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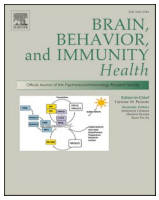

Seven robust and easy to obtain biomarkers to measure acute stress

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ABSTRACT

With the purpose of identifying a sensitive, robust, and easy-to-measure set of biomarkers to assess stress reactivity, we here study a large set of relatively easy to obtain markers reflecting subjective, autonomic nervous system (ANS), endocrine, and inflammatory responses to acute social stress (n = 101). A subset of the participants was exposed to another social stressor the next day (n = 48) while being measured in the same way. Acute social stress was induced following standardized procedures. The markers investigated were self-reported positive and negative affect, heart rate, electrodermal activity, salivary cortisol, and ten inflammatory markers both in capillary plasma and salivary samples, including IL-22 which has not been studied in response to acute stress in humans before. Robust effects (significant effect in the same direction for both days) were found for self-reported negative affect, heart rate, electrodermal activity, plasma IL-5, plasma IL-22, salivary IL-8 and salivary IL-10. Of these seven markers, the participants' IL-22 responses on the first day were positively correlated to those on the second day. We found no correlations between salivary and capillary plasma stress responses for any of the ten cytokines and somewhat unexpectedly, cytokine responses in saliva seemed more pronounced and more in line with previous literature than cytokines in capillary plasma. In sum, seven robust and easy to obtain biomarkers to measure acute stress response were identified and should be used in future stress research to detect and examine stress reactivity. This includes IL-22 in plasma as a promising novel marker.

1. Introduction

Acute stress protocols combined with sensitive, robust, and easy-to-obtain markers of stress reactivity are needed in a range of (research) areas. Application areas include the selection or evaluation of high-risk professionals, research on the relation between stress reactivity and health (Allen et al., 2017; Turner et al., 2020), and assessment of the effectiveness of interventions to reduce stress levels or increase stress resilience (Morton et al., 2020; Mücke et al., 2018). Acute stress induction protocols, such as the Trier Social Stress Test (TSST), affect a variety of pathways and responses, including the hypothalamic-pituitary-adrenal (HPA) axis, the autonomic nervous system (ANS), immune responses, cognitive responses, and self-reported emotional responses (Allen, et al., 2014; Campbell and Ehlert, 2012; Dickerson and Kemeny, 2004; Marsland et al., 2017). This expansive response pattern is best explained by viewing the underlying mechanisms of the stress response. Brain systems including areas of the prefrontal cortex, the amygdala and hippocampus are instrumental in determining if environmental stimuli are threatening or excessively demanding, hence stressful (McEwen and Gianaros, 2010; Haykin and Rolls, 2021). The sympathetic nervous system (SNS) is activated immediately following stress recognition by the brain, whereas the HPA axis activates more gradually, with cortisol levels peaking around 20–30 min post stressor onset (Dickerson and Kemeny, 2004; Sapolsky et al., 2000). Activation of the SNS is thought to mediate increases of pro-inflammatory cytokines (e.g., IL-1β, TNF-α) following acute stress, through the release of catecholamines and the activation of nuclear factor kB (NF-kB) (Bierhaus et al., 2003; Sapolsky et al., 2000). Acute stress also tends to stimulate circulating levels of cytokines with more anti-inflammatory (IL-10) or mixed (IL-6) properties, which may restore

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the inflammatory response. Furthermore, a delayed cortisol response subsequently decreases cytokine production, through glucocorticoid receptor-mediated down regulation of NF-kB (Lieberman, 2007; Sapolsky et al., 2000). From an evolutionary perspective it is proposed that these SNS and HPA-axis pathways function together to orchestrate adaptive peripheral inflammatory responses, to initially protect the organism from immediate injury or infection but to subsequently limit metabolic costs of prolonged inflammatory responses, respectively (Dhabhar, 2014; Segerstrom and Miller, 2004).

In modern societies the origins of stress that most human beings experience are different from those experienced by our ancient ancestors. Common threats that currently cause stress often relate to a person's self-esteem or social status. Especially when these threats are uncontrollable and challenge the social self (e.g., risk of negative social evaluation or embarrassment) they elicit psychological and physiological responses (Dickerson and Kemeny, 2004). Stress tests such as the Trier Social Stress Test (TSST) and the Sing a Song Stress Test (SSST) include these elements of uncontrollable threats that are challenging the social self and are frequently used to study acute stress responses in a standardized and controlled way (Allen et al., 2014; Brouwer and Hogervorst, 2014). Below we shortly discuss previous research on ANS, HPA and immune responses to such acute social stressors.

1.1. ANS responses to acute stress – HR and EDA

The heart is innervated by the SNS, where heart rate (HR) increases with increasing activity of the SNS, though effects are also mediated by the parasympathetic nervous system (PNS) which innervates the heart as well (Randall, 1994). Electrodermal activity (EDA) or skin conductance reflects the activity of sweat glands which are exclusively innervated by the SNS (Dawson et al., 2007). Acute social stress tests have been shown to elicit increases in both HR and EDA across a range of studies (Campbell and Ehlert, 2012; Man et al., 2023; Brouwer and Hogervorst, 2014; van der Mee et al., 2020; Mathissen et al., 2022; Toet et al., 2017).

1.2. HPA responses to acute stress - cortisol

Cortisol levels can be reliably assessed in either plasma or saliva. Plasma contains protein-bound cortisol as well as biologically active free cortisol (unbound). Salivary samples contain mainly free cortisol. Acute stress tasks containing both uncontrollable and social-evaluative elements have been shown to consistently elicit cortisol responses (Dickerson and Kemeny, 2004), irrespective of sampling method (plasma or saliva). Strongest increases are typically observed 20–40 min after stressor onset.

1.3. Immune responses to acute stress – cytokines in plasma and saliva

Cytokine responses to acute stress may be assessed in plasma or saliva. Compared to sampling plasma, saliva is less invasive, safer, cheaper, and less burdensome to collect, making saliva a desirable biosample for the assessment of inflammatory biomarkers (Szabo et al., 2020; Szabo and Slavish, 2021). Across studies of which the majority included healthy volunteers, systematic reviews and meta-analyses show acute stress-induced increases of IL-1β, IL-2, IL-6, IL-10 and TNF-α in plasma (Marsland et al., 2017) and increases of IL-1β, IL-6, IL-8, IL-10, and TNF-α in saliva (Szabo et al., 2020). Though different cytokines each have their own temporal dynamics, largest increases in plasma IL-1β, IL-2, TNF-α, or IL-10 have been observed 40–50 min after stress (Marsland et al., 2017). Increases in IL-6 were also evident 40–50 min after stress, but the response was more prolonged with largest increases seen at 90–120 min. In saliva, peak effect sizes were observed approximately 50 min (IL-1β, IL-6), 66 min (TNF-α), 85 min (IL-8), and 100 min (IL-10) after stressor onset (Szabo et al., 2020). Traditionally, plasma is collected by venipuncture. Capillary plasma obtained by a prick in the finger or ear may be a viable and less invasive alternative. Cullen et al. (2015) and Reichel et al. (2023) reported cytokine (IL-6) responses in both capillary plasma and venous plasma to physical exercise. They found responses to be correlated, be it weakly. However, there is a lack of studies on acute social stress and inflammatory responses in capillary plasma. We are not aware of any studies that compared immune responses to acute social stressors in capillary plasma with saliva.

1.4. Assessing ANS, HPA and immune markers in acute stress tests

While there is evidence for the sensitivity of a range of biomarkers from different domains to acute stress as described above, they have been recorded across a number of studies varying in the exact stress induction protocol, participant population and sample size. This makes it difficult to compare their sensitivity (Campbell and Ehlert, 2012; Man et al., 2023). With the present study we address this gap. Specifically, we.

1) Provide an overview of the stress response of a range of relatively easily obtained biomarkers from different domains to an acute social stressor (n = 101). These include inflammatory responses in capillary plasma, and the more novel inflammatory marker IL-22 (Bottenheft et al., 2023). To get an impression of the robustness of the investigated markers we examined their responses to a different type of acute social stressor, which was administered one day later to about half of the participants.
2) Check for possible correlations of acute cytokine stress responses in saliva and capillary plasma.
3) For the markers that showed a consistent response on both days, we investigated putative associations between responses in day 1 and day 2 over participants, providing a further indication of biomarker robustness.

2. Material and methods

The data reported here are part of a larger two-day study that is described in greater detail by Bottenheft et al. (2023) and Stuldreher et al. (2023).

2.1. Participants

Approval for the study was granted by an accredited medical research ethics committee (MREC Brabant, reference number: P2045, approval number NL74961.028.20). Prior to study start, all participants gave written informed consent.

One hundred and one participants took part in the study. Exclusion criteria were: smoking, drugs use in the last three months, signs of flue or viral infection in the last ten days, pregnancy, history of psychiatric illness, including sleep disorders, autoimmune disease and/or hyperactive thyroid and known heart, kidney or liver disease or neurological complaints. BMI ranged from 18 to 30 kg/m2. Ages ranged from 19 to 55 years old (M = 28.5, SD = 10.3). Participants were instructed to not consume any caffeine containing substances (e.g. coffee, chocolate, tea) from 6 p.m. the night prior to the day of the experiment. Of the 101 participants, 48 were randomly selected and invited to repeat the experiment on the next day with a different social stress test.

2.2. Materials

2.2.1. Social stress tests

On day one, stress was induced using the Sing-a-Song Stress Test (SSST) developed by Brouwer and Hogervorst (2014). In the presence of an experimental leader and a camera, participants were instructed to sit still in front of a laptop showing neutral messages. These messages lasted for 12 s and were followed by a counter counting down from 60 to 0 s. The last message was an instruction to remain sitting still for the

subsequent countdown interval, but to sing a song for 20 s directly after the counter reached zero, and that the video recording of their singing performance would be evaluated by music professionals. The message that the video was recorded only served the purpose to increase stress. Video recordings were not actually made.

On day two, stress was induced using the Trier Social Stress Test (TSST) (Kirschbaum et al., 1993). Before undergoing the stress test, a relaxation period took place to ensure minimum stress. The test consisted of three phases, each lasting 5 min: 1) anticipation, 2) presentation, and 3) mental arithmetic. The first phase consisted of anticipatory stress caused by the instruction to prepare a 5 min presentation for a job interview. Participants were instructed to talk about their own personality and convince a jury that they were the best candidate for the job. The participants were told that during the presentation, a camera would be present and that their taped performance would be judged by experts (adapted from Yim et al., 2010). During the second phase, the participant gave the presentation in front of a one-person jury. The jury was trained to maintain neutral expressions throughout the test and to instruct the participant to keep talking for 5 min. Participants were told that the jury would not answer questions or give feedback. The participants were oriented towards the camera. The participants did not know that this was part of the social stressor and that no video recordings were made. In the third phase, the participants responded verbally to a challenging arithmetic problem in the presence of the same jury.

2.2.2. Self-ratings of affect

Subjective affect was rated through the Positive and Negative Affect Scale (PANAS; Watson et al., 1988). The PANAS consists of the question "To what extent do you now feel … ?" followed by ten negative and ten positive emotions. Participants indicate to what extent they feel affiliated to that emotion on a five point Likert-scale, ranging from "Very slightly or not at all" (1) to "Extremely" (5). Outcomes are two total scores, one for positive affect (PA) and one for negative affect (NA), ranging from 10 (very low) to 50 (very high). The PANAS was administered twice: approximately 10 min before the stress test and directly after the stress test.

2.2.3. Autonomic nervous system: recording and processing HR and EDA

Heart rate (HR) data was obtained using an HR monitor with chest strap (TICKR heart rate monitor, Wahoo Fitness LLC, Atlanta, Georgia, USA). The HR monitor was connected via Bluetooth to a Wahoo Fitness Workout Tracker application (version 1.33.0.115) on an Android mobile phone for data collection. Electrodermal activity (EDA) responses were measured using a wrist band (EdaMove4, Movisens GmbH, Karlsruhe, Germany) through two disposable electrodes placed on the palm of the non-dominant hand of the participant.

EDA was processed to obtain the fast-changing phasic part, also referred to as skin conductance response (SCR), using Ledalab for MATLAB (Benedek and Kaernbach, 2010). This phasic response is considered to be an index of sympathetic nervous system activity (Benedek and Kaernbach, 2010; Boucsein, 2012). Mean HR and SCR pre-stress values were computed over the 3-min rest period prior to the SSST and TSST. Post-stress values were defined as the mean HR and SCR computed over the 1 min after stress onset (i.e. the countdown interval after the announcement that they had to sing a song in the SSST, and the anticipation phase in TSST).

2.2.4. Saliva sampling for cortisol and inflammatory markers

Saliva was collected three times by passive drool (SalivaBio Saliva Collection Aid, Salimetrics, USA): approximately 10 min before the stressor and 15 min and 30 min after stress onset (as recommended by Shields, 2020 for characterizing cortisol reactivity). The obtained samples were immediately stored at −20 °C and, at the end of each test day, moved to a −70 °C freezer for further storage until use in assays. Salivary cortisol was determined using assay number #KGE008B (R&D Systems, Abingdon, United Kingdom). Based on Pruessner et al. (2003) the 'Area Under the Curve with respect to increase' (AUC_i) as a measure of cortisol stress response was computed:

$$AUC_i = \left(\sum_{i=1}^{n-1} \left(\frac{(m_{(i+1)} + m_i)}{2} \right) \right) - (n-1) \bullet m_1$$

with m_i meaning the individual measurements and n denoting the total amount of measurements.

The first and third saliva samples were also used to determine levels of IFN-γ, IL-1β, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p70, IL-22, and TNF-α (Human 10plex Cytokine Panel 1, Quanterix, Billerica, USA).

2.2.5. Plasma sampling for inflammatory markers

In line with the study aim to define an easy-to-measure set of biomarkers, capillary plasma was obtained by finger prick instead of plasma collected by venipuncture. Capillary blood (130 μL) was collected twice by a finger prick: approximately 5 min before and 50 min after stress onset (Shields, 2020). The test leader collected capillary blood according to the protocol developed by McDade (2014). Blood samples were collected in EDTA-coated vials, put on ice and immediately centrifuged at 4 °C (14,000 rpm for 15 min). Obtained EDTA plasma was stored at −70 °C until use. The frozen samples were used for biomarker analysis following protocols described in previous literature (Schutte et al., 2022; Vreeken et al., 2022). This included an assessment of systemic inflammation using the same cytokine multiplex panel as for the saliva samples.

2.3. Procedure

During a training visit several days before the study, participants were informed about the outline and procedure of the study. They were not informed about the social stress tests. Participants arrived at 08:00 a.m. at the test location on the first testing day. After explanation of the procedure, the sensors for EDA and HR measurement were attached. After participants performed cognitive tasks (not reported here), they rested for 15 min, followed by taking the first saliva sample and filling in the PANAS questionnaire. Next capillary blood samples were collected by using a finger prick, followed by a 5 min rest period. Then the SSST started. Directly after the stress test a second subjective affect rating was collected, followed by saliva samples 15 and 30 min after stress onset and a second blood sample 50 min after stress onset. The procedure for day two was the same, except for the use of the TSST instead of the SSST.

2.4. Statistics

Data analysis was carried out using R statistics v4.3.1 (R Core Team, 2020). Wilcoxon Matched-Pairs Signed Rank tests were used to determine the effect of the stressor by comparing the value after the stressor to that before the stressor. This was done for all variables, except for cortisol AUC_i, for which a one sample Wilcoxon test was used to determine whether the AUC was larger than 0. Before testing, outliers were removed, where outliers are defined as values lower than Q1-1.5*IQR or higher than Q3 + 1.5*IQR (Q1 = 25th percentile; Q3 = 75th percentile; IQR = interquartile range or the distance between the 25th to the 75th percentile). The tables in results section show, for each measure and each day, the amount of data included in the analyses. This corresponds to the number of participants (101 on day 1 and 48 on day 2) minus misses and excluded outliers, and associated pre- or post-stressor data required in pairwise comparisons. Concerning misses, for subjective data (PA/NA), data of 2 participants were not recorded on day 2. There was no recorded data due to failing sensors or failure to start data recording for 15 (HR) and 9 (SC) participants on day 1; and for 4 (HR) and 9 (SCR) participants on day 2. For plasma, we missed data caused by drawing an insufficient amount of blood. This happened for 4 participants on day 1, and for 3 participants on day 2.

Spearman rank correlations were used to explore correlations

between cytokine responses (post-minus pre-stressor value) in plasma versus saliva for each of the 10 cytokines and each of the 2 days.

For the markers that showed significant stress responses in the same direction on day 1 and day 2 as indicated by the Wilcoxon tests (i.e., markers that show robustness), as a further test of robustness within individuals, we tested for spearman rank correlations between responses (post-minus pre-stressor value) on day 1 and day 2.

All statistical tests were performed at a significance level of alpha = 0.05.

3. Results

3.1. Self-reported affect, ANS, and cortisol responses to acute stress

Fig. 1 shows pre- and post-stressor values for affect and ANS variables, and cortisol AUC_i on day 1 (n = 101) and day 2 (n = 48, subset of day 1 sample). Table 1 shows the results of Wilcoxon Matched-Pairs Signed Rank tests, indicating the statistical significance of the stress responses. Day 1 did not show a change in PA, whereas the stress test on the second day led to a significant increase in PA, rather than the possibly expected decrease. Both days resulted in significant increases in NA. HR and SCR significantly increased in response to stress, on both day 1 and 2. For both stress tests, the cortisol AUC_i was not significantly different from zero.

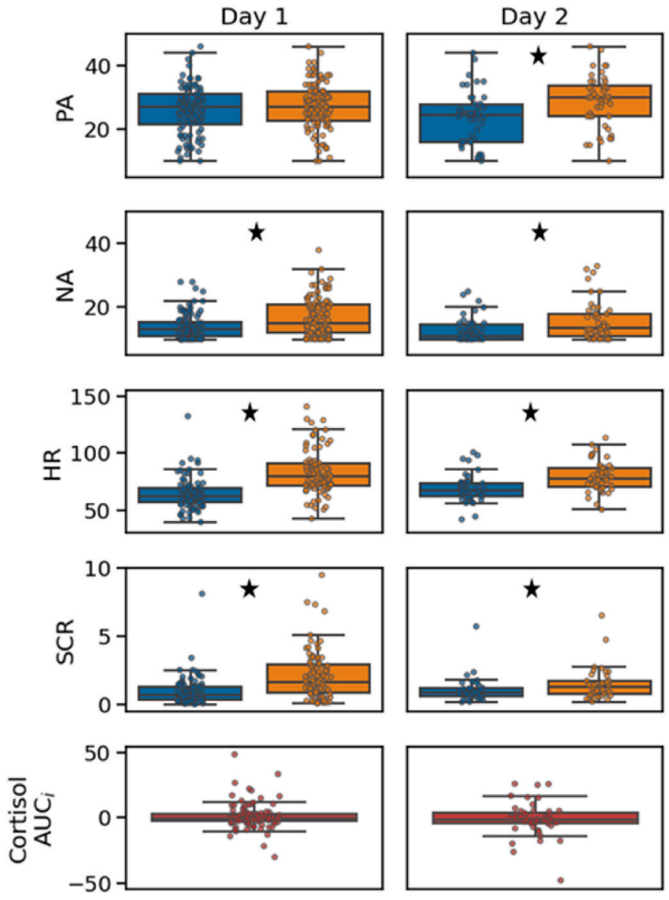

Fig. 1. Pre- and post-stressor values (coded in respectively blue and orange) of affect and ANS variables, and cortisol AUC in response to the acute stress test, for day 1 (left) and day 2 (right). Significant (within-participant) differences between pre- and post-stressor values (see Table 1) are indicated by stars. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 1
Wilcoxon test outcomes of changes in positive and negative affect, ANS variables, and cortisol in response to the acute stress test at day 1 and 2. Significant effects are printed in bold with a + indicating that the value after the stressor was higher than before the stressor. Significant effects in the same direction on both days are highlighted in gray.

	Day 1 (all)		Day 2 (subset)	
	n	p	n	p
PA	101	0.13	46	**< 0.001+**
NA	101	**< 0.001+**	46	**0.004+**
HR	79	**< 0.001+**	38	**< 0.001+**
SCR	82	**< 0.001+**	34	**0.001+**
Cortisol AUC_i	87	0.512	39	0.521

PA: Positive affect; NA: Negative Affect; HR: Heart rate; SCR: skin conductance response; amplitude AUC_i: Area under the curve with respect to increase.

3.2. Cytokine responses to acute stress

Fig. 2 shows pre- and post-stressor values for cytokines in plasma and saliva on day 1 and day 2. Table 2 shows the results of Wilcoxon Matched-Pairs Signed Rank tests, indicating the statistical significance of the stress responses. For many cytokines, especially in plasma, statistically significant effects as summarized in the table are not immediately obvious from the graphical illustrations of their absolute concentrations in Fig. 2. The boxplots illustrate the large biological variability that exists regarding initial setpoints (i.e. absolute concentrations at start) between participants, yet paired comparisons resulted in significant differences between pre- and post-levels for a number of markers.

In saliva, levels of IL-8 and IL-10 increased significantly both on day 1 and day 2 in response to the stressor. Levels of IL-6 increased significantly on day 1, but decreased significantly on day 2 in response to stress. IFN-γ increased on day 1, but there was no effect on day 2. Finally, acute stress led to significant increases in levels of IL-1β, IL-4, IL-22, and TNF-α, on day 2 but not day 1.

In plasma, levels of IL-5, IL-10, IL12p70 and IL-22 decreased significantly in response to the stressor on day 1. Such a decrease was replicated on day 2 for IL-5 and IL-22. Levels of IFN-γ decreased significantly on day 2, but there was no effect on day 1.

3.3. Cytokine responses: plasma vs saliva

The results presented in Table 2 indicate that cytokines do not respond to a stressor in similar ways in plasma as in saliva. None of the 10 cytokines show a significant effect in the same direction in plasma as in saliva, for either of the two days. This apparent difference in responses is supported by the absence of positive rank correlations between the cytokine responses in plasma and saliva.

3.4. Robust responses: day 1 vs day 2

As highlighted in Table 1 and Table 2, we found significant effects in the same direction in response to the acute stress tests on both days for the following variables: NA, HR, SCR, plasma IL-5, plasma IL-22, saliva IL-8 and saliva IL-10. To explore whether these robust responses were also similar at the level of individual participants, we analyzed the responses (i.e. post-minus pre-stressor values on an individual participant level) at day 1 against day 2 using Spearman correlations. These analyses indicated that for plasma IL-22, individuals showed similar acute social stress responses on day 1 compared to day 2 ($r_s = 0.47$, $p < 0.01$). There was no significant rank correlation for the other variables.

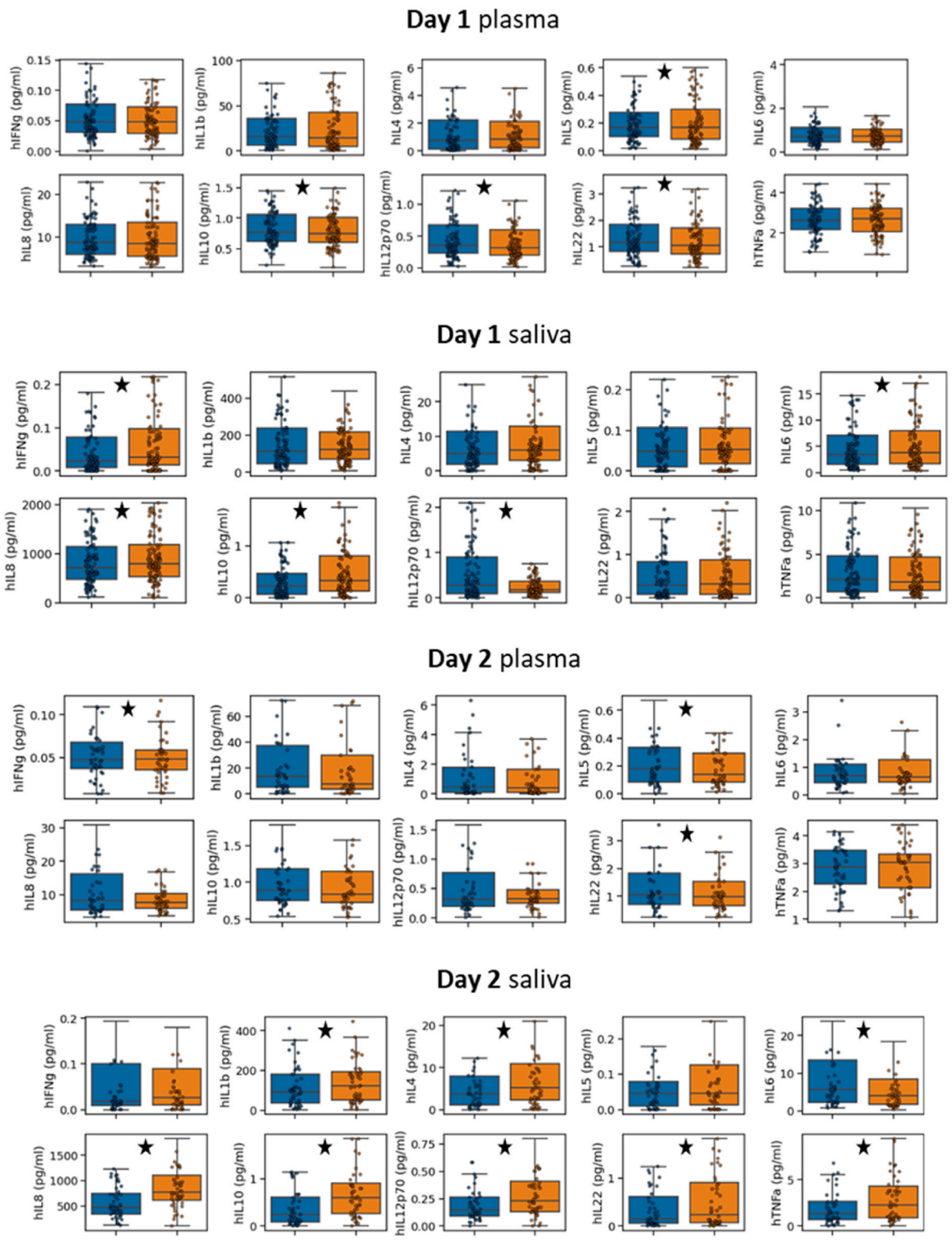

Fig. 2. Pre- and post-stressor values (coded in respectively blue and orange) for each of the ten cytokines in plasma and saliva on day 1 and day 2. Significant (within-participant) differences between pre- and post-stressor values (see Table 2) are indicated by stars. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

4. Discussion

The present study was designed to identify a sensitive, robust and easy-to-obtain set of markers to assess acute stress responses. Using standardized social stress induction protocols on two consecutive test days, robust responses were observed for HR, SCR, plasma IL-5 and IL-22, salivary IL-8 and IL-10 and self-reported negative affect.

The observed effect of social stress on positive affect (PA) on day 2, and the absence of an effect on cortisol may be unexpected. However, the increase rather than decrease in PA on day 2 has also been reported in other studies using TSST (Man et al., 2023). With respect to cortisol, we observed that AUCi values were not significantly different from zero

Table 2
Wilcoxon test outcomes of changes in cytokines for plasma and saliva in response to the acute stress test at day 1 and 2. Significant effects are printed in bold, and the direction of the effect is indicated with a + (higher value after acute stress than before) or a – (the reverse). Significant effects in the same direction on both days are highlighted in gray.

	Day 1				Day 2			
	Plasma		Saliva		Plasma		Saliva	
	n	p	n	p	n	p	n	p
IFN-γ	87	0.248	85	**0.001+**	41	**0.010-**	37	0.272
IL-1β	81	0.864	89	0.525	38	0.576	47	**0.044+**
IL-4	78	0.624	82	0.125	39	0.411	41	**0.001+**
IL-5	83	**0.015-**	82	0.615	39	**0.003-**	38	0.668
IL-6	82	0.185	88	**0.022+**	38	0.966	37	**0.000-**
IL-8	88	0.619	95	**0.027+**	36	0.423	43	**0.000+**
IL-10	89	**0.000-**	89	**0.000+**	41	0.291	44	**0.000+**
IL-12p70	82	**0.034-**	89	**0.009-**	35	0.273	42	**0.001+**
IL-22	87	**0.000-**	91	0.536	38	**0.018-**	43	**0.015+**
TNF-α	86	0.809	92	0.420	42	0.233	45	**0.000+**

on both days. In their meta-analysis and review, Dickerson and Kemeny (2004) found that the majority of acute social stress studies showed cortisol increases 15–30 min after stress onset. However, they also report that studies conducted in the morning, like our study, show significantly smaller effects than studies conducted in the afternoon, with an overall three times smaller effect size. Dickerson and Kemeny explain this finding by the effects of cortisol diurnal variation - in the morning cortisol levels naturally decrease which could make it more difficult to detect a cortisol response than in the afternoon, when levels are relatively stable. Additionally, our social stress test on the first day, the SSST, was of very short duration and in that sense may have been milder than the TSST that was used in research reviewed by Dickerson and Kemeny (2004). Furthermore, our participants performed the TSST on day 2, after they had been exposed to a social stress test the day before, which may have reduced the level of stress caused by the TSST. Given the detectable effects in other markers to the possibly relatively mild stressors, the observed lack in a cortisol response suggests that salivary cortisol measured in the morning is not a very sensitive marker.

We evaluated the responses to stress of 10 different inflammatory cytokines, both in capillary plasma and in saliva. Our findings in saliva are in agreement with a systematic review and meta-analysis by Szabo et al. (2020) who reported increases of IL-1β, IL-6, IL-8, IL-10, and TNF-α in saliva in response to acute stress. All of these cytokines increased significantly after the stressor on day 2, except for IL-6, which showed a statistically significant decrease. On day 1, three of these five cytokines increased (IL-6, IL-8 and IL-10). Although the majority of samples included in the meta-analysis by Szabo al. (2020) report stress-induced increases in IL-6 from saliva, a quarter of the samples reported negative effect sizes. Their analysis pointed out that IL-6 effects are heterogeneous, which is in line the opposite responses we find on day 1 and day 2. Our results in capillary plasma however are at odds with prior venous plasma research. In contrast to a systematic review and meta-analysis (Marsland et al., 2017), we did not find acute stress-induced increases in IL-1β, IL-6, IL-10 and TNF-α on either day. Stress-induced activation of the ANS - which was clearly observed in the increases in HR and SCR in the present study - is thought to mediate increases of pro-inflammatory cytokines (e.g., IL-1β, TNF-α) as well as anti-inflammatory cytokines (e.g., IL-10 and IL-6; Bierhaus et al., 2003; Lieberman, 2007; Sapolsky et al., 2000). In the present study we did not see such changes in plasma. We did however observe consistent reductions (at both days) for IL-5 and IL-22. The discrepancy between our study and previous ones may be explained by the different plasma sampling method. Despite previous research showing some correspondence between cytokine responses in venous and capillary plasma samples (Cullen et al., 2015; Reichel et al., 2023) to physical exercise, this difference in plasma sampling may underly the discrepancy with previous research on cytokine responses to acute social stress. The present study was focused on easy-to-obtain markers and did not include venous plasma sampling, hence this possible explanation remains open. Regardless of the sampling method, it is worth noting that although we observed no change in levels of IL-6 and TNF-α, a closer look at the studies included in the meta-analysis of Marsland et al. (2017) reveals that 13 out of the 28 studies in healthy subjects showed no significant change in IL-6 and even 8 out of the 10 studies in healthy subjects showed no significant change in TNF-α. This illustrates considerable heterogeneity across studies and that inductions of cytokines are less consistent than often assumed even when the sampling strategy was comparable, i.e. when venous plasma was used to determine cytokine responses to stress.

As may be expected by the differential results of acute social stress effects on inflammatory markers in capillary plasma and saliva, we did not find correlations between responses in capillary plasma and saliva for the different cytokines. Though some methodological choices in bio sampling may partly account for this (see limitations below), these findings, at least in part, suggest that that these cytokines are a result of different biological processes or have distinct temporal patterns in the two compartments.

IL-22 has not been studied in response to acute stress in humans before, and the robust IL-22 reduction in response to acute social stress (on both days, and robustly within individuals) is a new finding. IL-22 is a member of the IL-10 family and a pleiotropic cytokine that exhibits both proinflammatory and anti-inflammatory effects. It is critically involved in the control of epithelial homeostasis, epithelial barrier function and host protection (Keir et al., 2020). Its effects depend on the tissue microenvironment including the cytokine environment (Alabbas et al., 2018). IL-22 has been observed in both mice and humans to influence the production of other proinflammatory cytokines, including IL-6, IL-8 and TNF-α, to coordinate the inflammatory response (Andoh et al., 2005; Kong et al., 2012). Studies in mice showed that chronic stress stimulated IL-22 production in intestinal immune cells (Gomez-Nguyen et al., 2022) and plasma levels (Ilanges et al., 2022, preprint). Furthermore, IL-22 treatment increased resilience to anxiety like behavior in chronically stressed mice and it was proposed that IL-22 plays a role in an immune-to-brain axis to mitigate neuro-behavioral consequences of chronic stress (Ilanges et al., 2022, preprint). Mice deficient in IL-22 are sensitive to pathogens and infections, and have an exacerbated disease in comparison with wild-type mice that express IL-22 (Keir et al., 2020) suggesting that the observed reduction in IL-22 in response to acute stress is rather detrimental. Bottenheft et al. (2023) found a reduction of IL-22 in response to one night of sleep deprivation. The mechanism how an acute stressor causes a reduction in IL-22, as observed in the present study, remains unclear. Given the limited

knowledge on the role of IL-22 in stress reactivity, further research in humans is needed on this topic.

4.1. Limitations and future work

We explored correlations between cytokine responses in capillary plasma versus saliva, none of which were significant. It is possible that some limitations in plasma sampling may have contributed to these null-findings. First, as the present study was focused on easy-to-obtain markers we included capillary plasma but did not include venous plasma sampling. The difference in plasma sampling may underly the discrepancy with previous research on cytokine responses to acute social stress. Future studies are needed to shed more light on this. Second, to reduce the burden of plasma collection for the participant, we sampled blood only at a single post-stress timepoint, at approximately 50 min post-stressor onset, to measure cytokine responses. Most of the included cytokines have been reported to significantly increase at that timepoint (Marsland et al., 2017; Szabo et al., 2020), but future studies should be cautious and sample more frequently because there are considerable variations among the individual studies used in the meta-analysis of Marsland et al. It should therefore be concluded that the exact temporal dynamics of most cytokines in response to stress are not precisely known for all cytokines that we included, with practically no information on IL-22, and our sampling timing may have been sub-optimal for a part of them. In addition, differences in temporal dynamics likely exist between venous and capillary sampled plasma. Comprehensive dynamical cytokine profiling studies (preferably sampling venous, capillary blood and saliva) would help researchers to design their studies most optimally. In our study, timing of saliva sampling was in first place optimized for cortisol detection. It is therefore worth noting that we observed increases in salivary IL-8 and IL-10 on both days, despite that their peak effects have been reported by others to occur later, namely 85 min and 100 min after stressor onset, respectively (Szabo et al., 2020).

A strength of our study is that we recorded responses in a large amount of variables in the same individuals, to the same stressor, so that they can be compared. However, a limitation that comes with recording many variables is the problem of multiple comparisons. We found that 12 out of 25 variables responded to stress on day 1, and 15 out of 25 on day 2 which is considerably more than expected by chance. We used the consistency in stress responses on both days to deal with the multiple comparisons problem, resulting in the list of seven robust markers.

4.2. Conclusion

Measuring social stress responses in a wide range of modalities simultaneously, for two different stress tests, allowed us to identify a set of robust, relatively easy to obtain markers of acute social stress: self-reported negative affect, HR, SCR, capillary plasma IL-5 and IL-22, and salivary IL-8 and IL-10. We are the first to show the effect of acute stress on IL-22 in humans. We advise future studies on stress resilience, e.g. studies that test for effects of stress reducing interventions or selection of stress resilient individuals, to include these measures. Future studies on differences between capillary and venous plasma, as well as studies on the exact time course of effects in different populations and at different points during the day are required to further optimize the identified set of markers.

5. Ethics statement

The studies involving human participants were reviewed and approved by the MREC Brabant (reference number P2045, approval number NL74961.028.20). The participants provided their written informed consent to participate in this study.

CRediT authorship contribution statement

Koen Hogenelst: Writing – review & editing, Writing – original draft, Methodology, Investigation, Conceptualization. **Serdar Özsezen:** Visualization, Methodology, Formal analysis, Data curation. **Robert Kleemann:** Writing – review & editing, Supervision, Methodology, Funding acquisition. **Lars Verschuren:** Writing – review & editing, Methodology. **Ivo Stuldreher:** Writing – review & editing, Software, Methodology, Investigation, Formal analysis, Data curation. **Charelle Bottenheft:** Writing – review & editing, Methodology, Investigation, Data curation, Conceptualization. **Jan van Erp:** Writing – review & editing, Funding acquisition, Conceptualization. **Anne-Marie Brouwer:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Conceptualization.

Declaration of competing interest

The authors report there are no competing interests to declare.

Data availability

The authors do not have permission to share data.

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References

Alabbas, S.Y., Begun, J., Florin, T.H., Oancea, I., 2018. The role of IL-22 in the resolution of sterile and nonsterile inflammation. Apr 18 Clin Transl Immunology 7 (4), e1017. https://doi.org/10.1002/cti2.1017. PMID: 29713472; PMCID: PMC5905349.

Allen, A.P., Kennedy, P.J., Dockray, S., Cryan, J.F., Dinan, T.G., Clarke, G., 2017. The trier social stress test: Principles and practice. Neurobiology of Stress 6, 113–126.

Allen, A.P., Kennedy, P.J., Cryan, J.F., Dinan, T.G., Clarke, G., 2014. Biological and psychological markers of stress in humans: focus on the Trier Social Stress Test. Neurosci. Biobehav. Rev. 38, 94–124.

Andoh, A., Zhang, Z., Inatomi, O., Fujino, S., Deguchi, Y., Araki, Y., Tsujikawa, T., Kitoh, K., Kim-Mitsuyama, S., Takayanagi, A., Shimizu, N., Fujiyama, Y., 2005. Interleukin-22, a member of the IL-10 subfamily, induces inflammatory responses in colonic subepithelial myofibroblasts. Sep Gastroenterology 129 (3), 969–984. https://doi.org/10.1053/j.gastro.2005.06.071. PMID: 16143135.

Benedek, M., Kaernbach, C., 2010. A continuous measure of phasic electrodermal activity. J. Neurosci. Methods 190 (1), 80–91. https://doi.org/10.1016/j.jneumeth.2010.04.028.

Bierhaus, A., Wolf, J., Andrassy, M., Rohleder, N., Humpert, P.M., Petrov, D., Ferstl, R., von Eynatten, M., Wendt, T., Rudofsky, G., Joswig, M., Morcos, M., Schwaninger, M., McEwen, B., Kirschbaum, C., Nawroth, P.P., 2003. A mechanism converting psychosocial stress into mononuclear cell activation. Feb 18 Proc Natl Acad Sci U S A 100 (4), 1920–1925. https://doi.org/10.1073/pnas.0438019100. Epub 2003 Feb 10. PMID: 12578963; PMCID: PMC149934.

Bottenheft, C., Hogenelst, K., Stuldreher, I., Kleemann, R., Groen, E., van Erp, J., Brouwer, A.M., 2023. Understanding the combined effects of sleep deprivation and acute social stress on cognitive performance using a comprehensive approach. Brain, Behavior, & Immunity-Health, 100706.

Boucsein, W., 2012. Electrodermal Activity, second ed. Springer, New York. https://doi.org/10.1007/978-1-4614-1126-0. 2012.

Brouwer, A.M., Hogervorst, M.A., 2014. A new paradigm to induce mental stress: the Sing-a-Song Stress Test (SSST). Front. Neurosci. 8 (8 JUL), 1–8. https://doi.org/10.3389/fnins.2014.00224.

Campbell, J., Ehlert, U., 2012. Acute psychosocial stress: does the emotional stress response correspond with physiological responses? Psychoneuroendocrinology 37 (8), 1111–1134. https://doi.org/10.1016/j.psyneuen.2011.12.010. Epub 2012 Jan 18. PMID: 22260938.

Cullen, T., Thomas, A.W., Webb, R., Hughes, M.G., 2015. The relationship between interleukin-6 insaliva, venous and capillary plasma, at rest and in response to exercise. Cytokine. https://doi.org/10.1016/j.cyto.2014.10.011.

Dawson, M.E., Schell, A.M., Filion, D.L., 2007. The electrodermal system. In: Cacioppo, J. T., Tassinary, L.G., Berntson, G.G. (Eds.), Handbook of Psychophysiology. Cambridge University Press, Cambridge.

Dhabhar, F.S., 2014. Effects of stress on immune function: the good, the bad, and the beautiful. May Immunol. Res. 58 (2–3), 193–210. https://doi.org/10.1007/s12026-014-8517-0. PMID: 24798553.

Dickerson, S.S., Kemeny, M.E., 2004. Acute stressors and cortisol responses: a theoretical integration and synthesis of laboratory research. Psychol. Bull. 130 (3), 355.

Gomez-Nguyen, A., Gupta, N., Sanaka, H., Gruszka, D., Pizarro, A., DiMartino, L., Basson, A., Menghini, P., Osme, A., DeSalvo, C., Pizarro, T., Cominelli, F., 2022. Chronic stress induces colonic tertiary lymphoid organ formation and protection against secondary injury through IL-23/IL-22 signaling. Oct 4 Proc. Natl. Acad. Sci. U.S.A. 119 (40), e2208160119. https://doi.org/10.1073/pnas.2208160119. Epub 2022 Sep 26. PMID: 36161939; PMCID: PMC9546604.

Haykin, H., Rolls, A., 2021. The neuroimmune response during stress: a physiological perspective. Immunity 54 (9), 1933–1947.

Ilanges, A., Xia, M., Lu, J., Chen, L., Shiao, R., Wang, C., Jin, Z., Feng, R., Qi, Q., Yi, H., Li, J., Schneeberger, M., Lu, B., Friedman, J.M., Yu, X., 2022. Microbiota-stimulated Interleukin-22 regulates brain neurons and protects against stress-induced anxiety. bioRxiv. https://doi.org/10.1101/2022.09.16.508224, 2022.09.16.508224.

Keir, M., Yi, Y., Lu, T., Ghilardi, N., 2020. The role of IL-22 in intestinal health and disease. Feb 13 J. Exp. Med. 217 (3), e20192195. https://doi.org/10.1084/jem.20192195. PMID: 32997932; PMCID: PMC7062536.

Kirschbaum, C., Pirke, K.M., Hellhammer, D.H., 1993. The 'Trier Social Stress Test'–a tool for investigating psychobiological stress responses in a laboratory setting. Neuropsychobiology 28 (1–2), 76–81.

Kong, X., Feng, D., Wang, H., Hong, F., Bertola, A., Wang, F.S., Gao, B., 2012. Interleukin-22 induces hepatic stellate cell senescence and restricts liver fibrosis in mice. Sep Hepatology 56 (3), 1150–1159. https://doi.org/10.1002/hep.25744. Epub 2012 Jul 12. PMID: 22473749; PMCID: PMC3394879.

Lieberman, M.D., 2007. Social cognitive neuroscience: a review of core processes. Ann Rev Psychol 59, 259–289.

Man, I.S.C., Shao, R., Hou, W.K., Xin, Li S., Liu, F.Y., Lee, M., Wing, Y.K., Yau, S.Y., Lee, T.M.C., 2023. Multi-systemic evaluation of biological and emotional responses to the Trier Social Stress Test: a meta-analysis and systematic review. Jan Front. Neuroendocrinol. 68, 101050. https://doi.org/10.1016/j.yfrne.2022.101050. Epub 2022 Nov 18. PMID: 36410619.

Marsland, A.L., Walsh, C., Lockwood, K., John-Henderson, N.A., 2017. The effects of acute psychological stress on circulating and stimulated inflammatory markers: a systematic review and meta-analysis. Brain Behav. Immun. 64, 208–219. https://doi.org/10.1016/j.bbi.2017.01.011.

Mathissen, M., Hennes, N., Faller, F., Leonhardt, S., Teichmann, D., 2022. Investigation of three Potential stress Inducement tasks during on-Road Driving. IEEE Trans. Intell. Transport. Syst. 23 (5), 4823–4832. https://doi.org/10.1109/TITS.2021.3112811.

McEwen, B.S., Gianaros, P.J., 2010. Central role of the brain in stress and adaptation: links to socioeconomic status, health, and disease. Ann. N. Y. Acad. Sci. 1186 (1), 190–222.

McDade, T.W., 2014. Development and validation of assay protocols for use with dried blood spot samples. Am. J. Hum. Biol. : The Official Journal of the Human Biology Council 26 (1), 1–9. https://doi.org/10.1002/ajhb.22463.

Morton, M.L., Helminen, E.C., Felver, J.C., 2020. A systematic review of mindfulness interventions on psychophysiological responses to acute stress. Mindfulness 11, 2039–2054.

Mücke, M., Ludyga, S., Colledge, F., Gerber, M., 2018. Influence of regular physical activity and fitness on stress reactivity as measured with the trier social stress test protocol: a systematic review. Sports Med. 48, 2607–2622.

Pruessner, Jens C., Kirschbaum, C., Meinlschmid, G., Hellhammer, D.H., 2003. Two formulas for computation of the area under the curve represent measures of total hormone concentration versus time-dependent change. Psychoneuroendocrinology 28 (7), 916–931. https://doi.org/10.1016/S0306-4530(02)00108-7.

Randall, W.C., 1994. Efferent symphathetic innervation of the heart. In: Armour, J.A., Ardell, J.L. (Eds.), Neurocardiology. Oxford University Press, New York, pp. 77–94.

Reichel, T., Held, S., Schwarz, A., Hacker, S., Wesemann, F., Donath, L., Krüger, K., 2023. Acute response of biomarkers in plasma from capillary blood after a strenuous endurance exercise bout. Eur. J. Appl. Physiol. 123 (1), 179–189. https://doi.org/10.1007/s00421-022-05068-1. Epub 2022 Oct 13. PMID: 36227377; PMCID: PMC9813032.

Sapolsky, R.M., Romero, L.M., Munck, A.U., 2000. How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. Feb Endocr. Rev. 21 (1), 55–89. https://doi.org/10.1210/edrv.21.1.0389. PMID: 10696570.

Schutte, M.H., Kleemann, R., Nota, N.M., Wiepjes, C.M., Snabel, J.M., T'Sjoen, G., Thijs, A., Heijer, M. Den, 2022. The effect of transdermal gender-affirming hormone therapy on markers of inflammation and hemostasis. PLoS One 17 (3 March), 1–13. https://doi.org/10.1371/journal.pone.0261312.

Segerstrom, S.C., Miller, G.E., 2004. Psychological stress and the human immune system: a meta-analytic study of 30 years of inquiry. Jul Psychol. Bull. 130 (4), 601–630. https://doi.org/10.1037/0033-2909.130.4.601. PMID: 15250815; PMCID: PMC1361287.

Shields, G.S., 2020. Stress and cognition: a user's guide to designing and interpreting studies. Psychoneuroendocrinology 112, 104475.

Stuldreher, I.V., Maasland, E., Bottenheft, C., van Erp, J.B.F., Brouwer, A.-M., 2023. Physiological synchrony in electrodermal activity predicts decreased vigilant attention induced by sleep deprivation. Front. Neuroergon. 4, 1199347 https://doi.org/10.3389/fnrgo.2023.1199347.

Szabo, Y.Z., Slavish, D.C., Graham-Engeland, J.E., 2020. The effect of acute stress on salivary markers of inflammation: a systematic review and meta-analysis. Brain Behav. Immun. https://doi.org/10.1016/j.bbi.2020.04.078.

Szabo, Y.Z., Slavish, D.C., 2021. Measuring salivary markers of inflammation in health research: a review of methodological considerations and best practices. Psychoneuroendocrinology 124, 105069.

Toet, A., Bijlsma, M., Brouwer, A.-M., 2017. Stress response and facial trustworthiness judgements in civilians and military. Sage Open 7 (3), 1–11.

Turner, A.I., Smyth, N., Hall, S.J., Torres, S.J., Hussein, M., Jayasinghe, S.U., Ball, K., Clow, A.J., 2020. Psychological stress reactivity and future health and disease outcomes: a systematic review of prospective evidence. Psychoneuroendocrinology 114, 104599. https://doi.org/10.1016/j.psyneuen.2020.104599. Epub 2020 Feb 1. PMID: 32045797.

van der Mee, D.J., Duivestein, Q., Gevonden, M.J., Westerink, J.H.D.M., de Geus, E.J.C., 2020. The short Sing-a-Song Stress Test: a practical and valid test of autonomic responses induced by social-evaluative stress. Auton. Neurosci. 224, 102612.

Vreeken, D., Seidel, F., de La Roij, G., Vening, W., den Hengst, W.A., Verschuren, L., Özsezen, S., Kessels, R.P.C., Duering, M., Mutsaerts, H.J.M.M., Kleemann, R., Wiesmann, M., Hazebroek, E.J., Kiliaan, A.J., 2022. Impact of white Adipose tissue on brain Structure, Perfusion and cognitive function in Patients with Severe Obesity: the BARICO study. Neurology. https://doi.org/10.1212/wnl.0000000000201538.

Watson, D., Clark, L.A., Tellegen, A., 1988. Development and validation of brief measures of positive and negative affect: the PANAS scales. Journal of personality and social psychology 54 (6), 1063.

Yim, I.S., Quas, J.A., Cahill, L., Hayakawa, C.M., 2010. Children's and adults' salivary cortisol responses to an identical psychosocial laboratory stressor. Psychoneuroendocrinology 35 (2), 241–248.