva sample themselves.

The mental stress test is considered an intrusive procedure. It is however, a well-established, standardised stress test which will not involve the potential for psychological distress or pain greater than that of normal life for the participant. The stress test will not deviate from the standardised version of the Maastricht stress test.

The volunteer will be debriefed regarding the stress test immediately after completion and they will be allowed to rest comfortably in the physiological monitoring room until they are comfortable to leave.

M1.2) What procedures are in place for the appropriate referral of a participant who discloses an emotional, psychological, health, education or other issue during the course of the research or is identified by the researcher to have such a need?

The participant will be reminded that they are free to cease the study at any point if they feel that it is too stressful or personal for them. If they disclose any health related issues, they will be advised to speak to their GP or the City Counselling and Mental Health Services team with regards to their issues at +44 (0)20 7040 8094 or coun@city.ac.uk.

M2) Does the project involve any deceptive research practices?

No

M3) Is there a possibility for over-research of participants?
No

M4) Please upload copies of any questionnaires, topic guides for interviews or focus groups, or equivalent research materials.

M5) Will participants be provided with the findings or outcomes of the project? Yes

M5.1) Explain how this information will be provided.
The participant can request to be informed of the outcomes of the project. They will then be provided a lay summary of the results of the study at the end of the project.

M6) If the research is intended to benefit the participants, third parties or the local community, please give details.

M7) Are you offering any incentives for participating?
No

M8) Does the research involve clinical trial or clinical intervention testing that does not require Health Research Authority or MHRA approval? No

M9) Will the project involve the collection of human tissue or other biological samples that does not fall under the Human Tissue Act (2004) that does not require Health Research Authority Research Ethics Service approval?
No

M10) Will the project involve potentially sensitive topics, such as participants' sexual behaviour, their legal or political behaviour, their experience of violence?
No

M11) Will the project involve activities that may lead to 'labelling' either by the researcher (e.g. categorisation) or by the participant (e.g. 'I'm stupid', 'I'm not normal')?
No

Data

D1) Indicate which of the following you will be using to collect your data.
Questionnaire
Physiological measurements

D2) How will the the privacy of the participants be protected?
De-identified samples or data

D3) Will the research involve use of direct quotes?
No

D5) Where/how do you intend to store your data?
Data to be kept in a locked filing cabinet
Password protected computer files
Storage at City

D6) Will personal data collected be shared with other organisations?
No

D7) Will the data be accessed by people other than the named researcher, supervisors or examiners? Yes

D7.1) Explain by whom and for what purposes.
The data may also be accessed by researchers within the Biomedical Engineering Research Group with expertise in data analysis and statistics. It is the aim of the researchers working on this project to pursue onto the development of an in vivo sensor for cortisol monitoring and further research into the effects of cortisol on human saliva. Therefore, although there aren't any immediate plans to reuse the data, it is possible that the data will be used in future research relating to this work and/or to seek funding.

D8) Is the data intended or required (e.g. by funding body) to be published for reuse or to be shared as part of longitudinal research or a different/wider research project now or in the future?
No

D10) How long are you intending to keep the research data generated by the study?
10 years.

D11) How long will personal data be stored or accessed after the study has ended?
10 years.

D12) How are you intending to destroy the personal data after this period?
Paper records will be formally shredded and all electronically archived data will be erased.

Health & safety

HS1) Are there any health and safety risks to the researchers over and above that of their normal working life? No

HS3) Are there hazards associated with undertaking this project where a formal risk assessment would be required?
No

School of Mathematics, Computer Science & Engineering
Northampton Square
London EC1V 0HB
Tel. +44(0)20 7040 8131
Email: p.kyriacou@city.ac.uk

CONSENT FORM Title of Study: **Stress Monitoring Through Cortisol Measurements from Saliva Using Spectroscopic Methods**

Principal Investigator: Professor Panicos Kyriacou, Associate Dean for Postgraduate Studies,
Director of Biomedical Engineering Research Group, Reg No.: 90096558

REC Reference Number: ETH2122-0653

		Please initial box
1.	I confirm that I have had the project explained to me, and I have read the participant information sheet, which I may keep for my records. I understand this will involve (Please tick the appropriate boxes): • Providing eight samples of saliva ☐ • Completing a questionnaire asking me about my general mental health ☐ • Dedicating my time for participation in the study ☐ • Participating in a standardised stress test ☐ I understand that the collected saliva samples will be analysed for cortisol concentrations with the help of the following instruments: • A commercial UV-Vis Spectrophotometer from Perkin Elmer • Commercial ELISA analysis from R&D Systems • Commercial Dye analysis from Thermo Fisher Scientific	☐ ☐
2.	This information will be held and processed for the following purpose(s): • Scientific publications I understand that any information I provide is confidential, and that no information that could lead to the identification of any individual will be disclosed in any reports on the project, or to any other party. No identifiable personal data will be published. The identifiable data will not be shared with any other organisation. I understand that my data will be saved truly anonymous, and my personal information locked safely within the University to protect my identity from being made public. I understand that my data may be reused in the future as part of a wider research project relating to this work	☐ ☐ ☐

3.	I understand that my participation is voluntary, that I can choose not to participate in part or all of the project, and that I can withdraw at any stage of the project without being penalized or disadvantaged in any way.	☐
4.	I agree to City, University of London recording and processing this information about me. I understand that this information will be used only for the purpose(s) set out in this statement and my consent is conditional on City complying with its duties and obligations under the Data	☐

_____	_____	_____
Name of Participant	Signature	Date

_____	_____	_____
Name of Researcher	Signature	Date

When completed, 1 copy for participant; 1 copy for researcher file.

	Protection Act 2018 and the General Data Protection Regulation (GDPR).	
5.	I consent to my data will only be accessed by the investigators from the Research Centre for Biomedical Engineering laboratory at City University.	☐
6.	I agree to take part in the above study.	☐
7.	I understand that the personal data processed by City as data controller for the purposes as described in the participant information sheet for this research study are processed under the following lawful basis: City, University of London considers the lawful basis for processing personal data to fall under Article 6(1)(e) of GDPR (public task) as the processing of research participant data is necessary for learning and teaching purposes and all research with human participants by staff and students has to be scrutinised and approved by one of City's Research Ethics Committees.	☐
8.	I understand that the following special category data will be collected and retained as part of this research study: information about my health and biometric data. City considers the processing of special category personal data will fall under: Article 9(2)(g) of the GDPR as the processing of special category data has to be for the public interest in order to receive research ethics approval and occurs on the basis of law that is, inter alia, proportionate to the aim pursued and protects the rights of data subjects and also under Article 9(2)(a) (explicit consent) of the GDPR as the provision of these personal data is completely voluntary.	☐

School of Mathematics, Computer Science & Engineering.
Northampton Square London
EC1V 0HB
Tel. +44(0)20 7040 8131
Email: p.kyriacou@city.ac.uk

Research Centre for Biomedical Engineering (RCBE) City, University of London

Participant Information Sheet

Title of Study: Stress Monitoring Through Cortisol Measurements from Saliva

Name of Principal Investigator

Professor Panicos Kyriacou, Associate Dean for Postgraduate Studies, Director of
Biomedical Engineering Research Group, Reg No.: 90096558

Invitation paragraph

You are being invited to take part in a research study. Before you decide, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and talk to others about the study, if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether you wish to take part.

What is the purpose of the study?

The Research Centre for Biomedical Engineering (RCBE) has many years of experience in the development of biomedical sensors for physiological monitoring. Currently, the centre is focusing its efforts on the development of a stress monitoring sensor which measures cortisol concentrations from small volumes of saliva. The development of this sensor would give a quick and easy way to determine psychological stress in humans without the need for lengthy and expensive processes that often involve clinical visits and sending off samples to commercial laboratories. This can be a major inconvenience in a person's day-to-day life and often leads to the stigmatisations surrounding stress and mental wellbeing. As the RCBE has expertise in optical spectroscopy and the development of optical sensors, this project will be enhanced by the support of the research centre and expand the centre's expertise into the field of mental health and stress monitoring.

The project will involve the study of interactions between various dyes and their aide in the spectroscopic evaluation of known concentrations of stress hormones in saliva. Participants will be asked to complete a general mental health questionnaire and a stress test whilst supplying the investigator with 8 saliva swabs to evaluate the change in cortisol levels in saliva as the participant undergoes a standardised stress test. This research will provide new knowledge regarding the spectroscopic 'fingerprint' of cortisol in saliva and aid the conception and development of a sensor which can detect

changes in cortisol rapidly and with high sensitivity and accuracy. The standardised stress test that you will take part in is known globally as the Maastricht Acute Stress Test (MAST). This stress test involves submerging one hand in a container of ice-cold water, known as the cold-pressor test, and partaking in a mental arithmetic exercise. The stress test is known to induce stress in a safe manner which does not surpass any stresses that you may experience in your normal dayto-day activities.

The information collected about the health of the participant is classed as special category data under the General Data Protection Regulation. The research data will be retained for 10 years after the end of the study.

Why have I been invited to participate?

You have been invited to participate to this study as healthy volunteer aged between 18-65 years. The saliva samples provided by you will help us to test and verify the mathematical model that we have built in the lab to understand the fluctuations in cortisol concentration in response to external psychological stress. On successful trails of this project, the model will enable us to understand the optical properties of cortisol and facilitate the development of a handheld stress monitor that can monitor levels of psychological stress through saliva with real-time applications and at point-of-care sights.

You should not take part in this research if you do not meet the following inclusion criteria or prefer not to share information you consider too personal.

Inclusion Criteria

To be eligible for inclusion in this study, participants must meet the following criteria:

- Adult volunteers must be aged between 18-65 years who can give informed consent.
- Adult volunteers who are not taking any time of medication.
- Adult volunteers who do not suffer from any existing medical condition, including any heart conditions, peripheral vascular or arterial diseases, oral infections, or mental health disorders.
- Adult volunteers who have not used recreational drugs during the last month.
- Adult volunteers who do not excessively consume alcohol (more than 14 units per week).
- Adult volunteers who have not used stimulating supplements, either natural or synthetic, at least 12 hrs prior to taking part in the study i.e., caffeinated drinks including coffee.
- Potential participants will only be enrolled if they have a good understanding of spoken English.

Exclusion Criteria

The following are a set of criteria which determines a participant's ineligibility to take part in the study:

Any inclusion criteria not met.

- Persons who decline or cannot give consent.
- Pregnant women.
- Persons with acute illness/cold or COVID-19 on the day of the study.
- Participation in drug trials/studies in the last 6 months.
- Participant declaring a Mental Illness in their Participant Information Sheet.
- Participants with cold sensitivity.

Do I have to take part?

Participation in this study is entirely voluntary. You can avoid answering the questions which you may feel are intrusive or too personal by withdrawing. It is up to you to decide whether to take part. If you do, you will be asked to fill a questionnaire and sign a consent form. If you decide to take part, you are still free to withdraw at any time.

For students only: Your participation or withdraw will not affect your grade during your studies at City University.

What will happen to me if I take part?

Following the advertisement or email, if you wish to volunteer and hear back from us, (i.e. we identify you as a potential volunteer) you will have to come to the Biomedical Engineering lab on the particular date and time (as will be mentioned in your email) and meet the personal investigator. Thereafter, you will be given a questionnaire and a consent form. If you agree to be a volunteer, you will be asked to take part in the mentioned stress test and give eight saliva swab samples.

What do I have to do?

If you do agree to volunteer for the study, you can contact the principal investigator and will be asked to fill a questionnaire and sign a consent form. We will ask that you do not eat or drink (with exception of water) at least 2 hour prior to the study.

What are the possible disadvantages and risks of taking part?

There may be discomfort in placing the synthetic swab in the mouth of the volunteer. To eradicate this we will ask you to place the swab into your mouth by yourself. You are also reminded that you can stop participation the stress test during any time throughout the course of the study. ALL measurements and procedures will be stopped immediately if you express any discomfort and ask for the procedure to be stopped. You will be asked to fill out a questionnaire, however, if you wish not to answer some of the questions, then you will have to be excluded from the study.

What are the possible benefits of taking part?

This study will not have a direct benefit to you. However, we believe that the results of this study may contribute to clinicians and to the scientific community.

What will happen when the research study stops?

After the stress test is completed, you will be asked to supply 2 saliva swabs. Thereafter the reason for the stress test will be explained to you and you will be asked to make yourself comfortable in the physiological monitoring room for 20 minutes. After this you will be asked to supply a final two saliva swabs, after which you will be allowed to go home.

Will what I say in this study be kept confidential?

You will be asked to fill a form regarding your mental health and sign a consent form. Your personal information will not be saved except the consent form and the questionnaire which will be secured and locked in Research Centre for Biomedical Engineering inside the university. Your measurement data will be saved anonymously as a number (e.g. “Participant 1”) into a computer. Only your age and sex will be saved. Your measurement data will only be accessed by the investigators and some selected researchers from the biomedical research laboratory at City University.

What should I do if I want to take part?

If you do agree to volunteer for the study, you can contact the principal investigator.

What will happen to the results of the research study?

The study results will be used for thesis submission. Results may also be utilized for scientific publications. True anonymity of the participants will be kept when publishing the results.

The study is NOT considered a medical examination of your health status. Participation in this study should not be taken as substitution to regular medical examinations and your GP will not be notified.

What will happen if I do not want to carry on with the study?

In case of withdraw before commencing of the study no data will be taken and saved. If you withdraw during the study, data may be saved for future analysis.

Who is organising and funding the research?

The study will be conducted by the members of staff and students at Research Centre for
Biomedical Engineering at City, University of London who are directly involved with the EPSRC funded project:" Patient held device for cortisol monitoring in depressed patients." They are:

1. Professor Panicos Kyriacou
2. Dr Meha Qassem
3. Ms. Tashfia Ahmed

Who has reviewed the study?

This study has been approved by the Senate Research Ethics Committee at City, University of London.

Contact for Further Information

Professor Panicos Kyriacou (Principal Investigator and Point of Contact) Email: p.kyriacou@city.ac.uk
Phone Number: +44 (0)20 7040 8131

What are my rights under the data protection legislation?

City, University of London is the data controller for the personal data collected for this research project. Your personal data will be processed for the purposes outlined in this notice. The legal basis for processing your personal data will be that this research is a task in the public interest; and that City, University of London considers the lawful basis for processing personal data to fall under Article 6(1)(e) of GDPR (public task) as the processing of research participant data is necessary for learning and teaching purposes and all research with human participants by staff and students has to be scrutinised and approved by one of City's Research Ethics Committees.

City considers the processing of special category personal data – information about your health and biometric data will fall under Article 9(2)(g) of the GDPR as the processing of special category data has to be for the public interest in order to receive research ethics approval and occurs on the basis of law that is, inter alia, proportionate to the aim pursued and protects the rights of data subjects and also under Article 9(2)(a) (explicit consent) of the GDPR as the provision of special category data is completely voluntary.

For more information, please visit www.city.ac.uk/about/city-information/legal **What if I have concerns about how my personal data will be used after I have participated in the research?**

In the first instance you should raise any concerns with the research team, but if you are dissatisfied with the response, you may contact the Information Compliance Team at dataprotection@city.ac.uk or phone 0207 040 4000, who will liaise with City's Data Protection Officer Dr William Jordan to answer your query. If you are dissatisfied with City's response you may also complain to the Information Commissioner's Office at www.ico.org.uk

What if there is a problem?

Projects taking place in countries where it is possible that the participants will not be able to go through the standard complaint's procedure (e.g. if participants may feel inhibited or unable to complain to City, University of London, for reasons of cost, language, literacy and culture) a local contact needs to be identified. This should be someone who is not directly involved in the research. The name of this person should be provided to the research ethics committee approving the application. The local contact needs to be made aware that they must pass all written and verbal complaints/issues on to the Secretary to Senate Research Ethics Committee soon as possible.

For all research undertaken in the UK if you have any problems, concerns, or questions about this study, you should ask to speak to a member of the research team. If you remain unhappy and wish to complain formally, you can do this through City's complaints procedure. To complain about the study, you need to phone 020 7040 3040. You can then ask to speak to the Secretary to Senate Research Ethics Committee and inform them that the name of the project is: Patient held device for cortisol monitoring in depressed patients.

You could also write to the Secretary at:
Anna Ramberg
Research Integrity Manager, Research & Enterprise
City, University of London, Northampton Square, London, EC1 V0HB

You should add that if they have any concerns about the way in which the study has been conducted, they should contact the Vice-President (Research & Enterprise) at researchintegrity@city.ac.uk.

Thank you for reading the Information Sheet

Date

21-03-2022 (Version 2)

School of Mathematics, Computer Science & Engineering.
Northampton Square London
EC1V 0HB
Tel. +44(0)20 7040 8131
Email: p.kyriacou@city.ac.uk

Research Centre for Biomedical Engineering (RCBE) City, University of London

PARTICIPANTS NEEDED FOR RESEARCH IN BIOMEDICAL PHYSIOLOGICAL MEASUREMENT

We are looking for volunteers to take part in a study of

Stress Monitoring Through Cortisol Measurements from Saliva Using Spectroscopic Methods

As a participant in this study, you will be asked to:

- Complete a stress evaluation questionnaire
- Give saliva swab samples 8 times
- Participate in a standardised stress test involving a cold pressor hand immersion task and a mental arithmetic task

Your participation will involve *one* session, which will last approximately *1 hour,*

Your participation is considered voluntary. There will not be any monetary reward or travel expenses reimbursed.

For more information about this study, or to volunteer for this study, please contact:

Ms. Tashfia Ahmed
(Research Centre for Biomedical Engineering) At
020 7040 3878 or
Email: *tashfia.ahmed@city.ac.uk*

This study has been reviewed by and received ethics clearance through the *Senate Research Ethics Committee at* City, University of London.

If you would like to complain about any aspect of the study, please contact the Secretary to the Senate Research Ethics Committee on 020 7040 3040 or via email: Anna.Ramberg.1@city.ac.uk

City, University of London is the data controller for the personal data collected for this research project. If you have any data protection concerns about this research project, please contact City's Information Compliance Team at dataprotection@city.ac.uk

Stress Monitoring Through Cortisol Measurements from Saliva Using Spectroscopic Methods

Background

Clinical depression or major depressive disorder is one of the most prevalent mood disorders affecting over 3.8% of the global population[1]. It is considered by the World Health Organisation (WHO) as one of the main contributors to the global health and economic burden. With over one in five adults (19.7%) experiencing a form of depression in the UK since the start of the COVID-19 pandemic, the correct diagnosis and treatment of the disorder is of utmost significance[2]. This has doubled from 9.7% since before the pandemic (July 2019)[2]. Moreover, financial difficulties during the COVID-19 pandemic and the anticipation of unexpected expenses, has further accelerated the rate of development of depressive symptoms with over 21.2% of adults developing moderate to severe depressive symptoms by June 2020[2].

Currently, the diagnostic procedures surrounding mental health focus on a qualitative approach, through the utilisation of mood-evaluation and subjective questionnaires. Questionnaires and surveys such as the Hamilton Depression Rating Scale (HAM-D) have proven to be effective in the assessment of depression, however cases of misdiagnosis still exist[3]–[6]. Several factors can contribute to the misdiagnosis of human depression, such as the miscoding of mental illnesses as secondary disorders to more socially accepted illnesses like brain tumours and cardiovascular disease[6]. The stigmatisation of mental illnesses, as well as the economic burden associated with such disorders further deteriorate the diagnostic processes involved. It has been reported that often physicians will deliberately treat mental illnesses as secondary illnesses to ensure that patients are receiving rightful imbursement for treatment from insurance companies[7]. However, by doing so, physicians are also contributing to the stigmatisation of mental illnesses and preventing the advancements and acceptance of mental illnesses as disorders of great severity[8]. This destructive societal process is leading to the further burdening of global economy and health, as the population of humans affected by mental illnesses is increasing[2], [6].

To eradicate the issues of misdiagnosis and the subjective diagnoses of mental illnesses, the implementation of a quantitative system is necessary. Not only will this lead to greater confidence in making judgments regarding the depression diagnoses, but it will also encourage the de-stigmatisation of mental illnesses through the association of the illnesses with tangible and comprehendible information from the human body. Otherwise, the existing systems will continue to cause disadvantages for the patients who are misdiagnosed, as well as those who are correctly diagnosed due to the economic implications.

Cortisol is predominantly considered as the main biomarker of stress, showing a strong relationship with the manifestation of clinical depression in the human body[9]. However, the regular monitoring of this stress hormone has not yet been considered as an objective measure of clinical depression, which could facilitate the early identification of the mental illness in patients from home settings. This is primarily due to the current measurement techniques involved with the monitoring of cortisol, such as enzyme-linked immunoassays (ELISA) which can be time consuming, costly and require complex processing in the laboratory [10]. Therefore, regular and on-demand testing of cortisol levels under these conditions can be greatly troublesome for patients, especially for those in high-risk groups.

This project proposes the development of a point-of-care device which is capable of measuring cortisol levels from saliva samples, alongside complementing stress hormones, such as adrenaline, noradrenaline and dehydroepiandrosterone through optical techniques[9], [11]. The utilisation of infrared, ultraviolet-visible spectroscopy and colorimetric techniques will facilitate the rapid quantification of cortisol and stress hormone levels from different media. As there have been successful attempts in the measurement of different analytes in blood and saliva through these techniques, it is considered that the measurement of cortisol is feasible[12]. This will ultimately lead to the development of a system for the measurement of cortisol and stress hormones alongside physiological stress biomarkers. Further advancements within this field could lead to the development of non-invasive transcutaneous technology for the continuous measurement of stress levels in depressed patients through stress hormone quantification. Evidently, the design and development of this patient-held device will lead to improvements in the understanding of the pathophysiology of the course of depression and its relationship with psychological stress. Additionally, this device could empower patients through enabling greater patient involvement in the management of the mental illness.

Aims and Objectives

The project proposes the utilisation of spectroscopic techniques to overcome the current limitations of psychological stress monitoring. With UV-visible spectroscopy and colorimetric analysis, cortisol concentrations within human saliva can be determined rapidly and accurately. This will provide a novel technique for the measurement of stress biomarkers in salivary applications, eradicating the need for use of blood sampling and ELISA sensors. As the RCBE has expertise in optical spectroscopy and the development of optical sensors, this project will be enhanced by the support of the research centre and expand the centre's expertise into the field of mental health and stress monitoring. The project will involve the study of interactions between various dyes and their aide in the spectroscopic evaluation of known concentrations of stress hormones in saliva, before application of this knowledge in an in vivo protocol involving human participants. Participants will be asked to complete a stress test whilst supplying the investigator with 4 saliva swabs to evaluate the change in cortisol levels in saliva as the participant undergoes a standardised stress test. This research will provide new knowledge regarding the spectroscopic 'fingerprint' of cortisol in saliva and aid the conception and development of a sensor which can detect cortisol fluctuates rapidly and with high sensitivity and accuracy. The aims of this study are:

- Collection of human saliva from participants undergoing a standardised stress test.
- Optical analysis of human saliva using spectrophotometers for colorimetric determination of cortisol.
- Cross-validation of proposed technique of cortisol determination with the goldstandard i.e., ELISA analysis.
- Development of a portable sensor that measures cortisol concentrations from ultra-low volumes of human saliva.
- Development of a computational model that can determine and predict cortisol concentrations from ultra-low volumes of human saliva.

Protocol

- Participant Recruitment and General Health Screening

Once the participant has enrolled onto the study, they will be asked to fill out a consent form and general health questionnaire including COVID-19 Screening. Participants that fully meet the inclusion criteria as listed below will then be asked to take part in the study.

Inclusion Criteria

To be eligible for inclusion in this study, participants must meet the following criteria:

- Adult volunteers must be aged between 18-65 years who can give informed consent.
- Adult volunteers who are not taking any time of medication.
- Adult volunteers who do not suffer from any existing medical condition, including any heart conditions, peripheral vascular or arterial diseases, oral infections, or mental health disorders.
- Adult volunteers who have not used recreational drugs during the last month.
- Adult volunteers who do not excessively consume alcohol (more than 14 units per week).
- Adult volunteers who have not used stimulating supplements, either natural or synthetic, at least 12 hrs prior to taking part in the study i.e., caffeinated drinks including coffee.
- Potential participants will only be enrolled if they have a good understanding of spoken English.

Exclusion Criteria

The following are a set of criteria which determines a participant's ineligibility to take part in the study:

Any inclusion criteria not met.

- Persons who decline or cannot give consent.
- Pregnant women.
- Persons with acute illness/cold or COVID-19 on the day of the study.
- Participation in drug trials/studies in the last 6 months.
- Participant declaring a Mental Illness in their Participant Information Sheet.
- Participants with cold sensitivity.

- COVID-19 Screening

Upon arrival to the testing facility, the participant's temperature will be taken to ensure they do not have a fever. Furthermore, they will be asked a series of questions to ensure the correct COVID-19 precautions have been taken:

- Do you have any signs or symptoms associated with COVID-19 infection, such as a high temperature, a new, continuous cough or loss or change to your sense of smell or taste?
- Have you or any member of your household had a confirmed case of COVID-19 in the last 10 days?

If the participant answers yes to these questions, they will be asked to reschedule their session and leave the facility.

- Mental Health Screening

Participants will be asked to fill out the NHS depression self-assessment quiz on a laptop in the laboratory for mental health screening purposes. Participants who score highly on the depression severity rating scale will not be included in this study. They will be advised to seek help from an experienced health professional through contact with their GP or the City, University of London Student Counselling and Mental Health Service at +44 (0)20 7040 8094 or coun@city.ac.uk.

Protocol Design

This research will adopt a range of analytical techniques on human saliva samples in the laboratory.

These analytical methods comprise of the following:

a. Commercial ELISA analysis from R&D Systems

b. Commercial Dye analysis from Thermo Fisher Scientific

c. Commercial UV-Vis Spectrophotometer from PerkinElmer

1. The Standardised Stress Test and Sample Collection

This experiment will be conducted at 2pm for any given day to ensure that cortisol measurements are not affected by the cortisol awakening response or the diurnal variations of cortisol.

Participants are introduced to experimenter and the lab environment in which the experiment will be conducted i.e., the Physiological Monitoring Room (CLG13). After COVID-19 screening, the participant is asked to make themselves comfortable in the patient area of the Physiological Monitoring Room. The Physiological Monitoring Room is separate from the shared Biomedical Laboratory facility, with its own ventilation system. Therefore, the room will be well ventilated, and the blinds will ensure the privacy and confidentiality of the participants during the experimental procedure.

Upon participant informed consent, the participant is asked to complete a questionnaire to assess whether they meet inclusion criteria, as well as the NHS depression self-assessment quiz.

The participant is asked to provide 2 saliva swabs which will evaluate baseline cortisol levels. One sample will be used for spectroscopic evaluation and the other for ELISA analysis. The participant will be

reminded of the stress test (MAST), which was be previously outlined in the Patient Information Sheet, involving alternation between hand immersion cold pressor task and a mental arithmetic task of randomly varying durations – unknown to the participant.

Hand Immersion Task (HI): Immerse non-dominant hand into ice-cold water (maintained at 2°C with water bath). Mental Arithmetic Task (MA): Count backwards from 2043 in steps of 17. If a mistake is made or a response is not given in 5 seconds, negative feedback is given by experimenter and the participant is asked to start again.

Trial Duration and Order: HI (90s) -> MA (45s) -> HI (60s) -> MA (60s) -> HI (60s) -> MA (90s) -> HI (90s) -> MA (45s) -> HI (60s).

Immediately after the stress test, participant is asked to provide 2 saliva swabs.

The participant is debriefed in which they are informed that the purpose of the test was to create stress and that the results do not reflect the participant's abilities. They are asked to provide 2 saliva swabs within 10 minutes of the debriefing.

The participant is asked to make themselves comfortable by laying on the patient bed in the physiological monitoring room whilst classical music or nature sounds are played, based on the participant's choice. This relaxation phase will last 20 minutes and will be unmonitored by the experimenter, to ensure that the participant is comfortable

The participant is asked to provide the final 2 saliva swabs. The participant is informed that the study is complete and that they are free to leave. They are reminded that they can remain in contact if they have any queries.

After the procedure, the physiological monitoring room will be disinfected with disinfectant spray and surfaces will be wiped down with antibacterial wipes.

2. Acquisition of Saliva

Human saliva samples will be acquired from volunteers following informed, written consent. Two forms will be signed so that the participant is provided with a signed copy to keep for their personal records. The saliva will be acquired through a selfswabbing technique. Unstimulated whole human saliva will be acquired by placing a synthetic swab into the mouth of the participant. The participant will be asked to chew on the swab for 60 seconds before removal. The swab will then be placed into a sealable blue cap plastic vial for subsequent laboratory-based analysis. The swab type that will be utilised will be commercially purchased from Salivette, as it is specific for salivary

cortisol collection. Prior to saliva collection, the participant will be supplied with an instructional booklet to ensure they understand the procedure of saliva acquisition. The action of saliva collection will be conducted entirely by the participant throughout the duration of the study. This will ensure that covid-19 guidelines regarding social distancing are maintained throughout the investigation. No more than 1.1ml of saliva will be acquired from the participant during each saliva collection. Saliva collection will be required twice during 4 phases of the investigation, for a total of 8 saliva samples per participant. One batch of saliva samples will be used for ELISA analysis and the other will be used for UV-Vis spectroscopic analysis.

3. Saliva Handling and Preparation

Following acquisition of the saliva samples they will be centrifuged for 2 minutes at 1000 x g to yield clear saliva samples. Particles and mucus strands will be collected in the extended tip of the Salivette vial and disposed of. The recovered saliva will be frozen in a -80 °C freezer for subsequent analysis. Saliva will only be handled in a suitable, well-prepared area within the Biomedical Engineering Research Laboratory. The area will be equipped will the analysing equipment and tools needed for handling the samples. The samples will not be removed from the dedicated space for the saliva analysis. The area will have smooth, easily sterilisable surfaces, a sink, a first aid kit sterilising agents and kit, sharps disposal bin, biological fluids disposal bin, and suitable storage for saliva samples. The investigator handling saliva will wear suitable attire, such as a disposable gown, white lab coat, disposable nitrile gloves, n95 mask and safety goggles. Saliva samples will be analysed within a maximum time of 3 months, depending on the recruitment of participants for the study as ELISA analysis will be conducted on all samples after they have been collected from all participants recruited throughout the duration of the study. After analysis, the samples will be disposed of in a suitable biological fluids' disposal bin. Waste from the ELISA method will be disposed of as clinical waste. Within this time, the saliva samples will be stored in a dedicated, padlocked freezer awaiting analysis.

4. Analytical Procedures on Saliva Samples

• ELISA method:

Following preparation and thawing of samples, the saliva samples will be pipetted into a clear microtiter plate coated with an antibody. A cortisol-peroxidase conjugate is added to the wells. After 1-hour of incubation, the plate will be washed, and the substrate will be added. After a 20-minute incubation, the reaction is stopped, and the intensity

of the colour generated from the reaction is detected an analysed with a microtiter plate reader at 450nm.

- Spectroscopic method: Following preparation and thawing of samples, the saliva samples will be pipetted into individual vials containing a solution of tetrazolium blue dye and methanol. After 10 minutes, the solution will be pipetted into a disposable cuvette. The cuvette will be placed into the spectrophotometer where measurements will be taken. Optical absorption spectra of saliva samples will be taken from 400nm to 720nm. The concentration of cortisol in saliva will be measured by optical absorption spectrophotometry.

5. Disposal and Sterilisation

- All waste will be collected by a registered carrier on the day of sample analysis.
- Analysed saliva samples will be disposed of in appropriate biological fluids waste disposal bins (yellow biohazard-marked bin).
- All items that come into contact with biological fluids, such as used cuvettes and vials will either be disposed in the same manner or cleaned with sterilising solution.

6. Analysis of data

All acquired data will be de-identified and analysed. Signal processing techniques will be applied to the obtained optical spectra to acquire further information regarding salivary cortisol concentrations and its fluctuations in response to the undergone stress test. The specificity and sensitivity of the optical technique will be compared with the ELISA method through a cortisol calibration curve. Analysis of variance (e.g., t-test) will be conducted to compare measured variables using the different measurement techniques. The measurements will be saved on a computed in an encrypted and password protected folder. Artificial neural networks and machine learning based techniques will be applied to the results to determine data trends, for the development of a mathematical model.

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General Health & Safety Risk Assessment Form

School/Professional Service	SMCSE, Biomedical Engineering	**Highest Risk Rating** (given existing controls)	**High**	
Room/Equipment/ Activity Location(s)	Biomedical Laboratory & Physiological Monitoring Room		**Medium**	
Head of Department/Principal Investigator Responsible for the Area/Task	**Prof. Panicos Kyriacou**		**Low**	**1**
Assessor(s)	Ms. Tashfia Ahmed			

Previous Assessment Date:	-	**This Assessment Date:**	**1st January 2022**	**Recommended Review Period:**	**January 20 23**

See Appendix for supplementary information on how to complete this risk assessment

Description of Activity/Workplace/Equipment being assessed	**Picture**
Research project involving saliva sampling and stress testing.	

Hazard No	HAZARD (Potential for harm) Insert a description of the hazard and how harm might arise	RISK (without controls)			Groups of persons exposed to hazard see appendix	EXISTING CONTROL MEASURES (those already in place to reduce risk level) e.g. • *Electrical testing in date* *Guarding in place, suitable and functional*	RISK (with existing controls)			ADDITIONAL CONTROLS REQUIRED? (to reduce risk to acceptable level)
		Likelihood (L)	Severity(S)	Risk (See Appendix)			Likelihood (L)	Severity(S)	Risk (see appendix)	
H1	**Covid-19 Risk** Infection	3	6	18	Pa, S, UG, PG, V	• Participants and investigators will be asked to take a Covid-19 PCR or lateral flow test 48 hours prior to entering the lab. • Participants will be advised to not attend the lab if they are feeling ill or develop symptoms on the day of the study.	1	3	3	None
H2	**Risk of Swallowing Saliva Swab** Physical Injuries	2	3	6	Pa	• Participant will be supervised during saliva collection. • The saliva swab will be chewed by the participant instead of placed near back of the throat to reduce risk of inducing gag reflex and swallowing mechanism.	1	2	2	None
H3	**Unsafe Access/Exit to the Lab** Physical Injuries	2	2	4	UG, PG, S, Pa, V	• The participants will be guided to the lab by the researcher and in the case of a fire alarm will be guided to the appropriate fire exit. • The participants will be made aware of exits once they first enter the lab. • The investigation will take place in a dedicated section of the lab, cleared of any obstructive equipment and machines.	1	2	2	None

H4	**Unsafe for other members in the lab** Physical injuries	2	2	4	UG, PG, S, V	• Passages are kept free of obstructions. • Spills and/or leaks are dealt with promptly. • Storage and work areas are kept separate. • Floor surfaces are sound and kept clean.	1	2	2	None

Details of Personal Protective Equipment (P.P.E) identified as required for this activity		
Type	**Specification**	**Available to all users and in good condition A/X**
Lab Coat	**Standard knee length type with long sleeves**	**YES**
Mask	**Standard N95 face mask**	**YES**
Safety Goggles	**Standard goggles for eye protection**	**YES**
Gloves	**Sterile**	**YES**

Action Plan

Ensure you include all additional controls required above.

Hazard No.	Action required	Person Responsible and target date	Date Complete	Risk rating with additional Controls			Individual to sign off for completion of remedial action. Print name and sign
				L	S	R	
H1 – H6	None						

Signature(s) of assessor(s)		**Signature of Head of Department/Principal Investigator responsible**	
Signed:	Ms Tashfia Ahmed	*I confirm that I have seen and approved this risk assessment form*	
Date:	01/01/2022		
Signed:		Name:	Prof. Panicos Kyriacou
Date:		Signed:	
Signed:		Date:	01/01/2022
Date:			
Name of DSLO:			

Guidance	
• List any hazards that potentially exist • Use the risk matrix to assess the risk rating without any control measures in place. • Detail the control measures in place to control the hazard and reduce risk of injury or loss of another kind e.g. property damage • Use the matrix to assess the risk rating when the existing control measures are in place. • Complete an action plan for the additional control measures required to reduce risk to an acceptable level. • The highest risk rating is the highest single risk identified with existing control measures in place.	
Risk Matrix	**Groups of Persons Exposed to Hazard**

		SEVERITY				
		Slight (No First aid no or little damage)	**Minor** (First aid injury minor damage)	**Moderate** (Medical treatment off site or property damage)	**Major** (Lost time accident/major injury or major damage)	**Very Severe** (Long term disability or fatality)
		1	**2**	**3**	**4**	**5**
LIKELIHOOD	**Very Likely** (Common occurrence) **5**	**Low (5)**	**Medium(10)**	**High (15)**	**High (20)**	**High (25)**
	Likely (Easily foreseeable) **4**	**Low (4)**	**Medium (8)**	**Medium(12)**	**High(16)**	**High(20)**
	Possible (Foreseeable under unusual circumstances) **3**	**Low(3)**	**Low(6)**	**Medium(9)**	**Medium(12)**	**High(15)**
	Unlikely (Unlikely sequence of events / unplanned event) **2**	**Low(2)**	**Low(4)**	**Low(6)**	**Medium(8)**	**Medium(10)**
	Very Unlikely 1	**Low(1)**	**Low(2)**	**Low(3)**	**Low(4)**	**Low(5)**

Undergraduate	Ug
Postgraduate	Pg
Staff	S
Contractor	C
Visitor	V
Participant	Pa
General Public	Pu
Young Person	Yp
New/Expectant Mother	Nm