The Energetic Stress Marker GDF15 is Induced by Acute Psychosocial Stress

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Abstract

GDF15 (growth differentiation factor 15) is a marker of cellular and mitochondrial energetic stress linked to physical-mental illness, aging, and mortality. Here, we describe the psychobiological regulation of plasma and saliva GDF15 in four human studies including 3,599 samples from 148 healthy individuals. We report two main observations establishing GDF15 as a novel tractable biomarker of psychosocial stress. 1) In two experimental laboratory studies, socio-evaluative stress rapidly elevates GDF15 and lactate, two molecular markers of energetic/reductive stress. 2) Similar to other stress-related metabolic hormones, we also find that saliva GDF15 exhibit a robust awakening response, being highest at the time of waking up and declining by ~42-92% within 30-45 minutes. These data position GDF15 as a dynamic biomarker of psychosocial stress accessible in human blood and saliva, pointing towards a shared psychobiological pathway linking mental and mitochondrial energetic stress. These foundational observations open the door to large-scale studies using GDF15 to non-invasively probe how acute psychosocial factors promote cellular and mitochondrial and energetic stress contributing to the stress-disease cascade across the lifespan.

Keywords: stress response; psychobiology; mitochondria; lactate; stress physiology

Significance statement

This study uncovers the dynamic psychobiological regulation of plasma and saliva GDF15, establishing it as a biomarker of acute psychosocial stress. Using over 3,500 samples we demonstrate that acute socio-evaluative stress increases within minutes both GDF15 and lactate, reflecting reductive stress. High-intensity sampling over multiple weeks also confirms novel dynamics around the saliva GDF15 awakening response. These findings highlight a connection between acute psychosocial stress and mitochondrial energetic stress, offering new opportunities to investigate how stress becomes biologically embedded and contributes to health trajectories across the human lifespan.

1. Introduction

The circulating metabokine-cytokine growth differentiation factor 15 (GDF15) is produced by various somatic tissues and cell types, secreted and transported in the blood, eventually reaching the hindbrain (and possibly other tissues) where it signals energetic stress to alter behavior and whole-body energy expenditure [1-5]. GDF15 fulfills several criteria as a marker of systemic energetic stress: 1) GDF15 is the most robustly elevated circulating protein with aging [6,7], 2) it is induced by some medications targeting mitochondria (e.g., metformin [8]), and 3) is elevated in several chronic physical and mental illnesses including cardiovascular disease [9], cancer [10], mood disorders [11], Alzheimer's disease [12,13] autoimmune diseases [14], and others. Clinically, blood GDF15 has emerged as the top discriminatory plasma protein to identify individuals with various current [15] and future clinical illnesses [13]. Moreover, circulating GDF15 is among the best prognostic mortality indicators in clinical populations and older adults [6,16], often predicting death and disability better than well-established disease-specific biomarkers. Thus, resolving GDF15 biology is of high scientific and clinical significance.

GDF15 is also secreted in response to various non-disease states. Blood GDF15 increases with nutritional and physiological stressors such as exhaustive exercise [17,18], pregnancy [2,19], multi-day starvation [20], and infectious challenges [21]. This secretion pattern aligns with the canonical, energy-mobilizing glucocorticoid and sympathetic hormonal pathways, with which GDF15 interacts [1,5,22], and may contribute to GDF15-mediated increase in energy expenditure [1,23]. Taken together, the rapidly growing body of literature suggests that GDF15 may play a role in systemic energy homeostasis, resilience, and health, calling for a refined understanding of its natural dynamics and reactivity to the types of challenges that punctuate daily human lives, including mental or psychosocial stressors.

The human mind is connected to every organ such that physiology is rapidly altered by real or perceived threats [24]. Simple stressful thoughts increase heart rate and blood pressure within seconds and alter metabolic/stress hormones within minutes [25]. In mice, evoked stress was recently shown to elevate blood lactate levels [26], a marker of *reductive stress* (high $NADH/NAD^+$ ratio) [27]. Reductive stress originates in mitochondria when energy flow is limited relative to cellular energy demands, which acts upstream of GDF15 by mechanistically activating the integrated stress response (ISR)-GDF15 cascade [27-29]. GDF15 secretion allows cells to communicate their energetic state with the rest of the organism, including to the brain where it signals a dual energy conservation and mobilization response promote adaptation and survival [3,30]. But whether mental stress is linked to GDF15 has not been examined.

Although most GDF15 research has focused on blood, preliminary evidence also suggested that it may be detectable in human saliva [31,32]. Determining if saliva GDF15 is physiologically regulated together with, or independently from, circulating blood levels would expand our understanding of GDF15 biology.

Here, we integrate repeated-measures data across four studies including 3,599 samples from 148 individuals (see Supplemental Table 1) to define the dynamic properties, natural morning awakening response, and stress-evoked psychobiological regulation of blood and saliva GDF15 in healthy individuals. Our results establish the dynamic, psychobiological nature of blood and saliva GDF15.

2. Results

2.1 Psychobiological regulation of plasma and saliva GDF15

To examine whether plasma and saliva GDF15 are affected by acute mental stress in humans we evoked mental stress in healthy human participants using a validated socio-evaluative speech test (modified from the Trier Social Stress Test–TSST [33], see *Methods* for details). During the speech task, participants defend themselves against an alleged transgression in front of a white coat-wearing "evaluator", a mirror, and a camera. Unlike physical exercise, which increases whole-body energy expenditure several-fold [34], the socio-evaluative speech task does not elicit a large increase in metabolic rate [35]. It does however trigger a coordinated multi-system response, including elevations in heart rate and blood pressure, cortisol, cell-free mtDNA, inflammatory cytokines, and other stress mediators [36-39]. In *Study 1 (MiSBIE – Mitochondrial Stress, Brain Imaging, and Epigenetic Study [24])* both plasma and saliva were concurrently collected with a salivette in the mouth while blood was drawn through an intravenous catheter at eight timepoints: 5 min before and 5, 10, 20, 30, 60, 90, 120 min after the onset of the stressor (Figure 1A). This sampling strategy allows to capture both the reactivity and recovery phases of the stress responses.

We first confirmed that, similar to what was previously observed in mice [26], evoked mental stress elevated circulating lactate in humans. Lactate is produced by increasing the intracellular $NADH/NAD^+$ ratio (i.e., reductive stress), which pushes the lactate dehydrogenase reaction towards lactate production [27].In our cohort, the brief TSST mental stress increased plasma lactate by an average of 14.5% (p=0.0054, range: -39 to +151%, Figure 1B) from

baseline to 5 min. This result demonstrates that a brief mental stressor induces systemic reductive stress from in humans (see Figures S1A-B for full time course and absolute change).

Concurrently, the speech task significantly increased plasma GDF15 by 5.9% at 10min (Hedges' g=0.59, p=0.032) before declining by 6.8% from 10 to 30 min (g=-0.49, p=0.0010) after the stressor (Figures 1C, see Figures S1C-D for full time course and absolute change). This represents, to our knowledge, the first evidence that mental stress alters blood GDF15 concentration within minutes.

Saliva GDF15 also exhibited significant change over the stress reactivity-recovery time course. Acute mental stress increased saliva GDF15 by a striking 42.8% at 5 min (g=0.84, p=0.0014). This response was approximately 7 times larger than the one observed in plasma. Saliva GDF15 returned to baseline levels 5 min later, 10 min after the onset of the stressor. This demonstrates the rapid increase and decrease in saliva GDF15 concentration (Figures 1D, see Figures S1E-F for full time course and absolute change).

To validate the speech task-induced changes in plasma GDF15, we analyzed samples from *Study 2* (MaPS – *Mitochondria and Psychological Stress Study*, see Methods and Materials for details) where 70 healthy individuals (mean age 30.6 years, 51.2% female) were randomized to either a speech task or a control (no stressor other than the blood draw and laboratory setting) (Figures 1E). Plasma GDF15 was measured at the same 8 timepoints as in *Study 1*, but saliva was not collected to avoid potential interference with the participant's psychological state. Blood was drawn without the participant's awareness via an obscured IV line running through the wall behind the participant. This protocol variation minimizes psychological interference after the induction of the stressor. However, it also eliminated interpersonal interactions for 2 hours after the stressor, which could produce anxiety.

Study 2 validated the direction and magnitude of results from *Study 1* (Figures 1F, left, see Figure S2A for individual trajectories). Contrasting to the control visit, mental stress increased plasma GDF15 by +3.5% (paired t-test: p=0.0035, g=0.59) at the 5 min timepoint in the stress visit (Figure 1F, right). On both control and speech task visits, GDF15 gradually increased over the 2-hour post-stressor recovery period, reaching a maximum at 120min (+9.0% in the stress visit, +8.1% in the control visit, relative to baseline).

Notably, participants randomized to the stress condition on their first visit exhibited ~6.2% (p=0.45) higher baseline plasma GDF15 concentrations than those randomized to control on their first visit, consistent with a potential influence of novelty and anticipatory stress on GDF15

levels (Figure S2B and S2C). Interestingly, and perhaps consistent with this hypothesis, across the cohort, two participants who did not return for their second visits (lost to follow-up) presented with the two highest baseline GDF15 levels (Figures S2D-F). Combined with our *Study 1* results, these findings establish that psychosocial stress increases human plasma GDF15 levels within minutes.

Importantly, there was no significant correlation between plasma and saliva GDF15 responses within each person, pointing to their independent (psycho)biological regulation (Figure S3A). In relating GDF15 and lactate, we found a modest positive correlation between the saliva GDF15 response and the acute rise in blood lactate (r=0.32, p=0.045; Figure S3B), consistent with a potential link between systemic reductive stress and saliva GDF15 reactivity. Resting blood lactate levels were found to be negatively correlated with resting saliva levels (r=-0.39, p=0.013; Figure S3C). However, resting blood lactate levels explained no more than 5-15% of the variance in inter-individual differences in saliva and plasma GDF15 or plasma GDF15 reactivity (Figure S3C-E). No correlation was found between plasma or saliva GDF15 with other stress hormones such as cortisol, norepinephrine, epinephrine (Figures S3F-K).

2.3 Stability and AM-PM dynamic variation

To quantify and compare how much saliva and plasma GDF15 levels change over time, in *Study 1* we implemented a repeated-sampling design including 14 saliva collections over a 2-day period (Figure S4A). Frequent saliva sampling allowed for a refined description of GDF15 dynamics. We found that compared to plasma GDF15 concentrations, which have been shown to exhibit a moderate degree of stability over weeks [40], saliva GDF15 levels presented 10.0-fold higher within-person variations (Figure S4B-C). Thus, saliva GDF15 is approximately an order of magnitude more dynamic or changeable than plasma GDF15.

We then examined how much plasma and saliva GDF15 levels vary across consecutive days, and from morning to afternoon within a given day. In *Study 1*, morning fasting saliva collected under the same conditions on two subsequent days exhibited higher GDF15 levels on the first study visit day (when the hospital environment was novel) and decreased on average by 26% from Day 1 to Day 2 (p=0.032, Figures S4D), demonstrating significant within-person variation. This decline may also reflect possible habituation to the novel, and therefore psychologically stressful research environment.

We also had the opportunity to investigate changes in GDF15 from morning (9am, fasting) to afternoon (~1-2pm, fed) on the two consecutive days in *Study 1*. On Day 1, from morning to afternoon, GDF15 levels decreased on average by -12% ($p<0.0001$) in plasma but tended to increase +6.5% ($p=0.06$) in saliva (Figure S4E). On Day 2 (only saliva collected), saliva GDF15 levels again increased from morning to afternoon by an average of +20% ($p<0.0001$). The magnitude and direction of change in saliva GDF15 between days 1 and 2 were not significantly correlated ($r=0.05$, $p=0.76$, Figure S4F). Thus, both the fasting/fed state [20] and the time of day might influence blood and saliva levels differently, further highlighting the distinct regulation of saliva GDF15 from circulating blood levels.

2.4 Awakening response and day-night variation

Waking up from sleep is a psychophysiological challenge accompanied by a return to conscious awareness and elevations in multiple physiological parameters, including core body temperature, energy expenditure, blood pressure, cortisol, and several other metabolically-demanding processes [34,41]. As a result, several hormones detectable in saliva exhibit an "awakening response", meaning that they either spike or decline as one wakes up in the morning. Among these is the cortisol awakening response [36,42]. Therefore, we investigated whether saliva GDF15 also exhibited an awakening response.

Study 1 included a 3-day home-based saliva collection protocol optimized to detect awakening responses on three non-consecutive days (Monday, Wednesday, and Friday), including 4 samples per day: (1) awakening, (2) 30 min after awakening, (3) 45 min after awakening, and (4) bedtime (Figure 2A). Remarkably, saliva GDF15 was highest immediately at the time of waking up (i.e., awakening) across the three days. Relative to the awakening timepoint, saliva GDF15 levels fell sharply by an average of -40% at 30 min, and -46% at 45 min, reflecting a robust negative awakening response. At bedtime, saliva GDF15 remained on average 47% lower than the awakening value (average group trajectory, Figure 2B; individual participants trajectories, Figure S5A). Observed over the awakening time course, males tended to exhibit a 39-101% higher median saliva GDF15 levels than females (n.s.), although both exhibited a similar decline by 47-57% after waking up (Figures S5B-C). Thus, saliva GDF15 exhibits rapid and robust variation after waking up, with potential sexual dimorphism in the absolute saliva GDF15 levels.

Intensive daily repeated measures in n=2. Next, we examined the stability and magnitude of the awakening response over multiple weeks. In *Study 3 (*Intensively Sampled n=2 Study, see

Methods and Materials for details*)*, we performed an intensive repeated-sampling protocol for saliva on two male participants using the same 4-timepoints as in *Study 1*. Both participants collected saliva for 53 (Participant A) and 60 (Participant B) consecutive days, as described previously [43]. This yielded 199 and 221 GDF15 measures for each participant, shown in Figure 2C. Consistent with *Study 1* results, saliva GDF15 levels varied substantially both within and across days. The average combined within-person C.V. was 267%, reflecting substantial day-to-day changes within each individual.

Participant A (male, age 34, sedentary, stress score average: 0.90 out of 5 on the modified Differential Emotional Scale [44]) saliva GDF15 levels ranged from 2.3pg/mL to 54.3pg/mL (median: 10.5pg/ml, Figure 2D). Saliva GDF15 exhibited an average -57.1% decline from awakening to 30 min, and -60.5% at 45 min. At bedtime, saliva GDF15 levels were, on average -55.8% lower than at awakening (Figure 2E), consistent with group results in *Study 1*.

Participant B (male, age 35, competitive runner, stress score average: 2.57 out of 5 on the modified Differential Emotional Scale) saliva GDF15 levels ranged from 3.5pg/mL to 4,428pg/mL (median: 15.1pg/ml, Figure 2F). His awakening response was on average -85.5% at 30 min, -88.7% at 45 min, and -69.6% at bedtime (Figure 2G). During the data collection period, Participant B was actively training for a marathon (race on day 27, red arrow), possibly accounting for the high GDF15 values and the overall higher mean levels than Participant A. The highest GDF15 value (4,428pg/mL) was recorded at the 45 min timepoint the day after the marathon (day 28), consistent with prior reports linking exhaustive exercise with *blood* GDF15 [45], but here observed punctually in *saliva*, at a specific timepoint, *the day after* the thorough effort.

Within each participant, the three morning time points (awakening, 30, 45 min) were significantly correlated with one another ($r=0.36$-0.60, all $p<0.01$, Figure S5E). This result is consistent with robust day-to-day variation, marked by some low GDF15 mornings and some high GDF15 mornings. Morning and evening timepoints were not correlated, possibly pointing to different drivers of saliva GDF15 levels between morning and evening.

***Day-night variation in blood GDF15*.** The awakening responses observed in Studies 1 and 2 were observed in most individuals and were remarkably large effect sizes, suggesting potential night-day GDF15 changes. Unlike blood, which can be drawn through an intravenous catheter throughout the sleep-wake transition, saliva cannot be collected easily during sleep. To explore if the saliva GDF15 awakening response is also apparent in plasma, *Study 4* (Overnight Meal Alignment Study, see Methods and Materials for details), included collection of blood via indwelling catheter and intravenous (IV) line every 30 minutes overnight during sleep, and in the

morning after waking up, on two separate visits (n=6 participants). Some participants exhibited up to 25.6% variation in serum GDF15 levels between day and nighttime, consistent with a previous report [46]. However, in this small sample, we found no systematic diurnal variation across participants (Figure S6). Thus, unlike the robust saliva GDF15 awakening response, blood diurnal variation was either too small in magnitude or too idiosyncratic in its timing to be detected in this sample.

3. Discussion

We have established that both plasma and saliva GDF15 are rapidly inducible within minutes by socio-evaluative stress. For saliva GDF15, we also document for the first time a robust morning awakening response and mild morning-to-afternoon diurnal variation. These results across thousands of samples obtained by a combination of robust laboratory and ecological GDF15 measures in healthy women and men expand our understanding of the psychobiological regulation of GDF15, opening important questions around the intersection of mental and cellular energetic stress.

One of the most remarkable and robust findings from the studies described above is the reactivity of GDF15 in response to a simple and relatively mild 5-minute mental stressor. The GDF15 response was replicated across independent studies. It was relatively small in plasma (3-6% across *Studies 1 and 2*), whereas saliva GDF15 reactivity was ~8-10-times larger (42-71%).

Both plasma and saliva GDF15 levels increased and decreased remarkably quickly, within 5-10 minutes, or perhaps even faster than our measures could resolve. We note that the group averages (required to perform standard statistical analyses) obscure the true individual-level dynamics of human GDF15 biology. Individual-level dynamics can be appreciated from the individual participant trajectories (for example, see Figure S1E). In some participants, saliva GDF15 increased >300-400% over 10 min and declined/returned to normal levels within 5 min. Because the precision of the GDF15 assay is consistently <20%, we see no reason to doubt the observed magnitude nor the rate of change. The magnitude and rate of these responses appear consistent with pulsatile release, as for several stress hormones and neuromodulators, including cortisol [47] and catecholamines [48]. This insight afforded by repeated sampling at the scale of minutes suggests the need for studies examining the GDF15 release and signaling mechanisms.

In addition to the experimental mental stressor, four elements of our results suggest that psychosocial factors may generally modulate plasma GDF15 levels. First, in the control condition of *Study 4*, participants visited a novel laboratory environment and were instrumented with an IV line causing some discomfort, isolated in a quiet room without access to their personal communication device (phone), and monitored remotely without interpersonal interaction for 2 hours. Under these conditions, we observed a gradual elevation in plasma GDF15 from 60 to 120 min. This pattern was either not observed or observed in attenuated magnitude in MiSBIE *Study 1* participants, where interactions with the study nurse and study coordinator occurred at each measurement timepoint (8 times over the 2 hours, for blood and saliva collections). The basis for this discrepancy in GDF15 dynamics requires further research, but could relate to an unanticipated "social isolation" effect in *Study 2*.

Second, we note that two *Study 2* participants randomized to the stressful speech task on their first visit did not return for the second visit. Curiously, both participants exhibited abnormally high GDF15 levels throughout the protocol – the highest average GDF15 observed across our control cohorts (see Figure S2D-F). Given that high GDF15 levels signaling on the brainstem causes aversive responses and visceral malaise (e.g., nausea [4,49]), we speculate that their decision not to return for the second visit could have been influenced by their particularly aversive experience during their first visit, possibly either enhanced or provoked by abnormally high plasma GDF15 levels. In trials where individuals were administered recombinant GDF15, participants also complained of discomfort such as nausea and some trials were terminated early because of intolerance[50]. The absolute GDF15 concentrations in all healthy, non-pregnant individuals here are markedly lower than in pregnancy, possibly undermining our proposition. But in the absence of an actively GDF15 secreting placenta (the main source of GDF15 in pregnancy), the acute rise in GDF15 from other sources could be perceptible and contribute to adverse experiences. Additional studies are necessary to examine this hypothesis.

Third, the saliva GDF15 awakening response measured across three different days was highest on the first day (Monday), and progressively declined as the psychological novelty of the daily sample collection exercise subsided. This observation is not definitive, but also aligns with the notion that *novelty* (a hallmark of mental stress [51]) places the organism in an anticipatory state where energetic resources are preemptively mobilized [35]. The dynamic psychological regulation of saliva GDF15 levels calls for more research. In Supplemental File 1, we discuss three functional connections between GDF15 and the psychobiology of human stress responses.

Our results also align with three lines of evidence linking GDF15 to the psychobiology of stress responses. First, in the same manner that immune cytokines trigger sickness behavior [52], GDF15 may exist to induce energy conservation behaviors to support stress-related energy demands, promoting resilience to stressors. Second, human cortisol deficiency has been shown to elevate GDF15 levels [53], and GDF15 has been shown to direct increase circulating corticosterone and catecholamines [5], suggesting a functional interaction between the social stress-responsive hypothalamic-pituitary-adrenal (HPA) axis and the ISR-GDF15 axis. Third, we note that plasma GDF15 is elevated in some psychiatric conditions [11,54-56]. A recent study profiling human proteome in >52,000 adults from the UK Biobank showed that people diagnosed with depression and anxiety are more likely to have higher plasma GDF15 levels (Depression: Hazardous Ratio = 1.53, 95% CI = 1.45-1.61, $p = 6.7e^{-58}$; Anxiety: Hazardous Ratio = 1.50m 95% CI = 1.41-1.59, $p = 5.6e^{-43}$), further linking psychopathology and abnormal mental states to elevated blood GDF15 [57].

The lack of correlation between saliva and plasma GDF15 levels opens some exciting questions. Plasma GDF15 concentration is roughly an order of magnitude higher than saliva. Plasma GDF15 is a robust marker of aging, whereas saliva GDF15 is not. Following mental stress, saliva GDF15 increases more strongly and quickly than plasma GDF15, suggesting that saliva GDF15 could exhibit more robust state properties whereas blood GDF15 may represent a more stable trait – albeit nevertheless being robustly stress-inducible. Nevertheless, together these observations point towards a fairly independent regulation of GDF15 secretion between salivary glands and blood.

In summary, this work reveals that the energetic stress marker GDF15 is dynamically regulated by mental stress. Our strategic repeated-measures sampling across laboratory and home-based studies uncovers general dynamic principles for the diurnal and psychobiological regulation of both blood and saliva GDF15, broadening our understanding of human GDF15 psychobiology beyond disease and aging. These results point towards a shared psychobiological pathway or sets of pathways implicating GDF15 and cellular energetic regulation at the intersection of metabolic and mental stressors.

4. Methods

4.1 Study 1: MiSBIE cohort

4.1.1 Participants

Participants were enrolled in the Mitochondrial Stress, Brain Imaging, and Epigenetics (MiSBIE) study [24] in adherence to the directives outlined by the New York State Psychiatric Institute IRB protocol #7424, clinicaltrials.gov #NCT04831424. Recruitment was conducted both within our local clinic at the Columbia University Irving Medical Center, and nationally throughout the United States and Canada. All enrolled participants provided written informed consent, authorizing their participation in the investigative procedures and the potential dissemination of data. Recruitment occurred from June 2018 to May 2023 for a total of 70 healthy controls (females n=48, males n=22). This manuscript specifically reports on MiSBIE GDF15 dynamics in blood and saliva. Sex was determined by self-report. This single-step method is limited, does not distinguish between gender and sex, and can exclude both transgender and intersex people; current best practices include a two-step [58] measurement and broadening the available answer options.

Our inclusion criteria included healthy adults willing to provide saliva samples and have blood collected using an intravenous catheter during the hospital visits. Exclusion criteria included the presence of pronounced cognitive impairment precluding informed consent, concurrent neoplastic disease, recent occurrences of flu or other temporally pertinent infections within the four-week window preceding study participation, Raynaud's syndrome, engagement in ongoing therapeutic or exercise trials registered on ClinicalTrials.gov, and the existence of metallic elements within or on the body, alongside claustrophobia posing an impediment to magnetic resonance imaging (MRI). In-person laboratory procedures were performed over 2-days (Tuesday and Wednesday), followed by a 1-week home-based saliva collection protocol.

Non-stress plasma and saliva samples were collected both in the morning, under fasting conditions around 9:30 AM, or after lunch around 1:00 PM. Both the breakfast and lunch meals were selected from a restricted MiSBIE study menu to avoid large differences in meal types between participants.

4.1.2 Stress reactivity

A validated socio-evaluative speech stress task was conducted on all MiSBIE participants on Day 1, as described previously [59,60]. The 5-min test included 2 min of preparation and 3 min of delivering a simulated speech to a court judge where participants defend themselves against an alleged shoplifting transgression. The speech was delivered in front of a confederate evaluator wearing a white lab coat (adding gravity to the situation), a mirror where participants can see themselves, and a (non-operational) video camera. To minimize participant discomfort during the task and avoid venipuncture-related stress responses during the task, an

intravenous catheter was placed in the right arm >45 min before the initiation of the speech stress task and participants sat quietly, undisturbed for 30 minutes before receiving instructions for the speech task. Blood and saliva samples were collected at -5, +5, 10, 20, 30, 60, 90, and 120 min relative to the beginning of the TSST. Concurrently, during each blood collection, saliva samples were collected using salivettes (Starstedt Cat# 51.1534.500). On day 2, while in the MRI scanner, to avoid habituation to the speech task, participants were informed that their performance on the previous day was “below average compared to other participants” and asked to prepare a speech to a court judge in defense of another alleged transgression (running a stop sign). Saliva samples were collected using salivettes at 3 timepoints: ~30 minutes before the MRI session, 33 minutes into the session (~9 minutes before beginning the speech task), and 75 minutes into the session (~18 minutes after the beginning of the speech task).

4.1.3 Plasma and saliva collection

The blood and saliva collection protocols are illustrated in Supplemental Figure S2A. Plasma was collected via a central venous catheter at 9 time points: 1) day 1 morning fasting sample, 2) 8 afternoon samples collected during the stress reactivity protocol. A total of 16-20mL of whole blood was collected at the morning fasting timepoint in two 10 mL K_2EDTA blood collection tubes (BD #366643). A total of 5mL of whole blood was collected at each afternoon time point both before and after stress in 6mL K_2EDTA blood collection tubes (BD #367899).

Saliva was collected at 26 timepoints: 1) day 1 morning fasting sample, 2) 8 afternoon samples collected during the stress reactivity protocol, 3) 1 sample after a cold pressor test, 4) day 2 morning fasting sample, 5) 3 afternoon stress samples before and during the MRI, 6) 12 at home samples collected across 3 days: (i) immediately upon awakening, (ii) 30 min after waking up, (iii) 45 min after waking up, and (iv) at bedtime. A total of 1-2 mL of saliva was collected at each sample timepoint using a salivette (Starstedt # 51.1534.500) following the Biomarker Network recommended procedure [61]. Participants were directed to position the cotton swab at the center of their tongue within their mouth for 2 to 5 min. It was emphasized that they should refrain from biting down on the cotton and ensure the swab did not come into contact with their cheeks. Afterward, participants reinserted the cotton swab into the salivette recipient tube. Samples were collected on visit days 1 and 2, the salivettes were placed on ice (4°C) in a styrofoam box for transportation and further processing at the laboratory. For home-based sample collection, salivettes were frozen immediately after collection in a home freezer (typically -20°C). During the at-home morning collections, individuals were advised to ideally delay tooth brushing and eating until after the third sample (+45 minutes), while the nighttime sample was to

be taken prior to bedtime tooth brushing. If participants needed to, they were advised to eat breakfast within the 30min break of awakening sampling and note the time and content. Participants were instructed to avoid consuming water or any other liquids within 10 minutes of each saliva sample collection. This was monitored and controlled for the laboratory samples. Within 2 weeks of collection, participants either transported (~10%) or shipped them (~90%) to the laboratory in a non-temperature controlled, pre-stamped USPS priority shipping.

4.1.4 Biofluids (Plasma and Saliva) processing and storage

Whole blood tubes for morning fasting plasma samples were immediately inverted 10-12 times and centrifuged at 1000xg for 5 minutes at room temperature after collection. Samples were placed on ice (4°C) in a styrofoam box and transported to the laboratory for further processing. When samples reached the laboratory, they were immediately centrifuged at 2000xg for 10 minutes at 4°C. To avoid platelets and other cellular debris, approximately 80% of the plasma was collected from the upper portion of each tube, transferred and pooled into a fresh 15 mL conical tube. To further eliminate cellular components, pooled plasma tube was centrifuged at 2000xg for 10 minutes at 4°C. Around 90% of the resulting plasma supernatant was transferred to fresh 15 mL conical tube and mixed through inversion. The resulting cell-free plasma was aliquoted into 0.5-1.5 mL aliquots and stored at -80°C before being used for stress reactivity GDF15 measurements by ELISA.

Stress reactivity time points include 8 tubes for each timepoint, starting at -5 minutes to 120 minutes. Whole blood tubes for afternoon stress plasma samples were immediately inverted 10-12 times and centrifuged at 2000xg for 3.5 minutes at room temperature after collection. Samples were placed on ice (4°C) in a styrofoam box, and transported to the laboratory together for further processing. When samples reached the laboratory, they were immediately centrifuged at 2000xg for 10 minutes at 4°C. To avoid platelets and other cellular debris, approximately 80% of the plasma was collected from the upper portion of each tube and transferred into fresh 8*15 mL conical tubes. To further eliminate cellular components, plasma tubes were centrifuged at 2000xg for 10 minutes at 4°C. Around 90% of the resulting plasma supernatant was transferred to fresh 8*15 mL conical tubes and mixed through inversion. The resulting cell-free plasma was aliquoted into 0.5-1.5 mL aliquots for each timepoint and stored at -80°C before being used for stress reactivity GDF15 measurements by ELISA.

When saliva samples reached the laboratory, Salivettes were centrifuged at 1000xg for 5 min in a refrigerated centrifuge at 4°C. To avoid cellular contamination with leukocytes or epithelial buccal cells[62], supernatant was carefully removed from the top of the tube, transferred

to cryovials, and stored immediately at -80°C. Before use, saliva was thawed and centrifuged at 5000xg for 10 min to further eliminate cellular components. The resulting supernatant was collected as cell-free saliva and was used for subsequent GDF15 assessment.

4.2. *Study 2: The Mitochondria and Psychological Stress (MaPS) study*

4.2.1 Participants

Generally healthy community volunteers were recruited to participate in the MaPS study (clinicaltrials.gov #NCT04078035). Eligibility criteria were (1) 20-50 years of age (equal numbers within each decade of age); (2) for females, regular menstrual cycles over past 12 months, not lactating or pregnant; (3) generally physically healthy (no reported history of chronic systemic immune, metabolic or mitochondrial diseases, or chronic diseases that influence the central nervous, autonomic nervous or neuroendocrine systems); (4) not taking medications that influence the central nervous, autonomic nervous or neuroendocrine systems; (5) no reported history of psychotic illness or mood disorder; (6) resting blood pressure<140/90mmHg; (7) not a current smoker or user of illicit drugs; (8) weight>110lbs and BMI<30; and (9) fluent in English (used everyday in speaking and reading for at least 10 years). All enrolled participants provided written informed consent as approved by the University of Pittsburgh IRB. Recruitment occurred between July 2020- December 2022. A total of 72 participants were enrolled (n=34 self-identified as female; 38 as male). Participants were rescheduled if (1) they endorsed having an infection, cold or flu, taking antibiotics or glucocorticoids, or having received a vaccination or tattoo in the prior two weeks; (2) presented with symptoms in a range consistent with possible infection on the day of the visit; or (3) reported having taken glucocorticoids in the past 24 hours on the day of the visit.

4.2.2. Stress Reactivity

Participants attended two afternoon laboratory sessions scheduled at least one month apart at the Behavioral Physiology Laboratory, University of Pittsburgh. Both sessions started between 1:00-3:00 PM. Participants were asked to avoid eating or drinking after 12 pm prior to each session. The order of sessions was counterbalanced in randomized starting order across participants. At one session, they completed the same 5-minute socio-evaluative speech task that was used in Study 1, MiSBIE (see above). At the other session they rested quietly for the 5-minute task period and were not exposed to the stressor. On both occasions, an intravenous catheter was inserted and participants sat quietly for a 30-minute baseline (habituation) period before completing the task. Following the task period, participants watched a wildlife

documentary for a 120-minute recovery period. Participants were left alone as much as possible, except when the study nurse had to re-insert or reposition the catheter, during all periods except when performing the speech task. Blood samples were collected 5 minutes before and 5, 10, 20, 30, 45, 60, 75, 90, and 120min after the beginning of the task period. Saliva samples were not collected in this study.

4.3 *Study 3: Intensive repeated-measures n=2 study*

4.3.1 Participants and procedures

To evaluate the intra-individual fluctuations of salivary GDF15, repeated daily measurements were taken over periods of 53 and 60 days from two Caucasian male participants: author M.P., 34 years old (Participant A) and author G.G., 35 years old (Participant B, see [43]). Study investigators served as participants to mitigate the excessive demand on volunteer participants required to collect numerous daily samples over multiple consecutive weeks. The study was approved by the New York State Psychiatric Institute (Protocol #7748). Participants provided written informed consent for participating and publishing the results. As for the at-home collection protocol in the Study 1 MiSBIE cohort, participants collected saliva immediately upon awakening, 30min and 45min after waking up, and at bedtime. Participants delayed consuming liquid and tooth brushing until after the third sample (45 min), and the nighttime sample was taken prior to bedtime tooth brushing. On each morning and evening, participants completed a morning diary assessing positive and negative affective states, using items adapted from the modified Differential Emotional Scale (MDES), including ratings of stress levels on a 5-point Likert scale: 1 (Not at all), 2 (A little bit), 3 (Somewhat), 4 (Moderately) to 5 (Extremely) [44].

4.3.1. Saliva processing and storage

Salivette samples were stored in home freezer (-20°C) before being transported or shipped frozen to the laboratory. Upon arrival salivettes were thawed at room temperature and centrifuged at 1000 x g for 5 min in a refrigerated centrifuge operating at 4°C to extract saliva from the cotton swab. 200uL of saliva supernatant was taken from the top, transferred to a 1.5 ml Eppendorf tube and stored at −80°C. Prior to analysis, Eppendorf tubes was further centrifuged at 2000 x g for 10 min at 4°C to remove cellular debris, and the resulting supernatant was collected as cell-free saliva and was used for GDF15 measurements by ELISA. To control for the effect of saliva protein concentration on GDF15 results, total protein

concentration was measured using the Bicinchoninic acid (BCA) assay as described previously [43].

4.4. *Study 4: Overnight meal alignment study*

4.4.1 Participants and procedures

Healthy females and males were recruited to participate in a randomized crossover study testing the impact of varying mealtimes, relative to wake time, on energy balance regulation (clinicaltrials.gov #NCT03663530). Participants interested in the study underwent a multi-stage screening process. Inclusion criteria included being 20-49 years of age at the time of screening, having a body mass index between 20-34.9 kg/m^2, healthy habitual sleep patterns, and habitually consuming the first meal of the day within 1 h of waking. Individuals who were pregnant or lactating, smoked (currently or <3 years since stopping), reported excessive caffeine or alcohol intake, had extreme morning or evening chronotype, or who were actively engaging in intentional weight change or had a recent weight change >5% in the past 3 months were excluded. In addition, prospective participants with cancer; chronic cardiovascular, metabolic, autoimmune, neurologic, psychiatric, or sleep-related disorders/conditions; or who or take medications or supplements known to affect sleep or energy balance were not included cancer were not included. Individuals meeting all eligibility criteria competed a two-week outpatient screening period, which included daily assessment of sleep via wrist accelerometer (Actigraph GT3X+, Actigraph Corp., Pensacola, FL.) and daily sleep diaries along with assessment of daily meal timing/eating patterns. Those with average nightly total sleep time ≥7 h and average daily eating start times within 1 h of awakening were invited to participate in the study. All procedures were approved by the Columbia University Irving Medical Center Institutional Review Board, and all participants provided informed consent form prior to participating in the study.

The main study included two experimental conditions, circadian alignment and circadian misalignment, followed for 6 weeks each. During the circadian alignment condition, participants were instructed to consume their first meal within 1 h of awakening. Two subsequent meals and an evening snack were to be consumed at 5-, 10-, and 11-hours post-awakening, respectively. During the circadian misalignment condition, meals were delayed by 4 hours relative to the aligned condition. For the present analyses, only samples collected during the first 2 weeks of the aligned condition were used. This corresponded to a period during which all meals and

snacks were provided to participants based on weight maintenance energy needs, determined using the Mifflin-St. Jeor equation [63]. Participants were required to consume all of the foods provided each day and to refrain from consuming other foods or beverages. On days 3-4 and 14-15 of this controlled feeding period, participants underwent cardiometabolic and energy expenditure assessments in a whole-room indirect calorimeter. During these 24-h visits, blood samples were collected every 30 min, from 7 PM to 8:30 AM, via an indwelling catheter connected to an IV line that ran through a port to the outside of the room. Throughout the wake period, participants remained in a semi-recumbent position in dim light, wearing blue-blocking lenses. They were instructed to consume the meals and snack provided at the assigned times, and to turn the lights out at 11:30 PM, to initiate sleep, and were awoken via phone call at 8 AM and instructed to turn the lights back on.

4.4.2. Serum processing and storage

Whole blood was collected into 4mL BD vacutainer serum tubes (cat# 367812). Blood remained upright in the tube until clotted, after which it was centrifuged for 20 minutes at 3000 RPM and 4°C. Resultant serum was aliquoted into cryovials and stored at -80°C until GDF15 measurements by ELISA.

4.5. GDF15 assays

Plasma, serum and saliva GDF15 levels were quantified using a high-sensitivity ELISA kit (R&D Systems, DGD150, SGD150) following the manufacturer's instructions. Different lot numbers were used, and the average coefficient of variation (C.V.) between lot numbers was determined by reference samples for quality control. Plasma and serum samples were diluted with assay diluent (1:4 ratio) to maximize the number of samples within the dynamic range of the assay. Saliva samples were not diluted. Absorbance was gauged at 450nm, and concentrations were computed utilizing the Four Parameter Logistic Curve (4PL) model. Samples were run in duplicates, on separate plates when possible, and the concentration for each sample was computed from the average of the duplicates. Standard curve (5 samples per plate) and plasma reference samples (2-3 samples per plate, same sample per batch) were run with each individual assay and the inter-assay C.V. was monitored. All standard curves and references were overlaid on top of each other to monitor failed runs. Samples were run in duplicate plates when possible and those with C.V. larger than 15% were re-ran. When it was not possible to rerun (e.g., no sample left), sample sets with a C.V. >15% between the

duplicates were included. We performed sensitivity analyses excluding these samples, confirming that the results were unchanged by the presence of absence of these samples with lower reliability. Values below the mean minimum detectable dose (2.0pg/ml) were considered as non-detectable (reported as NA) and excluded in the graphs or statistical analyses. For the MiSBIE study, samples were run in 3 batches over 2 years, multiple quality control measures were applied to monitor batch-to-batch and within batch variability. For a full experimental setup, see Supplementary Table 4. Data-preprocessing and quality control measures was done using R Software (version 4.2.2) and are available as supplemental files.

Based on the 1000 Genomes Project, around 15-30% of the population assessed in the project carried the common H202D variant in GDF15 [64]. Compared to the Roche Elecsys assay, the R&D ELISA kits was found to report GDF15 concentrations that are 36% lower in individuals carrying one D allele or 61% lower in those that carry two D alleles at position 202 of the pro-peptide (or position 6 of the mature peptide) [65]. Despite this limitation, our within-person findings related to psychosocial stress reactivity, diurnal variation, and awakening responses would be unaffected by the underestimation of GDF15 concentration in some participants with the H202D variant.

4.6. Statistical analyses

The association between age and plasma/salivary GDF15 level at morning, fasting sample (baseline) was determined using Spearman rank correlation. Differences across two timepoints in the same individual (e.g. Morning to afternoon, day 1 to day 2 fasting) were assessed using paired non-parametric signed-rank Wilcoxon test. Diurnal pattern was assessed using Tukey multiple comparison post hoc test. Stress reactivity was operationalized as % change from baseline at each time point, and a mixed effect models were used to test whether acute stress affected plasma and saliva GDF15 levels over time. For awakening response analysis from immediate upon awakening to 45 min, we averaged GDF15 measures collected at the same timepoint during a day from three days (Mon, Weds, and Fri). Statistical analyses were conducted using GraphPad Prism (version 9.4.1) and R (version 4.2.2 and 4.3.0).

5. Code and data availability statement

All data is available and submitted along with his manuscript. The code for data analysis is available on GitHub at https://github.com/mitopsychobio/2024_GDF15_Dynamics_Huang

Financial competing interests

The authors have no competing interests to declare.

Author contributions

M.P., M.H., C.T., R.P.S., V.L., R.P.J. developed *Study 1* (MiSBIE). A.L.M., B.A.K., M.P. developed *Study 2* (MaPS). M.P., G.G., S.R., C.T. developed *Study 3* (intensively sampled n=2). M.P.S.O. developed *Study 4* (overnight meal alignment). C.K. and K.E. recruited MiSBIE participants. C.K. and S.R. enrolled participants, collected study visits data and samples. M.K. processed samples. S.L. and M.H. performed the medical examinations. J.D. and A.S.M. analyzed gene expression data. Q.H., R.H., S.R., A.S.M., C.C.L. performed GDF15 assays. E.T. performed the lactate assays in collaboration with J.M. Q.H., A.S.M., N.B.A., and C.C.L. performed statistical analyses. Q.H. and C.T. prepared the figures. S.W. and T.W. advised on statistical analyses. C.T., Q.H., M.P. drafted the manuscript. M.P. and C.T. conceived and supervised the overall project. All authors reviewed the final version of the manuscript.

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Figure legends

Figure 1. Socio-evaluative stress acutely increases plasma and saliva GDF15.
(**A**) Study 1 experimental design measuring psychological response in GDF15 after socio-evaluative stress elicited by a validated socio-evaluative speech task. (**B**) Plasma lactate levels 5 min before and 5min after the stressful speech task shown as percent change from baseline. N=47, 72% female, 94 observations. See Supplemental Figure S1A-B for the full time course. (**C,D**) Plasma (**C**) and Saliva (**D**) GDF15 levels before and after psychological stress shown as percent change from baseline (-5 min). For plasma N=65, 68% female, 512 observations, for saliva GDF15 N=57, 65% female, 442 observations. For individual trajectories and absolute change see Supplemental Figure S1. (**E**) Study 2 experimental design including two visits randomized to either socio-evaluative speech task (same as in Study 1) or a control session without stress exposure. Plasma GDF15 was measured at 8 timepoints before and after stress, following the same schedule as Study 1. (**F**) *Left:* Plasma GDF 15 levels for control and stress visit shown as percent change from baseline (-5min). N=70, 48% female, 916 observations. See Supplemental Figure S3A for individual trajectories), p-values from followed by Tukey multiple comparison test, corrected for multiple testing. *Right:* Focus on -5 vs 5min timepoints for plasma GDF 15 levels for control and stress visit shown as percent change from baseline, p-values from Wilcoxin paired t-test for stress group, effect size from Hedge's g comparing -5 to 5min for stress group. N=58 pairs, 48% female, 117 observations. Data shown as mean ± SEM. P-values from Wilcoxon paired t-test (B) or mixed-effect model followed by Tukey multiple comparison test, corrected for multiple testing (C,D) and *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Figure 2. Saliva GDF15 exhibits a robust negative awakening response and large within-person variation over days to weeks.
(**A**) Schematic of experimental design measuring GDF15 awakening response from human saliva samples. Saliva was sampled at awakening, 30 and 45 min after awakening, and at bedtime. (**B**) Saliva GDF15 diurnal variation. Data from the Study 1 healthy adults on three non-consecutive days four timepoints each day (N=710 observations, 68% female).(**C**) Study 3 saliva GDF15 concentrations (*left*, log10 scale; *right*, median on linear scale, data median ± 95% CI) in Participants A and B. (**D,F**) Intensive repeated sampling of saliva GDF15 levels showing day-to-day variation Participant A (Figure **D**, N=199 observations, repeated measures for 53 days) and Participant B (Figure **F**, N=221 observations, repeated measures for 60 days). The 4-day yellow highlight (Days 27-31) were relatively anomalous and showed no cortisol awakening response and no cell-free mtDNA awakening response[44]. (**E,G**) Diurnal profiles for saliva GDF15 in Participant A (**E**) and B (**G**). *Top*, individual days; bottom, average trajectory. One outlier timepoint, Day 28, 45 min, was removed from analyses. See figure S4D for median of diurnal pattern for Participant A and B. Data are mean ± SEM unless otherwise noted. Effect sizes and P-values from (B,F,G) Mixed-effect model and Tukey multiple comparison test corrected for multiple testing. All data were included in graph, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Figure 1

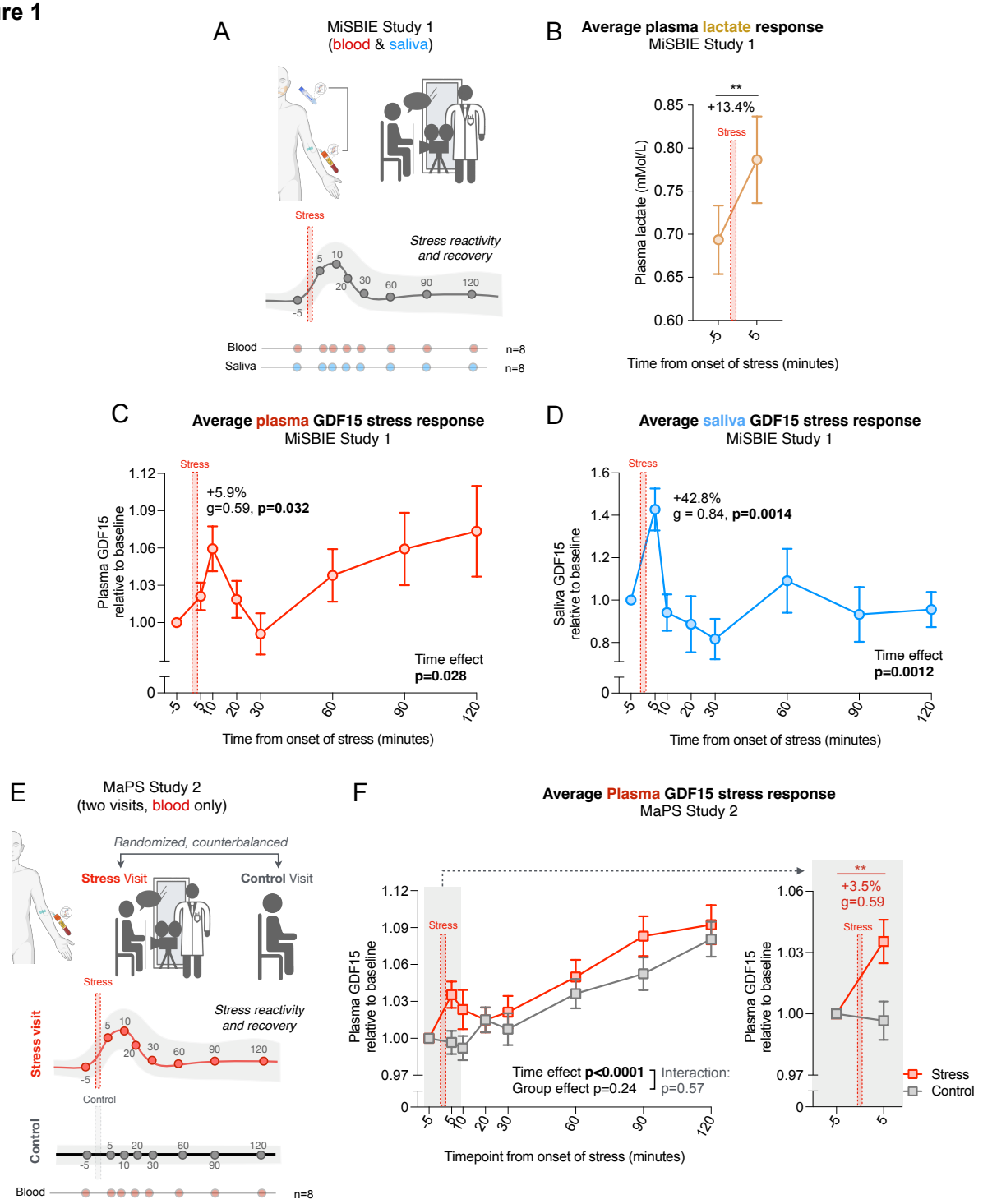

Figure 1. Socio-evaluative stress acutely increases plasma and saliva GDF15.

(**A**) Study 1 experimental design measuring psychological response in GDF15 after socio-evaluative stress elicited by a validated socio-evaluative speech task. (**B**) Plasma lactate levels 5 min before and 5min after the stressful speech task shown as percent change from baseline. N=47, 72% female, 94 observations. See Supplemental Figure S1A-B for the full time course.(**C,D**) Plasma (**C**) and Saliva (**D**) GDF15 levels before and after psychological stress shown as percent change from baseline (-5 min). For plasma N=65, 68% female, 512 observations, for saliva GDF15 N=57, 65% female, 442 observations. For individual trajectories and absolute change see Supplemental Figure S1.(**E**) Study 2 experimental design including two visits randomized to either socio-evaluative speech task (same as in Study 1) or a control session without stress exposure. Plasma GDF15 was measured at 8 timepoints before and after stress, following the same schedule as Study 1. (**F**) *Left:* Plasma GDF 15 levels for control and stress visit shown as percent change from baseline (-5min). N=70, 48% female, 916 observations. See Supplemental Figure S3A for individual trajectories), p-values from followed by Tukey multiple comparison test, corrected for multiple testing. *Right:* Focus on -5 vs 5min timepoints for plasma GDF 15 levels for control and stress visit shown as percent change from baseline, p-values from Wilcoxin paired t-test for stress group, effect size from Hedge's g comparing -5 to 5min for stress group. N=58 pairs, 48% female, 117 observations.
Data shown as mean ± SEM. P-values from Wilcoxon paired t-test (B) or mixed-effect model followed by Tukey multiple comparison test, corrected for multiple testing (C,D) and *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Figure 2

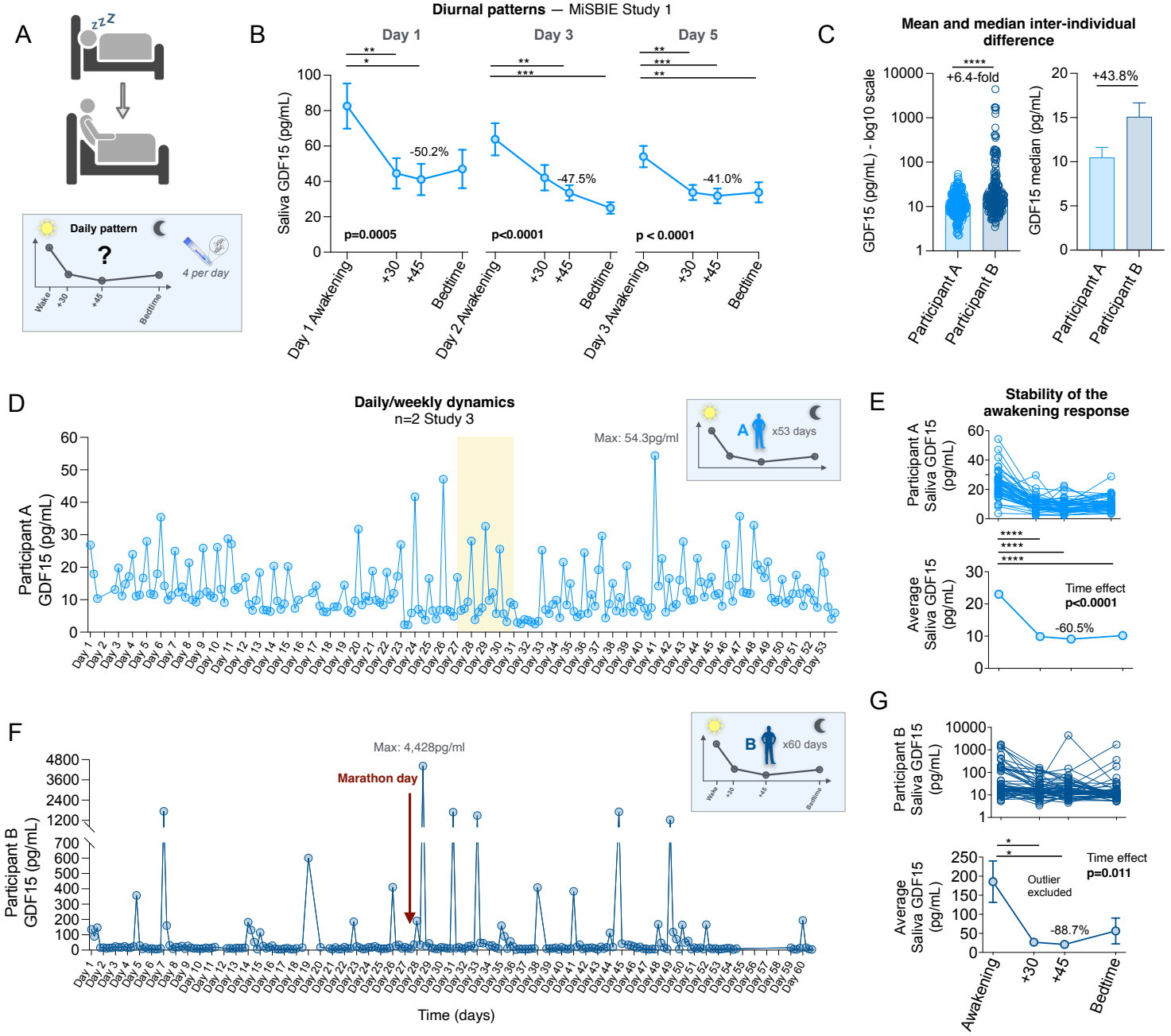

Figure 2. Saliva GDF15 exhibits a robust negative awakening response and large within-person variation over days to weeks.

(**A**) Schematic of experimental design measuring GDF15 awakening response from human saliva samples. Saliva was sampled at awakening, 30 and 45 min after awakening, and at bedtime. (**B**) Saliva GDF15 diurnal variation. Data from the Study 1 healthy adults on three non-consecutive days four timepoints each day (N=710 observations, 68% female).(**C**) Study 3 saliva GDF15 concentrations (*left*, log10 scale; *right*, median on linear scale, data median ± 95% CI) in Participants A and B. (**D,F**) Intensive repeated sampling of saliva GDF15 levels showing day-to-day variation Participant A (Figure **D**, N=199 observations, repeated measures for 53 days) and Participant B (Figure **F**, N=221 observations, repeated measures for 60 days). The 4-day yellow highlight (Days 27-31) were relatively anomalous and showed no cortisol awakening response and no cell-free mtDNA awakening response[44].
(**E,G**) Diurnal profiles for saliva GDF15 in Participant A (**E**) and B (**G**). *Top*, individual days; bottom, average trajectory. One outlier timepoint, Day 28, 45 min, was removed from analyses. See figure S4D for median of diurnal pattern for Participant A and B.
Data are mean ± SEM unless otherwise noted. Effect sizes and P-values from (B,F,G) Mixed-effect model and Tukey multiple comparison test corrected for multiple testing. All data were included in graph, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.