Salivary Biomarkers of Health and Disease from Childhood to Older Age

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Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Master in Philosophy by Orla Ellen Bright

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ABSTRACT

BACKGROUND

Cortisol, one of the body's main stress hormones and is released in a pulsatile state following our body's normal circadian rhythm. Cortisol is regulated by two isoforms of the enzyme 11β-hydroxysteroid dehydrogenase (11βHSD) type 1 and type 2. Deficiency of 11βHSD2 has been linked to increased blood pressure, evident in conditions such as apparent mineralocorticoid excess (AME) and Cushing's Syndrome. Currently, salivary cortisol is used as a diagnostic and physiological biomarker in a range of different clinical and research studies Blood sampling is a painful and invasive procedure affecting cortisol levels, especially in children. For many years, blood has been one of the tools used to measure a range of hormones, but only recently have researchers taken notice of saliva. The aim of this research is to therefore investigate salivary cortisol and cortisone and to achieve this I will undertake a systematic review and the SMILE study.

METHODS

Undertake a PROSPERO registered systematic review to examine the relationship between salivary cortisol and cortisone and hypertension. Set up and recruit to an observational cohort study (SMILE) to determine normative values of salivary cortisol and cortisone in children and young people. Participants collected samples of their saliva as they initially woke up and throughout the waking hours of a day. These samples were sent for cortisol and cortisone analysis.

RESULTS

The systematic review only found 3 papers. Kidambi et Al found that late night salivary ($p<0.01$) and early morning cortisol ($p<0.03$) levels were higher in hypertensive patients compared to normotensive (PM salivary (1.2nmol/ vs 1.8 nmol/L) AM Salivary (11.2nmol/l vs 14.4nmol/l). Walker 2002 didn't find any difference in concentrations of cortisol/ cortisone in normotensive and hypertensive patients at baseline and after administration of glycyrrhetinic acid. Similarly, Wirix et al. found that no significant differences in these parameters between hypertensive and normotensive children and salivary cortisol levels weren't significantly associated with systolic or diastolic blood pressure SDS.

Within SMILE, 54 participants, aged 5-18 years, 31 male (57.4%) and 23 females (42.6%), with a median age of 9 (range 12 years (5-17)) were recruited, to obtain 365 saliva samples. Both cortisol and cortisone exhibited a diurnal rhythm similar to that of serum. Cortisol mean concentration over 24 hours was 0.2 nmol/L (+/-4.225), median 1.4nmol. Cortisone mean concentration over 24 hours was 15.8 nmol/L (+/-12.83), median 11.5 nmol/L.

CONCLUSION

The research found within this thesis is a great starting point to discovering more about salivary cortisol and cortisone in healthy children and young people. The use of salivary cortisol as a non-invasive accessible method of measuring cortisol excess or deficiency in clinical practice looks promising and that it clearly follows the normal physiological diurnal rhythm of cortisol. But there is contradicting results surrounding the relationship of salivary cortisol and cortisone concentrations and blood pressure. Furthermore, we didn't find a significant relationship between salivary cortisol, cortisone and parameters such as sex, age, BMI, height or systolic blood pressure. Therefore, more research and participant recruitment needs to be carried out in order to investigate these relationships.

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Abbreviation	**Full Word**

α-MSH	Alpha-melanocyte stimulating hormone
[THF+allo-THF]:THE	ratio of tetrahydrometabolites of cortisol and cortisone
110HA4	11-Hydroxyandrostenedione
11βHSD	11-beta hydroxysteroid
17-OHP	17-hydroxyprogesterone
17-OHP	17αhydroxyprogesterone
ACTH	Adrenocorticotropin hormone
AE	Androstenedione
AI	Adrenal Insufficiency
Allo-THF	5α-tetrahydrocortisol
AMP	Adenosine monophosphate
BMI	Body mass index
CAH	Congenital adrenal hyperplasia
CAR	Cortisol Awakening Response
CBG	Cortisol binding globulin
CLIP	Corticotrophin-like intermediate lobe peptide
CRF	Clinical research facility
CRH	Corticotrophin releasing hormone
CRHR1	Corticotrophin releasing hormone receptor type I
CRHR2	Corticotrophin releasing hormone receptor type II
CT	Computed tomography
CYP11A1	Cytochrome P450 family 11 subfamily A member 1
CYP11B1	11b-hydroxylase
CYP17A1	17a-hydroxylase A1
CYP21A2	21-hydroxylase A2
CYP3A4	Cytochrome P450 3A4
DHEA	Dehydroepiandrosterone
ECLIA	Electrochemiluminescence immunoassay
EIA	Enzyme immunoassay
ELISA	Enzyme-Linked Immunosorbent Assay

ENT	Ear nose throat
FSH	Follicle Stimulating Hormone
GH	Growth Hormone
GnRH	Gonadotrophin releasing hormone
GPCR	G-protein coupled receptor
GR	Glucocorticoid Receptor
HPA	Hypothalamus pituitary adrenal
IMD	Index of multiple deprivation
Kg	Kilograms
LC-MS/MS	Liquid chromatography with tandem mass spectrometry
LH	Luteinising Hormone
LNSC	Late night salivary cortisol
MCR2	Adrenal melanocortin 2 receptor
MDU	Medical day case unit
mmHg	Millimetre of mercury
MR	Mineralocorticoid receptor
MRI	Magnetic resonance imaging
NETs	Pancreatic neuroendocrine tumours
Nmol/L	Nanomoles per litre
PCOS	Polycystic Ovarian Syndrome
POMC	Pro-opiomelanocortin
RE-1/NRSE	Restrictive element-1/neuron restrictive silencing element
REST	Restrictive element silencing transcription factor
RIA	Radioimmunoassay
SDS	Standard Deviation Score
THE	tetrahydrocortisone
THF	5 β-tetrahydrocortisol
TP	Time Point
TRH	Thyrotrophin releasing hormone
UFC	Urinary free cortisol
UFF:UFE	ratio of urinary free cortisol to cortisone

1. CHAPTER 1 INTRODUCTION

1.1 THE NEED FOR RESEARCH INTO ADRENAL HORMONE PROFILES IN SALIVA

Currently, salivary cortisol is used as a diagnostic and physiological biomarker in a range of different clinical and research studies including the investigation of psychological stress and related mental illness, circadian rhythms in preterm infants, monitoring hydrocortisone therapy and assessing adrenal function in patients with adrenal incidentalomas (1–3). Due to its circadian rhythm, continuous cortisol measurement is required, for example a late night sample is used in the investigation of Cushing Syndrome, an early morning sample for adrenal insufficiency and multiple cortisol samples to monitor hydrocortisone therapy. (4) The use of saliva has a number of advantages over serum in children. Salivary cortisol is free, representing the biologically active concentration within the body, in contrast to measurements made in serum which include free and bound (biologically inactive fraction). It is a non-invasive, less stressful test which doesn't require a hospital visit and thus no disruption to the family's normal day and minimises the risk of stress related changes in cortisol release. Furthermore, profiles of salivary cortisol reflect the diurnal changes that occur in plasma cortisol (5).

Early methods of measuring salivary cortisol used radioimmunoassay (RIAs), Enzyme-Linked Immunosorbent Assay (ELISA), enzyme immunoassay or automated immunoassays all of which relied on antibody-antigen binding. A lot of other steroids are naturally very similar in structure to cortisol including its inactive form cortisone. This results in cross-reactivity within the sample and makes the creation of a specific antibody to bind cortisol with high specificity more difficult (5). Moreover, immunoassays are lack sensitivity and specificity when dealing with low saliva volumes causing poor inter-laboratory reproducibility.(6) More recently, liquid chromatography methods coupled with mass spectrometry (LC-MS/MS) has become another option in many studies, due to its high specificity, its ability to simultaneously measure cortisol and cortisone and other steroids such as 17 αhydroxyprogesterone (17-OHP), aldosterone, androstenedione (AE) and oestradiol and is 10-100 times more sensitive in comparison to RIA (7,8). Recent studies are reporting that the 11-oxygenated C19 steroids such as 11-hydroxyandrostenedione (11OHA4), 11-hydroxysterone and 11-ketotestosterone can act as valuable markers for androgen excess in congenital adrenal hyperplasia (CAH) and in polycystic ovary syndrome (PCOS). Data have also shown that the measurements of these steroids made in saliva and plasma are tightly correlated (9). However, due to the range of different assays being used to test androgens in saliva, there is a lack of one validated reference range.

The enzyme 11βHSD type 2 is present within the salivary glands and is responsible for conversion of cortisol to its inactive form cortisone. Cortisone had been being reported as a superior marker over salivary cortisol as cortisone levels are 6-fold higher than those of cortisol. Salivary cortisol tends to become undetectable later in the day, reported when serum cortisol levels are below 74 nmol/L in

adults, but salivary cortisone still remains detectable. Salivary cortisone also has been shown to reflect serum cortisol levels as salivary cortisone is presumed to be generated from serum free cortisol.(10) Furthermore, many children who require regular cortisol testing are on hydrocortisone treatment which may contaminate saliva. However, salivary cortisone doesn't pose the risk of contamination allowing monitoring of hydrocortisone therapy. (4)

1.2 THE HYPOTHALAMIC PITUITARY ADRENAL AXIS (HPA)

The hypothalamic pituitary adrenal axis (HPA) is responsible for the release of circulating glucocorticoids throughout the body, primarily cortisol.(11) These hormones are essential for the maintenance of the body's homeostatic state in order for an organism to produce a rapid response to everyday stresses, whether that be physical or emotional.(12) The axis begins with the release of corticotrophin releasing hormone (CRH) from the master organ, the hypothalamus and ends with the release of cortisol from the zona fasciculata of the adrenal cortex. (11) Once within the circulation, glucocorticoids can bind to target tissues and exert their metabolic, cardiovascular, anti-inflammatory effects in order to promote processes that are required to cope with stress.(11) In order to prevent overstimulation of the HPA axis, there are negative feedback loops in place to maintain homeostasis of the body. High concentrations of cortisol suppresses CRH and adrenocorticotrophic hormone (ACTH) release from both the hypothalamus and the anterior pituitary thus cortisol release is stopped (13).

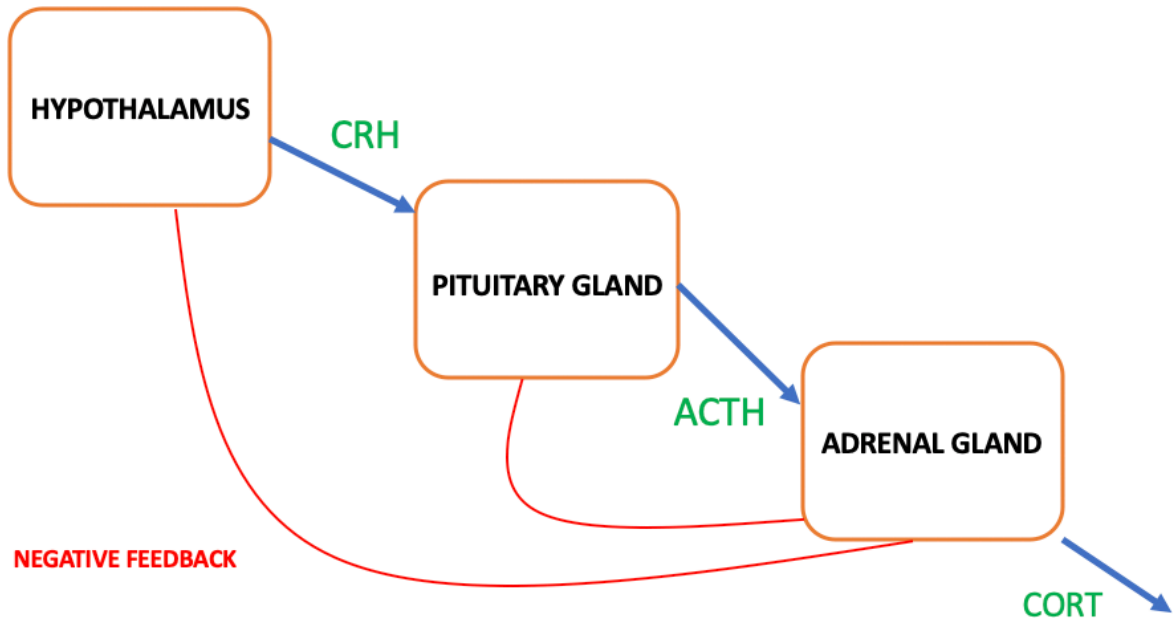


FIGURE 1: HYPOTHALAMIC PITUITARY ADRENAL AXIS. STRESS RESPONSE INITIATED BY CORTICOTROPHIN RELEASING HORMONE (CRH) FROM THE PARAVENTRICULAR NUCLEUS OF THE HYPOTHALAMUS. CORTICOTROPH CELLS WITHIN THE ANTERIOR PITUITARY ARE STIMULATED BY THE RELEASE OF CRH, CAUSING THE RELEASE OF ADRENOCORTICOTROPHIC HORMONE (ACTH) INTO THE BLOODSTREAM WHICH PROMOTES THE RELEASE OF GLUCOCORTICOIDS, PRIMARILY CORTISOL (CORT) FROM THE ADRENAL CORTEX.

1.2.1 HYPOTHALAMUS

Known previously as the 'integrator' of bodily functions by Italian anatomist Mondino de Liuzzi, the hypothalamus has a major role in many of our daily activities such as eating, drinking, sleeping and thermoregulation (14), and has a role in homeostasis. It is situated at the base of the brain below the thalamus and forms part of the diencephalon(15), which originates from the forebrain around the fifth week of development(16).

The hypothalamus which occupies 2% of brain volume has four surfaces (17). The lateral surface runs along the boundary of the thalamus, subthalamus nucleus and the internal capsule (18). Medially, the hypothalamus extends to the third ventricle, superiorly the hypothalamic sulcus (Sulcus of Monro) which marks the boundary between the thalamus and hypothalamus and inferiorly which is continuous with the floor of the third ventricle (17,19). Anteriorly it is bound by the optic chiasm and posteriorly bound by the mamillary bodies which are small round white structures involved in memory and sense of direction (19,20).

The hypothalamus gathers information from a range of sensors, set points and stressors to restore the body to its equilibrium, through connections to peripheral hormone secreting targets, the autonomic system and parts of the brain that control our emotional behaviour (18–20). In response, the hypothalamus synthesises and releases a range of hormones with different physiological properties.(11) These hormones are produced from different nuclei, all of which are grouped into four distinct regions, the preoptic, supraoptic, tuberal and mammillary regions.(16) Magnocellular cells within the supraoptic and paraventricular regions are responsible for secreting arginine vasopressin (AVP) and oxytocin. Parvocellular cells, from the paraventricular nucleus secrete factors into the hypophyseal portal system in order to regulate pituitary function, (16) including thyrotropin-releasing hormone (TRH), CRH, growth-hormone-releasing hormone (GHRH), gonadotrophin-releasing hormone (GnRH), dopamine and somatostatin (21).

The hypophyseal-portal system provides a rich supply of blood to the pituitary gland. It originates from branches of the internal carotid artery called the superior and inferior hypophyseal arteries with the superior supplying the anterior lobe and the latter providing the posterior (22). A primary capillary plexus is formed from the superior hypophyseal artery which originates within the hypothalamus, surrounds the infundibulum and median eminence and descends down to the pituitary gland. A secondary capillary plexus within the anterior pituitary then drains into the adenohypophyseal veins located at the sulcus. It then passes into the cavernous sinus, into the petrosal sinuses and finally into the jugular vein (14,22). The hypothalamus and corresponding glands also have input from the

autonomic system by direct innervation from parasympathetic and sympathetic nerves to further regulate hormonal control. (16)

1.2.2 Corticotrophin releasing hormone

Corticotrophin releasing hormone (CRH) is a 41 amino acid which belongs to a family of stress response regulating proteins which also include the urocortins (urocortin 1,2,3) (23). Present on the long arm of chromosome 8q13, the CRH gene is composed of two exons separated by an 800bp intron (18,24). One exon contains the 3' untranslated region and the prohormone sequence whilst the other exon contains the majority of the 5'-untranslated region in the messenger RNA (24). The CRH intron a restrictive element-1/neuron restrictive silencing element (RE-1/NRSE) sequence, then binds to the restrictive element silencing transcription factor (REST), which restricts CRH expression to neuronal cells (25).

CRH has a major role in the stress response through many different pathways (25). Present in the amygdala, a region of the brain responsible in behavioural stress and the cerebral cortex, CRH is involved in anxiety and fearful behaviour (25). CRH fibres also project into the brainstem and spinal cord having descended from the locus coeruleus in order to help regulate the autonomic system (26). CRH is synthesised in the medial parvocellular subdivision of the paraventricular nucleus, CRH is synthesised and released into the hypothalamo-hypophyseal portal system through the median eminence to reach the anterior pituitary (25,27,28). CRH is released in response to systemic or physiological stress including salt loading, haemorrhage, hypoglycaemia, fasting or cytokine release but also directly by CRH stimulators such as serotonin, histamine, norepinephrine, epinephrine, dynorphin or substance P (24). At a cellular level, two CRH receptors are present, CRHR1 and CRHR2 (CRHR2α/CRHR2β), both belonging to the G protein coupling family (25). CRHR1 is the predominant type of receptor present in pituitary corticotropes, which is also present in the adrenal medulla and to a lesser extent the cortex (18). Once bound to CRHR1, adenylate cyclase activity is stimulated, increasing cyclic adenosine monophosphate (AMP) levels within the corticotrope. This stimulates the release of ACTH, the next step of the HPA axis pathway (24).

1.2.3 The Pituitary Gland

The pituitary gland, a pea-sized organ weighing around 0.5 grams , is responsible for integrating signals that the hypothalamus and other organs have received to augment the release of anterior pituitary hormones into the bloodstream (16). It sits within the sella turcica of the sphenoid bone, (16) and it is bounded by the cavernous sinus laterally which contains the internal carotid artery and cranial nerves III,IV, V and VI, and the optic chiasm superiorly (14). The pituitary gland consists of an anterior (adenohypophysis) and a posterior (neurohypophysis) lobe. The anterior lobe can be divided into

three parts, the pars distalis which makes up the bulk of the anterior lobe, pars intermedia which is the Rathke's pouch remnant and the pars tuberalis which surrounds the infundibulum, the connection to the hypothalamus (14) The six primary hormones that are released from the pituitary gland are, prolactin, thyrotrophin (thyroid stimulating hormone: TSH), ACTH, growth hormone (GH), luteinising hormone (LH) and follicle stimulating hormone (FSH)(16). The posterior lobe, which passes through the diaphragm sellae, is derived from neural primordia as an outpouching of the third ventricle (29). It is composed of unmyelinated axons and axon terminals extending from the supraoptic and paraventricular nuclei of the hypothalamus down through the infundibulum stalk to the posterior lobe (19,30). It can be split into the pars eminens or median eminence and the pars nervosa (21).

1.2.4 ACTH

Once CRH binds to CRH type 1 receptor (CRH-R1) corticotrophs release ACTH stored in secretory vesicles. ACTH is a 39 amino acid structured hormone, released from corticotropes which are present in the median wedge, laterally, anteriorly and posteriorly (31). Corticotrophs are irregular cells with prominent neurosecretory granules, producing the products of the pro-melanocortin (POMC) gene, ACTH, opioid and melanotropic peptides(16). ACTH is also released from neurones of the mediobasal hypothalamus and within skin melanocytes (32).

The POMC gene is located on the short arm of the chromosome 2, 2p23 and consists of three exons, which undergo translation to produce the 266 amino acid POMC pre-prohormone(16). The POMC gene protein precursor generates a number of bioactive peptides (ACTH, α-, β-, and γ-melanocyte stimulating hormone (MSH), β-endorphin) through post-translational modifications. This prohormone is cleaved to give ACTH, and can be further converted to alpha-melanocyte stimulating hormone (α-

MSH) and corticotrophin-like intermediate lobe peptide (CLIP) (31,33).

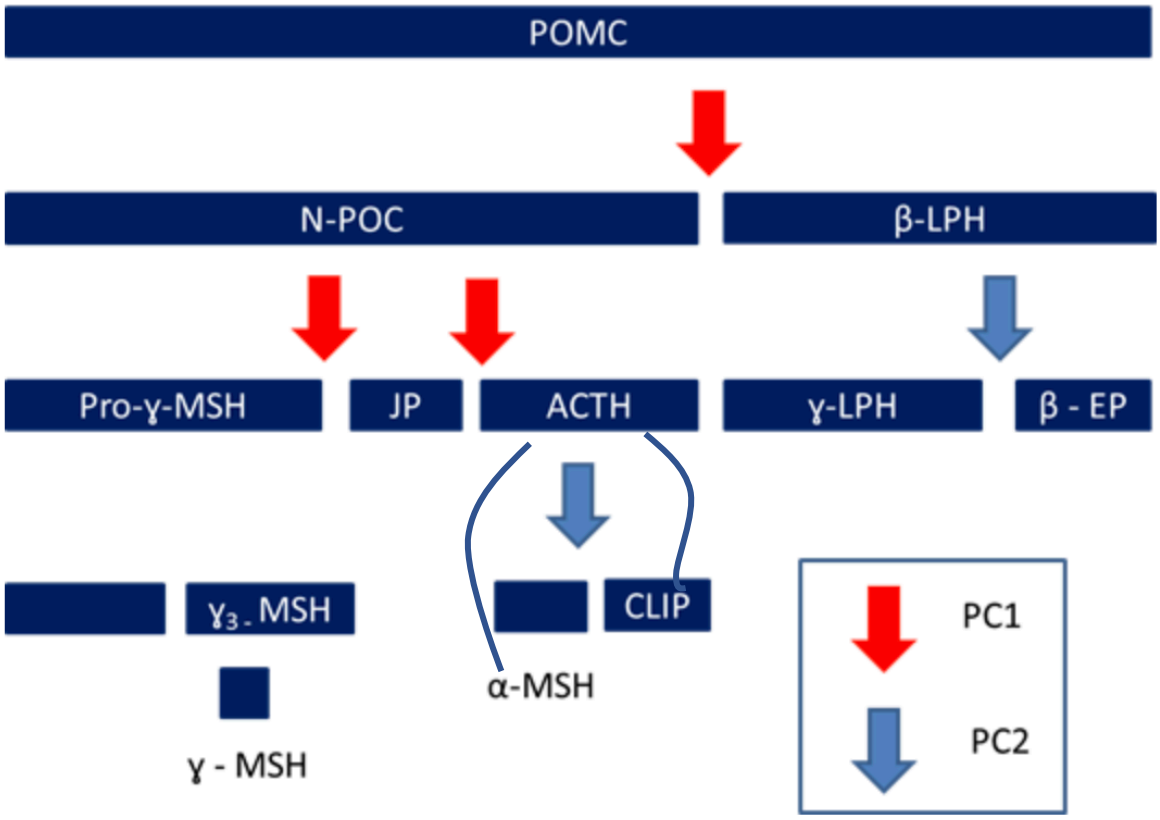


FIGURE 2: IMAGE ADAPTED FROM(16). ACTH: ADRENOCORTICOTROPHIC HORMONE, CLIP: CORTICOTROPIN-LIKE INTERMEDIATE LOBE PROTEIN, EP: ENDORPHIN, JP: JOINING PEPTIDE, LPH: LIPOPROTEIN, MSH:MELANOCYTE STIMULATING HORMONE: N-POC N-TERMINAL PRO POMC FRAGMENT. THE POMC GENE IS LOCATED ON THE SHORT ARM OF CHROMOSOME2, 2P23, AND CONSISTS OF THREE EXONS: THE FIRST ENCODES A LEADER SEQUENCE, THE SECOND THE SIGNAL INITIATION SEQUENCE AND N-TERMINAL PORTION OF THE POMC PEPTIDE AND THE THIRD CONTAINS MOST OF THE SEQUENCE FOR CORTICOTROPHIC, MELANOTROPHIC AND OPIOID PEPTIDES. TRANSLATION OF THE GENE RESULTS IN A 266 AMINO ACID PRE-PROHORMONE WHICH UNDERGOES EXTENSIVE MODIFICATION AND PROCESSING INCLUDING REMOVAL OF THE N TERMINAL SEQUENCE, GLYCOSYLATION OF THR45, N-LINKAGE OF ASN65 AND SERINE PHOSPHORYLATION (FIGURE 11). CLEAVAGE OF POMC AT PAIRS OF BASIC RESIDUES (LYS-LYS OR LYS-ARG) BY PROHORMONE CONVERTASE 1 (PC1) RELEASES N-TERMINAL PRO POMC FRAGMENT (N-POC) AND B-LPH. FURTHER CLEAVAGE OF N-POC BY PC1 RELEASES PRO-ɣMSH, JOINING PEPTIDE AND ACTH AND FURTHER CLEAVAGE OF B-LPH BY PC2 RESULTS IN ɣ-LPH AND B ENDORPHIN. ACTH MAY BE FURTHER CLEAVED BY PC2 TO A-MELANOCYTE STIMULATING HORMONE (A-MSH) AND CORTICOTROPHIN LIKE INTERMEDIATE LOBE PEPTIDE (CLIP).

Secretion of ACTH follows a circadian rhythm, and it is secreted in 40 pulses +/- 1.5 each day (31). ACTH secretion is reflected in release of cortisol, with a 15 minute delay. ACTH levels peak at 06:00h-09:00h and decline through the day to a nadir at 23:00-02:00h (31). The main function of ACTH is to bind with the adrenal melanocortin 2 receptor (MCR2), a G protein coupled receptor (GCPR) that signals through cyclic AMP, resulting in release of glucocorticoids from the adrenal cortex (32).

1.2.5 The Adrenal Gland

The adrenal glands sit atop the kidneys between the 10^{th}-12^{th} ribs lateral to the spinal column, suspended within the perineal space (34). Each gland weighs around 5 grams with the right gland being more pyramidal in shape whereas the left more cresenteric(35). The right adrenal sits behind the inferior vena cava, in front of the diaphragm and just below the liver. The left adrenal sits above the splenic artery and vein, in front of the diaphragm, medially to the spleen and laterally to the aorta (36). It is split into an outer cortex representing 90% of the gland's weight and an inner medulla representing 10%, both of which have different physiological functions and embryological origins (37–39).

Embryologically, the cortex begins as the urogenital ridge which is infiltrated by mesothelial cells which then penetrate the mesenchymal layer to form the foetal adrenal cortex which will eventually differentiate into the definitive adrenal cortex. The medulla derives from neuroectoderm cells from the neural crest (36,40). The superior, middle and inferior adrenal arteries provide a rich blood supply to the adrenals. Branches from these arteries penetrate the adrenal capsule at the hilus and reach both the cortex and medulla. Blood drains differently between the left and right adrenal with the left adrenal draining into the left adrenal vein and then into the left renal vein whereas the right adrenal drains directly into the inferior vena cava (36). Histologically, the adrenal cortex is comprised of three layers all involved within the process of steroidogenesis, producing glucocorticoids, mineralocorticoids and androgen precursors(41). The outer layer, the zona glomerulosa, produces aldosterone which is involved in sodium reabsorption within the kidney. The middle zone, zona fasciculata produces glucocorticoids, mainly cortisol, released during stress. The inner zone, zona reticularis secretes adrenal androgens primarily dehydroepiandrosterone (DHEA which are involved in production of sex steroids. (41) The adrenal medulla is responsible for releasing catecholamines, epinephrine and norepinephrine, involved within the body's fight or flight response. (41)

1.2.6 CORTISOL

1.2.6.1 METABOLISM

Cortisol, one of the body's main stress hormones, is a short polypeptide, belonging to the C21 steroid pregame family (34,42). It is synthesised from a pregnenolone (a derivative of cholesterol) by enzymes of the cytochrome P450, family 11, subfamily A, member 1 (CYP11A1) family within mitochondria in the zona fasciculata layer (42,43). ACTH interaction with the MCR2 receptor accelerates the delivery of cholesterol to the P450cc mitochondrial membrane. Pregnenolone then moves into the endoplasmic reticulum, to be converted to 17-hydroxypregnenolone or progesterone by 3β-hydroxysteroid dehydrogenase. 17-hydroxyprogesterone is synthesized from 17-hydroxypregnenolone by 3β-HSD, and progesterone by the enzyme 17-hydroxylase (CYP17). 11-deoxycortisol is then formed through by 21α-hydroxylase activity of 21-hydroxylase (CYP21) of 17-hydroxyprogesterone (43). In the final step of cortisol biosynthesis occurs, 11-deoxycortisol undergoes 11β-hydroxylation that is catalysed by 11 β -hydroxylase 1 (CYP11B1) or 11b-hydroxylase 2 (CYP11B2) (43).

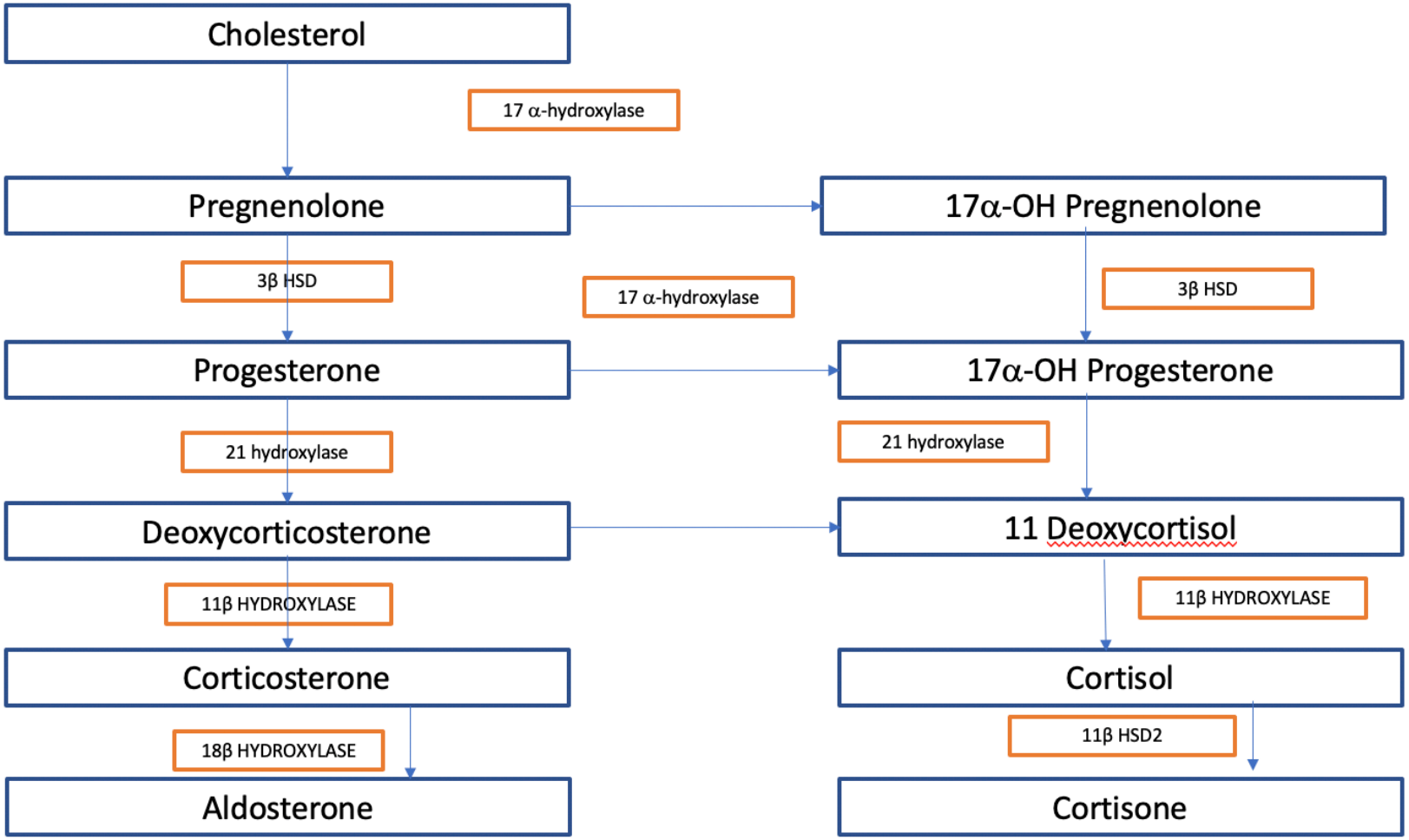


FIGURE 3 THE ADRENAL STEROIDOGENESIS PATHWAY. HSD: HYDROXYSTEROID DEHYDROGENASE. CORTISOL SYNTHESIS STARTS WITH CHOLESTEROL OCCURRING WITHIN THE ZONA FASICULATA OF THE ADRENAL CORTEX

1.2.6.2 Diurnal Rhythm / Cortisol Awakening Response

ACTH and cortisol are released in a pulsatile state and follows a circadian rhythm in line with CRH. A circadian rhythm ensures that cortisol is optimised at various points throughout a 24 hour period so the body can fight any type of stress(44). Cortisol levels are highest approximately 30 minutes after awakening (cortisol awakening response: CAR) (45) around 7am-8am and nadir around 2am-4am at night (13,43,46). Throughout the afternoon and evening cortisol levels steadily decrease until sleep onset, before rising again in preparation for subsequent waking in the morning (34,47). This rhythm is regulated by the pacemaker suprachiasmatic nucleus, located within the hypothalamus. It is synchronised to environmental cues such as light to dark cycles which are picked up by the retinal ganglion cells. It then enforces its rhythm to neuronal projections to the different hypothalamic nuclei for the release of ACTH and cortisol(48). This rhythm can be affected by a number of factors such as stress, burnout, depression and any other mental disorders (47).

The CAR is being considered to be a useful measure of acute reactivity of the HPA axis as it can be monitored via several salivary cortisol measurements in samples collected at home and doesn't require stimulation of the axis by exogenous agents (49). It has also been used as a marker for HPA dysfunction in conditions such as anxiety and depression, showing increased CARs within these patients(50). A range of different factors have been shown to affect the CAR such as age, sex, puberty, disease, smoking and sleep. For example, studies have shown that earlier awakening in the morning is associated with larger CARs and larger diurnal declines in comparison to later awakening. (51,52) In nurses, very early morning shifts (4-5:30am) have been associated with a greater and prolonged CAR when compared to waking before the late day shift (6-9am) or the night shift (11am-2pm)(53).

In children and adolescents, the diurnal profile may be influenced by a number of factors including age, sex, pubertal development, weight and sleep duration. In infants (1-2 months), the diurnal profile is characterised by two daily cortisol peaks, at age 2-3 months, the CAR can be observed (54). The usual diurnal decline in cortisol levels throughout the day isn't commonly seen until the age of 4 years. Sex differences include girls' CAR to be greater in comparison to boys of a similar age(54–56). Pubertal maturation has also been shown effect, but with inconsistent results. Some investigators have found an association with increased cortisol, a steeper diurnal slope and reduced awakening in those post-menarche and other studies reporting no association(54). Furthermore, in children who are overweight or obese, their average cortisol levels in early morning, late morning and evening were significantly lower in comparison to their normal weight counterparts(57).

1.2.6.3 Transportation and the Glucocorticoid Receptor

Cortisol is lipophilic so therefore requires proteins to be transported around the body. In circulation around 92% of cortisol is bound to cortisol binding globulin (CBG) or to albumin and the rest is considered 'free' (13,46). CBG is a glycoprotein with a molecular weight ranging from between 50 and 60kDa, which is released from the liver. It binds with cortisol stoichiometrically and with high affinity in order to transport it to glucocorticoid requiring tissues to act as a reservoir of cortisol and regulate release of free hormone (2,58). The cortisol-CBG complex binds to the membrane receptors, offering an alternative way of entering cells that are glycoform dependent (2). Once bound to the glucocorticoid receptor within the cytoplasm of target cells, cortisol dissociates from the CBG/albumin protein complex and translocates into the nucleus. There it binds to a specific DNA motif as a homodimer to either induce or inhibit gene transcription (43).

Cortisol is regulated by two isoforms of the enzyme 11β-hydroxysteroid dehydrogenase (11βHSD), a member of the short-chain dehydrogenase family, by conversion to the inactive glucocorticoid cortisone and back again (59). 11βHSD occurs in two forms, 11βHSD type 1 and 11βHSD type 2. 11βHSD type 1 enzyme is mainly expressed within the liver, adipose tissue and CNS and it is bi-directional meaning it is able to both reduce inactive cortisone to cortisol and dehydrogenase cortisol to cortisone (60)(61). 11βHSD type 1 also converts the synthetic glucocorticoid prednisone to its active hormone prednisolone(62). The 11βHSD type 2 enzyme catalyses the NAD+-dependant dehydrogenation of cortisol converting it to inactive cortisone and prednisone. It is mainly expressed in mineralocorticoid target tissues such as the salivary glands, colon and renal cortex (60). This process of inactivation gives protection to the mineralocorticoid receptor from high circulating levels of cortisol as the receptor has equal affinity for both cortisol and aldosterone(63). 11βHSD2 is also majorly expressed within the human placenta and dramatically reduces the concentration of cortisol reaching the foetus in order to protect it from high levels of glucocorticoids (64).

Cortisol and cortisone can then be acted on again by 5α and 5β-reductases and 3α-hydroxysteroid dehydrogenase, leading to the further inactive cortisol metabolites including tetrahydrocortisone (THE), 5β-tetrahydrocortisol (THF) and 5α-tetrahydrocortisol (allo-THF).(61) Measuring the ratio of tetrahydrometabolites of cortisol and cortisone ([THF+allo-THF]:THE), can give an idea of the rate of conversion of cortisol to cortisone is being achieved. This gives an indication of the activity of the hepatic isoform of 11βHSD1 most accurately. For 11βHSD2, which presents mostly within the kidney, the ratio of urinary free cortisol to cortisone (UFF:UFE) most accurately reflects renal 11βHSD activity.(61)

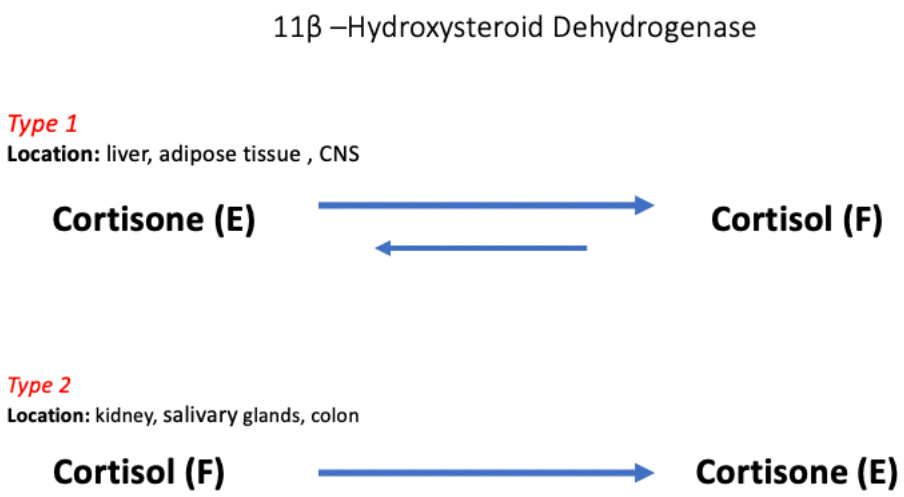


FIGURE 4: REGULATION OF CORTISOL BY TWO ISOFORMS OF 11BHSD. TYPE 1, GENERATES CORTISONE TO CORTISOL BUT ALSO CAN ACT IN REVERSE AS REACTION IS BIDIRECTIONAL. PRESENT WITHIN THE LIVER, ADIPOSE TISSUE AND CENTRAL NERVOUS SYSTEM. TYPE 2 CONVERTS CORTISOL TO CORTISONE. PRESENT WITHIN THE KIDNEY, SALIVARY GLANDS AND COLON.

The actions of cortisol are mediated by the glucocorticoid receptor (GR, NR3C1) which is part of the nuclear receptor superfamily of ligand-dependent transcription factors (60). Glucocorticoid receptors are expressed throughout the body whereas mineralocorticoid receptor (MCR) expression is limited to the limbic-forebrain which is involved in attention, memory retrieval and appraisal and drives expression of fear and aggression, distal renal tubule, parotid glands, colon, sweat glands and the nucleus tratus soliarii and circumventricular organs of the brain.

Activation of the GR either induces or suppresses the activation of target genes which represent 10-20% of the human genome. A GR is encoded from a single gene however, alternative splicing and translation results in a number of different GR isoforms, which are then subjected to post-translation modifications. This can then impact glucocorticoid signalling and thus the regulation of physiological processes. (65) GR polymorphisms may account for inter-individual variability in glucocorticoid sensitivity may influence the dose of glucocorticoid needed for cortisol replacement and has been an association with increased weight gain during glucocorticoid therapy has been reported (62).

1.2.6.4 Widespread Effects

Cortisol has a widespread effect on the body and has a critical role in homeostasis. It is a stress hormones, protecting our body from any type of aggression, whether that be biochemical, hormonal, mental or emotional(34)(66). Stress is an imbalance of the body's normal homeostasis and this is counteracted by a series of internal physiological and behavioural responses in order to re-establish an equilibrium (67). A central neurochemical circuit is responsible for orchestrating the stress response. This includes hypothalamic CRH and AVP neurones as well as central catecholaminergic neurones responsible for releasing adrenaline and noradrenaline (68). One of the main sources of energy to our skeletal muscles and brain during stress is glucose. Cortisol optimises the body's state of hyperglycaemia by upregulating glycogenolysis and gluconeogenesis, by activating enzymes within these biochemical pathways, for example the PEPCK enzyme in gluconeogenesis. Furthermore, cortisol blocks the action of insulin on glycogen synthesis and glucose uptake into cells via the GLUT4 transporter (69). Additionally, during stress, the body enters an immunosuppressive state. Cortisol decreases white blood cell count through a number of mechanisms.(69). It induces apoptosis of proinflammatory T cells, inhibits interleukin- 2 which is required in T cell proliferation, decreases neutrophil entering cells, redistributes lymphocytes to the spleen, lymph nodes and bone marrow and inhibits monocyte differentiation into macrophages. Cortisol also inhibits immunoglobulin synthesis and supresses local inflammation by inhibiting histamine production (46,69).

Moreover, cortisol is thought to have a role in the regulation of mood, sleep patterns and behaviour as those with Cushing's Syndrome suffer with depression, difficulty falling asleep, anxiety and irritability (69,70). Sleep loss confuses the HPA axis and there is a loss of negative feedback to the hypothalamus and pituitary. A study reported that after sleep deprivation the patient's plasma cortisol concentrations increased by 45% (71). This could have implications for immunity, cognition and metabolic health (42).

1.3 Illnesses affecting the HPA axis

1.3.1 Cortisol excess : Cushing's Syndrome

Prolonged exposure to supraphysiological concentrations has a profound effect on the body. Physical changes include moon face, centripetal weight gain, muscle wasting, acne, hirsutism, hypertension, poor bone health, congestive heart failure and skin fragility (72). Patients may also experience low mood, poor concentration and sleep disturbance and biochemical features include hypokalaemia, glucose intolerance and insulin resistance. Cushing's syndrome can be split into two types; endogenous (having an internal cause) vs exogenous (caused by factors outside the organism). Exogenous Cushing's Syndrome is more common with an incidence of 13 per million (73).

Endogenous Cushing Syndrome can again be further classified as ACTH dependent (known as Cushing Disease) or ACTH independent. Endogenous causes are rare, affecting 0.7-2.4 per million per year. ACTH dependent causes originate from either a pituitary or ectopic ACTH source. 75-80% of Cushing syndrome is caused by a solitary corticotrope adenoma (Cushing's disease) which produces excessive amounts of ACTH, causing increased production of cortisol, androgens and 11-deoxycorticosterone and adrenal hyperplasia (74). 15-20% of Cushing's Syndrome due to ACTH excess are caused by an ectopic ACTH syndrome characterised a tumour outside of the pituitary or adrenal glands which secretes excessive amounts of ACTH. Examples include small cell carcinoma of the lung, pulmonary carcinoid tumour, pancreatic neuroendocrine tumours (NETs) and medullary thyroid cancers (75,76).

ACTH independent causes of Cushing's Syndrome include adrenal adenoma (80%), adrenal carcinoma (20%), macronodular adrenal hyperplasia or primary pigmented nodular adrenal disease (75). In children, the cause of Cushing's Syndrome depends on age as children below the age of 7 tend to present with adrenocortical tumours whereas around half of prepubertal children will present with Cushing's disease and ectopic ACTH syndrome is especially rare (77).

Cushing's Syndrome commonly affects adults between the ages of 25-40 and one of the first and major symptoms is centripetal weight gain (73,75). This is then followed by fat accumulation within the face and supraclavicular fat pads giving the typical moon shape and buffalo hump (74). Protein wasting signs can also be seen, including wasting of the pelvic and proximal limb muscles for example, increased skin fragility with striae and increased fractures affecting the feet, ribs and vertebrae. Psychiatric symptoms including anxiety, depression and irritability also affect 70-85%.

The recommended diagnostic tests include measurements of 24 hour urinary free cortisol excretion, documentation of circadian profiles, together with measurement of ACTH to indicate whether cortisol excess results from ACTH excess, or whether ACTH is suppressed by autonomous cortisol release. The

1mg low dose dexamethasone suppression test assesses the integrity of the HPA axis: In patients with Cushing's Disease, suppression of ACTH by the administration of dexamethasone results in suppression of cortisol release, documented by early morning cortisol <50 nmol/L (78).

Imaging tests can also aid the identification of the location of the tumour including CT and MRI scans and inferior petrosal sinus sampling which localises the tumour to the pituitary gland (75). Tumour resection which removes the source of ACTH excess is the first line treatment and done via the transsphenoidal route or laparoscopic surgery (75). For patients unfit for surgery or for tumours that cannot be found on imaging, drug therapy can be offered to control hypercortisolism. Those drugs include steroidogenesis inhibitors such as mitotane, ketoconazole and metyrapone, those that modulate ACTH release like somatostatin and dopamine agonists and those which block glucocorticoid receptor action like mifepristone (79).

1.3.2 Cortisol Deficiency: Adrenal Insufficiency

Adrenal insufficiency (AI) can be classified into primary (disorders of the adrenal), secondary (disorders of the pituitary) or tertiary (disorders of the hypothalamus). AI may also result from glucocorticoid suppression of the HPA axis at the level of the hypothalamus and pituitary gland in patients who receive long term treatment with glucocorticoid medication (approximately 150 to 280 per million Europeans, more frequently women than in men (80). Chronic suppression of the HPA axis, causes ACTH deficiency, which in turn causes atrophy of the zonae fasciculata and reticularis of the adrenals (80).

Symptoms of chronic AI include general weakness and fatigue, vomiting, abdominal pain, failure to thrive in children and joint pain (81,82). Acute adrenal crisis can be a life threatening disorder characterised by severe hypotension and hypovolemic shock due to glucocorticoid and mineralocorticoid deficiency (80,83) AI can be life threatening and precipitation of a serious infection, adrenal haemorrhage or infarction can cause a condition called an adrenal crisis (84). It has a mortality rate of 1.5%, and early diagnosis and treatment are essential (84).

Tests of adrenal reserve can be classified as those that test the integrity of the hypothalamic pituitary adrenal axis, including the insulin tolerance test, glucagon stimulation test, metyrapone test, cortico-releasing hormone test, and those that stimulate the adrenal directly, the Synacthen tests, which rely on involution of the adrenal in the absence of stimulation by adrenocorticotropic hormone. A single measurement of cortisol, preferably taken as close to the CAR as possible, can be a helpful screening test.

A serum cortisol < 100nmol/L is considered diagnostic of AI however, if the value is between 100-450 nmol/L a formal test of adrenal function should be undertaken (85). Synacthen tests are used widely in clinical practice, as they are safer and better tolerated than other tests. A number of different protocols are in use, which differ according to dose of Synacthen and timing of samples. In the commonly used 'standard dose test' serum cortisol levels are measured before, 30 and 60 minutes after the administration of 250 micrograms of the synthetic analogue of ACTH tetracosactide (Synacthen) given intramuscularly or intravenously. Adrenal insufficiency is diagnosed in patients in whom peak cortisol concentrations are below 450 nmol/L. (85).

As AI is potentially life threatening treatment should start as soon as a diagnosis is made (83). The goal of treatment for steroid induced AI is to eventually wean the patient off exogenous steroids, while providing adequate cortisol replacement with hydrocortisone to allow the HPA axis to recover (86,87).

1.3.3 11βHSD AND HYPERTENSION

The ratio of serum cortisol to cortisone is a marker of 11βHSD activity enzyme which can change in different settings including inflammatory conditions or following administration of glycyrrhetinic acid (inhibitor of 11βHSD2) found in liquorice (88).

Mineralocorticoid receptors (MR) have equal affinity for cortisol and aldosterone however, cortisol may predominant binding as it normally circulates at levels 100-1000 times higher than those of aldosterone (60,89). Conversion to inactive cortisone protects the MR from high circulating levels of cortisol. However, in the absence, deficiency or saturation of this enzyme glucocorticoids activate the MR receptor thus mimicking the effects of aldosterone and increasing sympathetic activity within the brain(90). This can then cause increased renal sodium retention and intravascular volume expansion eventually leading to hypertension (60). Cortisol is also known to enhance vascular sensitivity to catecholamines and angiotensin II (91). Linking increased circulating cortisol levels to hypertension is evident in the condition Cushing's Syndrome, presenting in around 80% of adult patients and 47% in children (60). It is also evident in the condition of apparent mineralocorticoid excess which is an autosomal recessive disorder caused by a deficiency of 11β-hydroxysteroid dehydrogenase type 2 enzyme. Patients present with a triad of hypertension, hypokalaemia and suppressed aldosterone levels (89).

There has been evidence of change of 11βHSD activity during infancy and childhood. During the first year of life, plasma cortisol is shown to increase significantly, with no further increase until adulthood. Cortisone however, significantly decreased during the first year of life and then remain unchanged.

Due to increased cortisol secretion, there is a shift towards mineralocorticoid activity which may explain the physiologically rise in blood pressure seen during childhood. There is a theory that this may be due to altered activity of the 11βHSD isoenzymes; either type 2 (cortisol to cortisone) decreases or type 1 activity rises with age. (92)

1.4 Methods of Measuring cortisol

1.4.1 Serum / Urinary Cortisol

Measurement of cortisol can be made in both serum and urine (93). However, they present with both advantages and disadvantages. Serum cortisol is one of the earliest described methods of measuring cortisol within the body. As far back as the early 1960s, the first fluorometric assay was described which then was later replaced by gas chromatography in 1975 due to both improved specificity and sensitivity. In 2001, liquid chromatography with tandem mass spectrometry was developed and was able to measure a range of steroid hormones in patients with deficiencies in the 11-hydroxylase and 21-hydroxylase enzymes (46). However, using LC-MS/MS can come at a high cost and a need for specialised technical skillsets in comparison to immunoassays (94), which allow for high throughput and provide simplicity, speed and analytical sensitivity (94,95).

When measuring serum cortisol, it is important to remember that bound (biologically inactive) and unbound (biologically active) cortisol are quantified. Therefore, the measurement is influenced by concentrations of CBG and albumin within the blood (46). CBG is released from the liver and acts like a reservoir of cortisol which is available for release to interact with the GR. It is affected by a number of different factors including the contraceptive pill, pregnancy, infection and exogenous steroids (46,96). CBG is increased in both pregnancy and with the contraceptive pill due to oestrogen excess, meaning that more cortisol is bound to CBG and the total cortisol concentration within serum is increased (3). The Free Hormone Hypothesis, predicts that the biologically active part of a given steroid is the free unbound part rather than the total concentration (2). If free cortisol is to be measured by immunoassays, displacement of CBG before analysis is required through the use of steroid substitutes like danazol or through pH change. However, if there is an excess of CBG, as in situations of oestrogen excess, this is not always sufficient (46).

Urinary cortisol, like serum was first measured using a simple fluorometric assay, then radioimmunoassay (RIA), enzyme immunoassay (EIA) and currently LC-MS/MS is preferred due to its highly sensitive and specific assays used (46). The kidneys filter out and excrete cortisol, 80-90% of free, unbound cortisol is then partially reabsorbed back into the distal tubule of the kidneys and just 2% of it is excreted into the urine (3,46,97). The conjugated biologically inactive part, still bound to CBG or albumin is excreted within urine, stool and skin (3). Measuring urinary cortisol gives a good indicator of integrated free cortisol over a 24 hour period. It also allows assessment of night-time cortisol levels which can be useful when investigating conditions when day-time cortisol is not elevated as in anxiety or Cushing's Syndrome (93). In patients with Cushing's, they have a higher renal cortisol clearance than normal caused rapid rise of free cortisol once CBG binding has been exceeded

(5). However, urinary cortisol measurements aren't helpful in investigating the patient's acute response to stress. Furthermore, collecting samples over a 24 hour period can be inconvenient for patients, especially younger children and can then lead to poor compliance and inadequate collection (3,93).

The presence of other cortisol metabolites can cause issues when analysing urinary cortisol. These metabolites or variable salt content within the sample can cross-react with the antibodies used in immunoassays necessary for extracting free cortisol leading to falsely high results (93). This can be especially true if a large volume of urine is being used (2). Some laboratories will pre-treat the urine sample with an organic solvent like dichloromethane or ethyl acetate to remove these interfering substances (94).

Plasma clearance of cortisol occurs within the liver (through A-ring reductase) and kidney (conversion of cortisol to cortisone by 11β-hydroxysteroid dehydrogenase type 2) and occurs rapidly with a half-life of 66 minutes (98,99). Therefore using 24 hour urinary free cortisol (UFC) does not correlate strongly with serum free cortisol. Creatinine clearance should therefore be checked beforehand in these patients for adjustment of urinary cortisol levels (3).

1.4.2 SALIVA

As early as the 1980s salivary cortisol and its clinical utility have been used in a range of specialities including endocrinology, psychobiology and behavioural medicine and continues to grow in popularity. It has a number of advantages including non-invasive sampling which can be undertaken at home, avoiding stress induced artificial increases in cortisol and representing the biologically active form of serum cortisol (2,8). Like serum and UFC, historically salivary cortisol was measured mainly by immunoassays (RIA, ELISA, ECLIA) but more recently liquid chromatography methods coupled with mass spectrometry (LC-MS/MS) have become another option. During the pre-1990s, the first generation of salivary cortisol assays were developed, but these were modifications of commercially available serum assays. It was not until the late 1990s that assays were specifically produced for saliva (100). Immunoassay may be the preferred method of analysis due to convenience and accessibility in comparison to LC-MS/MS which require a high level of expertise However, LC-MS/MS is able to simultaneously measure other steroids such as 17 αhydroxyprogesterone (17-OHP), aldosterone, androstenedione (AE) and oestradiol and it is 10-100 times more sensitive in comparison to RIA (7,8).

1.4.2.1 Uses in Clinical Practice

Currently, salivary cortisol is used as a diagnostic and physiological biomarker in the clinical and research setting, investigating for example psychological stress and related mental illness, circadian rhythms in preterm infants, monitoring hydrocortisone therapy and in patients with adrenal incidentalomas (1–3). Cushing's Syndrome is a difficult condition to diagnose and distinguish from pseudo-Cushing, in which patients experience symptoms of severe obesity, high blood pressure and depression, and clinical and biochemical evidence can be vague and confusing. (101) The diagnosis of Cushing's Syndrome can also be difficult with young children and adolescents. Symptoms such as excessive weight gain and growth retardation are the most common features but the characteristic phenotype of centripetal obesity and protein wasting is seen less frequent in young people. (102) Thus, biochemical evidence is heavily relied on for a diagnosis. Guidelines from the Endocrine Society state that late night salivary cortisol (LNSC) is a preferred screening test for Cushing's, particularly in combination with 24 hour urine free cortisol (UFC) and serum cortisol, as salivary cortisol levels reflect the diurnal rhythm of serum cortisol (2,8).

1.4.2.2 Different ways of collecting saliva

There are many ways saliva can be collected whether that be within hospital or at home. Currently one of the most popular methods is via the Salivette which has been developed by the German brand Sarstedt. They state it is a more hygienic, more aesthetically pleasing, more precise method of collecting saliva for analysis (103). The Salivette device is a plastic tube, with a stopper, that can contain either a plain cotton swab, a cotton swab with citric acid preparation or a synthetic swab which the patient is instructed to chew on for 60 seconds (103). The swab is then placed into a small plastic tube, which is sealed by a blue stopper and sent for centrifugation. Even with the smallest volumes of saliva, the Salivette can produce precise analytical values (103). Limitations of this device include the risk of it being a choking hazard to younger children, the cotton swab being unpalatable, younger children unable to chew on it for 60 seconds therefore not achieving a sufficient sample (104).

Another method is via passive drool into a tube or straw. This is suitable for children and adolescents, less so for younger children who may lack the oral coordination to collect a sample (104). The patient allows saliva to pool into their mouth which they then drool into a vial (105). However, the resulting saliva sample can be thick and sticky, thus hindering pipetting and leading to an inaccurate result in the saliva analysed (106). Other methods of saliva collection include absorbent cotton pads, braided cotton dental wool, disposable mucous extractors or modified eye sponges (104).

There are a list of criteria that ensure saliva sample are of optimal quality, these include (107):

- No drinking or eating 30 minutes before sample
- No brushing or flossing teeth 30 minutes before taking sample
- Avoidance of caffeine and smoking
- Patients at risk of blood contamination from mouth ulcers, oral lesions or gingivitis should not collect saliva samples
- Cannot take inhaled corticosteroids before sample, must stop 24 hours before (not needed if LC-MS/MS is used)

If the patient is finding it hard to salivate or in populations with low spontaneous flow rates such as babies, the elderly or depressed patients, there are a number of agents that can be used stimulate saliva flow (93).These include including sugarless gum, flavoured drink crystals, wax pellets of parafilm to chew on or cotton swabs soaked in citric acid which is known to stimulate the salivary glands(103,107). However, there is a potential for these agents to alter the salivary pH and thus affect the reliability of the result(107).

1.4.2.3 Advantages of Salivary Cortisol / Cortisone

Within serum cortisol 95% of it is bound to a transporter protein such as albumin or CBG therefore considered biologically inactive, the rest (5%) is free and unbound. Whereas within saliva, cortisol is free and unbound therefore representing a direct measurement of the concentration of biologically active cortisol within the body (47). Salivary cortisol is believed to mimic changes and reflect the diurnal changes that occur in plasma cortisol (5). Goodyer et al reported that salivary cortisol levels correlate highly with serum levels (r= 0.6–0.9 or proportions of total variance ranging between R2= 0.36 and R2= 0.81) (2).

Throughout life we all must face needles whether that is through taking a vaccine or having our bloods taken. For some this can be a slightly fearful process but for others it can come with severe anxiety and symptoms of nausea, trembling, fainting or shortness of breath(108). In a large sample of children, those who were 6-8 yrs., 68% reported they had a fear of needles, 65% in 9-12 yrs., and 51% in 13-17 yrs. (109). This fear tends to decrease as they become older, but it is still prevalent for some with 22% of adults thought to be affected (110,111). This can be even more stressful for those children who need multiple blood tests such as those with type 1 diabetes, adrenal insufficiency or nephrotic syndrome (112). Using saliva as a method of testing removes this fearful and stressful situation for the child. It also removes the artificial increase of cortisol that is induced due to the child being stressed about the hospital visit or the blood test. Another advantage is that salivary cortisol can be collected from home therefore removing the disruption that is caused due to the child needing to be taken out

of school or the parent from work to visit hospital. Saliva collection can be fitted easily between the child's normal activities or family life. Repeated samples can be taken from an individual due to convenience of collection and this is especially useful in analysing conditions such as episodic Cushing's (8). There is ease with collection as the sample can then be kept at room temperature for around 30 minutes, other studies say 2-4 weeks and then can be stored within the family's fridge at 5°C for up to 3 months or -20/-80 °C for up to 1 year (113–115). No freezing or refrigeration is needed when transporting the samples back to hospital or to the labs(116). Also, salivary cortisol can withstand 5 repeated samples of thawing and freezing(117).

1.4.2.4 Cons of Salivary Cortisol / Cortisone

Most patients who need to take continuous cortisol samples are those on synthetic glucocorticoids such as prednisolone or dexamethasone as treatment for cortisol excess or deficiency. But the concentration of salivary cortisol can be influenced by these drugs, for example prednisolone cross-reacts with the antiserum when assaying cortisol causing inaccurate values and dexamethasone can significantly suppress the HPA axis (114). Furthermore, there is evidence that medications used to treat attention deficit hyperactivity disorder can also impact HPA activity and lower cortisol levels (107). Fruit juices which contain vitamin C can stimulate salivary flow which can then alter salivary pH, further altering the result. If a patient has drank before their sample, they are recommended to rinse out their mouth for 5 minutes in order to return the oral environment to the normal pH which is around 6.3-7.4 (107,114). The issue with collection at home is that there is a risk of human error either through incorrect method of collection or contamination. Also, as the release of cortisol follows a circadian rhythm, the time of day that the samples are collected is important and needs to be standardised for each patient (107).

There is an overall lack of a validated reference method of measurement that all laboratories can follow and use when assaying salivary cortisol. A lot of other steroids are naturally very similar in structure to cortisol including its inactive form cortisone and thus causes cross-reactivity within the sample and the creation of specific antibody to bind with more difficult (5). This therefore makes results unreliable in a clinical setting for patients with congenital adrenal hyperplasia or in Cushing's. Furthermore there is a lack of a validated reference range of salivary cortisol for both adults and children causing differences in results between studies (5).

Reference ranges have been developed by researchers for salivary cortisol, but they are still reluctant in applying them to data. There are many reasons for this including the majority of assays used within the different studies are developed from other biological matrices used for urine or serum. These assays usually aren't adjusted for salivary analysis, causing large intra-assay variability and

overestimation of cortisol concentrations(116). Many of the studies use different immunoassays and therefore their reference ranges cannot be used universally(53,117–119). Furthermore, reference data was from specific groups or age ranges (10-12 year olds (55) or female adolescents (120)) or from very small sample sizes(121). There is a lack of information available surrounding reference ranges of salivary cortisol in children and adolescents, that covers from the ages of 5-18 years of age.

1.4.2.5 Reference Intervals

Reference intervals are used by clinicians and laboratories to assist in the investigation and diagnosis of disease, hence are very useful in patient care(122). In order to find out what a reference range is, a large population of healthy individuals are required in order to figure out what the 'normal' range is. The range should be calculated as the set of values of which 95% of the population falls i.e., taking two standard deviations either side of the mean(122). Factors such as age, sex, BMI, ethnicity and puberty need to come into consideration when testing specific populations as these factors can have individual effects on the hormone and the range we are studying(123).

1.5 CONCLUSION

In conclusion, cortisol concentration tends to vary from childhood to adolescence, but studies are presenting contradicting findings or limited populations. An increase in the ratio of salivary cortisol/cortisone during the first year of life, followed by stability throughout childhood and into adolescence has been described, while salivary cortisol concentrations have variably been associated with sex, pubertal development and body mass index. Furthermore, 11βHSD isoenzymes play a pivotal role in controlling cortisol metabolism within the body. Studies are now showing that it may have a key involvement in physiological rising blood pressure in early childhood, Cushing's, central obesity and insulin resistance. There are many advantages in the use of saliva to measure cortisol, especially in children such as it being a non-invasive and efficient test, where artificial increases and disruption of daily life are avoided. Saliva is also now being seen as a tool in the diagnosis of diseases such as Cushing's, congenital adrenal hyperplasia and polycystic ovarian syndrome. However, there is still a lack of a validated reference range and very few data describing salivary cortisol and cortisone since introduction of new analytical methods like LC-MS/MS within a cohort of healthy children and young people.

1.6 Aims and Objectives

In order to address the issues outlined above, the aim of this project was to further investigate the concentration and ratio of these two hormones in healthy children and adolescents, and how they might relate to growth, weight gain and blood pressure.

Primary Objective

To define reference intervals for salivary cortisol and cortisone in healthy children and young people.

Secondary Objectives

1. Describe salivary cortisol and cortisone concentrations 30 minutes after waking, the 'area under the curve' for salivary cortisol and cortisone measured every two hours during waking hours, mean salivary cortisol and cortisone during waking hours, the frequency of samples in which either salivary cortisol or cortisone are below detectable limits
2. To further explore relationships between salivary biomarkers and the following parameters age, sex, body mass index standard deviation score, blood pressure centiles, weight and height standard deviation scores
3. To investigate relevant literature surrounding the relationship between cortisol, cortisone, cortisol-to-cortisone ratio and hypertension.

The primary objective and the first two secondary objectives will be detailed in the SMILE chapters and the third secondary objective will be discussed within the systematic review.

2.0 THE SMILE STUDY

For many years, saliva has been one of the tools used to measure the range of hormones present within the body, but only recently have researchers taken notice of saliva as a way of measuring cortisol excess or deficiency. The regulation of cortisol activity is carried out by two isoforms of the 11β hydroxysteroid dehydrogenase (11βHSD) enzyme. Type 2 is responsible for inactivating cortisol to cortisone and Type 1 carries out the reverse. Measuring the ratio of cortisol to cortisone is being researched as a potential medium to screen for abnormalities of the 11β hydroxysteroid dehydrogenase (11βHSD) enzyme.

Using saliva as a medium has a number of advantages, especially with children. Artificial increases in cortisol caused by the child's needle phobia or from stress of attending hospital is avoided. Samples can be collected anywhere such as at home or at school and reduces the number of hospital visits needed therefore school or work isn't disrupted. Cortisol within saliva is not bound to any proteins therefore considered 'free' and biologically active. Currently, there are few data describing salivary cortisol cortisone concentrations in healthy children and young people. This study recruited healthy children to take saliva samples throughout the day in order to create a reference range of what the normal levels of cortisol, cortisone is in the body, with the goal that it can be implemented in clinical practice in the future.

2.1. STUDY SETTING AND DESIGN

The study was conducted within the Clinical Research Facility (CRF) at Alder's Children Hospital. Founded in September 2018, the CRF is a facility which carries out a range of research in order to benefit the health care and wellbeing of children and young people. The facility comprises of 20 full time members of staff including paediatric doctors, research nurses, clinical trial pharmacists, play specialists and physiotherapists who work alongside children and their families from the initial and set up of the study right through till the end. (Alder Hey website) From October 2020, I met with the CRF team for an introduction and tour of the facility. Efforts were made to put forward the idea of the SMILE study and how we were going to execute it on the CRF. Our research team consisted of Dr Hawcutt, Professor Blair, Dr Parks, Mr Dliso and me.

2.2 STUDY PROTOCOL

The SMILE protocol below was developed by Dr. Hawcutt and Professor Blair.

Aims:

1. To define reference intervals for salivary cortisol and cortisone in healthy children and young people

Objectives:

To describe:

1. salivary cortisol and cortisone concentrations 30 minutes after waking
2. the 'area under the curve' for salivary cortisol and cortisone measured every two hours during waking hours
3. mean salivary cortisol and cortisone during waking hours
4. the frequency of samples in which either salivary cortisol or cortisone are below detectable limits

To further explore relationships between salivary biomarkers and the following parameters:

1. Age
2. Sex
3. Body mass index standard deviation score

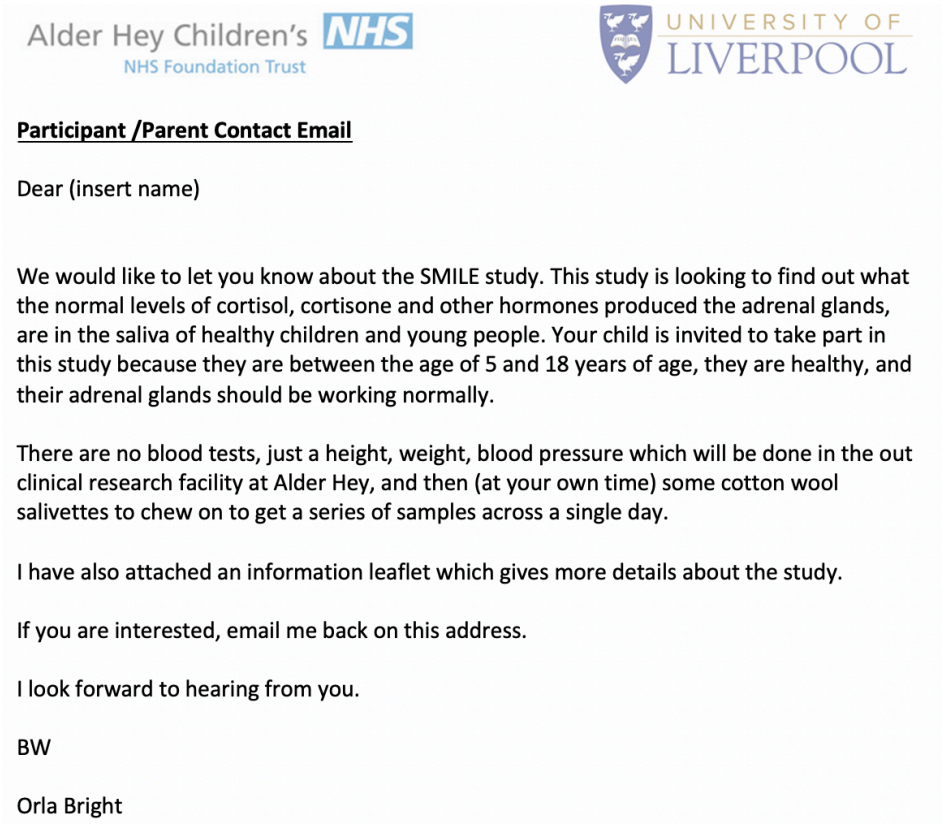
To consider new associations between salivary biomarkers and blood pressure centile, estimated according to age, sex, weight and height standard deviation score.

2.2.1 Participant Recruitment

In this observational cohort study, we planned to recruit 150 healthy children and young people from the ages of 5-18 years. By power calculation 150 are needed to complete the dataset with a relative error of 0.2. Recruitment was stratified by two-year age groups (5-6 year / 7-8 years / 9-10 years etc.) to ensure adequate representation across the age range. Due to the COVID-19 pandemic, we commenced recruitment later than planned and a lot of parents and children did not feel comfortable coming to a hospital therefore we had to reduce this goal to 50 children. Recruitment was carried out in 3 ways.

1. Children of Alder Hey Staff

This was our main form of recruitment. We began by sending the SMILE protocol via hospital email, the Trust website and through direct communication to the different specialties within the hospital. This was done by the whole research team. If the participant showed interest, me or the physician associate working on SMILE would make contact via email / telephone to check study eligibility and then their first visit to Alder Hey. The generic email sent to potential participants is detailed below:



Alder Hey Children's NHS
NHS Foundation Trust

UNIVERSITY OF LIVERPOOL

Participant /Parent Contact Email

Dear (insert name)

We would like to let you know about the SMILE study. This study is looking to find out what the normal levels of cortisol, cortisone and other hormones produced the adrenal glands, are in the saliva of healthy children and young people. Your child is invited to take part in this study because they are between the age of 5 and 18 years of age, they are healthy, and their adrenal glands should be working normally.

There are no blood tests, just a height, weight, blood pressure which will be done in the out clinical research facility at Alder Hey, and then (at your own time) some cotton wool salivettes to chew on to get a series of samples across a single day.

I have also attached an information leaflet which gives more details about the study.

If you are interested, email me back on this address.

I look forward to hearing from you.

BW

Orla Bright

FIGURE 5: PARTICPANT CONTACT EMAIL TEMPLATE USED TO CONTACT STAFF AT ALDER HEY AND THOSE FOUND FROM CLINIC ONTO SMILE.

2. Siblings of patients attending outpatient clinics

Due to the COVID-19 pandemic, we assumed that there would be a few people who would not be happy with attending the hospital for a research visit, so we extended recruitment to siblings of patients from outpatient clinics. I attended outpatient clinics such as ENT, orthopedics and MDU to find siblings of patients who were interested in participating in SMILE. Due to COVID social distancing rules, I met with patients in the waiting area and explained the background and purpose of the study. If interested, study eligibility was checked, and contact details were taken. I would then arrange a time over the telephone as to when they could come to the CRF.

3. Children attending outpatient visits

If a member of the clinical team saw a potential participant within their clinic, they would inform the research team. This participant would then be contacted by telephone by a member of the research team, with age appropriate information about SMILE.

Inclusion Criteria

- Children and young people aged 5-18 years. We didn't include children below the age of 5, as the Salivette device may present as a choking hazard

Exclusion Criteria

- Children with oral conditions likely to result in blood contamination of saliva samples including gingivitis, mouth ulcers and those undergoing dental procedures.
- Conditions likely to affect serum cortisol or CBG including abnormalities of thyroid or anterior pituitary hormone secretion, psychiatric pathology, type I or type 2 diabetes, cystic fibrosis, protein losing enteropathies, nephrotic syndrome and patients undergoing renal dialysis. Children with a family history of adrenal insufficiency due to an inherited condition will also be also excluded.
- Children receiving medications likely to affect serum cortisol or CBG including glucocorticoids, sex steroids, thyroxine, growth hormone, azole compounds, insulin and metformin.

The list of surgical conditions that would not exclude participation in the study, in children who meet the other eligibility criteria, include but are not limited to:

- Insertion of grommets
- Squint surgery

- Removal of birth marks or other skin lesions
- Circumcision
- Removal of foreign bodies
- Examination under anesthesia

2.2.2 Statistical Analysis

Simple associations between pairs of variables will be assessed using Spearman Rank's correlation coefficient. Linear regression will be used to assess the association between cortisol and cortisone concentrations and height SDS, BMI SDS, decimal age, sex and systolic blood pressure centiles. Since the distribution of the response variables (salivary cortisol, salivary cortisone, serum cortisol) is likely to be positively skewed, regression will be performed using log-transformed versions of the variables.

Based on the regression analysis, pointwise 95% prediction intervals for serum cortisol, salivary cortisol and the salivary cortisone/cortisol ratio will be constructed based on the critical values of the relevant t-distribution using the estimated residual standard deviation and back-transforming to the natural scale.

In cases where the measurement falls below the detectable limits, a value of half to the limit of detection will be imputed.

2.3 STUDY DESIGN MATERIALS

2.3.1 PATIENT INFORMATION LEAFLETS / CONSENT FORMS + ASSENTS

Professor Blair created 5 separate information booklets for each different age group and for the parents (under 8, 8-12 years, 13-15 years, 16+). Each information leaflet was written in a format that was understandable and appropriate to that specific age group. It included information on what the study was about, background to the adrenal glands and cortisol, why it was being done, what it would involve, risks/benefits of taking part and how we will use their information. These documents were shared with interested participants, mostly via email and through clinic.

As this study was using human participants, I created consent forms so that I had written proof that the potential participant had a clear understanding of why they were doing the study, what it would mean for them and the reasons the study was being carried out. It was proof that there had been a full discussion with the participants and his/her parents and a time for the participant to ask any questions. I made a parental consent form, 16+ consent form and an assent form (can be found in Appendices 2,3 and 4). If the physician associate or I deemed a child of under 16 years old to have Gillick competence, we used the 16+ consent form for them.

2.3.2 CASE RECORD FORM

In order to collect data from the participants, I created a case record form (found in appendix 5). I used the case record form to gain a general overview of who we were recruiting, including information such as their date of birth, ethnicity, sex, gestation and birth weight. It included information on their medical history and medications. This information was collected in order to check if they had a medical condition that would have excluded them from the study such as oral conditions that resulted in blood contamination (gingivitis, mouth ulcers), conditions that would affect serum cortisol (type 1 or 2 diabetes, cystic fibrosis, abnormalities of the thyroid or anterior pituitary). If the participant was taking any medications, did any of them affect serum cortisol such as glucocorticoids, sex steroids, growth hormone or insulin. It also included space to record down the participant's height, weight and blood pressure and information on the actual study done themselves.

2.3.3 PATIENT RECORD BOOKLET

I also designed a document for the participant to take home to record the process of saliva sample collection. I created a 5-page booklet which included the salivette method, instructions on how to carry out the saliva collection (Figure 6), a table to note times of taking the sample and putting into the fridge and times taking out of fridge to return to Alder Hey. This can be found in appendix 6.

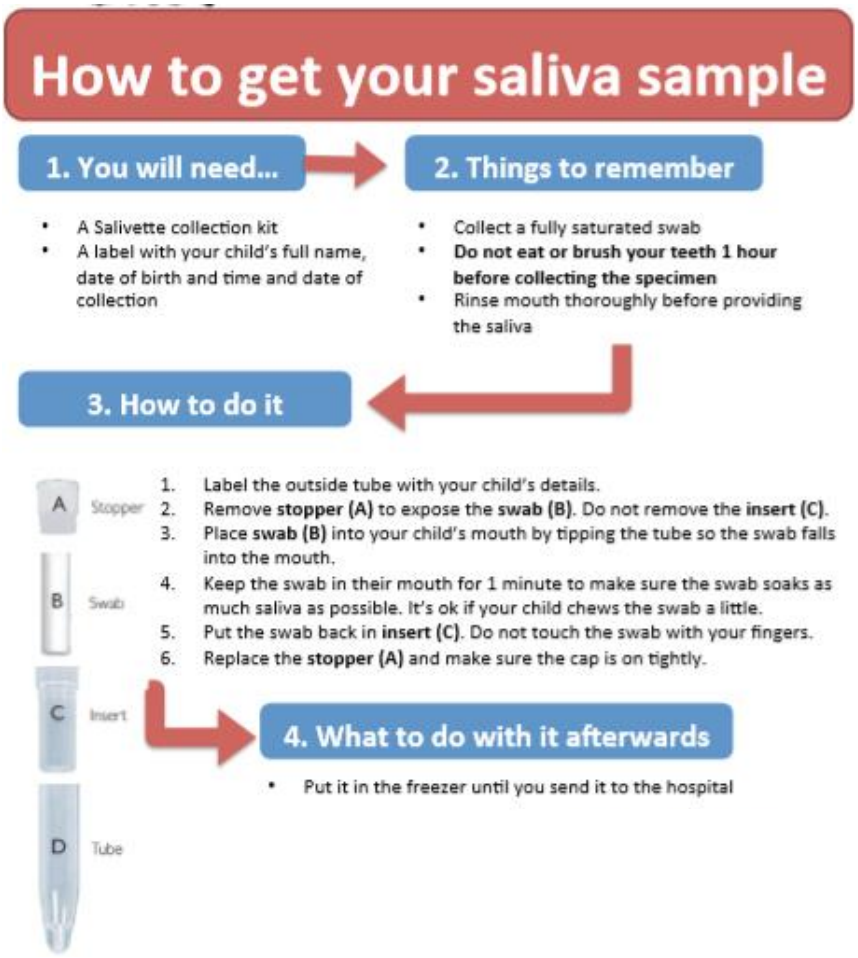


FIGURE 6: INSTRUCTIONS ON HOW TO USE THE SALIVETTE WHEN TAKING A SALIVA SAMPLE WHICH IS INCLUDED WITHIN THE PATIENT RECORD BOOKLET

2.3.4 CREATION OF SALIVA PACKS

The physician associate or I created saliva packs which consisted of;

- 7 labelled Salivette devices
- 7 labelled sealed bags
- Parental / 16+ consent / assent forms
- Case Record Booklet
- Patient Record Booklet

The Salivette devices were made by a company called Salimetrics, which we ordered in. The Salivette device consisted of a plastic tube, containing a cotton wool sample. (Figure 7) We decided on 7 saliva Salivettes as we calculated it would be enough to cover an average day that a child is awake. The laboratory in Alder Hey printed labels for each Salivette device that included the participants study ID and date and time the participant collected the sample. Participants were responsible for writing the date and time they took each sample onto the labelled Salivettes. Then they would put each sample into its own labelled separate bag and securely seal it before freezing. Labels were developed and printed within the lab at Alder Hey and also contained the participants study ID and the date and time the participant collected the sample.

FIGURE 7: IMAGE ADAPTED FROM SALIMETRICS (105) **PICTURE OF THE SALIVETTE DEVICE WHICH CONSISTS OF A PLASTIC TUBE AND SCREW CAP. THIS DEVICE WAS USED IN SMILE TO TAKE A SALIVA SAMPLE**

2.3.5 SITE FILE / RECRUITMENT POSTERS

The physician associate created a site file within the Alder Hey system in order for us to upload all documentation including signed consent forms, SMILE protocol, all of the information leaflets, case record form and patient record booklets. It also contained our HRA approval, documentation about funding and information about the SMILE team. I also created recruitment posters containing our contact details that we planned on putting up around Alder Hey to gain interest into the study (Appendix 7).

2.4 ETHICAL PROCESS

In September, Dr Blair registered the SMILE study with IRAS. I then checked and uploaded further documentation which I had created to the application and submitted for HRA and HRCW approval. This was approved by Cornwall and Plymouth Research Ethics Committee to have an ethical review on the 20th of October. During the evening of the 20th, Dr. Hawcutt and I met with the committee over Zoom to answer queries that the committee had about SMILE. Dr Hawcutt answered these questions. During the meeting, they had concerns about how COVID would impact the study, how we planned to recruit participants, reducing patient contact and travel costs towards participants have to travel far.

On the 27th of October, the committee gave us a response of changes that needed to be made (Figure 8). I carried out the corrections they submitted and gave the following response in the table below. This was done on the 17th of November through IRAS.

HRA and HCRW assessment - Further information required

In addition, please provide the following information in order to clarify points raised in the assessment of the application

Ethical Review - Further Information required	Response from the applicant
The consent for taking part in the study should be taken in person and not over the phone.	**This has been altered in the protocol, parent PIS and over 16 years PIS. Consent will be done in person before any investigations, when the patient is being seen face to face.**
Please provide a copy of the PIS for parents.	**I have attached this with the other documents.**
Please include a statement on the consent form with regards to retaining samples for the use in future ethically approved research. This should be an optional statement with a yes/no option.	**I have added this to both parent and over 16 consent form and assent.**
Please provide the instructions for freezing the samples for review.	**This has been provided.**
It should be clearly explained in the PIS that the samples will have to be returned to Alder Hey Hospital	**The parent and 16+ PIS have been updated to include this. The younger groups have not as they are not required to return in person, just the parent with the samples.**
The travel expenses should be offered if participants will have to make additional visits only for this study	**A sentence about this has been added to the 'what will it involve' section of the parent and 16+ information leaflet.**

HRA and HCRW assessment - Further Information Required	Response from the applicant
It is noted that human tissue samples would be sent to University Hospital Of South Manchester NHS Foundation Trust for analysis. Please clarify whether a material transfer agreement is in place.	**A material transfer agreement is being arranged – this is not active currently as COVID has slowed down the process. However, the samples would not be transported until the end of the study in a single batch, and we would not transfer them without this MTA being in place.**
The General Data Protection Regulation (GDPR) applied from 25 May 2018. The HRA has published recommended transparency wording which you can use to ensure that your Participant Information Sheet (PIS) is compliant with the GDPR. Please include this in the Patient Information sheet and remove any superseded wording. If you wish to use non-standard language, please advise and we will seek sponsor level approval for this. This would then be expected to be used in all studies from this sponsor. https://www.hra.nhs.uk/planning-and-improving-research/policies-standards-legislation/data-protection-and-information-governance/gdpr-guidance/templates/transparency-wording-for-all-sponsors/	**We have added the set test to the adult and 16+ patient leaflets. For the younger participants we do not feel that this information is relevant to the population and is not written in a child appropriate manner, so have not included it.**
IRAS A27-1 indicates that parents of children or siblings would be approached by their treating clinician or the study team. Where the approach is by the study team, please confirm that they would also be part of the direct care team. If not, please ensure the identification of and initial approach to potential participants, prior to consent, would always be by the direct care team.	**For patients of Alder Hey, who have a direct team, then the first approach will be from a member of this team However, children of staff or siblings of children attending the hospital, will not have a direct care team and the initial approach will therefore be from a member of the research team.**
Regarding children aged under 16, please clarify whether parental consent would be obtained in person and provide the PIS/Consent form.	**For those age less than 16 who are competent to consent for themselves, we would like to take consent from them. The 16+ patient information leaflet has been re-named "16+ or older participant consenting for themselves". If a young person less than 16 does consent for themselves then we will NOT have a separate parent consent form completed. However, we will ask that parents who wish to record that they are in agreement with the consent to sign the "optional parent signature" section at the end of the young person (<16y) consent form**
Please amend the PIS and ICF to include the IRAS ID on the documentation.	**This has been added to all the documents**
Note: We would expect that the PIS/ICF would be produced on headed paper.	**We have reviewed our documentation and all forms are now on headed paper.**

FIGURE 8: HRA AND HCRW ASSESSMENT REQUIRED CHANGES SUBMITTED TO US FOR SMILE. ON THE LEFT HAND SIDE OF THE TABLE ARE THE REQUIRED CHANGES AND ON THE RIGHT HAND SIDE OF THE TABLE IS THE RESPONSE WE GAVE AND CHANGES WE MADE.

2.4.1 TIMELINE OF FURTHER CHANGES / UPDATES

I corrected these further instructions that the committee had sent back to us to the relevant documentation.

20th November: Their response was that more transparency wording needed updated (Figure 9)

HRA and HCRW assessment - Further Information Required	Response from the applicant
The parent and 16+ Participant Information Sheets have not had the recommended transparency wording added. Please review and update.	These changes have been made to the parent and 16+ PILs.

FIGURE 9: ANOTHER CHANGE SUBMITTED BY THE ETHICAL BOARD AFTER WE HAD SUBMITTED ORIGINAL CHANGES. MORE TRANSPARENCY WORDING WAS REQUIRED IN THE PATIENT INFORMATION LEAFLETS

25th November: I emailed these corrections back to the committee

26Th November : Their response was that more wording needed to be added in regard to data protection (Figure 10)

Please update both information sheets with the following:

Include this missing wording

"Where can you find out more about how your information is used?

You can find out more about how we use your information

- at www.hra.nhs.uk/information-about-patients/
- our leaflet available from [**X**]
- by asking one of the research team
- by sending an email to [**email**], or
- by ringing us on [**phone number**].

NOTE: At least one of these sources must be able to point people directly to the sponsor's Data Protection Officer.

For [X] sponsors can either provide the HRA link: www.hra.nhs.uk/patientdataandresearch or if this is available on sponsor website, the sponsor may choose to include their own website link."

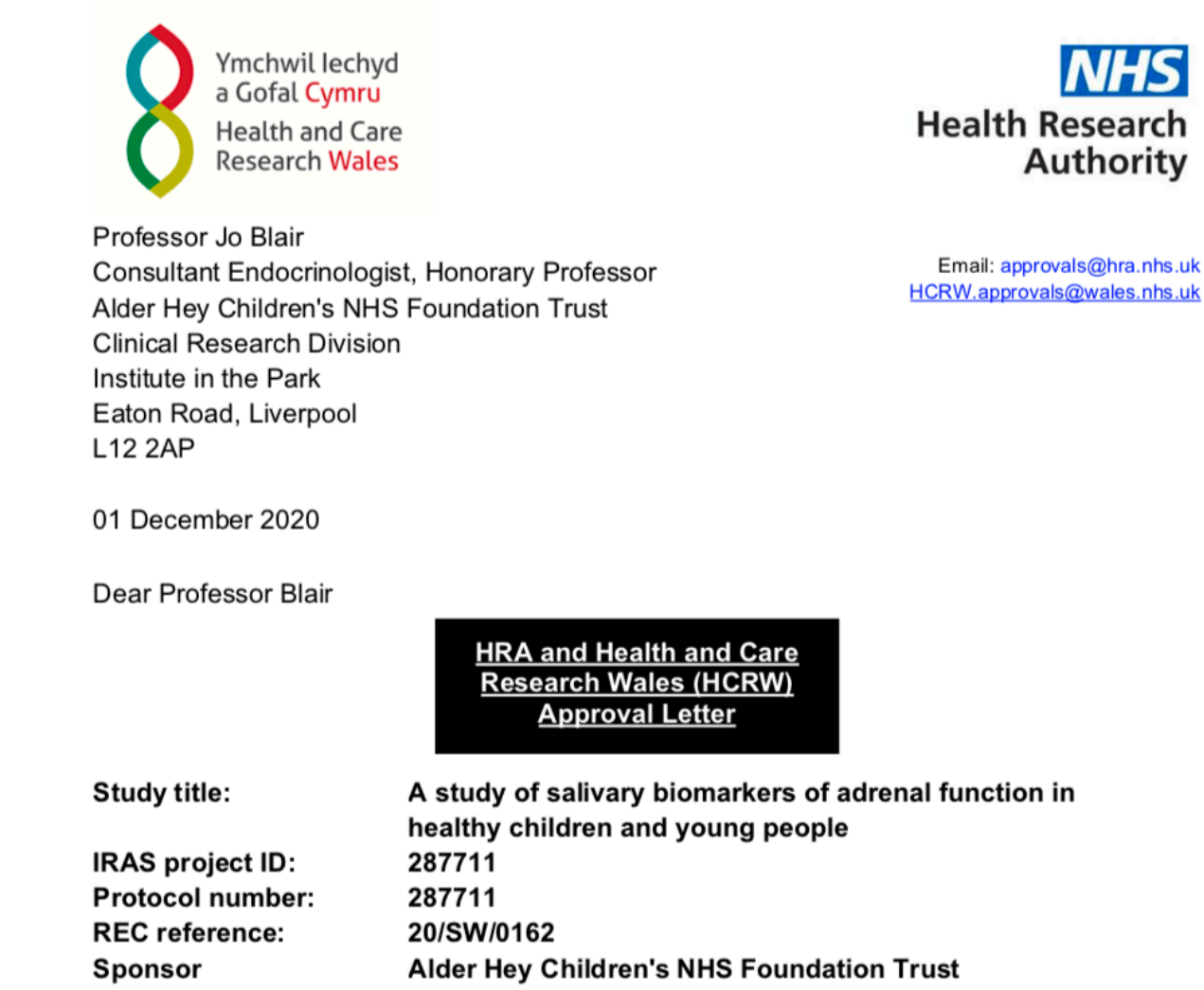
Update the information sheets with the actual number of years that data is kept at the moment you have only added for "XX" years.

FIGURE 10: COMMENT FROM THE ETHICAL BOARD TO ADD FURTHER WRITING TO THE PATIENT INFORMATION LEAFLETS

26th November: I updated these changes and sent back to ethics

30th November: I needed to change version number on the documents, this was re-submitted on same date

1st December: HRA approval given (Figure 11)



Ymchwil Iechyd a Gofal Cymru
Health and Care Research Wales

NHS
Health Research Authority

Professor Jo Blair
Consultant Endocrinologist, Honorary Professor
Alder Hey Children's NHS Foundation Trust
Clinical Research Division
Institute in the Park
Eaton Road, Liverpool
L12 2AP

Email: approvals@hra.nhs.uk
HCRW.approvals@wales.nhs.uk

01 December 2020

Dear Professor Blair

HRA and Health and Care Research Wales (HCRW) Approval Letter

Study title:	**A study of salivary biomarkers of adrenal function in healthy children and young people**
IRAS project ID:	**287711**
Protocol number:	**287711**
REC reference:	**20/SW/0162**
Sponsor	**Alder Hey Children's NHS Foundation Trust**

FIGURE 11: CERTIFICATE OF HRA AND HRCW APPROVAL GIVEN BY THE CORNWALL AND PLYMOUTH ETHICAL BOARD

In February we decided to submit amendments after receiving feedback from parents who didn't feel comfortable or safe coming into Alder Hey for consent and measurement of height, weight and blood pressure due to COVID-19. We decided to offer a telephone consent and an option of a home visit instead of making the trip to the hospital. I created the documentation to support this and information about this was added to the protocol and information leaflets. We received approval for this in early March.

2.5 First Initial Visit to the CRF

2.5.1 Consent Process / Measuring blood pressure / education on saliva taking

The physician associate or I gained consent / assent during the participants first visit to the CRF. Out of the 54 participants I gained the consent of 37 participants. We discussed SMILE in detail including the background, method and any risks / benefits to the participants. If they agreed, we took them through the consent form and checked that they understood each statement. If the child was under 16 or did not have Gillick competence at recruitment, a parental consent was needed, and the child signed an assent form. If the child was >16 years old or deemed to have Gillick competence, they signed their own consent form. Consent was also gained for future use of the participant's samples in other similar studies of measurement of hormones in saliva.

After consent was gained, we took the parents and participants through our case record form and asked for their patient demographics, medical and medication histories. The consent forms were photocopied, and the original was kept within the case file and a copy was given to the participant. Then the participant was weighed on electronic scales and their height measured on a calibrated stadiometer. Blood pressure was measured using WelchAllyn Propaq LT. We allowed the child to settle by waiting around 5 minutes before taking their blood pressure. We took 3 blood pressure measurements in total, waiting 1 minute between each, then calculated an overall average.

A saliva kit was given to the participants and the method of collection was explained. The first initial sample was to be taken 30 minutes after they woke up (to capture the 'early wakening response'), then every two hours after that until they went to bed. We requested that they note down the time of waking and the way in which they woke up either by themselves, an alarm clock or by their parents. Participants were instructed to place samples into a fridge after collection and to be kept there until returning them to Alder Hey. Once returned to Alder Hey, the samples were stored within the laboratory. We checked that they understood what they were required to do by asking them to re-call the information they had just heard.

Due to the COVID-19 pandemic, we also offered the choice of a home visit for participants who wanted to reduce the number of visits to Alder Hey. Mr Dliso or I visited the participant's home, gained consent whilst there and measured height, weight and blood pressure using validated equipment. Whilst there we complied with Alder Hey's lone worker policies. We decided before hand whether or not visiting the family home was suitable on an individual case to case basis. We also offered the option of collecting the saliva samples from the family home if they preferred not to make the trip to Alder Hey.

2.5.2 Collection of Saliva Samples

On completion of the SMILE study, participants were expected to return their samples within a 10 day time frame. If they had not been returned, I emailed or rang the parent directly to check for an update. The participants brought back their patient record booklet and samples to the CRF which were collected by the CRF nurses, or the SMILE team and the samples taken to the laboratory at Alder Hey for storing.

2.6 SMILE RESULTS

2.6.1 RECRUITMENT: PARTICIPANTS INCLUDED VS DROPOUTS

The physician associate and I approached 92 participants in total. 30 participants were excluded for reasons below (Figure 12);

- 15 participants didn't reply to the original email and several emails after asking for their participation
- 12 declined participation
    - 3 too far to travel to Alder Hey Hospital
    - 3 didn't want to take saliva swab due to recent upset of taking a COVID swab
    - 1 due to anxiety didn't want to take part
    - 5 declined without giving reason
- 3 didn't meet inclusion criteria
    - 2 found to be using a steroid inhaler
    - 1 was on the contraceptive pill

4 participants required home visits in order to reduce the risk of COVID. These home visits weren't done within the time frame I had for these set of saliva samples but will be done by the physician associate on the SMILE team who is continuing on recruitment when I return to Year 4 of medical school.

Similarly, 4 participants showed interest in participating but had to rearrange the date for their initial visit to the CRF and will be recruited by the physician associate on the SMILE team.

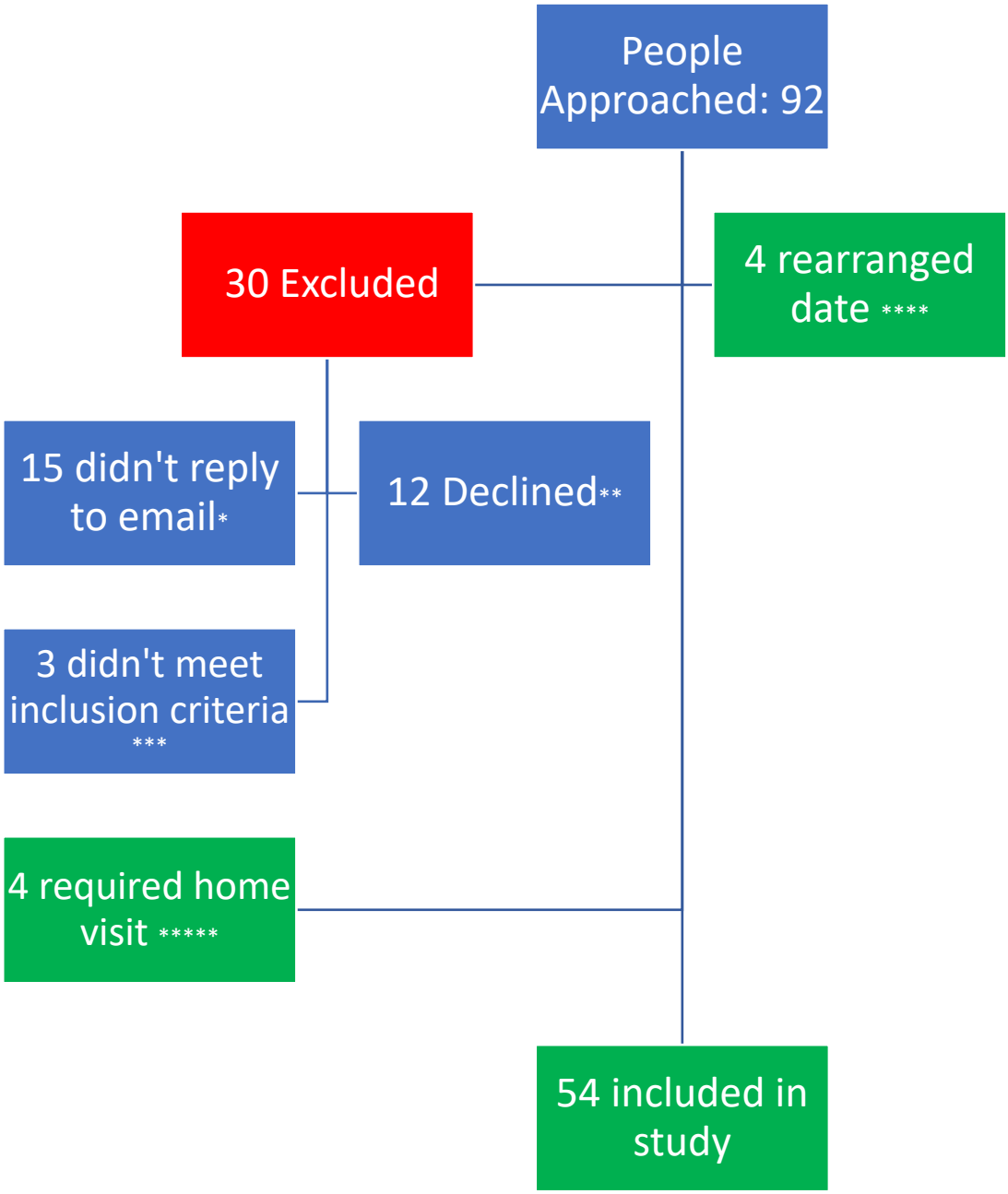


FIGURE 12: RECRUITMENT VS DROP OUT FLOW CHART. ORIGINALLY 92 PARTIPANTS WERE APPROACHED BY A MEMBER OF THE CLINICAL RESEARCH TEAM. 30 WERE EXCLUDED (15 DIDN'T REPLY TO OUR CONTACT EMAILS, 12 DECLINED, 3 DIDN'T MEET THE INCLUSION CRITERIA. 4 REQUIRED HOME VISITS AND 4 REARRANGED THEIR INITIAL CRF VISIT. OVERALL, 54 PARTICIPANTS WERE INCLUDED IN THE SMILE STUDY

2.6.2 Weekly Recruitment Rate and saliva collection

All recruitments were carried out within the CRF. We began recruitment in December 2020 and completed in April 2021. Our original goal was to reach 100 participants but due to the challenges of the COVID-19 pandemic, achieving 54 was a goal in itself. At the beginning recruitment was slow, mainly due to starting it over the Christmas and New Year period. However, by midterm (February), when children were off school, the rate of recruitment increased especially towards the end of January into the month of February as communication between specialities increased and from attending more outpatient clinics. See figure 13.

With participants returning saliva samples, we gave them a two week period to complete the saliva sample collection and return to the CRF. If not, we contacted them via telephone or email for an update. We noticed that this period of time was enough and many of the participants followed this time frame and all of them brought back their saliva samples.

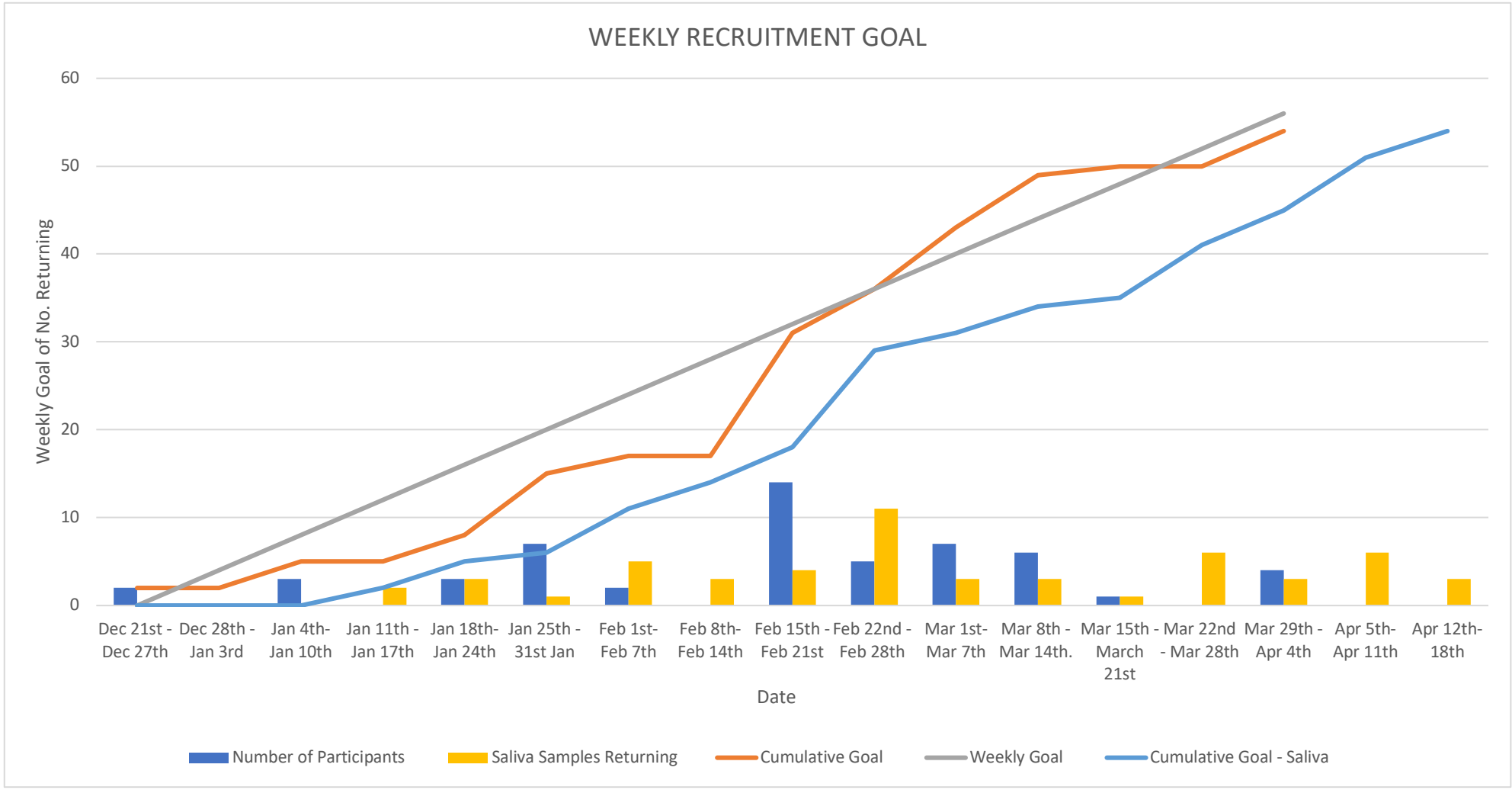


FIGURE 13: GRAPH SHOWING OUR WEEKLY RECRUITMENT AND SALIVA COLLECTION RATE. OUR RECRUITMENT PERIOD LASTED FROM LATE DECEMBER TO MID-APRIL. RECRUITMENT WAS SLOW, MAINLY DUE TO STARTING IT OVER THE CHRISTMAS AND NEW YEAR PERIOD. HOWEVER, BY MIDTERM (FEBRUARY), WHEN CHILDREN WERE OFF SCHOOL, THE RATE OF RECRUITMENT INCREASED ESPECIALLY TOWARDS THE END OF JANUARY INTO THE MONTH OF FEBRUARY. GREY LINE SHOWS OUR WEEKLY GOAL, THE ORANGE LINE IS THE TOTAL PARTICIPANTS WERE RECRUITED IN TOTAL AND THE BLUE LINE IS THE TOTAL SALIVA SAMPLES WE RECEIVED BACK IN TOTAL.

2.6.2 PATIENT DEMOGRAPHICS

We recruited a total of 54 patients over the period of December 2020 to April 2021, 31 male (57.4%) and 23 females (42.6%), with a median age in decimal years of 9.5, range of 5-17.5 to obtain 365 saliva samples. Age distribution was shifted to the left towards the younger age groups, with the highest number being aged 5 years, see figure 14. The majority of the participants were White British (74.1%), 5.6% were Indian, 1.9% were Sri Lankan, 3.7% were Lithuanian, 5.6% were British Indian, 3.7% were Egyptian, 1.9% were British Vietnamese and 3.7% were White Irish. All participants were born at term (37-<42 weeks), with an average gestational age of 39 weeks and 5 days.

Most of the children were from less socioeconomic deprived areas at 25.9% (IMD Decile 7-8) in areas such as Mossley Hill, Victoria and St Helens. Less of the children were from more deprived areas, with 16.7% (IMD Decile 1-2) coming from areas such as Old Swan, Knowsley and Broadheath.

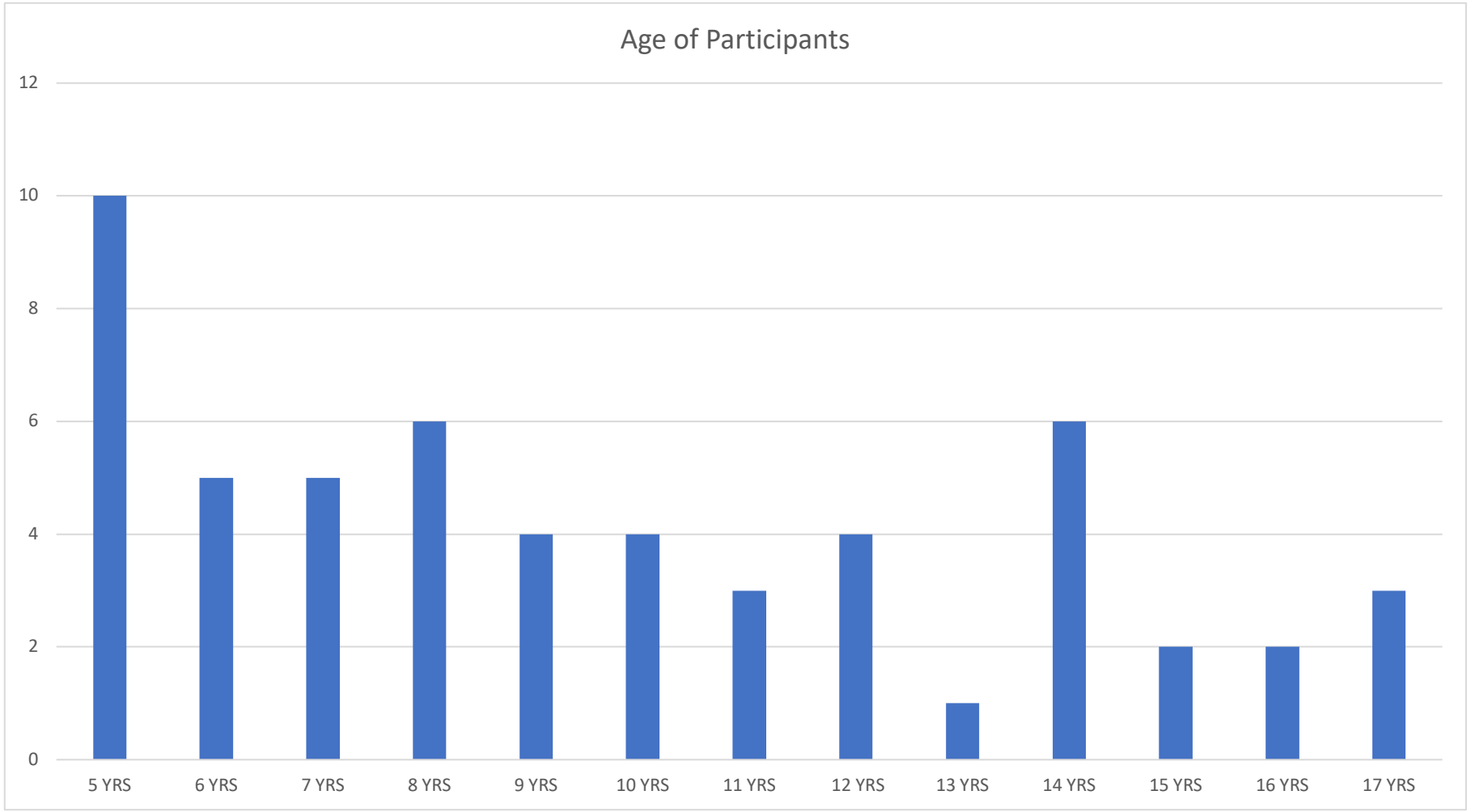


FIGURE 14: GRAPH SHOWING THE NUMBER OF SMILE PARTICIPANTS FOR EACH AGE (5-18 YEARS) . THE MOST COMMON AGE WE RECRUITED WAS 5 YRS (10 PARTICIPANTS) AND THE LOWEST WAS 13 YEARS (1 PARTICIPANT). OUR SMILE COHORT WAS PRODOMINANTLY YOUNGER PATIENTS

Participant Characteristic		Number (%)
Sex	Male	31(57.4)
	Female	23(42.6)
Ethnicity	British	40 (74.1)
	Indian	3 (5.6)
	Sri Lankan	1 (1.9)
	Lithuanian	2 (3.7)
	British Indian	3 (5.6)
	Egyptian	2 (3.7)
	British Vietnamese	1 (1.9)
	Irish	2 (3.7)
Deprivation Score (IMD Decile)*	1-2	9 (16.7)
	3-4	8 (14.8)
	5-6	12 (22.2)
	7-8	14 (25.9)
	9-10	11 (20.4)

TABLE 1: TABLE SHOWING PATIENT CHARACTERISTICS INCLUDING SEX, ETHNICITY AND DEPRIVATION SCORE IN THE SMILE PARTICIPANTS

***1- MOST DEPRIVED 9-10 LEAST DEPRIVED**

All of the participant measurements of the SMILE participants including height, decimal age birth weight, and BMI can be found in Table 2.

Participant Measurement	Mean (1SD)	Median	Maximum Value	Minimum Value
Height (SDS)	0.4 (0.9)	0.4	2.3	-1.4
Decimal Age (years)	10.2 (3.8)	9.5	17.5	5.0
Birth Weight (SDS)	0.1 (1.1)	-0.1	2.2	-2.3
BMI (SDS)	0.5 (1.1)	0.3	2.9	-1.6

TABLE 2: TABLE SHOWING PARTICIPANT MEASUREMENTS OF THE 54 SMILE PARTICIPANTS INCLUDING HEIGHT SDS, DECIMAL AGE, BIRTH WEIGHT SDS AND BMI SDS SCORES. TABLE INCLUDES MEAN, MEDIAN, MAXIMUM AND MINIMUM VALUE OF EACH MEASUREMENT.

Height

The median height of participants was 1.39m (1.05-1.85). The mean z -score for height standard deviation was 0.4. Table 3 contains the percentage of participants within each height percentile.

Figure 15 shows the distribution of height SDS scores of the 54 SMILE participants. 11.1% IN -1.99-(-1), 24.1% IN THE -0.99-0, 33% IN 0-1, 29.6% IN 1-2 AND 1.9% IN 2-3.

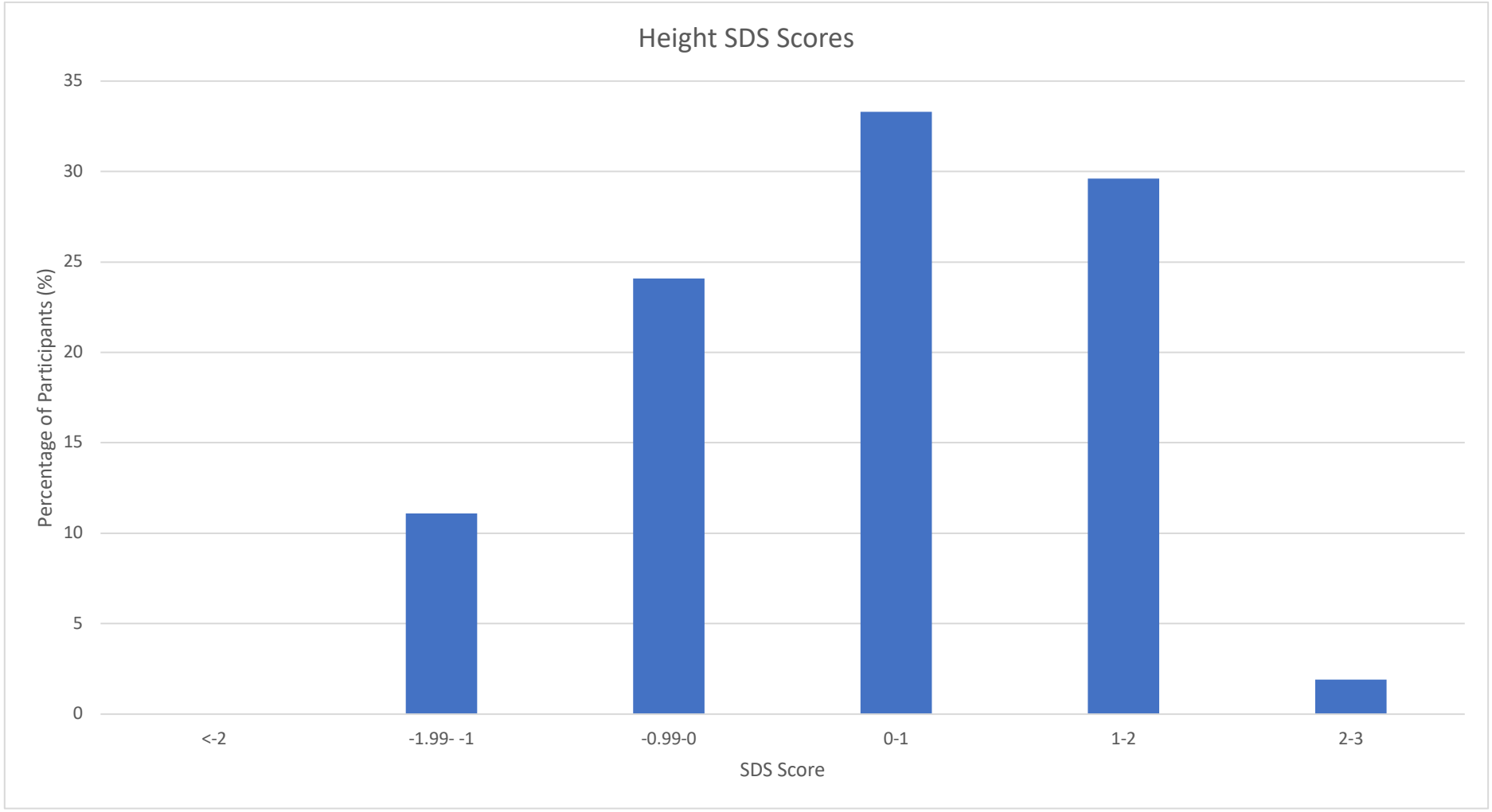


FIGURE 15: GRAPH REPRESENTS DISTRIBUTION OF HEIGHT SDS SCORES OF 54 SMILE PARTICIPANTS. GRAPH GIVES PERCENTAGE OF EACH PARTICIPANTS IN EACH SDS GROUP. 11.1% IN -1.99-(-1), 24.1% IN THE -0.99-0, 33% IN 0-1, 29.6% IN 1-2 AND 1.9% IN 2-3.

Gestation Age/ Birth Weight

All participants were born at term (37-<42 weeks), with an average gestation age of 39 weeks and 5 days. All participants were of a healthy birth weight of 0.1(+/-1.1), median was -0.1 and range 4.5 (2.2-(-2.3)). Figure 16 details the distribution of birth weight SDS scores in the 54 SMILE participants.

Body Mass Index (BMI)

The majority of the participants fell under the healthy weight category at 68.5%, with a mean BMI centile of 56. 1 participant was underweight, 13% were overweight and 16.7% were obese. More males (6) were overweight in comparison to females (1), but more females (6) were obese compared to males (3). We used CDC BMI centile categories and the NHS BMI calculator for children for BMI calculation.(124,125) See figure 17 for BMI centiles.

The average BMI SDS score was 0.4 (+/-1.1), median was 0.3 and range was 4.9. Figure 18 shows the distribution of BMI SDS scores between the 54 SMILE participants.

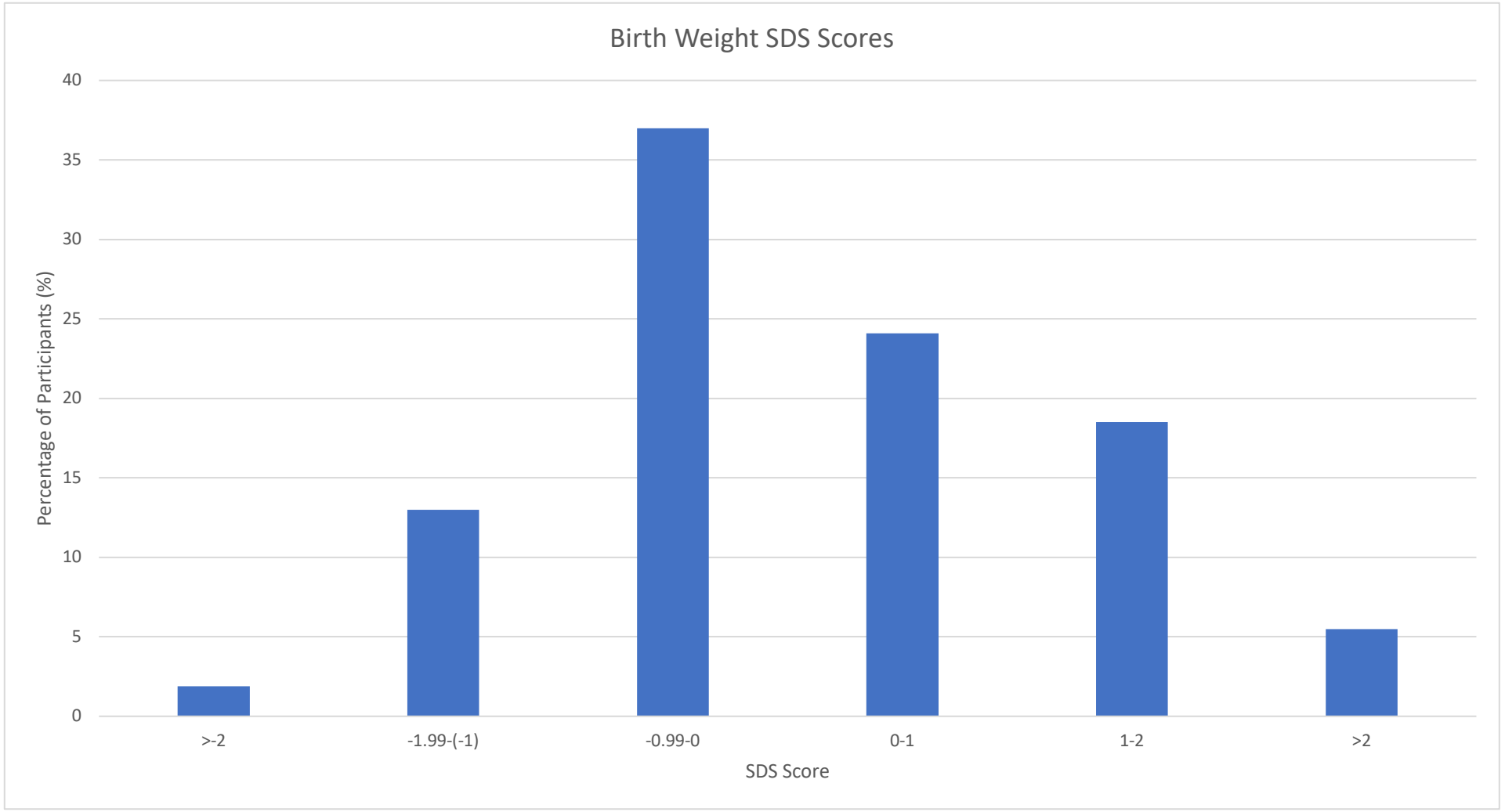


FIGURE 16: GRAPH SHOWING BIRTH WEIGHT SDS SCORES OF THE 54 SMILE PARTICIPANTS . 1.9% WERE <-2, 13% IN -1.99-(-1), 37% IN THE -0.99-0, 24.1% IN 0-1, 18.5% IN 1-2 AND 5.5% IN >2.

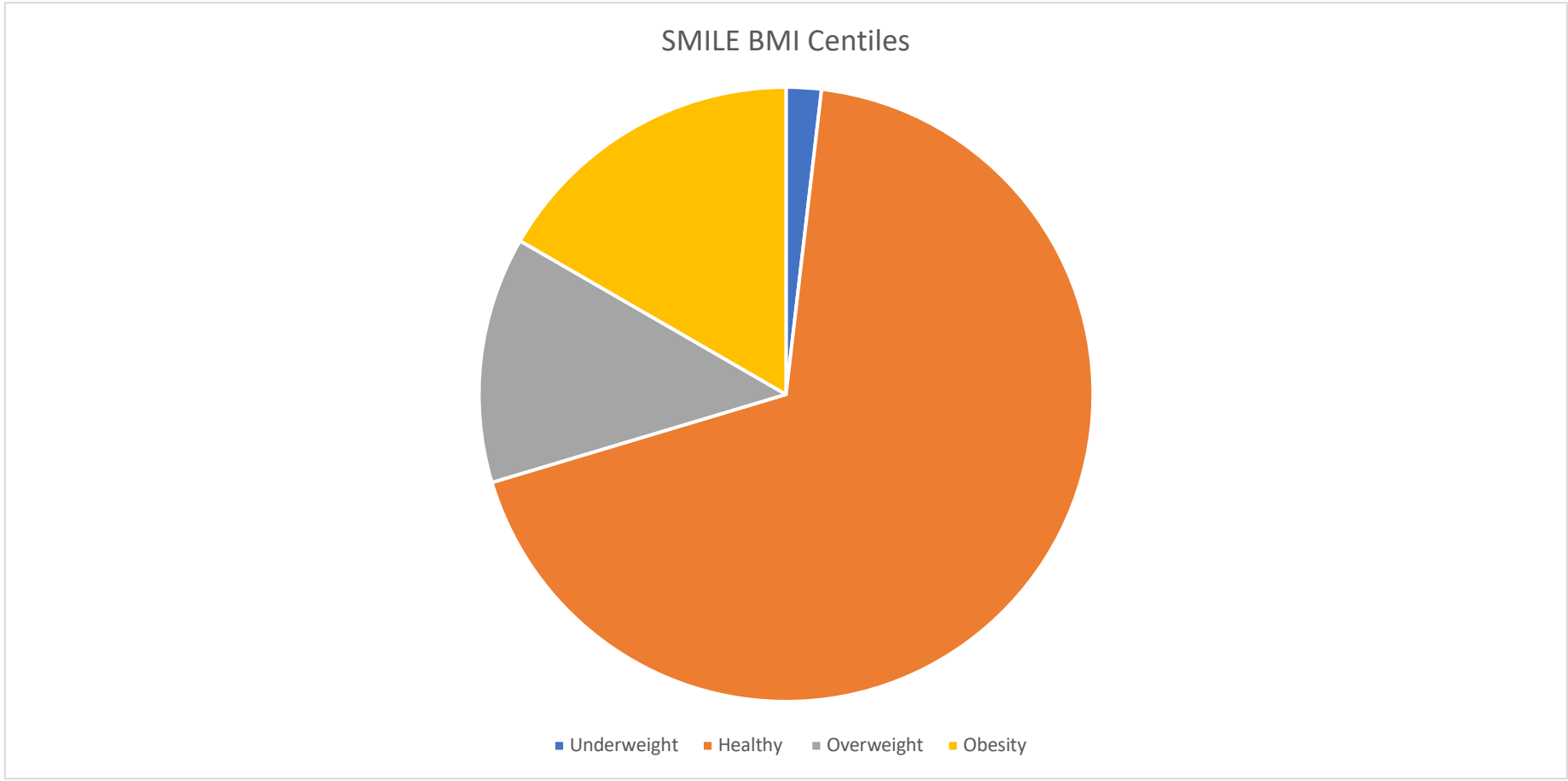


FIGURE 17: PIE CHART SHOWING PERCENTAGE OF PARTICIPANTS IN EACH BMI CATEGORY. 1.85% WERE UNDERWEIGHT, 68.5% WERE HEALTHY, 13% WERE OVERWEIGHT AND 16.7% WERE OBESE.

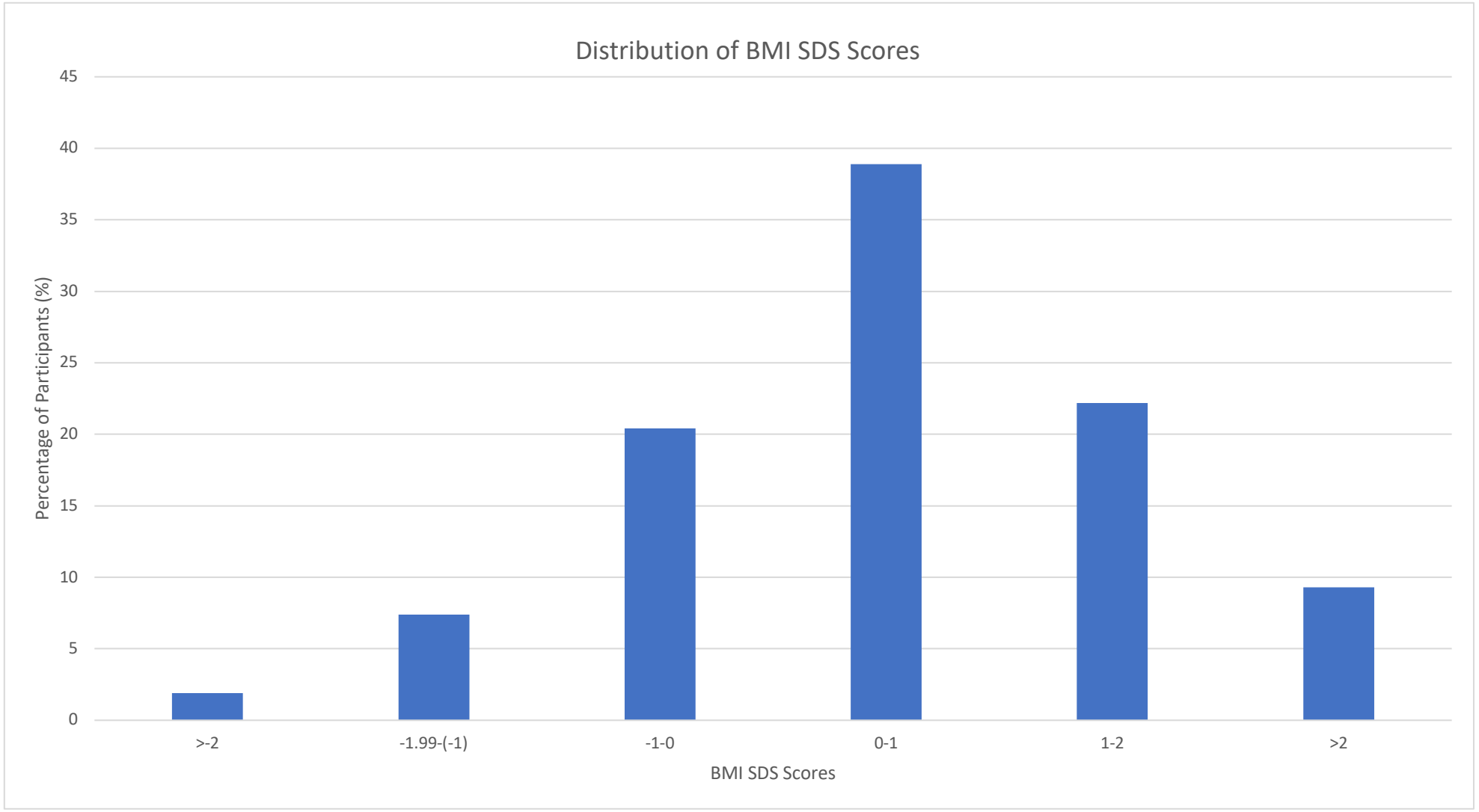


FIGURE 18: GRAPH SHOWING BMI SDS SCORES OF THE 54 SMILE PARTICIPANTS . 1.9% WERE <-2, 7.4% IN -1.99-(-1), 20.4% IN THE -0.99-0, 38.9% IN 0-1, 22.2% IN 1-2 AND 9.3% IN >2.

Blood Pressure

We calculated blood pressure centiles using the Age Based Paediatric Blood Pressure Reference Charts. (126) The mean systolic blood pressure centile was 66.2 mmHg and diastolic 44.3 mmHg, both considered within the normal limit. 83.3% of the children had a blood pressure below the 90th percentile, 5 children were between the 90-95th centile and 4 children were above the 95th centile, see Figure 19 below. All participants with blood pressure above 90th centile were discussed with a more senior clinician. We sent the below paragraph, in a letter, to all parents of these children.

“Thank you very much indeed taking part in the SMILE study.

You will remember that when came for the study visit, we measured XXX’s blood pressure. In young people, blood pressure is lower than it is in healthy adults. We work out whether it is normal or not by looking at the age of the person, their height and sex.
When we reviewed XXX's blood pressure readings, we found them to be just at the top of the range we consider to be normal for her age, height and sex. We often see that blood pressure is slightly high in patients coming to hospital, because of the stress of the hospital visit.
It would be sensible for XXX's blood pressure to be checked again at your GP surgery. If it is high on this measurement, we will make arrangements for you to be seen at Alder Hey.
Please can you make an appointment with the practice Nurse for a blood pressure measurement?
If you have any concerns about the information in this letter, please do not hesitate to contact me”

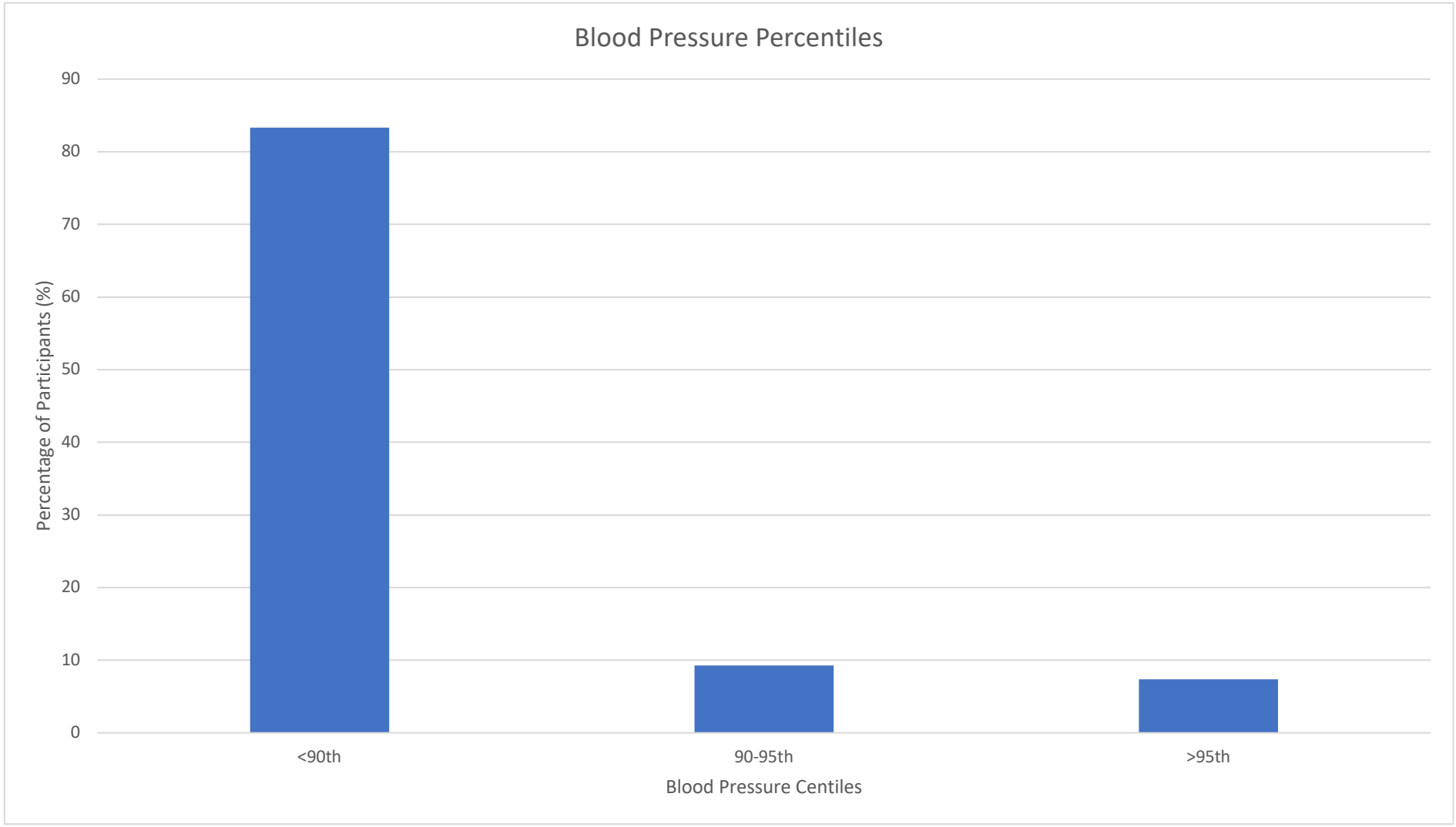


FIGURE 19: PERCENTAGE OF SMILE PARTICPANTS IN EACH BLOOD PRESSURE CENTILES . 83.3% <90TH, 9.3% IN 90-95TH AND 7.4% >95TH.

2.6.3 SALIVA SAMPLES

2.6.3.1 COLLECTION OF SALIVA SAMPLES

39 of the participants took all 7 of the Salivettes given to them, 9 took 6 samples and one participant only took 4 samples. 3 participants gave reason for being in bed for the last sample (TP7) which we can assume for the others who only took 6 as the timing of collection depended on the individual's awakening and bedtime. 2 of the participants stated that they dropped their final sample (TP7) on the ground and were therefore unable to use it. These were a female and male participants who were 5 and 7 years of age. We could assume that this was due to being of a young age as a reason for dropping their samples. One participant (14 years) only took 4 samples (TP4 taken at 3pm) but didn't give reason to why this was. We can assume that she may have not enjoyed taking the samples and decided to stop half-way through the collection.

The average wake up time of all 54 participants was 08:15am. Boys on average woke up earlier than girls at 07:42am with the earliest time of 05:50am and the latest of 11:00am. Girls on average woke up at 08:04am with the earliest waking up at 05:00am and the latest at 10:15am. The majority of the participants woke up by themselves naturally (79.6%), 9.6% got woken up by a parent, 9.6% woke up by an alarm and 1.9% got woken up by a sibling.

2.6.3.2 PARTICIPANTS STORAGE OF SAMPLES

The majority of the participants placed their saliva samples in the fridge within minutes of taking their saliva samples. One participant waited two hours to place one of her samples within the fridge which we can assume she forgot to do. Another participant put all 7 of their samples into the fridge at the same time at 20:40. The saliva samples had been sat at room temperature for 12 hours as they took their first sample at 08:24.

3. SMILE STUDY BIOCHEMICAL RESULTS

3.1 METHODS

363 samples from 54 participants were sent to the University Hospital of South Manchester in Wyenshawe for analysis. Prior to this the samples were centrifuged at 3000G for 10 minutes and then separated into 2ml tubes. The aliquots were then labelled with anonymized labels in a study folder. The samples were then stored at -80°C within the laboratory freezers at Alder Hey.

At Wythenshaw, samples were cleaned-up prior to analysis by protein precipitation using zinc sulphate and a methanolic internal standard to remove interfering substances and minimize matrix effects. Then the sample supernatant is then injected onto a C18 reverse phase chromatography column connected to the Waters TQS Micro tandem mass spectrometer with Waters Acquity i-Class HPLC.

Approximate transitions (m/z):

- cortisol 363>121 (qualifier 363>97)
- d4 cortisol 367>121 (internal standard)
- cortisone 361>163 (qualifier 361>145)
- d8 cortisone 369>169 (internal standard)

The LOQ for both analytes are 0.3 nmol/L, with intraassay CV of <=3.3 nmol/L for cortisol, <=3.2 nmol/L for cortisone and interassay CV of <=4.5% for cortisol and <=3% for cortisone at concentrations 5-150 nmol/L.

3.2 RESULTS

365 saliva samples were analysed at the University Hospital of South Manchester in Wyenshawe for cortisol and cortisone concentrations during the waking hours of our participants (TP1-TP7). 2 of the samples had insufficient volumes of saliva so were unable to be analysed.

24 hour measurements of cortisol, cortisone and cortisol: cortisone ratio presented for the whole population, are given in table 3.

	Mean concentration over 24 hours (nmol/L)	**Standard deviation concentration over 24 hours (nmol/L)**	**Median concentration over 24 hours (nmol/L)**
Cortisol	4.4	4.225	1.4
Cortisone	15.8	12.83	11.5
Cortisol: cortisone ratio	0.20	0.12477	0.125

TABLE 3: MEAN, STANDARD DEVIATION AND MEDIAN 24 HOUR MEASUREMENTS OF CORTISOL, CORTISONE AND CORTISOL-CORTISONE RATIO

The mean and standard deviation of cortisol at each time point can be found in Figure 20. The highest concentration was seen at TP1 which is when the participant awoke and then declined throughout the day to the lowest concentration at TP7. At TP1 the mean concentration of cortisol was 6.6nmol/L and at TP7 the mean concentration of cortisol was 0.3 nmol/L (+/- 2.04) and for cortisone it was 2.6nmol/L (+/-0.105). 15 of the samples went below detectable limits (<0.3nmol/L) and this occurred once at TP5, three times at TP6 and 11 times at TP7.

The mean and standard deviation of cortisone at each time point can be found in Figure 21. Like cortisol, the highest concentration was seen at TP1 and then declined throughout the day until TP5 where it increased and then to the lowest concentration at TP7. At TP1 the mean concentration of cortisone was 27.4 nmol/L (+/-1.19) and at TP7 the mean concentration of cortisone was 2.6 nmol/L and for cortisone it was 2.6nmol/L (+/-0.335). There isn't a steep decline in concentration throughout the day in comparison to cortisol. Salivary cortisone was detectable in all samples. The mean and standard deviation of cortisol: cortisone ratio can be found in Figure 22.

We analysed the mean and standard deviation of those who were normotensive (10th-90th), pre-hypertensive (90th-95th) and those who were hypertensive (>95th). The mean concentration of cortisol in the normotensive patients was 4.5 nmol/L and cortisone 16 nmol/L. The mean concentration of cortisol in those between the 90-95th percentile was 8 nmol/L and in cortisone 19.1 nmol/L. For the participants above the 95th percentile the mean cortisol concentration was 3.2 nmol/L and in cortisone 16.1 nmol/L. These results can be seen in Table 4.

Hormone	Whole Cohort (nmol/L) (n=54)	Normotensive (Blood pressure between 10th-90th centile) (nmol/L) (n=45)	Between the 90th-95th Centile (nmol/L) (n=5)	> 95th percentile (nmol/L) (n=4)
Cortisol	4.4 (4.25)	4.5 (4.07)	8.0 (7.84)	3.2 (3.1)
Cortisone	15.8 (12.8)	16.0 (11.69)	19.1 (16.84)	16.1 (13.2)
Cortisol: cortisone ratio	0.20 (0.125)	0.196 (0.105)	0.253 (0.187)	0.134 (0.082)

TABLE 4: TABLE SHOWS THE AVERAGE CORTISOL, CORTISONE AND CORTISOL-CORTISONE RATIO MEASUREMENT IN THE WHOLE COHORT (N=54), IN THE NORMOTENSIVE GROUP (N=45), IN THOSE BETWEEN 90-95TH CENTILE (N=5) AND IN >95TH (N=4).

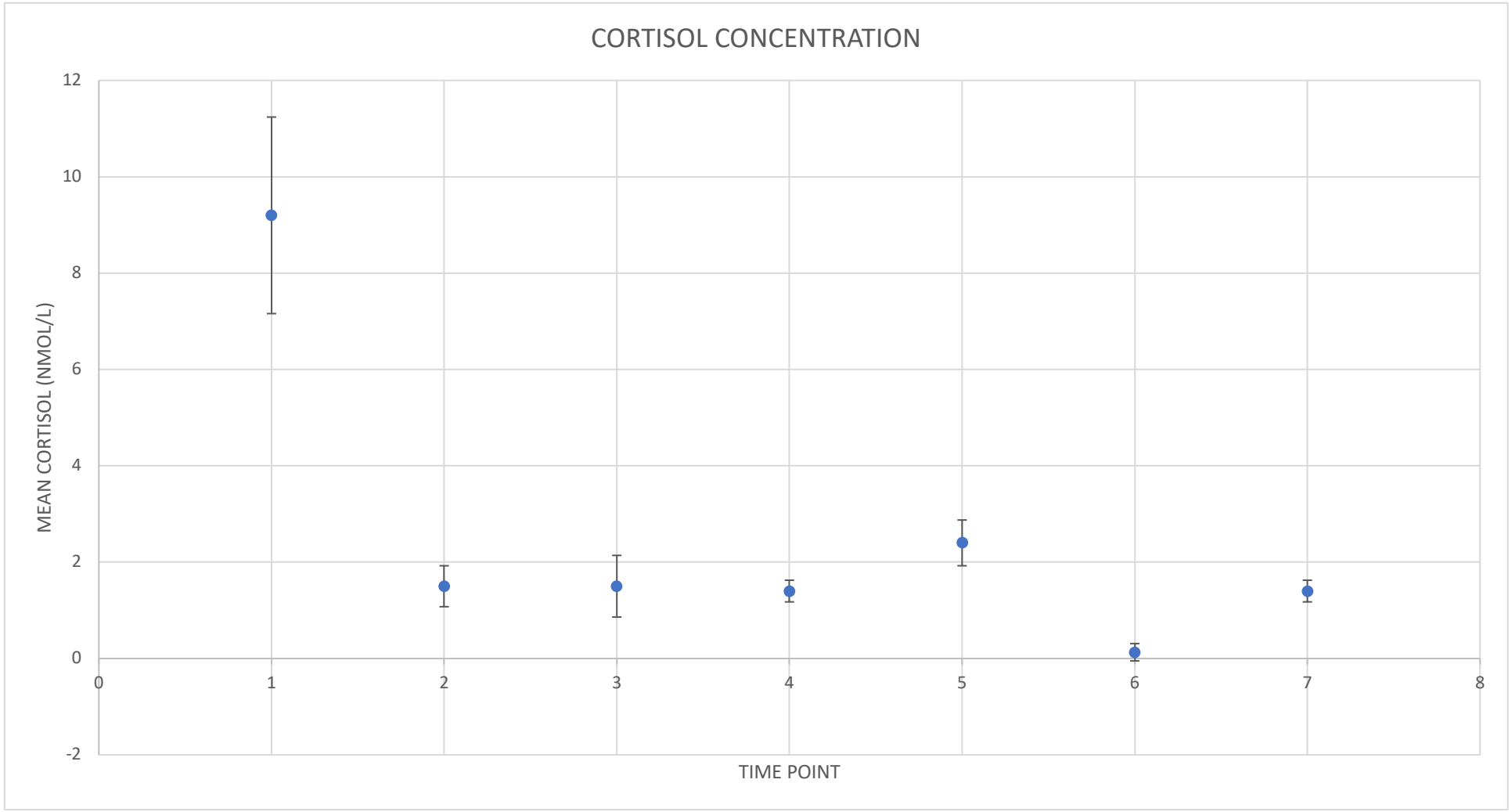


FIGURE 20: GRAPH SHOWING CORTISOL MEAN CONCENTRATIONS AT EACH TIME POINT (TP1-TP7) AND ONE STANDARD DEVIATION ABOVE AND BELOW

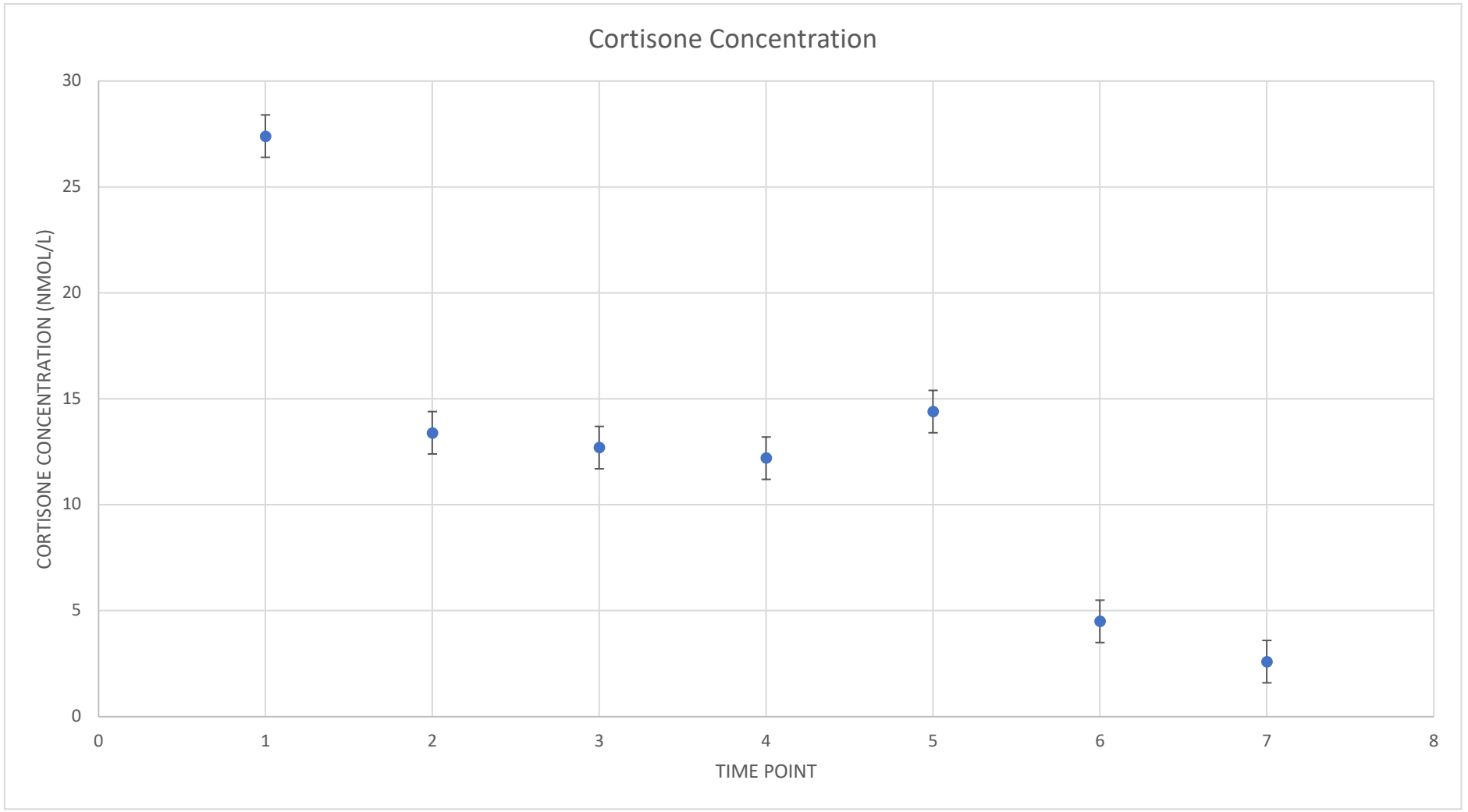


FIGURE 21: GRAPH SHOWING CORTISONE MEAN CONCENTRATIONS AT EACH TIME POINT (TP1-TP7) AND ONE STANDARD DEVIATION ABOVE AND BELOW

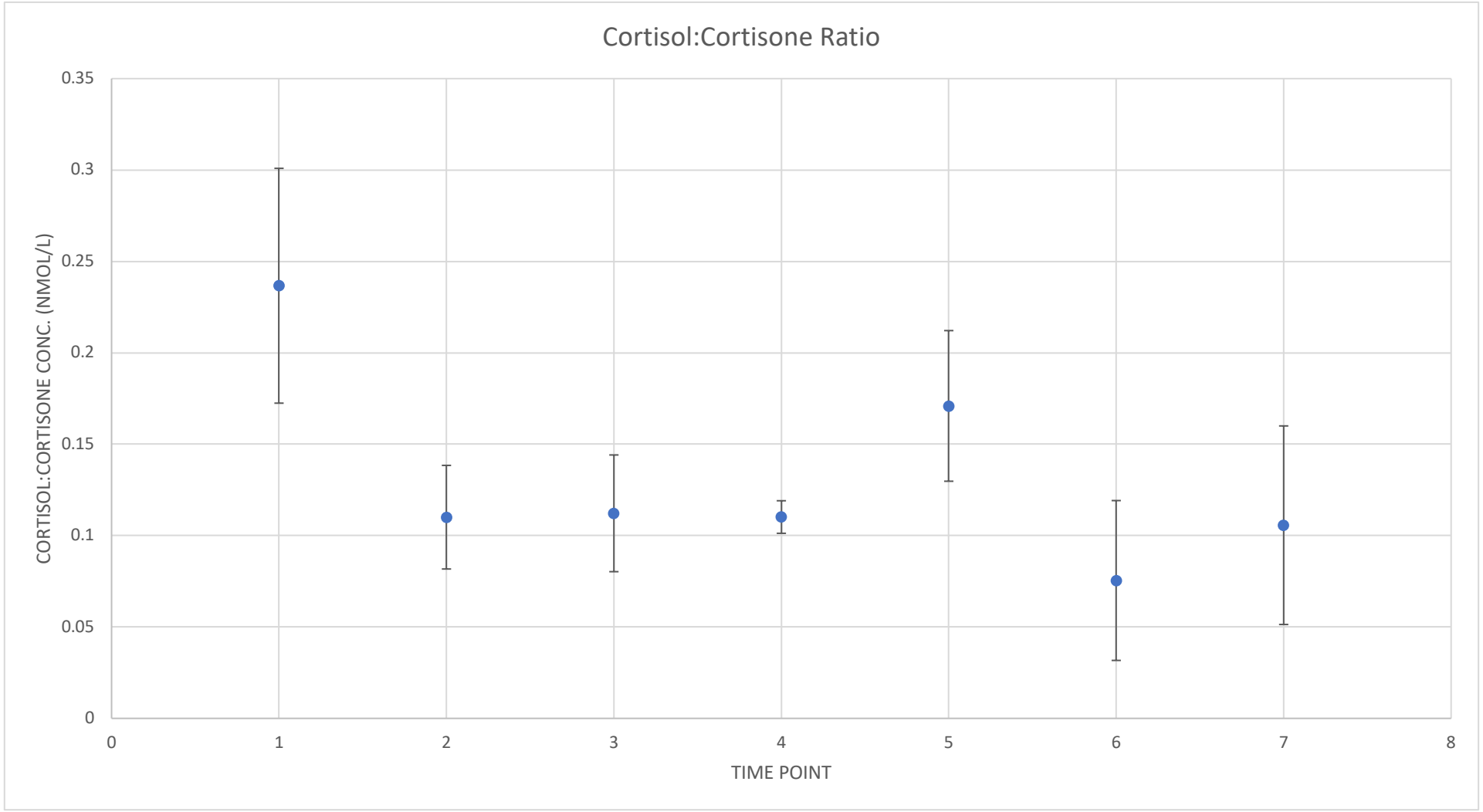


FIGURE 22: MEAN GRAPH SHOWING CORTISOL-TO-CORTISONE RATIO MEAN CONCENTRATIONS AT EACH TIME POINT (TP1-TP7) AND ONE STANDARD DEVIATION ABOVE AND BE

Linear regression was used to assess for an association between cortisol and cortisone concentrations and height SDS, BMI SDS, decimal age, sex and systolic blood pressure centiles. No clear relationships were found between the biomarker concentrations and demographic variables. However, looking at each scatter plots there seems to be a complete outlier. We believe this is due to TP1 being a lot higher than the other time points which is physiologically normal due to CAR, but it could be the reason for these outliers. For future analysis which is discussed in Section 3.3.3, we will exclude the TP1 concentrations to see if this changes the results to show a significant relationship. A Pearson coefficient test has been applied to each continuous variable and can be found on each graph. The scatter plots can be found below. For each dependent variable, there was no significant relationship found with any of the salivary biomarkers due to the Pearson's correlation being close to 0 for each.

Statistical advice was given by statistician Steven Lane. I plotted the scatter graphs of salivary biomarkers against demographic variables and carried out the Pearson correlation test for each. Steven Lane checked the scatter plots and gave advice on which correlation test to do.

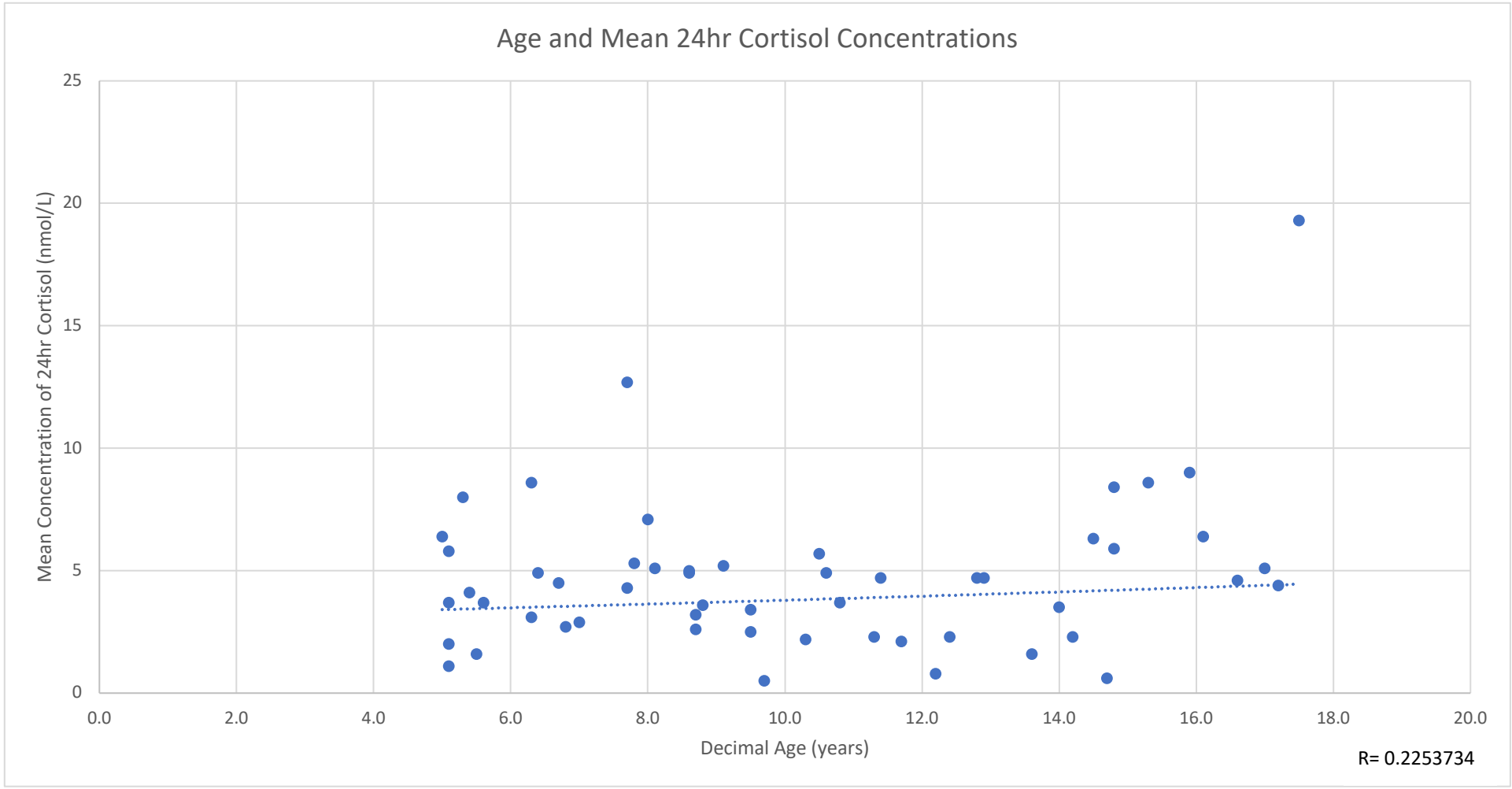


FIGURE 23: SCATTER PLOT SHOWING THE RELATIONSHIP BETWEEN DECIMAL AGE AND MEAN 24HR CORTISOL CONCENTRATION (TP1-TP7) OF THE SMILE PARTICIPANTS (R=PEARSON COEFFICIENT VALUE)

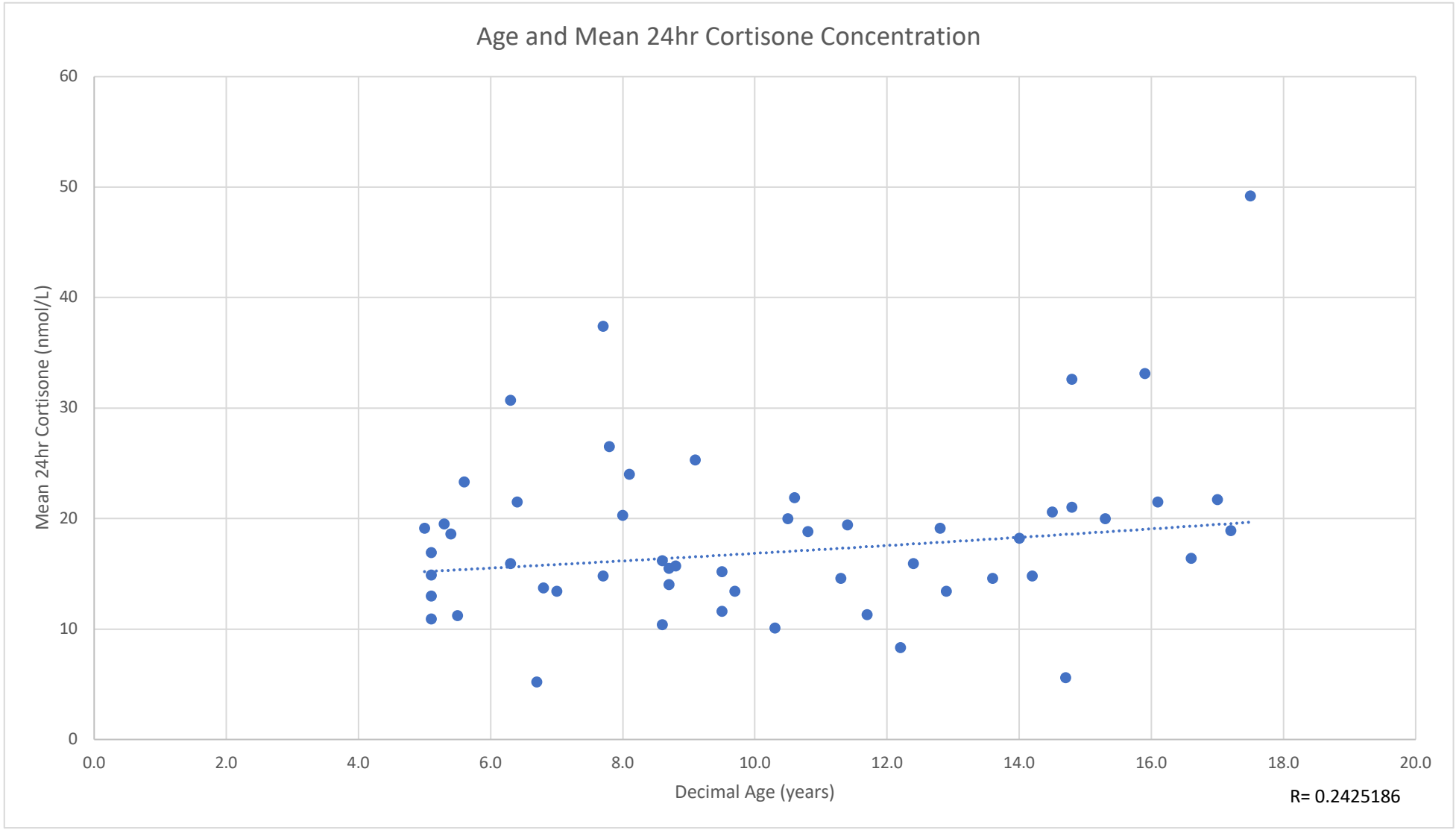


FIGURE 24: SCATTER PLOT SHOWING THE RELATIONSHIP BETWEEN DECIMAL AGE AND MEAN 24HR CORTISONE CONCENTRATION (TP1-TP7) OF THE SMILE PARTICIPANTS (R=PEARSON COEFFICIENT VALUE)

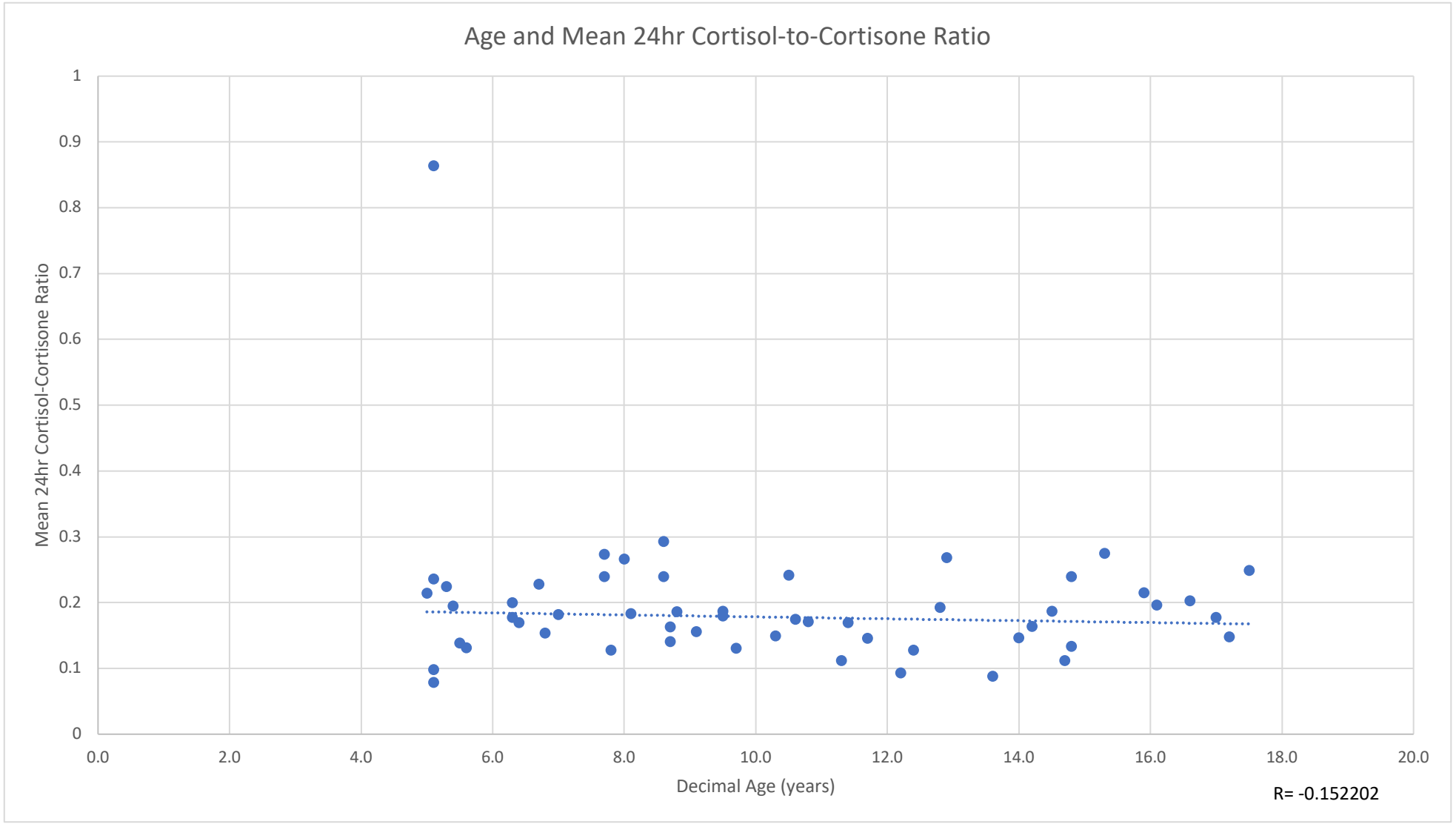


FIGURE 25: SCATTER PLOT SHOWING THE RELATIONSHIP BETWEEN DECIMAL AGE AND MEAN 24HR CORTISOL-TO CORTISONE RATIO (TP1-TP7) OF THE SMILE PARTICIPANTS (R=PEARSON COEFFICIENT VALUE)

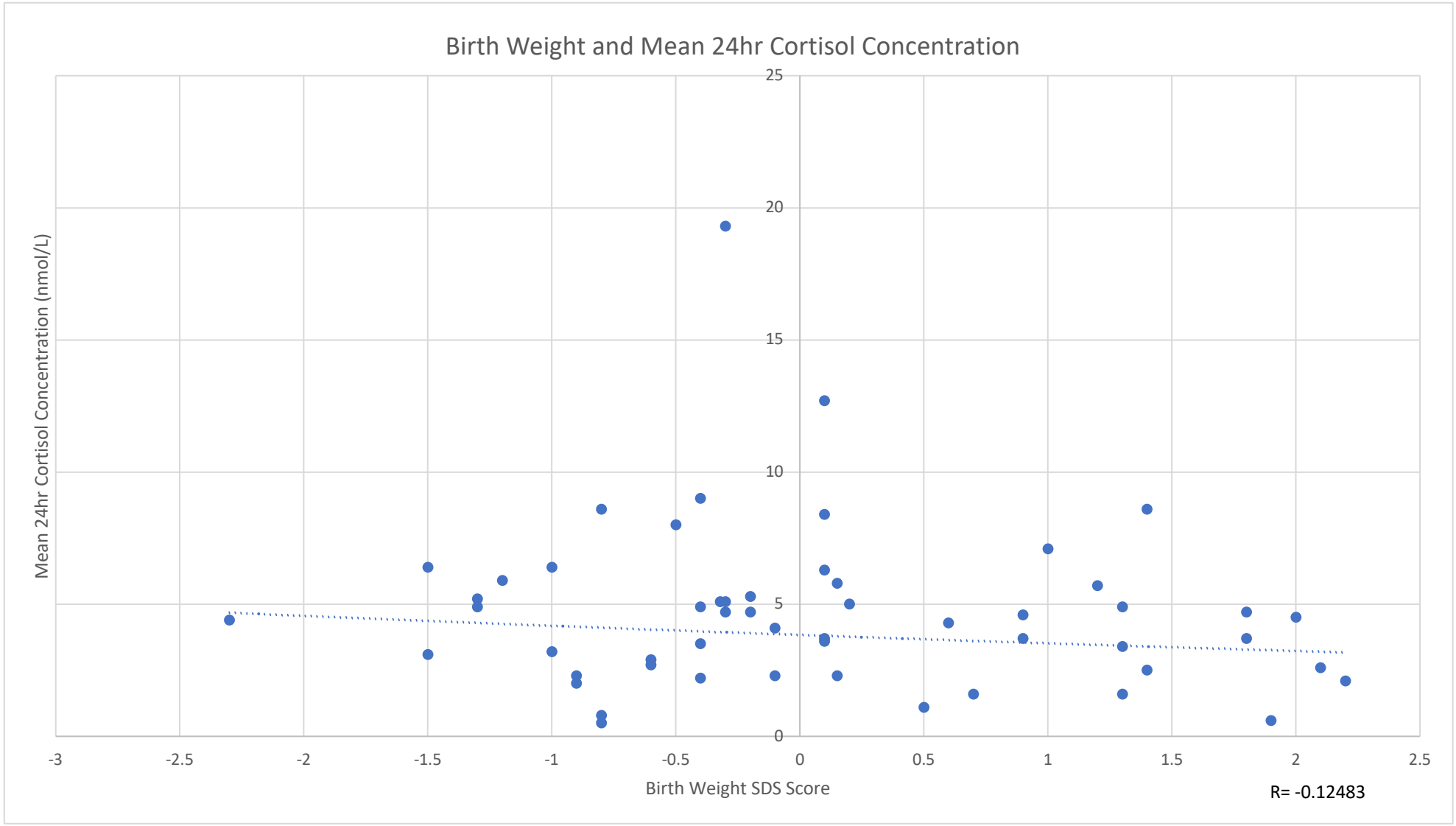


FIGURE 26: SCATTER PLOT SHOWING THE RELATIONSHIP BETWEEN BIRTH WEIGHT SDS AND MEAN 24HR CORTISOL CONCENTRATION (TP1-TP7) OF THE SMILE PARTICIPANTS (R=PEARSON COEFFICIENT VALUE)

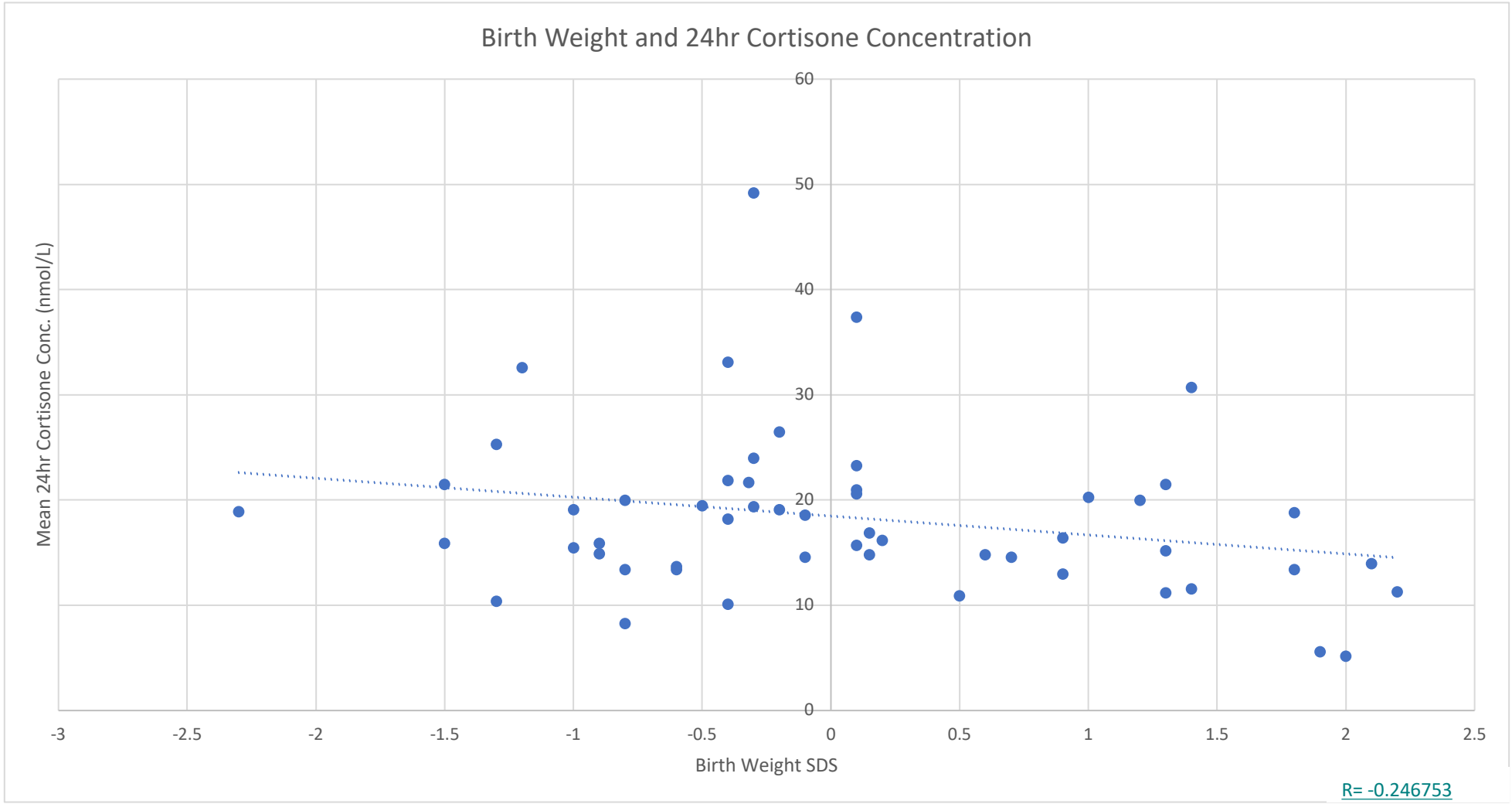


FIGURE 27: SCATTER PLOT SHOWING THE RELATIONSHIP BETWEEN BIRTH WEIGHT STANDARD DEVIATION SCORE AND MEAN 24HR CORTISONE CONCENTRATION (TP1-TP7) OF THE SMILE PARTICIPANTS (R=PEARSON COEFFICIENT VALUE)

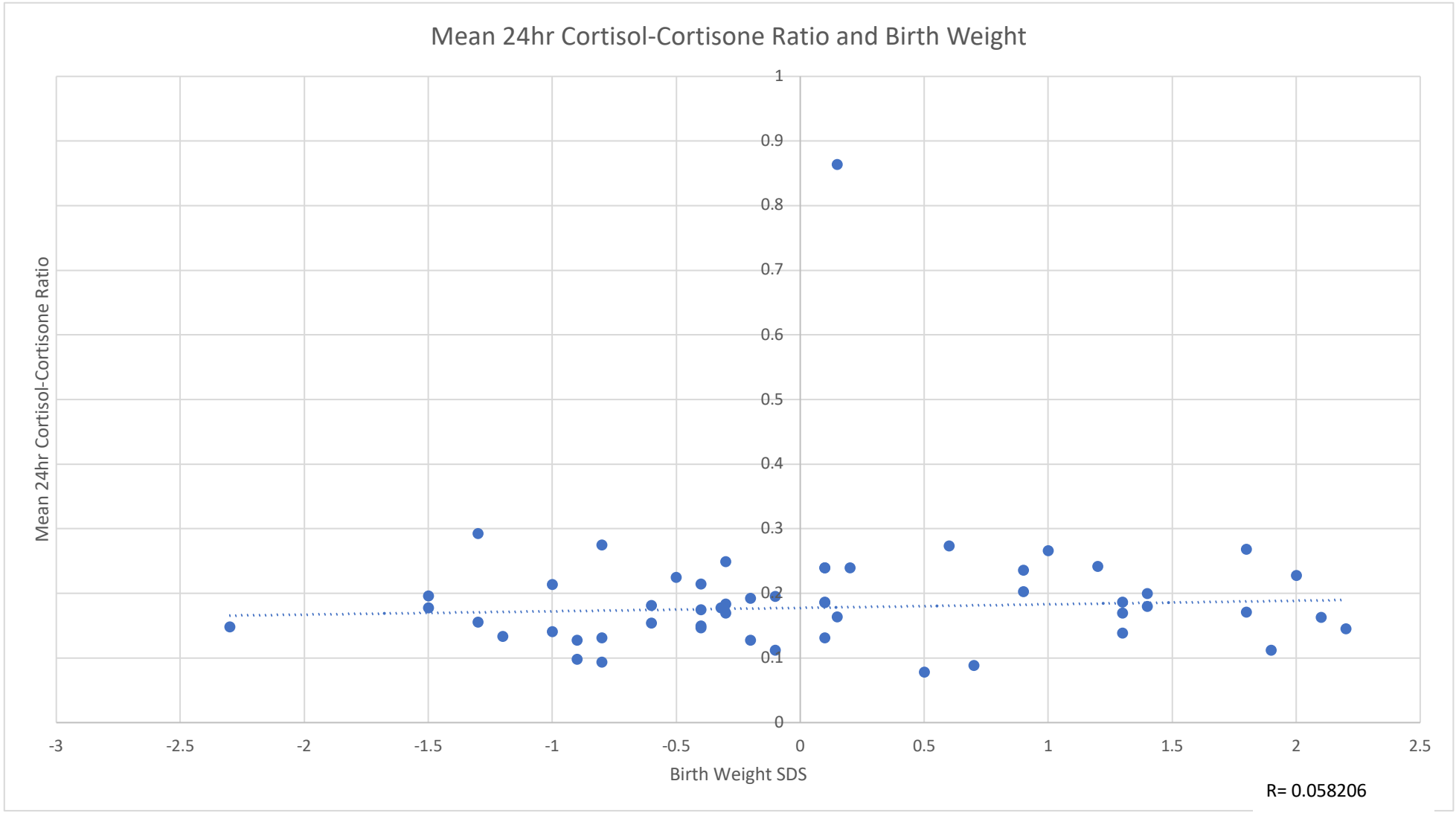


FIGURE 28: SCATTER PLOT SHOWING THE RELATIONSHIP BETWEEN BIRTH WEIGHT STANDARD DEVIATION SCORE AND MEAN 24HR CORTISOL-TO-CORTISONE RATIO (TP1-TP7) OF THE SMILE PARTICIPANTS (R=PEARSON COEFFICIENT VALUE)

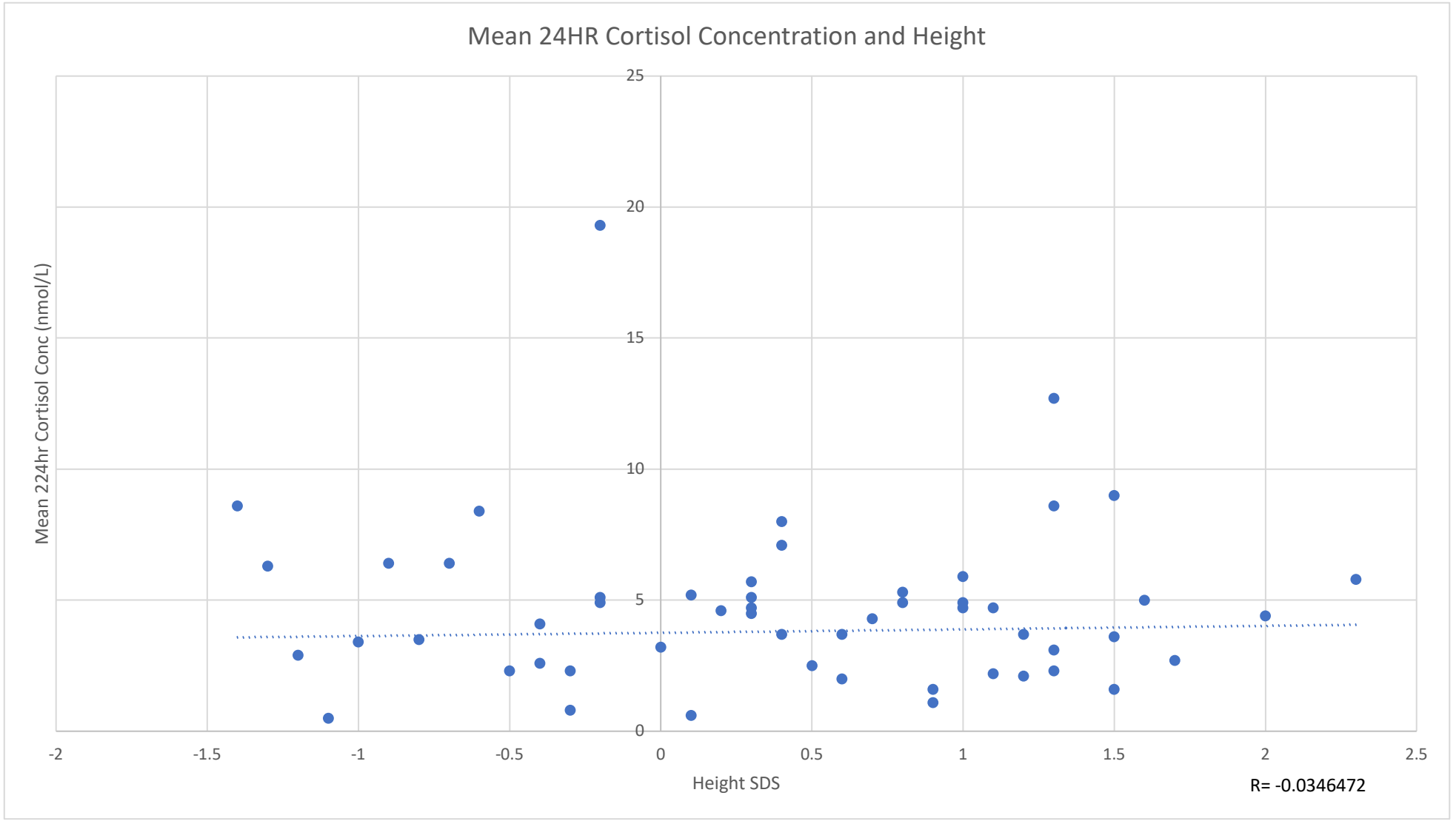


FIGURE 29: SCATTER PLOT SHOWING THE RELATIONSHIP BETWEEN HEIGHT STANDARD DEVIATION SCORE AND MEAN 24HR CORTISOL CONCENTRATION (TP1-TP7) OF THE SMILE PARTICIPANTS (R=PEARSON COEFFICIENT VALUE)

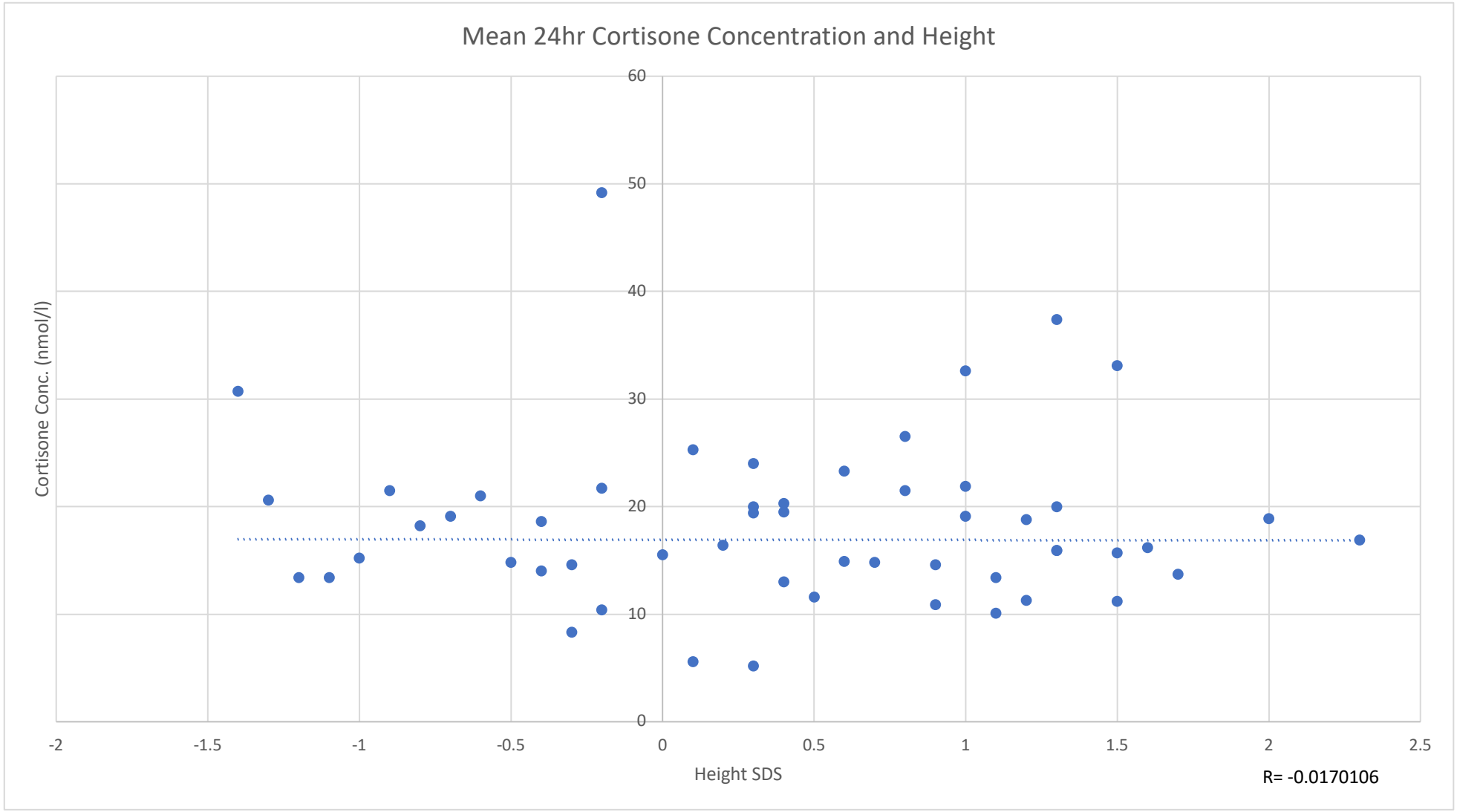


FIGURE 30: SCATTER PLOT SHOWING THE RELATIONSHIP BETWEEN HEIGHT STANDARD DEVIATION SCORE AND MEAN 24HR CORTISONE CONCENTRATION (TP1-TP7) OF THE SMILE PARTICIPANTS (R=PEARSON COEFFICIENT VALUE)

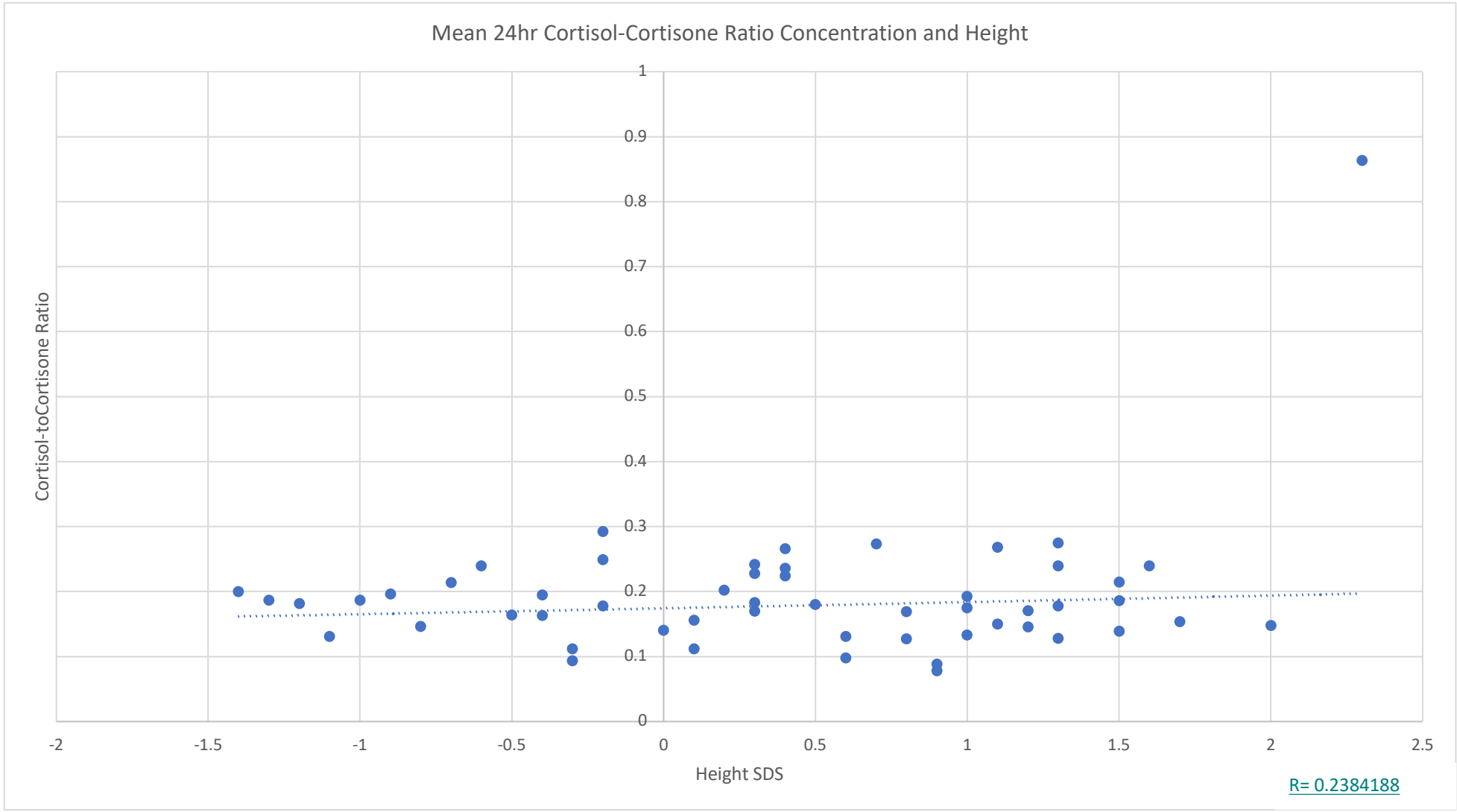


FIGURE 31: SCATTER PLOT SHOWING THE RELATIONSHIP BETWEEN HEIGHT STANDARD DEVIATION SCORE AND MEAN 24HR CORTISOL-TO-CORTISONE RATIO (TP1-TP7) OF THE SMILE PARTICIPANTS(R=PEARSON COEFFICIENT VALUE)

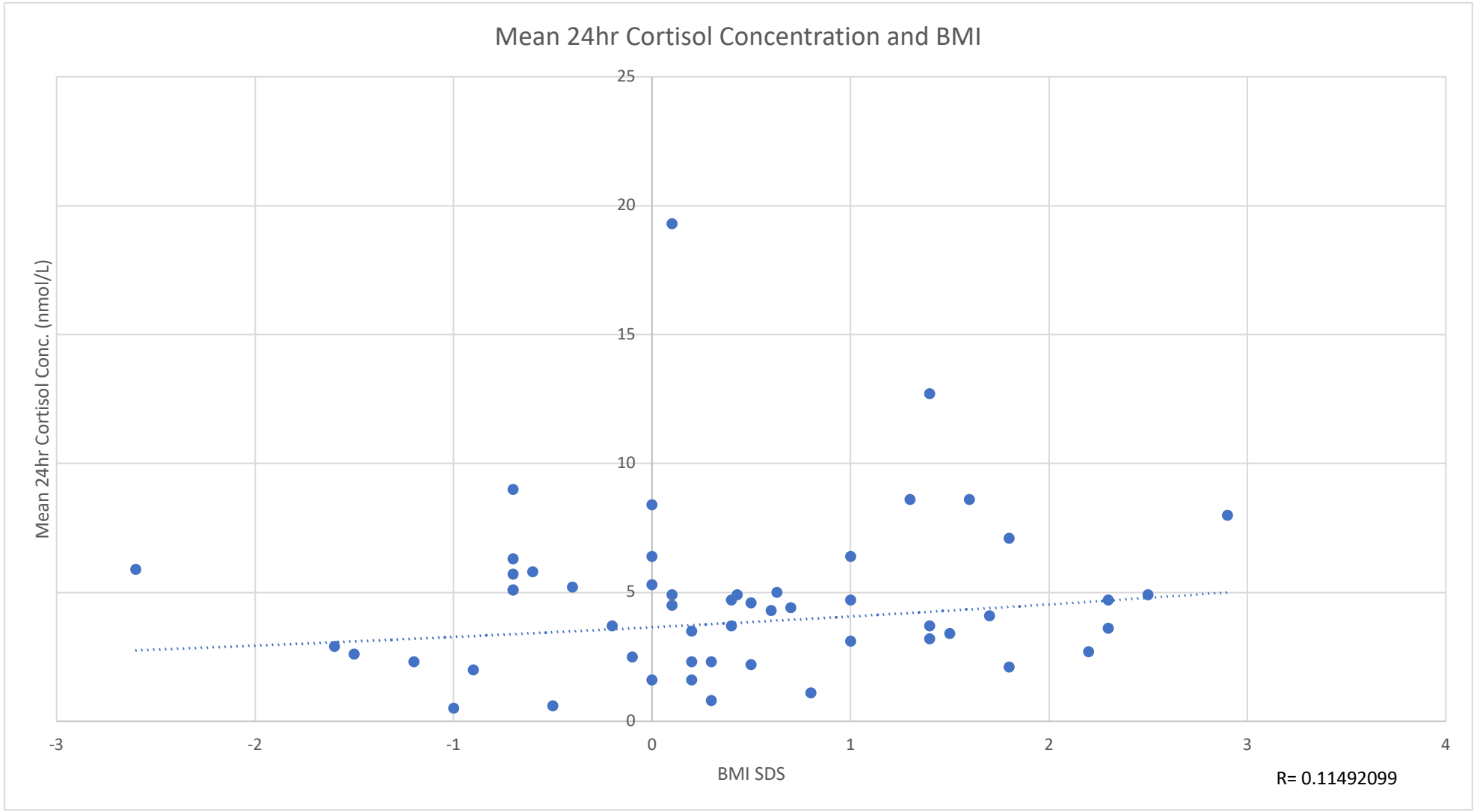


FIGURE 32: SCATTER PLOT SHOWING THE RELATIONSHIP BETWEEN BMI STANDARD DEVIATION SCORE AND MEAN 24HR CORTISOL CONCENTRATION (TP1-TP7) OF THE SMILE PARTICIPANTS (R=PEARSON COEFFICIENT VALUE)

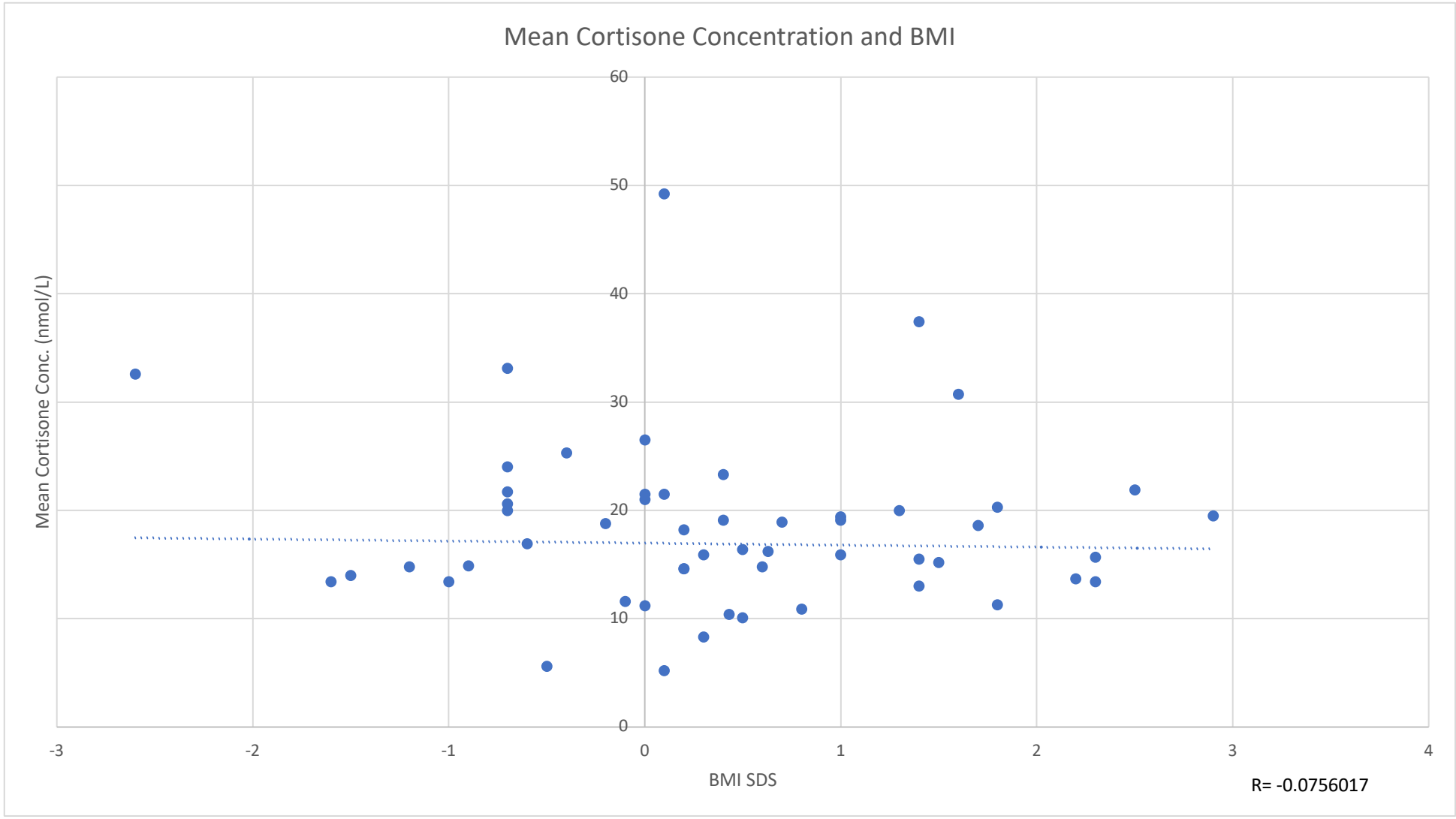


FIGURE 33: SCATTER PLOT SHOWING THE RELATIONSHIP BETWEEN BMI STANDARD DEVIATION SCORE AND MEAN 24HR CORTISONE CONCENTRATION (TP1-TP7) OF THE SMILE PARTICIPANTS(R=PEARSON COEFFICIENT VALUE)

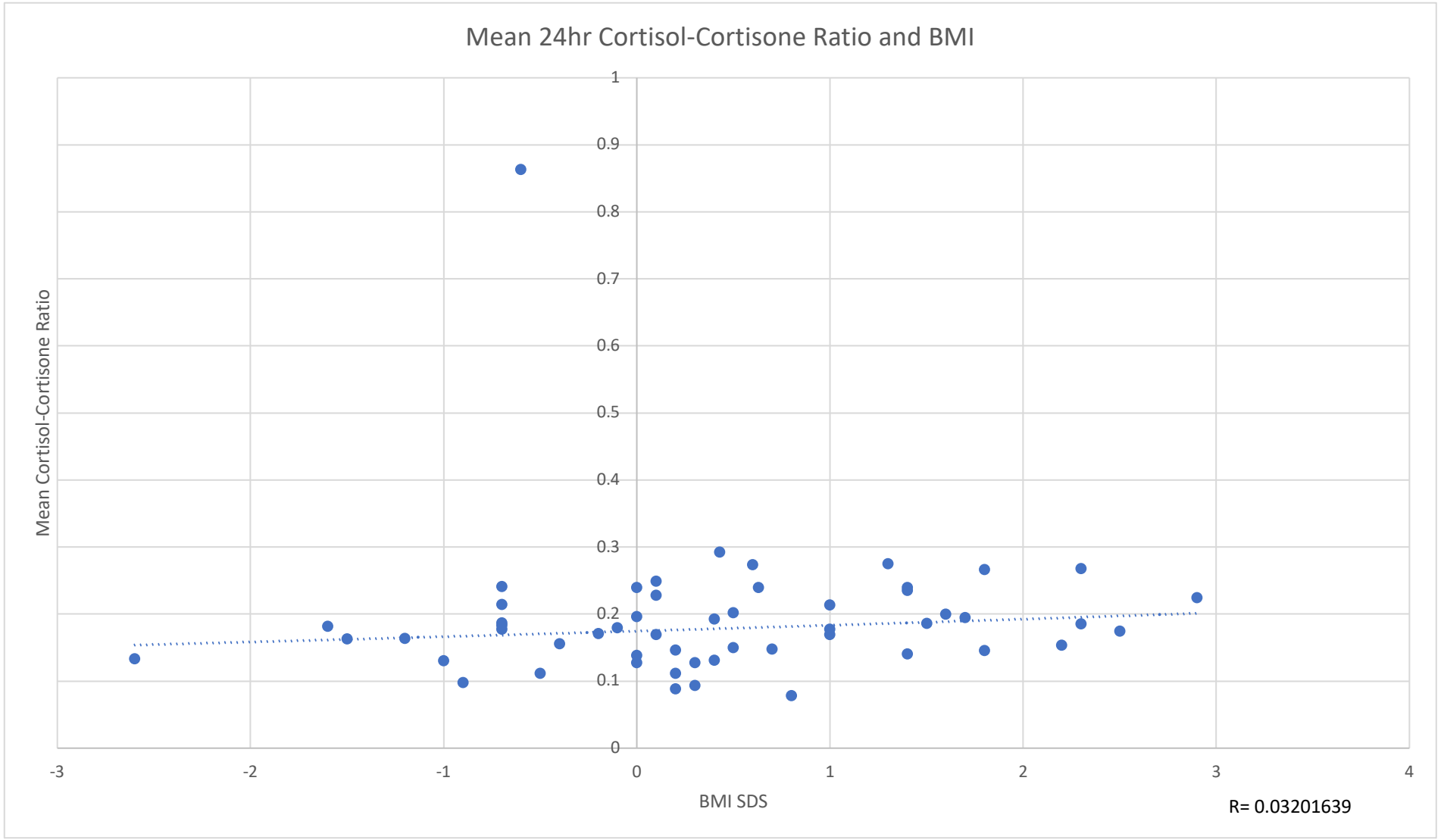


FIGURE 34: SCATTER PLOT SHOWING THE RELATIONSHIP BETWEEN BMI STANDARD DEVIATION SCORE AND MEAN 24HR CORTISOL-TO-CORTISONE RATIO (TP1-TP7) OF THE SMILE PARTICIPANTS (R=PEARSON COEFFICIENT VALUE)

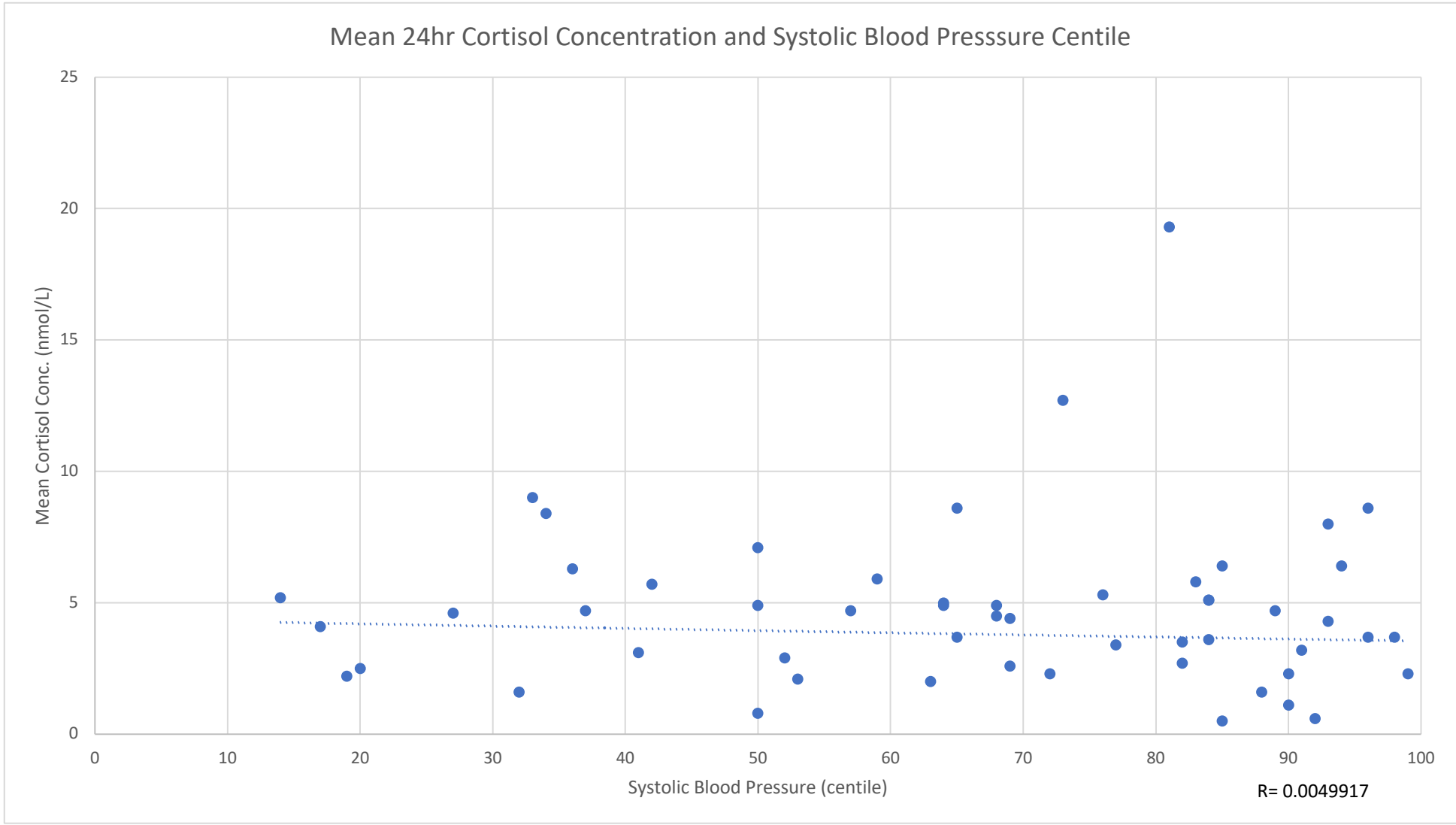


FIGURE 35: SCATTER PLOT SHOWING THE RELATIONSHIP BETWEEN SYSTOLIC BLOOD PRESSURE CENTILE AND MEAN 24HR CORTISOL CONCENTRATION (TP1-TP7) OF THE SMILE PARTICIPANTS (R=PEARSON COEFFICIENT VALUE)

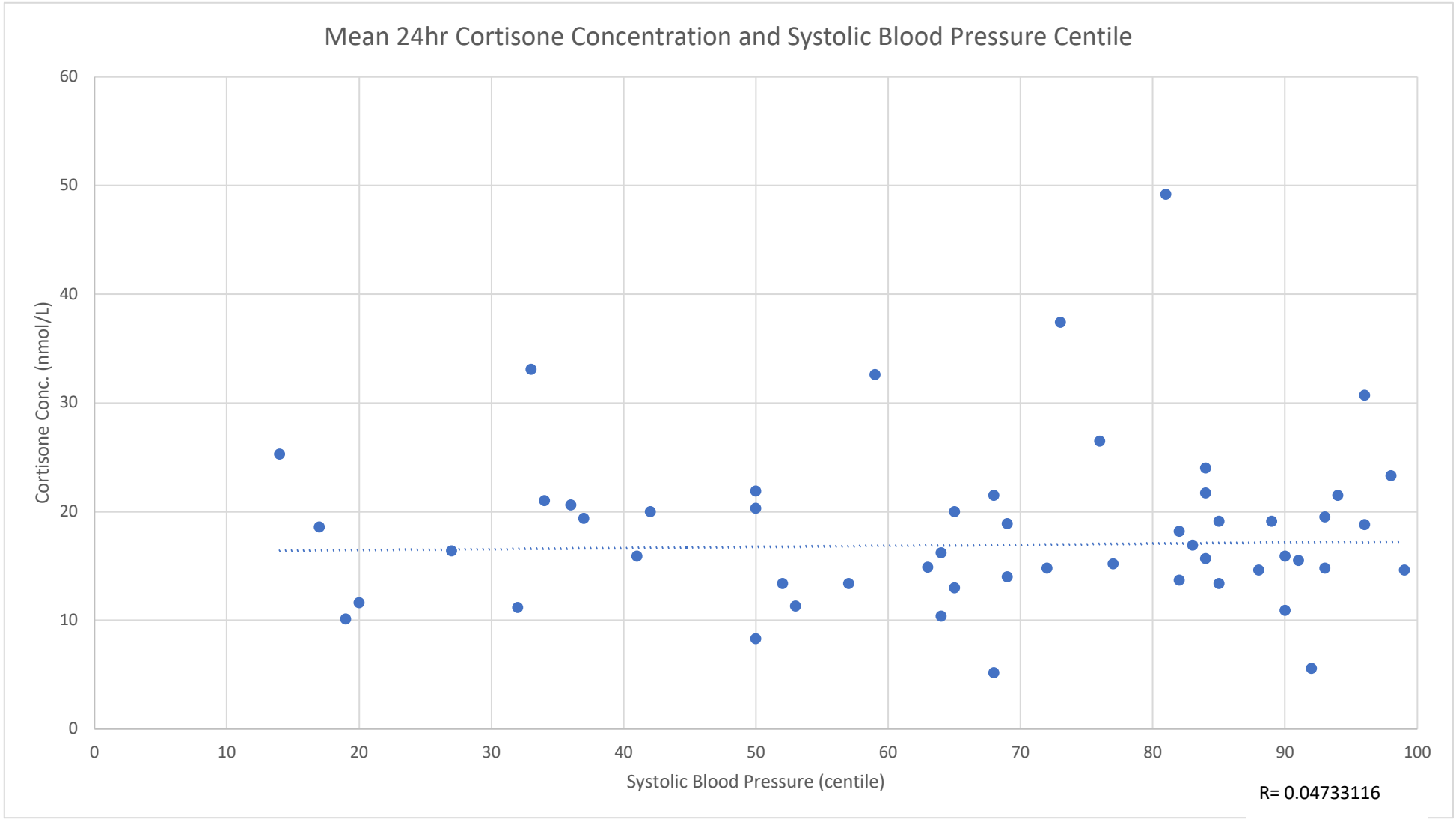


FIGURE 36: SCATTER PLOT SHOWING THE RELATIONSHIP BETWEEN SYSTOLIC BLOOD PRESSURE CENTILE AND MEAN 24HR CORTISONE CONCENTRATION (TP1-TP7) OF THE SMILE PARTICIPANTS (R=PEARSON COEFFICIENT VALUE)

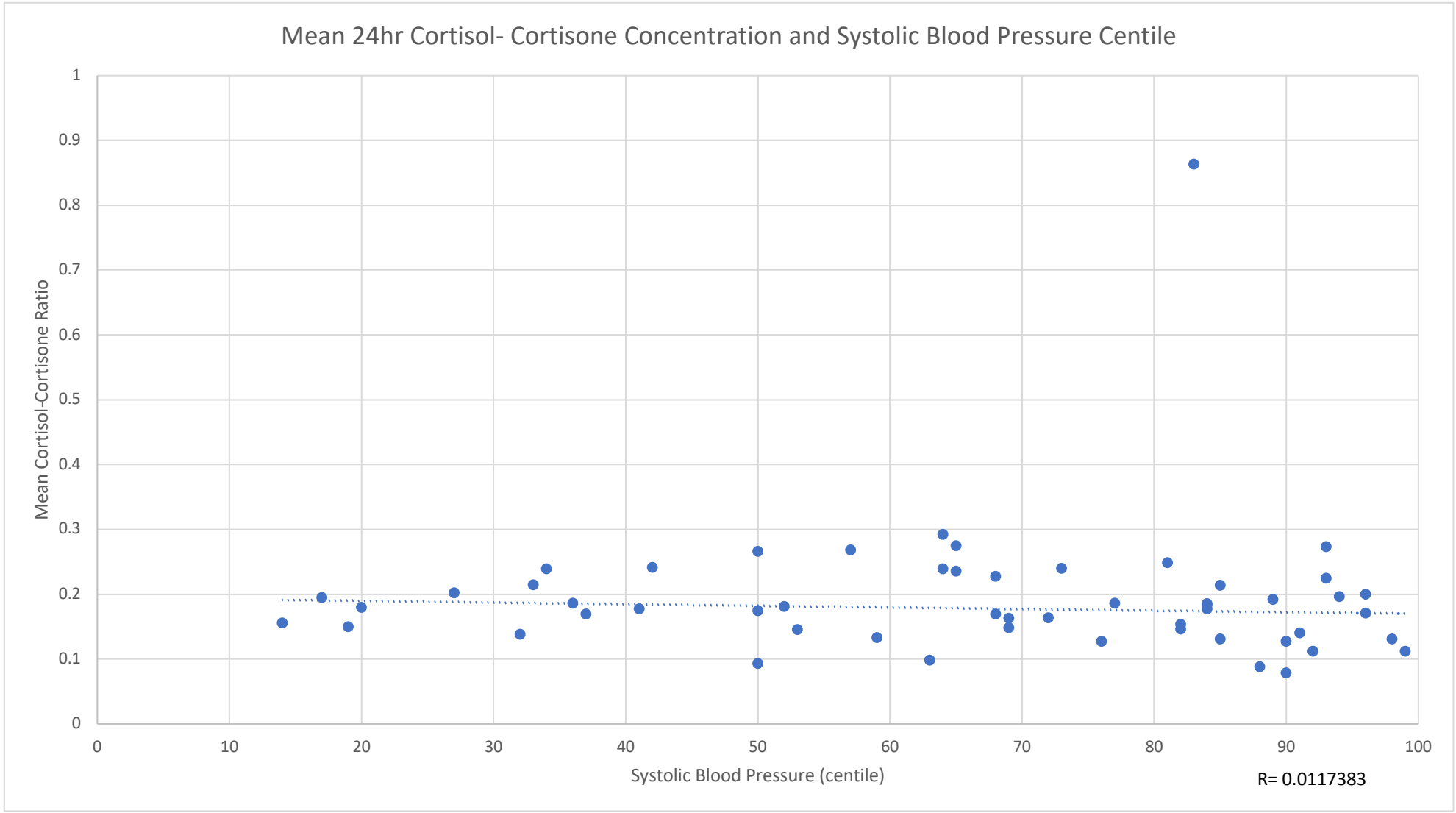


FIGURE 37: SCATTER PLOT SHOWING THE RELATIONSHIP BETWEEN SYSTOLIC BLOOD PRESSURE CENTILE AND MEAN 24HR CORTISOL-TO- CORTISONE RATIO (TP1-TP7) OF THE SMILE PARTICIPANTS (R=PEARSON COEFFICIENT VALUE)

To test the distribution of our dependent variable (salivary biomarker concentration) and gender, I used the Mann Whitney test. This test was a recommendation made by a statistician, Steven Lane. We hypothesised that there would be similar distribution between each salivary biomarker and gender. There was no significant difference between gender and 24hr salivary cortisol concentration (P=0.958), 24hr salivary cortisone concentration (P=0.720) and 24hr cortisol cortisone ratio (P=0.923).

3.3 DISCUSSION

The SMILE study is the first study to describe both cortisol and cortisone concentrations during waking hours in healthy children and young people. Even though there are clear advantages in the use of saliva, there was a lack of a knowledge regarding normal concentrations using current analytical methods and a validated reference range that could be used within clinical practice. This study has provided valuable initial data regarding the presence of a diurnal rhythm in salivary cortisol / cortisone similar to that seen in measurements made in serum.

This study obtained 365 salivary samples from 54 participants, two of which had insufficient volumes and were unable to be analysed. This is a small sample size in comparison to other studies that have been done in salivary cortisol in children and young adults(127,128) The participant population was mainly male (57.4%) in comparison to female (42.6%) and the age distribution was skewed towards the younger age groups which balances out the population from the pilor study which was mostly of the older ages(129).

Our results are similar to those in other paediatric studies which show that salivary cortisol follows a similar diurnal rhythm to serum(127,130,131). Both salivary cortisol and cortisone concentration levels were highest during the morning and then began to decrease throughout the day, to nadir during the evening. Similarly in the pilot study done by Professor Jo Blair, salivary cortisol became undetectable in 4.1% of the saliva samples, while salivary cortisone remained detectable in all the samples (129). Mean salivary cortisone was at least three times that of mean salivary cortisol concentrations. This makes sense due to the presence of 11βHSD type 2 enzyme within the salivary glands which is converting cortisol to inactive cortisone(61).

There was not a significant relationship between the salivary biomarkers and demographic variables we studied, in contrast to previous studies. Early morning cortisol levels have been associated with age but not to gender, ICS dose or treatment with alternate days of oral corticosteroids in children with asthma (132). Morning cortisol levels have been discovered to correlate with body weight and with the different pubertal stages irrespective of sampling time (133,134) Children with obesity have also been found to have significantly lower morning cortisol and higher evening cortisol (135). We recognise that this small sample size isn't sufficient to account for inter-variability between participants, so therefore we plan to continue recruitment to increase this number and analyse these relationships again.

Similarly, there was so significant difference between salivary biomarkers and gender. This contrasts with a study done by Netherton et al. which studied salivary cortisol levels in 129 normally developing participants aged 8-16 years. They found that there was a marked gender difference in morning salivary cortisol levels, girls being about 20-30% higher than boys(136) This was also found in adults subjects(137).

3.3.1 Strengths of SMILE

Study Design

The design and method of this study was influenced by a pilot study done by Professor Blair and Dr Hawcutt. This included one of the recruitment methods, saliva collection, some of the study materials and inclusion of participant's weight. They found that early morning serum cortisol correlated strongly with salivary cortisol and cortisone, $p<0.0001$ for both and serum cortisol, salivary cortisol and salivary cortisone correlated directly with age ($p<0.0001$, $p=0.002$ and $p=0.015$ respectively.(129) This was a good starting point in using salivary cortisol as a hormone measure in comparison to serum cortisol which is widely used in clinical practice. SMILE was then a good follow up to find a validated reference range to use in a younger population.

Conducting this study within Alder Hey Children's Hospital had many advantages, including the range of specialties of doctors and nurses who were very responsive and interested in telling their children about SMILE. Emailing the protocol through the Trust Website and through direct communication was an advantageous way of gaining interest. Alder Hey has a range of clinics available that I was able to attend and speak to parents and children about the study. In addition, having an extra member of staff working alongside me during patient recruitment was beneficial at the progression of the study. I believe that if I was working unaccompanied on recruitment, we would not have reached the goal of 50 in time. Despite a smaller period of recruitment than we wanted, reaching 54 participants during a global pandemic was an achievement. There was a worry that the children would not have liked the taste or feeling of the cotton wool when taking a sample, but we got great informal feedback from the parents and children saying that it was easy, efficient, and enjoyable. Only a very small number of samples were too small to be analysed. This further authenticates the use of saliva as a non-invasive method of measurement that is quick and easy in comparison to taking blood. Our results have shown that there is a clear diurnal rhythm present within saliva that mimics the rhythm within serum. This could mean less blood tests required in order to assess cortisol levels throughout the day which could be beneficial for those who are very scared of needles or those who require several tests. Time lost

from school and work would also be minimised and the sample results may be more representative of the patients' normal cortisol profile than those taken under the artificial environment of hospital.

In regard to existing literature, there is a paucity of data for the determination of a reference range which therefore further highlights the need for SMILE. However, there is research surrounding the relationship between cortisol and the demographic variables that we have studied as noted above. There is also evidence of the physiological diurnal rhythm of cortisol secretion and the CAR is present within children, which is a key finding to SMILE(107). It will be interesting to see, after further statistical analysis, how the SMILE cohort of participants' demographic variables relate to current literature.

3.3.2 LIMITATIONS OF SMILE

STUDY DESIGN AND RECRUITMENT

The method of recruiting patients either came from children of staff who worked at Alder Hey or siblings of outpatients from clinics within Alder Hey. Recruitment slowed down around March when we felt we had exhausted the list of doctors and nurses we could have used, and the rate of recruitment was decreasing. Furthermore, due to the COVID-19 pandemic we found it difficult to be able to sit in clinics as with many children's MDT teams there were already a number of members within the room. So due to restrictions we were unable to join. Instead, I sat in the waiting areas which I felt made interacting and building rapport with the parents and their children slightly more difficult. I believe widening patient recruitment to other hospitals within the Liverpool and Merseyside area or reaching out to local schools would have increased the number of recruits and allowed us to gain a larger sample size.

Allowing the children to carry out the study at home has many benefits but also a lot of limitations, including human error. Having taught how to take the saliva sample during their initial visit and giving instructions to them within their patient record booklet, we still are unsure whether or not they carried out the saliva sample collection correctly. There is also a high risk of contamination, especially in younger children. A small number of participants dropped at least one of their saliva samples and therefore we were unable to use it. The child could have potentially touched the cotton wool or ate something before therefore disrupting the sample. In future, I think the child should be monitored within a clinic setting to ensure that they are collecting their saliva correctly thus increasing sample result reliability. As our cortisol levels are heavily influenced by day-to-day stress such as getting upset, dealing with school or friends or hurting themselves, it is important to factor these into the end result. We did not ask the participants to record down any stressful things they encountered, therefore we are unsure if this caused an increase in cortisol levels during the afternoon or evening. Additionally, we didn't have set times for collection for each participant. However, we decided not to standardize the timepoints as we wanted to follow each participant's natural awakening diurnal rhythm that cortisol secretion follows.

3.3.3 COVID-19 PANDEMIC

During December and January there was uncertainty as to whether the project would continue because of the third national lockdown. There were around 60 studies at Alder Hey waiting to be activated and reviewed by an ethical board. There was a question mark over how we would recruit patients and whether or not patients were willing to make the trip to Alder Hey despite the risk of COVID-19. We did not commence recruitment until the end of December which worried the team whether we would reach our goal in time. Clinical appointments were still allowed to go ahead, and we received a great response from patients willing to come in and take part in the SMILE study. The SMILE team adhered to COVID hygiene guidelines and ensured both parents and child were happy throughout their visit. We obtained positive feedback from the children who seemed happy to come to Alder Hey for the day.

However, we did receive some negative response from participants who declined coming to the hospital which we felt was due to anxiety surrounding the pandemic. To combat this, we offered home visits or over the telephone consent to minimize the need to attend Alder Hey. Some participants people replied that their children did not want to take part due to having an uncomfortable experience with COVID swabs and did not like the idea of taking saliva samples throughout the day. Furthermore, there were two occasions where my housemate, who works as a physiotherapist, became positive with COVID, therefore causing our house to isolate. This meant I was unable to see patients for a two-week period thus slowing down the progression of recruitment. During this time the physician associate carried out any appointments made by potential participants which was a great help. Despite this I am very happy with the number of participants we recruited.

Overall, I feel that I still have had a great experience working on SMILE despite the drawbacks COVID has caused. We recruited the target amount needed for sample analysis. All the families that we met were very responsive and interested to take part which made the study much more enjoyable.

3.3.4 MEETING OUR AIMS/ FUTURE PLANS

Primary Aim

Our primary aim was to find a reference range for salivary cortisol and cortisone in healthy children and young people. Unfortunately, this has not been completed for a few reasons. Due to the COVID-19 pandemic, the recruitment period was shortened, and we did not reach the goal that we wanted to achieve. A larger cohort of children are needed in order to calculate this validated reference range, as it is one of the first calculated and there is limited knowledge surrounding variables that could potentially influence it. Therefore, SMILE will continue recruitment to reach the goal of 150 participants or more and this will be carried out by the Silothabo who was working with me. This would increase the number of samples to be analysed and further improve reliability and variability of our results. This set of results will also be combined with a set of 107 children from a previous pilot study done by Professor Blair and Dr. Hawcutt. Using a larger cohort of children will also allow a more varied population for the investigation into the associations between age, sex, BMI, blood pressure and height and salivary cortisol and cortisone.

Secondary Aims

Due to timing of submission of my thesis and returning to the MBChB programme, an in-depth statistical analysis has not been carried out and included within this thesis. We did investigate the relationship between salivary biomarkers and demographical parameters through linear regression which showed no significant relationship between these variables. However, there is a plan in place to carry out further tests to explore the secondary aims we set out including finding the area under the curve for salivary cortisol and cortisone. We will continue to receive advice from a university statistician (Steven Lane) at each stage of this plan for further recommendations.

Furthermore, we are still awaiting results which includes the adrenal androgens 17-hydroxyprogesterone, androstenedione, 11-hydroxyandrostenedione and 11-ketotestosterone. These results and analysis will be included within the research paper.

3.4 CONCLUSION

In conclusion, the research found within this study is a great starting point to discovering more about salivary cortisol and cortisone in healthy children and young people. The use of salivary cortisol as a non-invasive accessible method of measuring cortisol excess or deficiency in clinical practice looks promising and that it clearly follows the normal physiological diurnal rhythm of cortisol. However, we didn't find a significant relationship between salivary cortisol, cortisone and parameters such as sex, age, BMI, height or systolic blood pressure but this could be due to limited numbers. The next step would be to continue recruitment and gain a larger sample of people to further improve the validity of the results, so that we can calculate a reference interval.

4.0 SYSTEMATIC REVIEW

During the project, a protocol for a systematic review on the relationship between salivary cortisol and cortisone concentration and the ratio and high blood pressure was developed and can be found below. The search strategy was tested in four databases and the results found are detailed in the table below. An initial submission was accepted by PROSPERO and registered (PROSPERO 2020: CRD42021228670) and following examiner comments during the viva a revised protocol has been submitted and is pending . The initial submission can be found within Appendix 1.

4.1 BACKGROUND

Cortisol is the main glucocorticoid hormone that is released from the zona fasciculata of the adrenal cortex. It is tightly monitored by the activity of the hypothalamus-pituitary-adrenal axis in order to respond to the body's physiological demands and maintain its homeostasis (138) Cortisol is regulated by two isoforms of the enzyme 11β-hydroxysteroid dehydrogenase (11βHSD), a member of the short-chain dehydrogenase family, by conversion to the inactive glucocorticoid cortisone and back again (59). 11βHSD occurs in two forms, 11βHSD type 1 and 11βHSD type 2. The ratio of serum cortisol to cortisone is a marker of 11βHSD activity enzyme which can change in different settings including inflammatory conditions or following administration of glycyrrhetinic acid (inhibitor of 11βHSD2) found in liquorice (88). Mineralocorticoid receptors (MR) have equal affinity for cortisol and aldosterone however, cortisol may predominant binding as it normally circulates at levels 100-1000 times higher than those of aldosterone (60,89). Conversion to inactive cortisone protects the MR from high circulating levels of cortisol. However, in the absence, deficiency or saturation of this enzyme glucocorticoids activate the MR receptor thus mimicking the effects of aldosterone and increasing sympathetic activity within the brain (139). This can then cause increased renal sodium retention and intravascular volume expansion eventually leading to hypertension (60).

There has been evidence of change of 11βHSD activity during infancy and childhood. Due to increased cortisol secretion, there is a shift towards mineralocorticoid activity which may explain the physiologically rise in blood pressure seen during childhood. There is a theory that this may be due to altered activity of the 11βHSD isoenzymes; either type 2 (cortisol to cortisone) decreases or type 1 activity rises with age. (92)

Conventionally, the method that is used to measure cortisol in the body is total or free serum concentrations. (140) But salivary cortisol is becoming a more favourable method due to being a less invasive, stress free, cheaper method of measurement.(141) Salivary cortisol is measuring the free

biologically active form which passively diffuses through the parotid glands, in comparison with serum which is also including cortisol bound to cortisol binding globulin (CBG) or albumin. It is also found to be directly proportional to the free serum cortisol concentrations in both sexes and in women with elevated CBG. (140)

Current studies describing the relationship between salivary cortisol and cortisone and cortisol-to-cortisone ratio and hypertension are limited. Therefore, our aim is to systematically review relevant papers linked to salivary cortisol and cortisone and blood pressure.

4.1 AIMS

PRIMARY

To systematically review the literature examining the relationship between cortisol cortisone concentration and ratio in saliva and hypertension.

SECONDARY

To investigate any other comorbidities present, other patient parameters like BMI, age, causative lifestyle factors or concomitant medications.

4.2 METHODS

The protocol for this review was registered in the International Prospective Register of Systematic Review (PROSPERO 2020: CRD42021228670). See within Appendix The systematic review is reported in line with the Preferred Reporting Items for Systematic Reviews (PRISMA) guidelines.

4.2.1 ELIGIBILITY CRITERIA

Eligible studies contained patients, both children and adults who had their salivary cortisol and cortisone concentration measured (absolute values or ratio) as well as a blood pressure measurement. All types of study designs including cohort studies, case-control and randomised control trials regardless of language, publication year, publication type and publication status were considered for inclusion. Letters, editorials, commentaries and case reports were excluded.

4.2.2 SEARCH STRATEGY AND STUDY SELECTION

In November 2020, the following bibliographic databases were searched, PUBMED, MEDLINE, EMBASE and CINAHL without any date, language or age restrictions, for relevant studies. The primary author used the search strategy [‘high blood pressure OR ‘hypertension] AND [‘salivary cortisol’ OR ‘salivary cortisone’]. The primary author (OB) firstly screened the full titles of all identified studies and comparing to the eligibility criteria before reviewing the abstract and full text. This process was related by a secondary author (AT) in December 2020. Both authors (OB/AT) finalised the included studies.

References of the included papers were stored in an Excel spreadsheet and included each stage of screening as well as the reasons for exclusion (See in Table 8). All papers that reached the full-text screening were accessed and reviewed further, noting any reasons for exclusion in the table generated previously.

PAPER EXCLUSION METHOD

1) Review at title level

- Exclude if it does not study human participants
- Title clearly didn’t relate to primary outcome
- Exclude if study focuses on serum / urinary cortisol

2) Review at abstract level

- Exclude if it has no relevance to the systematic review i.e., no keywords relating to salivary cortisol or hypertension

3) Review at full publication level

Does the paper study a population that has both their salivary cortisol cortisone concentrations measured as well as their blood pressure – exclude if population not studied or unable to extract relevant data from study.

4.2.3 Data Extraction and Analysis

A) Data Extraction

Data was extracted independently by the primary author (OB) from the identified trials and entered into a separate Excel spreadsheet. Uncertainties encountered during data extraction were independently reviewed by a second author (DH). Extracted information included study setting; study population and participant demographics and baseline characteristics; aims of the study, total number of patients, inclusion and exclusion criteria, how saliva was sampled, number of samples taken, how saliva was analysed, details of the intervention and control conditions; study outcomes, and overall conclusions.

B) Quality Assessment

Independently, risk of bias was assessed by two researchers (OB/AT) and involved a third party assessment (DH) where consensuses couldn't be reached. Risk of bias was assessed independently by the primary author (OB) using the Newcastle-Ottawa Scale for evaluating the quality of nonrandomised studies in meta-analyses(142). Three factors are considered in scoring the quality of each included study, including selection (representativeness of the exposed cohort, selection of the non-exposed cohort, ascertainment of exposure and demonstration that at the start of the study the outcome of interest wasn't present), comparability (basis of study design and analysis and whether there was any confounding variables adjusted for) and outcome (follow up period and cohort retention). The quality of the studies (good, fair, poor) was rated by a score of awarding stars in each of the three domains. A "good" quality score required 3 or 4 stars in selection,1 or 2 stars in comparability, and 2 or 3 stars in outcomes. A "fair" quality score required 2 stars in selection, 1 or 2 stars in comparability, and 2 or 3 stars in outcomes. A "poor" quality score reflected 0or 1 star(s) in selection, or 0 stars in comparability, or 0 or 1 star(s) in outcomes. (Table 7)

There were no ethical issues as this was a systematic review of published papers.

4.4 RESULTS

PRISMA diagram showing the data identification and screening process. (Figure 38)

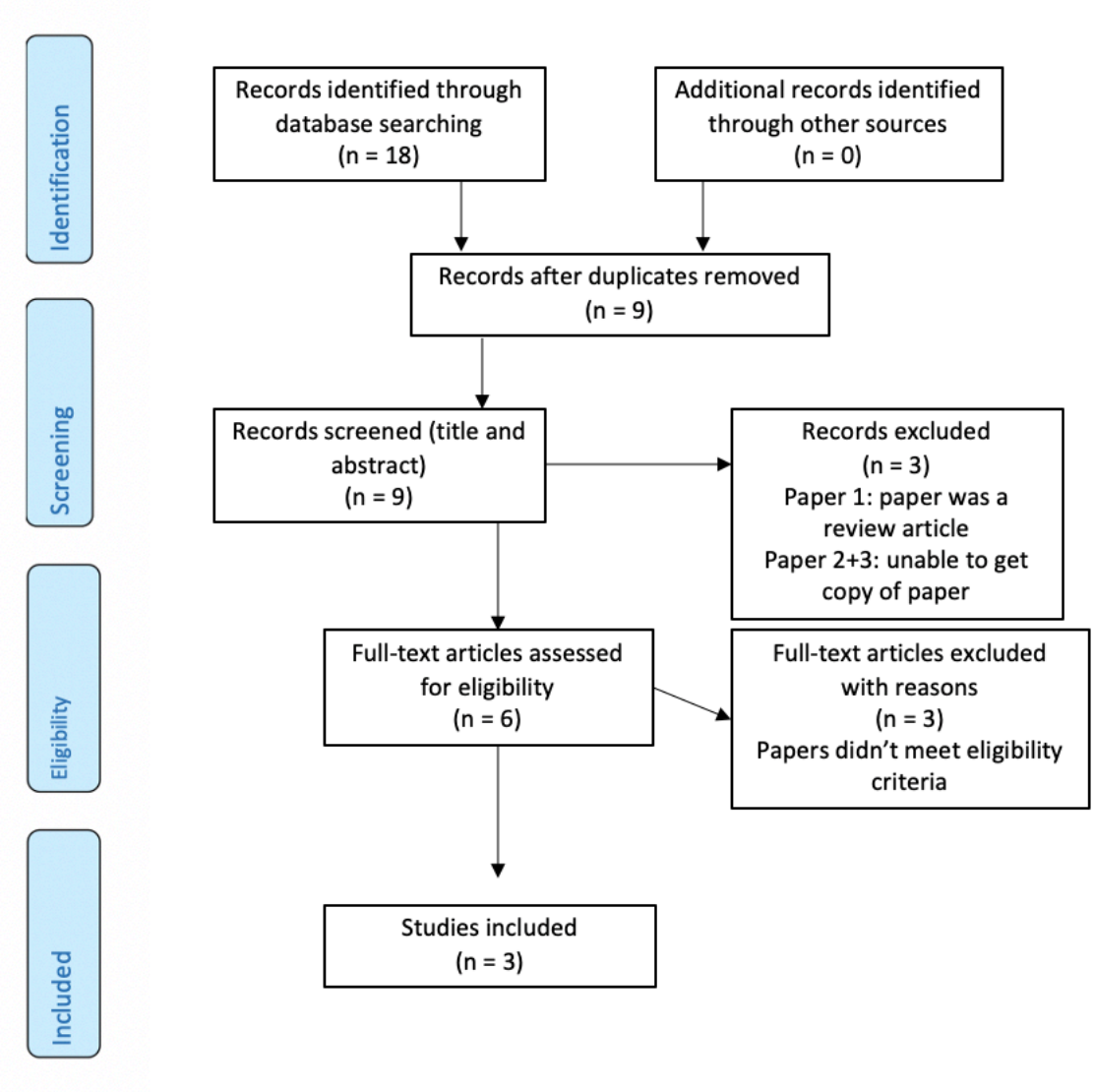


FIGURE 38: FIGURE SHOWING THE DATA IDENTIFICATION AND SCREENING PROCESS OF PAPERS FOUND THROUGH HDAS SEARCH. ORIGINALLY 18 PAPERS WERE FOUND, WHICH WHEN REMOVED OF DUPLICATES RESULTED IN 9 PAPERS. THESE WERE SCREENED BY TITLE AND ABSTRACT. 6 PAPERS WERE EXCLUDED AFTER NOT MEETING ELIGIBILITY. 3 PAPERS WERE INCLUDED IN THE FINAL SYSTEMATIC REVIEW

4.5.2 RESULTS TABLE

Name of Study	Authors	Date	Aim of Study	Total No. of Participants	How is Saliva Sampled/Analysed?	How was blood pressure taken?	Secondary Outcomes	Results
Association of adrenal steroids with hypertension and the metabolic syndrome in blacks(143)	Kidambi S.; Kotchen J.M.; Grim C.E.; Raff H.; Mao J.et al.	2007	To evaluate the hypothesis that adrenal steroids are associated with hypertension in blacks	97 (58 normotensive / 39 hypertensive	2 saliva samples (7am / 11pm) Enzyme immunoassay	Yes – measured over 24 hour period every 30 mins (6AM-8pm) then every 60 minutes (8PM-6AM)	BMI, triglycerides, cholesterol, insulin,	Higher late night and early morning salivary cortisol in hypertensive patients. Blood pressure correlated positively with late night salivary cortisol

Effect of glycyrrhetinic acid on 11beta-hydroxysteroid dehydrogenase activity in normotensive and hypertensive subjects(144)	Van Uum S.H.M.; Walker B.R.; Hermus A.R.M.M.; Sweep C.G.J.; Smits P. et al	2012	The effect of glycyrrhetinic acid on 11 beta-hydroxysteroid dehydrogenase activity in normotensive and hypertensive pts	40 (20 hypertensive 20 normotensive)	3 citric saliva samples (via salivette) (8:30am, 10am, 11am) RIA after prior extraction with organic solvents + paper chromatography	Yes – 1 BP pressure measurement taken	BMI	No differences between normotensive and hypertensive patients in at baseline salivary cortisol or after given glycyrrhetinic acid. No differences in clinical or laboratory parameters between the two groups apart from higher blood pressure in hypertensives.
Is there an association between cortisol and hypertension in overweight or obese children?(145)	Aleid JG Wirix1, Martijn JJ Finken2, Ines A von Rosenstiel-Jadoul3, Annemieke C Heijboer4, Jeroen Nauta5, Jaap W Groothoff6, Mai JM Chinapaw1, Joana E Kist-van Holthe1	2017	To investigate the association between cortisol parameters and hypertension in overweight or obese children.	126 50 hypertensive overweight 145 normotensive overweight 75 normotensive non-overweight	3 fasted saliva samples (salivette) Post awakening, between 6am-9am Prior to breakfast Isotope dilution LC-MS/MS	3 consecutive blood pressure measurements Electronic oscillometric blood pressure device	BMI	Salivary cortisol and cortisone levels, but not the salivary cortisol-to-cortisone ratio, were significantly lower in overweight or obese children than in non-overweight children. No significant differences between hypertensive and normotensive children Salivary cortisol levels were not significantly associated with systolic or diastolic blood pressure

TABLE 5: TBALE SHOWING RESULTS OF HDAS SEARCH. INCLUDES DATA EXTRACTION OF NAME OF STUDY, AUTHORS, AIM OF STUDY, HOW SALIVA WAS TAKEN AND ANALYSED, HOW BLOOD PRESSURE WAS TAKEN AND OVERALL CONCLUSIONS OF EACH STUDY

Study	Title	Reason for Exclusion
Ceccato et al(146) (2016)	Cushing Syndrome: Screening and Diagnosis	It is a review article
Tulloh et al (147) (2020)	Cortisol/ cortisone levels and quality of life in individuals with pulmonary arterial hypertension	Didn't take a blood pressure measurement
Ceccato et al (148) (2018)	Daily salivary cortisol and cortisone rhythm in patients with adrenal incidentaloma	Didn't take a blood pressure measurement.
Bachega T.A. et al (2017)	A case of AME responsible to dexamethasone caused by a novel HSD11B2 mutation	Unable to get a copy of the paper- conference abstract
Schäffer L et al. (2010)	Altered stress physiology in neonates with diabetic macrosomia	Unable to get a copy of the paper
Schäffer L et al. (149) (2009)	Blunted stress response in small for gestational age neonates	Didn't take a blood pressure measurement

TABLE 6: PAPERS THAT WERE EXCLUDED AFTER ANALYSIS OF FULL TITLE OR ABSTRACT. REASONS FOR EXCLUSION HAVE BEEN GIVEN.

Study	Representativeness of Exposed Cohort	Selection of the Non-Exposed Cohort from Same Source as Exposed Cohort	Ascertainment of Exposure	Outcome of Interest was not Present at Start of Study	Comparability of Cohorts	Assessment of Outcome	Follow up Long Enough for Outcome to Occur	Adequacy of Follow-Up	Quality Score
Kidambi et al. (2007)	Participants weren't representative as they included an enriched population of African Americans	Yes *	Designation of hypertension was based on screening systolic blood pressure >140 mmHg or diastolic >90 mmHg or taking antihypertensive medication*	Yes *	Multiple linear regression carried out for HOMA insulin resistance and other traits of metabolic syndrome adjusting for age and gender *	Independent blind assessment*	Yes*	Complete follow up *	Good
Walker et al. (2012)	20 of the hypertensive patients were taken from the community 6 were from outpatients*	Yes *	two occasions; Designation of hypertension was based on screening systolic blood pressure >160 mmHg or diastolic >90 mmHg or taking antihypertensive medication	Yes *	Differences in response to glycyrrhetinic acid and placebo between two groups assessed with repeated-measurements ANOVA *	Independent blind assessment*	Yes*	Complete follow up *	Good
Wirix et al (2017)	Participants were of a convenience sample so unsure to whether this was a best representation of the community at that time * somewhat average of 5-17 years old	Yes *	Medical reports *	Yes *	Differences in characteristics between hypertensive overweight and obese children, normotensive overweight and obese children, and normotensive non-overweight children	Independent blind assessment*	Yes*	Complete follow up *	Good

					were tested with ANOVA and post-hoc t-tests. Linear regression analysis was used to test associations with cortisol parameters between children with and without hypertension, adjusted for BMI SDS. **				

TABLE 7: TABLE SHOWING RESULTS FROM QUALITY ASSESSMENT OF INCLUDED PAPERS USING THE NEWCASTLE OTTAWA SCALE. THE QUALITY OF THE STUDIES (GOOD, FAIR, POOR) WAS RATED BY A SCORE OF AWARDING STARS IN EACH OF THE THREE DOMAINS. A "GOOD" QUALITY SCORE REQUIRED 3 OR 4 STARS IN SELECTION,1 OR 2 STARS IN COMPARABILITY, AND 2 OR 3 STARS IN OUTCOMES. A "FAIR" QUALITY SCORE REQUIRED 2 STARS IN SELECTION, 1 OR 2 STARS IN COMPARABILITY, AND 2 OR 3 STARS IN OUTCOMES. A "POOR" QUALITY SCORE REFLECTED 0OR 1 STAR(S) IN SELECTION, OR 0 STARS IN COMPARABILITY, OR 0 OR 1 STAR(S) IN OUTCOMES

Description of the Included Studies

There were 9 results after removal of duplicates, through the use of the search strategy, only 3 papers fit the eligibility criteria (Figure 25) studies were carried out in the Netherlands and the third was carried out in the US. Overall, 263 patients were used, 137 (52.1%) were adults and 126 (47.9%) were paediatric patients. Further characteristics of each study are presented in Table 7.

Salivary Sample Collection

See table 8 below on how each study differed in collection of salivary cortisol and cortisone. All studies took a similar number of salivary samples, all of which took a sample early in the morning which we can assume captured the cortisol awakening response. Only Kidambi et al. took a sample at 11pm at night whereas the others were during the morning hours. Both Walker et al. and Wirix et al. used Salivette devices which are saliva collection devices consisting of a plastic tube containing a cotton swab to chew on. Kidambi et al didn't specify their method of collection. All three studies used different forms of salivary analysis including enzyme immunoassay (143), radioimmunoassay (144) and isotope dilution liquid chromatography with tandem mass spectrometry (LC-MS/MS)(150)

Study ID	Number of Saliva Samples	Timing of Samples	How is saliva collected?	How is saliva analysed?
Kidambi et al. 2006	2	11pm then 7am the next morning	Not mentioned	Enzyme immunoassay
Walker et al. 2002	4	Day before experiment, 8am, 9:30am, 10:30am	Citric acid stimulated samples via Salivettes	RIA after prior extraction with organic solvents + paper chromatography
Wirix et al. 2017	3	Post-awakening, between 6-9am, prior to breakfast	Salivettes	Isotope dilution LC-MS/MS

TABLE 8: SALIVA COLLECTION METHOD IN EACH OF THE THREE INCLUDED STUDIES INCLUDING THE NUMBER OF SAMPLES TAKEN, THE TIMEPOINTS OF EACH SAMPLE, HOW IT WAS COLLECTED BY EACH PATIENT AND METHOD OF SALIVARY ANALYSIS

Kidambi et al.(143) only analysed saliva for cortisol concentration, Walker et al.(144) measured a cortisol cortisone ratio and Wirix et al. (150) measured all three (cortisol cortisone concentration and ratio). Kidambi et al.(143) found that late night salivary ($p<0.01$) and early morning cortisol ($p<0.03$) levels were higher in hypertensive patients compared to normotensive (PM salivary (1.2nmol/ vs 1.8 nmol/L) AM Salivary (11.2nmol/l vs 14.4nmol/l). Walker et al.(144) did not find any difference in concentrations of cortisol/ cortisone in normotensive and hypertensive patients at baseline and after administration of glycyrrhetinic acid. Wirix et al. found that salivary cortisol and cortisone levels, but

not cortisol-to-cortisone ratio, were significantly lower in the overweight or obese children than in non-overweight. Salivary cortisol, cortisone and cortisol-to-cortisone ratio results for Wirix et al. can be found in Table 9.

Hormone	Hypertensive Overweight Children (n=50)	Normotensive Overweight Children (n=145)	Normotensive Non-overweight children (n=75)
Cortisol Median Concentration (nmol/L)	5.0	5.9	7.4
Cortisone Median Concentration (nmol/L)	22.0	23.5	28.0
Cortisol-to-cortisone ratio Median Concentration (nmol/L)	0.24	0.26	0.27

TABLE 9: TABLE SHOWS MEDIAN CONCENTRATION OF CORTISOL, CORTISONE AND THE CORTISOL-TO-CORTISONE RATIO IN THE HYPERTENSIVE OVERWEIGHT, NORMOTENSIVE OVERWEIGHT AND NORMOTENSIVE NON-WEIGHT CHILDREN.

Blood Pressure Measurement

Each study differed in how they measured blood pressure, see table 10 below. Kidambi et al. measured blood pressure using an ambulatory blood pressure monitor over 24 hours taking measurements at intervals of 30 minutes from 8am-6pm and every hour from 6pm-8am. That totals to 30 blood pressure measurements taken over 24 hours. Whereas Walker et al. cannulated the brachial artery with 20 gauge catheter and took only one measurement at 8am. Overall, on average Walker's et al. patients were slightly more hypertensive than Kidambi et al (159/107 mmHg / 146/88 mmHg).

Study ID	**How was blood pressure measured?**	**Number of Measurements**	**Mean Morning Blood Pressure mmHg (Normotensive) (SD)**	**Mean Morning Blood Pressure mmHg (Hypertensive) (1SD)**
Kidambi et Al. 2006	Using Accutracker (ambulatory blood pressure monitor)	Measured over 24 hour period every 30 mins (6AM-8pm) then every 60 minutes (8PM-6AM)	116/70 (+/- 0.5/0.4)	146/88 (+/-0.8/0.6)
Walker et Al. 2002	Cannulation of brachial artery using a 20 gauge catheter	One measurement at 8am	Systolic: 124 +9 Diastolic:76 +7	Systolic: 159 +16 Diastolic: 101+8
Wirix et al. 2017	Electronic oscillometric blood pressure device	3 consecutive measurements	Systolic (SDS): 1.98 Diastolic (SDS): 1.03	Overweight : Systolic (SDS): 0.5 Diastolic (SDS): 1.03

				Non-overweight Systolic (SDS): 0.11 Diastolic (SDS): -0.09

TABLE 10: BLOOD PRESSURE MEASUREMENT METHOD IN EACH STUDY AND THE AVERAGE BLOOD PRESSURE BETWEEN HYPERTENSIVE AND NORMOTENSIVE. WIRIX ET AL. GAVE BLOOD PRESSURE IN A STANDARD DEVIATION SCORE

LINKING SALIVARY CORTISOL TO HYPERTENSION

Kidambi et al. found that salivary cortisol correlated positively with blood pressure. However, Walker et al. did not find any difference in concentrations of cortisol/ cortisone in normotensive and hypertensive patients at baseline and after administration of glycyrrhetinic acid. Walker et al. didn't provide cortisol values in the results. Similarly, Wirix et al. found no significant association between salivary cortisol and systolic or diastolic blood pressure SDS.

All three papers stated a p value <0.05 was statistically significant. Kidambi et al tested differences in the continuous variables between hypertensive and normotensive patients using t test or Wilcoxon rank sum test. Whereas Walker et al. used unpaired student's t test to assess baseline values. The correlation coefficient was calculated using Spearman's rank correlation. Wirix et al. used linear regression analysis to test associations with cortisol parameters between children with and without hypertension.

CO-MORBIDITIES

Kidambi et al. measured more variables in comparison to Walker et al. Kidambi et al hypertensive participants had higher BMI, larger waist circumference and waist: height ratio. They also had higher plasma concentrations of triglycerides, total cholesterol and LDL cholesterol and lower HDL cholesterol, higher serum insulin and were more insulin resistant. Both normotensive and hypertensive groups had similar plasma glucose levels (88.3 mg/dL / 90.3 mg/dL). Overall, triglycerides (89.9 mg/dL / 109.8 mg/dL) and total cholesterol (175.1mg/dL / 185.4 mg/dL) was higher in the hypertensive groups in comparison to normotensive.

Walker et al. measured far fewer variables in comparison to Kidambi et al. The only two characteristics that they both measured was BMI and plasma potassium. Results can be seen in Table 11. BMI was similar in both groups, with a mean totalled average of 26.7 which falls under the overweight BMI category. BMI tended to be higher in the hypertensive patients for both groups. Both papers had normal plasma potassium levels. Walker's normotensive and hypertensive participants had no differences in clinical or laboratory parameters.

Wirix et al paediatric patients were of an overweight/obese majority as this was their exposure. In the hypertensive overweight group, the average BMI was 27.1 with 28% of the children being overweight,

36% obese and 36% morbidly obese. In the normotensive overweight group, the average BMI was 26.6 with 35% being overweight, 37% obese and 29% morbidly obese. In the non-overweight group, the average BMI was 17.

Study ID	Mean BMI (kg/m2)		Mean Plasma Potassium (mmol/L)	
Kidambi et al. 2006	Normotensive	27.8	Normotensive	3.8
	Hypertensive	29.6	Hypertensive	3.8
Walker et al. 2002	Normotensive	24.2	Normotensive	4.3
	Hypertensive	25.3	Hypertensive	4.4

TABLE 11: TABLE COMPARING BMI AND PLASMA POTASSIUM FROM THE KIDAMBI ET AL AND WALKER ET AL PAPERS. BOTH PAPERS COMPARED NORMOTENSIVE AND HYPERTENSIVE GROUPS.

4.6 DISCUSSION

This is the first systematic review to examine the relationship between salivary cortisol cortisone and blood pressure. Unfortunately, this review only identified 3 papers in total, two including an adult population and the other paediatric patients. Studies have been done in serum and urinary cortisol and their relationship with hypertension but there is still a lack of evidence and research using salivary cortisol. (151–154) The relationship between cortisol and its effect on blood pressure has been studied previously(61,155,156). The 11βHSD2 enzyme protects the mineralocorticoid receptor from high circulating concentrations of cortisol by converting it to its inactive form cortisone. However, if there is a deficiency of this enzyme activates the MR receptor causing aldosterone effects such as renal sodium retention and increased sympathetic activity, resulting in raised blood pressure(155). This is evident in apparent mineralocorticoid excess (AME) which is characterised in sodium retention, hypokalaemia, and salt-dependent hypertension in children(155). Other studies have suggested essential hypertension could also be caused by decreased 11βHSD2 enzyme activity.

Kidambi et al. found that salivary cortisol was higher in hypertensive patients compared to normotensive whereas Walker at al. and Wirix et al. found no difference between groups. Both Walker et al and Wirix measured cortisol, cortisone and the cortisol-cortisone ratio. But Walker et al did not provide the exact concentration values of each within their paper, therefore we are unable to compare normotensive to hypertensive values or see how they changed over the morning. The cortisol-to-cortisone ratio can give us a rough estimate of the activity of 11βHSD2 enzyme(150). Kidambi et al. didn't measure the ratio therefore we are unable to see how this enzyme compared between normotensive and hypertensive patients. Kidambi et al. measured a lot more variables in comparison to Walker et al., allowing to view any correlation between cortisol and the co-morbidities that present in hypertensive patients.

The limitations to this review were firstly the lack of papers available which measured both salivary cortisol and blood pressure within the same study. Secondly, small sample sizes were used in each of the papers and the population that they used were of only one ethnicity (Kidambi et al). Kidambi et al included an enriched population of just African Americans due to the fact that they develop hypertension at a young age. Furthermore, Wirix et al. participants were majority either obese or overweight, which is known to influence cortisol levels(135). Also, So, to broaden this research, larger samples of participants need to be

recruited as well as a generalised group of those who are hypertensive from different ethnicities and of different ages. There is also few data describing salivary cortisol and cortisone in children and young people and research is now claiming that origins of cardiovascular disease can begin in early life (157–159). So, it is essential to include younger participants like Wirix et al. has done.

Thirdly, the number of saliva samples and times blood pressure was taken was insufficient in analysing the overall diurnal pattern in which cortisol acts and how this matches up to blood pressure. To further facilitate this research, saliva samples should be collected 2 hourly over a normal waking day and blood pressure taken every hour, like Kidambi et al. did, to see how each affect one another.

4.7 CONCLUSION

Currently, there is literature surrounding the use of serum and urine as methods of measuring cortisol but not of saliva. Despite knowing that high cortisol concentrations increases blood pressure there is insufficient evidence in cortisol being a hypertensive marker in clinical practice. More research needs to be carried out within this area to further explore the relationship, through the use of a larger, varied sample population.

5. CHAPTER 5 DISCUSSION

5.1PERSONAL DEVELOPMENT

Coming from 3rd year medicine, not having much paediatric experience, not knowing how to take consent or how a study is set up and carried out, this year has taught me so much. I have learnt how to speak and interact with different age groups of children and young people through learning new methods in explaining the SMILE study to them whilst making it engaging and interactive. Taking consent from any age is a key skill within medicine and having the opportunity to practice this at an early stage of my medical education has been beneficial. I gained knowledge in an array of areas - from the background and reason for taking consent to the process and ways of explaining it to younger children. This knowledge and practice I can now utilise throughout my career as a doctor. Furthermore, being able to participate in the setup of and the follow through of a study was a new and interesting experience. In addition, being able to witness a study passing through ethics, from submission of the IRAS form, to attending a board meeting further enhanced my research.

5.2 WHERE SHOULD THE SCIENCE GO NEXT?

SMILE has demonstrated promising data surrounding salivary biomarkers in children and young people, but further work is still needed in order to further investigate it for use in future clinical practice. This work includes continuing the recruitment of children in order to gain more normative values and increase the variability in demographic variables. In future studies, broadening recruitment to other parts of Liverpool and the Merseyside area will provide a more varied population which could provide a more generalised view of the participants we potentially want to treat. In order to cover the issue of human error, especially in the younger children monitored collection of the saliva samples should be carried out within the CRF or within another hospital so that we eliminate this risk.

COVID-19 majorly impacted this study and the completion of the aims. We didn't achieve our aim of calculating a validated reference range for salivary cortisol and cortisone. But this is due to us not reaching our intended recruitment goal therefore we didn't have enough participants to calculate a significant range. However, recruitment is being continued by Silothabo who worked on SMILE with me. We did achieve all of our secondary aims apart from the calculation of area under the curve but this was due to timing issues and will be calculated and shown once the research paper has been written. This thesis has been a great step in investigating salivary biomarkers and demographic variables and hopefully the beginning of more research within this area. Continuing this work is essential as the use of saliva for measuring hormones has so many advantages and could eliminate a lot of anxieties for both parents and young children in the future.

In regard to the systematic review, we did meet our third secondary aim about exploring the relationship of salivary biomarkers and blood pressure. It is clearly evident that there is a lack of papers that have been written surrounding salivary cortisol and blood pressure despite the well documented physiological link between the two. For future research, salivary cortisol and blood pressure measurements should be taken throughout the day in order to monitor how both influence one another. This should be done in a varied population including both adults and children.

5.3 CONCLUSION

The research found within this thesis is a great starting point to discovering more about salivary cortisol and cortisone in healthy children and young people. Even though we didn't meet our primary aim due to difficulties caused by the pandemic we still achieved beneficial research surrounding salivary biomarkers and demographic variables. The use of salivary cortisol as a non-invasive accessible method of measuring cortisol excess or deficiency in clinical practice looks promising and that it clearly follows the normal physiological diurnal rhythm of cortisol. But there is contradicting results surrounding the relationship of salivary cortisol and cortisone concentrations and blood pressure. Furthermore, we didn't find a significant relationship between salivary cortisol, cortisone and parameters such as sex, age, BMI, height or systolic blood pressure. Therefore, more research and participant recruitment needs to be carried out in order to investigate these relationships.

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APPENDICES

APPENDIX 1: PROSPERO REGISTRATION

PROSPERO
International prospective register of systematic reviews

NHS National Institute for Health Research

UNIVERSITY of York
Centre for Reviews and Dissemination

Systematic review

1. * Review title.

Give the title of the review in English

In adults, what is the relationship between cortisol:cortisone ratio in saliva and hypertension?

2. Original language title.

For reviews in languages other than English, give the title in the original language. This will be displayed with the English language title.

3. * Anticipated or actual start date.

Give the date the systematic review started or is expected to start.

30/10/2020

4. * Anticipated completion date.

Give the date by which the review is expected to be completed.

30/06/2021

5. * Stage of review at time of this submission.

Tick the boxes to show which review tasks have been started and which have been completed. Update this field each time any amendments are made to a published record.

Reviews that have started data extraction (at the time of initial submission) are not eligible for inclusion in PROSPERO. If there is later evidence that incorrect status and/or completion date has been supplied, the published PROSPERO record will be marked as retracted.

This field uses answers to initial screening questions. It cannot be edited until after registration.

The review has not yet started: No

Review stage	Started	Completed
Preliminary searches	Yes	Yes
Piloting of the study selection process	Yes	No
Formal screening of search results against eligibility criteria	Yes	No
Data extraction	No	No
Risk of bias (quality) assessment	No	No
Data analysis	No	No

Provide any other relevant information about the stage of the review here.

6. * Named contact.

The named contact is the guarantor for the accuracy of the information in the register record. This may be any member of the review team.

Orla Bright

Email salutation (e.g. "Dr Smith" or "Joanne") for correspondence:

Miss Bright

7. * Named contact email.

Give the electronic email address of the named contact.

Orla.Bright@alderhey.nhs.uk

8. Named contact address

Give the full institutional/organisational postal address for the named contact.

+44 (0)151 228 4811

9. Named contact phone number.

Give the telephone number for the named contact, including international dialling code.

10. * Organisational affiliation of the review.

Full title of the organisational affiliations for this review and website address if available. This field may be completed as 'None' if the review is not affiliated to any organisation.

Department of Women's and Children's Health, Institute of Life Course and Medical Sciences, University of Liverpool

Organisation web address:

11. * Review team members and their organisational affiliations.

Give the personal details and the organisational affiliations of each member of the review team. Affiliation refers to groups or organisations to which review team members belong. **NOTE: email and country now MUST be entered for each person, unless you are amending a published record.**

Miss Orla Bright. Department of Women's and Children's Health, Institute of Life Course and Medical Sciences, University of Liverpool
Mr Dan Hawcutt. Department of Women's and Children's Health, Institute of Life Course and Medical Sciences, University of Liverpool
Mrs Joanne Blair. Alder Hey Children's Hospital

12. * Funding sources/sponsors.

Details of the individuals, organizations, groups, companies or other legal entities who have funded or sponsored the review.

None

Grant number(s)

State the funder, grant or award number and the date of award

13. * Conflicts of interest.

List actual or perceived conflicts of interest (financial or academic).

None

14. Collaborators.

Give the name and affiliation of any individuals or organisations who are working on the review but who are not listed as review team members. **NOTE: email and country must be completed for each person, unless you are amending a published record.**

15. * Review question.

State the review question(s) clearly and precisely. It may be appropriate to break very broad questions down into a series of related more specific questions. Questions may be framed or refined using PI(E)COS or similar where relevant.

In adults who have their cortisol to cortisone ratio measured and blood pressure taken, is there a relationship between them both?

16. * Searches.

State the sources that will be searched (e.g. Medline). Give the search dates, and any restrictions (e.g. language or publication date). Do NOT enter the full search strategy (it may be provided as a link or attachment below.)

The following bibliographic databases will be searched, PubMed, MEDLINE, EMBASE and CINAHL without any date or language restrictions, for relevant studies.

Papers found in the search will be screened by title, abstract and full text.

Mendeley will be used to log and combine the searches from the individual databases.

17. URL to search strategy.

Upload a file with your search strategy, or an example of a search strategy for a specific database, (including the keywords) in pdf or word format. In doing so you are consenting to the file being made publicly accessible. Or provide a URL or link to the strategy. Do NOT provide links to your search **results**.

Alternatively, upload your search strategy to CRD in pdf format. Please note that by doing so you are consenting to the file being made publicly accessible.

Do not make this file publicly available until the review is complete

18. * Condition or domain being studied.

Give a short description of the disease, condition or healthcare domain being studied in your systematic review.

There is a plausible link between cardiovascular health and cortisol, as it is released in times of stress and stress is a commonly known risk factor for CVD (cardiovascular diseases). The long term development of

atherosclerosis and subclinical CHD, acute triggering of cardiac events and impaired recovery and quality of life can all be linked back to stress as a causative factor. Furthermore, cortisol and hypertension can be linked due to glucocorticoids playing a crucial role in blood pressure regulation. Cortisol increases GFR and acts on the distal tubule to increase sodium retention and potassium loss as well as increasing angiotensinogen synthesis and enhancing the vasopressor effects of catecholamines. Too little cortisol can lead to life threatening hypertension in Addison's patients whereas in Cushing's too much causes hypertension.

19. * Participants/population.

Specify the participants or populations being studied in the review. The preferred format includes details of both inclusion and exclusion criteria.

Inclusion:
The population will consist of adults aged 18 and above who have both their salivary cortisone to cortisol ratio measured and their blood pressure.

Exclusion:
Non-human patients, as well as studies that do not meet the age criteria (children);

Patients will any heart disease, varicose veins, liver or renal disease, COPD, diabetes, etc.

20. * Intervention(s), exposure(s).

Give full and clear descriptions or definitions of the interventions or the exposures to be reviewed. The preferred format includes details of both inclusion and exclusion criteria.

The intervention is patients who have a blood pressure measurement recorded which is classified as hypertensive. Patients with blood pressure above 140/90mmHg or higher and ABPM daytime average or HBPM average of 135/85mmHg or higher.

21. * Comparator(s)/control.

Where relevant, give details of the alternatives against which the intervention/exposure will be compared (e.g. another intervention or a non-exposed control group). The preferred format includes details of both inclusion and exclusion criteria.

Not relevant.

22. * Types of study to be included.

Give details of the study designs (e.g. RCT) that are eligible for inclusion in the review. The preferred format includes both inclusion and exclusion criteria. If there are no restrictions on the types of study, this should be stated.

Inclusion:
All types of study designs including cohort studies, case-control and randomised controlled trials regardless of language, publication year, publication type and publication status will be considered for inclusion.

Exclusion:

Letters, editorials, commentaries and case reports will be excluded.

23. Context.

Give summary details of the setting or other relevant characteristics, which help define the inclusion or exclusion criteria.

24. * Main outcome(s).

Give the pre-specified main (most important) outcomes of the review, including details of how the outcome is defined and measured and when these measurement are made, if these are part of the review inclusion criteria.

The main outcome is recorded blood pressures in patients who have salivary cortisol: cortisone ratios measured.

Measures of effect

Please specify the effect measure(s) for you main outcome(s) e.g. relative risks, odds ratios, risk difference, and/or 'number needed to treat.

Not relevant.

25. * Additional outcome(s).

List the pre-specified additional outcomes of the review, with a similar level of detail to that required for main outcomes. Where there are no additional outcomes please state 'None' or 'Not applicable' as appropriate to the review

Secondary outcomes are other patient parameters such as BMI, presence of co-morbidities, age, lifestyle factors, concomitant medications.

Measures of effect

Please specify the effect measure(s) for you additional outcome(s) e.g. relative risks, odds ratios, risk difference, and/or 'number needed to treat.

Not relevant.

26. * Data extraction (selection and coding).

Describe how studies will be selected for inclusion. State what data will be extracted or obtained. State how this will be done and recorded.

Titles (and abstracts where available) will be reviewed against the inclusion/exclusion criteria to determine eligibility. The full text of selected papers will then be assessed for inclusion, based on the types of participants, study design and outcome measures. Record of the selection process will be recorded and a PRISMA diagram will be created. Each study will be reviewed by two reviewers separately and then compared. If there are any disagreements, the inclusion/exclusion criteria will be discussed between the two reviewers to reach an agreement, and then a third reviewer if needed. References will be stored in an Excel spreadsheet and will include each stage of screening, as well as any reasons for exclusion. All papers that reach the full-text screening will be accessed and reviewed further, noting any reasons for exclusion in the table generated previously.

Data will be extracted independently from the identified trials and entered into a separate Excel spreadsheet. Extracted information will include study setting; study population and participant demographics and baseline characteristics; details of the intervention and control conditions; study methodology; recruitment and study completion rates; outcomes and times of measurement; suggested mechanisms of intervention action; information for assessment of the risk of bias.

27. * Risk of bias (quality) assessment.

State which characteristics of the studies will be assessed and/or any formal risk of bias/quality assessment tools that will be used.

Independently, risk of bias will be assessed by two researchers and will involve a third party assessment where consensuses cannot be reached. The necessary risk of bias tool will be used for each study where appropriate, and a statistician will.

28. * Strategy for data synthesis.

Describe the methods you plan to use to synthesise data. This **must not be generic text** but should be **specific to your review** and describe how the proposed approach will be applied to your data. If meta-analysis is planned, describe the models to be used, methods to explore statistical heterogeneity, and software package to be used.

A narrative synthesis of the findings of the included studies will be provided summarising the population characteristic, the outcomes and study quality. Where sufficient data is available studies that report the number of patients who have their cortisol to cortisone ratio measured as well as their blood pressure will be pooled in a meta-analysis using Review Manager 5.4 and the overall incidence event rate (with 95% confidence intervals) will be reported. Studies which report other comorbidities present, other patient parameters like BMI, age, causative lifestyle factors or concomitant medications will be pooled by outcome and random effects model meta-analysis will be used to pool the results by outcome. Risk ratios and 95% confidence intervals will be calculated for binary outcomes and standardised mean differences (and 95% CI) for continuous variables. Studies that use the same validated quality of life measure to assess this outcome will be pooled and a meta-analysis will also be undertaken, with standardised mean differences and 95% [illegible] heterogeneity will be assessed using the $?^2$ test and the I^2 statistic and an I^2 value greater than 50% will be indicative of substantial heterogeneity. If heterogeneity is high the results will be presented using a random effects model and if 50% using the fixed effects model.

29. * Analysis of subgroups or subsets.

State any planned investigation of 'subgroups'. Be clear and specific about which type of study or participant will be included in each group or covariate investigated. State the planned analytic approach.

None planned.

30. * Type and method of review.

Select the type of review, review method and health area from the lists below.

Type of review

Cost effectiveness
No

Diagnostic
No

Epidemiologic
Yes

Individual patient data (IPD) meta-analysis
No

Intervention
No

Living systematic review
No

Meta-analysis
Yes

Methodology
No

Narrative synthesis
Yes

Network meta-analysis
No

Pre-clinical
No

Prevention
No

Prognostic
No

Prospective meta-analysis (PMA)
No

Review of reviews
No

Service delivery
No

Synthesis of qualitative studies
No

Systematic review
Yes

Other
No

Health area of the review

Alcohol/substance misuse/abuse
No

Blood and immune system
No

Cancer
No

Cardiovascular
Yes

Care of the elderly
No

Child health
No

Complementary therapies
No

COVID-19
No

Crime and justice
No

Dental
No

Digestive system
No

Ear, nose and throat
No

Education
No

Endocrine and metabolic disorders
No

Eye disorders
No

General interest
No

Genetics
No

Health inequalities/health equity
No

Infections and infestations
No

International development
No

Mental health and behavioural conditions
No

Musculoskeletal
No

Neurological

No

Nursing
No

Obstetrics and gynaecology
No

Oral health
Yes

Palliative care
No

Perioperative care
No

Physiotherapy
No

Pregnancy and childbirth
No

Public health (including social determinants of health)
No

Rehabilitation
No

Respiratory disorders
No

Service delivery
No

Skin disorders
No

Social care
No

Surgery
No

Tropical Medicine
No

Urological
No

Wounds, injuries and accidents
No

Violence and abuse
No

31. Language.

Select each language individually to add it to the list below, use the bin icon to remove any added in error.
English

There is not an English language summary

32. * Country.

Select the country in which the review is being carried out. For multi-national collaborations select all the countries involved.

England

33. Other registration details.

Name any other organisation where the systematic review title or protocol is registered (e.g. Campbell, or The Joanna Briggs Institute) together with any unique identification number assigned by them. If extracted data will be stored and made available through a repository such as the Systematic Review Data Repository (SRDR), details and a link should be included here. If none, leave blank.

34. Reference and/or URL for published protocol.

If the protocol for this review is published provide details (authors, title and journal details, preferably in Vancouver format)

Add web link to the published protocol.

Or, upload your published protocol here in pdf format. Note that the upload will be publicly accessible.

No I do not make this file publicly available until the review is complete

Please note that the information required in the PROSPERO registration form must be completed in full even if access to a protocol is given.

35. Dissemination plans.

Do you intend to publish the review on completion?

Yes

Give brief details of plans for communicating review findings.?

Publication in a peer-review journal.

36. Keywords.

Give words or phrases that best describe the review. Separate keywords with a semicolon or new line. Keywords help PROSPERO users find your review (keywords do not appear in the public record but are included in searches). Be as specific and precise as possible. Avoid acronyms and abbreviations unless these are in wide use.

Cortisol; High blood pressure; Hypertension; Salivary biomarkers; CVS risk; Salivary cortisol; Cortisone

37. Details of any existing review of the same topic by the same authors.

If you are registering an update of an existing review give details of the earlier versions and include a full bibliographic reference, if available.

38. * Current review status.

Update review status when the review is completed and when it is published.New registrations must be ongoing so this field is not editable for initial submission.
Please provide anticipated publication date

Review_Ongoing

39. Any additional information.

Provide any other information relevant to the registration of this review.

40. Details of final report/publication(s) or preprints if available.

Leave empty until publication details are available OR you have a link to a preprint (NOTE: this field is not editable for initial submission). List authors, title and journal details preferably in Vancouver format.

Give the link to the published review or preprint.

APPENDIX 2: PARENTAL CONSENT FORM

UNIVERSITY OF LIVERPOOL

NHS Alder Hey Children's NHS Foundation Trust

INFORMED CONSENT DOCUMENT : Face-to-face consent

(Parents and Legal Guardians)

IRAS 287711

Subject Number:
Patient Recruitment Date:

Study Title: **A study of salivary biomarkers of adrenal function in healthy children and young people**

Study Sponsor: Alder Hey Foundation NHS Trust, Eaton Road, Liverpool, L12 2AP

Study Doctor: Professor Joanne Blair – Alder Hey Children's NHS Trust, Liverpool, UK

Please initial boxes

	INITIALS
1. I confirm that I have read and understand the Participant Information Sheet, version …… dated ……. for the above study. I have had the opportunity to consider the information and to ask questions about my child's/ward's participation and these were answered to my satisfaction. I will receive a signed and dated copy of this informed consent document.	
2. I understand that my child's/ward's participation is voluntary. I can terminate my participation in the trial at any time without giving reasons and without consequences for my medical care or legal rights.	
3. I give my consent that my data may child's/ward's be used by the investigator and his/her study staff for the purposes stated in this Patient Information Sheet. I understand that responsible persons from the sponsor or its representatives, members of the study staff, Institutional Review Boards, and government authorities might have access to parts of my medical files and data collected for the purposes of this trial. I give these persons permission to access my child's/ward's documents.	
4. I agree for my child's/ward to take part in the above trial.	

Consent for parents and guardians Version 1.0 dated 03.11.2020

5. I also agree for the information to be used and to gift the saliva samples collected for this study for future studies hormones in saliva.	YES ☐ NO ☐	

Participant's Name (printed) _____

CONSENT SIGNATURES

_____

Printed Name of Parent/Legal Guardian

_____ _____

Signature of Parent Legal Guardian Date

_____ _____

Printed Name of Person Conducting the Informed Consent Discussion

_____ _____

Signature of Person Conducting the Informed Consent Discussion

Date

(When completed: 1 for parent/legal Guardian; 1 original for researcher site file, 1 for medical records).

APPENDIX 3: 16+ CONSENT FORM

UNIVERSITY OF LIVERPOOL

NHS
Alder Hey Children's
NHS Foundation Trust

INFORMED CONSENT DOCUMENT : Face-to-face consent

(Participants aged 16 years and older or younger participants consenting for themselves)

IRAS 287711

Subject Number:

Patient Recruitment Date:

Study Title: **A study of salivary biomarkers of adrenal function in healthy children and young people**

Study Sponsor: Alder Hey Foundation NHS Trust, Eaton Road, Liverpool, L12 2AP

Study Doctor: Professor Joanne Blair – Alder Hey Children's NHS Trust, Liverpool, UK

Please initial boxes

	INITIALS
1. I confirm that I have read and understand the Participant Information Sheet, version …… dated ……. for the above study. I have had the opportunity to consider the information and to ask questions about my child's/ward's participation and these were answered to my satisfaction. I will receive a signed and dated copy of this informed consent document.	
2. I understand that my child's/ward's participation is voluntary. I can terminate my participation in the trial at any time without giving reasons and without consequences for my medical care or legal rights.	
3. I give my consent that my data may child's/ward's be used by the investigator and his/her study staff for the purposes stated in this Patient Information Sheet. I understand that responsible persons from the sponsor or its representatives, members of the study staff, Institutional Review Boards, and government authorities might have access to parts of my medical files and data collected for the purposes of this trial. I give these persons permission to access my child's/ward's documents.	
4. I agree for my child's/ward to take part in the above trial.	

Consent for participants aged 16 years and older Version 1.0 dated 03.11.2020

5. I also agree for the information to be used and to gift the saliva samples collected for this study for future studies hormones in saliva.	YES ☐ NO ☐	

Participant's Name (printed) _____

CONSENT SIGNATURES

_____

Printed Name of Participant

_____ _____

Signature of Participant. Date

_____ _____

Printed Name of Person Conducting the Informed Consent Discussion

_____ _____

Signature of Person Conducting the Informed Consent Discussion Date

_____ _____

Signature of Parent / Legal Guardian

(Optional for patients less than 16 years old) Date

(When completed: 1 for parent/legal Guardian; 1 original for researcher site file, 1 for medical records).

APPENDIX 4: ASSENT FORM

UNIVERSITY OF LIVERPOOL

NHS
Alder Hey Children's
NHS Foundation Trust

PARTICIPANT ASSENT FORM FOR RESEARCH
IRAS 287711

Subject Number: ☐

Patient Recruitment Date: DD/MMM/YYY

Title of Project: **A study of salivary biomarkers of adrenal function in healthy children and young people**

Name of Researcher: _____ **Job title:** _____

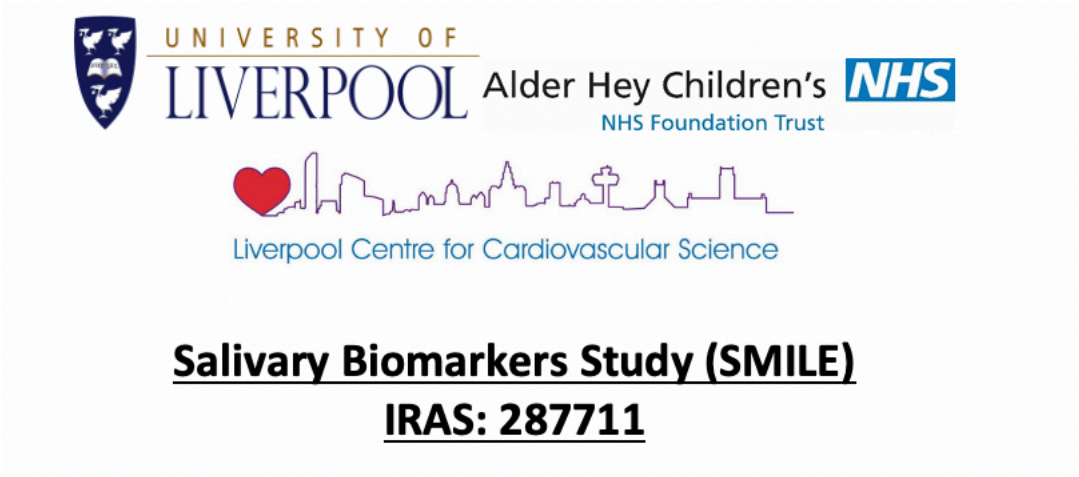
	Initial box
1. I am happy I know about the study and have asked any questions.	☐
2. I know being part of this study is up to me and I can change my mind at any time.	☐
3. I know that some people that are helping with the study will be able to see my results.	☐
4. I agree to take part in the above study and for some of my saliva to be kept for future studies.	YES ☐ NO ☐

_____
Name of participant

_____	_____	_____
Name of person taking assent (if different from researcher)	Date	Signature
_____	_____	_____
Researcher	Date	Signature

Assent form version 1. Date 03.11.2020

APPENDIX 5: CASE RECORD FORM



Salivary Biomarkers Study (SMILE)
IRAS: 287711

Case Record Form

Site:
Subject Number:
Patient Recruitment Date: DD/MMM/YYY
Recruiting Health Professional:
Name:
Hospital:
Work Telephone Number:
Work Email Address:

Chief Investigator:
Professor J Blair
Institute in the Park
Alder Hey Children's Hospital
Eaton Road
Liverpool
L12 2AP

Tel:
Email: jo.blair@alderhey.nhs.uk

Dr Dan Hawcutt, Senior Lecturer Paediatric Pharmacology
Email: d.hawcutt@liv.ac.uk

Orla Bright MPhil Student
Email: orla.bright@alderhey.nhs.uk

21 1

General Guidelines for CRF Completion

Please complete the Case Report Form (CRF) as thoroughly as possible and then post or fax a photocopy of the completed CRF to the lead coordinating centre, along with the anonymised discharge summary and/or additional anonymised reports

The structure of the CRF is shown in the following diagram;

Even Pages	**Odd Pages**
Contain notes on how to complete the adjacent → odd numbered page	To be completed

All forms should be completed in black ink in a clear manner. Any changes or corrections should be made by drawing a line through the data, entering the corrected information and initialling and dating the change.

Following standard notation should be used in the event that values or answers cannot be provided:

- NA: Not applicable
- NK: Not known
- ND: Not done
- NR: Not retrievable/Not available

Please ensure that when referencing medications and chemicals you detail the GENERIC name and not the brand name

Inclusion/Exclusion criteria – Notes

[1] The patient **must** be given a Patient Information Leaflet and Consent Form to be included in the study. If the patient is <16 it must be deemed whether a child has Gillick competence. If the child does not have Gillick competence, consent must be granted by Parent or Guardian/Nominated Parent or Guardian, else if >16 or deemed to have Gillick competence patient must give consent. For those <16 years who also have Gillick competence, gain consent from parents also.

Inclusion/Exclusion Criteria

Please tick 'yes' or 'no' to **all** question
ns

Inclusion Criteria

A		Yes	NA	No
1.	Patient/Participant willing to take part in study if ≥16 years of age or deemed to have Gillick competence at recruitment			
2.	Patients/Participants parent or guardian willing to consent to study if <16 years of age and deemed not to have Gillick competence at recruitment			
4.	Assent obtained from competent young person			
5.	Patient/Parent information leaflet provided and read by Patient/Parent (or guardian)**[1]** Version [___] Version dated [DD / MM / YYYY] Date given [DD / MM / YYYY]			
6.	Patient aged 5-18 years			
7.	Patient/parent has a good understanding of English and understands the requirements for Adverse Events to be reported to the investigators			
8.	Patient/family willing and able to comply with protocol including salivary sampling, measurement of blood pressure, height and weight if aged 5 years or older.			

Exclusion Criteria

B		Yes	No
10.	Patients/Participants parent or guardian unwilling to consent to study if <16 years of age at recruitment		
11.	Participant unwilling to consent/assent (competence assessed on a case by case basis)		
12.	Patient has an oral condition that is likely to result in blood contamination of saliva samples (gingivitis, mouth ulcers and those undergoing dental procedures)		
13.	Any condition that affects serum cortisol or CBG (abnormalities of thyroid or anterior pituitary hormone secretion, psychiatric pathology, type 1 or 2 diabetes, cystic fibrosis, protein losing enteropathies, nephrotic syndrome, undergoing renal dialysis)		
14.	Family history of adrenal insufficiency		
15.	Medications likely to affect serum cortisol or CBG (glucocorticoids, sex steroids, thyroxine, growth hormone, azole compounds, insulin and metformin		
16.	Patient is, in the opinion of the Investigator, not suitable to participate in the study.		

Please tick 'yes' or 'no' to **all** questions

Patient Eligible for study?	Yes ☐ **Patient included in the study Please complete CRF**	No ☐ **Patient NOT included in the study**

Recruitment Information – Notes

[1] Please enter **both** the patients date of birth and age at the time of recruitment.

[2] Ethnic origin as self-reported, by the patient or documented in case notes. Please note that we appreciate it may be difficult to obtain Parents information, so we would be very grateful for any information provided.

Please use codes as listed:

1. White
2. White Irish
3. Other White
4. Mixed: White and Black Caribbean
5. Mixed: White and Black African
6. Mixed: White and Asian
7. Other mixed background
8. Indian
9. Pakistani
10. Bangladeshi
11. Other Asian background
12. Caribbean
13. African
14. Other Black background
15. Chinese
16. Other ethnic group (please specify)
17. Not Known

[3] Please use table on the next page to convert pound measurements into kilograms.

Pounds to Kilograms Conversion Table

Pound	Kilogram	Pound	Kilogram	Pound	Kilogram	Pound	Kilogram
1	0.45359237	26	11.79340162	51	23.13321087	76	34.47302012
2	0.90718474	27	12.24699399	52	23.58680324	77	34.92661249
3	1.36077711	28	12.70058636	53	24.04039561	78	35.38020486
4	1.81436948	29	13.15417873	54	24.49398798	79	35.83379723
5	2.26796185	30	13.6077711	55	24.94758035	80	36.2873896
6	2.72155422	31	14.06136347	56	25.40117272	81	36.74098197
7	3.17514659	32	14.51495584	57	25.85476509	82	37.19457434
8	3.62873896	33	14.96854821	58	26.30835746	83	37.64816671
9	4.08233133	34	15.42214058	59	26.76194983	84	38.10175908
10	4.5359237	35	15.87573295	60	27.2155422	85	38.55535145
11	4.98951607	36	16.32932532	61	27.66913457	86	39.00894382
12	5.44310844	37	16.78291769	62	28.12272694	87	39.46253619
13	5.89670081	38	17.23651006	63	28.57631931	88	39.91612856
14	6.35029318	39	17.69010243	64	29.02991168	89	40.36972093
15	6.80388555	40	18.1436948	65	29.48350405	90	40.8233133
16	7.25747792	41	18.59728717	66	29.93709642	100	45.359237
17	7.71107029	42	19.05087954	67	30.39068879	125	56.69904625
18	8.16466266	43	19.50447191	68	30.84428116	150	68.0388555
19	8.61825503	44	19.95806428	69	31.29787353	175	79.37866475
20	9.0718474	45	20.41165665	70	31.7514659	200	90.718474
21	9.52543977	46	20.86524902	71	32.20505827	250	113.3980925
22	9.97903214	47	21.31884139	72	32.65865064	300	136.077711
23	10.43262451	48	21.77243376	73	33.11224301	500	226.796185
24	10.88621688	49	22.22602613	74	33.56583538	750	340.1942775
25	11.33980925	50	22.6796185	75	34.01942775	1000	453.59237

Recruitment Information

Participant Demographics

Sex	Male ☐	Female ☐		
Date of Birth [1]	DD/MM/YYYY		**Age** (in years)	
Postcode				NK ☐
Gestation	**weeks**			NK ☐
Birth Weight [3]	. **kg**	(2 decimal places)		NK ☐

Ethnic origin [2]

Own	☐	Specify	Country of Birth	
Mother	☐	Specify	Country of Birth	
Father	☐	Specify	Country of Birth	

Parent / Guardian Contact Details

Parent/Guardian Contact Number:

Parent/Guardian Email Address:

Previous Medical History - Notes

[1] Has patient ever had any medical or surgical interventions :

Previous Medical History

Not Recorded = section not relevant to study arm

1.	**Has patient ever had any medical or surgical problems? [1]**	Yes ☐ No ☐ Specify:
3.	**Has the patient any allergies?**	Yes ☐ No ☐ Specify ☐
4.	**Immunisations up to date?**	Yes ☐ No ☐

Medications

What medications does the patient take?

5.	**Does the patient take any meds?**	Yes ☐ No ☐ Specify ☐
6.	**Medications** *(Please specify Medication name, dose and units)*	Yes ☐ No ☐ Specify ☐ Specify ☐ Specify ☐
	7. Has patient been treated with a steroid in the last 12 months?	Yes ☐ No ☐ Specify When Stopped:

Blood Pressure- Notes

Notes

- Either automatic or manual blood pressure measurement can be taken
- Allow 5 minutes for the child to settle and is content before taking their blood pressure
- Ensure you are using the correct sized cuff on the participant
- Take three blood pressure measurements in total, each reading spaced by 1 minute

Blood Pressure

	Systolic Measurement (mmHg)	Diastolic Measurement (mmHg)	Not Done
1.			
2.			
3.			
Mean Measurement (mmHg)			

Height	. m	(2 decimal places)	ND
Weight	. kg	(2 decimal places)	ND

Date & time height collected	DD/MM/YYYY – HH:MM
Date & time weight collected	DD/MM/YYYY – HH:MM

Salivary Sample Collection
(For all patients)

Information leaflet given to participant?	Yes ☐ No ☐ N/A ☐

Method of collection explained to participant?	Yes ☐ No ☐ N/A ☐

Saliva kit collected by participant?	Yes ☐ No ☐ Date & time collected DD/MM/YYYY – HH:MM N/A ☐

Sample No.	Date Taken	Time Taken	Time Frozen

How was the patient awoken? **(Themselves / By Parents)**	DD/MM/YYYY – HH:MM

Saliva kits taken out of participant fridge?	Yes ☐ No ☐ Date & time removed DD/MM/YYYY – HH:MM N/A ☐

Saliva samples returned to CRF?	Yes ☐ Date & time collected DD/MM/YYYY – HH:MM No ☐ Reason not collected How many? ☐

Saliva samples returned to the lab?	Yes ☐ Date & time collected DD/MM/YYYY – HH:MM No ☐ Reason not collected How many? ☐

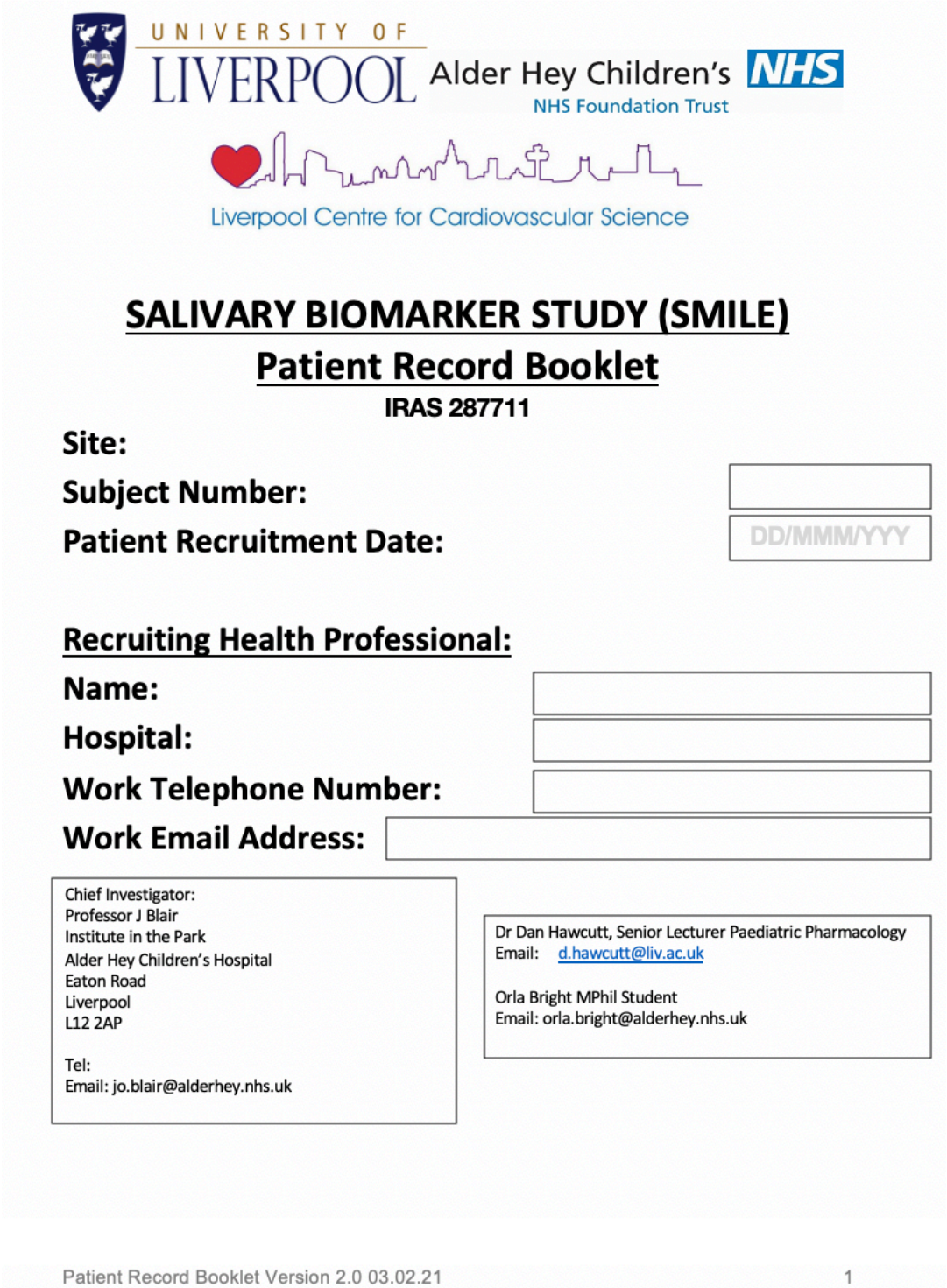
Saliva kits frozen by lab?	Yes ☐ No ☐ Date & time frozen DD/MM/YYYY – HH:MM N/A ☐
Saliva kits taken out of the labs freezer?	Yes ☐ No ☐ Date & time removed DD/MM/YYYY – HH:MM N/A ☐

Saliva samples taken to Manchester ?	Yes ☐ No ☐ Date & time N/A ☐	DD/MM/YYYY – HH:MM

Participant consented to extra testing?	Yes ☐ No ☐ N/A ☐

Participant has completed each section ?	Yes ☐ No ☐ Date & time complete N/A ☐	DD/MM/YYYY – HH:MM

APPENDIX 6: PATIENT RECORD BOOKLET



UNIVERSITY OF LIVERPOOL
Alder Hey Children's NHS Foundation Trust
Liverpool Centre for Cardiovascular Science

SALIVARY BIOMARKER STUDY (SMILE)

Patient Record Booklet

IRAS 287711

Site:

Subject Number:

Patient Recruitment Date: DD/MMM/YYY

Recruiting Health Professional:

Name:

Hospital:

Work Telephone Number:

Work Email Address:

Chief Investigator:
Professor J Blair
Institute in the Park
Alder Hey Children's Hospital
Eaton Road
Liverpool
L12 2AP

Tel:
Email: jo.blair@alderhey.nhs.uk

Dr Dan Hawcutt, Senior Lecturer Paediatric Pharmacology
Email: d.hawcutt@liv.ac.uk

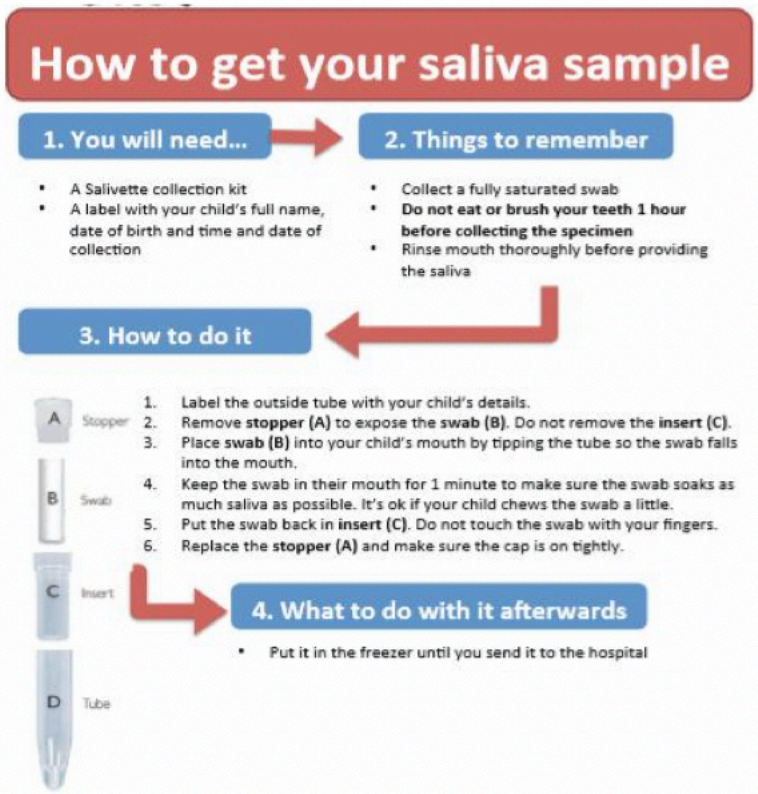
Orla Bright MPhil Student
Email: orla.bright@alderhey.nhs.uk

Patient Record Booklet Version 2.0 03.02.21 1

Salivette Method

Before taking your samples, please become familiar with how the salivettes work with the instructions below.

NOTE: We would like you to put samples in the fridge NOT freezer as it states in the instructions.



Obtaining the Saliva Samples (2)

1. Please note down on the next page the time your child wakes up and how they were awoken
2. We would like to ask you to collect the first sample **30 minutes after they wake up,** and then **every 2 hours** after that until they go to bedtime at night-time.

EXAMPLE

Wake Up: 8:00am

First Sample :8:30am

Second Sample: 10:30am

Third Sample: 12:30pm

Fourth Sample: 14:30pm and so on

3. Saliva samples are collected by chewing on a cotton wool roll in the mouth and you should have been shown by a member of staff how to do this. You can also refer to the instructions on the previous page

NOTE: It is important that they do not eat for one hour before a sample is collected.

4. After you have taken your sample note down the sample number, date taken and time taken in this booklet.
5. Each sample (TP1-TP7) has a corresponding bag with numbers 1-7. Please can you match these up and put the correct sample in each bag starting with 1 and going in ascending order.
6. Then place your sample into your home fridge, note down the time that you do this
7. The saliva samples can be stored in your fridge at home, until you are ready to bring them back to Alder Hey. Ideally, we would ask you to return them within ten days of them being collected.

Study Details

Saliva kit collected?	Yes ☐ No ☐ Date & time collected DD/MM/YYYY – HH:MM

How was the participant awoken? **(Themselves / By Parents)**	

Date & time patient woke up?	DD/MM/YYYY – HH:MM

Sample No.	Date Taken	Time Taken	Time Put In Fridge

Date & time samples taken out of fridge?	DD/MM/YYYY – HH:MM

Date & time returning to CRF?	DD/MM/YYYY – HH:MM

Please return samples and patient record booklet to the CRF.

If you have any queries about the study, you can email myself on orla.bright@alderhey.nhs.uk.

If you have a problem and you're not comfortable with speaking to the staff looking after you, contact the Patient and Family Support Team at Alder Hey on 0151 252 5374.

APPENDIX 7: RECRUITMENT POSTER

UNIVERSITY OF LIVERPOOL

Alder Hey Children's NHS
NHS Foundation Trust

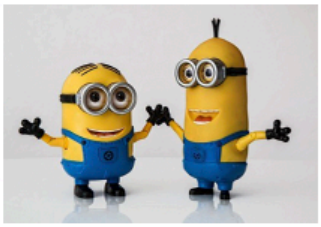
Liverpool Centre for Cardiovascular Science

Research Study Participants Needed!!

Are you between 5 and 18 years and healthy?

Are you willing to help us find out more about our hormones like cortisol in our saliva ?

If so, you may be eligible to participate in the study, CALL for DETAILS!!!



Travel expenses reimbursed

Contact:

Orla at orla.bright@alderhey.nhs.uk

Orla Orla.bright@@alderhey.nhs.uk
Orla Orla.bright@@alderhey.nhs.uk
Orla Orla.bright@@alderhey.nhs.uk
Orla Orla.bright@@alderhey.nhs.uk
Orla Orla.bright@@alderhey.nhs.uk
Orla Orla.bright@@alderhey.nhs.uk
Orla Orla.bright@@alderhey.nhs.uk
Orla Orla.bright@@alderhey.nhs.uk
Orla rla.bright@@alderhey.nhs.uk
Orla rla.bright@@alderhey.nhs.uk

Version 1, 03/02/21